Short title: Ubiquitination is involved in corolla senescence

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Article title: Proteomes and Ubiquitylomes Analysis Reveals the Involvement of Ubiquitination in Protein Degradation in Petunias

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


Supporting information: 12 figures and 13 excel tables.

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Abstract

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

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

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

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

Protein degradation is one of the hallmarks of senescence (Shahri and Tahir, 2014). Ubiquitination, a well-known PTM, plays important roles in protein degradation (Wilkinson, 2000). Ubiquitin is a highly conserved 76-amino-acid polypeptide that is found throughout the eukaryotic kingdom. In vivo, poly-ubiquitin chains are most frequently linked through K48 and the canonical ubiquitin signal is recognized by the 26S proteasome and thereby targets tagged proteins for degradation (Peng et al., 2003). Among six other lysine residues of ubiquitin, at least four (K6, K11, K29 and K63) can function as a linkage for poly-ubiquitin chains (Arnason and Ellison, 1994; Peng et al., 2003). K11- and K29-linked poly-ubiquitin chains may target proteins to the proteasome (Johnson et al., 1995; Baboshina and Haas, 1996). Conjugation of mono-ubiquitylation is a regulatory modification involved in diverse processes including transcription, histone function, endocytosis, DNA repair, viral budding and membrane trafficking (Passmore and Barford, 2004; Schnell and Hicke, 2003).
The attachment of the ubiquitins to proteins involves three classes of enzyme: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (Hochstrasser, 1995). Ubiquitinated substrates may be degraded to peptides by the multisubunit 26S protease. However, no attempts have been made to perform PTM analysis to characterize the ubiquitination of the proteome or the association between modifications and the proteome during flower senescence in response to ethylene.

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

Results and Discussion

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

The evaluated petunias (Mitchell) exhibited the first visible symptom of senescence, the wilting of the corolla, at approximately 16 h after 2 µL L⁻¹ ethylene treatment. The margins of the corollas began to involute, and a few translucent dots appeared in the corollas (Fig. 2A); however, the corolla fresh weight and protein content remained constant. At 32 h after ethylene treatment, the petunias exhibited obvious symptoms of senescence (Figs. 2B, 2C), and the corolla fresh weight and protein content decreased to approximately 87% and 88%,
respectively, compared to air-treated, control corollas. These decreases coincided with corolla wilting. Air-treated petunia corollas were fully turgid 0-48 h after flower opening, exhibited no symptoms of senescence and were visually indistinguishable from flowers at anthesis (Fig. 1).
We selected a 2 µL L⁻¹, 16-h ethylene treatment (Eth) and a 16-h air treatment (Air) to perform transcriptome, proteome, and ubiquitylome analyses.

**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 ±SE. Experiments were conducted at least twice with similar results.
Ethylene treatment increases ubiquitin in petunia corollas at the protein level

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

RNA sequencing and assembly

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

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

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

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

Significant pathway enrichment analysis showed that plant hormone signal transduction was the most important pathway in the Eth vs. Air comparison, and plant hormone signal transduction was the key biological event. Plant hormone signal transduction is very important for hormone-induced biochemical changes during plant growth, development, and
environmental information processing pathways. A previous study showed that ethylene interacts with plant hormones at different levels to form a network of signaling pathways connected by antagonistic and synergistic interactions (Sun et al., 2006; Stepanova et al., 2007). Our evidence indicated that the genes involved in plant hormone signal transduction play important roles in ethylene-induced senescence in petunia corolla.

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

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

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

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

To elucidate the functional differences between the down-regulated and up-regulated proteins, the quantified proteins were analyzed for GO enrichment based on clustering analysis (SM Fig. S4; SD Exc4 Sheet1-3). In the cellular component category, many of the down-regulated proteins were enriched in the ribosome and ribosomal subunit category, whereas the up-regulated proteins were not enriched in any cellular component category. In iris, one of the earliest ultrastructural senescence symptoms is the loss of the majority of ribosomes (Van Doorn et al., 2003). In harvest-induced senescence in detached *Arabidopsis* plants, genes involved in ribosome biogenesis and assembly are down-regulated (Chang et
al., 2015). These results suggest that protein processing might be suppressed during senescence in plants.

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

The analysis of molecular functions showed that many of the up-regulated proteins were highly enriched for the following: oxidoreductase activity, acting on paired donors, iron ion binding, transferase activity, hexosyl groups, transition metal ion binding, cysteine-type peptidase activity, UDP-glucosyltransferase activity, sucrose synthase activity, heme binding, transferase activity, transferring glycosyl groups, tetrapyrrrole binding, glucosyltransferase activity, and UDP-glycosyltransferase activity. The down-regulated proteins were enriched in transferase activity, transferring alkyl or aryl groups, structural constituent of ribosome, methionine adenosyltransferase activity, and 3-deoxy-7-phosphoheptulonate synthase activity. The term transferase activity was observed to occur among both up-regulated and down-regulated proteins in the ontology of molecular
functions, suggesting the impact of ethylene on protein modification and the important role of protein modification during corolla senescence in petunia.

**Comparative analysis of proteome and transcriptome data**

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

Numerous reports have suggested that RNA transcript accumulation is not always conveyed to the final product-protein (Shemesh-Mayer et al., 2015). For example, a negative correlation between mRNA and protein accumulation patterns was found in Arabidopsis in response to cold treatment (Nakaminami et al., 2014). The lack of correlation between mRNA and protein levels has been attributed to differences in translational efficiency, codon usage/bias, and mRNA versus protein stability, post translational modifications, sequencing depth and proteomic approach (Alberch, 1991; Gygi et al., 1999; Pigliucci, 2010; Ghazalpour et al., 2011; Rodrigues et al., 2012). In this study, the number of mRNA copies in the sample and the subcellular localization of the protein restricted the number of identified proteins relative to the detection of their cognate transcripts (SM Fig. S6). Comparing the number of reads recorded for transcripts corresponding to identified and not identified proteins, a transition is reached at around 20 reads, under which the products of the majority of transcripts was not
detected (SM Fig. S6A). In addition, proteins tightly associated with membranes are underrepresented in the pool of identified proteins relative to the predicted proteome (SM Fig. S6B).

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

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

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

To elucidate the functions of the proteins that underwent ubiquitination, KEGG pathway analysis was performed. A number of vital pathways, including those related to the spliceosome, RNA transport, mRNA surveillance pathway, endocytosis and ABC transporters, were enriched among proteins with lysine ubiquitination (Kub) sites (SD Exc7 Sheet1). These results suggested that ubiquitination might be highly associated with RNA metabolism, endocytosis and ABC transporters. Alternative pre-mRNA splicing is thought to
provide a mechanism to increase the complexity of the proteome and introduce additional layers to regulate gene expression in different cell types and during development (Zhou and Fu, 2013). A previous study showed that the ubiquitination of histone H2B modulates spliceosome assembly and function in budding yeast (Zhou and Fu, 2013). The ubiquitination of proteins associated with the spliceosome may change the alternative pre-mRNA splicing that takes place during corollas senescence.

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

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

In the molecular function analysis, proteins with binding activity, catalytic activity, and transporter activity were enriched among proteins containing both up-regulated and down-regulated Ub sites. Previous studies have shown that ions and amino acids are
transferred to vegetative organs during senescence in unpollinated petunia petals (Shibuya et al., 2013). These results suggested that proteins demonstrating changes in ethylene-mediated ubiquitination are connected to protein interactions, DNA transcription, and ion and protein...
KEGG pathway analysis of proteins whose ubiquitination quantitatively changed revealed a number of vital pathways. The protein processing pathways in the endoplasmic reticulum, stilbenoid and diarylheptanoid biosynthesis, phagosome, fatty acid elongation, flavonoid biosynthesis, cysteine metabolism, methionine metabolism, phenylpropanoid biosynthesis, phenylalanine metabolism, proteasome, ABC transporters and others were enriched among proteins with up-regulated Kub sites. Proteins with down-regulated Kub sites were enriched in pathways involving Ras signaling, ether lipid metabolism, cysteine metabolism, methionine metabolism and others (Figs. 3C, 3D). These results indicate that ubiquitination was associated with protein processing, protein degradation and secondary metabolites.

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

Sequence Properties of Ubiquitinated Proteins

To understand the properties of the identified Kub sites in petunia, we used the Motif-X
program to compare the position-specific frequencies of the amino acid residues surrounding all ubiquitinated lysine residues.

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

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

In addition to primary sequences around Kub sites, protein secondary structure has been found to be informative in Kub site prediction (Gnad et al., 2011). Therefore, we integrated protein secondary structure features using NetSurfP software (Muller et al., 2010). The probabilities of different secondary structures (coil, α-helix, and β-strand) near ubiquitinated lysine sites were compared with the secondary structure probabilities of all lysine sites on
proteins identified in this study. Ubiquitinated lysine sites occurred significantly more frequently in unstructured regions of proteins ($p=6.74E-07$ for coil) and less frequently in structured regions ($p=6.32E-09$ for $\alpha$-helix and $p=4.29E-07$ for $\beta$-strand) (Fig. 4D). However,
in mammals, ubiquitinated lysines are marginally, yet significantly, more frequently present in
structured regions of proteins than in unstructured regions (Wagner et al., 2011), indicating a
difference in ubiquitinated lysine sites between plants and mammals.

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

The correlation between the global proteome and ubiquitylome

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

The correlation between the whole proteome and ubiquitylome during senescence in corollas
was analyzed based on the quantitative results obtained in this study. There were 985
quantified proteins that were also found to undergo ubiquitination, and 2,270 Kub sites in
1,221 proteins were quantified. Of the 985 quantified proteins, 66 proteins were
down-regulated and 96 were up-regulated. Quantitative ratios from the proteome and
ubiquitylome were compared upon ethylene treatment, as shown in Fig. 4. Pearson's
correlation coefficient, a statistical measure of the strength of a linear relationship between
paired data, is denoted by r and is by design constrained between -1 and 1. Positive values
denote positive linear correlation, negative values denote negative linear correlation, and a
value of 0 denotes no linear correlation. The closer the value is to 1 or -1, the stronger the
linear correlation. The Pearson's correlation coefficient was calculated as -0.38 when all significantly altered proteins were considered in terms of their ubiquitination, regardless of the direction of the change (Figs 5A, 5F). In addition, the overlap between differentially

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

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

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

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

The degradation of proteins in developing tissues is a notable process during senescence (Shahri and Tahir, 2014). In the transcriptome obtained in this study, 144 unigenes encoding
putative ubiquitin-protein ligases (35 E3 ubiquitin-protein ligases, 72 F-box protein and 37 U-box proteins), 6 unigenes encoding ubiquitin proteins and 7 unigenes encoding 26S proteasome subunits up-regulated by ethylene were identified (SD Exc10 Sheet1-5). In the proteome, ethylene treatment resulted in 284 down-regulated and 233 up-regulated proteins, and among them, four putative ubiquitin ligases were up-regulated (SD Exc11 Sheet1). Moreover, 246 quantified proteins also underwent ubiquitination, and their up-regulated Kub sites were identified; among them, 44 proteins were down-regulated, and only 8 proteins were up-regulated with respect to protein concentration. In addition, 118 quantified proteins underwent ubiquitination, and their down-regulated Kub sites were identified in this study; among these, 23 proteins were up-regulated, and only 2 proteins were down-regulated with respect to protein concentration following ethylene treatment (SD Exc9 Sheet1). Of the 18 ubiquitinated proteins identified only in the control, 17 were up-regulated and only one was down-regulated by ethylene at the protein level, while of the 11 ubiquitinated proteins identified only in corollas following ethylene treatment, 9 were down-regulated and only 2 were up-regulated by ethylene at the protein level (SD Exc9 Sheet1). Silencing the expression of a gene homolog to MjXB3 in petunia resulted in an extension in flower life (Xu et al., 2007). Proteomic analysis of pollination-induced corolla senescence in petunia identified a ubiquitin-conjugating enzyme (E2) that was up-regulated by pollination, accelerating flower senescence (Bai et al., 2010). These results indicate the involvement of ubiquitination in protein degradation during ethylene-mediated corolla senescence in petunia. In addition, the proteasome system was apparently up-regulated during petal senescence in daylily (Courtney et al., 1994; Müller et al., 2004) and daffodil (Hunter et al., 2002). In carnation, several transcripts homologous to genes encoding various components of the 26S proteasome machinery, including RPT6, RPN2, a RING finger protein and a U-box containing protein, were all induced during carnation petal senescence (Hoeberichts et al., 2007). Feeding isolated Iris petals with Z-Leu-Leu-Nva-H, an inhibitor of proteasome activity, led to a significant delay in the time to visible senescence (Pak and van Doorn, 2005), indicating that proteasome action is limiting senescence. In addition, Arabidopsis UPL5, a HECT E3 ubiquitin ligase, negatively regulates leaf senescence through degradation of WRKY53 and ensures that senescence is executed in the correct time frame.
To elucidate the function of proteins with opposite trends in protein and ubiquitination levels, KEGG pathway enrichment-based clustering analyses were performed (SM Fig. S7). The protein processing pathways in the flavonoid biosynthesis, phenylalanine metabolism, phenylpropanoid and secondary metabolites biosynthesis and others were enriched among proteins with up-regulated Kub sites and down-regulated protein levels. Previous studies suggested that ethylene treatment reduced the biosynthesis of phenylpropanoid and secondary metabolites in petunia (Negre et al. 2003; Underwood et al., 2005; Schuurink et al., 2006). It is possible that ubiquitination could be involved in degradation of the proteins in these pathways during ethylene-mediated flower senescence. Proteins with down-regulated Kub sites and up-regulated protein levels were enriched in pathways involving SNARE interaction in vesicular transport and galactose metabolism.

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

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

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

Changes of the autophagy proteins after ethylene treatment

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

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

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

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

ACC synthase (ACS) is the rate-limiting enzyme of ethylene synthesis. Previous research has suggested that ACS family proteins are up-regulated by ethylene and that ETO1/EOL,
calcium-dependent protein kinase (CDPK), 14-3-3 and mitogen-activated protein kinase (MAPK) interact with ACS family proteins, modulating their stability in plants (Xu and Zhang, 2014). However, in this study, in both protein and ubiquitination analyses, ACS

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; CT1, 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.
family proteins were not identified.

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

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

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

Ethylene-insensitive protein 2 (EIN2) acts downstream of ethylene receptors and upstream of EIN3/EIL and is involved in the regulation of flower senescence. Qiao et al. (2009) reported
that the stability of EIN2 is modulated by the two F-box proteins ETP1/2 via ubiquitination, but the ubiquitination of PhEIN2 was not observed in this study. In addition, it was proposed that EIN3 is targeted by the F-box proteins EBF1/2 in Arabidopsis (Potuschak et al., 2003). However, PhEILs, PhEBF1 and PhEBF2 were not identified in this study at either the protein or ubiquitination levels.

A recent study showed that Arabidopsis cytochrome b$_5$ (Cb5) proteins are involved in ethylene signaling, and REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1) physically interacts with AtCb5-B, -C, -D and -E (Chang et al., 2014). The Kub sites of two Cb5s (PhCb5B, Unigene0023698, K35; PhCb5E, Unigene0016038, K51) were up-regulated more than 4-fold by ethylene in this study, which further supported the involvement of ubiquitination in ethylene signaling in petunia.

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

Ethylene appears to be a negative regulator of ABA action during germination, although it was confirmed to exert a positive synergistic effect on ABA action by modulating the overall carbon status in Arabidopsis roots (Ghassemian et al., 2000; Gazzarrini and McCourt, 2001; Cheng et al., 2009). In carnations, ABA has been found to accelerate flower senescence (Ronen and Mayak, 1981). A large increase in ABA levels was observed in the gynoecium prior to or concomitant with the upsurge in ethylene (Onoue et al., 2000). In this study, the enzymes related to ABA biosynthesis, PhDXS (Unigene0009358), PhPDS3 (Unigene0017870), PhNCED4 (Unigene0037462), and PhSDR (Unigene0012764), were down-regulated between 1.5 and 3.0-fold at the protein level by ethylene (SM Fig. S10A; SD Exc13 Sheet2). Additionally, the ABA signaling component PP2C, a major negative regulator of ABA signaling, inhibits SnRK2, a positive regulator of ABA signaling, thus inhibiting activation of the ABA pathway (Umezawa et al., 2010). In this study, PP2C (PhPP2C,
Unigene0006325; PhPP2C58, Unigene0014490 and SnRK2A (Unigene0014500) increased at the protein level after ethylene treatment. These results hinted that ethylene likely negatively regulates ABA biosynthesis and signaling transduction in petunia corollas. In rose petals, the external application of ethylene accelerated senescence and induced a rise in endogenous abscisic acid-like activity (Mayak and Halevy, 1972). In petunia, ethylene might directly affect senescence in petals without requiring involvement of the ABA pathway.

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

In summary, during ethylene-mediated corolla senescence, ethylene appeared to affect the biosynthesis and signal transduction pathways of plant hormones such as ABA, auxin, and
ethylene itself at the transcript, protein, and ubiquitination levels in this study. In addition, it should be noted that the omics changes in this study may be directly or indirectly caused by ethylene treatment.

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

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

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

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

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

In the ubiquitylome, the ubiquitination levels of shikimate 5-dehydrogenase (PhSDH, Unigene0001508, K114, K504, >15-fold), cinnamate-4-hydroxylase (PhC4H1, Unigene0023326, K268), coniferyl alcohol acetyltransferase (PhCFAT1, Unigene0011295, K176, >11-fold), isoeugenol synthase (PhIGS1, Unigene0003787, K39; PhIGS1,
Unigene0015809, K47), eugenol synthase (EGS, Unigene0016673, K85), benzoic acid/salicylic acid carboxyl methyltransferase (PhBSMT1, Unigene0029058, K274, K188, >10-fold), CCOMT (PhCCOMT1, Unigene0026144, K159, >35-fold), and cinnamyl alcohol dehydrogenase (PhCAD5, Unigene0026909, K354, >35-fold) increased after ethylene treatment (SM Fig. S11). These results implied that, aside from alterations at the mRNA level, ethylene regulated the abundance of proteins associated in floral scent biosynthesis at the ubiquitination level in petunia, and ubiquitination might play an important role in floral scent biosynthesis.

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

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

Ethylene treatment increases the ubiquitination levels of proteins involved in ERAD

In yeast, mammalian, and plant cells, unfolded or misfolded proteins generated in the rough ER are predominantly degraded by ER-associated degradation (ERAD), which involves ubiquitination, retrotranslocation, and degradation by the cytosolic proteasome (Smith et al., 2011). In ERAD, the family of ER-localized HSP70 proteins (known as BiPs) recognizes and binds to exposed hydrophobic patches of incompletely folded or misfolded proteins in an ATP-dependent manner (Buck et al., 2007). Arabidopsis BiPs were thought to contribute to the ER retention of two mutant BR receptors (Hong et al., 2008). BiPs and their associated factor, ERdj3B (an Arabidopsis ER-localized DNAJ homolog), were also involved in the
biogenesis and folding control of EFR (Nekrasov et al., 2009). In this study, ethylene
treatment increased the ubiquitination levels of PhHSP70 (Unigene0027213, K560, K91) and
a DnaJ homolog subfamily A member (PhDnaJ2, Unigene0027373, K66, >10-fold) (SM Fig.
S13; SD Exc13 Sheet6).

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

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

The ER is a well-controlled microenvironment that facilitates proper protein synthesis and
folding and is highly susceptible to stress conditions (Liu and Howell, 2010). The
accumulation of unfolded or misfolded proteins activates the unfolded protein response
pathway and, if unsuccessful, leads to cell death (Deng et al., 2013). The above results
implied the important role of ethylene in the regulation of ERAD in plants. To our knowledge, this is the first report of a relationship between ethylene and ERAD in plants, particularly in the context of ubiquitination regulation. Further exploration of these Kub protein targets may provide insight into previously unknown effectors of the ethylene signaling pathway. In addition, ERAD might be associated with corolla senescence in petunia as the ubiquitination abundance of several proteins involved in ERAD was significantly changed during ethylene-mediated senescence.

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

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

**Conclusions**

This study provides a global and comparative analysis of transcriptome, proteome, and ubiquitylome regulation by ethylene and offers further insights into the dynamics of individual Kub sites. Our results revealed Kub site motifs not previously observed in plants and these novel plant Kub site motifs could lead to future discoveries of novel ubiquitin ligase-substrate interactions. We also revealed that the global proteome and ubiquitylome
were negatively correlated because of the important function of ubiquitination in protein
degradation (Wilkinson, 2000). Several putative ubiquitin ligases were up-regulated by
ethylene at the protein and transcription levels. These results demonstrated the important
roles of ubiquitination in the degradation of proteins during ethylene-mediated corolla
senescence in petunias. We analyzed the effects of ethylene on several aspects of flower
senescence. For the first time, our results revealed the effects of ethylene on proteins
involved in ERAD and identified many novel ubiquitination sites in several proteins,
including PhETR2, PhACO, PhCb5s and PhAUX1. The provided data set may serve as an
important resource for the functional analysis of lysine ubiquitination in petunia and
facilitate the elucidation of the senescence process in this model petunia. In addition, it
should be pointed out that some changes unveiled by omics in this study could be an
outcome of senescence and the ubiquitination type (poly-ubiquitination or
mono-ubiquitination) of the specific Kub site of proteins in this study needs further study.

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SUPPLEMENTAL INFORMATION

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

Supplemental Materials and Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

Supplemental Figure 14: Confirmation of proteome and ubiquitylome data.

Supplemental Table 1: Summary of Illumina Paired-end sequencing and assembly
Supplemental Table 2: The predicted amino acid sequences of the CDS unigenes

Supplemental Table 3: The differently expressed genes of ethylene treatment or not

Supplemental Table 4: Ethylene treatment changes proteome profile in petunia corollas

Supplemental Table 5: GO enrichment of proteins with Kub sites down-regulated

Supplemental Table 6: Protein and mRNA

Supplemental Table 7: Ethylene treatment changes ubiquitylome profile in corollas in petunia

Supplemental Table 8: KEGG pathway enrichment of all ubiquitination proteins

Supplemental Table 9: Ubiquitination sites in petunia

Supplemental Table 10: Differentially expressed proteins and ubiquitination overlap.

Supplemental Table 11: The putative E3 ubiquitin-protein ligases up- or down-regulated by ethylene

Supplemental Table 12: Proteasome and nonproteasome proteases were probably involved in the degradation of proteins during ethylene-mediated corollas senescence in petunia

Supplemental Table 13: 37 non-proteasomal proteases up-regulated by ethylene

Supplemental Table 14: Autophagy proteins

Figure legends

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

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 ±SE. Experiments were conducted at least twice with similar results.

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.

**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, $\alpha$-helix and $\beta$-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*.

**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.

**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.
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