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SHORT COMMUNICATIONS

Application of Digital Image Analysis System for Fine Evaluation of Varietal Differences and the Role of Ethylene in Visible Petal Senescence of Morning Glory

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Abstract We detected differences in both onset and progression of visible petal senescence among morning glory cultivars by application of a digital image analysis system. The system is based on semiautomated time-lapse measurement of corolla areas. The system could also be applied to evaluate the effects of ethylene and its inhibitor on visible petal senescence. Both onset and progression of visible petal senescence were accelerated by ethylene treatment in all six cultivars tested. Treatment with aminooxyacetic acid (AOA), an ethylene biosynthesis inhibitor, prolonged time to onset of visible petal senescence in three of the six tested cultivars. In contrast, AOA treatment had no effect on duration of visible petal senescence in any tested cultivars. These data suggested differences among morning glory cultivars in the role of endogenous ethylene in controlling onset of visible petal senescence. In addition, we propose a new application of image analysis to fine quantification of time-lapse changes in the shape of plant organs.

Keywords Flower longevity · *Ipomoea nil* · Morphological character · Phenotypic variation · Quantitative determination · Shape analysis

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Introduction

Flower longevity is one of the important quality factors in ornamental plants. In several species, visible symptoms of petal senescence such as deformation and discoloration mainly restrict flower longevity. To prolong flower longevity of these species, molecular breeding focusing on genes that regulate visible petal senescence may be a beneficial approach. In addition, visible petal senescence is also a symptom of developmental programmed cell death (PCD) in petals (van Doorn and Woltering 2008). Thus, the study of genes that regulate visible petal senescence leads to the characterization of petal PCD.

Genes that exhibit enhanced expression during senescence in plant organs have been called senescence-associated genes (SAGs) (Nam 1997). Many SAGs have been identified in senescing petals of flowers such as *Hemerocallis* (Panavas and others 1999), *Narcissus* (Hunter and others 2002), and *Ipomoea* (Yamada and others 2007) using subtractive and/or differential methods. However, few genes have been demonstrated to regulate visible petal senescence. Thus, we have planned another approach which is to identify genes of the quantitative trait loci (QTLs) responsible for variation in the parameters of visible petal senescence such as time to onset and rate of progression of senescence symptoms. Such an approach must discriminate slight differences in these parameters among a large number of individuals from a segregating population. However, this is very time- and energy-consuming work, and it is not easy to evaluate these visual parameters quantitatively.

Digital image analysis has emerged as a powerful tool for plant phenotypic analysis. For example, systems have been developed for quantitative evaluation of the shape of plant organs. Bylesjö and others (2008) developed one system to provide indicators of leaf shape such as blade dimensions

and area on scanned images of leaves. They showed that their system allowed semiautomated and large-scale determination of leaf shape. We developed another system for efficient measurement of the area of selected portions of digital photographs of plant organs (Tanabata and others 2010). The system can also be used in semiautomated mode to analyze time-lapse changes in the shape of plant organs. Measurements of the corolla area in time-lapse photographs of cut *Ipomoea nil* flowers suggested that our system is able to quantify visible petal senescence as a decrease in corolla area.

Ephemeral morning glory flowers (including *I. nil*, *I. purpurea*, and *I. tricolor*) show clear visible senescence as petal inrolling, which is a type of petal deformation, and have been used as a model of petal senescence (Matile and Winkenbach 1971; Kende and Baumgartner 1974; Yamada and others 2006; Shibuya and others 2009). In Japan, many cultivars of morning glory have been produced and collected; thus, varietal differences in visible petal senescence may be detectable. To detect varietal differences in visible petal senescence as indicated by petal inrolling, we investigated eight cultivars of morning glory, including *I. nil* and the closely related *I. hederacea*, using the previously developed digital image analysis system (Tanabata and others 2010). Furthermore, we applied this system to evaluate the role of ethylene, which is a major accelerator of plant senescence, in visible petal senescence of morning glory cultivars.

Materials and Methods

Plant Material

Seven *I. nil* cultivars [Peking Tendan (Q61), Nepal line (Q62), Shishi Botan line (Q438), Uzu line (Q851), Tokyo Kokei Standard (Q1065), Africa line, and Violet] and one *I. hederacea* cultivar [American Standard (Q65)] were grown in a controlled chamber at 24 ± 1°C with 12 h per day of fluorescent light (color temperature of 5,000 K) at a light intensity of 100 μmol m^{-2} s^{-1}. The light period was from 12:00 to 0:00.

Digital Image Analysis

To detect differences in visible petal senescence, flower buds were cut at 22:00 on the day prior to anthesis. Cut buds were then put in microtubes containing distilled water and held in a controlled chamber with the same conditions. For digital image analysis, the petals were photographed horizontally every 30 min using an interval program of a digital camera (PowerShot S3 IS, Canon, Tokyo, Japan), and the image data were stored in JPEG format at 180 dpi (dot per inch) resolution. The distance between the camera and flowers was 60 cm. The corolla area was measured as a pixel value using automatic segmentation software (free to download at http://www.kazusa.or.jp/picasos/). The relative corolla area was calculated as the ratio to the maximum corolla area, defined as 1.0. Time to onset and rate of progression of visible petal senescence were evaluated based on the length of time during which the relative corolla area ranged between fixed values, as described in the "Results and Discussion" section. To investigate the effects of ethylene and the ethylene biosynthesis inhibitor aminooxyacetic acid (AOA) on visible petal senescence, flower buds were excised at 10:00 on the day of anthesis and then transferred to sterile distilled water as controls or to aqueous solutions of 5 mM AOA. For ethylene treatment, cut buds were put into closed 12-l clear plastic chambers containing fresh air for controls or 1 μl l^{-1} ethylene at 12:00. Time to onset and the progression rate of visible petal senescence were evaluated as described above.

Ethylene Measurement

Flowers of Violet were treated with sterile distilled water as controls or aqueous solutions of 5 mM AOA as mentioned above. Ethylene production was measured after careful excision of the petal from the flowers and then enclosed in a 15-ml glass vial. After 1 h of incubation under darkness at 23°C, ethylene in 1 ml of headspace gases was detected using a gas chromatograph (model GC-13B, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a 150-cm stainless-steel column packed with activated alumina. The column temperature was 80°C and the detector was 200°C, with a nitrogen gas pressure of 95 kPa.

Statistical Analysis

Statistically significant differences among cultivars were examined using Scheffe's F test and between chemical treatment and control using Student's t test. The relationship between time to onset and duration of visible petal senescence was examined using Pearson's correlation coefficient test.

Results and Discussion

Figure 1a, b shows a representative flower of each cultivar in time-lapse photographs and the corresponding value of relative corolla area, calculated as the ratio to the maximum corolla area, defined as 1.0. Although the values of relative corolla area increased rapidly to about 0.7 by 12:00, the subsequent rate of increase of the value varied depending on the cultivar. Based on these data, we defined

the time point when the relative corolla area exceeded 0.7 as anthesis. In a similar manner, we also defined the time point when the value fell below 0.9 after reaching a maximum as the onset of visible petal senescence, and when it fell below 0.4 to be essentially the completion of visible petal senescence.

Time to onset of visible petal senescence was calculated as the time from anthesis to onset of visible petal senescence. The mean time to onset of visible petal senescence of Violet, a commonly used cultivar in many studies, was 10.9 h (Fig. 1b). The mean time for Peking Tendan, which had the shortest time among these cultivars, was 4.2 h or approximately 39% the value of Violet. The mean time for the Uzu line, which had the longest time, was 20.1 h or approximately 185% the value of Violet. The rate of progression of visible petal senescence was evaluated based on the duration of visible petal senescence, which was calculated from onset to near completion of visible petal senescence. The mean duration of visible petal senescence of the Shishi Botan and the Uzu lines was longer than the other cultivars (Fig. 1c), indicating that they had a lower rate of progression of visible petal senescence. In addition, a positive correlation was observed between time to onset and duration of visible petal senescence among individual flowers ($r = 0.58$, $P < 0.01$, Pearson's correlation coefficient test). Based on time-lapse measurement of the corolla

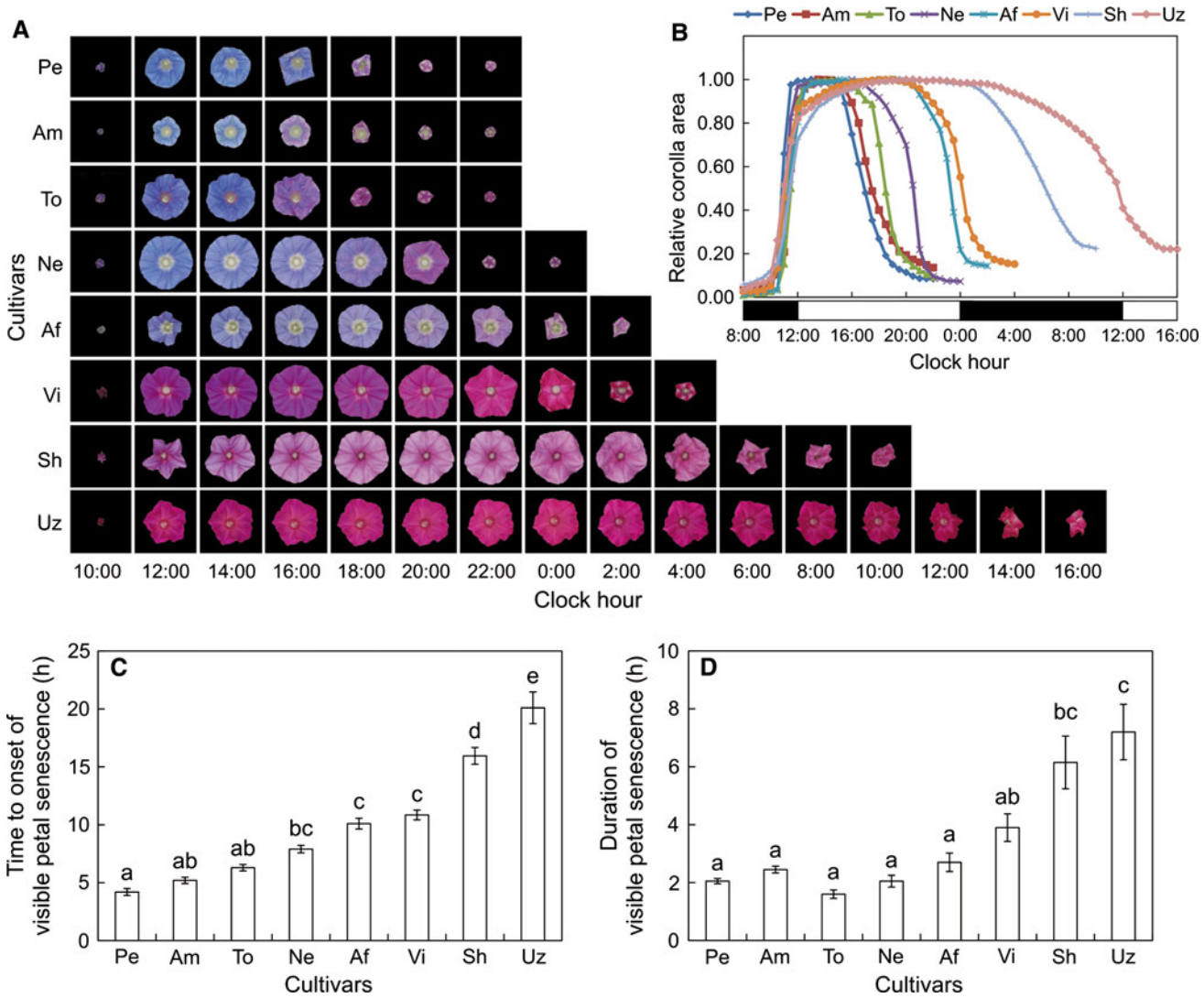

Fig. 1 Time to onset and rate of progression of visible petal senescence in eight cultivars of morning glory. **a** A representative flower of each cultivar in time-lapse photographs. **b** Changes in the value of relative corolla area. Values shown are the ratio to the maximum corolla area, defined as 1.0, of the flowers in panel A. **c** Difference in time to onset of visible petal senescence in cut flowers. **d** Difference in duration of visible petal senescence in cut flowers. The values are mean ± SE of ten flowers. Different letters indicate that the values were significantly different at $P < 0.05$ (Scheffe's F test). *Pe* Peking Tendan, *Am* American Standard, *To* Tokyo Kokei Standard, *Ne* Nepal line, *Af* Africa line, *Vi* Violet, *Sh* Shishi Botan line, *Uz* Uzu line

area, the system used revealed differences in visible petal senescence of morning glory cultivars, and the relationship between onset and progression of senescence.

Petal senescence is regulated differently in different flower species. In one group of flowers, petal senescence is highly ethylene sensitive and regulated by ethylene, whereas in another group it is ethylene insensitive and not regulated by this molecule (van Doorn and Woltering 2008). In a previous study, exogenous ethylene induced premature petal senescence in Violet (Yamada and others 2006). We also applied the digital image analysis system to evaluate the effects of ethylene treatment on both onset and progression of visible petal senescence in six cultivars, including *I. nil* and *I. hederacea*. In all tested cultivars, 1 μl l^{-1} ethylene reduced time to onset of visible petal senescence to a similar level (Fig. 2a), suggesting that the ethylene sensitivities of the cultivars were not significantly different. Furthermore, ethylene treatment shortened the duration of visible petal senescence in all tested cultivars (Fig. 2b), indicating that it accelerated the progression of visible petal senescence. These results also indicated that the differences in both onset and progression of visible petal senescence among these cultivars did not result from lack of ethylene sensitivity. The image analysis system clearly showed that exogenous ethylene can function as an accelerator of both onset and progression of visible petal senescence in morning glory flowers. Although ethylene can accelerate the onset of visible petal senescence, few studies have investigated whether ethylene accelerates progression of the visible symptoms of petal senescence.

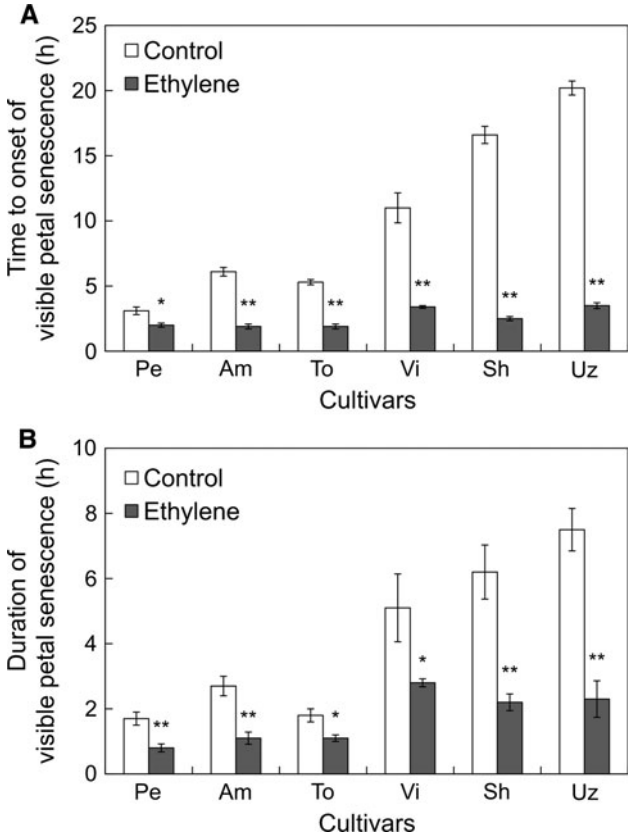

Fig. 2 Effects of ethylene treatment on onset and progression of visible petal senescence in six cultivars of morning glory. **a** Time to onset of visible petal senescence in cut flowers treated with 1 μl l^{-1} ethylene. **b** Duration of visible petal senescence in cut flowers treated with 1 μl l^{-1} ethylene. The values are mean ± SE of five flowers. Statistical significance is indicated by asterisks at $*P < 0.05$ and $**P < 0.01$ (Student's *t* test). Abbreviations of cultivar names are the same as those used in Fig. 1

Yamada and others (2006) showed that ethylene inhibitors had no effect on the visible petal senescence of Violet, suggesting that natural senescence is not controlled by endogenous ethylene in *I. nil*. To investigate the role of endogenous ethylene in petal senescence of other morning glory cultivars from *I. nil* and *I. hederacea*, we evaluated the effects of AOA on visible petal senescence with the digital image analysis system. Inhibition of ethylene biosynthesis by AOA treatment was preliminarily confirmed in petals of Violet (Fig. 3). Two peaks of ethylene production were detected in petals of control flowers at 12:00 and 4:00, corresponding to the time of anthesis and completion of visible petal senescence in this cultivar (Fig. 1a, b). The former peak of ethylene production might be involved in corolla unfolding (Koning 1986) and the latter one in petal senescence (Kende and Baumgartner 1974). In petals of flowers treated with 5 mM AOA, the ethylene production was very low at all tested time points and no peaks of ethylene production were detected. These data suggested that AOA treatment almost completely inhibited ethylene biosynthesis in petals of morning glory.

Effects of 5 mM AOA on time to onset of visible petal senescence varied depending on the cultivar (Fig. 4a). The mean time to senescence of flowers of Peking Tendan,

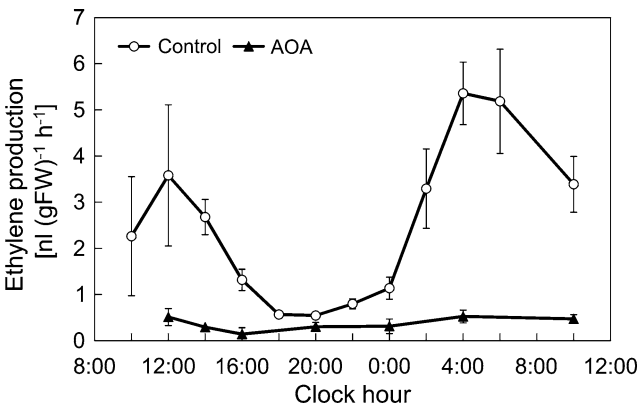

Fig. 3 Ethylene production in Violet flowers treated with an ethylene inhibitor. Flowers were excised at 10:00 and transferred to sterile distilled water (control) or aqueous solution of the ethylene biosynthesis inhibitor AOA. Ethylene production was measured using gas chromatography. The values are mean ± SE of three flowers

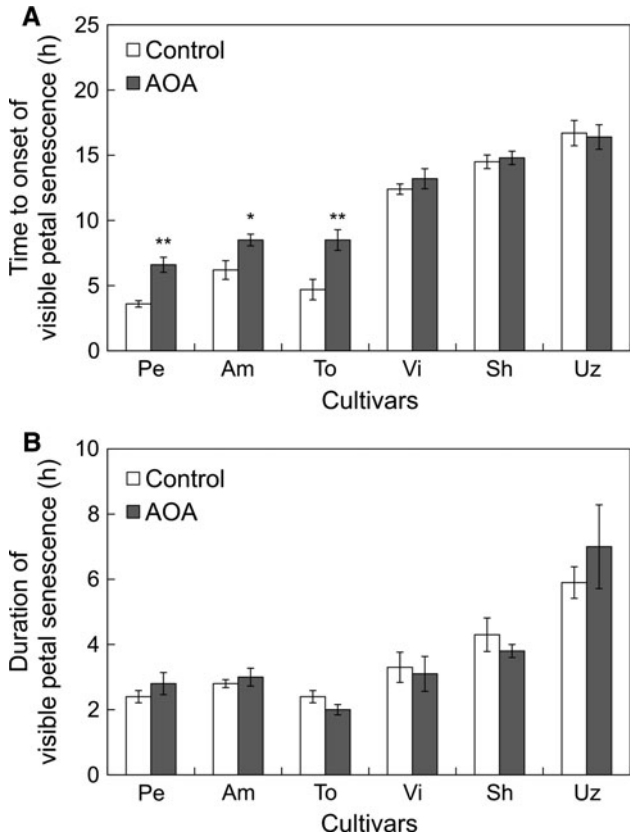

Fig. 4 Effects of AOA on onset and progression of visible petal senescence in six cultivars of morning glory. **a** Time to onset of visible petal senescence in cut flowers treated with 5 mM AOA. **b** Duration of visible petal senescence in cut flowers treated with 5 mM AOA. The values are mean ± SE of five flowers. Statistical significance is indicated by asterisks at $*P < 0.05$ and $**P < 0.01$ (Student's *t* test). Abbreviations of cultivar names are the same as those used in Fig. 1

American Standard, and Tokyo Kokei Standard treated with AOA was prolonged to approximately 183, 137, and 181% the value for the control flowers, respectively. In contrast, the mean time of Violet, the Shishi Botan line, and the Uzu line showed no significant change. These results suggest that endogenous ethylene is involved in the time to onset of visible petal senescence of two *I. nil* (Peking Tendan and Tokyo Kokei Standard) cultivars and one *I. hederacea* (American Standard) cultivar. Interestingly, these cultivars showed relatively short mean time to onset and duration of visible petal senescence among those tested (Fig. 1c, d). In contrast, AOA had no effect on duration of visible petal senescence in any tested cultivars (Fig. 4b), suggesting that endogenous ethylene does not accelerate progression of visible petal senescence in morning glory. The image analysis system thus suggested that among morning glory cultivars there are differences in the role of endogenous ethylene in controlling the onset of visible petal senescence. In addition, our results imply that factors other than ethylene are related to varietal differences detected in visible petal senescence of morning glory. The genes associated with such factors may regulate visible petal senescence and be available for molecular breeding of flower longevity.

We have applied a digital image analysis system to quantify visible petal senescence in morning glory cultivars. Methods for evaluating the degree of morphological senescence symptoms in petals generally have been subjective (Kende and Baumgartner 1974; Hunter and others 2004; Onozaki and others 2004; Xu and others 2007). These evaluation methods are based on qualitative judgment of the degree of senescence symptoms and may be insufficient when discriminating the slight differences among a large number of individuals, especially with respect to the relatively complex traits of visible petal senescence, such as its rate of progression. An earlier method based on detecting changes in corolla diameter accompanied by petal inrolling (Yamada and others 2006) was relatively objective and quantitative but required many manual procedures and much time for evaluation. However, the analysis system used in our study, which was based on semiautomated quantification of time-lapse changes in corolla area as an indicator of flower shape, is able to discriminate slight differences in both time to onset and rate of progression of visible petal senescence in a shorter amount of time and with less reliance on manual procedures. Large-scale application of this system will enable analysis of QTLs associated with onset as well as progression of visible petal senescence using morning glory cultivars with significant differences in these parameters, for example, Peking Tendan and the Uzu line. This system may be applicable to other species that show petal deformation. We have already confirmed that this system can be used to quantify visible petal senescence of nonephemeral snapdragon (*Antirrhinum majus*) flowers (Takahashi and others unpublished data). In addition, the system may also be useful for quantitative analysis of time-lapse changes in other plant organs in situ, such as the growth of leaves or fruit.

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