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The Application of Quercetin to Study the Effect of Hsp70 Silencing on Plant Virus Infection in *Nicotiana benthamiana* Plants

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[Abstract] *Pepino mosaic virus (PepMV*) is a mechanically-transmitted pathogen affecting tomato plants worldwide. Like with other plant viruses (Verchot, 2012), the heat shock cognate protein 70 homolog (Hsc70) was identified as an interactor of the *PepMV* coat protein (CP) (Mathioudakis *et al*., 2012). Here, we describe a pharmacological approach to silence Hsp70 in plants using quercetin (Mathioudakis *et al*., 2014), an Hsp70 protein expression flavonoid inhibitor (Hosokawa *et al*., 1990; Manwell and Heikkila 2007). In the case of Hsp70, this methodology represents a faster and easier approach than silencing of Hsp70 by reverse genetics assays, such as VIGS methodology. Fully expanded leaves of 2 to 3 weeks old *Nicotiana benthamiana* plants were infiltrated, using a syringe, with either quercetin (dissolved in DMSO) or DMSO (control plants). The plants were mechanically inoculated with *PepMV* virus inocula. The accumulation of Hsp70 and *PepMV* were analyzed on local leaves by immunoblot analysis 4 days post inoculation.

Materials and Reagents

1. 1 ml syringe without a needle (BD, Nipro, catalog number: SY3 1 C100U ET)
2. Mortar and pestles (Carl Roth GmbH + Co., catalog number: XL96.1 and XP01.1)
3. Pelet pestles (Nippon Genetics, catalog number: NG006)
4. 13 ml round base tubes (SARSTEDT AG & Co, catalog number: 62.515.006)
5. 1.5 ml microtubes (SARSTEDT AG & Co, catalog number: 72.690.001)
6. *Nicotiana benthamiana* leaves from 2 to 3 weeks old seedlings
7. *PepMV*-Sp13 isolate infected material
8. Quercetin hydrate (Sigma-Aldrich, catalog number: 337951)
9. Dimethyl sulfoxide (DMSO) (AppliChem GmbH, catalog number: A3009)
10. 10 mM Sodium carbonate (Na_2CO_3) (Sigma-Aldrich, Riedel-de Haen, catalog number: 31432)
11. Sodium dihydrogen phosphate monohydrate (0.5 M, pH 7.0) (AppliChem GmbH, catalog number: A3559)
12. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S0876)
13. Potassium dihydrogen phosphate (KH_2PO_4) (Merck Millipore Corporation, catalog number: 104873)

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14. Potassium chloride (KCl) (Sigma-Aldrich, Riedel-de Haen, catalog number: 31248)
15. Sodium Chloride (NaCl) (AppliChem GmbH, catalog number: A1149)
16. Magnesium Chloride 6-hydrate ($MgCl_2$) (AppliChem GmbH, catalog number: A4425)
17. Silicon carbide (carborundum) 400 mesh (Sigma-Aldrich, catalog number: 357391)
18. Bromophenol blue (Sigma-Aldrich, catalog number: B-8026)
19. Coomassie Brilliant Blue (AppliChem GmbH, catalog number: A1092)
20. Glycerol (Sigma-Aldrich, catalog number: G6279)
21. Tris ultrapure (1.5 M, pH 8.8; 1 M, pH 6.8) (AppliChem GmbH, catalog number: A1086)
22. β-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
23. 30% 37.5:1 Acrylamide/Bis solution (Bio-Rad Laboratories, AbD Serotec®, catalog number: 161-0158)
24. 10% Ammonium persulfate (APS) (AppliChem GmbH, catalog number: A2941)
25. N, N, N´, N´-tetramethylethylenediamine (TEMED) (AppliChem GmbH, catalog number: A1148)
26. 10% Sodium dodecyl sulfate (SDS) (AppliChem GmbH, catalog number: A2263)
27. Glycine (AppliChem GmbH, catalog number: A1067)
28. Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
29. Methanol (Thermo Fisher Scientific, catalog number: M/4000/17)
30. Acetic acid (Sigma-Aldrich, catalog number: 33209)
31. PepMV CP polyclonal antibody (Neogen/Adgen Phytodiagnostics, catalog number: 1127-01)
32. HSP70 monoclonal antibody (Enzo Life Sciences, Stressgen, catalog number: N27F3-4)
33. Anti-rabbit IgG, Alkaline phosphatase-conjugated antibody (Promega Corporation, catalog number: S3731)
34. Anti-mouse IgG, Alkaline phosphatase-conjugated antibody (Promega Corporation, catalog number: S3721)
35. BCIP/NBP Color Development Substrate (Promega Corporation, catalog number: S3771)
36. PVDF membrane, Westran Clear Signal (Thermo Fisher Scientific, Whatmann, catalog number: 10485289)
37. Tween-20 (Sigma-Aldrich, catalog number: P2287)
38. Phosphate buffer saline (PBS) (see Recipes)
39. Protein extraction buffer (see Recipes)
40. 4x Laemmli buffer (see Recipes)
41. SDS-PAGE buffers: Separation and Stacking gel buffers (see Recipes)
42. Western Blot buffers: Running, Transfer, Wash and Detection buffers (see Recipes)

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Equipment

1. Plant growth chamber (25 °C, light:dark =16:8 h)
2. Biofuge stratos highspeed table centrifuge (Thermo Fisher Scientific, Heraeus, catalog number: 75005282)
3. Fixed angle rotor #3334 (Thermo Fisher Scientific, Heraeus, catalog number: 75003334)
4. Fixed angle microliter rotor #3332 (Thermo Fisher Scientific, Heraeus, catalog number: 75003332)
5. Mini-PROTEAN 3 Cell Electrophoresis System (Bio-Rad Laboratories, AbD Serotec®, catalog number: 165-3301)
6. Mini Trans-Blot Electrophoretic Transfer Cell apparatus (Bio-Rad Laboratories, AbD Serotec®, catalog number: 170-3930)
7. Power Pack Supply model 200/2.0 (Bio-Rad Laboratories, AbD Serotec®, catalog number: 165-4761)
8. Western blot membranes were visualized using the Gel Doc™ XR Molecular Imager & System (Bio-Rad Laboratories, AbD Serotec®, catalog number: 170-8195 EDU)
9. Julabo Water-bath TW12 (Sigma-Aldrich, catalog number: Z615498)
   Note: Pricing & availability is not currently available.

Software

1. Quantification of the band intensity as absorbance units was conducted by Quantity One analysis software (Bio-Rad Laboratories, AbD Serotec®, catalog number: 170-9600)

Procedure

A. Preparation of *PepMV* inocula

Infected freeze dried material with the *PepMV*-Sp13 isolate (Aguilar *et al.*, 2002, kindly provided from Dr. M. Aranda, CSIC-CEBAS Murcia, Spain) was homogenized with a ratio 1:10 (w/v) in NaH_2PO_4 0.5 M phosphate buffer using a mortar and pestle, and used as a fresh virus inocula.

B. Silencing of Hsp70 by quercetin application

1. Quercetin was easily dissolved in DMSO in order to prepare an initial stock of 200 mM. The quercetin stock was subsequently diluted in a final concentration of 1 mM using a 10 mM Na_2CO_3 solution (*e.g.*, 5 µl of 200 mM quercetin per 1 ml). DMSO alone was diluted accordingly to quercetin using the 10 mM Na_2CO_3 solution. Stock and working solutions of quercetin were always freshly prepared.

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2. One fully expanded leaf of 2 to 3 weeks old *N. benthamiana* plants (Figure 1) was totally (entirely) infiltrated, from the bottom side, using a syringe (Figure 2) either with 1 mM quercetin solution or diluted DMSO (control plants). Approximately to infiltrate an entire fully expanded leaf 1 ml of the corresponding solution was used.

C. Viral infection by rub inoculation

1. One hour after the plant infiltration (kept at room temperature), the treated leaves with quercetin or DMSO were slightly dusted with carborundum.
2. Five minutes later the leaves were mechanically inoculated with equal amount (~30 µl) of freshly prepared virus inocula. After the inoculation, plants were kept at growth chambers for four days at 25 °C and 16-h light and 8-h dark cycle.

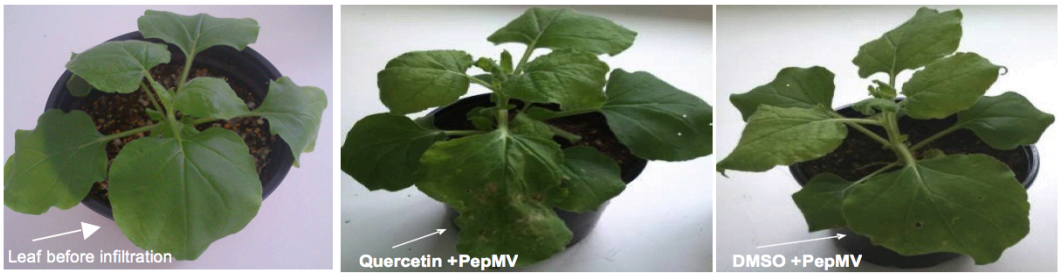

Figure 1. *N. benthamiana* leaf before infiltration with DMSO or quercetin (left panel), and DMSO- or quercetin-treated *N. benthamiana* plants 4 days after *PepMV* rub inoculation (arrows indicate the DMSO- or quercetin-infiltrated leaves)

Figure 2. The left panel shows the infiltration method by syringe in *N. benthamiana* leaves (bottom side) and the right panel shows the leaves after infiltration

D. Analysis of the Hsp70 expression levels in Hsp70-silenced plants and the effect in viral Infection

1. Four days post *PepMV* inoculation the leaves of *N. benthamiana* plants treated with quercetin or DMSO were collected (Figure 1). Control plants did not show any phenotype.
2. Total proteins were extracted from 0.1 g of plant tissue using 300 µl of protein extraction buffer. Briefly: after the grinding of the infiltrated leaves to a fine powder

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using liquid nitrogen with a mortar and pestle, 0.1 g of tissue powder was homogenized using 300 µl of extraction buffer in 1.5 ml microtube (using pellet pestle sticks) and good vortex (2 min). 150 µl were mixed with 50 µl of 4x Laemmli buffer and boiled at 95 °C for 5 min. After a centrifugation at 12,000 rpm for 1 min the supernatant of the samples was used in the following steps.

3. Small Rubisco unit protein was used for quantification of equal loading of total protein extracts in Coomassie Brilliant Blue staining (Figure 3C). After the SDS-polyacrylamide gel electrophoresis (PAGE) on 12% gels, the proteins were electrophoretically transferred onto PVDF membrane. Western Blot analysis was performed under standard conditions. Briefly, the blotted membranes were firstly incubated with the blocking buffer (1x TBS-T, 4% milk) for 1 hour at room temperature and subsequently with the primary antibodies (all conjugated to alkaline phosphatase [AP]), overnight at 4 °C. The following morning the membranes were washed 3 times and then incubated with the secondary AP-conjugated antibodies for 1 h at room temperature. Finally, after washing the membranes 3 times, the blotted proteins were detected using the NBT/BCIP substrate.
4. In this given example, the results showed that the application of quercetin for silencing Hsp70 reduced its protein levels up to 73% (Figure 3A). This reduction of Hsp70 in the case of our example on *PepMV* infection had a corresponding negative effect on viral accumulation up to 92% (Figure 3B).

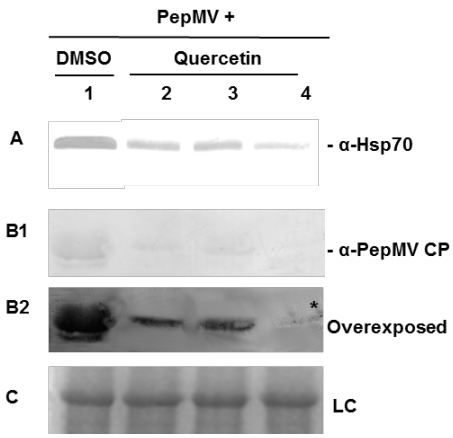

Figure 3. Immunoblot analysis of Hsp70 expression levels and *PepMV* accumulation in *PepMV*-inoculated leaves treated either with DMSO (lane 1) or quercetin (lanes 2 to 4, corresponding to different replicate plants), using α-Hsp70 (A panel) and α-*PepMV* CP (B1 panel) antibodies. B2 panel is an overexposure of B1 and the asterisk indicates the faint CP band. In panel C the small unit of Rubisco protein stained with Coomassie brilliant blue served as protein loading control (LC).

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Recipes

1. 10x PBS
   40 g NaCl
   1 g KCl
   7.2 g Na_2HPO_4
   1.2 g KH_2PO_4
   dd H_2O to 1 L
   Fix pH to 6.8
2. Protein extraction buffer
   For 10 ml: Mix 1 ml 10x PBS
   2% β-mercaptoethanol
   1 mM PMSF
3. 4x Laemmli buffer
   250 mM Tris-HCl (pH 6.8)
   8% SDS
   40% glycerol
   20% β-mercaptoethanol
   0.02% Bromophenol Blue
4. SDS-PAGE buffers
   a. Separation gel:
      0.375 M Tris-HCl (pH 6.8)
      12% Acrylamide/Bis solution
      0.1% SDS
      0.1% APS
      0.01% TEMED
   b. Stacking gel:
      0.125 M Tris-HCl (pH 6.8)
      4% acrylamide/Bis solution
      0.1% SDS
      0.1 M APS
      0.01% TEMED
5. Western Blot buffers
   a. Running buffer
      3 g Tris base
      14.4 g glycine
      1 g SDS
      dd H_2O to 1 L, fix pH to 8.3
   b. Transfer buffer (1x TBS)
      3.03 Tris base

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14.4 g glycine

200 ml methanol

dd H_2O to 1 L

c. 1x TBS buffer

6.57 g Tris base

8.76 g NaCl

dd H_2O to 1 L, fix pH to 7.4

d. Wash buffer (1x TBS-T)

1x TBS

10% Tween-20

Antibodies were diluted in 1x TBS-T and 4% milk powder

e. Detection buffer

0.1 M Tris base

0.15 M NaCl

1 mM $MgCl_2$

33 µl NBT and 16.5 µl BCIP substrates were mixed in 5 ml of detection buffer

Acknowledgments

This protocol was adopted from previous work (Hafrén *et al*., 2010; Wang *et al*., 2009). Matthaios Mathioudakis was a recipient of an Onassis Foundation doctoral fellowship.

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