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# Landscape Features Impact on Soil Available Water, Corn Biomass, and Gene Expression during the Late Vegetative Stage

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# Landscape Features Impact on Soil Available Water, Corn Biomass, and Gene Expression during the Late Vegetative Stage

Stephanie Hansen, Sharon A. Clay, David E. Clay, C. Gregg Carlson, Graig Reicks, Youssef Jarachi, and David Horvath\*

## Abstract

Crop yields at summit positions of rolling landscapes often are lower than backslope yields. The differences in plant response may be the result of many different factors. We examined corn (*Zea mays* L.) plant productivity, gene expression, soil water, and nutrient availability in two landscape positions located in historically high (backslope) and moderate (summit and shoulder) yielding zones to gain insight into plant response differences. Growth characteristics, gene expression, and soil parameters (water and N and P content) were determined at the V12 growth stage of corn. At tassel, plant biomass, N content,  $^{13}\text{C}$  isotope discrimination ( $\Delta$ ), and soil water was measured. Soil water was 35% lower in the summit and shoulder compared with the lower backslope plots. Plants at the summit had 16% less leaf area, biomass, and N and P uptake at V12 and 30% less biomass at tassel compared with plants from the lower backslope. Transcriptome analysis at V12 indicated that summit and shoulder-grown plants had 496 downregulated and 341 upregulated genes compared with backslope-grown plants. Gene set and subnetwork enrichment analyses indicated alterations in growth and circadian response and lowered nutrient uptake, wound recovery, pest resistance, and photosynthetic capacity in summit and shoulder-grown plants. Reducing plant populations, to lessen demands on available soil water, and applying pesticides, to limit biotic stress, may ameliorate negative water stress responses.

**I**N ANY GIVEN YEAR or landscape position, yield can be limited by multiple reasons including both resource-dependent factors (e.g., light, water, nutrients) and resource-independent factors (e.g., downregulation of specific genes). Understanding the relationships among factors is critical for implementing appropriate farming practices that increase profitability and reduce agricultural impacts on the environment. Information collected using yield monitors, grid soil sampling, and remote sensing can help identify problem areas. However, this information often does not adequately characterize a specific yield limiting factor or factors. In addition, statistical analysis of measured soil and plant parameters are poorly correlated and may be attributed to collecting data at inappropriate scales or times, inaccurate recommendation algorithms, inability to accurately measure plant responses to stress, not fully understanding the complexity of the system, or a combination of these factors. In the field, biotic and abiotic stresses impact gene expression simultaneously. An improved understanding of gene expression of field-grown plants is needed to develop better adaptive management practices to improve plant growth and, ultimately, yield. Molecular biology techniques, such as transcriptome analysis, may provide clues to resolve these issues.

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**Abbreviations:** ABA, abscisic acid; AM, arbuscular mycorrhizae; aRNA, amplified RNA; cDNA, complementary DNA; Ct, cycle threshold; JA, jasmonic acid; PCR, polymerase chain reaction; qPCR, real-time polymerase chain reaction; RNA, ribonucleic acid.

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The influence of stress on physiological and transcriptomic responses in higher plants and corn have been investigated in many studies (Reddy et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2007; Valliyodan and Nguyen, 2006). However, most studies have focused on seedlings, embryos, or plants grown under controlled conditions with application of a single stress or controlled well-orchestrated stress combinations (Fernandes et al., 2008; Yu and Setter, 2003; Zheng et al., 2004, 2010). This study examines field stress responses in an uncontrolled environment.

In whole field studies conducted at this dryland research site, many different tools including remote sensing, modeling, targeted experiments, and  $^{13}\text{C}$  isotopic discrimination have been used to assess the causes of crop yield variation within the site (Mishra et al., 2008, Clay et al., 2003; Kharel et al., 2011). Although different field positions can have different soil compositions and nutrient profiles, based on previous research, it has been shown that water availability is a critical factor that influences yield in this dryland system (Clay et al., 2001; Mishra et al., 2008; Kharel et al., 2011). For example, in a targeted field study, Mishra et al. (2008) showed that by adding about 2.5 cm of water per week from mid July to mid August (total approximately 12.5 cm) to corn plants in the summit and shoulder areas of this field, grain yield increased from 87 to 145 g per plant in a year with approximately 22 cm of rain from May through August (long-term average rainfall 34 cm). However, adding water to corn in foot and backslope positions did not increase yield. Likewise, soybean [*Glycine max* (L.) Merr.] growing in the summit and shoulder positions responded to water addition, with grain yields increasing from 4.7 to 7.2 g per plant when water was added in a dry year (Clay et al., 2003). Due to changes in  $^{13}\text{C}$  isotopic discrimination, the landscape differences in Mishra et al. (2008) and Clay et al. (2003) were attributed to water stress. However, these studies did not mechanistically define how water stress reduced yield or what other process were affected (Kim et al., 2008; Kharel et al., 2011).

When plants are water stressed, stomates close and  $\text{C}_3$  becomes limiting. Many plants engage mechanisms that limit photosynthesis to protect cells from detrimental effects of free  $\text{O}_2$  (Chaves et al., 2009). Plants with reduced photosynthesis also have downregulation of genes related to N uptake and use, with numerous reports linking C and N status within plants (Foyer et al., 1998; Hirel et al., 2001; Li et al., 2003; Noctor et al., 2004). In addition, water stressed plants often have an increase in proline biosynthesis, carbohydrate accumulation in the plant tissues (Valliyodan and Nguyen, 2006), and abscisic acid (ABA) signaling that induces drought-responsive transcription factors such as dehydration-responsive element or C-repeat binding factors (DREB or CBF) (Shinozaki and Yamaguchi-Shinozaki, 2007). Other plant responses to water stress include increased root growth, slowed shoot growth, and increased susceptibility to pests and diseases, which may result in decreased production of  $\text{C}_6\text{H}_4(\text{OH})\text{COOH}$  (salicylic

acid), jasmonic acid (JA), and pathogenic proteins (Loake and Grant, 2007; Howe and Jander, 2008).

Studies of corn grown under field conditions, such as this one, could identify patterns of gene expression not previously observed or expected—but nonetheless associated with recognized physiological processes. If the likely responses can be anticipated, adaptive management techniques could be used to buffer plant stress responses. Preventative seed treatments, increased fertilizer rates, or pesticide applications could be targeted to specific landscape positions where plants are likely to develop a reduced capacity to respond to pathogen, nutrient, insect, or other stresses. The objective of this study was to compare gene expression of corn growing in moderate and high yielding landscape positions. Because rainfall was minimal during the study period, plants in water-stressed vs. adequate water conditions could be visualized easily for sampling comparison. A transcriptomics approach was used to identify genomic indicators of the stresses induced by landscape variation.

## Materials and Methods

### Field and Yield History

This research was conducted in a 65 ha corn field located in eastern South Dakota with latitude and longitude values of  $44^{\circ}10' \text{ N}$  and  $96^{\circ}37' \text{ W}$  and elevations ranging from 518 to 534 m. The soil texture at both sites was silty clay loams. The soil series in the lower backslope was a Wauby (fine-silty, mixed, superactive, frigid pachic hapludolls) whereas the soil series in summit and shoulder was a Vienna (fine-loamy, mixed, superactive, frigid calcic hapludolls). Soils in the lower backslope and summit and shoulder areas had organic C contents of 16.4 and 13.5 g C  $\text{kg}^{-1}$ , respectively. This field has been grid soil sampled at various times in the past and nutrient status information for the site has been reported in Clay et al. (2001, 2003) and Chang et al. (2004). Previous studies conducted in this field have reported that differential water stress was a major cause of yield variability across landscape positions (Clay et al., 2001, 2003; Mishra et al., 2008).

Based on 5 yr (2002–2007) of yield monitor information collected before this study, areas that routinely produced high and moderate yields were identified (Kleinjan et al., 2007). The lower backslope landscape position averaged 9920 kg corn grain  $\text{ha}^{-1}$  (SD = 1920 kg grain  $\text{ha}^{-1}$ ) whereas the summit and shoulder position averaged 8020 kg grain  $\text{ha}^{-1}$  (SD = 828 kg  $\text{ha}^{-1}$ ).

Fertilizers were applied as broadcast applications in the fall 2007 and spring 2008 based on soil test recommendations. The fall application consisted of 20 kg N  $\text{ha}^{-1}$ , 23 kg P  $\text{ha}^{-1}$  (51.4 kg  $\text{P}_2\text{O}_5$ ), and 36 kg K  $\text{ha}^{-1}$  (44.7 kg  $\text{K}_2\text{O}$ ). A 102 kg N  $\text{ha}^{-1}$  rate was surface broadcast in spring 2008. A commercially available genetically modified 97-d corn hybrid that contained glyphosate resistance and Western corn rootworm (*Diabrotica virgifera virgifera*) and/or European corn borer (*Ostrinia nubilalis*) resistance stacked traits was planted in 76-cm

rows on 16 May 2008. Five individual plot areas, four rows wide by 5 m long, were chosen and identified in one high yielding zone and two moderate yielding zones.

### Plant and Soil Sampling

At V12 (plants with about 15 leaf tips) (18 July), soil samples from study locations were collected from the 0- to 15- and 15- to 60-cm depths. Each soil sample consisted of 10 individual 0.7-cm diameter cores that were combined. A 10-g subsample was weighed, air dried, and reweighed to calculate soil water content. The remaining soil was air dried, ground, and analyzed for total N, total C, NH<sub>3</sub> (ammonia)-N, NO<sub>3</sub><sup>-</sup> (nitrate) N, Olsen-P, and K using standard techniques (Chang et al., 2004; Whitney, 1998; Frank et al., 1998).

On the same day, plant populations were determined by counting plants along 5-m of three plant rows and averaging. Five leaf tips, 8 cm long, from the most recently emerged leaves were collected from the five replicates per area and immediately placed in liquid N and stored at -80°C for ribonucleic acid (RNA) transcriptome analysis (see subsequently in the manuscript and Clay et al., 2009). Ten plants (two per area) were collected from the high yielding zone and 20 plants were collected from moderate yielding zones. Leaf area per plant was measured using a leaf area meter (Li-Cor 3100 C; Li-Cor Biosciences) and averaged by zone. Plants were dried at 60°C to constant weight, dry weights were measured, and average plant dry weight calculated (Clay et al., 2009).

On 6 August when corn was at the VT growth stage, soil samples were taken as described previously to determine soil water content. Plants in a 3.47 m<sup>2</sup> area were counted in each plot and total plant biomass was removed by cutting plants in the area at soil level. These plants were dried at 60°C to constant weight, weighed, and biomass per plant calculated.

Due to dry conditions (32.5 cm of rainfall from planting to VT), the 65 ha field was harvested for silage on 31 August, so that grain yield could not be determined. The growing degree days (base 10°C) from planting to silage harvest totaled 1018.

Dry plant samples from the V12 and VT growth stages were ground and analyzed for total P, N, δ<sup>13</sup>C, and δ<sup>15</sup>N (Clay et al., 2001, 2003). The data from the two moderate yielding zones were similar and these data were combined for analysis and compared with data from the high yielding zone. Differences were considered significant between parameter means when paired *t* test analysis had a *P*-value ≤ 0.10.

### Ribonucleic Acid Extraction and Microarray Analysis

Three of the five biological replicates of leaf tissue at V12 collected from the high yield zone, and 6 of the 10 biological replicates collected from the medium yield zones were used for gene expression analysis. One gram of frozen leaf material per replicate was finely ground to a talc-like

powder in liquid N using a precooled porcelain mortar and pestle. Total RNA was extracted using Trizol reagent (Invitrogen) and purified using a Qiagen RNeasy Min-Elute cleanup kit (Qiagen), following the manufacturer's protocol. First-strand complementary DNA (cDNA) synthesis was performed using 1900 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 Message Amp II kit (Ambion). Amplified RNA (aRNA) was synthesized using the resulting double-stranded cDNA, and technical replicates from each treatment were then labeled with Alexa Fluor 647 (Invitrogen) or Alexa Fluor 555 (Invitrogen) dye. An Alexa Fluor 647-labeled sample from one yield zone was mixed with an Alexa Fluor 555-labeled sample from another zone and the mixture hybridized to a 46,000-element microarray chip developed by the University of Arizona using their protocol (Gardiner et al., 2005). A full description of the array layout and design is available on the NCBI Gene Expression Omnibus (series accession number GSE33494). Microarray chips were washed according to the manufacturer's protocols.

Within each biological replicate, a rolling circle balanced dye swap hybridization scheme (Churchill, 2002) was used to quantify gene expression among replicates of the medium and high yield zones. A detailed explanation of the hybridization scheme and experimental design is stored in the NCBI Gene Expression Omnibus (series accession number GSE33494) and the samples that were hybridized to each array are listed in Supplemental Table S1). The amount of aRNA from different treatments hybridizing to each probe was visualized by fluorescence intensities and quantified with a GenePix scanner (Molecular Devices) and GenePix Pro software (Molecular Devices). Individual sample hybridizations were treated as independent events for analysis. GeneMaths XT software (Applied Maths, 2010) was used to log transform (log 2) the intensity readings and normalize all arrays against each other. Probes that had hybridization intensity less than two times the standard deviation plus the average of the nonplant and blank-spot negative controls from both treatment groups were deleted (Horvath et al., 2006, 2007) and technical replicates for each probe were averaged to reduce any dye bias that existed. The MA plots (intensity ratio: *y* axis and average intensity: *x* axis) following normalization confirmed excellent quality and appropriate normalization (data not shown). Within-array LOWESS normalization was tested; however, it did not significantly alter the number of differentially expressed genes nor impact the gene ontology assessments. GeneMaths XT software was then used to identify *P*-values based on individual *t* tests between zones. 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 (the minimum false discovery rate at which the test may be called significant) are reported.



## Regulatory Network Analysis

The gene network of differentially expressed genes was analyzed by Pathway Studio software 8.0 (Ariadne Genomics, 2012) (Nitikin et al., 2003). Probable gene ontologies were based on most similar arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] homolog using BlastX (Altschul et al., 1990) with a cut off of  $E \leq 10^{-5}$ . The regulatory network was constructed by gene set enrichment analysis, based on microarray data to examine interactions and signaling among promoter binding, expression, regulation, and binding (Subramanian et al., 2005). False interactions and signal genes without interaction were removed if the pathway did not pass  $P \leq 0.05$ .

## Validation of Microarray Finding using Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (qPCR) assay and analyses 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 controls for each analysis. This gene was chosen because it showed minimal differential expression in all treatments based on hybridization in the microarray experiments. Using the same RNA samples used in microarray analysis, cDNA was synthesized using Invitrogen Superscript First-Strand Synthesis System (Invitrogen).

Manufacturer's protocols were followed, using supplied Oligo (dT) primers and 5  $\mu\text{g}$  total sample RNA for each 25  $\mu\text{L}$  reaction. Primers were designed for select genes using Primer Express software (Applied Biosystems, 2004). Quantitative polymerase chain reaction (PCR) using Go Taq Promega Master Mix Kit (Promega Corp.) was performed on high throughput ABI 7900 PCR system (Applied Biosystems) following manufacturer's protocols (established protocols are found in the GoTaq qPCR Master Mix Technical Manual #TM318 [Promega Corporation, 2011]). Threshold values were determined with SDS2.4 software (Applied Biosystems, 2010). Samples were run in three replicates and averaged for data analysis.

The resulting cycle threshold (Ct) values were normalized to the average of an ubiquitin gene and relative quantification was conducted when PCR efficiency calculated by

$$\frac{\text{slope of the standard curve of the target gene/}}{\text{slope of the reference gene}} \times 100$$

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  $\Delta\Delta\text{Ct}$  method in which

$$\frac{\text{target gene } \Delta\text{cycle threshold (Ct)} - \text{control gene } \Delta\text{Ct, in which } \Delta\text{Ct} = \text{target gene Ct} - \text{reference gene Ct}}{\text{reference gene Ct}}$$

was used to compare differential gene expression among treatments (Livak and Schmittgen, 2001).

## Results and Discussion

### Field Results

From planting until V12 plant sampling on 18 July, rainfall totaled 19.5 cm; however, during the 35 d before this sampling, the area received only 5.3 cm of rain. The inorganic soil N on 18 July averaged 72 kg N ha<sup>-1</sup> in both zones. Although both soil types have similar water holding capacities, the soil water content was 17 cm in the high yielding zone (Table 1) whereas soil water content in the moderate yielding zone (shoulder and summit) averaged 11 cm, 35% lower. These data confirm that differential water availability across this field landscape in 2008 was similar to that previously reported (Mishra et al., 2008). Other research conducted at this site also indicated that corn and soybean yield losses due to differential water variability could be recovered by applying water to plants growing in lower yielding zones (Clay et al., 2003; Mishra et al., 2008). However, Clay et al. (2003) and Mishra et al. (2008) did not identify the impact of the water on the ability of the plant to use soil nutrients or respond to other stresses.

Soil P differed between zones. The moderate zone had twice as much P in the 0- to 15-cm depth (Table 1) and tended to be higher in the 15- to 60-cm depth ( $P = 0.18$ ). Based on soil P calibration work in the region, the P content in the moderate yield zone was in the medium range and the P content in the high yield zone was in the low range. Although 23 kg P ha<sup>-1</sup> was broadcast applied the previous fall, the low P soil values suggest that adding a higher amount of P fertilizer would have increased yields in both zones. The whole plant P concentrations at V12 averaged 1.9 g kg<sup>-1</sup> in both zones (data not shown), lower than the reported critical P level of 2.2 g kg<sup>-1</sup> in whole plant samples when collected at early vegetative growth stages (Rehm et al., 1983) and 2.0 to 2.5 in the ear leaf at silking (Schulte and Kelling, 1991). Because the plant P concentration and soil P values were both low, it is likely that plant growth was P limited. A comparison between the soil and plant P values suggests that there was a disconnect between the soil content and plant uptake values. If soil test P controlled plant P, then P concentration and uptake should have been higher in the moderate than the high yield zone as soil P was greater in the moderate areas (Table 1), yet this was not the case. Plant rooting depth and density were not measured in this study but may have differed between locations enough to influence P uptake and tissue concentrations. In addition, arbuscular mycorrhizae (AM) colonization often facilitates P uptake in soils that have low to moderate P levels. We did not examine roots for AM; however, based on previous reported findings (Liu et al., 2000) the N and P inputs applied to this field would not have been expected to inhibit colonization, if AM were present.

At the V12 growth stage, plant populations in the two zones were similar (Table 1). Growth rates of plants were 15% lower in the moderate yield zone (0.81 g d<sup>-1</sup>) compared with the high yield zone (0.96 g d<sup>-1</sup>). Corn

**Table 1. Soil water and P, plant population, biomass, leaf area, P and N content, and  $\delta^{15}\text{N}$  and  $^{13}\text{C}\%$  of tissue taken from the historically high yielding and moderate yielding zones of the Moody Co., SD field.**

Yield zone and growth stage	Soil (0- to 15-cm)		Plant						
	Water	P	Population	Biomass	Leaf area	P content	N content	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$
V12	cm	$\mu\text{g g}^{-1}$	plant $\text{m}^{-1}$ row	g per plant	cm per plant	$\text{kg ha}^{-1}$	$\text{g kg}^{-1}$	$\%$	$\%$
High (lower backslope)	17.0	4.0	10.7	60.6	2466	9.2	0.28	1.44	-11.83
Moderate (summit and shoulder)	11.0	8.5	10.9	50.9	2086	8.6	0.23	3.45	-12.31
<i>P</i> -value		0.0027		0.0136	0.01258	0.08	0.025	0.06	0.0016
VT (tassel)									
High	16.9			195			0.17	3.38	-11.72
Moderate	11.0			138			0.14	4.15	-11.96
<i>P</i> -value				0.00065			0.004	0.023	0.0045

growing in the moderate yield zone also had 15% less leaf area and 16% less biomass per plant compared with corn growing in the high yield zone (Table 1). Plants collected from the moderate yield zone had lower plant N concentrations, 33% lower total N per plant, but higher  $\delta^{15}\text{N}$  values than plants collected in the high yield zone (Table 1). Higher  $\delta^{15}\text{N}$  value was attributed to an increased uptake of soil derived N in the moderate yielding zone compared with more fertilizer uptake (fertilizer  $\delta^{15}\text{N}$  value typically 0 or -1) in the high yielding zone. The  $\delta^{13}\text{C}$  value in the moderate yield zone was more negative compared with the high yielding zone. This would indicate greater stomatal closure and more reliance on  $\text{CO}_2$  trapped in the stomata for photosynthesis than with atmospheric  $\text{CO}_2$ . These data suggest that plants in the moderate yield zone were under more stress (Clay et al., 2009).

From V12 to VT, plant growth rates were  $7.1 \text{ g d}^{-1}$  in the high yield zone and  $4.6 \text{ g d}^{-1}$  in the moderate yield zone, a 35% slower growth rate. Total biomass per plant at VT was 29% lower in the moderate compared with high yield zone (Table 1). There was a decrease in the concentration of plant N from V12 to VT in both zones, with total N per plant 42% lower in the moderate yield zone plants compared with the high yield zone plants. These decreases were attributed to a drier soil, which reduced the plants ability to use inorganic N (Kim et al., 2008). The  $\delta^{15}\text{N}$  values were greater in VT tissue from both zones compared with V12 indicating more reliance on mineralized N rather than fertilizer N source. The  $\delta^{13}\text{C}$  was lower in the moderate yield than the high yield zone indicating more stomatal closure and greater plant stress.

### Transcriptome Analysis

Plants growing in the moderate yield zone had 37 genes that were differentially expressed ( $P < 0.05$ ) when compared with gene expression of plants grown in the high yield zone. The entire dataset has been deposited in the Gene Expression Omnibus database (accession #GSE33494) with selected pathways of interest reported in Table 2 and Supplemental Table S1. Specific genes selected to confirm differential expression from microarray analysis using reverse transcription PCR are reported in Table 3.

Eleven of the 12 selected genes showed similar expression levels when compared across the two methods. Therefore, the differences observed between plants grown in different zones were reliable and not an analysis artifact.

Several different gene ontologies and gene regulatory networks were impacted based on yield zone (Table 2). These included genes involved in (i) abiotic stress (processes associated with environmental stress such as cold and drought stress), (ii) biotic stress (processes associated with plant microbe interactions such as JA signaling and pathogen responses), (iii) circadian responses (processes and pathways associated with day-length signaling such as phytochrome signaling and clock genes), (iv) growth and development (processes associated with hormone responses and organ formation), and (v) nutrient and energy (processes associated with photosynthesis and C and N flow).

The transcriptome analysis also helped explain the apparent contradiction between comparatively high soil P levels in the moderate yield zone but low plant P uptake in this same area (Tables 3 and 4; Supplemental Table S1). This apparent contradiction was attributed to the downregulation of several acid phosphates, P transporters (*P*-values between 0.08 and 0.20), the phosphate inducible gene, and the P membrane transporter (antigen peptide transporter 2 [APT2]) in moderate yield zone plants (Table 4). Assuming that the differences in phosphate transporters were manifested in the roots, these results suggest that the plant may preferentially manage water shortages at the expense of managing P limitations. Several ion transporters were noted as differentially expressed in transcriptome analyses of drought stress under nonfield conditions; however, reduced phosphate uptake was not noted (Zheng et al., 2010). It is unclear if these differences were due to the severity or longevity of the stress or to differences in developmental stages of the plants in these analyses. However, it is impossible to rule out that the reduced phosphate levels in the summit and shoulder grown plants was due to factors other than reduced expression of phosphate transporters, and could have resulted from differences in solubilized phosphate due to reduced soil moisture, or to potential developmental

**Table 2. The influence of management zone (summit and shoulder relative to lower backslope) on regulation of selected pathways and homologs of indicated genes (also see Supplemental Table S1 and Gene Expression Omnibus database accession #GSE33493) as determined by gene set and subnetwork enrichment analysis.**

Functional category	Pathway name <sup>†</sup>	Pathway expression	P-value	Examples of genes with differential expression compared with control used to determine overall pathway expression
Abiotic stress	Response to water deprivation	Down	<0.001	ERD7, PIP2B, F18C1.3, F18C1.9, PIP1.4, ANNAT7, and F2P16.10
	Response to abscisic acid stimulus	Down	<0.001	T3P4.5, ATAIB, ATMYB94, F2N1.20, PRT6, MQL5.25, and AtMYB78
	Neighbors of drought	Down	0.013	GOLS1, STZ, ZAT6, PYRD, ATJ2, ITN1, PPK, and GAMMA-TIP
	Regulation of stomatal movement	Down	0.023	MYB61, JAR1, EIN2, AHA1, OST1, ATRBOH_F, TPC1, and CPK3
	Response to cold	Down	0.042	ERD7, T13L16.11, T29E15.24, KCS11, PAG1, DRT102, and AT3G17020
	Abscisic acid signaling	Down	0.043	ABF3, ADH1, HAB1, AGB1, PRN, ABH1, and F7K24.80
	Flavonoid biosynthesis	Down	0.045	AT3G21420, TT4, EFE, OPCL1, 4CL1, and 4CL3
	Response to desiccation	Up	0.015	RD2, F6E13.19, RD22, RD20, RD19, ALDH7B4, and RCI3
Biotic stress	Response to cold	Up	0.028	T13L16.11, PAG1, F15K20.19, T1B8.10, F19I3.27, and T2N18.5
	Neighbors of MYC2	Up	0.029	LOX3, RBCS1A, JAZ1, COI1, RD22, ADH1, TT4, RD20, and ERD15
	Response to wounding	Down	<0.001	F11A3.11, T7F6.4, ATAIB, ATPERK1, T12G13.10, VEP1, and atnudt8
	Defense response to fungus	Down	<0.001	LCR69, LCR68, T7F6.4, PDF1.5, ATHCHIB, AGB1, EIN2, and WRKY33
Circadian	Systemin signaling	Down	<0.01	AT3G07400, CYP71B20, AT3G49050, T6G15.100, and DL4435W
	Jasmonic acid biosynthesis	Down	0.019	AOS, LOX1, LOX3, LOX2, OPR3, OPR2, OPR1, HPL1, LOX5, and SGR2
	Red or far red light signaling pathway	Down	0.01	SOUL-1, APRR9, PRR7, ARR4, HY5, RAP2.4, and PHYD
	Binding partners of CCA1	Up	0.01	TEJ, ZTL, GI, APRR9, TOC1, and LHY
Growth development	Neighbors of TOC1	Up	0.028	APRR9, CCR2, GI, LHY, and ZTL
	Pollination	Down	<0.01	rbcl, psbA, MYC2, SUT2, and AGP19
	Ethylene mediated signaling pathway	Down	<0.01	F25G13.130, CRF3, ERF4, ERF2, ARA3, CTR1, RCD1, EIN2, and EIN3
	Meristem structural organization	Down	0.018	AP1, AFO, RPT2A, ATSK11, and ATSK12
	Meristem identity	Down	0.046	AG, AP2, AP1, SVP, SEP3, AFO, AGO1, SEP1, PUCHI, and LD
	Root growth	Up	0.01	SHY2, PAC, STM, PIN1, TT4, ERF4, IAA7, SOS3, and PLDALPHA1
	Leaf initiation	Up	0.011	SE, ABP1, ARP, F27K7.6, and CKS1
Nutrient and energy	Leaf morphogenesis	Up	0.033	TCP5, PAC, FVE, AXR1, HUB1, ATHB-15, AN, ATHB-1, and RPT2A
	Photosynthesis	Down	<0.001	PSAE-2, LHB1B2, F22F7.15, ATPPC3, PSBQ-2, F7A7.50, and FTRAI
	Light-harvesting complex	Down	<0.01	LHB1B2, F7A7.50, CAB1, LHCB2.1, psbC, LHCB2.3, LHCB5, and LHCA1
	Light response	Down	0.014	ERD9, SHY2, PHYA, RAX2, ABCB1, CRY1, APRR9, IAA7, and CCA1
	Amino acid transmembrane transporter activity	Down	0.033	T7F6.1, AT3G30390, F22I13.20, AAP7, CAT3, F5M6.16, and F11I4.17
	Pyrimidine nucleotide metabolism (phosphotransfer and nucleotide modification)	Up	0.014	emb2742, AT3G18680, T32A16.70, NDPK2, and NDPK1
	Secondary cell wall biogenesis	Up	0.017	ERF38, IRX6, GUT1, CESA4, TED7, IRX9, and GUT2
	Sucrose biosynthetic process	Up	0.023	F11F19.25, SUS4, SBPASE, SUS1, and SUS3
Sucrose degradation	Up	0.033	ATHXK4, AT3G54090, MJB21.12, T21E18.7, F4H5.18, and T22J18.18	
Starch catabolic process	Up	0.049	F4P13.6, ABI4, SEX1, CT-BMY, SEX4, ISA3, GWD3, ATLDA, and DPE2	

<sup>†</sup>MYC2, myelocytomatosis 2; CCA1, circadian clock associated 1; TOC1, timing of cap expression 1.

differences in root structure that could result from soil differences between the two landscape features. That said, based on transcriptome analysis, it appears that neither the Liebig law of the minimum (Liebig, 1855) nor the multiple limitation theory (Bloom et al., 1985; Rubio et al., 2003; Ågren et al., 2012) adequately explained plant responses and this new information may be useful in mechanistically defining multiple plant stress response.

The findings also suggest that plants grown in the moderate yield zone had altered circadian rhythm responses (Table 2). Cold and drought stress have both been implicated in altering the circadian response in other systems (Legnaioli et al., 2009; Ibañez et al., 2008), and these alterations could impact corn growth

and development under moderate water stress. It has been estimated that over a third of the transcriptome is modified by the circadian response in arabidopsis (Michael and McClung, 2003) and very likely will impact both flowering and seed development (Jain et al., 2007). Unfortunately, this dataset does not indicate if the circadian cycle itself is altered or if just the level of expression of these circadian regulatory and response genes is simply higher in the moderate yield zone plants. Therefore, more work is needed to provide an adequate explanation for the observation that circadian-responsive genes are upregulated in the moderate yield zone plants.

An analysis of genes with reduced expression in moderately water stressed plants indicates that genes



**Table 3. A comparison between microarray analysis and real-time polymerase chain reaction (qPCR). Log fold differences (summit–shoulder and backslope) and associated *P*-values are shown for the microarray and qPCR data.**

Gene	Function <sup>†</sup>	Microarray	<i>P</i> -value	qPCR	<i>P</i> -value
MZ00018541	EREBP-like protein	−0.9289	0.0084	Down	0.094
MZ00018542	EREBP-like protein	−1.2677	0.0011	Down	0.073
MZ00020242	Circadian rhythm	−0.9173	0.0004	Down	0.028
MZ00024893	Abscisic acid-induced protein	−0.2202	0.1193	Down	0.563
MZ00041134	Ferredoxin	−0.0788	0.6754	Down	0.610
MZ00043643	Putative anion transporter	0.2582	0.2027	Up	0.425
MZ00048663	Putative high-affinity potassium transporter	0.2670	0.2715	Up	0.378
MZ00041292	Photosynthesis (PSI-N subunit, chloroplastic precursor) seq 2	−0.5546	0.0819	Down	0.277
MZ00024895	Abscisic acid-induced protein	−0.4904	0.0162	Down	0.082
MZ00036315	Photosystem I (PSI-N) seq 1	−0.7927	0.051	Down	0.309
MZ00017722	Iron transport protein 2	−0.2073	0.5312	Down	0.852
MZ00039137	Ferredoxin III, chloroplast precursor	0.2674	0.1346	Down	0.889

<sup>†</sup>EREBP, ethylene-responsive element binding protein; PSI-N, photosystem I subunit N; seq, sequence.

involved in photosynthetic processes and production of biotic and abiotic stress response processes such as JA, flavonoids, ethylene, and systemin signaling were downregulated. Photosynthetic processes are commonly observed to be downregulated in response to drought stress (Chaves et al., 2009), and therefore this would be expected if the moderate yield zone plants had experienced lower water levels. Unexpectedly, ABA signaling components appear to be downregulated in moderate water stressed plants (see below for further discussion of this observation).

In contrast to the expected downregulation of photosynthetic processes, the downregulation of ABA, ethylene, and systemin along with downregulation of JA and flavonoids suggests that major defense responses against pests were reduced in plants grown in moderately yielding zones. Systemin signaling is generally associated with defense against microbial pathogens (Ryan, 2000). It is unclear if this response was due to reduced insect and pathogen attack or to reduced ability of the crop to respond to attack. The presumably more exposed and less moist conditions in the moderate yield zone could decrease pathogen levels. However, although quantitative data were not collected, no obvious differences in disease incidence were noted between the yield zones. If insect and pathogen stress were similar between the summit and backslope, the inability to respond to these stresses under the presumably dryer conditions at the summit could have resulted in reduced yields. Indeed, the downregulation of plant defenses against diseases may help explain mycotoxin problems often associated with increased insect predation during drought conditions (Parsons and Munkvold, 2010).

Ethylene, JA, and flavonoid biosynthesis are usually associated with wound responses and are often observed to be elevated in response to insect attack (Baldwin, 2001). However, there was no indication of less insect damage in moderately water stressed plants.

Even more surprising was the observed reduction in ABA signaling. Abscisic acid is generally associated with

**Table 4. Selected phosphate-related genes expression status on moderate yield zone (summit and shoulder position) as compared to the high yield zone (lower backslope) plants. *P*-values of gene expression differences based on *t* tests are as indicated.**

Gene	Putative annotation	Gene expression	<i>P</i> -value
MZ00046532	Acid phosphatase	Down	0.1806
MZ00019894	Acid phosphatase	Down	0.1555
MZ00017800	Acid phosphatase	Down	0.0426
MZ00000571	Acid phosphatase-like	Down	0.0829
MZ00023866	Inorganic diphosphatase	Down	0.1246
MZ00023951	Inorganic diphosphatase	Up	0.0280
MZ00015877	Phosphate transport protein	Down	0.0816
MZ00003527	Phosphate transporter 2-1	Down	0.1785
MZ00042137	Phosphate-induced protein 1-like protein	Down	0.0237

drought stress, and the observed downregulation in leaf material collected near midday from plants that appeared to be water stressed was quite unexpected. In nearly every previous transcriptomic analysis of drought stress, ABA signaling was increased and subsequently buffered plants against the effects of drought stress (Jiang and Huang, 2002). It is possible that these plants may have initially induced ABA signaling as water became limiting but that during the long sustained drought stress conditions, the plants had become fully acclimated and no longer needed to respond to the drought—unlike the plants growing in the foot slope areas that may indeed have been in the active process of responding to the short-term drought stress that occurred at the hottest point of the day.

In general, repression of biotic stress responses results in higher growth. A previous study by Hao et al. (2011) comparing the transcriptome between fast and slow growing poplar (*Populus* spp.) indicated that low levels of biotic defense response gene expression were observed in the fast-growing variety even though neither variety was subjected to biotic stress.

Therefore, one might expect to see higher growth and yield in plants grown in the moderate yield zone based on the transcriptome alone. However, the repression of photosynthesis due to reduced soil water appears to override the potential gains observed by downregulation of the biotic stress responses. Based on these observations, we hypothesize that adding water to the summit-grown plants would allow them to produce higher yields than the plants grown on the backslopes—assuming that additional watering did not directly or indirectly trigger higher expression of defense response genes.

## Conclusions

Plants growing in production fields can have multiple factors, both resource dependent and resource independent, interacting to influence their yields. The field data suggest that in plants growing in the moderate yield zone (summit and shoulder areas) had growth limited by P, N, and water stress in the samples tested. In response to stress, plants can up- or downregulate genes. In this study, 840 genes were differentially regulated. Differential expression of critical genes of interest was confirmed using qPCR. The up- and downregulated genes extended beyond the genes generally reported for water stressed conditions. Differences between controlled studies and this project may be related to long-term multiple stress interacting to influence plant response. Key processes associated with nutrient uptake, especially P, were downregulated in moderately water stressed plants. This downregulation might contribute to an apparent contradiction in the ability of corn to use P. Critical plant processes associated with pathogen protection were also downregulated. The differential regulation of genes may provide clues on how to better manage problems in fields where crops are influenced by multiple stresses. Plants growing under moderate water stress in this study had a reduced protection capacity against plant diseases and insects and therefore may have lower action thresholds than predicted if using recommendations for integrated pest management systems. These findings are contrary to a general perception that a single economic pest threshold is appropriate for relatively large regions. Additional studies are needed to determine if the responses observed in this experiment are common to other corn hybrids or other locations with similar landscape features or if they are subject to modification by genetic variation as well as climactic differences that might occur between years.

## Supplemental Information Available

Supplemental material is included with this manuscript.

Supplemental Table S1. List of genes present on the array along with their annotation, expression, and significance.

Supplemental Table S2. Results of gene set and sub-network enrichment analysis.

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