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Comparative Proteomics of Peanut Gynophore Development under Dark and Mechanical Stimulation

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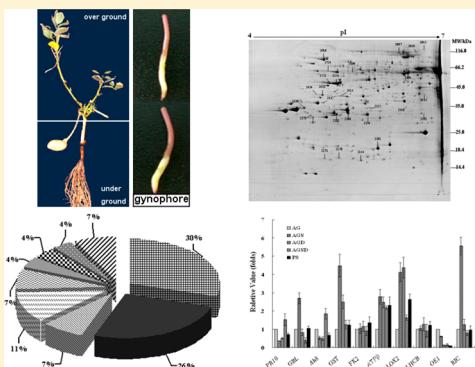
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Supporting Information

ABSTRACT: Peanut (*Arachis hypogaea*. L) is an important leguminous crop and source of proteins and lipids. It has attracted widespread attention of researchers due to its unique growth habit of geocarpy, which is regulated by geotropism, negative phototropism, and haptotropism. However, the protein expression pattern and molecular regulatory mechanism underlying the physiological processes of peanut remain unknown. In this study, the peanut gynophores under five treatment conditions were used for proteomic analysis, including aerial growth of the gynophores, the gynophores penetrated into the soil, as well as aerial growth of the gynophores under mechanical stimulation, dark, and mechanical stimulation combined with dark. The analysis of protein abundances in peanut gynophores under these conditions were conducted using comparative proteomic approaches. A total of 27 differentially expressed proteins were identified and further classified into nine biological functional groups of stress and defense, carbohydrate and energy metabolism, metabolism, photosynthesis, cell structure, signaling, transcription, protein folding and degradation, and function unknown. By searching gene functions against peanut database, 10 genes with similar annotations were selected as corresponding changed proteins, and their variation trends in gynophores under such growth conditions were further verified using quantitative real-time PCR. Overall, the investigation will benefit to enrich our understanding of the internal mechanisms of peanut gynophore development and lay a foundation for breeding and improving crop varieties and qualities.

KEYWORDS: peanut, gynophore, dark and mechanical stimulation, proteomics



1. INTRODUCTION

Peanut, one of the most important oil and economic leguminous crops in the world, is mainly cultivated in tropical, subtropical, and warm temperate climates as the main resource of vegetable oil and protein for daily life.¹ Peanut has a fascinating growth habit of geocarpy, such as flowers that develop in the air while fruits develop in the soil.^{2,3} Unlike other legume crops, after aerial fertilization, the peanut ovary is brought into the soil by geotropic elongation of the ovary handle (also called gynophore) and swells in the gynophore tip, and this process is crucial in determining the peanut yield.^{4,5} On the basis of the growth characteristics of the peanut gynophore, its role in the peanut reproductive growth process also has been investigated by other researchers. It was thought that the elongation of the peanut gynophores was affected by auxin, gibberellin, and other phytohormones.^{6,7} The distribution of auxin in different developmental stages and the effect of external stimuli on the distribution of auxin in the peanut gynophores were investigated. The notion that geotropism

could impact the distribution of auxin and thereby affect the growth and development of the gynophore was proposed.^{6,8} In addition, the impact of related enzymes on the lignification and the activities of polyamine oxidases and peroxidases during the growth and development of gynophores of six peanut varieties were measured, and the effects of enzymes on the subterranean gynophores were also studied. The results showed that different parts of the gynophores had different enzyme activities, and the biggest activities appeared at the tips of the gynophores.⁹ It was also proved that the enzyme activities were mainly affected by the peanut varieties but less affected by the growth time of gynophore.⁹ Under field condition, effects of AnM cultivate technique on bud differentiation and gynophore elongation of peanut were studied. The results indicated that this method could boost bud differentiation in seedling stage, favor to form strong seedling, reduce the content of gibberellin acid (GA3)

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and indole-3-acetic acid (IAA) of gynophores, and obviously regulate the elongation and growth rate of gynophore.^{10,11} In recent years, with the development of molecular biology, the release of peanut genome ($\sim 2.8 \times 10^9$ bp) information, as well as the establishment of different peanut databases, studies related to peanut gene cloning and transformation have increased. However, the formation mechanisms and prominent characteristics of peanut gynophore elongation and geotropic growth after chasmogamy have received little attention.

Previous research indicated that gynophore elongation and embryo development are controlled by growth regulators, such as light, touch, gravity, and phytohormones.^{2,12} In addition, the physiological changes that occur during gynophore development have decisive effects.^{13–15} Studies have shown that dark, mechanical stimulation and gravitation were necessary for peanut development.² Light stimulates gynophore elongation and inhibits embryo and pod growth, while dark arrests gynophore elongation and promotes the development of ovary into a pod.^{2,16,17} Meanwhile, the tip of the gynophore is highly sensitive and responsive to mechanical stimulation.^{18,19} After gynophore penetrated into the soil, normal mechanical stimulation occurred and subsequently lead to the arrest of gynophore elongation, while it promoted the development of pod underground.¹⁸ If the elongating peanut gynophore hung in the air and did not penetrate into the soil, the ovary would not grow and the tip of the gynophore would not swell, even if the growing gynophore was in the dark without mechanical stimulation. Thus, mechanical stimulus is supposed to be necessary for pod development, and the penetration into the soil of the gynophore is prerequisite for the fructification of the peanut.^{12,18} Moreover, a combination of IAA and gibberellin was reported to be responsible for normal gynophore elongation, and cytokinin was involved in the early stage of cell division. IAA is a key regulator and may play the most important role throughout the whole process of gynophore development.^{19–21} However, the mechanisms of molecular changes and key regulators involved in special physiological processes during the peanut gynophore formation and development have not been studied in detail.

Recently, significant numbers of peanut EST/cDNA libraries have been released to the public, but the protein expression data are lacking.^{3,22,23} High-throughput comparative proteomic approaches have been widely applied in the investigation of metabolisms in plant development and environmental responses.^{24–28} Because proteins serve as the bridge between genetic information encoded in the genome and the phenotype and proteomic analysis is a direct approach to define the function of their associated genes, proteomic research will reveal the plasticity of gene expression of plants under particular conditions.²⁵ The link of proteins to genome sequences is very useful for functional genomic studies.²⁶

To uncover the crucial proteins that participate in the process of gynophore penetrated into the soil after flowering and pod formation, we used the environmental factors, such as darkness, mechanical stimulation, and darkness combined with mechanical stimulation, to mimic the light, touch, and combined factors encountered during the normal growth of the gynophores. The tip region including important component of ovules and meristem of the gynophores was harvested for proteomic analysis, and 27 identified proteins were further classified into nine functional groups. In addition, the expression patterns of 10 representative genes were analyzed using quantitative real-time PCR. This research provided

further evidence at the protein level for understanding the regulatory and metabolic mechanisms in the development of peanut gynophores and legumes and has shed some light on the biological functions of geotropism, negative phototropism, and haptotropism during peanut gynophore development under different light and mechanical stimulation.

2. MATERIALS AND METHODS

2.1. Plant Materials and Growth Conditions

Peanut cultivar '*Luhua 14*' seeds were sown in plastic trays (20 cm in length and 13 cm in width) filled with perlite. Hoagland medium was added until the surface of perlite was humidified. The trays were placed in a growth chamber (Ningbo Jiangnan Instrument Factory, Hangzhou, China) with temperature of 28/25 °C (day/night), 15 h light/9 h dark, and relative humidity of 75%.³ 3 cm long tissues from the tip of gynophore were sampled at 4 days after treatments (DAT). The materials for each biological replicate were rinsed in ddH₂O to remove the contaminants, quickly dried with paper towels, and then frozen in liquid nitrogen and stored at –80 °C prior to protein extraction.

2.2. Mechanical and Light Treatments on Gynophores

Peanut seedlings were cultured to blossom and fertilized into gynophores. When gynophores grew to nearly 3 cm long, uniform gynophores that were about 5 to 10 cm above the ground were selected and treated separately as: (1) aerially grown (AG); (2) aerially grown plus exogenous mechanical stimulation (AGS), that is, using tweezers to clamp the tip of gynophore 10 times each day; (3) aerially grown plus dark (AGD), that is, the aerially grown gynophore was wrapped with aluminum foil with holes; (4) aerially grown plus mechanical stimulation combined with dark (AGSD); and (5) gynophore that was naturally grown and penetrated into soil (PS). The aerially grown (AG) peanut gynophore was set as the control. There were about 15 gynophores for each treatment with three replicates.

2.3. Protein Extraction, Preparation, and 2-DE Analysis

The total protein for each sample was extracted according to a previous method.²⁵ Protein samples were independently prepared from three different batches of plants, and the concentrations were determined using a Quant-kit according to manufacturer's instruction (GE Healthcare, USA). For protein profiling, the extracted proteins were prepared, separated, and visualized as previously described.²⁷ Three biological replicates for each sample were subjected to 2-DE gel. Gel image acquisition and analysis were conducted according to the previous method.²⁵ For quantitative analysis, the average vol % values were calculated from three technical replicates to represent the final vol % values of each sample. Comparison and statistical analysis were performed using the calculated average values of each biological replicate among the different samples. Spots with greater than two-fold change (compared with the control) and a *p* value less than 0.05 were considered as differentially expressed.

2.4. Protein Identification

According to a previous method,²⁴ the differentially expressed protein spots were excised from the 2-DE gels and digested with trypsin. The MS and MS/MS spectra were acquired on a 4800 Proteomics Analyzer (MALDI TOF/TOF mass spectrometer, AB Sciex Inc., USA). A Mass standard kit (Applied Biosystems) and a standard BSA digest (Sigma-Aldrich) were

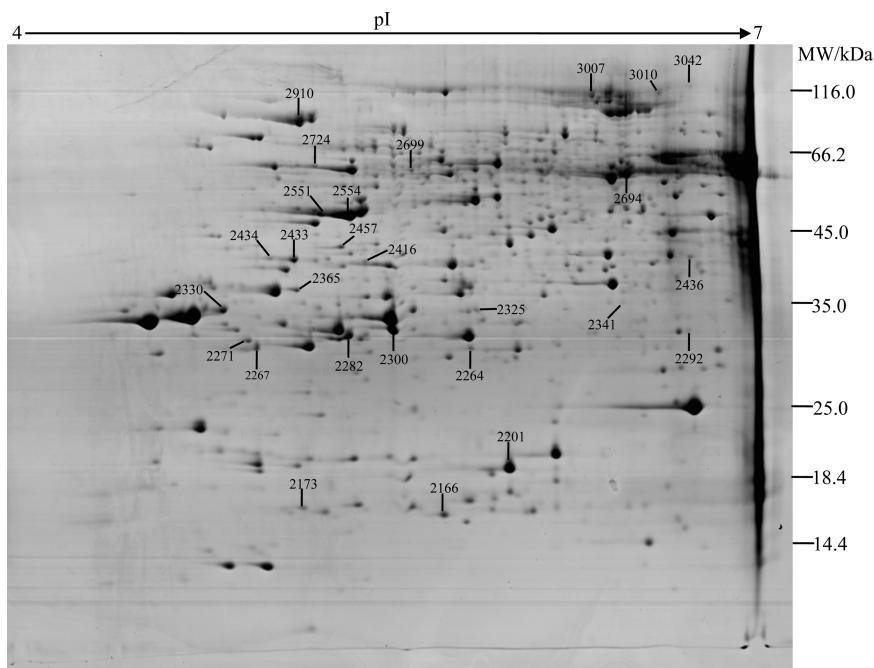


Figure 1. Representative 2-DE profile of proteins in peanut gynophore under various treatments. Total proteins in gynophore were separated by 2-DE stained with CBB R250. Twenty seven stress-responsive protein spots were marked on the gel. They represented the differentially expressed proteins in gynophore under aerially grown (AG) when compared with the proteins in gynophore under aerially grown plus exogenous mechanical stimulation (AGS), natural growing and penetrated into soil (PS), aerially grown plus dark (AGD), and aerially grown plus mechanical stimulation combined with dark (AGSD), respectively. Molecular weight (MW) and pI of proteins are indicated on the right and top of gel, respectively. For detailed information, please refer to Supporting Information Figures S1 and S2.

used for MS and MS/MS calibrations and fine-tuning the resolution and sensitivity of the system. The mass error was less than 30 ppm at both MS and MS/MS mode, and the resolution was more than 25 000. Proteins were identified by searching the MS/MS spectra against NCBI nr protein databases (<http://www.ncbi.nlm.nih.gov/>) (10 348 164 sequences entries in NCBI in Jan 20, 2010) using Mascot software (Matrix Sciences, U.K.). The taxonomic category Viridiplantae (Green Plants, including 730,150 sequences), and the search criteria were set according to Wang et al (2010).²⁵ The mass accuracy of 0.3 Da, one missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine as a variable modification. Proteins had to meet the following criteria: (1) the top hits on the database searching report, (2) a probability-based MOWSE score greater than 43 ($p < 0.05$), and (3) the matched peptides with nearly complete y-ion series and complementary b-ion series present.

2.5. Protein Functional Classification and Hierarchical Cluster Analysis

Protein motifs were obtained by blasting against the NCBI and UniProt database (<http://www.ebi.uniprot.org/>). Combined with knowledge from the literature, proteins were classified into different categories according to the functions or predicted functions.

2.6. Quantitative Real-Time PCR Analysis

To verify the results of 2-DE gel, we used the MS identified peptide sequences of randomly picked 10 differentially expressed proteins to search the homologues in other species in NCBI database, and the DNA sequences encoding the corresponding proteins were used for further investigation by quantitative real-time PCR (qRT-PCR) analysis.^{28,29} All homologous proteins and specific primers used for qRT-PCR

were listed in the Supporting Information Table S1. Total RNA was separately isolated from sample aliquots of the peanut gynophores under AG, AGS, AGD, AGSD, and PS according to the method described by Huang et al. (2012).³⁰ First-strand cDNA was synthesized from 5 μ g of total RNA with an oligo(dT) primer using a PrimeScript first-strand cDNA synthesis kit (D6110A; TaKaRa, Dalian). The PCR was performed in 96-well plates using a qRT-PCR system (Bio-Rad) with SYBR Green PCR Master Mix (Applied Biosystems). Each 20 μ L reaction mixture contained 1 μ L (\sim 50 ng) of first-strand cDNA, 0.5 μ L of 10 mmol/L gene-specific primers, 10.0 μ L of 2X SYBR Green Master Mix, and 8 μ L of ddH₂O. The PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. The amplification of *AhTUB* was used as an internal control. Each sample was replicated three times. Quantifying the relative changes in gene expression was performed using the 2^{-ΔΔCT} method, as described.³¹

2.7. Statistical Analysis

All results were presented as means \pm standard deviation (SD) of at least three replicates. Data were analyzed by one-way ANOVA using the statistical software SPSS 17.0 (SPSS, Chicago, IL). The treatment mean values were compared by the post hoc least significant difference (LSD) test. A p value less than 0.05 was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Protein Expression Profiles of the Peanut Gynophores under Dark and Mechanical Stimulation

The protein samples were extracted from the peanut gynophores under AG, AGS, AGD, AGSD and PS, respectively, and then were separated by 2-DE. After the gels for each

Table 1. Differentially Expressed Proteins in the Peanut Gynophores under Various Treatments

Spot No. ^(a)	Protein name ^(b)	Plant Species ^(c)	gi Number ^(d)	Thr. MW(Da) /pi ^(e)	Exp. MW(Da) /pi ^(f)	Changed patterns of protein V%±SD ^(g)
AGS responsive proteins						
2271	Chalcone isomerase 4B	<i>Glycine max</i>	51039632	30,719/4.89	15,641/4.64	
2282	Galactose-binding lectin precursor	<i>Arachis hypogaea</i>	951112	31,232/5.33	26,140/5.14	
2300	Hypothetical protein	<i>Zea mays</i>	195647188	31,905/5.53	30,867/5.26	
2433	Putative fructokinase 2	<i>Petunia integrifolia</i> subsp. <i>inflata</i> <i>Zea</i>	33329198	41,501/5.09	34,981/5.20	
AGD responsive proteins						
2325	Mannose/glucose-binding lectin	<i>Arachis hypogaea</i>	951116	34,470/5.87	28,185/5.95	
2330	Mannose/glucose-binding lectin precursor	<i>Arachis hypogaea</i>	951118	34,442/4.78	28,372/5.36	
3010	Os03g0136900, containing cd01586 Aconitase A catalytic domain	<i>Oryza sativa</i> (<i>japonica</i> cultivar-group)	115450595	81,539/6.64	106,235/6.45	
3007	Lipoxygenase	<i>Arachis diogoi</i>	217038660	80,857/6.32	22,348/8.05	
2416	Hypothetical protein, containing cd00200 WD40 domain	<i>Vitis vinifera</i>	147838769	41,128/5.41	47,626/6.44	
2724	ATP synthase subunit beta, mitochondrial	<i>Nicotiana plumbaginifolia</i>	114421	63,767/5.18	59,819/5.95	
2434	Putative fructokinase 2	<i>Petunia integrifolia</i> subsp. <i>inflat</i>	33329198	41,701/4.99	34,981/5.20	
2166	Putative glycine-rich RNA-binding protein	<i>Prunus avium</i>	34851124	15,971/5.74	17,374/7.82	
2554	Unnamed protein, containing cd00012 Actin domain	<i>Oryza sativa</i>	20322	47,097/5.32	41,926/5.22	
2551	Hypothetical protein OsJ_16821 containing cd00012 Actin domain	<i>Oryza sativa</i>	222629907	36,861/5.01	43,132/5.24	
2365	Oxygen-evolving enhancer protein 1	<i>Solanum lycopersicum</i>	12644171	37,153/5.10	34,926/5.91	
2267	Light-harvesting chlorophyll a/b-binding protein	<i>Prunus persica</i>	556367	30,024/4.93	28,314/5.30	
AGSD responsive proteins						
2325	Mannose/glucose-binding lectin	<i>Arachis hypogaea</i>	951116	34,470/5.87	28,185/5.95	
2173	Ara h 8 allergen isoform	<i>Arachis hypogaea</i>	145904610	16,392/5.13	16,402/5.07	
2436	Unknown.CHL00194, Ycf39	<i>Glycine max</i>	255637531	41,827/6.76	34,290/5.73	
2910	Luminal binding protein	<i>Gossypium hirsutum</i>	211906506	75,018/5.12	73,338/5.13	
2292	Triosephosphate isomerase	<i>Glycine max</i>	48773765	31,905/6.76	27,213/5.87	
2694	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	<i>Lopezia riesenbachia</i>	168312	52,998/6.50	52,778/6.23	
2699	ATP synthase beta subunit	<i>Eucryphia milligani</i>	7708286	63,012/5.61	52,850/5.03	
2416	Hypothetical protein. cd0020 containing WD40 domain	<i>Vitis vinifera</i>	147838769	41,128/5.41	47,626/6.44	

Table 1. continued

Spot No. ^(a)	Protein name ^(b)	Plant Species ^(c)	gi Number ^(d)	Thr. MW(Da) /pI ^(e)	Exp. MW(Da) /pI ^(f)	Changed patterns of protein V%±SD ^(g)
2554	Unnamed protein product containing cd00012 Actin	<i>Oryza sativa</i>	20322	47,097/5.32	41,926/5.22	
2551	Hypothetical protein OsJ_16821 containing cd00012 Actin domain	<i>Oryza sativa</i>	222629907	36,861/5.01	43,132/5.24	
	PS responsive proteins					
3042	Aconitase	<i>Arabidopsis thaliana</i>	599625	100,755/5.98	83,446/6.76	
3010	Os03g0136900 containing cd01586 Aconitase A catalytic domain	<i>Oryza sativa (japonica cultivar-group)</i>	145904610	16,587/5.38	16,402/5.07	
2264	Glutathione-s-transferase	<i>Ricinus communis</i>	255578691	24,964/6.24	29,859/5.84	
2341	Mannose/glucose-binding lectin	<i>Arachis hypogaea</i>	255637531	41,827/6.76	34,290/5.73	
2325	Mannose/glucose-binding lectin	<i>Arachis hypogaea</i>	951116	34,470/5.87	28,185/5.95	
2457	Drought inducible 22 kD protein.	<i>Saccharum officinarum</i>	15667623	43,106/5.30	15,923/5.78	
2201	Unknown containing pfam00407 protein Pathogenesis-related protein Bet v I family domain	<i>Astragalus membranaceus</i>	168312	52,998/6.50	52,778/6.23	
2554	Unnamed protein product containing cd00012 Actin domain	<i>Oryza sativa</i>	20322	47,097/5.32	41,926/5.22	
2551	Hypothetical protein OsJ_16821 containing cd00012 Actin domain	<i>Oryza sativa</i>	222629907	36,861/5.01	43,132/5.24	

^aAssigned spot number as indicated in Figure 1. ^bThe name and functional categories of the proteins identified by MS. ^cThe plant species that the peptides matched from. ^dDatabase accession numbers from NCBIInr. ^eTheoretical mass (kDa) and pI of identified proteins. ^fExperimental mass (kDa) and pI of identified proteins. ^gThe mean values of relative protein abundances. The left column represents average protein abundance in gynophores under aerial growth (AG), and the right column represents that in exogenous stimulation (AGS), aerial growth plus dark conditions (AGD), aerial growth plus stimulation combined with dark conditions (AGSD), and natural growth and penetration into the soil (PS). Error bars indicate \pm standard deviation SD.

treatment were scanned and analyzed by Image Master 5.0 software, five sets of protein expression profiles of different gynophore samples, including 782 ± 8 , 773 ± 8 , 726 ± 14 , 771 ± 3 , and 708 ± 20 protein spots, were generated (Figure 1, Supporting Information Figures S1 and S2). Among these protein spots, spots with more than two-fold changes ($p < 0.05$) in three replicates were defined as differentially expressed protein spots, and totally 48 differentially expressed protein spots were detected. Among them, 35 protein spots were further identified by MS/MS (Table 1). Therein, four were identified from five AGS-responsive protein spots, and 10 proteins were identified from 15 AGSD-responsive protein spots. Twelve proteins were identified among 17 differentially expressed protein spots under AGD, and 9 were identified among 11 differentially expressed protein spots under PS.

Meanwhile, among the 35 protein spots, spot 3010 was changed under AGD and PS, and spot 2416 was changed under AGD and AGSD. In addition, three spots (spot 2325, spot 2554, and spot 2551) were all changed under AGD, AGSD, and PS. Besides, in the initial stage of gel analysis and data acquisition, spot 2178 was found to contain more than one protein (Supporting Information Table S2). In this case, it was difficult to determine which protein changed in abundance of spot 2178. Therefore, only the rest of the 27 proteins were

defined as changed proteins of gynophores under certain treatment conditions, and they were further used for functional analysis (Figure 1). On the basis of the functional domain annotation and reference of the GO classification criteria, these proteins were classified into nine major functional groups (Figure 2 and Supporting Information Table S3), including photosynthesis, sugar and energy metabolism, metabolism, stress and defense, signal transduction, cell structure, transcription, protein folding and degradation, and function unknown. Among these, proteins involved in stress and defense, sugar and energy metabolism, metabolism, and photosynthesis were over-representative, which accounted for 30, 26, 11, and 7% of the differentially expressed proteins, respectively.

3.2. Common and Different Responsive Proteins under AGS and AGD of the Gynophores

Among the differentially expressed proteins, four proteins were mechanical stimulation-responsive in the peanut gynophores under AGS (Table 1 and Supporting Information Table S2). While in the peanut gynophores under AGD, there were 12 proteins changed (Table 1 and Supporting Information Table S2). The difference in the above data indicated that dark, or other factors related to light and illumination, may be much

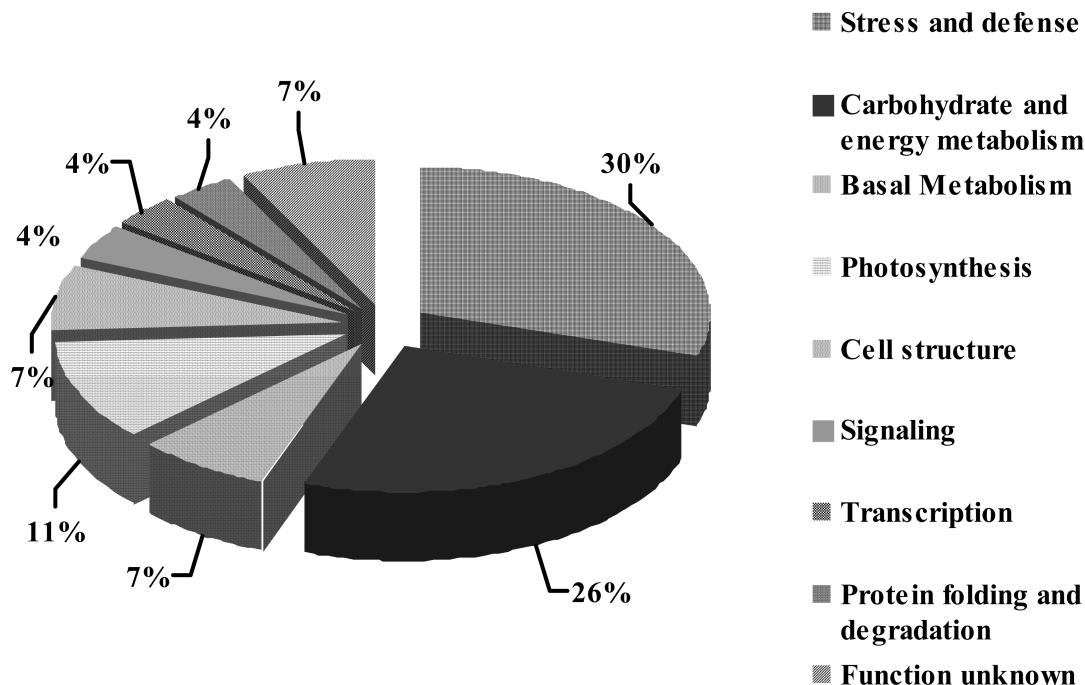


Figure 2. Functional classification of differentially expressed proteins identified in different samples of the peanut gynophores. Please refer to Supporting Information Table S3 for details of the nine functional groups.

more important and have more influence than mechanical stimulation during gynophore growth and development.

Among the mechanical stimulation-responsive proteins, the abundances of chalcone isomerase 4B (spot 2271), galactose-binding lectin precursors (spot 2282), and an unknown protein (spot 2300) were significantly increased. Of the dark-responsive proteins, four proteins were increased, including mannose/fructose-binding lectin (spot 2325), mannose/fructose combination of lectin precursor (spot 2330), *cis*-aconitase A (spot 3010), and lipoxygenase enzyme (spot 3007). It is known that galactose-binding lectin precursor, as a member of plant lectin family, plays an important role in the promotion of cell differentiation, regulation of cell growth, and resistance to apoptosis. It also acts in the recognition of external stimuli and resistance to outside stresses.³² Additionally, mannose/fructose-binding lectin and its precursors have abilities for stress resistance and adaptation and may participate in plant stress defense.³³ Aconitase in the citric acid cycle could play a role in nutrient metabolism, energy supply, and disease resistance.³⁴ Lipoxygenases catalyze unsaturated fatty acids into saturated fatty acids under the action of oxygen, and they are also involved in external stress resistance.³⁵ The abundance of chalcone isomerase 4B increased only in the gynophores under AGS compared with that under AGD. Considering that its effect of rate limitation in the flavonoid biosynthetic pathway, the results derived here implied that the mechanical stimulation may promote the biosynthetic of flavonoids in the gynophores under AGS. As a class of secondary plant metabolic compounds, flavonoids are known to be endogenous regulators of auxin transport and could block the process of auxin transport by inhibition of the auxin efflux carrier complexes and influence the formation of the auxin gradient.³⁶ As the auxin gradient contributes to gravity signal transduction in the statocyte, the change of the chalcone isomerase 4B may impact gynophores formation and tropism under AGS through hormone metabolism and responsive pathway.

In addition, the abundances of two isoforms of fructokinase (spot 2433 and spot 2434) were reduced in the gynophores under AGS and AGD. Fructokinase, which is related to substrate and energy metabolism, is a key enzyme in the fructose metabolism pathway and also regulates the metabolism, growth, and development as the sugar sensing and signaling element. In addition, previous studies have shown that sucrose signaling pathway could regulate metabolism in cells and photoassimilate translocation and partitioning and plant development. It also regulates gene expression in plants at the transcription and translation levels, while a higher level of sucrose inhibits gene expression.^{37,38} Because sucrose is easily cleaved to glucose and fructose, the mechanical stimulation and dark-reduced fructokinase imply that alone with the degradation of sucrose slowing down, the sucrose accumulation increased in the gynophores under AGS and AGD when compared with that under AG, and the sucrose and fructose metabolism were proposed to be affected by external mechanical stimulation and light, but the relationship between sugar metabolism and gynophore growth regulation needs to be investigated in the future.

The eight spots representing seven changed proteins were solely inhibited and reduced by dark treatment, such as WD40 domain proteins (spot 2416) for signaling transduction, ATP synthase subunit (spot 2724) for energy conversion, glycine-rich RNA-binding protein (spot 2166) for maintenance of morphology and internal structure of eukaryotic cells, actins (spot 2544 and spot 2551), oxygen-evolving enhancer protein 1 (spot 2365), and light-harvesting chlorophyll *a/b*-binding protein (spot 2267) for capturing and stabilizing light energy. Among them, the expression of light-harvesting chlorophyll *a/b*-binding protein (spot 2267) had the biggest decline, and the ranges of reduced proteins were from 58 to 90%. These inhibited proteins under dark verified that photosynthesis was mainly affected by light. The reduction of the ATP synthase subunit and chlorophyll *a/b*-binding protein also led to the

changes of energy capture and internal oxygen-evolving complex subunits stabilization.³⁹ Chen et al. reported that the photosynthesis pathway was specifically expressed in young aerial peanut pods compared with young subterranean pods, which indicates that the activity of corresponding genes of photosynthesis in aerial pods is higher than those in subterranean pods.⁵ In comparison with the natural growth conditions of the peanut gynophores under AG, the applied dark and mechanical stimulation to the aerially grown gynophores may act as environmental stress factors, which could mimic the effect of soil resistance and obstruction when the gynophores penetrated into the soil. The change of these enzymes showed that dark and mechanical stimulation were able to influence the energy supply and anti-stress-related processes. This leads to the content alteration of the corresponding products in the gynophores that would be beneficial to the gynophore of penetrating into the soil and then smoothly developing into the pod.

3.3. Changed Proteins in the Gynophores under PS

Compared with the gynophores under AG, nine changed proteins were identified in the gynophores under PS, including five induced proteins and four reduced proteins (Table 1 and Supporting Information Table S2). The increased proteins were aconitase (spot 3042 and spot 3010), glutathione-S-transferase (spot 2264), and mannose/glucose-binding lectins (spot 2341 and spot 2325). Among them, glutathione-S-transferase, which participates in reduction and conversion of harmful H₂O₂ to harmless H₂O and in protection against oxidative stress,^{40–42} was induced to transcription and translation only in the gynophores under PS. The other four proteins were all identified in the gynophores under AGS, AGD, and AGSD. The increase in such stress and defense-related proteins could reduce the damage caused by oxidative stress and external stimuli. In addition, the reduced proteins included drought-inducible 22 kD protein (spot 2457), pathogenesis-related protein (spot 2201), and actins (spot 2551 and spot 2554). Because such proteins were reported to participate in resistance to various external stresses,^{43–45} the mechanisms of decreased expression in the gynophores under PS need to be further investigated.

3.4. Common and Different Responsive Proteins under AGSD and PS of the Gynophores

Different from nine changed proteins in the gynophores under PS, 11 differentially expressed proteins were found in the gynophores under AGSD (Table 1 and Supporting Information Table S2). Among them, six proteins were increased. As the common increased protein in the gynophores under AGSD and PS, mannose/fructose-binding lectin (spot 2325) was speculated to have similar effects to participate in plant stress resistance and alleviate the injury under mechanical stimulation combined with dark. Other increased proteins in the gynophores under AGSD include Ara h 8 allergen isoforms (spot 2178 and spot 2173), unknown protein (spot 2436), luminal binding protein (spot 2910), and triosephosphate isomerase (spot 2292). Ara h 8 allergen isoforms (spot 2178, and spot 2173) and luminal binding protein (spot 2910) were reported to be induced when gynophores were grown gravitropically. They were proposed to protect gynophores from damage of reactive oxygen species and participate in resistance to stimulation of soil microorganisms or allergens.^{40,46} Triosephosphate isomerase (spot 2292), as a catalyst for glycerol aldehyde 3-phosphate and dihydroxyacetone

phosphate internal conversion, could participate in glycolytic pathway and the calvin cycle. The increase in this enzyme under AGSD could meet the energy requirements of the gravitropic growth of gynophore and sustain the activities of the entire body when the gynophore was grown under disadvantageous photosynthesis conditions.⁴⁷

In addition to common decreased proteins of actins (spot 2551 and spot 2554) in gynophores under AGSD and PS, there were three distinctive proteins decreased under AGSD: ATP synthase β subunit (spot 2699) and hypothetical protein with the WD40 domain (spot 2416), which are reported to participate in energy conversion and signal transduction processes, respectively,³⁹ and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (spot 2694), which could effect as indispensable dioxygenase during photorespiration and its down-regulation, probably due to the lack of light and the inhibition of the photoreaction under dark condition.³⁹ It is known that actin is an essential membrane component participating in cytoskeleton rearrangements. It could play an important role in cytoplasm streaming, cell-shape determination, cell division, organelle movement, and extension growth.^{48,49} The change of actin-related proteins was speculated to ultimately influence morphogenesis and development of gynophores under AGSD and PS by participating in cytoskeleton rearrangement processes.^{26,49} The common changes of mannose/fructose-binding lectin and actin-related proteins implied that they would be the key factors during gynophore development and lignification.

3.5. Quantitative Real Time PCR Analysis of Partial Functional Related Genes during Peanut Gynophore Development

To further validate the variation trends of the identified proteins and the corresponding genes at the transcription level during peanut gynophore development, the total RNA of each sample was extracted and analyzed by qRT-PCR. Ten peptides were used to search against the green plant protein database in NCBI and matched with known plant proteins. Because there are relatively few known peanut proteins and genomic sequences available,^{3,28,29} only three proteins matched peanut proteins by database searching (Supporting Information Table S1). The gene sequences corresponding to 10 randomly selected proteins were further divided into four major functional groups according to their biological functions, including stress- and defense-responsive, sugar and energy metabolism, metabolism, and photosynthesis.

The results of qRT-PCR revealed that (Figure 3) in the group of stress- and defense-responsive proteins, the coding genes of pathogenesis-related protein (spot 2201) *RP10* and Ara h 8 allergen isoform (spot 2173) *Ah8* displayed similar expression patterns between mRNA and protein expression. Compared with the expression levels in gynophores under AG, *RP10* and *Ah8* were moderately decreased (36–50%) in the gynophores under AGS and AGD, while almost no obvious change could be detected in abundance of the corresponding proteins, but in the gynophores under AGSD, these two genes were induced to express, while there was only a slight increase in corresponding proteins. There were also changes in the consistency of genes and relevant proteins in the gynophores under PS.

Expression of galactose-binding lectin precursors (*GBL*) was about 1.7-fold induced in the gynophores under AGS compared with that in the gynophores under AG, which was in conformity

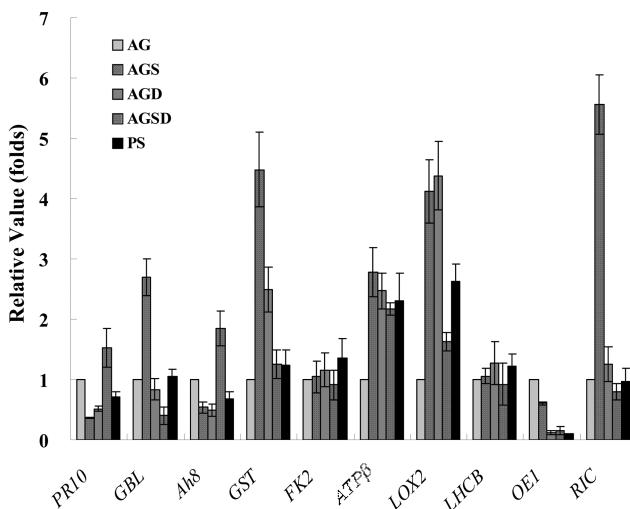


Figure 3. qRT-PCR analysis of gene expression patterns in the gynophores under different growth conditions. The *AhTUB* gene was used as the internal control. Bars represent the mean \pm standard deviation from three independent experiments. Please refer to Figure 1 legend for abbreviations.

with the expression pattern of protein (spot 2282) under similarity conditions. For the glutathione-S-transferase (GST) gene, the expression level was induced to 3.5-and 1.5-folds, respectively, in the gynophores under AGS or AGD compared to that of the gynophores under AG, and only slightly induced to express in the gynophores under AGSD or PS. But the abundance of corresponding protein (spot 2264) stayed at a relatively high level in the gynophores under AGSD or PS.

Expression of lipoxygenase enzyme (*LOX2*) was increased in the gynophores under AGS and AGD, with an increased rate of 310 and 300%, respectively, and expression of *LOX2* increased 160% in the gynophores under PS compared with that under AG. The variation of the gene was in accordance with the protein expression pattern in the gynophores under AGD.

In the sugar and energy metabolism-related group, expression of the gene *fructokinase 2* (*FK2*) showed almost no change in the gynophores under various treatments, while expression of its coding proteins (spot 2433 and spot 2434) decreased about half in the gynophores under AGS and AGD. For ATP synthase β subunit (*ATP β*), the mRNA level was identified as slightly up-regulated under different treatments, with an increased rate of more than 120%, but expressions of the corresponding protein (spot 2724) of the *ATP β* in the corresponding treatments were decreased.

Photosynthesis-related proteins, such as light-harvesting chlorophyll *a/b* binding protein (spot 2267) and RuBisCO large subunit (spot 2694), were decreased compared with those in the gynophores under AG, but their gene expression levels did not show similar trends. Gene expression of the selected ribulose-1,5-bisphosphate carboxylase/oxygenase large (*Ric*) was up-regulated in the gynophores under AGS, while expression of light-harvesting chlorophyll *a/b* binding gene (*LHCb*) showed almost no change compared with that under AG. These probably were due to the inhibition of photosynthesis that affects gene transcription and translation and finally lead to the reduction or degradation of the proteins. However, the transcription level of oxygen-evolving enhancer protein 1 (*OE1*) decreased 80–90% in the gynophores under AGD and

PS, which was in accordance with the protein (spot 2365) expression pattern in the gynophores under AGD.

4. CONCLUSIONS

This work is a comprehensive report of proteomic analysis on the impact of dark and mechanical stimulation on peanut gynophores. The gravitropic growth of the peanut gynophore was one of the most critical characters for peanut fructification. The results demonstrated the molecular and metabolic regulatory mechanisms of peanut from vegetative growth to reproductive growth and also gave insight into internal mechanisms of physiological phenomena of peanut gynophore. The 27 unique proteins were ultimately identified when peanut gynophores were grown under dark and mechanical stimulation. On the basis of the homology searching and analysis, three functional groups of sugar and energy metabolism, stress resistance and defense, and basal metabolism were the large proportion in the changed ones. These proteins, such as the common changed proteins of actin and mannose/fructose-binding lectin, triosephosphate isomerase, ATP synthase, pathogen-related, and allergen isoforms, could participate in the processes of morphogenesis and energy production. They were proposed to prevent toxic substances from injuring the gynophores by participating in cell-shape determination and oxidizing substances, and further provide the metabolic substrates for the development and gravitropic growth. Verification of changed genes by qRT-PCR further confirmed the trend of some genes. These results provide further evidence of previous views that the changes of regulatory mechanisms and physiological processes occurred during gynophore penetrated into the soil, and the gene expression patterns do not always correlate with the levels of protein. More detailed analysis of proteins/genes is undergoing to further identify their possible relationships and functional roles in the process of gynophore growth and development under dark and mechanical stimulation.

■ ASSOCIATED CONTENT

S Supporting Information

Representative 2-DE profiles of differentially expressed proteins in the peanut gynophores under various treatments. 2-DE profiles of the peanut gynophores under various treatments. Genes and their specific primers used in qRT-PCR. Differentially expressed proteins and the sequences of peptides identified using MALDI TOF-TOF MS. List of proteins identified in gynophores and their relative expression abundances in response to various conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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