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# Detecting the Interaction of Double-stranded RNA Binding Protein, Viral Protein and Primary miRNA Transcript by Co-immunoprecipitation *in planta*

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**[Abstract]** MicroRNAs (miRNAs) play important roles in plant growth, development, and response to infection by microbes. Double-stranded RNA binding protein 1 (DRB1) facilitates the processing of primary miRNA transcripts into mature miRNAs. Recently, we found that NS3 protein encoded by *rice stripe virus* (RSV) associates with DRB1 and promotes miRNA biogenesis during RSV infection (Zheng *et al.*, 2017). RNA co-immunoprecipitation (RIP) method was applied to identity association patterns among DRB1, NS3, and miRNA transcript.

**Keywords:** *Rice stripe virus*, NS3, Double-stranded RNA binding protein, Primary-miRNA, miRNA, Plant-microbe interaction

**[Background]** MicroRNAs (miRNAs) are processed from their primary transcripts (pri-miRNAs) by the RNase III enzyme DICER-LIKE 1 (DCL1) with the help of the double-stranded RNA (dsRNA) binding protein HYPONASTIC LEAVES1 (DRB1/HYL1) and the zinc finger protein SERRATE (SE). *Rice stripe virus* (RSV) infection broadly perturbs miRNA accumulation. We found that RSV-encoding nonstructural protein 3 (NS3) promotes miRNA accumulation by downregulating pri-miRNAs through interaction with DRB1 in rice (Zheng *et al.*, 2017). To reveal how NS3 enhances pri-miRNA processing, we used co-immunoprecipitation (Co-IP) to illustrate the relationship of NS3, DRB1 and pri-miRNA *in vivo*. This protocol contributes to understand association patterns between two proteins and one RNA transcript.

## Materials and Reagents

1. Pipette (RNase free 1 ml, 0.2 ml and 0.02 ml, Axygen)
2. Miracloth (Merck, Calbiochem, catalog number: 475855)
3. Centrifuge tube (1.5 ml, 2 ml and 50 ml) (Corning)
4. 4- to 6- weeks old *Nicotiana benthamiana* (leaves, grow in green house)
5. *Agrobacterium tumefaciens* (EHA105 strain, preserved in our lab)
6. Expression plasmids: pEarleyGate202-*DRB1*, *-mutant DRB1*, pEarleyGate203-*NS3*, *-mutant NS3* and pCAMBIA-*artificial primary miRNA transcript*, *-mutant primary miRNA transcript* (Constructed by ourselves)

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7. Double-distilled or MilliQ water ($ddH_2O$)
8. Formaldehyde (Sigma-Aldrich, catalog number: F8775)
9. PBS (Thermo Fisher Scientific, Gibco™, catalog number: 70011069)
10. Glycine (Sigma-Aldrich, catalog number: V900144)
11. Liquid nitrogen
12. Anti-Myc (9E10) monoclonal antibodies (Sigma-Aldrich, catalog numbers: M4439) and mouse IgG control (Thermo Fisher Scientific, Invitrogen, catalog number: 02-6502)
13. Protein G-agarose (Roche Diagnostics, catalog number: 11243233001)
14. Trizol
15. Chloroform (Sigma-Aldrich, catalog number: 613304)
16. GlycoBlue™ coprecipitant (15 mg/ml) (Thermo Fisher Scientific, Invitrogen™, catalog number: AM9516)
17. Ethanol (Sigma-Aldrich, catalog number: E7023)
18. Ethanol (Aladdin, catalog number: E111963)
19. DNase I (Promega, catalog number: M6101)
20. Sucrose (Sigma-Aldrich, catalog number: V900116)
21. Ficoll 400 (Sigma-Aldrich, catalog number: F9378)
22. Dextran T40 (Sigma-Aldrich, catalog number: 1179708)
23. HEPES (Sigma-Aldrich, catalog number: RDD002)
24. KOH
25. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
26. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: M8266)
27. EDTA-free protease inhibitor cocktail (Roche Diagnostics, catalog number: 05892953001)
28. DTT (DL-Dithiothreitol) (Sigma-Aldrich, catalog number: 43817)
29. Tris-HCl (Sigma-Aldrich, catalog number: V900312)
30. NP-40 (Sigma Aldrich, catalog number: NP40S)
31. RNase inhibitor (RNaseOUT) (Thermo Fisher Scientific, Invitrogen™, catalog number: 10777019)
32. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: V900312)
33. Diethyl pyrocarbonate (Sigma-Aldrich, catalog number: 40718)
34. Honda buffer (see Recipes)
35. High salt nuclear lysis buffer (see Recipes)
36. Dilution buffer (see Recipes)
37. IP buffer (see Recipes)

## Equipment

1. Eppendorf pipettes suite (1 ml, 0.2 ml, 0.02 ml, 0.01 ml and 0.0025 ml)
2. Thermomixer C (Eppendorf, model: Thermomixer® C, catalog number: 5382000023)

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3. Vacuum pump (FJC, model: 6912)
4. Rotator (Glas-Col, model: 099A MR1512)
5. Centrifuge (Eppendorf, models: 5424 R and 5804 R)
6. Pico Ultrasonicator (Diagenode, model: Bioruptor® Pico, model: 4486126)
7. Vortex (Kylin-Bell Lab Instruments, model: VORTEX-5)
8. Pestle

## Procedure

A. Material preparation

*Agrobacterium tumefaciens*-mediated transient co-expression of corresponding proteins and RNAs was performed with 4- to 6-week old *N. benthamiana*. *N. benthamiana* leaves (~8 g). Harvest the samples at 3 dpi (days post inoculation).

B. Nuclei isolation

1. Cross-link *N. benthamiana* leaves with 1% formaldehyde in 1x PBS for 15 min.
   *Note: Make sure the sample is submerged in the buffer, apply the vacuum for 5 min, release, Reapply vacuum, repeat this for 10 times.*
2. Add glycine to a final concentration of 0.125 M, mix well the buffer and apply vacuum for 5 min to stop cross-linking.
3. Rinse the plants three times with sterile water. Remove excess water, freeze the plants in liquid nitrogen. If not proceed to the next step immediately, store the frozen plants at -80 °C.
4. Grind the plant material into fine powder with liquid nitrogen in mortar. Transfer the power to a 50 ml centrifuge tube.
5. For each sample (2 g), add 15 ml Honda buffer, rotate at 4 °C for 5 min.
6. Filter through one layer of Miracloth, then wash the Miracloth by adding another 10 ml of Honda buffer (in a 50 ml centrifuge tube).
7. Centrifuge at 3,500 *x g* for 5 min at 4 °C. Discard the supernatant, softly resuspend the pellet in 1 ml Honda buffer with 1 ml pipet with end-cut tip.
8. Centrifuge at 3,500 *x g* for 5 min, wash the pellet once more with 1 ml Honda buffer. Centrifuge at 10,000 *x g* for 1 min and remove the supernatant thoroughly.

C. Immunoprecipitation

1. Add 2.5 volumes/weight high salt nuclear lysis buffer in nuclear pellets, resuspend nuclei and sonicate (30 sec long pulses, 30 sec intervals, 10 cycles) by using a Diagenode Bioruptor.
2. Centrifuge (16,000 *x g*, 4 °C) for 10 min, take supernatant (400 µl) into a 2 ml centrifuge tube, add four volumes of dilution buffer, mix well, take out 1/20, and keep it as input.
3. Add anti-Myc (10 µg/2 ml) antibody, rotate at 4 °C for 2 h, then add 50 µl of Protein G and rotate for another 2 h.

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4. Centrifuge at 1,500 *x g* for 5 min at 4 °C. Remove supernatant carefully and add IP buffer. Wash beads with IP buffer for 3 times.
5. Take out 1/10 of IP extract for Western blot detection.
6. Centrifuge at 1,500 *x g* for 5 min at 4 °C, remove the supernatant thoroughly.

D. RNA extraction and RT-PCR
1. Add 1 ml Trizol, 65 °C for 10 min.
2. Add 200 µl chloroform, vortex, incubate the tubes on ice for 5 min.
3. Centrifuge at 16,000 *x g* for 5 min.
4. Take 400 µl aqueous phase.
5. Add 1 µl GlycoBlue and 40 µl sodium acetate, then add 1 ml anhydrous ethanol, incubate at -80 °C, 2 h.
6. Centrifuge at 16,000 *x g* for 5 min, discard the supernatant.
7. Suspend the RNA pellet in 89 µl of nuclease-free water.
8. Add 10 µl 10x DNase I buffer and 1 µl DNase I, 37 °C for 10 min.
9. Add 300 µl nuclease-free water then 400 µl chloroform, vortex.
10. Centrifuge at 16,000 *x g* for 5 min.
11. Take 400 µl aqueous phase.
12. Add 1 µl GlycoBlue and 40 µl sodium acetate, then add 1 ml anhydrous ethanol, -80 °C, 2 h.
13. Centrifuge at 16,000 *x g* for 5 min, discard the supernatant.
14. Suspend the RNA pellet in 20 µl of nuclease-free water.
15. Reverse transcript by SuperScriptIII (follow the manufacturer's instructions) with gene-specific primer, and detect RNAs by RT-PCR (melting temp.: 95 °C, annealing temp.: 60 °C, cycle numbers: 30 cycles).

## Data analysis

Co-IP products were reversely transcribed using gene specific primer. Reverse transcripts were separated by electrophoresis on a 2% agarose gel. As shown in Figure 1, both OsDRB1a and mOsDRB1a interacted with apri-miR528, but neither of them recognized mapri-miR528; and NS3 and mNS3 were associated with OsDRB1a instead of mOsDRB1a. With the expression of NS3, both OsDRB1a and mOsDRB1a interacted with apri-miR528. However, with the expression of mNS3, only mOsDRB1a was associated with apri-miR528. These results indicate that NS3 may act as a scaffold to mediate the interaction between OsDRB1 and pri-miRNA.

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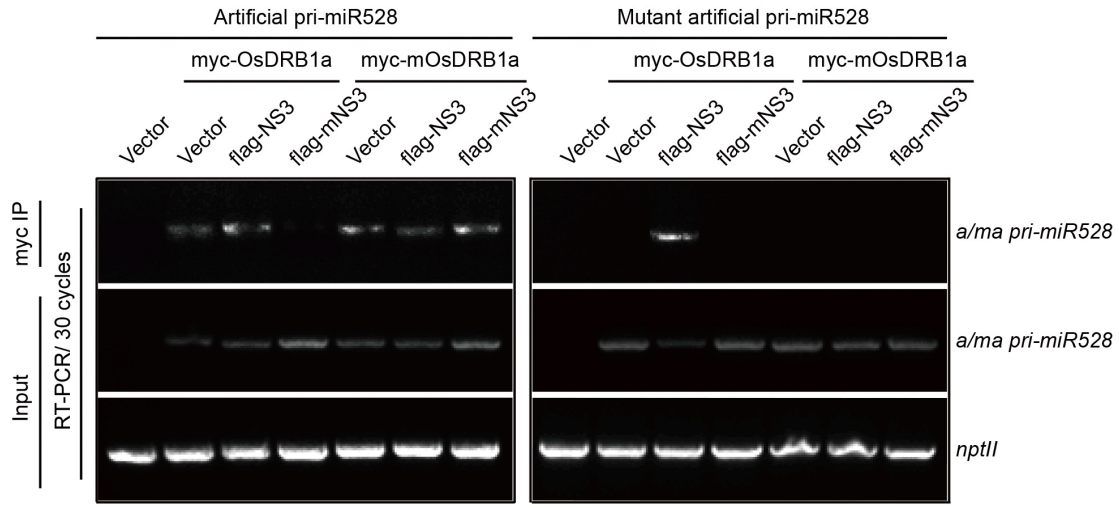


**Figure 1. NS3 acts as a scaffold between DRB1 and pri-miRNA.** RT-PCR detection of coimmunoprecipitated and input products of transiently co-expressed protein (DRB1a or mDRB1a), pri-miRNA (aprimiR528 or mapri-miR528), and protein (empty vector, NS3, or mNS3) in *N. benthamiana*. For more information, see Zheng *et al.*, 2017.

## Notes

1. Keep all the buffers RNase-free.
2. If you have many samples, isolates nuclei one by one, and freeze the nuclear pellet in the liquid nitrogen.
3. More detailed information on nuclei isolation and immunoprecipitation can be obtained from Saleh *et al.*, 2008 and Terzi *et al.*, 2009.

## Recipes

1. Honda buffer
   0.44 M sucrose
   1.25% Ficoll
   2.5% Dextran T40
   20 mM HEPES-KOH, pH7.4
   0.5% Triton X-100
   10 mM $MgCl_2$
   20 U/ml RNase inhibitor
   5 mM DTT (freshly added)
   1x Cocktail Roche cOmplete (freshly added)
2. High salt nuclear lysis buffer
   20 mM Tris-HCl, pH = 7.5
   500 mM NaCl

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4 mM $MgCl_2$
20 U/ml RNase inhibitor (freshly added)
0.2% NP-40 (add fresh)
5 mM DTT (add fresh)
1x Cocktail Roche cOmplete (freshly added)

3. Dilution buffer
   20 mM Tris-HCl, pH = 7.5
   4 mM $MgCl_2$
   0.2% NP-40 (freshly added)
   5 mM DTT (freshly added)
   20 U/ml RNase inhibitor (freshly added)
   1x Cocktail Roche cOmplete (freshly added)
4. IP buffer
   20 mM Tris-HCl, pH = 7.5
   100 mM NaCl
   4 mM $MgCl_2$
   0.2% NP-40 (add fresh)
   5 mM DTT (add fresh)
   20 U/ml RNase inhibitor (add fresh)
   1x Cocktail Roche cOmplete (freshly added)

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## References

1. Saleh, A., Alvarez-Venegas, R. and Avramova, Z. (2008). An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants. *Nat Protoc* 3(6): 1018-1025.
2. Terzi, L. C. and Simpson, G. G. (2009). *Arabidopsis* RNA immunoprecipitation. *Plant J* 59(1): 163-168.
3. Zheng, L., Zhang, C., Shi, C., Yang, Z., Wang, Y., Zhou, T., Sun, F., Wang, H., Zhao, S., Qin, Q., Qiao, R., Ding, Z., Wei, C., Xie, L., Wu, J. and Li, Y. (2017). Rice stripe virus NS3 protein

 

regulates primary miRNA processing through association with the miRNA biogenesis factor OsDRB1 and facilitates virus infection in rice. *PLoS Pathog* 13(10): e1006662.