Involvement of Ca\textsuperscript{2+} in Vacuole Degradation Caused by a Rapid Temperature Decrease in Saintpaulia Palisade Cells: A Case of Gene Expression Analysis in a Specialized Small Tissue

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Saintpaulia (African violet) leaves are known to be damaged by a rapid temperature decrease when cold water is applied to the leaf surface; the injury is ascribed to the chloroplast damage caused by the cytosolic pH decrease following the degradation of the vacuolar membrane in the palisade cells. In this report, we present evidence for the involvement of Ca\textsuperscript{2+} in facilitating the collapse of the vacuolar membrane and in turn in the temperature sensitivity of Saintpaulia leaves. In the presence of a Ca\textsuperscript{2+} chelator (EGTA) or certain Ca\textsuperscript{2+} channel inhibitors (Gd\textsuperscript{3+} or La\textsuperscript{3+}) but not others (verapamil or niledipine), the pH of the vacuole, monitored through BCECF (2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein) fluorescence, did not increase in response to a rapid temperature drop. These pharmacological observations are consistent with the involvement of mechanosensitive Ca\textsuperscript{2+} channels in the collapse of the vacuolar membrane. The high level of expression of an MCA- (Arabidopsis mechanosensitive Ca\textsuperscript{2+} channel) like gene, a likely candidate for a mechanosensitive Ca\textsuperscript{2+} channel(s) in plant cells, was confirmed in the palisade tissue in Saintpaulia leaves by using a newly developed method of gene expression analysis for the specialized small tissues.

Keywords: Ca\textsuperscript{2+} • MCA • Mechanosensitive channel • Saintpaulia • Temperature stress • Vacuole degradation.

Abbreviations: BCECF, 2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein; Lhcb, light-harvesting Chl a/b-binding protein; MCA, mid1-complementing activity; rbcL, large subunit of Rubisco (ribulose-1,5-bisphosphate carboxylase-oxygenase); RNA-seq, RNA sequencing; ROS, reactive oxygen species; TRP, transient receptor potential.

Introduction

Saintpaulia sp. (saintpaulia hereafter), commonly known as African Violet, is an important ornamental species belonging to the family Gesneriaceae. It is native to Kenya and Tanzania, and is highly valued for its beautiful violet flowers produced throughout the year. It was reported over half a century ago that saintpaulia plants are damaged when cold water is applied to the leaf surface. This feature has also been observed in certain other members of the Gesneriaceae family and those of the Acanthaceae. The leaf tissue damage caused by cold water application leads to a typical leaf color change from dark green to an unattractive brown. This has undesirable consequences for the cultivation and marketing of saintpaulia plants. Obviously, in view of the immense economic importance of the species, it has been of interest to obtain insight into the mechanism of injury and to find means of minimizing this injury. Initially, the stated leaf tissue damage was thought to be a consequence of chilling injury. However, the injury was caused even by pouring water at a temperature of 25°C when the leaf temperature was maintained above 35°C, suggesting that the temperature difference between the leaf itself and the applied water was an important factor responsible for the injury (Maekawa et al., 1987). Furthermore, the injury did not occur when the leaf temperature was slowly decreased (Suzuki et al., 1998). Apparently, the leaf injury of saintpaulia differs from a typical chilling injury.

Analysis of saintpaulia leaves damaged due to the application of cold water revealed the injury to be restricted to only palisade mesophyll cells, particularly the organelles such as the nuclei, mitochondria and chloroplasts resulting in cell death (Yun et al., 1996). The typical leaf color change from green to brown could be ascribed to the damaged chloroplasts. However, to date, the precise mechanism(s) of this injury are largely unknown, except for a few reports suggesting the involvement of reactive oxygen species (ROS) (Yasuda et al., 1997, Yang et al., 2001).

We recently reported that the rapid temperature decrease induced the acidification of the cytosol of palisade mesophyll cells accompanied by degradation of the vacuole in saintpaulia leaves. The temperature change indeed led to vacuolar membrane degradation, facilitating the release of toxic vacuolar contents (protease or excess acidic substance, i.e. organic acid, Cl\textsuperscript{−} and SO\textsubscript{4}\textsuperscript{2−}) into the cytoplasm and in turn the damage to diverse organelles during the initial phase (Kadohama et al., 2013).
At present, we do not know the molecular signaling mechanism involved in the temperature change-dependent collapse of the vacuolar membrane in saintpaulia. In various organisms, several specific molecular changes have been reported to occur rapidly in response to the temperature changes, which might constitute the signaling component(s). These include the changes in membrane fluidity, induction of ROS and the increase of the cytoplasmic Ca$^{2+}$ level. In animal cells, it is reported that the changes in ambient temperature activate transient receptor potential (TRP) channels, and the increase in the cytoplasmic Ca$^{2+}$ levels via these channels is involved in the regulation of various cellular phenomena (Ramsey et al. 2006). An increase of the cytoplasmic Ca$^{2+}$ level due to the environmental temperature decrease is also reported in other plant cells (Knight et al. 1991). Since the integrity of the vacuolar membrane is known to be strongly dependent on the cytoplasmic Ca$^{2+}$ level (Tazawa et al. 1987), we hypothesized that the cytoplasmic Ca$^{2+}$ level increased in the palisade mesophyll cells of saintpaulia plants during a rapid temperature decrease, which was responsible for the degradation of the vacuolar membrane.

We here report evidence for the involvement of Ca$^{2+}$ in the collapse of the vacuolar membrane in response to the rapid temperature decrease in saintpaulia leaves through experiments involving a pH-dependent fluorescent indicator, 2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein (BCECF), and various Ca$^{2+}$ channel inhibitors. Pharmacological experiments suggest that the mechanosensitive Ca$^{2+}$ channels, like TRP channels in animal cells, might be functioning as the temperature sensors in plant cells too. In saintpaulia plants, a stable transformation technique has not yet been developed. Therefore, reporter genes to detect the localization of gene expression could not be used. We attempted to measure the expression levels of a target gene by using a recently developed gene expression analysis method for a specialized small tissue.

Results

Changes in the subcellular distribution of BCECF fluorescence in palisade cells due to rapid temperature decrease

It has previously been reported that the injury to saintpaulia leaves due to a rapid temperature decrease is restricted to the palisade cells as evident from the disappearance of Chl fluorescence therein. This could be ascribed to the chloroplast damage as a consequence of cytosolic acidification (Kadohama et al. 2013). To begin with, we monitored the pH changes in the palisade cells using BCECF-AM as a fluorescent pH indicator. BCECF is a very widely used intracellular pH probe that is highly water soluble. BCECF-AM is a membrane-permeant acetoxymethyl ester of BCECF enabling the easy loading of BCECF into cells and is readily hydrolyzed by endogenous esterases into a free acid form of BCECF in the cell. The dye shows weak fluorescence at low pH, but stronger fluorescence with an increase in pH. The saintpaulia leaf sections (200 µm in thickness) were stained with BCECF-AM at 25°C for 1 h and examined for the distribution of BCECF fluorescence in the palisade cells by laser scanning confocal microscopy prior to the temperature decrease (Fig. 1A, B), immediately following the onset of treatment (Fig. 1C, D) and after 10 min of treatment (Fig. 1E, F). The temperature treatment was applied on the stage of the confocal microscope. The temperature treatment comprised three steps, namely a rapid decrease from 25 to 5°C within 4 min, maintaining 5°C for 1 min and an increase from 5 to 25°C within 2 min.

Prior to the rapid temperature decrease, BCECF fluorescence was observed in the cytosol only (Fig. 1A, B), but, just after the treatment, it was visible both in the cytosol and in the vacuole (Fig. 1C, D). After 10 min of the treatment, the BCECF fluorescence was observed in both the cytosol and the vacuole (Fig. 1E, F). BCECF fluorescence intensity is linear between pH 6.4 and 8.0, whereas under the acidic condition, BCECF fluorescence is weak. Before the rapid temperature decrease, BCECF fluorescence in the cytosol is strong (lining the cell) (Fig. 1A, B) which shows that cytosolic pH was kept around neutral. After temperature treatment, BCECF fluorescence was detected in the vacuole and the bright BCECF fluorescence in the cytosol became weaker which shows that the vacuolar pH increased and the cytosolic pH decreased (Fig. 1C–F). Apparently, during the rapid temperature decrease, the vacuolar pH increased concomitant with a decline in that of the cytosol in the palisade cells as a result of vacuolar membrane disruption (Kadohama et al. 2013).

Influence of EGTA and exogenous Ca$^{2+}$ on the subcellular distribution of BCECF fluorescence in palisade cells during rapid temperature decrease

Since the temperature decrease is known to lead to the elevation of the cytoplasmic Ca$^{2+}$ level that is also an important determinant of vacuolar membrane integrity, we tested whether the extracellular Ca$^{2+}$ levels affect the distribution of BCECF fluorescence by using EGTA, a Ca$^{2+}$ chelator. Accordingly, saintpaulia leaf sections were pre-treated with 5 mM EGTA prior to the temperature treatment. At all stages of treatment, the BCECF fluorescence was observed in the cytosol only (Fig. 2A, B). As an exception, after 10 min of treatment, in the case of one only palisade cell, the BCECF fluorescence was evident in both the cytosol and vacuole (Fig. 2E, F). Thus, the application of EGTA prevented the acidification of the cytosol induced by a drop in the temperature. Next, we tested whether the observed EGTA effect could be reversed by the application of exogenous Ca$^{2+}$. For this, after 10 min of temperature treatment of the EGTA-pre-treated sections (BCECF fluorescence restricted to the cytosol only) (Fig. 2G, H), 10 mM CaCl$_2$ was added (final concentration: 5 mM). The CaCl$_2$ addition did not change the fluorescence pattern immediately (Fig. 2I), but, after 7 min, the fluorescence was observed in both the vacuole and the cytosol (Fig. 2K–N). The numbers of cells exhibiting BCECF fluorescence both in the vacuole and in the cytosol after temperature treatment were counted (Fig. 2O). We regarded the number of cells exhibiting BCECF fluorescence in the cytosol before temperature treatment as the number of living cells.
In approximately 80% of the cells, BCECF fluorescence was observed in the vacuole after temperature treatment. Under EGTA treatment, this was reduced to about 50%. Upon addition of CaCl$_2$ to the EGTA-pre-treated sections, the proportion of cells exhibiting fluorescence was restored to about 80% (Fig. 2O). These results suggest that the cytosolic Ca$^{2+}$ elevation may induce the vacuolar membrane disruption.

Influence of a Ca$^{2+}$ ionophore on the subcellular distribution of BCECF fluorescence in palisade cells

In order to ascertain further whether Ca$^{2+}$ influx into the cytosol induced the vacuolar membrane disruption, the effect of a Ca$^{2+}$ ionophore (A23187) on the subcellular distribution of BCECF fluorescence in palisade cells was determined. The fluorescence was observed only in the cytosol without treatment with the Ca$^{2+}$ ionophore (Fig. 3A, B). The addition of the Ca$^{2+}$ ionophore A23187 (10 μM) to the leaf sections at 25°C led to the distribution of BCECF fluorescence both in the cytosol and in the vacuole (Fig. 3C, D). We regarded the number of cells exhibiting BCECF fluorescence in cytosol before A23187 treatment as the number of living cells (100%). After the A23187 treatment (without temperature treatment), approximately 35% of the cells exhibited BCECF fluorescence both in the vacuole and in the cytosol (Fig. 3E). Clearly, the Ca$^{2+}$ influx into the cytosol induced the vacuolar membrane disruption that was independent of a rapid temperature decrease. Similar changes in distribution of BCECF fluorescence in cells other than the palisade cells were not evident.

Influence of a Ca$^{2+}$ ionophore on the subcellular distribution of BCECF fluorescence in palisade cells during rapid temperature decrease

Since Ca$^{2+}$ influx into the cytosol is essentially mediated by the Ca$^{2+}$ channels on the plasma membrane, we examined the effects of various Ca$^{2+}$ channel inhibitors, namely verapamil, nifedipine (both are voltage-dependent calcium channel inhibitors), La$^{3+}$ (a non-selective calcium channel inhibitor) and Gd$^{3+}$ (a mechanosensitive calcium channel inhibitor), on the subcellular distribution of BCECF fluorescence in palisade cells. Under 100 μM verapamil or nifedipine pre-treatment prior to the rapid temperature decrease, BCECF fluorescence was observed in the cytosol only (Fig. 4A, B, E, F), but, immediately following the temperature treatment, it was observed in both the vacuole and the cytosol (data not shown) and after 10 min of treatment could be observed only in the vacuole (Fig. 4C, D, G, H). However, under 1 mM LaCl$_3$ or GdCl$_3$ treatment, BCECF fluorescence was observed in the cytosol at all three stages of observation, i.e. prior to the temperature treatment, and immediately and 10 min following the treatment (Fig. 4I–L for La$^{3+}$; Fig. 4M–P for Gd$^{3+}$). The numbers of cells showing BCECF fluorescence which were observed in the vacuole, were...
Fig. 2 Influence of EGTA and exogenous Ca\(^{2+}\) on the subcellular distribution of BCECF fluorescence in palisade cells during a rapid temperature decrease. Staining, temperature treatment and observation were conducted as shown in Fig. 1. After staining, sections were soaked in buffer solution containing 5 mM EGTA for 15 min before measurement. Before rapid temperature treatment, BCECF fluorescence was observed in the cytosol (A, B), and just after the temperature treatment the fluorescence remained in the cytosol (C, D). At 10 min after the temperature treatment, most BCECF fluorescence still remained in the cytosol (E, F). The arrow indicates the cell in which BCECF fluorescence remained in the cytosol. The arrowhead indicates the cell in which BCECF fluorescence remained in the cytosol. Scale bar = 30 μm. n = 13. At 10 min after the temperature treatment, the BCECF fluorescence still remained in the cytosol (G, H). Then 10 mM CaCl\(_2\) were added to the section on the slide glass (the final concentration of CaCl\(_2\) was 5 mM). At 2 min after the treatment with CaCl\(_2\), the fluorescence remained in the cytosol (I, J). Then the fluorescence was observed both in the vacuole and in the cytosol (K, L after 7 min, M, N after 12 min). Scale bar = 30 μm. n = 3. The number of the cells in which BCECF fluorescence moved into the vacuole from the cytosol after temperature treatment was counted (O). Control, n = 23, total number of cells counted = 116; +EGTA, n = 13, total number of cells counted = 55; +EGTA + Ca\(^{2+}\), n = 3, total number of cells counted = 9.
retained in the cytosol or were quenched were counted after temperature treatment under no inhibitor (control) and each Ca\(^{2+}\) channel inhibitor treatment (Fig. 4Q). We regarded the number of cells exhibiting BCECF fluorescence in the cytosol before temperature treatment (under each inhibitor treatments) as the number of living cells (100%). Under control, verapamil or nifedipine treatment, 70–85% cells showed BCECF fluorescence in the vacuole. However, under lanthanum treatment, about 50% cells showed BCECF fluorescence in the cytosol and that increased to 90% in the case of gadolinium treatment (Fig. 4Q). These results suggest that the changes in the vacuolar and cytosolic pH due to the disruption of the vacuolar membrane might involve the Ca\(^{2+}\) influx into the cell via the mechanosensitive Ca\(^{2+}\) channels.

### Mechanosensitive channels in saintpaulia leaves

In order to search the mechanosensitive calcium channels in saintpaulia leaves, we performed next-generation sequencing of RNA from saintpaulia leaves. Forty-six billion nucleotide bases were generated in total. In the assembly results, 128,250 contigs were detected. From the RNA sequencing (RNA-seq) data, we could detect one contig sequence homologous to the MCA (mid1-complementing activity) gene, which is known as the Arabidopsis mechanosensitive calcium channel (Nakagawa et al. 2007). The homologous sequence showed 66% and 61% identity to MCA1 and MCA2 at the amino acid level, respectively (Supplementary Fig. S1). We then sought to confirm whether the MCA-like gene is expressed in saintpaulia palisade tissue. Since a stable transformation for saintpaulia is not established, we could not use reporter genes to detect the localization of expression of the MCA-like gene. Thus, we attempted to measure the expression levels of the MCA-like gene by using a recently developed gene expression analysis method for a specialized small tissue (Hitachi method by Kajiyama et al. 2015). A small (130 \(\mu\)m diameter) piece of tissue was excised from a section of saintpaulia leaf with the help of a needle (Supplementary Fig. S2). From this tissue, employing the Hitachi method, cDNAs could be synthesized. The difference between palisade and spongy tissues was confirmed by the expression of rbcL (large subunit of Rubisco) and Lhcb (light-harvesting Chl a/b-binding protein) genes, because the palisade cells contain far more chloroplasts than spongy cells (Supplementary Tables S1, S2). By using real-time PCR, we compared the expression levels of the MCA-like gene in palisade, spongy and whole tissues. In palisade tissue, the MCA-like gene was expressed more strongly than in the spongy and whole tissues (Table 1). The expression level of the MCA-like gene in palisade tissue was about 15 times higher than that in whole tissue and about 20 times higher than that in spongy tissue (Table 1). A high expression level of the MCA-like gene in the palisade tissue might be associated with vacuolar membrane disruption occurring specifically in palisade tissue due to a rapid temperature decrease.

### Discussion

#### Changes in ambient temperature and plants

The aim of the present study was to gain insight into the molecular mechanism of leaf damage in saintpaulia due to a sudden change in temperature upon application of water to the leaf surface. Indeed, temperature is one of the most important factors affecting plant growth and geographical distribution (Lyon et al. 1973, Criddle et al. 1994, Sage and Kubien 2007). Since plants are immobile, the acquisition of specific physiological and molecular tolerance strategies is crucial for them to withstand the unfavorable temperatures including those...
Fig. 4 Influence of Ca²⁺ channel inhibitors on the subcellular distribution of BCECF fluorescence in palisade cells during rapid temperature decrease. Staining, temperature treatment and observation were conducted as shown in Fig. 1. After staining with BCECF, sections were soaked in buffer solution containing 100 μM verapamil (A–D), 100 μM nifedipine (E–H), 1 mM LaCl₃ (I–L) or 1 mM GdCl₃ (M–P) before temperature treatment. Before the rapid temperature decrease, BCECF fluorescence was observed in the cytosol (A, B, E, F, I, J, M, N). At 10 min after the temperature treatment, the BCECF fluorescence was observed in the vacuole (C, D for verapamil, n = 8; G, H for nifedipine, n = 14), or remained in the cytosol (K, L for LaCl₃, n = 9; O, P for GdCl₃, n = 8). Scale bar = 30 μm. The number of cells in which BCECF fluorescence moved into the vacuole from the cytosol, remained in the cytosol or were quenched after temperature treatment under no inhibitor (control) and each Ca²⁺ channel inhibitor (Q) was counted. Control, n = 23, total number of cells counted = 116; verapamil, n = 8, total number of cells counted = 28; nifedipine, n = 14, total number of cells counted = 40; lanthanum, n = 9, total number of cells counted = 27; gadolinium, n = 8, total number of cells counted = 23.
increase in the cytoplasmic Ca\(^{2+}\). Rapid changes in temperature have been shown to cause an increase of the cytoplasmic Ca\(^{2+}\) levels in response to temperature decrease-induced leaf injury. This apparently implies a role for a possible mechanosensitive Ca\(^{2+}\) channel in the stated phenomenon.

That the EGTA effect on the vacuolar membrane degradation due to temperature change was reversed by the application of exogenous Ca\(^{2+}\) (Fig. 2) suggests that the Ca\(^{2+}\) channels were opening following the temperature decrease. The maintenance of the open status of Ca\(^{2+}\) channels has been reported under osmotic stress in a brachish characean algae <i>Lamprothamnium</i> (Okazaki et al. 2002).

Addition of the Ca\(^{2+}\) ionophore A23187 seems to have induced the collapse of the vacuole only in the palisade cells (Fig. 3). It was actually difficult to ascertain whether the vacuolar collapse also occurred in the epidermal and spongy cells. It is well known that the increase of the cytoplasmic Ca\(^{2+}\) level causes the vacuolar membrane to become unstable (Tazawa et al. 1987). It would be of interest to investigate whether the vacuolar features of palisade cells differ from those of other cells. Since we are also interested in the lipid components of the saintpaulia vacuolar membrane, we tried to isolate the vacuoles of saintpaulia palisade cells. However, it was not possible in this study to isolate the vacuoles from palisade cells. We have also tried to analyze the lipid component of saintpaulia palisade cell by gas chromatography–mass spectrometry and imaging–mass spectrometry. However, no remarkable features have been detected so far. Analysis of the lipid components of the saintpaulia vacuolar membrane will be the subject of a future work.

**Mechanosensitive channels in plants**

MCA1 and MCA2 are well characterized as the mechanosensitive channels in Arabidopsis. Both of them have been reported to function in the detection of stimuli such as mechanical touch or gravity. They have also been demonstrated to act as the mechanosensors using the patch-clamp technique (Furuichi et al. 2012). Very recently, another mechanosensitive channel (OSCA) was reported in Arabidopsis (Yuan et al. 2014) that is likely to be the osmotic stress sensor. By RNA-seq, it was shown that saintpaulia also possesses a homolog of the OSCA gene; this, however, remains to be characterized.

In animal cells, certain TRP channels are known to be mechanosensitive channels that act as temperature sensors (Ramsey et al. 2006). Whereas TRPA1 (<17°C) and TRPM8 (<23–28°C) are sensors of low temperature, TRPV1 (>43°C),

### Table 1 Expression analysis of the MCA-like gene in the saintpaulia leaf

<table>
<thead>
<tr>
<th>Tissue</th>
<th>(\Delta Ct), MCA-like – actin</th>
<th>(\Delta \Delta Ct), (\Delta Ct – \Delta Ct) whole tissue</th>
<th>MCA-like expression relative to whole tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole tissue</td>
<td>7.35 ± 1.80</td>
<td>0.00</td>
<td>1.0</td>
</tr>
<tr>
<td>Palisade tissue</td>
<td>3.46 ± 1.64</td>
<td>–3.89</td>
<td>14.8</td>
</tr>
<tr>
<td>Spongy tissue</td>
<td>7.78 ± 1.71</td>
<td>0.432</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The expression levels of the MCA-like gene in palisade tissue, spongy tissue and whole tissue were compared. The MCA-like gene expression level in palisade tissue is about 15 times greater than that in whole tissue and about 20 times greater than that in spongy tissue. \(n = 3\) or 5. Mean ± SE.
TRPV2 (>52 °C) and TRPV3 (>30–39 °C) are sensors of higher temperature than usual condition (20–25 °C) (Montell 2005). In saintpaulia, a possible mechanosensitive channel might be functioning in the temperature injury. Whether a homolog of TRP channels exists in planta is not known to date. No homolog of MCA channels is found in the animal genome. In planta MCA may act as a temperature sensor. We thought that at least in saintpaulia palisade cells the MCA-like Ca2+-channel might be involved in sensing the rapid temperature decrease. Therefore, we attempted to measure the expression levels of the MCA-like channel in saintpaulia leaves.

Measurements of the gene expression in very small tissues in plants

If mechanosensitive Ca2+ channels are functioning as the temperature sensors, they must be expressed in the palisade cells more strongly than in the other cells. From RNA-seq data of the whole leaf of saintpaulia, we found a single gene expressed in the leaf which is a homolog of MCA1 and MCA2 in Arabidopsis (Supplementary Fig. S1). A comparison of its expression in the palisade cells and other cells in the saintpaulia leaves could be expected to provide an explanation of the mechanistic basis of temperature injury. However, thus far, there are no reports of stable transformation of saintpaulia plants. It is nearly impossible to use reporter genes to detect the localization of target gene expression. Although a few methods of gene expression analysis in cells or tissues of such materials are available, including in situ hybridization, laser microdissection and PCR of target mRNA, we successfully used a method recently developed by the Hitach group (Kajiyama et al. 2015). This allows the isolation of RNA from a very small piece of tissue. We compared the expression levels of the MCA-like channel between palisade and spongy cells in saintpaulia; a much stronger expression in the former than in the latter was evident (Table 1). The data imply that the MCA-like channel is functioning in the rapid temperature decrease-induced injury in the palisade cells of saintpaulia. To ascertain further the involvement of the MCA-like channel in this phenomenon, we plan to construct and analyze knock-down or knock-out plants of MCA-like channels in saintpaulia. Work on developing a stable transformation system for saintpaulia plants is currently in progress.

Materials and Methods

Plant material

Saintpaulia sp. cv. Iceberg was cultured in damp artificial soil (vermiculite : perlite, 3:1) at 25 °C under a 14h light period. Hiponex solution (1/1,000) was supplemented as the nutrient once a week.

Monitoring of intracellular pH with pH-sensitive fluorescent dye

Leaf cross-sections, 200 μm thick, were prepared using a microtome (Plant Microtome MT-3; Nippon Medical and Chemical Instruments) and were stained with 10 μM BCECF-AM (Calbiochem) in a buffer solution (50 mM MES-Tris, pH 6.0) for 60 min at 25 °C. After staining, sections were washed twice with 50 mM MES-Tris (pH 6.0). The sections were placed in a fluorescent dye-free buffer solution of 50 mM MES-Tris (pH 6.0) for a MATS-SSSR-IM temperature controller (Tokai Hit) on the stage of a confocal laser scanning microscope (FV1000-D; Olympus). For EGTA treatment, after washing sections stained with BCECF-AM, the sections were soaked in 50 mM MES-Tris (pH 6.0) containing 5 mM EGTA for at least 15 min until temperature treatment. For EGTA and Ca2+ treatment, 10 mM CaCl2 was added to the section on the slide glass (the final concentrations of CaCl2 was 5 mM) after EGTA and temperature treatment. For Ca2+ channel inhibitor treatment, after washing sections stained with BCECF-AM, the sections were soaked in 50 mM MES-Tris (pH 6.0) containing verapamil (40 or 100 μM), nifedipine (40 or 100 μM), 1 mM LaCl3 or 1 mM GdCl3 on the slide glass just before temperature treatment. The temperature treatment included three steps, a rapid decrease from 25 to 5 °C within 4 min, maintenance at 5 °C for 1 min, a return from 5 to 25 °C within 2 min using the MATS-SSSR-IM temperature controller. In order to increase thermal conduction, the copper plates were placed between the slide glass and the temperature controller. For A23187 treatment, after washing sections stained with BCECF-AM, the sections were placed in 50 mM MES-Tris (pH 6.0), and then A23187 was added to the section on the slide glass (the final concentration of A23187 was 10 or 50 μM) without temperature treatment. The number of cells in which BCECF fluorescence was observed both in the vacuole and in the cytosol before and after each treatment was counted using the captured image.

Expression analysis of the MCA-like gene in saintpaulia leaves

Total RNA from saintpaulia leaf (FW approximately 100 mg) was isolated using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. Total RNA was submitted to the Beijing Genomics Institute (BGI) for de novo transcriptome sequencing (RNA-seq analysis). Sequencing was carried out on the Illumina HiSeq 2000 platform. From RNA-seq data, an MCA-like gene was detected by blast search (Altschul et al. 1997) using Arabidopsis MCA1 and MCA2 amino acid sequences. cDNA synthesis from a small piece of tissue followed the Hitachi method (Kajiyama et al. 2015). Palisade tissue, spongy tissue and whole tissue were collected using a special needle having a 130 μm inner diameter which was supplied by Hitachi Co. (Supplementary Fig. S2). Real-time PCR amplification was performed using the SYBR® Premix Ex Taq™ (TAKARA) and real-time PCR detector (TAKARA Smart Cycler II system). Primers used for amplification of the actin 315 bp sequence were Saint-Actin1F (5'-CGGATGTTTCTGAGGCTTG-3') and Saint-Actin1R (5'-GGGACACACTGTCCATTTAT-3') (Sato et al. 2011). The same sequence was conserved in Saintpaulia sp. cv. Iceberg. Other primers were designed using RNA-seq data. Primers used wereSaint-MCA-likeFP1 (5'-TGCATGTTGTCCTCCTGTT-3') and Saint-MCA-likeRP1 (5'-ATGAACTCTCCACTCCTCGCC-3') and Saint-MCA-likeRP1 (5'-ATGAACTCTCCACTCCTCGCC-3') and Saint-MCA-likeRP1 (5'-ATGAACTCTCCACTCCTCGCC-3') and Saint-MCA-likeRP1 (5'-ATGAACTCTCCACTCCTCGCC-3') for amplification of the MCA-like 152 bp sequence, Saint-rbcLFP1 (5'-TACCAGGGCGCTCAGGGGT-3') and Saint-rbcLRP1 (5'-ACGCCACCTGGCCATTACCC-3') for amplification of the rbcL 130 bp sequence, and Saint-LhcbFP1 (5'-TTGAACGACACGGCTGTCGGC-3') and Saint-LhcbRP2 (5'-TTGAACGACACGGCTGTCGGC-3') for amplification of the Lhcb 150 bp sequence. For discrimination between the palisade and the spongy tissues, expression of rbcL and Lhcb genes was used as a major palisade tissue marker (Supplementary Tables S1, S2).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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