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Microarray and Growth Analyses Identify Differences and Similarities of Early Corn Response to Weeds, Shade, and Nitrogen Stress

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Weed interference with crop growth is often attributed to water, nutrient, or light competition; however, specific physiological responses to these stresses are not well described. This study's objective was to compare growth, yield, and gene expression responses of corn to nitrogen (N), low light (40% shade), and weed stresses. Corn vegetative parameters from V2 to V12 stages, yield parameters, and gene expression using transcriptome (2008) and quantitative polymerase chain reaction (qPCR) (2008/09) analyses at V8 were compared among the stresses and with nonstressed corn. N stress did not affect vegetative parameters, although grain yield was reduced by 40% compared with nonstressed plants. Shade, present until V2, reduced biomass and leaf area > 50% at V2, and recovering plants remained smaller than nonstressed plants at V12. However, grain yields of shade-stressed and nonstressed plants were similar, unless shade remained until V8. Weed stress reduced corn growth and yield in 2008 when weeds remained until V6. In 2009, weed stress until V2 reduced corn vegetative growth, but yield reductions occurred only if weed stress remained until V6 or later. Principle component analysis of differentially expressed genes indicated that shade and weed stress had more similar gene expression patterns to each other than they did to nonstressed or N-stressed tissues. However, corn grown in N-stressed conditions shared 252 differentially expressed genes with weed-stressed plants. Ontologies associated with light/photosynthesis, energy conversion, and signaling were down-regulated in response to all three stresses. Shade and weed stress clustered most tightly together, based on gene expression, but shared only three ontologies, O-METHYLTRANSFERASE activity (lignification processes), POLY(U)-BINDING activity (posttranscriptional gene regulation), and stomatal movement. Based on morphologic and genomic observations, weed stress to corn was not explained by individual effects of N or light stress. Therefore, we hypothesize that these stresses share limited signaling mechanisms.

Nomenclature: Corn, *Zea mays* L.

Key words: Weed competition, genomics, transcriptome analysis, critical weed free period.

Plant growth is influenced by various environmental factors that modify morphology and physiology, making generalizations of plant responses to stress difficult (Kropff 1993). Weed interference during the critical weed-free period (CWFP), often results in irreversible negative effects to growth and, in many cases, yield (Hall et al. 1992; Knake and Slife 1969; Knezevic et al. 2002; Maddonni and Otegui 2004; Massinga et al. 2001; Mohler 2001; Nieto et al. 1968; Norsworthy and Oliveira 2004; Weaver et al. 1992; Zimdahl 2004). Yield reductions are attributed to competition for one or more growth-limiting resources, such as light, water, CO_2, and mineral nutrients. Many weeds consume resources more quickly and grow more rapidly than do the crop (Deen et al. 2003; Li 1960). However, in most managed agricultural fields, there are sufficient resources during the first few weeks of the growing season to support both weed and crop growth (Kropff 1993; Norsworthy and Oliveira 2004). Although deficiencies can develop later in the season if continued demand for limited resources occurs, weed presence during the early developmental stages (within the CWFP), even if physically separated from the crop and subsequently removed, is known to reduce growth and yield (Liu et al. 2009; Rajcan et al. 2004). These observations suggest that competition for nutrients or water may not be the major mechanism of early season weed stress.

Light energy, both quantity and quality, is often cited as a primary environmental resource that drives weed–crop competition (Donald 1963; Zimdahl 2004). No reservoir of light exists in the soil, atmosphere, or plant, unlike other environmental resources, such as water, nutrients, or CO_2. A photon of light is either captured and converted to chemical energy through photosynthesis or is dissipated as heat (Patterson 1995). After the light is captured by a leaf, however, the spectral quality of the reemitted light in a plant canopy is altered and causes perceptible differences in the ratio of red to far-red (R : FR) light. The altered photon energies can be perceived at a limited distance and can affect plant growth and morphology and alter plant strategies for continued competition for light (Ballaré et al. 1990; De la Torre and Burkey 1990; Patterson 1995). The specific changes in R : FR ratios of reemitted light are often cited as the most important mechanism for early detection of neighboring plants and an important factor during the CWFP in plant–plant interactions (Ballaré et al. 1987; 1990; Kasperbauer and Karlen 1994; Merotto et al. 2009; Rajcan and Swanton 2001; Rajcan et al. 2004). However, during early season competition, weeds are not often directly competing for sunlight, and weed densities as low as 1 plant m^{-2} can reduce corn yield (Weaver 2001).

At present, quantitative physiological data that clarify the mechanisms involved in crop–weed interaction and the weed stress effect on gene expression or signaling pathways in crops, especially during the CWFP, is unknown. The object of this study was to examine the impact of N and light stresses individually to determine whether, at the whole-plant or molecular level, these stresses produced results similar to those of direct weed stress. Corn plants were subjected to season-long N stress and to weed and light stresses from emergence (VE) to the eight collar (V8)(~11-leaf tip) growth stage. Growth parameters at various growth stages, during and after stress and at end-of-season yield, and gene expression in leaf tissue at V8 were compared with the responses of nonstressed plants.

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Table 1. Herbicide treatments, rates, and application dates for weed control in corn, 2008 and 2009, Aurora, SD.

Treatment	Application timing	Date of application	Herbicide	Rate kg ai ha^{-1}
Weed free	PRE	May 9, 2008	*S*-Metolachlor	2.06
Weed free and V2 weed removal[a]	POST	June 5, 2008	Glyphosate	0.92
V4 weed removal	POST	June 17, 2008	Glyphosate	0.92
V6 weed removal	POST	June 26, 2008	Mesotrione + glyphosate	0.10 + 0.92
Grass control all plots	PRE	April 23, 2009	Acetochlor	2.68
Weed free	POST	May 11, 2009	Glyphosate	0.73
Weed free and V2 weed removal	POST	June 2, 2009	Carfentrazone + glyphosate	0.07 + 0.73
V4 removal	POST	June 19, 2009	Tembotrione + glyphosate	0.08 + 1.45
V6 weed removal	POST	June 26, 2009	Tembotrione + glyphosate	0.08 + 1.45
V8 weed removal	POST	June 2, 2009	Tembotrione + glyphosate	0.08 + 1.45

[a] After initial application for the vegetative stage, weed-free plots and plots from lower vegetative stages were hand-weeded and hoed as necessary to maintain weed-free conditions.

Materials and Methods

Field experiments were conducted in 2008 and 2009 at South Dakota State Research Farm about 15 miles east of Brookings, SD. The soil parent materials were loess over glacial outwash, and the soil series was Brandt silty clay loam (fine-silty, mixed, superactive, frigid Calcic Hapludolls)(https://soilseries.sc.egov.usda.gov/OSD_Docs/B/BRANDT.html; Clay et al. 2009). The Brandt soil has high water availability and is well drained (USDA-NRCS 2004). Field capacity (−0.03 MPa) and permanent wilting point (−1.5 MPa) of this soil are about 0.3 and 0.1 g g^{-1}, respectively.

The gravimetric water content at planting was about 23%, and soil pH averaged 6.3 (using 1 : 2 soil to 0.01 M $CaCl_2$ method). Soil N at the beginning of the season was 44.5 (2008) and 41 (2009) kg ha^{-1} (South Dakota State University Soil and Plant Tissue Testing Laboratory, Brookings, SD). A commercially available 97-d corn hybrid that had glyphosate-resistance and corn rootworm/corn borer stacked traits was planted on May 10, 2008, and April 28, 2009. The seeding rate was about 79,000 seeds ha^{-1}. Row spacing was 76 cm.

Treatments were weed-free corn with full N-rate application of 236 kg ha^{-1} (based on SDSU soil test recommendations for a yield goal of 13,000 kg ha^{-1})(nonstressed control), no N application (N stress), and corn grown under 40% black shade cloth (NTG Products, Erie, PA) and in competition with weeds—rapeseed (*Brassica napus* L.) in 2008 and velvetleaf (*Abutilon theophrasti* Medik.) in 2009—with removal at V2, V4, V6, and V8 stages of corn growth. The 40% commercially available shade cloth (PAR reduction verified using a line quantum sensor [LI-191 Line Quantum Sensor, Li-Cor Biosciences, Lincoln, NE]) that did not preferentially reduce any given wavelength of light (verified using CropScan [CropScan, Inc., Rochester, MN] equipped with blue, green, red, and near-infrared band sensors) was placed on frames at corn emergence (VE) and maintained 25 to 30 cm above the crop. Rapeseed was drilled 10-cm from the corn row at 7 kg ha^{-1} at corn planting. Velvetleaf developed from a seedbank that had been established for studies conducted in previous years (Horvath et al. 2006, 2007).

The experimental design was a randomized complete block with four replications. Plots, except shade, were 12-m long by eight rows wide (6 m). Shade plots were 3 by 3 m (3 m long by four rows). The nonstressed, N-stressed, and shaded plots were maintained weed-free for the entire season using herbicides (Table 1), with any surviving or newly emerged plants hand-pulled or hoed from the plots. Weed-stressed plots were maintained weed-free after the weed-removal timing using the same methods. All plots were irrigated as needed based on the checkbook method of irrigation management (Werner 1993) to limit water stress. Water was needed during tasseling (VT) in 2008 and 2009 and early grain fill (R2) in 2008 but not during earlier vegetative stages of growth. In 2008, the two water applications totaled 4.4 cm, and in 2009, a single application of 2.5 cm was applied.

In-Season Sampling. Corn growth stage was recorded using the leaf collar system in which the leaf after the cotyledon is counted as V1 (Agronomy Extension 2007; Evans et al. 2003a and b). When at least 90% of corn plants in the weed-free, high-N treatment were at the designated growth stage, sampling occurred for selected plots with measurements taken at V2, V4, V6, V8, and V12 (Table 2). Plant height to the uppermost leaf tip and leaf greenness (starting at V4) of the uppermost expanded leaf, using a SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan) were measured on 10 corn plants plot^{-1}.

Four corn plants at V2, V4, and V6, and two plants at V8 and V12, per plot were harvested from areas that had treatment influence but would not affect areas designated for yield measurements. Plants from the nonstressed and N stress treatments were sampled at each sampling time. Plants in V2 weed- and shade-removal treatments were collected at all sampling times, plants in V4, V6, and V8 weed-removal treatments were sampled starting at the corn vegetative stage when the stress was removed. Plants were separated into leaves and stems, with total leaf area measured using a leaf area meter (Li-Cor 3100 C, Li-Cor Biosciences). Total plant biomass dry weight was determined after drying at 60 C for 1 wk or until constant weight.

Weed density in weedy plots at the designated removal time was estimated by counting and averaging weeds in four 1-m row areas in 2008 and in four 0.10-m^2 quadrats in 2009. Weed biomass was determined by clipping weeds at the soil surface where densities were enumerated. The vegetation was combined, dried to constant weight at 60 C, and weighed.

Soil samples were taken at V8 and at physiological maturity from the 0- to 15-cm depth. The 15- to 60-cm depth was also sampled after harvest. Samples were dried at 38 C, and percentage of soil moisture was calculated. A 10-g subsample was analyzed for NO_3^- and NH_4^+ after extraction with 1.0 M KCl (Mulvaney 1996) using an Astoria nitrate/ammonia analyzer (Astoria-Pacific, Inc., Clackamas, OR).

Table 2. Corn physiological growth stage and corresponding sampling dates in 2008 and 2009, Aurora, SD.[a]

Corn growth stage	Sampling date	
	2008	2009
	Planted: May 10	Planted: April 28
VE	May 17	May 15
V2	June 4	June 1
V4	June 16	June 17
V6	June 26	June 25
V8	July 2	June 30
V12	July 14	July 15
Physiological maturity (harvest)	October 6	October 8

[a] Abbreviation: VE, emergence.

At corn physiological maturity, corn ears and stover were hand-harvested from 3-m sections of four middle rows for all plots, except in shade treatments, where 2-m sections of the middle two rows were harvested. The number of ears and plants was counted, and ears and stover from the harvest area were weighed. A 20-ear subsample per plot was weighed and dried at 60 C to constant weight, and grain was shelled from the ear and weighed. Grain weight was adjusted to 15.5% moisture content. A 20-plant subsample for stover was weighed and chopped, and a subsample was dried at 60 C to constant weight and weighed. Grain yield and stover biomass were calculated on a per-plant basis.

SAS version 9.1 (SAS Institute Inc., Cary, NC) was used for data analyses on leaf area and plant biomass. Class values were year, replication, and treatment. Treatments included nonstress, N stress, shade-stress removal (SSR) V2, SSR V4, SSR V6, SSR V8, and weed stress removal (WSR) V2, WSR V4, WSR V6, and WSR V8. PROC GLM/PROC MIXED test was used to determine whether measurable differences in corn growth occurred between years and among treatments. Fisher's Protected LSD tests were calculated at the 95% level for all treatments.

Sampling for Gene Expression Analyses. Leaf samples were collected at V8 each year in V8 weed removal, V8 shade removal, low nitrogen, and nonstressed control treatments. Samples were collected between 11:00 A.M. to 2:00 P.M. Each sample was composed of about 8 cm of the topmost fully expanded leaf of four plants per plot combined in one tube. Four sample replicates of each treatment were collected. The plant samples were frozen in liquid N immediately after removal from the plant and stored at −80 C until RNA extraction.

RNA Extraction, Hybridization, and Microarray Analysis. In 2008, microarray analysis was conducted on V8 stressed vs. nonstressed treatments. With a precooled, porcelain mortar and pestle, about 1 g of frozen leaf sample was homogenized in liquid N and finely ground to a talc-like powder. Total RNA was extracted using Trizol reagent and Superscript First-Strand Synthesis System (Invitrogen, Life Technologies, Inc., Carlsbad, CA) and purified using Qiagen RNeasy MinElute cleanup kit (Qiagen, Valencia, CA) following the manufacturer's protocol. First-strand complementary DNA (cDNA) synthesis was performed using 1,900 ng total RNA, and second-strand cDNA synthesis was performed using the resulting first-strand cDNA sample to make double-stranded cDNA using the Aminoallyl MessageAmp II kit (Ambion, Austin, TX). Amplified RNA (aRNA) was synthesized using the resulting double-stranded cDNA. Technical replicates from each treatment were labeled with Alexa Fluor 647 (Molecular Probes, Inc., Eugene, OR) or Alexa Fluor 555 dye. An Alexa Fluor 647-labeled sample from one treatment was mixed with an Alexa Fluor 555-labeled sample from another treatment. This mixture was hybridized to the 46,000-element microarray chip developed by the University of Arizona using their protocol (Gardiner et al. 2005). This procedure used three biological replicates (i.e., leaf tissue samples from three of the four field replications) and the two technical replicates (obtained by the dye swap procedure).

Microarray chips were washed according to the manufacturer's protocols. A rolling circle–balanced, dye-swap hybridization scheme (Churchill 2002) was used to compare gene expression among replicates of stressed and nonstressed treatments. Intensities based on fluorescence for each probe were visualized and quantified with a GenePix scanner and GenePix Pro software (MDS Analytical Technologies, Sunnyvale, CA). GeneMaths XT software (Applied Maths Inc., Gales Ferry, CT) was used to log-transform (log 2) the intensity readings and normalize the arrays against each other. Probes that had hybridization intensity less than 2 times the standard deviation plus the average of the negative controls were deleted (Horvath et al. 2007), and technical replicates for each probe were averaged to reduce any dye bias that existed. GeneMaths XT software was then used to identify P values based on ANOVA and individual *t* tests between treatments. Probes were considered differentially expressed if P values for any test were ≤ 0.05. False discovery rates for each probe were also determined, and the resulting *Q* values are reported (Supplemental Table 1). The raw data set can be downloaded from the Gene Expression Omnibus (Accession GSE29132).

Regulatory Network Analysis. Gene set enrichment analysis (GSEA)(Subramanian et al. 2005) and subnetwork analysis (Nikitin et al. 2003) were analyzed by Pathway Studio software 7.0 (Ariadne Genomics, Rockville, MD). Probable gene ontologies were based from most similar *Arabidopsis* homologue using BLASTX with an E-value cutoff of e^{-5}. Interactions, processes, and signaling networks were not reported if the enrichment did not pass $P < 0.05$ (Supplemental Tables 2a–c).

Real-Time PCR Assay and Analysis. In 2008 and 2009, real-time quantitative polymerase chain reaction (qPCR) assay and analysis were performed on selected genes of interest from the differentially expressed genes based on microarray analysis. The ubiquitin-conjugating enzyme was included as the endogenous control for each analysis. This gene was chosen because it showed minimal differential expression in all treatments based on hybridization in the microarray experiments. The same RNA samples used in microarray analysis were used for cDNAs synthesized with Invitrogen Superscript First-Strand Synthesis System.

Manufacturer's protocols were followed, using supplied Oligo (dT) primers and 5 μg total sample RNA for each 25-μl reaction. Primers were designed for select genes using Primer Express software (ABI 7900 PCR system, and SDS2.4 software, Applied Biosciences, Life Technologies, Inc., Foster City, CA). Quantitative PCR using Go Taq Promega Master Mix Kit (Promega, Madison, WI) was performed on a high-throughput

Table 3. Corn vegetative and yield responses to N, shade, and weed stress in 2008 and 2009 at Aurora, SD.

Treatment	Leaf area					Plant biomass					Harvest	
	V2	V4	V6	V8	V12	V2	V4	V6	V8	V12	Grain	Stover
	$cm^2\ plant^{-1}$					$g\ plant^{-1}$						
2008												
Nonstressed control	52	257	1,245	1,750	4,253	0.23	2.00	9.8	18.7	59.2	190	134
N stress	46	223	1,106	1,457	3,871	0.19*	1.56*	8.3	14.8	51.6	149*	110*
Shade removed V2	22*	124*	625*	969*	3,223*	0.11*	0.81*	4.4*	9.9*	39.0*	189	129
Shade removed V4		94*	591*	766*	2,943*		0.52	4.0*	6.8*	34.2*	181	141
Shade removed V6			463*	734*	2,536*			2.4*	6.4*	27.5*	176	121
Shade removed V8				656*	2,338*				5.6*	24.7*	168*	122
Weeds[a] removed V2	48	270	1,099	1,762	3,999	0.20*	1.81	9.9	18.8	52.5	183	133
Weeds removed V4		261	1,030	1,451	3,497		1.91	7.8	14.5	43.4	186	125
Weeds removed V6			686*	1,434	2,799*			5.2*	13.8*	35.7*	163*	96*
Weeds removed V8				970*	2,297*				9.0*	27.3*	153*	96*
$LSD_{(0.05)}$	7.5	58	282	476*	940	0.03	0.33	2.4	4.3	16.8	21	20
2009												
Nonstressed control	61	379	1,786	2,362	5,582	0.41	2.9	15.0	27.0	203	201	133
N stress	62	339	1,554	1,809	5,063	0.40	2.6	13.4	22.8	177	158*	113
Shade removed V2	28*	225*	1,203*	1,964	4,936	0.13*	1.3*	8.0*	21.4	165*	191	139
Shade removed V4		132*	736*	1,550*	4,643*		0.6*	5.6*	13.5*	145*	184	122
Shade removed V6			560*	1,121*	4,075*			3.0*	11.0*	128*	184	137
Shade removed V8				1,025*	3,959*				10.1*	115*	175*	118
Weeds[a] removed V2	41*	212*	1,247*	1,874	5,305	0.26*	1.6*	8.8*	19.3*	172	207	138
Weeds removed V4		267*	1,202*	1,442*	4,311*		1.7*	9.4*	15.1*	165*	188	126
Weeds removed V6			692*	1,104*	3,001*			5.0*	10.6*	105*	174*	99*
Weeds removed V8				1,161*	2,987*				10.7*	96*	154*	88*
$LSD_{(0.05)}$	14.8	95	455	596	881	0.13	0.64	4.5	5.9	37	21	29

[a] For the weed response in 2008, rapeseed was planted in rows 10 cm from the corn row at the time of corn planting. The weed in 2009 was velvetleaf that was indigenous to the field and was scattered throughout the plot.

* Significantly different from control at $P \leq 0.05$.

ABI 7900 PCR system following manufacturer's protocols (established protocols are found in the GoTaq qPCR Master Mix Technical Manual TM318 available online at: www.promega.com/tbs). Threshold values were determined with SDS2.4 software (Primer Express software, Applied Biosciences, Life Technologies). Samples were run in three replicates and averaged for data analysis.

The resulting threshold cycle (C_T) values were normalized to the average of ubiquitin for each sample, and relative quantification was conducted when PCR efficiency calculated by Equation 1:

$$(\text{Slope of the standard curve of the target gene}/\text{Slope of the reference gene}) \times 100 \quad [1]$$

The results of Equation 1 was between 95 and 105% or had an R^2 close to 0.99 (Livak and Schmittgen 2001), an indication that the efficiency of the target and reference genes were comparable. The fold-change method was used to compare differential gene expression among treatments, as shown in Equation 2:

$$\Delta\Delta C_T(\text{treated sample}) - \Delta C_T(\text{control sample}) \quad [2]$$

and the fold change equals Equation 3 (Livak and Schmittgen 2001):

$$\log 2^{-\Delta\Delta C_T} \quad [3]$$

Results and Discussion

Corn Growth and Yield Responses. Corn growth in nonstressed treatments differed between years. Shoot biomass was almost 50% greater at each sampling period in 2009 than it was in 2008 (Table 3), possibly because of differences in temperature and rainfall (data not shown). However, the stress effects to corn plants relative to nonstressed plants generally were similar.

Response to N Stress. Plants grown in the N-stress treatment had similar biomass but 20% less leaf area and 12% less N at V8 compared with measurements from nonstressed plants each year (Table 3). Total soil N content $\left(NO_3^- \text{ and } NH_4^+\right)$ was 73% lower in the N stress (total N – 18.5 μg g^{-1}) than it was in the nonstressed treatment (total N – 69 μg g^{-1}) at V8. By V12, N-stressed plants were about 12% shorter than nonstressed plants (data not shown). End-of-season grain yield and stover biomass were each reduced by about 22% each year compared with nonstressed plants (Table 3) with N content of grain and stover 17 and 32% lower, respectively, than the contents measured in nonstressed plants.

Response to Shade Stress. Shade stress resulted in nearly a 70% biomass loss at V8 in 2008 and a 59% loss in 2009, and the leaf area in both years was 58% less when compared with nonstressed plants. Shade stress also reduced plant height from 25 to 50% compared with plants grown under full light conditions (data not shown). These results agree with those reported by Clay et al. (2009). For the 40% shade treatment, the critical shade-free period, when yield losses were reduced from nonstressed plants, was V8 in both years. Grain yield and stover biomass were reduced by about 15 and 10%, respectively, compared with nonstressed plants. The N contents of leaves at V8, the grain, and the stover from all shade treatments were similar to the N contents measured in nonstressed plants.

Table 4. Weed biomass and density at vegetative corn sampling stages V2, V4, V6, and V8 in 2008 and 2009.[a]

Treatment	2008		2009	
	Weed biomass	Weed density	Weed biomass	Weed density
	g m of row $^{-1}$ (SEM)	plants m of row^{-1} (SEM)	g m^{-2} (SEM)	plants m^{-2} (SEM)
Weeds removed V2	6 (0.2)	119 (30)	52 (10)	760 (240)
Weeds removed V4	29 (7.0)	391 (60)	120 (10)	600 (35)
Weeds removed V6	98 (21)	320 (40)	214 (27)	570 (200)
Weeds removed V8	134 (10)	367 (40)	246 (34)	610 (70)

[a] For the weed response in 2008, rapeseed was planted in rows 10 cm from the corn row at the time of corn planting. The weed in 2009 was velvetleaf that was indigenous to the field and was scattered throughout the plot.

Response to Weed Stress. Weed species, density (Table 4), and plant distribution differed between years; however, weed pressure was high enough each year to cause stress to corn. Weed biomass per plant at V2 was similar between years (about 0.05 g plant^{-1})(Table 4) although rapeseed per meter of row averaged 119 plants and velvetleaf density average 760 plants m^{-2}. At V8, rapeseed averaged 0.36 g plant^{-1}, and velvetleaf averaged 0.40 g plant^{-1}. These weedy plants never overtopped corn and ranged from about 25 to 50% shorter than the weed-stressed corn (Moriles 2011).

At V4, corn grown with weeds until V4 had similar height, leaf area, and biomass as nonstressed V4 corn; however, at V6, even though the weeds were removed at V4, those plants were shorter and had less leaf area and biomass than the nonstressed corn had. When weeds remained until V6, height reductions of weed-stressed plants were observed. At V8, plants grown with weeds until V8 had about a 50% biomass reduction and a 53% leaf area reduction, which was similar to shade-stressed plants. Leaves had 20% lower N content, similar to N-stressed plants. Yield per plant and end-of-season stover losses occurred when weeds remained until V6, thus marking the beginning of the critical weed-free period. This indicates that even though climate conditions and weed species differed between years, the critical weed-free period remained constant. When weeds remained until V8, grain yield and stover biomass losses were 15 and 33% in 2008 and 2009, respectively. Grain and stover N contents from corn under weed stress until V8 in 2008 were similar ($P > 0.05$) to the nonstressed treatment and were reduced by about 10% in 2009.

Comparison of Corn Response to N, Shade, and Weed Stresses. In both 2008 and 2009, leaf area was greatly reduced by shade at V2, whereas leaf area was nearly twice as great under weed stress and, as noted above, only significantly different from the control in 2009. Likewise, a similar reduction in biomass was also observed for shade-stressed corn at V2 in both years. Yields were nearly equal to nonstressed corn, even when shade was allowed to remain until V8, whereas weed stress permanently reduced yields by V6.

These data suggest possible overlap but also clear differences in growth and yield parameter responses among weed, N, and shade stress. Clearly, by the V8 stage, weed stress morphologically resembled shade stress. However, the lack of shading of the corn by the weeds (data not shown) and the critical weed-free period beginning earlier (V6) than the critical shade-free period (V8 each year) suggest that mechanisms controlling yield losses from weed stress differ from those caused by shade stress. In addition, these results strongly suggest that competition for light is not a major factor during the critical weed-free period.

Mechanisms of corn–weed interaction are at least partially independent of competition for light or N. The lack of any differences in corn grown under N-limiting conditions (about 40 kg ha^{-1} [residual] in N-stress treatment vs. about 280 kg ha^{-1} [residual + fertilizer] in the nonstressed control) until harvest suggests that early season weed stress was independent of N competition. These observations are consistent with the results of Liu et al. (2009) that clearly demonstrate that weeds can negatively affect corn yield, even without direct competition for light or nutrients. However, the permanent effect of weeds and low light on corn development and the similar losses in specific growth characteristics suggest overlap in mechanisms between these stresses. Thus, a transcriptomics approach was undertaken to identify specific genes or signaling pathways that were affected by N, shade, and weed stresses.

Effect of Early Season Weed Stress on Gene Expression in Corn. Gene expression at V8 for corn experiencing stress due to rapeseed, shade, and low N was investigated using microarray chip data analysis, which provides information on ''global gene expression'' in any analyzed tissue. In these assays, 11,494 probes hybridized to greater than twofold the standard deviation of the background levels in at least two out of three biological samples from at least one of the four treatments (Supplemental Table 1). Expression levels of a gene were considered up-regulated or down-regulated compared with the control for the sampling period only if the hybridization intensity ratio was different at $P < 0.05$. ANOVA of the treatments indicated that 420 genes were differentially expressed.

Principle component analysis of the differentially expressed genes indicates that expression patterns between corn grown

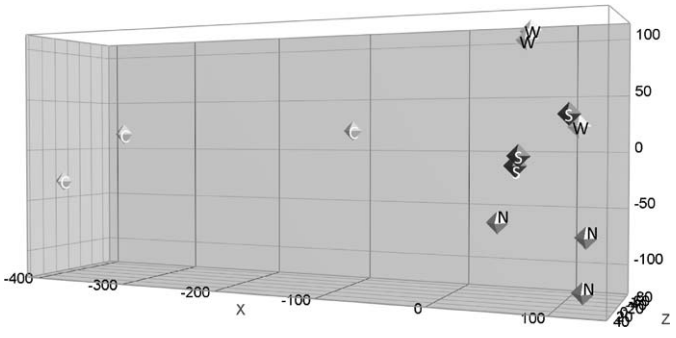

Figure 1. Principle component analysis of gene expression among genes with a significant differential expression as determined by ANOVA. Shapes marked with a C correspond to control samples, shapes marked with an N correspond to low-N samples, shapes marked with an S correspond to shaded samples, and shapes marked with a W correspond to weedy samples. Percentages of variation due to the x, y, and z axes are as indicated.

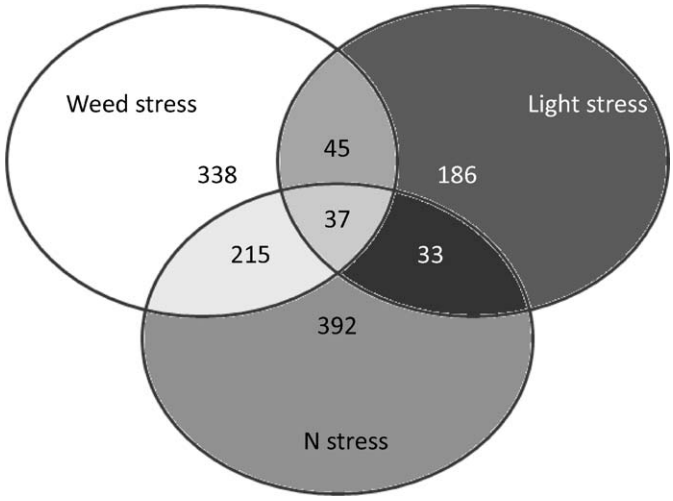

Figure 2. Venn diagram showing the number of genes differentially expressed in response to the weed, shade, and low-N stress as indicated.

under shade stress and weed stress were more similar to each other than they were to nonstressed or N-stressed corn (Figure 1). These data are consistent with the overlap in developmental responses to both shade and weed stress. Indeed, of the 635 genes differentially expressed under weed stress, 82 were shared with shaded plants (Figure 2). There were also many genes that were present in both weedy and N-stressed plants as well (252 genes), and 70 genes were often expressed in both shade-stressed and N-stressed plants. Thirty-seven genes were differentially expressed between the control and all three stresses. These observations demonstrate the robustness of the assay. Based on chance ($P < 0.05$), probably no more than 29 genes would be differentially expressed in any two treatments, and only one gene would be expected to be differentially expressed in all three treatments. In a direct comparison, 259 genes were differentially expressed between shade-stressed and weed-stressed corn, suggesting that the mechanisms responsible for these responses were not identical.

Gene Set and Subnetwork Enrichment Analysis. The data set was subjected to gene set and subnetwork enrichment analysis using the Pathway Studio 7.0 program (Supplemental Tables 2a–c). Analyses of the results indicate that there are several ontologies associated with all stresses (Table 5). These include the 13-LOX and 13-HPL pathway and the nutrient reservoir activity ontologies, which were up-regulated in response to stress, and a number of ontologies associated with light/photosynthesis, energy conversion, and signaling (transcription activity and protein kinase activity), which were all down-regulated in response to stress. These results suggest that all three stresses negatively affected photosynthetic processes. The negative effect of weeds on photosynthesis was also observed for corn during late-season weed stress (Horvath et al. 2006). This is in contrast to what was observed in a comparison between high-density and shaded corn, where shading appeared to induce some photosynthetic gene expression (Clay et al. 2009). However, in the earlier study, the ability to perform GSEA was not available, and it is possible that manual interpretation of the data was less accurate. The induction of *13-LOX* suggests there may be some effect on oxidative stress or jasmonic acid (JA) signaling

Table 5. Statistically significant ($P < 0.05$), overrepresented ontologies associated with the three stress responses as indicated by gene set and subnetwork enrichment analysis. The values indicate the median log 2 expression ratio between the treated and the control for all genes associated with the significantly overrepresented ontology. If there is no value indicated, then the ontology was not statistically significant for the stress indicated.

Ontology	Weed stress	Shade stress	N stress
13-LOX and 13-HPL pathway	1.1	1.3	1.3
Binding partners of photosystem I reaction center	−1.2	−1.4	−1.3
Chloroplast thylakoid membrane	−1.2	−1.2	−1.3
Gluconeogenesis	−1.3	−1.3	−1.4
Neighbors of heme catalase	−1.3	−1.2	−1.4
Nutrient reservoir activity	1.4	1.3	1.4
Photosynthesis	−1.3	−1.3	−1.3
Photosynthesis, light reaction	−1.3	−1.3	−1.3
Plastoglobule	−1.3	−1.4	−1.4
Protein kinase activity	−1.2	−1.1	−1.0
Response to light stimulus	−1.3	−1.2	−1.5
Transcription activator activity	−1.1	−1.2	−1.2
O-methyltransferase activity	−1.3	−1.4	
Poly(U)-binding	−1.1	−1.6	
Stomatal movement	−1.2	−1.3	
Apoplast	−1.3		−1.2
Calvin cycle	−1.3		−1.4
Expression targets of heme catalase	−1.3		−1.4
Phosphatases	−1.1		−1.1
Removal of superoxide radicals	−1.2		−1.6
Sucrose degradation to ethanol and lactate	−1.2		−1.3
Superpathway of starch degradation to pyruvate	−1.2		−1.2
Superpathway of sucrose degradation to pyruvate	−1.2		−1.2

because 13-LOX activity is required for JA biosynthesis (Wasternack 2007) in all three treatments.

Commonalities were observed among ontologies associated with sugar, starch, and lactate/ethanol degradation as well as pathways associated with other energy conversion processes in both weedy and N-stressed plants. Differential expression of genes involved in carbon use pathways are well known to be controlled under differing N levels (Coruzzi and Zhou 2001). Indeed, soil samples in 2008 revealed that NO_3^- was reduced by about 50%, compared with soil from nonstressed plots, when weeds remained until V8 (Moriles 2011). It is unclear whether weed stress is acting through the same signaling mechanisms as N stress on these systems or if signaling cross talk was responsible for those similarities.

Although shade and weed stress clustered most tightly together, only three ontologies were uniquely shared by those stresses. These were O-METHYLTRANSFERASE activity, POLY(U)-BINDING activity, and stomatal movement. O-METHYLTRANSFERASE activity is associated with lignification processes (Boerjan et al. 2003) and was down-regulated in both high-density and shaded corn in an independent experiment (Clay et al. 2009). Stomatal movement suggests some response involving water relations, and POLY(U)-BINDING suggests altered regulation of posttranscriptional gene regulation.

Numerous ontologies were unique to each of the individual stresses (26 for weedy, 29 for shaded, and 42 for low nitrogen; for a full list, see Supplemental Table 3). Notable differences in response to weeds include ontologies associated with several meristem identity genes, such as *AP1 (APETALA1)*, *AFO (ABNORMAL FLORAL ORGAN*), and *ANT* (*AINTEGUMENTA*). Likewise ontologies associated with growth

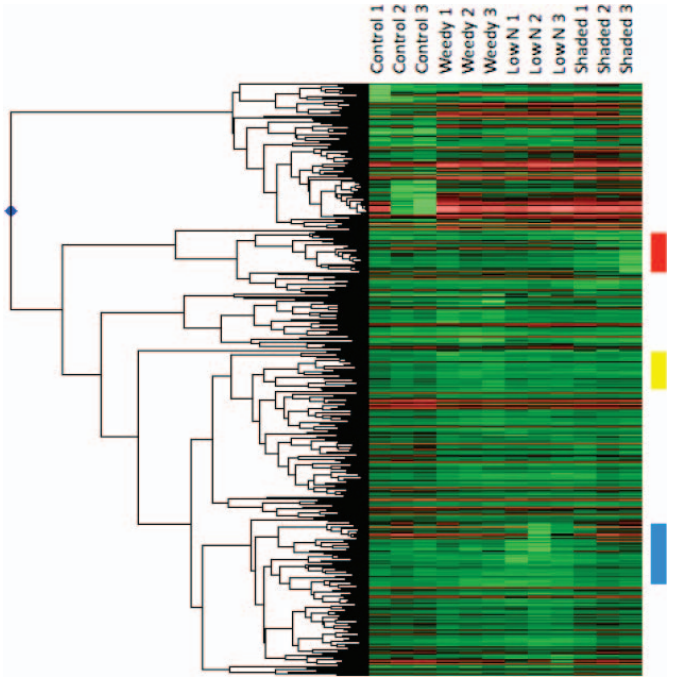

Figure 3. Cluster analysis of genes with a significant differential expression as determined by ANOVA. Intensity of expression is indicated by color with the lightest green being low expression and bright pink being most highly expressed genes. Black indicates a moderate level of gene expression. The bars indicate the clusters of genes with low expression in weedy (yellow), shaded (red), or low-N (blue) stress.

regulating systems, such as CYTOKININS-*O*-GLUCOSIDE biosynthesis (cytokinin sequestration), binding partners of COI1 (CORONATINE INSENSITIVE 1, involved in JA signaling), binding partners of TIR1 (TRANSPORT INHIBITOR RESPONSE 1, involved in auxin signaling), and binding partners of ASK1 (*Arabidopsis* SERINE/THREONINE KINASE 1, involved in cell-division regulation) were also noted to be overrepresented in the weed-stressed corn. These data indicate possible targets of the signals generated by weeds. Likewise, shade primarily influenced regulation of circadian responses, as indicated by ontologies associated with known circadian and light regulatory genes, such as expression targets of CCA1 (CIRCADIAN CLOCK ASSOCIATED 1), expression targets of LHY (LATE ELONGATED HYPOCOTYL), neighbors of TOC1 (TIMING OF CHLOROPHYLL A/B-BINDING APOPROTEIN CP24 PRECURSOR [CAB] EXPRESSION 1), and binding partners of COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1). These observations would be consistent with a shade response. The lack of these signals in the weedy corn suggests a different mechanism for shade and weed effects on corn. N stress, unsurprisingly, specifically affected genes with ontologies associated with N use, such as 4-aminobutyrate degradation I, alanine biosynthesis I, alanine degradation, and neighbors of ammonium ion. Interestingly, genes with ontologies associated with sugar metabolism and energy use were also noted such as superpathway of glycolysis, pyruvate dehydrogenase, superpathway of acetyl-COA biosynthesis, and malate dehydrogenase activity. As noted above, there has long been a known association between N and C signaling (Coruzzi and Zhou 2001).

Cluster Analysis and Identification of Coordinately Expressed Genes. Microarray analysis opens the opportunity to identify conserved binding sites for specific transcription factors that regulate clusters of coordinately regulated genes. This information could lead to the identification of signaling processes through which weeds or other stresses affect corn growth and development. This could result in development of compounds or procedures that may be used to make corn blind to weeds. Genes that were considered differentially expressed in response to any of the three stresses were identified and subjected to cluster analysis (Figure 3; details of the expression analysis are available in Supplemental Table 1). A cluster of 37 coordinately regulated genes that were preferentially down-regulated in response to weeds was identified (yellow bar, Figure 3). Interestingly, this cluster is rich in stress-related and regulatory genes. Likewise, a cluster of genes that were coordinately down-regulated under conditions of N stress (blue bar) was characterized by several genes with known function in oxidative stress responses. The coordinately down-regulated cluster of genes in shaded corn (red bar) did not appear to have any obvious linkage to known processes. However, before promoter analysis is warranted, the expression pattern of these genes will need to be confirmed.

Specific Gene Expression Using qPCR Analysis. A few individual genes of interest were quantified and compared between the V8 control and stress treatments that remained until the V8 sampling using qPCR analysis to confirm differences in gene expression observed in microarray data (Table 6). Some of the specific genes selected for qPCR that were confirmed as down-regulated in microarray analysis of weed, shade, and N stressed corn included *FRUCTOSE 1,6-BISPHOSPHATASE*; *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE*, *CYTOSOLIC* 2; *PHOTOSYSTEM II STABILITY/ASSEMBLY FACTOR HCF 136; PHOTOSYSTEM II OE 17*; *PHOTOSYSTEM II 10K PROTEIN*; *CIRCADIAN OSCILLATOR COMPONENT*; and *CHLOROPLAST 50S RIBOSOMAL PROTEIN L31. DEHYDRATION-RESPONSIVE PROTEIN RD22 PRECURSOR* was confirmed as up-regulated in all three stress treatments (Table 6). Confirmed genes in weedy and N-stressed treatments only included *GLYCOSYLTRANSFERASE FAMILY 8*; and *PHOTOSYSTEM I REACTION CENTER SUBUNIT V*. Down-regulation of the *PHOTOSYSTEM I REACTION CENTER SUBUNIT VI* and *PHOTOSYSTEM I REACTION CENTER SUBUNIT N* genes were confirmed for the shade and low N stresses only. *RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN* and one of the family members of *CHLOROPHYLL A/B-BINDING APOPROTEIN CP24 PRECURSOR (CAB)* were down-regulated only in weed-stressed corn. Only a few of the selected genes were up-regulation in the weed-stressed corn at V8 and included a different family member of *CAB*, *SENESCENCE-ASSOCIATED PROTEIN*, and *GLYCOSYLTRANSFERASE FAMILY 8* (data not shown). Similar regulation patterns were confirmed for genes in 2009, when corn was competing with velvetleaf (data not shown). Some of these same genes were down-regulated in corn competing with velvetleaf until V12 in previously reported studies (Horvath et al. 2006).

In gene-subset analysis (above), photosynthesis was affected by weed, shade, and N stress. The qPCR data confirm these results because the selected genes were, for the most part, crucial for photosynthesis activity. The down-regulation of these genes confirms that weed, N, and shade stress limit

Table 6. Fold differences in gene expression between non-stressed corn (control) and weed (canola)-stressed corn in 2008. Negative values indicate down-regulation and positive values indicate up-regulation.

		Array log ratio treated over control			qRT-PCR log ratio treated over control		
Gene function	Maize ID	weedy	shaded	low N	weedy	shaded	low N
chlorophyll a-b binding protein CP24	MZ00057327	−0.567	−0.303	−0.408	−0.769	−1.415	−1.921
fructose-1,6-bisphosphatase	MZ00017229	−0.376	−0.991	−0.816	−0.644	−0.074	−0.377
Snf1-related protein kinase	MZ00017125	−0.386	−0.493	−0.759	1.039	−3.366	−2.911
chlorophyll a/b-binding apoprotein	MZ00036465	−0.706	−0.300	−0.850	1.704	−3.012	−2.358
unknown protein	MZ00022737	−0.519	−0.353	−0.576	−0.269	−0.105	−0.269
Nuclear-encoded plastid gene	MZ00047899	−0.714	−0.433	−0.719	−0.120	0.202	0.322
chloroplast 50S ribosomal protein L31	MZ00014452	−0.734	−0.137	−0.656	−0.535	−0.761	−1.120
dehydration-responsive protein RD22	MZ00035862	1.476	1.259	0.856	0.856	0.444	1.753
thioredoxin h-like protein	MZ00025932	−0.473	−0.375	−0.433	0.189	−0.358	−0.184
circian oscillator component	MZ00014271	−0.412	−0.606	−0.534	−0.186	−2.556	−2.582
Photosystem I reaction center subunit VI	MZ00014709	−0.497	−0.206	−0.699	0.541	−1.355	−0.715
Photosystem II stability/assembly factor HCF136	MZ00031900	−0.546	−0.104	−0.529	−2.427	−3.626	−3.211
photosystem II 10K protein	MZ00036116	−0.092	−0.021	−0.175	−1.047	−3.943	−6.381
Photosystem I reaction centre subunit N	MZ00041292	−0.594	−0.299	−0.631	0.073	−1.029	−1.742
SET domain protein SDG111	MZ00015659	−0.319	−0.133	−0.598	3.385	2.696	3.586

photosynthetic capabilities of the plant. This ultimately caused yield losses and smaller plants (Table 3).

The exact signaling mechanism by which the corn plant perceives weed stress has not been elucidated in this study. However, based on both morphologic and genomic observations, results suggest that the responses of corn to shade, N, and weed stress are generally regulated by different mechanisms, although there is likely some overlap in down-stream physiological responses that are manifested in plant development, yield, and gene expression patterns.

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