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Quantification of Plant Cell Death by Electrolyte Leakage Assay

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[Abstract] We describe a protocol to measure the electrolyte leakage from plant tissues, resulting from loss of cell membrane integrity, which is a common definition of cell death. This simple protocol is designed to measure the electrolyte leakage from a tissue sample over a time course, so that the extent of cell death in the tissue can be monitored dynamically. In addition, it is easy to handle many tissue samples in parallel, which allows a high level of biological replication. Although the protocol is exemplified by cell death in *Arabidopsis* in response to pathogen challenge, it is easily applicable to other types of plant cell death.
Keywords: Plants, Cell death, Electrolyte leakage, Electrolytic conductivity

[Background] When a cell dies and loses the integrity of the cell membrane, electrolytes, such as K^+ ions, leak out of the cell. Thus, we can use the amount of electrolytes leaked from a tissue as a proxy for the extent of cell death in the tissue. A simple way to quantify such electrolytes leaked from a tissue is to measure the increase in electrolytic conductivity of water that contains the tissue with dying cells. This electrolyte leakage assay has been applied to plant tissues to assess the relative quantity of cells that died in response to biotic and abiotic stresses, such as pathogen challenge, insect herbivory, wounding, UV radiation, oxidative stress, salinity, drought, cold and heat stress (Demidchik *et al.*, 2014).

The original method was designed to measure the conductivity of the aqueous bathing solution containing plant tissues before and after boiling it, in which the conductivity after boiling was used to normalize tissue size differences (Whitlow *et al*., 1992). Here we describe a procedure to dynamically monitor electrolyte leakage from leaf disks by measuring at multiple time points the electrolytic conductivity of water on which the leaf disks float in a 12-well plate. It is reasonable to assume that the total amount of electrolytes from tissue samples of the same size, such as disks of the same area punched out from leaves of a similar developmental stage, is comparable and that it is not necessary to measure the electrolytic conductivity after boiling the tissues. We inoculated leaves with the bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 pVSP61-*avrRpt2* (*Pto* DC3000 *avrRpt2*) in order to trigger a type of programmed cell death, known as hypersensitive cell death. The protocol presented here has been applied to our studies (Igarashi *et al*., 2013; Bethke *et al.*, 2016; Hatsugai *et al.*, 2016; Hatsugai *et al.*, 2017) and could also be used to quantify plant cell death triggered by any other stimuli. If detailed comparisons of the time courses of the electrolyte leakage are needed, fitting polynomial regression to the time courses is possible (Van Poecke *et al.*, 2007; Qi *et al.*, 2010), as it is easy to obtain electrolytic conductivity measurements at many time points with many replicates.

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Materials and Reagents

1. 2 ml microcentrifuge tubes (Fisher Scientific, catalog number: 05-408-138)
2. Sterilized tubes for liquid bacterial cultures (Evergreen Scientific, catalog number: 222-2094-050)
3. Disposable 1-ml needleless syringes for bacterial inoculation (BD, catalog number: 309659)
4. 12-well cell culture plates (flat bottom with lid) (Corning, Costar®, catalog number: 3513)
5. 1-200 µl Pipette tips (Sorenson BioScience, catalog number: 3211)
6. 50-1,250 µl pipette tips (Sorenson BioScience, catalog number: 3205)
7. *Arabidopsis thaliana* accession Col-0 (Figure 1A)
   Note: Arabidopsis thaliana accession Col-0 carries the R gene RPS2, which confers resistance to Pto DC3000 avrRpt2 (Bent et al., 1994; Mindrinos et al., 1994).
8. *Pseudomonas syringae* pv. *tomato* DC3000 pVSP61-*avrRpt2* (*Pto* DC3000 *avrRpt2*)
   Note: Pto DC3000 avrRpt2 delivers the AvrRpt2 effector into plant cells, thereby inducing hypersensitive cell death in Arabidopsis Col-0.
9. Sterilized ultrapure water (*e.g.*, Milli-Q)
10. Conductivity standard solution 1.41 mS/cm (HORIBA, model: Y071L, catalog number: 514-22)
11. Antibiotics
    a. Kanamycin sulfate (Thermo Fisher Scientific, Gibco™, catalog number: 11815032)
    b. Rifampicin (Sigma-Aldrich, catalog number: R3501)
12. Bacto proteose peptone No. 3 (BD, catalog number: 211693)
13. Glycerol (Fisher Scientific, catalog number: G33-500)
14. Dibasic potassium phosphate (K_2HPO_4) (Fisher Scientific, catalog number: BP363-500)
15. Bacto agar (BD, catalog number: 214010)
16. Hydrochloric acid (HCl) (Fisher Scientific, catalog number: A508-P500)
17. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Sigma-Aldrich, catalog number: 230391)
18. King's B liquid medium (see Recipes)

Equipment

1. Walk-in *Arabidopsis* growth chamber (22 °C, 70% relative humidity, and 12-h/12-h day/night photoperiod) (Conviron, model: BDR40)
2. Tissue culture roller rotator drum for bacterial culture at 28 °C (New Brunswick Scientific, model TC-7)
3. Centrifuge (Eppendorf, model: 5415 D)
4. Spectrophotometer to determine the density of bacterial culture (Beckman Coulter, model: DU-800)
5. Cork borer (size 4, diameter = 7.5 mm)
6. Electrolytic conductivity meter (HORIBA, model: B-173)

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7. Single-channel micropipettor (Eppendorf, 20-200 µl and 100-1,000 µl)
8. Autoclave

Software

1. Microsoft Excel

Procedure

A. Inoculation of *Arabidopsis* leaves with *Pto* DC3000 *avrRpt2*
   1. Culture the bacterial strain in 2 ml King's B liquid medium supplemented with 50 µg/ml kanamycin and 50 µg/ml rifampicin at 28 °C for 16-18 h.
      Note: The bacterial culture used should be in a late log phase of growth (OD_{600} = 1.5 - 2.0).
   2. Transfer 1 ml of the liquid culture to a 2-ml microcentrifuge tube.
   3. Harvest bacteria by centrifugation at 3,000 *x g* for 5 min at room temperature.
   4. Remove supernatant and then suspend bacteria in 1 ml sterilized ultrapure water.
   5. Repeat Steps A3-A4 one more time.
   6. Adjust the optical density of the bacterial suspension with sterilized ultrapure water to OD_{600} = 0.1 (measured by a spectrophotometer).
   7. Pressure-infiltrate the density-adjusted bacterial suspension (Katagiri *et al*., 2002) into two leaves per plant in a walk-in *Arabidopsis* growth chamber.
      Note: Gently pressure-infiltrate the bacterial suspension through the stomatal openings on the abaxial side of the leaf using a 1-ml needleless syringe. The infiltrated area of a leaf is readily visible as the appearance of the infiltrated area turns dark green due to flooding of the intercellular space of the leaf with bacterial suspension.

B. Measurement of electrolytic conductivity
   1. Cut two leaf disks (7.5 mm diameter) from one plant (one disk per leaf) using a cork borer on paper towels (Figure 1B). Leaf disks should be cut out before the infiltrated bacterial suspension from Step A7 dries out from the intercellular space of the leaves, which occurs approximately 20 min after infiltration.
      Note: We typically use 4-weeks old plants grown under the growth conditions described in Equipment section and select the 7th and 8th leaves of each plant to obtain leaves of comparable developmental stages. Selecting leaves in similar developmental stages is important not only to have a similar amount of tissue for each leaf disk but also to have a similar response to the pathogen.
   2. Float two leaf disks (adaxial surface down) from a single plant on 2 ml of sterilized ultrapure water in one well of a 12-well plate, immediately after the leaf disks are cut out in Step B1 (Figure

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1C). Use as many wells and 12-well plates as needed according to the number of plants to be assayed.

3. Cover the plate with the lid and incubate it for 30 min in a walk-in *Arabidopsis* growth chamber.
4. Replace the water in the well with 2 ml of fresh sterilized ultrapure water.
   Note: Steps B3 and B4 are to remove electrolytes initially leaked from the damaged cells on the edges of the leaf disks.
5. Incubate the plate in a walk-in *Arabidopsis* growth chamber.
6. Calibrate the electrolytic conductivity meter before the first use with the conductivity standard solution to 1.41 mS/cm.
7. Wash the sensor of the electrolytic conductivity meter with sterilized ultrapure water.
8. Sample 100 µl of the water per well at the determined time point and measure its conductivity using the sensor of an electrical conductivity meter (Figure 1D).

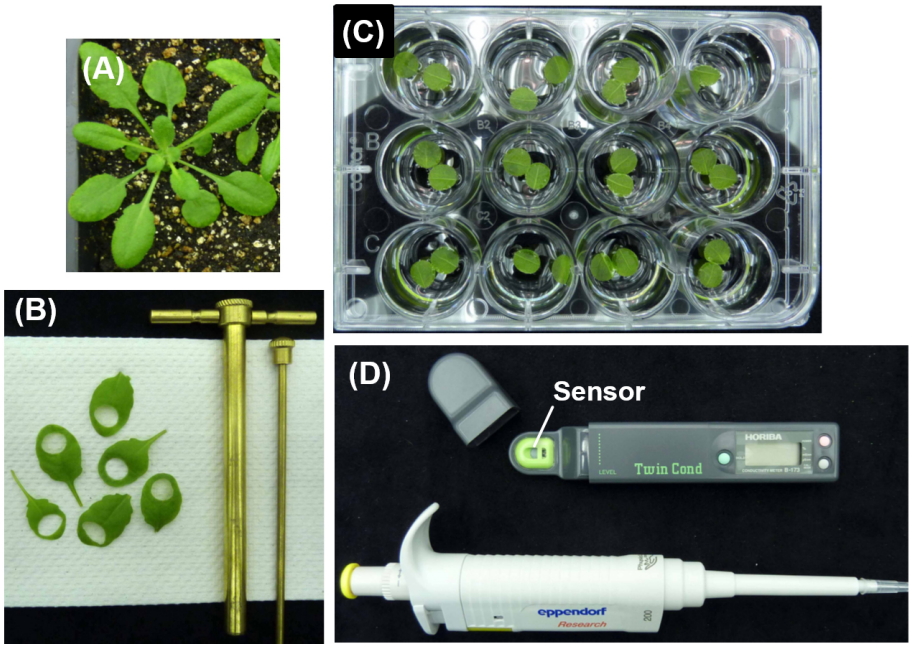

Figure 1. Experimental procedure to measure the electrolytic leakage from *Arabidopsis* leaf disks. A. 4-weeks old *Arabidopsis thaliana* accession Col-0 grown under the growth conditions described in Equipment section. B. Cut two leaf disks (7.5 mm diameter) from one plant (one disk per leaf) using a cork borer on paper towels. C. Float two leaf disks (adaxial surface down) on 2 ml of sterilized ultrapure water in one well of a 12-well cell culture plates. D. Drop 100 µl of the water from one well with a pipette onto the sensor of an electrical conductivity meter and measure its conductivity.

9. Return the sampled water to the well to maintain the water at constant volume during the time course.
10. Continue incubating the plate and repeating Steps B8-B9 at additional time points.

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Note: The sensor of the electrical conductivity meter should be rinsed with sterilized ultrapure water between one sample and the other.

Data analysis

1. For data analysis, directly use the values of the conductivity value obtained from the sensor of the electrical conductivity meter. We recommend inclusion of uninoculated leaf disks in the experiment as a negative control.
   Note: Data analysis is done using Microsoft Excel.
2. The experiments were performed at least three independent times, each of which had four to six biological replicates. For statistical analysis, Student's *t*-test was used for comparison between two genotypes and a one-way analysis of variance (ANOVA) was used for comparison of more than two genotypes. The increased electrolytic conductivity is interpreted as the extent of cell death (Figure 2).

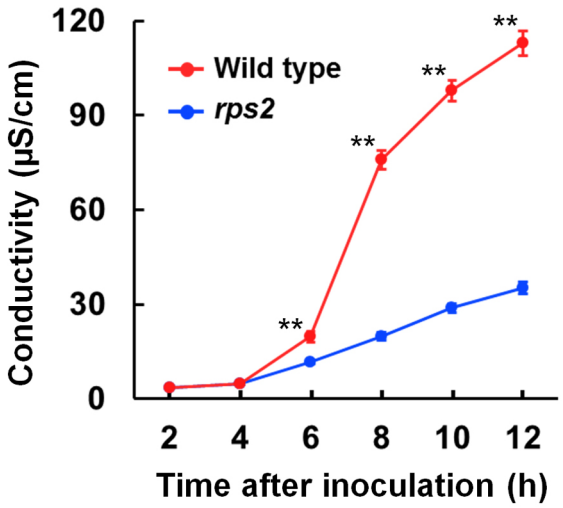

Figure 2. Electrolyte leakage from leaf disks at 2 to 12 h after bacterial inoculation. Representative data of electrolyte leakage from leaf disks. *Pto* DC3000 *avrRpt2* was infiltrated into *Arabidopsis* leaves of wild-type and *rps2* mutant plants. Electrolytic conductivity dramatically increased with wild-type plants after bacterial inoculation, whereas the increase was limited with *rps2* mutant plants. Error bars indicate standard errors of three independent experiments, each with four biological replicates. Asterisks indicate significant differences compared with *rps2* mutant plants (**, $P < 0.01$, two-tailed *t*-tests).

Recipes

1. King's B liquid medium
   20 g of Bacto proteose peptone No. 3
   15 ml of glycerol

1.5 g of dibasic potassium phosphate (K_2HPO_4)
15 g of Bacto agar
Dissolve the ingredients in distilled water (1 L), adjust the pH of the solution to 7-6.8 with hydrochloric acid (HCl), and then autoclave it
After autoclaving, add 6 ml of 1 M filter-sterilized magnesium sulfate heptahydrate ($MgSO_4·7H_2O$) to the 1-L medium
Note: $MgSO_4·7H_2O$ is added after autoclaving to avoid precipitation. If storing liquid KB medium, do not add $MgSO_4$ until just before use.

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