The Effects of Elevated CO_2 Concentration on Soybean Gene Expression. An Analysis of Growing and Mature Leaves[1][W][OA]

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Improvements in carbon assimilation and water-use efficiency lead to increases in maximum leaf area index at elevated carbon dioxide concentration ($[CO_2]$); however, the molecular drivers for this increase are unknown. We investigated the molecular basis for changes in leaf development at elevated $[CO_2]$ using soybeans (*Glycine max*) grown under fully open air conditions at the Soybean Free Air CO_2 Enrichment (SoyFACE) facility. The transcriptome responses of rapidly growing and fully expanded leaves to elevated $[CO_2]$ were investigated using cDNA microarrays. We identified 1,146 transcripts that showed a significant change in expression in growing versus fully expanded leaves. Transcripts for ribosomal proteins, cell cycle, and cell wall loosening, necessary for cytoplasmic growth and cell proliferation, were highly expressed in growing leaves. We further identified 139 transcripts with a significant $[CO_2]$ by development interaction. Clustering of these transcripts showed that transcripts involved in cell growth and cell proliferation were more highly expressed in growing leaves that developed at elevated $[CO_2]$ compared to growing leaves that developed at ambient $[CO_2]$. The 327 $[CO_2]$-responsive genes largely suggest that elevated $[CO_2]$ stimulates the respiratory breakdown of carbohydrates, which provides increased energy and biochemical precursors for leaf expansion and growth at elevated $[CO_2]$. While increased photosynthesis and carbohydrate production at elevated $[CO_2]$ are well documented, this research demonstrates that at the transcript and metabolite level, respiratory breakdown of starch is also increased at elevated $[CO_2]$.

By 2050, soybean (*Glycine max*) will grow in an atmosphere with a 50% higher carbon dioxide concentration ($[CO_2]$) (Prentice et al., 2001). As the world's most widely grown seed legume, the physiological responses of soybean to elevated CO_2 have been well characterized. Elevated $[CO_2]$ increases carbon (C) uptake, foliar carbohydrate content, plant growth, and yield, while decreasing stomatal conductance (for review, see Ainsworth et al., 2002). A Free Air CO_2 Enrichment (FACE) experiment was established in one of the world's most productive soybean-growing areas, Central Illinois, in 2001. This facility allows investigation of the response of field-grown soybean to an atmosphere predicted for 2050 without alteration of the microclimate (Long et al., 2004). Across the growing season, daily integrals of leaf photosynthetic CO_2 uptake increased by approximately 25%, even as midday stomatal conductance decreased by approximately 20% (Rogers et al., 2004; Bernacchi et al., 2006). Improvements in C assimilation and water-use efficiency spurred increases in maximum leaf area index (LAI) and aboveground biomass in elevated $[CO_2]$ (Morgan et al., 2005; Dermody et al., 2006). The combination of increased photosynthesis and increased LAI provided the inputs for significant increases in soybean seed yield (Ort et al., 2006).

Increased leaf growth, leading to larger individual leaf size, is one component of increased LAI measured at elevated $[CO_2]$ in field experiments (Taylor et al., 2003; Tricker et al., 2004; Dermody et al., 2006). At the molecular level, the basis for changes in LAI at elevated $[CO_2]$ is largely unknown. Both cell production rates and cell expansion have been shown to be sensitive to elevated $[CO_2]$ (Taylor et al., 1994, 2003). Transcript analysis of growing poplar (*Populus* spp.) leaves exposed to elevated $[CO_2]$ showed that genes involved in cell wall loosening and synthesis were

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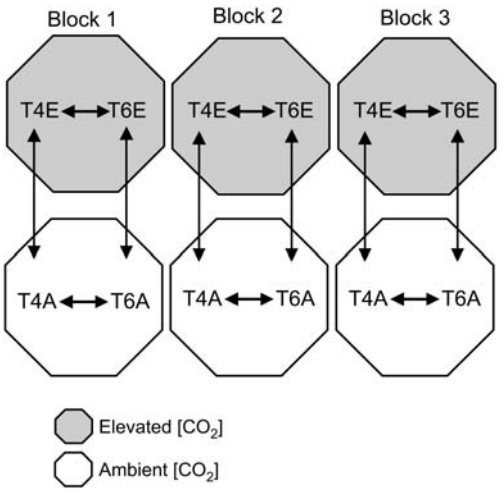


Figure 1. Design of the cDNA microarray experiment. Each double-headed arrow represents four microarrays per library, two biological replicates and two technical replicates. Each biological replicate included pooled RNA from six individual plants. A total of 96 microarrays were analyzed. Four treatments were compared: 1, T4 versus T6 in ambient [CO_2] (T4A versus T6A); 2, T4 versus T6 in elevated [CO_2] (T4E versus T6E); 3, T4 in ambient [CO_2] versus T4 in elevated [CO_2] (T4A versus T4E); 4, T6 in ambient [CO_2] versus T6 in elevated [CO_2] (T6A versus T6E).

up-regulated (Taylor et al., 2005; Druart et al., 2006). Therefore, at least in poplar, elevated [CO_2] appears to stimulate or prolong expansion in leaves (Ferris et al., 2001; Taylor et al., 2003; Druart et al., 2006).

Leaf growth is a spatially and temporally dynamic process (for review, see Schurr et al., 2006). To understand the mechanisms controlling leaf growth in dicots, experiments must account for the spatial and diel variations in growth (Trainotti et al., 2004; Matsubara et al., 2005). Growing leaves do not expand at all times throughout the diel cycle, nor do they necessarily expand homogeneously. Tobacco (*Nicotiana tabacum*) leaves show a base-to-tip gradient in developmental stage of the tissue, and differential expression of genes in apical and basal tissues (Trainotti et al., 2004). While the functional maturation of dicot leaves has been described to progress with a base-to-tip gradient (Avery, 1933; Turgeon, 1989), recent experiments using digital image sequence processing revealed that some dicot species lack a base-to-tip gradient in relative expansion rates (Ainsworth et al., 2005; Matsubara et al., 2005). Prolonged cytoplasmic growth as opposed to vacuolated growth dominated leaf expansion in *Populus deltoides*, a species that lacks a base-to-tip gradient in leaf growth (Matsubara et al., 2005). Soybean also lacks a pronounced base-to-tip gradient in leaf growth rates (Ainsworth et al., 2005). Yet, soybean has a clear diel pattern of leaf expansion, with maximum rates occurring at night (Bunce, 1977; Ainsworth et al., 2005). How elevated [CO_2] alters the dynamics of leaf expansion in soybean has not been examined to date. However, there is evidence in poplar species that altered carbohydrate status may change the fine-scale temporal and spatial patterns of growth in response to elevated [CO_2] (Walter et al., 2005), and experiments with transgenic plants clearly show a link between leaf C metabolism and leaf development (Raines and Paul, 2006).

The first objective of this research was to investigate molecular changes in growing and fully expanded soybean leaves developed at elevated [CO_2] under fully open-air conditions. Research has shown that the response of soybean to elevated [CO_2] in the field is less than predicted from chamber studies (Long et al., 2006). One approach for maximizing future production of soybean is to increase LAI and, therefore, the potential for C uptake. Thus, the other objectives of our study were to identify transcripts that control leaf growth and elongation, and to investigate how elevated [CO_2] alters the expression of those transcripts. This research provides an overview of the soybean transcriptome response to elevated [CO_2] in both fully expanded and growing leaves.

RESULTS AND DISCUSSION

The transcriptome response of fully expanded trifoliate 4 (T4) and growing trifoliate 6 (T6) soybean leaves to elevated [CO_2] was analyzed using cDNA microarrays (Fig. 1). On July 7, T4 leaflets were fully expanded and longer in elevated [CO_2] compared to ambient [CO_2] (Fig. 2). T6 leaflets were growing with a relative increase in length of 42% ± 6% per day in both ambient and elevated [CO_2] (Fig. 2). We were specifically interested in how elevated [CO_2] alters genes related to leaf development, so samples were taken between 1 and 2 AM, which corresponded to the time of maximum leaf expansion rate (Ainsworth et al., 2005). Analysis of variance revealed 1,146 genes with different expression ($P < 0.05$) in growing leaves compared to fully expanded leaves (Supplemental Table I), 139 transcripts with a significant CO_2 × development interaction (Supplemental Table II), and 327 transcripts that responded to CO_2 (Supplemental Table

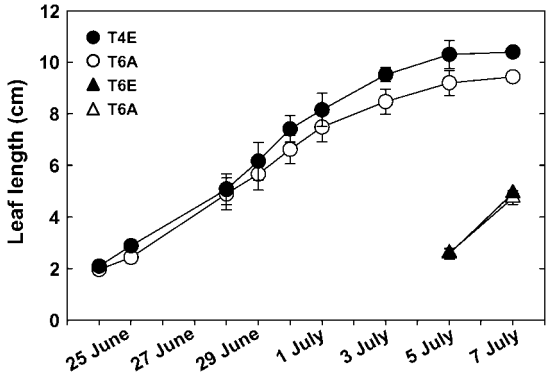


Figure 2. Increase in length of the fourth (T4, circles) and sixth (T6, triangles) trifoliate lateral leaflets grown at ambient (A; white symbols) and elevated (E; black symbols) [CO_2]. Samples for microarray analysis and leaf carbohydrates were taken on July 8, 2004, between 1 and 2 AM, when T4 leaflets were fully expanded and T6 leaflets were expanding in length at 42% ± 6% d^{-1}.

III). The changes in transcript abundance were up to 3.5-fold in developmentally regulated transcripts (Table I) and up to 2-fold in transcripts regulated by [CO_2] (Table II). This result is similar to results from two other FACE experiments where only small changes in transcript expression were detected at elevated [CO_2] (Gupta et al., 2005; Taylor et al., 2005). This likely reflects the chronic nature of FACE treatment. In the FACE experiment, we analyzed transcript profiles of plants acclimated to an environmental change (a higher [CO_2]) rather than observing the response of gene expression to an acute change, e.g. an herbivore attack.

Genes Associated with Leaf Development

Expression of 1,146 genes was significantly different in growing versus fully expanded leaves, irrespective of growth [CO_2] (for a complete list of transcripts, see Supplemental Table I). A total of 178 transcripts, encoding genes for a wide variety of functions, showed 1.5 times lower gene expression in growing leaves compared to fully expanded leaves. This group included genes involved in secondary metabolism, transport, stress and metal handling, and major and minor carbohydrate metabolism. Notably, starch phosphorylase (Gm-r1088-8633), a glucan-metabolizing enzyme (Smith et al., 2005), was expressed at lower levels in growing leaves compared to fully expanded leaves (T6/T4 = 0.595). This correlated with measured amounts of leaf carbohydrates (Fig. 3), where fully expanded, mature leaves had between 4 to 5 times the amount of starch as developing leaves (Fig. 3, C and D) and, therefore, more substrate for degradation.

A total of 132 transcripts showed at least 1.5 times higher expression in growing leaves and, therefore, represent our best estimates of control points of leaf

Table I. *Selection of 38 genes that were differentially expressed between growing and fully expanded leaves*

We report a description based on matching BLASTX hits from sequences of both the 3′ and 5′ ends of each clone unless only the 5′ information is available (5′), relative expression in growing (T6) versus fully expanded leaves (T4), and results of the statistical analysis (*F*, *P*). The entire list of 1,146 genes that were differentially expressed between growing and fully expanded leaves is provided in Supplemental Table I.

Clone ID	Description	T6/T4	*F*	*P*
Gm-r1070-3452	Ribosomal protein L35 (*Arabidopsis thaliana*)	1.505	12.85	0.01158
Gm-r1088-5863	Ribosomal protein (*Petunia x hybrida*)	1.508	18.25	0.00272
Gm-r1088-5173	40s ribosomal protein S23 (*Euphorbia esula*)	1.509	18.56	0.00259
Gm-r1070-8126	Putative ribosomal protein (*Capsicum annuum*)	1.514	21.67	0.00349
Gm-r1070-8058	ADP-ribosylation factor (*Hyacinthus orientalis*)	1.516	15.86	0.00726
Gm-r1070-1257	60S ribosomal protein L7A (*A. thaliana*)	1.521	18.69	0.00497
Gm-r1070-8966	Putative histone H2A protein (*Oryza sativa*)	1.525	20.58	0.00395
Gm-r1088-6691	Ribosomal protein L36 (*Triticum aestivum*)	1.528	27.57	0.00077
Gm-r1070-7448	α-Expansin 3 (*Cicer arietinum*)	1.532	9.93	0.01979
Gm-r1070-7790	Putative 60S ribosomal protein L10A (RPL10aC) (*O. sativa*)	1.538	18.35	0.00519
Gm-r1088-4318	Histone H2B (*C. arietinum*)	1.541	20.91	0.00182
Gm-r1070-8158	Ribosomal protein L14-like protein (*A. thaliana*)	1.576	9.41	0.02203
Gm-r1070-4867	30S ribosomal protein S16-like (*O. sativa*)	1.580	13.66	0.01013
Gm-r1070-3909	Ribosomal protein L11, putative (*A. thaliana*)	1.586	17.14	0.00608
Gm-r1088-164	Putative 40S ribosomal protein (*O. sativa*)	1.590	26.71	0.00086
Gm-r1070-970	Histone H2A (*E. esula*)	1.624	7.12	0.03709
Gm-r1070-3576	Ribosomal protein L37 (soybean)	1.628	12.90	0.01149
Gm-r1088-7068	60S ribosomal protein L13E (*Picea abies*)	1.639	7.67	0.02431
Gm-r1070-5626	Putative ribosomal protein L19 (*O. sativa*)	1.641	22.99	0.00302
Gm-r1088-4541	Tubulin family protein (*A. thaliana*)	1.646	10.33	0.01236
Gm-r1088-7215	Ribosomal protein L15 (*P. x hybrida*)	1.653	11.23	0.01007
Gm-r1070-8151	40S ribosomal protein S21, putative (*O. sativa*)	1.655	54.66	0.00031
Gm-r1070-8751	Ribosomal protein L29 (*Panax ginseng*)	1.660	45.72	0.00051
Gm-r1070-7659	α-Tubulin (*Gossypium hirsutum*)	1.683	14.48	0.00891
Gm-r1070-8444	Ribosomal protein S26 (*Pisum sativum*)	1.685	17.01	0.00619
Gm-r1070-4817	60S ribosomal protein L7 (*A. thaliana*)	1.703	31.44	0.00137
Gm-r1088-4320	Histone H2B1 (*G. hirsutum*)	1.711	6.22	0.03727
Gm-r1070-9008	40S ribosomal protein S25 (soybean)	1.740	14.28	0.00919
Gm-r1070-8414	40S ribosomal protein S14	1.760	33.04	0.00121
Gm-r1070-8326	Histone H2B-3 (*Lycopersicon esculentum*)	1.780	17.70	0.00564
Gm-r1070-3758	Ribosomal protein small subunit 28 (*Helianthus annuus*)	1.801	26.41	0.00214
Gm-r1088-7080	60S ribosomal protein L34 (*Solanum demissum*)	1.820	11.78	0.00893
Gm-r1070-5890	40S ribosome protein S7 (*Avicennia marina*)	1.843	34.44	0.00108
Gm-r1070-7481	Putative cytoplasmic ribosomal protein S15a (*A. thaliana*)	1.862	10.00	0.01950
Gm-r1070-8254	Acidic ribosomal protein (*H. orientalis*)	1.908	21.80	0.00343
Gm-r1070-5505	Expansin (*Pyrus communis*)	1.941	6.12	0.04814
Gm-r1070-8979	Acidic ribosomal protein P0 (soybean)	2.041	7.42	0.03447
Gm-r1070-9002	40S ribosomal protein S20-like protein (*A. thaliana*)	2.416	27.29	0.00197

Table II. *Selection of 85 genes that were differentially expressed between ambient and elevated [CO_2]*

We report a description based on matching BLASTX hits from sequences of both the 3′ and 5′ ends of each clone unless only the 5′ information is available (5′), relative expression in elevated [CO_2]/ambient [CO_2] (E/A), and results of the statistical analysis (*F*, *P*). The numbers in the final column refer to the transcripts illustrated in Figure 6. The entire list of 327 genes that were differentially expressed between ambient and elevated [CO_2] is provided in Supplemental Table III.

Clone ID	Description	E/A	*F*	*P*	Figure 6
C Metabolism					
Gm-r1070-5613	β-Amylase (soybean)	1.434	15.39	0.0078	1
Gm-r1070-7748	Granule-bound starch synthase Ib precursor (*Phaseolus vulgaris*)	1.829	10.60	0.0173	
Gm-r1070-5070	β-Amylase (soybean)	1.360	16.41	0.0067	2
Gm-r1070-8759	Chain A, crystal structure of soybean β-amylase mutant (M51t)	1.313	14.97	0.0083	3
Gm-r1070-8484	Chain A, crystal structure of soybean β-amylase mutant (M51t)	1.401	11.88	0.0137	3
Gm-r1088-4020	*myo*-Inositol-1-P synthase (soybean)	1.668	6.21	0.0375	4
Gm-r1070-5201	Inositol-3-P synthase (soybean)	1.714	7.01	0.0382	5
Gm-r1088-2682	α-Galactosidase preproprotein (*Cyamopsis tetragonoloba*)	1.476	5.85	0.0419	
Gm-r1070-6564	Phosphofructokinase (*Medicago truncatula*)	1.241	13.13	0.0111	6
Gm-r1070-4852	Glyceraldehyde-3-P dehydrogenase (*Solanum tuberosum*)	1.244	11.27	0.0153	7
Gm-r1070-7510	Phosphoglycerate mutase (*Ricinus communis*)	1.352	10.75	0.0169	8
Gm-r1070-6442	Enolase, isoform 2 (*Hevea brasiliensis*)	1.214	10.73	0.0169	9
Gm-r1070-5429	Cytosolic phosphoglycerate kinase (*Pisum sativum*)	1.142	10.60	0.0173	10
Gm-r1088-3248	Plastidial phosphoglucomutase (*P. sativum*)	1.901	6.49	0.0343	11
Gm-r1070-7756	Putative pyruvate dehydrogenase E1 β-subunit (*Oryza sativa*)	1.322	7.03	0.0379	12
Gm-r1070-7077	Phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44)	1.454	6.54	0.0338	13
Mitochondrial e-Transport					
Gm-r1070-6065	Hydrogen-transporting ATPase, rotational mechanism (*Arabidopsis thaliana*)	1.198	6.16	0.0476	
Gm-r1070-4318	F1-ATP synthase δ-subunit (*Ipomoea batatas*)	1.168	6.08	0.0488	
Cell Wall Metabolism					
Gm-r1070-5796	Putative NAD-dependent epimerase (*A. thaliana*)	1.268	18.47	0.0051	14
Gm-r1070-6616	Cellulose synthase (EC 2.4.1.-) catalytic chain celA2	1.346	8.64	0.0260	15
Gm-r1070-4646	UDP-D-apiose/UDP-D-Xyl synthase (*A. thaliana*)	1.303	8.61	0.0261	16
Lipid Metabolism					
Gm-r1070-9006	Palmitoyl-acyl carrier protein thioesterase (*Gossypium hirsutum*)	1.308	18.55	0.0051	17
Gm-r1070-7584	Enoyl-ACP reductase (tobacco)	1.263	7.71	0.0321	18
Gm-r1070-3469	Omega-3 fatty acid desaturase (EC 1.14.99.-) GMD	1.145	7.02	0.0380	
Gm-r1070-5787	Microsomal omega-3 fatty acid desaturase (soybean)	1.348	6.82	0.0400	
Gm-r1070-5473	Acyl-CoA thioesterase (*A. thaliana*)	1.244	6.27	0.0462	
N Metabolism					
Gm-r1070-5797	P-protein precursor (*S. tuberosum*)	1.417	15.94	0.0072	
Gm-r1070-9038	Putative urease accessory protein G (*O. sativa* [japonica cultivar group])	1.167	7.46	0.0342	
Gm-r1088-2099	Indole-3-glycerol phosphate synthase (*A. thaliana*)	1.409	5.49	0.0472	19
Gm-r1070-6244	Gln synthetase precursor (soybean)	1.328	8.98	0.0241	20
Transport					
Gm-r1070-2965	Ca2+/H+-exchanging protein (*Vigna radiata*)	1.373	51.02	0.0004	
Gm-r1070-5393	Ca2+/H+-exchanging protein (*V. radiata*)	1.616	40.58	0.0007	
Gm-r1070-5594	Vacuolar H+-ATPase B subunit (*Citrus unshiu*)	1.424	27.29	0.0020	
Gm-r1088-3648	Aquaporin (*Vitis vinifera*)	1.998	9.82	0.0139	
Gm-r1070-5792	Vacuolar ATPase subunit E (*Phaseolus acutifolius*)	1.294	10.96	0.0162	
Gm-r1070-2941	Vacuolar H+-ATPase c subunit (*C. unshiu*)	1.339	10.53	0.0176	
Gm-r1070-6113	Putative nitrate transporter NRT1-3 (soybean)	1.734	8.90	0.0245	21
Gm-r1070-4599	Putative nitrate transporter NRT1-3 (soybean)	1.259	6.73	0.0410	21
Gm-r1070-8647	Core protein (*P. sativum*)	1.222	6.71	0.0412	
Gm-r1088-1920	ADP, ATP carrier-like protein (*A. thaliana*)	1.600	5.75	0.0433	
Gm-r1088-2089	Cation diffusion facilitator 9 (*Stylosanthes hamata*)	1.349	5.36	0.0494	
Gm-r1088-1149	Outer envelope protein (*P. sativum*)	1.390	5.35	0.0494	

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Clone ID	Description	E/A	*F*	*P*	Figure 6
Hormone Metabolism					
Gm-r1070-6109	Lipoxygenase (*P. vulgaris*)	1.293	25.18	0.0024	
Gm-r1070-8913	Auxin response factor-like protein (*Mangifera indica*)	1.376	15.32	0.0079	
Gm-r1070-5659	Response reactor 4 (*A. thaliana*)	1.215	7.72	0.0321	
Secondary Metabolism					
Gm-r1070-1714	Homogentisate phytylprenyltransferase (soybean)	1.224	14.87	0.0084	
Gm-r1070-3733	1-Deoxy-D-xylulose 5-P reductoisomerase (*Pueraria montana*)	1.347	9.01	0.0240	22
Gm-r1070-3522	Putative caffeic acid methyl transferase (*Arachis hypogaea*)	1.327	8.15	0.0290	23
Gm-r1070-8296	Putative cinnamoyl-CoA reductase (*Solanum demissum*)	1.439	7.62	0.0328	24
RNA Regulation of Transcription					
Gm-r1070-5468	MYB transcription factor (*A. thaliana*)	1.282	43.60	0.0006	
Gm-r1070-8564	mRNA-binding protein precursor (tobacco)	1.316	24.43	0.0026	
Gm-r1070-7770	DNA-binding protein (*A. thaliana*)	1.218	17.97	0.0054	
Gm-r1070-6997	Remorin (*S. tuberosum*)	1.389	13.30	0.0107	
Gm-r1070-7595	LHY protein (*P. vulgaris*)	1.287	11.64	0.0143	
Gm-r1070-6170	NAM-like protein (*Prunus persica*) (5′)	1.272	11.64	0.0143	
Gm-r1088-2878	bZIP DNA-binding protein (*Antirrhinum majus*)	1.643	8.12	0.0215	
Gm-r1070-4141	SEU3B protein (*A. majus*)	1.370	9.40	0.0221	
Gm-r1070-4704	Double WRKY-type transfactor (*S. tuberosum*)	1.389	8.38	0.0275	
Gm-r1070-8883	Nucleoid DNA-binding-like protein (*A. thaliana*)	1.173	8.30	0.0280	
Gm-r1070-1976	DNA-binding protein 4 (tobacco) (5′)	1.340	8.24	0.0284	
Gm-r1070-7398	Putative RING zinc-finger protein (*A. thaliana*)	1.162	7.60	0.0330	
Gm-r1070-7935	Dc3 promoter-binding factor-3 (*Helianthus annuus*)	1.228	7.38	0.0348	
Gm-r1070-6989	Nucleic acid-binding/transcription factor (*A. thaliana*)	1.276	7.06	0.0377	
Gm-r1070-5991	Putative AP2-binding protein (*Jatropha curcas*)	1.139	6.74	0.0409	
Gm-r1070-8292	Homeodomain-Leu zipper protein 56 (soybean)	1.355	6.41	0.0445	
Gm-r1070-3954	MYB family transcription factor (*A. thaliana*)	1.237	6.41	0.0446	
Gm-r1070-7416	LEC1-like protein (*Phaseolus coccineus*)	1.136	6.36	0.0452	
Gm-r1070-8125	HMG-1-like protein gene (soybean)	1.263	6.32	0.0456	
Gm-r1070-6056	BEL1-related homeotic protein 29 (*S. tuberosum*)	1.225	6.25	0.0465	
Protein Degradation					
Gm-r1070-7908	Probable aminopeptidase F24D7.4 (imported) (*A. thaliana*)	1.516	41.24	0.0007	
Gm-r1070-5823	Ubiquitin-specific protease 16 (*A. thaliana*)	1.244	13.62	0.0102	
Gm-r1070-8169	Skp1 (*Medicago sativa*)	1.315	10.24	0.0186	
Gm-r1070-8143	Pentameric polyubiquitin	1.287	9.91	0.0199	
Gm-r1070-3489	Cullin 1C (tobacco)	1.304	9.85	0.0201	
Gm-r1070-5188	Subtilisin-like protease C1 (soybean)	1.418	9.21	0.0229	
Gm-r1070-3592	Cys proteinase (soybean)	1.536	8.71	0.0256	
Gm-r1070-4236	Cys proteinase (soybean)	1.250	8.19	0.0287	
Gm-r1070-8885	Aspartyl protease family protein (*A. thaliana*)	1.192	7.72	0.0321	
Gm-r1070-4830	Peptidase C1A, papain; Somatotropin hormone (*M. truncatula*)	1.223	7.61	0.0329	
Gm-r1070-6672	hyuC-like protein (*A. thaliana*)	1.235	7.29	0.0356	
Gm-r1088-4646	Ubiquitin-conjugating enzyme E2 (*Gossypium raimondii*)	1.116	6.33	0.0360	
Gm-r1088-1518	Metal-dependent hydrolase-like protein (*O. sativa* [japonica cultivar group])	1.338	6.22	0.0373	
Gm-r1070-5742	Putative PRT1 protein (*A. thaliana*)	1.227	7.01	0.0381	
Gm-r1070-5457	Peptidase (*A. thaliana*)	1.258	6.99	0.0384	
Gm-r1070-8504	Proteasome subunit α type 3	1.187	6.39	0.0448	

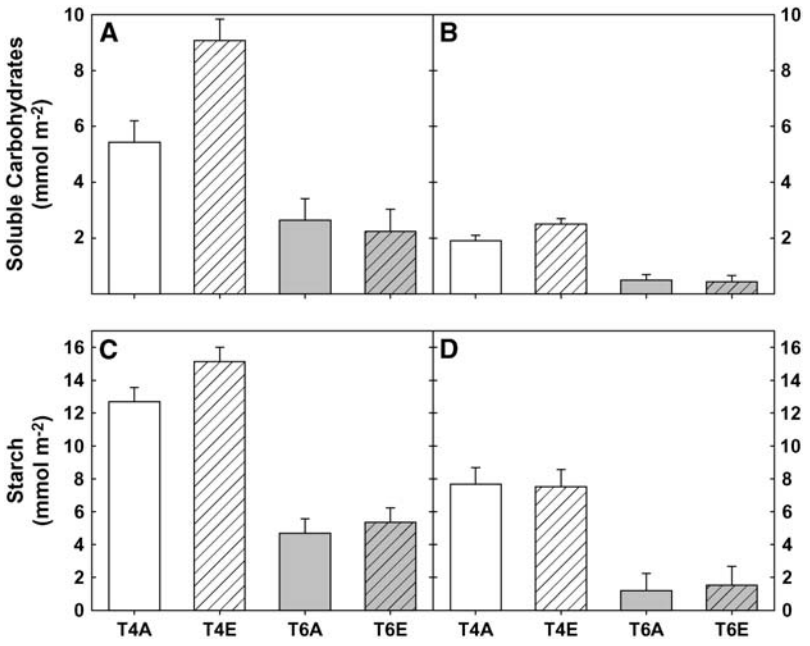


Figure 3. Leaf level contents of soluble carbohydrates (Suc, Glc, Fru; A and B) and starch (C and D) in fully expanded (T4) and growing (T6) leaves in ambient (A) and elevated (E) [CO_2]. Leaves were sampled at dusk on July 7, 2004 (A and C), and between 1 and 2 AM on July 8, 2004 (B and D). At dusk, there was a significant buildup of carbohydrates in mature leaves grown at elevated [CO_2] ($P < 0.05$), but there was no significant effect of [CO_2] treatment on soluble carbohydrates or starch ($P > 0.05$) in the middle of the night. There was a highly significant effect of development on both carbohydrate pools ($P < 0.001$).

expansion. The major family of genes that were highly expressed in growing tissues included ribosomal proteins (Table I). Some cell-cycle genes (histones) and cell wall-loosening genes (expansins) were also included in this group (Table I). Tubulin genes, necessary for regulating the direction of diffuse growth in plants (Abe et al., 2004), were also highly expressed in growing tissues (Table I). The T6 soybean leaflets in this experiment were expanding rapidly (Fig. 2). It is likely that this expansion was due to cytoplasmic growth and cell proliferation, which require significant ribosome biosynthesis (Sugimoto-Shirasu and Roberts, 2003). These findings are supported by previous work with poplar that highlighted up-regulation of ribosome biosynthesis as the primary process underlying nocturnal variations in leaf growth (Matsubara et al., 2005).

CO_2 × Development Interaction

We identified 139 transcripts with a CO_2 × development interaction ($P < 0.05$). These were of particular interest because they represent potential genes involved in growth that may be altered by [CO_2] treatments. These transcripts were clustered into four groups using *k*-means clustering (Saeed et al., 2003). The first cluster of 32 transcripts showed lower expression in developing leaves grown at ambient [CO_2] (T6A) compared to developing leaves grown at elevated [CO_2] (T6E; i.e. T6A/T6E < 1), higher expression in fully expanded leaves grown at ambient [CO_2] (T4A) compared to developing leaves at ambient [CO_2] (T6A; i.e. T4A/T6A > 1), and no change in other comparisons (Fig. 4A). These transcripts included DAG (Gm-r1070-6478), a gene involved in chloroplast development and leaf palisade differentiation (Chatterjee et al., 1996), and a putative Mob-1 like protein, which likely functions in cell proliferation (Citterio et al., 2006). Some transcription factors and DNA-binding proteins were also included in this cluster (Fig. 4A). This provides some evidence that cell proliferation and development are increased in growing leaves at elevated [CO_2] compared to growing leaves at ambient [CO_2].

The second cluster contained transcripts with lower expression in T4E compared to T6E (Fig. 4B) and rather subtle changes in other comparisons. This group contained a number of ribosomal proteins (Gm-r1070-3758, Gm-r1070-6640, Gm-r1070-8751, Gm-r1070-3694) involved in protein synthesis, as well as a binding protein (BiP; Gm-r1070-7989), a highly conserved endoplasmic-reticulum luminal protein that functions as a molecular chaperone (Kalinski et al., 1995). Other genes involved in transcription and regulation of transcription were clustered in this group, lending further evidence to increased levels of cell proliferation and development in young leaves exposed to elevated [CO_2].

The third cluster included 43 transcripts that showed higher expression in T4E compared to T6E (Fig. 4C). This cluster included genes with a wide range of functions, including amino acid synthesis and transport, carbohydrate and cell wall metabolism, protein degradation, redox, and stress response (Fig. 4C). The fourth cluster included 23 genes that showed lower expression in T4A compared to both T4E and T6A (Fig. 4D), including two genes involved in cell wall metabolism, a putative NAD-dependent epimerase and a glycosyl hydrolase family 17 protein (Gm-r1070-5796 and Gm-r1070-767). Glycosyl hydrolase family 17 proteins hydrolyze 1,3-β-glucan polysaccharides in the cell wall matrix and are involved in many stages of plant

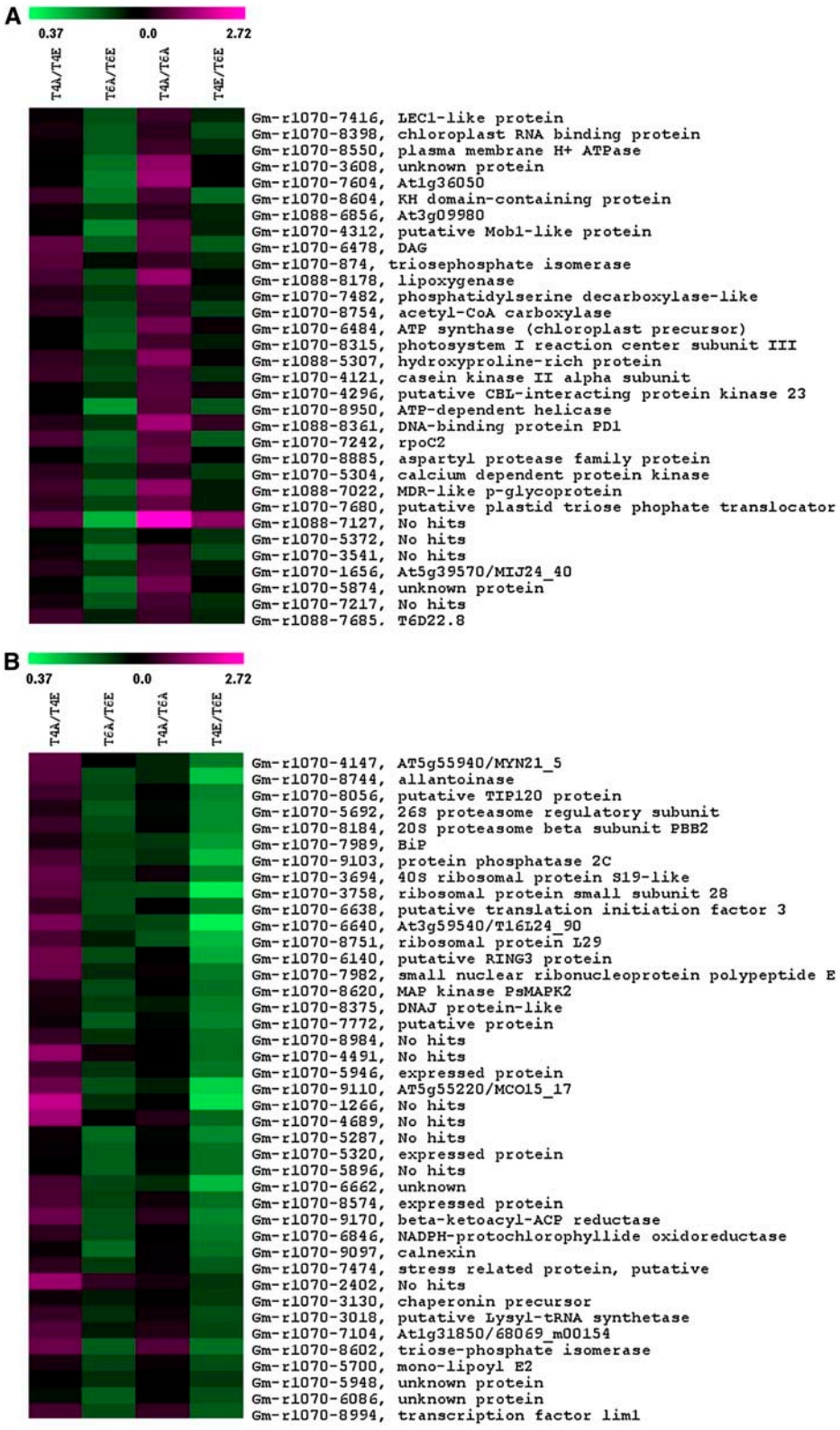
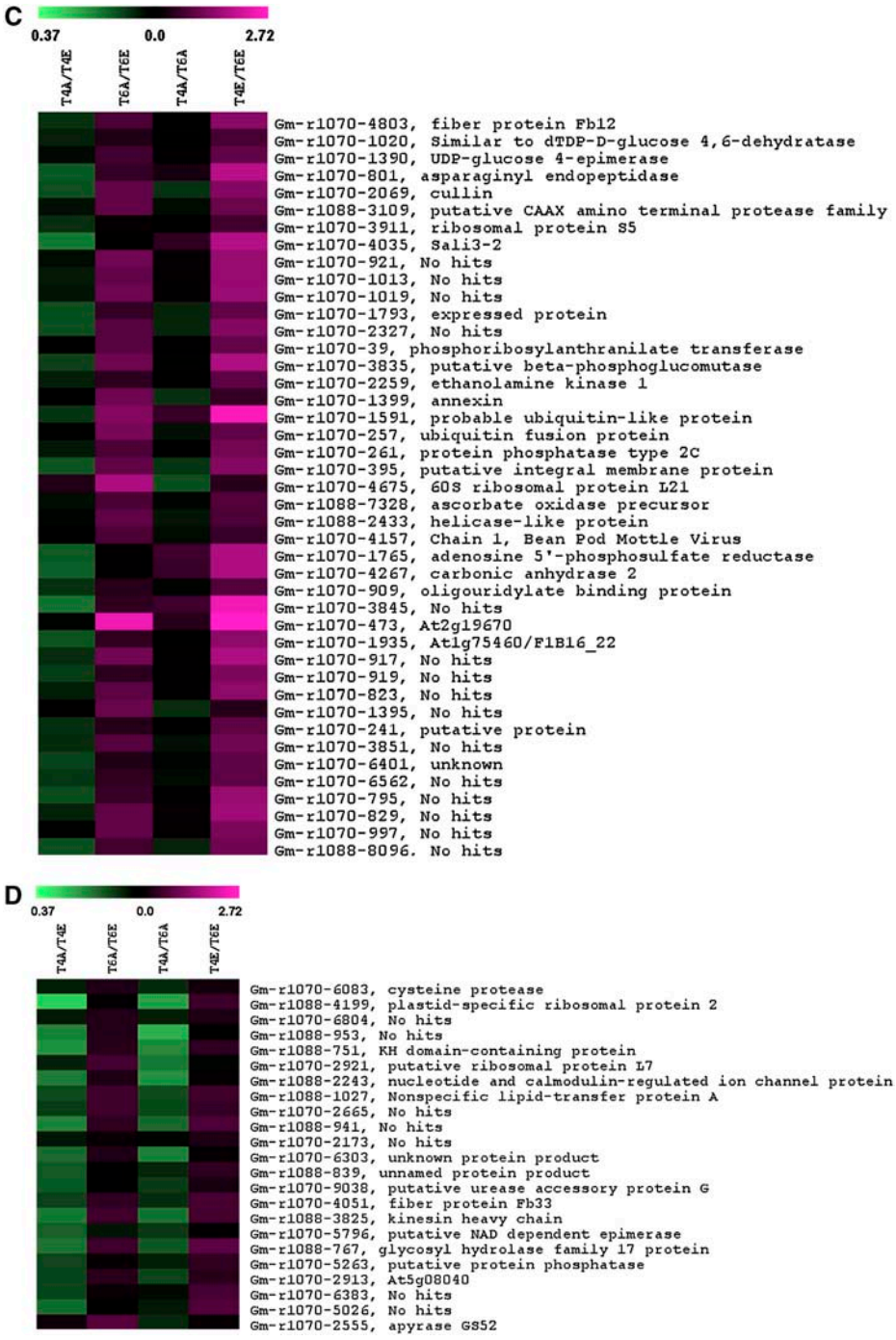


Figure 4. Heat map of transcripts with significant [CO_2] × trifoliate interaction ($P < 0.05$). Transcripts were clustered into four distinct clusters (A–D) using *k*-means clustering (TIGR MeV version 3.1). Values for each ratio are expressed by color intensity, where higher expression is indicated by shades of magenta and lower expression by shades of green. Comparisons between [CO_2] treatments and developmental stages are described in Figure 1.

Figure 4. (*Continued.*)



development, including cell division (Thomas et al., 2000). Previous transcriptome analysis of growing poplar leaves showed that glycosyl hydrolase was up-regulated in growing leaves at the time of maximum expansion (Matsubara et al., 2005), supporting our finding that glycosyl hydrolase was expressed at lower levels in fully expanded leaves compared to developing leaves. In general, the clustering of genes with a CO_2 × development interaction led to the identification of a number of transcripts involved in growth and cell proliferation with high expression in young leaves grown at elevated [CO_2].

CO_2 Response

The 327 CO_2-responsive genes were assigned to different functional categories (Fig. 5). Many genes with roles in cellular functions (i.e. cell cycle, RNA regulation of transcription, DNA synthesis, and cell organization) showed higher expression in elevated

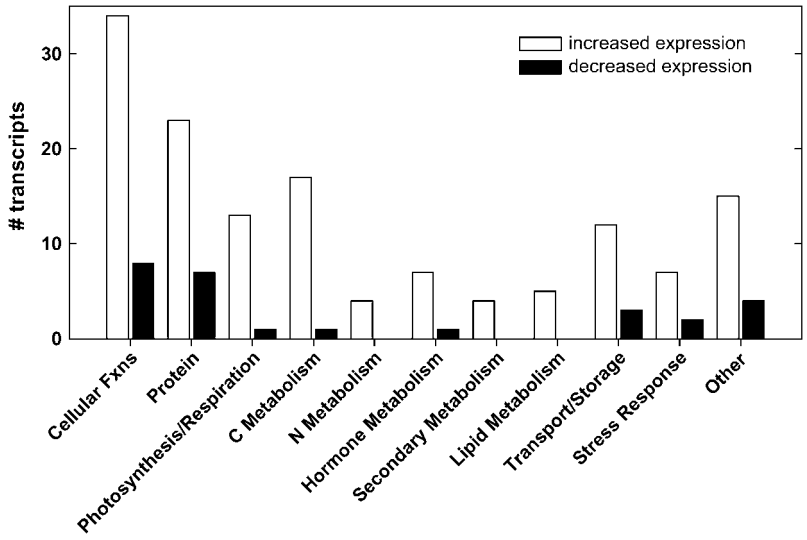


Figure 5. Categorical distribution of genes showing differential expression under elevated [CO_2].

[CO_2]. Within this category, most of the genes with higher expression in elevated [CO_2] were transcription factors (Table II). While increased expression of transcription factors suggests increased protein synthesis, most transcripts in the protein category (Fig. 5) were involved in protein degradation. These included ubiquitin-specific proteases, Cys proteinases, and different proteosome subunits (Table II). Therefore, we might hypothesize that growth at elevated [CO_2] accelerates protein turnover. Other categories where genes were differentially expressed in elevated [CO_2] included nitrogen (N) metabolism, hormone metabolism, secondary metabolism (in particular lignin biosynthesis), and transport (Fig. 5; Table II).

Growth of soybeans at elevated [CO_2] stimulates photosynthesis during the day and results in marked and significant accumulations of soluble carbohydrates and starch at the end of the photoperiod (Fig. 3, A and C; Rogers et al., 2004). Experiments on the day preceding sampling for microarray analysis showed that soybeans grown at elevated [CO_2] had increased photosynthesis and a marked end-of-day accumulation of soluble carbohydrates and starch (Fig. 3C). However, measurements of carbohydrate content made in the middle of the night showed no effect of elevated [CO_2] (Fig. 3, B and D), suggesting that plants at elevated [CO_2] were more rapidly utilizing the accumulated carbohydrate. This period of rapid carbohydrate utilization between dusk and the middle of the night coincided with the time of maximum leaf expansion in young leaves (Ainsworth et al., 2005). Therefore, increased carbohydrate utilization at elevated [CO_2] may provide more energy and biochemical precursors to fuel leaf expansion.

The transcripts of genes encoding enzymes of central C metabolism support this hypothesis. Figure 6 depicts a representation of central C metabolism, annotated with the steps where transcript levels indicated that they were up-regulated at elevated [CO_2] (Heldt, 1997; Dennis and Blakeley, 2000). There were increased levels of β-amylase, an exoamylase involved in starch degradation (Fig. 6, nos. 1–3; see also Table II). Markedly increased levels of phosphoglucomutase (Fig. 6, no. 11; see also Table II) suggest that the hexose phosphate pool was larger in leaves grown at elevated [CO_2], consistent with an increased availability of hexose from starch degradation. Moving downstream from the hexose phosphate pool, there were increased transcript levels of phosphofructokinase, the first committed step in glycolysis (Fig. 6, no. 6; see also Table II), and all of the enzymes required to make phosphoenolpyruvate from glyceraldehyde-3-P (Fig. 6, Pentose P/Triose P Pool). Elevated [CO_2] also increased transcript levels of a putative pyruvate dehydrogenase (Fig. 6, no. 12; see also Table II), which provides acetyl-CoA for the tricarboxylic acid (TCA) cycle. There was also a marked increase in phosphogluconate dehydrogenase (Fig. 6, no. 13; see also Table II) at elevated [CO_2]. This enzyme is part of the oxidative pentose phosphate (OPP) pathway that is used to provide reductant for biosynthesis and pentose phosphates for nucleotide and nucleic acid biosynthesis. In short, elevated [CO_2] clearly increased the transcript levels of genes encoding enzymes of glycolysis, entry to the TCA cycle, and the OPP pathway. Davey et al. (2004) found that long-term growth at elevated [CO_2] led to a stimulation of foliar respiration. These data suggest one potential explanation for this observation, i.e. increased flux through glycolysis and the TCA cycle fueled by higher substrate availability (starch) at elevated [CO_2]. Figure 6 and Table II also illustrate that a significant proportion of the C presumably flowing through the glycolytic pathway was diverted into secondary metabolism, in particular, cell wall, lignin, and fatty acid biosynthesis. Increases in transcripts associated with enzymes in inositol phosphate and isoprenoid biosynthesis also increased at elevated [CO_2] (Fig. 6; Table II).

While developing leaves at both ambient and elevated [CO_2] had similarly high relative leaf expansion rates, mature leaves reach a larger final area at elevated

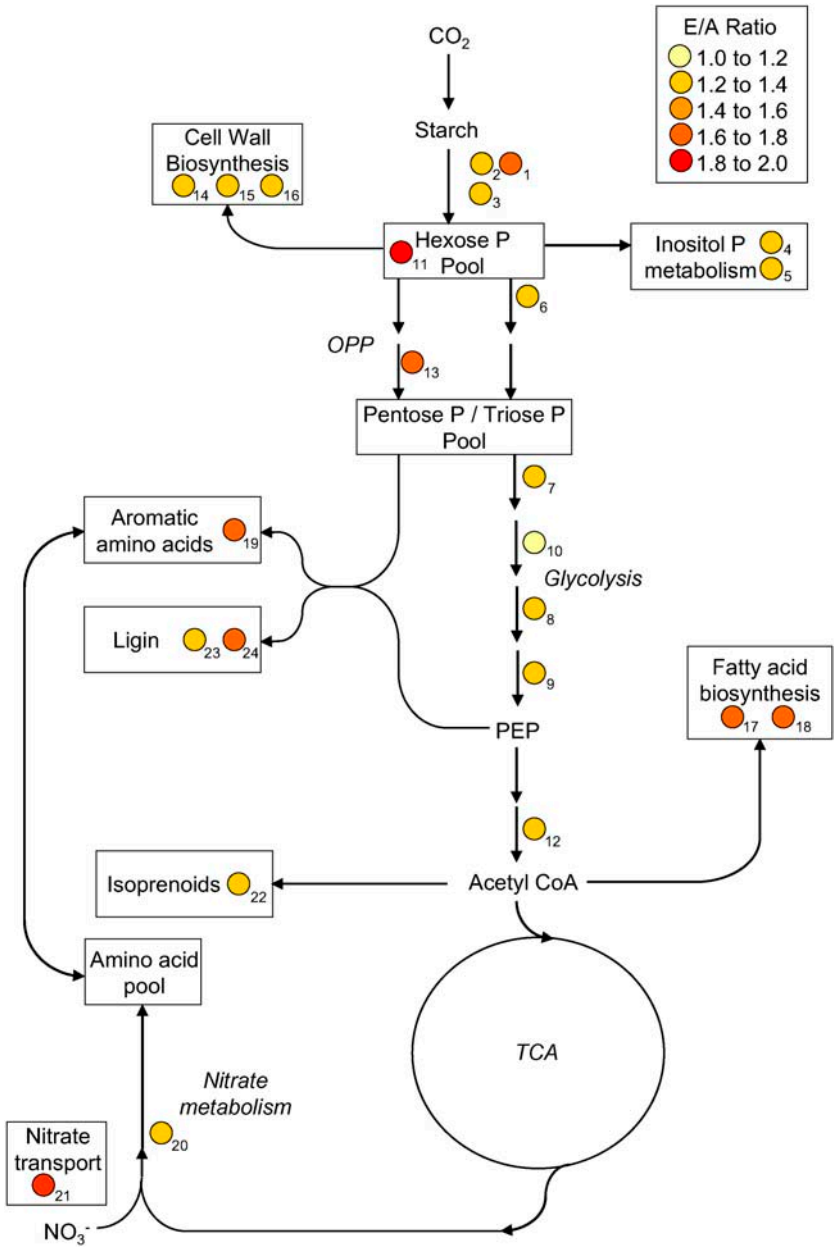


Figure 6. Graphical representation of selected gene transcripts up-regulated in response to growth at elevated [CO_2]. The arrows and boxes indicate metabolic steps, pathways, or metabolite pools in central C and N metabolism. The colored dots indicate that a gene encoding an enzyme for that step or pathway is significantly up-regulated at elevated [CO_2]. The color of the dot indicates the degree of up-regulation at elevated [CO_2] relative to ambient [CO_2] controls (E to A ratio): the darker the color, the greater the up-regulation (see insert). Each spot signifies an individual gene and is coded to the complete list of clones in Table II.

[CO_2] (Fig. 2). Data at the transcript level are consistent with the carbohydrate data (Fig. 3) and provide further evidence that biochemical precursors and energy from soluble carbohydrate and starch degradation may stimulate increased leaf growth and area at elevated [CO_2]. Carbon from carbohydrate and starch degradation may be used along with other substrates to produce cell walls and phospholipid membranes. This was supported by the increased transcript levels of genes associated with fatty acid biosynthesis and desaturases (Fig. 6; Table II).

Soybeans get most of their N through their association with N-fixing bacteria (Ritchie et al., 1997). However, early in the season when N fixation is low, plants can be N limited and this is exacerbated at elevated [CO_2] (Rogers et al., 2006). We made our measurements early in the season when soybeans at elevated [CO_2] are N limited and may be more dependent on soil-borne nitrate than fixed N. Some transcript levels of genes associated with nitrate transport and assimilation (Fig. 6, nos. 20 and 21; see also Table II) were increased at elevated [CO_2], and a transcript associated with aromatic amino acid biosynthesis (Fig. 6, no. 19; see also Table II) was also increased at elevated [CO_2]. The reduced levels of transcripts for protein synthesis and increased levels for protein degradation, cell wall biosynthesis, lignin, and fatty acid production suggest that there may be a shift away from N-rich proteins to biosynthetic products with higher C to N ratios occurring at elevated [CO_2]. This

is supported by the increased levels of the OPP enzyme phosphogluconate dehydrogenase, indicating that, at elevated [CO_2], C is being utilized for biosynthesis rather than simply increased energy production.

CONCLUSION

In this field study, we investigated the transcriptome response of soybean to elevated [CO_2] in growing and fully expanded leaves. We tested the hypothesis that increased C assimilation in plants grown at elevated [CO_2] altered pools of carbohydrates and transcripts that control growth and expansion of young leaves. It is well established that elevated [CO_2] increases photosynthetic C fixation and carbohydrate synthesis (Long et al., 2004); however, this research suggests that at the transcript level, elevated [CO_2] also stimulates the respiratory breakdown of carbohydrates, which likely provides increased fuel for leaf expansion and growth at elevated [CO_2].

MATERIALS AND METHODS

Experimental Site

Soybeans (*Glycine max* cv 93B15; Pioneer Hi-Bred) were grown at the SoyFACE facility, located in Champaign, IL (40°02′N, 88°14′W, 228 m above sea level). SoyFACE was established on a tile-drained field that has been in continuous cultivation for more than 100 years. The 32-ha site has organically rich Flanagan/Drummer series soil. Following standard agronomic practice in the region, no fertilizer was applied. The crop was planted on May 28, 2004, and measurements were made on July 8, 2004, when the crop was in the vegetative growth phase (Ritchie et al., 1997). The experiment consisted of four blocks, each containing two 20-m-diameter octagonal plots. One plot was fumigated from sunrise to sunset to an elevated target [CO_2] of 550 μmol mol^{-1}, using the FACE design of Miglietta et al. (2001); the other plot provided a current ambient [CO_2] control (375 μmol mol^{-1}). In 2004, the actual elevated [CO_2] averaged across the growing season was 550 μmol mol^{-1}. One-minute averages of [CO_2] within the plots were within $\pm$20% of the 550 μmol mol^{-1} target 93% of the time (T. Mies, personal communication).

Leaf Growth

The length of T4 and T6 lateral leaflets was tracked with a ruler ($\pm$0.1 cm) approximately every other day from initiation of T4 until sampling of both developmental stages on July 7, 2004. Growth of 12 leaflets on six randomly selected plants per plot was followed. Leaf development in field-grown plants was similar to leaf development of plants raised in growth chambers, where a homogeneous distribution of growth along the leaf blade and a distinct diurnal rhythm of expansion were described for leaflets of a similar developmental stage (Ainsworth et al., 2005).

Leaf Carbohydrates

Leaf discs from T4 and T6 middle leaflets of three plants within each plot were sampled for analysis of carbohydrates between 1 and 2 AM on July 8, 2004. Therefore, 12 leaflets per developmental stage and [CO_2] treatment were sampled. Each disc (approximately 1.8 cm^2) was removed from a vein-free area of a middle leaflet, wrapped in foil, and plunged immediately into liquid N. Samples were lyophilized prior to analysis.

Individual leaf discs were powdered in liquid N. Foliar contents of carbohydrates were extracted from ground leaf tissue in 80% (v/v), buffered (2 mM HEPES, pH 7.8) ethanol at 80°C. Four 20-min incubations were needed to recover the soluble carbohydrates. Glc, Fru, and Suc were determined using a continuous enzymatic substrate assay (Jones et al., 1977). For starch determination, pellets of the ethanol extraction were solubilized by heating to 95°C in 0.1 M NaOH. The NaOH solution was then acidified to pH 4.9, and starch content was determined as Glc equivalents (Hendriks et al., 2003). For the comparison of carbohydrates, a mixed-model ANOVA was performed with trifoliate and CO_2 treatment as fixed effects and block as a random effect (SAS Institute).

Microarray Analysis

T4 and T6 lateral leaflets from 12 individual soybeans within each plot were harvested between 1 and 2 AM. Entire leaflets were cut, wrapped in foil, plunged immediately into liquid N, and then lyophilized (Multi-Dry Lyophilizer; FTS Systems) and stored at −20°C. Total RNA was extracted from six pooled freeze-dried leaflets from each plot and developmental stage using a SDS/phenol chloroform method and lithium chloride precipitation (Wang and Vodkin, 1994). RNA content was quantified by spectrophotometry, and the integrity was confirmed using agarose gel electrophoresis (Sambrook et al., 1989). RNA was further purified using RNeasy columns (Qiagen) according to the manufacturer's instructions. Prior to labeling, purified RNA was concentrated in a Speed Vac (Savant Instruments). The cDNA synthesis, probe labeling, hybridization conditions, and slide scanning followed Vodkin et al. (2004). Microarrays from two reracked libraries, Gm-1070 and Gm-1088, were probed. The experimental design for the microarray experiment is illustrated in Figure 1. Three of the four experimental blocks in the FACE experiment were used. Each double-headed arrow represents four arrays per library, two biological samples of RNA (from six pooled leaflets), and the dye swaps (technical replicates). Therefore, a total of 96 separate hybridizations were made.

Spot intensities were quantified using Imagene 6.1 (Biodiscovery). The local background was subtracted for each spot, and spots were normalized to the median intensity of each dye on each slide. The natural log of the background-corrected median signal was used for all statistical analyses. Spots flagged by the Imagene image analysis software were removed from subsequent analyses (Prakash and Petrov, 2004). Reliability of the data was evaluated with Pearson correlation coefficients and kappa statistics on pairwise comparisons of arrays. Five slides from library Gm-1070 and three slides from library Gm-1088 had low-weighted kappa values ($<$0.50) and were dropped from the analysis. Gm-1070 contained 9,216 cDNA clones from various developmental stages of immature cotyledons, flowers, pods, and seed coats, and Gm-1088 contained 9,216 cDNA clones from cotyledons and hypocotyls of germinating seedlings and other plant parts subjected to various pathogens or environmental stress conditions (Vodkin et al., 2004). Transcripts that had missing data points on more than 20% of the arrays were also dropped from the analysis. Therefore, 5,314 transcripts from library Gm-1070 and 5,831 transcripts from library Gm-1088 were included in the analysis of variance.

Biological and technical replications were averaged for each plot for statistical analysis. A mixed-model ANOVA was performed, with trifoliate and CO_2 treatment as fixed effects and block as a random effect. The model was tested for conformation to the assumption of normality of the residuals using the Shapiro-Wilkes Test. A Bonferroni significance level was used as an initial criterion for rejecting the null hypothesis of a significant treatment effect (0.05/5,314 for Gm-1070 and 0.05/5,831 for Gm-1088). No genes were significant at the Bonferroni level, so we used a second nominal threshold of $\alpha <$ 0.05 because type I and II errors are inversely related and because Bonferroni correlation is overly conservative (Kerr and Churchill, 2001; Wayne and McIntyre, 2002). If no evidence for departure from normality of the residuals was evident and the P value for the test of differences was $\leq$0.05, the gene was considered significant, following the methods of Li et al. (2004). All analyses were performed in SAS. Reproducibility of the hybridizations and degree of variation between technical and biological replicates and experimental blocks in the field are illustrated in Supplemental Figure 1.

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