Material and methods

Plant material

Petunia (*Petunia hybrida* 'Mitchell') plants were grown under greenhouse conditions (22-25°C) and a natural photoperiod (Liu et al., 2011). Flowers were emasculated 1 d before they were fully open to prevent self-pollination. Eight to ten petunia flowers were harvested at the anthesis stage (corollas 90° reflexed) and were then placed immediately in tap water. Buds were collected at about the 3rd week after the first flower opened. Stems, leaves and roots were collected from plants at the vegetative stage when the plants were ~10 cm in height. All tissues were frozen in liquid nitrogen and stored at –80°C until used for RNA extraction. Fresh weights were measured immediately before freezing (Liu et al, 2011). All experiments were conducted at least three times with independently collected and extracted tissues unless otherwise noted.

Ethylene treatment

Petunia flowers were treated with ethylene according to previously described protocols (Tan et al., 2014). Petunia flowers were harvested at anthesis, and their stems were re-cut to 5 cm, placed in flasks with distilled water, and subsequently treated with 2 μl l^{-1} ethylene for 0, 8, 16, 24, 32, 40 and 48 h. Corollas from 8–10 flowers were collected at each time point, immediately frozen in liquid nitrogen, and stored at –80°C for subsequent RNA or protein extraction.

Proteasome inhibitor studies were performed by spraying corollas with an 80 μm MG132 solution (8% DMSO) 4 h prior to 16 h ethylene treatment. Control corollas were sprayed with an 8% DMSO solution (Kevany te al, 2007).

RNA extraction, library construction and sequencing

The total RNA of each of above listed samples was isolated using the Trizol Kit (Promega, USA) following by the manufacturer's instructions. Then the total RNA was treated with

RNase-free DNase I (Takara Bio, Japan) for 30 min at37℃ to remove residual DNA. RNA quality was verified using a2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA) and were also checked by RNase free agarose gel electrophoresis. Next, Poly (A) mRNA was isolated using oligo-dT beads (Qiagen). All mRNA was broken into short fragments by adding fragmentation buffer. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by the synthesis of the second-strand cDNA using RNase H and DNA polymerase I. The cDNA fragments were purified using a QIA quick PCR extraction kit. These purified fragments were then washed with EB buffer for end reparation poly (A) addition and ligated to sequencing adapters. Following agarose gel electrophoresis and extraction of cDNA from gels, the cDNA fragments were purified and enriched by PCR to construct the final cDNA library. The cDNA library was sequenced on the Illumina sequencing platform (Illumina HiSeq™ 2000) using the paired-end technology by Gene Denovo Co. (Guangzhou, China).A Perl program was written to select clean reads by removing low quality sequences (there were more than 50% bases with quality lower than 20 in one sequence), reads with more than 5% N bases (bases unknown) and reads containing adaptor sequences.

Reads alignment and Normalization of gene expression levels

Sequencing reads were mapped to reference sequence by the SOAPaligner/soap2 (Li et al, 2009), a tool designed for short sequences alignment. Coverage of reads in one gene was used to calculate expression level of this gene. Using this method we obtained the expression levels of all genes detected.

Reads that could be uniquely mapped to a gene were used to calculate the expression level. The gene expression level was measured by the number of uniquely mapped reads per kilobase of exon region per million mappable reads (RPKM). The formula was defined as below:

$$\mathrm{RPKM}=\frac{10^6 C}{NL/10^3}$$

In which C was the number of reads uniquely mapped to the given gene; N was the number of reads uniquely mapped to all genes; L was the total length of exons from the given gene. For genes with more than one alternative transcript, the longest transcript was selected to calculate the RPKM. The RPKM method eliminates the influence of different gene lengths and sequencing discrepancies on the gene expression calculation. Therefore, the RPKM value can be directly used for comparing the differences in gene expression among samples. All expression data statistic and visualization was conduction with R package (http://www.r-project.org/).

Quantitative real-time PCR assays

Quantitative real-time PCR assays were performed according to the methods of Liu et al. (2011). Total RNA was extracted from the samples of corollas and digested with RNase-free DNAase I followed by reverse transcription (RT) according to the manufacturer's instruction. PCR analysis was performed with the cDNA extracted of different samples as a template.

The quantitative PCR was performed on a LightCycler® 480 Real-Time PCR system (Roche). Samples were subjected to thermal-cycling conditions of DNA polymerase activation at 95°C for 4 min, 40 cycles of 45 s at 95°C, 45 s at 52°C or 55°C, 45 s at 72°C, and 45 s at 80°C; a final elongation step of 7 min at 72°C was performed. The amplicon was analyzed by electrophoresis and sequenced once for identity confirmation. The sequences of all primers used for real-time PCR analysis are described in Supplemental Table S1. Petunia *ACTIN* was used as the housekeeping gene to quantify cDNA abundance. Primer specificity was determined by melting curve analysis; a single, sharp peak in the melting curve ensured that a single, specific DNA species had been amplified. Quantification was based on analysis of the threshold cycle (Ct) value as described by Pfaffl (2001).

Protein Extraction

Petunia corollas were ground in liquid nitrogen, then the cell powder was transferred to 5 mL centrifuge tube and sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Triton-100, 65 mM DTT and 0.1% Protease Inhibitor

Cocktail). The remaining debris was removed by centrifugation at 20,000 g at 4 °C for 10 min. Finally, the protein was precipitated with cold 15% TCA for 2 h at -20 °C. After centrifugation at 4 °C for 10 min, the supernatant was discarded. The remaining precipitate was washed with cold acetone for three times. The protein was redissolved in buffer (8 M urea, 100 mM TEAB, pH 8.0) and the protein concentration was determined with 2-D Quant kit according to the manufacturer's instructions. Three biology replicates were performed.

Preparation of Specific Antibodies against proteins and western blot analysis

The synthetic peptides of proteins were used as an antigen for antibody production in rabbit from Abmart (www.ab-mart.com.cn/). These antibodies were used for blotting analysis.

Western Blot Analyses were performed according to the methods of Tatsuki et al. (2001). Proteins were separated using SDS-polyacrylamide gel electrophoresis (PAGE; 10% acrylamide gels) and blotted onto nitrocellulose membranes (BA-S 85; Schleicher & Schuell). The membrane was blocked with 5% skim milk, 0.05% Tween 20 in Tris-buffered saline (50 mM Tris-HCl, pH 8.0, 150 mM NaCl). Purified ubiquitin antibody or anti-GAPDH antibody (internal reference) were used at a concentration of 50 mg/ml. The membrane was washed with 0.05% Tween 20 in Tris-buffered saline and then reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) at a dilution of 1:20,000. Detection was achieved using Super Signal West FemtoTM (Pierce). Three biological replicates were performed.

Trypsin Digestion

For digestion, the protein solution was reduced with 10 mM DTT for 1 h at 37 °C and alkylated with 20 mM IAA for 45 min at room temperature in darkness. For trypsin digestion, the protein sample was diluted by adding 100 mM TEAB to urea concentration less than 2M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion. Approximately 100 μg protein for each sample was digested with trypsin for the following experiments.

TMT Labeling

After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 6-plex TMT kit. Briefly, one unit of TMT reagent (defined as the amount of reagent required to label 100 μg of protein) were thawed and reconstituted in 24 μl ACN. The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted and dried by vacuum centrifugation.

HPLC Fractionation

The sample was then fractionated into fractions by high pH reverse-phase HPLC using Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 2% to 60% acetonitrile in 10 mM ammonium bicarbonate pH 10 over 80 min into 80 fractions, Then, the peptides were combined into 18 fractions and dried by vacuum centrifuging.

Affinity Enrichment

To enrich Kub peptides, tryptic peptides dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) were incubated with pre-washed antibody beads (PTM Biolabs) at 4°C overnight with gentle shaking. The beads were washed four times with NETN buffer and twice with ddH2O. The bound peptides were eluted from the beads with 0.1% TFA. The eluted fractions were combined and vacuum-dried. The resulting peptides were cleaned with C18 ZipTips (Millipore) according to the manufacturer's instructions, followed by LC-MS/MS analysis.

LC-MS/MS Analysis

Three parallel analyses for each fraction were performed. LC-MS/MS Analysis was performed according to previously described protocols(Wu et al., 2015). Peptides were dissolved in 0.1% FA, directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific). Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). The gradient was comprised of an increase from 8% to 25% solvent B (0.1% FA in 98% ACN) over 26 min, 25% to 38% in 8

min and climbing to 85% in 4 min then holding at 85% for the last 4min, all at a constant flow rate of 280 nl/min on an EASY-nLC 1000 UPLC system. The resulting peptides were analyzed by Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using NCE setting as 30; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1.5E4 in the MS survey scan with 30.0s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800. Fixed first mass was set as 100 m/z.

Database Search

The resulting MS/MS data were processed using MaxQuant with an integrated Andromeda search engine (v.1.4.1.2). Tandem mass spectra were searched against a database (40,341 sequences) made from RNA sequencing of petunias in this study.

For proteomic peptides, Trypsin/P was used as a cleavage enzyme, allowing up to 2 missed cleavages. Mass error was set to 10 ppm for precursor ions and to 0.02 Da for fragment ions. Carbamidomethyl on Cys, TMT-6plex (N-term) and TMT-6plex (K) were specified as fixed modifications, and oxidation on Met was specified as a variable modification. FDR was adjusted to < 1%, and peptide ion score was set to > 20.

For Kub peptides, Trypsin/P was specified as a cleavage enzyme, allowing up to 3 missed cleavages. First, the search range was set to 5 ppm for precursor ions, and the main search range was set to 5 ppm and 0.02 Da for fragment ions. Carbamidomethyl on Cys was specified as a fixed modification, and GlyGly on lysine and oxidation on Met were specified

as variable modifications. The label-free quantification method was LFQ, and FDR was adjusted to < 1%, while the minimum score for modified peptides was set to > 40.

Bioinformatic Analysis

Bioinformatic analysis was performed according to previously described protocols (Wu et al., 2015; Xie et al., 2015). GO term association and enrichment analysis were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID). The KEGG database was used to annotate protein pathways (Kanehisa and Goto, 2000). The KEGG online service tool KAAS was used to annotate the proteins' KEGG database descriptions. The annotation results were mapped on the KEGG pathway database using the KEGG online service tool KEGG Mapper. The domain annotation was performed with InterProScan on the InterPro domain database via web-based interfaces and services. WoLF PSORT was used for predicting subcellular localization (Horton et al., 2007). The CORUM database was used to annotate protein complexes. Motif-X software was used to analyze the models of the sequences with amino acids in specific positions of ubiquityl-21-mers (ten amino acids upstream and downstream of the Kub site) in all of the protein sequences. In addition, the IPI Arabidopsis proteome was used as the background database, and the other parameters were set to the default values. The setting parameters for searching motifs using Motif-X software were 'occurrences 20' and 'the Bonferroni corrected $P = 0.005$'. Protein–protein interaction networks were analyzed with the IntAct database (http://www.ebi.ac.uk/intact/). The protein–protein interaction network map was generated with the Cytoscape software (Shannon et al., 2003).

Protein quantitative ratio analysis

Protein quantitative ratio was calculated as median of all unique peptides' ratio. Student's t test was performed to investigate differentially expressed proteins (DEPs) effects. In order to meet the condition of student's t test, logarithmic transformation was performed to achieve the ratio of all peptides. Then, Student's t test was explored to calculate the p value.

Ubiquitinated Protein Secondary Structure Analysis

In this study, we show the distribution of ubiquitinated and non-ubiquitinated amino acids in protein secondary structures. Probabilities for different secondary structures (alpha-helix, beta-strand and coil) of ubiquitinated lysine were compared with the secondary structure probabilities of all lysine in all identified proteins. We further investigated the local secondary structures of proteins using NetSurfP, a software that predicts the surface accessibility and secondary structure of amino acids in an amino acid sequence.

Conservation analysis of ubiquitinated proteins

To analyze the conservation of ubiquitinated proteins, first, proteins homologous to the ubiquitinated proteins identified in this study were obtained by blasting in UniprotKB database against eight species, including *Oryza sativa* japonica, *Brachypodium distachyon*, *Sorghum bicolor*, *Zea mays, Arabidopsis thaliana*, *Glycine max*, *Solanum lycopersicum*, and *Vitis vinifera*. All of the protein sequences of these 8 species were downloaded from the UniprotKB database. The sequence alignment software BLASTp was used to obtain the homologous proteins of the ubiquitinated proteins in this study. To find conserved sites, the multiple sequence alignment software muscle was used to align homologs.

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