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Real-time PCR Analysis of PAMP-induced Marker Gene Expression in *Nicotiana benthamiana*

Fan Liu, Yuanpeng Xu, Yan Wang and Yuanchao Wang*

Department of Plant Pathology, Nanjing Agriculture University, Nanjing, China
*For correspondence: wangyc@njau.edu.cn

[Abstract] Perception of pathogen-associated molecular patterns (PAMPs) often triggers various innate immune responses in plants. The transcriptional changes of defense-related genes are often used as a marker to assay PAMP-triggered plant immune response. Here we described a protocol to monitor the relative expression level of marker genes in *Nicotiana benthamiana* upon treatment with PAMPs. The procedure includes leaf treatment using PAMPs, total RNA isolation, cDNA synthesis, quantitative real-time PCR and data analysis. This protocol is applicable to monitor marker gene expression triggered by different PAMPs in *N. benthamiana.*

Keywords: PAMPs, PTI, Real-time PCR, *Nicotiana benthamiana,* Defense-related marker gene

[Background] Pathogen-associated molecular patterns, namely PAMPs, are a class of molecules derived from pathogens and are relatively conserved across microorganisms. Multiple PAMPs such as flg22 and XEG1 (Felix *et al*., 1999; Ma *et al.*, 2015), have been characterized that can be detected by plant cell surface localized pattern-recognition receptors (PRRs) and thereby induce PAMP-triggered immunity (Couto and Zipfel, 2016). One of the predominant PAMP-triggered responses is the activation of defense-related maker genes (Navarro *et al.*, 2004; Zipfel *et al.*, 2006). *Nicotiana benthamiana* has been used extensively as a model plants and is sensitive to multiple PAMPs. In *N. benthamiana*, the marker genes, such as *NbCYP71D20, NbACRE31 and NbWRKY22*, were previously found that are rapidly activated upon PAMP treatment (Heese *et al.*, 2007; Segonzac *et al*., 2011; Wang *et al.*, 2018). Here, we describe a detailed protocol for checking the PAMP-triggered marker gene expression in *N. benthamiana*. The relative gene expression was also determined in parallel using a negative control to exclude the background noise.

Materials and Reagents

1. MicroAmp™ Splash-Free 96-Well Base (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4312063)
2. 1,000 µl pipette tips (Corning, Axygen®, catalog number: TF-1000-R-S)
3. 200 µl pipette tips (Corning, Axygen®, catalog number: TF-200-R-S)
4. 10 µl pipette tips (Corning, Axygen®, catalog number: TF-300-R-S)
5. 1 ml needless syringe (BD, catalog number: 309659)
6. 1.5 ml RNase-free tube (Corning, Axygen®, catalog number: MCT-150-C)
7. Axygen® 0.2 ml Polypropylene PCR Tube Strips (8-Tubes/Strip) (Corning, Axygen®, catalog

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number: PCR-0208-C)

8. MicroAmp™ Optical 96-Well Reaction Plate with Barcode (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4306737)
9. MicroAmp™ Optical Adhesive Film (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4360954)
10. Tissue paper
11. Healthy 5-6 weeks *N. benthamiana* plants (see Figure 1)
12. Any PAMPs of interest and corresponding control solution
13. Liquid nitrogen
14. β-mercaptoethanol (Solarbio, catalog number: M8210)
15. E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, catalog number: R6834-01)
16. DNase/RNase-free ddH_2O (Solarbio, catalog number: R1600)
17. PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Clontech, catalog number: RR047A)
18. TB Green™ Premix Ex Taq™ (Tli RNase H Plus) (Takara Bio, Clontech, catalog number: RR420A)
19. 1 mM flg22 stock solution (Gene Script, RP19986) (see Recipes)
20. 1 mM XEG1 stock solution (see Recipes)
21. 70% ethanol (see Recipes)

Equipment

1. Mortar and pestle
2. Growth chamber
3. NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, model: NanoDrop™ 1000)
4. Vortexer (Scientific Industries, model: Vortex-Genie 2, catalog number: SI-0246)
5. Cold Centrifuge (Eppendorf, model: 5424 R, catalog number: 5404000014)
6. Pipettes 100-1,000 µl (Eppendorf, catalog number: 3120000062)
7. Pipettes 10-100 µl (Eppendorf, catalog number: 3120000046)
8. PCR Thermal Cyclers (Thermo Fisher Scientific, Applied Biosystems™, model: 2720, catalog number: ED000651)
9. MicroAmp™ Adhesive Film Applicator (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4333183)
10. MPS 1000 Mini PCR Plate Spinner (Labnet International, catalog number: C1000)
11. ABI 7500 fast real-time PCR system (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4351107)

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Software

1. Applied Biosystems Sequence Detection Software v1.4.0

Procedure

A. Infiltration of *Nicotiana benthamiana*

1. *N. benthamiana* plants were grown in a growth chamber at 19-22 °C under long-day conditions (14-h-light/10-h-dark) and 70-80% relative humidity for 5-6 weeks as shown in Figure 1.

Figure 1. *Nicotiana benthamiana* plant used for PAMP treatment

2. *N. benthamiana* leaves of the same position and similar size were treated with PAMPs (*e.g.*, flg22) or control (*e.g.*, ddH_2O) by infiltration using 1 ml needleless syringe (Figure 2). The infiltrated plant were dried gently using the tissue paper and kept in the growth chamber for 3-6 h before collected and frozen in liquid nitrogen.

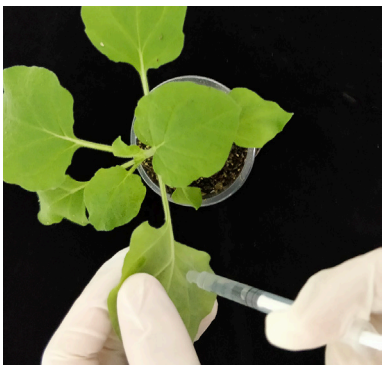

Figure 2. Infiltration of *N. benthamiana* using the needless syringe

B. Total RNA isolation

Total RNA was isolated from *N. benthamiana* leaves using E.Z.N.A.® Total RNA Kit I isolation kit according to the manufacturer's instructions with proper modifications.

1. Harvest the *N. benthamiana* leaves at 3-6 h after PAMP treatment. Grind the frozen tissues into

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powder with a mortar and pestle in liquid nitrogen.

2. Transfer the leaf powder into the 1.5 ml RNase-free tube with 700 µl TRK Lysis Buffer and 14 µl β-mercaptoethanol, vortex the samples for 30 sec, and maintain the sample at 4 °C for 5-10 min.
3. Centrifuge the samples at 15,000 *x g* for 5-10 min at 4 °C, carefully transfer 600 µl supernatant into a new 1.5 ml RNase-free tube.
4. Add equal volume (600 µl) 70% ethanol (see Recipes).
5. Add the mixed liquid into the HiBind® RNA Mini Column (assembled 2 ml collection tube), centrifuge at 10,000 *x g* for 30 sec at 4 °C. Discard the filtrate and reuse the collection tube.
6. Add 300 µl RNA Wash Buffer I to the HiBind® RNA Mini Column, centrifuge at 10,000 *x g* for 30 sec at 4 °C. Discard the filtrate and reuse the collection tube.
7. Add 500 µl RNA Wash Buffer I to the HiBind® RNA Mini Column, centrifuge at 10,000 *x g* for 30 sec at 4 °C. Discard the filtrate and change a new 2 ml collection tube.
8. Add 500 µl RNA Wash Buffer II, centrifuge at 10,000 *x g* for 30 sec at 4 °C. Discard the filtrate and reuse the collection tube.
9. Repeat Step B8.
10. Centrifuge at 10,000 *x g* for 2 min to completely dry the HiBind® RNA Mini Column.
11. Put the HiBind® RNA Mini Column into a new 1.5 ml RNase-free tube, add 50 µl DNase/RNase-free ddH_2O into Column, and let it sit at room temperature for 1 min. Centrifuge at 10,000 *x g* for 1 min at 4 °C to elute RNA from the HiBind® RNA Mini Column.
12. Measure RNA concentration using Nanodrop™ 1000. Store the eluted RNA at -70 °C.

C. First-strand cDNA synthesis

Total RNA was reverse transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time).

1. Remove gDNA reaction

   Reaction mixture:

5x gDNA Eraser Buffer	2 µl
gDNA Eraser	1 µl
Total RNA	700 ng (calculate volume)
DNase/RNase-free ddH_2O	up to 10 µl

   Reaction program:

42 °C	2 min
4 °C	∞

2. Reverse transcription reaction

   Reaction mixture:

The reaction solution of Step C1	10 µl
PrimeScript™ RT Enzyme Mix I	1 µl
RT Primer Mix	1 µl

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5x PrimeScript™ Buffer 2 (for Real Time)	4 µl
DNase/RNase-free ddH_2O	up to 20 µl

Reaction program:

37 °C	15 min
85 °C	5 sec
4 °C	∞

3. Dilute the obtained cDNA 20 times with 180 µl RNase-free H_2O.

D. Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was performed using TB Green™ Premix Ex Taq™ Kit (Tli RNase H Plus):

1. qRT-PCR reaction mix:

2x TB Green™ Premix Ex Taq™ (Tli RNase H Plus)	10 µl
50x ROX Reference Dye II	0.4 µl
qRT-PCR Forward Primer	0.4 µl
qRT-PCR Reverse Primer	0.4 µl
The diluted first-strand cDNA	5 µl
DNase/RNase-free ddH_2O	up to 20 µl

2. Add the qRT-PCR reaction mix into each wall of MicroAmp™ Optical 96-Well Reaction Plate with Barcode with three replicates for each sample. Seal the plate using MicroAmp™ Optical Adhesive Film, and centrifuge the plate at room temperature for 3 min using the MPS 1000 Mini PCR Plate Spinner.
3. qRT-PCR program:

Stage 1: Pre-denaturation at 95 °C for 30 sec

Stage 2: PCR amplification (Reps: 40)

95 °C 5 sec

60 °C 34 sec

Stage 3: Melting Curve

95 °C 15 sec

60 °C 1 min

95 °C 15 sec

Data analysis

Ct (cycle threshold) values were collected and exported from the Sequence Detection Software v1.4.0. Relative expression of marker genes in each sample was normalized against the internal control (Endo), such as the housekeeping genes *NbActin* or *NbEF1α*, and was calculated using the formula: $2^{-\Delta CT} = 2^{-(Ct_{target} - Ct_{Endo})}$ calculated as ΔOD1-ΔOD2.

The relative expression level of marker genes in PAMP-treated samples to the control-treated sample was calculated using the following formula: $2^{-\Delta\Delta CT} = 2^{-(\Delta CT_{PAMP} - \Delta CT_{control})}$ (Figure 3).

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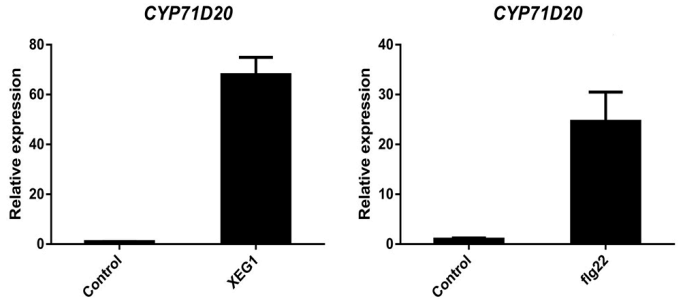

Figure 3. Transcription level changes of PTI marker gene *CYP71D20* upon XEG1 or flg22 treatment. Transcript levels were quantified by qRT-PCR and normalized to internal control *NbEF-1α*. Bars represent the mean fold changes (± S.E.M.) of the XEG1-treated or flg22-treated leaves relative to the value in control-treated leaves, which was set as 1.

Notes

1. It is important to use plants of similar size. Leaves of different plants should be collected at the same position with similar size.
2. The negative control should be included to measure the background level.
3. Cross-contamination of the PAMPs and negative control should be avoided during infiltration.
4. For each experiment, at least three replicates should be included for statistical analysis.
5. Different PAMPs may induce the defense-related marker gene expression at different time points.
6. The qRT-PCR program applies to Applied Biosystems 7500 fast Real-Time PCR System.

Recipes

1. 1 mM flg22 stock solution
   2.27 mg of flg22 (QRLSTGSRINSAKDDAAGLQIA) (Molecular Weight: 2272.52) was dissolved in 1 ml ddH_2O, and store at -20 °C. The stock was diluted to a final concentration of 200 nM
2. 1 mM XEG1 stock solution
   2.73 mg of XEG1 was dissolved in 1 ml ddH_2O, and store at -20 °C. The stock was diluted to a final concentration of 200 nM
3. 70% ethanol
   Add 30 ml DNase/RNase-free ddH_2O to 70 ml of absolute ethanol

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Competing interests

The authors declare that they have no conflicts of interest or competing interests.

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