Acceleration of Senescence by High Temperature Treatment in *Lycoris* (*L. traubii × L. sanguinea*) Leaf Sections

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*Lycoris* plants are extensively cultivated as ornamental plants in Japan and China. Some *Lycoris* (for example, a hybrid; *L. traubii × L. sanguinea*) plants grown in temperate zones are likely to show high sensitivity to high temperatures. We have investigated the effect of high temperatures on chlorophyll degradation in *Lycoris* plants, using leaf sections under 12 h-light and 12 h-dark conditions. A rapid decrease in chlorophyll levels was observed when all sections were exposed to continuous high temperature at 35°C or 45°C, in contrast to control sections at 15°C. The high temperature treatment (for 12 or 18 h at 35°C), followed by incubation at 20°C for 1 to 3 days induced significant chlorophyll degradation. Experiments using two kinds of protease inhibitors showed that 10 mM Phenylmethylsulfonyl fluoride (PMSF) and 4 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) significantly suppressed the decrease in chlorophyll content and in the cell viability of leaf sections treated with the high temperature (35°C for 18 h). We found that the remarkable decrease in chlorophyll levels was followed by DNA laddering, which was induced by high temperature treatment at 35°C for 18 h. Other characteristic events, such as the activation of caspase-3-like activity and release of cytochrome *c* to the cytosolic fraction, were also observed in this system. These results implied that high temperatures accelerated leaf senescence, including the programmed cell death (PCD)-like phenomenon in *Lycoris* leaves.

**Key Words:** caspase-3-activity, DNA laddering, high temperature treatment, *Lycoris* plant, programmed cell death.

**Introduction**

*Lycoris* is a genus of 13–20 species of flowering plants in the family Amaryllidaceae. They are native to eastern and southern Asia and are extensively cultivated as ornamental plants in Japan and China. In Japan, one of them, *L. radiate* is widely used at the edges of rice paddy fields to provide a strip of bright flowers in late summer, and over 230 cultivars have been selected for garden use and have recently become popular commercial products. A type of *Lycoris* plant (a hybrid; *L. traubii × L. sanguinea*) is a cool-temperate plant and the leaves grow vigorously in the winter season (Ueno et al., 1994). This monocotyledonous plant shows a unique life cycle; after flowering in autumn the plants sprout only green leaves and they grow actively through the winter season. Then, when the temperature rises in late spring, the leaves suddenly start to show yellowing and then all aerial parts of the plant wither at the end of spring. On the other hand, the underground parts of the plant survive in the soil; only the bulbs and roots stay alive and grow underground through the hot and dry summer (Campbell, 1986; Howard, 2001). We have tried to elucidate the molecular mechanism of springtime leaf withering in the *Lycoris* plant. Generally, two major factors in spring are suggested to be involved in affecting and inducing leaf withering in this plant; change in day length and rise in temperature at the end of spring. We therefore focused on the effect of temperature rise under a constant photoperiodic condition when we started our study. We assumed that the temperature rise in spring, especially higher temperatures at the end of spring, may induce yellowing and cell death of *Lycoris* leaves, which may be related to senescence, including programmed cell death (PCD) or a PCD-like phenomena.
However, in our preliminary experiment using intact whole leaves, it was difficult to obtain consistent results because chlorophyll degradation in leaves did not proceed uniformly throughout but depended on the site of leaves. In this study, we used leaf sections to investigate the effect of high temperature treatment on leaf withering in vitro under a constant photoperiodic condition (12 h light and 12 h dark), which was similar to the photoperiod in the Japanese spring.

To gain some insight into the mechanisms by which Lycoris leaves show withering and die at the end of spring, we examined the high temperature sensitivity of Lycoris leaf sections. We also investigated characteristic biochemical events associated with PCD during high temperature treatment.

**Materials and Methods**

*Plant material, high temperature treatment and chemicals*

Leaves of Lycoris plants (a hybrid; L. traubii × L. sanguinea) (Ueno et al., 1994) were harvested during the winter and spring in 2003 to 2006 from a garden at the Faculty of Agriculture, Kagoshima University (Kagoshima, Japan). The maximum temperatures in daytime in the garden field differed greatly, ranging from about 8°C to 22°C. Fresh mature-green leaves of nearly 25-cm length and 2-cm width were selected and the extreme parts of harvested leaves, 5-cm lengths of leaf apex and leaf base, were trimmed to prepare uniform sections. They were washed three times with sterilized water and 80% ethanol, cut into 3 cm length sections and laid on moist filter paper in Petri dishes which had been heat-sterilized. These processes were completed within 1 hour after cutting the leaves. None of the sections showed any wilting after 4 days and were considered not to be water-stressed, even at 45°C.

The plant's sensitivity to high temperature was investigated using leaf sections incubated in Petri dishes under 12-h-light and 12-h-dark conditions. The leaf sections were exposed continuously at 15, 25, 35 and 45°C for 4 days, respectively, in an air incubator. For high temperature treatment, Lycoris leaf sections were incubated at 35°C for various periods from 12 h to 48 h and then transferred to 20°C. The physiological and biochemical changes were examined regularly; that is, 1, 2, 3, and 4 days after transfer to 20°C.

We examined the effect of protease inhibitors on chlorophyll degradation and cell viability in Lycoris leaf sections after high temperature treatment (35°C, 18 h). Phenylmethylsulfonyl fluoride (PMSF) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) are popular inhibitors of endogenous cysteine and serine proteases, which significantly suppressed cell death in tobacco (Lam and Del Pozo, 2000) and block menadione-induced PCD in tobacco protoplasts (Sun et al., 1999). In this research, treatment with 2.5, 5, 10 mM PMSF (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) and 2, 4 mM TPCK (Sigma-Aldrich Co., Ltd., St. Louis, USA) was performed on leaf sections using vacuum infiltration before exposure to high temperature treatment.

Using this system, we also investigated the effects of endonuclease inhibitors. Two kinds of endonuclease inhibitors, zinc chloride (ZnCl₂) and aurin tricarboxylic acid (ATA) (Nacalai Tesque Co., Ltd., Kyoto, Japan) were administered at concentrations of 2.5 mM to 10 mM and 0.25 mM to 1 mM, respectively. Sun et al. (2000) found that 5 mM ZnCl₂ can inhibit the fragmentation of DNA completely in tobacco suspension cells. Batistatou and Greene (1991) reported that ATA inhibited apoptosis in a wide variety of systems, including inhibition of DNA fragmentation by the withdrawal of trophic support in rat PC-12 pheochromocytoma-12 cells.

**Chlorophyll measurement**

One gram of chilled Lycoris leaf sections was cut into small pieces (about 1 mm wide) with a razor blade, and then the leaf sections were macerated and finely ground in 85% acetone (Nacalai Tesque Co., Ltd.) with a mortar and pestle. The mixture was transferred to a funnel and filtered through Whatman No. 1 filter paper. The residue was washed with 85% acetone, returned to the mortar with more 85% acetone and ground again. This process was repeated until the tissue was devoid of any green, and washings were colorless. The supernatant was assayed with a UV-VIS spectrophotometer (UV mini 1240, Shimadzu Co. Ltd., Kyoto, Japan) at 644 nm and 662 nm, respectively (Askar and Treptow, 1993). The chlorophyll content was calculated using the following formulas:

Chlorophyll a (mg L⁻¹) = (9.784 × E662) – (0.99 × E644)  
Chlorophyll b (mg L⁻¹) = (21.426 × E644) – (4.65 × E662)

The chlorophyll levels in the leaf sections were expressed as mg·g⁻¹ FW.

**Measurement of cell viability by WST-1 reduction assay**

WST-1, (4-[3-(4-iiodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzenes disulfonate), is a tetrazolium salt that has been known to be metabolically reduced to highly colored products called formazans by actively viable cells (Altman, 1976). The WST-1 reduction assay is a colorimetric determination of the bioreducibility of cells, which originates from various mitochondrial dehydrogenase (Ishiyama et al., 1995; Rappeneau et al., 2000; Pipaon et al., 2002), in particular, by measuring cell viability (Ishiyama et al., 1993).

Sodium salt of WST-1 and 1-methoxy phenazine methosulfate (PMS) were purchased from Dojindo Co., Ltd. (Kumamoto, Japan). Stock solutions of 8 mM WST-1 and 0.4 mM PMS were prepared. Lycoris leaf sections were washed three times with distilled water, and transferred to 18 mm × 150 mm test tubes containing 3 mL of 50 mM Hepes phosphate buffer (pH 7.4) containing 0.1% Tween 20. The samples were vacuum
infiltrated for 10 min. Cell viability of *Lycoris* leaf sections was determined by measuring the absorbance of the yellowish solution with a UV-VIS spectrophotometer at 450 nm at 1 h intervals up to 5 h after the start of incubation at 30°C. The values were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. Background absorbance in solutions without *Lycoris* sections was subtracted in all assays. The data are usually expressed as temporary absorbance (ΔOD = OD at 450 nm-OD at 600 nm). For details of this assay refer to Chucheep et al. (2005).

**DNA isolation and Electrophoresis**

Genomic DNA from the heat-induced and control sections of *Lycoris* leaves was extracted using Isoplant kit (Nippon Gene Co., Ltd., Tokyo, Japan) daily after high temperature treatment (35°C, 18 h). DNA samples were treated with DNase-free RNase for 1 h at 37°C and then 50 μg of the DNA fragments were separated by electrophoresis on a 2% (w/v) agarose gel, followed by visualization with ethidium bromide staining.

**Measurement of caspase-3-like protease activity and release of cytochrome c to cytosol**

Caspase-3-like protease activity assays were performed according to Lam and Del Pozo (2000) with some modifications. During the high temperature treatment (35°C, 18 h), caspase-3-like activity was measured at 6, 12, and 18 h, respectively. *Lycoris* leaf sections were ground in buffer (50 mM Hepes, pH 7.5; 1 mM DTT; 1 mM PMSF; 20% glycerol; 1 mM EDTA) to extract soluble proteins, and the homogenate was centrifuged at 6700 × g for 60 min with a RS-71A angle rotor (Tomy Seiko Co., Ltd., Tokyo, Japan) to give a clear supernatant. Fifty microliters of lysate (100 μg protein) were mixed with 50 μl of caspase assay buffer (caspase extraction buffer with 150 μM Ac-DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-7-Amino-4-trifluoromethylcoumarin (fluorogenic substrate for caspase-3-enzyme). The reaction was stopped by adding 20 μl of 1 M HCl after incubation at 37°C for 1 h, the fluorescence intensity of AFC hydrolyzed from the peptide substrate was quantified at Excitation: 405 nm and Emission: 505 nm using a microplate reader (Mithras LB 940; Berthold Technologies Co. Ltd., Bad Wildbad, Germany). To determine the inhibition of caspase-3-like activity by Ac-DEVD-CHO (caspase-3-inhibitor), an assay was conducted in the presence of 75 μM inhibitor (final concentration). The fluorescence intensity of hydrolyzed AFC was measured. Each assay was carried out with 3 sets of each sample.

Mitochondrial and cytosolic fractions were isolated from lysates of protoplasts prepared from leaf sections, as described by De Pinto et al. (2000) with some modifications. *Lycoris* leaf sections were cut into small strips (width: 1–2 mm) with a razor blade after the middle vein was removed after washing and sterilizing. Ten grams of leaf section strips were washed twice with solution A (composed of 10 mM CaCl₂ and 0.5 M mannitol) and the solution was removed by filtering through a stainless steel filter. All of the strips were immersed in a digesting enzyme solution (4% Cellulase Onozuka R-10, 2% Macerozyme R-10, 0.1% Pectolyase Y-23 and 1% potassium dextran sulfate in solution A) and then were subjected to vacuum infiltration for 5 min to facilitate penetration of the enzyme solution into the tissue of the strips. The strips were incubated at 35°C for 4 h to aid digestion. The enzyme solution was filtered through Miracloth to remove cellular debris. The filtrate was then centrifuged at 1127 × g for 5 min at 4°C and the pellet was gently suspended in 1 mL of chilled 10 mM MOPS-KOH buffer (pH 7.2) containing 0.3 M mannitol, 1 mM EDTA Na, 0.6% soluble PVP, and a protease inhibitor cocktail (Sigma-Aldrich Co., Ltd., St. Louis, USA) at a concentration of 30 μL per 3 mL. All protoplasts prepared from leaf section strips were ruptured by ten gentle strokes with a Teflon homogenizer. The solution was centrifuged at 420 × g for 10 min at 4°C to remove cellular debris and nuclei, and further centrifuged at 6708 × g for 15 min to collect the mitochondrial fraction. The fraction was kept at −80°C for further analysis. The supernatant was ultracentrifuged at 104000 × g for 60 min at 4°C to obtain a cytosolic fraction. Protein concentration in the fraction was determined by the Bradford method (Bradford, 1976).

Protein (20 μg) in the cytosolic fractions was separated by electrophoresis on SDS-polyacrylamide gel (16% w/v) and transferred to a Bio-Rad Immun-Blot PVDF membrane. Protein gel blot analysis was carried out using a primary antibody against cytochrome c (c-20) (SC-8385, Santa Cruz Biotechnology Co., Ltd., CA, USA) and a secondary antibody donkey anti-goat IgG-HRP (Santa Cruz Biotechnology Co., Ltd., CA, USA). Labelled proteins were detected by chemiluminescence (LAS-1000, Image Reader, Fuji Co., Ltd., Tokyo, Japan) using the ECL Western blotting detection system (GE Healthcare Bio-Science Co., Ltd., NJ, USA).

**Statistical analysis**

The data of chlorophyll contents, cell viability and caspase-3-like activity in *Lycoris* leaf sections were subjected to analysis of variance (ANOVA), and mean values were evaluated at the *P* < 0.05 level of significance using Tukey’s test.

**Results**

**Induction of chlorophyll degradation in Lycoris leaf sections treated with various temperatures**

We first investigated the plant’s sensitivity to high temperature using leaf sections incubated in Petri dishes under 12 h-light and 12 h-dark conditions. In our preliminary experiment, the sections showed severe damage after continuous high temperature of 45°C and 50°C
for 4 days, that is, all of the sections became dark green and waterlogged. As shown in Figure 1, the chlorophyll content of fresh green *Lycoris* sections was 0.68 mg·g$^{-1}$ FW at 0 day. A rapid decrease in chlorophyll levels was observed in all sections exposed to continuous high temperature of 35°C and 45°C. The sections incubated at 20°C for 3 days showed only a small decrease in total chlorophyll levels (Fig. 2). During incubation at 20°C after the high temperature treatment at 35°C for 12 h, the levels of chlorophyll did not decrease significantly for the first 2 days and then decreased to 0.51 mg·g$^{-1}$ FW 3 days after high temperature treatment. Further, the pattern of decrease varied depending on the duration of each high temperature treatment. The high temperature treatment (for 12 and 18 h at 35°C) induced a significant decrease in chlorophyll levels of the sections after 3 days of treatment. High temperature treatment of 35°C for 36 and 48 h caused a rapid decrease in total chlorophyll.

**Effect of two protease inhibitors on the cell viability and chlorophyll content of Lycoris sections treated by high temperature**

Some proteases are known to play important roles in PCD (Gray, 2004). In plants, cysteine proteases, serine proteases, and caspase-like proteases are known to be linked with PCD. Here, the effect of two protease inhibitors on the chlorophyll degradation of leaf sections was investigated under the same conditions as shown in Figures 3 and 4.

As shown in Figure 3A, PMSF (an inhibitor of serine protease) and Aprotinin (an inhibitor of cysteine protease) significantly inhibited the chlorophyll degradation. The effect of these inhibitors was dose-dependent, with higher concentrations showing greater inhibition. However, a higher concentration of Aprotinin caused slight inhibition at 36 and 48 h after treatment, suggesting potential toxicity at these concentrations.

![Figure 1](image1.png)

**Fig. 1.** Effect of various temperatures on chlorophyll content of *Lycoris* leaf sections. The six sections, on moisture filter paper in Petri dishes, were subjected to temperatures of 15, 25, 35, and 45°C respectively, in an air incubator with a photoperiod condition (12 h light and 12 h dark), and chlorophyll content was measured every 2 days until the leaf sections became dark green. Data represent the mean (±SE) of three independent measurements. Value represents the means of three replicates. Means followed by different letters within the same column are significantly different at $P<0.05$ (L.S.D. Tukey’s test).

![Figure 2](image2.png)

**Fig. 2.** Effect of various high temperature treatments on chlorophyll degradation of leaf sections from *Lycoris* plants. After high temperature treatment of 35°C for 12, 18, 24, 36, and 48 h for each treatment, the sections were transferred to 20°C and then incubated for 3 days. A sample was taken every day after high temperature treatment to determine total chlorophyll levels. Data represent the mean (±SE) of three independent measurements.
proteases) suppressed the decrease in chlorophyll levels effectively within the range of 2.5 mM to 10 mM. No addition of PMSF showed that the level of chlorophyll declined to 0.28 and 0.18 mg·g⁻¹ FW at 2 and 3 days after the high temperature treatment, respectively. Suppression of the decrease in chlorophyll levels was observed to be dose-dependent. All leaf sections treated with 10 mM PMSF remained green and maintained almost the original level of chlorophyll, which even 3 days after the high temperature treatment was nearly the same as in leaf sections not exposed to high temperature (0.62 mg·g⁻¹ FW). Only PMSF administration without high temperature showed no large differences compared to control leaf sections.

Cell viability of the leaf sections treated with PMSF was examined after high temperature treatment. Leaf sections treated with 10 mM PMSF had the highest cell viability, as shown by the highest △OD in WST-1 reduction method. Data represent the mean (±SE) of three independent measurements. Value represents the means of three replicates. Means followed by different column letters are significantly different at \( P < 0.05 \) (L.S.D. Tukey’s test).

Fig. 3. Suppressive effect of a proteinase inhibitor, phenylmethylsulfonyl fluoride (PMSF), on chlorophyll degradation and decreased cell viability of leaf sections from Lycoris plants treated by high temperature at 35°C for 18 h and then transferred to 20°C. Chlorophyll content (A) was measured every day for 3 days after high temperature treatment. Cell viability (B) of the sections was determined 2 days after the high temperature with the WST-1 reduction method. Data represent the mean (±SE) of three independent measurements. Value represents the means of three replicates. Means followed by different column letters are significantly different at \( P < 0.05 \) (L.S.D. Tukey’s test).

Fig. 4. Suppressive effect of a proteinase inhibitor, N-tosyl-L-phenylalanine chloromethylketone (TPCK), on chlorophyll degradation and decreased cell viability of leaf sections from Lycoris plants treated by the high temperature at 35°C for 18 h and then transferred to 20°C. Data represent the mean (±SE) of three independent measurements. Value represents the means of three replicates. Means followed by different column letters are significantly different at \( P < 0.05 \) (L.S.D. Tukey’s test).
compared to non-TPCK-treated sections (Fig. 4A). The treatments also maintained higher cell viability than control leaf sections ($\Delta OD = 0.863$ in contrast to 0.461), as shown in Fig. 4B. Administration of TPCK without high temperature showed no large difference from control leaf sections. The involvement of $Ca^{2+}$, $Mg^{2+}$-endonuclease activation in chlorophyll degradation of leaf sections was investigated using two inhibitors of endonucleases, ZnCl$_2$ and ATA, $Ca^{2+}$ $Mg^{2+}$-endonucleases. The results indicated that ZnCl$_2$ and ATA treatments were effective in maintaining higher cell viability than the control but did not suppress chlorophyll degradation in leaf sections (Figs. 5 and 6).

**Induction of DNA laddering in Lycoris leaf sections by high temperature**

DNA laddering is a feature of the late stages of PCD. Genomic DNA was prepared from sections of *Lycoris* leaf incubated at 20°C for 1 and 2 days after heat treatment. DNA fractions prepared from control green sections by the procedure described above did not indicate distinct fragmentation of DNA on 2% agarose gel electrophoresis, as shown in Figure 7. After staining with ethidium bromide, DNA laddering was observed clearly in fractions prepared from leaf sections incubated for 1 and 2 days after high temperature treatment. Fragments of genomic DNA were regularly observed in the range of about 180 bp to over 2.3 kbp on the gel.

**Other characteristic biochemical events of PCD-like phenomenon induced by high temperature**

We investigated the presence of caspase-3-like protease activity, which was monitored by measuring fluorometrically the hydrolysis of Ac-DEVD-AFC, the caspase-3-like protease-specific fluorogenic substrate. As shown in Figure 8, caspase-3-like activity in the

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**Fig. 5.** Suppressive effect of a endonuclease inhibitor, zinc chloride (ZnCl$_2$), on chlorophyll degradation and decreased cell viability of leaf sections from *Lycoris* plants treated by high temperature at 35°C for 18 h and then transferred to 20°C. Chlorophyll content (A) and cell viability (B) were measured as described in Fig. 3. Data represent the mean (±SE) of three independent measurements. Value represents the means of three replicates. Means followed by different column letters are significantly different at $P < 0.05$ (L.S.D. Tukey’s test).

**Fig. 6.** Suppressive effect of an endonuclease inhibitor, ATA, on chlorophyll degradation and decreased cell viability of leaf sections from *Lycoris* plants treated by high temperature at 35°C for 18 h and then transferred to 20°C. Chlorophyll content (A) and cell viability (B) were measured as described in Fig. 3. Data represent the mean (±SE) of three independent measurements. Value represents the means of three replicates. Means followed by different column letters are significantly different at $P < 0.05$ (L.S.D. Tukey’s test).
The cytosolic fraction of leaf sections increased with time during high temperature treatment. The results indicated the significant activation of caspase-3-like protease in *Lycoris* leaves treated with high temperature.

Western blot analysis of extracts probed with a rabbit cytochrome *c* antibody demonstrated the presence of recognizable cytochrome *c* protein bands in the cytosolic fraction of high temperature-treated leaf sections 6–12 h after high temperature treatment, whereas control and high temperature-treated leaf sections followed by 0 h high temperature treatment did not show a detectable cross-reacting protein band, as shown in Figure 9.

### Discussion

*Lycoris* is a cool-temperature plant and the leaves grow vigorously in the winter season. It shows a unique life cycle; all leaves grow vigorously through winter and then the leaves start to yellow. They disappear in spring while the bulbs and roots remain alive in the soil. This unique life cycle may be a defense system for survival in hot and dry summers (Campbell, 1986; Howard, 2001); therefore, we considered that the *Lycoris* plant has an extremely different physiological survival strategy from plants which grow vigorously in spring and summer. Thus, we have been interested in the molecular mechanism of the springtime leaf withering in this plant. We investigated the high temperature sensitivity of *Lycoris* leaf sections characteristic biochemical events associated with PCD during high temperature treatment.

Under photoperiodic conditions (12 h light and 12 h dark), we found that a high temperature (35°C for 18 h) evidently accelerated remarkable chlorophyll degradations of the plant in experiments using leaf sections (Figs. 1 and 2). In the system, the administration of two protease inhibitors (10 mM PMSF and 4 mM TPCK) had an evident effect on the chlorophyll degradation of leaf sections (Figs. 3 and 4) and a significant increase of caspase-3-like activity was found during high temperature treatment (Fig. 8). Since the activation of some proteases is essential in the onset of PCD, we suggest that this chlorophyll degradation in the sections may be related to PCD accelerated by high temperature. Further, DNA laddering, one of the key events in plant PCD (Balk et al., 1999; Fan and Xing, 2004; LoSchivo et al., 2000; McCabe et al., 1997; Tian et al., 2000; Vacca et al., 2004; Zuppini et al., 2006) was also observed in our system, as shown in Figure 7. DNA laddering appears to follow the remarkable decrease in chlorophyll levels. Cytochrome *c* in the cytosolic fraction was also observed in this system using *Lycoris* leaf sections. High temperature treatment (35°C) caused the release of cytochrome *c* from mitochondria to cytosol, which is a typical key event in PCD (Vacca et al., 2006; Zuppini et al., 2006).
These results imply that high temperature treatment might accelerate leaf senescence, including PCD-like phenomena leading to the chlorophyll degradation of *Lycoris* leaf sections. This is the first report that *Lycoris* plant senescence, including PCD-like phenomena is accelerated by high temperature. In our system, high temperature treatment may accelerate and synchronize the senescence program of the leaf or, independently, a PCD-like response. Little is known, however, about the molecular difference between these responses. In many experiments using suspension-cultured cells, a close correlation of high temperature treatments with the onset of PCD has been recognized (McCabe et al., 1997; Vacca et al., 2004, 2006; Zuppini et al., 2006). Some papers have indicated that PCD induced by heat is accompanied by DNA fragmentation (laddering), and/or the release of cytochrome c to cytosol (Balk et al., 1999; Fan and Xing, 2004; LoSchivo et al., 2000; McCabe et al., 1997; Tian et al., 2000; Vacca et al., 2004, 2006; Zuppini et al., 2006). It was also evident that the release of cytochrome c was followed by the activation of caspase-3-like activity in a system using tobacco BY2 cells treated by short 55°C treatment (Vacca et al., 2006).

At present we have no evidence to connect senescence, including PCD, to the natural chlorophyll degradation of intact *Lycoris* leaves at the end of spring. Further studies on the homology and differences between chlorophyll degradation in leaf sections and that in intact leaves are thus required. In our preliminary experiment using intact whole leaves, it was difficult to obtain consistent results because leaf yellowing did not proceed uniformly throughout but depended on the site of leaves; however, we speculate that a rise in temperature is an important factor for accelerating the yellowing and withering of *Lycoris* leaves in spring.

Next, it is an interesting question why only the aerial parts of this plant wither and disappear in spring; that is to say, as spring arrives and temperatures increase gradually, the leaves of the plant start to show yellowing and then at the end of spring all of the leaves disappear. Only the aerial part of the *Lycoris* plant disappears before the hot and dry summer but, in the same plant, the underground part (bulbs and roots) is still alive in the soil, maintaining the ability to sprout in autumn. Sensitivity against high temperatures may be largely different between aerial and underground parts in this plant, and it is speculated that their temperature sensing systems may be different. A more extensive study is now being conducted to find evidence to solve this interesting problem.

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