JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Proteomic Analysis of Shade-Avoidance Response in Tomato Leaves

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The aim of this project was to investigate the molecular mechanisms of shade-avoidance response in tomato (*Solanum lycopersicum*) plants. Plants were grown in direct sunlight in ambient temperature and in an adjacent environment under shade cloth. Leaves were harvested, and protein expression differences were investigated using two-dimensional differential in-gel electrophoresis and nanoflow high-performance liquid chromatography–tandem mass spectrometry. Striking differences in plant physiology and protein expression were observed. Plants grown in the shade grew very tall but bore almost no fruit and displayed a dramatic reduction in the accumulation of Rubisco and a number of other metabolic enzymes. We have identified, quantified, and classified 59 protein features found to be up- or down-regulated as part of a shade-avoidance response in *S. lycopersicum* and correlated these with phenotypic data. A large group of proteins related to metabolism and respiration were greatly reduced in accumulation in shade-grown plants, and there was also evidence of significant proteolysis occurring. Four stress-related proteins appear to be constitutively expressed as a result of heat acclimation, while three distinct stress-related proteins appear to accumulate as part of the shade-avoidance response. The identification and functional classification of all 59 differentially accumulating proteins is presented and discussed.

KEYWORDS: Differential in-gel electrophoresis; differential protein expression; liquid chromatography–tandem mass spectrometry; tomato; shade-avoidance

INTRODUCTION

Naturally occurring plant communities, as well as agricultural crops, are frequently resource-limited, causing competition between individuals that often results in developmental responses to the specific resource shortage. Higher plants are photoautotrophs that rely upon the acquisition of light energy for survival; hence, competition for light is characteristic of plant communities. Monitoring changes in the quantity, quality, and direction of light enables plants to optimize both the timing of germination and the subsequent growth and development for the optimal acquisition of light energy to drive photosynthesis. Plants are sessile organisms that cannot choose their surroundings and therefore must adapt their growth and development to the ambient light environment. Furthermore, light signals enable plants to monitor day length (photoperiod) and adapt to changing seasonal environments, including the timing of the transition from vegetative to reproductive development. This high degree of developmental plasticity in response to light signals is mediated by highly specialized information-transducing photoreceptors (*1*, *2*).

Solar light is the source of most biological energy and is vital to the molecular processes and physiological development of plants. The chemical reactions of photosynthesis directly involve the light-harvesting complexes in the chloroplast and the components of the photosystems, but without them, many other cell processes would cease to occur properly. ATP synthesis, amino acid biosynthesis, and carbohydrate metabolism are all affected by light-dependent reactions (*3*). Exactly how the aforementioned metabolic processes and others are modified by a substantial change in sunlight exposure is examined in this report.

Investigations into the response mechanisms to abiotic stresses in a variety of plant species have traditionally been focused on specific cellular activities or physiological responses, rather than

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10.1021/jf0713049 CCC: $37.00
Published on Web 09/18/2007

global protein expression patterns. Examples of specific cellular activities studied include the analysis of heat stress transcription factors in several plant species (*4*), chloroplast heat shock proteins in response to heat stress (*5*), and iron metabolism in heat-stressed tomato and watermelon (*6*). Examples of studies focused on physiological response to abiotic stress include the analysis of photosynthetic activity in tomato and *Arabidopsis* in response to heat stress (*7*, *8*), photosynthetic response in transgenic soybeans subjected to heat and drought stress (*9*), and respiration and growth in tomatoes subjected to prolonged darkness (*3*).

Proteomics in the plant family has gained much ground since early studies in *Arabidopsis* (*10*), and this has been aided by the completion of the genome sequences of both *Arabidopsis* (*11*) and rice (*12*, *13*). A wide variety of proteomic studies have now been completed in higher plants investigating the effects of environmental or abiotic stress conditions such as drought in sugar beet (*14*) and maize (*15*); cold shock in rice (*16*); heat shock in wheat (*17*), barley (*18*), and festuca (*19*); and ozone exposure in rice (*20*). Other proteomic studies have focused on biotic stresses such as the interactions of *Medicago truncatula* roots with soil pathogens (*21*) and symbiotic nitrogen-fixing bacteria (*22*) or the effects of bacterial challenges in *Arabidopsis* leaves (*23*). Protein separation by two-dimensional (2D) gel electrophoresis used in conjunction with identification by mass spectrometry has been a key component of many of these studies (*24*).

Despite the increasing interest in plant proteomics (*25*, *26*), few reports have been published as yet on tomato proteomics. Published reports concern the proteome of developing tomato seeds (*27*), the impact of heat stress on fruit protein expression (*28*), the effect of a physiological disorder (blossom-end rot) on the fruit proteome (*29*), and comparison of fruit proteins of two different tomato ecotypes (*30*).

Tomatoes (*Solanum lycopersicum*) are one of the most widely consumed vegetables in the world, and fulfill an important role in human food consumption. Tomatoes have long served as a model system for plant genetics, development, physiology, pathology, and fleshy fruit ripening. Many genomic tools are now available on this *Solanaceous* species including mapping populations, mapped DNA markers, and Bacterial Artificial Chromosome (BAC) and Expressed Sequence Tag (EST) collections (*31*). There are currently 184000 tomato ESTs available, including 37000 fruit ESTs, corresponding to an estimated 30000 unigenes. Numerous mutants of fruit development and ripening exist, and genome sequencing is in progress (*32*).

Tomatoes are an economically important commercial crop in Southern Arizona, even though it is considered a marginal growing region because the intense heat of even early summer may trigger flower drop and cause poor yields (*33*). Various strategies have been suggested to alleviate this problem, but most attempts to reduce ambient temperature also involve limitation of ambient light, which introduces a different set of problems. The classical shade-avoidance response in plants is to grow taller, in an attempt to access more sunlight, which requires increased metabolic effort in vegetative growth at the expense of reproductive success (*34*). These phenomena have been investigated at the physiological level (*35*–*37*), but little is known regarding the molecular level response of plants grown in such conditions.

The present study takes a broader approach to studying the shade-avoidance response in tomato plants by identifying and quantifying the differentially expressed proteins in shade-grown tomato leaves. Plants were grown in direct sunlight or under shade cloth at outdoor ambient temperatures in April, May, and June in Tucson, Arizona. Protein extracts were prepared from leaf tissue of mature plants grown in either direct sunlight or shade conditions. Two-dimensional differential gel electrophoresis (2D-DIGE) analysis was used to quantify protein expression differences; 47 protein spots were found to be significantly down-regulated as a result of shade growth, and a further 12 protein spots were found to be significantly up-regulated under the same conditions. Nanoflow high-performance liquid chromatography–tandem mass spectrometry (nanoLC-MS/MS) was used to identify all 59 of the differentially expressed protein spots. We demonstrate that an accurate representation of protein accumulation differences can be generated using this suite of technologies and also show that this enables us to undertake a molecular level investigation into shade-avoidance in tomato plants.

MATERIALS AND METHODS

Chemicals, Buffers, and Solutions. Trichloroacetic acid, mercaptoethanol, acetone, acetonitrile, and glycerol were from EMD Chemical (Gibbstown, NJ). Tris-HCl and lysine were from Sigma (St. Louis, MO). Thiourea, urea, dithiothreitol (DTT), mineral oil, iodoacetamide, agarose, glycine, and sodium dodecyl sulfate (SDS) were from BioRad (Hercules, CA). Immobiline DryStrips, pH 3–10, NL (24 cm), 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate (CHAPS), CyDye DIGE Fluors, Destreak Reagent, and IPG buffers, pH 3–10, were from GE Healthcare (Piscataway, NJ). Formic acid was from Spectrum (Gardena, CA).

Tomato Growth. Tomato plants (*S. lycopersicum*, Better Boy variety) were purchased as 14 day seedlings (Mesquite Valley Growers, Tucson, AZ) and transferred to pots (25 cm diameter, 30 cm height) with a 3:2 mix of sunshine #3 and vermiculite coarse #3 soils (Mesquite Valley Growers), fertilized with Miracle Gro (Mesquite Valley Growers), and watered twice daily. Two groups of 10 tomato plants each were grown in two distinct environmental conditions varying by sunlight exposure. The first group was grown outdoors exposed to ambient temperatures and direct sunlight in Tucson in the April–June growing season, while the second group was grown outside underneath a green shade cloth canopy, which reduced incident light intensity by 95%. The temperature was recorded in the center of each group on a minimum/maximum thermometer with a probe placed immediately above soil level, and light readings were taken in the center of each group immediately above the uppermost leaves of the plants. The height of each plant in both groups was measured and recorded individually.

Harvesting and Protein Extract Preparation. Samples of fully expanded, mature, nonsenescing leaves were taken from each group 72 days after germination and immediately frozen. Protein extracts were prepared from the leaf tissue by acetone and trichloroacetic acid (TCA) precipitation (*38*, *39*). The protein-containing precipitate was re-extracted with 2D gel sample buffer [7 M urea, 2 M thiourea, 0.012% DeStreak reagent (GE Healthcare), and 0.5% IPG buffer, pH 3–10]. Samples were then processed using a 2D Clean-Up kit (GE Healthcare) and resuspended in 2D gel sample buffer for protein quantitation.

Cyanine Dye Labeling and Two-Dimensional Electrophoresis (2-DE). Protein extracts were labeled using CyDye 2D-DIGE Fluor minimal dyes (GE Healthcare). Working dye solutions [0.04 mM in dimethyl sulfoxide (DMF)] were added to the prepared protein samples at a final ratio of 50 μg protein:400 pmol dye. Samples were incubated on ice for 30 min in the dark, and labeling was stopped by addition of 10 mM lysine (*40*).

For the first two replicates, 50 μg quantities of sunlight-grown (Cy3-labeled) and shade-grown (Cy5-labeled) samples were mixed with an additional 50 μg of Cy2-labeled pooled internal standard (prepared by mixing equal amounts of the two samples) and supplemented to a final volume of 250 μL of 2D-DIGE sample buffer [7 M urea, 2 M thiourea, 0.012% DeStreak reagent (GE Healthcare), and 0.5% IPG buffer, pH

3–10]. The final solution was loaded onto a Bio-Rad IEF unit using 24 cm IPG pH 3–10 strips, actively rehydrated at 50 V for 12 h, and focused for 72000 Vh. The IEF strips were re-equilibrated, including reduction with DTT and alkylation with iodoacetamide, loaded onto 12% linear SDS–polyacrylamide gel electrophoresis (PAGE) gels, and electrophoresed until the dye front eluted. For the third replicate, the experiment was identical except that the sunlight-grown material was labeled with Cy5 and the shade-grown material was labeled with Cy3.

Image Acquisition and Analysis. CyDye-labeled protein gels were imaged directly in low-fluorescence glass cassettes (GE Healthcare) using a Typhoon variable mode imager (GE Healthcare) at 100 nm resolution. The resulting gel images were processed and analysed using DeCyder Biological Variation Analysis (BVA) software version 5.0 (GE Healthcare). A paired *t*-test was performed using the normalized average spot volume ratios for all spots detected from three replicate experiments, and 59 protein spots showing an expression level change greater than three-fold with a significance value $p < 0.05$ were selected for nanoLC-MS/MS analysis.

Spot Excision and In-Gel Trypsin Digestion. Following image analysis as described above, the gels were poststained with silver (*41, 42*) to allow visualization of protein spots for manual excision with a scalpel. Because lower abundance protein spots were not all clearly visible by this approach, the gels were repeated using a higher protein load (400 μg total) and stained with silver to allow additional spot picking where required. Silver-stained gel spots were destained (*43*) and digested (*44*) using a Multiprobe-II liquid handling system (Perkin Elmer, Shelton, CT). Following digestion, tryptic peptides were extracted from the gel pieces with 5% formic acid/50% CH_3CN (*45*). The combined extracted peptides were concentrated to 10 μL using a Speedvac vacuum centrifuge (Savant, Farmingdale, NY).

NanoLC-MS/MS. A microbore HPLC system (TSP4000, Thermo, San Jose, CA) was modified to operate at capillary flow rates using a simple T-piece flow-splitter. Columns (8 cm × 100 μm i.d.) were prepared by packing 100 Å, 5 μm Zorbax C18 resin at 500 psi pressure into columns with integrated electrospray tips made from fused silica, pulled to a 5 μm tip using a laser puller (Sutter Instrument Co., Novato, CA). An electrospray voltage of 1.8 kV was applied using a gold electrode via a liquid junction upstream of the column. Samples were introduced onto the analytical column using a Surveyor autosampler (Surveyor, Thermo, San Jose, CA). The HPLC column eluent was eluted directly into the electrospray ionization source of a Thermo LCQ Deca ion trap mass spectrometer (Thermo).

Peptides were eluted in a gradient using buffer A (0.1% formic acid) and buffer B (acetonitrile and 0.1% formic acid), at a flow rate of 500 nL/min. Following an initial wash with buffer A for 10 min, peptides were eluted with a linear gradient from 0 to 50% buffer B over a 60 min interval, followed by 50–98% B over 5 min and a 5 min wash at 98% B. Automated peak recognition, dynamic exclusion, and daughter ion scanning of the top three most intense ions were performed using the Xcalibur software as previously described (*46*): (i) full mass survey scan 400–1500 amu, (ii) MS/MS of most abundant ion from survey scan, (iii) MS/MS of second most abundant ion from survey scan, and (iv) MS/MS of third most abundant ion from survey scan. Other instrument parameters included the following: collision energy, 39%; activation Q, 0.25; activation time, 30 ms; isolation width, 2.0 amu; dynamic exclusion enabled with repeat count, 2; duration, 0.5 min; exclusion duration, 5 min; and exclusion mass width low, 1.5 amu, and high, 1.5 amu.

Database Searching and Result Filtering and Validation. MS/MS data were analyzed using SEQUEST run under Bioworks 3.1 (Thermo) (*47, 48*). All spectra were searched against the combined Plant Protein database downloaded from the *Arabidopsis* Information Resource Web site (www.arabidopsis.org). This FASTA format database contains 514092 plant protein sequence entries in the version downloaded September 2006. Dynamic peptide modification by oxidation of methionine and static modification by carbamidomethylation of cysteine were considered. The peptide and fragment mass tolerances were 2 and 0.2 Da, respectively. Database search results were filtered and organized using DTAselect and Contrast (*49*).

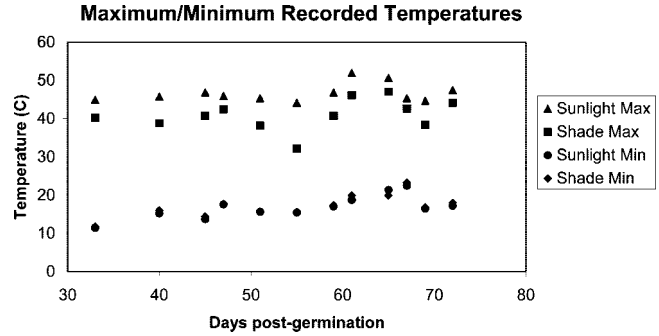

Figure 1. Diurnal temperature range through the growth cycle. Maximum and minimum temperatures recorded in °C as a function of plant age measured in days postgermination. Triangles represent the maximum temperature of plants grown in sunlight, squares represent the maximum temperature of plants grown in shade, circles represent the minimum temperature of plants grown in sunlight, and diamonds represent the minimum temperature of plants grown in shade.

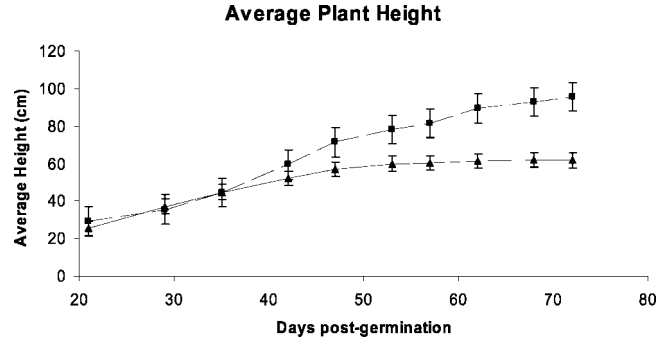

Figure 2. Kinetics of tomato plant growth. Whole plant growth estimated by measurement of apical stem height. The height of each cohort of 10 plants in centimeters is given as a function of plant age measured in days postgermination. Values shown represent the mean, and error bars indicate the standard error ($n = 10$). Triangles represent plants grown in sunlight, and squares represent plants grown in shade.

In this work, the initial criteria for a preliminary positive peptide identification for a doubly charged peptide were a correlation factor (Xcorr) greater than 2.5, a δ-cross-correlation factor (dCn) greater than 0.1, a minimum of one tryptic peptide terminus, and a high preliminary scoring (*50*). For triply charged peptides, the correlation factor threshold was set at 3.5, and for singly charged peptides, the threshold was set at 1.8. False positive rates for protein identification were assessed using reversed database searching (*51, 52*). Imposing a minimum of three peptides per protein on each nanoLC-MS/MS data set (*53*), no proteins fitting the criteria were detected in the reversed database searches, indicating a protein identification confidence level of greater than 99% (*54*).

Data Interpretation and Assembly. The Plant Protein database was used because there is no complete genome sequence available for tomato. In many instances where there was no tomato protein sequence present, unambiguous protein identification was made using multiple peptides identified from a single protein but found in different species. These high-quality peptide matches were manually grouped under descriptive protein headers, to create a nonredundant final data assembly. Protein functions were assigned using information from the Swissprot (http://www.expasy.ch), Munich Information Center for Protein Sequences (MIPS, http://mips.gsf.de), and National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) databases.

RESULTS

Measurement of Environmental Variables. Both groups of plants were exposed to a wide diurnal temperature range as shown in **Figure 1**. During the growth cycle, the plants grown

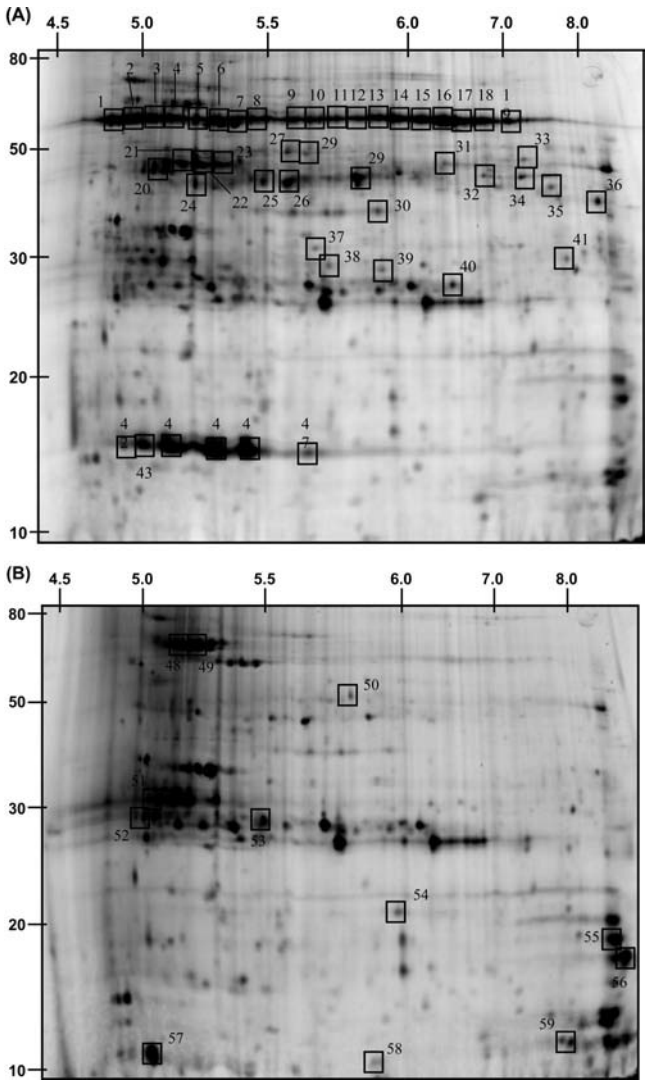

Figure 3. Representative 2D-DIGE gel images of leaf tissue from plants grown in sunlight and shade. (**A**) Leaf tissue prepared from plants grown in sunlight, labeled with Cy3. (**B**) Leaf tissue prepared from plants grown in shade, labeled with Cy5. Up- and down-regulated spots are indicated by boxes and numbered to correspond with the identifications listed in **Table 1**.

in sunlight recorded an average daily maximum temperature of 46.6 °C and an average overnight minimum temperature of 16.8 °C, while the shaded plants recorded an average maximum daily temperature of 40.9 °C and an average overnight minimum temperature of 17.2 °C. Analysis of the temperature measurements recorded throughout the growth cycle indicated that both the maximum temperature readings and the recorded diurnal temperature ranges were statistically significant between the two groups using a paired *t*-test ($p < 0.05$). In contrast, the minimum temperature readings recorded were not significantly different between the two groups ($p > 0.05$).

Plants grown in sunlight experienced intense light exposure with an average of 99800 lux during the day, while those under shade cloth were severely limited in terms of light exposure with an average reading of only 2400 lux, a reduction in incident light intensity of more than 95%. Clearly, the shade cloth had a much greater impact on incident light intensity difference between the two groups than on the ambient temperature.

Plant Physiological Parameters. The two groups of plants displayed obvious physiological differences during the growth season. The average height for the plants measured during the course of this experiment, with the standard error as indicated, is shown in **Figure 2**. Plants grown in sunlight reached a final average height of 64.3 cm, while those grown in the shade grew

Table 1. Quantification of Proteins Differentially Expressed in Shade-Grown Tomatoes

Up-Regulated in Sunlight-Grown Plants

spot no.[a]	#1[b]	#2	#3	mean	SD[c]	CV %[d]
1	13.91	8.15	11.13	11.06	2.35	21.3
2	11.66	9.69	11.57	10.97	0.91	8.3
3	11.28	9.98	11.82	11.03	0.77	7.0
4	14.67	14.67	11.28	13.54	1.60	11.8
5	10.1	13.9	11.83	11.94	1.55	13.0
6	6.73	6.39	7.5	6.87	0.46	6.8
7	8.34	6.53	7.92	7.60	0.77	10.2
8	6.17	8.22	7.49	7.29	0.85	11.6
9	21.98	20.18	17.91	20.02	1.67	8.3
10	19.68	17.23	17.61	18.17	1.08	5.9
11	15.77	18.39	15.78	16.65	1.23	7.4
12	17.38	14.59	15.01	15.66	1.23	7.8
13	15.95	18.05	15.55	16.52	1.10	6.6
14	6.91	8.59	6.35	7.28	0.95	13.1
15	9.38	10.96	9.31	9.88	0.76	7.7
16	3.01	4.48	3.87	3.79	0.60	15.9
17	3.68	3.25	3.11	3.35	0.24	7.2
18	13.05	15.19	12.61	13.62	1.13	8.3
19	8.72	7.76	6.69	7.72	0.83	10.7
20	3.53	3.55	3.06	3.38	0.23	6.7
21	7.49	10.56	8.59	8.88	1.27	14.3
22	4.64	4.25	3.39	4.09	0.52	12.8
23	7.81	7.14	5.01	6.65	1.19	17.9
24	4.16	3.08	5.01	4.08	0.79	19.3
25	5.43	5.06	4.14	4.88	0.54	11.1
26	4.21	4.01	5.18	4.47	0.51	11.4
27	6.92	8.91	7.82	7.88	0.81	10.3
28	4.08	4.26	3.51	3.95	0.32	8.1
29	5.55	5.47	3.88	4.97	0.77	15.5
30	3.91	3.62	4.85	4.13	0.52	12.7
31	7.15	5.62	8.42	7.06	1.14	16.2
32	6.38	8.05	5.88	6.77	0.93	13.7
33	4.12	4.39	5.77	4.76	0.72	15.2
34	10.04	9.54	7.35	8.98	1.17	13.0
35	4.93	4.15	4.03	4.37	0.40	9.1
36	5.15	5.98	3.81	4.98	0.89	18.0
37	3.19	3.01	4.26	3.49	0.55	15.8
38	3.02	4.03	3.05	3.37	0.47	13.9
39	4.39	3.15	5.13	4.22	0.82	19.3
40	3.41	2.88	3.41	3.23	0.25	7.7
41	4.1	3.95	2.69	3.58	0.63	17.7
42	9.71	13.69	13.43	12.28	1.82	14.8
43	12.87	13.68	11.16	12.57	1.05	8.4
44	26.48	20.16	16.48	21.04	4.13	19.6
45	18.67	24.08	14.26	19.00	4.02	21.1
46	24.23	19.21	18.96	20.80	2.43	11.7
47	14.18	9.44	12.33	11.98	1.95	16.3

Up-Regulation in Shade-Grown Plants

spot no.[a]	#1[e]	#2	#3	mean	SD	CV %
48	3.87	3.63	3.33	3.61	0.22	6.1
49	3.99	4.07	4.81	4.29	0.37	8.6
50	4.69	3.73	4.49	4.30	0.41	9.6
51	3.76	3.57	4.89	4.07	0.58	14.3
52	3.76	3.5	4.84	4.03	0.58	14.4
53	3.57	4.43	3.15	3.72	0.53	14.3
54	6.28	4.36	6.47	5.70	0.95	16.7
55	4.1	2.97	3.27	3.45	0.48	13.9
56	15.46	14.19	13.14	14.26	0.95	6.7
57	12.78	18.43	13.39	14.87	2.53	17.0
58	6.44	7.68	7.02	7.05	0.51	7.2
59	13.13	11.03	12.83	12.33	0.93	7.5

[a] Corresponds to spot number indicated on **Figure 3**. [b] Normalized spot volume ratio from each of three replicate experiments (1–3) expressed as unlight/shade. [c] Standard deviation. [d] Coefficient of variation. [e] Normalized spot volume ratio from three replicate experiments, expressed as shade/sunlight.

to be almost 50% taller, reaching a final average height of 94.4 cm. Further analysis of the recorded plant height measurements again indicated that both the average height readings and the height of individual plants considered in pairwise fashion were statistically significant between the two groups using a paired *t*-test ($p < 0.05$).

In striking contrast, the total number of fruit produced by the shade-grown plants was only two, an average of 0.16 fruits per plant, while the total number of fruits produced by the plants grown in sunlight was 45, an average of 3.75 fruits per plant.

Quantification of Differentially Expressed Proteins Using 2D-DIGE. Image analysis detected approximately 1180 spots in each of three replicate gels within the pH range of 3–10 and a size range of 8–100 kDa, with a high degree of reproducibility between gels. A representative gel of the sunlight- and shade-grown leaf material is shown in **Figure 3**, with the fluorescent image acquired from the Cy3 (sunlight) and Cy5 (shade) channels displayed separately. Statistical analysis showed that the expression level of 47 protein spots was significantly down-regulated ($p < 0.05$) in the shade-grown plants, while an additional 12 proteins were significantly up-regulated. The quantitation from triplicate gels using normalized spot volume ratios for each of these protein spots is shown in **Table 1**. Two points are obvious on visual inspection of the gels: several large features indicated by the software as multiple spots appear to represent a large group of closely related proteins, and the same features were almost completely absent in the shade-grown plants.

Identification of Differentially Expressed Proteins Using nanoLC-MS/MS. The proteins present in each of the 59 differentially expressed spots were identified by nanoLC-MS/MS and database searching and are presented in **Table 2**. In several cases where more than one protein was found in a single spot, the number of peptides identified from each of the identified proteins is given. A more detailed accounting of these, showing the peptide identification parameters and NCBI accession numbers of each individual peptide match, is included in the Supporting Information, Table S1. The most immediately obvious feature of the identified proteins was that the two large protein features found to be greatly reduced in the plants grown under shade cloth corresponded to the small and large subunits of ribulose bisphosphate carboxylase (Rubisco). The 47 spots up-regulated in sunlight-grown plants corresponded to 26 unique protein identifications, including multiple isoforms of both the large and the small chains of Rubisco, while the 12 spots up-regulated in shade-grown plants corresponded to 12 unique protein identifications. Rubisco large chains and small chains were identified in 18 and five protein spots, respectively. The distribution of these two proteins across multiple spots on the gels may be due to the presence of post-translational modifications or charged amino acid polymorphisms, as evidenced by the fact that the multiple spots appear to be very similar in molecular weight, while differing markedly in isoelectric point. A similar degree of microheterogeneity for both the large and the small chains of Rubisco has been previously observed in leaf tissue from both tomato (*55*) and rice (*39*) plants.

DISCUSSION

Both groups of tomato plants examined in this study were grown at sustained high temperatures, which reflect normal environmental conditions in Southern Arizona. The plants were acclimated to these conditions throughout their growth cycle; hence, this represents high temperature adaptation rather than

Table 2. Identification of Proteins Differentially Expressed in Shade-Grown Tomatoes

spot no.[a]	protein descriptive header	peptides[b]
	up-regulated in sunlight-grown plants	
1	Rubisco large chain	4
2	Rubisco large chain	4
3	Rubisco large chain	8
4	Rubisco large chain	7
5	Rubisco large chain	6
	calreticulin	6
6	Rubisco large chain	7
7	Rubisco large chain	7
8	Rubisco large chain	6
9	Rubisco large chain	7
	UDP-glucose pyrophosphorylase	3
10	Rubisco large chain	7
	neutral leucine aminopeptidase	4
11	UDP-glucose pyrophosphorylase	6
	Rubisco large chain	5
12	Rubisco large chain	4
	neutral leucine aminopeptidase	5
13	Rubisco large chain	8
	neutral leucine aminopeptidase	3
14	Rubisco large chain	7
15	Rubisco large chain	11
16	Rubisco large chain	11
17	Rubisco large chain	11
18	Rubisco large chain	15
19	catalase	7
	glycine hydroxymethyl transferase	5
20	ATP synthase β-subunit	5
	photosystem II stability/assembly factor	4
21	ATP synthase β-subunit	21
22	ATP synthase β-subunit	20
23	ATP synthase β-subunit	14
24	sedoheptulose-1,7-bisphosphatase	3
	low-temperature-induced cysteine proteinase	3
25	Rubisco activase	16
	phosphoglycerate kinase	5
26	fructose bisphosphate aldolase	5
27	Rubisco activase	13
	phosphoribulokinase	4
28	malate dehydrogenase	4
29	fructose bisphosphate aldolase	4
30	fructose bisphosphate aldolase	4
31	ribulose phosphate epimerase	4
32	ATP synthase γ-chain	3
33	glycolate oxidase	4
34	nucleoside diphosphate kinase	4
35	oxidoreductase	4
36	glycolate oxidase	12
37	peroxidase	15
38	wound-inducible carboxypeptidase	12
39	glyceraldehyde-3-phosphate dehydrogenase	5
40	ferredoxin-NADP reductase	7
41	osmotin	3
42	Putative Rieske Fe-S protein	8
43	Rubisco small chain	4
44	Rubisco small chain	3
45	Rubisco small chain	4
46	Rubisco small chain	6
47	Rubisco small chain	3
	up-regulated in shade-grown plants	
48	threonine dehydratase	13
	aminopeptidase I	3
49	threonine dehydratase	9
50	oxygen-evolving enhancer protein 3	3
51	chlorophyll a/b binding protein	6
52	hypothetical protein precursor (tomato clone TPP11)	5
53	Clp protease proteolytic subunit	4
54	superoxide dismutase	5
55	proteinase inhibitor type II K	3
56	pathogenesis-related protein P2	6
57	Rubisco small chain	4
58	ERT10	3
59	plastocyanin B/B″	3

[a] Corresponds to spot number indicated on **Figure 3**. [b] Number of peptides identified for each protein, using criteria described in the Materials and Methods.

a short-term heat shock response. The diurnal temperature range, however, was sufficient to satisfy the requirement originally noted by Went and colleagues that a minimum of 10 °C difference between daytime and night-time temperatures is needed to achieve vigorous growth and maximum fruiting (*56*). Differences observed in protein accumulation between the two groups of plants analyzed in this study are thus principally due to the difference in incident light, although the relatively small difference in daytime maximum temperatures may also have some effect on protein expression and accumulation.

The growth of plants in an outdoor setting presents problems not usually encountered in the laboratory, including the loss of plants due to animal predation and the fact that such experiments can only be repeated on an annual basis due to the relatively short growing season. In this study, we were able to grow to maturity a single cohort of plants in each environment, and leaf material harvested from these was analyzed in triplicate to establish the statistical significance of observed differences.

Our phenotypical and morphological observations (**Figures 1** and **2**) confirmed that the plants grown in sunlight appeared vigorous and produced a normal amount of fruit as expected. The plants grown in shade conditions exhibited features of the classical shade-avoidance syndrome that has been described in numerous species (*34*, *57*–*59*). The average height of the shade-grown plants at maturity was approximately 50% taller than those grown in sunlight, and almost no fruit was produced. Also, the leaves of the shade-grown plants were observed to be noticeably more vertical in orientation, when compared to their counterparts grown in full sunlight, as has been reported previously in *Arabidopsis thaliana* (*57*). The phenotypic variation of increased height and elevated leaf inclination, coupled with reduced levels of fruit reproduction, is also observed in studies of plant submergence response (*60*). In both cases, the plants appear to be diverting the majority of their metabolic energy into vegetative growth rather than reproduction, to access more sunlight necessary for photosynthesis (*36*) or to escape a submerged environment and thus optimize gas-exchange capacity (*61*, *62*).

To investigate the observed shade-avoidance response phenotype at the molecular level, proteins differentially expressed between the tomato plants grown in sunlight and shade were first examined quantitatively using 2D-DIGE (**Table 1** and **Figure 3**) and subsequently identified using nanoLC-MS/MS (**Table 2**). Tandem mass spectra were searched against the Plant Protein database to identify peptides, and protein identifications were inferred from minimally redundant assembly of the identified peptides. Nine of the identified proteins were matched exclusively to *S. lycopersicum* gene sequences, a further 11 were identified from other solanaceous species (*Solanum tuberosum*, *Solanum melongena*, *Solanum pennellii*, *Nicotiana tabacum*, and *Petunia* spp.), and the remainder were from a wide variety of other plant species (Supporting Information, Table S1). Using this composite plant protein sequence database (*63*), we were able to successfully identify proteins in all of the 59 differentially expressed spots observed in 2D-DIGE experiments, even in the absence of a complete tomato genome sequence. Substantial manual data analysis was required, but this problem will be alleviated when a complete tomato genome sequence becomes available in the near future (*32*). To facilitate a functional and mechanistic analysis of our protein identification results, the identified proteins were categorized according to their annotated cellular functions, as shown in **Table 3**. A majority of the proteins identified are known to be of relatively high abundance, which may be attributed to the fact that we have chosen to focus on those with protein expression level differences of three-fold or more. More subtle changes in protein level, observed for less abundant proteins, have been previously reported using the 2D-DIGE approach (*40*, *64*) .

Table 3. Functional classification of proteins differentially expressed in shade-grown tomatoes

up-regulated in sunlight	up-regulated in shade
respiration	**respiration**
Rubisco (large and small chain)	oxygen-evolving enhancer protein 3
Rubisco activase	chlorophyll a/b binding protein
putative Rieske Fe–S protein	Rubisco small chain
PSII stability/assembly factor	
ferredoxin-NADP reductase	
Calvin cycle	**light harvesting**
sedoheptulose 1,7-bisphosphatase	plastocyanin B/B″
ribulose phosphate epimerase	ERT10
phosphoribulokinase	
glyceraldehyde-3-phosphate dehydrogenase	
glycolysis	**cell maintenance**
fructose bisphosphate aldolase	threonine dehydratase
phosphoglycerate kinase	aminopeptidase I
glycolate oxidase	Clp protease proteolytic subunit
biosynthesis	**stress response**
malate dehydrogenase	superoxide dismutase
oxidoreductase	proteinase inhibitor type II K
ATP synthase	pathogenesis-related protein P2
glycine hydroxymethyl transferase	
nucleoside diphosphate kinase	
UDP-Glc pyrophosphorylase	
cell maintenance	**unclassified**
calreticulin	hypothetical protein (clone TPP11)
catalase	
peroxidase	
stress response	
low-temp-induced cysteine proteinase	
wound-inducible carboxypeptidase	
osmotin	
neutral leucine aminopeptidase	

The plants grown in full sunlight clearly have much higher levels of Rubisco, ATP synthase, and other metabolic and respiratory proteins. These plants appear to be compensating for the large amount of light, and consequently energy, by accelerating assimilation of carbon dioxide and processing of simple sugars and amino acids. Rubisco is employed as a means of storage for excess carbon fixation products that can then be metabolized into energy when required. The much lower levels of Rubisco detected in the leaves of shade-grown plants provide a strong indication that these plants were investing all available energy resources into stem elongation, leaving little excess for storage (*36*, *62*, *45*). The reduced levels of metabolic proteins detected in the leaves of the shade-grown plants also support this hypothesis, as it suggests that there was much less metabolic activity occurring in these leaves than those grown in full sunlight. The shade-grown plants were still able to survive and even grew to be taller than the plants grown in the sun, but the fruiting process was substantially arrested.

The shade-grown plants displayed an increase in light-harvesting proteins such as chlorophyll a/b binding protein and oxygen-evolving enhancer 3 protein. There was also a similar up-regulation of a group of proteins involved in copper and electron transport, related to light harvesting, including ERT10, plastocyanin B/B″, and superoxide dismutase. These plants seemed to be responding to the lack of sunlight by boosting their ability to convert light energy to chemical energy using the small amount of light available, possibly even going through state 1 to state 2 transitions known to occur in low-light conditions (*23*).

Rubisco small chains were detected as being both up-regulated and down-regulated in response to shade growth.

The five protein spots (spots 43–47) assigned to Rubisco small chains with apparent molecular masses of 18 kDa in the plants grown in full sunlight were strongly down-regulated in the shade-grown plants. A prominent spot with an apparent molecular mass of 12 kDa (spot 57) was also identified as a Rubisco small chain but was only present in the shade-grown plants. As shown in Supporting Information Table S1, the four peptides identified from the smaller isoform of the protein found in spot 57 were located in residues 69–150 of the Rubisco small chain protein sequence; the theoretical molecular mass of this fragment is 10.7 kDa. In contrast, the peptides identified from the Rubisco small chain sequence in spots 43–47 encompass the region from residue 69 to the carboxy-terminal tyrosine residue at position 181. This difference in identified peptides, along with the shift in gel mobility, indicates that the Rubisco small chain isoform in spot 57 is a breakdown product of the original protein cluster.

These data suggest that shade growth may evince a protein degradation phenotype, as has been demonstrated previously in cold-stressed rice (*16*). This is supported by the observed up-regulation of the Clp protease proteolytic subunit in the shade-grown plants (spot 53), as this is known to play a major role in the degradation of misfolded proteins in senescing *Arabidopsis* leaves (*65*).

There are a series of proteins identified as being up-regulated in the plants grown in full sunlight that are annotated as stress response proteins, including the low-temperature-induced cysteine proteinase (spot 24) (*66*), wound-inducible carboxypeptidase (spot 38) (*67*), and osmotin antifungal protein (spot 41) (*68*). Our data show that all three of these proteins were constitutively expressed in leaves at higher levels as a result of heat acclimation, rather than as a stress response, as these plants were not subjected to low temperature, wounding, or fungal challenge. The low-temperature-induced cysteine proteinase may have been named as such when it was first detected in cold stress experiments, but it appears that it can also be up-regulated by other temperature variations.

Conversely, a different set of stress response proteins was found to be up-regulated in the shade-grown plants, including superoxide dismutase (spot 54) (*69*), proteinase inhibitor type II K (spot 55) (*70*), and pathogenesis-related protein P2 (spot 56) (*71*). Therefore, these proteins can now also be considered as subject to induction as part of the shade-avoidance response. There was also one hypothetical protein from tomato (spot 52) that was found to be up-regulated in the shade-grown plants. It is not possible to infer any information regarding the precise function of this protein at this stage, but our data suggest that it may play a role in light harvesting, protein degradation, or stress response. At the very least, it can now be considered as a protein that is up-regulated as part of the shade-avoidance syndrome.

In conclusion, we have shown that by using a combination of 2D-DIGE and nanoLC-MS/MS it is feasible to investigate changes in protein expression in *S. lycopersicum* at the molecular level in response to the imposition of abiotic stress. Our results provide valuable insights into how plants adapt to growth in low-light conditions and will enhance our understanding of the shade-avoidance response at the molecular level. We have shown that using this approach, we were able to dissect distinct molecular mechanisms of response to imposed environmental stresses, which are characterized by differential expression of complementary gene networks.

ABBREVIATIONS USED

2-DE, two dimensional electrophoresis; 2D-DIGE, two dimensional differential in-gel electrophoresis; BAC, Bacterial Artificial Chromosome; CHAPS, 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate; DMF, Dimethyl formamide; EST, Expressed Sequence Tag; IEF, isoelectric focusing; nanoLC-MS/MS, nanoflow high pressure liquid chromatography tandem mass spectrometry; RP, reversed phase; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis.

ACKNOWLEDGMENT

We thank Vicki Chandler and the Bio5 Institute for support; P.H. acknowledges the NSW Office of Science and Medical Research Biofirst Scheme for support and thanks Ben Riggs, Peter Lehmann, and Kellen Haynes for continued support and encouragement.

Supporting Information Available: Sequest output results for each peptide used to identify proteins listed in **Table 1**, including NCBI accession number, species name, protein description, Xcorrelation value, δ-Xcorrelation value, peptide mass ($M + H^+$), peptide charge state, and peptide sequence. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review May 3, 2007. Revised manuscript received June 18, 2007. Accepted July 9, 2007. This work was supported in part by the Australian Government through the Major National Research Facilities program funding of the Australian Proteome Analysis Facility.

JF0713049