Proteomic analysis of differently expressed proteins in sex differentiation phases of flower buds in monoecious *Pistacia chinensis* Bunge

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ABSTRACT

The *Pistacia chinensis* Bunge is traditionally dioecious, and the female trees are more required to grow in practice for oil seed production. The discovery of monoecious *P. chinensis* Bunge in North China provided good raw materials to study the sex differentiation process. The objective of this study was to identify the differently expressed proteins in flower buds in two key sex differentiation phases in monoecious *P. chinensis* Bunge. Morphological observation and paraffin section were used to determine the key phenophases, and label-free quantitative technique was used for proteomic analysis. The results showed that the proteins related to oxidative stress resistance up-regulated while proteins involved in photosynthesis down-regulated during the female primordium differentiation in bisexual flower buds of the monoecious *P. chinensis* Bunge in early March, while proteins related to oxidative stress resistance, ribosome activity, and photosynthetic function up-regulated during the male primordium differentiation in bisexual flower buds of the monoecious *P. chinensis* Bunge in late May. The most up-regulated proteins all involved in the photosynthesis pathway in both kind of flower buds in late May compared to those in early March, and the down-regulated proteins all involved in the ribosome pathway. The identified differentially expressed proteins such as the Cu/Zn superoxide dismutases may be possible molecular markers for sex determination in monoecious *P. chinensis* Bunge.

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Introduction

Most flowering plants are monoecious, with flowers containing both male and female parts (Xiong et al. 2013). However, some economic forest tree species are dioecious, with only male or female flowers in individuals. The low yield of these species can be largely attributed to the unbalanced sex ratio of their flowers. Therefore, studies on sex determination mechanisms and sex ratio regulations in dioecious plants have always been of great concern, considering its important biological meanings and economic values.

It is believed that the sex determination of dioecious plants could be attributed to both genetic factors (e.g. sexual chromosomes, sexual determination genes) and environmental factors (e.g. light, temperature, CO₂) (Milewicz and Sawicki 2012). Most dioecious plants such as maize (*Zea mays*), cucumber (*Cucumis sativus*) and watermelon (*Citrullus lanatus*) undergoes the development process of asexuality, bisexuality, and

unisexuality. The sex determination takes place during the bisexual process, resulting from the development or degradation of the primordium of sex organs in flowers (Milewicz and Sawicki 2012). However, the key sexual determination genes have not been identified in any dioecious plants by now (Xiong et al. 2013).

The Chinese *Pistacia* tree (*Pistacia chinensis* Bunge) belongs to *Pistacia* genus (Anacardiaceae). It is widely planted in China and is the most preferred biodiesel tree (Wang 2011). The *Pistacia* tree is generally believed as strictly dioecious, therefore, the female plants are more required to grow in practice for oil seed production. Even though a few studies have been conducted on *Pistacia* grafting, hybridization, and molecular markers (Gercheva et al. 2008; Isfendiyaroglu and Oze-ker 2009; Kafkas et al. 2003; Marra et al. 2007), the sex determination mechanisms of *Pistacia* is still unclear. In recent years, the monoecious *P. chinensis* has been discovered in North China (Wang 2015), providing

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good raw materials to study the sex differentiation and regulation of *P. chinensis*.

Proteomics is a powerful tool to analyze the protein profile quantitatively, which can provide fundamental understanding of the biological changes (Tsou et al. 2010). The label-free proteomic approach quantifies protein expression using multiple LC-MS/MS analyses directly without any labeling techniques. The protein quantification is based on either the peptide peak intensity or spectral count in chromatography, and changes in protein abundance are calculated via a direct comparison between different analyses (Zhu et al. 2010). Compared to the isotope or fluorescent labeling techniques, the label-free quantitative proteomic techniques can provide fast and low-cost measurements of protein expression in complex biological samples (Zhu et al. 2010). Therefore, it has been widely used in recent years in plant biology to explore the protein expression dynamics in conditions such as plant development or environmental stress (Szymanski et al. 2017; Wang et al. 2017; Zhang et al. 2017).

The relative stable sex phenotypes of female and bisexual flowers have been observed on monoecious *P. chinensis* by our research group in Tangxian (Hebei province, China) in the year of 2013, 2014 and 2015, while there was presence of fruits on some pure male inflorescences (Bai et al. 2016). This phenomenon took place within only 1 year, and has been confirmed using markers. Furthermore, the statistics showed that the overall trend in recent years was that the male branches turned into bisexual gender, with no female turning into male (Bai et al. 2016). Accordingly, these plants could be utilized to explore the underlying mechanisms of sex differentiation in *P. chinensis*.

The sex differentiation process usually starts from the asexual status, goes through the bisexual process and finally stays at this stage (monoecious plants) or enters into unisexual stage (dioecious plant) with the development or degradation of the primordium of sex organs. Based on the continuous observation by our research group starting from 2013, obvious and stable sex differentiation in monoecious *P. chinensis* plants takes place every year (Bai et al. 2016). The stamen primordium (male primordium) of the monoecious *P. chinensis* Bunge developed in late May after blooming, and differentiated to anthers to form male or bisexual flowers, while the stamen primordium in female flower buds degraded to form female flowers. Then the pistil primordium (female primordium) of the monoecious

P. chinensis Bunge in both bisexual flower buds and female flower buds developed in early March next year before blooming, and differentiated to the stigma and the ovary. Because the differentiation process of the stamen primordium in male flower buds in male tree and in bisexual flower buds in monoecious tree are similar (Bai et al. 2016), we may get some clues of the sex determination process in *P. chinensis* Bunge by comparing the bisexual flowers and female flowers in monoecious trees.

Therefore, the aim of this study was to identify and quantitate the differently expressed proteins between female *P. chinensis* and bisexual *P. chinensis* in sex differentiation phases of flower bud using label-free quantitative technique. Together with the undergoing genomic and transcriptomic studies, these data could provide some possible clues to depict the sex determination process in *P. chinensis* Bunge.

Materials and methods

Plant materials

The monoecious *P. chinensis* Bunge sampled in this study were located in Tangxian, Hebei province, China. The branches with stable sex phenotypes (female or bisexual flowers) in successive years were selected based on the previous record from our research group. Flower buds with different sex were identified through external morphological observation, and further confirmed through internal anatomical observation via optical microscope. Based on the continuous monitoring and observation, both female flower buds and bisexual flower buds (Fig. 1A, B) were sampled during two key phenophases for sex differentiation: (I) from May 27, 2016 to May 31, 2016 (labelled as 5F and 5B, respectively), and (II) from March 3, 2017 to March 7, 2017 (labelled as 3F and 3B, respectively). The male primordium in the bisexual flower buds developed during phenophase I, while the female primordium in both the female flowers and the bisexual flowers developed during phenophase II. The male primordium that developed in the male flower buds went through the same differentiation process as that in bisexual flower buds (Bai et al. 2016), thus no male flower buds were sampled. Approximately 2g of flower buds were randomly collected from 3 different trees for each sex phenotype in each phenophase, and the flower buds were kept in liquid nitrogen for further analysis.

Figure 1. orphological changes of female and bisexual flower buds in different phenophases in paraffin sections. (A) Morphology of the bisexual flower bud. (B) Morphology of the female flower bud. (C) Paraffin sections of the bisexual flower bud in late May, 2016, when the male primordium in the bisexual flower bud started to develop. (D) Paraffin sections of the female flower bud before differentiation of the female primordia in late May, 2016. (E) Paraffin sections of the bisexual flower bud in early March, 2017, when the female primordium in the bisexual flower bud started to develop. (F) Paraffin sections of the female flower bud in early March, 2017, when the female primordium started to develop. (G) Timelines of different phenophases and time-points for sample collection in this study. Abbreviations: br: bract; fl: floret; fp: female primordia; in: inflorescence axis; mp: male primordia; pr: primordia; te: tepal. Red arrows are representative male/female primordia developed in that phenophase. The scale represents 200 μ m.

Paraffin section of flower buds

Several female and bisexual flower buds in different phenophases were selected and soaked in FAA solution (6 mL formalin, 4 mL acetic acid, 90 mL 70%

ethanol and 5 mL glycerin) for fixation about 48 hours. Then the fixed flower buds were dehydrated through a series of graded ethanol baths to displace the water, infiltrated with paraffin, and embedded into paraffin blocks. The flower buds in paraffin blocks were cut

by microtome into 8–12 μm sections, and then made into slides. The paraffin around the plant materials was washed out using a series of xylene and ethanol baths, and the flower buds were stained with 1% safranin and PIHCH successively. The flower buds sections were observed with OLYMPUS-BH microscope and pictures were captured using camera.

Total protein extraction

Approximately 0.5 to 1 g flower buds (3F, 3B, 5F, or 5B) were separately ground and homogenized with a -20°C pre-cooled mortar and pestle in the urea extraction buffer, which contained 150 mM Tris-HCl (pH 7.6), 8 M urea, 0.5% SDS, 1.2% Triton X-100, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 1% glycerol 2-phosphate, 1 mM PMSF, 5 mM DTT, 0.5% phosphatase inhibitor mixture 2 (Sigma), an EDTA-free protease-inhibitor mixture (Complete™), 5 mM ascorbic acid, and 2% polyvinylpyrrolidone. The ratio of flower buds to urea extraction buffer was 1:3 (w/v). Plant cell debris were removed via centrifugation at 10,000 g for 1 h at 4°C and then 110,000 g for 2 h at 13°C . The protein supernatant was then mixed with 3 volumes of a cold acetone: methanol (12:1 v/v) organic mix for urea-methanol protein precipitation. The protein pellet was rinsed with 15 volumes (v/w) of a cold acetone: methanol: H_2O mix (12:1:1.4 v/v) to remove residual pigment and urea precipitates. The protein pellet was air-dried and re-dissolved in a 1/3 volume of resuspension buffer, which contains 50 mM Tris-HCl (pH 6.8), 8 M urea, 5 mM DTT, 1% SDS, and 10 mM EDTA. This was followed by another round of protein precipitation and re-suspension. The cycling of protein precipitation and resuspension was repeated twice. The resulting total protein content was measured via protein DC assay (Bio-Rad) and calculated according to a bovine serum albumin (BSA) protein standard curve.

Protein digestion

Digestion of protein were performed according to the procedures described by Zhu et al. (2013). Briefly, total protein were excised from the preparative tube and destained with 50 mM NH_4HCO_3 to the final concentration 0.5 mg/mL. With the addition of 100 mmol/L DTT to its final concentration of 10 mmol/L, the protein fractions were mixed at 56°C for 60 min, then diluted 10 $\times$ with 250 mmol/L Iodoacetamide (IAM) and kept in dark for 60 min. Finally, the samples were digested

with trypsin (substrate to enzyme mass to mass ratio at 50:1) at 37°C for 12 h. Digested supernatant fractions were stored at -80°C without further treatment until MS analysis.

Liquid chromatography (LC) – Tandem mass spectrometry (MS/MS) analysis

Each sample loaded for LC-MS/MS was a mixture of 3 bio-replicates (plant materials from 3 different trees) of each kind of flower bud (3F, 3B, 5F, or 5B). Digested peptide mixtures from each sample were pressure-loaded onto a fused silica capillary column packed with 3- μm dionex C18 material (RP; Phenomenex). The RP sections with 100 $\text{\AA}$ were 15 cm long, respectively, and the column was washed with buffer A (water, 0.1% formic acid) and buffer B (acetonitrile, 0.1% formic acid). After desalting, a 5-mm, 300- μm C18 capture tip was placed in line with an Agilent 1100 quaternary high performance liquid chromatography (HPLC) and analyzed using a 12-step separation. The first step consisted of a 5-min gradient from 0% to 2% buffer B, followed by a 45-min gradient to 40% buffer B. Next, the buffer B flowed by 3-min gradient from 40% to 80% and 10-min 80% buffer B. After 2-min buffer B gradient from 80% to 2%, approximately 100 μg of tryptic peptide mixture was loaded on to the columns and was separated at a flow rate of 0.5 $\mu\text{L}/\text{min}$ by using a linear gradient. As peptides were eluted from the micro-capillary column, they were electrosprayed directly into a micro TOF-Q II mass spectrometer (BRUKER Scientific) with the application of a distal 180°C source temperature. The mass spectrometer was operated in the MS/MS (auto) mode. Survey MS scans were acquired in the TOF-Q II with the resolution set to a value of 20,000. Each survey scan (50–2,500) was followed by five data-dependent tandem mass (MS/MS) scans at 2HZ normalized scan speed.

Sequence database searching and data analysis

Tandem mass spectra were analyzed using Proteome Discover™ software (Thermo Scientific) against the NR_database_Sapindales_Genebank databases. The search results were then filtered using a cutoff of 1% for peptide false identification rate. Peptides with Z score <4 or Delta-Mass >5 ppm were rejected. Furthermore, the minimum number of peptides to identify a protein was set to 1. The differentially expressed proteins (DEPs) were considered to be up-regulated if the fold

change (FC) was greater than 2 and down-regulated if the FC was less than 0.5.

Bioinformatics analysis was carried out to categorize proteins based on biological processes, cellular component and molecular function using annotations in Protein Analysis Through Evolutionary Relationships (PANTHER) database v6.1 (www.pantherdb.org) (Thomas et al. 2003), which is in compliance with gene ontology (GO) standards. Signaling pathway analysis were performed with the tools on the Kyoto Encyclopedia of Genes and Genome (KEGG) database (<http://www.genome.jp/kegg/pathway.html>), respectively. Identified proteins in different groups were analyzed for molecular function, biological process or pathway term in PANTHER using the binomial test (Cho and Campbell 2000).

Results

Morphological observation showed various differentiation processes of female and bisexual flower buds of monoecious *P. chinensis* trees

In late April and early May, the flower buds on the monoecious *P. chinensis* trees started to grow and showed distinguished appearance (bigger but still green) from leaf buds. The morphological differentiation of flower buds began from the growing point which located at the middle bottom of the buds, and the bract, deputy panicle, secondary bract, secondary deputy panicle and floret primordium formed sequentially. During this period, there was no morphological differences between male primordium and female primordium. In late May (phenophase I), the sex differentiation emerged, with bulges arising around the floret primordium base. The bulges in the middle was treated as the female primordium (pistil primordium), while the bulges in the peripheral was treated as the male primordium (stamen primordium) (Bai et al. 2016). As the florets continued to develop, the pistil primordia sunk down (Fig. 1C, red arrow) and the stamen primordia began to differentiate in bisexual flower buds, whereas the stamen primordia in female buds degenerated into tepals (Fig. 1D). After phenophase I, internal structures of both flower buds were relative stable, thus entering into the dormancy stage. In early March next year (phenophase II), their dormancy was disrupted, with pistil primordia in both bisexual and female flower buds bulging (Fig. 1E, F, red arrows). As a result, the pistil primordia came into the differentiation stage. In

early April, both flower buds entered into the blooming period. Therefore, female and bisexual flower buds of monoecious *P. chinensis* trees demonstrated various differentiation processes during phenophase I and phenophase II. More detailed description of the flower bud differentiation processes were included in another manuscript (Bai et al. submitted).

Proteomic analysis of DEPs in both flower buds in different phenophases

Through the label-free quantitative technique, a total of 3750 proteins was successfully identified in bisexual flower buds and female flower buds in two phenophases (Supplementary Table 1). Of these, 3267 (87.1%) proteins were identified in the bisexual flower buds in two phenophases, while 3332 (88.9%) proteins were identified in the female flower buds in two phenophases (Fig. 2A). When compared between 3B and 3F, 5B and 5F, 5B and 3B, and 5F and 3F, the number of DEPs classified are 516, 232, 806 and 637, respectively, with 146, 128, 417, and 303 up-regulated proteins, and 370, 104, 389, and 334 down-regulated proteins, respectively (Fig. 2B). The GO analysis were performed to gain a better understanding of the DEPs involved in the biological process, cellular components, and molecular function in flower buds with different sex and in different phenophases. Compared 3B to 3F, majority of DEPs were down-regulated and were annotated to cellular and metabolic process, molecular binding and cellular component (e.g. cell, cell part, membrane, organelle) (Fig. 3A). Compared 5B to 5F, majority of DEPs were annotated to cellular and metabolic process, cell and organelle components, and molecular binding and catalytic activity (Fig. 3B). Compared to 5B and 3B, and 5F and 3F, majority of DEPs were up-regulated and annotated to cellular and metabolic process, cellular component, and molecular binding and catalytic activity (Fig. 3C, D). In sum, the DEPs annotated to cellular and metabolic process, and molecular binding and catalytic activity accounted for approximately 60% of all the DEPs. Moreover, a large proportion of these DEPs take functions in more than one process. Among these DEPs, 9 DEPs were involved in cellular or metabolic process only, 9 DEPs involved in molecular binding and catalytic activity only, while 43 DEPs involved in both processes (Table 1). Furthermore, copper/zinc-superoxide dismutase was the most up-regulated DEPs when compared 3B to 3F, and 5F to 3F, whereas

Table 1. Differentially expressed proteins involved in cellular/metabolic process or molecular binding/catalytic activity when compared bisexual and female flower buds in different phenophases.

Accession ¹	Peptide count	Score	Fold change ²				Description ³
			3B/3F	5B/5F	5B/3B	5F/3F	
Cellular/metabolic process only							
AHF81486.1	13	428	0.3↓	1.3	3.7↑	0.7	small GTP Rab-GDI [Mangifera indica]
JAV45330.1	6	225	1.4	1.4	0.5↓	0.5↓	Ribosomal L27e protein family [Citrus limon]
ABY27084.1	3	55	0.2↓	0.9	1.6	0.4↓	limonoid UDP-glucosyltransferase [Citrus maxima]
AJW74933.1	2	47	0.4↓	0.8	0.8	0.4↓	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Mischocarpus australis]
ADA70346.1	2	44	3.5↓	0.9	0.7	2.6↑	glutaredoxin [Litchi chinensis]
ABV59096.2	5	40	1.0	2.6↑	0.3↓	0.1↓	ribosomal protein S7 (chloroplast) [Mangifera indica]
JAV44889.1	2	37	1.2	1.5	0.2↓	0.2↓	Ribosomal protein S14p/S29e family protein [Citrus limon]
AHF71481.1	6	35.5	4.3↓	–	0.1↓	–	50S ribosomal protein L2 (chloroplast) [Acer buergerianum subsp. ningpoense]
ACH87659.3	4	8.7	0.4↑	–	–	0.02↓	somatic embryogenesis receptor kinase [Dimocarpus longan]
Both cellular/metabolic process and molecular binding/catalytic activity							
AGE44289.1	24	1142	0.6	0.6	0.3↓	0.3↓	beta-tubulin [Dimocarpus longan]
ACN59435.1	14	834	0.8	2.3↑	3.3↑	1.1	glyceraldehyde-3-phosphate dehydrogenase [Dimocarpus longan]
ACC66148.3	31	779	1.0	0.9	0.4↓	0.4↓	cell division cycle protein [Dimocarpus longan]
ANV82689.1	25	718.1	–	0.4↓	–	–	Hsp90 [Citrus maxima]
AAP13994.1	14	662.9	5.3↓	–	–	–	S-adenosylmethionine synthetase [Litchi chinensis]
AAD11697.1	10	605.2	–	–	10.6↑	–	ribulose 1,5-bisphosphate carboxylase, partial (chloroplast) [Atalantia ceylanica]
ARV86757.1	18	567	0.5	1.4	9.0↑	3.1↑	ATP synthase CF1 alpha subunit (chloroplast) [Anacardium occidentale]
ARJ62870.1	14	532	0.1↓	1.3	24.9↑	2.8↑	photosystem II protein C (plastid) [Boswellia sacra]
AMB65109.1	13	463	0.2↓	1.2	20.6↑	4.1↑	photosystem II 47 kDa protein (chloroplast) [Boswellia sacra]
AIN75617.1	20	401	0.7	0.7	4.0↑	3.8↑	argonaute 1 [Dimocarpus longan]
APT69248.1	13	342	0.7	0.9	4.9↑	3.8↑	PetA (chloroplast) [Rhus chinensis]
AMB65062.1	6	318	0.2↓	1.8	26.1↑	3.1↑	photosystem II protein D1 (chloroplast) [Boswellia sacra]
AGH13332.1	12	297	1.3	0.9	1.7	2.7↑	chalcone synthase [Rhus chinensis]
AQZ55555.1	13	294	0.1↓	1.1	25.0↑	1.9	acyl-CoA-oxidase-1 [Mangifera indica]
ADM35974.1	9	277	1.3	0.7	0.4↑	0.7	protein disulfide isomerase [Citrus limon]
AHJ91313.1	5	274	0.2↓	1.2	30.0↑	5.0↑	photosystem II protein D2 (chloroplast) [Azadirachta indica]
ARS43799.1	11	272	0.7	1.0	2.2↑	1.5	acetyl-CoA carboxylase carboxyltransferase beta subunit (plastid) [Pistacia vera]
ADY75750.1	13	272	0.2↓	1.1	15.2↑	2.2↑	phospholipase D alpha [Dimocarpus longan]
ABI49019.1	9	248.8	–	1.3	–	9.1↑	photosystem I P700 apoprotein A2 (chloroplast) [Citrus sinensis]
AQZ55556.1	10	210.3	–	1.5	–	4.3↑	9-lipoxygenase [Mangifera indica]
AEO51756.1	8	201	0.2↓	1.3	21.8↑	4.0↑	chloroplast chlorophyll A/B binding protein [Dimocarpus longan]
AIY24975.1	11	192.1	–	2.2↓	–	–	phenylalanine ammonia lyase [Mangifera indica]
ACA49723.1	9	192	0.2↓	1.1	13.1↑	2.6↑	phospholipase D alpha [Citrus sinensis]
AHL43880.1	9	188	1.0	0.6	0.3↓	0.5↓	putative inorganic pyrophosphatase [Citrus maxima]
AEO21912.1	4	159	1.0	0.6	2.1↑	3.3↑	chloroplast oxygen-evolving enhancer protein 1 [Dimocarpus longan]
APT69235.1	12	151.7	–	1.4	–	11.0↑	PsaA (chloroplast) [Rhus chinensis]
AEQ30069.1	5	149	0.2↓	0.9	14.6↑	2.6↑	sucrose synthase, partial [Mangifera indica]
AFJ53077.1	8	130	0.2↓	1.1	10.6↑	2.0↑	farnesyl pyrophosphate synthase [Mangifera indica]
ALS20376.1	9	129	0.8	0.6	2.0	2.7↑	1-deoxy-D-xylulose-5-phosphate reductoisomerase, partial [Mangifera indica]
AFM08812.1	8	120	1.1	1.3	0.3↓	0.2↓	NADP-dependent malic protein [Dimocarpus longan]
AKU77159.1	15	114	0.2↓	1.0	9.6↑	1.8	structural maintenance of chromosomes protein 1, partial [Rhus chinensis]
ANI86868.1	4	105	1.3	1.5	2.1↑	1.9	photosystem I subunit VII (chloroplast) [Spondias tuberosa]
AOP12358.2	3	98.5	–	0.19↓	–	–	(+)-limonene synthase [Citrus sinensis]
AKA09592.1	6	98	2.1↑	0.6	0.4↓	1.3	stearoyl-ACP desaturase [Xanthoceras sorbifolium]
AIT96751.1	2	94.4	–	1.0	–	6.4↑	photosystem II phosphoprotein (chloroplast) [Sapindus mukorossi]
ANC29206.1	3	87	0.5↑	1.1	18.2↑	7.6↑	photosystem II cytochrome b559 alpha subunit (chloroplast) [Leitneria floridana]
ADV59923.1	5	79	0.6	1.4	2.1↑	1.0	putative GDP-mannose pyrophosphorylase [Citrus unshiu]
ADO62712.1	4	73	0.7	0.2↓	0.2↓	0.7	p-hydroxyphenylpyruvate dioxygenase [Mangifera indica]
APT69267.1	5	65	0.5↓	0.4↓	3.4↑	4.3↑	RpoA (chloroplast) [Rhus chinensis]

Accession ¹	Peptide count	Score	Fold change ²				Description ³
			3B/3F	5B/5F	5B/3B	5F/3F	
ACK57683.1	3	61	0.7	2.6↑	2.5↑	0.7	peroxidase 4 [<i>Litchi chinensis</i>]
APT69271.1	5	57	1.4	4.3↑	0.4↓	0.1↓	ribosomal protein L14 (chloroplast) [<i>Rhus chinensis</i>]
ANC29218.1	3	47.0	–	1.1	–	5.3↑	cytochrome b6 (chloroplast) [<i>Leitneria floridana</i>]
ARS43830.1	2	39	1.8	0.3↓	1.1	7.3↑	ATP synthase CF1 epsilon subunit (plastid) [<i>Pistacia vera</i>]
Molecular binding/catalytic activity only							
AEQ45960.1	21	1189	0.3↓	1.1	1.2	0.3↓	actin 4 [<i>Mangifera indica</i>]
ADB43615.1	8	320	1.1	1.0	4.2↑	4.8↑	alcohol dehydrogenase 3 [<i>Mangifera indica</i>]
ATL77049.1	8	288	1.3	1.3	2.0↑	2.0↑	ascorbate peroxidase 3 [<i>Phellodendron amurense</i>]
JAV44882.1	6	245	1.3	1.2	0.2↓	0.2↓	cold, circadian rhythm, and rna binding [<i>Citrus limon</i>]
AIY24994.1	9	233	2.6↑	0.7	0.7	2.6↑	flavanone 3-hydroxylase 1 [<i>Mangifera indica</i>]
AAV86033.1	9	227	1.0	1.2	4.7↑	3.8↑	alcohol dehydrogenase [<i>Citrus x paradisi</i>]
ADB43613.1	8	227	1.0	0.9	5.0↑	5.5↑	alcohol dehydrogenase 1 [<i>Mangifera indica</i>]
ABV65355.1	3	217	20.3↑	0.7	1.5	41.0↑	copper/zinc-superoxide dismutase [<i>Litchi chinensis</i>]
ADX97325.1	13	212	1.2	0.8	2.0↑	2.9↑	glycolate oxidase [<i>Mangifera indica</i>]
AHF81485.1	9	202	0.5↓	1.1	3.1↑	1.4	small GTP Rab11 [<i>Mangifera indica</i>]
ABZ79406.1	7	178	0.5↓	1.2	3.2↑	1.4	ascorbate peroxidase, partial [<i>Litchi chinensis</i>]
JAV45212.1	7	172	0.4↓	1.1	2.6↑	0.9	RAS 5 [<i>Citrus limon</i>]
ACV72277.1	8	156	1.3	1.0	0.4↓	0.6	ubiquitin-conjugating enzyme 13 [<i>Citrus sinensis</i>]
AIY25001.1	8	146	1.4	1.0	2.9↑	4.2↑	dihydroflavonol 4-reductase [<i>Mangifera indica</i>]
AFB18780.1	5	125	1.1	0.8	0.3↓	0.5	glutathione peroxidase, partial [<i>Dimocarpus longan</i>]
AIY25006.1	2	96	0.9	1.2	5.3↑	4.0↑	anthocyanidin reductase [<i>Mangifera indica</i>]
AFB18803.1	4	90	2.3↑	2.5↑	10.5↑	9.7↑	dehydroascorbate reductase, partial [<i>Dimocarpus longan</i>]
AEI72269.1	3	82	1.3	0.7	0.5↓	0.9	histone H2A [<i>Citrus trifoliata</i>]
AQZ55552.1	3	79	0.2↓	0.7	11.8↑	3.0↑	epoxide hydrolase-2 [<i>Mangifera indica</i>]
ADK97702.1	2	71	1.6	0.8	1.0	2.0↑	putative caffeic acid O-methyltransferase [<i>Citrus aurantium</i>]

¹Accession: accession number according to the NR_database_Sapindales_Genebank databases.

²5B: bisexual flower buds sampled in late May, 2016; 3B: bisexual flower buds sampled in early March, 2017; 5F: female flower buds sampled in late May, 2016; 3F: female flower buds sampled in early March, 2017. ↑: up-regulated proteins (fold-change ≥ 2.0); ↓: down-regulated proteins (fold-change ≤ 0.5); –: not detected in either or both samples.

³Names in brackets (“[]”) represent plant species.

ribosomal protein L14 (chloroplast) and photosystem II protein D2 (chloroplast) were the most up-regulated DEPs when compared 5B to 5F, and 5B to 3B, respectively. The most down-regulated DEPs comparing 3B to 3F, 5B to 5F, 5B to 3B, and 5F to 3F were acyl-CoA-oxidase-1, (+)-limonene synthase, 50S ribosomal protein L2 (chloroplast), and somatic embryogenesis receptor kinase, respectively (Table 1).

The KEGG pathway analysis revealed that photosynthesis and ribosome were two pathways that enriched in the most DEPs. In addition, other pathways that enriched in more than 10 DEPs included phenylpropanoid biosynthesis, flavonoid biosynthesis, alpha-linolenic acid metabolism, carbon metabolism, glutathione metabolism, glycolysis/gluconeogenesis, and phenylalanine metabolism (Fig. 4). A total of 13 DEPs participated in photosynthesis pathway, with all DEPs up-regulated when compared 5B to 3B and 5F to 3F, and almost all DEPs down-regulated when compared 3B to 3F (Table 2). The photosystem II protein D2 (chloroplast) and PsaA (chloroplast) were the most up-regulated DEPs for 5B/3B and 5F/3F comparison, respectively, while photosystem II protein C

(plastid) was the most down-regulated DEPs for 3B/3F comparison in the photosynthesis pathway. A total of 9 DEPs participated in ribosome pathway, with all DEPs down-regulated when compared 5B to 3B and 5F to 3F (Table 2). The 50S ribosomal protein L2 (chloroplast) and ribosomal protein L14 (chloroplast) were the most down-regulated DEPs when making this comparison in the ribosome pathway.

Discussion

Due to the lack of defined sex chromosomes, there is no reliable methods for sex determination of the dioecious *P. chinensis* plants prior to flowering. Moreover, the underlying sex determination mechanisms in *P. chinensis* are still unknown, making it difficult to breed female plants at juvenile stage for seed production (Xiong et al. 2013). The discovery of wild-type monoecious *P. chinensis* in China provided good materials to study the sex differentiation mechanisms of *P. chinensis* Bunge. The morphological and paraffin section observations in this study confirmed the sex differentiation process of monoecious *P. chinensis* trees reported by

Figure 2. Distribution of identified proteins and differentially expressed proteins in bisexual flower buds or female flower buds in different phenophases. (A) Venn diagram showing the number of overlapping proteins that were identified in bisexual flower buds in late May, 2016 (5B) or in early March, 2017 (3B), and in female flower buds in late May, 2016 (5F) or in early March, 2017 (3F). (B) The number of differentially expressed proteins (up-regulated proteins with fold change ≥ 2.0 ; down-regulated proteins with fold change ≤ 0.5) and proteins not commonly expressed when compared among the four groups.

Bai et al. (2016). Therefore, the female and bisexual flower buds in late May 2016 (phenophase I) and early March 2017 (phenophase II) were sampled to study the difference during sex differentiation at the proteome level. Considering the male *Pistacia chinensis* Bunge tree is rare grown in the wild, and the sex phenotype

of male flowers in sequential years were unstable, no male flower buds were collected in this study.

The label-free quantitative technique identified hundreds of DEPs when compared different flower buds in the same phenophase (3B vs. 3F, and 5B vs. 5F) or the same kind of flower bud in different phenophases (5B

Figure 3. Gene Ontology (GO) analysis of differentially expressed proteins (DEPs) in bisexual flower buds or female flower buds in different phenophases. (A) GO analysis of DEPs between bisexual flower buds in early March, 2017 (3B) and female flower buds in early March, 2017 (3F). (B) GO analysis of DEPs between bisexual flower buds in late May, 2016 (5B) and female flower buds in late May, 2016 (5F). (C) GO analysis of DEPs between 5B and 3B. (D) GO analysis of DEPs between 5F and 3F. UP: up-regulated (fold change ≥ 2.0); DOWN: down-regulated (fold change ≤ 0.5); 3B/3F/5B/5F: expression only detected in 3B/3F/5B/5F.

Figure 4. Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis of differentially expressed proteins in bisexual flower buds or female flower buds in different phenophases. 5B: bisexual flower buds sampled in late May, 2016; 3B: bisexual flower buds sampled in early March, 2017; 5F: female flower buds sampled in late May, 2016; 3F: female flower buds sampled in early March, 2017.

Table 2. Differentially expressed proteins involved in major pathways when compared bisexual and female flower buds in different phenophases.

Accession ¹	Peptide count	Score	Fold change ²				Description ³
			3B/3F	5B/5F	5B/3B	5F/3F	
Photosynthesis							
ARV86757.1	18	567	0.5↓	1.4	9.0↑	3.1↑	ATP synthase CF1 alpha subunit (chloroplast) [<i>Anacardium occidentale</i>]
ARJ62870.1	14	532	0.1↓	1.3	24.9↑	2.8↑	photosystem II protein C (plastid) [<i>Boswellia sacra</i>]
AMB65109.1	13	463	0.2↓	1.2	20.6↑	4.1↑	photosystem II 47 kDa protein (chloroplast) [<i>Boswellia sacra</i>]
APT69248.1	13	342	0.7	0.9	4.9↑	3.8↑	PetA (chloroplast) [<i>Rhus chinensis</i>]
AMB65062.1	6	318	0.2↓	1.8	26.1↑	3.1↑	photosystem II protein D1 (chloroplast) [<i>Boswellia sacra</i>]
AHJ91313.1	5	274	0.2↓	1.2	30.0↑	5.0↑	photosystem II protein D2 (chloroplast) [<i>Azadirachta indica</i>]
ABI49019.1	9	248.8	–	1.3	–	9.1↑	photosystem I P700 apoprotein A2 (chloroplast) [<i>Citrus sinensis</i>]
AEO21912.1	4	159	1.0	0.6	2.1↑	3.3↑	chloroplast oxygen-evolving enhancer protein 1 [<i>Dimocarpus longan</i>]
APT69235.1	12	151.7	–	1.4	–	11.0↑	PsaA (chloroplast) [<i>Rhus chinensis</i>]
AIT96751.1	2	94.4	–	1.0	–	6.4↑	photosystem II phosphoprotein (chloroplast) [<i>Sapindus mukorossi</i>]
ANC29206.1	3	87	0.5↓	1.1	18.2↑	7.6↑	photosystem II cytochrome b559 alpha subunit (chloroplast) [<i>Leitneria floridana</i>]
ANC29218.1	3	47.0	–	1.1	–	5.3↑	cytochrome b6 (chloroplast) [<i>Leitneria floridana</i>]
ARS43830.1	2	39	1.8	0.3↑	1.1	7.3↑	ATP synthase CF1 epsilon subunit (plastid) [<i>Pistacia vera</i>]
Ribosome							
JAV45330.1	6	225	1.5	1.4	0.5↓	0.5↓	ribosomal L27e protein family [<i>Citrus limon</i>]
ARV86811.1	6	76.5	–	0.7	–	–	ribosomal protein S3 (chloroplast) [<i>Anacardium occidentale</i>]
APT69271.1	5	57	1.4	4.3↑	0.4↓	0.1↓	ribosomal protein L14 (chloroplast) [<i>Rhus chinensis</i>]
ABV59096.2	5	40	1.0	2.6↑	0.3↓	0.1↓	ribosomal protein S7 (chloroplast) [<i>Mangifera indica</i>]
JAV44889.1	2	37	1.2	1.5	0.2↓	0.2↓	ribosomal protein S14p/S29e family protein [<i>Citrus limon</i>]
AHF71481.1	6	35.5	4.4↑	–	0.1↓	–	50S ribosomal protein L2 (chloroplast) [<i>Acer buergerianum</i> subsp. <i>ningpoense</i>]
ARS43823.1	3	21.6	–	1.4	–	–	ribosomal protein L22 (plastid) [<i>Pistacia vera</i>]
ABI49009.1	2	19.6	–	1.0	–	0.1↓	ribosomal protein S2 (chloroplast) [<i>Citrus sinensis</i>]
APT69237.1	2	8.7	–	–	–	–	ribosomal protein S4 (chloroplast) [<i>Rhus chinensis</i>]

¹Accession: accession number according to the NR_database_Sapindales_Genebank databases.

²5B: bisexual flower buds sampled in late May, 2016; 3B: bisexual flower buds sampled in early March, 2017; 5F: female flower buds sampled in late May, 2016; 3F: female flower buds sampled in early March, 2017. ↑: up-regulated proteins (fold-change ≥ 2.0); ↓: down-regulated proteins (fold-change ≤ 0.5); –: not detected in either or both samples.

³Names in brackets (“[]”) represent plant species.

the same kind of flower buds. The most up-regulated proteins all involved in the photosynthesis pathway in both kind of flower buds, and the down-regulated proteins all involved in the ribosome pathway, indicating the improved photosynthesis and reduced protein synthesis in late May compared to those in early March, which is also reasonable.

In this study, we reported the differently expressed proteins in key sex differentiation phases of flower buds in monoecious *P. chinensis* Bunge for the first time. The only previous literature that could be found on proteomic analysis of *P. chinensis* Bunge is from Xiong et al. (2013), who discovered that several functional and stress proteins change in abundance greatly between male dioecious *P. chinensis* and female dioecious *P. chinensis*, including NB-ARC domain containing protein

[*Oryza sativa*], light harvesting chlorophyll a/b-binding protein [*Arabidopsis thaliana*], temperature-induce lipocalin [*Solanum lycopersicum*], asorbate peroxidase (APX) [*Pennisetum americanum*], eukaryotic translation initiation factor 5A2 (eIF-5A2) [*Tamarix androssowii*], and phosphoglycerate kinase 2 (PGK) [*Vitis vinifera*]. Similarly, we also observed the different expression of some stress-related proteins in this study. However, the two-dimensional electrophoresis approach was used for protein detection, and the green plants (NCBI nr 20120922) database was used for DEPs searching in the study of Xiong et al. (2013), which may be not as accurate as ours from the methodology aspect.

The DEPs in flower buds with different sex and in various phenophases identified in this study could help to understand the mechanisms of sex differentiation

vs. 3B, and 5F vs. 3F) in *P. chinensis*. As the *P. chinensis* genome has not yet been sequenced, the identified proteins were searched against the database of Sapindales (*P. chinensis* Bunge belongs to Sapindales order) to identify the proteins of interest, as in other unsequenced plant species (Renuse et al. 2012). Large amount of proteins classified are from *Mangifera indica* (Mango), *Citrus*, *Litchi chinensis* (Litchi), *Dimocarpus longan* (Longan), *Rhus chinensis* (Sumac), etc. Majority of the identified DEPs involved in cellular/metabolic process or molecular binding/catalytic activity, especially when compared between different phenophases, indicating that these biological and cellular processes changed drastically during the sex differentiation of *P. chinensis* Bunge.

During the key phase for female primordium differentiation (phenophase II), the most up-regulated proteins in bisexual flower buds included copper/zinc-superoxide dismutase [*Litchi chinensis*] and S-adenosylmethionine synthetase [*Litchi chinensis*] compared to female flower buds. The plant Cu/Zn superoxide dismutases (SODs) is one of the three SODs in plant which could catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide, and plays a major role in the defense against toxic-reduced oxygen species, thus important for plant stress tolerance (Bowler et al. 1994). The S-adenosylmethionine (SAM) synthetase is an enzyme that creates SAM by reacting methionine and ATP (Horikawa et al. 1990). Meanwhile, the most down-regulated proteins in bisexual flower buds included acyl-CoA oxidase 1 [*Mangifera indica*] and epoxide hydrolases-2 [*Mangifera indica*] compared to female flower buds. Acyl-CoA oxidase was reported to be the key enzyme catalyzing the first step in the fatty acid β -oxidation pathway, and showed a positive relationship with the lactone formation in plant fruits (Zhang et al. 2017). Epoxide hydrolases could transform the epoxide-containing lipids to 1,2-diols by the addition of a molecule of water, and many of these oxygenated lipid substrates are associated with host defense responses (Morisseau 2013). Therefore, it seems that the female primordium differentiation in the bisexual flower bud of the monoecious *P. chinensis* Bunge in early March was associated with enhanced superoxide removal, and reduced fatty acid oxidation and epoxide-containing lipid removal in comparison to the female flower bud taken at the same time. Furthermore, our research also revealed that many proteins in the photosynthesis pathway were down-regulated during this process, e.g. photosystem II protein C (plastid), photosystem II protein D1

(chloroplast), photosystem II protein D2 (chloroplast), indicating the reduced photosynthesis function.

During the key phase for male primordium differentiation (phenophase I), the most up-regulated proteins in bisexual flower buds included ribosomal protein L14 (chloroplast) [*Rhus chinensis*], ribosomal protein S7 (chloroplast) [*Mangifera indica*], peroxidase 4 [*Litchi chinensis*], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [*Dimocarpus longan*] and dehydroascorbate reductase (DHAR) [*Dimocarpus longan*] compared to female flower buds, while the most down-regulated proteins was (+)-limonene synthase [*Citrus sinensis*]. Peroxidase was reported to be responsible for eliminating peroxides to increase the plant's defenses against pathogens (Karthikeyan et al. 2005). The GAPDH was usually involved in the glycolysis pathway, and the inactivation of GAPDH would lead to the consequences of oxidative stress (Hwang et al. 2009). The DHAR could regulate the cellular ascorbic acid redox state, which in turn affects cell responsiveness and tolerance to environmental stress, and increased DHAR expression maintained higher levels of photosynthetic functioning, resulting in delayed leaf aging (Chen and Gallie 2006). The (+)-limonene synthase is one of the synthases for limonene, a kind of terpenes that serve as chemical defense agents against microbial infection and predation and as attractants for pollinators (Pichersky and Gershenzon 2002). To summarize, the male primordium differentiation in the bisexual flower bud of the monoecious *P. chinensis* Bunge in late May was associated with higher oxidative stress resistance, the ribosome activity, and the possible enhanced photosynthetic function in comparison to the female flower bud taken at the same time.

When compared the above two processes, the increased expression of oxidative stress-resistance proteins during both female and male primordium differentiation were observed. This can support the theory of Horandl and Speijer (2018), who stated that in the evolutionary order of events, repair of oxidative damage was the first step as a response to endogenous reactive oxygen species production in eukaryotes, and this happens during prophase I of meiosis, the most indispensable phase of sex. On the other hand, the opposite expression pattern of photosynthesis-related proteins during these two processes could be attributed to the differences in light and temperature between the differentiation phases.

To study the effect of sex differentiation phases, the DEPs in different phenophases were also compared on

of flower buds in monoecious *Pistacia chinensis* Bunge. Moreover, the DEPs could act as molecular markers for sex determination. Many sex-linked molecular markers such as AFLP, RAPD, RFLP and microsatellite have been generated for sex determination in dioecious plants. However, these techniques need to design and test different sets of random primers and the function of amplified products is often unclear (Esfandiyari et al. 2012), while the proteins classified through proteomic analysis could be quantified directly with the function known. Therefore, our research results may have certain prospects in application.

Conclusion

Our study identified the differently expressed proteins in flower buds in two key sex differentiation phenophases in monoecious *P. chinensis* Bunge. In the bisexual flower bud of the monoecious *P. chinensis* Bunge, the female primordium differentiation in early March was associated with enhanced oxidative stress resistance and reduced photosynthesis function, while the male primordium differentiation of *P. chinensis* Bunge in late May was associated with increased oxidative stress resistance, the ribosome activity, and the enhanced photosynthetic function, in comparison with the female flower bud taken at the same time. The identified differentially expressed proteins such as the Cu/Zn superoxide dismutases could act as a possible molecular marker for sex determination in monoecious *P. chinensis* Bunge. With the results of the undergoing genomic and transcriptomic studies, this proteomic study may help to depict the underlying sex determination mechanisms of *P. chinensis* Bunge.

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Supplementary material

Supplementary material is available online at: <https://doi.org/10.6084/m9.figshare.8229137>

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