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In vivo Monitoring of the Incorporation of Chemicals into Cucumber and Rice Leaves by Chlorophyll Fluorescence Imaging

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Abstract

Chlorophyll (Chl) fluorescence imaging was used to investigate the effectiveness of *in vivo* incorporation methods for two chemicals, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) and methyl viologen (MV) in rice, a monocot, and cucumber, a dicot, leaves. Four different methods (vacuum infiltration, floating, transpiration-aided incorporation through petiole and spraying) were compared, and F_i and F_v/F_m were chosen for the imaging of the DCMU- and MV-treated leaves, respectively. The effects of the chemicals in plants were generally heterogeneous over the whole leaf area. Moreover, the effectiveness of the treatment of a chemical in plant leaves was dependent on chemical species, plant species, concentration of the chemical, the treatment method, the duration of the treatment, the existence of light and detergent, etc. In conclusion, we suggest that to achieve the proposed effects of chemicals in plants for an actual experiment, these factors must be considered before the chemical treatment, and the best method for the treatment of the chemicals tested was floating and vacuum infiltration in the dicot and the monocot leaves, respectively, as drawn from Chl fluorescence imaging analysis.

Introduction

Since the classical experiments of Kautsky and Hirsch (1931), chlorophyll (Chl) fluorescence has come to occupy an important position in the study of plant physiology as non-destructive, non-invasive, and highly sensitive probe of photosynthesis. The Chl fluorometry was developed to an imaging technique by Omasa et al. (1987). The imaging has proved to be indispensable whenever the experimental object exhibits a substantial heterogeneity of fluorescence emission over its area (Govindjee and Nedbal, 2000). Currently, the Chl fluorescence imaging is used not only to study heterogeneity of photosynthesis in leaves, but also to monitor localized infection by viruses and other pathogens (before visible symptoms appear) (Balachandran et al., 1994; Ning et al., 1995; Scholes and Rolfe, 1996; Osmond et al., 1998) as well as to study the local effects of irradiance, temperature, and heavy metal stress (Genty and Meyer, 1995; Siebke and Weis, 1995; Lichtenthaler et al., 1996; Oxborough and Baker, 1997; Nedbal et al., 2000; Lichtenthaler et al., 2000; Buschmann et al., 2000; Langsdorf et al., 2000; Lee et al., 2001).

There have been few reports about the methods of chemical incorporation into plants, although the chemical treatment is generally required for many *in vivo* or *in planta* experiments. Since the treatment methods seemed too fundamental to be considered, they might be frequently overlooked in actual experiments. However, in *in vivo* or *in planta* experiments using plant materials, the chemical treatment has an important meaning in carrying out the experiments in more predictable ways and thereby improving the quality of the results, because it can frequently work as an error-producing step. In general, the chemical treatment to plants has been accomplished by vacuum infiltration, floating, transpiration-aided incorporation through a petiole or spraying. The former two methods are mainly used for leaf discs or segments, while the latter two ones are often used for plant seedlings or whole

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plants. Regardless of the methods employed for the chemical treatment, the most important criterion for determining the treatment conditions would be the homogeneity of the chemicals over the whole area of the samples under investigation.

In the present study, we attempted to compare these traditional methods for the chemical treatment to plant leaves using two widely-used chemicals affecting leaf photosynthesis in a monocot and in a dicot plant species. For this purpose, Chl fluorescence imaging was used to quantitatively map photosynthesis yields over the whole area of plant leaves to reveal a substantial heterogeneity over its area.

Materials and Methods

Plant materials

Cucumber (*Cucumis sativus* L.) and rice (*Oryza sativa* L. cv. Dongjin-byeo) plants were grown in a growth chamber with photosynthetic photon flux density (PPFD) at pot level of 100 μmol photons m^{-2} s^{-1} supplied by fluorescence lamps and metal halides, respectively. The growth chambers were maintained at 25℃ with a 12 h photoperiod for cucumber and at 28/23℃ (day/night) with a 14 h photoperiod for rice. For the experiments, cucumber and rice plants were used at the developmental stages of 3 to 4 and 4 to 6 weeks, respectively.

Incorporation of chemicals

Leaf samples were treated with an inhibitor of photosystem II (PSII) electron transport activity, 50 μM 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), and with 10 μM methyl viologen (MV) for photooxidation of two photosystems. The chemicals were incorporated into plant tissues by the four traditional methods as follows; ***1. Vacuum infiltration (M1)*** - Cucumber leaf discs (0.8 cm in diameter) or rice leaf segments (2 cm in length) were vacuum infiltrated with DCMU, MV or water using a 20-mL syringe. To help the chemical incorporation, all the leaf samples were repetitively exposed to about 0.5 atmosphere of negative pressure until they became nearly translucent. Immediately after the vacuum infiltration, samples treated with DCMU were used for Chl fluorescence imaging, but samples treated with MV were further incubated on a sheet of wet paper towels in the light with PPFD of 150 μmoL photons m^{-2} s^{-1} at 28℃ for 3 h for photooxidation before Chl fluorescence imaging. ***2. Floating (M2)*** - Cucumber leaf discs and rice leaf segments were floated on a DCMU solution in darkness at room temperature for 3 h or on a MV solution under the light with PPFD of 150 μmol photons m^{-2} s^{-1} at 28℃ for 3 h. Immediately after the treatment, all the leaf samples were used for Chl fluorescence imaging. ***3. Transpiration-aided incorporation through petiole (M3)*** - Cucumber and rice leaves were excised and allowed to take up DCMU or MV by submerging their petioles. The incorporation of DCMU was done in darkness and that of MV was done in the light with the PPFD of 150 μmol photons m^{-2} s^{-1} at 28℃ for 3 h. To help the transpiration stream, all the leaves were air-blown using a fan for the whole period of incorporation time. After the treatment, leaves were cut into several leaf discs or segments and used for Chl fluorescence imaging. ***4. Spray (M4)*** - whole plants of cucumber and rice were continually sprayed with the DCMU solution in darkness or with the MV solution under the PPFD of 150 μmol photons m^{-2} s^{-1} at 28℃, having 15-min intervals for 3 h. After the treatment, cucumber and rice plants were excised into a seedling and several leaf segments, respectively, and used for Chl fluorescence imaging.

Chl Fluorescence Imaging

Chl fluorescence images were taken using an imaging fluorometer (FluorCam 700MF, P.S.Instruments, Brno, Czech Republic) referring to the operating manual. To measure *in vivo* or *in planta* slow fluorescence induction kinetics, the fluorometer was operated by a standard protocol template for quenching analysis included in the operating software. However, the template was slightly modified to have 'Sensitivity' as 50% and 'Actinic light intensity' as 30% (about 100 μmol photons m^{-2} s^{-1}) by a preliminary experiment in the present study. For analysis of experimental results, Chl fluorescence images corresponding to F_0, F_m and F_t were further processed to F_v/F_m and $(F_m - F_t)/F_t$, and a new parameter, F_i, was first introduced here to imply the intermediate fluorescence, that was measured 0.8 s after actinic light is on, which is greater in the DCMU-treated cells than that in the control cells. According to Nedbal et al. (2000) and Oh et al. (2001), the other parameters can be generally defined as follows; F_0 - the initial Chl fluorescence before actinic light is on, F_m - the maximal yield of fluorescence, F_v - the maximal variable fluorescence and F_t - the steady-state fluorescence level under the continuous actinic illumination. Two processed parameters, F_v/F_m and $(F_m - F_t)/F_t$, were used to imply the maximal PSII quantum yield (or the photochemical efficiency of PSII) and the relative fluorescence density (Rfd) at the steady-state level, respectively. Before the Chl fluorescence imaging, all the plant materials were dark-adapted for 10 min at room temperature.

Results

Chl fluorescence imaging of DCMU-incorporated leaves through their petioles

After transpiration-aided incorporation through petioles, the degree of DCMU incorporation throughout the cucumber leaves could be monitored by Chl fluorescence imaging. DCMU increases the Chl fluorescence yield even in low intensity light, because it blocks electron transport from Q_A to Q_B sites, leading to the photoreduction of Q_A. Therefore, the difference between the DCMU-affected cells and the control cells was prominent in intermediate fluorescence, F_i, images (Figure 1), because fluorescence

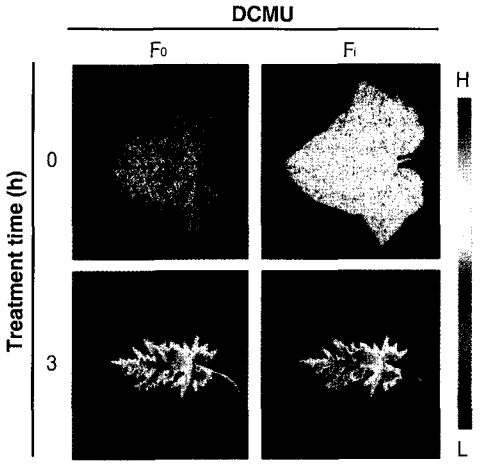

Figure 1. Chl fluorescence images of cucumber leaves incorporated with DCMU through their petioles by transpiration in darkness. The images were taken at 0 h and 3 h after the DCMU treatment. Each leaf is colored in a relative scale based on the fluorescence intensities of the parameter, F_0 or F_i.

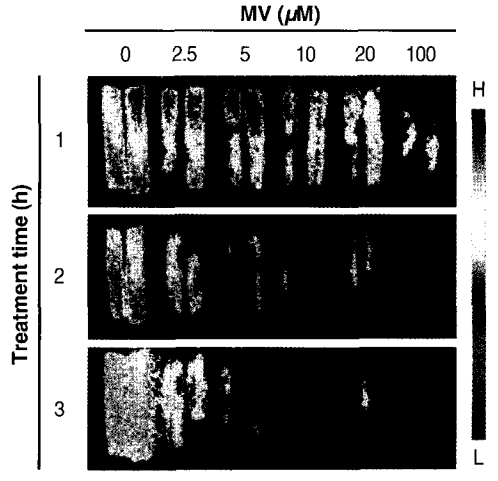

Figure 2. Chl fluorescence imaging of MV incorporation into rice leaf segments by floating under illumination. A Chl fluorescence parameter, F_v/F_m, was used for imaging. Leaf segments were treated with 0 to 100 μM MV for 1 to 3 h. The control image at 0 h was omitted, because no leaf segments were affected. Each row is colored in a relative scale based on the fluorescence intensities.

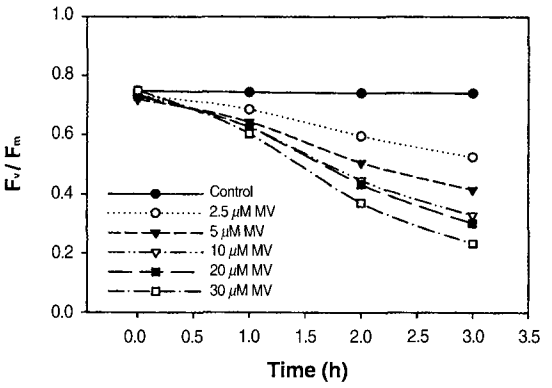

Figure 3. Photooxidative effect of MV depending on its concentration and duration of the treatment. Data used for this plot are the average of F_v/F_m values on an image of leaves treated with a given concentration for a given time in Figure 2.

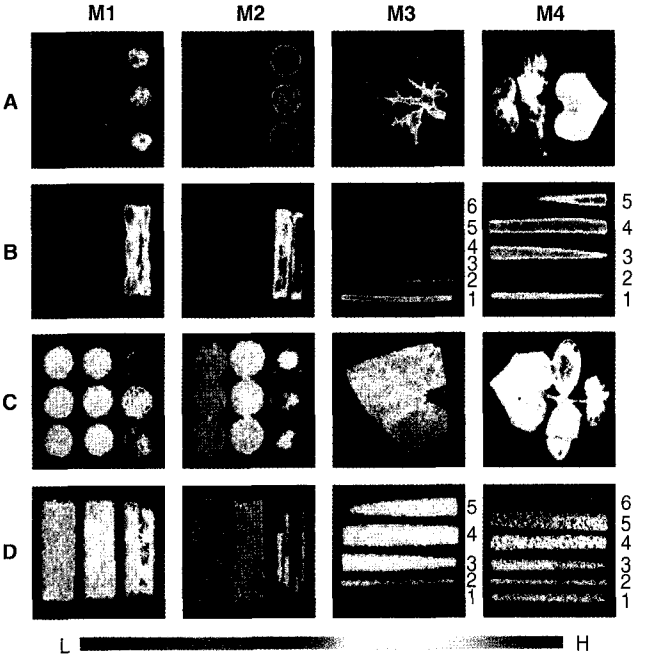

Figure 4. Chl fluorescence imaging of chemical incorporation by four traditional methods. As stated in Materials and Methods, M1 to M4 represent vacuum infiltration, floating, transpiration-aided incorporation through petiole and spraying, respectively. A and C, Cucumber; B and D, Rice. A and B, F_i images for DCMU; C and D, F_v/F_m images for MV. In all the images of M1 and M2, the first, second and third columns represent control, water- and chemical-treated samples, respectively. Among the images of M3 and M4, the numbers inside B and D represent the location of each segment from bottom to the tip of a rice leaf.

increased more rapidly in DCMU-affected cells. In the F_0 images in Figure 1, Chl fluorescence was slightly higher in the DCMU-affected cells than that in the control cells, as expected.

As shown in Figure 1, DCMU is penetrating through veins of the leaf, revealing the existence only in vicinity of the veins after 3 h. Even after an extended incubation for 18 h, the effect was not spread throughout the leaves, but only nearer to the margin of the leaf. Moreover, the leaf was completely wilted after the extended incubation (data not shown).

Chl fluorescence imaging of MV incorporation by floating

Figure 2 shows the constructed images showing photochemical efficiency, F_v/F_m, of leaves treated with increasing concentrations (2.5 to 100 μM) of MV for 1 to 3 h by floating rice leaf segments under illumination to induce photooxidative damages in photosystems. MV mediates reactive oxygen species (ROS) cascade by producing superoxide ($\bullet O_2^-$) at PSI sites through photoreduction of dioxygen. As ROS attack the photosystems, the fluorescence yield typically decreases in both F_0 and F_m, but the decrease is much more pronounced in F_m. Therefore, F_v/F_m could be better for visualization of the changes, and the usage of ratio has an additional advantage of canceling differences in the amount of Chl among different spots on a leaf, because the magnitudes of the two parameters are roughly proportional to the amount of Chl.

When the effect of MV was visualized by F_v/F_m imaging, it was very heterogeneous over the leaf segments (Figure 2). When the average values of F_v/F_m over the whole leaf area were plotted, we could see a conventional plot showing the changes in F_v/F_m depending on the concentration of MV and the treatment times (Figure 3).

These results suggest that the *in vivo* or *in planta* incorporation of chemicals into plants would be substantially heterogeneous over the whole plant materials, depending on the incorporation methods as well as the chemical concentrations and the duration of the treatment. In general, such heterogeneity in the materials may affect all the actual experiments after the chemical incorporation. Therefore, four traditional methods were tested and compared for their efficiencies in the chemical incorporation into plant leaves, using two chemicals (DCMU and MV) and two plant species (cucumber and rice). For better comparison, the parameters, F_i and F_v/F_m, for Chl fluorescence imaging, were chosen by a preliminary experiment for DCMU and MV, respectively.

Comparison of four traditional methods for chemical incorporation into plants

M1: When vacuum infiltration using a syringe was used for chemical incorporation, the most critical problem was physical damages in leaf materials by the repeated and abrupt pressure changes. The damages could be observed by comparing the images of samples in the first column with those in the second column in M1-A picture for cucumber and in M1-B picture for rice in Figure 4. In other words, the initial fluorescence, F_i, decreased even in the water-infiltrated cases, indicating some damages to photosynthetic machinery. Especially, in the case of rice, a monocot plant, this method seemed to be very erroneous, because the leaf segments were easily broken into pieces by this method.

When this method was used for the incorporation of DCMU, the effect appeared to be prominent at the center of both leaf materials, having heterogeneity over the materials, as shown in the samples in the third column of the picture M1-A and those in M1-B in Figure 4. In the case of MV, the effect was much milder in the rice leaf segments (M1-D in Figure 4) than in the cucumber leaf discs (M1-C in Figure 4). This means that the amount of MV incorporated into the rice leaf segments was not enough to induce significant photooxidative damages in photosystems.

M2: In cucumber leaf discs incorporated with DCMU by floating for 3 h, the effect of DCMU was relatively homogeneous and seemed saturated (M2-A in Figure 4), when compared with M1. However, in the rice leaf segments, cells in the marginal parts of the segments were affected more severely (i.e. higher F_i) (M2-B in Figure 4). As MV was also more effective (i.e. lower F_v/F_m) in the marginal parts of both leaf materials (M2-C and M2-D in Figure 4), it could be thought that the chemicals entered into the leaf materials through the cut area rather than through the stomatal pores.

When the leaf materials were floated on water under illumination, they could be often exposed to photoinhibition. For example, F_v/F_m values of the cucumber leaf discs in the second column (water-treated controls) in the picture M2-C in Figure 4 were lower than those in the first columns (untreated controls). This photoinhibition effect was severer in cucumber than that in rice (M2-D in Figure 4), implying that the light intensity used for the MV-treatment was more stress-inducible in cucumber leaves.

M3: As mentioned above, the most serious problem in this 'transpiration through petiole' method was that detached leaves were apt to wilt unless enough humidity was provided. However, this problem seems to be somewhat unavoidable, because high humidity can reduce the

incorporation efficiency by inhibiting the transpiration stream. After 3 h-incubation, the effect of DCMU and MV was restricted to the area in the vicinity of the veins in a cucumber leaf (M3-A and M3-C in Figure 4), and in a rice leaf, it was visible only in the segment (in the lowest parts of the leaves) (M3-B-1 and M3-D-1) which were submerged in the DCMU or MV solution during incorporation. Therefore, in this method, a prolonged incubation under an optimal humidity condition is generally required.

M4: When the whole plant of cucumber was sprayed with DCMU or MV, the effect was dependent on the developmental stage. DCMU affected older leaves more, while MV did younger ones (M4-A and M4-C in Figure 4). However, this might be due to the positional differences. In a rice plant, the effect of DCMU seemed to be relatively homogeneous over the leaf, but the effect of MV was not observed by spraying (M4-B and M4-D in Figure 4). These results suggest that the chemical incorporation by spraying should be largely dependent on the chemical properties as well as the developmental stage of the sample used.

The effect of Tween-20 on the chemical treatment

To help the uptake of chemicals in aqueous phase by reducing the surface tension of water and disturbing the membrane integrity of plant cells, detergents have been mixed in chemical solutions used for many physiological studies. Generally, detergents were applied at a very low concentration to minimize its artifacts.

In Figure 5, the effects of Tween-20 at different concentrations were tested for the incorporation efficiencies of the two chemicals using two different incorporation methods. In the case of DCMU test, all the data were not from a single image, and therefore F_i cannot be used, because it depends on the Chl concentration. In stead, a new parameter, Rfd $((F_m - F_t)/F_t)$, was used which does not depend on Chl concentration, because it is in the form of ratio of Chl fluorescence parameters. DCMU causes an increase of the steady-state fluorescence, F_t, resulting in a decrease in Rfd by blocking the electron transfer chain in the acceptor side of PSII. When rice leaf segments were treated with DCMU in the presence of Tween-20 by floating, DCMU was effective with higher concentrations of Tween-20, but when treated by vacuum infiltration method, it couldn't induce any additional decrease of Rfd (Figure 5A).

When Tween-20 was treated with MV, F_v/F_m decreased further in 0.001% and 0.01% Tween-20 in the case of vacuum infiltration and floating methods, respectively (Figure

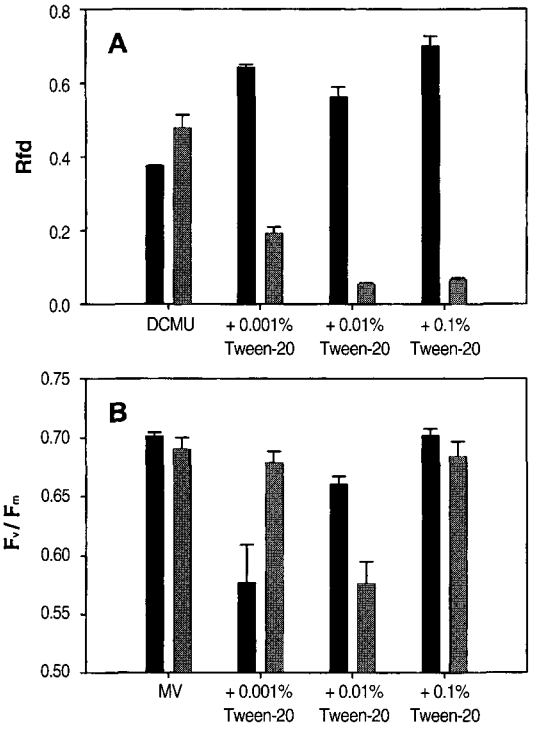

Figure 5. The effect of Tween-20 on the chemical treatment by vacuum infiltration and floating. A, the effect of Tween-20 on the decrease of Rfd by DCMU. B, the effect of Tween-20 on the decrease of Fv/Fm by MV. The average values of Rfd and F_v/F_m before the chemical treatment were about 1.0 and 0.81, respectively. Black and gray bars represent vacuum infiltration and floating, respectively. All the materials used for the experiments were rice leaf segments. SE (n=3) was shown for each value.

Table 1. Summary of the evaluation of 4 traditional methods for chemical incorporation of DCMU and MV into cucumber and rice leaves. E1, E2 and E3 represent an arbitrary evaluation for the efficiency of each method in the aspect of evenness, effectiveness and artifacts, respectively. The evaluation was based primarily on the images shown in Figure 4.

		M1			M2			M3			M4		
		E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3
Cucumber	DCMU	++	++	+	+++	+++	++	+	+	+++	++	+++	-
	MV	+++	++	+	++	+++	++	+	+	+++	-	-	-
Rice	DCMU	+++	+++	+++	++	++	+	+	+	-	+++	+++	-
	MV	+	+	+++	+++	+++	+	+	+	-	-	-	-

5B). However, the effect of Tween-20 was diminished at a higher concentration (0.1%) of MV. In the case of cucumber, the presence of Tween-20 with DCMU and MV did not cause any further changes in Chl fluorescence parameters (data not shown), suggesting that the effect of Tween-20 in the chemical incorporation was also dependent on the plant species.

Discussion

The expression of "Seeing is believing." by Govindjee and Nedbal (2000) was used to emphasize how Chl fluorescence imaging can be used to get a scientific belief in the experiments. In the current experiments, the Chl fluorescence imaging was used to show how the *in vivo* treatment of DCMU and MV to cucumber and rice leaves depends on the chemical incorporation methods. Through some preliminary experiments, F_i and F_v/F_m were chosen for the imaging of the DCMU- and MV-treated leaves, respectively. As shown in Figures 1 and 2, the incorporation of DCMU and MV was very heterogeneous among the leaf cells in the whole leaf area. The incorporation pattern was dependent on the treatment methods as well as the chemical concentrations and the duration of the treatment. Supposing that parts of the leaves were used for the following experiments, the heterogeneity might cause serious problems by decreasing the reproducibility of the results or by making the results ambiguous by increasing the variations of the results. Therefore, to find a proper method and condition for chemical treatments could be often decisive in determining the quality of experimental results.

In general, four traditional methods have been used for chemical treatment in plants. According to the results shown in Figure 4, the efficiency of each method was dependent on the chemical and plant species. The detailed evaluation for the efficiency of the 4 methods was summarized in Table 1. Although the evaluation was not quantitative, the 4 methods were evaluated in the aspect of the evenness (homogeneity) of the incorporation, the effectiveness of chemical treatment and the degree of physical damages causing on leaves. In the case of M1 and M3, the most serious artifact was mainly physical damages and wilting, respectively. According to Kim et al. (2001), the authors used a spherical container for desiccation with a vacuum pump instead of a syringe for vacuum infiltration of chemicals into cucumber leaf discs, probably minimizing the physical damages. However, the latter would be favorable especially in rice, because the hydrophobic nature of the leaf surface necessitates repeated changes of the pressure for the effective absorption of the chemical solution. The easy way to protect from wilting in using the M3 method was to provide enough humidity during the treatment, but too much humidity will delay the chemical incorporation by inhibiting the transpiration stream.

As stated above, the surface of monocot leaves has the hydrophobic nature and thereby hinders the uptake chemical solutions. Tween-20 has been used to solve such a problem, because it helps the chemical incorporation by reducing the surface tension of the chemical solutions in the aqueous phase and disturbing the cell membranes. However, unexpectedly as shown in Figure 5, the effect of Tween-20 was largely dependent on the treatment methods, the chemicals and plant species to be treated as well as its concentrations. These effects should be considered carefully in using detergents including Tween-20 for chemical incorporation.

In conclusions, we have shown that the effectiveness of the treatment of a chemical in plant leaves was dependent on chemical species, plant species, concentration of the chemical, the incorporation method, the duration of the treatment, the existence of light and detergent, etc. Especially, the best method for the treatment of the chemicals tested was floating and vacuum infiltration in dicot (cucumber) and monocot (rice) leaves, respectively. Therefore, to achieve the proposed effects of chemicals in plants for an actual experiment, these factors must be considered before the chemical treatment. Furthermore, as the effects of chemicals in plants were generally uneven over the whole leaf area, the exact evaluation of the effectiveness of the chemical treatment is necessary for *in vivo* experiments using plant materials to produce reproducible results with low variations. For this purpose, Chl fluorescence imaging is a very useful tool, and the development of proper Chl fluorescence imaging methods by choosing parameters specific for a certain chemical with a specific effect is necessary.

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