Published in final edited form as:

Planta. 2012 May ; 235(5): 939–954. doi:10.1007/s00425-011-1551-4.

Experimental sink removal induces stress responses, including shifts in amino acid and phenylpropanoid metabolism, in soybean leaves

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Abstract

The repeated removal of flower, fruit, or vegetative buds is a common treatment to simulate sink limitation. These experiments usually lead to the accumulation of specific proteins, which are degraded during later stages of seed development, and have thus been designated as vegetative storage proteins. We used oligonucleotide microarrays to assess global effects of sink removal on gene expression patterns in soybean leaves and found an induction of the transcript levels of hundreds of genes with putative roles in the responses to biotic and abiotic stresses. In addition, these data sets indicated potential changes in amino acid and phenylpropanoid metabolism. As a response to sink removal we detected an induced accumulation of γ -aminobutyric acid, while proteinogenic amino acid levels decreased. We also observed a shift in phenylpropanoid metabolism with an increase in isoflavone levels, concomitant with a decrease in flavones and flavonols. Taken together, we provide evidence that sink removal leads to an up-regulation of

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Electronic supplementary material The online version of this article (doi:10.1007/s00425-011-1551-4) contains supplementary material, which is available to authorized users.

stress responses in distant leaves, which needs to be considered as an unintended consequence of this experimental treatment.

Keywords

Flavonoid; γ -Aminobutyric acid; Isoflavone; Sink removal

Introduction

Sink-limitation experiments involving the removal of young fruit or growing vegetative shoots have been an important tool for researchers studying nitrogen metabolism in soybean, especially in relation to the mobilization of stored nitrogen from vegetative tissues to maturing seeds (Wittenbach 1983; Staswick 1989; Saravitz and Siedow 1996; Bunker et al. 1995). These experiments led to the discovery of proteins that increased in abundance within soybean leaves with sink removal, including two glycosylated proteins of 27 and 29 kDa and another (non-glycosylated) protein of approximately 94 kDa. Because these proteins accumulated in experimental sink removal studies and were degraded during later stages of seed development (seed filling), they were designated as vegetative storage proteins Vsp27 (or Vsp α), Vsp29 (or Vsp β) and Vsp94 (Franceschi and Giaquinta 1983a, b; Staswick 1988; Tranbarger et al. 1991). Wittenbach (1983) demonstrated that both Vsp27 and Vsp29 were acid phosphatases, which were subsequently shown to exert broad substrate specificity for low molecular weight polyphosphates (DeWald et al. 1992). Vsp94 was found to consist of five separate isozymes of soybean vegetative lipooxygenase (Vlx), designated as Vlx(A–E).

Subsequently, several immunocytochemical localization studies indicated that, following pod removal, both Vsp and Vlx proteins accumulated within the vacuoles of paraveinal mesophyll (PVM) cells (Franceschi et al. 1983; Stephenson et al. 1998; Fischer et al. 1999). The PVM is a distinctive, laterally elongated, layer of cells situated between the palisade and spongy mesophyll regions of soybean leaves (Fisher 1967). The occurrence of putative storage proteins within its vacuoles appeared to support a protein storage function for the PVM. However, we recently found a different subcellular localization pattern of three soybean vegetative lipooxygenase isozymes (Turner et al. 2011). Contrary to previous immunocytochemical studies, we demonstrated that none of the Vlx isozymes was present in PVM storage vacuoles. Isozymes VlxB and VlxC were localized to the cytoplasm and nucleoplasm of PVM cells, while VlxD was found only in the cytoplasm and nucleoplasm of mesophyll chlorenchyma cells. These subcellular locations are not consistent with putative roles of Vlx isozymes as storage proteins, and alternative functions thus need to be considered. In the present study we demonstrate that, based on evidence obtained with oligo-nucleotide microarrays in combination with targeted metabolite profiling, sink removal has significant transcriptional and metabolic effects beyond the intended accumulation of putative storage proteins.

Materials and methods

Plant materials

Soybean plants (*Glycine max* (L.) Merr. cv. Wye) were maintained in growth chambers under the following conditions: light intensity 360–400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (measured at 50 cm above pot level), diurnal cycle 16 h (day)/8 h (night), temperature 25°C (day)/18°C (night). Plants were kept individually in 4 L pots filled with potting compost and were fertilized once a week by alternating between Peter's Excel and Peter's Professional at 4 g L⁻¹ (Grace-Sierra Horticultural Products). Five replicate sets of experimental and control plants were grown for 30 days. Experimental plants were then subjected to a daily removal of all

axillary and terminal buds for a period of 15 days ('Detip' period), whereas controls were left untreated (C0-15) during the same time period. Following the Detip period, shoot tips on experimental plants were allowed to regrow for another 15 days (R16-30; 'Retip' period) while control plants continued to be grown without treatment (C16-30). During the Retip period plants were not fertilized but were watered on a regular schedule. Generally, each sample consisted of two leaflets taken from mature, main stem leaves that were present prior to the experimental treatment. Sample leaves (pools of two equivalent leaflets each) were immediately snap-frozen in liquid nitrogen and stored at -80°C prior to further analyses.

Microarray analysis

Three replicate sets of experimental and control plants were used to extract RNA for our microarray analysis. Because of the high costs of performing microarray experiments, the three replicate samples were chosen at random from a total of five replicates. Samples were harvested from both Detip/Retip-treated plants and untreated controls of the same age, which were maintained side-by-side under the same growth conditions. RNA was extracted from leaf samples using a method developed for tissues containing abundant polysaccharides and polyphenols (Gehrig et al. 2000). In addition, the extracted RNA was further purified using the RNeasy kit (Qiagen). Each RNA sample (C0, C15, C20, C30, D15, R20, R30; 3 independent biological replicates each; 21 samples total) was converted to cRNA and hybridized to Affymetrix soybean GeneChips according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA, USA). Data (.cel files) from these hybridizations were loaded into R using the *affy* package (Gautier et al. 2004). The data sets were pre-processed using the *gcrma* package, which uses sequence information to correct for background signal, while employing quantile normalization and median polishing to produce $\log_2$ transformed probe set expression values (Wu et al. 2004). First 'unexpressed' genes were eliminated by removing probe sets for which the statistical means of all seven sample sets fell below the 99% quantile of all absent values. This value was 3.265. It was determined from PMA calls, with marginal calls also scored as absent for our analysis. 28,028 probe sets had group mean expression values above this cutoff. Next, filtering by gene inter-quartile range (IQR) was used to remove probe sets with low variability. Probe sets with IQRs less than 0.5 were removed from further consideration (24,095 probe sets passed this criterion). Then, the *limma* package (linear models for microarray data; Smyth 2005) was used to calculate differences between each group and C0 controls. The model was based on a randomized design where the C0 control was considered the intercept. *Limma* further adjusted the variance using an empirical Bayes method. Through these procedures we obtained raw *P* values for each comparison. These *P* values were then corrected (also within *limma*) using the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995). Probe sets with an adjusted *P* value < 0.05 were considered to be differentially expressed. This criterion was met by 2 probe sets in C15, 619 probe sets in D15, 2,843 probe sets in C20, 963 probe sets in R20, 1,582 probe sets in C30, and 563 probe sets in R30 (Supplemental Material S1). Meta data for the experimental design and the microarray analysis are provided as Supplemental Material S2. All raw data files (.cel and .chp) were submitted to NCBI's Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/geo/>; accession number GSE23128).

Quantitative real-time PCR analysis of transcript abundance

Total RNA was extracted from frozen soybean leaves (five replicate sets of experimental and control plants) using the ConcertTM Reagent (Invitrogen) and further purified using the RNeasyTM Mini Kit (Qiagen) according to the manufacturer's instructions. Isolated RNA (300–700 ng) was treated with RNase-free DNase (Fermentas Life Science) and first-strand cDNA synthesized using reverse transcriptase III (Invitrogen). RNA isolation and cDNA synthesis were carried out with three independent biological replicate samples. In a 10 μL

qPCR reaction, concentrations were adjusted to 150 nM (primers), 2.5 μ M (oligo dTs) and 0.5 mM (dNTPs). Reactions were performed in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60° for 10 min in a 7500 Real-Time PCR system (Applied Biosystems). Fluorescence intensities of three independent measurements (technical replicates) were normalized against the ROX reference dye. For each sample, the amount of target and endogenous control (β -actin gene; U60499) were determined using the comparative CT method according to the manufacturer's instructions (Applied Biosystems). For one replicate set of samples we used new enzymes and reagents, which turned out to be of insufficient quality and the acquired data were unusable. We are thus only reporting data for four replicate sets of samples.

Amino acid analysis

Frozen tissue samples were dry-milled for 2 min under liquid nitrogen and extracted twice for 1 h each at 23°C with 500 μ L 80% aq. EtOH containing 50 nmol *L*-norvaline as an internal standard. Supernatants for each sample were pooled, loaded onto a Dowex (H^+ form) column and washed twice with 1 ml 80% aq. EtOH and 1 mL H_2O (also twice). Amino acids were eluted from the Dowex column using 6 M aqueous ammonia. Extracts were evaporated to dryness under reduced pressure, the remaining pellet was resuspended in 50 μ L DMF and 20 μ L MTBSTFA (*N*-methyl-*N*-[tert-butyldimethylsilyl]trifluoroacetamide), and heated to 74°C for 1 h. Aliquots of 1 μ L were used for analysis on a GC/MS instrument (Leap Technologies CombiPal Autosampler and Agilent Technologies 6890N GC interfaced with an Agilent Technologies 5973 inert MSD). Separation of amino acids was achieved using an HP5-MS column (J&W Scientific; 0.25 mm $\times$ 30 m $\times$ 0.25 μ m). GC settings were as follows: inlet 270°C, split 15:1, column flow 1.0 mL/min. The GC oven program had an initial temperature of 130°C, which was held for 2 min, ramped to 290 at 5°C per min, and held at the final temperature for 4 min. The MS source was set to 230°C, the MS quadrupole to 150°C, and the MS transfer line to 280°C. Peak areas for each amino acid were adjusted for sample weight and recovery. Absolute quantification of amino acids was performed based on calibration curves with authentic standards. Student *t* tests to assess statistical significance of differences between control and experimental samples were performed in Microsoft Excel.

Flavonoid/isoflavone aglycone analysis

Roughly 100 mg of frozen leaf tissue was homogenized in a ball mill (Retsch) for 2 min at in the presence of liquid nitrogen. The homogenate was extracted for 2 h at 23°C with 2 mL 80% aq. MeOH containing 3.15 mg/L 9-anthracene carboxylic acid as an internal standard. The mixture was centrifuged at 8,000*g* for 2 min, the supernatant removed and filtered through a 0.45 μ m PTFE filter. The filtered supernatant was dried under reduced pressure using an EZ-Bio system (GeneVac). The residue was resuspended in 3 mL 1 M HCl and then incubated at 90°C for 2 h. The mixture was allowed to cool, 3 mL EtOAc were added, and the mixture vortexed for 5 min at 23°C. The EtOAc phase was removed with a Pasteur pipette and the residue extracted against another 3 mL EtOAc for 5 min at 23°C. The combined EtOAc extracts were dried under reduced pressure at 23°C. The dried residues were either stored at -80°C until further use or directly resuspended in 1 mL MeOH with 0.2% acetic acid. The suspensions were filtered through a 0.45 μ m PTFE filter into 2 mL glass vials. LC/MS analysis was performed using a 1200 Rapid Resolution system interfaced with a 6520 series Quadrupole Time-of-Flight Mass Spectrometer (Agilent Technologies). The filtered sample solution (5 μ L) was loaded onto a C8 cartridge column (2.1 $\times$ 30 mm, 3.5 μ m internal diameter) connected to a Zorbax SB-Aq (2.1 $\times$ 100 mm, 1.8 μ m internal diameter) analytical column (both from Agilent Technologies). Separation was achieved at 60°C with a flow rate of 0.6 mL/min using gradient of water with 0.2% acetic acid (A) and methanol with 0.2% acetic acid (B). The gradient started at 2% B (hold for 2 min) with a

linear increase to 98% B at 16 min (hold for 3 min). Before the injection of the next sample, the column was re-equilibrated under the initial conditions for 5 min. The mass spectrometer was operated in 2 GHz high gain acquisition mode with an ESI source in negative ion polarity. Analytes were identified by comparison of retention time, mass-to-charge ratio and mass fragmentation patterns with those obtained with authentic standards: daidzein ($[M-H]^- = 253.0506$), genistein ($[M-H]^- = 269.0455$), glycitein ($[M-H]^- = 283.0612$), kaempferol ($[M-H]^- = 285.0405$), apigenin ($[M-H]^- = 269.0455$), biochanin A ($[M-H]^- = 283.0612$), naringenin ($[M-H]^- = 271.0612$), formononetin ($[M-H]^- = 267.0663$), 7,4'-dihydroxyflavone ($[M-H]^- = 253.0506$) and coumestrol ($[M-H]^- = 267.0299$). Signals for target analytes were integrated and resulting peak areas were adjusted for internal standard loading and initial tissue sample weight. Quantitative values were calculated based on calibration curves with authentic standards. Matrix interference was determined to be negligible for all analytes.

Analysis of total phenolics, anthocyanins, chlorophylls, and carotenoids

Total phenolics were assayed with the Folin-Ciocalteu method (Singleton et al. 1999), total anthocyanins according to Lichtenthaler (1987), and total chlorophylls and carotenoids based on Lange et al. (1971).

Results

Phenotypic differences between controls and plants subjected to sink removal

The experimental design is summarized in Fig. 1a (for details see “Materials and methods”). Experimental bud removal caused evident phenotypic changes of the morphology of the experimental plants. When compared to control plants, leaves of ‘detipped’ plants (subjected to daily bud removal) appeared to be considerably thicker and darker (Fig. 1b). During the Retip period, this dark color was partially lost and leaves showed some yellowing. The leaves of control plants exhibited yellowing much earlier than those of plants subjected to a sink removal treatment. In order to quantitatively investigate the visually apparent differences in pigmentation between controls and detipped samples we determined the levels of total chlorophylls (TChl) and carotenoids (TCar). TChl levels in controls remained constant at 2.0–2.2 μg per mg fresh weight (FW) during the first 15 days of the experimental period (C0 to C15), and then decreased sharply to 1.0 μg per mg FW at C22 (Fig. 1c). In plants subjected to daily sink removal, TChl levels increased steadily from 1.9 μg per mg FW at D0 to 2.6 μg per mg FW at D15, before decreasing slightly to 2.3 μg per mg FW at R22. TChl differences between controls and detipped plants were statistically significant (P values of 2.73×10^{-8} and 2.22×10^{-5} , respectively) for the R20 and R22 time points. In controls, TCar levels increased for the first 10 days of the experimental period (from 0.29 to 0.35 μg per mg FW), and then dropped to 0.19 μg per mg FW at C22 (Fig. 1d). In Detip/Retip-treated plants TCar levels remained constant (0.33–0.35 μg per mg FW) throughout the experimental period, and were significantly higher than in controls at the R20 and R22 time points (P values of 1.44×10^{-6} and 1.49×10^{-4} , respectively).

Gene expression changes in response to sink removal

Leaf RNA isolated from experimental and control soybean leaves was converted to cRNA and hybridized to Affymetrix soybean GeneChip microarrays. Microarray raw data were filtered to eliminate genes with low expression values and low variance, and then analyzed in a comparative model to select genes with significantly different expression levels compared to C0 control samples harvested at the beginning of the experimental period (P value <0.05) (Benjamini and Hochberg 1995; Smyth 2005) (Supplemental Material S1). At the D15 time point this list contained 619 probe sets, while only 2 probe sets were in the corresponding C15 list. These gene lists were sorted into functional categories by gene

ontology (GO) annotation using AgriGO, an online tool for agricultural crops, including soybean (Du et al. 2010). A singular enrichment analysis was employed to evaluate the enrichment of a GO term in a query list of genes (i.e. genes up-regulated by sink removal treatment) in comparison to the prevalence of the same GO term in a reference list (all genes represented on the soybean genome microarray). The most significant positive enrichment (D15 up-regulated list) was determined for the GO terms 'cellular amino acid derivative biosynthetic process' (GO 0042398; P value 7.5×10^{-16}), 'aromatic compound biosynthetic process' (GO 0019438; P value 1.6×10^{-17}) and 'phenylpropanoid metabolic process' (GO 0009698; P value 6.4×10^{-18}) (Table 1). A BlastX search with sequences represented by these probe sets against the non-redundant NCBI protein database confirmed that many of these genes had putative roles in the phenylpropanoid metabolic pathway, thus confirming the results of the GO analysis (Table 1). Genes related to phenylpropanoid metabolism were also found in the lists of the most enriched GO categories of the other experimental samples (R20 and R30) (Supplemental Material S1). The second most positively enriched GO term in all experimental samples was 'oxylipin biosynthetic process' (GO 0031408; P value 0.014), a category that includes the genes that encode Vlx isoforms (Table 2). We also used the MapMan bioinformatic tool (Rotter et al. 2007) to identify functionally related genes up-regulated by sink removal (Supplemental Material S1). This analysis indicated a treatment-induced enrichment in genes related to biotic and abiotic stress responses (GO categories 0009607 and 0009628, respectively), including proteinase inhibitors, putative stress-induced proteins, disease resistance-related proteins, pathogenesis-related proteins, and heat shock proteins (Table 2).

To evaluate these findings with an independent technique, we used quantitative real-time PCR (qPCR) to determine the relative expression levels of a representative of each functional subcategory of stress-related genes in Table 2. Compared to untreated controls at C15 the expression levels of the genes encoding Vlx isozymes VlxD and VlxE were 25.6- and 13.0-fold higher, respectively, in leaves of Detip-treated plants at D15 (Fig. 2). The expression coefficients shown in Table 2 translate to a 17-fold higher expression level of VlxE and a 5-fold higher transcript abundance of VlxD (Detip vs. control). The serine proteinase inhibitor gene (probe set Gma.3612 on the oligonucleotide microarray) increased 25-fold (based on qPCR) (Fig. 2) by the sink removal treatment compared to controls (40-fold based on microarray results). The stress-induced gene represented by probe set Gma.11119 on the microarray was induced 4.7-fold (based on qPCR) (Fig. 2) in Detip samples when compared to controls (22-fold higher based on microarray results). Other stress-related genes up-regulated (compared to untreated controls) by sink removal treatment included a gene with homology to disease resistance response proteins (probe set GmaAffx.91558 on the oligonucleotide microarray; 3.6-fold increased by qPCR; 11-fold higher based on microarrays), a class 10 pathogenesis-related protein (probe set GmaAffx.18040 on the oligonucleotide microarray; 3.7-fold increased by qPCR; 18-fold higher based on microarrays), and a gene encoding a putative 70 kDa heat shock protein (probe set GmaAffx.92270 on the oligonucleotide microarray; 1.1-fold increased by qPCR; 4.1-fold higher based on microarrays) (Fig. 2). Overall, the trend toward an increase of genes with putative functions in stress responses following sink-removal, as observed with microarray analyses, was confirmed by qPCR experiments (with the exception of a heat shock-related transcript that was only marginally increased).

Gene expression changes in untreated controls

The sink removal experiment spanned a 30 days time frame and, as described above (Fig. 1b), phenotypic changes were visible in both experimental and control samples. Toward the end of this time period we detected a loss of pigments, a process that appeared to be delayed in experimental samples (Fig. 1c, d). We investigated if these developmental changes in

leaves of untreated control plants were reflected in our microarray data set. Compared to the samples harvested at the beginning of the experimental period (C0), 932 unique genes were significantly down-regulated in C20 leaves, while 673 genes were listed with increased expression levels at C30 (Supplemental Material S1). An analysis of our time course microarray data with the AgriGO tool revealed an enrichment of three GO categories in the lists of down-regulated transcripts in C20 and C30 samples: GO 0015979 ('photosynthesis'; P value 8.79×10^{-41}); GO 0042440 ('pigment metabolic process'; P value 3.66×10^{-5}); and GO 0019497 ('hexachlorocyclohexane metabolic process'; P value 3.84×10^{-3}). The 'photosynthesis' category consisted of genes related to photosystems and photosynthate metabolism in plastids (primarily Calvin/Benson cycle) (Table 3). The 'pigment metabolic process' category was comprised of genes involved in the metabolism of chlorophylls and carotenoids (Table 3). These findings are in full agreement with phenotypic and phytochemical observations described above (yellowing and loss of chlorophylls and carotenoids). Lastly, the 'hexachlorocyclohexane metabolic process' category contained genes encoding members of the haloacid dehydrogenase superfamily (Table 3), which includes Vsp and other acid phosphatases (DeWald et al. 1992). In this case the GO category annotation is misleading (Vsp does not metabolize hexachlorocyclo-hexane but is part of a larger family of proteins with members that do metabolize this chemical). However, our annotation was derived by searches against various plant annotation databases and the categorization of these genes as acid phosphatases was straightforward. Relative to C0 there were 1,048 genes with increased expression levels at C20 and 342 genes at C30 (Supplemental Material S1). The only enriched functional category in the data set of up-regulated genes at C20 was GO 0034641 ('cellular nitrogen compound metabolic process'; P value 7.89×10^{-14}). This category primarily contained genes involved in amino acid metabolism and the formation of aminoacyl transfer RNAs (Table 4).

Effect of sink removal treatment on amino acid levels

The enrichment of genes of the GO category 'cellular nitrogen compound metabolic process' in control but not experimental samples (Table 4) indicated that there might be differences in the homeostasis of amino acids. To investigate this further we analyzed both total protein and soluble amino acid levels. The total protein content of control leaves decreased dramatically from 7.6 μ g per mg FW at C0 to 0.5 μ g per mg FW at C22 (92% loss) (Fig. 3). In contrast, the total protein content rose in Detip/Retip-treated plants (13% up at D10, corresponding to 8.6 μ g per mg FW), and then dropped slightly to 83% of D0 levels at R22 (6.3 μ g per mg FW). To evaluate the effects of the Detip/Retip treatment on metabolic processes related to protein accumulation, we also quantified soluble amino acid levels (Fig. 3). The total concentration of proteinogenic amino acids in controls decreased sharply from 5,446 pmol per mg FW at C0 to 742 pmol per mg FW at C22, while a much less pronounced and delayed decrease was detected in experimental samples (from 5,855 pmol per mg FW at D0 to 1,589 pmol per mg FW at R22). At C0/D0, the amino acids L-Asn, L-Gln, L-Arg, L-Glu and L-Asp, which have been implicated in nitrogen transport (Rentsch et al. 2007), were highly abundant (up to 1,447 pmol per mg FW) (Fig. 3). During the course of the experimental period the concentrations of L-Asn, L-Gln, L-Arg and L-Asp decreased sharply in control samples, whereas the L-Glu concentration decreased more gradually. In Detip/Retip-treated samples, L-Gln and L-Arg concentrations increased transiently, before sharply falling to control levels (Fig. 3). In comparison to controls, the decline of L-Asn was delayed in experimental samples. L-Asp and L-Glu concentrations decreased in plants subjected to sink removal (from 1,100 to 200 pmol per mg FW and 1,500 to 900 pmol per mg FW, respectively) at the end of the experimental period, but this decrease was much less pronounced than in control samples (at C22 L-Asp was at <10 and L-Glu at 500 pmol per mg FW). Medium abundance amino acids at C0/D0 (50–1,000 pmol per mg FW) included L-Ser, L-Ala, L-Thr, and L-Phe (Fig. 3). The decline of these amino

acids was slower in experimental samples compared to controls. L-Phe concentration increased transiently in Detip/Retip-treated samples (2.9-fold up at D5; P value 3×10^{-5}). The concentrations of low abundance amino acids, such as L-Gly, L-Val, L-Leu, L-Ile, L-Pro and L-Tyr (10–40 pmol per mg FW at C0/D0), decreased in controls over time but to a lesser extent than medium and high abundance amino acids (Fig. 3). The concentrations of these amino acids were generally higher in Detip/Retip-treated samples (compared to controls). The concentrations of L-Leu, L-His, L-Trp, L-Cys and L-Lys remained at extremely low levels (<10 pmol per mg FW) throughout the experimental period in both controls and plants subjected to sink removal. The concentrations of γ -aminobutyric acid (GABA), which is derived metabolically from L-Glu, fluctuated between 70 and 110 pmol per mg FW in controls throughout the experimental period. In contrast, a very strong induction of GABA occurred in experimental plants (Fig. 3) with increases up to 360 pmol per mg FW at R20 (4.1-fold compared to C0/D0; P value 3×10^{-6}) and 7.6-fold compared to C20; P value 2×10^{-7}).

Effect of sink removal treatment on phenylpropanoid levels

Guided by the observation of a differential induction of various genes related to phenylpropanoid metabolism by the sink removal treatment (Table 1), we quantified representative metabolic end products of this pathway. The concentrations of total phenolics were very similar in controls and Detip/Retip-treated plants (Fig. 4a) and did not change significantly during the experimental period. In plants subjected to shoot tip removal total anthocyanin concentrations increased slightly above the levels of C0 controls. In contrast, anthocyanin concentrations in controls increased dramatically during the experimental period (15.9-fold higher at C30 compared to C0) (Fig. 4b). Differences between controls and experimental samples were statistically significant ($P < 0.001$) at C10 (2.0-fold higher than D10), C15 (2.9-fold higher than D15), C20 (4.3-fold higher than R20), and C22 (7.6-fold higher than R22) (Fig. 4c). Flavonoids extracted from C15 and D15 samples were separated by Rapid Resolution HPLC and detected, identified and quantified using a QTOF mass spectrometer. The concentration of the most abundant flavonoid, kaempferol, was 25% lower in D15 (1,462 pmol per mg FW) compared to C15 (1,833 pmol per mg FW) (P value 0.03). A trend toward lower concentrations in experimental samples (D15 vs. C15) was also observed for naringenin (P value 0.05) and, to a lesser extent, apigenin (P value not significant) (Fig. 4c). In contrast, isoflavone concentrations were higher in experimental samples (D15 vs. C15), with genistein increased by 29% (P value 0.13), daidzein by 86% (P value 0.01), formononetin by 107% (P value 0.04), biochanin A by 100% (P value 0.07), glycitein up by 138% (P value 0.04), and coumestrol up by 170% (P value 0.07) (Fig. 4c).

Discussion

Vegetative shoot tip removal induces the expression levels of genes putatively involved in stress responses in distant leaves

The sink removal protocol was originally developed to study the dynamics of source-sink relationships, including the transient accumulation of putative vegetative storage proteins in leaves (Franceschi and Giaquinta 1983a, b; Wittenbach 1983; Bunker et al. 1995; Murphy et al. 2005). However, this procedure involves repeated mechanical wounding (removal of vegetative shoot tips or seed pods), which has potential to induce stress or defense responses in distant leaves. In this context it is important to compare our microarray results obtained with soybean plants subjected to a sink removal treatment with those reported for experiments involving mechanical wounding, pathogen attack or herbivory. Reymond et al. (2000) used *Arabidopsis* cDNA microarrays to assess global transcript patterns following wounding, dehydration, and cabbage butterfly feeding. Genes identified as specifically responsive to wounding included those involved in phenylpropanoid metabolism and

pathogenesis-related (PR) genes. De Vos et al. (2005) used full-genome microarrays to monitor the dynamics of gene expression following challenge of *Arabidopsis* plants with phytopathogenic bacteria, fungi, caterpillars, thrips or aphids. Although they documented clear differences in the accumulation of the signaling metabolites jasmonic acid, ethylene, and salicylic acid, they were not able to distinguish treatments based on gene expression patterns, because changes in the expression levels of hundreds of genes related to stress-responses were reported for all treatment regimes (De Vos et al. 2005). Similarly, the infection of soybean with a nematode resulted in the induction of 429 transcripts, including genes involved in phenylpropanoid metabolism and general stress responses (Ithal et al. 2007). The responses of poplar to feeding by forest tent caterpillars included the up-regulation of homologs of Vlx lipoxygenases identified as putative storage proteins in the context of soybean sink-limitation experiments as well as genes involved in phenylpropanoid metabolism (Major and Constabel 2006; Ralph et al. 2006a). Furthermore, wounding or herbivory in spruce led to the global induction of transcripts involved in the phenylpropanoid and oxylipin metabolic pathways (Ralph et al. 2006b). Li et al. (2008) found that two soybean cultivars responded to aphid infestation with a strong induction of genes encoding lipoxygenase, PR1, and enzymes involved in phenylpropanoid metabolism. Finally, Mithöfer et al. (2005) concluded that repeated mechanical wounding of lima bean elicited patterns of volatile emissions similar to those induced by caterpillar feeding. The repeated 'detipping' in our experiments appeared to have had similar effects. In our Detip/Retip experiments with soybean, we observed a strong up-regulation of transcripts encoding the same classes of proteins (Vlx and Vsp) and genes that belong to the same metabolic pathways (phenylpropanoid metabolism) that have been identified as being induced by wounding or insect feeding (Fig. 2; Table 2).

Vegetative shoot tip removal induces a redistribution of flux through the phenylpropanoid pathway and an accumulation of γ -aminobutyric acid

Our sink removal treatment led to an increase in the concentrations of total isoflavones (71% higher in D15 compared to C15; P value 0.004) and a concomitant decrease in total flavone/flavonol levels (27% lower in D15 compared to C15; P value 0.009) (Fig. 5). Interestingly, the concentration of all flavonoids and isoflavones combined was roughly the same in controls (2,569 pmol per mg FW at C15) and Detip/Retip-treated samples (2,518 pmol per mg FW at D15), indicating that the experimental treatment may have led to a general redirection of carbon flux within the phenylpropanoid pathway (Fig. 5). These patterns at the metabolite level correlated with highly significant differences in the expression levels of genes with putative and established functions in the phenylpropanoid pathway (Fig. 4b). The up-regulation of isoflavone biosynthesis, based on cDNA microarray data, has also been reported for soybean leaves infected with the bacterial pathogen *Pseudomonas syringae* (Zou et al. 2005; Zabala et al. 2006), the fungal pathogen *Phakospora pachyrhizi* (Alvord et al. 2007) and the aphid *Aphis glycines* (Li et al. 2008). An increased accumulation of isoflavones was observed in soybean leaves after various experimental treatments, including wounding (Wegulo et al. 2005), inoculation with fungi (Morris et al. 1991; Wegulo et al. 2005), infection with phytopathogenic bacteria or nematodes (Ingham et al. 1981; Osman and Fett 1983; Fett 1984; Itah et al. 2007), treatment with abiotic and biotic elicitors (Davis et al. 1986), and application of herbicides (Cosio et al. 1985). Considering these responses of soybean to experimental stress conditions, our observation of the sink removal-associated shift toward isoflavone accumulation, at the expense of flavones and flavonols, appears to be consistent with the activation of stress responses in soybean leaves. The potential reasons for such a metabolic shift remain to be elucidated.

We also observed dramatic increases of GABA (Fig. 3), while the concentrations of most proteinogenic amino acids decreased, in leaves of Detip/Retip-treated soybean plants.

GABA has been demonstrated to accumulate as a response to various stresses, including acidosis (Crawford et al. 1994), mechanical damage (Wallace et al. 1984), cold (Wallace et al. 1984; Cholewa et al. 1997), anoxia (Aurisano et al. 1995), heat (Mayer et al. 1990), drought (Raggi 1994), and salt (Bolarin et al. 1995). The concentration of GABA under stress can increase to match or exceed those of amino acids involved in protein synthesis (Wallace et al. 1984; Mayer et al. 1990), as observed in the experiments described in this study. However, the role of GABA in such stress responses has remained elusive. Proposed functions include the regulation of cellular pH, maintaining the carbon/nitrogen balance, or as an osmolyte (reviewed in Fait et al. 2007). Newer work has suggested a possible role of GABA as an antioxidant during stress responses (Liu et al. 2011). GABA is an important neurotransmitter in insects and plant-produced GABA has been demonstrated to be highly effective in the defense against insects (reviewed in Huang et al. 2011). Although an in-depth analysis of the role of GABA in the context of our sink removal experiments is beyond the scope of this study, there are striking parallels to GABA accumulation observed under various stress conditions, in particular insect herbivore attack.

Conclusions

We present several lines of evidence that the removal of vegetative shoot tips from soybean plants results in the up-regulation of stress responses in distant leaves: (i) microarray and qPCR data indicated increases (compared to untreated controls) of genes involved in stress responses; (ii) metabolite profiling analyses provided evidence for a metabolic shift in phenylpropanoid metabolism toward isoflavone synthesis, concomitant with a decrease in flavones and flavonols; and (iii) dramatic increases in GABA concentration were observed, while proteinogenic amino acid levels decreased. Although the immediate functional roles of these responses are currently unknown, we have demonstrated that a sink removal treatment similar to those used to induce the accumulation of putative vegetative storage proteins has unintended consequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a Grant from the Department of Energy (DE-FG02-05ER15672 (H.D.G.)). We are grateful to Mr. Derek Pouchnik (School of Molecular Biosciences) for performing microarray hybridizations and assisting in data analysis. We also thank Ms. Sue Vogtman (Institute of Biological Chemistry) and Mr. Charles Cody (School of Biological Sciences) for help with growing plants.

Abbreviations

PVM	Paraveinal mesophyll
Vlx	Vegetative lipoxygenase
Vsp	Vegetative storage protein
FW	Fresh weight
PCR	Polymerase chain reaction

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Fig. 1.

Design of sink removal experiment and phenotypic characterization of plants. **a** Soybean plants were maintained in climate-controlled growth chambers for 30 days. Experimental plants were then subjected to a daily removal of vegetative shoot tips ('Detip' period) for 15 days (time points designated D0 to D15). Following this treatment shoots were allowed to regrow for another 15 days ('Retip' period) (time points designated R16 to R30). Controls remained untreated through the experimental period (time points designated C0 to C30). Plants were watered with fertilizer for 45 days (up to the C15/D15 time point; indicated by *light blue* background), after which the fertilizer was omitted. **b** Phenotypic response of plants to sink removal. 'Detipped' plants at D15 are smaller than controls (C15) and have darker leaves. Controls show signs of senescence at C30, whereas Detip/Retip-treated plants (at R30) maintain darker leaves and only the new growth shows sign of senescence. **c** Total chlorophyll levels. **d** Total carotenoid levels. Patterns observed with control plants are depicted by *hollow circles*, while *filled black triangles* are used to indicate values for Detip/Retip-treated plants; mean values ($n = 3$) $\pm$ SD

Fig. 2.

Quantitative real-time PCR analysis of putative stress-related genes up-regulated by sink removal treatment (fold change of D15 Detip-treated plants vs. C15 controls $\pm$ standard deviation). The level of significance is indicated by one ($P < 0.05$), two ($P < 0.01$) or three ($P < 0.001$) asterisks; mean values ($n = 4$) $\pm$ SD. The identifiers in parentheses refer to the corresponding probe set IDs on Affymetrix soybean oligonucleotide microarrays (for details see Table 2). As a reference, a *broken line* indicates the expression level of genes in untreated controls

Fig. 3. Effect of sink removal on protein content and the concentration of soluble amino acids. Patterns observed with control plants are depicted by *hollow circles*, while *filled black triangles* are used to indicate values for Detip/Retip-treated plants; mean values ($n = 5$) $\pm$ SD. Various shades of *black and gray* are used as background to indicate the abundance levels of different amino acids

Fig. 4.

Effect of sink removal on total phenolics (a), total anthocyanins (b), and flavonoids/isoflavones (c). *P* values for the comparison of C15 and D15 metabolite concentrations are listed; the level of significance is indicated by one (*P* < 0.05), two (*P* < 0.01) or three (*P* < 0.001) asterisks; *n.s.* not significant; mean values (*n* = 5) ± SD

Fig. 5. Redirection of carbon flux during sink removal. **a** Outline of the flavonoid/isoflavone pathway. Differences of metabolite levels in C15 and D15 samples are indicated with a red/blue color code (see image for details). **b** Comparison of total flavonoid and total isoflavone levels at C15 (hollow bars) and D15 (gray bars) ($n = 4$; standard deviations are indicated). P values for the C15 vs. D15 comparison are listed. The level of significance is indicated by one ($P \leq 0.05$), two ($P \leq 0.01$) or three ($P \leq 0.001$) asterisks

Table 1

Gene ontology (GO) analysis, using the AgriGO tool (Wu et al. 2004), of genes affected by sink removal treatment

Functional annotation	Affymetrix soybean genome microarray probe set ID	GO sub-category
Flavonoid/anthocyanin biosynthesis		
Naringenin-chalcone synthase EC 2.3.1.74	Gma.4300.3.S1_s_at	GO:0042967 acyl-carrier-protein biosynthetic process
	Gma.4300.3.S1_x_at	GO:0016210 naringenin-chalcone synthase activity
	Gma.4300.1.S1_s_at	GO:0008415 acyltransferase activity
	Gma.4300.1.A1_at	GO:0009813 flavonoid biosynthetic process
Chalcone/6'-deoxychalcone reductase (EC 2.3.1.170)	GmaAffx.91944.1.S1_s_at	GO:0016740 transferase activity
		GO:0044464 cell part
		GO:0009813 flavonoid biosynthetic process
		GO:0016491 oxidoreductase activity
Chalcone isomerase EC 5.5.1.6	Gma.2826.1.S1_x_at	GO:0045430 chalcone isomerase activity
	Gma.3562.1.S1_at	GO:0009813 flavonoid biosynthetic process
	GmaAffx.92003.1.S1_x_at	
	Gma.15492.1.S1_s_at	
	GmaAffx.92003.1.S1_s_at	
	Gma.2826.1.S1_at	
Putative dihydroflavonol 4-reductase (substrates: dihydromyricetin, dihydrokaempferol, dihydroquercetin)	Gma.2712.1.S1_at	GO:0050662 coenzyme binding
		GO:0009813 flavonoid biosynthetic process
		GO:0042406 extrinsic to endoplasmic reticulum membrane
		GO:0045552 dihydrokaempferol 4-reductase activity
Putative flavonol 3-O-glucosyl-transferase (substrates of <i>Arabidopsis</i> homologue: myricetin, kaempferol, quercetin)	GmaAffx.58893.1.S1_at	GO:0009809 lignin biosynthetic process
		GO:0009805 coumarin biosynthetic process
		GO:0009636 response to toxin
		GO:0009811 stilbene biosynthetic process
		GO:0047209 coniferyl-alcohol glucosyltransferase activity
		GO:0042178 xenobiotic catabolic process
Isoflavone biosynthesis		
Isoflavone reductase EC 1.3.1.51	Gma.5863.2.S1_s_at	GO:0017053 transcriptional repressor complex
	Gma.2686.1.S1_s_at	GO:0016481 negative regulation of transcription
	GmaAffx.90339.1.A1_at	GO:0016564 transcription repressor activity
	Gma.2712.1.S1_at	GO:0009813 flavonoid biosynthetic process
	Gma.15664.1.S1_at	GO:0047526 2'-hydroxyisoflavone reductase activity

Functional annotation	Affymetrix soybean genome microarray probe set ID	GO sub-category
Isoflavone 7- <i>O</i> -methyl-transferase EC 2.1.1.150 (substrates: daidzein, 6,7,4'-trihydroxyisoflavone)	Gma.7727.1.A1_at	GO:0006808 regulation of nitrogen utilization
		GO:0008171 <i>O</i> -methyltransferase activity
Putative 2,7,4'-trihydroxy-isoflavanone 4'- <i>O</i> -methyltransferase	GmaAffx.32612.1.S1_at	GO:0009809 lignin biosynthetic process
		GO:0008171 <i>O</i> -methyltransferase activity
Phenylpropanoid/flavonoid/anthocyanin pathway regulation	GmaAffx.92550.1.S1_s_at	GO:0009809 lignin biosynthetic process
		GO:0009809 lignin biosynthetic process
MYB92 (<i>Arabidopsis</i> homologue (AtMYB4) is a known repressor of C4H)	GmaAffx.89269.1.A1_s_at	GO:0045941 positive regulation of transcription
		GO:0003700 transcription factor activity
		GO:0009800 cinnamic acid biosynthetic process
		GO:0009813 flavonoid biosynthetic process
		GO:0006355 regulation of transcription, DNA- dependent
		GO:0016563 transcription activator activity
		GO:0005667 transcription factor complex
		GO:0010224 response to UV-B
		GO:0016481 negative regulation of transcription
		GO:0009611 response to wounding

A list of genes in GO categories 0009699 and 0009813, which are likely involved in phenylpropanoid metabolism, is shown

Table 2

Gene ontology (GO) functional category 0016165 ('Lipoxygenase activity') and MapMan functional categories 'biotic stress' and 'abiotic stress' are enriched in the list of genes up-regulated by sink removal treatment

Functional annotation	Probe set ID	Expression coefficient	P value
AgriGO molecular function category enrichment (GO 0016165; lipoxygenase activity)			
Lipoxygenase	Gma.16500.1.S1_at	4.116	0.007
Lipoxygenase (VIXE, LOX7)	GmaAffx.24359.1.A1_at	3.927	0.019
Lipoxygenase	Gma.10969.3.S1_x_at	3.674	0.031
Lipoxygenase (LOX9)	Gma.11166.1.S1_at	2.487	0.029
Lipoxygenase (VIXD)	Gma.7625.1.S1_s_at	2.330	0.023
MapMan functional category enrichment (biotic and abiotic stress)			
Proteinase inhibitors			
Serine proteinase inhibitor	Gma.3612.1.S1_s_at	5.310	0.037
Trypsin inhibitor	Gma.9202.1.S1_at	4.445	0.000
Kunitz inhibitor ST1-like protein	Gma.11026.1.S1_at	3.605	0.019
Stress-induced proteins			
Stress-induced protein SAM22	Gma.6999.3.S1_s_at	5.457	0.022
Stress-induced protein SAM22	GmaAffx.91442.1.S1_at	5.283	0.034
mRNA from stress-induced gene	Gma.11119.2.S1_s_at	4.503	0.044
Disease resistance-related proteins			
Similarity to disease resistance response protein	GmaAffx.91558.1.S1_s_at	3.503	0.004
Disease resistance-responsive family protein	Gma.4332.1	3.117	0.025
Similarity to disease resistance response protein	GmaAffx.89785.1	1.638	0.049
Pathogenesis-related proteins			
Pathogenesis-related transcriptional factor	GmaAffx.15015.1.S1_at	5.083	0.027
Class 10 PR protein	GmaAffx.18040.1.S1_s_at	4.207	0.041
Pathogenesis-related transcriptional factor	GmaAffx.23351.1.S1_at	3.871	0.043
Pathogenesis-related protein	GmaAffx.86745.1.S1_s_at	3.250	0.025
Stress and pathogenesis-related protein	GmaAffx.93487.1.S1_x_at	2.867	0.024
Heat shock and chaperone-related proteins			
Heat shock protein DnaJ	GmaAffx.22152.1.A1_at	2.281	0.049
Heat shock 70 kDa protein, mitochondrial precursor	GmaAffx.92270.1.S1_at	2.072	0.024
Heat shock 70 kDa protein	GmaAffx.17961.3.S1_at	1.935	0.041
Putative heat-shock protein	GmaAffx.71557.1.S1_at	1.921	0.037
Heat shock protein	Gma.17525.2.S1_at	1.744	0.047
Endoplasmic homolog precursor	Gma.18032.1.S1_at	1.628	0.022
Putative heat-shock protein	GmaAffx.78362.1.S1_at	1.557	0.032
Chaperonin GroEL	Gma.2903.1.S1_at	1.486	0.042
Heat shock protein DnaJ	Gma.6793.1.A1_at	1.419	0.021

Genes are listed in order of decreasing expression coefficients (log₂-transformed fold-change of D15 vs. C0 samples) and are associated with the corresponding probe set IDs for the Affymetrix soybean genome array

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Gene ontology (GO) functional categories 0015979 (‘Photosynthesis’), 0042440 (‘Pigment Metabolic Process’), and 0019497 (‘Acid Phosphatases’) are enriched in the list of genes down-regulated in control samples over time

Table 3

Functional annotation	Probe set ID	Expression coefficient	P value
<i>Photosystem I (part of GO 0015979)</i>			
P subunit	Gma.11189.1.S1_at	-4.973	0.009
D1 subunit	Gma.11667.1.S1_at	-4.037	0.020
F subunit	Gma.11333.1.S1_s_at	-2.708	0.005
H subunit	Gma.16838.1.S1_at	-2.486	0.015
Light harvesting antenna complex component	Gma.10766.1.S1_at	-2.416	0.005
K subunit	Gma.1992.1.S1_at	-2.265	0.002
PsaD protein	Gma.10852.2.S1_at	-2.228	0.006
P subunit	Gma.17589.1.S1_at	-2.019	0.030
G subunit	Gma.15377.1.S1_at	-1.834	0.021
Light harvesting antenna complex component	Gma.12238.1.S1_at	-1.784	0.046
Light harvesting antenna complex component	Gma.4613.1.A1_a_at	-1.775	0.027
<i>Photosystem II (part of GO 0015979)</i>			
PsbX protein	Gma.1319.1.S1_at	-6.748	0.004
Lhcb6 protein	Gma.Affx.57226.1.S1_at	-5.770	0.004
Lhcb3 protein	Gma.10867.1.S1_at	-5.248	0.003
Lhcb3 protein	Gma.4593.1.S1_x_at	-4.733	0.003
Lhcb4.3 protein	Gma.2362.1.S1_at	-4.262	0.002
Lhcb2.1 protein	Gma.Affx.86110.1.S1_at	-4.018	0.035
PsbY protein	Gma.12488.1.S1_at	-3.673	0.021
Lhcb6 protein	Gma.11123.1.S1_at	-3.370	0.011
PsbW protein	Gma.8778.1.S1_at	-3.058	0.036
Lhca2.1 protein	Gma.13395.1.A1_s_at	-2.986	0.017
CP43 subunit	Gma.Affx.4691.1.S1_at	-2.914	0.012
Lhcb CP26 protein	Gma.2360.1.S1_at	-2.893	0.007
Lhcb2.1 protein	Gma.Affx.92935.1.S1_s_at	-2.847	0.015
PsbY protein	Gma.11676.1.S1_at	-2.288	0.020
Photosystem II component	Gma.14466.1.S1_at	-2.771	0.010

Functional annotation	Probe set ID	Expression coefficient	P value
PsbO protein	GmaAffx.32256.1.S1_at	-2.168	0.029
PsbP protein	Gma.15006.1.A1_at	-2.151	0.036
Light-harvesting complex II component	GmaAffx.93190.1.S1_s_at	-2.105	0.011
PsbP protein	Gma.5672.3.S1_a_at	-2.067	0.024
PsbQ subunit	Gma.1955.4.A1_at	-2.012	0.004
Chlorophyll binding protein D1	GmaAffx.20469.1.A1_at	-1.943	0.033
PsbY protein	Gma.4613.4.S1_at	-1.866	0.005
Lhcb2.1 protein	Gma.17554.1.S1_a_at	-1.685	0.012
PsbO protein	Gma.10731.1.S1_x_at	-1.591	0.018
PsbO protein	Gma.11034.1.S1_at	-1.363	0.012
Lhcb4.2 protein	Gma.1166.2.S_a_at	-1.181	0.023
PsbO protein	GmaAffx.47232.1.A1_at	-1.087	0.024
<i>Cytochrome b6/f complex (part of GO 0015979)</i>			
Subunit V of the cytochrome b6/f complex	GmaAffx.32146.1.S1_at	-2.877	0.045
Subunit IV	GmaAffx.14048.2.S1_at	-1.943	0.026
<i>Other (part of GO 0015979)</i>			
NADH dehydrogenase	GmaAffx.28886.1.A1_at	-3.197	0.021
NADH dehydrogenase	GmaAffx.17247.1.S1_at	-3.122	0.006
<i>Photosynthetic metabolism (part of GO 0015979)</i>			
Phosphoenolpyruvate carboxylase	Gma.60.1.A1_at	-2.944	0.002
Ribulose-5-phosphate-3-epimerase	Gma.6333.3.S1_at	-2.492	0.004
Glyceraldehyde-3-phosphate dehydrogenase	GmaAffx.82328.1.S1_at	-2.242	0.005
Fructose-1,6-bisphosphatase	Gma.168331.S1_s_at	-1.879	0.038
Phosphoribulokinase	Gma.10851.1.S1_at	-1.738	0.027
Transketolase	Gma.9720.1.S1_at	-1.299	0.034
Glyceraldehyde-3-phosphate dehydrogenase	Gma.13868.2.S1_s_at	-1.056	0.034
<i>Pigment metabolism (part of GO 0042440)</i>			
Glutaryl-tRNA reductase	Gma.9248.1.S1_at	-6.779	0.006
Phytoene synthase	GmaAffx.58990.1.S1_at	-5.103	0.021
Chlorophyllase	Gma.13454.1.A1_at	-4.636	0.002
Magnesium chelatase	Gma.1796.1.S1_at	-4.415	0.023

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Functional annotation	Probe set ID	Expression coefficient	P value
Magnesium chelatase	Gma.12621.1.A1_at	-3.605	0.044
Chlorophyllase	GmaAfx.40675.1.S1_at	-3.415	0.013
GUN4 regulator of chlorophyll biosynthesis	Gma.13003.1.S1_s_at	-3.397	0.014
Mg-protoporphyrin IX monomethyl ester cyclase	Gma.1711.1.A1_at	-3.154	0.011
Mg-protoporphyrin IX monomethyl ester cyclase	GmaAfx.49796.2.S1_at	-2.836	0.017
Mg-protoporphyrin IX methyltransferase	Gma.15868.2.A1_a_at	-2.338	0.030
Geranylgeranyl reductase	Gma.1279.1.S1_a_at	-2.239	0.023
NADPH:protochlorophyllide oxidoreductase A	Gma.2542.1.S1_at	-2.030	0.006
1-Deoxy-D-xylulose 5-phosphate synthase	Gma.11205.1.S1_a_at	-1.795	0.043
NADPH:protochlorophyllide oxidoreductase A	Gma.10890.1.S1_at	-1.312	0.050
<i>Acid phosphatases (part of GO 0019497); contains putative vegetative storage proteins</i>			
Vsp-alpha	GmaAfx.89772.17.S1_s_at	-8.349	0.021
Vsp-alpha	Gma.5793.1.S1_s_at	-7.400	0.011
Vsp-alpha	Gma.1822.1.S1_s_at	-6.550	0.034
Acid phosphatase	Gma.8520.1.S1_at	-3.921	0.049
Acid phosphatase	GmaAfx.89772.8.S1_at	-3.346	0.029
Vsp-beta	GmaAfx.89772.9.A1_s_at	-2.986	0.022

Genes are listed in order of increasing expression coefficients (log2-transformed fold-change of C20 vs. C0 samples) and are associated with the corresponding probe set IDs for the Affymetrix soybean genome array

Table 4

Gene ontology (GO) functional category 0034641 ('cellular nitrogen compound metabolic process') is enriched in the list of genes up-regulated in control samples over time

Functional annotation	Probe set ID	Expression coefficient	P value
<i>Amino acid metabolism</i>			
Alanine-glyoxylate aminotransferase, putative	GmaAffx.61931.1.S1_at	6.052	0.017
Alanine-glyoxylate aminotransferase, putative	Gma.6895.1.S1_a_at	3.927	0.006
Homocysteine S-methyltransferase	Gma.398.1.A1_at	3.827	0.044
Bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase	GmaAffx.10497.1.S1_at	3.388	0.049
Serine hydroxymethyltransferase, putative	GmaAffx.23195.1.S1_at	2.343	0.020
Glutamine synthetase, cytosolic	Gma.4375.2.S1_at	2.309	0.005
Glutamate synthetase, cytosolic	GmaAffx.91964.1.S1_s_at	1.996	0.033
Sarcosine oxidase family protein	GmaAffx.27758.1.S1_at	1.971	0.017
Lysine decarboxylase family protein	GmaAffx.57846.1.S1_at	1.944	0.034
Glycine/serine hydroxymethyltransferase family protein	Gma.4108.1.S1_a_at	1.811	0.024
Asparagine synthetase type III	Gma.7400.2.S1_at	1.723	0.030
Glutamine synthetase, cytosolic	Gma.2105.1.S1_at	1.556	0.038
Acetylornithine aminotransferase, mitochondrial precursor	GmaAffx.92829.1.S1_s_at	1.529	0.046
Anthranilate synthase-alpha subunit	Gma.13056.1.S1_at	1.277	0.039
Dihydroxy-acid dehydratase, putative	Gma.6764.1.S1_at	1.172	0.040
<i>tRNA synthetases</i>			
Alanyl-tRNA synthetase, mitochondrial precursor	GmaAffx.83421.1.S1_at	2.073	0.019
Methionyl-tRNA synthetase, putative	GmaAffx.7504.1.S1_at	1.822	0.044
Tryptophanyl-tRNA synthetase, putative	GmaAffx.38011.1.S1_at	1.452	0.025
tRNA synthetase class II family protein	GmaAffx.7362.1.S1_at	1.136	0.010
Glutaminyl-tRNA ligase	Gma.3544.1.S1_at	1.023	0.041
<i>Other</i>			
Sphingosine-1-phosphate lyase, putative	GmaAffx.84961.1.S1_at	2.046	0.028
Molybdenum cofactor biosynthesis protein, putative	GmaAffx.79966.1.S1_at	1.750	0.033
Glutathione S-transferase	GmaAffx.91535.1.S1_s_at	1.273	0.039

Genes are listed in order of decreasing expression coefficients (log₂-transformed fold-change of C20 vs. C0 samples) and are associated with the corresponding probe set IDs for the Affymetrix soybean genome array. Only genes with a likely direct involvement in nitrogen metabolism are included