Differentially Expressed Proteins of Soybean (*Glycine max*) Pulvinus in Light and Dark Conditions

Hakme Lee[a], Wesley M. Garrett[b], Joe H. Sullivan[a], Irwin Forseth[a] and Savithiry Natarajan[c,*]

[a]*Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD 20742, USA*

[b]*USDA-ARS, Animal Bioscience and Biotechnology Laboratory, Beltsville, MD 20705, USA*

[c]*USDA-ARS, Soybean Genomics and Improvement Laboratory, Beltsville, MD 20705, USA*

Abstract: Some plant species both track and avoid the sun through turgor changes of the pulvinus tissue at the base of their leaves, maximizing light reception in dim conditions and minimizing cellular damage due to excessive light. Pulvinar response is known to be affected by both diurnally varying environmental factors and circadian patterns. Differential expression of the proteins between light and darkness are not well-known. In this study we used two-dimensional gel electrophoresis and mass spectrometry to separate and identify proteins in the soybean leaf pulvinus that were differentially expressed in the light compared to a dark control. Out of 165 protein spots previously identified (data not shown) 11 were found to have decreased expression in the light and 7 had increased light expression. The proteins that were more highly expressed in the light were mostly stress response proteins, while the under-expressed proteins were categorized as energy proteins. While the higher levels of expression of stress response proteins in the light align with other studies, the under-expressed light proteins require further examination to rule out artefactual results. These findings can provide a better understanding of the circadian pattern of protein expression in the legume pulvinus proteome.

Keywords: Heliotropism, LC-MS/MS, nyctinasty, proteomics, soybean.

INTRODUCTION

Plant tissues respond to a variety of environmental variables by reversible cell turgor changes and irreversible differential growth [1-3]. The reversible bending response of a plant to a light stimulus is called heliotropism and can be further classified as paraheliotropism (light-avoiding) and diaheliotropism (light-seeking) [4, 5]. The benefits of heliotropism include the reduction of excessive leaf temperature and transpiration, avoidance of photoinhibition, increased light and water use efficiency, and maximal photosynthetic rates, depending upon the particular conditions [4-7]. For many plants, particularly leguminous species, the site of light perception is in the pulvinus, a histologically distinct tissue located at the base of the leaf [4]. Potassium and chloride influx coupled with proton efflux leads to turgor increase in abaxial, shaded pulvinar motor cells; while on the adaxial, lighted side of the pulvinus ion efflux results in turgor decrease [8, 9]. The asymmetric turgor gradient formed between the adaxial and abaxial regions of the pulvinus results in leaf movement.

The specific mechanisms leading to opposing responses in the two motor regions are not yet fully understood. Some studies isolating adaxial and abaxial pulvinus protoplasts have shown the same physiological response to a given stimulus for both regions. *Phaseolus vulgaris* adaxial and abaxial motor cells both responded to blue light irradiance with cell shrinkage; the only difference between the two regions was the intensity of the response which was likely dependent on the fluence rate [9]. Likewise, application of the auxin indole-acetic acid (IAA) increased turgor in both adaxial and abaxial cells of *P. vulgaris* pulvinus; similarly, abscisic acid decreased turgor for both regions [10]. However, other studies of intact pulvini have shown that a given stimulus produces opposing activity between the two regions, as is expected from the visible bending response. *Samanea saman* pulvinusapoplasts had a pH difference between adaxial and abaxial motor cells which was exaggerated by treatments of white light, blue light, and darkness [11]. Kim *et al*. [12] found that red light pulses preceding darkness opened K^+ channels in *S. saman* adaxial cells and closed channels in the abaxial cells. They concluded that pulvinus responses to red and blue light differ not only by the tissue region but also by the time in the overall photoperiod.

In general, it appears that unique signaling pathways in the two motor cell regions result in complementary cell responses for light or darkness. What are presently unknown are the gene products preferentially expressed in light over darkness and the

*Address correspondence to this author at the Soybean Genomics and Improvement Laboratory, ARS-USDA, 10300 Baltimore Avenue, Beltsville, MD 20705, USA; Tel: 301-504-5258; Fax: 301-504-5728;
E-mail: savi.natarajan@ars.usda.gov

ISSN: 1814-8085 / E-ISSN: 1927-5129/13

nature of the regulation of those differentially expressed proteins. Protein levels may vary as a result of a light stimulus, as would be assumed in heliotropic leaf movement, or by a rhythm endogenous to the plant and at least partially independent of external stimuli, such as daily nyctinastic leaf folding of *S. saman* and *Mimosa pudica*, among other species [13]. There is evidence for the latter form of regulation in the expression of ion transporters. In a review by Haydon *et al*. [13] multiple transporters in *Arabidopsis* were found to be regulated by circadian rhythms, including aquaporins, sugar transporters, K^+ channels, and other cation channels. In a previous study by our lab, ion transport proteins such as potassium channels and voltage-gated anion channels were not detected in the soybean pulvinus due to the difficulty of separating plasma membrane proteins using the trichloroacetic acid/acetone extraction methods. Other enzymes implicated in pulvinar functions that were detected by our lab could also be regulated in a similar circadian fashion as the transport proteins.

The objective of this study was to compare pulvinus protein levels expressed during the light to that in darkness to identify areas of differential expression. Tissue samples were taken of the whole pulvinus both before and after the dark-light transition. Proteins were separated using 2-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by tandem mass spectrometry (LC-MS/MS). Gels from both time periods were compared to each other to detect the effect of light on protein expression relating to pulvinar function. The differentially expressed spots were discussed in the context of their roles in diurnal and circadian rhythms.

METHODS

Plant Material

Soybean (*G. max* cv. Clark) seeds were soaked overnight in tap water before they were planted in 6-inch pots (2-3 per pot) with an LC1 soil mixture (Sun Gro Horticulture, Vancouver, BC, Canada). The plants were grown in a growth chamber at the University of Maryland, College Park, set to a 16:8 photoperiod with 750-900 µmol m^{-2} s^{-1} of photosynthetic active radiation (PAR) measured at the top of the plants. The temperature was set to 25°C during the day and 20°C at night, with 50%/60% humidity. The plants were watered to avoid water stress and received 100 ppm N fertilizer once a week. The plants were harvested after the appearance of 6-7 trifoliolate leaves (between 6-8 weeks after planting). For light-harvested pulvini, the terminal and lateral pulvini from the second through sixth trifoliolate leaves were separately excised with a razor one to three hours into the light period and frozen in liquid nitrogen; dark-harvested pulvini were harvested one to two hours prior to the end of the dark period. The pulvini were stored in a -80°C freezer until further use.

Protein Extraction

Trichloroacetic acid (TCA)/acetone precipitation, described previously by Natarajan *et al*. [14] was used to extract pulvinar protein. For each of three biological replicates approximately 2.0 g of pulvinus tissue was ground into a powder using a mortar and pestle with liquid nitrogen, then extracted with a 10% TCA/0.07% β-mercaptoethanol in acetone mixture. Following a minimum of one hour incubation at -20°C and centrifugation at 14,000 rcf in 4°C for 20 minutes, the supernatant was discarded. The pellet was rinsed with 0.07% β-mercaptoethanol in acetone solution followed by centrifugation at 14,000 rcf (4°C) for 20 minutes; the rinsing and centrifugation steps were repeated until the supernatant was clear. After vacuum drying, the pellet was resolubilized in a 9M urea, 2M thiourea, 4% (w/v) CHAPS, 1% DTT solution and sonicated on ice for 45 minutes. The supernatant was collected after centrifugation at 14,000 rcf (4°C) for 20 minutes and the protein concentration was quantified using the Bradford method with bovine standard albumin as spectrophotometric standard [15].

2D-PAGE

For first dimension electrophoresis, 500 µg of protein in a solution of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, 1% (v/v) IPG buffer (pH 4-7), and 0.002% bromophenol blue were loaded onto 13 cm IPG strips, pH 4-7, and run on a flatbed EttanIPGphor II (GE Healthcare, Piscataway, NJ) under conditions described earlier by Natarajan *et al*. [14]: 30 V for 13 hours, 500 V for one hour, 1000 V for one hour, 8000 V gradually for 1:30 hours, 8000 V for 24000 Vhr, and 500 V for ten hours. The final step was truncated if the protein appeared to be sufficiently separated. Prior to second dimension SDS-PAGE the IPG strips were equilibrated twice to reduce the disulfide bridges, first in DTT and then in IAA, 15 minutes each in equilibration buffer (50 mMTris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT). The strips were loaded onto 12.5 % polyacrylamide gels using a Hoefer SE 600 Ruby electrophoresis unit and

run for 15 mA per gel for 30 minutes and 25 mA per gel for up to five hours. The gels were stained for two days using Coomassie Blue G-250. Gels were scanned on an Image Scanner III (GE Healthcare), and analyzed using Progenesis Same Spots software (Nonlinear Dynamics, Durham, NC).

Significant differences in protein expression were calculated through the Progenesis Same Spots software program for each gel replicate. All gels were subject to quality control assessment to and image alignment. It was found that lateral and terminal gels were not significantly different from one another and so the lateral dark-harvested pulvinus gel was used as the control condition. Between-subject design experiment setting was used for comparing the lateral light and lateral dark. Significance values were calculated by one-way Analysis of Variance (ANOVA) where each gel replicate was examined for only a single treatment, that is, the presence or absence of light during harvesting. Significance in ANOVA is determined when the ratio of mean squares between treatments and mean squares within treatments (the F-statistic) exceeds a threshold value that is dependent on the number of samples and treatments. Fold changes are calculated as the ratio of the treatment volumes.Only spots with both ANOVA p-values ≤ 0.05 and max fold change ≥ 1.5 were considered differentially expressed. False positives and false negatives were manually examined for exclusion and inclusion, respectively, from the dataset. Gels were stored in a 17.5% ammonium sulfate solution until further use.

In-Gel Digestion

Trypsin digestion of selected spots was based on methods by Shevchenko *et al*. [16] and Gharahdaghi *et al*. [17]. Spots were excised with a 1.5 mm picker and rinsed twice with 50% methanol, ten minutes each, before methanol removal and storage at -20°C. The gel pieces were reconstituted and subsequently dehydrated in solutions of 25 mM NH_4CHO_3 and CH_3CN respectively, by placement on a shaker for ten minutes per solution. The prior step was repeated for a second time. The spots were further dried in a speed vac concentrator for about 15 minutes. Each gel piece was then reswollen with a 20 µL aliquot of 10 ng/µL porcine trypsin (sequencing grade, Promega, Madison, WI) in 25 mM NH_4CHO_3 and refrigerated for one hour in 4°C before overnight incubation at 37°C.

The supernatants were transferred to new tubes and the remaining peptides were extracted from the gel pieces by 50% CH_3CN/5% trifluoroacetic acid (TFA). 50 µL of the extraction mixture was added to each gel piece and placed in a shaker for an hour. The supernatants were added to the original trypsin digests and the extraction was repeated once more with another 50 µL aliquot. The supernatants were then dried for up to two hours on a speed vac concentrator. The peptides were solubilized in a 20 µL solution containing 5% CH_3CN/0.1% HCO_2H.

Mass Spectrometry

The peptides were run through an LTQ Orbitrap XL hybrid linear ion trap Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with reverse-phase chromatography on a 100 x 0.18 mm BioBasic-18 column and 3 µL/min flow rate. The 30-minute linear gradient was 5-40% acetonitrile in a 0.1% formic acid solution. The resolution survey scan was over the range 400-1600 m/z (4=30000 at m/z 400) and the MS/MS spectra of the five most abundant ions were recorded. The electrospray voltage was 3.5 kV with normalized collision energy set to 30% and a minimum ion count of 5000. Mascot Distiller version 2.3.00 was employed for producing searchable peak lists.

Data Analysis

MS/MS data were analyzed by the Scaffold toolkit version 3 (Proteome Software, Portland, OR). Scaffold searches MS/MS data against several database search engines and computes a peptide probability incorporating similar results among the search engines. Based on the peptide distribution, a protein probability is computed and the peptides are identified as parts of the computed protein [18]. The MS/MS data was searched against the UniProt Knowledgebase. The results were limited to *G. max*, with minimum values of two significant peptide matches, 80% peptide identification probability, and 95% protein identification probability. Uncharacterized proteins were identified by examining the homologous protein clusters at 100%, 90%, and 50% homology as curated by UniRef. Proteins without a name at the 50% homology level remained uncharacterized.

RESULTS

In this study we have undertaken 2-D PAGE and LC-MS/MS analysis for protein characterization of soybean pulvinus expressed under dark and light conditions under a controlled environment. A representative gel of pH 4-7 and all differentially

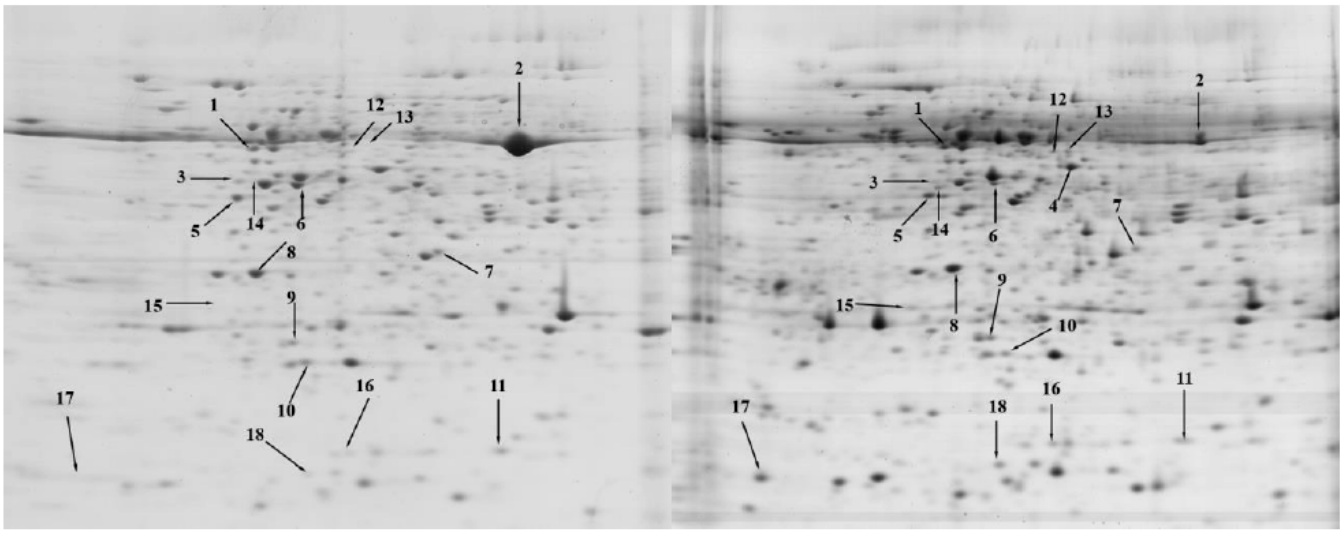

Figure 1: Comparative protein expression levels of light and dark harvested pulvinus. **A**) on the left is the dark harvested sample, excised about six to seven hours into the dark period. **B**) on the right is the light harvested pulvinus, harvested about one to three hours into the beginning of the light period. Differential expression was measured by ProgenesisSameSpots software and required a minimum 1.5-fold increase/decrease and ANOVA p≤0.05.

expressed proteins are indicated by arrows (Figure **1**). Out of 165 previously identified protein spots (data not shown), 7 increased (spots 12-18, corresponding to 11 possible proteins) and 11 (spots 1-11, corresponding to 16 possible proteins) decreased expression in the light (Figure **1**). Information for the spots, including volumes of the protein spot under light and dark conditions, protein name, fold difference, and p-value are listed (Table **1**).

Proteins that had higher protein expression in the light included four involved in stress response: cytosolic ascorbate peroxidase (spot 15), superoxide dismutase (spot 18), an elicitor-inducible protein (spot 17) and an uncharacterized protein that had gene ontology (GO) annotation in biological process for stress response (spot 16). Several of the spots with increased expression contained more than one potential protein. Spot 12 contained a mixture of four proteins: argininosuccinate synthase (nine peptides), elongation factor 1-alpha (two peptides), alanine aminotransferase 2 (two peptides), and 26S protease regulatory subunit 6B homolog (five peptides, also found in other spots) (Table **1**, peptide data not shown). Spot 13 was also a mixture of proteins: DEAD-box ATP-dependent RNA helicase 56 and an uncharacterized protein. Beta-amylase (spot 14) was the final protein that was more highly expressed in the light.

Among the spots that had higher protein expression in the dark were seven containing proteins associated with photosynthesis or the Calvin cycle: phosphoribulo kinase (spot 5), rubisco large subunit (spot 2), rubisco activase beta form (spots 3, 6), cytochrome b_6f iron-sulfur subunit (spot 11), ferredoxin-NADP reductase (spot 7), oxygen-evolving enhancer protein 1 (spot 8), and an oxygen evolving enhancer protein 2-1 (spot 10). Several of those protein spots had multiple peptide sources. Phosphoribulokinase in two protein forms and an uncharacterized protein GO annotated for proteolysis were both detected in spot 5. Rubisco activase and an uncharacterized protein with a molecular function annotation for ATP binding appeared in the same spot (spot 6; rubisco activase was also detected in spot 3). The dark-expressed proteins not involved in photosynthesis and the Calvin cycle included a chloroplast 20 kDa chaperonin (spot 9), S-adenosylmethionine synthase (spot 4), and a multi-peptide mixture containing three tubulin A proteins (spot 1).

DISCUSSION

Leaf movements in soybean occur as the adaxial and abaxial regions of pulvinar motor cells adjust their levels of turgor relative to each other. Illumination from above results in greater turgor pressure in the abaxial motor cells compared to the adaxial motor cells; while leaves in the dark have the reverse turgor gradient [4, 19, 20]. Motor cells of both regions are structurally the same, but react with opposing movements to the same light/dark stimulus. A detailed explanation for the

Table 1: Differentially Expressed Proteins in Light and Dark Harvested Soybean Pulvini

Spot no.	Protein	UniProtKB acces. no.	Volume Light + SD	Volume Dark + SD	p-value	Fold change
1	Tubulin alpha-6 chain	I1K2I1	4.98E+06	7.70E+06	0.013	1.5
	Tubulin alpha-2 chain	I1JV03				
	Tubulin A	I1K3X9				
2	Ribulose bisphosphate carboxylase large chain	P27066	1.83E+07	5.70E+07	0.033	3.1
3	Rubisco activase	D4N5G3	1.55E+06	3.27E+06	0.039	2.1
4	S-adenosylmethionine synthase	I1MHR0	1.66E+07	2.51E+07	0.038	1.5
5	Phosphoribulokinase	I1L540	7.86E+06	1.43E+07	0.024	1.8
		I1J4L6				
	Uncharacterized protein	I1JGY9				
6	Rubisco activase	D4N5G3	5.98E+06	1.31E+07	0.01	2.2
	Uncharacterized protein	I1MZA9				
7	Ferredoxin--NADP reductase, leaf isozyme 2, chloroplastic	I1JCG8	3.55E+06	5.79E+06	0.016	1.6
8	Oxygen-evolving enhancer protein 1, chloroplastic	I1MNK0	2.02E+07	3.00E+07	0.011	1.5
9	20 kDa chaperonin, chloroplastic	C6TJG0	3.72E+06	5.46E+06	0.016	1.5
10	Oxygen-evolving enhancer protein 2-1, chloroplastic	I1N123	4.93E+06	1.01E+07	0.018	2
11	Cytochrome b6-f complex iron-sulfur subunit	I1LUB3	6.37E+06	1.19E+07	0.009	1.9
12	Alanine aminotransferase 2	I1J547	2.05E+06	1.32E+06	0.005	1.6
	Argininosuccinate synthase, chloroplastic	I1K099				
	26S protease regulatory subunit 6B homolog	I1MZN7				
	Elongation factor 1-alpha	P25698				
13	DEAD-box ATP-dependent RNA helicase 15	I1JUD5	3.44E+06	1.94E+06	0.002	1.8
	AT5g63860/MGI19_6	I1JXY7				
14	Beta-amylase	P10538	1.98E+06	6.90E+05	0.018	2.9
15	Cytosolic ascorbate peroxidase 1	I1LKA6	3.39E+06	1.60E+06	6.23E-04	2.1
16	Uncharacterized protein	C6SVX3	4.32E+06	2.87E+06	0.039	1.5
17	Elicitor-inducible protein EIG-J7	I1LNL7	1.01E+07	2.34E+06	0.003	4.3
18	Superoxide dismutase [Cu-Zn]	I1LKZ3	7.00E+06	4.47E+06	0.027	1.6

Note: 18 spots with ANOVA values p≤0.05 and a minimum 1.5-fold change were positively identified as 25 unique proteins using ProgenesisSameSpots and Scaffold protein identification software programs. All proteins are from the species *Glycine max*.

complementary mechanisms that occur in similar cells differing only in their location within the pulvinus has yet to be offered. It is possible that some level of differential protein expression between light- and dark-harvested tissues could be detected, especially if the level of activity of one region is disproportionate to the level in the other region. Studies in pulvinar water potential differences between the two regions have indicated a more dominant role of the adaxial motor cells in enabling leaf movement. Measurements on overall water potential, osmotic potential, and hydrostatic pressure in *S. saman* and *P. coccineus* have indicated that the water potential gradients formed between adaxial and abaxial motor cells are largely the result of hydrostatic pressure changes in the adaxial cells [19]. Measurements in *S. saman* apoplasts found that the pH gradient across abaxial plasma membranes was smaller during the light period than adaxial plasma membranes. In darkness, the abaxial pH gradient all but disappeared while the adaxial pH gradient was exaggerated [20]. The development of the proton motive force across the plasma membrane energizes the transport of K^+ and Cl^-; the increasing pH gradient across adaxial plasma

membranes between light and dark periods could correlate to a greater energy requirement in that motor region that could be detected at the translational level even with whole pulvinus samples. If the two regions complement each other in activity then net protein expression may not be significantly different between light and darkness if the whole pulvinus is examined, as increased expression in one region would be balanced by decreased expression in the other region. It is also possible that changes in transcript levels would not resemble changes at the level of protein synthesis. Surprisingly, none of the proteins we detected as differentially expressed in the soybean pulvinus were directly involved in processes of ion transport and the development of a proton gradient.

Pulvinus Proteins More Highly Expressed in the Light Include Stress Response Proteins

Many of the proteins that were identified in the pulvinus proteome were categorized as stress response proteins (data not shown). Though a number of those proteins are active in response to stimuli such as cold or pathogen attack, the activity of the majority of the stress response proteins were in response to light-dependent reactions. Therefore the increased protein levels of four stress response proteins in the light is in agreement with their functions as light-mediated scavenging enzymes. Enzymes involved in disarming reactive oxygen species (ROS) were over-expressed in the light compared to dark control, a result supported by prior studies on antioxidant activity [21, 22]. Ascorbate peroxidase and superoxide dismutase activity were found to be positively correlated with photosynthetic photon flux density (PPFD) in *Curcubita pepo*, *Vinca major,* and *Nicotiana sylvestris* leaves [23, 24]. The increased expression in light-harvested pulvinus was greater for ascorbate peroxidase than superoxide dismutase (2.1-fold to 1.6-fold, respectively), which is the reverse of what Logan *et al*. [23] found for *C. pepo* and *N. sylvestris*, but their measurements were of sun-acclimation from shaded plants to fully illuminated plants while this report had the same lighting conditions for the duration of the experiment. Furthermore other studies cited by Logan *et al*. [23] had higher expression for ascorbate peroxidase over superoxide dismutase, consistent with the results of the current study. The antioxidant glutathione, which induces the activity of superoxide dismutase, requires arginine formed by argininosuccinate synthase (spot 12) [25, 26]. Argininosuccinate synthase showed a 1.6-fold increase in the light-harvested pulvinus. DEAD-box RNA helicase (spot 13, 1.8-fold increase), like the proteins mentioned above, has been implicated in stress response in plants among numerous other functions [27]. Interestingly, the pulvinus contains many ROS-mediating proteins but only the few mentioned above increased expression in the light.

The Increased Expression of Beta Amylase in the Light May Reflect Greater Sucrose Demand by the Pulvinus

Beta amylase (spot 14, 2.9-fold increase) degrades starch into sucrose, which is then utilized for pulvinar function. For example, sucrose was found to support the endogenous rhythm of *S. saman* pulvini as well as serving as a mechanism for short term (non- circadian) turgor flux [28-30]. However Rieger *et al*. [31] found insignificant carbohydrate flux in *P. coccineus* pulvini between light and dark phases, which is not consistent with the results found in soybean pulvini. One possible explanation for the discrepancy is the isoform of beta amylase that was detected in our study. Transcript levels of nine beta amylase genes in *Arabidopsis* were found to have varying patterns of expression; for some beta amylase genes transcript activity peaked at the dark-light transition, but other genes exhibited lower levels of activity during the transition and higher levels several hours into the light period [32]. Furthermore, the beta amylase isoforms that demonstrated high activities were not always the more functionally significant isoforms for plant metabolism. Only one form of beta amylase was detected in our soybean pulvinus, and its significance in starch breakdown is currently unknown. That there is a significant change between spots in the light versus the dark is noteworthy, because Lu *et al*. [33] noted while transcription levels suggested circadian regulation, protein content did not reflect a similar fluctuation.

Proteins with Increased Expression in the Dark May be a Result of Multiple Factors

Of the sixteen proteins (11 spots) with higher expression in the dark seven were related to photosynthesis or the Calvin cycle. Other proteins with higher expression in the dark included α-tubulin and *S*-adenosylmethionine synthetase. The latter is involved in forming a stress response precursor. The former is a cytoskeletal protein shown to rearrange in pulvinus cells in response to electricity and cold [3, 34], as well as in response to darkness in guard cells [35]. The physiological significance of α-tubulin in soybean is not as well characterized at this point as β-tubulin. In etiolated soybean seedlings β-tubulin had decreased

transcript levels in the hypocotyls upon light illumination. However the levels of β-tubulin in etiolated soybean cotyledons were not affected by illumination [36].

Rubisco activase Expression Levels are Possibly a Result of Circadian Rhythms

Rubisco activase activates the rubisco enzyme by the removal of sugar phosphates in complex with rubisco, and is also reported to function as a chaperone under high temperature conditions (Ayala-Ochoa *et al.* and sources therein) [37]. Rubisco activase in dark-harvested pulvini had over a two-fold increase in volume over light-harvested pulvini in two spots (Figure **1**). Studies have shown higher levels of rubisco activase mRNA both before and after the dark-light transition, which can indicate species-specific behavior or possibly the light-dark division within a 24-hour cycle. For example, apple cultivars grown on a 16:8 photoperiod had maximum mRNA expression two hours into the light period while *Arabidopsis* mRNA expression was at its peak one hour prior to light induction, or nine hours into the dark period, in a 14:10 photoperiod [38, 39]. The C_4 plant maize also had its highest levels before dawn for two different rubisco activase transcripts, though the actual point within the dark period was not specified [37]. They proposed the fluctuations in transcript levels to be regulated in a circadian pattern. Dark expression was also shown at the translational level, where a study of tomato plants grown in a 14:10 photoperiod demonstrated protein synthesis of rubisco activase eight hours into the dark period and lower levels later on in the afternoon of the light period [40]. Circadian regulation has also been demonstrated in other photosynthetic proteins detected in the pulvinus. For example, a protein that is part of the cytochrome b_6f complex (spot 11) was shown to have higher expression in dark controls over light samples in cyanobacteria [41]. In addition, maize mRNA levels of chlorophyll a/b protein increased towards the end of its dark period and maintained higher levels in the morning compared to other hours of the day and night [42]. Several chlorophyll a/b proteins have been identified in the soybean pulvinus although in the present study there was no evidence of differential expression.

Other Explanations for Diminished Photosynthetic and Carbon-Fixing Proteins in the Light

The decrease of the photosynthetic/ Calvin cycle proteins in light samples, other than the ones mentioned above, is somewhat puzzling given the dependence of these proteins on light. There are several possible explanations for the increased levels of photosynthetic proteins in the dark compared to the light in soybean pulvinus. These increased levels of photosynthetic protein expression in the dark may have been an artefact of the tissue homogenization protocol. In *Pisum sativum* chloroplasts, Stieger and Feller [43] found that a β-mercaptoethanol extraction buffer degraded protein levels of rubisco large subunit exposed to light, while protein levels remained unaffected in darkness. The β-mercaptoethanol extraction method did not appear to break down phosphoribulokinase as severely. They also detected a decrease in stromal enzymes as a result of reactive oxygen species generated under light conditions, which is in agreement with the higher levels of superoxide dismutase and ascorbate peroxidase detected in the light-harvested soybean pulvinus. If these reactions took place in our procedure then we may have underestimated the actual amounts of these proteins in the light.

Another explanation could be that our results were confounded by the inclusion of leaves of various ages in the samples. Suzuki *et al*. [44] found that rubisco protein synthesis was higher in younger leaves than older leaves, which maintained rather constant protein levels. They also found that rubisco degradation began at about 10 days after leaf emergence in rice plants. Our study combined pulvini from the second through sixth or seventh leaves due to the small size of soybean pulvini and lower protein levels than other soybean tissues. Samples that were collected over a period of months did not always contain the same combination of plant phenological stages and leaf ages. Therefore it is possible that light-harvested samples contained more pulvini from older leaves with reduced protein levels than dark-harvested samples. Further studies are needed in order to determine whether the differences in expression of rubisco large subunit and other light-dependent proteins in the dark were a result of an endogenous circadian rhythm.

CONCLUSION

Pulvinar tissue respond to differing light conditions with reversible bending and different levels of protein expression. An examination of nearly 200 soybean pulvinus proteins found less than twenty with significantly different expression levels between light and dark periods. Light-favored proteins fall largely in the functional category of oxidative stress response

occurring during light-dependent reactions. Proteins more highly expressed in the dark also appear to belong to light-dependent pathways. Explanations for lower expression during the day rather than night for these light-dependent proteins include artifactual elements arising from the experimental design and underlying circadian rhythmicity. Further investigations to clarify the nature of dark expression in light-dependent proteins will shed some understanding on the mechanisms of heliotropic leaf bending.

ACKNOWLEDGEMENTS

We thank Dr. William Kenworthy for providing the seeds for this project. This work was carried out with the support of a Cooperative Agreement between the University of Maryland, College Park, and USDA in Beltsville, MD.

APPENDIX

Table A1: Peptide Sequences and Mass Spectrometry Data of the Differentially Expressed Proteins

Spot number	Protein	Sequence	Ion score	Delta ion score	Obs. m/z	m/z mr (amu)	z	Δmass/ppm
24 (1)	Tubulin alpha-6 chain	(R)AIFVDLEPTVIDEVR(T)	56.2	30	858.46	1704.91	2	-2.8
		(K)DVNAAVGIIK(T)	79.8	46.7	200.3	998.58	2	-0.56
		(K)EDAANNFAR(G)	60.5	43.2	504.23	1006.45	2	-0.74
		(K)EIVELcLDR(I)	35.7	33.5	573.79	1145.58	2	-0.2
		(K)FDLmYAK(R)	49.9	33.3	452.22	902.42	2	-1.2
		(R)IHFmLSSYAPVISAEK(A)	50	27.6	603.65	1807.91	2	-2.3
		(R)LSVDYGKK(S)	45	27.5	455.26	908.5	2	-1.2
		(R)LVSQVISSLTASLR(F)	104.6	99.1	737.43	1472.85	2	-1.9
		(R)SLDIERPTYTNLNR(L)	34.2	20.1	846.44	1690.86	2	-1.3
		(K)TVGGGDDAFNTFFSETGAGK(H)	75.5	67.7	989.44	1976.87	2	-2.4
		(K)YmAccLmYR(G)	33.8	33.8	650.25	1298.49	2	-0.74
		(K)DVNAAVGIIK(T)	49.9	30.9	999.58	998.58	1	-0.84
		(R)EDLAALEK(D)	38.9	10.8	888.47	887.46	1	-1.7
		(R)eDLAALEK(D)	42.4	20.5	870.45	869.45	1	-3.6
		(R)LSVDYGK(K)	33.7	22.2	781.41	780.4	1	-1.1
		(K)EDAANNFAR(G)	30.6	12.5	1007.45	1006.45	1	-0.14
		(R)GHYTIGK(E)	27.7	9.4	775.41	774.4	1	-0.99
		(K)FDLmYAK(R)	24.7	13.3	903.43	902.42	1	-0.91
	Tubulin alpha-2 chain	(K)DVNAAVATIK(T)	71.2	50.6	501.28	1000.55	2	-0.56
		(K)EDAANNFAR(G)	60.5	43.2	504.23	1006.45	2	-0.74
		(K)FDLmYAK(R)	49.9	33.3	452.22	902.42	2	-1.2
		(R)IHFmLSSYAPVISAEK(A)	50	27.6	603.65	1807.91	2	-2.3
		(R)LSVDYGKK(S)	45	27.5	455.26	908.5	2	-1.2
		(R)LVSQVISSLTASLR(F)	104.6	99.1	737.43	1472.85	2	-1.9
		(R)SLDIERPTYTNLNR(L)	34.2	20.1	846.44	1690.86	2	-1.3
		(K)TVGGGDDAFNTFFSETGAGK(H)	75.5	67.7	989.44	1976.87	2	-2.4
		(K)YmAccLmYR(G)	33.8	33.8	650.25	1298.49	2	-0.74
		(R)EDLAALEK(D)	38.9	10.8	888.47	887.46	1	-1.7
		(R)eDLAALEK(D)	42.4	20.5	870.45	869.45	1	-3.6
		(R)LSVDYGK(K)	33.7	22.2	781.41	780.4	1	-1.1
		(K)DVNAAVATIK(T)	32	22	1001.56	1000.55	1	-1.8
		(K)EDAANNFAR(G)	30.6	12.5	1007.45	1006.45	1	-0.14
		(R)GHYTIGK(E)	27.7	9.4	775.41	774.4	1	-0.99

(Table A1). Continued.

Spot number	Protein	Sequence	Ion score	Delta ion score	Obs. m/z	m/z mr (amu)	z	Δmass/ppm
		(K)FDLmYAK(R)	24.7	13.3	903.43	902.42	1	-0.91
	Tubulin A	(K)DVNAAVATIK(T)	71.2	50.6	501.28	1000.55	2	-0.56
		(K)EDAANNFAR(G)	60.5	43.2	504.23	1006.45	2	-0.74
		(K)FDLmYAK(R)	49.9	33.3	452.22	902.42	2	-1.2
		(R)LSVDYGKK(S)	45	27.5	455.26	908.5	2	-1.2
		(R)SLDIERPTYTNLNR(L)	34.2	20.1	846.44	1690.86	2	-1.3
		(K)YmAccLmYR(G)	33.8	33.8	650.25	1298.49	2	-0.74
		(R)EDLAALEK(D)	38.9	10.8	888.47	887.46	1	-1.7
		(R)eDLAALEK(D)	42.4	20.5	870.45	869.45	1	-3.6
		(R)LSVDYGK(K)	33.7	22.2	781.41	780.4	1	-1.1
		(K)DVNAAVATIK(T)	32	22	1001.56	1000.55	1	-1.8
		(K)EDAANNFAR(G)	30.6	12.5	1007.45	1006.45	1	-0.14
		(K)FDLmYAK(R)	24.7	13.3	903.43	902.42	1	-0.91
32 (2)	Ribulosebisphosphate carboxylase large chain	(R)AVYEcLR(G)	58.5	30.1	455.73	909.44	2	-0.03
		(K)DDENVNSQPFmR(W)	70.6	70.6	734.31	1466.61	2	-0.53
		(R)DDFVEKDR(S)	43.1	21.1	512.24	1022.47	2	-0.65
		(K)DTDILAAFR(V)	58.1	49	511.27	1020.52	2	-0.67
		(R)LSGGDHVHAGTVVGK(L)	104.5	96.2	717.38	1432.74	2	-0.18
		(K)LTYYTPDYETK(D)	53.4	50	697.33	1392.64	2	-0.23
		(R)VALEAcVQAR(N)	85.1	77.5	558.79	1115.57	2	-0.83
		(R)DDFVEK(D)	37	12	752.34	751.34	1	-2.5
		(R)AVYEcLR(G)	33.6	17.8	910.45	909.44	1	0.68
		(R)EGNEIIR(E)	30.2	15.8	830.44	829.43	1	-0.13
		(R)GGLDFTK(D)	29.8	11.3	737.38	736.38	1	-0.5
		(R)IPTAYIK(T)	25.2	5.2	805.48	804.47	1	-0.0074
		(R)LSGGDHVHAGTVVGK(L)	23.7	11.7	478.59	1432.74	3	-0.96
		(K)SQAETGEIK(G)	22.7	11.2	962.48	961.47	1	0.025
52 (3)	Rubiscoactivase	(K)DGPPTFEQPK(M)	64.4	41.6	558.27	1114.53	2	-1.1
		(R)IGVcTGIFR(T)	57.8	37.5	511.78	1021.54	2	0.052
		(K)NFmTLPNIK(V)	37.5	22.2	547.29	1092.56	2	-1.1
		(K)SFQcELVFAK(M)	36.3	18.5	614.8	1227.59	2	-1.6
		(R)EAADLIK(K)	28	3.1	759.42	758.42	1	-0.39
		(R)TDSIPEQDVVK(I)	70.1	62.3	615.81	1229.61	2	-1.3
58 (4)	S-adenosylmethionine synthase	(K)ANVDYEK(I)	44.2	26.3	419.7	837.39	2	0.022
		(K)EHVIKPVIPEK(Y)	60.3	48.3	644.88	1287.75	2	-0.42
		(R)FVIGGPHGDAGLTGR(K)	48.6	27.7	485.26	1452.75	3	-0.78
		(R)FVIGGPHGDAGLTGR(K)	48.9	30.7	485.26	1452.75	3	-0.99
		(R)FVIGGPHGDAGLTGR(K)	71.5	61.4	727.38	1452.75	2	-0.9
		(R)FVIGGPHGDAGLTGR(K)	103.6	89.6	727.38	1452.75	2	-0.46
		(K)IPDKEILSIVK(E)	58.3	48.1	418.93	1253.76	3	-0.33
		(K)IPDKEILSIVK(E)	47.8	34.1	627.89	1253.76	2	-0.96
		(R)NIGFVSDDVGLDADNcK(V)	116.6	107.5	919.91	1837.81	2	-0.68
		(K)SIVANGLAR(R)	62.2	42.9	450.77	899.52	2	-0.31
		(K)TAAYGHFGR(D)	66.5	59.2	490.24	978.47	2	-0.37

(Table A1). Continued.

Spot number	Protein	Sequence	Ion score	Delta ion score	Obs. m/z	m/z mr (amu)	z	Δmass/ppm
		(K)TIFHLNPSGR(F)	50.7	35	571.31	1140.6	2	-1.4
		(K)TIFHLNPSGR(F)	56	42.9	571.31	1140.6	2	-0.91
		(K)TNmVmVFGEITTK(A)	49.7	40.9	751.86	1501.71	2	-1.1
		(K)TNmVmVFGEITTK(A)	101.6	89.7	751.86	1501.71	2	-0.83
		(K)TQVTVEYYNDK(G)	67.4	60.7	680.33	1358.64	2	0.62
		(K)TQVTVEYYNDK(G)	70.1	60.1	680.33	1358.64	2	0.6
		(K)EILSIVK(E)	35.8	0	801.51	800.5	1	-0.23
		(K)EHVIKPVIPEK(Y)	31.9	19.5	430.26	1287.75	3	-0.33
		(K)EILSIVK(E)	30.5	0	401.26	800.5	2	-0.0024
		(K)eILSIVK(E)	26.4	0	783.5	782.49	1	-0.74
		(K)EHVIKPVIPEK(Y)	25.7	13.7	430.26	1287.75	3	-0.47
		(R)FVIGGPHGDAGLTGR(K)	25.5	12.2	485.26	1452.75	3	-0.95
61 (5)	Phosphoribulokinase	(R)ANDFDLmYEQVK(A)	98.9	86.8	744.84	1487.66	2	-0.94
		(R)DLYEQLIASK(A)	49.6	39.7	590.32	1178.62	2	-0.82
		(K)FYGEVTQQmLK(H)	47	40.8	680.33	1358.66	2	0.9
		(K)FYGEVTQQmLK(H)	88.8	79.9	680.34	1358.66	2	1.8
		(R)GHSLESIK(A)	39.6	25.3	435.74	869.46	2	0.14
		(K)GVTALDPR(A)	43.8	27.8	414.73	827.45	2	-0.051
		(K)GVTALDPR(A)	46.4	30.9	414.73	827.45	2	-0.15
		(K)GVTALDPR(A)	55	35.2	414.73	827.45	2	-0.17
		(R)KLTcSYPGIK(F)	48.1	30	583.81	1165.61	2	-2.3
		(R)KPDFEAYIDPQK(Q)	71	58.5	725.86	1449.71	2	-0.72
		(R)LTSVFGGAAEPPK(G)	80.1	69.7	637.34	1272.67	2	-0.5
		(R)LTSVFGGAAEPPK(G)	94.6	85.9	637.34	1272.67	2	-0.14
		(K)ILVIEGLHPmFDSR(V)	39.2	8.8	548.29	1641.85	3	-1.2
		(K)GVTALDPR(A)	31	11	414.73	827.45	2	-0.58
		(R)KPDFEAYIDPQK(Q)	33.2	20.9	484.25	1449.71	3	-0.49
	Phosphoribulokinase (Gma. 55320)	(R)ANDFDLmYEQVK(A)	98.9	86.8	144.84	1487.66	2	-0.94
		(K)FYGEVTQQmLK(H)	47	40.8	680.33	1358.66	2	0.9
		(K)FYGEVTQQmLK(H)	88.8	79.9	680.34	1358.66	2	1.8
		(R)GHSLESIK(A)	39.6	25.3	435.74	869.46	2	0.14
		(K)GVTALDPR(A)	43.8	27.8	414.73	827.45	2	-0.051
		(K)GVTALDPR(A)	46.4	30.9	414.73	827.45	2	-0.15
		(K)GVTALDPR(A)	55	35.2	414.73	827.45	2	-0.17
		(R)KLTcSYPGIK(F)	48.1	30	583.81	1165.61	2	-2.3
		(R)KPDFEAYIDPQK(Q)	71	58.5	725.86	1449.71	2	-0.72
		(R)LTSVFGGAAEPPK(G)	80.1	69.7	637.34	1272.67	2	-0.5
		(R)LTSVFGGAAEPPK(G)	94.6	85.9	637.34	1272.67	2	-0.14
		(K)GVTALDPR(A)	31	11	414.73	827.45	2	-0.58
		(R)DLYEQLIATK(A)	30.6	23.8	597.32	1192.63	2	0.0032
		(R)KPDFEAYIDPQK(Q)	33.2	20.9	484.25	1449.71	3	-0.49
		(K)ILVIEGLHPmYDSR(V)	28.2	17.9	553.62	1657.85	3	-0.5
	Uncharacterized protein	(R)GGFDTcFVK(T)	52.3	17.2	515.74	1029.46	2	0.012
		(K)GSGTILDSGTVITR(F)	79.5	0	688.87	1375.73	2	-1.1
		(R)VLFDTVNNR(V)	60.8	37.6	539.29	1076.56	2	-0.35

(Table A1). Continued.

Spot number	Protein	Sequence	Ion score	Delta ion score	Obs. m/z	m/z mr (amu)	z	Δmass/ppm
66 (6)	Rubiscoactivase	(K)DGPPTFEQPK(M)	67.4	52.2	558.27	1114.53	2	-1.7
		(R)IGVcTGIFR(T)	48.9	39	511.78	1021.54	2	-2.1
		(K)NFmTLPNIK(V)	40.8	16.1	547.29	1092.56	2	-1.5
		(R)TDSIPEQDVVK(I)	31.6	25.3	615.81	1229.61	2	-1.3
	Uncharacterized protein	(K)DGPPTFEQPK(M)	67.4	52.2	558.27	1114.53	2	-1.7
		(R)IGVcTGIFR(T)	48.9	39	511.78	1021.54	2	-2.1
		(K)NFmTLPNIK(V)	40.8	16.1	547.29	1092.56	2	-1.5
		(R)TDGIPEQDIVK(L)	58.2	45.2	607.82	1213.62	2	-1.6
		(K)WISGVGVDSVGK(K)	60.3	48.2	602.32	1202.63	2	-0.97
108 (7)	Ferredoxin--NADP reductase, leaf isozyme 2, chloroplastic	(K)DNTFVYmcGLK(G)	71.6	62.4	682.3	1362.59	2	-1
		(K)KQDEGVVVNK(F)	65.6	47.7	558.31	1114.6	2	-0.65
		(R)LDFAVSR(E)	45.7	20.1	404.22	806.43	2	0.57
		(R)LVYTNENGEIVK(G)	81.5	70.1	689.86	1377.71	2	-1.6
		(K)mFFEK(H)	25.5	0.1	717.33	716.32	1	-2.3
112 (8)	Oxygen-evolving enhancer protein 1, chloroplastic	(K)DGIDYAAVTVQLPGGER(V)	90.3	72.5	880.94	1759.87	2	-1
		(R)GASTGYDNAVALPAGGR(G)	81.6	77.2	788.89	1575.77	2	0.53
		(R)GDEEELAKENNK(S)	62.9	45.3	688.32	1374.63	2	-0.38
		(R)GSSFLDPK(G)	42.7	29.3	425.72	849.42	2	-0.45
		(K)GTGTANQcPTIDGGLDSFAFKPGK(Y)	50.7	11.6	813.72	2438.15	3	-1.6
		(K)KLcLEPTSFTVK(A)	40	28	711.88	1421.75	2	-2.9
		(K)LcLEPTSFTVK(A)	46	16.5	647.84	1293.66	2	-0.92
		(R)LTFDEIQSK(T)	55.2	31.7	540.78	1079.55	2	-1
		(R)LTYTLDEIEGPFEVSSDGTVK(F)	65.7	58.4	1150.56	2299.1	2	-4.6
		(K)NAPLEFQNTK(L)	59	45.3	581.3	1160.58	2	-0.45
		(K)NAPLEFQNTK(L)	73.7	56.2	581.3	1160.58	2	-0.6
		(K)qLVASGKPDSFSGEFLVPSYR(G)	67.4	61.4	1134.07	2266.12	2	-1.7
		(K)RLTFDEIQSK(T)	59.2	17	618.83	1235.65	2	-0.55
		(K)TKPETGEVIGVFESVQPSDTDLGAK(A)	53.9	50	868.77	2603.29	3	-1.4
		(R)VPFLFTIK(Q)	39.8	25.8	482.8	963.58	2	-0.98
		(K)NAPLEFQNTK(L)	33	20.9	581.3	1160.58	2	-0.071
		(K)RLTFDEIQSK(T)	36.4	15.7	412.89	1235.65	3	3.1
		(K)TYLEVK(G)	35	13.7	752.42	751.41	1	-1
		(R)VPFLFTIK(Q)	31.8	27.9	482.8	963.58	2	-0.79
		(R)GDEEELAK(E)	27.5	22.5	890.41	889.4	1	-0.6
		(K)ITLSVTK(T)	25.9	7.1	761.48	760.47	1	-1.3
		(K)QLVASGKPDSFSGEFLVPSYR(G)	24.1	19.2	762.06	2283.15	3	-0.8
148 (9)	20 kDachaperonin, chloroplastic	(K)NNVEISVK(T)	52.1	39.9	451.75	901.49	2	-1
		(K)TEGGILLPSTAQTKPQGGEVVAVGEGK(T)	41.9	39.7	875.13	2622.38	3	-1.6
		(K)TSGGLLLTEATK(D)	79.4	69	595.83	1189.65	2	-1.4
		(R)VSDVmAVLS(-)	39.7	39.7	468.74	935.46	2	-0.83
		(K)YAGTEVDFDGTK(H)	88.4	79.2	651.8	1301.58	2	-1.5
		(K)YTAIKPLGDR(V)	51.7	46.9	567.32	1132.62	2	-2.1
		(K)YAGNDFK(G)	32	31.6	814.37	813.36	1	-1
		(K)TGAQVVYSK(Y)	30.9	17.9	952.51	951.5	1	-0.3

(Table A1). Continued.

Spot number	Protein	Sequence	Ion score	Delta ion score	Obs. m/z	m/z mr (amu)	z	Δmass/ppm
		(K)DLKPLNDR(V)	36	23.7	485.77	969.52	2	-1.6
		(K)NNVEISVK(T)	27.6	18.2	902.49	901.49	1	-0.47
		(K)DGSDYITLR(V)	27.2	16	1039.51	1038.5	1	-0.14
		(R)VSDVmAVLS(-)	24.5	24.5	936.47	935.46	1	-0.097
		(R)VSDVmAVLS(-)	23.3	23.3	936.47	935.46	1	-0.18
152 (10)	Oxygen-evolving enhancer protein 2-1, chloroplastic	(K)EVEYPGQVLR(Y)	56	27.6	595.31	1188.61	2	-1.3
		(R)FVESTASSFSVA(-)	69.9	61.5	616.3	1230.58	2	-0.75
		(K)QYYSLTVLTR(T)	35.1	24.7	622.34	1242.66	2	-1.5
		(R)TADGDEGGKHQLITATVK(D)	45.3	40	614.32	1839.93	3	-1.9
		(K)HQLITATVK(D)	30.5	14.2	505.8	1009.59	2	-0.56
		(R)RFVESTASSFSVA(-)	28.6	28.5	694.35	1386.68	2	-1.1
166 (11)	Cytochrome b6-f complex iron-sulfur subunit	(K)GDPTYLVVEK(D)	43.2	23.5	560.8	1119.58	2	-0.93
		(K)GDPTYLVVEKDR(T)	48	32.7	696.36	1390.71	2	-0.33
45 (12)	Elongation factor 1-alpha (2)	(K)IGGIGTVPVGR(V)	56.4	49.5	513.31	1024.6	2	-0.63
		(R)LPLQDVYK(I)	38.6	22.2	488.28	974.54	2	-3
	26S protease regulatory subunit 6B homolog (5)*	(K)AVANHTTAAFIR(V)	54.8	49	424.57	1270.68	3	-0.23
		(K)AVANHTTAAFIR(V)	60.3	52.5	636.35	1270.68	2	-0.41
		(R)ELLKPSASVALHR(H)	48.3	43.4	474.28	1419.82	3	0.77
		(R)GVLLYGPPGTGK(T)	69.8	52	579.83	1157.64	2	-0.99
		(K)KPDTDFEFYK(-)	45.6	33	645.31	1288.6	2	-0.9
		(R)VVGSEFVQK(Y)	61.2	43.2	496.77	991.53	2	-0.91
		(R)ILSTINR(E)	28.5	6.9	408.75	815.49	2	-0.49
	Argininosuccinate synthase, chloroplastic (9)	(R)EDAIEYAK(K)	62.3	45.7	469.73	937.44	2	-1.3
		(K)ELEGLEQK(A)	37.9	26.2	473.25	944.48	2	-1.3
		(R)ESmDAFmQK(I)	535.5	27.5	559.73	1117.44	2	-1.8
		(K)EVGADAVSHGcTGK(G)	74.7	67.7	694.32	1386.62	2	-1.9
		(K)ITETTTGSVTLK(L)	87.3	57.2	625.84	1249.67	2	-1.6
		(R)SDTEVVSETTK(A)	81.9	67.7	598.29	1194.56	2	-1.5
		(R)LYGLPVR(V)	30.6	14.7	409.25	816.48	2	-1.2
		(K)GSVTVTSR(T)	30.4	16.5	403.72	805.43	2	-0.97
		(R)AmLEQGI(-)	31.1	21.2	777.38	776.37	1	-0.8
	Alanine aminotransferase 2 (2)	(R)ESTGILESLR(R)	49.6	31.3	552.8	1103.58	2	-0.83
		(K)EVAEFILR(R)	30.7	10.5	488.78	975.54	2	-0.82
46 (13)	DEAD-box ATP-dependent RNA helicase 15	(R)HFILDEcDK(M)	62.2	52.2	588.77	1175.53	2	-0.11
		(R)ILVATDLVGR(G)	87.3	76.6	528.82	1055.63	2	-0.85
		(K)mLESLDmR(K)	47.6	25.8	513.73	1025.45	2	-0.15
		(K)NEcPHIVVGTPGR(I)	55.9	44.6	479.24	1434.7	3	-1.2
		(R)DKDLSLK(N)	33.5	14.3	409.73	817.45	2	-0.76
		(K)DVQDIFK(M)	33.8	0	864.45	863.44	1	-0.7
		(R)FEVDIK(Q)	32	3	750.4	749.4	1	1.2
		(K)VAVFYGGVNIK(V)	29.5	21.1	583.83	1165.65	2	-1.2
	AT5g63860/MGI19_6	(K)SGVSLSER(Y)	66.7	45.7	417.72	833.42	2	0.022
		(R)YGNLGLGDR(N)	65.4	16.2	482.75	963.48	2	-0.81
		(R)GTNGQLGHGDTIDR(N)	23.6	8.5	480.9	1439.67	3	-0.85

(Table A1). Continued.

Spot number	Protein	Sequence	Ion score	Delta ion score	Obs. m/z	m/z mr (amu)	z	Δmass/ppm
62 (14)	Beta-amylase	(R)TAIEIYSDYmK(S)	71.8	69.5	675.32	1348.62	2	-0.71
		(K)YNDVPESTGFFK(S)	38.2	28.2	702.33	1402.64	2	-1.6
		(K)EQLLQLR(A)	26.1	10.8	450.27	898.52	2	-0.65
126 (15)	Cytosolic ascorbate peroxidase 1	(K)SYPTVSADYQK(A)	41	28	629.8	1257.59	2	-1
		(K)TGGPFGTIK(H)	71.9	48.9	439.24	876.47	2	0.16
		(K)LSELGFADA(-)	26.8	26.3	922.45	921.44	1	-0.55
		(R)LLEPLK(A)	22.5	0	712.46	711.45	1	0.58
163 (16)	Uncharacterized protein	(K)IVEAVGDLK(L)	78.8	41.3	472.28	942.54	2	-0.6
		(K)LDSLVmGSR(G)	76.3	53.2	497.25	992.5	2	-0.98
		(R)qKQVNVVAK(L)	57.8	44.8	498.79	995.58	2	-1.3
		(K)IGVALDFSK(G)	38.9	24	475.27	948.53	2	-0.91
		(K)IGVALDFSK(G)	30.8	20.7	949.54	948.53	1	-0.069
		(K)qVNVVAK(L)	27.8	16.8	740.43	739.42	1	-1.1
		(K)QVNVVAK(L)	28.5	15.4	757.46	756.45	1	-0.074
		(R)GLGAIQR(V)	18.2	7	714.43	713.42	1	-0.23
169 (17)	Elicitor-inducible protein EIG-J7	(K)GGTDSVIGLK(L)	56.5	13.5	473.76	945.51	2	-0.95
		(K)GGTDSVIGLK(L)	76.2	44	473.76	945.51	2	-1
		(R)GNLDIFSGR(G)	35.9	24.4	978.5	977.49	1	-0.66
		(K)GGTDSVIGLK(L)	31	21.3	946.52	945.51	1	-0.75
173 (18)	Superoxide dismutase [Cu-Zn]	(R)ALVVHELEDDLGK(G)	75.8	65.6	719.38	1436.75	2	-0.82
		(K)GGHELSLTTGNAGGR(L)	96	85.5	713.85	1425.69	2	-1.1
		(K)GTSAVEGVATLIQEDDGPTTVSVR(I)	52.8	51.3	801.41	2401.19	3	-1.1
		(K)GTSAVEGVATLIQEDDGPTTVSVR(I)	78.8	69	1201.6	2401.2	2	-0.97
		(R)LAcGVVGLTPA(-)	61.3	52.7	1057.57	1056.56	1	-0.96
		(K)LTHGAPEDEVR(H)	38.7	37.5	612.31	1222.6	2	1.2
		(R)ALVVHELEDDLGK(G)	26.5	16.3	479.92	1436.75	3	-1.6

Note: For spot number, the parenthetical numbers correspond with numbers in Figure **1**. The non-parenthetical numbers refer to an earlier numbering system from a prior study. Only peptides with a minimum significant probability of 80% were considered. Protein identification probability was set to 95%. All data were collected from the Scaffold protein identification software program. *26S protease regulatory subunit 6B homolog also appeared in spots 43 and 47 but those spots did not significantly change expression levels between the light and the dark.

REFERENCES

[1] Galen C, Stanton ML. Sunny-side up: flower heliotropism as a source of parental environmental effects on pollen quality and performance in the snow buttercup, *Ranunculus adoneus*(Ranunculaceae). Am J Bot 2003; 90: 724-9. http://dx.doi.org/10.3732/ajb.90.5.724

[2] Kanzawa N, Hoshino Y, Chiba M, Hoshino D, Kobayashi H, Kamasawa N, *et al*. Change in the actin cytoskeleton during seismonastic movement of *Mimosa pudica*. Plant Cell Physiol 2006; 47: 531-9. http://dx.doi.org/10.1093/pcp/pcj022

[3] Herrera R, Krier C, Lalanne C, Ba EH, Stokes A, Salin A, *et al*. (Not) Keeping the stem straight: a proteomic analysis of maritime pine seedlings undergoing phototropism and gravitropism. BMC Plant Biol 2010; 10: 217. http://dx.doi.org/10.1186/1471-2229-10-217

[4] Koller D. The Restless Plant. Cambridge, MA, and London, England: Harvard University Press 2011.

[5] Ehleringer J, Forseth I. Solar Tracking by Plants. Science 1980; 210: 1094-8. http://dx.doi.org/10.1126/science.210.4474.1094

[6] Zhang YL, Zhang HZ, Feng GY, Tian JS, Zhang WF. Leaf diaheliotropic movement can improve carbon gain and water use efficiency and not intensify photoinhibition in upland cotton (*Gossypium hirsutum* L.). Photosynthetica 2009; 47: 609-15. http://dx.doi.org/10.1007/s11099-009-0087-3

[7] Kao WY, Forseth IN. Diurnal Leaf Movement, Chlorophyll Fluorescence and Carbon Assimilation in Soybean Grown under Different Nitrogen and Water Availabilities. Plant Cell Environ 1992; 15: 703-10. http://dx.doi.org/10.1111/j.1365-3040.1992.tb01012.x

[8] Satter RL, Applewhite PB, Galston AW. Rhythmic Potassium Flux in Albizzia - Effect of Aminophylline, Cations, and Inhibitors of Respiration and Protein-Synthesis. Plant Physiol 1974; 54: 280-5. http://dx.doi.org/10.1104/pp.54.3.280

[9] Okazaki Y. Blue light inactivates plasma membrane $H^{(+)}$-ATPase in pulvinar motor cells of *Phaseolus vulgaris* L. Plant Cell Physiol 2002; 43: 860-8. http://dx.doi.org/10.1093/pcp/pcf099

[10] Iino M, Long C, Wang XJ. Auxin- and abscisic acid-dependent osmoregulation in protoplasts of *Phaseolus vulgaris* pulvini.Plant Cell Physiol 2001; 42: 1219-27. http://dx.doi.org/10.1093/pcp/pce157

[11] Lee YS, Satter RL. Effects of White, Blue, Red-Light and Darkness on Ph of the Apoplast in the *Samanea* Pulvinus. Planta 1989; 178: 31-40. http://dx.doi.org/10.1007/BF00392524

[12] Kim HY, Cote GG, Crain RC. Potassium Channels in *Samanea saman* Protoplasts Controlled by Phytochrome and the Biological Clock. Science 1993; 260: 960-62. http://dx.doi.org/10.1126/science.260.5110.960

[13] Haydon MJ, Bell LJ, Webb AAR. Interactions between plant circadian clocks and solute transport. J Exp Bot 2011; 62: 2333-48. http://dx.doi.org/10.1093/jxb/err040

[14] Natarajan S, Xu C, Caperna TJ, Garrett WM. Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. Anal Biochem 2005; 342: 214-20. http://dx.doi.org/10.1016/j.ab.2005.04.046

[15] Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. Anal Biochem 1976; 72: 248-54. http://dx.doi.org/10.1016/0003-2697(76)90527-3

[16] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996; 68: 850-8. http://dx.doi.org/10.1021/ac950914h

[17] Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis 1999; 20: 601-5. http://dx.doi.org/10.1002/(SICI)1522-2683(19990301)20:3<601::AID-ELPS601>3.0.CO;2-6

[18] Searle B. Scaffold: A bioinformatics tool for validating MS/MS-based proteomic studies. Proteomics 2010; 10: 1265-9. http://dx.doi.org/10.1002/pmic.200900437

[19] Gorton HL. Pulvinar Water Relations in Nyctinastic Plants. In: Satter RL, Gorton HL, Vogelmann TC, Eds. The Pulvinus: Motor Organ for Leaf Movement. Rockville, MD: American Society of Plant Physiologists 1990; pp. 214-22.

[20] Lee YS. Ion Movements that Control Pulvinar Curvature in Nyctinastic Legumes. In: Satter RL, Gorton HL, Vogelmann TC, Eds. The Pulvinus: Motor Organ for Leaf Movement. Rockville, MD: American Society of Plant Physiologists 1990; pp. 130-41.

[21] Pfannschmidt T, Allen JF, Oelmuller R. Principles of redox control in photosynthesis gene expression. Physiol Plant 2001; 112: 1-9. http://dx.doi.org/10.1034/j.1399-3054.2001.1120101.x

[22] Svensson AS, Rasmusson AG. Light-dependent gene expression for proteins in the respiratory chain of potato leaves. Plant J 2001; 28: 73-82. http://dx.doi.org/10.1046/j.1365-313X.2001.01128.x

[23] Logan BA, Demmig-Adams B, Adams WW. Antioxidants and xanthophyll cycle-dependent energy dissipation in *Cucurbita pepo* L. and *Vinca major* L. upon a sudden increase in growth PPFD in the field. J Exp Bot 1998; 49: 1881-8.

[24] Dutilleul C, Garmier M, Noctor G, Mathieu C, Chetrit P, Foyer CH, de R Paepe. Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. Plant Cell 2003; 15: 1212-26. http://dx.doi.org/10.1105/tpc.009464

[25] Dalton DA, Russell SA, Hanus FJ, Pascoe GA, Evans HJ. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. Proc Natl Acad Sci USA 1986; 83: 3811-5. http://dx.doi.org/10.1073/pnas.83.11.3811

[26] Knorzer OC, Lederer B, Durner J, Boger J. Antioxidative defense activation in soybean cells. Physiol Plant 1999; 107: 294-302. http://dx.doi.org/10.1034/j.1399-3054.1999.100306.x

[27] Umate P, Tuteja R, Tuteja N. Genome-wide analysis of helicase gene family from rice and Arabidopsis: a comparison with yeast and human. Plant Mol Biol 2010; 73: 449-65. http://dx.doi.org/10.1007/s11103-010-9632-5

[28] Racusen RH, Galston AW. Electrical Evidence for Rhythmic Changes in Cotransport of Sucrose and Hydrogen-Ions in *Samanea* Pulvini. Planta 1977; 135: 57-62. http://dx.doi.org/10.1007/BF00387976

[29] Fromm J, Eschrich W. Transport processes in stimulated and non-stimulated leaves of *Mimosa pudica* III. Displacement of ions during seismonastic leaf movements. Trees-Structure Function 1988; 2: 65-72.

[30] Moran N. Osmoregulation of leaf motor cells. FEBS Lett 2007; 581: 2337-47. http://dx.doi.org/10.1016/j.febslet.2007.04.002

[31] Rieger A, Lutz A, Hampp R. Compartmentation of Soluble Carbohydrates, of Starch and of Malate in Motor Organs (Pulvini) and Other Parts of *Phaseolus-Coccineus* L Leaves. Planta 1992; 187: 95-102. http://dx.doi.org/10.1007/BF00201629

[32] Smith SM, Fulton DC, Chia T, Thorneycroft D, Chapple A, Dunstan H, *et al*. Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in Arabidopsis leaves. Plant Physiol 2004; 136: 2687-99. http://dx.doi.org/10.1104/pp.104.044347

[33] Lu Y, Gehan JP, Sharkey TD. Daylength and circadian effects on starch degradation and maltose metabolism. Plant Physiol 2005; 138: 2280-91. http://dx.doi.org/10.1104/pp.105.061903

[34] Yao H, Xu Q, Yuan M. Actin dynamics mediates the changes of calcium level during the pulvinus movement of *Mimosa pudica*. Plant Signal Behav 2008; 3: 954-60.

[35] Lahav M, Abu-Abied M, Belausov E, Schwartz A, Sadot E. Microtubules of guard cells are light sensitive. Plant Cell Physiol 2004; 45: 573-82. http://dx.doi.org/10.1093/pcp/pch067

[36] Jongewaard I, Colon A, Fosket DE. Distribution of the transcripts of the tubB1 β-tubulin gene in developing soybean (*Glycine max* [L.]Merr.) seedling organs. Protoplasma 1994; 183: 77-85. http://dx.doi.org/10.1007/BF01276815

[37] Ayala-Ochoa A, Vargas-Suarez M, Loza-Tavera H, Leon P, Jimenez-Garcia LF, Sanchez-de-Jimenez E. In maize, two distinct ribulose 1,5-bisphosphate carboxylase/ oxygenaseactivase transcripts have different day/night patterns of expression. Biochimie 2004; 86: 439-49. http://dx.doi.org/10.1016/j.biochi.2004.06.007

[38] Watillon B, Kettmann R, Boxus P, Burny A. Developmental and Circadian Pattern of Rubisco Activase Messenger-Rna Accumulation in Apple Plants. Plant Mol Biol 1993; 23: 501-9. http://dx.doi.org/10.1007/BF00019298

[39] Pilgrim ML, Mcclung CR. Differential Involvement of the Circadian Clock in the Expression of Genes Required for Ribulose-1,5-Bisphosphate Carboxylase Oxygenase Synthesis, Assembly, and Activation in *Arabidopsis thaliana*. Plant Physiol 1993; 103: 553-64.

[40] Martino-Catt S, Ort DR. Low temperature interrupts circadian regulation of transcriptional activity in chilling-sensitive plants. Proc Natl Acad Sci USA 1992; 89: 3731-5. http://dx.doi.org/10.1073/pnas.89.9.3731

[41] Stockel J, Jacobs JM, Elvitigala TR, Liberton M, Welsh EA, Polpitiya AD, *et al*. Diurnal Rhythms Result in Significant Changes in the Cellular Protein Complement in the Cyanobacterium Cyanothece 51142. PLoS One 2011; 6(2). http://dx.doi.org/10.1371/journal.pone.0016680

[42] Kloppstech K. Diurnal and circadian rhythmicity in the expression of light-induced plant nuclear messenger RNAs. Planta 1985; 165: 502-6. http://dx.doi.org/10.1007/BF00398095

[43] Stieger PA, Feller U. Requirements for the light-stimulated degradation of stromal proteins in isolated pea (*Pisum sativum* L.) chloroplasts. J Exp Bot 1997; 48: 1639-45.

[44] Suzuki Y, Kihara-Doi T, Kawazu T, Miyake C, Makino A. Differences in Rubisco content and its synthesis in leaves at different positions in *Eucalyptus globulus* seedlings. Plant Cell Environ 2010; 3: 1314-23.

Received on 11-01-2013 Accepted on 05-03-2013 Published on 13-03-2013

http://dx.doi.org/10.6000/1927-5129.2013.09.22