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RNAseq reveals weed-induced PIF3-like as a candidate target to manipulate weed stress response in soybean

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Summary

- Weeds reduce yield in soybeans (*Glycine max*) through incompletely defined mechanisms. The effects of weeds on the soybean transcriptome were evaluated in field conditions during four separate growing seasons.
- RNASeq data were collected from six biological samples of soybeans growing with or without weeds. Weed species and the methods to maintain weed-free controls varied between years to mitigate treatment effects, and to allow detection of general soybean weed responses.
- Soybean plants were not visibly nutrient- or water-stressed. We identified 55 consistently downregulated genes in weedy plots. Many of the downregulated genes were heat shock genes. Fourteen genes were consistently upregulated. Several transcription factors including a *PHYTOCHROME INTERACTING FACTOR 3*-like gene (*PIF3*) were included among the upregulated genes. Gene set enrichment analysis indicated roles for increased oxidative stress and jasmonic acid signaling responses during weed stress.
- The relationship of this weed-induced *PIF3* gene to genes involved in shade avoidance responses in Arabidopsis provide evidence that this gene may be important in the response of soybean to weeds. These results suggest that the weed-induced *PIF3* gene will be a target for manipulating weed tolerance in soybean.

Introduction

Weeds have long been known to reduce crop yields (Zimdahl, 2004). However, the mechanisms through which weeds cause these losses are unresolved. Although weeds undoubtedly compete with crops for water, nutrients and light when these resources are limiting, in modern agricultural conditions these resources are generally abundant. Weeds reduce crop yields most when they are present early in the growing season, and can reduce yields even if they are removed several weeks after crops emerge. Such observations led to the concept of a critical weed-free period (CWFP) – the generally narrow portion of the growing season where weed presence has a significant impact on end of season yield. CWFPs often occur before weeds have any significant impact on soil nutrient or moisture status (Kropff *et al.*, 1993). In soybean this occurs between the vegetative second-leaf (V2) and V4 stages of growth (Van Acker *et al.*, 1993). This CWFP generally occurs too early in the growing season for the weed to be directly competing with crops for resources. These data suggest that weeds primarily reduce yield via mechanisms other than direct competition for soil resources. Thus, researchers have hypothesized that exposure to weeds during early growth may alter crop developmental trajectories such that yield is reduced (Afifi & Swanton, 2012).

This hypothesis is supported by research that indicates weeds can reduce crop growth and yields when present during the CWFP even if crop and weed roots are physically separated (Green-Tracewicz *et al.*, 2011, 2012). These results imply that weeds produce a signal that alters crop development without requiring direct physical contact between the weed and crop plants. Chlorophyll absorbs red light strongly but reflects far-red light. Consequently, light microenvironments proximal to plants are depleted in red light (*c.* 660 nm), but have far-red light (*c.* 730 nm) content similar to that of ambient light. This reduced ratio of red: far red (R : FR) light is detected by plants via phytochrome photoreceptors. Reduced R : FR has been shown to induce developmental responses such as decreased root-to-shoot ratios, increased specific leaf area, reduced photosynthetic capacity and early flowering, which are often referred to collectively as 'shade avoidance syndrome' (reviewed in Franklin, 2008; Casal, 2012). Shade avoidance syndrome has been proposed as a major cause of early developmental changes associated with some weed-induced yield losses (Rajcan & Swanton, 2001; Afifi & Swanton, 2012).

Recent studies in Arabidopsis (*Arabidopsis thaliana*) and corn (*Zea mays*) have identified numerous genes that are differentially regulated during plant–plant interactions (Horvath *et al.*, 2006; Masclaux *et al.*, 2012; Moriles *et al.*, 2012). In corn, expression of genes involved in photosynthesis, auxin signaling and responses to pathogens were downregulated in response to weed presence compared with plants grown in weed-free conditions (Horvath *et al.*, 2006; Moriles *et al.*, 2012). The downregulation of some of these genes could be observed as early as V2, and even if weeds were removed at this time, gene expression never fully reverted to match expression patterns of plants growing weed-free.

To date, gene expression changes that occur in soybean (*Glycine max*) grown with weeds during the CWFP have not been characterized. Therefore, the objective of this study was to characterize soybean growth, gene expression and yield as influenced by weed presence or absence during the CWFP. With this work, we test the hypothesis that transcriptome responses of soybeans to weed pressure are the result of direct competition for resources. If weeds were directly competing for resources, we would expect to find differences in expression of genes involved in resource gathering or use. Instead, however, we identified a small number of genes suggesting alterations in light quality perception and hormone signaling which are more indicative of altered developmental responses in crops grown with weeds during the CWFP.

Materials and Methods

Plant material

A commercially available, commonly planted late group I soybean (*Glycine max* Willd.) (cv AG1631) was planted at the Aurora, SD, USA farm in east-central South Dakota in 2008–2011, between 12 and 22 May depending on the year (Table 1). The soil at this location is loess over glacial outwash, and the soil series is Brandt silty clay loam (Clay *et al.*, 2009). The crop was grown under natural rainfall conditions. Accumulated growing degree days (GDD) from planting until tissue collection date (V3 of soybean growth) ranged from 426 to 608 GDD (base 10°C) (Table 1). Rainfall from planting to collection ranged from 10 to 24 cm. Plot sizes were 3 × 6 m with four rows. Row spacing was either 76 cm or 18 cm in the case of one replicate each in 2010 and 2011.

Treatments consisted of control (weed-free), weedy, and weed removal early during the CWFP, and were arranged in a randomized complete block design with four replications. During 2008 and 2009, a naturally occurring weed population consisting primarily of velvetleaf (*Abutilon thoephrasti*) and wild buckwheat (*Polygonum convolvulus)* was the weed competition source. At the V3 soybean growth stage in 2008 and 2009, weed densities were 300 m^{-2} and 48 m^{-2}, respectively. During 2010 flax was seeded as a weed proxy at a half normal rate used for production (23 kg ha^{-1}) on 19 May (1 d after soybean planting), and reached a density of 600 plants m^{-2} by the soybean V3 stage. During 2011 naturally occurring velvetleaf and weedy common sunflower (*Helianthus annuus*) populations served as the source of weed competition, with average densities of 160 m^{-2}. Weeds were controlled in weed-free treatments in 2008 with applications of s-metolachlor (Dual II Magnum; Syngenta Crop Protection LLC, Greensboro, NC, USA) (1.9 l ha^{-1}) on 22 May and fluazifop-P (Fusilade DX; Syngenta Crop Protection) (584 ml ha^{-1}) on 26 June. In 2009, weeds were controlled by application of sethoxydim (Poast; BASF Ag Products, Research Triangle Park, NC, USA) (1.8 l ha^{-1}) on 26 June. Glyphosate (Roundup Weather Max; Monsanto Co., St Louis, MO, USA) was used at 1.2 l ha^{-1} in 2010 for midseason weed control. In 2011, Roundup Weather Max, Dual II Magnum, Poast and imazethapyr (Pursuit; BASF Ag Products, Research Triangle Park, NC, USA) were used at manufacturers suggested rates and times. Differences in weed types and herbicides were used to intentionally dilute the effects of these variables.

The day after sampling at V3, weeds were removed from four plots of the eight weedy plots (designated as WRV3 for weeds removed at V3) using herbicide application, followed by hand-weeding (starting *c.* 2 wk after application) for the rest of the season. Weeds remained until the end of the season in the remainder of the plots (weedy).

Soybean height was measured from the soil concentration to the top emerging trifoliate at V3 and at canopy closure (determined visually, when leaves from neighboring rows touched). Soybean leaf area was measured destructively using a LiCor LI-3100C area meter with all trifoliates of four plants per plot clipped for measurement. Soybean plants were harvested in October from 33 m of row by a plot combine at physiological maturity and yield was estimated after threshing pods and cleaning seeds as metric tonnes per hectare (MT ha^{-1}) from each plot.

Table 1 Soybean (*Glycine max*) planting dates, sampling dates and growing conditions for each study year

Year	Weed species	Herbicide	Plant date	Sampling date V3 stage of growth	Precip to V3 (cm)	Growing degree days at time of sampling (V3)	Air temperature at collection V3 (°C)
2008	Velvetleaf and buckwheat	S-metolachlor and fluazifop-P	22 May	23 June	12.4	426	23.9
2009	Velvetleaf and buckwheat	sethoxydim	12 May	25 June	9.9	562	27.2
2010	Flax	Glyphosate	18 May	22 June	19.4	576	25.6
2011	Velvetleaf and sunflower	S-metolachlor, sethoxydim, and imazethapyr	12 May	29 June	24.2	608	26.1

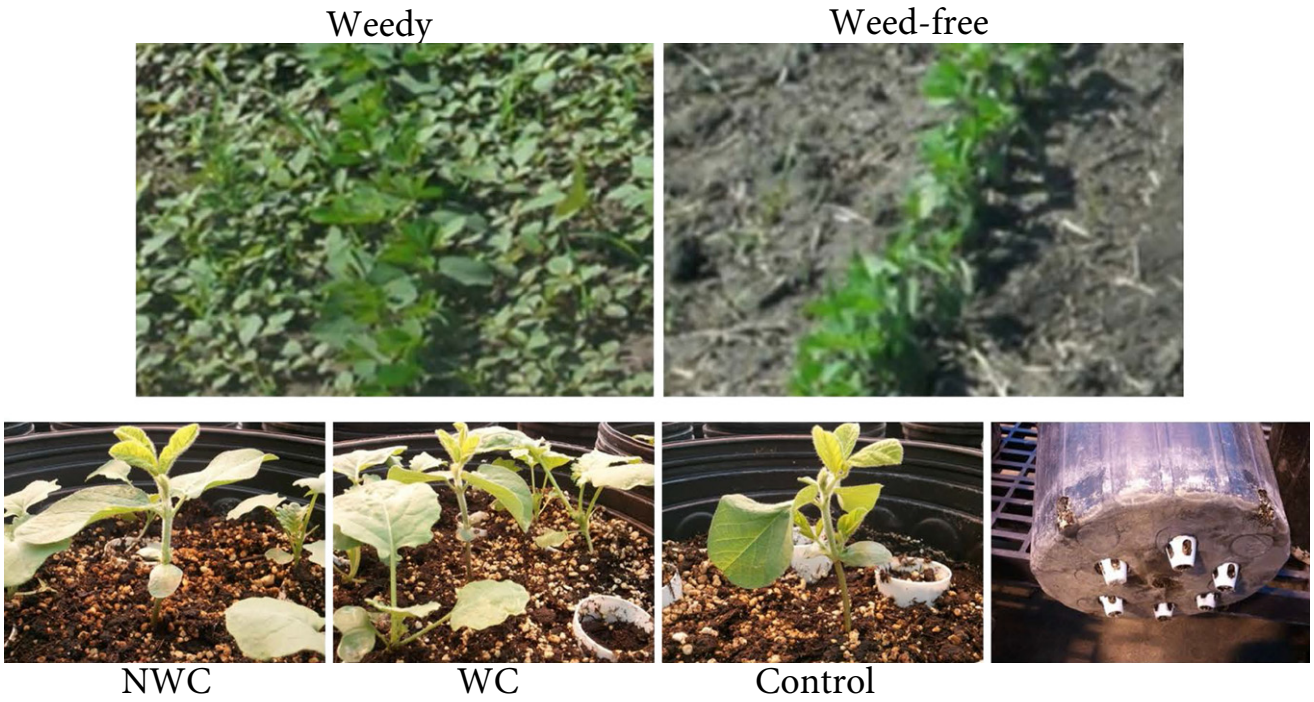

Fig. 1 Photos of soybean (*Glycine max*) plants growing under field or glasshouse weedy or weed-free conditions. Upper panels, a representative set of photos of field-grown soybean plants at the V3 stage of growth growing under weedy (left panel) and weed-free (right panel) conditions. Lower panels, a representative set of photos of plants with surrounding canola as the weed either in with no direct root-to-root contact between the soybean and the weeds (NWC), with direct root-to-root contact between the soybean and the weeds (WC) or growing alone (control). Also included is a view of the bottom of the pot demonstrating that the conetainers used to separate the root systems of the canola and soybean fully pass through the pot and thus prevent any soil-borne signaling or competition for resources.

Data (leaf area, plant height and yield) were averaged within treatment and Student's *t*-tests were used to determine significant differences ($P < 0.05$) between treatments for plant height and leaf area at V3. ANOVA was used to determine significant differences ($P < 0.05$) among treatments for plant height and leaf area, and yield response variables at sampling dates. In order to detect changes associated with both development and nutrient status, we collected newly emerged unfolded trifoliate < 2 cm in length, along with the associated meristem from at least eight plants per plot between 11:00 and 13:00 h at the soybean V3 (three fully expanded trifoliate leaflets) growth stage. Tissue was placed immediately in liquid nitrogen for storage.

In order to examine the expression of genes under more controlled conditions, soybean plants were grown in 8-l pots with potting soil (sunshine mix) in the glasshouse under 16 h photoperiod at 20–25°C. To examine the requirement of direct competition for resources or soil-transmitted signals between the soybean plant and the weed (winter canola in this case), a single soybean plant was placed in the center of each pot (control), or with 5–6 canola plants grown either in conetainers that protruded through the pot with separate drainage (no root-to-root contact) or that were planted between the conetainers (with root-to-root contact). Canola seeds were planted *c.* 8 cm away and surrounding the central soybean plant at the same time and emerged within a day before or after the soybean emergence. Conetainers were present in the control pots as well. When plants were at the V3 stage of growth, the top emerging trifoliates were harvested into liquid nitrogen. Two biological replicates with each replicate consisting of 3–4 pooled individuals from each treatment were harvested for quantitative reverse transcription (qRT)-PCR analysis as described later. Photos of plants growing in field and glasshouse are shown to provide a visual representation of the growth conditions for the reader (Fig. 1).

RNA cDNA Library construction

RNA extraction was performed by grinding *c.* 0.1 g of frozen tissue in liquid nitrogen to a fine powder, and adding 1 ml Trizol reagent (Ambion). Chloroform: Isoamyl (24:1) was added to the Trizol/tissue mixture, centrifuged, and RNA was extracted from the resulting supernatant using an RNeasy Plant Mini kit (Qiagen). Quality control was assessed using a nanodrop (ND-1000 Spectrophotometer; ThermoScientific, Waltham, MA, USA) for quantitation and assurance of minimal carbohydrate and protein contamination, and then on a bioanalyzer (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA) using the RNA setting for size determination and assessment of integrity.

cDNA libraries were created following the Illumina TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA, USA), which is briefly summarized here. Four thousand nanograms of total RNA was purified and mRNA was extracted, followed by

first and second strand cDNA synthesis, and fragmentation. End repair and adenylation of 3′ ends preceded adapter ligation and PCR amplification. Library quality was assessed using an Agilent Bioanalyzer, and quantified for pooling by qRT-PCR using the PhiX Control Kit v2 according to manufacturer specifications. Libraries were paired-end sequenced over four lanes (with other unrelated samples) on an Illumina HiSeq2000 for 100 base reads per end. However, the second paired read files were generally poor in quality, and resulted in reduced mapping of the sequences to the reference genome. Consequently, we chose to use only the first read files from each fragment (see later).

Transcriptome analysis

Illumina sequences were analyzed using the Tuxedo suite of programs (Trapnell *et al.*, 2012) in the iPlant infrastructure (Goff *et al.*, 2011). Briefly, single-end reads were mapped to the soybean genome (*Glycine max* (Soybean) (Ensembl 14)) using the Tophat-SE program in the iPlant discovery environment with an anchor weight of 8, 0 mismatch, 70–50 000 base intron length, 0.15 minimum isoform fraction, maximum 20 alignments, two mismatches for independently mapped reads, and minimum read length per segment of 20. The Cufflinks program was used to produce the fragments per kb per million reads (FPKM) data for the individual genes and Cuffdiff was used for statistical analyses using default settings. Gene set and subnetwork enrichment analyses were run on the data set using Pathway Studio 9.0 (Nikitin *et al.*, 2003), on default settings with gene functions based on top Arabidopsis hits using the BlastX program ($E > 10^{-5}$). Over-represented sequences present in the promoters of genes that were consistently up- or downregulated in response to weed stress were identified using the program ELEMENT 2.0 (Mockler *et al.*, 2007) with 2000 bases of promoter sequence indicated for the gene clusters. Two thousand bases 5′ to the start of transcription as designated by soybean gene models in phytozome 9.0 were analyzed using the MEME program (Bailey & Elkan, 1994) to identify over-represented sequences in the putative promoters of genes consistently up- and downregulated in response to weed stress. Gene set and subnetwork enrichment analysis were accomplished using Pathway Studio 8.0. Raw data and metadata have been deposited in the Gene Expression Omnibus (accession number GSE59875).

Gene expression analysis by qRT-PCR

Primers were designed to specifically amplify 12 upregulated genes and 16 downregulated genes based on sequences in Phytozome 9.0. Internal control genes, *Glyma12g02790* (encoding CYCLOPHILIN3), *Glyma03g25200* (unknown) and *Glyma01g40950.2* (encoding a phosphoacetylglucosamine mutase-like isoform X4 protein) were chosen from the RNAseq data as having changed little across the samples tested and having passed the PCR analysis as reasonable control genes. Where possible, primers were designed such that at least one spanned an intron junction. Primer and amplicons sequences for these genes can be found in Supporting Information Table S1.

qRT-PCR for the *PIF3a* gene was performed on RNA extracted from leaf material (as described earlier) of plants at the V3 stage of growth. Treatments were control, weedy and weeds removed at V3 from 2008 samples. Four biological replicates were analyzed for control and weedy plants and three biological replicates were examined from plants where weeds had been removed at V3. Additionally, all of these primers were used to assess transcript accumulation in two biological replicates from glasshouse grown samples at the V3 stage of development that were either grown with direct root-to-root contact or when roots of the soybean plants were isolated from the weeds grown in the same pot. The $2^{-}\ \Delta\Delta^{C}{}_{\mathrm{T}}$ values from three technical replicates from each biological replicate were determined using an average of all three control genes to normalize expression between samples.

Results

Weed presence altered growth and yield of soybean

Weed responses of soybean under field conditions can be variable (Van Acker *et al.*, 1993), and thus it was important to confirm that exposure to weeds effectively altered soybean growth during all 4 yr. Plant height at V3 was similar in weedy and weed-free treatments (Table 2a). Leaf area was reduced by 50% V3 in 2010, and 18% in 2011 ($P < 0.05$), with similar downward trends in 2008 and 2009 ($P = 0.11$) (2009 had only moderate weed pressure compared with other years) (Table 2a). Plants competing with weeds at canopy closure were shorter than weed-free plants, and even if weeds were removed at V3 the plants did not grow as tall as controls (Table 2b). Leaf area at canopy closure was reduced from 6% (2010) to 35% (2011) compared with the weed-free control plants although the weeds had been removed at V3 (Table 2b). If weeds remained until canopy closure, leaf area was reduced from 43% (2011) to 70% (2010) compared with weed-free soybean. Soybean yield was not reduced (ranging from 3% in 2010–2011, 8% in 2008, to 24% in 2009) in 3 of the 4 yr when weeds were removed at V3 compared with weed-free treatments. However, soybean yield was reduced from 24% (2011) to 80% (2008) when subjected to season-long weed pressure compared with weed-free soybean yield. Soil nitrogen and moisture were measured in 2008 and showed no significant difference between weedy and weed-free plots, even though these measurements were taken at the end of the growing season (Table 2a).

RNAseq identifies differentially expressed genes

RNAseq produced between 10 and 49 million reads per library, with all libraries having > 85% reads unambiguously mapping to the soybean genome (Table 3). Approximately 30 500 transcripts were identified and quantified to annotated soybean genes. No genes were significantly differentially expressed ($q < 0.05$) when data were averaged over all 4 yr of the study (Table S2). However, when biological replicates were examined within years (2010 and 2011; note, no replication of samples were collected in 2008 or

Table 2 (a) Soybean (*Glycine max*) plant height and leaf area measured at V3 during 2008, 2009, 2010 and 2011 in weedy and control (weed-free) plots, and soil % moisture and total nitrogen (N; NO_3-N + NH_4-N) at harvest in 2008; (b) data collected at soybean canopy closure for plant height, leaf area and yield in weedy or control (weed-free) plots or in plots where weeds were present up to V3 stage of growth and then removed (WRV3) for all four years

V3 Year	Plant height (cm) Control	Plant height (cm) Weedy	Leaf area (cm^2) Control	Leaf area (cm^2) Weedy	Weed density (plants m^{-2})	% Soil moisture (0–15 cm) Control	% Soil moisture (0–15 cm) Weedy	Soil N μg g^{-1} (0–15 cm) Control	Soil N μg g^{-1} (0–15 cm) Weedy
2008	11[a]	11[a]	47[a]	41[a]	300	22.47[a]	22.32[a]	20.56[a]	28.20[a]
2009	52[a]	48[a]	207[a]	163[a]	48				
2010	25[a]	27[a]	159[a]	87[b]	600				
2011	22[a]	22[a]	135[a]	110[b]	160				

CC Year	Plant height (cm) Control	Plant height (cm) Weedy	Plant height (cm) WRV3	Leaf area (cm^2) Control	Leaf area (cm^2) Weedy	Leaf area (cm^2) WRV3	Yield (MT ha^{-1}) Control	Yield (MT ha^{-1}) Weedy	Yield (MT ha^{-1}) WRV3
2008	54[a]	59[a]	48[b]	1510[a]	512[b]	1235[a]	2.4[a]	0.5[c]	2.2[b]
2009	80[a]	75[ab]	68[b]	901[a]	475[b]	676[b]	3.3[a]	1.7[a]	2.5[a]
2010	89[a]	70[b]	76[b]	1417[a]	438[b]	1330[a]	3.1[a]	1.1[b]	3.0[a]
2011	83[a]	78[b]	76[b]	1438[a]	819[b]	953[b]	2.9[a]	2.2[b]	2.8[a]

Different letters indicate differences between treatments in given years at $P < 0.05$.

Table 3 Mapping of single-end reads to the soybean (*Glycine max*) genome

Sample	Number of reads	Accepted hits	Reads that unambiguously mapped	Percentage unmapped	Percentage unambiguously mapped
2008_control	12 438 478	11 369 532	9848 797	9.40	86.62
2008_weedy	12 847 367	11 957 941	10 359 450	7.44	86.63
2009_control	12 905 636	11 785 766	10 519 237	9.50	89.25
2009_weedy	11 977 124	10 791 081	9442 660	10.99	87.50
2010_control	27 034 933	25 868 028	23 013 035	4.51	88.96
2010_control	35 948 748	34 501 608	30 725 584	4.19	89.06
2010_weedy	22 387 036	21 551 910	19 245 697	3.87	89.30
2010_weedy	22 797 221	21 936 845	19 788 511	3.92	90.21
2011_control	26 588 285	25 571 618	22 708 463	3.98	88.80
2011_control	20 398 664	19 592 086	17 284 582	4.12	88.22
2011_weedy	52 312 036	48 664 381	42 767 628	7.50	87.88
2011_weedy	43 991 009	40 689 221	35 657 448	8.11	87.63

Number of reads, number of reads following trimming of the libraries for quality (PHRED value > 20 with at least 70 bases in size). Accepted hits, number of reads that mapped to the soybean genome. Reads that unambiguously mapped, reads that mapped to a single location in the soybean genome. Percentages of unmapped and unambiguously mapped soybean sequences are also indicated (relative to number of reads).

2009), 751 and 2339 genes were identified as significantly differentially expressed, respectively. Among the genes identified with differential expression for either 2010 or 2011 data, 145 were significant in both years and had the same expression trends. Of these, 69 had the same expression trend over all 4 yr with 55 downregulated in response to weeds and 14 upregulated (Table 4). The list of consistently downregulated genes was dominated by heat shock response genes, although several transcription factors (such as MYB113-like and a zinc finger C-x8-C-x5-C-x3-H type family protein) and JAZ1, a negative regulator of jasmonate (JA) signaling, were notable. Two transcription factors involved in phytochrome signaling (one encoded by a *PIF3*-like gene and another by a *B-BOX DOMAIN PROTEIN 19*-like gene) were present in the consistently upregulated gene list. A majority of the upregulated genes are known to play a role in various oxidative stress responses.

In order to confirm the differential expression of the most likely regulator of the weed-induced responses (see Discussion on *PIF* gene expression later), we examined the expression of this weed-induced *PIF3* gene in field-grown plants by qRT-PCR (Fig. 2). In three biological replicates from 2008 samples, this weed-induced *PIF3* gene (*PIF3a*) was clearly upregulated when weeds were present, but the expression of the weed-induced *PIF3a* did not remain high if weeds were removed at V3. Additionally, not only was the *PIF3a* gene upregulated by weeds consistently under field conditions, but it was also upregulated under glasshouse conditions – even when soybean plants were grown using the indirect competition method that prevented any

Table 4 List of soybean (*Glycine max*) genes that were differentially expressed during 2010 and 2011 and which have the same pattern of expression in 2008 and 2009

		Average log_2 fold ratios by year			
Locus ID	Putative function	2008	2009	2010	2011
Glyma14g11420	17.6 kDa class II heat shock protein	−0.93	−2.81	−1.78	−1.90
Glyma04g05720	17.6 kDa class II heat shock protein	−0.89	−2.50	−1.58	−1.21
Glyma14g11430	17.6 kDa class II heat shock protein	−0.77	−3.05	−1.63	−1.70
Glyma06g05740	17.6 kDa class II heat shock protein	−0.26	−2.18	−1.48	−1.16
Glyma20g01930	17.6 kDa class II heat shock protein	−0.21	−1.64	−1.34	−1.36
Glyma03g03270	Arginase/deacetylase superfamily protein	−0.31	−1.58	−0.65	−1.34
Glyma18g52150	BCL-2-associated athanogene 5	−0.14	−0.06	−0.85	−1.01
Glyma04g06610	Casein lytic proteinase B4	−0.23	−1.06	−1.04	−0.87
Glyma19g41760	Chaperone DnaJ-domain superfamily protein	−0.08	−0.54	−1.40	−0.61
Glyma02g18090	Concanavalin A-like lectin protein kinase family protein	−0.28	−1.51	−0.61	−2.10
Glyma06g44300	DNAJ heat shock family protein	−0.15	−0.24	−0.73	−0.69
Glyma03g37650	DNAJ heat shock family protein	−0.04	−0.81	−0.71	−0.72
Glyma19g40260	DNAJ heat shock family protein	−0.03	−0.63	−0.68	−0.69
Glyma18g43430	DNAJ heat shock N-terminal domain-containing protein	−0.19	−0.74	−1.38	−1.17
Glyma07g18550	DNAJ heat shock N-terminal domain-containing protein	−0.13	−0.92	−1.08	−0.92
Glyma11g17930	DNAJ homologue 2	−0.22	−0.35	−0.57	−1.00
Glyma02g47820	emp24/gp25L/p24 family/GOLD family protein	−0.36	−0.62	−0.31	−0.62
Glyma03g17870	Fes1A	−0.30	−0.61	−1.05	−1.09
Glyma05g28260	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	−0.32	−1.30	−1.46	−1.78
Glyma09g36250	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	−0.21	−0.26	−0.52	−0.53
Glyma08g11240	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	−0.09	−1.17	−1.24	−1.30
Glyma18g10760	Heat shock protein 21	−0.24	−2.17	−1.14	−1.06
Glyma11g37450	Heat shock protein 21	−0.13	−1.10	−1.39	−0.89
Glyma05g36600	Heat shock protein 70 (Hsp 70) family protein	−0.01	−0.64	−0.83	−1.03
Glyma17g08020	Heat shock protein 70B	−0.18	−2.82	−1.30	−1.25
Glyma02g36700	Heat shock protein 70B	−0.03	−3.21	−1.52	−1.19
Glyma08g03690	Heat shock protein 81-2	−0.03	−0.17	−0.92	−0.58
Glyma16g29750	Heat shock protein 90.1	−0.51	−1.73	−1.69	−2.50
Glyma09g24410	Heat shock protein 90.1	−0.35	−0.88	−1.75	−1.78
Glyma17g34540	Heat shock transcription factor A2	−0.75	−1.82	−1.35	−0.89
Glyma01g44910	Heat-shock protein 70T-2	−0.16	−1.06	−1.20	−0.88
Glyma19g36460	Homolog of mamallian P58IPK	−0.09	−1.07	−0.87	−0.84
Glyma07g32030	HSP20-like chaperones superfamily protein	−0.83	−2.58	−1.18	−1.24
Glyma08g07340	HSP20-like chaperones superfamily protein	−0.52	−2.53	−1.28	−1.47
Glyma19g01440	HSP20-like chaperones superfamily protein	−0.49	−2.77	−1.73	−1.23
Glyma08g07330	HSP20-like chaperones superfamily protein	−0.46	−0.34	−0.74	−1.53
Glyma08g07350	HSP20-like chaperones superfamily protein	−0.43	−2.79	−1.25	−1.41
Glyma07g32070	HSP20-like chaperones superfamily protein	−0.28	−1.11	−1.16	−1.48
Glyma13g24490	HSP20-like chaperones superfamily protein	−0.20	−1.29	−1.47	−1.53
Glyma06g16490	HSP20-like chaperones superfamily protein	−0.03	−0.10	−0.88	−1.02
Glyma04g04230	HXXXD-type acyl-transferase family protein	−0.10	−1.83	−1.08	−1.57
Glyma11g04130	Jasmonate-zim-domain protein 1	−0.06	−0.99	−0.85	−0.92
Glyma12g01580	Mitochondrion-localized small heat shock protein 23.6	−0.36	−1.62	−1.61	−1.33
Glyma09g37010	Myb domain protein 113	−0.98	−0.80	−1.75	−1.06
Glyma03g38150	NAD(P)-binding Rossmann-fold superfamily protein	−0.83	−0.98	−0.88	−0.90
Glyma01g24950	NAD(P)-linked oxidoreductase superfamily protein	−0.01	−0.07	−0.55	−0.29
Glyma18g36840	PPPDE putative thiol peptidase family protein	−0.11	−0.29	−0.87	−0.62
Glyma11g11670	Prohibitin 2	−0.25	−0.58	−0.10	−0.51
Glyma04g12320	Ribosomal L5P family protein	−0.13	−0.21	−0.06	−0.30
Glyma12g28990	SecE/sec61-gamma protein transport protein	0.00	−0.76	−0.70	−0.78
Glyma17g03520	Secretion-associated RAS super family 2	−0.27	−0.98	−1.02	−1.05
Glyma01g05170	UDP-galactose transporter 3	−0.05	−1.00	−0.79	−0.74
Glyma12g30590	Zinc finger C-x8-C-x5-C-x3-H type family protein	−0.33	−0.47	−0.70	−0.33
Glyma10g15160	Unannotated	−0.16	−0.93	−0.68	−0.36
Glyma02g04600	Unannotated	−0.13	−0.07	−0.34	−0.37
Glyma11g07930	B-BOX DOMAIN PROTEIN 19 (BBX19)	0.37	0.38	0.42	0.39

Table 4 (Continued)

Locus ID	Putative function	Average log_2 fold ratios by year			
		2008	2009	2010	2011
Glyma07g01680	GDSL-like Lipase/Acylhydrolase superfamily protein	0.46	0.78	0.64	0.63
Glyma06g16080	Gibberellin 20-oxidase 3	0.35	0.62	0.72	0.97
Glyma05g09920	Gibberellin 2-oxidase 8	0.28	1.39	1.25	1.34
Glyma11g04000	HXXXD-type acyl-transferase family protein	0.13	1.76	0.66	1.06
Glyma09g02210	Leucine-rich repeat protein kinase family protein	0.30	1.84	0.98	0.97
Glyma19g43940	Li-tolerant lipase 1	0.95	2.44	0.61	2.82
Glyma15g10870	lupeol synthase 2	1.18	0.99	1.32	0.83
Glyma19g40980	phytochrome interacting factor 3 (PIF3)	0.66	0.80	0.61	0.48
Glyma11g10460	proline-rich protein 4	0.72	1.43	1.10	1.59
Glyma15g13430	Pyridoxal phosphate (PLP)-dependent transferases superfamily	1.10	0.65	0.72	1.01
Glyma06g08190	UDP-N-acetylglucosamine (UAA) transporter family	0.27	0.49	0.68	0.67
Glyma20g35980	YELLOW STRIPE like 1	0.14	1.28	0.59	0.85
Glyma01g32370	unannotated	0.60	1.11	1.03	0.73

Relative expression pattern (log_2 fold ratio: weedy − control) is indicated with green highlighting downregulated genes and orange highlighting upregulated genes.

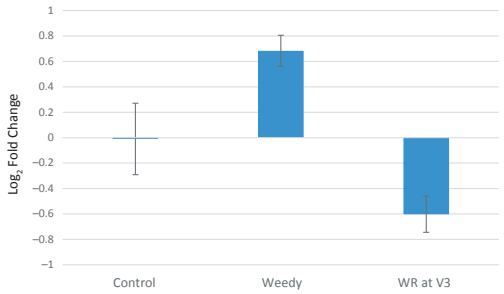

Fig. 2 $2^{-}\Delta\Delta^{C}_{T}$ of soybean (*Glycine max*) PIF3a in control or weedy plots at V5 stage of growth, or in plots following weed removal at V3 (WR at V3). Error bars represent ± SE of $2^{-}\Delta\Delta^{C}_{T}$ values.

transmission of soil-born signals or direct competition for soil nutrients (Fig. 3). Similar significant upregulation was observed for nine of the 12 tested upregulated genes even without root-to-root contact between the soybean plants and the adjacent weeds. Only two of the 16 tested genes identified as downregulated by RNAseq analysis (one, *Glyma03g03270* encoding an arginase/deacetylase superfamily protein, and the other *Glyma09g37010* encoding the MYB domain protein 113 protein) showed consistent downregulation under glasshouse conditions as measured by qRT-PCR. Both of these genes were only significantly downregulated when soybeans were grown in direct root-to-root contact between the soybean plants and the adjacent weeds.

Gene set enrichment analysis (GSEA) and subnetwork enrichment analysis (SNEA) identify processes and signals affected by weed stress

In order to produce a more complete assessment of the physiological and signaling processes affected by weed stress, GSEA and SNEA were run on data from each year (Table S3). GSEA identified 112 ontologies as significantly over-represented ($P < 0.005$) when only genes that were upregulated on average were used in the analysis and only 48 significantly over-represented ontologies were identified when only downregulated genes were analyzed. Eight of the top 10 ontologies for genes that are upregulated during weed stress are associated with oxidative stress or oxygen production (Table 5). Likewise, although the top ontology was 'response to heat', of the top 10 ontologies from downregulated genes, six are associated with protein synthesis. SNEA identified eight significant ($P < 0.005$) ontologies associated with genes that were upregulated by weed stress and seven associated with downregulated genes. The top 10 ontologies associated with genes that are upregulated during weed stress complement the GSEA in that several signals such as neighbors of CO_2, gluconeogenesis and neighbors of HY5 could be related to over-representation of genes with ontologies associated with photosynthesis. Additionally, in concurrence with functional analyses of the significant and consistent differentially expressed genes, five of the top 10 ontologies play a role in JA signaling or heat shock signaling.

Analysis of promoters identifies common elements

The top five over-represented sequences in the promoters for each group (up in weedy and down in weedy soybeans) are reported in Table 6. A search for similarities to known transcription factor binding sites using the PLANT CARE database (Lescot *et al.*, 2002) identified two transcription factor binding sites involved in directing expression in vascular tissues, and one involved in anthocyanin signaling among the genes upregulated by weed stress. No homologies to known transcription factor binding sites were identified among the sequences over-represented in the promoters of the downregulated genes. An investigation of

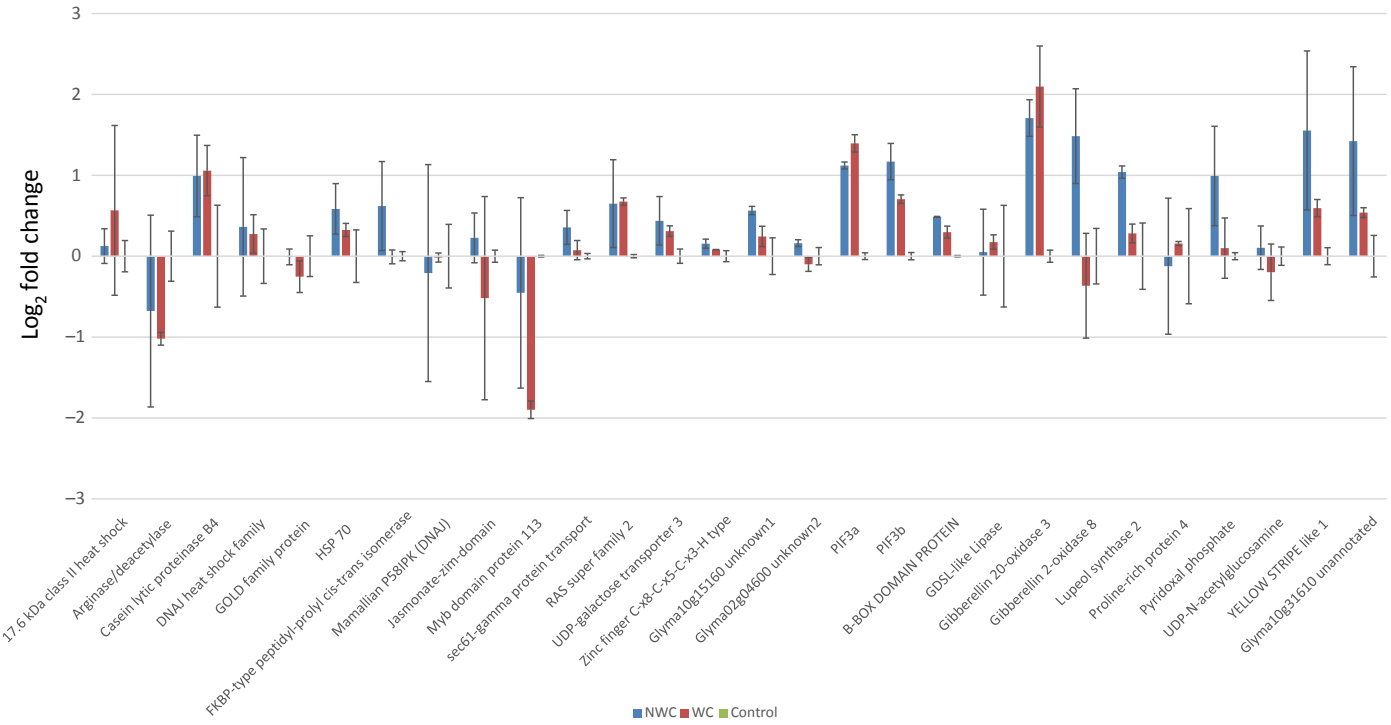

Fig. 3 $^{-}\Delta\Delta^{C}{}_{T}$ of selected genes in soybean (*Glycine max*) when grown under glasshouse conditions with no root-to-root contact (NWC) with the surrounding weeds, or when in direct root-to-root contact with the surrounding weeds (WC) or as a single plant in a pot (control, normalized to zero). Error bars represent range of $2^{-}\Delta\Delta^{C}{}_{T}$ from the two replicates.

Table 5 Top 10 ontologies from soybean (*Glycine max*) gene set enrichment analysis (GSEA) or subnetwork analyses (SNEA) associated with genes upregulated by weeds (top) or downregulated by weed (bottom) as indicated using the Pathway Studio 9.0 program

Top 10 ontologies associated with genes upregulated by weeds	
GSEA	SNEA
Oxidation–reduction process	Lignification
Extracellular region	Neighbors of HY5
Chloroplast thylakoid membrane	Binding partners of ISP
Endomembrane system	Gluconeogenesis
Oxygen binding	Respiratory chain
Iron ion binding	Fatty acid elongation
Monooxygenase activity	Flavonol metabolism
Photosynthesis	Anthesis
DNA binding transcription factor activity	Neighbors of sulfur
Response to salicylic acid stimulus	Neighbors of CO_2
Top 10 ontologies associated with genes downregulated by weeds	
GSEA	SNEA
Response to heat	Neighbors of tunicamycin
Structural constituent of ribosome	Neighbors of COI1
Ribosome	Neighbors of HSF
Translation	Neighbors of heat shock
Cytosolic ribosome	Targets of COI1
Cytosolic large ribosomal subunit	Respiratory chain
Cell wall	Neighbors of radicicol
Cytosolic small ribosomal subunit	Ripening
Response to hydrogen peroxide	Chloroplast division
Response to chitin	Binding partners of JAZ10

over-represented promoter motifs was also completed (Table 6) using the program ELEMENT (Mockler *et al.*, 2007). The most over-represented sequences in the promoters of genes downregulated by weed stress were similar to heat shock binding sites, and G-Box ABRE-like sequences. An AC-rich vascular specific regulatory element was among the top five most significant sequences identified by ELEMENT; however, none of the *P*-values were highly significant among the genes upregulated by weed stress as determined by the ELEMENT program.

Discussion

We examined the transcriptomic changes associated with weed presence during the CWFP in soybeans. Although the CWFP is variable and dependent on planting density, weed density and other growing conditions in soybean (Hock *et al.*, 2006), our data on year-end yield loss following weed removal is indicative of the weed impact at the time of sampling. However, our goal was not to establish a CWFP for soybean, but rather to examine the transcriptome changes associated with weed presence during the early CWFP under field conditions. Because false discovery rates are dependent on the amount of variation in expression of the entire dataset, it is desirable to reduce the concentration of environmental variation to maximize the number of significantly differentially expressed genes. However, field conditions among years resulted in high concentrations of expression variation in many genes and thus necessitated an alternative approach. A reasonable number of significantly differentially expressed genes

Table 6 Over-represented motifs in the promoters of soybean (*Glycine max*) genes that are upregulated or downregulated in soybeans in response to weeds as identified using the programs MEME and ELEMENT

Upregulated genes MEME Putative function	Motif	*e*-value
RNFG2O phloem-specific gene expression	GTGTGTCCC	3.10E+00
Unknown-found in Arabidopsis SAS	CACCACAACNCC	1.20E+02
ARELIKEGHPGDFR2 Anthocyanin signaling	GTGGGAGGGGG	9.90E+03
Unknown	ATGGCTTCAAG	6.20E+04
ACIIPVPAL2	CACATACACAC	1.20E+05

Upregulated genes ELEMENT	Motif	*P*-value
Unknown	AAGTGATC	0.001274
Unknown	GAGTACTA	0.002809
ACIIPVPAL2	ACACACAC	0.135118
ACIIPVPAL2	CACACACA	0.160082
ACIIPVPAL2	ACACACA	0.197529

Downregulated genes MEME	Motif	*e*-value
Unknown	CCCACCTC	6.50E+03
Unknown	CACTCTCnTACC	7.30E+03
Unknown	TGACGTGG	1.50E+04
Unknown	GCACGCGTTGTC	3.00E+04
Unknown	TGGNCTCTGGTA	4.80E+04

Downregulated genes ELEMENT	Motif	*P*-value
HSF	TCCAGAA	7.25E-08
HSF	TCTAGAA	5.52E-07
ABA responsive element	ACGTGTAT	5.54E-07
Unknown	TCCAGA	4.01E-06
Unknown	TCCAGAAA	4.37E-06

Putative motif function, motif sequence and significance values generated by each program are shown. Top five over-represented promoter motifs using MEME and ELEMENT. Upregulated or downregulated refers to the expression in soybeans from the weedy plots.

were identified within these 2 yr of replication (2010 and 2011), indicating that reducing variation between years resulted in sufficiently consistent gene expression to identify genes that were responsive to weeds under the given conditions at the time of sampling. However, we expected that many responses to weeds could be modified by any given environmental condition. By only recognizing genes that had consistent expression over four growing seasons, we are assured that these genes are responding to the only variable that was constant (weed presence vs weed-free conditions). Thus, differences in the methods used to control the weeds, differences in weed species and weed density, and differences in weather and soil conditions between years are expected to result in high concentrations of noise, but should not result in consistent gene expression differences across samples. The only consistent difference between samples was the presence or absence of weeds. Thus, the limited number of genes that we identified as weed-responsive is the result of a robust and highly selective screen for such genes. Indeed, at least for most of the upregulated genes, glasshouse experiments where weeds were not controlled by herbicide treatment further indicate that the method of weed control had little impact on weed-induced gene expression. This work suggests that responses to weeds occur by the V3 stage of soybean growth. However, additional experiments examining the response of soybeans to weeds at earlier and later stages of growth and following weed removal to examine legacy effects of weed presence are of interest.

Consistent gene expression responses to weeds were bolstered by the fact that expression of nearly every upregulated gene tested in glasshouse experiments was differentially expressed in response to weed pressure under controlled environmental conditions. However, the limited confirmation under glasshouse conditions of downregulated genes was unexpected. It is unclear if this indicates a high concentration of false positives in the RNAseq data for downregulated genes, or if glasshouse conditions altered the response of soybeans to weeds. In general, RNAseq data generally correlate well with qRT-PCR (see Glaus *et al.*, 2012), and more replicates were done in the field than in the glasshouse. This implies that the differences between field and glasshouse are real. Thus, these observations, combined with the fact that many of the upregulated genes could be induced under glasshouse conditions – even when no direct competition for nutrients or allelopathic impacts was possible, strongly suggest that many of the upregulated genes are controlled by light quality signals.

Mechanisms underlying yield loss due to weeds

Shade avoidance responses are manifest in most crops and in a large variety of wild species, including the genetic model plant arabidopsis (Ballaré, 1999; Franklin, 2008; Galstyan *et al.*, 2011; Casal, 2012; Gommers *et al.*, 2013). Several transcriptomics studies have been done on the shade avoidance responses of model plants such as Arabidopsis which have identified key components of the signaling processes. Recent studies have identified numerous genes that are differentially regulated in Arabidopsis during intraspecies plant–plant interactions (Masclaux *et al.*, 2012). Although shade avoidance responses enable wild plants to escape from shade originating from neighboring plants in dense vegetation, these are a wasteful investment for crops because the energy used in stems comes at the expense of yield (Robson *et al.*, 1996, 2010; Boccalandro *et al.*, 2003) and combined with the reduced root investments (e.g. Kasperbauer, 1987; Morelli & Ruberti, 2002; Green-Tracewicz *et al.*, 2011) will stimulate lodging and increase drought sensitivity (Page *et al.*, 2011). Furthermore, these responses lead to a more open crop canopy, which results in greater light penetration through the canopy, potentially facilitating weed growth (Weiner *et al.*, 2010). Finally, shade avoidance responses in a variety of species are accompanied by suppression of defenses against herbivorous insects and pathogens (Ballaré *et al.*, 2012; Cerrudo *et al.*, 2012; De Wit *et al.*, 2013). Therefore, total yield potential is typically reduced by shade avoidance responses.

Although our study did not include an analysis of branching that would have provided better evidence for a classic shade

avoidance response, soybean growing in the presence of weeds exhibited reduced leaf area which is indicative of reduced branching commonly observed in shade avoidance responses in this species (Green-Tracewicz *et al.*, 2011), and reduced yield commonly associated with weed stress (Table 2). However, the fact that the weed-stressed plants were not taller than controls and were in some cases significantly shorter (Table 2b, 2010 and 2011) might seem inconsistent with a classic shade avoidance response. Soybean does not always show increased length as part of their shade avoidance response. On the one hand, Pausch *et al.* (1991) studied responses of soybean seedlings to low red : far-red light ratios (R : FR) and found no increased stem length after 4 wk of growth. This was consistent under both glasshouse and growth chamber conditions. On the other, Green-Tracewicz *et al.* (2011, 2012) showed a clear stimulation of plant height by reduced R : FR ratios in all vegetative stages of soybean development, whereas this effect was lost upon transition to the reproductive phase. Data from this field study were confirmed under controlled growth chamber conditions (Green-Tracewicz *et al.*, 2012). The principal difference between the Pausch *et al.* (1991) study and the two studies by Green-Tracewicz *et al.* (2011, 2012) is that the latter lower R : FR in the light reflected from below through non-interfering weeds, whereas Pausch *et al.* (1991) used filters to lower R : FR of the incoming light from above. It remains to be studied whether uniform low R : FR conditions (Pausch *et al.*, 1991) have a different impact on vegetative soybean plant height than does a local R : FR decrease (Green-Tracewicz *et al.*, 2011, 2012). Soybean carries the genes encoding phytochrome photoreceptors (including PhyB) needed to detect R : FR (Wu *et al.*, 2013), which is consistent with the R : FR-driven changes in branching (Green-Tracewicz *et al.*, 2011, 2012) and FR-induced changes in gene expression of etiolated soybean seedlings (Li *et al.*, 2011).

GSEA specifically identified 'shade avoidance' as an over-represented ontology ($P = 0.003$) among upregulated genes (Table S3). Very few genes that were significantly up- or downregulated in response to weeds were identified as differentially expressed in a microarray analysis of Arabidopsis seedlings exhibiting shade avoidance syndrome (Devlin *et al.*, 2003). Indeed, only genes encoding CYCLING DOF FACTOR 3 (*Glyma17g10920*/AT3G47500) and GIBBERELLIN 20-OXIDASE 3 (*Glyma06g16080*/AT5G07200) were upregulated in both datasets (using similar criteria for significance of $P < 0.05$ all years) and a gene encoding a NAD(P)-linked oxidoreductase superfamily protein (*Glyma01g24950*/AT2G37770) and PLASMA MEMBRANE INTRINSIC PROTEIN 2 (*Glyma16g27140*/AT2G37170) were significantly downregulated in both systems. In a more recent publication (Leivar *et al.*, 2012), 1216 Arabidopsis genes were found to be differentially expressed in simulated shade (R : FR ratio of 6.48) following 24 h of treatment. A comparison of this dataset indicated that eight genes had sequence similarity to our list of consistently differentially expressed transcripts (in at least three of the 4 yr) from soybean. Included in this comparison were genes encoding PIF3, HSP81-2, LTL1 (LI-TOLERANT LIPASE 1), a haloacid dehalogenase-like hydrolase encoding gene, the eukaryotic translation initiation factor SUI1, a nodulin MtN21 family protein, BIP2, and a gene of unknown function. However, only two genes (*HSP81-2* and *BIP2*) had the same expression trend (downregulation in response to weeds). Additionally, in a study on Arabidopsis petioles responding to increased FR light (Cerrudo *et al.*, 2012; De Wit *et al.*, 2013), 14 genes were commonly differentially expressed with five genes (AT3G30180, *BR6OX2*; AT1G07570, *APK1*; AT4G23060, *Q-DOMAIN 22*; AT1G01950, *ARK2*; AT5G65380, a MATE efflux family gene) showing similar patterns of upregulated expression. Thus, although shade avoidance responses resulting from altered light quality are likely occurring in response to weed presence, there may be other signals generated by weeds that could evoke plant responses. For example, allelopathic compounds produced by weeds could influence gene expression in the crop. Likewise, volatile signals produced by the weeds may be sensed by the soybean and could induce changes in gene expression or even modify the shade avoidance response (for a review of volatile plant–plant signaling, see Kegge & Pierik, 2010). Additional experiments controlling for these factors are needed to determine what effect, if any, these factors may have on the changes in gene expression observed in our field-grown samples. Finally, most microarray studies on shade avoidance have addressed responses to low R : FR conditions. However, the soybean interactions with weeds occur at high density with fully grown plants that not only affect the R : FR, but also induce reduction of blue light amounts and reduced light intensity. These additional light signals are sensed through other photoreceptors (such as cryptochromes) and can regulate shade avoidance responses through partially similar pathways compared with low R : FR (Keller *et al.*, 2011; Keuskamp *et al.*, 2011). So far, it is not well understood what the relative contributions of these different pathways are in determining the shade avoidance transcriptome during competition with neighbors. We must also acknowledge that we only examined a single variety, and there are indications that different soybean varieties may respond differentially to R : FR light ratios (Cober *et al.*, 1996). Thus, additional observations to examine the response of other soybean varieties are needed to confirm the generality of our observations.

Our results are highly correlated with the response of Arabidopsis to intraspecific competition (Geisler *et al.*, 2012). As in Arabidopsis we also observed upregulation of photosynthesis processes in soybean as indicated in our GSEA and SNEA results. We also observed a similar depression of defense response genes, particularly those involved in JA signaling (Table 5). It has been hypothesized that resource redirection to photosynthesis under competition comes at the expense of defense pathways and that this also might lead to reduced yield (Geisler *et al.*, 2012). The observed over-representation of ontologies associated with photosynthetic processes is the opposite of what we observed in earlier studies on maize under weed stress (Moriles *et al.*, 2012). Indeed, classic shade avoidance responses were not observed in maize in response to weed pressure in several related studies (Horvath *et al.*, 2006; Moriles *et al.*, 2012), suggesting that maize may have fundamentally different responses to weeds than soybeans. The commonalities observed in both intraspecific competition in Arabidopsis and interspecific completion in soybeans suggests hat

perhaps that broadleaved (or perhaps C_3) plants activate common signaling response pathways when under weed stress. The commonalities in expression observed between Arabidopsis responding to itself and soybean responding to different weed species give support to the possibility that the signaling mechanisms implicated by our study may also be important signaling mechanisms involved in planting density responses observed in soybean. However, these hypotheses need testing.

Transcriptional regulators involved in shade avoidance

R : FR light signals are perceived and transduced by the photoreceptor phytochrome. In the model plant Arabidopsis, there are several phytochrome receptors (PHYA, PHYB, PHYC, PHYD and PHYE; Franklin, 2008). These various receptors have partially overlapping roles in the shade avoidance syndrome, circadian regulation, seed germination, and seasonal developmental changes such as bud dormancy and flowering. Numerous additional components of the various signaling pathways altered by phytochrome have been identified such as phytochrome interacting factors (PIFs; Leivar & Quail, 2011; Hornitschek *et al.*, 2012; Li *et al.*, 2012a,b), several other basic helix-loop-helix (bHLH) transcription regulators (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2007; Galstyan *et al.*, 2011) and several homeodomain-leucine zipper (HD-Zip) class-II subfamily transcription factors (Steindler *et al.*, 1999; Ruberti *et al.*, 2011). These transcription factors also interact with other hormonal signaling networks including auxin, brassinosteroids, jasmonic acid (JA) and gibberellic acid (GA) (reviewed in Ruberti *et al.*, 2011; Casal, 2012; Gommers *et al.*, 2013).

In our study, one *PIF3*-like gene was consistently upregulated in response to weed presence. PIF3 is a basic helix-loop-helix transcription factor (Ni *et al.*, 1998) that is regulated diurnally primarily through post transcriptional mechanisms (Soy *et al.*, 2012). White light reduces *PIF3* gene expression (Yamashino *et al.*, 2003) and abscisic acid (ABA) induces *PIF3* in Arabidopsis, whereas JA and salicylic acid (SA) have no effect (Li *et al.*, 2012a, b). In Arabidopsis, PIF3 binds to G-box motifs and interacts with PHYB to regulate gene expression (Martínez-García *et al.*, 2000). *PIF*s *4*, *5* and *7* appear to play the most significant role in the shade avoidance response of Arabidopsis (Keller *et al.*, 2011; Li *et al.*, 2012a; Casal, 2013), however, the weed-induced *PIF3* may be playing a functionally equivalent role in soybean in our study, albeit without affecting height, targeting instead other shade avoidance components such as branching. A phylogenetic analysis of various *PIF* genes (using Clustal W) from soybean and Arabidopsis indicates that the weed-induced *PIF3*-like gene from soybean cluster scloser to *PIF1*, *PIF4* and *PIF7* from Arabidopsis than to Arabidopsis *PIF3* (*PIF3a* in Fig. 3), although the branch point is unsupported. Other phylogenetic analyses (i.e. using the Clustal Omega program) place these two weed-induced genes as outliers. From this perspective, the observation of weed-induced *PIF3* expression was somewhat expected. However, PIFs are not usually transcriptionally regulated by low R : FR conditions, but rather regulated at the protein concentration through phosphorylation and degradation. This may suggest that PIFs could be regulated differently in soybean than in Arabidopsis, or that other signals exist that do lead to enhanced expression (e.g. Zhong *et al.*, 2012).

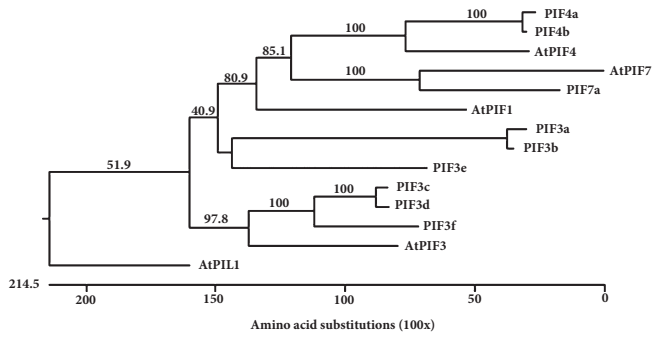

Fig. 4 Phylogenetic tree of the soybean (*Glycine max*) and Arabidopsis PIF gene family.

The phylogenetic analysis also indicates that there are six different *PIF3* genes in soybean. Two additional genes cluster with the weed-induced *PIF3a* gene. One, (designated as *PIF3e*) showed very little change in expression in response to weeds. The other is a likely paralog of the weed-induced *PIF3a* gene (denoted as *PIF3b* in Figs 3, 4), and was upregulated in 2009, 2010 and 2011 samples, but was slightly downregulated relative to the control in 2008 (Table S2). This *PIF3b* gene also was significantly upregulated in response to weeds either with or without root-to-root contact between the weeds and the soybean plants under glasshouse conditions (Fig. 3). Thus, it is likely that this paralogous gene may have a similar role to *PIF3a* during weed-induced stress responses. Other *PIF3*-like genes in soybean were not all induced – indeed, two (*Glyma10g28290* and *Glyma20g22280*) showed consistent if not significant downregulation (Table S2). Because of the importance of PIFs in phytochrome responses, we hypothesize that weed presence causes a shift in the expression of these four genes that results in differential expression of downstream targets. and altered growth and yield of soybean under weed pressure. This hypothesis could be tested by engineering variant soybean types with these genes silenced. If such plants were unresponsive to neighboring weeds compared with unaltered soybean, the link between these specific genes and weed-induced shade avoidance responses would be demonstrated.

In addition to the gene encoding PIF3-like transcription factors, we observed upregulation of a transcription factor encoding gene similar to *B-BOX DOMAIN PROTEIN 19* (*BBX19*). This transcription factor has been associated with regulation of circadian responses (Kumagai *et al.*, 2008) and anthocyanin biosynthesis (TAIR). *BBX19* has also been associated with the shade avoidance syndrome and is upregulated in Arabidopsis growing under a canopy (Crocco *et al.*, 2011). Thus, consistent upregulation of this gene in response to weed pressure was expected. However, BBX19 is a negative regulator of the R : FR response in Arabidopsis (Kumagai *et al.*, 2008). Thus the fact that it is upregulated following extended growth under low R : FR light regimes may suggest that induction of this gene is part of a negative

feedback loop that allows more normal growth in response to weed pressure. Other well-known negative regulators of low R : FR-induced shade avoidance in Arabidopsis include HFR1 and PAR1 and -2 (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2007), but these were not significantly induced in response to weed competition in our study. Altering the expression of *BBX19* to see exactly what role it has in soybean growth and yield when weeds are present during the CWFP would provide additional information about the precise role of this gene in weed-induced shade avoidance.

MYB113 is another transcription factor associated with anthocyanin biosynthesis that was consistently differentially expressed in response to weed pressure. MYB113 is upregulated in response to various stresses (Ambawat *et al.*, 2013) and mutations in this gene negatively affect the accumulation of anthocyanins in Arabidopsis (Gonzalez *et al.*, 2007). The fact that it is downregulated in response to weeds is consistent with the shade response because anthocyanins are needed to protect the plant from high light conditions. Consequently, it seems likely that downregulation of this gene may simply be a response by soybean to denser canopies associated with weed infestations.

The downregulation of a gene related to Arabidopsis *HEAT SHOCK TRANSCRIPTION FACTOR A2* (*HSTFA2*) was also observed in our study. This transcription factor is involved in positive regulation of numerous heat shock genes (Nishizawa-Yokoi *et al.*, 2009). One other observation that suggests functional cross-talk between many of these weed-responsive transcription factors is the observation that BBX18, which antagonizes the heat shock response, is induced by heat stress (Wang *et al.*, 2013). *HSTFA2* is also upregulated in response to oxidative stress (Nishizawa *et al.*, 2006). Thus, the fact that it is downregulated in our study was unexpected because GSEA indicated that various ontologies associated with oxidative stress were over-represented among genes that are upregulated by weed stress. However, the coordinate downregulation of numerous heat shock genes is consistent with a downregulation of protein production as noted by over-representation of ontologies such as structural constituents of ribosome, translation, endoplasmic reticulum lumen, among others noted as repressed in the GSEA. Recently there were several reports linking the high-temperature response to PIF4 function in Arabidopsis (Karayekov *et al.*, 2013; Proveniers & Van Zanten, 2013). However, in Arabidopsis, PIF4 is positively associated with heat shock induction. Thus, the downregulation of these heat shock genes in response to weed stress was not expected. The functional significance of this observation is a mystery. However, of the 1216 genes identified in Arabidopsis as differentially expressed in response to 24 h simulated shade, 15 were heat shock genes, nine of which were downregulated (Leivar *et al.*, 2012).

Hormone responses associated with weed stress

Among the hormones identified by GSEA as associated with the weed response, the most significant associated with upregulated genes were SA, followed by GA, JA, karrikin, brassinosteroids, auxin, ethylene, phytochrome, ABA and cytokinin, in that order. Many of the hormones associated with genes that were upregulated by weed stress have been previously associated with the shade avoidance syndrome. GA and brassinosteroids, (Chory & Li, 1997) have long been associated with increased shoot growth and etiolation responses commonly associated with the shade avoidance syndrome. Likewise, recent studies on shade-induced elongation growth have identified a major involvement of auxin in this process (e.g. Carabelli *et al.*, 2007; Tao *et al.*, 2010; Keuskamp *et al.*, 2011). Plants exhibiting the shade avoidance syndrome also show less branching, which corroborates the importance of auxins in the shade avoidance syndrome (Morelli & Ruberti, 2000; Green-Tracewicz *et al.*, 2011). In tobacco, the shade avoidance syndrome was shown to be inhibited in mutants that have reduced response to ethylene, and this response was dependent on the plants' ability to respond to GA (Pierik *et al.*, 2004). Although both hormones are also involved in shade avoidance regulation in Arabidopsis, ethylene action does not seem to rely on GA in this species (Pierik *et al.*, 2009). Despite the GSEA association of GA with genes upregulated by weed stress, we identified only two genes that regulate GA concentrations as significantly upregulated with the same expression trend in all four years. Expectedly, one was a *GA20ox3*-like gene. *GA20ox* genes encode proteins that play a role in production of active GA and have been shown to be low R : FR-inducible in Arabidopsis (Hisamatsu *et al.*, 2005). The anticipated enhanced endogenous GA concentrations might lead to degradation of growth-inhibiting DELLA proteins to facilitate shade avoidance growth (Djakovic-Petrovic *et al.*, 2007). However, the other consistently upregulated gene was a *GA2-ox8*-like gene known to be involved in GA catabolism (Hedden & Phillips, 2000). This observation suggests that there may be a negative feedback response active in controlling the concentrations of GA production in response to weed stress.

The strong response of genes associated with SA among genes upregulated by weed stress was notable. SA is generally involved in plant responses to pathogens and acts by inducing biosynthesis of defense chemicals such as phytoalexins and reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2; Torres, 2010). Consistent with this association, GSEA also identified numerous ontologies associated with oxidative stress as being significant among genes upregulated by weed-induced stress. Earlier transcriptomics studies on weed responses of corn to weed stress found the opposite response to oxidative stress responses following season-long weed stress (Horvath *et al.*, 2006). However, studies on shade avoidance in maize seedlings indicated that H_2O_2 production was stimulated by weed presence (Afifi & Swanton, 2012). It will be interesting to examine the expression of these weed-responsive genes to see how early they are induced and whether their expression pattern continues if weeds are removed during or after the CWFP. Previous work on other plant systems has also found an association with plant defense responses and shade avoidance signaling (reviewed in Ballaré, 2014), but these studies unanimously showed downregulation of defense concurrent with the shade avoidance syndrome. This observation suggests that, as in the case with antagonistic interactions between SA and JA (Spoel & Dong, 2008), weed stress may

enhance soybean resistance to disease, while making it more vulnerable to insect attack. However, a recent study of Arabidopsis responses to low R : FR showed that both the SA- and JA-mediated defense routes are inhibited by low R : FR at both the transcriptome and functional defense concentrations (De Wit *et al.*, 2013). Also, Masclaux *et al.* (2012) identified an induction of defense-related transcripts by competition and showed that feeding by *Spodoptera littoralis* larvae was reduced by competition at high and low planting density in Arabidopsis. Thus, it appears that even though low R : FR conditions downregulate defenses, the complex environment of plant competition – involving a variety of other signals – may change and sometimes even overrule these patterns.

In conclusion, we have identified consistent changes in gene expression, such as induction of the soybean *PIF3*-like gene, that implicate phytochrome signaling as being involved in early season weed responses of soybean. These changes in gene expression occur concordantly with the CWFP, and functional analyses of similar genes in Arabidopsis are consistent with the role of the soybean *PIF3* genes in a shade avoidance response that could alter growth and development in ways leading to reduced soybean yields. Based on gene set enrichment analysis and the probable function of the consistently differentially expressed genes, we hypothesize that weeds induce a shade avoidance response in soybean very early in the growing cycle, before direct competition for resources would occur. This response is likely to be mediated through reduced R : FR light ratios and the resultant signal transduction and altered gene expression. Reduced R : FR resulting from weed presence could be responsible for the observed weed-induced *PIF3* gene(s) expression. However, additional studies that examine the nature of the signal and quantitation of classic shade avoidance responses such as reduced branching are needed to confirm this hypothesis. Given that many of the deleterious responses of soybeans to weeds, such as reduced yield and increased lodging, could be explained by the developmental changes induced by a shade avoidance response such as early flowering, elongated stems and reduced leaf area, reducing expression of these weed-induced *PIF3* genes could reduce the response of soybeans to weeds. Indeed, over 30 yr ago, manipulating the shade avoidance response was suggested as a means to improve weed tolerance in crops (Smith, 1992). However, if weeds remain later in the season, direct competition for resources may also play a significant role in weed-induced yield losses. Additional work will be required to determine the relative effect of competition for resources (if any) and altered plant development (shade avoidance responses) have on yield losses in soybean, and if the weed-induced *PIF3* genes play any significant role in these processes.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 List of primers used for quantitative reverse transcription (qRT)-PCR

Table S2 Normalized expression data and significance values from the soybean transcriptome in response to weeds

Table S3 Gene set and subnetwork enrichment analysis of soybean gene expression

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