Effects of mitochondria-selective fluorescent probes on mitochondrial movement in Arabidopsis mesophyll cells evaluated by using the quantification

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Abstract  Mitochondria-selective fluorescent probes such as MitoTracker are often used for mitochondria imaging in various plants. Although some of the probes are reported to induce mitochondria dysfunction in animal cells, the effect on plant cells remains to be determined. In the present study, we applied quantitative methods to analyze mitochondrial movement, speed frequency, and speed-angle changes, based on trajectory analysis of mitochondria in mesophyll protoplast cells of Arabidopsis thaliana expressing the mitochondria-localized fluorescent protein. Using the quantitative method, we assessed whether MitoTracker Red (FM and CMXRos) induce mitochondria dysfunction in A. thaliana. Although both the fluorescent probes well-stained mitochondria, the CMXRos probe, not the FM probe, gave a severe effect on mitochondrial movement at the low concentration (10 nM), indicating a MitoTracker-induced mitochondria dysfunction in A. thaliana. These results revealed that our quantitative method based on mitochondrial movement can be used to determine the appropriate concentrations of mitochondria-selective fluorescent probes in plants.

Key words:  Arabidopsis thaliana, Cytotoxicity, Mitochondrion, MitoTracker, Movement.

The mitochondria-selective fluorescent probes are widely used for staining mitochondria in eukaryotic cells such as animal and plant cells. Various types of the mitochondria-selective fluorescent probe are commercially available, e.g., MitoTracker, and also essential tools to evaluate the mitochondrial modifications including genetic and biochemical modifications (Chuah et al. 2015; Kazama et al. 2019; Yoshizumi et al. 2018). However, in animal cells, some of the MitoTracker® probes sometimes affect cell viability via mitochondria dysfunctions (Minamikawa et al. 1999; Pendergrass et al. 2004). An example is the CMXRos probe, which has a strong photosensitizing action inducing depolarization of the inner mitochondrial membrane and mitochondria swelling, resulting in the generation of reactive oxygen species (Minamikawa et al. 1999). Meanwhile, in plant cells, it is unclear whether mitochondria-selective fluorescent probes induce mitochondrial dysfunction.

Plant mitochondria are known to move at various speeds depending on cell types, tissues, and plant species (Doniwa et al. 2007; Logan 2006; Sheahan et al. 2005; Van Gestel et al. 2002; Zheng et al. 2009, 2010). Mitochondria have two major types of movement: a long-distance movement which depends on filamentous actin (F-actin), and a short-distance movement remaining at almost the same position (Van Gestel et al. 2002; Zheng et al. 2009, 2010).

We have recently clarified two types of mitochondrial movements: F-actin-dependent directional movement and F-actin-independent wiggling, based on the trajectory analysis of the mitochondrial movement, leading to two criteria: speed and angle changes (Oikawa et al. 2021). The directional movement consists of high speed and low angle changes, whereas the wiggling consists of low speed and high angle changes (Oikawa et al. 2021). In the present study, by using the method, we evaluated whether two MitoTracker Red (FM and CMXRos) induce mitochondrial dysfunction in leaf mesophyll protoplast cells of Arabidopsis thaliana.

As plant materials, true leaves of 3-week-old transgenic A. thaliana, which visualizes mitochondria

Abbreviations: FM, MitoTracker Red FM; CMXRos, MitoTracker Red CMXRos; A. thaliana, Arabidopsis thaliana; MTS, mitochondria-targeting sequence; CLSM, confocal laser scanning microscopy; DMSO, dimethyl sulfoxide; F-actin, filamentous actin.
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with Citrine (yellow fluorescent protein) fused to the mitochondria-targeting sequence (MTS-Citrine), were used to monitor and visualize the mitochondria position. To prepare protoplasts from the transgenic *A. thaliana*, we used an enzyme cocktail containing 1.5% Cellulase R10 (Yakult Pharmaceutical Industry, Japan), 0.4% Macerozyme R10 (Yakult Pharmaceutical Industry), 0.4 M mannitol, 20 M KCl, 10 M CaCl₂, 20 M MES-KOH (pH 5.7), and 0.1% bovine serum albumin (Yoo et al. 2007). Before evaluating mitochondrial movement, the isolated protoplasts were adapted to a white light condition (100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) for 1 h. To stain mitochondria by MitoTracker Red, FM (Thermo Fisher Scientific M22425) and CMXRos (Thermo Fisher Scientific M22425) and CMXRos (Thermo Fisher Scientific M22425) and CMXRos (Thermo Fisher Scientific M22425).
Fisher Scientific M7512), protoplasts were treated for 15 min at 22°C with different concentrations (1 nM, 10 nM, 100 nM, and 1 µM). 0.1% dimethyl sulfoxide (DMSO) was used for resolving the probes. The stained protoplasts were washed three times with MES buffer (pH 5.7) containing 70 mM CaCl₂ and 0.4 M mannitol by centrifugation for 5 min at 50 × g. For tracking the mitochondrial movement, confocal laser scanning microscopy (CLSM) analysis was performed using a Zeiss LSM880 equipped with a 63× NA 1.4 oil immersion objective (Carl Zeiss, Germany). To observe Citrine fluorescence and chlorophyll autofluorescence, the 488 nm excitation laser was used, and 509–580 nm and 630–700 nm were used as emission for Citrine and chlorophyll, respectively. The mitochondrial movement was evaluated by tracking more than 200 mitochondria in time-lapse images acquired every 250 ms for 30 s using MTrackJ (Meijering et al. 2012) with a plugin of Fiji (Schindelin et al. 2012). The brightness centroid of each mitochondrion was determined as the mitochondrial position. From the serial images of the mitochondrial trajectories, each value for the mitochondrial position was acquired at coordinate (xₙ (µm), yₙ (µm))ₙ=1–30, speed (µm s⁻¹), and angle change (Δθ) at each time point.

First, we observed MTS-Citrine-expressed protoplasts stained with the FM or CMXRos at the indicated concentration (Figure 1). Both the probes could stain mitochondria in the protoplasts, and the fluorescence of the probes was co-localized with that of Citrine in mitochondria. However, the two probes' staining ability was different; mitochondria stained with the CMXRos probe were brighter than that with the FM probe at all tested-concentrations. Mitochondria were invisible with the FM probe at 1 nM (Figure 1B), whereas visible with the CMXRos probe at the same concentration (Figure 1C).

Figure 2 shows the trajectories images of mitochondrial movement within 30 s taken by CLSM (Supplementary Movies S1–S18). The images were created from the plots of all mitochondria positions with a formula: (xₙ (µm), yₙ (µm))ₙ=1–30. Similar to the control treatment with DMSO (Figure 2A, Supplementary Movies S1, S2), the...
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trajectories of mitochondria stained with the FM probe (1 nM–1 µM) showed both a short- and a long-distance movement in the cells (Figure 2B, Supplementary Movies S3–S10). On the other hand, the trajectories of mitochondria stained with the CMXRos probe showed mostly a short-distance movement at more than 10 nM and completely lost a long-distance movement at 1 µM (Figure 2C, Supplementary Movies S11–S18). In the CMXRos-treated cells, most mitochondria were swelling at higher than 10 nM and spherical at 1 µM (Figure 2C, Supplementary Figure S1, Supplementary Movies S13, S14, S17, S18).

Using the trajectory data of mitochondrial movement (n = 200) in the three different protoplasts, we acquired the value of speed (µm s⁻¹) at every time point. Frequency analysis on the mean speed of mitochondrial movement in the DMSO-treated cells revealed a broad range (0.05-over 1.0 µm s⁻¹) (Figure 3A). Similar patterns were also observed in the FM-treated cells (1–100 nM), but speeds of more than 0.40 µm s⁻¹ were decreased at 1 µM (Figure 3B). On the other hand, the CMXRos-treated cells reduced speeds of more than 0.50 µm s⁻¹ at 10 nM, and of more than 0.20 µm s⁻¹ at 100 nM (Figure 3C). Coincidentally, speed frequencies of lower than 0.05 µm s⁻¹ were increased in the CMXRos-treated cells at more than 10 nM (Figure 3C).

Next, we focused on speed and angle changes of mitochondria to further clarify the effect of the mitochondria-selective fluorescent probes on mitochondrial movement, that is, whether these probes affect F-actin-dependent directional movement or wiggling. Scatter plots of the speed (µm s⁻¹) and angle changes (Δθ) of mitochondrial movement in the DMSO-treated protoplasts showed a broad range of plot patterns (Figure 4A). The scatter plots in the FM-treated cells at 1–100 nM exhibited a similar pattern to that in the DMSO-treated cells, but a small reduction in plots of high speed-low angle changes at 1 µM (Figure 4B). The scatter plots in the CMXRos-treated cells at 100 nM–1 µM severely reduced the plots of high speed-low angle changes (Figure 4C). The CMXRos probe at 1 nM could stain mitochondria brighter than the FM probe at 1 nM with normal mitochondrial movement (Figure 1, Supplementary Movies S11, S15). However, based on the

Figure 3. Speed distributions of mitochondrial movement in Arabidopsis mesophyll protoplasts treated with the mitochondria-selective fluorescent probes. The speeds frequency was acquired from trajectories data of mitochondrial movement in the protoplasts treated with 0.1% DMSO (A), MitoTracker Red FM (FM) (B), and MitoTracker Red CMXRos (CMXRos) (C). Three independent protoplasts were analyzed (each protoplast is represented as blue-, green-, or orange-colored boxes).
quantitative analysis of the mitochondrial movement, it is suggested that the CMXRos probe severely induces mitochondrial dysfunction at 100 nM concentration, as compared with the FM probe, which did at 1 µM (Figures 2–4). In the CMXRos-treated cells at more than 100 nM, most of the mitochondria showed low speed and high-angle changes, suggesting that directional movement was lost (Figure 4C, Supplementary Movies S13, S14, S17, S18). The results agreed with the statistical analysis of speed and angle changes of the mitochondrial movement treated with the mitochondria-selective fluorescent probes (Supplementary Figure S2). Mitochondrial movement of the CMXRos-treated cells at more than 100 nM was similar to that of the F-actin-disrupted A. thaliana cells (Doniwa et al. 2007; Sheahan et al. 2005; Van Gestel et al. 2002; Zheng et al. 2009, 2010). In animal cells, the cytotoxicity of the CMXRos probe has been well characterized (Minamikawa et al. 1999); it gave severe phototoxicity to mitochondria function at 100 nM, as compared with other probes such as tetramethylrhodamine ethyl ester (200 nM), rhodamine 123 (10 µM), and MitoTracker Green (100 nM) (Minamikawa et al. 1999). Here, we confirmed the cytotoxicity of the CMXRos probe by assessing the mitochondria swelling, resulting in that the size of mitochondria was apparently increased at more than 10 nM (Supplementary Figure S3). Thus, the CMXRos probe might induce damage to F-actin and phototoxicity to mitochondria in A. thaliana cells.

In summary, our quantitative analysis of mitochondrial movement could evaluate the two MitoTracker Red, FM and CMXRos, in A. thaliana, and determined their appropriate concentrations that exhibited normal mitochondrial movement (FM: 100 nM or less, CMXRos: 1 nM or less). Given the appropriate concentrations of the probes, our quantitative analysis with the probes is considered to be useful for evaluating whether mitochondrion is normal or not in non-transgenic plants under various experimental conditions (e.g., media, nutrients, chemicals, light, and temperature).

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