ABSTRACT

Title of Document: IDENTIFYING MOLECULAR FUNCTIONS OF HELIOTROPIC MOTOR TISSUE THROUGH PROTEOMIC ANALYSIS OF SOYBEAN PULVINI

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Heliotropic and nyctinastic leaf movement are mediated in soybean through turgor changes in the motor cells of the pulvinus, located at the base of the leaves. While some elements of the signaling pathways have been studied, a broad-scale protein identification has not yet been reported. In my research pulvini proteins were extracted in light- and dark-harvested soybean using the TCA/acetone method and identified by LC-MS/MS. Gene ontology analysis revealed proteins involved in proton transport were enriched in the soybean pulvinus proteome compared to a background soybean proteome. Proteins more highly expressed in the light were mostly stress response proteins, while under-expressed proteins were categorized as energy proteins. Further investigations using more sensitive extraction protocols and a multitude of sample times will build on these initial results to provide a thorough examination of heliotropic mechanisms at the molecular level.

IDENTIFYING MOLECULAR FUNCTIONS OF HELIOTROPIC MOTOR TISSUE THROUGH PROTEOMIC ANALYSIS OF SOYBEAN PULVINI

By

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Dedication

I would like to thank my parents and my siblings for loving me just as I am.

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Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction and Literature Review

Heliotropism overview

Introduction

Plants respond to light, gravity, touch, and other environmental signals by both temporary and permanent differential growth [1-3]. The directional growth of plants as a response to an external stimulus is called tropism. 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]. Many species exhibit both heliotropic responses that are specific to the time of day. For those species paraheliotropic movement is greatest around midday, while diaheliotropic response occurs in the early morning and late afternoon [5].

Benefits of heliotropism

Diaheliotropic leaf movement following the solar azimuth maximizes absorption of solar energy to the laminar surface. By preferentially angling leaves to receive maximum light during the early morning hours when the vapor pressure difference is lower, evapotranspiration that occurs in conjunction with carbon assimilation is limited. Diaheliotropism therefore results in both increased water use efficiency and high rates of carbon assimilation compared to horizontal controls, which has been shown in plants such as *Abutilon theophrasti* [6], cotton [7], *Strophostyles helvola* [8], and soybean [9]. Morning diaheliotropic bending also

allows shaded plants to receive higher levels of irradiance, as was shown with *Amphicarpa bracteata* grown both at the edge and within a forest canopy [8].

Paraheliotropism in leaves adjusts the lamina parallel to incident light to reduce absorption of excess solar radiation, lowering leaf temperatures and consequently reducing evapotranspiration. In *Phaseolus*, increases in temperature and irradiance were shown to increase leaf angles in an additive manner [10]. Bielenberg et al. [10] found that leaf angles in *P. vulgaris*, a crop species, increased up until 1000 µmol $m^{-2}s^{-1}$ over a range of temperatures; higher irradiance did not increase leaf angles over all temperatures tested. Along with an upper limit to irradiance a minimum irradiance is required to induce leaf movement; this was found to be at least 50% of natural sunlight for *Robinia* leaves [11]. Paraheliotropism further benefits plants by reducing photoinhibition while maintaining high levels of photosynthetic quantum yield under stressed conditions [12] and reducing UVB radiation levels [13].

Modulating factors in heliotropism

Donahue and Berg [14] suggested that the photoreceptors involved in diaheliotropism and paraheliotropism are the same, and that other factors influence the nature of the heliotropism. These factors include water potential, nutrient levels, and irradiance. When isolated, each factor appears to affect leaf angle movement independently but the extent of independence is unknown because the factors tend to be found together in nature. Furthermore, similar species that naturally inhabit different climates have different degrees of paraheliotropism. For some plants, arid species are more paraheliotropic than their mesic cousins [15, 10], but for others the opposite appears to be the case [16].

Leaves with diaheliotropic movement respond to directional sunlight dynamically according to the position of the leaves within the plant. North-facing *Abutilon* leaves had reduced movement near midday and west-facing leaves took longer for its diaheliotropic response in the early morning [6]. West-facing *Lavatera cretica* leaves had the opposite turgor flux at the end of the photoperiod than an east-facing leaf. Plants transferred out of phase took several days to reorient their leaves in response to the new light regime, indicating an endogenous element to solar tracking [17]. However, *Ranunculus adoneus* flowers did not share this internal memory element [1]. The differences in responses in nighttime plant reorientation suggests that heliotropism can be either strictly light-induced or co-regulated with circadian clocks. Nyctinastic folding responses, which are regulated by circadian patterns, will be discussed in a later section.

As previously stated, it is difficult to separate the depth of influence by each abiotic factor. In soybeans, Rosa et al. [18] found that water stress elicited a stronger response than the level of irradiance. Water- and nutrient-limited plants were more paraheliotropic than their well-watered, well-fertilized counterparts [12, 19]; moreover, the deficiencies in either water or nutrients were compensated by increased efficiency in use of the other factor [12]. The type and duration of the heliotropic response over the period of a growing season is also affected by the soil conditions in addition to drought adaptability by the plant itself. Forseth and Ehleringer [20] compared two desert heliotropic plants over a season and found that *Malvastrum rotundifolium* exhibited diaheliotropic movement throughout the season if the plants were drought-tolerant, whereas the more drought-sensitive *Lupinus arizonicus* was

diaheliotropic in the mornings and early in the season but paraheliotropic later in the day and during drier months. They concluded that because a drought-tolerant species could maintain high water use efficiency over a longer range of soil water conditions the carbon gains from diaheliotropic movement would outweigh the water loss. Kao and Forseth [21] found that N_2-fixing soybean plants had greater leaf angles than soybean supplemented by NO_3 fertilizers. They hypothesized that the greater paraheliotropic response by nutrient-limited soybean plants relieved some of the resource burden off of already-stressed plants by reducing the rate of photorespiration that would occur in leaves receiving high amounts of irradiance. Heliotropism adjusts leaf angles in accordance to solar azimuth, temperature, soil nutrient and water levels, and vapor pressure difference in order to balance the costs of photosynthetic carbon gain and evapotranspiration over a range of conditions.

Heliotropism and implications for agriculture

Many leguminous plants, including several important crop species, exhibit heliotropic behavior. As climate change will likely lead to higher temperatures and increased UV radiation reaching the surface, there may be an agricultural shift in the type of crop species grown to favor plants with greater paraheliotropic ability. Understanding the biological processes leading to heliotropism can assist science and industry to respond to these impending changes.

Pulvinar Structure and Mechanisms

Histology

The structural nature of heliotropism differs among plant species. In some

species the leaf lamina contains the photoreceptors [22, 23] but for many plants, particularly leguminous species, the photoresponsive site is in the pulvinus, an enlarged organ located at the base of the leaf [14, 24]. The structure of the pulvinus reveals its specialized role in leaf movement. In contrast to the stem and petiole, the pulvinus has a relatively larger cortex, composed of motor cells, and smaller pith. The proportion of pulvini comprising cortex and vascular cylinder tissue appear to be specific at least to the level of the subfamily. Rodrigues and Machado [25] revealed through histological examinations of nine legume species that plants in the subfamily *Faboideae* are 60% cortex and nearly 40% vascular cylinder, contrasting with *Mimosoideae* species which have more equal proportions of the two tissues. However the proportion of the vascular cylinder that is pith varies even within subfamilies [25].

In soybean, a member of the *Faboideae*, the pulvinus has a continuous vascular cylinder with functional phloem cells both outside and inside of the xylem. The presence of the second internal phloem in pulvinus is not matched in stems or petioles [26]. There are also two types of vacuoles in pulvini. Tannin-rich vacuoles have been previously mentioned as a major source of cellular volume change [27-29]. Indeed, calcium movement has been measured in tannin vacuoles in conjunction with volume flux in *Mimosa pudica* pulvini [30]. Others have found the primary volume changes to be from the non-tannin containing vacuoles [31].

Basic mechanism

The wavelengths of light that trigger heliotropism in plants are located within the blue region. Blue light filters enabled diaheliotropism in *R. adoneus* though red and green lights had no effect [1], while an action spectrum for soybeans found two

peaks at 410-400 nm and 470-490 nm [14]. Upon light exposure, an asymmetric turgor gradient formed between the adaxial and abaxial motor cells leads to leaf movement. Potassium ion influx coupled with Cl^- is powered by a proton gradient and results in osmotic influx [32]. Some studies isolating adaxial and abaxial pulvinus protoplasts have shown both regions to respond similarly to blue light and auxin, suggesting that turgor gradient is a result of differential exposure to the stimulus [33, 34]. This hypothesis is supported in studies of whole heliotropic plants [6, 17]. However, the pulvinus participates in nyctinastic leaf folding in addition to heliotropic leaf bending, and nyctinasty studies have indicated that both blue and red light produce opposing activity between the two regions [35, 36]. It may be that water potential and nutrient composition, as previously mentioned for paraheliotropism, are the final determinants for the turgor gradient. Most studies examining the mechanisms of pulvinar bending have focused on nyctinastic rather than heliotropic movement, and their major findings are summarized below.

Ion channels

Potassium is the ion whose movement most dictates the turgor response of pulvinus motor cells. To illustrate, a study using potassium blockers found reduced turgor flux in *Samanea* pulvini [37]. The concentration of malate, an ionic form of malic acid, is connected to the level of K^+ in *Phaseolus* pulvinus; both ions are inhibited by K^+ blockers. Furthermore, an increase in malate concentration in abaxial cells is accompanied by a decrease in adaxial cells, and vice versa [38, 39].

The movement of potassium and chloride are facilitated through ion channels located on the plasma membrane and the tonoplast. Distinct channels for inward and

outward ion flux have been identified for potassium; the channels are activated by differences in membrane potential triggered by blue and/or red light [36, 37, 40]. There is also evidence by Yu et al. [41] for a voltage-independent potassium channel. Studies have also suggested the presence of aquaporins, channels that facilitate the movement of water following the movement of ions [42, 43].

Induction factors for ion movement

Studies on the effect of light on membrane potentials and pH have produced contradictory results, showing that the relationships among the three are not clearly understood. In some cases blue light resulted in the depolarization of membranes [39], while in others membranes were hyperpolarized by blue light [44]. Research has also suggested the necessity of extracellular K^+ concentrations for altering membrane potentials [44]. That depolarization is triggered by excess K^+ and not by light is at odds with the studies indicating rapid polarization response followed by the slower ion transport.

The extent of hyperpolarization of blue light-exposed pulvini is reduced by H^+-ATPase blockers [44], indicating the importance of a photoreceptor-induced proton motive force in enabling the level of K^+-Cl^- movement. H^+-ATPase proteins are found in high numbers in mature motor cells compared to surrounding regions and proton activity can be roughly estimated by examination of pH flux in the pulvinus [43, 41]. Research has shown pH changes in motor cell apoplasts when unilaterally or completely exposed to light. Lee and Satter [35] found that abaxial apoplasts were more alkaline than adaxial apoplasts in both white and blue light. Okazaki [34] found transient and sustained pH increases in *P. vulgaris* pulvinus

apoplasts across both adaxial and abaxial regions when adaxial motor cells were unilaterally exposed to blue light. Red light (or darkness) appears to heighten the pH difference between the two regions, with abaxial apoplasts becoming more alkaline and adaxial apoplasts more acidic Lee and Satter [35].

Other molecules implied in turgor change

In addition to potassium, chloride, and protons, calcium is also implicated in the pulvinus bending response, particularly in the swelling of abaxial motor cells. *Phaseolus* abaxial cells in a Ca^{2+}-free solution did not swell in the light, and the use of Ca^{2+} blockers produced the same response [45]. They also found that Ca^{2+} activators led to increased turgor in the dark for abaxial tissue. The concentration of calcium in the cytoplasm fluctuates on different time scales depending on the stimulus: circadian oscillations occur over a roughly 24-hour period while fluctuations from a direct stimulus could take as soon as 15 seconds [46].

Inositol phospholipids are also elements of the signaling transduction pathway in the pulvinus that contribute to differential turgor response and have distinct tissue-specific responses to light stimuli and circadian rhythm. Brief exposure to white light towards the end of the dark period in *Samanea* pulvini promoted inositol phospholipid turnover (from membrane-bound complexes to aqueous metabolites) [47, 48]. Increased levels of IP_3 correlated with a decrease of K^+ influx and resultant turgor loss [40]. In *Phaseolus* pulvini IP inhibitors prevented turgor decrease in abaxial cells that occurs in the dark [45].

Plant hormones involved in irreversible growth also have an impact on reversible pulvinar activity. Auxin applied late in the photoperiod of *Phaseolus*

enhanced adaxial turgor increase following a short time lag [49]. Abscisic acid decreased the concentration of malic acid, a proton charge-balancing anion, though when applied toward the end of the dark period the drop was stalled until the light period began [50]. Finally jasmonate enhanced cellulase activity in *Phaseolus* pulvini, higher than adjacent tissues, while auxin inhibited activity [51]. While we have some understanding on the roles of various factors on pulvinus motor mechanisms, the understanding is fragmented. Determining the underlying molecular framework will connect what has been found up until now from individual studies on pulvinus response.

Proteins involved in heliotropism

The proteome-level identification of the molecules involved in the photoreception, signaling cascade, and bending response have yet to be identified. Up until recently information on mostly small non-protein elements have been studied. Ueda and Nakamura [52] isolated leaf movement factors from five species and found them to be genus specific. They detected interaction of the movement factors in *Cassia mimosoides* plants with 210 and 180 kDa proteins, but the structure was not known. The localization of *Albizzia* opening and closing factors were to extensor motor cells. The changes in concentration of leaf movement factors over 24 hours differed among species but appeared to be dependent on the concentration of the protein β-glucosidase. The influence of movement factors may be due to a ratio flux regulated by circadian rhythm. Proteins annotated for tropic responses in the roots and shoots may serve similar functions in the pulvinus. An overview of proteomics,

the exploration of molecular functions from the expression of proteins, is given below.

Proteomics

History of Protein Analysis

Knowledge of plant genomes does not equal knowledge of the gene products because more than one protein can be synthesized from a single gene through post-translational modifications [53]. Furthermore many species have not yet had their genomes fully sequenced. The field of proteomics complements existing genomic data and also provides functional analysis in species where genomic information is not yet available. Proteome databases specific to developmental stages of the plant have been assembled for a number of model plant species that exhibit some sort of tropic behavior, including *Medicago truncatula* root [54]; *Arabidopsis thaliana* guard cell [55]; and *Glycine max* (soybean) xylem sap and apoplast, seedlings, and root [56-59].

Procedures for protein identification have changed since its inception. An early method of protein identification was the Edman degradation method, an accurate yet time-consuming method that characterized a sequence amino acid by amino acid. Currently mass spectrometry (MS), which identifies protein fragments by their mass-to-charge ratio (*m*/*z*), is the predominant method for protein identification though Edman degradation is still used for confirmation of identified peptides [60]. After extraction and purification of protein samples, two-dimensional gel

electrophoresis separates the proteins by isoelectric point (pI) and molecular weight (M_r). The separated proteins are then prepared for analysis by MS [61].

Two widely-used forms of MS are matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). MALDI-TOF utilizes the methods of electrospray ionization and laser desorption to identify proteins of higher molecular masses (into the 100 kilodalton range). Resonant wavelengths from short-pulse lasers hit the sample in an energy-modulating matrix, ionizing the proteins into small fragments. The fragments are then identified by time of flight analysis where ions hit the detector at a time proportional to the square root of the m/z ratio [62]. LC-MS/MS, a form of tandem mass spectrometry, physically separates the proteins in a column before running multiple mass spectrometry stages. LC-MS/MS allows protein identification even from partial sequences [60], allowing for database searches with different levels of sensitivity. MS identification coupled with biochemical analysis can characterize proteins as part of greater complexes; comparison with orthologs from similar species can offer insight into the mechanism of the complex [63]. Weaknesses to the various identification technologies include instrument bias/noise, distortion by the conversion of gels to digital images or the consistency of the gels themselves, and limitations from the various algorithms used by the software for analysis of the peptide fragments [60, 61]. Nevertheless, mass spectrometry provides a powerful and effective means to analyze active gene products from which their functions can be determined.

Study objectives

While previous studies have identified a number of genes involved in a variety of tropic responses, there remains a great dearth of knowledge on the specific proteins present and responsive in the pulvinus. The purpose of this study was to map the proteome of the soybean (*Glycine max*) pulvinus using trichloroacetic acid/acetone extraction, 2-dimension electrophoresis separation (2-DE), identification by LC-MS/MS, and to compare levels of protein expression between light-harvested and dark-harvested pulvini.. The soybean has been selected because of its importance as a crop species as well as the numerous studies that have highlighted soybean paraheliotropic response. In this study I will map the proteome in the soybean pulvinus and compare this to the proteins detected in guard cells, which function in similar ways to the pulvinus. Following the proteome map, I will also assess changes in the proteome in response to light by mapping the proteome in periods of dark and in the light.

Chapter 2: Proteomic analysis of the Pulvinus, a Heliotropic Tissue, in *Glycine max*

Abstract

Certain plant species respond to light, dark, and other environmental factors by leaf movement. Leguminous plants both track and avoid the sun through turgor changes of the pulvinus tissue at the base of leaves. Mechanisms leading to pulvinar turgor flux, particularly knowledge of the proteins involved, are not well-known. In this study we used two-dimensional gel electrophoresis and liquid chromatography-tandom mass spectrometry to separate and identify the proteins located in the soybean pulvinus. A total of 183 spots were separated and 195 proteins from 165 spots were identified and functionally analyzed using single enrichment analysis for gene ontology terms. The most significant terms were related to proton transport. Comparison with guard cell proteomes revealed similar significant processes but a greater number of pulvinus proteins are required for comparable analysis. To our knowledge, this is a novel report on the analysis of proteins found in soybean pulvinus. These findings provide a better understanding of the proteins required for turgor change in the pulvinus.

Introduction

Plants respond to light, gravity, touch, and other environmental signals by both temporary and permanent differential growth [2, 3]. The directional growth of plants as a response to an external stimulus is called tropism. The movement of leaves by sunlight, or heliotropism, can angle leaf lamina both toward (diaheliotropism) and away from (paraheliotropism) the light depending on the intensity of the irradiance, circadian rhythms (nyctinasty), and environmental stresses. Diaheliotropic movement has been shown to increase water use efficiency and carbon assimilation compared to horizontal controls in cotton and soybean [7, 9]. Paraheliotropism benefits plants by maintaining high levels of photosynthetic quantum yield under stressed conditions and reducing UVB radiation levels [12, 13].

The pulvinus is an enlarged motor organ at the base of leaves found in many leguminous plants. It has been observed to force the movement of leaves in heliotropic, seismonastic, and nyctinastic patterns [4, 32]. Unlike pulvini found in maize and oat that respond to gravity by permanent growth, the movement of leaf pulvini is reversible [4, 64]. In heliotropism, upon light exposure, an asymmetric turgor gradient formed between the adaxial and abaxial motor cells leads to leaf movement. Potassium ion influx coupled with chlorine ion is powered by a proton gradient and results in osmotic influx [32]. In addition to heliotropism, the pulvinus changes turgor for nyctinastic leaf folding, and is affected by alterations in the length of the photoperiod [65].

The structure of the pulvinus reveals its specialized role in leaf movement. In contrast to the stem and petiole, the pulvinus has a relatively larger cortex and smaller

pith. The motor cells are part of the cortex [25]. Two types of pulvinar vacuoles found in many species participate in the volume flux. Tannin-rich vacuoles have been previously mentioned as a major source of cellular volume change [29]. Still other studies have found the primary volume changes to be from the type of vacuole that does not contain tannin [31].

A number of proteins have been linked with pulvinar heliotropism and nyctinasty. H^+-ATPase activity increases turgor by H^+ efflux and consequent K^+ influx, and H^+-ATPase inhibitors reduce diaheliotropic response in soybean pulvinus [66]. Blue light is a deactivator of H^+-ATPase in *Phaseolus vulgaris* motor cells which leads to decreased turgor pressure on the illuminated region rather than an activator of H^+-ATPase on the opposite region [34]. Furthermore, studies on gravitropic grass pulvini proteins have begun to identify differentially expressed proteins including one demonstrating MAPK-like activity [67].

While studies have identified a number of genes involved in a variety of tropic responses, there remains a great dearth of knowledge on the gene products and their expression patterns. The purpose of this study was to map the proteome of the soybean (*Glycine max*) pulvinus using trichloroacetic acid/acetone extraction, 2-dimension gel electrophoresis (2-DE) separation, and identification by liquid chromatography-tandom mass spectrometry (LC-MS/MS). This map would highlight the molecular and functional characterization of the pulvinus at the protein level. The soybean has been selected because of its importance as a food crop, the relative size of its pulvinus, and ease in growing samples. After profiling the pulvinus proteome, it will be compared to previously identified proteomes of functionally-similar guard

cells.

Experimental

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). 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 six or seven trifoliolate leaves (between six and eight weeks). The terminal and lateral pulvini from the second through sixth trifoliolate leaves were separately excised with a razor one to two hours into the light period and frozen in liquid nitrogen. 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 (2005) was used to extract pulvinar protein [68]. For each of three biological replicates approximately 2.0 g of pulvinus were 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 [69].

2D gel electrophoresis

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 Ettan IPGphor II (GE Healthcare, Piscataway, NJ) under conditions described earlier by Natarajan et al (2005): 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 5000 V for ten hours [68]. 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 mM Tris-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

ImageScanner III (GE Healthcare), and analyzed using Progenesis SameSpots software (Nonlinear Dynamics, Durham, NC). 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 [70] and Gharahdaghi et al [71]. 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 (ThermoFisher 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 [72]. 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.

Biological process gene ontology (GO) terms as listed in the Gene Ontology Annotation (GOA) Database, a collaboration with the UniProt Knowledgebase, were retrieved for the proteins and input into the agriGO version 1.2 GO analysis program (China Agricultural University, Beijing) for single enrichment analysis. The analysis used as a default a hypergeometric statistical method with the Benjamini-Yekutieli correction method and a minimum five terms for significance [73]. The reference GO term list was the soybean locus genome provided by Phytozome (US Department of Energy, Joint Genome Institute, Walnut Creek, CA).

Results and Discussion

Protein Extraction, Identification, and Functional Analysis

To investigate pulvinus protein functional analysis, 2-DE was used to separate and extract the proteins. A total of 183 spots were then excised for LC-MS/MS analysis (Figure 1). MS/MS data run through Scaffold resulted in the identification of 195 proteins; 18 spots of the 183 total either did not contain any peptides or had a protein identification probability <95%. Fifty-five proteins were named directly as a result of the Scaffold analysis workflow. An additional 116 proteins were named through the UniRef sequence clusters at the 100%, 90%, or 50% homology level. The remaining 24 proteins were not named even at the 50% homology level. Of the 195 proteins, 129 had GO terms for biological processes. Protein or cluster names, UniProtKB accession numbers, UniRef accession numbers, molecular weight, corresponding gel spots, and genetic information are listed in Table I.

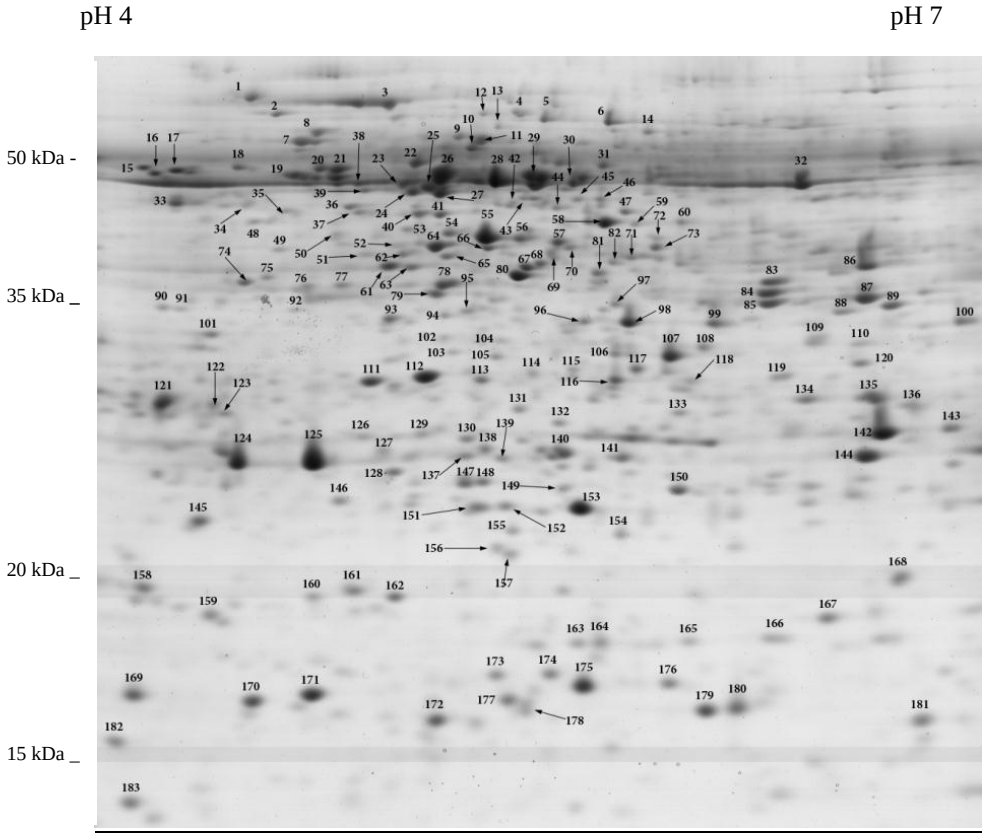

Figure 1. Representative SDS-PAGE gel of soybean lateral pulvinus, pH 4-7. The lateral and terminal pulvinus did not significantly differ from one another (data not shown). The numbers correspond to the proteins identified through LC-MS/MS and listed in Table I. Eighteen spots did not produce significant hits.

Table I. Soybean pulvinus proteins as identified by LC-MS/MS and Scaffold analysis program. The MS/MS spectra were examined against the UniProt database in order to retrieve GO terms for functional analysis. Fifty-five proteins had names; a further 116 proteins initially identified as "uncharacterized" were named using UniRef cluster homology and 24 proteins remained uncharacterized even at the 50% homology level.

Identified Proteins	Gel Spot(s)	UniProtKB	MW kDa	GO Term(s)	
40S ribosomal protein S12	178	C6SVV7	15	GO:0006412	
Alpha-form rubisco activase	40	D4N5G0	52	GO:0010150	GO:0009753
Aminomethyltransferase	49	I1KNF5	44	GO:0006546	
Annexin	110	C6TFT8	36		
ATP synthase subunit alpha, chloroplastic	22	Q2PMS8	56	GO:0015991	GO:0015986

ATP synthase subunit alpha, mitochondrial	17	Q01915	55	GO:0015991	GO:0015986	
ATP synthase subunit beta	26, 28, 29	I1NFS4	60	GO:0015991	GO:0015986	
ATP synthase subunit beta	26	I1LEP2	59	GO:0015991	GO:0015986	
ATP synthase subunit beta, chloroplastic	29	Q2PMV0	54	GO:0015991	GO:0015986	
Beta-amylase	15, 16, 17, 28, 29, 30, 51, 62	P10538	56	GO:0000272		
Betaine aldehyde dehydrogenase	10	B0M1A6	55	GO:0009737	GO:0009651	GO:0009414
Cysteine synthase	96	C6TMX6	34	GO:0006535		
Cytochrome b6-f complex iron-sulfur subunit	166	I1LUB3	24	GO:0022900	GO:0015979	
Elongation factor 1-alpha	16, 19, 20, 23, 25, 27, 39, 45	P25698	49	GO:0006184		
Ferritin	147	C6TI81	28	GO:0006879	GO:0006826	
Fructose-bisphosphate aldolase	85, 97	I1JSJ3	43	GO:0006096		
Glucose-1-phosphate adenylyltransferase	42, 44	I1M6B5	55	GO:0005978		
Glutamine synthetase	63	C6T8F0	39	GO:0006542	GO:0006807	
Glutamine synthetase	67	I1MZF3	39	GO:0006542	GO:0006807	
Glutamine synthetase	67, 68, 80	Q9FUK4	39	GO:0006542	GO:0006807	
Glutamine synthetase	57, 72	I1M170	48	GO:0006542	GO:0006807	
Glutathione peroxidase	167	I1KP94	25	GO:0006979		
Glycinin G1	75	P04776	56			
In2-1 protein	128, 137	Q9FQ95	27			
Late-embryogenesis abundant protein 1	74, 75, 90, 91	C6TLT7	36	GO:0009269		
Late-embryogenesis abundant protein 2	90, 91	C6T750	34	GO:0009269		
Malate dehydrogenase	84, 85	I1JB84	33	GO:0044262	GO:0006108	GO:0006099
Malate dehydrogenase	83, 84, 85, 87	H2D5S3	35	GO:0044262	GO:0006108	GO:0006099
Malate dehydrogenase	83, 84, 85, 87	Q9SPB8	36	GO:0044262	GO:0006108	GO:0006099
Malic enzyme	13	I1JMI9	69	GO:0006108		
Nucleoside diphosphate kinase	181	I1KJI7	17	GO:0006241	GO:0006183	GO:0006228
Phosphoglycerate kinase	71, 82	I1MJC6	39	GO:0006096		
Phosphoribulokinase	61	I1L540	45	GO:0005975		
Phosphoribulokinase	61	I1J4L6	45	GO:0005975		
Profilin	183	A7XZJ7	14	GO:0030036		
Proteasome subunit alpha type	138, 140	C6TH59	26	GO:0006511		
Proteasome subunit alpha type	93	I1L3S2	31	GO:0006511		
Proteasome subunit beta type	144	I1JJI4	29	GO:0051603		
Protein disulfide isomerase L-3b	7	C6KXH6	59	GO:0045454		
Ribulose bisphosphate carboxylase large chain	3, 16, 17, 18, 19, 23, 30, 32, 38, 39	P27066	53	GO:0009853	GO:0019253	
Rubisco activase	40, 41, 51, 52, 64, 66	D4N5G3	49	GO:0010150	GO:0009753	

S-adenosylmethionine synthase	46, 47, 58, 59, 60	I1MHR0	43	GO:0006730	GO:0006556	
S-adenosylmethionine synthase	59, 60	I1KMK1	43	GO:0006730	GO:0006556	
S-adenosylmethionine synthase	47	I1JQV8	43	GO:0006730	GO:0006556	
Serine hydroxymethyltransferase	19	I1K3S6	45	GO:0006544	GO:0006563	GO:0006730
SGT1-2	53	B6EBD5	41			
Stem 31 kDa glycoprotein	135, 142, 143	P10743	29			
Sulfurtransferase	102, 104	I1KTY9	42			
Superoxide dismutase [Cu-Zn]	179	I1JRI7	15	GO:0006801		
Superoxide dismutase [Cu-Zn]	173	I1LKZ3	21	GO:0006801		
Superoxide dismutase	139	I1LCI3	28	GO:0006801		
Triosephosphate isomerase (Fragment)	141, 144	I1M4W9	27			
Triosephosphate isomerase	140	I1NAD0	33			
Triosephosphate isomerase	140	C6TK33	33			
Triosephosphate isomerase	138, 140	C6T7V6	33			

Proteins identified by UniRef

Cluster Name	**Gel spot(s)**	**UniProtKB**	**MW kDa**	**GO Terms**	
(+)-pulegone reductase	88	C6TIQ5	38		
1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	146	I1LE06	26		
20 kDa chaperonin, chloroplastic	148, 149	C6TJG0	27	GO:0006457	
26S protease regulatory subunit 6A homolog A	36, 37	I1LBA2	47	GO:0030163	
26S protease regulatory subunit 6B homolog	43, 45, 47	I1MZN7	47	GO:0030163	
2-Cys peroxiredoxin BAS1-like, chloroplastic	145	I1JQ73	24		
3-oxo-Delta(4,5)-steroid 5-beta-reductase	37	I1K843	44		
40S ribosomal protein S19-like protein	182	C6SVR8	16	GO:0006412	
60S acidic ribosomal protein P0	93, 95	C6TGA6	34	GO:0042254	GO:0006414
60S ribosomal protein L23	182	C6T0H9	15	GO:0006412	
Acid phosphatase-like protein	123	I1KJR7	28		
Actin	53, 55	I1MDT4	42		
Actin depolymerizing factor 1	180	C6SYJ7	16		
Actin-11	53, 55, 57	I1JY56	42		
Actin-11	55	I1M4W2	42		
Alanine aminotransferase 2	45	I1J547	54	GO:0009058	
Allantoinase	134, 135	I1M261	57	GO:0000256	
Alpha-form rubisco activase	41	I1M841	52		
Alpha-form rubisco activase	66	I1MZA9	47		
Apyrase 2	33, 91	I1ML46	50		
Argininosuccinate synthase, chloroplastic	45	I1K099	52	GO:0006526	
Aspartic proteinase	33	I1LAZ3	57	GO:0006629	GO:0006508
AT5g63860/MGI19_6	46	I1JXY7	47		
ATP synthase subunit d, mitochondria	161, 162	C6SZJ2	20	GO:0015986	
ATP synthase subunit d, mitochondria	162	C6SYC1	20	GO:0015986	

Caffeic acid 3-O-methyltransferase	68	C6TIJ7	40	GO:0032259	
Caffeoyl-CoA O-methyltransferase	131	I1JE07	37		
Chaperonin CPN60-1, mitochondrial	10	I1LCI1	61	GO:0042026	
Chlorophyll a-b binding protein 21, chloroplastic	124	C6TNE6	28	GO:0009765	
Chlorophyll a-b binding protein 6, chloroplastic	154, 155	I1JCY0	27		
Chloroplast HSP70	24	I1MJU7	74	GO:0006457	
Cystatin (Fragment)	105	I1M778	23		
Cysteine proteinase RD19a	118	I1MC68	40	GO:0006508	
Cysteine proteinase RD21a	122	I1M8Q5	51	GO:0006508	
Cytochrome c oxidase subunit 5b-2, mitochondrial	182	C6TAW8	18		
Cytosolic ascorbate peroxidase 1	126, 129, 130	I1LKA6	27	GO:0006979	
D-3-phosphoglycerate dehydrogenase	6	I1NFX7	63	GO:0006564	
DEAD-box ATP-dependent RNA helicase 15	46	I1JUD5	48		
Dihydroflavonol reductase	106, 107	I1LLS1	30	GO:0044237	
Dihydrolipoyllysine-residue acetyltransferase component 3 of pyruvate dehydrogenase complex, mitochondrial	29	I1KH71	59	GO:0006090	
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 2, mitochondrial	60	I1JJB5	50	GO:0006099	
Disease resistance response protein 1	164	C6SX05	21		
Elicitor-inducible protein EIG-J7	169	I1LNL7	20		
Enolase 2	29	I1JPW5	48	GO:0006096	
Eukaryotic initiation factor 4A-1	42, 44	I1KUR0	48		
Eukaryotic initiation factor 4A-1	42, 44	I1L1K7	47		
FAM10 family protein At4g22670	39	I1MUT2	44		
Ferredoxin--NADP reductase, leaf isozyme 2, chloroplastic	108	I1JCG8	40		
Fructokinase-2	95	I1M561	35	GO:0006014	
Fumarylacetoacetase	72	I1KZY9	46	GO:0009072	
Glutathione S-transferase DHAR3, chloroplastic	150	I1NJ59	23		
Glutathione S-transferase GST	141	I1KK66	26		
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Fragment)	74, 75, 76, 77, 83, 84	I1JXG9	37	GO:0006006	
Glycosyl hydrolase family 18 protein	35	I1MKL0	48		
Heat shock 70 kDa protein (Fragment)	1, 3	I1JPC5	68		
Heat shock 70 kDa protein 3	1, 3	I1LQR4	71		
Heat shock 70 kDa protein 3	3	I1N557	71		
Heat shock 70 kDa protein, mitochondrial	4, 12	I1M2K9	72	GO:0006457	
Heat shock 70 kDa protein, mitochondrial	4	I1KL72	72	GO:0006457	
Heat shock 70 kDa protein, mitochondrial	4	I1ME25	72	GO:0006457	
Hydroxyacylglutathione hydrolase, mitochondrial	133	I1MB50	29		
Inositol-3-phosphate synthase 1	31	I1K4L3	56	GO:0006021	GO:0008654
Isoflavone reductase homolog 2	98, 99	I1JSN3	28		
Lactoylglutathione lyase	116	I1KND9	27		
L-ascorbate peroxidase T, chloroplastic	115, 117	I1JZ45	45	GO:0006979	
Malate dehydrogenase [NADP], chloroplastic	70	I1LCM5	46	GO:0005975	GO:0006108

Mediator of RNA polymerase II transcription subunit 37a	1	I1K668	73		
Methyl binding domain protein	48	I1JWB8	32		
MLP-like protein	171	C6SZA9	17	GO:0006952	GO:0009607
MLP-like protein	170, 171	C6SYW1	17	GO:0006952	GO:0009607
NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	136	C6TIE6	28		
Oxygen-evolving enhancer protein 1, chloroplastic	111, 112, 113	I1MNK0	35	GO:0015979	GO:0042549
Oxygen-evolving enhancer protein 2-1, chloroplastic	153	I1JJ05	28	GO:0015979	
Oxygen-evolving enhancer protein 2-1, chloroplastic	152	I1N123	28	GO:0015979	
Peroxiredoxin	174, 176	C6SWE0	17	GO:0055114	
Phosphoglycerate mutase	6, 14	I1L6W0	61	GO:0006007	
Probable mitochondrial-processing peptidase subunit alpha-2	43	I1KGH6	54	GO:0006508	
Probable mitochondrial-processing peptidase subunit alpha-2	23, 43	I1KPV1	55	GO:0006508	
Probable nucleoredoxin 1-2	2	I1KXE7	64	GO:0045454	
Protein disulfide isomerase-like 1-1	7, 8, 68	I1KAB7	56	GO:0045454	GO:0006662
Protein disulfide isomerase-like 1-1	8	I1JZ42	48	GO:0045454	GO:0006662
Putative DNA repair protein RAD23-3	48	C6TIN6	41	GO:0006289	GO:0043161
Putative DNA repair protein RAD23-3	34, 48	I1LEX5	43	GO:0006289	GO:0043161
Putative RNA-binding protein rbpA	172	I1LJC9	16		
Pyruvate dehydrogenase, putative	79	C6T827	39		
Pyruvate dehydrogenase, putative	79	I1JJD2	39		
Quinone oxidoreductase-like protein At1g23740, chloroplastic	89	I1MLW9	35		
Quinone oxidoreductase-like protein At1g23740, chloroplastic	89	I1N5R5	34		
RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)	7	I1LL25	62	GO:0042026	
RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)	7, 8	I1LR60	62	GO:0042026	
RuBisCO large subunit-binding protein subunit beta, chloroplastic	9, 10, 11	I1MJ28	63	GO:0042026	
Sedoheptulose-1,7-bisphosphatase, chloroplastic	75, 76, 77	I1LMN5	42	GO:0005975	
Sedoheptulose-1,7-bisphosphatase, chloroplastic	77	I1MZ53	42	GO:0005975	
Serpin-ZX	63	I1K7W5	43		
SGT1-2	53	I1MPF6	40		
SHOOT1 protein	93	I1K480	38		
Soluble inorganic pyrophosphatase	141	C6SYN5	24	GO:0006796	
Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	65	I1L8G3	45		
Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	65	I1LYN0	45		
Transaldolase (Fragments)	50	I1KZJ0	48	GO:0006098	
Translationally-controlled tumor protein homolog	158	I1L0Z3	15		
Trypsin inhibitor A	145	I1KYX1	23	GO:0010951	
Tubulin A	24, 25, 27, 39, 43	I1K3X9	50	GO:0007018	GO:0051258
Tubulin alpha-2 chain	24	I1JV03	50	GO:0007018	GO:0051258
Tubulin alpha-6 chain	24, 27	I1K2I1	50	GO:0007018	GO:0051258
Tubulin beta-2 chain	18, 19, 20, 21, 38	I1JT28	51	GO:0007018	GO:0051258
Tubulin beta-2 chain	38, 39	I1KPA1	50	GO:0007018	GO:0051258
Tubulin beta-2 chain	21	I1J752	51	GO:0007018	GO:0051258

Tubulin beta-2 chain	19	I1LDR2	50	GO:0007018	GO:0051258
Tubulin beta-2 chain	19, 21, 38	I1K2Y0	50	GO:0007018	GO:0051258
Uncharacterized protein At5g39570	15, 16, 17	I1N675	41		
Uncharacterized protein At5g39570	17	I1LWM2	43		
UTP--glucose-1-phosphate uridylyltransferase	23, 25, 27, 28	I1MBR7	51		
V-type proton ATPase catalytic subunit A	5, 6	I1KVU0	69	GO:0015991	
V-type proton ATPase subunit B2	20, 21	I1L655	54	GO:0015991	
V-type proton ATPase subunit E3	119, 120	I1KPH2	27	GO:0015991	

Unknown or uncharacterized proteins

Gel spot(s)	UniProtKB	MW kDa	Gene	GO Term(s)	
96	I1JPE6	45		GO:0009089	
157	I1KJH7	21	Gma.11258	GO:0015986	
17	C6TGU6	45	Gma.13206	GO:0006886	
43	I1L281	59	Gma.13759	GO:0005975	
95	I1JN31	55	Gma.14675	GO:0006508	
69, 82	I1JEI0	44	Gma.2190	GO:0007047	GO:0030244
49, 51, 61, 63, 65, 67, 68, 86	I1JGY9	49	Gma.24854	GO:0006508	
30	I1N7G4	61	Gma.27992	GO:0006508	
132	C6THR8	36	Gma.31275		
163	C6SVX3	18	Gma.31311	GO:0006950	
51	C6T9R1	42	Gma.32429	GO:0006629	
138	I1LU40	26	Gma.3521		
106, 107	I1LLS2	28	Gma.37094	GO:0044237	
165	I1JHP9	23	Gma.3898	GO:0008152	
86	I1KAJ5	37	Gma.55012		
160	C6T2Y2	19	Gma.6437	GO:0022904	
78	I1LZ92	38	Gma.6545	GO:0006167	GO:0006166
76	I1L340	30	Gma.9083		
55	C6TKV3	46		GO:0006222	
68, 69, 81	I1JBH8	40		GO:0045454	GO:0006662
156	I1JML1	21		GO:0015986	
130	I1K4S0	28			
50	I1KYR2	40		GO:0006508	
95	I1JVA6	33			

AgriGO single enrichment analysis of 129 proteins compared to a background soybean genome locus identified 74 significant GO terms (data not shown). Most of the 74 terms fell under three broad parent terms: nucleobase, nucleoside, and nucleotide metabolic processes (23 child GO terms), nitrogen compound metabolic processes (13 child GO terms), and transport (11 child GO terms). All terminal significant child GO terms are listed in Table II. The top two significant biological process GO terms were related to proton transport. The third significant term was a negative regulator of proton transport (oxidative phosphorylation, GO:0006119). Photosynthesis and respiration were also enriched compared to the reference background.

Table II. Statistically significant child GO Terms for biological process as determined by agriGO GO analysis program. A GO term had to appear at least five times for statistical significance. Seventy-four GO terms were found to be significant, many of which were ancestor terms for the same pathway. The terms listed here are the most terminal child terms and are listed in order of lowest e-values.

Child GO Term	Process	E-value	Number
GO:0015991	ATP hydrolysis coupled proton transport	2.74E-13	8/129
GO:0015986	ATP synthesis coupled proton transport	9.93E-08	9/129
GO:0006119	oxidative phosphorylation	2.04E-06	9/129
GO:0051258	protein polymerization	6.44E-06	8/129
GO:0006108	malate metabolic process	1.71E-05	5/129
GO:0007018	microtubule-based movement	3.52E-05	8/129
GO:0009119	ribonucleoside metabolic process	4.59E-04	6/129
GO:0009084	glutamine family amino acid biosynthetic process	1.91E-03	5/129
GO:0006457	protein folding	2.99E-03	9/129
GO:0045333	cellular respiration	8.18E-03	5/129
GO:0045454	cell redox homeostasis	1.29E-02	5/129
GO:0015979	photosynthesis	1.56E-02	6/129
GO:0006508	proteolysis	4.48E-02	14/129
GO:0006007	glucose catabolic process	4.99E-02	5/129

Proton transport

The two most significant (lowest e-values) GO terms were related to the transport of protons through hydrolysis and synthesis of ATP (Table II). This would indicate a higher demand for proton transport in the pulvinus relative to the overall soybean plant and confirms our understanding of turgor change in pulvinar motor cells. The changes in the different turgor pressures between the adaxial and abaxial motor cells that result in leaf movement are triggered by K^+ and Cl^- flux across the plasma membranes. Proton concentrations dictate the level of activity in ion channels and consequent osmotic movement [32]. Vacuolar H^+-ATPases are believed to also assist in Ca^{2+} accumulation through the generation of the electrochemical gradient across the tonoplast [74]. In their examination of apoplast pH levels in the nyctinastic *S. saman* pulvini, Lee and Satter [35] confirmed that swelling of motor cells corresponded to proton extrusion, and the same light treatment resulted in opposing proton fluxes between the adaxial and abaxial motor cells. Okazaki [34] found similar pH changes in *P. vulgaris* pulvini and noted that an increase of extracellular pH were accompanied by inhibition of plasma membrane H^+-ATPase activity which would lead to turgor decrease. Unlike Lee and Satter [35], Okazaki [34] found that blue light increased extracellular pH in both adaxial and abaxial protoplasts and speculated that the relative differences in turgor between the two regions resulted from the differences in the amount of blue light received. It is important to remember that the responses measured by the two groups of researchers were not the same; Lee and Satter [35] were looking at the leaf movements of a nyctinastic plant while Okazaki

was examining the heliotropic leaf movement of *P. vulgaris*. The apparent differences in pH response to blue light may be a result of a photoreceptive signaling pathway unique to heliotropic plants.

Malate metabolism

Malate metabolism was the fifth most-common significant GO term as calculated by agriGO (Table II). In the pulvinus the dissociated anion of malic acid, malate, is believed to serve as an additional source of charge balance in the cell, as the co-transport of Cl^- does not account for all the positive charge associated with K^+ movement. Malic acid appears in higher concentrations during the day in the whole pulvinus of *P. coccineus* motor cells than at night, and increased concentrations correspond to regions of K^+ accumulation. The majority of malic acid occurs through synthesis (~80%), not by transport between the abaxial and adaxial regions [75]. Malate is also known to be an important counter-ion in guard cells [76]. Four of the five proteins with the malate metabolism annotation were various isoforms of malate dehydrogenase (spots 83-85, 87). The fifth, malic enzyme (spot 13), is involved in the synthesis of malic acid. A precursor to malate is sucrose, believed to be present in motor cells by import from nearby tissues and also by the degradation of starch. One protein identified as the starch degrading enzyme beta-amylase was detected in various amounts across eight spots in the pulvinus gel (spots 15-17, 28-30, 51 and 62).

Cytoskeletal processes

Two significant GO terms, protein polymerization and microtubule-based movement, were both annotated to eight tubulin proteins (spots 18-21, 24, 25, 27, 38, 39 and 43). The multiple tubulin proteins are an example of the tetraploid nature of soybean; despite having over 86% sequence homology five tubulin beta-2 chain proteins are encoded by genes located on five different chromosomes (data not shown). While tubulin is the protein of note in GO analysis, studies have found actin (spots 53, 55, 57) to be more integral to pulvinar bending. The actin proteins in the soybean pulvinus, however, did not have GO terms to input for analysis. Kanzawa et al. [2]and Yao et al. [77] detected the fragmentation of actin filaments in response to cold and electrical stimuli, respectively in *Mimosa pudica pulvini*; Kanzawa et al. [2] also detected microtubule fragmentation but microtubule modulators did not appear to affect pulvinar bending while actin modulators did. Yao et al. [77] noted that both actin rearrangement and the presence of intracellular Ca^{2+} were required for bending. Several other proteins with Ca^{2+}-dependent activity were detected in the pulvinus, including inositol 3-phosphate synthase (spot 31), apyrase (spots 33 and 91), and annexin (spot 110). These three proteins could function as early participants in signal-transduction pathways that lead to ion movement across the plasma membrane.

Ion transporters and Aquaporins

Along with proton ATPases was the expectation of finding aquaporins and K^+ transport proteins in the pulvinus. Previous studies have suggested the presence of aquaporins, and at least two K^+ transporters for inward and outward ion movement [78, 37]. The channels, conventionally labeled as K_H and K_D for hyperpolarized and

depolarized K^+ channels, respectively, correspond to inward and outward ion movement. However, Yu et al. [41] found evidence for a voltage-independent K_H channel not affected by extracellular pH levels that appears to serve not a minor role in K^+ influx. Fluerat-Lessard et al. [43] detected high levels of a 23kDa aquaporin in addition to V-H^+ATPases in the aqueous vacuole of mature *M. pudica* pulvini compared to juvenile, non-functioning pulvini. At least two aquaporin-like gene products were successfully cloned from *S. saman* pulvini; one of the two, SsAQP2, also demonstrated diurnal and circadian patterns and was not found to an appreciable degree in nearby leaf tissue [78]. Unfortunately we did not detect any other transporters (other than ferritin, spot 147) or any proteins localized to the plasma membrane in the soybean pulvinus. There could be several explanations for the lack of these proteins. For one, aquaporins have an isoelectric point (~8) higher than the range that was used in this study. Furthermore, plasma membrane proteins typically are not recovered from traditional protein extraction methods, requiring an additional partitioning step and special extraction buffers [79]. V-type H^+-ATPases, such as the three detected in the soybean pulvinus, are not only localized in vacuoles but may also be located in the plasma membrane. The GO annotation for cellular component of the three V-H^+ATPases did not specify plasma membrane or the tonoplast; given the dearth of other plasma membrane proteins it can safely be assumed that the H^+ATPases detected were from endomembranes.

Pulvinus proteome compared to guard cells

Besides the pulvinus motor cell, another cell demonstrating rapid turgor changes is the guard cell. Like the pulvinus, guard cells require H^+-ATPase activity

triggered by light for its ion transport and subsequent water movement. In guard cells H^+-ATPase activity in the plasma membrane increases when exposed to blue light, with a corresponding decrease of extracellular pH and turgor increase [76]. However the opposite response occurred in Okazaki's examination of *P. vulgaris* motor cells, where blue light illumination led to the inhibition of H^+-ATPase [34]. This suggests that, despite functional similarities, guard cells and heliotropic pulvinus motor cells have distinct mechanisms for their turgor responses. It could be that guard cells, more often located on the abaxial leaf surface, have a similar photoreceptive mechanism to abaxial motor cells of nyctinastic plants such as *S. saman*. Therefore comparing the proteomes of the two tissues could assist in determining the degree of similarity of guard cells to pulvini of either heliotropic or nyctinastic plants.

Zhao et al. [80] examined the diploid *Arabidopsis* guard cell proteome. Their top GO term was related to stress response (response to cold), with energy categories making up three of the top eight. In the soybean pulvinus GO terms for photosynthesis and respiration were less significant than in the *Arabidopsis* guard cell. The most abundant protein found in the *Arabidopsis* guard cell was the stress response protein TGG1, which functions as a defense against pathogen attack [80]. In the soybean pulvinus protein abundance was not measured and so no similar observations could be made. One common term between the pulvinus and guard cell was protein folding. The different rankings of significant GO terms may reflect different mechanisms between the two, or many simply reflect the tetraploid nature of soybean, where multiple functionally homologous proteins would contribute an amount of GO terms not proportional to its functional significance.

Zhu et al. [81] compared mesophyll and guard cells from the tetraploid crop species *Brassica napus* using a combination of GO analysis and functional classification following the method of Bevan et al. [82]. They [81] found the most highly enriched guard cell proteins relative to the mesophyll cell fell under the categories of energy, photosynthesis, membrane and transport, metabolism, and stress response. Categorization of the soybean pulvinus proteins using the Bevan et al. [82] method found similar results for the major groups (data not shown). In contrast to the guard cell proteome, cytoskeletal and other structural proteins appeared to factor in more strongly in the pulvinus. Both Zhao et al. [80] and Zhu et al. [81] recovered around 10-fold more proteins than recovered in soybean pulvinus proteins, including many of the plasma membrane proteins that were not extracted using the methodology of this study. The magnitude of detected proteins in the guard cells compared to the pulvinus conceivably skews the GO analysis. Additional comparisons of the two proteomes would require a higher amount of protein extraction from soybean pulvinus, and care must be taken to compare the soybean pulvinus with the guard cell profile of another tetraploid organism.

Conclusion

To our knowledge, this is a novel report on the analysis of protein in soybean pulvinus. These findings provide a better understanding of the molecular basis of pulvinar protein function. In summary, 195 proteins were extracted and positively identified from 165 spots from the pulvinus gel. Gene ontology analysis of significant terms found the top dominant GO biological processes were related to proton

transport, malate metabolism, and cytoskeletal movement, which are in good agreement with previous studies on pulvinar response. Regrettably many highly basic proteins were not detected, including proteins at isoelectric points above pH 7 and plasma membrane proteins. In order to recover the latter, methods such as two-phase partitioning and the use of special buffers would be necessary to recover the integral membrane proteins. Finally, the number of proteins recovered from the pulvinus will have to increase for future comparative studies with proteins of the guard cell.

Chapter 3: Differentially Expressed Proteins of Soybean (*Glycine max*) Pulvinus in Light and Dark Conditions

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 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 artifactual results. These findings can provide a better understanding of the circadian pattern of protein expression in the legume pulvinus proteome.

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) [24, 4]. 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 [24, 4, 7, 12]. 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 [24]. 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 [32, 34]. 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. *P. 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 [34]. 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 [33]. 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. *S. saman* pulvinus apoplasts had a pH difference between adaxial and abaxial motor cells which was exaggerated by treatments of white light, blue light, and darkness [35]. Kim et al. [36] 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 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 [83]. There is evidence for the latter form of regulation in the expression of ion transporters. In a review by Haydon et al. [83] 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.

Experimental

This chapter followed the protocol established in Chapter 2 up through Mass Spectrometry. Additional steps are described below.

2-D PAGE

Gel replicates were compared against each other using the Progenesis SameSpots software program. 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. Between-subject design experiment setting was used for comparing the lateral light and lateral dark. Only spots with both ANOVA p-values ≤ 0.05 and max

fold ≥ 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.

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 expressed proteins are indicated by arrows (Figure 2). Out of 165 previously identified protein spots, 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 2). 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 III).

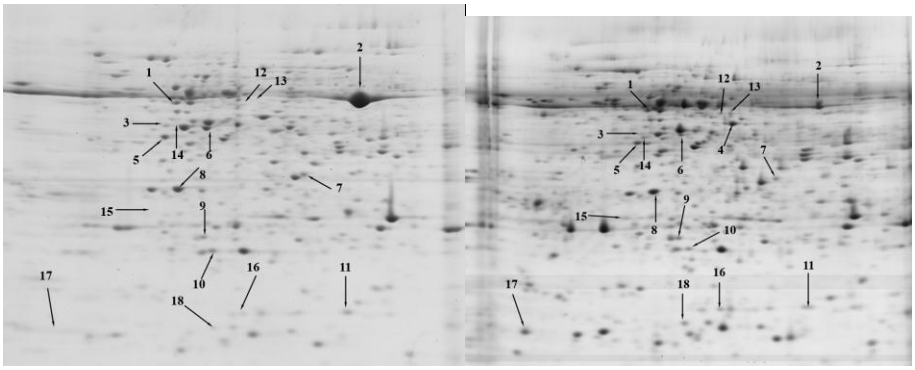

Figure 2. Comparative protein expression levels of light and dark harvested pulvinus. The left is the dark harvested sample, excised about six to seven hours into the dark period. The right is the light-harvested pulvinus, harvested about one to three hours into the beginning of the light period. Differential expression was measured by Progenesis

SameSpots software and required a minimum 1.5-fold increase/decrease and ANOVA p ≤0.05.

Table III. Differentially expressed proteins in light and dark harvested soybean pulvini.18 spots with ANOVA values p≤0.05 and a minimum 1.5-fold change were positively identified as 25 unique proteins using Progenesis Samespots and Scaffold protein identification software programs. All proteins are from the species Glycine max.

Spot no.	Protein	UniProt KB acces. no.	Volume Light + SD	Volume Dark +SD	p-value	Fold
1	Tubulin alpha-6 chain	I1MJU7	4.98 E+06	7.70 E+06	0.013	1.5
	Tubulin alpha-2 chain	I1K2I1				
	Tubulin A	I1JV03				
2	Ribulose bisphosphate carboxylase large chain	I1K3X9	1.83 E+07	5.70 E+07	0.033	3.1
3	Rubisco activase	P27066	1.55 E+06	3.27 E+06	0.039	2.1
4	S-adenosylmethionine synthase	D4N5G3	1.66 E+07	2.51 E+07	0.038	1.5
5	Phosphoribulokinase	I1MHR0	7.86 E+06	1.43 E+07	0.024	1.8
		I1J4L6				
	Uncharacterized protein	I1JGY9				
6	Rubisco activase	D4N5G3	5.98 E+06	1.31 E+07	0.01	2.2
	Uncharacterized protein	I1MZA9				
7	Ferredoxin--NADP reductase, leaf isozyme 2, chloroplastic	I1JCG8	3.55 E+06	5.79 E+06	0.016	1.6
8	Oxygen-evolving enhancer protein 1, chloroplastic	I1MNK0	2.02 E+07	3.00 E+07	0.011	1.5
9	20 kDa chaperonin, chloroplastic	C6TJG0	3.72 E+06	5.46 E+06	0.016	1.5
10	Oxygen-evolving enhancer protein 2-1, chloroplastic	I1N123	4.93 E+06	1.01 E+07	0.018	2.0
11	Cytochrome b6-f complex iron-sulfur subunit	I1LUB3	6.37 E+06	1.19 E+07	0.009	1.9
12	Alanine aminotransferase 2	I1J547	2.05 E+06	1.32 E+06	0.005	1.6

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	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	3.44 E+06	1.94 E+06	0.002	1.8
	AT5g63860/MGI19_6	I1JXY7				
14	Beta-amylase	P10538	1.98 E+06	6.90 E+05	0.018	2.9
15	Cytosolic ascorbate peroxidase 1	I1LKA6	3.39 E+06	1.60 E+06	6.23E-04	2.1
16	Uncharacterized protein	C6SVX3	4.32 E+06	2.87 E+06	0.039	1.5
17	Elicitor-inducible protein EIG-J7	I1LNL7	1.01 E+07	2.34 E+06	0.003	4.3
18	Superoxide dismutase [Cu-Zn]	I1LKZ3	7.00 E+06	4.47 E+06	0.027	1.6

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 III, 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: phosphoribulokinase (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 and a chloroplastic heat shock 70 kDa protein (spot 1).

Discussion

Overview

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 [24, 84, 85]. 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

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 [84]. 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 [85]. 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. 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 [86, 87]. 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 [88, 89]. 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. [88] 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. [88] 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) [90, 91]. 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 [92]. 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 [90-95]. However Rieger et al. [39] 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 [96]. 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 [97] 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, 77], as well as in response to darkness in guard cells [98]. 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 [99].

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 [100 and sources therein]. Rubisco activase in dark-harvested pulvini had over a two-fold increase in volume over light-harvested pulvini in two spots (Figure 2). 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 [101, 102]. 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 [100]. 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 [103]. 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 [104]. 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 [105]. 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 artifact of the tissue homogenization protocol. In *Pisum sativum* chloroplasts, Stieger and Feller [106] 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. [107] 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

While the increased expression in light-harvested pulvini is consistent with previous studies, the conflicting results of under expressed proteins in the light period warrants further analysis. Specifically, it would be important to determine whether the differentially expressed proteins were based on a photoperiod which can be manipulated or an underlying circadian rhythm independent of the photoperiod. Also it is not known how much of the expression can be altered by other abiotic factors such as temperature, water stress or nutrient levels since these factors have been shown to affect paraheliotropic leaf angles in *Phaseolus* and soybean [18, 15]. Finally, the extent that protein expression differs between the abaxial and adaxial regions of the pulvinus as has been previously demonstrated is unknown [102]. For example, if one region is over expressed while the other is complementarily under expressed than studies such as this that have not separated abaxial from adaxial portions of the pulvini may miss some key differences in protein levels. Isolating the pulvinar tissue into its two component halves, harvesting light and dark samples at multiple times within the same 24-hr photoperiod and altering the light/dark hours of the photoperiod are some ways these questions can be further explored.

Chapter 4: Conclusions

Overview

The pulvinus enables leaf movement in response to light by complementary osmotic flux in the abaxial and adaxial portions occurring as a result of K^+ and Cl^- movement. The main biological process expected from the pulvinus proteome is the transportation of the ions through the establishment of a proton gradient. This proton gradient is energized by ATP, which is formed during photosynthesis and respiration. The pulvinus proteome includes many proteins that participate in photosynthesis and respiration. The ten proteins that were identified as ATP synthases or V-ATPases reflect the high demand for energy and the importance of the proton gradient to power the turgor flux. Functional analysis also indicated that processes relating to proton transport were the two most significant GO terms in the pulvinus proteome. Surprisingly, though, no aquaporins or ion channels (specifically K^+ and a general anion transporter channel) were identified in this study, which could indicate a limitation of the protein extraction. The comparatively smaller numbers of pulvinus proteins detected compared to turgor-mediated guard cells, in addition to the ploidy level of soybean, made direct functional comparisons of the two proteomes difficult.

A comparison of light- and dark-harvested pulvini found increased expression of stress response proteins in the light and decreased expression of several Calvin-Benson cycle proteins. While the increased expression of antioxidant proteins was expected I was surprised that more of them—such as malate dehydrogenase and glutathione S-transferase, among others—were not overexpressed given the many

stress-response proteins that were identified. A possible explanation is the proteins found with increased expression in the day are the only proteins that are light-regulated. Schaffer et al [108], in a broad examination of *A. thaliana* genes, found only 11% of the 7800 genes demonstrated diurnal regulation with an even smaller percentage regulated by a circadian rhythm (2%). Given that one major function of these stress response proteins would be scavenging ROS brought on by photosynthetic activity, itself dependent on a diurnal pattern, this explanation is unlikely. It is possible that the multiple isoforms of the various ROS proteins that were detected in the pulvinus have differing temporal patterns of transcript and/or protein expression, which has been noted in *Arabidopsis* beta amylase (not an ROS protein) genes [96]. Another explanation is that the levels of protein expression would not change significantly despite changes in transcript levels, which have been shown previously [97].

A final likely explanation then would be time-lag differences in expression. For example, two antioxidant enzymes in *Arabidopsis* plants had higher levels of activity in response to high ozone levels—while many other enzymes were unaffected, it must be noted—but one enzyme increased activity immediately while the other took a day before altering expression [109]. In my study, in the light, pulvinus samples were harvested about an hour after the lights were turned on. Perhaps by harvesting at a later hour more stress response proteins would have been found overexpressed, whether by their own diurnal pattern or in response to the diurnal activity of photosynthetic proteins.

I was originally surprised at the higher protein levels of rubisco activase in the dark versus light pulvinus but then found many studies indicating a strong circadian pattern in transcript expression for that particular protein. Peak rubisco activase mRNA expression has been detected in both light and dark portions of the photoperiod depending on the species and possibly also the partition of the light-dark cycle (i.e. 14:10 light/dark vs. 16:8 light/dark). Other studies on rubisco large subunit and other photosynthetic proteins such as phosphoribulokinase did not appear to suggest circadian rhythms, however. The diminished expression in the light for those proteins could be a result of harvesting and experimental artifacts.

A note on methodology

Plant protein extraction is more difficult than animal protein extraction because of the strength and structural complexity of the plant cell wall; furthermore, many secondary metabolites can interfere with proteins when the tissue is being homogenized [110]. The method I utilized in this research, a combination TCA/acetone extraction, largely mitigates protein interactions with secondary metabolites by inactivation by TCA [111]. Alternatively, increasing the amount of protein loaded for 2-DE or trying a different extraction method may recover a greater number of transporter proteins. To be fair, the lack of channel proteins may be misleading because many proteins found in the pulvinus were uncharacterized even at a level of 50% homology. A natural follow-up to this work would be to query unknown protein sequences in a 3D protein structure database, such as NCBI Structure, to find alignments with similar proteins.

New directions

In my study the proteome of the whole pulvinus was examined without dividing the tissue into its adaxial and abaxial regions. This was done to provide a general overview of the proteins that are found in the pulvinus. However, this indiscrimination may have masked evidence of differential expression. Many pulvinus studies have shown differences between the abaxial and adaxial regions, including opposing molecular activities, when exposed to light or dark. Therefore any additional attempts to identify the pulvinus proteome should be done with the two regions separated. Other directions to take this work could be altering the light-dark photoperiod, adjusting the light level, and exposing the plants to short- and long-term stressors such as drought or limited nutrients. These directions would reveal more information on nyctinastic and paraheliotropic mechanisms. In order to gauge diaheliotropic protein responses the light source should simulate daily solar movement and abaxial/adaxial portions of the pulvinus would be harvested at different points in the light period. Harvesting pulvini from leaves facing different directions would add another layer of understanding. In all cases protein expression should be examined alongside mRNA expression levels, as many earlier studies have focused on the latter and not the former.

The establishment of the pulvinus proteome is the first step in elucidating the molecular function that enables leaf movement. As we continue to uncover the biological processes of heliotropism and nyctinasty via pulvini we can apply this knowledge toward breeding crops to favor cultivars that can adjust to more extreme

growth conditions. Nyctinastic signaling pathways may also be applicable to other non-plant systems which are also regulated by an endogenous rhythm.

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