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Note

Handmade leaf cutter for efficient and reliable ROS assay

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Abstract Reactive oxygen species generation is one of the most popular index of plant immune responses. Leaf disk assay has been commonly used for MAMP/elicitor-induced ROS analysis by many groups. However, the reproducibility of the leaf disk assay relies on the skills of the people engaged in the experiments and the experiment itself seems not suitable for some plant species, which had a tough leaf structure and lower penetration efficiency of MAMPs/elicitors. In this study, we prepared a handmade leaf cutter to cut out the leaf fragments with uniform size and slits. The use of such fragments obtained by the new leaf cutter as well as the increase of the number of leaf fragments for each experiment improved the reliability and reproducibility of the leaf disk assay. This cutter was also successfully applied to rice leaf disk assay, indicating the applicability to other plant spices.

Key words: defense response, elicitor, leaf disk, MAMP, ROS.

Plants have the ability to induce reactive oxygen species (ROS) in response to pathogen infection or elicitor treatment (Kadota et al. 2015; Qi et al. 2017). The biological function of this oxidative burst in plant immunity is still not fully understood and several possibilities have been discussed. For example, ROS could directly attack pathogens or act as a second messenger in immune signaling (Boller and Felix 2009; O'Brien et al. 2012). Apart from its intrinsic function, ROS generation associated with microbial infection/elicitor treatment has been used as a popular index of plant defense response because ROS can be easily detected and quantified (Sang and Macho 2017).

To evaluate pathogen infection using ROS generation, infected leaves are stained with 3,3′-diaminobenzidine (DAB) to visualize ROS (Torres et al. 2005). Classically, these stained images are directly compared with the mock-treated leaves. In recent years, the levels of ROS accumulation in the DAB-stained leaves have been quantified using image analysis (Lee et al. 2017). On the other hand, ROS generation induced by elicitor or microbe-associated molecular pattern (MAMP) treatment can be easily detected and quantified by luminol chemiluminescence assay and has been widely used to evaluate the degree of defense response in plants (Albert et al. 2006; Desaki et al. 2006; Sang and Macho 2017; Yamaguchi and Kawasaki 2017).

In Arabidopsis, leaf disk assay is a popular method to analyze MAMP-induced ROS generation, although seedling assay has also been reported (Albert et al. 2006; Sang and Macho 2017; Zhang et al. 2010). Typically, leaf disks are cut from rosette leaves and each one disk is floated on the medium, which contains luminol and MAMPs, in each well of 96-well plates (Sang and Macho 2017; Zhang et al. 2010). The 96-well plates can be directly set into a microplate reader and the amount of ROS is quantified. It is known that the addition of "slits" in the leaf fragments helps the responsiveness probably because of the increase of penetration of MAMPs/elicitors (e.g., Jian-Min Zhou, private communication). However, the reproducibility of such experiments largely relies on the skills of the people engaged in the experiments.

On the other hand, in rice, ROS generation has often been evaluated by using cultured cells, which are incubated in the medium containing appropriate MAMPs (Desaki et al. 2006). Aliquots of the medium can be taken at various time points and used for the luminol assay. The experimental error obtained by this method is usually small because of the homogeneity of

Abbreviations: CERK1, chitin elicitor receptor kinase 1; DAB, 3,3′-diaminobenzidine; MAMP, microbe-associated molecular pattern; ROS, reactive oxygen species.

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cell density and population in the case of cultured cells. Although leaf disk assay has also been reported for rice, it is not very popular so far (Kouzai et al. 2014). The major difficulty in the use of rice leaves comes from their tough structure that makes penetration of MAMPs/elicitors less efficient compared to Arabidopsis. Preparation of leaf disks suitable for ROS assay in a reproducible manner is also a potential problem for rice.

To resolve such potential problems and establish more reliable leaf disk assay, we prepared a special leaf cutter that enabled the stable ROS assay both in Arabidopsis and rice. This leaf cutter enabled to cut out the leaf fragments with uniform size and slits both in Arabidopsis and rice.

The cutter was made from the commercial art knife blades (Figure 1A, https://www.artstuff.net/Olfa-AK4-Art-Knife_p_519.html) that were then combined together by welding (Figure 1A, prepared by Bio Tools Inc.; http://www.biotools.jp/index.html). At first, thirteen blades were arranged as described below and combined by welding. Then the combined blades were fixed vertically onto another blade, again by welding (Figure 1A). Those combined blades other than the central and two side blades were arranged to leave 0.8 mm spaces between the top/vertically fixed blades. These spaces are important to maintain the integrity of the leaf fragments when they are cut out from the leaves (Figure 1B).

To cut out leaf fragments from the rosette leaves of Arabidopsis plants, the leaves were firstly cut with a knife to form a straight edge as shown in Figure 1B. The

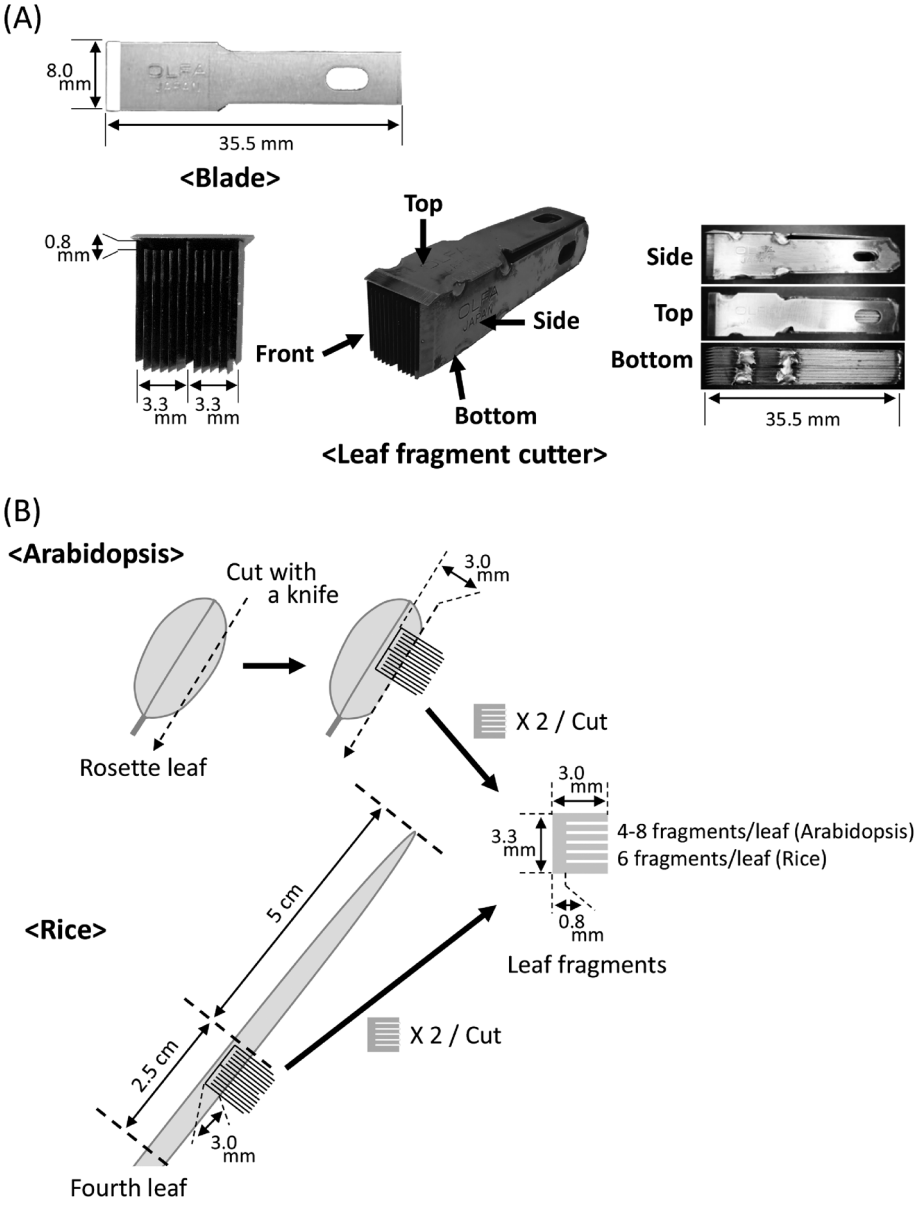

Figure 1. Structure of the leaf cutter and the images of cutting leaves. (A) Structure of the leaf cutter. (B) Scheme for the preparation of *Arabidopsis* and rice leaf fragments.

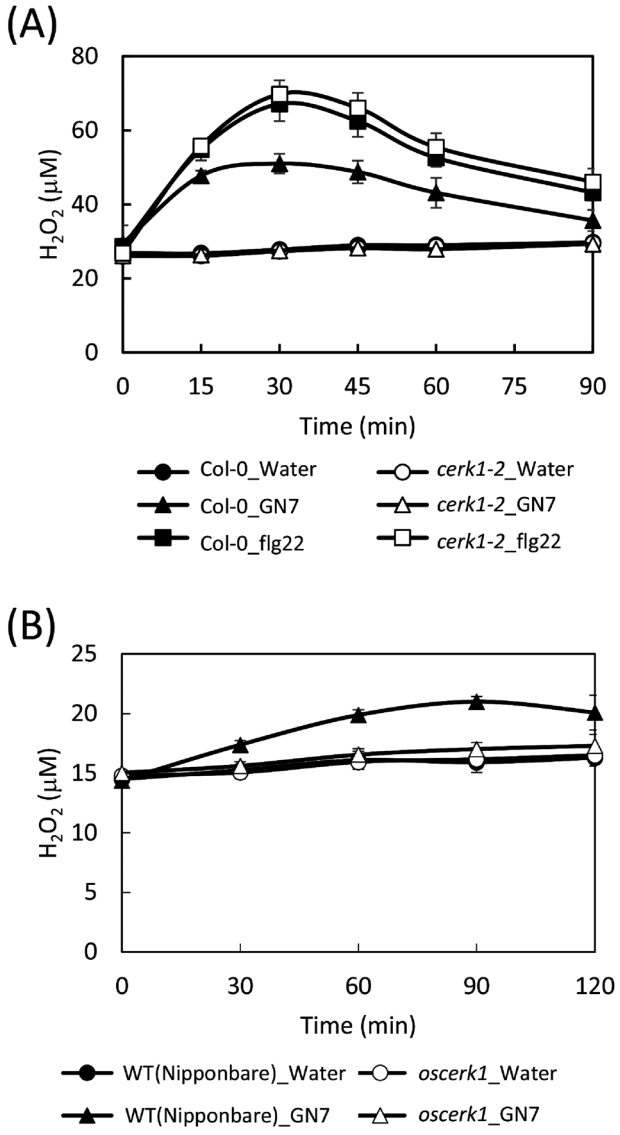

Figure 2. Evaluation of ROS production in *Arabidopsis* and rice. (A) *Arabidopsis* plants were grown in soil in a growth chamber with 16 h light (22°C)–8 h dark (16°C) for 5–6 weeks. The CERK1 knockout mutant *cerk1-2* (GK_096F09) was previously described (Miya et al. 2007). (B) Rice plants were grown in one-eight liquid Chu basal salt solution in a growth chamber with 16 h light (24°C)–8 h dark (24°C) for 5 weeks. The OsCERK1 knockout mutant *oscerk1-2* was previously described (Kouzai et al. 2014). (A, B) The medium was replaced with each fresh medium 2 h before the MAMPs treatment. Three replicates were prepared for each treatment. After the MAMP treatments, 10 μl aliquots of the medium were transferred to 96-well microtiter plates at various times. Immediately, 50 μl of 1.1 mM luminol, which dissolved in 50 mM potassium phosphatate buffer (pH 7.8), and 100 μl of 14 mM potassium hexacyanoferrate solution were supplemented using a programmable injector attached to the luminometer and ROS was measured as a chemiluminescence by a microplate luminometer model TR717 (Berthold Technologies). The amount of ROS was estimated by using a standard curve for hydrogen peroxide. The data are given as means±SD of three replicates.

leaf cutter was set on the leaf so that the position of the "top" blade was adjusted to 3 mm from the straight edge (Figure 1B). By doing so, the cutter generated two leaf fragments of 3×3.3 mm per one cut. As the thickness of the blade was 0.55 mm, the leaf fragment had 5 slits at intervals of 0.5–0.6 mm (Figure 1B). Four to eight leaf fragments could be obtained from one rosette leaf, avoiding the central vein.

For ROS assay, five leaf fragments were floated on the 200 μl of MGRL medium containing 1% sucrose in each well of a 24-well plate (Albert et al. 2006; Naito et al. 1994). After kept overnight in the dark, the medium was replaced with the flesh medium and the leaf fragments were preincubated for 2 h on a rotary shaker at 300 rpm. After the addition of 40 μM *N*-acetylchitoheptaose (GN7) or 100 nM flg22, each 10 μl of the medium was taken and transferred to 96-well plate at various time points and used for luminol assay. While the wild type plants showed a typical GN7- or flg22-induced ROS generation, the *cerk1-2* mutants, which lack the receptor-like kinase essential for chitin responses, lost the chitin-induced ROS generation (Figure 2A). These data were reproducible and well corresponded with our previous results obtained by seedling assay (Miya et al. 2007). The use of new leaf cutter enabled to generate leaf fragments with the reproducible size and shape efficiently, and thus resulted in the data with minimal SD/SE.

In the case of rice, the middle portion of the fourth leaf, ranging from 5 to 7.5 cm from the tip, was used to cut out the fragments (Figure 1B). As the leaf edge of this area was mostly linear, the leaf cutter was set on the leaf without forming a linear edge with the knife. By using the leaf cutter, six leaf fragments could be obtained per leaf (Figure 1B). Six leaf fragments were pre-incubated overnight with 200 μl modified-N6D medium in each well of a 24-well plate (Kuchitsu et al. 1997), then the medium was replaced with the fresh medium as described for Arabidopsis leaf fragments. One nM GN7 was added to the medium and each 10 μl of the medium was taken at various time points and used for luminol assay. As shown in Figure 2B, the wild type rice plants showed a typical GN7-induced ROS generation but the *oscerk1* mutants did not (Kouzai et al. 2014). Compared to Arabidopsis, leaf disk ROS assay has not been so popular in rice, partly because of the tough structure of rice leaves that seems to form a barrier for the penetration of MAMPs. The use of leaf cutter reported here could be applicable to other plant species that have similar problems.

In conclusion, the use of new leaf cutter is useful for efficient and reliable leaf disk ROS assays in both Arabidopsis and rice. The use of multiple leaf fragments in each well also improved the sensitivity and stability of the assay.

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