Response mechanisms to a rapid temperature decrease in plant cells

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Response mechanisms to a rapid temperature decrease in plant cells
（植物細胞の急激な温度降下に対する応答機構の解析）

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Contents

Chapter 1

General Introduction

1.1 The response of plants to temperature

Temperature is one of the most important factors affecting plant growth and geographical distribution. All biological processes, such as metabolism, photosynthesis, or membrane function, are strongly affected by changes in environmental temperature (Criddle *et al*. 1994; Nishida and Murata 1996; Allen and Ort 2001; Sage and Kubien 2007). Plants, being immobile, acquire temperature tolerance by altering their physiological conditions before exposure to unfavorable temperatures, such as heat in summer or freezing temperatures in winter (Badger *et al*. 1982; Sung *et al*. 2003; Kaplan *et al*. 2004; Hannah *et al*. 2006; Penfield 2008). The ability to minimize damage and ensure protection of cellular homeostasis against unfavorable temperature is called acclimation.

During acclimation under seasonal temperature changes, plants alter their fatty acid compositions to stabilize membrane fluidity (Falcone *et al*. 2004; Bohn *et al*. 2007). At higher temperatures, plants accumulate heat shock proteins (HSPs) (Kotak *et al*. 2007), while at lower temperatures, plants sometimes alter the quantity and type of membrane proteins (Kawamura and Uemura 2003), accumulate antifreeze proteins (AFPs) (Griffith and Yaish 2004), and/or synthesize compatible solutes against osmotic stress (Withers and King 1979). It is well established that various transcription factors are involved in changes in the expression levels of genes associated with temperature-dependent acclimation (Fowler and Thomashow 2002; Yamaguchi-Shinozaki and Shinozaki 2006; Urano *et al*. 2010).

1.2 Temperature injury caused by unfavorable temperature

In addition to seasonal temperature changes, plants are often exposed to sudden changes in the environmental temperature. When plants are exposed to drastic changes in temperature, they often become damaged, irrespective of acclimation. This damage is referred to as temperature injury and can be mediated by effect on membrane fluidity, causing lethal damages to cells (Daniell *et al*. 1969; Lyon 1973; Havaux *et al*. 1991; Kratsch and Wise 2000). Likewise, the growth of extracellular ice crystals under freezing conditions results in cell injury (Brown *et al*. 1974; Fujikawa *et al*. 1999). Leaf temperatures change within seconds to minutes in response to changes in sunlight, shade, wind, rainfall, and air temperature (Ansari and Loomis 1959; Mellor *et al*. 1964). Despite considerable research on temperature stress in plant cells, response to sudden changes in temperature has gone largely unnoticed.

1.3 Leaf damage of Saintpaulia induced by a rapid temperature decrease

Leaves of African violet (*Saintpaulia* sp., Fig. 1-1), popular potted plants of Gesneriaceae native to Kenya and Tanzania (Johanson 1978; Baatvik 1993), was reported over half a century ago to be damaged by irrigation of cold water onto the leaf surface (Poesch, 1942; Elliot, 1946). This leaf damage was considered to be due to chilling injury (Larcher and Neuner 1989). However, the damage is also caused by water at 25°C if the leaf temperature was maintained above 35°C, indicating that temperature difference between the leaf and the water was a more likely cause of the injury (Maekawa *et al*. 1987). Moreover, the injury was not observed even at 5°C when

the leaf temperature was reduced at a rate of -5°C min^{-1} (Suzuki *et al*. 1998, Fig. 1-2b), although 5°C is a stressful temperature for chilling sensitive plants (Lyon 1973). *Saintpaulia shumensis*, one of the original species identified, inhabits a mountainous region of Tanzania where the mean minimum temperature of the coldest month is about 3°C with extreme minima below 0°C (Bodner and Larcher 1989). If this species was chilling sensitive, it would be impossible for it to survive in this habitat. Injury was not induced when the ambient air temperature decreased rapidly, because it took more than 15 min to lower the leaf temperature from 30°C to 15°C (Maekawa *et al*. 1987), although typical chilling injury is induced by moderate changes in leaf temperature (Lyon 1973). These observations suggest that the injury to *Saintpaulia* leaves is different from a typical chilling injury.

1.4 Temperature conditions resulting in the leaf injury

In previous studies, this leaf injury in *Saintpaulia* had been observed in various strains; e.g. the original species *S. ionantha* (Bodner and Larcher 1987; Maekawa *et al*. 1987), cultivar 'Ritali' (Yun *et al*. 1996). I first performed replication studies to confirm that a rapid temperature decrease induced leaf injury in *Saintpaulia* in my measuring system. Figure 1-2a shows the leaf damage of *Saintpaulia* cultivar 'Iceberg', which was used as my experimental material, resulting from a rapid temperature decrease from 25°C to 5°C. Leaves became brown 24 h after the temperature decrease. When the leaf temperature was decreased slowly from 25°C to 5°C at the rate of 5°C min^{-1}, the leaf did not exhibit any noticeable change (Fig. 1-2b). Even though the

decrease in temperature was within the same range, visible damage was only observed with rapid decreases.

The severity of leaf injury after the temperature treatments was estimated as the proportion of damaged area to total leaf area (Fig. 1-2c). When the leaf temperature rapidly decreased from 30°C to 5, 10, 15, 20, and 25°C, the injury severities were 90.9, 85.7, 63.9, 17.9, and 1.3%, respectively. The severities resulting from lower initial temperatures were also measured. These results agreed with the former observations that the injury severity at a given temperature was affected by the initial leaf temperature.

1.5 Changes in chlorophyll fluorescence during the leaf injury

In damaged *Saintpaulia* leaves, palisade mesophyll cells are injured, resulting in cell death (Fig. 1-3; Yun *et al*. 1996). In the leaves after 60 min of temperature decrease, chloroplasts of palisade mesophyll cells change their color from green to yellow green, whereas no noticeable changes are observed in the upper and lower epidermis, and spongy mesophyll cells. One week after the temperature decrease, the palisade mesophyll cells shrink and the chloroplasts become brown, although the upper and lower epidermal cells and spongy mesophyll cells did not show any noticeable changes. In particular, chloroplasts in palisade mesophyll cells are damaged and their chlorophyll fluorescence decline after the temperature decrease (Fig. 1-4; Yun *et al*. 1997; Yun *et al*. 1998). To reproduce previous studies, the decline of chlorophyll fluorescence during injury was assessed using two indices of photosynthesis, the quantum yield of PS II (ϕII) and non-photochemical quenching (NPQ), using a pulse amplitude modulated fluorometer

(PAM). Following a rapid temperature decrease from 25°C to 15°C, ϕII decreased by 28% but increased to the initial value when the leaf temperature returned to 25°C, whereas NPQ did not change (Fig. 1-5a). When the leaf temperature was reduced slowly from 25°C to 5°C at a rate of 5°C min^{-1}, ϕII decreased by 50% and recovered to ~90% when the leaf temperature returned to 25°C, whereas NPQ did not change (Fig. 1-5b). When the temperature was decreased rapidly from 25°C to 5°C and was kept at 5°C for 5 min, ϕII decreased by 85% and continued to decrease after the temperature returned to 25°C. Concurrently, NPQ increased and remained high (Fig. 1-5c). Similar changes of ϕII and NPQ were also observed when the temperature was decreased rapidly from 25°C to 5°C, kept constant at 5°C for 30 s, and then returned to 25°C (Fig. 1-5d). These results were consistent with previous reports showing decline of chlorophyll fluorescence during injury.

1.6 Purpose of this study

I attempted to obtain new findings on responses to a rapid temperature decrease in plant cells by investigation of leaf damage and temperature sensitivity of *Saintpaulia*. Even though there are a few reports showing leakage of electrolytes and production of oxygen radicals in discolored leaves of *Saintpaulia* (Maekawa *et al*. 1990; Yasuda *et al*. 1997), the mechanism of this damage is largely unknown. The physiological events during the leaf damage in *Saintpaulia* leaves were investigated and described in chapter 2. In chapter 3, the molecular basis of temperature sensitivity was investigated using proteomic approach. Chapter 4 deals with correlation of leaf

sensitivity to a rapid temperature decrease and phylogenic relationships in Gesneriaceae plants. Finally, I discuss the further consideration in chapter 5.

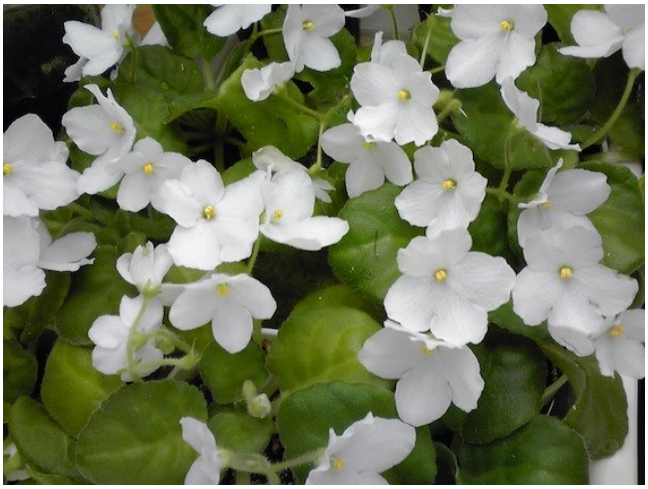


Figure 1-1. *Saintpaulia* species mainly used in this study (*Saintpaulia* sp. cv. 'Iceberg').

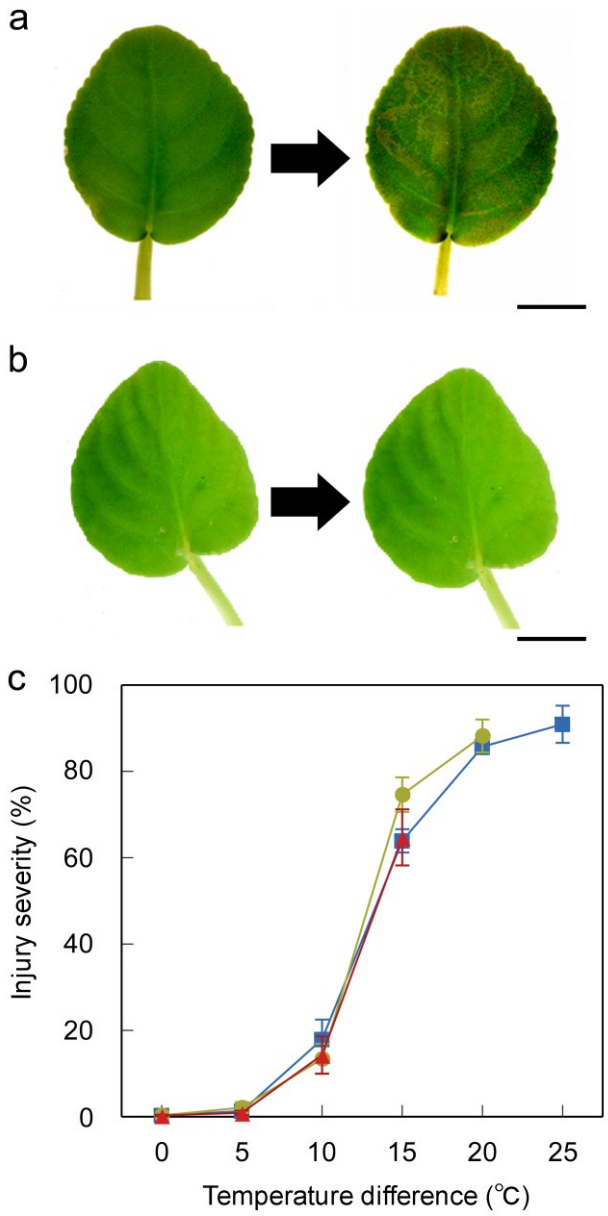


Figure 1-2. Leaf discoloration and injury severity of *Saintpaulia* cultivar 'Iceberg'. (a) Leaf color change of *Saintpaulia* before (left) and 24 hours after (right) a rapid temperature decrease from 25°C to 5°C. (b) Leaf before (left) and 24 hours after (right) a moderate temperature decrease from 25°C to 5°C. In (a) and (b), scale bars = 2 cm. (c) Severity of injury after rapid temperature decrease of leaves initially kept at 30°C (*squares*), 25°C (*circles*), and 20°C (*triangles*). The points and associated bars indicate mean severity and standard error (n = 5).

Leaves of *Saintpaulia* sp. cv. 'Iceberg' growing at 25°C were used. To change leaf temperatures, leaves detached just before the experiment were pre-incubated in temperature-controlled water in a circulation bath (Thermo Minder SM-05, TAITEC, Koshigaya, Japan). Each leaf was tightly packed in a plastic bag to prevent the leaves

from coming into direct contact with the water. Leaf surface temperature was measured by a thermocouple placed on the surface of the leaf. When a leaf pre-incubated in a bath at a particular temperature (T1) for 60 min was transferred to another bath of different temperature (T2) for 1 min, leaf temperature decreased from T1 to T2 within 10 s. When cold water was added gradually to the water bath, both bath and leaf temperatures decreased simultaneously at -5°C min^{-1}. After the temperature change treatment, the treated leaf was wrapped with aluminum foil and kept at 22°C for an appropriate period to avoid leaf shrinkage. The leaf was then removed from the aluminum foil and images were taken using a digital camera (COOLPIX4500, Nikon. Tokyo, Japan). The images were analyzed with the PC program LIA for Win32, (http://www.agr.nagoya-u.ac.jp/~shinkan/LIA32/index-e.html). The ratio of damaged leaf area to the total leaf area (referred to as 'injury severity') was used to estimate relative injury.

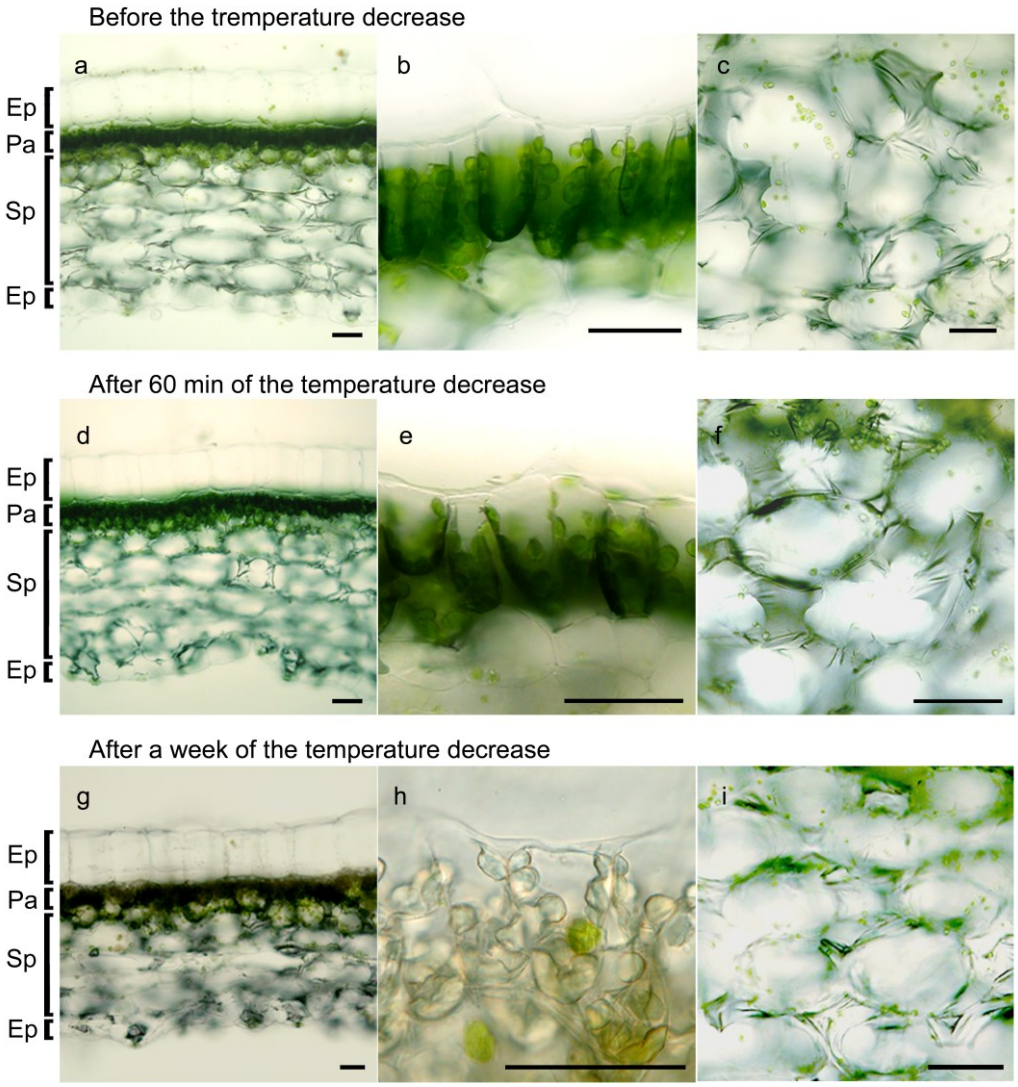


Figure 1-3. Light microscopic observation of cells in *Saintpaulia* leaves before and after a rapid temperature decrease. Whole-leaf section before (a), 60 min after (d) and a week (g) after a temperature decrease from 25°C to 5°C. Magnified view of palisade mesophyll (b, e, h) and spongy mesophyll cells (c, f, i) were also observed. Ep, epidermis; Pa, palisade mesophyll; Sp, spongy mesophyll. Scale bars = 100 μm.

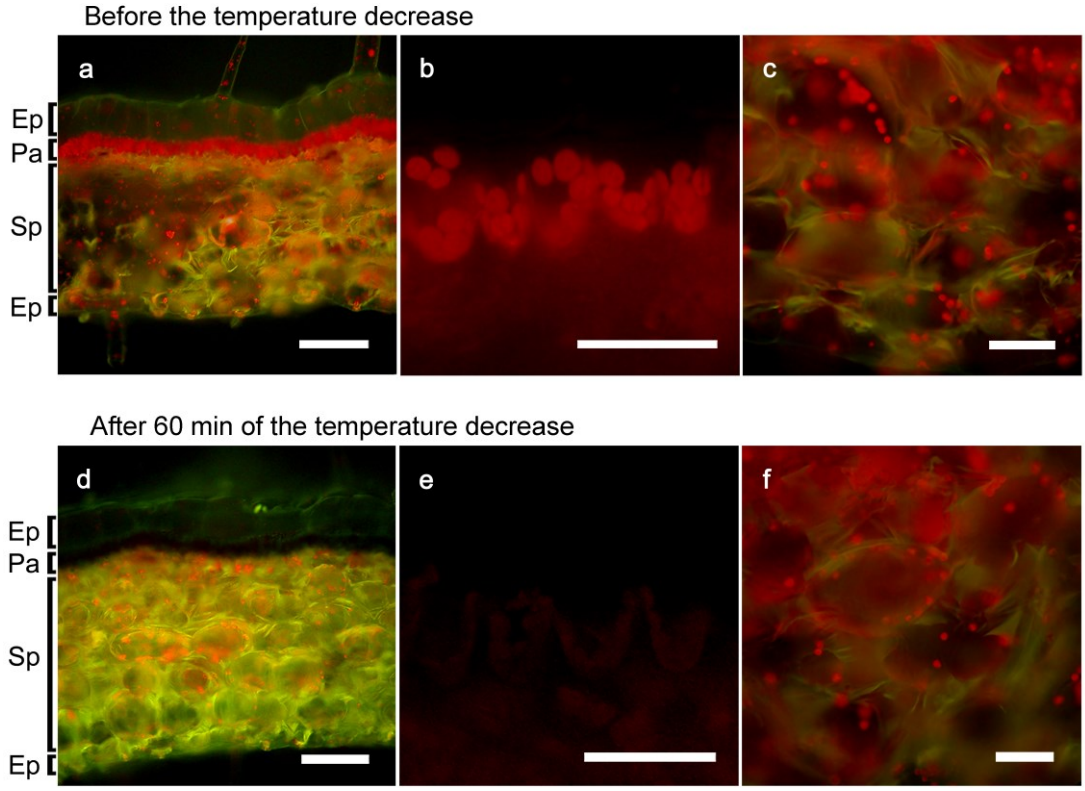


Figure 1-4. Changes in chlorophyll fluorescence of chloroplasts in *Saintpaulia* leaves induced by a rapid temperature decrease. Whole leaf sections before (a), 60 min after (d) a temperature decrease from 25°C to 5°C were observed with a fluorescence microscope. Magnified view of palisade mesophyll and spongy mesophyll cells before (b, c) and after (e, f) a temperature decrease were also observed. Ep, epidermis; Pa, palisade mesophyll; Sp, spongy mesophyll. Scale bars = 100 μm.

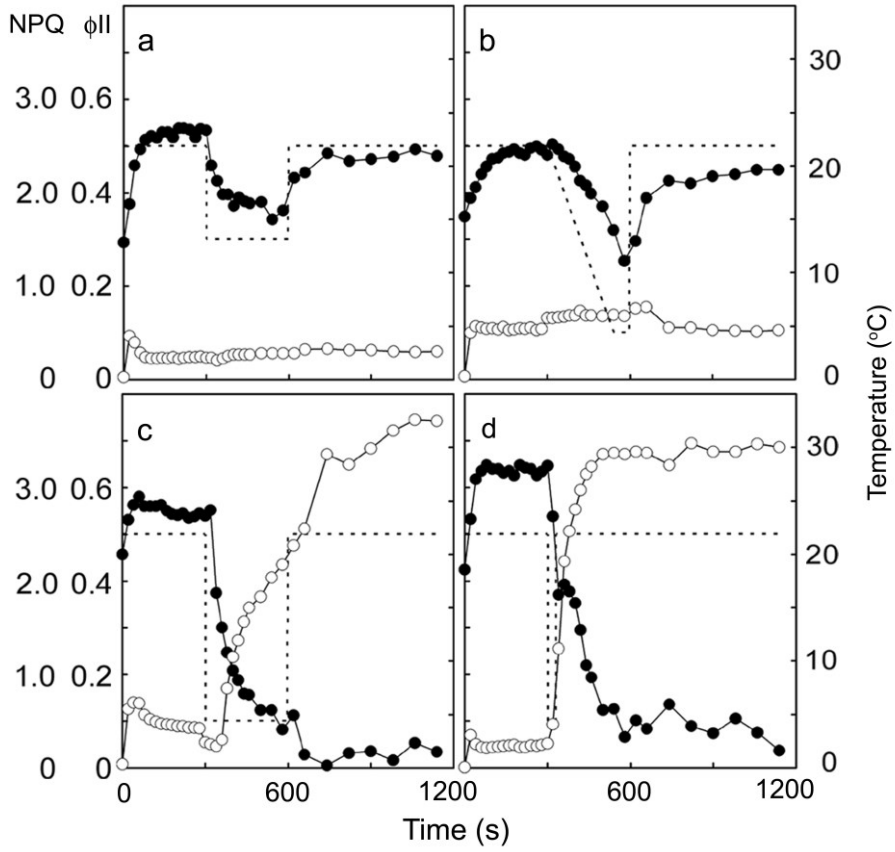


Figure 1-5. Changes in chlorophyll fluorescence during injury in *Saintpaulia* leaf. Quantum yield of PSII (ϕII) and non-photochemical quenching (NPQ) were estimated from changes in chlorophyll fluorescence. Solid lines show ϕII (*closed circles*) and NPQ (*open circles*), while broken lines indicate leaf temperature. These indices were measured during a rapid temperature decrease from 25°C to 15°C followed by rewarming to 25°C (a), during a gradual decrease from 25°C to 5°C (b), during a rapid decrease from 25°C to 5°C and kept at 5°C for 5 min followed by rewarming to 25°C (c), and during a rapid decrease from 25°C to 5°C and kept at 5°C for 30 s followed by rewarming to 25°C (d).

Chlorophyll fluorescence of detached leaves was determined with a pulse amplitude modulated fluorometer (PAM; PAM-200, Heinz Walz GmbH, Effeltrich, Germany). A leaf sample (10 × 10 mm) was cut with a razor and the adaxial side was set on the PAM measuring head. A glass chamber, whose temperature was controlled by circulating water, was set on the abaxial side of the piece. To change the leaf temperature, the water temperature circulating within the chamber was controlled. The leaf temperature measured by a thermocouple was quickly adjusted to the water

temperature in the chamber. The leaf piece was kept in the dark at 25°C for 60 min before the measurement. Minimum fluorescence (F_0 and F_0') was determined by measuring light of 3 μmol photons m^{-2} s^{-1} in the dark adapted leaf and by actinic light (which facilitates photosynthesis) of 80 μmol photons m^{-2} s^{-1} in light adapted leaf, respectively. Maximal fluorescence (Fm and Fm') was determined by a saturating pulse of 0.6 s duration. Changes in the quantum yield of photosystem II (ϕII) = (Fm' – F)/Fm' and in the non-photochemical quenching (NPQ) = (Fm – Fm')/Fm' during the temperature change were estimated with the DA-TEACH software (Heinz Walz GmbH).

Chapter 2

Sudden collapse of vacuoles in *Saintpaulia* sp. palisade cells induced by a rapid temperature decrease

Abstract

Saintpaulia (*Saintpaulia* sp. cv. 'Iceberg') leaves were damaged and discolored when subjected to a rapid decrease in temperature, but not when the temperature was decreased gradually. Sensitivity to the rapid temperature decrease increased within 10 to 20 min during pre-incubation at higher temperature. Damage was restricted to the palisade mesophyll cells, where there was an obvious change in the color of the chloroplasts (see chapter 1). During a rapid temperature decrease, chlorophyll fluorescence monitored by a pulse amplitude modulated fluorometer diminished and did not recover even after rewarming to the initial temperature. In contrast, the decline in chlorophyll fluorescence during a gradual temperature decrease was reversible. Isolated chloroplasts were not directly affected by the rapid temperature decrease. Intracellular pH was monitored with pH-dependent fluorescent dye. In palisade mesophyll cells damaged by a rapid temperature decrease, the cytosolic pH decreased and the vacuolar membrane collapsed. In isolated chloroplasts, chlorophyll fluorescence declined when the pH of the medium was lowered. These results suggest that a rapid temperature decrease directly or indirectly affects the vacuolar membrane, resulting in a pH change in the cytosol that subsequently affects the chloroplasts in palisade mesophyll cells.

2.1 Introduction

When leaf temperature of *Saintpaulia* rapidly decreases, only palisade mesophyll cells are injured, resulting in palisade cell death. As mentioned in chapter 1-3, in injured palisade mesophyll cells, organelles such as nuclei, mitochondria and chloroplasts, were damaged (Yun *et al*. 1996). In particular, chloroplasts in palisade mesophyll cells rupture and there is a decline in their chlorophyll fluorescence within a very short time after the temperature decrease (Yun *et al*. 1997; Yun *et al*. 1998). However, the mechanism of this cell injury is largely unknown, except for a few reports suggesting that reactive oxygen species (ROS) may be involved (Yasuda *et al*. 1997; Yang *et al*. 2001). The injury is restricted to palisade mesophyll cells; therefore, it is predicted that physiological events resulting in the injury may specifically occur in the palisade mesophyll cells, even though the meaning of such a physiological response is unclear.

To investigate response mechanism to a rapid temperature decrease in plant cells, it is important to understand injury mechanisms of palisade mesophyll cells of *Saintpaulia* leaves. In this study, I focused on organelles such as chloroplasts and vacuoles, that are involve in various types of plant cell death (Obara *et al*. 2001; Kim *et al*. 2012) to investigate the injury mechanisms in palisade mesophyll cells of *Saintpaulia.* I report that a rapid temperature decrease may initially affect vacuolar membrane, resulting in cellular pH changes followed by organelle damages in the initial phase. Stabilization of the vacuolar membrane may be essential to tolerate rapid decreases in temperature.

2.2 Materials and methods

2.2.1 Plant material

Saintpaulia sp. cv. Iceberg was used. Cut leaves of one plant were rooted in water for four weeks and then cultured in damp artificial soil (vermiculite:perlite, 3:1). After 20 weeks, genetically homogenous plants with seven to eight leaves were obtained. These plants were cultured at 22°C or 25°C under a 12 h (22°C) or 14 h (25°C) light period each day at 50 μmol m^{-2} s^{-1} (FL20SS BPN, National, Osaka, Japan). There was no difference between two different growth conditions for control plants. Fully expanded leaves were detached just before each experiment. In preliminary experiments, we confirmed that leaf detaching did not affect on injury severity.

2.2.2 Effects of pre-incubation period on leaf sensitivity

Leaves, detached from potted plants growing at 25°C just before the experiment, were pre-incubated in temperature-controlled water in a circulation bath (Thermo Minder SM-05, TAITEC, Koshigaya, Japan). Each leaf was tightly packed in a plastic bag to prevent the leaves from coming into direct contact with the water. After pre-incubation at 30°C or 20°C for 5, 10, 20, 30, 60 min, leaves were transferred to another bath maintained at 10°C for 1 min. After the temperature change treatment, the treated leaf was wrapped with aluminum foil and kept at 22°C for an appropriate period to avoid leaf shrinkage. The leaf was then removed from the aluminum foil and images were taken using a digital camera (COOLPIX4500, Nikon. Tokyo, Japan). The images were analyzed with the PC program LIA for Win32,

(http://www.agr.nagoya-u.ac.jp/~shinkan/LIA32/index-e.html). The ratio of damaged leaf area to the total leaf area (referred to as 'injury severity') was used to estimate relative injury.

2.2.3 Chloroplast isolation

Chloroplasts were isolated with a continuous Percoll gradient centrifugation method as described previously (Miyadai *et al*. 1990). Fully expanded leaves (10 g) were infiltrated into enzyme solution [2% Cellulase Onozuka R-10 (Yakult pharmaceutical industry, Tokyo, Japan), 1% Macerozyme R-10 (Yakult pharmaceutical industry), 10 mM Mes, 1 mM $CaCl_2 \cdot 2H_2O$, and 0.6 M sorbitol] at 30°C. After 10 min of the filtration, the adaxial side of leaves (palisade layer and upper epidermis) was separated from the abaxial side (spongy layers and lower epidermis) with tweezers. The separated adaxial suefaces were collected and homogenized at room temperature in 30 ml of a buffer solution [0.35 M sorbitol, 50 mM HEPES-Tris (pH 7.5), 0.1 % BSA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 1 mM NaH_2PO_4, 2 mM EDTA (2Na), and 2 mM Na iso-ascorbate] using an homogenizer. The homogenates were filtered through four-layer cheesecloth, and the filtrate was centrifuged at 2,500 ***g*** for 2 min. The pellet was resuspended in the buffer solution and centrifuged again. The washed pellet was resuspended in 1 ml of the buffer solution and mixed with 30 ml of Percoll solution [50% (v/v) Percoll, 5%(w/v) PEG 6000, 1%(w/v) Ficoll 400, and the same ingredients as the buffer solution] in a tube. The tube was then centrifuged at 39,800 ***g*** for 20 min. Chloroplasts were separated as two green layers near to the top and the bottom of the

tube. The bottom layer containing intact chloroplasts was collected and resuspended in 15 ml of the buffer solution. The suspension was centrifuged at 2,500 ***g*** for 2 min. The pellet was resuspended in 2 ml of the buffer solution. Chloroplast intactness was assessed as 79.1% (n = 3) by the Hill reaction (Lilley *et al*. 1975).

2.2.4 Measurements of chlorophyll fluorescence of isolated chloroplasts during a temperature decrease

Chlorophyll fluorescence of isolated chloroplasts was determined with a pulse amplitude modulated fluorometer (PAM; PAM-200, Heinz Walz GmbH, Effeltrich, Germany). An aliquot of the chloroplast suspension on a well slide glass was set on the PAM measuring head. A glass chamber, whose temperature was controlled by circulating water, was set on the well slide glass. To change the leaf temperature, the water temperature circulating within the chamber was controlled. The leaf temperature measured by a thermocouple was quickly adjusted to the water temperature in the chamber. The chloroplast suspension was kept in the dark at 25°C for 60 min before the measurement. Minimum fluorescence (F_0 and F_0') was determined by measuring light of 3 μmol photons m^{-2} s^{-1} in the dark adapted chloroplasts and by actinic light of 80 μmol photons m^{-2} s^{-1} which facilitates photosynthesis in light adapted chloroplasts, respectively. Maximal fluorescence (Fm and Fm') was determined by a saturating pulse of 0.6 s duration. Changes in the quantum yield of photosystem II (ϕII) = (Fm' – F)/Fm' and in the non-photochemical quenching (NPQ) = (Fm – Fm')/Fm' during the temperature change were estimated with the DA-TEACH software (Heinz Walz GmbH).

For comparison, the fluorescence of chloroplasts suspended in a similar buffer solution, but at different pH [0.35 M sorbitol with 50 mM MES-NaOH (pH 5.0 and 6.0) or 50 mM HEPES-KOH (pH 7.6), as well as 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2mM EDTA] was also measured. Isolated chloroplasts were resuspended in the buffer solutions and left for 10 min prior to observation.

2.2.5 Monitoring of intracellular pH with pH-sensitive fluorescent dye

Leaf cross-sections, 100 μm thick, were prepared using a microtome (Plant Microtome MT-3, Nippon Medical and Chemical Instruments, Osaka, Japan) and were stained with 1 μM BCECF-AM (Molecular Probes, Eugene, OR, USA) in a buffer solution (50 mM HEPES-Tris, pH 7.8) for 60 min at 25°C. The sections were placed in a fluorescent dye-free buffer solution on a MATS-555R-IM temperature controller (Tokai Hit, Shizuoka, Japan) on the stage of a confocal laser scanning microscope (FV1000-D, Olympus, Tokyo, Japan). The temperature was kept at 25°C for 10 min and then decreased to 5°C for 1 min followed by rewarming to 25°C. BCECF-AM fluorescence intensities were measured using the ImageJ software (W. Rasband, National Institutes of Health, Bethesda, MD, USA).

2.2.6 Observation of vacuolar dynamics during cell injury

To visualize the dynamics of vacuoles during cell injury, vacuolar membrane from palisade mesophyll and spongy mesophyll cells were labeled with the fluorescent membrane probe FM1-43 (Molecular Probes). FM1-43 dye is first taken up by the

plasma membrane, and then by the vacuolar membrane via endocytosis (Emans *et al*. 2002). Leaf cross-sections of 100 μm thick were prepared with a microtome and then stained with 5 μM FM1-43 in a buffer solution (50 mM HEPES-Tris, pH 7.8) for 60 min at 25°C. To label the vacuolar membrane, the stained sections were incubated in dye-free buffer solution for 180 min at 25°C. The sections were placed in a fluorescent dye-free buffer solution on the MATS-555R-IM temperature controller on the stage of the confocal laser scanning microscope. The temperature was kept at 25°C for 10 min and then decreased to 5°C for 1 min followed by rewarming to 25°C.

2.3 Results

2.3.1 Effects of pre-incubation period on the leaf injury

Saintpaulia leaves pre-incubated at higher temperature show severe damages (Fig. 1-2). In this chapter, to determine the effects of pre-incubation period on the leaf injury, leaves detached from plants growing at 25°C were pre-incubated at 30°C or 20°C for various periods. When leaves incubated at 30°C for 5, 10, 20, 30, 60 min were rapidly cooled to 10°C, the injury severities were 17.6, 55.6, 60.7, 58.6, and 71.3%, respectively (Fig. 2-1). The severity of injury to leaves that were pre-incubated at 20°C and cooled to 10°C was mild; pre-incubation of leaves for 60 min resulted in an injury severity of 15.1%. Although the ranges in temperature decrease were the same, the injuries were more severe following pre-incubation at 30°C for over 10 min. These results suggested that the physiological conditions of the leaves were drastically changed within 10 min of pre-incubation.

2.3.2 Changes in chlorophyll fluorescence during the injury

In damaged leaves, chloroplasts in palisade mesophyll cells are damaged and their chlorophyll fluorescence decline after the temperature decrease (Fig. 1-4, 1-5). To confirm whether chloroplasts were directly affected by the temperature decrease, chloroplasts were isolated from *Saintpaulia* leaves and monitored ϕII and NPQ. Because the chloroplasts of palisade mesophyll cells were bigger than those of spongy mesophyll cells (Fig. 1-2), they could be clearly distinguished under the microscope. The chloroplasts suspensions used in this study contained 90.2% palisade mesophyll cells

chloroplasts (n = 3); therefore, I can assume that changes in chlorophyll fluorescence originated in the chloroplasts of these cells. Under the microscope, chlorophyll fluorescence was observed even after 10 min of the temperature decrease (Fig. 2-2a). ϕII and NPQ, monitored using PAM, did not change when the temperature was maintained at 25°C for 15 min (Fig. 2-2b). During the rapid temperature decrease from 25°C to 5°C, ϕII decreased by 59% but recovered to 93% after rewarming, and NPQ did not change markedly (Fig. 2-2c). These results indicated that the damage of chloroplasts was not directly induced by the temperature decrease.

2.3.3 Changes in intracellular pH induced by a rapid temperature decrease

Since isolated chloroplasts were not directly damaged by decreasing temperature, I suppose that the intracellular environment of the chloroplasts may be altered by the temperature decrease. Changes in cytosolic pH were estimated with a pH-sensitive fluorescent dye, BCECF-AM, which emitted green fluorescence (λ = 535 nm) above pH 6.4 when excited at 473 nm. Prior to the rapid temperature decrease from 25°C to 5°C, green fluorescence of BCECF was observed in the cytosol, but was low in the vacuole of palisade mesophyll cells (Fig. 2-3a). Immediately after the rapid temperature decrease, the fluorescence from the thin layer inside the palisade mesophyll cells became weaker, and virtually disappeared within 10 min. The fluorescence intensity decreased by 87% after 10 min of the temperature decrease (Fig. 2-3b), whereas the intensity was maintained without the temperature decrease and after a slow temperature decrease at -5°C min^{-1} (Fig. 2-4). In the spongy mesophyll and epidermal

cells, the fluorescence of BCECF-AM did not decrease even after the temperature decrease (Fig. 2-3). These results showed that the pH in the cytosol of palisade mesophyll cells became more acidic after the temperature decrease.

2.3.4 Effects of surrounding pH on isolated chloroplasts

A decline of chlorophyll fluorescence was observed concurrently with intracellular pH changes in palisade mesophyll cells (Fig. 2-3). To confirm whether chloroplasts were affected by the surrounding solution pH, the chlorophyll fluorescence was observed when isolated chloroplasts were suspended in buffer solution at various pH levels for 10 min. Under the fluorescence microscope, the chlorophyll fluorescence of isolated chloroplasts suspended in buffer solution was lower at pH 6.0 than at pH 7.6, and even lower at pH 5.0 (Fig .2-5). These results indicated that the decline of chlorophyll fluorescence during the rapid temperature decrease could be induced by acidification of the cytosol.

2.3.5 Vacuolar dynamics during cell injury

Since the cytosolic pH changed during cell injury, I predicted that there may have been leakage of acidic contents from the vacuoles in palisade mesophyll cells. To determine whether the vacuoles of the palisade mesophyll cells were affected by the rapid temperature decrease, I observed the vacuolar dynamics during cell injury using FM1-43. Prior to the rapid temperature decrease from 25°C to 5°C, the FM1-43 fluorescence was observed from the membrane surrounding the large vacuole in

palisade mesophyll cells (Fig. 2-6a). Chloroplasts emitting autofluorescence were seen at the outer surface of fluorescently labeled membrane. After 10 min of the temperature decrease, FM1-43 fluorescence also appeared inside the vacuole and around the outside of the chloroplasts (Fig. 2-6b). In spongy mesophyll cells, where cytosolic acidification was not observed (Fig. 2-3), the fluorescence pattern of FM1-43 remained unchanged even after the rapid temperature decrease (Fig. 2-6c, d). The vacuolar membrane of palisade mesophyll cells showed dynamic changes during the cell injury, which suggested that the vacuolar membrane may have collapsed following the rapid temperature decrease.

2.4 Discussion

2.4.1 Response to ambient temperatures in Saintpaulia leaves

I found that the severity of injury depended on the duration of the pre-incubation period. The data presented in Fig. 2-1 indicate that the sensitivity to rapid temperature decrease was enhanced by pre-incubation at 30°C for over 10 min. These results suggested that the physiological conditions of the leaf are drastically changed within 10 min of the pre-incubation; *Saintpaulia* leaves might rapidly respond to ambient temperatures. Even in *Arabidopsis thaliana*, which has relatively rapid acclimation mechanisms, it took 24 h to increase in freezing tolerance (Gilmour *et al*. 1988). In *Saintpaulia*, leaves kept at 30°C for over 10 min might be hardened to 30°C and more sensitive to 10°C than those kept for 5 min. Taking into consideration a previous report showing rapid hardening of *Saintpaulia* leaves (Suzuki *et al*. 1998), I speculate that *Saintpaulia* leaves have unique mechanisms to respond to ambient temperatures and reduce the extent of injury when temperature decreases slowly.

2.4.2 Process of cell injury monitored with chlorophyll fluorescence

Chlorophyll fluorescence has been widely used to assess temperature stresses (Smillie and Hetherington 1983). A decline in chlorophyll fluorescence after the leaf injury in *Saintpaulia* has also been reported (Larcher and Neuner 1989; Yun *et al*. 1997). In replication study, I continuously monitored the chlorophyll fluorescence during the injury process and showed that decrease of ϕII and increase of NPQ occurred concurrently in *Saintpaulia* leaves (Fig. 1-5). Under environment stresses that limit

fixation of CO_2 such as heat, cold, and salt, an excess of light energy suppress the repair of photodamage of PSII (Takahashi and Murata 2008) and these stress might accelerate photoinhibition by inhibiting the repair of photodamage to PSII (Al-Taweel *et al* 2007; Grennan and Ort 2007; Yang *et al* 2007). Photoinhibition under stresses can be detected using chlorophyll fluorescence technique (Krause and Weis 1991). At unfavorable temperatures, decrease in ϕII and increase in NPQ is observed in rice, barley, and a dipterocarp tree (Xu *et al* 1999; Kitao *et al*. 2000). In this study, I also observed low ϕII and high NPQ values in *Saintpaulia* leaves after a rapid temperature deceases (Fig. 1-5). This might be the result of photoinhibition and, if so, *Saintpaulia* plants lack mechanisms to prevent photodamage when the temperature decreases rapidly. Interestingly, however, chloroplasts isolated from *Saintpaulia* palisade mesophyll cells were not affected by a temperature decrease (Fig. 2-2). Chlorophyll fluorescence did not decline, and ϕII decreased reversibly even when the temperature was rapidly decreased, suggesting that the chloroplasts themselves may not be directly damaged by the rapid temperature decrease. These results indicated that, in palisade mesophyll cells, other cellular changes affecting chloroplasts occurred after the rapid temperature decrease.

2.4.3 Intracellular pH change during cell injury occurrence

Ii is known that various cytosolic factors such as pH, Ca^{2+}, and reactive oxygen species (ROS) can have an impact on photosynthesis (Espie and Colman, 1981; Meloni *et al*. 2003; Stael *et al*. 2012). Among these factors, I showed that cytosolic pH changed after a rapid decrease in temperature. Intracellular pH changes can be caused

by various environmental stresses, such as low temperature, salt, high CO_2, and hypoxia (Yoshida 1994, 1995; Roberts *et al*. 1984; Heber *et al*. 1994; Katsuhara *et al*. 1989). It is suggested that, in mung bean seedlings, chilling-induced cytoplasmic acidification is caused by failure of pyrophosphate (PPi)-dependent H^+ accumulation (Kawamura 2008). In this case, cytoplasmic and vacuolar pH monitored with a pH-sensitive fluorescent dye changed from 7.5 to 6.6 and from 5.1 to 5.7 after 8 h of exposure to 0°C, respectively (Yoshida 1995). Here, I observed that, in palisade mesophyll cells of *Saintpaulia* leaves, the cytosolic pH changed within 10 min of the temperature change (Fig. 2-3). I could not determine changes in the absolute pH value, because palisade mesophyll cells have very thin cytosol and it is not easy to determine an exact pH value using fluorescence dye. However, an abrupt decrease of fluorescence intensity implies rapid changes of intracellular pH. It is predicted that, in the palisade mesophyll cells of *Saintpaulia*, acidic vacuolar contents leak into the cytosol, resulting in large changes of intracellular pH. Cytosolic pH changes after a rapid temperature decrease were observed only in palisade mesophyll cells (Fig. 2-3), indicating that sensitivity to a rapid temperature decrease varied between different leaf tissues. However, at present I do not have any evidence to explain these differences in sensitivity.

Cytosolic acidification disrupts various functions of organelles. Cytosolic acidification under high CO_2 inhibits photosynthesis and causes a reduction in chlorophyll fluorescence (Savchenko *et al*. 2000). In isolated spinach chloroplasts, O_2 evolution reached a maximum around pH 7.6, but decreased when chloroplasts were suspended in media of higher or lower pH (Heber *et al*. 1994). I showed that, in

chloroplasts isolated from *Saintpaulia* leaves, chlorophyll fluorescence declined at weakly acidic pH (Fig. 2-5). Hence chloroplasts in intact palisade mesophyll cells of *Saintpaulia* may show a similar reduction in fluorescence upon acidification. From chloroplasts in spongy mesophyll cells, where intracellular pH did not change (Fig. 2-3), chlorophyll fluorescence was still observed even after a rapid temperature decrease (Yun *et al*. 1998).

2.4.4 Vacuolar dynamics during cell injury

The collapse of the vacuolar membrane was observed 10 min after the temperature decrease in palisade mesophyll cells (Fig. 2-6). Vacuole and vacuolar contents play a key role in programmed cell death (PCD) in plants (Hara-Nishimura and Hatsugai 2011). Vacuolar processing enzyme (VPE), a protease that degrades vacuolar membranes, is involved in virus-induced hypersensitive cell death in tobacco plants (Hatsugai *et al*. 2004). After the collapse of the vacuolar membrane, vacuolar contents are released into the cytosol, resulting in rapid cell death. My results strongly suggest that, in palisade mesophyll cells of *Saintpaulia*, the collapse of the vacuolar membrane is the event proceeding chloroplast injury because cytosolic pH changed rapidly (Fig 2-3). However, it is not clear whether VPE is involved in the *Saintpaulia* leaf injury. There is a report of electron microscope observations in *Saintpaulia* leaf cells after a rapid temperature decrease (Yun *et al*. 1996), in which the author suggested that some invagination of vacuolar membrane into the vacuole filled with cytosol could be seen. I suppose at present that these structures seems to suggest collapse of the vacuolar

membrane, and this is supported by direct observations of the vacuolar membrane with fluorescent dye in present study (Fig. 2-6).

The identity of the signal for the collapse of vacuolar membrane is unclear. One possibility is the rapid temperature change itself. It is well known that the lipid components of the bio-membrane change depending on the temperature, and therefore membrane fluidity may play a role in temperature sensing. Alternatively, temperature effects on the chloroplasts may lead to damage to the vacuole. ROS, which are thought to signal molecules that trigger PCD, is produced in chloroplasts when plants are exposed to temperature changes (Danon 2012), and these may trigger collapse of vacuolar membrane resulting in intracellular pH changes. The involvement of ROS in *Saintpaulia* leaf damage has been strongly implicated (Yasuda *et al*. 1997; Yang *et al*. 2001), but it is not known how ROS damage the vacuolar membrane. The nature of the initial event is unclear because both changes in chlorophyll fluorescence and pH occur rapidly following a temperature change. There is a possibility that vacuole and chloroplasts are damaged at the same moment by some physiological change. Here, I showed that the vacuoles collapsed during the initial phase of injury in addition to the chloroplasts injury.

2.4.5 Conclusions

In conclusion, my results strongly suggested that the vacuolar membrane of palisade mesophyll cells collapse during the initial phase of leaf injury. From these results, I speculate the mechanisms of leaf injury as follows; (1) vacuolar membrane in

palisade mesophyll cells is disordered by a rapid temperature decrease followed by collapse of vacuole; (2) vacuolar contents are leaked to the cytoplasm and intracellular pH homeostasis is altered; and (3) chloroplasts and other organelles are damaged by the pH changes, resulting in palisade mesophyll cell injury. Stabilization of the vacuolar membrane may be essential to tolerate rapid decreases in temperature. Whether such temperature sensitivity has an important physiological role, or is only a genetic variation left during evolution, remains to be resolved.

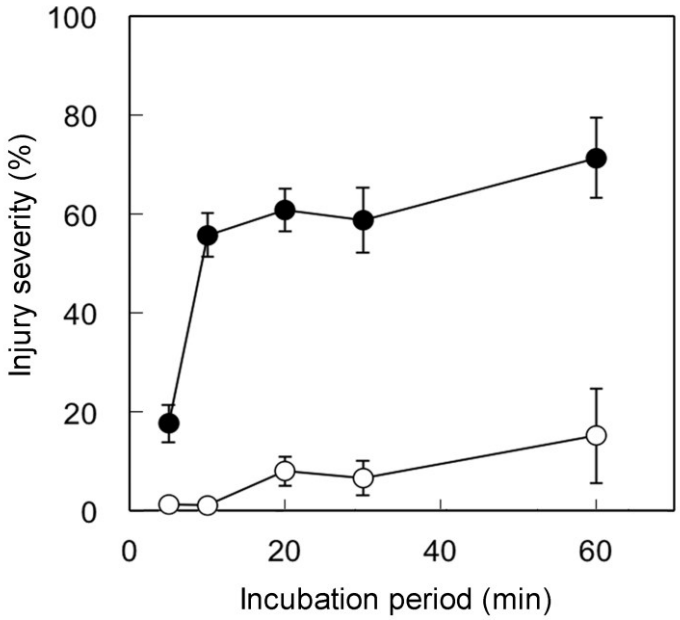


Figure 2-1. Injury severity of *Saintpaulia* leaves after various pre-incubation periods. Severity of injury when leaves pre-incubated at 30°C (*closed circles*) or 20°C (*open circles*) for 5, 10, 20, 30, 60 min were rapidly decreased to 10°C. The points and associated bars indicate mean severity and standard error (n = 5).

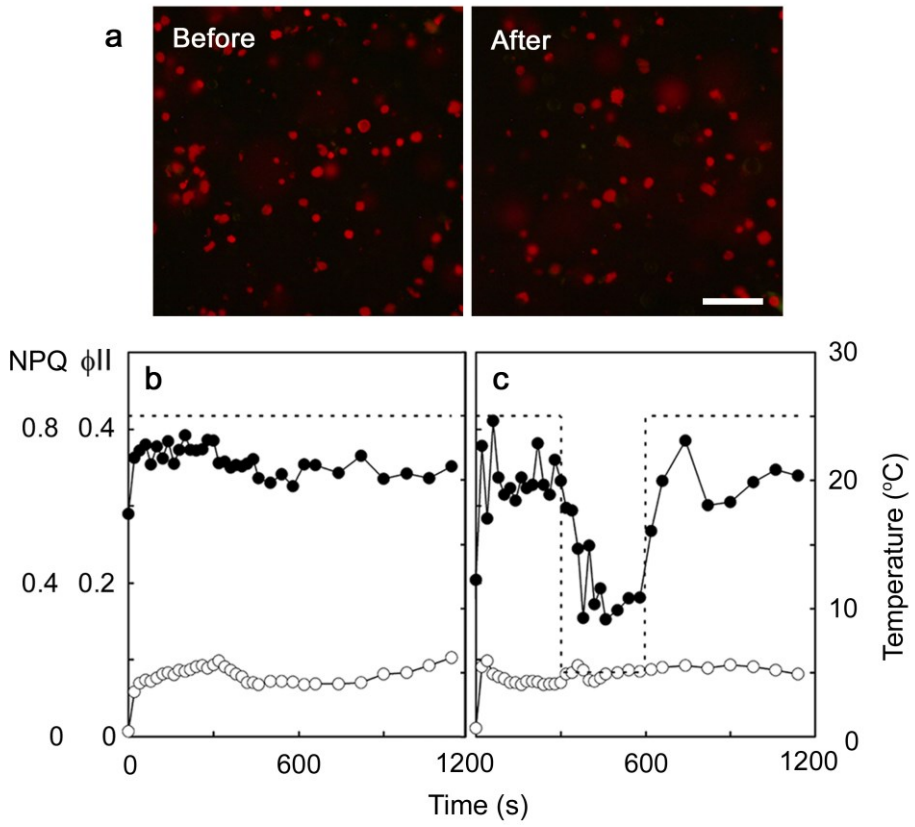


Figure 2-2. Chlorophyll fluorescence of chloroplasts isolated from palisade mesophyll cells of *Saintpaulia* leaf. (a) Fluorescent microscopic images of isolated chloroplasts observed with fluorescence microscope before (left) and 10 min after (right) a temperature decrease from 25°C to 5°C. Scale bar = 50 μm. (b), (c) Quantum yield of PSII (ϕII) and non-photochemical quenching (NPQ) of isolated chloroplasts estimated from changes in chlorophyll fluorescence. Solid lines show ϕII (*closed circles*) and NPQ (*open circles*), while broken lines show temperature. These indices were measured at 25°C (b), and during a rapid decrease from 25°C to 5°C and kept at 5°C for 5 min followed by rewarming to 25°C (c).

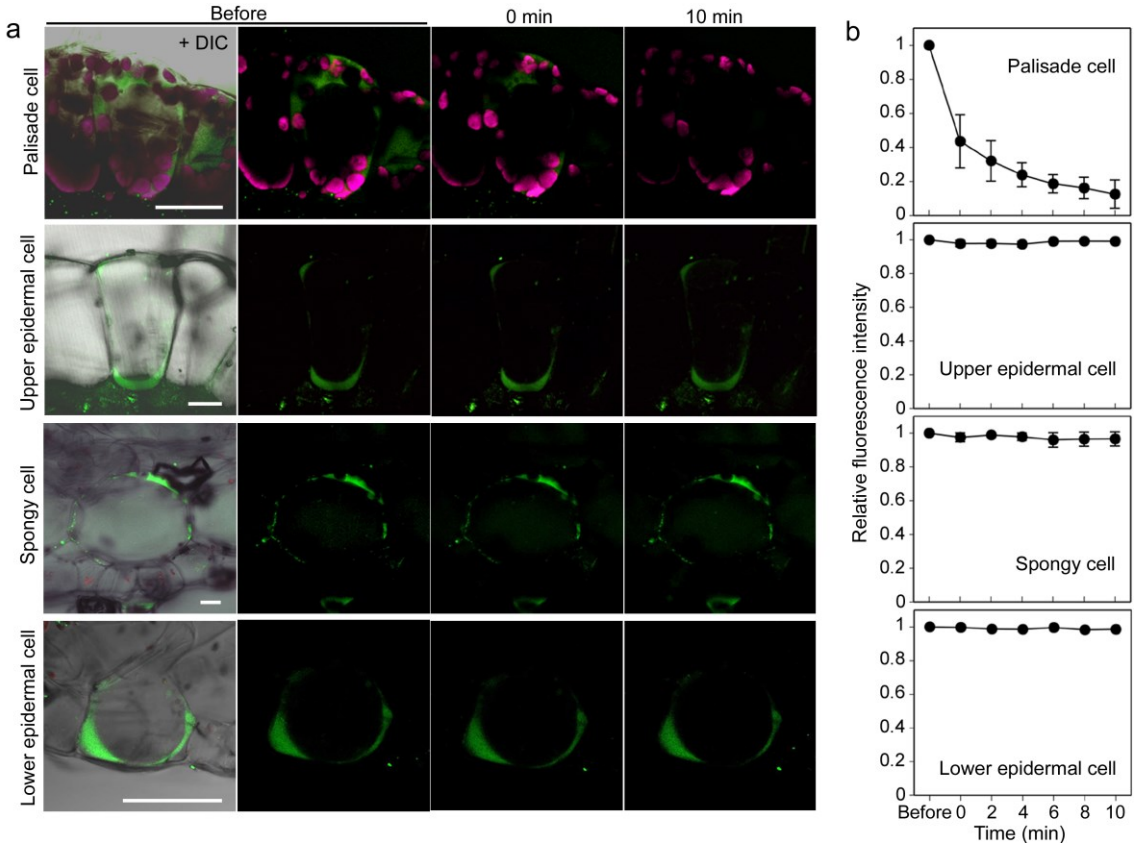


Figure 2-3. Cytosolic pH changes in palisade mesophyll, spongy mesophyll, and epidermal cells of *Saintpaulia* leaf. (a) Palisade mesophyll, spongy mesophyll, and upper and lower epidermal cells stained with BCECF-AM. BCECF-AM (green) and chlorophyll fluorescence (magenta) were observed before, immediately after (0 min), and 10 min after the temperature was decreased from 25°C to 5°C and kept at 5°C for 1 min. Scale bar = 40 μm. (b) Relative changes of BCECF-AM fluorescence intensity after a temperature decrease. The points and associated bars indicate mean intensity and standard error (n = 3).

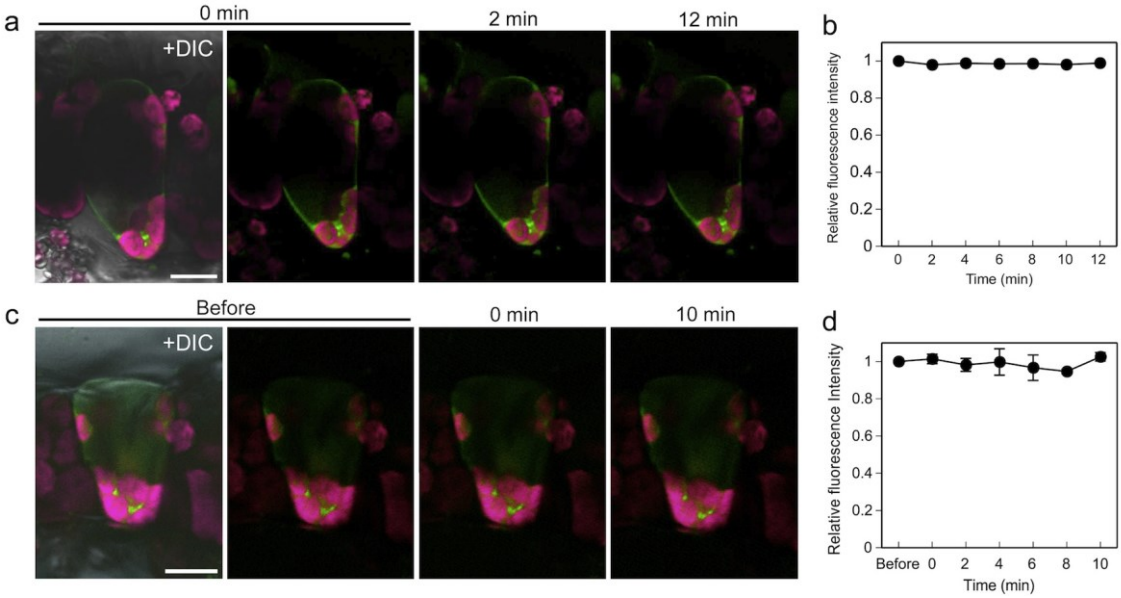


Figure 2-4. Cytoplasmic pH in palisade mesophyll cells of *Saintpaulia* at constant temperature and after a slow temperature decrease. (a) Palisade mesophyll cells of *Saintpaulia* leaf stained with BCECF-AM. Images of BCECF-AM (green) and chlorophyll fluorescence (magenta) were captured 0, 2, and 12 min after the start of observation at 25°C, respectively. (b) Relative changes of BCECF fluorescence intensity at 25°C. (c) Palisade mesophyll cells of *Saintpaulia* leaf stained with BCECF-AM. Images were captured before, immediately after (0 min), and 10 min after a slow temperature decrease (-5°C min^{-1}) from 25°C to 5°C. (d) Relative changes of BCECF fluorescence intensity after a slow temperature decrease. Scale bar = 40 μm. The points and associated bars in (b) and (d) indicate mean intensity and standard error (n = 3).

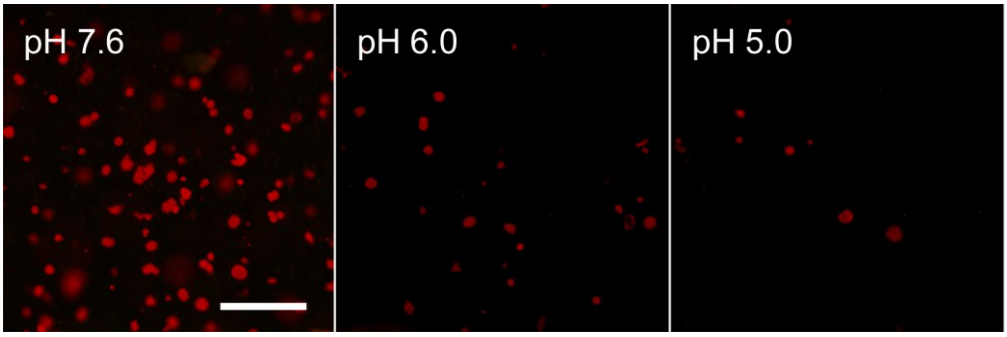


Figure 2-5. Effects of surrounding pH on isolated chloroplasts. Fluorescent microscopic images of chloroplasts isolated from *Saintpaulia* leaf suspended in buffer solution at pH 7.6 (left), pH 6.0 (middle), and pH 5.0 (right). Scale bar = 50 μm.

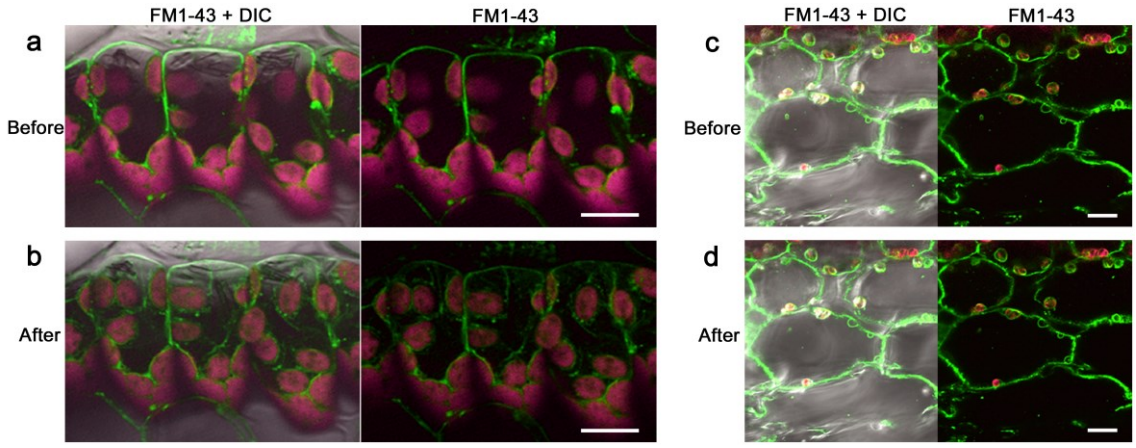


Figure 2-6. Vacuolar dynamics after a rapid temperature decrease. Vacuolar membrane labeled with FM1-43 (green) and chlorophyll fluorescence (magenta) in palisade mesophyll cells of *Saintpaulia* leaf were observed before (a) and 10 min after (b) a rapid temperature decrease from 25°C to 5°C and kept at 5°C for 1 min. Scale bar = 20 μm. FM1-43 and chlorophyll fluorescence in spongy mesophyll cells observed before (c) and after (d) a rapid temperature decrease from 25°C to 5°C. Scale bar = 40 μm.

Chapter 3

Molecular basis of sensitivity to a rapid temperature decrease in *Saintpaulia* leaves

Abstract

Saintpaulia leaf is sensitive to a rapid temperature decrease, whereas insensitive to a slow temperature decrease. In addition, leaves pre-incubated at 30°C exhibited more severe injury that at 20°C (see chapter 2). It is predicted that molecular mechanisms of temperature sensitivity are different in each temperature condition. To investigate molecular basis of sensitivity to a rapid temperature, changes in protein contents were analyzed using comparative proteomic approach. A number of proteins, which are involved in redox homeostasis, molecular chaperon, protein degradation, photosynthesis, and metabolic pathway, showed different accumulation patterns in leaves exhibited different sensitivity. Among identified proteins, 32 proteins was more than 2 times in abundance, and 41 proteins was less than 0.5 times in abundance in leaves that became more sensitive. In addition, levels of proteins that are possibly responsive to the phytohormone abscisic acid and harmful methylglyoxal changed significantly. The changes in abundances of these proteins might alter physiological conditions of leaves and subsequently confer sensitivity to a rapid temperature decrease in *Saintpaulia* leaves. These overall responses of proteins provide the fundamental information for further studies on response mechanisms to a rapid temperature decrease in plant cells.

3.1 Introduction

Plants develop protective mechanisms against temperature stresses and recover from unfavorable conditions. For example, under stressful temperatures, plants develop detoxification systems that break down toxic reactive oxygen species (ROS), which is one of the factors triggering temperature injury (Vickers *et al*. 2009). During cold acclimation, complex processes accompanied by changes in membrane structure and functions, gene expression, primary and secondary metabolite compositions were induced (Kawamura and Uemura, 2003; Kaplan *et al*. 2007; Urano *et al*., 2010; Krasensky and Jonak 2012).

To investigate physiological responses to temperature stress in plant, proteomic analysis has been performed to identify alternations the levels of various proteins, post-translational modifications, and protein-protein interactions (Timperio *et al*. 2008; Neilson *et al*. 2011). Proteomic analyses in subcellular compartment have elucidated various proteins that participate in response to temperature stress at the subcellular level in plant cells. In chloroplasts of *Arabidopsis thaliana*, levels of proteins functioning in photosynthesis, stress sensing, plastid metabolisms, and hormone synthesis changed under low temperature exposure (Goulas *et al*. 2006). Proteomic analyses of plasma membrane, which is a critical site for acquisition of freezing tolerance, showed differential accumulation patterns of membrane proteins during cold acclimatization in *Arabidopsis thaliana*, oat (*Avena sativa*) and rye (*Secale cereale*) (Uemura *et al*. 2006; Takahashi *et al*. 2012). In vacuolar membrane of *Arabidopsis*, protein contents of the phosphatase and subunits of vacuolar V-ATPase

increased during cold acclimation (Schulze *et al*. 2012).

Leaves of *Saintpaulia*, which are sensitive to a rapid temperature decrease, are damaged and discolored when leaf temperature rapidly decreases. In damaged leaves, only palisade mesophyll cells are injured and those organelles such as chloroplasts and mitochondria are dysfunctional and result in cell death (Yun *et al*. 1996). The organelle dysfunction supposed to be induced by rapid changes in the cytosolic pH caused by collapse of the vacuole (chapter 2). In addition, leaves pre-incubated at 30°C drastically enhanced injury after rapid exposure to 10°C, whereas leaves were not injured when temperature decreased slowly from 30°C to 10°C (Fig. 1-2, 2-2). In *Saintpaulia* leaves, molecular mechanisms of temperature sensitivity are modulated during pre-incubation and/or a slow temperature decrease.

In this chapter, proteomic analyses were conducted to elucidate the molecular mechanisms of temperature severity changes responding to ambient temperature in *Saintpaulia* leaves. The results show a new insight into the response to a rapid temperature decrease in *Saintpaulia* leaves and provide the fundamental knowledge for further studies on the response mechanisms within the subcellular fractions.

3.2 Materials and Methods

3.2.1 Plant material and temperature treatment

Saintpaulia sp. cv. Iceberg was used. Growth conditions were same as described in 2.2.1. The middle part of the leaf blade (0.35 g), which was tightly packed in a plastic bag to prevent direct contact with the water, was pre-incubated in the water at 30°C or 20°C in a circulation bath (Thermo Minder SM-05, TAITEC, Koshigaya, Japan) for 60 min. After pre-incubation, the leaf was transferred to another bath of 10°C for 5 min to decrease leaf temperature within 10 s. Leaf temperature was also slowly decreased at -5°C min^{-1} by gradual adding of cold water to the water bath. Comparative proteomic analyses were conducted between leaves pre-incubated at 30°C and 20°C, leaves after a rapid temperature decrease from 30°C to 10°C and from 20°C to 10°C, and leaves after a rapid temperature decrease from 30°C to 10°C and slow decrease from 30°C to 10°C, respectively. Three independent experiments were performed with leaves detached from three independent plants in each experiment.

3.2.2 Protein extraction

Pre-incubated leaves were ground down to a fine powder with a mortar and pestle in liquid nitrogen and suspended by adding 500 μl of buffer solution [50 mM HEPES, 5 mM EDTA, 400 mM sucrose, pH 7.8 (KOH)] supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). After centrifugation at 8,000 ***g*** at 4°C for 5 min, resulting supernatant was again centrifuged at 15,000 ***g*** at 4°C for 15 min. The second supernatant was used as a protein sample.

The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA).

Protein digestion was performed according to Li *et al* (2012). To purify the proteins, the protein sample was subjected to one-dimensional SDS-PAGE. The protein sample (2 μg) was suspended in detergents [2% (w/v) SDS, 50 mM Tris-HCl (pH6.8), 6% (v/v) β-mercaptoethanol, 10% (w/v) glycerol and bromophenol blue]. Electrophoresis was carried out at 50 V for 2 min on a 7.5% polyacryl-amide gel (E-R7.5L, ATTO, Tokyo, Japan). The gel was cut and digested according to Minami *et al*. (2009), followed by dehydration in acetonitrile. After drying in a vacuum centrifuge, 10 mM dithiothreitol in 100 mM NH_4HCO_3 was used to reduce proteins at 56°C for 60 min. To alkylation, the gel was incubated with 55 mM iodoacetoamide in 100 mM NH_4HCO_3 at room temperature for 45 min in dark, followed by washing with 100 mM NH_4HCO_3 for 10 min. The gel was dehydrated by addition of acetonitrile and then rehydrated in 100 mM NH_4HCO_3, followed by redehydration by acetonitrile. After drying in a vacuum centrifuge, the gel was swollen in a digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$) with 12.5 ng μl^{-1} of trypsin (sequencing grade, Boehringer Ingelheim GmbH, Ingelheim, Germany) in an ice-cold bath for 45 min. The digestion buffer was replaced with the same buffer in the absence of trypsin and incubated overnight at 37°C. Subsequently, the peptides were extracted by 20 mM NH_4HCO_3, and then three changes of 5% (v/v) formic acid in 50 % (v/v) acetonitrile for 20 min at room temperature.

3.2.3 Mass spectrometric analysis

Digested peptide solutions were subjected to nano-LC-MS/MS analysis according to Takahashi *et al*. (2012). Peptide solutions were loaded on a trap column (L-column Micro 0.3 × 5 mm; CERI, Japan) using a HPLC pump system (MICHROM Bioresources, Auburn, CA, USA). After elution from the column with 0.1% (v/v) formic acid in acetonitrile, peptides were separated with a column (Magic C18 AQ nano column 0.1 × 150 mm, MICHROM Bioresources) using a liner gradient of acetonitrile from 5% (v/v) to 45% (v/v) at a flow rate of 500 nl min^{-1}. Peptides were ionized with an ADVANCE spray source (MICHROM Bioresources) at a voltage of 1.8 kV. The mass spectrometer LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA) operated with Xcalibur software (Ver. 2.0.7, Thermo Fisher Scientific) was used. Full scan mass spectra were obtained in the range of 400 to 1,800 *m*/*z* with a resolution of 30,000 under data-dependent scanning mode. The experiments were repeated two or three times with biological replicates obtained independently.

The obtained spectra were converted to the mgf format using Proteome Discoverer (Ver. 1.1.0.263, Thermo Fisher Scientific) with the parameter conversions, that are as follows: precursor mass range, *m/z* 350-5,000; highest and lowest charge state, 0; lower and upper RT limit, 0; the minimum number of peaks in a spectrum, 1. MS/MS spectra from features with peptide charge (+1, +2, and +3), and then compared with the NCBI non-redundant Green Plants database (Ver. 2013217, comprising 23,214,025 sequences and 7,977,717,942 residues) using MASCOT server (version 2.3.02, Matrix Science, London, UK). The following Mascot search parameters were

used: enzyme, trypsin; fixed modification, carbamidomethyl and oxidation; peptide mass tolerance, 5 ppm; fragment ion mass tolerance, 0.6 Da. In semiquantitative analysis of proteins, average of protein abundance values, which were exported from Progenesis LC-MS software (version 4.0, Nonlinear Dynamics, New Castle, U.K.), was applied. In this process, peptides were filtered with ANOVA ($p < 0.05$). The relative abundance of proteins was significantly higher (fold change > 2) or lower (< 0.5) between groups were reported to be higher or lower in abundance. The identified proteins were classified by their functions according to the MapMan Bin classification (Thimm *et al*. 2004). For the proteins that were annotated either as unknown or as hypothetical proteins, their homologues were searched with protein-protein BLAST (http://blast.ncbi.nlm.nih.gov/) using their amino acid sequences to obtain the functional information. Semiquantitative analysis of mass spectrometry was supported by Mr. Daisuke Takahashi and Professor Matsuo Uemura (Iwate University).

3.3 Results

3.3.1 Identification and classification of proteins

The comparative proteomic analysis was performed in leaves subjected to three different temperature conditions: pre-incubated at 30°C or 20°C (pre-30°C/20°C), pre-incubated at 30°C or 20°C and then a rapid temperature decrease to 10°C (rapid-30°C/20°C), and pre-incubated at 30°C and then a rapid or slow temperature decrease to 10°C (rapid/slow). These approaches allowed the identification of 73 proteins that were statistically significant in abaundance, more abundant (> 2-fold) or less abundant (< 0.5-fold) with an ANOVA score of $p < 0.05$. The identified proteins were classified into functional categories according to the MapMan Bin classification (Fig. 3-1). The largest category of functions was photosynthesis proteins in all temperature conditions, indicating that they were greatly affected by pre-incubation and/or a rapid temperature decrease. The second most abundant proteins were function-unknown or hypothetical proteins. In addition, proteins involved in protein synthesis and folding and cell organization showed different accumulation pattern in all temperature conditions. Proteins showed significant changes in abundance in pre-30°C/20°C were categorized into cell wall synthesis, one-carbon metabolism, TCA cycle, and mitochondrial electron transport. Amino acid metabolism proteins were identified both in pre-30°C/20°C and rapid-30°C/20°C. Nucleotide metabolism protein was identified in the rapid-30°C/20°C. Proteins involved in cellular transport and stress response were identified both in rapid-30°C/20°C and rapid/slow. Redox and glycolysis involving proteins also showed significant changes in abundance. Only one stress-responsive protein showed changes in

abundance although all temperature conditions were stressful for *Saintpaulia* leaves.

3.3.2 Comprehensive analysis of proteins in leaves after pre-incubation at 30°C or 20°C

Comprehensive proteomic analysis was conducted in leaves after pre-incubation at 30°C or 20°C to identify proteins that show changes in abundance during pre-incubation. In total, 30 proteins showed significantly different abundance between leaves pre-incubated at 30°C and at 20°C (Table 3-1). Among the identified proteins, two glycolytic proteins (glyceraldehyde-phosphate dehydrogenase, gi|2494076 and gi|435103), were enriched in leaves pre-incubated at 30°C. In contrast, proteins involved in TCA cycle (malate dehydrogenase, gi|14269473 and gi|310689549), one-carbon metabolism (glycine dehydrogenase, gi|121083) were low in relative abundant in leaves pre-incubated at 30°C. Ribosomal protein (gi|5923684) and oligopeptidase A-like protein (gi|357144067), which has the function of protein synthesis and degradation, respectively, was also low abundant at 30°C. Subunits of mitochondrial F1-ATPase (gi|11583) and chloroplastic CF1-ATPase (gi|7525018) showed low abundance at 30°C. In addition to these proteins, redox, amino acid metabolism, cell organization, and photosynthesis involving proteins showed different accumulation in leaves pre-incubated at 30°C and 20°C. In proteins involved in redox homeostasis, cytosolic ascorbate peroxidase (gi|82941451) was more abundant whereas monodehydroascorbate reductase (gi|255537579) was less abundant in leaves after pre-incubation of 30°C. Photosynthetic proteins involved in light reaction (gi|131167, gi|11387206, gi|130270, gi|16374, gi|7271947, and gi|131289) and Calvin cycle

(gi|364285063, gi|16194, and gi|7525018) also showed differential accumulation. Two proteins involved in amino acid metabolisms were identified; methionine synthase (gi|59380482) was enriched at 30°C, whereas adenosylhomocysteinase (gi|417744) was enriched at 20°C. Among identified proteins of cytoskeleton, actin was enriched in leaves pre-incubated at 30°C, whereas tubulin alpha-3 chain (gi|6094431) and beta-1 chain (gi|135444) was enriched at 20°C. In addition, five function-unknown proteins (gi|21491, gi|307111057, gi|218199972, gi|351725153, and gi|118487652) were identified as significantly changed proteins.

3.3.3 Proteomic changes after a rapid temperature decrease to 10°C from 30°C or 20°C

To identify proteins that show changes in abundance in the process of the temperature injury, proteomic analysis was carried out in leaves, which pre-incubated at 30°C or 20°C subsequently subjected to a rapid temperature decrease to 10°C. As shown in Table 3-2, 19 proteins showed significantly different abundance between each temperature condition. Photosynthetic proteins involved in light reaction (gi|224114357, gi|169193, gi|4689382, and gi|110377793), histone H4 (gi|224293), and vacuolar-type H^+-ATPase (V-ATPase) subunit A (gi|137460) increased to more than 2.0 times in abundance. Stress-responsive uncharacterized protein (gi|18394049) appeared after the rapid temperature decrease. In contrast, lower abundance was found in 12 proteins involved in Calvin cycle (putative triosephosphate isomerase, gi|353441012 and gi|13431949; fructose-bisphosphatase, gi|255573935 and gi|11242), protein folding and degradation (chaperonin 10, gi|3057150; chloroplast protease, gi|52075839), cell cycle

(peptidyl-prolyl cis-trans isomerase, gi|10720315), and metabolism of amino acid and nucleotide (methionine synthase, gi|974782; adenine phosphoribosyltransferase, gi|1402894). In addition, three function-unknown proteins (gi|118487691, gi|255645535, and gi|9828630) showed significant changes in abundance.

3.3.4 Proteomic changes after a rapid or slow temperature decrease

To reveal proteins that show changes in abundance during the slow temperature decrease, the comparative proteomic analysis was performed in leaves subjected to a rapid or a slow temperature decrease to 10°C. Among 24 identified proteins, 15 proteins were 2.0 times higher in abundance and nine proteins were 0.5 times lower in abundance after the rapid temperature decrease (Table 3-3). Stress-responsive uncharacterized protein (gi|18394049) appeared after the rapid temperature decrease. Highly abundance was also found in proteins including catalase (gi|33146311), histone H4 (gi|224293), adenosine nucleotide translocator (gi|16160), V-ATPase subunit A (gi|401322), and elongation factor 1-alpha (gi|232029). An unknown protein (gi|12592) also increased in leaves after the slow temperature decrease. In contrast, proteins involved in glycolysis (glycerate hydrolase, gi|533474), protein folding (chaperonin 60 alpha subunit, gi|3790441), and metabolism of ammonia (ferredoxin-dependent glutamate synthase, gi|414979; glutamine synthetase leaf isozyme, gi|121353) and hormone (lipoxygenase, gi|213876486) showed low abundance after the rapid temperature decrease. Among the proteins involved in photosynthesis light reaction, oxygen-evolving enhancer protein, (gi|302595736), chlorophyll a/b binding protein (gi|4689382, gi|671737, and gi|12582)

was high in abundance, whereas chlorophyll a/b binding protein (gi|115766) was low in abundance. In addition, of the proteins involved in Calvin cycle, phosphoglycerate kinase (gi|255544584), RubisCO (gi|37574476 and gi|335059521) was high in abundance, whereas transketolase (gi|2501353), and chloroplastic F-ATPase subunit proteins (gi|5001577 and gi|7525018) were low in abundance.

3.3.5 Predicting protein functions from homology research

Some of the proteins were annotated either as unknown or as hypothetical proteins that are not specified functions in the database. To obtain the functional information about these proteins, their homologues were searched with protein-protein BLAST using their amino acid sequences. Corresponding homologues of nine function-unknown proteins with high homology are listed in Table 3-4. All proteins shared more than 75 % positives with their homologues in the amino acid sequence, indicating that these proteins might possess similar function. Predicted functions of identified proteins were classified into categories of photosynthesis (gi|21491, gi|118487691, gi|255645535, and gi|12592), cell wall precursor synthesis (gi|118487652), biodegradation of xenobiotics (gi|9828630), misc (gi|351725153), and not assigned (gi|218199972) according to the MapMan Bin system. However, one protein (gi|307111057) did not share homology with any other proteins.

3.4 Discussion

In this study, comparative proteomic analysis was conducted in *Saintpaulia* leaves to identify the proteins involved in conferring temperature sensitivity. In leaves damaged by a rapid temperature decrease, only palisade mesophyll cells were injured, indicating that temperature sensitivity may be determined by susceptibility of palisade mesophyll cells to a rapid temperature decrease. Consequently proteomic analysis conducted in palisade mesophyll cells may be an effective approach for identifying the molecular basis of temperature sensitivity. However, at present I have no effective method to isolate intact palisade mesophyll cells from leaves. Further, there is a possibility that temperature sensitivity of palisade mesophyll cells is altered by extracellular factors. Therefore, proteomic analysis in whole leaf is an essential first step in elucidating molecular basis of temperature sensitivity of *Saintpaulia* leaves. Although there is no genomic information of *Saintpaulia* at present, proteomic analysis using database obtained from other plant species provides the fundamental information for further studies on response mechanisms to a rapid temperature decrease in *Saintpaulia* leaves.

3.4.1 Classification of identified proteins

The identification of proteins that display different abundance patterns under various temperature conditions is an important step in elucidating the response mechanisms to a rapid temperature decrease in *Saintpaulia* leaves. To achieve this purpose, comparative proteomic analysis was performed in leaves exhibit different

sensitivity to a rapid temperature decrease. In this study, 73 candidate proteins that might involve in molecular mechanisms of sensitivity to a rapid temperature decrease. Most of them were not classified into stress-responsive protein (Table 3-1 to 3-4), suggesting that the molecular mechanism of temperature sensitivity in *Saintpaulia* leaves is possibly different from the well-known response mechanisms against environmental stresses. Photosynthetic proteins were greatly affected by temperatures (Fig. 3-1). Because the photosynthetic components are functionally linked each other, they might be wholly affect, resulting in abundance alterations of many photosynthetic proteins. Consequently, photosynthesis might be inhibited by a rapid temperature decrease (Fig. 2-3).

3.4.2 Changes in abundance of proteins during pre-incubation at 30°C or 20°C

Cytosolic ascorbate peroxidase (APX, gi|82941451) and monodehydroascorbate reductase (MDAR, gi|255537579), that is an enzyme component of the ascorbate-glutathion cycle that is antioxidant system against production of reactive oxygen species (Noctor and Foyer 1998), is identified in leaves pre-incubated at 30°C or 20°C (Table 3-1). Overexpression of APX and MDAR enhances tolerance to high or low temperature in tomato (*Lycopersicon esculentum*) and rice (*Oryza sativa*) plants (Li *et al*. 2010; Zhang *et al*. 2013). On the other hand, several reports suggest complexity of regulation mechanisms of antioxidant system in plant cells. In rice plants, low temperature stress induces rapid increases of APX activity and then gradual increase of MDAR activity (Oidaira *et al.* 2000). On the other hand, in pepper (*Capsicum annuum*) plants, APX and MDAR activities increase after one day of low

temperature treatment, but then no significant differences are observed in MDAR activity after two days of low temperature (Airaki *et al*. 2012). In *Saintpaulia* leaves pre-incubated at 30°C, APX was 44.214 times more abundant whereas putative MDAR was 0.137 times less abundant than leaves pre-incubated at 20°C. Less abundant of MDAR, which catalyzes reduction of monodehydroascorbate produced by APX, implying that monodehydroascorbate radical might be accumulated in *Saintpaulia* leaves pre-incubated at 30°C. This is possibly one factor increasing sensitivity to a rapid temperature decrease at 30°C. Considering previous reports suggesting that reactive oxygen species (ROS) may be involved (Yasuda *et al*. 1997; Yang *et al*. 2001), I may speculate contributions of the ascorbate-glutathion cycle to temperature sensitivity, although there is no direct evidence at present.

In *Saintpaulia* leaves, methionine synthase (gi|59380482) was enriched in leaves pre-incubated at 30°C (Table 3-1), which were high sensitivity to a rapid temperature decrease (Fig. 2-1). It has shown that, in barley leaves, the high expression of methionine synthase gene was induced under salt, drought, cold stresses and by ABA or H_2O_2 treatments (Narita *et al*. 2004). Methionine and its derivatives are crucial for substrates in one-carbon metabolisms and syntheses of betaine and polyamine, which have important roles in plant responses to environmental stresses (Hanson *et al*. 2000; Sakamoto and Murata 2002; Gii and Tuteja 2010). These findings suggest that highly abundance of methionine synthase alters stress tolerance in plant. Methionine and their derivatives may play a key role to regulate temperature sensitivity in *Saintpaulia* leaves.

Hypothetical protein (gi|218199972), which possesses abhydrolase domain, was highly enriched in after pre-incubation at 30°C. Some studies have shown a link between abhydrolase domain-containing protein and plant responses to environmental stresses. Expression of gene encoding abhydrolase domain-containing protein (gi|223944293) increases to 2-fold under salinity stress in maize plants (Kravchik and Bernstein 2013). Comparative analysis of nucreotide sequences isolated from pathogen-sensitive and tolerant genotypes of tubacco (*Nicotiana tabacum*) and Indian mustard (*Brassica juncea*) reveals that pathogen-responsive proteins with abhydrolase domain (HSR203J) plays a role in pathogen-induced hypersensitive cell death, although molecular machanisms for these roles remain unclear (Pontier *et al*. 1998; Mishra *et al*. 2010). In *Saintpaulia* leaves pre-incubated at 30°C, high abundance of the abhydrolase domain-containing protein might facilitate the palisade mesophyll cell death (Fig. 1-2) leading to leaf injury.

Function-unknown protein (gi|118487652) was enriched in pre-incubated leaves at 20°C. This protein has 93% positives compared to Arabidopsis AT1G09340, which is the abscisic acid (ABA)-responsive RNA-binding protein that participates in ABA signaling under various environmental stresses (Raab *et al*. 2006). In Arabidopsis, this protein is localized at chloroplasts and down-regulated during processes such as water stress and senescence that are known to involve ABA signaling (Raab *et al*. 2006). ABA signaling is also involved in the mechanisms that increase low temperature tolerance (Thomashow 1999). One possibility is that, in *Saintpaulia* leaves pre-incubated at 30°C, low abundance of this RNA-binding protein leads to disturbance

of transcriptions in chloroplasts during a rapid temperature decrease. Involvement of ABA in *Saintpaulia* leaf injury is unclear, however, disturbance of chloroplastic transcription might cause more severe injuries because chloroplasts carry out important processes of fatty acid, amino acid, and starch metabolisms.

The uncharacterized protein (gi|351725153), which has an AAI_LTSS domain found in plant trypsin-alpha amylase-inhibitor, plant lipid-transfer and seed-storage proteins, showed significant different patterns of accumulation in response to pre-incubation temperatures. Arabidopsis plants overexpressing gene of wheat alpha-amylase inhibitor, which has AAI_LTSS domain, show more tolerant to salinity and drought stresses and lower alpha-amylase activity than non-transgenic plants (Xiao *et al*. 2012). It has also been shown that proteins belong to the AAI_LTSS family may be involved in responses to pathogen, drought, and oxidative stress (Seo *et al*. 2010). *Saintpaulia* leaves pre-incubated at 20°C, where this protein was more abundant than leaves at 30°C, exhibited mild injuries (Fig. 2-1). Proteins belongs this family might participate in molecular mechanisms conferring sensitivity to a rapid temperature decrease.

Leaves pre-incubated at 30°C contains low abundance of predicted oligopeptidase A-like protein (gi|357144067), which may play a role in the converting signaling peptides precursor into their active form (Novak and Dev 1988). Changes in oligopeptidase A abundance under stress conditions has been reported: oxidative stress alters abundance of oligopeptidase A in rice leaves (Wan and Liu 2008), while heavy metal stress induces increase in oligopeptidase A-like protein in *Catharanthus roseus*

(Kumar *et al*. 2011). Lower abundance of oligopeptidase A-like protein may alter temperature perception and signal transduction of *Saintpaulia* leaves, resulting in sensitivity increase.

3.4.3 Proteins showed different accumulation patterns during the injury

Uncharacterized protein (gi|18394049) was found in leaves after the rapid temperature decrease to 10°C from 30°C (Table 3-2, 3-3). In Arabidopsis, this uncharacterized protein is encoded by At1g13930 gene, of which knockout mutants are sensitive to salt stress (Du *et al*. 2010). In *Saintpaulia* leaves, it is predicted that this protein might have some role in temperature stresses and progress leaf injuries because it was not found in leaves after pre-incubated at 20°C that showed mild injuries and in leaves after a slow temperature decrease that exhibited no injuries (Fig. 2-1), although molecular function of this protein remains unknown.

In leaves after temperature decrease from 30°C to 10°C, F1N21.10 protein (gi|9828630), which is putative lactoylglutathione lyase, showed lower abundance. Lactoylglutathione lyase is one of the enzymes comprising the glyoxalase system detoxifying methylglyoxal, which is harmful by-product of several metabolic pathways and accumulated to higher levels under environmental stresses in plant cells (Yadav *et al*. 2008). The transgenic tobacco plants overexpressing this enzyme resist an increase of methylglyoxal level and enhance tolerance to salinity stress (Yadav *et al*. 2005), indicating that lactoylglutathione lyase has an important role under environmental stresses that cause methylglyoxal increase in plant cells. Low abundance of

lactoylglutathione lyase in *Saintpaulia* leaves might cause severe injuries if methylglyoxal is accumulated after a rapid temperature decrease.

After the temperature decrease, abundance of adenine phosphoribosyltransferase (gi|1402894) decreased in leaves pre-incubated at 30°C. Adenine phosphoribosyltransferase is involved in the purine nucleotide salvage pathway, and catalyzes the reaction between adenine and phosphoribosyl pyrophosphate to AMP (Moffatt and Ashihara 2002). Changes in abundance and activity of adenine phosphoribosyltransferase alters the level of adenine conditions and tolerance to oxidative stress and high temperature in Arabidopsis, suggesting that adenine might play a important role in signaling in response to environmental stress (Sukrong *et al*. 2012). Different accumulation patterns of adenine phosphoribosyltransferase in *Saintpaulia* leaves might modulate sensitivity to a rapid temperature decrease.

3.4.4 Proteins showed different accumulation patterns during a slow temperature decrease

Temperature sensitivity is shown to be associated to activity of V-ATPase (Dietz *et al*. 2001). At low temperature, V-ATPase is inactivated and, as a consequence, the formation of pH gradients between vacuoles and cytoplasmic matrix is inhibited (Yoshida *et al*. 1999). Increase in V-ATPase amount has been reported under salinity stress in *Suaeda salsa* (Wang *et al*. 2001). In *Saintpaulia* leaves, V-ATPase subunit A (gi|137460 and gi|401322) was enriched in leaves after a rapid temperature decrease and subsequent incubation, exhibited severe injuries (Table 3-2 and 3-3). Taking into account

the results indicating intracellular pH changes (Fig. 2-3) and vacuolar collapse (Fig. 2-6) during injury in palisade cells, V-ATPase might have some role in pH homeostasis and stabilization of vacuolar membrane under temperature stress in *Saintpaulia* leaves, although it is not yet evident whether this enzyme is involved in injury or not.

Chaperonin 60 and 10 are essential molecules that are responsible for protein folding and assembly under stress conditions (Boston *et al*. 1996). Deletion of chaperonin 60 gene accelerates cell death to heat stress in Arabidopsis *lesion initiation 1* (*len 1*) mutant, leaves of which show discoloring, H_2O_2 accumulation, and low abundant of chlorophyll a/b binding proteins (Ishikawa *et al*. 2003). In *Saintpaulia*, abundance of chaperonin 60 (gi|3790441) and chaperonin 10 (gi|3057150) decreased to 0.289- and 0.456-fold, respectively, in leaves after a rapid temperature decrease from 30°C to 10°C and subsequent incubation (Table 3-2 and 3-3), suggesting that dysfunctions of cellular chaperon systems might be caused and thereby accelerate injuries that lead to leaf discoloring (Fig. 2-1). In addition, significant changes in abundance of many chloroplast proteins including chlorophyll a/b binding proteins were found after pre-incubation and a rapid temperature decrease (Table 3-1 to 3-3). Taken together with the report on *len 1* mutant, chaperonin 60 may be essential components that preventing disturbance of photosynthetic proteins under temperature stresses. Chaperonin 60 possibly protects RubisCO activase against thermal denaturation during photosynthetic acclimation to heat (Salvucci 2008).

3.4.5 Conclusions

In this study, the molecular responses to a rapid temperature decrease were investigated at the protein level in *Saintpaulia* leaves. Proteins involved in redox homeostasis, hypersensitive response, and signaling pathways, might play a key role in conferring sensitivity to a rapid temperature decrease. During a rapid temperature decrease, absence of putative stress-responsive proteins may facilitate the injury occurrence. Molecular chaperon may have function of the injury prevention during a slow temperature decrease. The V-ATPase may be involved in stabilization of vacuolar membrane and in pH homeostasis against a temperature decrease. These results show a overall view of changes in protein abundance in *Saintpaulia* leaves in response to a rapid temperature decrease, providing the framework for further studies on their functions.

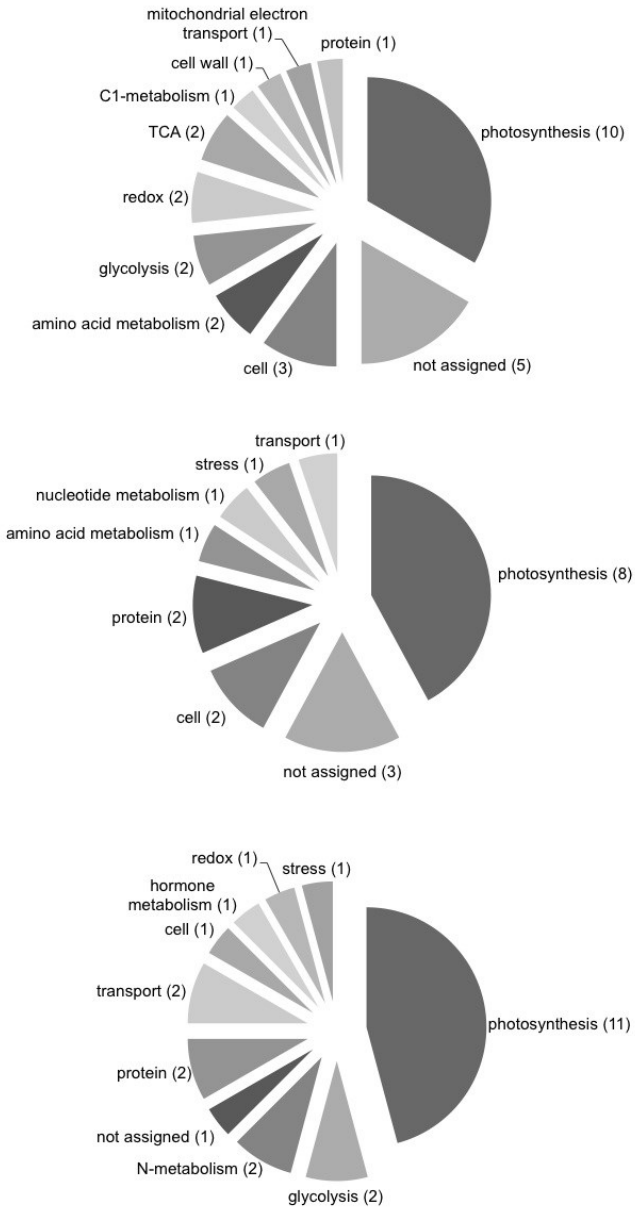


Figure 3-1. The functional categories of the proteins showed abundance changes. Proteins showed different abundances between (a) after pre-incubation at 30°C and 20°C, (b) after a rapid temperature decrease from to 10°C from 30°C and 20°C, and (c) after a rapid and slow temperature decrease from 30°C to 10°C. The classification is based on MapMan BIN (Thimm *et al*. 2004). The numbers of proteins classified into each functional category are shown.

Table 3-1. Proteins with significantly different abundance after pre-incubation at 30°C or 20°C

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	30℃/20℃[e]	Normalized abundance					
						30℃			20℃		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|82941451	43.10	0.003	cytosolic ascorbate peroxidase [Codonopsis lanceolata]	redox	44.214	64195.035	123447.866	-	2998.344	1142.218	2225.464
gi|59380482	27.39	0.042	methionine synthase [Helianthus annuus x Helianthus debilis subsp. debilis]	amino acid metabolism	20.487	150438.646	525051.370	-	36946.532	8416.239	4093.664
gi|21491	42.11	0.018	unnamed protein product [Solanum tuberosum]	not assigned	8.859	2074.210	2121.337	-	232.986	101.271	376.144
gi|2494076	51.37	0.009	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	glycolysis	6.784	128299.378	87688.827	-	23396.891	13267.414	11095.476
gi|162424651	58.08	0.027	actin, partial [Eritrichium sericeum]	cell. organisation	2.972	31195.804	21302.244	-	6024.743	9835.345	10638.445
gi|364285063	100.88	0.013	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Adiantopsis sp. MLP-2011-1]	photosynthesis. Calvin cycle	2.838	158455.230	169154.283	-	69616.476	61564.263	41960.093
gi|307111057	36.15	0.031	hypothetical protein CHLNCDRAFT_137638 [Chlorella variabilis]	not assigned	2.572	51879.161	57951.682	-	17352.808	30330.996	16380.936
gi|435103	102.25	0.034	glyceraldehyde-phosphate dehydrogenase [Pinus sylvestris]	glycolysis	2.338	72715.958	81962.131	-	32707.278	43318.300	23204.508
gi|131167	41.82	0.038	Photosystem I reaction center subunit II, chloroplastic	photosynthesis. light reaction	2.194	85980.090	83162.431	-	53029.199	33020.242	29569.823
gi|218199972	14.93	0.021	hypothetical protein OsI_26767 [Oryza sativa Indica Group]	not assigned	2.131	15224.627	19606.916	-	10003.884	7632.912	6880.461

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	30℃/20℃[e]	Normalized abundance					
						30℃			20℃		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|115461679	48.00	0.017	Os05g0110300 [Oryza sativa Japonica Group]	cell wall. precursor synthesis	0.498	19463.601	22927.119	-	36218.582	40361.141	51050.393
gi|14269473	60.42	0.043	malate dehydrogenase [Zea mays]	TCA	0.485	14571.949	20136.104	-	31144.973	46045.215	30170.306
gi|351725153	46.04	0.023	uncharacterized protein LOC100499687 precursor [Glycine max]	not assigned	0.447	84354.286	77073.607	-	143875.549	168176.393	230202.234
gi|5923684	40.00	0.020	putative 60S acidic ribosomal protein, 5' partial [Arabidopsis thaliana]	protein.synthesis	0.446	7221.724	11034.770	-	19182.780	18771.258	23414.551
gi|118487652	34.35	0.044	unknown [Populus trichocarpa]	not assigned	0.430	26800.980	18185.599	-	69128.828	47083.363	40718.428
gi|417744	81.60	0.017	Adenosylhomocysteinase	amino acid metabolism	0.417	43024.353	37491.487	-	72682.916	110588.192	106068.042
gi|310689549	87.63	0.011	malate dehydrogenase [Pinus sylvestris]	TCA	0.346	103551.667	94857.872	-	214605.921	303930.409	341547.538
gi|357144067	60.68	0.025	PREDICTED: oligopeptidase A-like [Brachypodium distachyon]	protein. degradation	0.331	19177.827	20650.651	-	51121.627	44425.890	84783.962
gi|11387206	45.60	0.022	Thylakoid lumenal 29 kDa protein, chloroplastic	photosynthesis. light reaction	0.272	2539.682	4407.087	-	15559.864	8912.571	13893.712
gi|130270	58.79	0.006	Plastocyanin	photosynthesis. light reaction	0.150	3121.978	2327.286	-	12637.171	24217.562	17627.125
gi|255537579	49.09	0.005	monodehydroascorbate reductase, putative [Ricinus communis]	redox	0.137	15427.867	19980.327	-	87188.873	135548.387	163718.501
gi|121083	47.90	0.016	Glycine dehydrogenase [decarboxylating], mitochondrial	C1-metabolism	0.118	9193.658	20228.093	-	70804.713	156340.312	146616.048

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	30℃/20℃[e]	Normalized abundance					
						30℃			20℃		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|11583	128.10	0.033	ATPase, beta subunit [Hordeum vulgare]	mitochondrial electron transport	0.077	251879.970	99946.870	-	824485.583	2397135.817	3625236.716
gi|16374	29.07	0.013	chlorophyll a/b binding protein (LHCP AB 180) [Arabidopsis thaliana]	photosynthesis. light reaction	0.057	716.084	3030.923	-	45366.075	28693.298	25278.700
gi|135444	38.82	0.045	Tubulin beta-1 chain	cell. organisation	0.045	1495.573	1493.371	-	6624.561	42536.644	51471.034
gi|7271947	52.69	0.001	chlorophyll a/b-binding protein type III [Alonsoa meridionalis]	photosynthesis. light reaction	0.004	694.432	318.689	-	146784.393	91388.427	112625.493
gi|16194	34.83	0.046	ribulose bisphosphate carboxylase [Arabidopsis thaliana]	photosynthesis. Calvin cycle	0.004	0	219.597	-	60146.411	13679.457	17326.684
gi|7525018	31.43	0	ATP synthase CF1 alpha subunit [Arabidopsis thaliana]	photosynthesis. Calvin cycle	0	0	0.110	-	28348.776	20019.220	10035.136
gi|131289	54.66	0.009	Photosystem II CP43 chlorophyll apoprotein;	photosynthesis. light reaction	0	0	0	-	167.950	984.406	4793.197
gi|6094431	44.35	0.016	Tubulin alpha-3 chain	cell. organisation	0	0	0	-	418.695	66550.823	15250.314

[a]Accession no. indicates NCBI GI number. [b]Confidence score indicates numbers generated from Progenesis software based on each peptide. [c]Proteins name was identified using NCBI protein database with green plant. [d]Functional classification according to MapMan BIN (Thimm *et al*. 2004). [e]The values were calculated as the mean ratio (30°C/20°C) of normalized abundance.

Table 3-2. Proteins with significantly different abundance after a rapid temperature decrease to 10°C from 30°C or 20°C

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	30℃/20℃[e]	Normalized abundance					
						30℃			20℃		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|18394049	55.19	0.045	uncharacterized protein [Arabidopsis thaliana]	stress. abiotic. drought/salt	Infinity	6.009	176.296	-	0	0	0
gi|224114357	48.04	0.028	light-harvesting complex II protein Lhcb1 [Populus trichocarpa]	photosynthesis. light reaction	9.617	1021565.310	1302821.953	-	43901.344	93656.542	224988.698
gi|169193	121.05	0.021	Major Cab protein [Petunia x hybrida]	photosynthesis. light reaction	4.428	45867.935	88590.139	-	12767.697	12168.462	20615.423
gi|224293	48.45	0.038	histone H4	cell. organisation	3.825	50496.849	28219.398	-	10249.751	6200.189	14415.560
gi|4689382	98.32	0.005	chlorophyll a/b binding protein CP29 [Vigna radiata]	photosynthesis. light reaction	2.947	113150.761	153879.506	-	41931.463	42585.924	51404.968
gi|110377793	71.88	0.040	chloroplast pigment-binding protein CP26 [Nicotiana tabacum]	photosynthesis. light reaction	2.663	358765.185	372244.613	-	90687.501	121159.645	199884.318
gi|137460	44.39	0.006	V-type proton ATPase catalytic subunit A	transport	2.281	21130.411	19668.918	-	9470.591	7469.698	9886.078
gi|118487691	158.19	0.039	unknown [Populus trichocarpa]	not assigned	0.494	601614.610	480373.923	-	1224250.421	1248365.019	815935.829
gi|353441012	136.37	0.049	putative triosephosphate isomerase [Elaeis guineensis]	photosynthesis. Calvin cycle	0.493	474051.484	372588.369	-	989524.687	965773.011	620908.719
gi|255645535	130.8	0.041	unknown [Glycine max]	not assigned	0.493	527188.625	427180.760	-	1089438.312	1098424.524	713186.547
gi|3057150	43.52	0.005	chaperonin 10 [Arabidopsis thaliana]	protein. folding	0.456	53184.703	58842.412	-	124539.725	138201.170	105888.360

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	30℃/20℃[e]	Normalized abundance					
						30℃			20℃		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|9828630	173.88	0.030	F1N21.10 [Arabidopsis thaliana]	not assigned	0.435	100631.542	74963.639	-	199832.958	250896.657	154864.809
gi|10720315	72.76	0.036	Peptidyl-prolyl cis-trans isomerase, chloroplastic	cell cycle	0.430	48397.054	35282.382	-	128348.598	84253.401	79180.808
gi|255573935	33.16	0.024	fructose-1,6-bisphosphatase, putative [Ricinus communis]	photosynthesis. Calvin cycle	0.421	133629.662	91240.726	-	243672.101	330669.217	226489.576
gi|11242	46.29	0.029	fructose-bisphosphatase [Arabidopsis thaliana]	photosynthesis. Calvin cycle	0.373	26449.937	22749.521	-	62565.765	88387.932	47131.976
gi|52075839	33.22	0.027	putative chloroplast protease [Oryza sativa Japonica Group]	protein. degradation	0.347	1757.945	2715.398	-	4610.392	7590.106	7146.0920
gi|13431949	32.54	0.039	Triosephosphate isomerase, chloroplastic	photosynthesis. Calvin cycle	0.337	52108.945	37524.598	-	137010.956	176312.586	85519.677
gi|974782	46.99	0.037	cobalamine-independent methionine synthase [Solenostemon scutellarioides]	amino acid metabolism	0.277	7297.700	6858.117	-	16542.978	39522.865	20573.154
gi|1402894	33.06	0.046	adenine phosphoribosyltransferase [Arabidopsis thaliana]	nucleotide metabolism	0.023	19.193	0	-	756.649	328.765	193.787

[a]Accession no. indicates NCBI GI number. [b]Confidence score indicates numbers generated from Progenesis software based on each peptide. [c]Proteins name was identified using NCBI protein database with green plant. [d]Functional classification according to MapMan BIN (Thimm *et al*. 2004). [e]The values were calculated as the mean ratio (30°C/20°C) of normalized abundance.

Table 3-3. Proteins with significantly different abundance after a rapid or a slow temperature decrease from 30°C to 10°C

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	Rapid/Slow[e]	Normalized abundance					
						Rapid			Slow		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|18394049	55.19	0.045	uncharacterized protein [Arabidopsis thaliana]	stress. abiotic. drought/salt	Infinity	6.009	176.296	-	0	0	0
gi|302595736	34.02	0.028	Oxygen-evolving enhancer protein 2	photosynthesis. light reaction	826.875	26839.344	5844.755	-	0.001	59.290	0
gi|255544584	31.53	0.001	phosphoglycerate kinase, putative [Ricinus communis]	photosynthesis. Calvin cycle	93.028	93674.445	44565.160	-	830.109	492.128	906.760
gi|37574476	39.93	0.009	ribulose 1,5-bisphosphate carboxylase/oxygenase [Pogonatum nipponicum]	photosynthesis. Calvin cycle	4.527	29986.343	27899.352	-	8775.922	5932.976	4471.676
gi|33146311	90.05	0.032	catalase [Acacia ampliceps]	redox	3.945	31038.521	21685.744	-	9638.794	6642.211	3768.310
gi|224293	48.45	0.039	histone H4	cell.organisation	2.836	50496.849	28219.398	-	12297.965	11158.030	18185.233
gi|4689382	67.92	0.006	chlorophyll a/b binding protein CP29 [Vigna radiata]	photosynthesis. light reaction	2.834	8490.356	10099.820	-	3542.673	2623.915	3674.476
gi|335059521	44.26	0.032	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Streptocarpus holstii]	photosynthesis. Calvin cycle	2.805	148132.612	169520.470	-	80553.889	51092.050	38222.593
gi|120666	45.89	0.042	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	glycolysis	2.660	4807.248	3177.119	-	1937.972	1013.919	1550.363
gi|16160	53.44	0.041	adenosine nucleotide translocator [Arabidopsis thaliana]	transport	2.354	49937.160	41878.192	-	24297.450	21327.548	12878.925
gi|671737	70.37	0.014	Chloropyll a/b binding protein [Amaranthus hypochondriacus]	photosynthesis. light reaction	2.176	772518.054	700317.965	-	268316.050	350288.815	396499.221

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	Rapid/Slow[e]	Normalized abundance					
						Rapid			Slow		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|12592	105.09	0	unnamed protein product [Hordeum vulgare subsp. vulgare]	not assigned	2.137	216595.338	218038.838	-	101564.835	103338.024	100199.343
gi|401322	54.96	0.031	V-type proton ATPase catalytic subunit A	transport	2.111	29737.649	19888.346	-	12807.185	9727.349	12734.426
gi|12582	44.64	0.036	light harvesting chlorophyll a /b binding protein [Hedera helix]	photosynthesis. light reaction	2.096	154679.514	174327.513	-	91853.003	55781.759	87769.594
gi|232029	42.01	0.013	Elongation factor 1-alpha	protein. synthesis	2.018	205180.017	276223.546	-	130393.729	108329.340	119143.357
gi|533474	67.73	0.014	2-phospho-D-glycerate hydrolase [Mesembryanthemum crystallinum]	glycolysis	0.466	228567.821	290286.141	-	493719.436	516052.143	660830.111
gi|2501353	122.36	0.004	Transketolase, chloroplastic	photosynthesis. Calvin cycle	0.464	343395.071	268027.860	-	655900.028	677056.221	644304.044
gi|115766	67.5	0.005	Chlorophyll a/b binding protein, chloroplastic	photosynthesis. light reaction	0.450	3080.245	2630.026	-	5493.618	6690.663	6844.784
gi|414979	41.45	0.004	ferredoxin-dependent glutamate synthase, partial [Spinacia oleracea]	metabolism. ammonia metabolism	0.386	110020.506	99438.837	-	228090.854	304145.740	281617.762
gi|213876486	55.98	0	lipoxygenase [Camellia sinensis]	hormone metabolism. jasmonate	0.361	276578.574	251173.633	-	719408.454	705132.452	770312.587
gi|3790441	25.07	0.006	chaperonin 60 alpha subunit [Canavalia lineata]	protein. folding	0.121	22884.020	20395.354	-	217871.758	108714.749	208859.228
gi|5001577	54.79	0.001	ATP synthase beta subunit [Podophyllum peltatum]	photosynthesis. Calvin cycle	0	0	0	-	1631.009	825.839	422.479

Accession no.[a]	Confidence score[b]	Anova (*p*)	Protein name[c]	Classification MapMan BIN[d]	Rapid/Slow[e]	Normalized abundance					
						Rapid			Slow		
						Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
gi|7525018	31.43	0	ATP synthase CF1 alpha subunit [Arabidopsis thaliana]	photosynthesis. Calvin cycle	0	0	0	-	307.929	603.652	313.811
gi|121353	34.29	0.002	Glutamine synthetase leaf isozyme, chloroplastic	metabolism. ammonia metabolism	0	0	0	-	20587.934	2002.255	21124.060

[a]Accession no. indicates NCBI GI number. [b]Confidence score indicates numbers generated from Progenesis software based on each peptide. [c]Proteins name was identified using NCBI protein database with green plant. [d]Functional classification according to MapMan BIN (Thimm *et al*. 2004). [e]The values were calculated as the mean ratio (rapid temperature decrease/slow temperature decrease) of normalized abundance.

Table 3-4. The homologues of the unknown proteins. The protein-protein BLAST was used to search the homologues of unknown-protein in Table 3-1, 3-2, and 3-3. The function-annotated homologues with the highest homology are shown.

Accession no.[a]	Homologue					
	Accession no.[b]	Protein name	Organism	Classification MapMan BIN	Ident. (%)[c]	Pos. (%)[d]
gi|21491	gi|131399	Photosystem II 10 kDa polypeptide, chloroplastic	*Solanum tuberosum*	photosynthesis. light reaction	99	99
gi|218199972	gi|474164461	putative hydrolase yugF	*Triticum urartu*	not assigned	73	80
gi|351725153	gi|357518817	Non-specific lipid-transfer protein-like protein	*Medicago truncatula*	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein[e]	64	77
gi|118487652	gi|255542956	RNA binding protein	*Arabidopsis thaliana*	not assigned	84	93
gi|118487691	gi|255576721	triosephosphate isomerase, putative	*Ricinus communis*	photosynthesis. light reaction	84	88
gi|255645535	gi|356572486	PREDICTED: triosephosphate isomerase, chloroplastic-like	*Glycine max*	photosynthesis. light reaction	99	99
gi|9828630	gi|15220397	putative lactoylglutathione lyase, chloroplast	*Arabidopsis thaliana*	biodegradation of xenobiotics	98	98
gi|12592	gi|118430287	photosystem II protein D2	*Agrostis stolonifera*	photosynthesis. light reaction	99	99

[a]Accession no. of the unknown proteins in Table 3-1, 3-2, and 3-3. [b]Accession no. of the homologues. [c]Identities. [d]Positives. [e]misc is category gathered enzymes that can not be attributed to any pathway.

Chapter 4

Sensitivity to temperature-decrease and phylogenic relationship in Gesneriaceae plants

Abstract

Temperature sensitivity to a rapid temperature decrease of Gesneriaceae plants was investigated. In this study, leaves of 29 species were exposed to a rapid temperature decrease from 30°C to 10°C, of which 12 species were sensitive to the rapid temperature decrease and discolored after 24 h. In the discolored leaves, only palisade mesophyll cells were injured and those chloroplasts were disordered. During the rapid temperature decrease, chlorophyll fluorescence declined and did not recover; ϕII (quantum yield of photosystem II) decreased and at the same time, NPQ (non-photochemical quenching) increased. In palisade mesophyll cell of sensitive species, intracellular pH homeostasis altered by the rapid temperature decrease. The cytosolic acidification was observed using pH sensitive fluorescent dyes. These results suggest that the changes in the intracellular pH damaged the chloroplasts resulting in injury of palisade mesophyll cells, as observed in *Saintpaulia* leaves. Although species injured by a rapid temperature decrease from 30°C to 10°C belong to different phylogenetic groups in Gesneriaceae and have different structures of leaves, all of them showed injury of palisade mesophyll cells, decline of chlorophyll fluorescence, and intracellular pH changes. These results imply that the sensitivity to a rapid temperature decrease from 30°C to 10°C is not correlated with phylogenic relationships.

4.1 Introduction

The leaves of *Saintpaulia* sp. are damaged and discolored by a rapid temperature decrease when cold water irrigated onto the surface of the leaf (Poesch 1942) as shown in chapter 1 to 3. In discolored leaves of S*aintpaulia*, palisade mesophyll cells and those organelles were damaged, resulting in cell injury (Yun *et al*. 1996). In particular, chloroplasts disordered and their chlorophyll fluorescence declined within a very short time after the temperature decrease (Yun *et al*. 1998). In chapter 2, I reported that damages of *Saintpaulia* leaves are triggered by collapse of vacuoles in palisade mesophyll cells; the rapid temperature decrease may initially affect vacuolar membrane, resulting in intracellular pH changes followed by organelle damage.

Leaves of other plants, some species or cultivars of Gesneriaceae, which is a large family comprising ca. 2000 species in ca. 150 genera distributed mostly in the tropic with a few species in temperate Europe and Asia (Good 1974), were also damaged by the same temperature conditions which cause discoloring of *Saintpaulia* leaves (Yun *et al*. 2001). However, the injury mechanisms of those plants have not been studied. Comparative investigation of this leaf injury may lead to understand the role of such a physiological response to rapid temperature changes.

In this capture, I investigate mechanisms of leaf damage of Gesneriaceae plants and discuss whether the mechanisms are the same as in *Saintpaulia* leaves or not. I report here that 12 species of 29 investigated plants were sensitive to the rapid temperature decrease and exhibited the same damage mechanisms as in *Saintpaulia* leaves. Although sensitive species belong to different phylogenetic groups in

Gesneriaceae and have different anatomical structures of leaves, all of them showed injury of palisade mesophyll cells, decline of chlorophyll fluorescence, and intracellular pH changes.

4.2 Materials and Methods

4.2.1 Plant material and growth condition

The 29 species in the 19 associated genera of Gesneriaceae (Table 4-1) were obtained from Hyogo Prefectural Flower Center (Hyogo, Japan) and commercial growers. These plants were cultured in artificial soil (vermiculite:perlite, 3:1) at 22°C to 25°C under a 12 (22°C) to 14 h (25°C) light at about 50 μmol m^{-2} s^{-1} (FL20SS BRN, National, Osaka, Japan). There was no difference between the two different growth conditions for control plants. Fully expanded leaves were detached just before each experiment. In preliminary experiments, I confirmed that leaf detachment did not affect the injury process.

4.2.2 Estimation of leaf injury after a temperature decrease

This experiment was performed as described in chapter 2.2.2. To change leaf temperature, detached leaves immersed in water kept at 30°C in a circulation bath (Thermo Minder SM-05, TAITEC, Koshigaya, Japan). Each leaf was packed in a plastic bag to prevent the leaves from coming into direct contact with the water. Leaf surface temperature, measured using a thermocouple placed on the surface of the leaf, was adjusted to that of the water bath. Leaf surface temperature was rapidly or gradually decreased from 30°C, 25°C, 20°C, 15°C to 10°C. When a leaf kept in a bath at 30°C, 25°C, 20°C, 15°C for 60 min was transferred to another bath at 10°C and kept for 1min, leaf temperature decreased to 10°C within 10 s. When cold water was added gradually to the water bath, both bath and leaf temperatures decreased simultaneously at -5°C

min^{-1}. After the temperature change treatment, the tested leaf was wrapped with aluminum foil and were kept at 22°C for appropriate period, while avoiding leaf shrinking. Damaged areas were estimated with the PC program (LIA for Win32).

4.2.3 Measurement of chlorophyll fluorescence during a temperature decrease

Changes in chlorophyll fluorescence during a rapid or a slow temperature decrease from 30°C to 10°C in 10 Gesneriaceae species was determined with the PAM as described in chapter 2.2.7. Changes in ϕII and NPQ were estimated in leaves of temperature-decrease sensitive species (*Achimenes patens*, *Chirita tamiana*, *Columnea linearis*, *Columnea* sp., *Kohleria warszewiczii*, *Nematanthus maclatus*, and *Streptocarpus saxorum*) and insensitive species (*Streptocarpus* sp. and *Sinningia speciosa*).

4.2.4 Preparation and observation of leaf sections

Leaves before and after 24 h of a rapid temperature decrease from 30°C to 10°C were fixed in FAA (formalin:acetic acid:50% ethanol, 5:5:90) and embedded in a methacrylate resin (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim, Germany). Sections of 5 μm thickness were made using a microtome (Nippon Optical Works, Tokyo, Japan) and stained with 0.1% toluidine blue before being covered with a mounting solution (Entellan neu, Merck KGaA, Darmstadt, Germany). The sections were observed under a light microscope (BX51, Olympus, Tokyo, Japan).

4.2.5 Monitoring of intracellular pH with pH-sensitive fluorescent dyes

Intracellular pH changes during a rapid temperature decrease were monitored with pH-sensitive dye, BCECF-AM (Molecular Probes, Eugene, OR, USA) in leaves of temperature-decrease sensitive species (*Columnea* sp.; *Kohleria warszewiczii*) and insensitive species (*Sinningia speciosa*; *Streptocarpus* sp.). Staining and observations were conducted as described in chapter 2.2.8.

4.2.6 Phylogenetic analyses

The phylogenic relationships of 18 species of Gesneriaceae were examined based on ribosomal DNA internal transcribed spacer 1 (ITS1) sequences. ITS is variable region which mutate frequently, so it is considered to be informative for analyses of relationships at the species to genus level (Suh *et al*. 1993). All ITS1 sequence data were obtained from GenBank (accession numbers are listed Table 1). The ITS1 sequences were aligned using the ClustalW option in the analysis software package MEGA5 (Tamura *et al*. 2011). A molecular phylogenetic tree was constructed with MEGA5 using the neighbor-joining (Saitou and Nei 1987) and maximum likelihood methods (Tamura and Nei 1993). The robustness of the resulting phylogenetic tree was tested by bootstrap analysis with 1000 replicates (Felsenstein 1985). Two species of Rubiaceae, *Pentas ulugurica* and *Triainolepis tomentella* (GenBank accession nos. AM267061 and AM267069), were used as an outgroup.

4.3 Results

4.3.1 Leaves of Gesneriaceae species injured by a rapid temperature decrease

The leaves of 29 species in the 19 associated genera of Gesneriaceae were tested to investigate their sensitivity to a rapid temperature decrease. The leaves of 12 species were sensitive to the rapid temperature decrease from 30°C to 10°C, leading to leaf injury (Fig. 4-1). The injured areas discolored within 10 min of the treatment. When the leaf temperature of the 12 sensitive species was slowly decreased from 30°C to 10°C at a rate of 5°C min^{-1}, the leaves were not damaged (data not shown). Although the decrease of temperature was within the same range, a slow decrease did not result in leaf discoloring.

The injury severity after the temperature decrease was estimated in three sensitive species; *Columnea* sp., *Kohleria warszewiczii*, and *Streptocarpus saxorum* (Fig. 4-2). When leaf temperature was rapidly decreased from 30°C to 10°C, 95.5%, 65.8%, and 49.9% of the total leaf area was discolored in *Columnea* sp., *K. warszewiczii*, and *S. saxorum* leaves, respectively. When leaf temperatures were decreased from 15°C to 10°C, the injury severities were 8.2%, 2.7%, and 0.4%, respectively. The lower the initial temperature of the leaves, the lower the injury severities were, even though the leaf temperatures were decreased to the same level (10°C). The injury severity at 10°C was affected by the initial leaf temperature before the temperature decrease. The result was in good agreement with the temperature conditions resulting in leaf injury of *Saintpaulia* shown in Fig. 1-2c.

4.3.2 Changes in chlorophyll fluorescence during a rapid temperature decrease

The rapid temperature decrease was thought to affect chloroplasts, because leaves were discolored within 24 h of the temperature decrease (Fig. 4-1). To investigate the photosynthetic activity during the leaf damage occurrence, changes in chlorophyll fluorescence in leaves of 10 species during the temperature decrease were monitored with PAM and two indices of photosynthesis, ϕII and NPQ, were estimated. The maximum values of F_v'/F_m' at 30°C after the actinic light exposure were 0.62-0.76. When leaf temperature was maintained at 30°C for 15 min, ϕII and NPQ was maintained at a constant value (Fig. 4-3, left columns). When the temperature slowly decreased from 30°C to 10°C at a rate of -5ºC min^{-1}, ϕII decreased reversibly with temperature decrease and had minimum value at 10°C (Fig. 4-3, middle columns). ϕII subsequently increased after rewarming to 30°C, whereas NPQ did not change during the temperature decrease. In *Columnea* sp. leaves, ϕII deceased by 35% at 10°C and increased to ~90% of the pretreatment value after rewarming (Fig. 4-3e, middle column). This result indicated that temperature of 10°C itself did not harmful to chloroplasts. When the temperature rapidly decreased from 30°C to 10°C and was maintained at 10°C for 1 min before returning to 30°C, ϕII continued to decrease in leaves of 8 sensitive species: *Achimenes patens*, *Chirita tamiana*, *Columnea linearis*, *Columnea* sp., *Kohleria warszweiczii*, *Nematanthus maculates*, *Saintpaulia* sp., *Streptocarpus saxorum*. Concurrently, NPQ increased and remained high value after the rapid temperature decrease (Fig. 4-3a-h, right columns). In *Saintpaulia* sp. leaves, ϕII decreased by 57% at the end of period of 10°C and continued to decrease by 90%

following rewarming to 30°C (Fig. 4-3a). NPQ increased to 2.4 times during the temperature decrease. Conversely, in leaves of *Streptocarpus* sp. and *Sinningia speciosa,* which were insensitive to the rapid temperature decrease, exhibited reversible decreases in ϕII even when the leaf temperature was rapidly decreased from 30°C to 10°C (Fig. 4-3i,j). Decreased ϕII was subsequently increased to the initial value after rewarming to 30°C. NPQ did not change during the temperature decrease. These results indicated that chloroplasts in leaves of sensitive species were damaged by the rapid temperature decrease in a short time prior to leaf discoloring.

4.3.3 Observation of transverse section of discolored leaves

Transverse sections of damaged leaves were observed microscopically and compared with those of intact leaves (Fig. 4-4). I observed 10 species; *Achimenes patens*, *Chirita. tamiana*, *Columnea linearis*, *Columnea* sp., *Gesneria pedicellaris*, *Kohleria warszewiczii*, *Nematanthus maclatus*, *Saintpaulia sp*., *Streptocarpus formosus,* and *Streptocarpus saxorum*. The leaves of *C*. *tamiana*, *C*. *linearis*, *Columnea.* sp., *N. maculatus*, *G. pedicellaris*, and *S.* s*axorum* had one to five cell layers of hypodermis beneath the upper epidermis (Fig. 4-4a-f). *A. patens*, *K. warszewiczii*, *Saintpaulia* sp., and *S. formosus* did not have a hypodermis layer (Fig. 4-4g-j). Palisade mesophyll was layered beneath the upper epidermis or hypodermis. Between the palisade mesophyll and lower epidermis, three to six layers of spongy mesophyll were observed. Both palisade mesophyll and spongy mesophyll cells contained chloroplasts. After 24 h following the temperature decrease from 30°C to 10°C, the palisade mesophyll cells

were injured and the chloroplasts disordered, whereas no notable changes were observed in the upper and lower epidermis, hypodermis, and spongy mesophyll (Fig.4-4). Injury of the palisade mesophyll cells was observed in all discolored leaves, regardless of the variation in leaf anatomical structure. This result indicated that only palisade mesophyll cells were sensitive to a rapid temperature decrease and that the difference in tissue structures was not correlated with temperature sensitivity.

4.3.4 Changes in intracellular pH during injury occurrence

Changes in cytosolic pH were observed in the palisade mesophyll cells of Gesneriaceae species that are sensitive or insensitive to a rapid temperature decrease. Prior to the rapid temperature decrease from 30°C to 10°C, fluorescence of pH-sensitive dye BCECF was observed in the cytosol of palisade mesophyll cells (Fig. 4-5a, b). The fluorescence declined 10 min after the rapid temperature decrease in palisade mesophyll cells of sensitive *Columnea* sp. and *Kohleria warszewiczii* leaves (Fig. 4-5a); 10 min after the temperature decrease, the fluorescence intensity decreased by 76% and 70% in *Columnea* sp. and *K. warszewiczii,* respectively (Fig. 4-5c). A fluorescence decrease of BCECF was not observed when the temperature was maintained at 30°C (Fig. 4-6). Conversely, in the insensitive leaves of *Sinningia speciosa* and *Streptocarpus* sp., BCECF fluorescence was strongly emitted even after the temperature decrease (Fig. 4-5b) and its intensity was constant (Fig. 4-5d). As with *Saintpaulia* leaves, injury to the palisade mesophyll cells of sensitive Gesneriaceae also correlated with intracellular pH changes induced by a rapid temperature decrease from 30°C to 10°C.

4.3.5 Phylogenetic relationships of investigated species

The phylogenetic tree was constructed based on ITS1 sequences of ribosomal DNA to examine whether the physiological features of palisade mesophyll cells are correlated with phylogenetic relationships or not. I constructed the neighbor-joining and maximum likelihood trees (Fig. 4-7). Despite variation in trees, Gesneriaceae was separated into two subfamilies: Gesnerioideae and Cyrtandroideae, which mainly distributed in the New World and the Old World (Wiehler 1983). This cladistics analysis was well supported with monophyletic Gesnerioideae and Cyrtandroideae subfamilies based on chloroplast *ndhF* (NADPH dehydrogenese subunit F) sequences (Smith *et al*. 1997). The sensitive species to a rapid temperature decrease were placed within both subfamilies. This result indicated that classification based on sensitivity to a rapid temperature decrease from 30°C to 10°C is disagreement with phylogenic relationships based on ITS1 sequences.

4.4 Discussion

4.4.1 Sensitivity to a rapid temperature decrease in Gesneriaceae plants

In this study, I examined 29 Gesneriaceae species in 19 genera, and showed 12 species to be sensitive to rapid decreases in temperature resulting in injuries (Fig. 4-1). According to phylogenetic analysis (Smith *et al*. 1997, Fig. 4-7), genera with sensitive species were not necessarily genetically similar to each other. Indeed, both sensitive and insensitive species were found within the same genus in *Chirita* and *Streptocarpus*. These data suggest that differences in sensitivity to a rapid temperature decrease may have evolved with climatic conditions in their individual habitats. The correlation between differences in sensitivity and environmental temperatures cannot be resolved without more microclimate data from each habitat.

4.4.2 Anatomical variations of leaves and temperature-decrease sensitivity

The leaves of sensitive plants exhibited variations in shape, size, and thickness (Fig. 4-1, 4-4). The thick leaves of *Chirita tamiana*, *Columnea linearis*, *Columnea* sp., *Nematanthus maculatus*, *Gesneria pedicellaris*, and *Streptocarpus saxorum* had single or multiple layer of hypodermis beneath the upper epidermis (Fig 4-4a-f). The hypodermis may act as a barrier against oscillation of heat when the leaf is exposed to sunflecks (Madison 1977) or a water-storage tissue for adaptation to a dry climate (Burtt 1998). However, leaves with hypodermis were also injured by a rapid temperature decrease (Fig. 4-4). Conversely, the leaves of *Seemania sylvatica* and *Streptocarpus* sp., which lack a hypodermis, exhibited insensitivity and were not injured.

Hence, sensitivity to a rapid temperature decrease may not be determined by anatomical variations in leaves. Considering that only palisade mesophyll cells were injured in discolored leaves (Fig. 4-4), sensitivity may be determined by susceptibility of palisade mesophyll cells to a rapid temperature decrease.

4.4.3 Chlorophyll fluorescence as an indicator of leaf damage

Chlorophyll fluorescence has been used as an indicator of environmental stress in plants, such as heat and cold stress, and herbicides (Smillie and Hetherington 1983; Kudoh and Sonoike 2002; Barbagallo *et al*. 2003; Tang *et al*. 2007). In this study, I continuously monitored chlorophyll fluorescence during the injury process and showed that decreases of ϕII and increases of NPQ occurred concurrently in leaves of sensitive plants (Fig. 4-3). This irreversible decline of chlorophyll fluorescence was detected within a few seconds of the temperature decrease, although leaf discoloration could not be observed until a few minutes later. Chlorophyll fluorescence is also useful as an indicator to detect cell injury induced by rapid temperature decreases.

4.4.4 Altering of intracellular pH in injured cells

I showed here that, in palisade mesophyll cells of *Columnea* sp. and *Kohleria warszewiczii* leaf, intracellular pH was altered by rapid temperature decrease (Fig. 4-5). This result is consistent with changes in intracellular pH in *Saintpaulia* palisade mesophyll cells induced by collapse of vacuoles after temperature decrease (chapter 2), suggesting that stabilization of the vacuolar membrane to tolerate rapid decreases in

temperature may be essential in Gesneriaceae plants. Sensitivity to rapid temperature decrease may be determined by stability of pH homeostasis in palisade mesophyll cells.

4.4.5 Phylogenetic relationships

Phylogenetic analysis indicated that classification based on sensitivity to temperature differences of 20°C is disagreement with phylogenic relationships based on ITS1 sequences. However, there is a possibility that species identified as insensitive are damaged by more differences in temperature than 20°C and that invisible physiological changes are induced by smaller differences in temperature than 20°C. Phylogenies based on physiological responses to temperature differences are further subject.

4.4.6 Conclusions

In Gesneriaceae plants, the initial event of leaf injury caused by a rapid temperature decrease may be common; in palisade mesophyll cells, the cytosolic pH altered by a temperature decrease, consequently, chloroplasts are damaged by pH changes resulting in leaf discoloring. Considering the physiological response in *Saintpaulia* leaves (chapter 2), vacuolar membrane in palisade mesophyll cells might have key role to confer sensitivity to a rapid temperature decrease in plants, regardless of their genetic background.

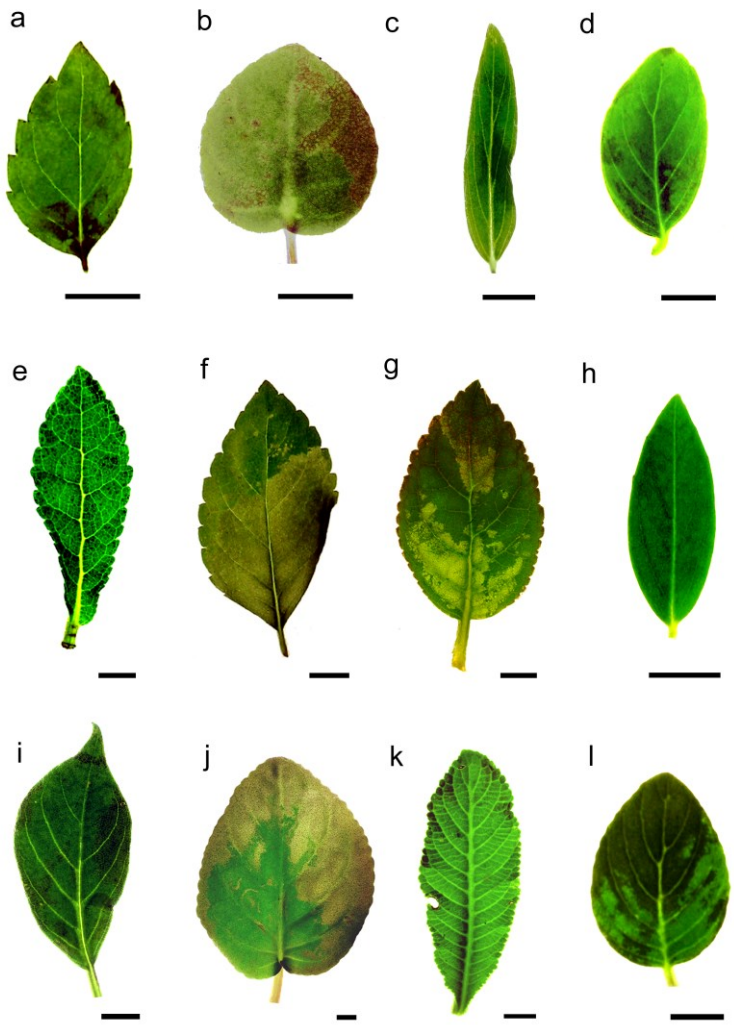


Figure 4-1. Leaf discolored leaves of 12 Gesneriaceae plants after a rapid temperature decrease from 30°C to 10°C. (a) *Achimenes patens*; (b) *Chirita tamiana*; (c) *Columnea linearis*; (d) *Columnea* sp.; (e) *Gesneria pedicellaris*; (f) *Kohleria warszewiczii*; (g) *Kohleria* sp.; (h) *Nematanthus gregarius*; (i) *Nematanthus maclatus*; (j) *Saintpaulia* sp.; (k) *Streptocarpus formosus*; (l) *Streptocarpus saxorum*. Scale bars = 1 cm.

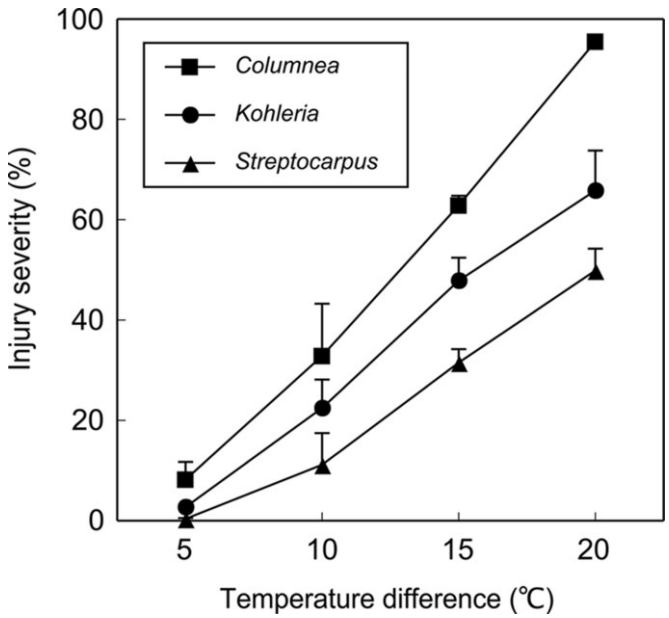
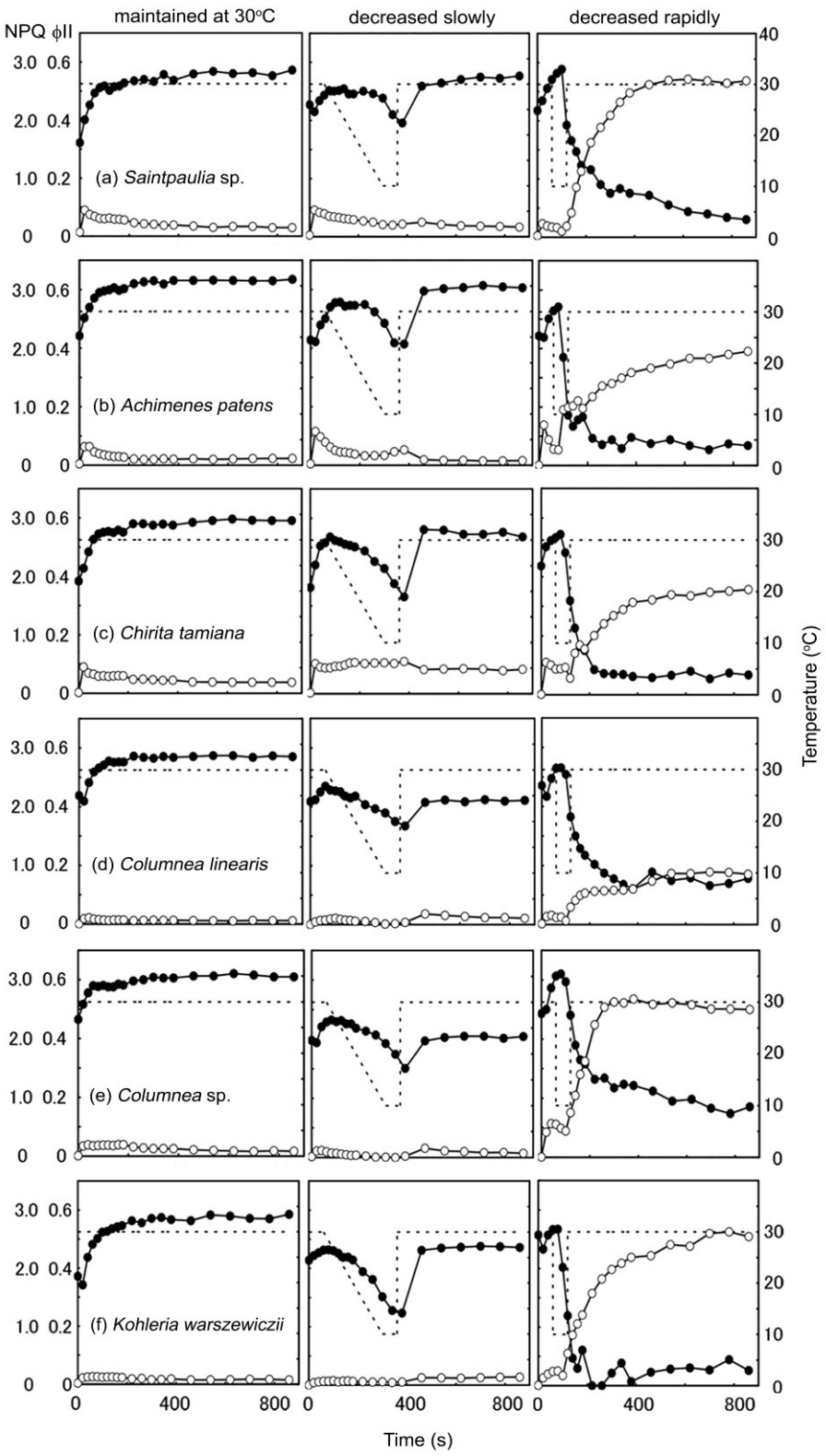


Figure 4-2. Severity of leaf injury in *Columnea* sp. (*squares*), *Kohleria warszewiczii* (*circles*), and *Streptocarpus saxorum* (*triangles*) after a rapid temperature decrease. Leaves initially kept at 30°C to 15°C were rapidly decreased to 10°C. The points and associated bars indicate mean severity and standard error (n = 3).


maintained at 30°C
decreased slowly
decreased rapidly
NPQ
ϕII
Temperature (°C)
(a) Saintpaulia sp.
(b) Achimenes patens
(c) Chirita tamiana
(d) Columnea linearis
(e) Columnea sp.
(f) Kohleria warszewiczii
Time (s)

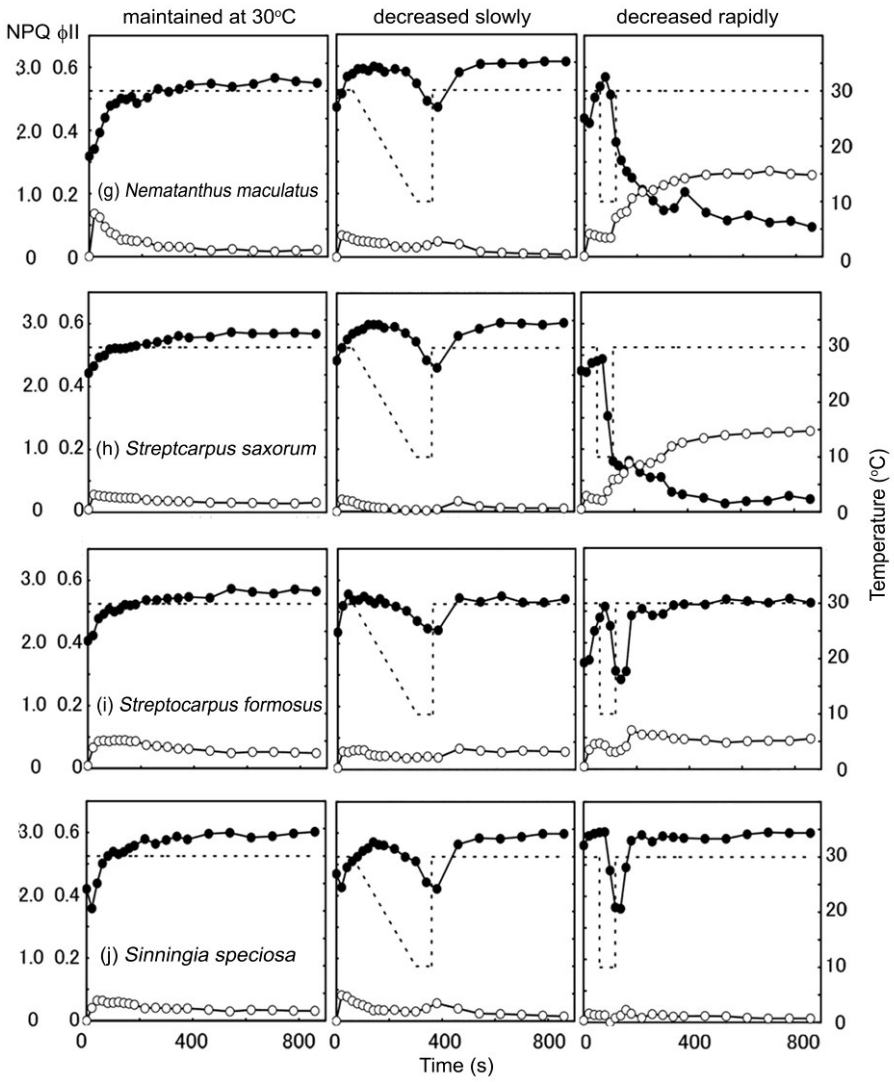
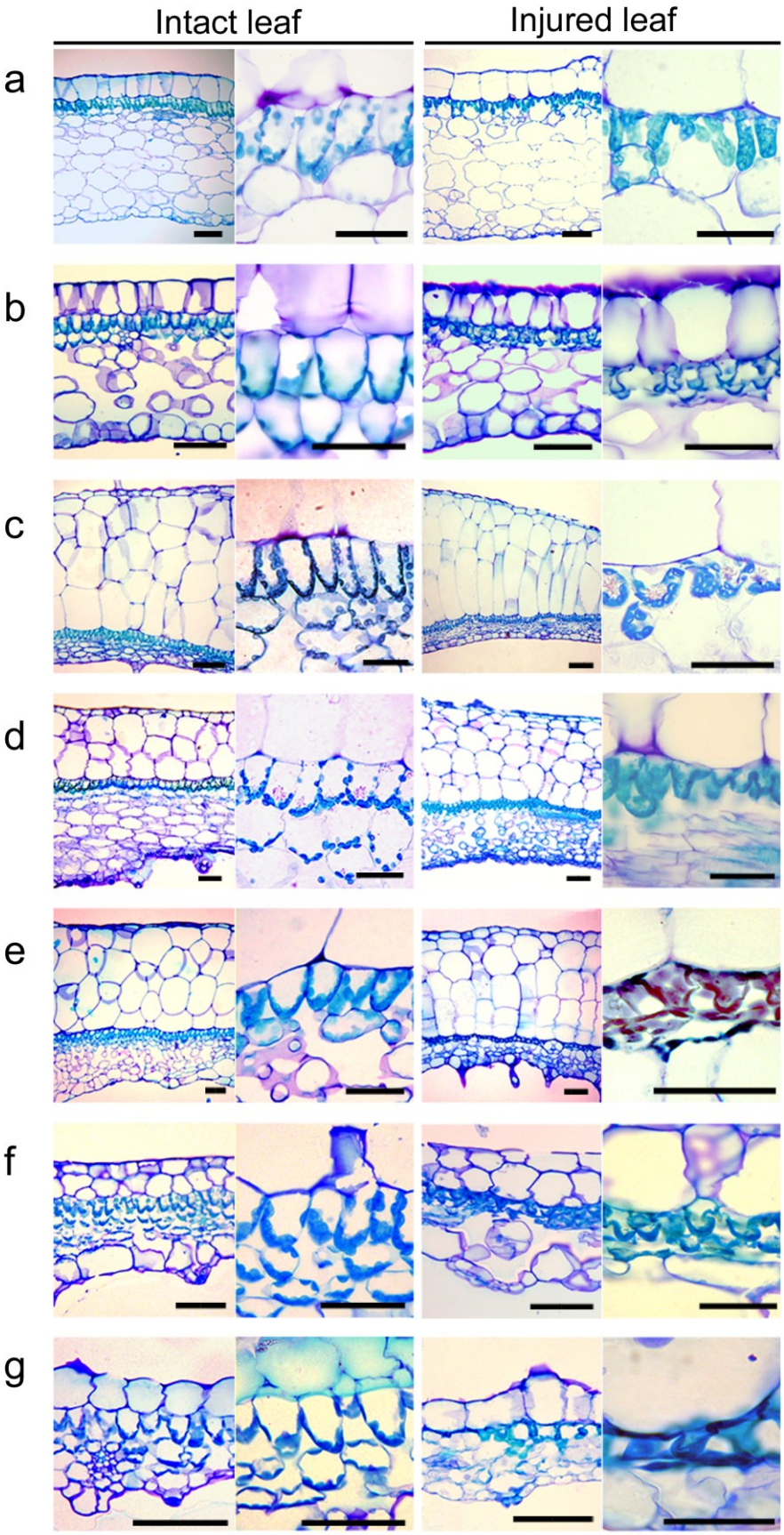


Figure 4-3. Changes in chlorophyll fluorescence in leaves of 10 Gesneriaceae species during a temperature decrease. Quantum yield of PSII (ϕII) and non-photochemical quenching (NPQ) were estimated from changes in chlorophyll fluorescence. Solid lines show quantum yield of ϕII (*closed circles*) and NPQ (*open circles*), while broken lines indicate leaf temperature. These indices were measured at 30°C (left panels), during a slow temperature decrease from 30°C to 10°C (middle panels), and during a rapid temperature decrease from 30°C to 10°C followed by rewarming to 30°C (right panels). (a) *Saintpaulia* sp.; (b) *Achimenes patens*; (c) *Chirita tamiana*; (d) *Columnea linearis*; (e) *Columnea* sp.; (f) *Kohleria warszewiczii*; (g) *Nematanthus maclatus*; (h) *Streptocarpus saxorum*; (i) *Streptocarpus* sp.; (j) *Sinningia speciosa*.


Intact leaf
Injured leaf
a
b
c
d
e
f
g

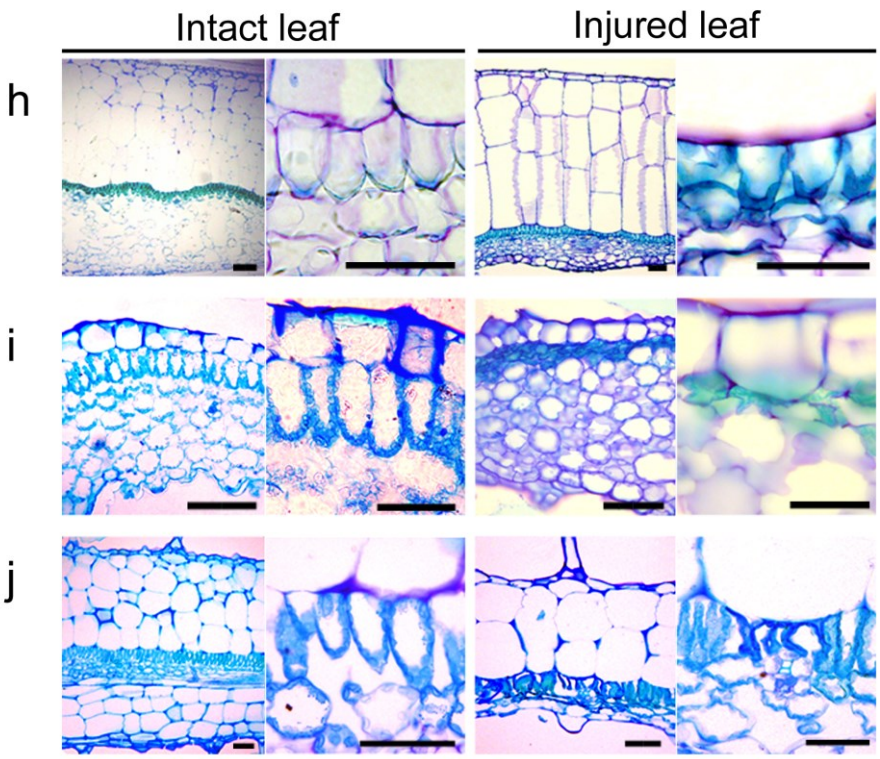


Figure 4-4. Cross sections of intact or damaged leaves of 10 Gesneriaceae plants. From left to right: shows a whole leaf sections of intat leaves, magnified view of palisade mesophyll cells of intact leaves, whole leaf sections of discolored leaves, and magnified view of palisade mesophyll cells of discolored leaves. (a) *Chirita tamiana*; (b) *Columnea linearis*; (c) *Columnea* sp.; (d) *Nematanthus maclatus*; (e) *Gesneria pedicellaris*; (f) *Streptocarpus saxorum*; (g) *Achimenes patens*; (h) *Kohleria warszewiczii*; (i) *Saintpaulia* sp.; (j) *Streptocarpus formosus*. Scale bars = 200 μm in whole-leaf images and 100 μm in magnified palisade mesophyll images, respectively.

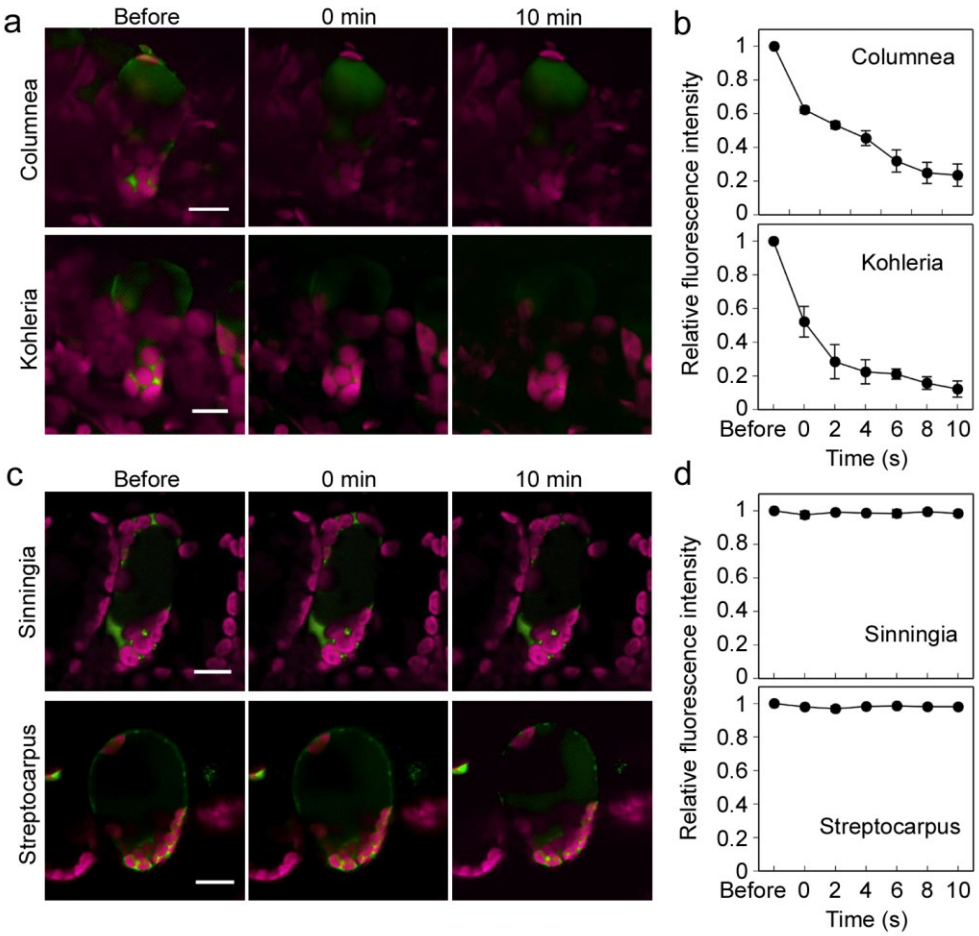


Figure 4-5. Cytosolic pH changes in palisade mesophyll cells of Gesneriaceae plants. Cytosolic pH changes in palisade mesophyll cell of sensitive species (*Columnea* sp. and *Kohleria warszewiczii*) and insensitive species (*Sinningia speciosa* and *Streptocarpus* sp.) induced by a rapid temperature decrease. (a) Palisade mesophyll cells of *Columnea* sp. and *Kohleria warszewiczii* stained with pH-sensitive fluorescent dye BCECF-AM. (b) Relative changes of BCECF-AM fluorescence intensity in palisade mesophyll cell of *Columnea* sp. and *K. warszewiczii*. (c) Palisade mesophyll cells of *Sinningia speciosa* and *Streptocarpus* sp. stained with BCECF-AM. (d) Relative changes of BCECF-AM fluorescence intensity of BCECF-AM in palisade mesophyll cell of *S. speciosa* and *Streptocarpus* sp. Images in (a) and (c) were taken before, immediately after (0 min), and 10 min after a temperature decrease from 30°C to 10°C and kept at 10°C for 1 min. Scale bar = 40 μm. The points and associated bars in (b) and (d) indicate mean intensity and standard error (n = 3).

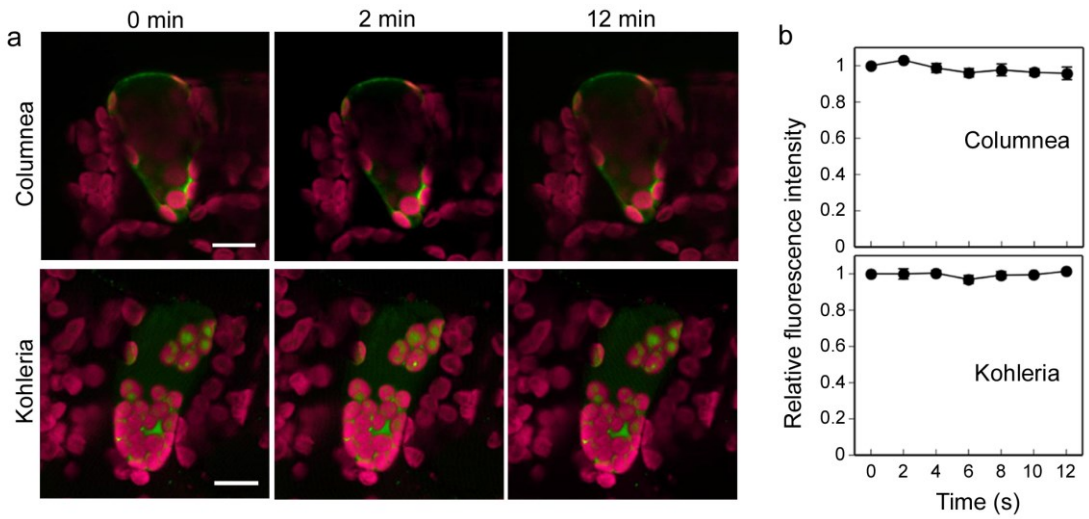


Figure 4-6. Cytoplasmic pH in palisade mesophyll cells of Gesneriaceae plants at a constant temperature. (a) Palisade mesophyll cells of *Columnea* sp. and *Kohleria warszewiczii* leaves stained with a pH-sensitive fluorescent dye, BCECF-AM. Images of BCECF-AM (green) and chlorophyll fluorescence (magenta) were captured 0, 2, and 12 min after the start of observation at 30°C. Scale bar = 40 μm. (b) Relative changes of fluorescence intensity of BCECF-AM at 30°C. The points and associated bars indicate mean intensity and standard error (n = 3).

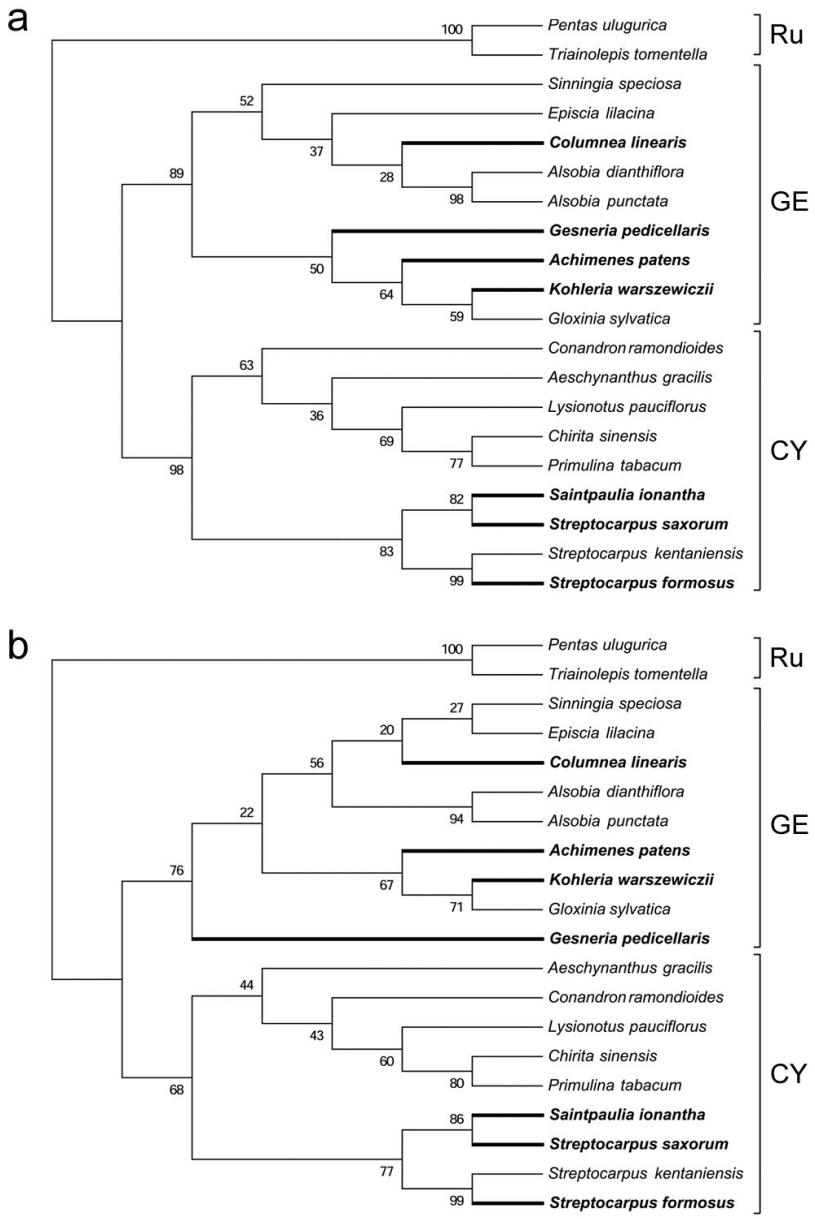


Figure 4-7. Phylogenies of a subset of Gesneriaceae plants. Phylogenetic trees based on ribosomal DNA internal transcribed spacer 1 (ITS1) sequences were constructed using the neighbor-joining (a) and maximum likelihood (b) methods. Numbers above branches are bootstrap percentages (1000 replicates). Bold branches indicate lineages iunjred by a rapid temperature decrease from 30°C to 10°C. *Pentas ulugurica* and *Triainolepis tomentella*, which belong to Rubiaceae, were used as an outgroup. Ru, Rubiaceae (outgroup taxa); CY, Cyrtandroideae subfamily; GE, Gesnerioideae subfamily.

Table 4-1. List of Gesneriaceae species used in this study, their distribution, and GenBank accession numbers for their internal transcribed spacer 1 (ITS1) sequences.

Taxa	Distribution[a]	GenBank accession no.
Achimenes patens Benth.	Guatemala, Mexico	AY182182
Aeschynanthus gracilis Parish ex C.B. Clarke	India, Bhutan, China, N. Burma, N. Vietnam, Thailand	AF349207
Alsobia dianthiflora (H.E. Moore et R.G. Wilson) Wiehler	Costa Rica, El Salvador, Mexico	DQ211160
Alsobia punctata (Lindl.) Hanst.	Belize, Guatemala, Mexico	DQ211159
Chirita sinensis Lindl.	China	FJ501348
Chirita tamiana B. L. Burtt	N. Vietnam	-
Chirita Buch.-Ham. sp. cv. Moon Walker	-	-
Codonanthe gracilis (Mart.) Hanst.	Brazil	-
Codonatanthus W.R.Saylor sp. cv. Vista	-	-
Columnea linearis A. S. Oersted	Belize, Costa Rica, Honduras, Nicaragua	AF543240
Columnea L. sp. cv. Chanticleer	-	-
Conandron ramondioides Sieb. et Zucc. var. *ramondioides*	Japan	GU350649
Episcia lilacina Hanst.	Colombia, Costa Rica, Nicaragua, Panama	AY047091
Gesneria pedicellaris Alain	Dominican Republic	AY047049
Kohleria warszewiczii (Regel) Hanst.	Columbia	AY702379
Kohleria Regel sp. cv. Bach	-	-
Lysionotus pauciflorus Maximowicz var. *pauciflorus*	China	AB498566
Nematanthus gregarius D.L. Denham	Brazil	-
Nematanthus maculatus (Fritsch) Wiehler	Brazil	-
Petrocosmea flaccida Craib	China	-
Primulina tabacum H. F. Hence	China	GQ497202
Saintpaulia Wendl sp. cv. Iceberg	Kenya, Tanzania	AF316923[b]
Seemannia sylvatica (Kunth) Hanst.	Bolivia, Brazil, Ecuador, Paraguay, Peru	AY702365
Sinningia speciosa (Lodd.) Hiern	Brazil	AY372337
Sinningia Nees sp. cv. Tinkerbells	-	-
Streptocarpus formosus (Hilliard et B.L. Burtt) T.J. Edwards	S. Africa Republic	AF316983
Streptocarpus kentaniensis Britten et Story	S. Africa Republic	AF316974
Streptocarpus saxorum Engler	Tanzania, Kenya	AF316914
Streptocarpus Lindl sp. cv. Popsicle	-	-

[a]Distribution is given by Wiehler (1976, 1978), Li (1996), and Skog and Boggan (2007).

[b]Saintpaulia cultivars are mainly derived from original species *Saintpaulia ionantha* (Baatvik 1993). Therefore, we used ITS 1 sequence of *Saintpaulia ionantha* to estimate phylogenetic trees.

Chapter 5

General Discussion

In this study, I investigated physiological response to a rapid temperature decrease in Gesneriaceae plant cells. A number of studies on physiological response to temperature have been focused on the temperature levels such as high or low temperature. As shown in chapter 4, photosynthetic capacity dropped after a rapid temperature decrease in both sensitive and tolerant Gesneriaceae plants (Fig. 4-3), indicating that a rapid temperature decrease is potentially stressful even for tolerance plants, although these plants did not show leaf discoloring. Results of this study strongly suggest that not only the levels of temperature but also the rate of temperature changes should be considered when examining the physiological response to temperatures in plants.

There were differences between temperature injury of *Saintpaulia* leaves and well-known temperature injury in plants. After a rapid temperature decrease, *Saintpaulia* leaves are largely damaged, however, rest of the leaf area remains intact; for example, when leaf temperature rapidly decreased from 30°C to 15°C, 63.9% of the total leaf area was damaged and discolored whereas the rest of 36.1% was intact (Fig. 1-2). *Saintpaulia* plants damaged by a temperature decrease dose not shed damaged leaves, of which intact areas show high photosynthetic capacity (Yun *et al*. 1998). Indeed, *Saintpaulia* can regenerate the individual from the damaged leaf. These things indicate that the damaged leaves have an ability to maintain nutritional status and a certain level of photosynthetic performance.

Most interesting question is the meaning of the physiological response to a rapid temperature decrease. In *Saintpaulia* leaves, not only higher temperature but also wounding of leaves possibly enhances temperature sensitivity. There has also been

reported that only wounding induced leaf browning which is similar to symptoms of injury caused by a rapid temperature decrease (Yang *et al.* 2002). Additionally, I found that temperature sensitivity diminished in wilted leaves by water shortage (data not shown). These findings imply physiological complexity of temperature-response mechanisms in *Saintpaulia* leaves. Leaf damage caused by a temperature decrease might be one aspect of physiological response to various environmental stresses. For further investigations, it should be considered that temperature sensitivity might be altered not only by temperature conditions but also by other factors including wound and drought.

Calcium (Ca^{2+}) has been reported to regulate plant responses to temperature stress. In plant cells, rapid elevation of the cytosolic calcium is one of the earliest events in response to cold temperature (Knight and Knight 2012). There is also some report that calcium has a role in regulation of expression of the major cold-induced gene *CRT* (C-repeat) and *ABRE* (Abscisic Acid Responsive Element) (Whalley *et al*. 2011). In addition to temperature response, several signaling pathways in biotic and abiotic stresses use calcium as a second messenger (Qiu *et al*. 2012). In this study, proteins involved in calcium signaling pathway such as calmodulins and calmodulin-related proteins were not identified by comparative proteomic approach (chapter 3). However, considering physiological complexity of temperature response of *Saintpaulia* leaves as described above, calcium is one of the candidates conferring temperature sensitivity in *Saintpaulia* leaves, because it participates in a number of physiological processes.

In this study, proteomic analysis revealed that abundance of a number of

proteins changed during pre-incubation or a temperature decrease (chapter 3). However, there is very little information about functions of the identified proteins in molecular mechanisms of temperature sensitivity. In model organisms, one of the effective approaches for identification of genes involved in environmental stress-induced physiological changes can be combined proteome, transcriptome, and metabolome analyses (Komatsu *et al*. 2013). However, there is no genomic information of *Saintpaulia* at present. Recently, high-throughput sequencing comes to be powerful tool for generating genomic data for non-model organisms. Linking proteomic data obtained in this study to gene expression and metabolite accumulation facilitates the identification of genes involved in temperature sensitivity if genomic information of *Saintpaulia* is available in the future.

Proteomic analysis in whole leaf was performed in this study (chapter 3). For more detailed analysis of the response mechanism, it is necessary to develop the isolation method of palisade mesophyll cells from *Saintpaulia* leaves because only palisade mesophyll cells were responsible to a rapid temperature decrease (chapter 2). I have attempted to separate palisade mesophyll cells from spongy mesophyll cells by the enzymatic treatment (Fig. 5-1). These isolated cells are expected to allow the comparative analysis of palisade and spongy mesophyll cells, which exhibit a differential sensitivity to a rapid temperature decrease. However, the temperature sensitivity of isolated cells was not sufficiently confirmed because it was very difficult to judge whether single palisade mesophyll cell is intact and reactive to a rapid temperature decrease or not. At first, I made an attempt to assess the intactness by

monitoring cytosolic pH changes using BCECF-AM as described in chapter 2.2.6, however, staining of the fluorescent dye could not be successfully applied to the experimental system. One conceivable way to check for intactness is microscopic measurement of irreversible decrease in chlorophyll fluorescence during cell injury occurrence using PAM fluorometer equipped with a microscope (Microscopy-PAM; Heinz Walz GmbH). The assessment based on chlorophyll fluorescence can be suitable for confirming intactness because other experimental manipulations such as fluorescent staining are not necessary.

General conclusions

1. The vacuolar membrane of palisade mesophyll cells collapsed during the initial phase of leaf injury of *Saintpaulia*. Stabilization of the vacuolar membrane may be essential to tolerate rapid decreases in temperature. It is predicted that acidic vacuolar contents are leaked into the cytoplasm and subsequently chloroplasts and other organelles are damaged by alteration of intracellular pH, resulting in the cell injury.

2. The proteins that are involved in redox homeostasis, molecular chaperon, and ABA signaling, showed different accumulation patterns in *Saintpaulia* leaves with different temperature sensitivity. These proteins might play a key role in molecular mechanisms and confer sensitivity to a rapid temperature decrease in *Saintpaulia* leaves.

3. Some Gesneriaceae plants showed similar temperature injury as *Saintpaulia* leaf. In palisade mesophyll cells of temperature decrease-sensitive plants, declines of chlorophyll fluorescence, cytosolic pH changes, and cell injuries were observed whereas were not in those of tolerance plants. Leaf injury of Gesneriaceae plants is possibly caused by similar mechanisms as *Saintpaulia* leaf.

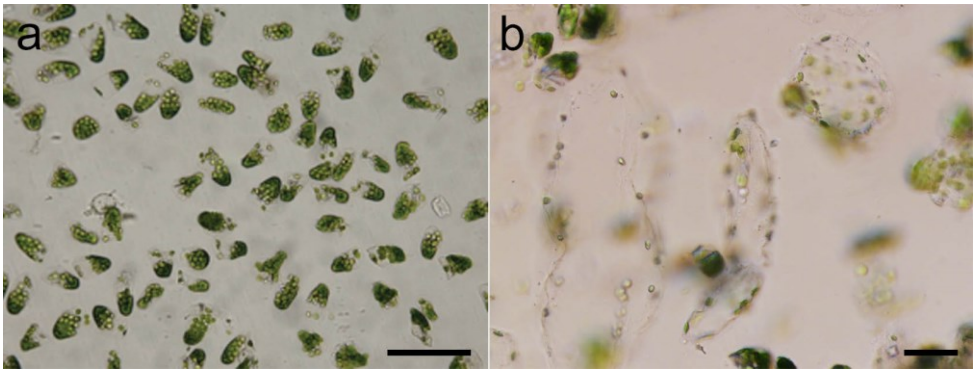


Figure 5-1. Isolated palisade mesophyll cells (a) and spongy mesophyll cells (b) from *Saintpaulia* leaves. Fully expanded leaves (2.5 g fresh weight) were infiltrated into enzyme solution [1% Cellulase "Onozuka" R-10 (Yakult pharmaceutical industry, Tokyo, Japan), 2% Macerozyme R-10 (Yakult), 10 mM Mes, 1 mM $CaCl_2 \cdot 2H_2O$, and 0.1 M sorbitol] at 25°C. After 5 min of the filtration, adaxial side of leaves (palisade layer and upper epidermis) was separated from abaxial side (spongy layers and lower epidermis) with tweezers. The adaxial sides of leaves were collected and further incubated in the enzyme solution by shaking at 70 rpm for 30 min at 25°C. After incubation, the resultant cell mixture was filtrated through 120 μm-pore and 80 μm-pore nylon filters. The filtrate was centrifuged at 200 ***g*** for 2 min. The resulting pellet was resuspended in the buffer solution (0.1 M sorbitol, 50 mM HEPES-Tris, 1 mM $CaCl_2 \cdot 2H_2O$, pH 7.8) and centrifuged again. The washed pellet of palisade cells was resuspended in the buffer solution. Scale bars = 100 μm.

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