

1 **Short title: Ubiquitination is involved in corolla senescence**

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5 **Article title:** Proteomes and Ubiquitylomes Analysis Reveals the Involvement of

6 Ubiquitination in Protein Degradation in Petunias

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21 **One-sentence summary:** The global proteome and ubiquitylome were negatively correlated
22 and ubiquitination could be involved in the degradation of proteins during
23 ethylene-mediated corolla senescence in petunias.

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27 **Authors' contributions**

28 Yu Y, Liu J, designed research; Liu J, Ma Y, Wang R, Yang W, performed research; Guo J,
29 Wei Q, analyzed data; Yu Y, Chen G, Liu J, wrote paper.

30 **Supporting information:** 12 figures and 13 excel tables.

31 * These authors contributed equally to this work.

32

33 **Abstract**

34 Petal senescence is a complex programmed process. It has been previously demonstrated
35 that treatment with ethylene, a plant hormone involved in senescence, can extensively alter
36 transcriptome and proteome profiles in plants. However, little is known regarding the impact
37 of ethylene on post-translational modification (PTM) or the association between PTM and
38 the proteome. Protein degradation is one of the hallmarks of senescence, and ubiquitination,
39 a major PTM in eukaryotes, plays important roles in protein degradation. In this study, we
40 first obtained reference petunia transcriptome data via RNA sequencing. Next, we
41 quantitatively investigated the petunia proteome, ubiquitylome, and the association between
42 them in petunia corollas following ethylene treatment. In total, 51,799 unigenes, 3,606
43 proteins, and 2,270 ubiquitination sites were quantified 16 hours after ethylene treatment.
44 Treatment with ethylene resulted in 14,448 down-regulated and 6,303 up-regulated unigenes
45 (absolute log₂-fold change >1 and FDR<0.001), 284 down-regulated and 233 up-regulated
46 proteins, and 320 up-regulated and 127 down-regulated ubiquitination sites using a 1.5-fold
47 threshold (P<0.05), indicating that global ubiquitination levels increase during
48 ethylene-mediated corolla senescence in petunia. Several putative ubiquitin ligases were
49 up-regulated at the protein and transcription levels. Our results showed that the global
50 proteome and ubiquitylome were negatively correlated and that ubiquitination could be
51 involved in the degradation of proteins during ethylene-mediated corolla senescence in
52 petunias. Ethylene regulates hormone signaling transduction pathways at both the protein
53 and ubiquitination levels in petunia corollas. In addition, our results revealed that ethylene
54 increases the ubiquitination levels of proteins involved in ER-associated degradation
55 (ERAD).

56 **Key words: Ethylene; Ubiquitination; Senescence; Petunia; Protein degradation**

57

58 **Introduction**

59 Flowers have limited lifespans and are irreversibly programmed to undergo senescence;
60 therefore, they represent an excellent model system to study senescence (Jones et al., 2005).
61 Post-harvest longevity is an important characteristic of cut flowers. Studying petal senescence
62 may provide insight into the mechanisms of plant senescence in general and provide a means
63 to improve the vase-lives of cut flowers (Borochoy et al., 1997).

64 Senescence is regulated at several levels, including mRNA, protein and post-translational
65 modification (PTM) (van Doorn and Woltering, 2008; Woo et al., 2013). The gaseous plant
66 hormone ethylene exerts significant effects on flower senescence (Abeles FB, 1992; Ecker,
67 1995; Douglas, 2014). Many flowers are classified as ethylene-sensitive, including petunias
68 (*Petunia hybrida*) and carnations (*Dianthus caryophyllus*) (Woltering and Van Doorn, 1988).
69 In these flowers, ethylene production peaks close to senescence. The application of exogenous
70 ethylene enhances this process, whereas inhibition of ethylene synthesis or activity slows
71 senescence (Reid and Wu, 1992). Previous studies have demonstrated that ethylene treatment
72 can extensively alter transcriptome and proteome profiles in plants (Mayuoni et al., 2011)
73 (Prayitno et al., 2006; Mayuoni et al., 2011; Slade et al., 2012; Cheng et al., 2013).

74 Protein degradation is one of the hallmarks of senescence (Shahri and Tahir, 2014).
75 Ubiquitination, a well-known PTM, plays important roles in protein degradation (Wilkinson,
76 2000). Ubiquitin is a highly conserved 76-amino-acid polypeptide that is found throughout
77 the eukaryotic kingdom. In vivo, poly-ubiquitin chains are most frequently linked through
78 K48 and the canonical ubiquitin signal is recognized by the 26S proteasome and thereby
79 targets tagged proteins for degradation (Peng et al., 2003). Among six other lysine residues
80 of ubiquitin, at least four (K6, K11, K29 and K63) can function as a linkage for
81 poly-ubiquitin chains (Arnason and Ellison, 1994; Peng et al., 2003). K11- and K29-linked
82 poly-ubiquitin chains may target proteins to the proteasome (Johnson et al., 1995; Baboshina
83 and Haas, 1996). Conjugation of mono-ubiquitylation is a regulatory modification involved
84 in diverse processes including transcription, histone function, endocytosis, DNA repair, viral
85 budding and membrane trafficking (Passmore and Barford, 2004; Schnell and Hicke, 2003).

86 The attachment of the ubiquitins to proteins involves three classes of enzyme:
87 ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin
88 ligases (E3) (Hochstrasser, 1995). Ubiquitinated substrates may be degraded to peptides by
89 the multisubunit 26S protease. However, no attempts have been made to perform PTM
90 analysis to characterize the ubiquitination of the proteome or the association between
91 modifications and the proteome during flower senescence in response to ethylene.

92 Petunia has served as a model plant for the molecular and biochemical analysis of flower
93 senescence (Gerats and Vandenbussche, 2005). In this study, a reference transcriptome
94 dataset from petunia was first obtained via RNA sequencing. Then, using iTRAQ and a
95 label-free quantitative strategy involving antibody-based affinity enrichment and
96 high-resolution LC-MS/MS analysis, we generated proteome and ubiquitylome analyses of
97 petunia corollas with and without ethylene treatment (Fig. 1). In total, 51,799 unigenes, 3,606
98 proteins, and 2,270 ubiquitination sites were quantified in response to 16 h of ethylene
99 treatment. Ethylene treatment altered the proteome and ubiquitylome profiles of petunia
100 corollas. The correlation between the proteome and ubiquitylome was also described. Finally,
101 the function of ubiquitination in protein degradation during ethylene-mediated corolla
102 senescence in petunia and the effects of ethylene on proteins involved in hormone
103 biosynthesis, signaling transduction, amino acid biosynthesis, ER-associated degradation
104 (ERAD) and other processes were discussed.

105 **Results and Discussion**

106 **Ethylene treatment accelerates corolla wilting and decreases fresh weight and total** 107 **protein content**

108 The evaluated petunias (Mitchell) exhibited the first visible symptom of senescence, the
109 wilting of the corolla, at approximately 16 h after 2 $\mu\text{L L}^{-1}$ ethylene treatment. The margins of
110 the corollas began to involute, and a few translucent dots appeared in the corollas (Fig. 2A);
111 however, the corolla fresh weight and protein content remained constant. At 32 h after
112 ethylene treatment, the petunias exhibited obvious symptoms of senescence (Figs. 2B, 2C),
113 and the corolla fresh weight and protein content decreased to approximately 87% and 88%,

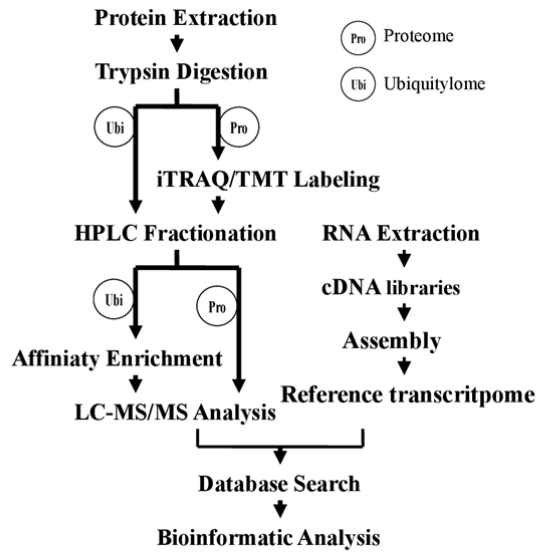


Figure 1. The systematic workflow for quantitative profiling of the global proteome and ubiquitylome in petunia corollas upon ethylene treatment.

1

114 respectively, compared to air-treated, control corollas. These decreases coincided with corolla
 115 wilting. Air-treated petunia corollas were fully turgid 0-48 h after flower opening, exhibited
 116 no symptoms of senescence and were visually indistinguishable from flowers at anthesis (Fig.

6

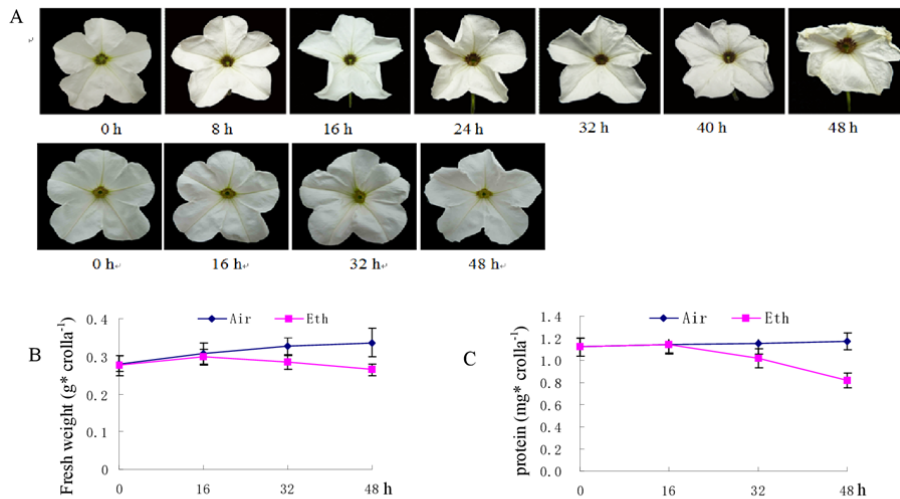


Figure 2. Effect of ethylene on flowers of petunia ‘Mitchell’. A, Flower profile with ethylene treatment (top) or without (bottom). B, Fresh weights of corollas with or without ethylene treatment. C, Protein contents of corollas with or without ethylene treatment. Corollas were collected from at least five flowers on various days after flower opening. Total protein was determined using the Bradford assay. Data represent the means of three replicates \pm SE. Experiments were conducted at least twice with similar results.

1

117 2A). We selected a $2 \mu\text{L L}^{-1}$, 16-h ethylene treatment (Eth) and a 16-h air treatment (Air) to
 118 perform transcriptome, proteome, and ubiquitylome analyses.

119 **Ethylene treatment increases ubiquitin in petunia corollas at the protein level**

120 To examine the effects of ethylene on the ubiquitin protein, western blotting was performed to
121 examine the expression patterns of ubiquitin in petunia corollas in response to ethylene
122 treatment. As shown in the Supplementary Materials (SM) Fig. S1, ethylene treatment
123 significantly increased the expression of ubiquitin at the protein level in petunia corollas. The
124 results implied that the ubiquitin-proteasome system may play a role during
125 ethylene-mediated corolla senescence.

126 **RNA sequencing and assembly**

127 To comprehensively construct the complete transcriptome of the ‘Mitchell’ petunia, eight
128 tissues, including the roots, stems, leaves, buds (0.4 cm), buds (0.8 cm), corollas (8 h post
129 ethylene treatment), corollas (16 h post ethylene treatment) and corollas (16 h post air
130 treatment) were harvested for RNA isolation. Shotgun libraries were constructed and
131 sequenced on an Illumina High-Seq 2000 platform according to the manufacturer’s
132 instructions (Illumina, San Diego, CA, USA). In total, ~247.25 million paired-end reads with
133 read lengths of 100 bp were generated (SM Table S1). After quality checks, adapter trimming,
134 and size selection, de novo assembly was performed using Trinity. A final high-quality
135 dataset of 72,249 unigenes longer than 200 bp with an average length of 820 bp and an N50
136 of 1,379 bp was obtained (SM Table S2; SRA accession: SRP077541).

137 To perform functional annotation of the petunia transcriptome, the unigene sequences were
138 BLAST searched against the NCBI non-redundant (Nr) protein database and the SwissProt,
139 COG and KEGG protein databases with a cutoff E value of 10^{-5} . A total of 41,035 unigenes
140 (56.8% of the total assembled unigenes) were aligned to the four protein databases (SM Table
141 S3; SM Fig. S2). The 40,341 predicted amino acid sequences of the unigenes are shown in
142 Supplementary Data (SD) Exc1 Sheet1. Tandem mass spectra were searched against these
143 sequences to analyze the proteome and ubiquitylome, the analysis of which we focused on in
144 this study.

145 **Ethylene treatment alters the transcriptome in petunia corollas**

146 To quantify the expression levels of the transcripts of 16-h ethylene and air treatment
147 corollas, HTseq was used to count the read numbers mapped to each gene based on the
148 72,249 genes in the petunia reference transcriptome. These data were then normalized to
149 reads in a given unigene per million mapped reads (RPKM). A total of 51,799 unigenes
150 available for both ethylene and air treatment were analyzed. This analysis indicated that
151 20,751 unigenes were differentially expressed (absolute log-fold change greater than one
152 and False Discovery Rate (FDR) <0.001), including 14,448 (69.6%) down-regulated and
153 6,303 (30.4%) up-regulated unigenes, whereas 31,048 unigenes were not differentially
154 expressed. Of the 20,751 differentially expressed unigenes (DEGs), 15,472 DEGs were
155 annotated, including 10,753 down-regulated and 4,719 up-regulated unigenes after ethylene
156 treatment (SD Exc2 Sheet1). Previous studies showed that ethylene treatment resulted in 935
157 down-regulated and 1,666 up-regulated genes in the auxiliary bud tissue of soybean (*Glycine*
158 *max*) (Prayitno et al., 2006), and ethylene treatment resulted in 331 (50%) down-regulated
159 and 330 (50%) up-regulated genes in *Citrus reticulata* fruits (Mayuoni et al., 2011), which
160 suggested a differential impact of ethylene on different species and tissues or differences
161 attributable to ethylene treatment time or concentration.

162 To investigate the influence of the DEGs on pathways, statistical pathway enrichment
163 analysis of ethylene and air treatment corollas was performed based on the KEGG database
164 using FoldChange and FDR. The DEGs from 16-h ethylene and air treatment corollas were
165 enriched in 22 KEGG metabolic pathways (SD Exc2 Sheet2). The top ten $P < 0.05$
166 metabolic pathways of the DEGs in ethylene and air treatment corollas were: Plant hormone
167 signal transduction, Photosynthesis, Carotenoid biosynthesis, Inositol phosphate metabolism,
168 Photosynthesis-antenna proteins, Homologous recombination, Ubiquinone and other
169 terpenoid-quinone biosynthesis, Flavonoid biosynthesis, Phenylalanine, tyrosine and
170 tryptophan biosynthesis.

171 Significant pathway enrichment analysis showed that plant hormone signal transduction was
172 the most important pathway in the Eth vs. Air comparison, and plant hormone signal
173 transduction was the key biological event. Plant hormone signal transduction is very
174 important for hormone-induced biochemical changes during plant growth, development, and

175 environmental information processing pathways. A previous study showed that ethylene
176 interacts with plant hormones at different levels to form a network of signaling pathways
177 connected by antagonistic and synergistic interactions (Sun et al., 2006; Stepanova et al.,
178 2007). Our evidence indicated that the genes involved in plant hormone signal transduction
179 play important roles in ethylene-induced senescence in petunia corolla.

180 **Confirmation of DEG data by qRT-PCR**

181 To confirm the results of the gene expression analysis obtained using DEG data,
182 transcriptional regulation revealed by RNA-Seq was assessed in a biologically independent
183 experiment using quantitative real-time PCR (qRT-PCR). We randomly selected 20 genes as
184 candidate genes. The results for the 20 candidate genes are shown in SM Fig. S3. Overall,
185 the qRT-PCR data were in agreement (pair-wise correlation coefficient of 0.87,
186 $P=5.1092E-7$) with the DEG results. Thus, our data showed that the DEG technique for
187 counting transcripts reflects transcript abundance and can be used for gene expression
188 analysis in an organism lacking genome information.

189 **Ethylene treatment changes the proteome profile in petunia corollas**

190 To examine the whole proteome in corollas in response to ethylene, three biological
191 replicates were analyzed for each treatment. In total, 5,189 protein groups were identified
192 from petunia, among which 3,606 proteins were quantified. A total of 233 proteins were
193 up-regulated and 284 proteins were down-regulated (with a threshold of 1.5-fold) in
194 response to ethylene ($P < 0.05$) with a high degree of repeatability (SD Exc3 Sheet1-2).

195 To elucidate the functional differences between the down-regulated and up-regulated
196 proteins, the quantified proteins were analyzed for GO enrichment based on clustering
197 analysis (SM Fig. S4; SD Exc4 Sheet1-3). In the cellular component category, many of the
198 down-regulated proteins were enriched in the ribosome and ribosomal subunit category,
199 whereas the up-regulated proteins were not enriched in any cellular component category. In
200 iris, one of the earliest ultrastructural senescence symptoms is the loss of the majority of
201 ribosomes (Van Doorn et al., 2003). In harvest-induced senescence in detached *Arabidopsis*
202 plants, genes involved in ribosome biogenesis and assembly are down-regulated (Chang et

203 al., 2015). These results suggest that protein processing might be suppressed during
204 senescence in plants.

205 In terms of biological processes, a large portion of the up-regulated proteins were highly
206 enriched in the heterocycle catabolic process, cellular nitrogen compound catabolic process,
207 aromatic compound catabolic process, disaccharide metabolic process, organic cyclic
208 compound catabolic process, sucrose metabolic process, and others. In petunia, it has been
209 found that elements such as carbon, nitrogen, phosphorus, potassium and some metal ions
210 are reduced in corollas during pollination-induced senescence (Paul and Frigerio, 2007).
211 These results suggest that a different nutrient remobilization program operates during
212 pollination- or ethylene-induced senescence. Moreover, it has been shown that
213 carbohydrates are primarily transported in the phloem during petal senescence (van Doorn
214 and Woltering, 2008). In our results, down-regulated proteins were enriched in the organ
215 nitrogen compound biosynthetic process, aromatic amino acid family metabolic process,
216 aromatic amino acid family biosynthetic process, cellular amino acid biosynthetic process,
217 small molecule biosynthetic process, organic acid biosynthetic process, carboxylic acid
218 biosynthetic process, aromatic compound biosynthetic process, and others. These results
219 suggest that ethylene treatment likely promotes many catabolic processes while inhibiting
220 certain biosynthetic processes, suggesting an intrinsic role for ethylene as a senescence
221 enhancer.

222 The analysis of molecular functions showed that many of the up-regulated proteins were
223 highly enriched for the following: oxidoreductase activity, acting on paired donors, iron ion
224 binding, transferase activity, hexosyl groups, transition metal ion binding, cysteine-type
225 peptidase activity, UDP-glucosyltransferase activity, sucrose synthase activity, heme
226 binding, transferase activity, transferring glycosyl groups, tetrapyrrole binding,
227 glucosyltransferase activity, and UDP-glycosyltransferase activity. The down-regulated
228 proteins were enriched in transferase activity, transferring alkyl or aryl groups, structural
229 constituent of ribosome, methionine adenosyltransferase activity, and
230 3-deoxy-7-phosphoheptulonate synthase activity. The term transferase activity was observed
231 to occur among both up-regulated and down-regulated proteins in the ontology of molecular

232 functions, suggesting the impact of ethylene on protein modification and the important role
233 of protein modification during corolla senescence in petunia.

234 **Comparative analysis of proteome and transcriptome data**

235 To compare the proteome with the transcriptome, all significantly differentially expressed
236 mRNAs were first matched with quantifiable proteins (SD Exc5 Sheet1), and then the
237 proteins were compared with their cognate mRNAs by sorting the proteins according to their
238 Eth/Air ratio. A positive correlation of $r = 0.39$ was observed when all significantly changed
239 mRNAs with a cognate protein were considered, regardless of the direction of the change
240 (SM Figs. S5A, S5F). Restricting the analysis to pairs in which the mRNA was up-regulated
241 markedly increased the correlation ($r = 0.49$; SM Figs. S5B, S5F), while no correlation ($r = 0.08$)
242 between transcript and protein abundance was observed for transcripts with significantly
243 decreased abundance upon ethylene treatment (SM Fig. S5C). This indicates that, contrary to
244 expectations, the vast majority of the down-regulated mRNAs were not associated with
245 lower-abundance proteins. For protein/mRNA pairs in which the protein was significantly
246 up-regulated, the highest positive correlation ($r = 0.53$) between the two levels was calculated
247 (SM Figs. S5D, S5F). A weak positive correlation was observed between protein and mRNA
248 for significantly down-regulated proteins ($r = 0.21$) (SM Figs. S5E, S5F).

249 Numerous reports have suggested that RNA transcript accumulation is not always conveyed
250 to the final product-protein (Shemesh-Mayer et al., 2015). For example, a negative correlation
251 between mRNA and protein accumulation patterns was found in Arabidopsis in response to
252 cold treatment (Nakaminami et al., 2014). The lack of correlation between mRNA and protein
253 levels has been attributed to differences in translational efficiency, codon usage/bias, and
254 mRNA versus protein stability, post translational modifications, sequencing depth and
255 proteomic approach (Alberch, 1991; Gygi et al., 1999; Pigliucci, 2010; Ghazalpour et al.,
256 2011; Rodrigues et al., 2012). In this study, the number of mRNA copies in the sample and
257 the subcellular localization of the protein restricted the number of identified proteins relative
258 to the detection of their cognate transcripts (SM Fig. S6). Comparing the number of reads
259 recorded for transcripts corresponding to identified and not identified proteins, a transition is
260 reached at around 20 reads, under which the products of the majority of transcripts was not

261 detected (SM Fig. S6A). In addition, proteins tightly associated with membranes are
262 underrepresented in the pool of identified proteins relative to the predicted proteome (SM Fig.
263 S6B).

264 **Ethylene treatment changes the ubiquitylome profile in petunia corollas**

265 Ubiquitination is a post-translational mechanism that is important for protein quality control,
266 DNA repair, cell survival and cell death in eukaryotes (Kerscher et al., 2006). Ethylene is an
267 important senescence hormone and has been observed to induce a drop in protein content. In
268 previous studies, ubiquitin E3 ligase was found to be closely related to ethylene in plants
269 (Potuschak et al., 2003; Xu et al., 2007; Qiao et al., 2009); therefore, the effects of ethylene
270 treatment on the protein ubiquitylome were investigated in this work.

271 Proteome-wide enrichment of ubiquitination is based on its distinct di-glycine remnant
272 (K-ε-GG). In this work, we combined label-free immunoaffinity enrichment using a
273 high-quality anti-K-ε-GG antibody (PTM Biolabs) and high-resolution mass spectrometry to
274 quantify protein ubiquitination in petunia corollas with and without ethylene treatment. In
275 total, after obtaining three replicates for each treatment, 3,263 lysine ubiquitination (Kub)
276 sites in 1,611 protein groups were identified, among which 2,270 sites in 1,221 proteins
277 were accurately quantified, possessing consistent quantification ratios in at least two of the
278 three LC-MS/MS analyses. From these, 127 (28.4%) sites in 118 proteins were quantified as
279 down-regulated targets, and 320 (71.6%) sites in 246 proteins were quantified as
280 up-regulated targets at a threshold of 1.5 ($P < 0.05$) (SD Exc6 Sheet1 and Sheet2). These
281 results suggested that ethylene treatment greatly increased the level of ubiquitination in
282 petunia corollas.

283 To elucidate the functions of the proteins that underwent ubiquitination, KEGG pathway
284 analysis was performed. A number of vital pathways, including those related to the
285 spliceosome, RNA transport, mRNA surveillance pathway, endocytosis and ABC
286 transporters, were enriched among proteins with lysine ubiquitination (Kub) sites (SD Exc7
287 Sheet1). These results suggested that ubiquitination might be highly associated with RNA
288 metabolism, endocytosis and ABC transporters. Alternative pre-mRNA splicing is thought to

289 provide a mechanism to increase the complexity of the proteome and introduce additional
290 layers to regulate gene expression in different cell types and during development (Zhou and
291 Fu, 2013). A previous study showed that the ubiquitination of histone H2B modulates
292 spliceosome assembly and function in budding yeast (Zhou and Fu, 2013). The
293 ubiquitination of proteins associated with the spliceosome may change the alternative
294 pre-mRNA splicing that takes place during corollas senescence.

295 To elucidate the functional differences between proteins with up-regulated and
296 down-regulated ubiquitination, enrichment-based clustering analyses were performed (Fig. 3;
297 SD Exc7 Sheet2-8). In the cellular component analysis, we found that proteins associated
298 with vesicles were highly enriched among proteins with down-regulated Kub sites. Coated
299 vesicles represent vital transport intermediates in all eukaryotic cells (Paul and Frigerio,
300 2007). The down-regulated ubiquitination of proteins associated with vesicles may play
301 important roles in cell death or senescence. Conversely, proteins with up-regulated Kub sites
302 were observed in the nucleosome, DNA binding complex, DNA packaging complex, and
303 protein-DNA complex. The degradation of nucleic acids by specific nucleases during flower
304 senescence has been observed in various flower systems, and a range of transcription factors
305 have been found to be differentially regulated during development and senescence in various
306 flower systems (Shahri and Tahir, 2014). These results suggest that ubiquitination might
307 play an important role in the nucleus, including in transcription regulation and DNA repair,
308 during ethylene-mediated senescence in petunia.

309 In the biological process analysis of ubiquitination, up- and down-regulated Kub proteins
310 were enriched in 28 processes, including proteasome-mediated ubiquitin-dependent protein
311 catabolic process, proteasomal protein catabolic process, and others, implying that
312 ubiquitinated proteins may be involved in a wide range of biological processes in plants (Figs.
313 3A and 3B).

314 In the molecular function analysis, proteins with binding activity, catalytic activity, and
315 transporter activity were enriched among proteins containing both up-regulated and
316 down-regulated Kub sites. Previous studies have shown that ions and amino acids are

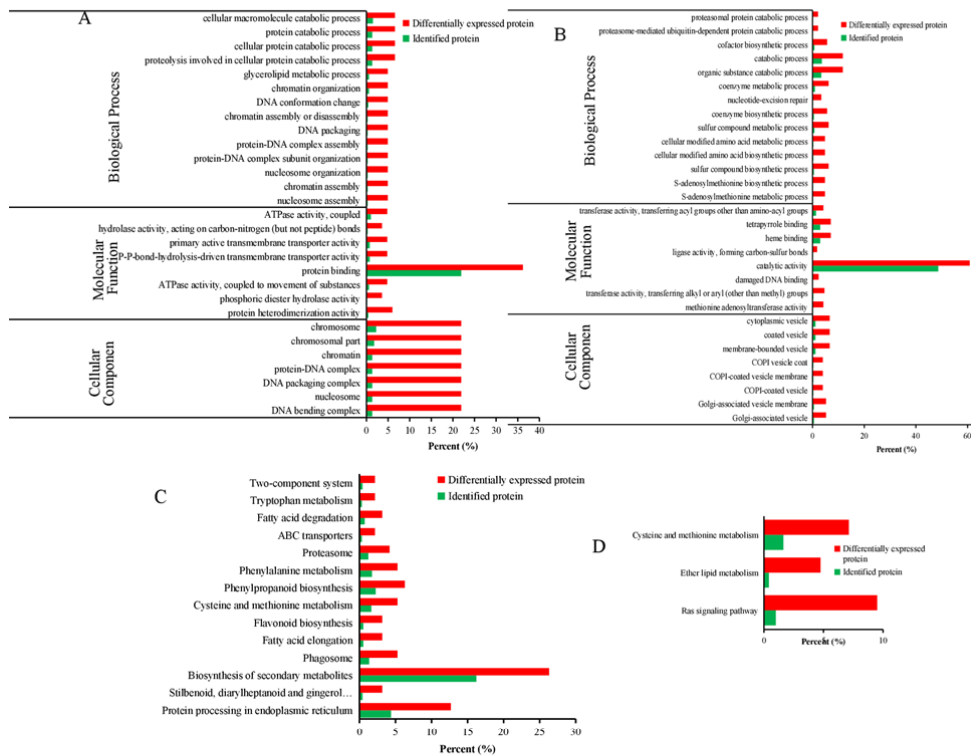


Figure 3. Functional enrichment analysis of proteins with up-regulated and down-regulated Kub sites. A and B, GO-based enrichment analysis of proteins with up-regulated (A) and down-regulated (B) Kub sites. C and D, KEGG pathway-based enrichment analysis of proteins with up-regulated (C) and down-regulated (D) Kub sites. The percent of differentially expressed proteins indicates the ratio of the mapping proteins to all mapping proteins. The percent of identified proteins indicates the ratio of the background proteins to all background proteins. The significance level was set at $P < 0.05$ (Fischer's exact test). The data come from the SD Exc7 Sheet2-5.

1

317 transferred to vegetative organs during senescence in unpollinated petunia petals (Shibuya et
 318 al., 2013). These results suggested that proteins demonstrating changes in ethylene-mediated
 319 ubiquitination are connected to protein interactions, DNA transcription, and ion and protein

320 transport.

321 KEGG pathway analysis of proteins whose ubiquitination quantitatively changed revealed a
322 number of vital pathways. The protein processing pathways in the endoplasmic reticulum,
323 stilbenoid and diarylheptanoid biosynthesis, phagosome, fatty acid elongation, flavonoid
324 biosynthesis, cysteine metabolism, methionine metabolism, phenylpropanoid biosynthesis,
325 phenylalanine metabolism, proteasome, ABC transporters and others were enriched among
326 proteins with up-regulated Kub sites. Proteins with down-regulated Kub sites were enriched
327 in pathways involving Ras signaling, ether lipid metabolism, cysteine metabolism,
328 methionine metabolism and others (Figs. 3C, 3D). These results indicate that ubiquitination
329 was associated with protein processing, protein degradation and secondary metabolites.

330 From protein domain analysis, we observed that protein domains associated with
331 S-adenosylmethionine synthetase, Ubiquitin-like, NmrA, and Small GTP-binding, and
332 others were enriched in proteins with up-regulated Kub sites, whereas histone core and
333 histone-fold, ubiquitin-like, zinc finger and others protein domains were enriched in
334 down-regulated quantiles (SD Exc7 Sheet6-7). We also identified 27 Kub sites in 14 histones,
335 including in H1D, H1.2, H2B, H2A, H3, H4 and various histone isoforms, in this study,
336 among which 16 sites in 10 histones were quantified (SD Exc7 Sheet8). The ubiquitination
337 levels of 6 Kub sites in 5 histones decreased. Five Kub sites were even down-regulated by
338 over 10-fold, whereas no up-regulated Kub sites were identified, suggesting that ethylene
339 negatively regulates the ubiquitination of histones and may play critical roles in regulating
340 many processes within the nucleus, including transcription initiation and elongation,
341 silencing, and DNA repair, by decreasing the ubiquitination levels of histones in petunia
342 corollas. In *Drosophila*, *Tetrahymena* and mammalian cells, the ubiquitylated forms of
343 histones H2A and H2B were associated specifically with actively transcribed genes, making
344 histone ubiquitination one of the first markers of transcriptionally active chromatin to be
345 recognized (Muratani and Tansey, 2003).

346 **Sequence Properties of Ubiquitinated Proteins**

347 To understand the properties of the identified Kub sites in petunia, we used the Motif-X

348 program to compare the position-specific frequencies of the amino acid residues surrounding
349 all ubiquitinated lysine residues.

350 Of the 3,265 Kub peptides, we identified a total of five conserved motifs for 1,373 unique
351 sites, which accounted for approximately 42% of the sites identified (SD Exc8 Sheet1 in
352 Supporting Data). The five unique sites were
353 designatedEK.....,E...K.....,KD.....,KE.....,
354 andK..E....., and they exhibited different abundances (. indicates any amino acid) (Fig.
355 4A). Among them,EK..... has been reported previously (Xie et al., 2015), while the
356 other four motifs are novel (Fig. 4B, red column), which may provide insight into ethylene
357 signaling in petunias, as well as in plants in general. A survey of these motifs revealed that
358 only two distinct residues are found upstream or downstream of the ubiquitinated lysine (Fig.
359 4A), including acidic aspartic acid (D) and glutamic acid (E), whereas in rice, only neutral
360 alanine (A) and acidic glutamic acid (E) were observed surrounding ubiquitinated lysines (Xie
361 et al., 2015). These results show the differences in ubiquitinated lysine motifs between
362 dicotyledon petunias and monocotyledon rice.

363 To further examine the properties of amino acids surrounding ubiquitination sites, the
364 frequencies of neighboring amino acid residues were analyzed for ubiquitinated lysines using
365 iceLogo (Colaert et al., 2009). We observed a significant preference for hydrophilic residues
366 such as Glu and Asp at positions adjacent to ubiquitinated lysines (+1, +3, -1, and -3) (Fig.
367 4C). In mammals, a significant preference for hydrophobic residues, such as Phe, Tyr, Trp,
368 Leu, Ile, and Val, adjacent to ubiquitinated lysines has been observed (Wagner et al., 2011).
369 These results indicate the different properties of amino acids surrounding ubiquitination sites
370 when comparing plants and mammals.

371 In addition to primary sequences around Kub sites, protein secondary structure has been
372 found to be informative in Kub site prediction (Gnad et al., 2011). Therefore, we integrated
373 protein secondary structure features using NetSurfP software (Muller et al., 2010). The
374 probabilities of different secondary structures (coil, α -helix, and β -strand) near ubiquitinated
375 lysine sites were compared with the secondary structure probabilities of all lysine sites on

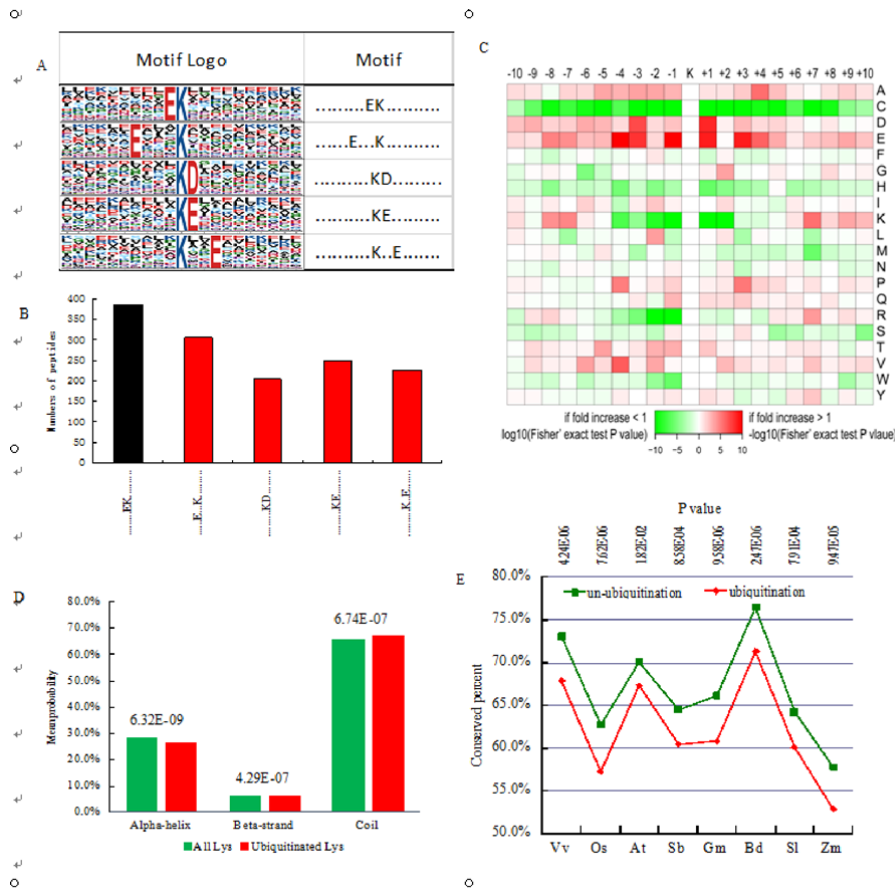


Figure 4. Motif analysis of all the identified Kub sites in petunia. A, Ubiquitination motifs and the conservation of Kub sites. The height of each letter corresponds to the frequency of that amino acid residue in that position. The central K refers to the ubiquitinated lysine. B, The number of identified peptides containing ubiquitinated lysine in each motif. The red columns represent novel motifs. C, Amino acid sequence properties of ubiquitylation sites. The heat map shows significant position-specific under- or over-representation of amino acids flanking the modification sites. D, Predicted protein secondary structures near Kub sites. Probabilities for different secondary structures (coil, α -helix and β -strand) of modified lysines were compared with the secondary structure probabilities of all lysines or all Ser/thr/Tyr on all proteins identified in this study. E, Evolutionary conservation of ubiquitylated and nonubiquitylated lysines on protein orthologs in selected eukaryotic species. Abbreviations: Vv, *Vitis vinifera*; Os, *Oryza sativa japonica*; At, *Arabidopsis thaliana*; Sb, *Sorghum bicolor*; Gm, *Glycine max*; Bd, *Brachypodium distachyon*; Sl, *Solanum lycopersicum*; Zm, *Zea mays*.

1

376 proteins identified in this study. Ubiquitinated lysine sites occurred significantly more
 377 frequently in unstructured regions of proteins ($p=6.74E-07$ for coil) and less frequently in
 378 structured regions ($p=6.32E-09$ for α -helix and $p=4.29E-07$ for β -strand) (Fig. 4D). However,

379 in mammals, ubiquitinated lysines are marginally, yet significantly, more frequently present in
380 structured regions of proteins than in unstructured regions (Wagner et al., 2011), indicating a
381 difference in ubiquitinated lysine sites between plants and mammals.

382 In mammals, ubiquitinated lysine is significantly more conserved than non-ubiquitinated
383 lysine (Wagner et al., 2011). To study the evolutionary conservation of ubiquitinated lysine
384 and non-ubiquitinated lysine in plants, we aligned petunia proteins with their respective
385 orthologues from 8 other plant species. The results unexpectedly showed that ubiquitinated
386 lysines are significantly less conserved than non-ubiquitinated lysines, suggesting that
387 ubiquitinated lysines do not maintain a stronger selective pressure compared with
388 non-ubiquitinated lysines in plants (Fig. 4E). It appears that ubiquitination primarily occurs in
389 non-conserved lysine positions in petunia corollas, and further experiments are required to
390 validate this possible evolutionary mechanism.

391 **The correlation between the global proteome and ubiquitylome**

392 Ubiquitination is well known for its role in proteasome-mediated protein degradation. The
393 expression of proteins in corollas may also be regulated by ubiquitination. In this work,
394 among the 5,189 proteins identified, 1,161 were ubiquitinated (SM Fig. S8). The quantitative
395 proteome and ubiquitylome of ethylene-treated corollas were both obtained to study the
396 interaction between the proteome and ubiquitylome.

397 The correlation between the whole proteome and ubiquitylome during senescence in corollas
398 was analyzed based on the quantitative results obtained in this study. There were 985
399 quantified proteins that were also found to undergo ubiquitination, and 2,270 Kub sites in
400 1,221 proteins were quantified. Of the 985 quantified proteins, 66 proteins were
401 down-regulated and 96 were up-regulated. Quantitative ratios from the proteome and
402 ubiquitylome were compared upon ethylene treatment, as shown in Fig. 4. Pearson's
403 correlation coefficient, a statistical measure of the strength of a linear relationship between
404 paired data, is denoted by r and is by design constrained between -1 and 1. Positive values
405 denote positive linear correlation, negative values denote negative linear correlation, and a
406 value of 0 denotes no linear correlation. The closer the value is to 1 or -1, the stronger the

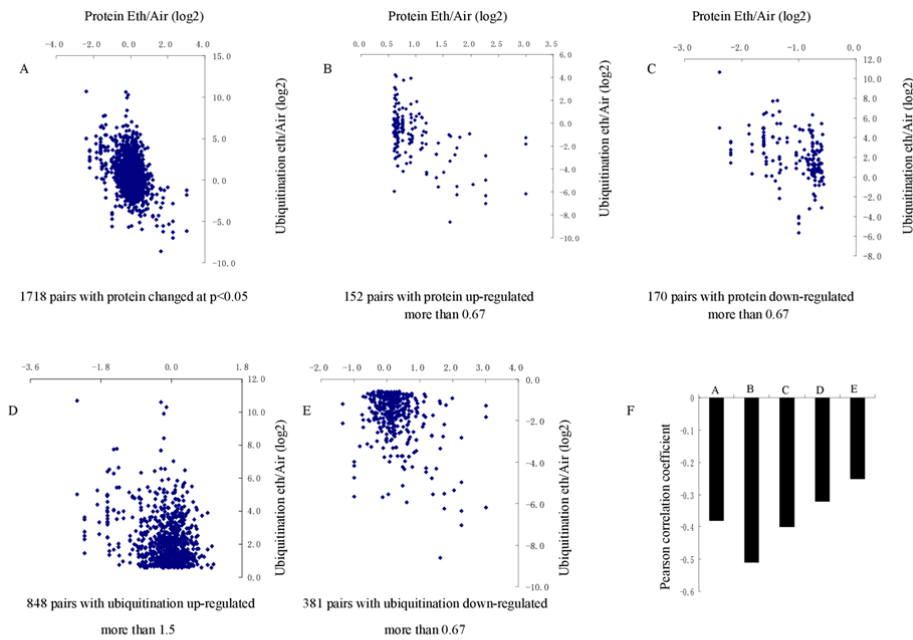


Figure 5. Concordance between changes in proteins and their ubiquitination. A–E, Correlation between protein and ubiquitination fold-changes upon ethylene treatment for all ubiquitination/protein pairs A, significantly up-regulated proteins B, significantly down-regulated proteins C, significantly up-regulated ubiquitination D, significantly down-regulated ubiquitination E. F, Pearson correlations of the comparisons shown in A–E.

1

407 linear correlation. The Pearson's correlation coefficient was calculated as -0.38 when all
 408 significantly altered proteins were considered in terms of their ubiquitination, regardless of
 409 the direction of the change (Figs 5A, 5F). In addition, the overlap between differentially

410 expressed proteins and ubiquitination is shown in Fig. 4B; SD Exc9 Sheet1-7. A total of 67
411 proteins exhibited opposing changes in protein and ubiquitination levels, whereas only 10
412 proteins demonstrated consistent changes. Therefore, the global proteome and ubiquitylome
413 were negatively correlated, which implies that, to a certain extent, the changing pattern of the
414 proteome was opposite that of the ubiquitylome following ethylene treatment. Restricting the
415 analysis to pairs of up-regulated proteins and pairs of down-regulated proteins increased the
416 correlation ($r=-0.51$ and -0.4 , respectively; Figs. 5B, 5C, 5F). For ubiquitination/protein pairs
417 with significantly up-regulated and with significantly down-regulated ubiquitination, two
418 weak negative correlations were observed ($r=-0.32$ and -0.25 , respectively; Figs. 5D, 5E, 5F).
419 These results suggested that proteome expression levels were negatively regulated by
420 ubiquitination.

421 It should be noted that the ubiquitylome reveals the status of proteins that are ubiquitinated
422 but not those already subjected to 26S proteasome degradation because these degraded
423 proteins will not be detectable in the ubiquitylome. Thus, the ubiquitylome does not truly
424 reflect the status of protein degradation. If ones takes into account these proteins already
425 subjected to 26S proteasome degradation, the ubiquitylome value is higher than the present
426 total value; however, this does not change the conclusion regarding the negative correlation
427 between the global proteome and ubiquitylome but rather supports this conclusion. In addition,
428 aside from proteasome-mediated degradation, ubiquitination has many other roles in protein
429 modification, such as altering biochemical properties and subcellular protein localization
430 (Shabek and Zheng, 2014); this partially explains why the negative correlation observed
431 between the proteome and ubiquitylome was not very strong.

432 Several spectra corresponding to sites from proteins that undergo ubiquitination are presented
433 in SM Fig. S9.

434 **Involvement of ubiquitination in the degradation of proteins during ethylene-mediated** 435 **corolla senescence in petunias**

436 The degradation of proteins in developing tissues is a notable process during senescence
437 (Shahri and Tahir, 2014). In the transcriptome obtained in this study, 144 unigenes encoding

438 putative ubiquitin-protein ligases (35 E3 ubiquitin-protein ligases, 72 F-box protein and 37
439 U-box proteins), 6 unigenes encoding ubiquitin proteins and 7 unigenes encoding 26S
440 proteasome subunits up-regulated by ethylene were identified (SD Exc10 Sheet1-5). In the
441 proteome, ethylene treatment resulted in 284 down-regulated and 233 up-regulated proteins,
442 and among them, four putative ubiquitin ligases were up-regulated (SD Exc11 Sheet1).
443 Moreover, 246 quantified proteins also underwent ubiquitination, and their up-regulated Kub
444 sites were identified; among them, 44 proteins were down-regulated, and only 8 proteins
445 were up-regulated with respect to protein concentration. In addition, 118 quantified proteins
446 underwent ubiquitination, and their down-regulated Kub sites were identified in this study;
447 among these, 23 proteins were up-regulated, and only 2 proteins were down-regulated with
448 respect to protein concentration following ethylene treatment (SD Exc9 Sheet1). Of the 18
449 ubiquitinated proteins identified only in the control, 17 were up-regulated and only one was
450 down-regulated by ethylene at the protein level, while of the 11 ubiquitinated proteins
451 identified only in corollas following ethylene treatment, 9 were down-regulated and only 2
452 were up-regulated by ethylene at the protein level (SD Exc9 Sheet1). Silencing the
453 expression of a gene homolog to *MjXB3* in petunia resulted in an extension in flower life
454 (Xu et al., 2007). Proteomic analysis of pollination-induced corolla senescence in petunia
455 identified a ubiquitin-conjugating enzyme (E2) that was up-regulated by pollination,
456 accelerating flower senescence (Bai et al., 2010). These results indicate the involvement of
457 ubiquitination in protein degradation during ethylene-mediated corolla senescence in petunia.
458 In addition, the proteasome system was apparently up-regulated during petal senescence in
459 daylily (Courtney et al., 1994; Müller et al., 2004) and daffodil (Hunter et al., 2002). In
460 carnation, several transcripts homologous to genes encoding various components of the 26S
461 proteasome machinery, including RPT6, RPN2, a RING finger protein and a U-box
462 containing protein, were all induced during carnation petal senescence (Hoeberichts et al.,
463 2007). Feeding isolated Iris petals with Z-Leu-Leu-Nva-H, an inhibitor of proteasome
464 activity, led to a significant delay in the time to visible senescence (Pak and van Doorn,
465 2005), indicating that proteasome action is limiting senescence. In addition, Arabidopsis
466 UPL5, a HECT E3 ubiquitin ligase, negatively regulates leaf senescence through
467 degradation of WRKY53 and ensures that senescence is executed in the correct time frame

468 (Miao and Zentgraf, 2010).

469 To elucidate the function of proteins with opposite trends in protein and ubiquitination levels,
470 KEGG pathway enrichment-based clustering analyses were performed (SM Fig. S7). The
471 protein processing pathways in the flavonoid biosynthesis, phenylalanine metabolism,
472 phenylpropanoid and secondary metabolites biosynthesis and others were enriched among
473 proteins with up-regulated Kub sites and down-regulated protein levels. Previous studies
474 suggested that ethylene treatment reduced the biosynthesis of phenylpropanoid and
475 secondary metabolites in petunia (Negre et al. 2003; Underwood et al., 2005; Schuurink et
476 al., 2006). It is possible that ubiquitination could be involved in degradation of the proteins
477 in these pathways during ethylene-mediated flower senescence. Proteins with
478 down-regulated Kub sites and up-regulated protein levels were enriched in pathways
479 involving SNARE interaction in vesicular transport and galactose metabolism.

480 The canonical view of protein ubiquitination posits that the entire pool of a targeted protein
481 becomes ubiquitinated and is subsequently degraded. However, Kim et al. (2011) and
482 Swaney et al. (2013) showed that most cases of increased ubiquitination were not
483 accompanied by corresponding reductions in protein abundance. Similarly, in this study, 221
484 and 96 proteins demonstrating increased and decreased ubiquitination, respectively, were not
485 accompanied by corresponding reductions and increases in protein abundance. One
486 reasonable explanation is that complex signaling may be at play, in which specific Kub sites
487 are utilized as degradation markers, whereas others serve to modulate protein function.

488 The regulatory pathways in flower senescence were divided into three phases: the signaling
489 phase, regulatory phase and execution phase (Tripathi and Tuteja, 2007). Protein
490 degradation, as well as the hydrolysis of nucleic acids, lipids and carbohydrates, takes place
491 in the execution phase (Tripathi and Tuteja, 2007). Our results suggested that the
492 involvement of ubiquitination in the degradation of proteins during ethylene-mediated corolla
493 senescence in petunias. Taken together, the large amounts of protein ubiquitination underlie
494 corolla senescence. Moreover, *PhXB3* silencing delayed flower senescence in petunia (Xu et
495 al., 2007).

496 **Involvement of non-proteasomal proteases in the degradation of proteins during**
497 **ethylene-mediated corolla senescence in petunias**

498 The activity of non-proteasomal protease has been found to increase prior to visible
499 senescence (Stephenson & Rubinstein, 1998; Pak & van Doorn, 2005). Of these proteases,
500 cysteine proteases have been exclusively reported to be involved and thought to mediate
501 remobilization of essential nutrients from senescing floral tissues. In this study, in the
502 transcriptome, 37 non-proteasomal proteases, including 6 cysteine proteases, 3
503 metalloproteases, 2 serine proteases, 3 subtilisin proteases, and 9 aspartic proteases, were
504 up-regulated by ethylene in petunia corollas (SD Exc12 Sheet1). Proteomic analysis showed
505 that three cysteine proteases, two metalloproteases, and one aspartic proteinase were
506 up-regulated by ethylene in this study (SD Exc11 Sheet1). Cysteine protease genes have
507 been reported to be up-regulated during senescence in petunia (Jones et al., 2005). These
508 results implied that non-proteasomal proteases, including cysteine proteases,
509 metalloproteases and aspartic proteinases, are likely also involved in the degradation of
510 proteins during ethylene-mediated corolla senescence in petunias.

511 **Changes of the autophagy proteins after ethylene treatment**

512 Autophagy is one of the main mechanisms of degradation and remobilization of
513 macromolecules (Shahri and Tahir, 2011). Shibuya et al (2013) suggested that ethylene is a
514 key regulator of autophagy in petal senescence of petunia. Ethylene inhibitor treatment in
515 pollinated flowers delayed the induction of homologues of autophagy-related gene
516 (*PhATG8*), and ethylene treatment rapidly up-regulated *PhATG8* homologues in petunia
517 petals. Arabidopsis *AtATG8* mRNA levels increase in senescing leaves (Doelling et al., 2002;
518 Yoshimoto et al., 2004; Thompson et al., 2005). In Arabidopsis, a number of autophagy
519 genes (ATG) had been knocked out, which resulted in hastened leaf yellowing (Hanaoka et
520 al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005). In this study,
521 PhATG8b (Unigene0018716) and PhATG11 (Unigene0069693) were increased in protein
522 level after ethylene treatment. In addition, PhATG18H (Unigene0007523), PhATG3
523 (Unigene0031140), and PhATG2 (Unigene0011829) were identified. No autophagy-related
524 protein down-regulated was identified (SD Exc13 Sheet1). These results suggested

525 autophagy occurs during senescence of corollas, is promoted by ethylene and plays an
526 important role in petal senescence.

527 In mammal and yeast, two ubiquitin-like systems, the autophagy-defective 12 (Apg12)
528 system and the Apg8 system, are required for autophagy (Ohsumi, 2001). Phosphorylation
529 and ubiquitination were crucial for autophagy induction, regulation and fine-tuning, and
530 were influenced by a variety of stimuli (McEwan and Dikic, 2011). In this study, for the first
531 time, the ubiquitination of ATG8b (K11), a ubiquitin-like protein, were up-regulated by
532 3.486-fold by ethylene, suggesting that ubiquitination could be involved in ethylene-induced
533 autophagy in plant.

534 **Effects of ethylene treatment on hormone biosynthesis and signaling transduction** 535 **pathways**

536 S-AdoMet, a precursor for ethylene biosynthesis and polyamine synthesis, is the methyl group
537 donor for many cellular molecules, including nucleic acids, proteins, and lipids (Yang and
538 Hoffman, 1984; Schuurink et al., 2006). The formation of S-AdoMet is catalyzed by SAM
539 synthetases (SAMS). In this study, we found 11 Kub sites in 5 SAMSs (PhSAMS1a,
540 Unigene0023828, K169, K175, K226, K340; PhSAMS3a Unigene0028250, K78;
541 PhSAMS3b, Unigene0028252, K67, K364; PhSAMS1b, Unigene0023825, K94; PhSAMS1c,
542 Unigene0023827, K67, K71, K120) that were significantly up-regulated by ethylene. Among
543 them, 8 Kub sites were up-regulated by more than 10-fold (Unigene0023828, K226, K169,
544 K175; Unigene0028250, K78; Unigene0028252, K67, K364; Unigene0023825, K94;
545 Unigene0023827, K120). Accordingly, in the proteome, the abundance of 5 SAMSs
546 (Unigene0023828, Unigene0028250, Unigene0028252, Unigene0023825, Unigene0023827)
547 decreased following ethylene treatment (Fig. 6; SD Exc13 Sheet2), suggesting that ethylene
548 negatively regulates SAM abundance. However, ethylene treatment did not result in a general
549 decrease in ethylene biosynthesis. It is possible that the SAM cycle and polyamine
550 biosynthesis are negatively regulated by ethylene.

551 ACC synthase (ACS) is the rate-limiting enzyme of ethylene synthesis. Previous research has
552 suggested that ACS family proteins are up-regulated by ethylene and that ETO1/EOL,

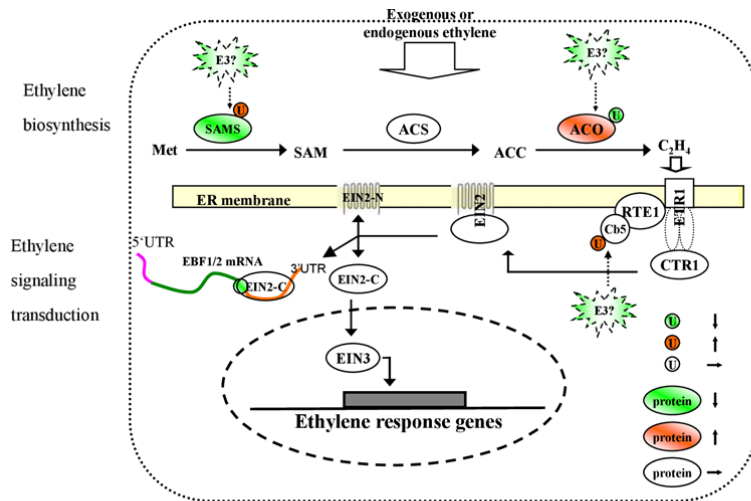


Figure 6. Effects of ethylene on the proteins engaged in ethylene biosynthesis and signaling transduction pathway in petunia. Differentially expressed proteins based on statistical significance in this study are framed in oval boxes, and differentially ubiquitinated and phosphorylated proteins have round boxes. The red box indicates up-regulation; the green box indicates down-regulation; and the blue indicates no significant changes upon ethylene treatment. Abbreviations: U, ubiquitination. ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; Cb5, cytochrome b₅; CTR1, CONSTITUTIVE TRIPLE-RESPONSE1; EIN, ETHYLENE INSENSITIVE; EIN2-C, EIN2 C end; EIN2-N, EIN2 N end; ETR1, ETHYLENE RESPONSE1; RTE1, REVERSION-TO-ETHYLENE SENSITIVITY1; SAM, S-adenosylmethionine; SAMS, S-AdoMet synthetase.

1
 553 calcium-dependent protein kinase (CDPK), 14-3-3 and mitogen-activated protein kinase
 554 (MAPK) interact with ACS family proteins, modulating their stability in plants (Xu and
 555 Zhang, 2014). However, in this study, in both protein and ubiquitination analyses, ACS

556 family proteins were not identified.

557 The discovery of two plant MAPK substrates, ACS2 and ACS6, which are two Type I ACS
558 isoforms, revealed ACS phosphorylation regulation by AtMPK3 and AMPK6, two
559 functionally redundant stress/pathogen-responsive MAPKs in Arabidopsis. In this study, two
560 Kub sites in PhMAPK6 (Unigene0025211, K57 and K95), a homolog of AtMAK6, were
561 identified. The ubiquitination levels of 14-3-3 (Unigene0024326, K48) and PhCDPK30
562 (Unigene0029654, K389, >4-fold) increased after ethylene treatment, which may maintain
563 protein abundance and promote the activity of ACS to alter their biochemical properties.

564 ACC oxidase (ACO) is another key enzyme in ethylene biosynthesis, and antisense *ACO*
565 RNA delayed flower senescence in transgenic carnations (Savin et al, 1995). In this study,
566 for the first time, the ubiquitination of PhACO3 (Unigene0022854, K41) was identified and
567 was found to be down-regulated more than 15-fold by ethylene treatment. Accordingly,
568 PhACO3 (Unigene0022854) protein levels were up-regulated following ethylene treatment,
569 suggesting ubiquitination could be involved in PhACO3 degradation and in ethylene
570 biosynthesis. In consistent with these results, ethylene production increases in corollas
571 during flower senescence in petunia (Liu et al, 2011).

572 Ethylene receptors are encoded by a multigene family that can be divided into subfamilies 1
573 and 2. Kevany et al. (2007) suggested that the receptors LeETR4 or LeETR6 were rapidly
574 degraded in the presence of ethylene and that degradation likely occurs through the 26S
575 proteasome-dependent pathway in tomato plants. In Arabidopsis, the ethylene-induced
576 decrease in ETR2 levels is not affected by cycloheximide, an inhibitor of protein
577 biosynthesis, but is affected by proteasome inhibitors, indicating a role for the proteasome in
578 ETR2 degradation (Chen et al, 2007). However, these authors did not provide direct
579 evidence of ubiquitination of ethylene receptors. In our study, a Kub site on PhETR2
580 (Unigene0010512, K359) was identified. These results suggested the involvement of
581 ubiquitination in ethylene receptors degradation and in ethylene signaling.

582 Ethylene-insensitive protein 2 (EIN2) acts downstream of ethylene receptors and upstream of
583 EIN3/EIL and is involved in the regulation of flower senescence. Qiao et al. (2009) reported

584 that the stability of EIN2 is modulated by the two F-box proteins ETP1/2 via ubiquitination,
585 but the ubiquitination of PhEIN2 was not observed in this study. In addition, it was proposed
586 that EIN3 is targeted by the F-box proteins EBF1/2 in Arabidopsis (Potuschak et al., 2003).
587 However, PhEILs, PhEBF1 and PhEBF2 were not identified in this study at either the protein
588 or ubiquitination levels.

589 A recent study showed that Arabidopsis cytochrome b₅ (Cb5) proteins are involved in
590 ethylene signaling, and REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1) physically
591 interacts with AtCb5-B, -C, -D and -E (Chang et al., 2014). The Kub sites of two Cb5s
592 (PhCb5B, Unigene0023698, K35; PhCb5E, Unigene0016038, K51) were up-regulated more
593 than 4-fold by ethylene in this study, which further supported the involvement of
594 ubiquitination in ethylene signaling in petunia.

595 Ethylene is an important regulator of flower senescence. The results mentioned above
596 illustrated protein and ubiquitination levels in ethylene biosynthesis and demonstrated that
597 signaling pathways can be regulated by ethylene. These findings, including the ubiquitination
598 of PhACO3, PhETR2, PhCb5B and PhCb5E, significantly advance our understanding of the
599 mechanisms underlying ethylene biosynthesis and signaling transduction (Fig. 6).

600 Ethylene appears to be a negative regulator of ABA action during germination, although it was
601 confirmed to exert a positive synergistic effect on ABA action by modulating the overall
602 carbon status in Arabidopsis roots (Ghassemian et al., 2000; Gazzarrini and McCourt, 2001;
603 Cheng et al., 2009). In carnations, ABA has been found to accelerate flower senescence
604 (Ronen and Mayak, 1981). A large increase in ABA levels was observed in the gynoecium
605 prior to or concomitant with the upsurge in ethylene (Onoue et al., 2000). In this study, the
606 enzymes related to ABA biosynthesis, PhDXS (Unigene0009358), PhPDS3
607 (Unigene0017870), PhNCED4 (Unigene0037462), and PhSDR (Unigene0012764), were
608 down-regulated between 1.5 and 3.0-fold at the protein level by ethylene (SM Fig. S10A; SD
609 Exc13 Sheet2). Additionally, the ABA signaling component PP2C, a major negative regulator
610 of ABA signaling, inhibits SnRK2, a positive regulator of ABA signaling, thus inhibiting
611 activation of the ABA pathway (Umezawa et al., 2010). In this study, PP2C (PhPP2C,

612 Unigene0006325; PhPP2C58, Unigene0014490) and SnRK2A (Unigene0014500) increased
613 at the protein level after ethylene treatment. These results hinted that ethylene likely
614 negatively regulates ABA biosynthesis and signaling transduction in petunia corollas. In rose
615 petals, the external application of ethylene accelerated senescence and induced a rise in
616 endogenous abscisic acid-like activity (Mayak and Halevy, 1972). In petunia, ethylene might
617 directly affect senescence in petals without requiring involvement of the ABA pathway.

618 Many components of the auxin efflux (but not influx) system have been shown to be activated
619 by PTM (Delbarre et al., 1998; Zourelidou et al., 2014). In this study, ethylene did not change
620 the abundance of proteins involved in auxin signaling or that of efflux or influx transporters.
621 However, two Kub sites on IAA/AUX repressors (PhIAA14, Unigene0023390; K26 and
622 K106) were up-regulated more than 20 and 7-fold by ethylene, respectively (SM Fig. S10B;
623 SD Exc13 Sheet2). Leitner et al. (2012) showed that ubiquitination of the PIN2 auxin carrier
624 protein governs hormonally controlled adaptation of Arabidopsis root growth. Ethylene
625 treatment significantly increased the ubiquitination level of PhPIN4 (Unigene0020360,
626 K331, K438). It is noteworthy that the auxin influx transport proteins, AUX1/LAX
627 (Unigene0019926; Unigene0070491), were ubiquitinated, and ethylene treatment
628 significantly increased the ubiquitination of PhAUX1 (Unigene0019926, K5, >5-fold). To
629 the best of our knowledge, the ubiquitination of AUX1 has not been reported previously. In
630 addition, a third class of auxin transporters includes phospho-glycoproteins (PGPs) that
631 belong to the ABCB subgroup of the ATP Binding-Cassette (ABC) transporter superfamily.
632 ABCB1 and ABCB19 have been shown to play direct roles in the cellular efflux of auxin
633 (Titapiwatanakun and Murphy, 2009). In this study, the ubiquitination level of PhABPB2
634 (Unigene0047722, K882) increased, whereas the ubiquitination level of another site in
635 PhABPB2 (K315) decreased after ethylene treatment. These results suggested that, in
636 petunia corollas, ethylene might play an important role in auxin transport, including both
637 influx and efflux. It is possible that the inhibition of auxin transport, a process that inhibits
638 senescence (Teale et al., 2006), accelerated corolla senescence.

639 In summary, during ethylene-mediated corolla senescence, ethylene appeared to affect the
640 biosynthesis and signal transduction pathways of plant hormones such as ABA, auxin, and

641 ethylene itself at the transcript, protein, and ubiquitination levels in this study. In addition, it
642 should be noted that the omics changes in this study may be directly or indirectly caused by
643 ethylene treatment.

644 **Changes of proteins involved in sucrose biosynthesis and transport after ethylene**
645 **treatment**

646 During petal senescence in *Alstroemeria* (Breeze et al., 2004) and *Iris* (Van Doorn et al.,
647 2003), the transcript abundance of a gene encoding a triose phosphate isomerase and that of
648 genes encoding sucrose synthase increased. In *Alstroemeria*, the transcripts of a gene
649 encoding a cell wall invertase also became more abundant (van Doorn and Woltering, 2008).
650 In this study, three sucrose synthases (PhSS7, Unigene0008278; PhSS6, Unigene0012766;
651 PhSS1, Unigene0025892) were increased in protein level after ethylene treatment. Two Kub
652 sites in sucrose synthases (PhSS1, K190; PhSS2, Unigene0011388, K65) were
653 down-regulated by ethylene (SD Exc13 Sheet3), which may alter the activity of sucrose
654 synthase. These data suggested an increase in sucrose synthesis in corollas after ethylene
655 treatment.

656 Petal senescence was accompanied by a high sugar concentration in the phloem (van Doorn
657 and Woltering, 2008). In order to reach the phloem, the sugars must be transferred, at some
658 point, through a membrane. Several genes encoding sugar transporters were up-regulated
659 during *Alstroemeria* and carnation petal senescence (Breeze et al., 2004; Hoeberichts et al.,
660 2007). In this study, Five Kub sites in three sugar transporters (PhERD6, Unigene0030195,
661 K277; PhSWEET10a, Unigene0064435, K28, K44K, K22; PhSWEET10b, Unigene0027205,
662 K225) were down-regulated by ethylene. PhSWEET10a and PhSWEET11
663 (Unigene0027207) were increased in protein level after ethylene treatment (SD Exc13
664 Sheet3). These data suggested that ethylene-mediated petal senescence was probably
665 accompanied by a high sugar concentration and the sugar was transported to the developing
666 tissues in petunia.

667 **Changes of proteins involved in the biosynthesis of volatile organic compounds after**
668 **ethylene treatment**

669 Petunia has become a model to study the biosynthesis and regulation of floral volatile
670 benzenoids and phenylpropanoids, which are produced from shikimate-derived
671 L-phenylalanine (Boatright et al., 2004). Several genes encoding shikimate enzymes
672 (Colquhoun et al., 2010; Maeda et al., 2010) and subsequent branched pathways have been
673 identified and characterized in petunias. Underwood et al. (2005) demonstrated that multiple
674 components of the emission of volatile benzenoids and phenylpropanoids and the transcripts
675 of genes involved in benzenoid and phenylpropanoid biosynthesis are negatively regulated
676 by ethylene in the petunia 'Mitchell'. In this study, seven of the eight enzymes related to
677 phenylalanine biosynthesis decreased at the protein level in the presence of ethylene,
678 including 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (PhDAHPS,
679 Unigene0014414), 3-dehydroquinate synthase (PhDHQS, Unigene0006116), 5-enolpyruvate
680 shikimate-3-phosphate (PhEPSPS, Unigene0021752), 3-dehydroquinate synthase
681 (Unigene0006116), and chorismate synthase (PhCS, Unigene0026072). In the
682 phenylpropanoid pathway, phenylalanine ammonia-lyase (PhPAL1, Unigene0017590,
683 PhPAL1, Unigene0035641, >3-fold), 4-coumarate:CoA ligase (Ph4CL1, Unigene0030548),
684 phenylacetaldehyde synthase (PhPAAS, Unigene0024129), acyl-activating enzyme
685 (PhAAE11, Unigene0028342) and two caffeoyl-CoA O-methyl transferases (PhCCOMT1,
686 Unigene0026144; PhCCOMT2, Unigene002614) were also down-regulated at the protein
687 level by ethylene (SM Fig. S11; SD Exc13 Sheet4). These results suggested that ethylene
688 negatively regulates the biosynthesis of phenylalanine, benzenoids and phenylpropanoids,
689 which is consistent with a previous report (Underwood et al., 2005).

690 To confirm the reduction of these proteins by ethylene treatment, specific antibodies against
691 PhCS, PhPAL1, Ph4CL1, PhAAE11, PhEPSPS proteins were prepared, and western blotting
692 was performed. The results showed that all eight proteins were reduced by ethylene
693 treatment (SM Fig. S14A), which is consistent with the iTRAQ results.

694 In the ubiquitylome, the ubiquitination levels of shikimate 5-dehydrogenase (PhSDH,
695 Unigene0001508, K114, K504, >15-fold), cinnamate-4-hydroxylase (PhC4H1,
696 Unigene0023326, K268), coniferyl alcohol acetyltransferase (PhCFAT1, Unigene0011295,
697 K176, >11-fold), isoeugenol synthase (PhIGS1, Unigene0003787, K39; PhIGS1,

698 Unigene0015809, K47), eugenol synthase (EGS, Unigene0016673, K85), benzoic
699 acid/salicylic acid carboxyl methyltransferase (PhBSMT1, Unigene0029058, K274,
700 K188, >10-fold), CCOMT (PhCCOMT1, Unigene0026144, K159, >35-fold), and cinnamyl
701 alcohol dehydrogenase (PhCAD5, Unigene0026909, K354, >35-fold) increased after
702 ethylene treatment (SM Fig. S11). These results implied that, aside from alterations at the
703 mRNA level, ethylene regulated the abundance of proteins associated in floral scent
704 biosynthesis at the ubiquitination level in petunia, and ubiquitination might play an
705 important role in floral scent biosynthesis.

706 **Ethylene treatment decreases the abundance of proteins involved in amino acid** 707 **biosynthesis**

708 In addition to the enzymes in the phenylalanine biosynthesis pathway mentioned above,
709 ethylene treatment significantly decreased the protein abundance of enzymes related to the
710 biosynthesis of other amino acids, including histidine biosynthesis, tyrosine biosynthesis,
711 methionine biosynthesis, serine biosynthesis, and lysine biosynthesis (SM Fig. S12; SD
712 Exc13 Sheet5). In contrast, previous studies have revealed considerable synthesis of specific
713 amino acids in cells undergoing senescence in *Sandersonia aurantiaca* and carnations, as well
714 as the accumulation of these amino acids in the phloem (van Doorn and Woltering, 2008).
715 These results illustrate the different levels of amino acid synthesis that occur in different
716 species undergoing senescence.

717 **Ethylene treatment increases the ubiquitination levels of proteins involved in ERAD**

718 In yeast, mammalian, and plant cells, unfolded or misfolded proteins generated in the rough
719 ER are predominantly degraded by ER-associated degradation (ERAD), which involves
720 ubiquitination, retrotranslocation, and degradation by the cytosolic proteasome (Smith et al.,
721 2011). In ERAD, the family of ER-localized HSP70 proteins (known as BiPs) recognizes and
722 binds to exposed hydrophobic patches of incompletely folded or misfolded proteins in an
723 ATP-dependent manner (Buck et al., 2007). Arabidopsis BiPs were thought to contribute to
724 the ER retention of two mutant BR receptors (Hong et al., 2008). BiPs and their associated
725 factor, ERdj3B (an Arabidopsis ER-localized DNAJ homolog), were also involved in the

726 biogenesis and folding control of EFR (Nekrasov et al., 2009). In this study, ethylene
727 treatment increased the ubiquitination levels of PhHSP70 (Unigene0027213, K560, K91) and
728 a DnaJ homolog subfamily A member (PhDnaJ2, Unigene0027373, K66, >10-fold) (SM Fig.
729 S13; SD Exc13 Sheet6).

730 In ERAD, processed substrates are delivered to the cytosolic proteasome by Cdc48 in
731 association with RAD23 and DSK2, two ubiquitin receptors (Raasi and Wolf, 2007).
732 UBX-containing proteins likely recruit AtCDC48A to the ER membrane (Rancour et al.,
733 2004). In Arabidopsis, RAD23 proteins also play an important role in the cell cycle,
734 morphology, and fertility of plants through their delivery of substrates to the 26S proteasome
735 (Farmer et al., 2010). In this study, ethylene treatment increased the ubiquitination levels of
736 PhCDC48C/P19 (Unigene0026112, K280) and three PhRAD23d proteins (Unigene0018393,
737 K51, >10-fold; Unigene0018392, K18, K28, K62, K9; Unigene0020741, K18).

738 In Arabidopsis, ERAD substrates may be processed through antagonistic interactions
739 between Ufd2 and Ufd3, along with unknown enzymes and the deubiquitinating enzyme
740 Otu1, and/or through deglycosylation by the cytoplasmic peptide *N*-glycanase (PNGase)
741 PNG1 (Raasi and Wolf, 2007). AtPNG1 may contain suspected PNGase activity and could
742 stimulate the degradation of two mutant variants of RTA in an *N*-glycan-dependent manner in
743 yeast cells (Diepold et al., 2007; Masahara-Negishi et al., 2012). Here, ethylene treatment
744 increased the ubiquitination levels of PhPNG1P (Unigene0025382, K104) and PhOUT2
745 (Unigene0047836, K57, K161). In addition, ethylene treatment altered the ubiquitination
746 abundance of S-phase kinase-associated protein 1 (PhSKP1, Unigene0020623, K79, K51),
747 molecular chaperone Hsp90 (PhHsp90a, Unigene0029683, K212, K277; PhHsp90b,
748 Unigene0029681, K376), and B-cell receptor-associated protein 31 (PhBRA31,
749 Unigene0007191, K84; Unigene0003563; K419).

750 The ER is a well-controlled microenvironment that facilitates proper protein synthesis and
751 folding and is highly susceptible to stress conditions (Liu and Howell, 2010). The
752 accumulation of unfolded or misfolded proteins activates the unfolded protein response
753 pathway and, if unsuccessful, leads to cell death (Deng et al., 2013). The above results

754 implied the important role of ethylene in the regulation of ERAD in plants. To our knowledge,
755 this is the first report of a relationship between ethylene and ERAD in plants, particularly in
756 the context of ubiquitination regulation. Further exploration of these Kub protein targets may
757 provide insight into previously unknown effectors of the ethylene signaling pathway. In
758 addition, ERAD might be associated with corolla senescence in petunia as the ubiquitination
759 abundance of several proteins involved in ERAD was significantly changed during
760 ethylene-mediated senescence.

761 **Confirmation of the ubiquitination of certain proteins by western blotting**

762 To confirm the ubiquitination of proteins utilizing the K- ϵ -GG antibody, we performed
763 western blotting. Proteins whose ubiquitination was not previously reported were selected as
764 candidates. More evidence has indicated that ER-associated degradation plays important
765 roles in plant development, including senescence (Guerra and Callis, 2012). We selected
766 three proteins, PhCDC48C/P19 (Unigene0026112), PhRAD23d (Unigene0018393), and
767 PhPNG1P (Unigene0025382), which were involved in ERAD, to further examine their
768 ubiquitination by western blotting. Two additional proteins, PhACO3 (Unigene0022854) and
769 PhAUX1 (Unigene0019926), were also selected. Synthetic peptide versions of these proteins
770 were used as immunogens to immunize rabbits for antibody production. Total proteins were
771 extracted from corollas treated with air, ethylene, and both ethylene and MG132. Western
772 blotting using the antibodies raised against these proteins showed that protein abundance was
773 higher in plants treated with both ethylene and MG132 compared to plants treated only with
774 ethylene (SM Fig. S14B), which further confirmed the ubiquitination of these proteins
775 (Kevany et al., 2007).

776 **Conclusions**

777 This study provides a global and comparative analysis of transcriptome, proteome, and
778 ubiquitylome regulation by ethylene and offers further insights into the dynamics of
779 individual Kub sites. Our results revealed Kub site motifs not previously observed in plants
780 and these novel plant Kub site motifs could lead to future discoveries of novel ubiquitin
781 ligase-substrate interactions. We also revealed that the global proteome and ubiquitylome

782 were negatively correlated because of the important function of ubiquitination in protein
783 degradation (Wilkinson, 2000). Several putative ubiquitin ligases were up-regulated by
784 ethylene at the protein and transcription levels. These results demonstrated the important
785 roles of ubiquitination in the degradation of proteins during ethylene-mediated corolla
786 senescence in petunias. We analyzed the effects of ethylene on several aspects of flower
787 senescence. For the first time, our results revealed the effects of ethylene on proteins
788 involved in ERAD and identified many novel ubiquitination sites in several proteins,
789 including PhETR2, PhACO, PhCb5s and PhAUX1. The provided data set may serve as an
790 important resource for the functional analysis of lysine ubiquitination in petunia and
791 facilitate the elucidation of the senescence process in this model petunia. In addition, it
792 should be pointed out that some changes unveiled by omics in this study could be an
793 outcome of senescence and the ubiquitination type (poly-ubiquitination or
794 mono-ubiquitination) of the specific Kub site of proteins in this study needs further study.

795

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800

801 **SUPPLEMENTAL INFORMATION**

802 Supplemental Information includes Supplemental Information includes (1) 14 figures, (2) 4
803 table, (3) material and methods, and (4) 13 excel tables.

804 **Supplemental Materials and Methods**

805 **Supplemental Figure 1:** Effects of ethylene on the expression of ubiquitin in petunia corollas
806 in protein level.

807 **Supplemental Figure 2:** Venn diagram of annotation results against four protein databases.

808 **Supplemental Figure 3:** Confirmation of digital gene expression data by qRT-PCR.

809 **Supplemental Figure 4:** Functional enrichment analysis of differently expressed proteins.

810 **Supplemental Figure 5:** Concordance between changes in the abundance of mRNA and its
811 encoded protein.

812 **Supplemental Figure 6:** Detection of mRNAs and their cognate proteins.

813 **Supplemental Figure 7:** KEGG pathway enrichment heat map of proteins with opposite
814 trends in protein and ubiquitination levels.

815 **Supplemental Figure 8:** Venn diagram of proteomics and ubiquitinomic identification.
816

817 **Supplemental Figure 9:** MS/MS spectra of ethylene receptor PhETR2 (Unigene0010512)
818 (left) and ABC transporter B (Unigene0017904) (right) ubiquitination.

819 **Supplemental Figure 10:** Effects of ethylene on the proteins engaged in ABA (A) and auxin
820 (B) signaling transduction pathway in petunia.
821

822 **Supplemental Figure 11:** Effects of ethylene on floral scent biosynthesis in petunia.
823

824 **Supplemental Figure 12:** Effects of ethylene on amino acid biosynthesis pathway (KEGG:
825 map01230) in petunia. The green line indicates down-regulation, and the red line indicates
826 up-regulation in protein level upon ethylene treatment based on the statistical significance.
827

828 **Supplemental Figure 13:** Effects of ethylene on ERAD (KEGG Pathway: ko04141) in
829 petunia.

830

831 **Supplemental Figure 14:** Confirmation of proteome and ubiquitylome data.

832

833 **Supplemental Table 1:** Summary of Illumina Paired-end sequencing and assembly

834

835 **Supplemental Table 2:** The predicted amino acid sequences of the CDS unigenes
836
837 **Supplemental Table 3:** The differently expressed genes of ethylene treatment or not
838
839 **Supplemental Table 4:** Ethylene treatment changes proteome profile in petunia corollas
840
841 **Supplemental Table 5:** GO enrichment of proteins with Kub sites down-regulated
842
843 **Supplemental Table 6:** Protein and mRNA
844
845 **Supplemental Table 7:** Ethylene treatment changes ubiquitylome profile in corollas in
846 petunia
847 **Supplemental Table 8:** KEGG pathway enrichment of all ubiquitination proteins
848
849 **Supplemental Table 9:** Ubiquitination sites in petunia
850
851 **Supplemental Table 10:** Differentially expressed proteins and ubiquitination overlap.
852
853 **Supplemental Table 11:** The putative E3 ubiquitin-protein ligases up- or down-regulated by
854 ethylene
855
856 **Supplemental Table 12:** Proteasome and nonproteasome proteases were probably involved in
857 the degradation of proteins during ethylene-mediated corollas senescence in petunia
858
859 **Supplemental Table 13:** 37 non-proteasomal proteases up-regulated by ethylene
860
861 **Supplemental Table 14:** Autophagy proteins
862
863
864
865 **Figure legends**
866
867 **Figure 1** The systematic workflow for quantitative profiling of the global proteome and
868 ubiquitylome in petunia corollas upon ethylene treatment.
869
870 **Figure 2** Effect of ethylene on flowers of petunia ‘Mitchell’. A, Flower profile with ethylene
871 treatment (top) or without (bottom). B, Fresh weights of corollas with or without ethylene
872 treatment. C, Protein contents of corollas with or without ethylene treatment. Corollas were
873 collected from at least five flowers on various days after flower opening. Total protein was
874 determined using the Bradford assay. Data represent the means of three replicates \pm SE.
875 Experiments were conducted at least twice with similar results.
876
877 **Figure 3** Functional enrichment analysis of proteins with up-regulated and down-regulated
878 Kub sites. A and B, GO-based enrichment analysis of proteins with up-regulated (A) and
879 down-regulated (B) Kub sites. C and D, KEGG pathway-based enrichment analysis of
880 proteins with up-regulated (C) and down-regulated (D) Kub sites. The percent of
881 differentially expressed proteins indicates the ratio of the mapping proteins to all mapping

870 proteins. The percent of identified proteins indicates the ratio of the background proteins to all
871 background proteins. The significance level was set at $P < 0.05$ (Fischer's exact test). The data
872 come from the SD Exc7 Sheet2-5.

873 **Figure 4** Motif analysis of all the identified Kub sites in petunia. A, Ubiquitination motifs and
874 the conservation of Kub sites. The height of each letter corresponds to the frequency of that
875 amino acid residue in that position. The central K refers to the ubiquitinated lysine. B, The
876 number of identified peptides containing ubiquitinated lysine in each motif. The red columns
877 represent novel motifs. C, Amino acid sequence properties of ubiquitylation sites. The heat
878 map shows significant position-specific under- or over-representation of amino acids flanking
879 the modification sites. D, Predicted protein secondary structures near Kub sites. Probabilities
880 for different secondary structures (coil, α -helix and β -strand) of modified lysines were
881 compared with the secondary structure probabilities of all lysines or all Ser/thr/Tyr on all
882 proteins identified in this study. E, Evolutionary conservation of ubiquitylated and
883 nonubiquitylated lysines on protein orthologs in selected eukaryotic species. Abbreviations:
884 Vv, *Vitis vinifera*; Os, *Oryza sativa japonica*; At, *Arabidopsis thaliana*; Sb, *Sorghum bicolor*;
885 Gm, *Glycine max*; Bd, *Brachypodium distachyon*; Sl, *Solanum lycopersicum*; Zm, *Zea mays*.

886 **Figure 5** Concordance between changes in proteins and their ubiquitination. A–E, Correlation
887 between protein and ubiquitination fold-changes upon ethylene treatment for all
888 ubiquitination/protein pairs A, significantly up-regulated proteins B, significantly
889 down-regulated proteins C, significantly up-regulated ubiquitination D, significantly
890 down-regulated ubiquitination E. F, Pearson correlations of the comparisons shown in A–E.

891 **Figure 6.** Effects of ethylene on the proteins engaged in ethylene biosynthesis and signaling
892 transduction pathway in petunia. Differentially expressed proteins based on statistical
893 significance in this study are framed in oval boxes, and differentially ubiquitinated and
894 phosphorylated proteins have round boxes. The red box indicates up-regulation; the green box
895 indicates down-regulation; and the blue indicates no significant changes upon ethylene
896 treatment. Abbreviations: U, ubiquitination. ACC, 1-aminocyclopropane-1-carboxylic acid;
897 ACO, ACC oxidase; ACS, ACC synthase; Cb5, cytochrome b₅; CTR1, CONSTITUTIVE
898 TRIPLE-RESPONSE1; EIN, ETHYLENE INSENSITIVE; EIN2-C, EIN2 C end; EIN2-N,
899 EIN2 N end; ETR1, ETHYLENE RESPONSE1; RTE1, REVERSION-TO-ETHYLENE
900 SENSITIVITY1; SAM, S-adenosylmethionine; SAMS, S-AdoMet synthetase.

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