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Vacuole Structure Analysis during Cell Death Subsequent to Application of *Erwinia carotovora* Culture Filtrates to Cell Cultures of *Nicotiana tabacum*

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[Abstract] We recently established an experimental model system for efficient defense-related cell death using tobacco BY-2 cultured cells treated with culture filtrates of the pathogenic bacterium *Erwinia carotovora* (*E. carotovora*) (Hirakawa *et al.*, 2015). Applying this experimental system to transgenic BY-2 cells stably expressing the vacuolar membrane marker GFP-VAM3 (Kutsuna and Hasezawa, 2002) allowed us to monitor changes in vacuolar membrane structures including a decrease of transvacuolar strands during cell death (Hirakawa *et al.*, 2015). Our model system can help to investigate organelle dynamics in defense-related cell death. Here, we show protocol for applying *E. carotovora* filtrates to BY-2 cells and confocal observation of vacuolar membrane dynamics and subsequent cell death. We used cell cycle synchronized BY-2 cells to effectively monitor invaginated vacuolar membranes such as transvacuolar strands in our recent report (Hirakawa *et al.*, 2015); however, we do not describe the protocol for cell cycle synchronization in this article. For the step-by-step protocol for BY-2 cell synchronization, please refer to previous protocol papers (Nagata and Kumagai, 1999; Kumagai-Sano *et al.*, 2006).

Materials and Reagents

1. Sterile filter with pore size 0.22 µm (Merck Millipore Corporation, Millex®–GV Filter unit, catalog number: SLGV033RS)
2. 50 ml syringe (TERUMO CORPORATION, catalog number: SS-50ESZ)
3. 12 well plate (Sumitomo Bakelite Co., catalog number: MS-80120)
4. *Erwinia carotovora* subsp. carotovora (National Institute of Technology and Evaluation, catalog number: 103133)
5. Transgenic tobacco BY-2 cell culture (*N. tabacum* L. cv. Bright Yellow 2) stably expressing GFP-VAM3 (RIKEN Bioresource Center, catalog number: RPC00039)
6. Aphidicolin (Wako Pure Chemical Industries, Siyaku, catalog number: 015-09814)
7. Yeast extract (KANTO KAGAKU, KANTO Chemical, catalog number: 712021-5)
8. Bacto™ Tryptone (BD bioscience, catalog number: 211705)
9. NaCl (KANTO KAGAKU, KANTO Chemical, catalog number: 37144-01)
10. Murashige and Skoog plant salt mixture (Wako Pure Chemical Industries, Siyaku, catalog number: 392-00591)

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11. Sucrose (Wako Pure Chemical Industries, Siyaku, catalog number: 196-00015)
12. *myo*-Inositol solution (Wako Pure Chemical Industries, Siyaku, catalog number: 094-00281)
13. Thiamine hydrochloride (Wako Pure Chemical Industries, Siyaku, catalog number: 201-00852)
14. Sodium 2,4-Dichlorophenoxyacetate Monohydrate (Tokyo Chemical Industry, catalog number: D1319)
15. Lysogeny broth (LB) medium (see Recipes)
16. Modified Linsmaier and Skoog (LS) medium (see Recipes)

Equipment

1. Rotary shaker for *E. carotovora* culture (TAITEC CORPORATION, model: BR-40LF)
2. 300 ml flask for *E. carotovora* culture (Sansyo, Iwaki, catalog number: 82-0087)
3. Spectrophotometer (Beckman Coulter, model: DU® 640)
4. Centrifuge (TOMY SEIKO CO, model: MX-300)
5. Deep freezer (Nihon Freezer, catalog number: CLN-30U)
6. 100 ml flask for BY-2 culture (Sansyo, Iwaki, catalog number: 82-0085)
7. Rotary shaker for the untreated BY-2 culture (TAITEC CORPORATION, catalog number: 300LF)
8. Rotary shaker for the treated BY-2 culture (TAITEC CORPORATION, catalog number: NR-30)
9. Incubator for the treated BY-2 culture (Sanyo, catalog number: MIR-553)
10. Glass base dish (Sansyo, Iwaki, catalog number: 3911-035)
11. Confocal laser scanning microscope (OLYMPUS, model: FV300)

Procedure

A. Preparation of *E. carotovora* culture filtrate
   1. Culture 100 ml of *E. carotovora* in liquid LB medium in a 300-ml flask at 37 °C overnight with rotary shaking at 150 rpm.
   2. Check OD_{600} with a spectrophotometer. The OD_{600} value should be in the range 0.8-1.0 for appreciable cell death induction.
   3. Centrifuge the bacterial cell culture for 10 min at 4,000 *x g* at room temperature.
   4. Collect the supernatant and sterile filter it with a syringe filter.
   5. The filtrate can be kept at -80 °C in a deep freezer for several months.

B. Application of the filtrate to cell cycle synchronized tobacco culture cells
   1. Prepare the S-phase synchronized transgenic BY-2 cells expressing GFP-VAM3 by DNA polymerase inhibitor (aphidicolin) treatment and wash out with fresh modified LS

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medium (Nagata and Kumagai, 1999; Kumagai-Sano *et al.*, 2006).

2. Just after the washout, place 30 ml of the synchronized culture cells in a 100-ml flask on a rotary shaker culture at 27 °C for 30 min at 130 rpm.
3. Add 1.6 ml of the cell cycle synchronized culture into a well of the 12-well plate.
4. Add 0.4 ml of the *E. carotovora* culture filtrate into the cell culture well [final concentration: 20% (v/v)].
5. Culture the treated cells on a rotary shaker in an incubator at 27 °C for two hours.
6. Collect 150 µl of the cells into a microscope glass dish base for observation.
7. Place the dish on the stage of the confocal laser scanning microscope.
8. Acquire time-lapse images of GFP-VAM3 at 5-min intervals with 488 nm excitation lasers and 524-546 nm emission filters according to the microscope manufacturer's instructions. See Figure 1 for representative results.

Representative data

The representative time-lapse images of GFP-VAM3 were shown in Figure 1. After the filtrate treatment, the invaginated vacuolar membrane structures including transvacuolar strands gradually decreased (Figure 1). The percentage of transvacuolar strand-less cells reached 12.8±3.00% 4 h after the filtrate treatment while 1.52±1.48% in the mock treatment (Hirakawa *et al.*, 2015).

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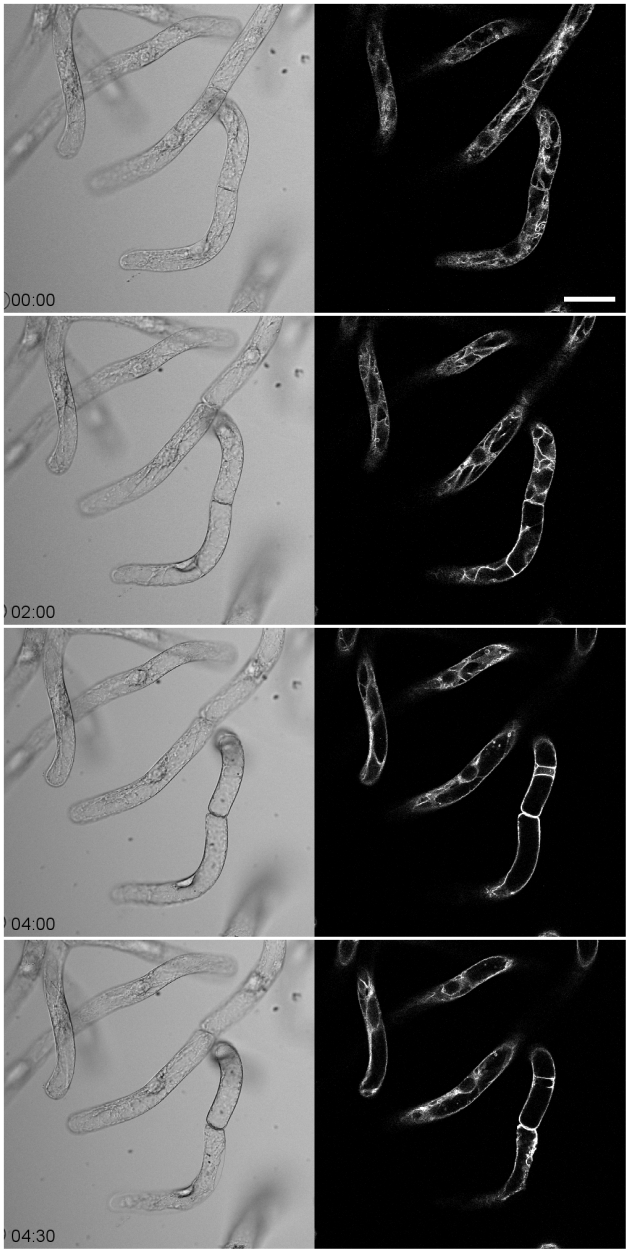

Figure 1. Time-lapse observation of tobacco BY-2 cells expressing GFP-VAM3 treated with culture filtrates of *Erwinia carotovora*. Note that the invaginated vacuolar membranes gradually decreased before cell death with cell shrinkage. The time-lapse images were obtained at 5-min intervals for 4.5 h. Scale bar indicates 50 μm.

Notes

You should wait around 10 min after the placement of a microscope glass dish base on the microscope stage to start a time-lapse imaging. The cells would be completely settled down to the bottom while waiting, resulting in appreciated results without the cell displacement.

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Recipes

1. LB medium

   Note: No need to adjust pH.

Yeast extract	5 g/L
Bacto™ tryptone	10 g/L
NaCl	5 g/L

2. Modified LS medium

Murashige and Skoog plant salt mixture	4.6 g/L
Sucrose	30 g/L
myo-Inositol solution	200 mg/L
Thiamine hydrochloride	1 mg/L
2,4-Dichlorophenoxyacetic acid	0.2 mg/L

   Adjust pH to 5.8 with KOH

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