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Novel Evaluation Method of Flower Senescence in Freesia (*Freesia hybrida*) Based on Apoptosis as an Indicator

Tetsuya YAMADA[1,2,*], Yasumasa TAKATSU[1], Masakazu KASUMI[1], Toru MANABE[1], Mikio HAYASHI[1], Wataru MARUBASHI[2] and Masaru NIWA[2]

[1] *Plant Biotechnology Institute, Ibaraki Agricultural Center, Ago, Iwama, Nishi-ibaraki, Ibaraki, 319-0292, Japan*
[2] *School of Agriculture, Ibaraki University, Ami, Inashiki, Ibaraki 300-0393, Japan*
**Corresponding author* E-mail address: teyamada@d9.dion.ne.jp

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Abstract

Flower longevity is an important character for cut flower quality. However it is hard to evaluate flower senescence objectively, and critical measurement method is required. To improve measurement methods of flower senescence, we tried to estimate the wilting process of freesia petals based on apoptotic cell death as an indicator. Apoptotic changes such as chromatin condensation, nuclear fragmentation and DNA fragmentation were observed in the petals undergoing senescence. A good correlation (r=0.84) was also observed between the rate of nuclear fragmentation detected by flow cytometry and the rate of cell death indicated by trypan blue staining in petals. These results suggested that the rate of nuclear fragmentation reflected flower senescence, and that it was possible to evaluate flower senescence using flow cytometry.

One of the most important characteristics to be considered in the improvement of cut flower quality is vase life. The senescence of cut flowers induced by ethylene has been studied and mechanisms have been well clarified (Van Altvorst and Bovy, 1995; Reid and Wu, 1992). Chemicals considered to be inhibitors of ethylene action such as silver thiosulphate (STS) complex (Reid and Wu, 1992) have been used to extend the vase life of ethylene-sensitive flowers. In addition, improvement of post-harvest longevity of ethylene-sensitive flowers has been attempted by conventional breeding (Onozaki *et al.*, in press), molecular breeding (Chang *et al.*, 1993) and application of chemicals (Beyer, 1976; Veen and Geijin, 1978) in ethylene-sensitive flower species such as *Dianthus caryophyllus*.

On the contrary, the senescence of petals of ethylene-insensitive flowers has not been studied, and the mechanism has not been clarified in detail (Reid and Wu, 1992). Yamane *et al.* (1999) reported that the activities of superoxide, catalase and peroxidase changed sharply during senescence of gladiolus florets. Otsubo and Iwaya-Inoue (2000) reported that trehalose preserved cell viability in gladiolus spikes, and prolonged the vase-life of the cut flowers. Although these studies were unique and useful, they did not have sufficient information for breeding for flower longevity, because the fundamental factors of flower senescence have not been clarified. In addition, we are trying to improve flower longevity of ethylene-insensitive flower such as freesia by breeding techniques, but it is hard to measure flower senescence objectively. The senescence of petals was visually categorized into five or six classes in former studies. However the categorization might be influenced by researcher subjectivity, so a critical measurement method is required for flower senescence evaluation.

Recently, apoptosis has been studied even in plants, and has received attention in the field of leaf senescence (Yen and Yang, 1998), ethylene-sensitive flower senescence (Orzaez and Granell, 1997), lethality in hybrid seedlings (Marubashi *et al.*, 1999; Yamada *et al.*, 2000, 2001) and host-parasite interaction (Sasabe *et al.*, 2000). Apoptosis is defined as the active cell death in a life cycle of animals and plants, and ethylene-insensitive flower senescence may be explained by this phenomenon as well. To improve evaluation methods of flower senescence, we tried to estimate the wilting process of freesia perianth using apoptotic cell death as an indicator, in this study.

Freesia hybrida L. H. Bailey 'Elegance' was kindly provided by Dr. Takeshi Motozu, Horticul-

tural Institute, Ibaraki Agricultural Center. Plants were grown in a green house at 12 ℃ to 30 ℃ under natural light conditions. Stages of flower development were visually categorized into five classes: I. bursting bud; II. half-opened; III. fully opened; IV. slightly wilted whole petal; V. severely wilted and browned whole petal. Typical examples of these stages were presented in **Fig. 1A**.

For detection of apoptotic change of nuclei, protoplasts were isolated from flower petals. Petals were collected from the flowers at each stage (I to V). Petals were treated by the enzyme cocktail for four hours at room temperature, and were filtered through a 50 μm nylon sieve. The enzyme cocktail was composed by 0.4% cellulase 'Onozuka' R-10 (Yakult Co., Japan), 0.2% macerozyme R200 (Yakult Co., Japan) and 300 mM Sucrose. Isolated protoplasts were stained with 0.5% 4,6-diamino-2-phenylindole 2HCl (DAPI) and changes of nuclei structures were observed under a fluorescence microscope (BH2; Olympus, Japan). For detection of DNA fragmentation, genomic DNA was extracted from fresh petals of flowers at each stage (I to V) using the modified CTAB method (Yamada *et al.*, 2000). To detect DNA fragmentation, extracted DNA was separated on 3% agarose gel and was elucidated using SYBR Gold Gel Stain (Molecular Probe, Oregon, USA) and UV light.

For flow cytometric analysis, 25 mm^2 portions of the blade of petals were collected from flowers at each stage (I to V) and petals heated at 85 ℃ for 10 min were used as a control for necrosis. These portions were chopped in 0.5 m*l* nuclei extraction buffer (solution A of the High Resolution Kit for Plant DNA, Partec, Münster, Germany). After filtration through a 30 μm nylon sieve, 2 m*l* of staining solution containing the dye (DAPI, solution B of the Kit) was added. The analysis was performed by a PA flow cytometer (Partec). Rate of nuclear fragmentation was calculated by the formula {(Area of typical peak / Area of total count) × 100} provided by Win MDI (ver. 2.8) software. For detection of cell death in petals, protoplasts were isolated from flower petals at each stage (I to V) by the method mentioned above, and were stained by 0.4% trypan blue (Wako, Japan). Stained cells were scored as dead ones. Rate of cell death was calculated as follows: (Number of dead cells / Total number of cells) × 100.

Apoptotic change of nuclei was detected in petals of freesia undergoing senescence. **Fig. 1B** showed progressive changes of nuclear structures. Normal structure of chromatin was observed in the isolated protoplasts from petals at stage I and II. Chromatin condensation was observed at stage of flowering

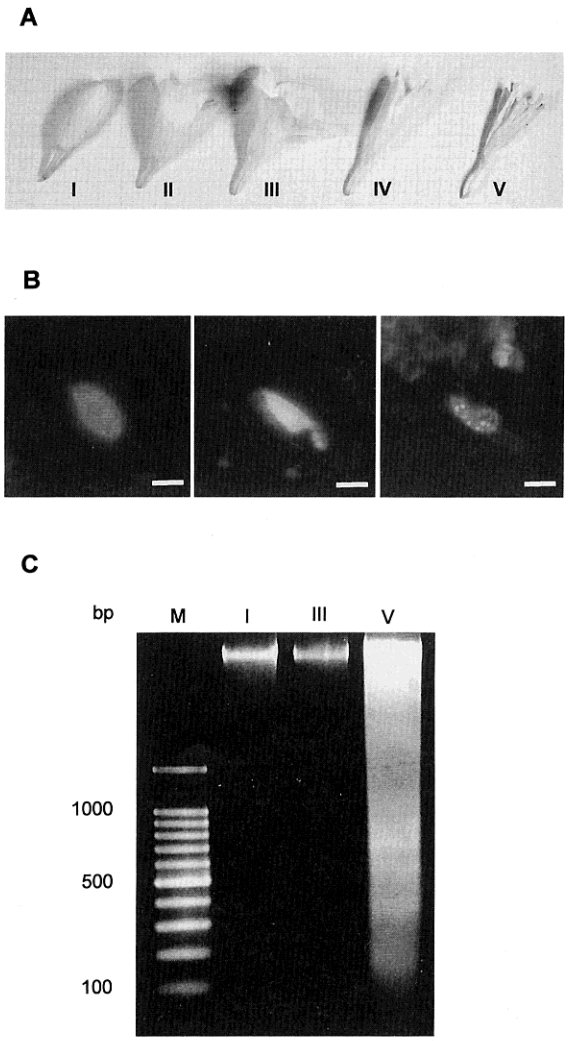

Fig. 1 Detection of apoptosis in petals of freesia (*Freesia hybrida*) undergoing senescence. (A) Stages of flower development: I. bursting bud; II. half-opened; III. fully opened; IV. slightly wilted whole petal; V. severely wilted and browned whole petal. Flowers were detached from plants that were grown in a green house at 12 ℃ to 30 ℃ under natural light conditions. (B) Apoptotic changes of nuclei detected in the petals. Protoplasts were stained with 0.5% DAPI, and then observed under a fluorescence microscope (Bars are 25 μm). Photographs show progressive changes of nuclear structure in the isolated protoplasts, left: normal structure of chromatin observed in petal at stages of I and II, middle: chromatin condensation observed in petal at stages of III to V, right: nuclear fragmentation observed in petals at stages of IV and V. (C) DNA fragmentation detected in the petals. DNA solution was electrophoresed in a 3% agarose gel and stained with SYBR Gold (Molecular Probes), M: 100 bp DNA ladder marker. The senescent petals at stage V generated DNA ladders as a typical characteristic of apoptosis.

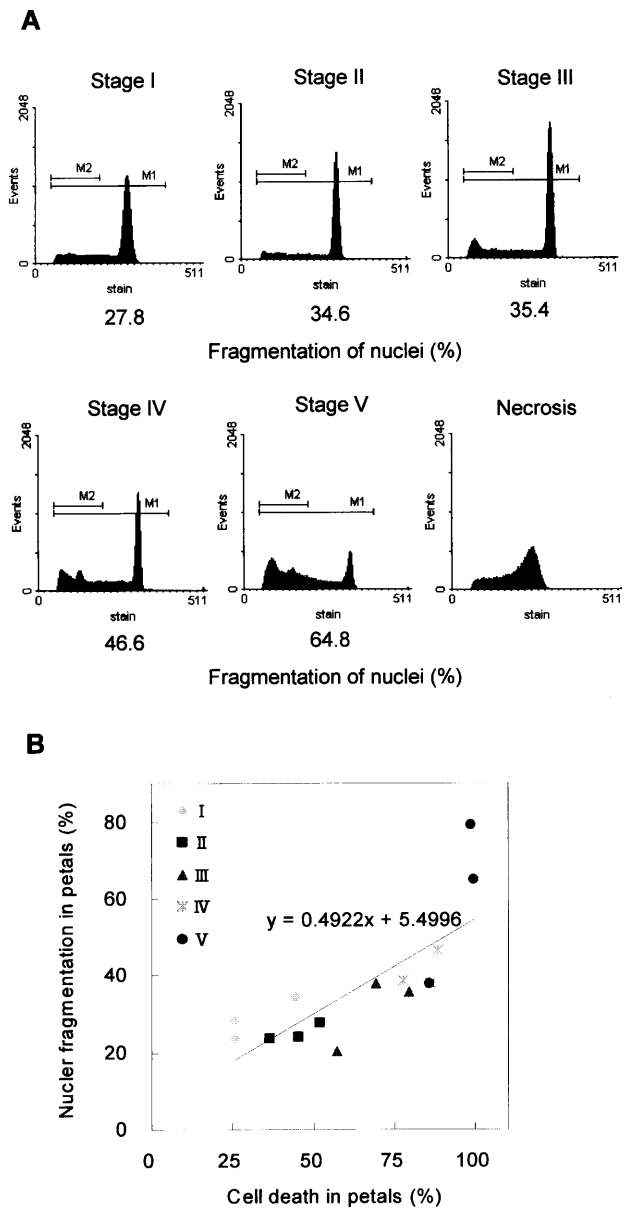

Fig. 2 Quantitative measurement of nuclear fragmentation in petals of freesia undergoing senescence. (A) Changes of nuclei in the petals. Data were obtained from flow cytometry of nuclei isolated from the petals of each stage (I-V). Total count is about 50,000 nuclei. Fragmentation rate of nuclei was calculated from histograms, as a relative percentage of fragmented nuclei in total count of nuclei (M2/M1), using Win MDI (ver. 2.8) software for flow cytometric analysis. Necrosis was induced in the petals of stage I by heat shock method (85 ℃ for 10 min) as a control. (B) Correlation between the rate of nuclear fragmentation and the rate of cell death detected in the petals (r=0.84, obtained after arcsine transformation). Nuclear fragmentation rate was estimated by flow cytometry of about 50,000 nuclei isolated from each petal. The rate of cell death was measured by 0.4% trypan blue staining about 1,000 protoplasts isolated from each petal.

(III, IV and V), and nuclear fragmentation was observed in petals at stage IV and V. DNA fragmentation was also detected in petals at stage of wilting (IV and V), and generated DNA ladders were observed. However this phenomenon was not observed in petals at stage I, II and III (**Fig. 1C**). Chromatin condensation, nuclear fragmentation and DNA fragmentation were considered as typical phenomena of apoptosis (Kerr *et al.*, 1974; Wyllie *et al.*, 1984). These results suggested that the process of cell death inducing senescence in petals of freesia was indeed apoptosis.

To improve evaluation methods of flower senescence, the relationship between the rate of nuclear fragmentation and the rate of cell death was examined. Quantitative measurement of nuclear fragmentation was performed by flow cytometric analysis (**Fig. 2A**). Typical peak reflected nuclear fragmentation was observed in normal petals at stage I to V, but the peak was not observed in petals showing necrosis (control). Rates of nuclear fragmentation was 27.8%, 34.6%, 35.4%, 46.6% and 64.8% in petals at stage I, II, III, IV and V, respectively. The rate increased gradually, according to flower development. While, the rate of cell death was also detected by trypan blue staining in petals at each stage. **Fig. 2B** showed changes of the rate of dead cells in petals, and a good correlation (r=0.84, P=0.05) was observed between the rate of nuclear fragmentation and the rate of cell death. These results suggested that it was possible to evaluate flower senescence using nuclear fragmentation detected in petals of freesia. Senescence of petals has usually been visually categorized, but the categorization might be influenced by subjectivity of a researcher. So, it has been hard to measure flower vase-life objectively. Our study showed a novel evaluation method of flower senescence using flow cytometric analysis. Flow cytometry required low technical input and a short time to analyze, and may provide a quantitative and objective measurement of flower vase-life. We are trying to evaluate senescence and vase-life of other ethylene-insensitive flowers by this method. In addition, the apoptotic cell death that was observed in this experiment may also play a role in ethylene-insensitive flower senescence, and we will improve a vase-life by controlling apoptotic cell death in flower petals.

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