**Nicotiana mutabilis** – a Novel System for Studying Ethylene-Mediated Floral Senescence

A.J. Macnisha and M.S. Reid  
Department of Plant Sciences  
University of California  
Davis, CA 95616  
USA

C.-Z. Jiang  
Crops Pathology & Genetics Research Unit  
USDA-ARS  
Davis, CA 95616  
USA

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**Abstract**  
*Nicotiana mutabilis,* a tobacco species recently discovered in southern Brazil, has flowers that change color during their display life. Opening buds are white, and as the flower ages, the color changes, at first gradually, and then rapidly through light pink to dark pink and even red. The onset of the pigment change is associated with a marked increase in ethylene production, and the change is delayed by treating the flowers with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action. Expression of the gene encoding chalcone synthase increased slightly in association with a substantial rise in concentrations of anthocyanins in the petals. Likewise, expression of a homolog of SAG12, a leaf senescence-associated gene first isolated from *Arabidopsis,* increased as the color change advanced and ethylene production increased. The numerous genetic and experimental tools available for *N. tabacum* can readily be applied to this close relative to provide an interesting new model for studying flower senescence.

**INTRODUCTION**

Exposure to ethylene greatly reduces the postharvest life of many cut flowers (Woltering and van Doorn, 1988; Reid, 2002). Ethylene binds to membrane-bound receptors and initiates changes at the gene level that accelerate rates of senescence (Bleecker and Kende, 2000; van Doorn and Woltering, 2008). Considerable research has been directed at identifying genes whose abundance changes during the onset of flower senescence (Eason, 2006). Genes associated with ethylene-mediated floral senescence have been isolated from a variety of flowers including carnation (Lawton et al., 1990), daffodil (Hunter et al., 2004), four o’clock (Xu et al., 2007) and petunia (Jones et al., 2005).

Recently, a close relative of tobacco, *Nicotiana mutabilis,* was discovered in southern Brazil (Stehmann et al., 2002). As its specific epithetic suggests, the petals of *N. mutabilis* change in color from white through pink to red as the flowers age. Pollination of flowers was reported to reduce their longevity (Kaczorowski et al., 2005), suggesting that ethylene may regulate flower senescence. By virtue of its close relationship to the model plant tobacco, *N. mutabilis* potentially represents an interesting alternative system for molecular studies of ethylene-mediated flower senescence.

In the current study, we confirm the involvement of ethylene in mediating senescence of *N. mutabilis* flowers. We also report on the role of ethylene in eliciting changes in expression of key genes associated with flower senescence and pigment synthesis.

**MATERIALS AND METHODS**

**Plant Material**

*N. mutabilis* plants were grown from seed in the greenhouse at the University of California, Davis using standard growth conditions and cultural practices. Flower buds

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a ajmacnish@ucdavis.edu
opened with white petals that changed to light pink and then through to dark pink and red as the flower aged during a typical 7-day lifespan (Fig. 1). Depending upon the particular experiment, flowers were harvested daily after opening at each of these seven stages of opening and senescence.

**Anthocyanin Content**

Petal samples were excised from floral tubes at each of the seven opening and senescence stages described above. They were frozen in liquid nitrogen and stored at -80°C. Frozen tissue samples (100 mg) were ground to a powder, extracted in 1 ml methanolic HCl (1%) and held overnight at 4°C (Ferrante et al., 2006). The anthocyanin concentration was then determined with a spectrophotometer at 535 nm and expressed as cyanidin-3-glucoside equivalents.

**Ethylene Production**

Flowers were harvested on the day of opening and placed into individual micro-centrifuge tubes containing deionized water. They were maintained under standard vase life conditions of 20°C, 50% relative humidity and 18 µmol/m².s⁻¹ light (12 h/day). Flowers in tubes were sealed individually into plastic 55 mL capacity vials with screw-on lids every 8 h. An air sample from within the sealed vials was withdrawn by syringe through a rubber port in each lid. The ethylene concentration in gas samples was quantified by gas chromatography to enable calculation of rates of ethylene production by flowers.

**Changes in Gene Expression**

Petal samples at each of the seven stages of flower opening and senescence were frozen in liquid nitrogen and stored at -80°C until needed. Total RNA was isolated from petal samples using TRIzol® (Invitrogen Corp.) and treated with RNase-free DNase I (Promega Corp.) to remove any contaminating genomic DNA. First-strand cDNA was synthesized from 2 µg total RNA using SuperScript™ III reverse transcriptase (Invitrogen). This cDNA was used in semi-quantitative PCR assays of the abundance of gene transcripts in the different flower stages. The PCR primers for the target genes ACO1 (1-aminocyclopropane-1-carboxylic acid oxidase), CHS (chalcone synthase) and SAG12 (senescence-associated gene) are shown in Table 1. The abundance of 18S rRNA served as an internal control (Table 1). PCR was conducted for 25-40 sequential cycles of 30 s each at 94, 55 and 72°C using a thermo cycler (Applied Biosystems). PCR products were separated and visualized on 1% agarose gel.

**Ethylene and 1-Methylcyclopropene (1-MCP) Treatment**

Flowers were harvested upon opening and placed into tubes containing deionized water. Selected flowers were sealed into a glass chamber and treated with 1 µl/L ethylene for 12 h at 20°C. For 1-MCP treatment, matching flowers were exposed to 500 nL/L 1-MCP (released from EthylBloc®, Floralife Inc.) for 12 h at 20°C in another chamber as previously described (Serek et al., 1994). Additional flowers held in air served as controls. All flowers were then held in the vase life conditions described above.

**RESULTS**

The development of pink and red coloration of *N. mutabilis* petals during the later stages of flower opening and senescence (Fig. 1) was strongly correlated with a substantial increase in the anthocyanin content of petals (Fig. 2). Between days 2 and 6 of vase life, rates of ethylene production by detached flowers increased by 2.4 to 3.7-fold relative to freshly harvested flowers (Fig. 3). Increased rates of ethylene synthesis accompanied the development of pink coloration in the petals and reached a maximum at the onset of flower senescence on days 5 to 6.

The *ACO1* gene encoding 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), an enzyme that catalyzes the final step of ethylene biosynthesis, was detected in
petals at all seven stages of flower opening and senescence (Fig. 4). The greatest abundance of ACO1 transcripts was recorded in flowers that produced elevated rates of ethylene production (Figs. 3 and 4). The gene encoding the flavonoid biosynthesis enzyme CHS was also expressed in all seven flower stages (Fig. 4). CHS transcript levels were highest in stage 1-5 flowers that were changing color (Figs. 1 and 2). A homologue of a senescence-associated gene (SAG12) was only detected in petals at stages 3, 4, 5 and 6 (Fig. 4). The sharp increase in SAG12 abundance at stage 3 corresponded to development of mid-pink coloration in petals (Figs. 1 and 4).

Exposure to 1 µL/L ethylene for 12 h at 20°C reduced the vase life of newly opened flowers by 1 day relative to control flowers (Fig. 5). Ethylene treatment accelerated the development of visible symptoms of petal senescence (wilting) and pigmentation, and transcripts of SAG12 were detectable 1 day after the start of ethylene treatment (data not shown) in comparison to 3 days for the controls (Fig. 4). In this particular experiment, the display life of detached flowers was shorter than that of flowers left attached to the plant. Pre-treatment of flowers with 500 nL/L 1-MCP for 12 h at 20°C delayed the change in petal color and senescence; flower display life was 3 days longer than control flowers (Fig. 5). 1-MCP treatment also delayed the expression of SAG12 transcripts in petals until day 6 of vase life (data not shown).

DISCUSSION

N. mutabilis represents a new and interesting model system for the study of ethylene-mediated flower senescence. Flowers are short-lived and their petals change color and wilt in association with elevated rates of ethylene production and ACO1 gene expression (Figs. 1, 3 and 4). Floral senescence was also accelerated upon exposure to exogenous ethylene (Fig. 5). These findings support the role of ethylene as a modulator of N. mutabilis flower senescence.

The petals of N. mutabilis flowers change color during their development from white through light to dark pink and ultimately red by the onset of senescence (Fig. 1). This change in coloration was associated with a substantial rise in the anthocyanin concentration in petals (Fig. 2). However, only a slight increase in expression of CHS (chalcone synthase) accompanied petal coloration (Fig. 4). CHS acts early in the flavonoid and anthocyanin biosynthesis pathway, and an increase in expression of downstream genes such as anthocyanidin synthase is likely to be more closely correlated with changes in petal color (Farzad et al., 2003). Increased rates of ethylene production by N. mutabilis flowers also accompanied the change in petal pigmentation (Fig. 3). Moreover, exposure to exogenous ethylene accelerated petal coloration while treatment with the anti-ethylene agent, 1-MCP, retarded the process. Ethylene has been associated with changes in petal color in relatively few flower species such as Lupinus and Cymbidium (Stead and Reid, 1990; Woltering and Somhorst, 1990). Accordingly, N. mutabilis flowers may be a useful system for studies of the role of ethylene in regulating anthocyanin biosynthesis.

A number of senescence-associated genes (SAGs) have been isolated from leaf tissues of several plant species including Arabidopsis thaliana (Lohman et al., 1994), Brassica napus (Noh and Amasino, 1999) and N. tabacum (Grbic, 2002). Of the Arabidopsis SAGs, SAG12 is considered the one of best molecular markers for leaf senescence as it is only present in senescing leaves (Lohman et al., 1994; Weaver et al., 1998). A homologue of the Arabidopsis SAG12 was also strongly up-regulated in senescing N. tabacum flowers (Grbic, 2002). In the present study, we showed that SAG12 transcripts began to accumulate in N. mutabilis petals 3 days prior to visible flower senescence (wilting) in association with increasing rates of ethylene biosynthesis (Figs. 3 and 4). This expression pattern is consistent with other leaf and petal SAGs (e.g. SAG13, PhCP8) and suggests that this particular homologue of SAG12 acts early in the N. mutabilis floral senescence process (Lohman et al., 1994; Jones et al., 2005).

Exposure of newly opened N. mutabilis flowers to ethylene significantly advanced the onset of SAG12 up-regulation, while treatment with 1-MCP delayed its expression and
associated flower senescence. Exogenous ethylene also induces an increase in the abundance of senescence-associated cysteine protease genes (e.g. \textit{SAG12}, \textit{pDCCP1}) in Arabidopsis leaves and carnation and petunia petals (Jones et al., 1995, 2005; Weaver et al., 1998). In Arabidopsis, ethylene treatment initiated substantial yellowing and \textit{SAG12} expression only in older leaves (Weaver et al., 1998). \textit{SAG12} expression in \textit{N. mutabilis} petals was also associated with an increase in transcript levels of \textit{ACO1}, a gene encoding the enzyme ACO which catalyzes the last step of ethylene synthesis (Fig. 4). Collectively, our results imply that \textit{SAG12} is an ethylene-up-regulated gene that might coordinate expression of other senescence-associated proteins that directly accompany wilting of \textit{N. mutabilis} petals.

Owing to its close taxonomic relationship to \textit{N. tabacum} and other members of the Solanaceae (e.g. petunia), a number of well established genetic and experimental tools (expressed sequence tags, transformation protocols, virus-induced gene silencing) could readily be applied to \textit{N. mutabilis} (Chen et al., 2004). For example, a newly devised protocol known as TASSEL (transposon-associated, senescence-specific enhancer-linked) activation tagging may aid the identification of senescence-specific genes in \textit{N. mutabilis} petals (S. Gan, pers. commun.). The novel features of ethylene-mediated floral senescence in \textit{N. mutabilis} whereby petals change color in association with an increase in \textit{SAG12} expression highlights interesting opportunities for further study of this species.

**Literature Cited**


Noh, Y.-S. and Amasino, R.M. 1999. Regulation of developmental senescence is conserved between \textit{Arabidopsis} and \textit{Brassica napus}. Plant Mol. Biol. 41:195-206.


**Tables**

Table 1. Primers used for PCR of target genes in *Nicotiana mutabilis* petals.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>PCR primers</th>
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<tbody>
<tr>
<td>ACO1</td>
<td>F 5’-TTTTACAATCCAGGAAGTGATGC-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-ATCTTGCTTCTTAGCTTGAAC-3’</td>
</tr>
<tr>
<td>CHS</td>
<td>F 5’-TATGATGTACCAAAAGGTTG-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-ACAACAGTCTGACTGGAAGCC-3’</td>
</tr>
<tr>
<td>SAG12</td>
<td>F 5’-AGTGGCTAATCAACCTGTTC-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-ATGCGCATATCCACTGTCAC-3’</td>
</tr>
<tr>
<td>Nt18S rRNA</td>
<td>F 5’-CATGGCCGTTCATGATGAGG-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-AAAGAAGCTTGAGGGGATAC-3’</td>
</tr>
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Figures

Fig. 1. Photographs of individual *Nicotiana mutabilis* flowers and the typical change in petal color from white to red over a 7 day period in planta. Scale bar represents 1 cm.

![Fig. 1](image)

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
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Fig. 2. Change in anthocyanin concentration of methanolic extracts of *Nicotiana mutabilis* petals. Data are presented as means±s.e.m. (*n*=5).

![Fig. 2](image)

Cyanidin-3-glucoside equivalents (µg/g)

Time (days)

0 1 2 3 4 5 6

0 100 200 300 400
Fig. 3. Change in rates of ethylene production at 20°C by detached individual flowers of *Nicotiana mutabilis*. Data are presented as means±s.e.m. (n=10).

Fig. 4. Photographs of 1% agarose gels showing expression of genes isolated from *Nicotiana mutabilis* petals at seven sequential stages of flower opening and senescence.
Fig. 5. Display life at 20°C of detached individual flowers of *Nicotiana mutabilis* in tubes of water following treatment with 1 µl/L ethylene or 500 nL/L 1-MCP for 12 h at 20°C. Flowers held in air acted as controls. Data are presented as means±s.e.m. (*n*=5).