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Ethylene signaling is required for pollination-accelerated corolla senescence in petunias

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ABSTRACT

Senescence in *Petunia* × *hybrida* 'Mitchell Diploid' (MD) flowers is characterized by petal wilting, which is observed approximately 8 days after flower opening. Pollination accelerates corolla senescence and associated wilting symptoms are observed within 48 h. This pollination-accelerated corolla senescence was not observed in transgenic petunias with reduced ethylene sensitivity (35S::*etr1-1*). The growth of the pollinated *etr1-1* ovary physically separated the corolla from the receptacle and the corolla was shed. Under most conditions the growing ovary resulted in the separation of a nearly turgid corolla. When this occurred, the up-regulation of the senescence specific cysteine protease, *PhCP10*, and the endonuclease, PhNUC1, was not detected. While a reduction in the N, P, and K content of the corollas accompanied the senescence of wild type MD flowers, a similar decrease was not detected in *etr1-1* corollas. Lowering production temperatures increased the time until pollinated *etr1-1* corollas were shed, and the growing ovary did not begin to separate the corolla from the receptacle until after the petals were wilting. Senescence was induced in wilted *etr1-1* corollas, as evidenced by the induction of *PhCP10* gene expression and PhNUC1 activity. A reduction in the macronutrient content of the wilted, pollinated *etr1-1* corollas was also observed, although P levels were not reduced as much as in pollinated MD flowers.

1. Introduction

Pollination regulates a series of developmental events that prepare the flower for fertilization while terminating the organs that have completed their functions in pollinator attraction and pollen dispersal and reception [1]. In some flowers, pollination reduces floral attraction by down-regulating scent production [2], altering perianth pigmentation, and accelerating the senescence or abscission of floral organs [3]. These changes are believed to function as visual cues that direct pollinators to unpollinated flowers in order to increase pollination efficiency. The continued maintenance of floral structures that are no longer needed is also costly in terms of respiratory energy and water loss [4]. Accelerating the senescence of the pollinated corolla, once it has completed its role in pollinator attraction, allows the plant to salvage valuable nutrients from the petals before they are shed. Many of the senescence up-regulated genes that have been identified from petals encode catabolic enzymes that play a role in the large scale degradation of cellular constituents, which allows nutrients to be recycled [5,6]. In support of this recycling function, the N, P, and K content of petunia petals is significantly reduced during pollination-accelerated senescence [7].

Pollination in ethylene-sensitive species is often accompanied by increased ethylene production from the style, then the ovary and petals [8]. This endogenous ethylene production mediates pollination-accelerated corolla senescence. The accelerated senescence response can be prevented in these species by treating flowers with inhibitors of ethylene synthesis or action [9–11]. Interestingly, treating the pollinated style with an inhibitor of ethylene action is sufficient to prevent pollination-accelerated senescence in carnation flowers [12]. These results indicate that the senescence response in the corolla is dependent on ethylene signaling within the gynoecium. More recently, the pollination response has been studied in transgenic petunias that are insensitive to ethylene [13–16]. Ethylene sensitivity has been reduced in transgenic petunias by constitutively expressing the mutant *etr1-1* ethylene receptor from *Arabidopsis* (35S::*etr1-1*) [16], as well as down regulating the expression of the endogenous *EIN2* gene [15]. *EIN2* is a component of the ethylene signaling pathway that is downstream of the receptor. Pollination does not accelerate corolla senescence in any of these ethylene-insensitive petunias. The effective life of the corollas is terminated when the growth of the pollinated ovary physically separates the corolla from the receptacle [13–16].

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This paper presents a comparative analysis of senescence associated gene expression and enzyme activity and changes in the nutrient content of wild type *Petunia* × *hybrida* 'Mitchell Diploid' (MD) and transgenic *etr1-1* (line 44568) corollas following pollination. Studies of unpollinated, naturally senescing flowers have determined that the senescence program is similar in MD and *etr1-1* flowers, except that the up-regulation of transcripts and associated enzyme activities is delayed in flowers with reduced ethylene sensitivity [17,18]. The purpose of this study was to investigate ethylene's role in mediating the timing of post-pollination responses in the corolla.

2. Materials and methods

2.1. Plant materials and growth conditions

Wild-type *Petunia* × *hybrida* 'Mitchell Diploid' (MD) and transgenic ethylene-insensitive plants (35S::*etr1-1*, line 44568; [16]) were used in all experiments. Seeds were treated with 100 mg L^{-1} GA_3 for 24 h and sown in cell-packs on top of soil-less mix (Promix BX, Premier Horticulture, Quebec, Canada). Plants were established in the greenhouse after germination and transferred to 16-cm pots after 4 weeks. Plants were fertilized at 150 mg L^{-1} N with Jacks Professional Petunia FeED 20N-3P-19K (J.R. Peters Inc., Allentown, PA, USA). A one time treatment of Soluble Trace Element Mix (S.T.E.M., The Scotts Co., Marysville, OH, USA) was applied four weeks after transferring to 16-cm pots. Growing conditions were 24/16 °C (day/night) with a 13 h photoperiod supplemented by high pressure sodium and metal halide lights. In a separate experiment, petunias were grown at 20/16 °C. These growing conditions will be referred to as higher temperatures and lower temperatures. Plants were arranged in the greenhouse using a random block design.

Flowers were emasculated 1 day before flower opening to prevent self-pollination. Newly open flowers were pollinated by brushing self-pollen from freshly dehisced anthers onto the stigma. Corollas were collected at various times after pollination from MD and etr-44568 plants grown at both higher temperatures (24/16 °C) and lower temperatures (20/16 °C). These corollas were used for the determination of dry weight (DW) and nutrient content, cysteine protease (*PhCP10*) gene expression and endonuclease activity (PhNUC1). Zero hour represents the day of flower opening. Corollas were also collected from unpollinated MD and etr-44568 flowers grown at 20/16 °C. These corollas were used for nutrient analysis to determine the N, P, and K content of nonsenescing and senescing corollas. Each collection was repeated three times and included at least 12 corollas. Corolla dry weights were determined following oven drying at 60 °C for 3 days.

2.2. Protein extraction and endonuclease activity assays

Protein extraction and endonuclease activity assays were as previously described [18]. Corollas were powdered in liquid N_2 and extracted by vortexing in 0.5 mL homogenization buffer (50 mM Tris–HCl [pH 7.6], 2 mM DTT) per corolla. SDS-PAGE was performed using a 15% (w/v) resolving gel that contained 100 μg mL^{-1} BSA. To identify DNase activity, gels contained either 15 μg mL^{-1} double stranded salmon sperm DNA (Stratagene, La Jolla, CA, USA) or DNA that had been made single stranded by boiling for 3 min. RNase activity gels contained 40 μg mL^{-1} petunia petal total RNA. Samples were equalized by loading 20 μL of total protein extract (equal volume per corolla) to correct for the large decreases in total proteins that accompany corolla senescence. Gels were run at 120 V for 2 h at 25 °C. After electrophoresis, nucleases were renatured by incubating gels in renaturation buffer (0.1 M Tris–HCl [pH 7.4], 1% Triton X-100) at 37 °C with gentle shaking for 1 h. Gels were rinsed twice in 0.1 M Tris–HCl (pH 7.4) and incubated in development buffer (50 mM Tris–HCl [pH 7.5], 20 mM NaCl, 100 μM $CoCl_2$) overnight at 37 °C. To visualize bands of nuclease activity, gels were stained for 1 h at room temperature in 50 mM Tris (pH 7.0) containing 0.5 μg mL^{-1} ethidium bromide. Cobalt was included in the development buffer to enhance the activity of a senescence-specific endonuclease (PhNUC1) that was previously identified from petunia corollas [18].

2.3. RNA extraction and gel blot analysis

RNA extraction and gel blot analyses were as previously described [17]. Total RNA from petunia corollas was isolated using Trizol (GibcoBRL, Rockville, MA, USA). Fifteen micrograms of total RNA was separated on a denaturing 1.2% agarose gel containing formaldehyde and transferred onto Hybond N membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by capillary transfer in 10 × SSC. A radio-labeled, cDNA probe for the petunia cysteine protease, *PhCP10* (GenBank number AY662996) was generated by PCR using gene specific primers. Primers included F 5′-TCCGTACAAGGGAGAAGACG-3′ and R 5′-CGGATAAGAAGCTTCCGTTG-3′. Hybridization signals were visualized using a Storm 860 PhosphoImager, quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA), and values were normalized to rRNA levels to correct for any differences in RNA loading.

2.4. Nutrient analysis

Corollas were dried at 60 °C for 3 days and ground to pass a 2 mm sieve. Nutrient measurements were completed at the Service Testing and Research Laboratory (The Ohio State University/OARDC, Wooster, OH, USA). Total N was analyzed on a 100 mg DW sample by combustion using the Dumas method [19]. Following microwave digestion, a 250 mg DW sample was analyzed for P and K using an inductively coupled plasma spectrophotometer (model PS3000, Leeman Labs Inc., Hudson, NH, USA) as described in Isaac and Johnson [20]. All data are presented as the amount of an individual nutrient per corolla.

3. Results

3.1. ETR1-mediated signaling is required for pollination-accelerated corolla senescence

Pollination accelerated the senescence of *Petunia* × *hybrida* 'Mitchell Diploid' (MD) flowers. MD flowers showed symptoms of corolla wilting within 48 h (Fig. 1A), while unpollinated flowers do not wilt until 192 h (8 days) after flower opening [17,18]. Once the corollas were completely wilted and starting to dry, they were shed. In contrast, corolla senescence was not accelerated by pollination in ethylene-insensitive transgenic petunias (35S::*etr1-1*, line 44568). In these flowers, the growth of the pollinated ovary split the corolla tube and eventually separated the corolla from the receptacle. Flowers from etr-44568 petunias grown at temperatures of 24/16 °C did not show symptoms of wilting until the corollas were almost completely separated from the receptacle by the growth of the ovary. By 240 h after pollination, etr-44568 corollas had only a single vascular connection to the receptacle, but the corollas were still turgid. At 288 h after pollination, the edges of the etr-44568 corolla limbs were starting to curl.

While the abscission of turgid corollas is the phenotype most often observed following the pollination of *etr1-1* flowers, we have also observed pollinated etr-44568 flowers that showed symptoms of corolla wilting before the growing ovary had split the corolla

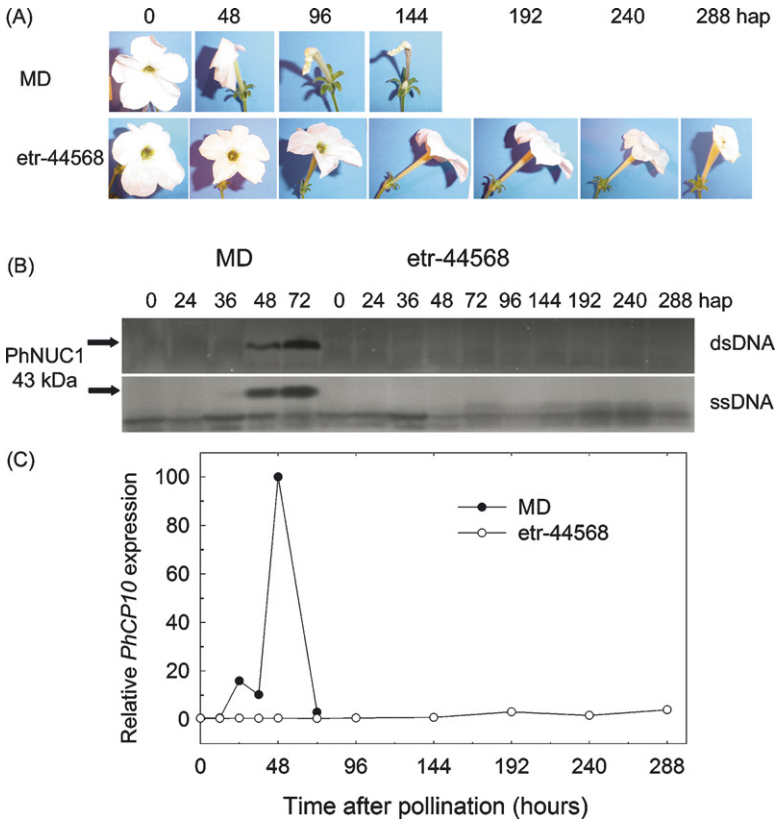

Fig. 1. The post-pollination response in ***Petunia* × *hybrida*** 'Mitchell Diploid' (MD) and 35S::***etr1-1*** transgenic petunias (etr-44568) grown at 24/16 °C (day/night). Visual characteristics of representative flowers at various times after pollination (hap, hours after pollination). Pollinated etr-44568 flowers had a nonwilting phenotype when grown at these higher temperatures (A). Temporal patterns of enzyme activity for the 43 kDa bifunctional endonuclease, PhNUC1. PhNUC1 activity was determined using SDS-PAGE nuclease activity gels containing either ssDNA (single-stranded DNA) or dsDNA (double-stranded DNA). Total proteins were extracted from corollas at various times after pollination as indicated (B). Temporal patterns of gene expression for the cysteine protease, ***PhCP10***, were determined using RNA gel-blot analysis. PhosphorImager data is expressed as a percentage of the maximum signal detected on that blot. Transcript levels were normalized to rRNA to correct for any differences in RNA loading (C).

tube. We have observed this wilting phenotype under a variety of environmental conditions, but we were able to reproduce this response by growing plants at lower temperatures (20/16 °C). Growing petunias at lower temperatures resulted in a similar acceleration of corolla senescence in pollinated MD flowers, with corolla wilting apparent by 48 h (Fig. 2A). Similar to the response observed at higher temperatures, flower longevity in etr-44568 was not reduced to 48 h. In these flowers, the expansion of the ovary was delayed and the corolla tube was not being detached at 144 h, as was observed at higher temperatures. At 240 h after pollination the edges of the etr-44568 corollas were clearly wilted and curling. The expansion of the etr-44568 ovaries and subsequent splitting of the corolla tubes was apparent at 336 h when the corollas were fully wilted. These two *etr1-1* flower phenotypes will be referred to as the wilting and nonwilting pollination responses.

3.2. Hallmarks of corolla senescence are not detected in etr-44568 flowers when turgid corollas are shed after pollination

The senescence of petunia corollas is accompanied by the induction of senescence associated proteases and nucleases that are believed to function in the execution of protein and nucleic acid catabolism during the death of the petals [17,18]. The previously characterized senescence specific nuclease, PhNUC1 [18] and the cysteine protease, *PhCP10* [17] were therefore used as molecular markers of corolla senescence in etr-44568 and MD flowers. In-gel activity assays indicated that PhNUC1 activity was induced in MD corollas at 48 and 72 h after pollination. PhNUC1 activity against dsDNA, ssDNA (Fig. 1B) and RNA (data not shown) was detected in senescing MD corollas. In contrast, PhNUC1 activity was not induced by pollination in etr-44568 corollas that were shed while still turgid (nonwilting pollination response). These pollinated etr-44568 corollas did not have any detectable PhNUC1 activity, even at 288 h, which was just before they were shed. *PhCP10* transcript abundance increased slightly in MD corollas at 24 h after pollination (Fig. 1C). Peak expression was detected at 48 h after pollination, corresponding with corolla wilting. Transcripts then decreased and were barely detectable at 72 h. Transcripts for the senescence-specific cysteine protease (*PhCP10*) were not detected in etr-44568 corollas that exhibited the nonwilting pollination response.

When ovary expansion was delayed by lower temperatures, and the etr-44568 corolla response following pollination was more similar to the senescence response in unpollinated *etr1-1* flowers (i.e. they wilted before being shed), molecular markers of senescence were detected in the corollas (Fig. 2B and C). In etr-44568 flowers grown at lower temperatures, the induction of PhNUC1 activity was detected at 192 h after pollination and corresponded with the first symptoms of corolla wilting. Maximum activity was detected at 336 h after pollination when corollas were completely wilted. Similarly, *PhCP10* transcripts were detected at 192 h and peaked at 288 h.

*3.3. Nutrient remobilization in pollinated **etr1-1** flowers depends on the timing of corolla separation*

Previous studies determined that nitrogen, phosphorus and potassium were the major nutrients remobilized during the

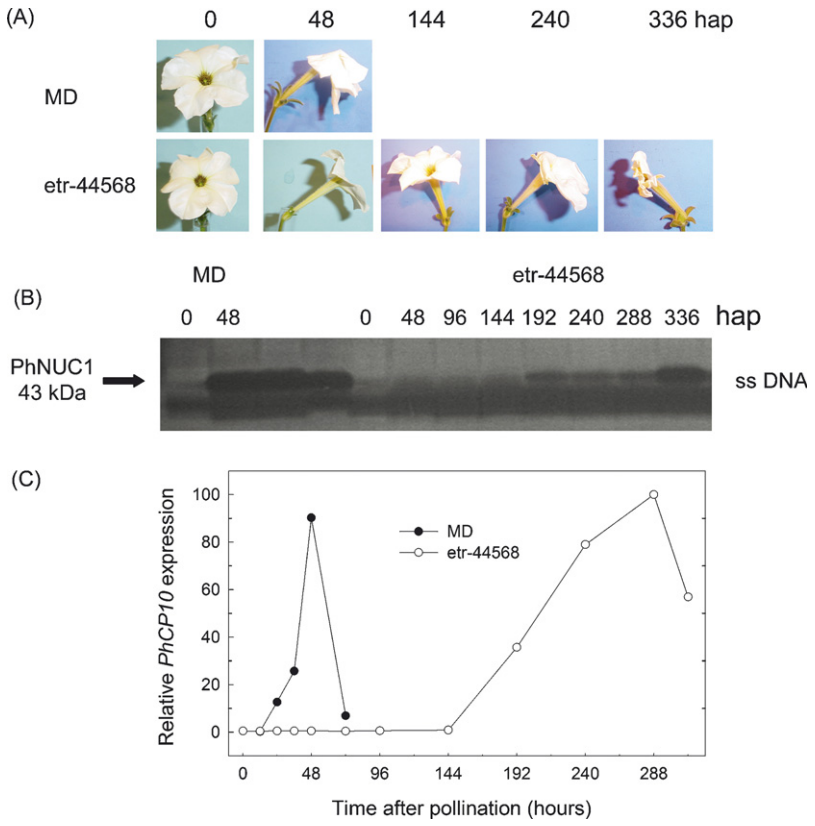

Fig. 2. The post-pollination response in ***Petunia* × *hybrida*** 'Mitchell Diploid' (MD) and 35S::***etr1-1*** transgenic petunias (etr-44568) grown at 20/16 °C (day/night). Visual characteristics of representative flowers at various times after pollination (hap, hours after pollination). Pollinated etr-44568 flowers had a wilting phenotype when grown at these lower temperatures (A). Temporal patterns of enzyme activity for the 43 kDa bifunctional endonuclease, PhNUC1. PhNUC1 activity was determined using SDS-PAGE nuclease activity gels containing ssDNA (single-stranded DNA) as described in Fig. 1B (B). Temporal patterns of gene expression for the cysteine protease, ***PhCP10***, were determined using RNA gel-blot analysis (C).

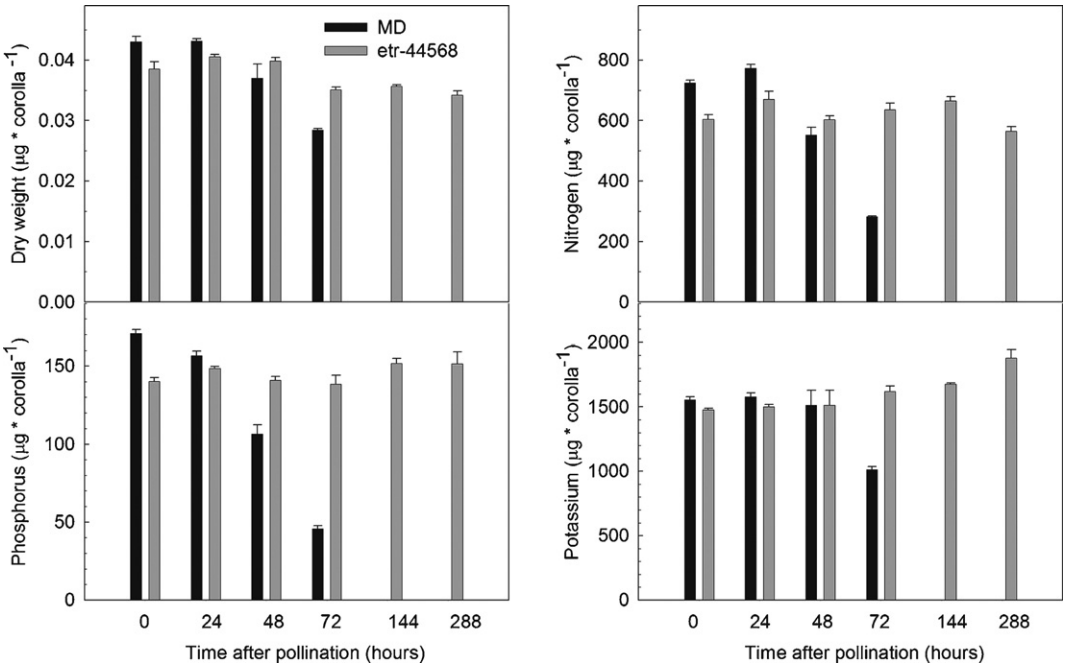

Fig. 3. Dry weight and nitrogen, phosphorus and potassium content of corollas from pollinated ***Petunia* × *hybrida*** 'Mitchell Diploid' (MD) and 35S::***etr1-1*** (etr-44568) flowers grown at higher temperatures. Petunias were grown at 24/16 °C and the longevity of etr-44568 flowers was terminated at 288 h when the growing ovary separated the nonwilted corolla from the receptacle. Zero hour (0 h) represents an unpollinated flower on the day of flower opening. Values are the mean ± S.D. for three independent nutrient measurements. Each nutrient measurement included at least 12 corollas.

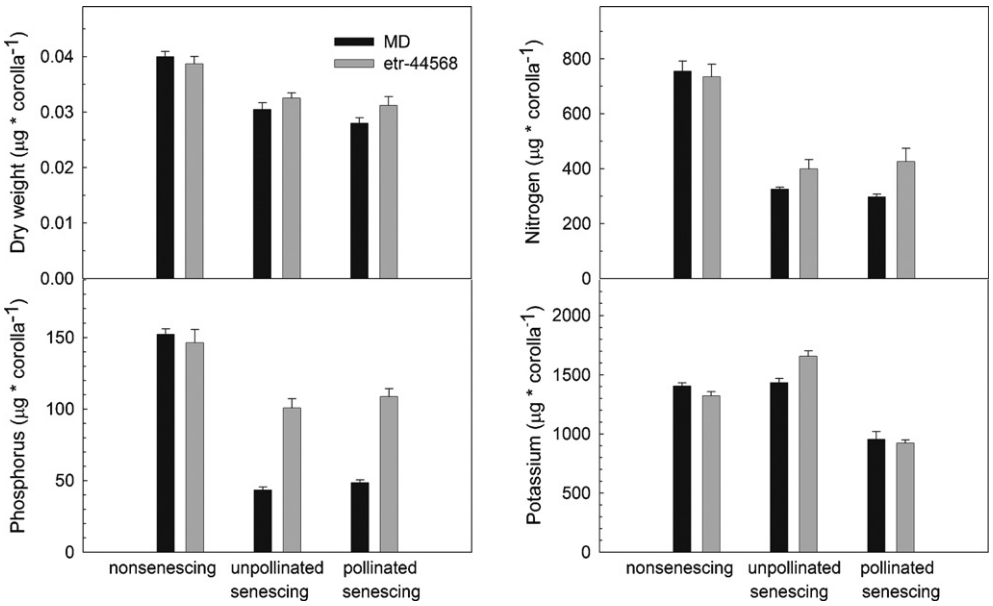

Fig. 4. Changes in the dry weight, nitrogen, phosphorus, and potassium content of ***Petunia* × *hybrida*** 'Mitchell Diploid' (MD) and 35S::***etr1-1*** corollas during the senescence of unpollinated and pollinated flowers grown at lower temperatures. Petunias were grown at 20/16 °C and the longevity of pollinated etr-44568 flowers was terminated when fully wilted corollas were shed at 432 h. DW and nutrient content was determined from nonsenescing corollas on the day of flower opening and from unpollinated senescing and pollinated senescing corollas. Corollas were collected from pollinated senescing flowers at 72 and 432 h after pollination for MD and etr-44568, respectively. Corollas were collected from unpollinated senescing flowers at 240 and 432 h after flower opening in MD and etr-44568, respectively. Values are the mean ± S.D. for three independent nutrient measurements. Each nutrient measurement included at least 12 corollas.

pollination-induced and natural senescence (i.e. the age-related senescence of unpollinated flowers) of MD petunia corollas [7,21]. Changes in the dry weight (DW) and the N, P, and K content of corollas were therefore measured to determine if nutrients were remobilized from etr-44568 corollas during the two pollination responses. The overall dry weight (DW) of MD corollas at 24/16 °C declined by 34%, from the day of flower opening to 72 h after pollination, when corollas were fully wilted (Fig. 3). A similar decline (30%) in corolla dry weight was detected from pollinated MD flowers grown at 20/16 °C (Fig. 4). Similar changes in the nutrient content of MD corollas were observed in flowers grown at the lower and higher temperatures. The N, P and K content of MD corollas at 72 h after pollination was less than on the day of flower opening (nonsenescing; 0 h). The largest decreases were seen in P content (72%), followed by N (61%) and K (34%).

When etr-44568 petunias were grown at higher temperatures and exhibited a nonwilting phenotype, there was little change in the nutrient status of the corollas from the day of flower opening until 288 h after pollination, just before they were shed (Fig. 3). The overall decline in corolla DW was only 11%. In contrast, when etr-44568 flowers were grown at lower temperatures, the delayed wilting phenotype observed in pollinated flowers was accompanied by a larger decrease in the DW and the nutrient content of the petals (Fig. 4). In the lower temperature experiment, the nutrient content of corollas was compared on the day of flower opening (nonsenescing; 0 h) and at the advanced stages of senescence in both pollinated and unpollinated flowers to determine the extent of nutrient remobilization. Pollinated senescing corollas were compared from MD and etr-44568 flowers at 72 and 432 h (18 days) after pollination, respectively. Unpollinated senescing corollas were compared from MD and etr-44568 flowers at 240 h (10 days) and 432 h (18 days), respectively. At lower temperatures, the N content of etr-44568 corollas was reduced to similar levels in senescing pollinated (reduced by 42%) and unpollinated (45%) flowers. The final N content of the etr-44568 corollas was slightly higher than MD corollas, which was reduced by 57 and 61% in unpollinated and pollinated flowers, respectively. The largest decreases were in the P levels within the MD petals, which decreased by 71% in unpollinated and 68% in pollinated flowers. While the P levels in the wilted (lower temperature) etr-44568 corollas were decreased compared to their higher temperature, nonwilting counterparts, the reduction in P levels was much less than that detected in MD corollas. In pollinated etr-44568 flowers P levels were reduced by only 26%. A 31% reduction in the total P content of the corollas was detected in senescing unpollinated etr-44568 corollas. Potassium levels in the corollas of pollinated flowers at lower temperatures were decreased by 32 and 30% in MD and etr flowers, respectively. In contrast, neither the etr-44568 nor the MD corollas showed a decrease in the K content of unpollinated corollas during senescence.

4. Discussion

Ethylene's role in flower senescence can be investigated by comparing visual, biochemical and molecular markers of senescence between wild type and 35S::*etr1-1* transgenic petunias. Transgenic petunias that constitutively express the mutant ethylene receptor *etr1-1* from *Arabidopsis* exhibit decreased ethylene sensitivity and delayed senescence [16]. The senescence of ethylene-insensitive corollas can be visualized as wilting and drying of the petal margins, while the entire corolla limb wilts and collapses in wild type flowers [18]. Despite the differences in visual symptoms, the senescence of both wild type (MD) and etr-44568 corollas is accompanied by similar hallmarks of senescence. These include DNA fragmentation, reduced protein levels and increased protease and nuclease activities [17,18]. The senescence of *etr1-1* corollas also involves the delayed induction of senescence-associated genes, but the maximum mRNA levels do not differ from those in wild type flowers [17]. This evidence would indicate that a similar senescence program is being executed during the delayed senescence of ethylene-insensitive flowers.

Similar studies in *Arabidopsis etr1-1* mutants have lead to the conclusion that ethylene operates in conjunction with age-related factors to regulate the timing of leaf senescence [22]. In *etr1-1* petunia flowers, similar age-related factors may induce the senescence program in the absence of the ethylene signal. Transgenic *etr1-1* petunia lines have been shown to have varying levels of ethylene sensitivity [15]. If these plants are not completely insensitive, the accumulation of ethylene during the extended lifespan of the flower in combination with age-related changes in the corolla's sensitivity to ethylene may lead to the induction of corolla senescence.

Pollinated flowers are often used in studies of flower senescence because pollination reliably accelerates senescence and synchronizes the symptoms among flowers. This pollination-accelerated senescence is not observed in *etr1-1* flowers, and their functional life is effectively ended when the growth of the pollinated ovary physically separates the corolla from the receptacle. The longevity of pollinated etr-44568 flowers has been reported to vary from 9.6 to 16.9 days [14–16]. This variability is due, at least in part, to differences in the growth rate of the ovary, which is influenced by temperature [13,14,23]. The number of days to fruit ripening in MD petunias is 20.6 and 30.3 days when grown at 29/24 and 24/18 °C, respectively [13]. Similar temperature effects have been observed in etr-44568 fruits, with ripening occurring at 31.9 days and 39.3 days. While accelerated ovary development at higher temperatures decreases the longevity of pollinated *etr1-1* flowers, it has no effect on MD longevity because the corollas have wilted long before the growing ovary splits the corolla tube.

The maintenance of petals is costly in terms of respiratory energy and water loss. It is therefore advantageous for the plant to remove the petals as soon as they have served their function in attracting a pollinator. Removing the showy petals may also reduce the incidence of herbivory, thereby protecting the developing ovary [4]. The programmed senescence of the corolla allows the plant to degrade macromolecules and remobilize essential nutrients before it is shed [4,7]. When ovary growth was slowed by lower temperatures, etr-44568 corollas wilted before the growing ovary began to separate them from the flower. This wilting was accompanied by hallmarks of senescence, including induction of the senescence-specific cysteine protease *PhCP10* and the nuclease PhNUC1, and a subsequent reduction in the macronutrient content of the petals. At higher temperatures, the growing ovary resulted in the shedding of a turgid corolla. The senescence program was not induced in these corollas, or it had not yet progressed to the point of inducing the senescence-specific catabolic enzymes used as molecular markers in this experiment, before the connection with the corolla was severed. Either way these experiments provide further evidence that the post-pollination senescence response involves the translocation of a signal from the gynoecium to the petals [8].

These experiments also indicate that the connection with the corolla must be maintained late into the senescence program to allow for nutrient remobilization. While corolla nutrient levels decreased following pollination in both genotypes grown at lower temperatures (Fig. 4), the reduction in the N and P content was less in etr-44568 corollas. This may indicate that the vascular connections needed for nutrient translocation were not maintained long enough to allow for complete remobilization of the N and P released during the pollination-induced catabolism of nucleic acids, proteins and other cellular constituents. The greatest differences in nutrient remobilization between etr-44568 and MD involved the macronutrient phosphorus. This may indicate that P is remobilized later in the senescence program than N, which is in keeping with the idea that large scale nucleic acid catabolism must be a component of the very latest stages of programmed cell death [5]. While the extent of remobilization and its control by ethylene is complicated in the study of pollinated etr-44568 flowers by the timing of corolla separation from the receptacle, this is not a concern in unpollinated *etr1-1* flowers. Since the large differences in P changes during senescence were also observed between unpollinated MD and etr-44568 flowers, it would be easy to conclude that phosphorus remobilization might be directly controlled by ethylene. The differences in nutrient remobilization between MD and *etr1-1* flowers represent the first quantitative differences that we have observed in the senescence of ethylene-sensitive and -insensitive petunias and they are worthy of further investigation.

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