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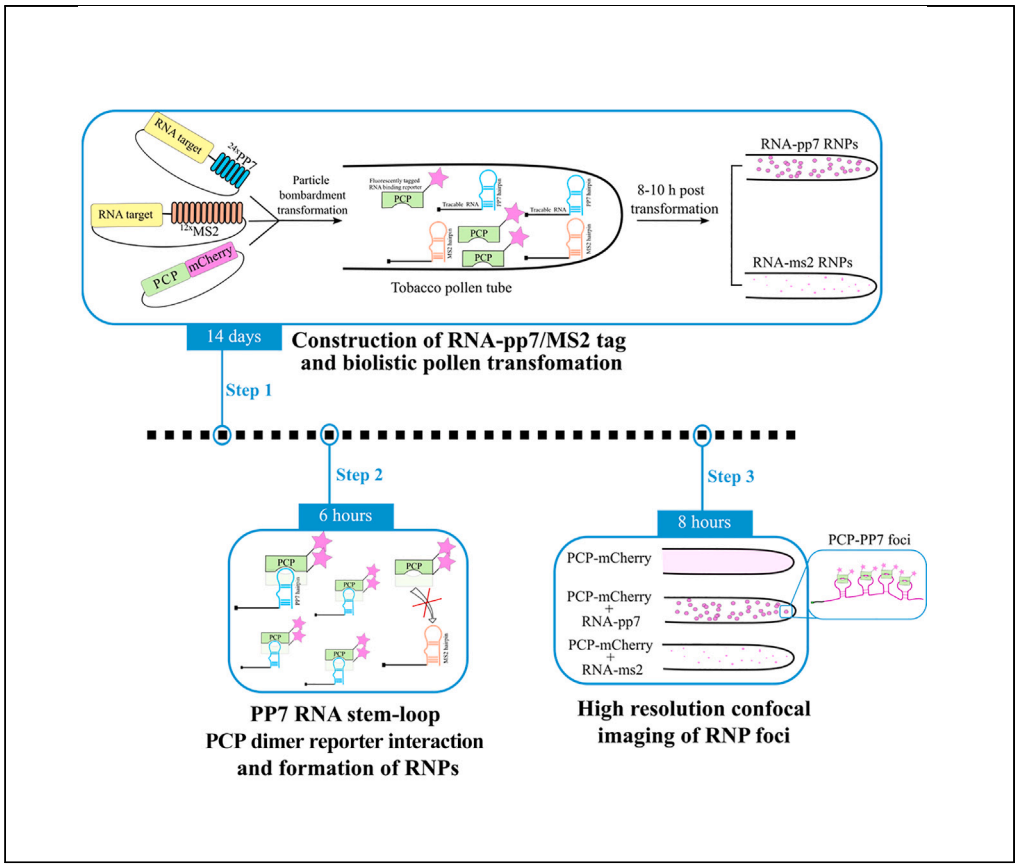
A protocol for *in vivo* RNA labeling and visualization in tobacco pollen tubes



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Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Instructions for generation of fluorescence *in vivo* PP7 aptamer RNA labeling

Steps for visualizing RNP complexes in transformed tobacco pollen tubes

Tips for checking the specificity of aptamer-reporter RNPs in pollen tubes

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A protocol for *in vivo* RNA labeling and visualization in tobacco pollen tubes

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SUMMARY

Here, we present a protocol for labeling and live visualization of RNA-protein complexes in the form of ribonucleoprotein particles (RNPs) in tobacco pollen tubes. We describe steps for constructing RNA-pp7/MS2 tag and biolistic gene-gun-mediated pollen transformation. We then provide detailed procedures for RNA labeling using PP7 aptamer nascent RNA tagging and a fluorescently labeled *Pseudomonas aeruginosa* PP7 bacteriophage coat protein (PCP) reporter that binds to PP7 RNA stem loops. This protocol is adaptable to other cell types by employing tissue-specific promoters.

BEFORE YOU BEGIN

Pollen tube is a single cell model system that is easy to maintain and manipulate with a range of experimental techniques for investigating diverse cellular processes including tip growth, post transcriptional gene regulation, cell morphology and stress responses in plants. Pollen sequesters mRNAs in the form of translationally repressed RNPs to facilitate rapid tip growth of the pollen tube. Therefore, visualizing RNPs dynamics in real-time combining with high resolution microscopy is of great biological importance and can address fundamental cell biology questions.

To track RNAs and study the nature of RNPs, different approaches have been used to label nascent RNA. The most widely used RNA labeling system is the coat protein from *Escherichia coli* (E. coli) bacteriophage MS2. MS2 coat proteins (MCPs) are usually 129 amino acid long polypeptide that binds strongly to 21-nucleotide RNA stem-loop region present in the MS2 RNA.[1–5] Analogous to MS2, a PP7 reporter system was also acquired from the bacteriophage coat protein in which a 127 amino acid long PP7 coat protein (PCP) recognizes and binds 25-nt RNA stem loop bulge region.[6–8] Despite their similarities, MCP and PCP are distinctive in identifying and discriminating their own RNA stem loops and therefore PP7 can be used alongside MS2 to reduce the background in a split system (Figures 1A and 1B).[9,10] To date, no RNA labeling method for live cell visualization of RNPs has been developed in gametophytic tissues. Here, we have used PP7 and MS2 aptamers to tag B1 RNA motif sequences recognized by the pollen-specific LARP6C RNA binding protein to demonstrate RNPs visualization in tobacco pollen tubes by live cell imaging.

Where applicable, this protocol can also be used to investigate and provide valuable insights into cis and trans regulatory elements of target mRNAs of interest in pollen tubes. The protocol is easily extendable to study RNA dynamics in any cell type by adopting cell-specific promoters and in complex multi-tissue models by generating stable transgenic lines.

Generation of PP7/MS2 aptamer-tagged target RNA and PCP fluorescently labeled reporter constructs

Timing: 10 days (for step 1)

Timing: 10 days (for step 2)

The first step of RNA-reporter assay is to amplify the RNA target sequence with PP7 aptamer repeats.[6,11–13] Subsequently, the RNA-PP7 tag and reporter protein (PCP) binary plasmids are transiently co-transformed to tobacco pollen tube via biolistic gene gun transformation.[14,15] To serve as control and to distinguish background RNPs in the absence of target RNA, we expressed either reporter PCP-mCherry alone or untagged target RNA with reporter PCP-mCherry or we expressed PCP-mCherry reporter in combination with the alternative MS2 tagged RNA.[2–5]

This protocol describes stepwise workflow from cloning to imaging labeled RNP complexes in tobacco pollen tubes. Furthermore, we have used this protocol in the transient transformation of tobacco pavement cells with similar results and in stable *Arabidopsis thaliana* plants.

Note: The sequences for PP7, MS2 and PCP reporter protein are provided in key resources table 1.

1. Construction of RNA target expressing PP7 aptamer.
   a. First step PCR amplification of the target RNA and aptamer sequences.
      i. Amplify the target RNA (in this study a B1 RNA motif) and 2 kb Ubiquitin 10 (UBQ10, AT4G05320) promoter fragment from cDNA and genomic DNA respectively with gene specific primers containing partial overhang attB sites (attB1, attB2, attB4) using high fidelity Phusion polymerase (Thermo Fisher). For PCR reaction mixture, cycler program and amplification (see Figure S1).

   Note: Any alternative cloning method is compatible with this protocol to achieve the same results.

   b. Second step PCR amplification and generation of entry clone modules.
      i. Use the first step PCR product (no PCR purification is necessary) to complete the full-length sequences of the attB overhangs for efficient recombination reaction (see key resources table 1).
      ii. After amplification resolve the PCR products on 1.2% agarose gel.
      iii. Gel purify the fragment of interest using kit of choice.
      iv. Ligate the purified fragment into pDONR vectors with P1-P2 and P4-P1 overhang recombination sites to create entry clones pDONR modules with L1-L2 B1 motif DNA fragment and L4-R1 UBQ10 promoter fragment using BP clonase Gateway BP Clonase II Enzyme mix (Thermo Fisher Scientific, Waltham, USA) at 25°C in the PCR cycler or incubator overnight.
      v. Similarly, subclone PP7/MS2 aptamers repeats from template plasmid (see key resources table 1) and ligate into pDONR with P2-P3 recombination sites to generate R2-L3 PP7 aptamer entry clones pDONR modules (see Figures S1 and S2 for gateway cloning workflow).
      vi. Transform 5 μL of the ligation product into *Escherichia coli* (E. coli) strain DH5 alpha.
      vii. Select positive clones on appropriate antibiotics.
      viii. Perform Sanger sequencing verification of all the entry clone modules (Approximately 3–4 days).

ix. Generate the expression clones by recombining the above three entry clones pDONR modules (^{L4}proUBQ10^{R1}, ^{L1}B1 motif^{L2} and ^{R2}PP7/MS2^{L3}) with binary destination vector (such as ^{R4}pB7m34GW, 0^{R3} or equivalent) using Multisite Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific, Waltham, USA) at 25°C overnight in the PCR cycler. The gateway cloning workflow is summarized in Figure S1 as previously outlined.[16]
x. Transform 5 μL of the ligation product into E. coli strain DH5 alpha.
xi. Select positive clones on appropriate antibiotics and verify the clones by Sanger sequencing.
xii. Isolate the plasmid with 6 mL culture ideally using plasmid isolation kit of choice to have a sufficient concentration of plasmid (> 0.5 μg/μL) and purity for the transient transformation of tobacco pollen.

Note: When selecting destination vectors, keep in mind antibiotic selection marker combinations that you will need for co-expressing target RNA with a reporter as well as multiple target matrix combinations in stable lines.

2. Construction of PCP fluorescently labeled coat protein constructs.
In this section we describe the cloning strategies to generate the PCP reporter for RNP visualization.
a. First step PCR amplification of the PCP reporter.
i. Amplify reporter PCP CDS and mCherry sequences using high fidelity Phusion polymerase (Thermo Fisher) from template plasmids (e.g., Addgene #101161) with respective attB1/B2 and attB2/attB3 overhang recombination sites with gene specific primers to generate first step PCR product (Figures S1 and S2).
b. Second step PCR reaction and construction of entry modules to generate the expression clone for PCP reporter protein.
i. Use the first step PCR product (no PCR purification is necessary) to complete the full-length sequences of the attB recombination sites for efficient recombination purposes (key resources table 1; Figures S1 and S2).
ii. After amplification resolve the PCR products on 1.2% agarose gel.
iii. Gel purify the fragment of interest using kit of choice.
iv. Ligate the purified fragments into pDONR vectors with P2-P3 recombination sites using BP clonase Gateway BP Clonase II Enzyme mix (Thermo Fisher Scientific, Waltham, USA) at 25°C in the PCR cycler or incubator overnight to create the entry clones pDONR constructs with L1-L2 and R2-L3 overhangs (Figure S2).
v. Perform Sanger sequencing verification of all the entry clone modules (Approximately 3–4 days).
vi. Once the intermediate entry clones are verified by Sanger sequencing, recombine the above generated entry modules, ^{L4}proUBQ10^{L1} (Ubiquitin 10 promoter), ^{L1}PCP^{L2} (bacteriophage coat protein) and ^{R2}mCherry^{L3} (red fluorescent protein) into binary destination vector (^{R4}pB7m34GW, 0^{R3} or equivalent) using Multisite Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific, Waltham, USA) at 25°C in the PCR cycler or incubator overnight to create an expression clone.
vii. Follow the same steps for transformation and sequence verification as in 1b (vi-viii) above.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Nicotiana tabacum pollen freshly collected or frozen at −20°C	This study	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sucrose	Lach:ner	40135-APO
H_3BO_3	Duchefa Biochemie	B0503.1000
$Ca(NO_3)_2.4H_2O$	Sigma	C5676
$MgSO_4.7H_2O$	Sigma	M1880-500G
KNO_3	Merck	1.05063.0500
MES	Duchefa Biochemie	M1503.0250
Experimental models: Cell lines		
DH5 alpha	Invitrogen	Cat #18263012
Experimental models: Organisms/strains		
Nicotiana tabacum	This study	N/A
Oligonucleotides		
Adapter primer: attB1 forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTA	This study	Sigma
Adapter primer: attB2 reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTG	This study	Sigma
Adapter primer: attB3 forward: GGGGACAACTTTGTATAATAAAGTTGCAATG	This study	Sigma
Adapter primer: attB2 reverse: GGGGACAACTTTGTATAGAAAAGTTGGGTG	This study	Sigma
Adapter primer: attB4 reverse: GGGGACTGCTTTTTTGTACAAACTTG	This study	Sigma
Adapter primer: attB1 reverse: GGGGACAACTTTGTATAGAAAAGTTG	This study	Sigma
Adapter primer: attB4-UBQ forward: TGTATAGAAAAGTTGCTCAACAACAAACTTTCCATT	This study	Sigma
Adapter primer: attB1-UBQ reverse: TTTGTACAAACTTGCTGTTAATCAGAAAAACTCAG	This study	Sigma
Flanking primer: M13 forward: GTAAAACGACGGCCAGT	This study	Sigma
Flanking primer: M13 reverse: CAGGAAACAGCTATGAC	This study	Sigma
Recombinant DNA		
Aptamer PP7, 24× AUAUGG	Johansson et al.; Peabody; Wu et al.; Karimi et al.[3,4,13,16]	Synthetic, This study
Aptamer MS2, 12× ACATGAGGATCACCCATGT	Johansson et al.; Peabody; Wu et al.; Karimi et al.[3,4,16]	Synthetic, This study
pUBQ10::PCP:mcherry (UPMC) Reporter	Alamos et al.[1]	Addgene; #161004/
Plasmid p35S::B1-motif:YFP	Billey et al.[17]	N/A
Plasmid pHSP101::H2B:pp7	Alamos et al.[1]	Addgene: #161014
pDONR221-P1-P2	Invitrogen	Cat#12536017
pDONR221-P3-P2	Invitrogen	Cat# V011823
pDONR221-P4-P1	Invitrogen	Cat#1 V011825
pB7m34WG, 0-R4-R3	VIB-UGent Center for Plant Systems Biology	https://vectorvault.vib.be/collection/pb7m34gw0
pDONR-L2-R3-PP7	This study, custom	Thermo Fisher
pDONR-L2-R3-MS2	This study, custom	Thermo Fisher
Other		
Rupture discs (1100 psi)	Bio-Rad	Cat#1652329
Macrocarrier	Bio-Rad	Cat#1652335
Macrocarrier holder	Bio-Rad	Cat#1652322
1.6 µm microcarrier gold particles	Bio-Rad	Cat#1652264
Stopping screen	Bio-Rad	Cat#1652336
0.45 µm, MCE membrane	MF-Millipore	Cat#HABP02500
Round petri dishes	Carl Roth	Cat#*N* 223.2
$CaCl_2$	Sigma	C5670-100G
Protamine	Sigma	P4505-1G
Absolute ethanol 99%	Lach:ner	Cat#20025-A96

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biolistic gene gun	Bio-Rad	165-2257 and 1652250LEASE to 165-2255LEASE

⚠ CRITICAL: While using the Biolistic gene gun, it is recommended to wear the protection Millennia glass (P-LAB, Cat# R675871) to avoid any possible injuries.

MATERIALS AND EQUIPMENT

Recipe for 2× SMM-MES media reagents

Reagent	Final concentration	Amount (grams)
Sucrose	0.175 M	59.9
H_3BO_3	1.6 mM	0.099
$Ca(NO_3)_2.4H_2O$	3 mM	0.708
$MgSO_4.7H_2O$	0.8 mM	1.97
KNO_3	1 mM	0.101
MES	23 mM	4.9
H_2O	N/A	N/A
Total	N/A	1 L

Note: The pH of the media should be 5.9 adjusted with 0.1 M KOH. Boil the 2× SMM-MES media at 100°C twice for 45 min each on two different days to ensure complete dissolution of the components and sterilization. Store the media in a cold room or at 4°C for long term use. To obtain 1× SMM-MES working media, simply dilute the 2× SMM-MES with deionized sterile water.

STEP-BY-STEP METHOD DETAILS

Biolistic gene-gun transformation of tobacco pollen tubes

Timing: 1 h 40 min

Timing: 20 min (for step 1)

Timing: 10 min (for step 2)

Timing: 10 min (for step 3)

Timing: 1 h (for step 4)

Timing: 10 min (for step 5)

Timing: 25 min (for step 6)

Timing: 10 min (for step 7)

Timing: 20–30 min (for step 8)

Timing: 10 h (for step 9)

This section outlines the necessary chemicals, media and biological sample preparation and describes the steps for the transient transformation of tobacco pollen.

1. Preparation of the gold particles:
   a. Weigh 30 mg of 1.6 μm gold particles (BIO-RAD) and rinse them in absolute ethanol by continuous vortexing for 3 min.
   b. Promptly centrifuge the particles and decant the ethanol.
   c. Rinse the gold particles twice in 1 mL of distilled water with continuous vortexing for 3 min each.
   d. Resuspend the equilibrated gold particles in 1 mL 50% glycerol and store at 4°C.
2. Preparation of 2.5 M $CaCl_2$:
   a. Weigh 2.774 g of $CaCl_2$ (Sigma) and dissolve in 10 mL of autoclaved deionized water.
   b. Filter sterilize the solution and store 1 mL aliquots at −20°C. The working stock is stored at 4°C for up to a month.
3. Preparation of the protamine:
   a. Dissolve 10 mg of protamine sulfate in 10 mL of autoclaved water.
   b. Filter sterilize the solution and store 1 mL aliquots at −20°C. The working solution is stored at 4°C for up to a month.
4. Preparation of 1× SMM solid pollen germination medium:
   a. Take out the 2× SMM liquid media from the 4°C and acclimatized to room temperature to avoid fast solidification of the media upon mixing with warm phytagel.
   b. Weigh 0.25 g of phytagel into 50 mL of distilled H_2O.
   c. Resuspend the phytagel by dissolving the powder cautiously by heating it in a microwave. Once fully dissolved, keep the media in hot water bath to stop phytagel solidification upon cooling.
   d. Mix 2× SMM-MES media and phytagel solution in a 1:1 ratio (25 mL + 25 mL) in a laminar flow box in a 50 mL sterile falcon tube.
   e. After thorough mixing, proceed immediately and pour 5 mL of the mixture into 5 cm round petri dishes (Φ55, H15 mm).
   f. Let the media dry in the flow box and store plates at 4°C for up to one week.
5. Biological material:
   a. Tobacco pollen preparation

Collect mature pollen from 3 months old flowering *Nicotiana tabacum* plants and stored them at −20°C in standard 1.5 mL Eppendorf tubes.

⚠ CRITICAL: Before use, frozen pollen must be acclimatized to room temperature for 10 min.

Note: Stored frozen tobacco pollen can be used for 3–4 years without compromising their germination if handled appropriately. Acclimatize only pollen aliquot required for transformation and do not thaw the complete frozen tube to avoid freeze-thaw cycles of the pollen.

6. Procedure for coating of the gold particles:
   Prior to transformation, coat gold particles with plasmids carrying PCP reporter alone or mixed with either B1-RNA motif tagged with PP7 or MS2 aptamers at 3:1 M concentration (Figure 2A).
   a. Mix 25 μL of gold particles with RNA-PP7/MS2 (6 μg) and PCP reporter (2 μg) plasmids at a concentration of 1 μg/μL to obtain a 3:1 M ratio of the two plasmids in an Eppendorf tube.

   ⚠ CRITICAL: The plasmids concentrations must be approximately 1 μg/μL. If a higher concentration can be obtained it is desirable.

   b. Add 25 μL of $CaCl_2$ (2.5 M) and 10 μL (1 mg/mL) of protamine to the same Eppendorf tube.
   c. Mix by thorough vortexing for 3 min, then briefly spin down for 30 s at full speed.

d. Remove the supernatant and wash the coated gold particles with 200 μL of absolute ethanol.
e. Vortex again for 3 min and remove the ethanol by pipetting after a quick centrifugation for 30 s at maximum speed.
f. Dissolve the pellet in 18 μL of absolute ethanol and spread it on the macrocarrier.

Pause point: Allow the macrocarrier to air dry on a clean dust-free bench top at room temperature before proceeding to the next step.

7. Pollen resuspension and pollen medium preparation:
   a. Pre-wet the nylon filter disc (0.45 μm, 22 mm, MCE Membrane) assembled on the filtration apparatus with distilled water and filter-out the water using a vacuum pump (see Figure 2A).
   b. For one transformation, resuspend 5 mg of tobacco pollen per 5 mL of 1× SMM-MES medium diluted from 2× SMM-MES with sterile water.
   c. Vortex the mixture thoroughly for 30 s to ensure full resuspension of the pollen.
   d. Pour the pollen suspension onto the pre-wet nylon filter disc (0.45 μm, 22 mm, MCE Membrane) placed on the filtration apparatus and filter-out the media using a vacuum pump (see Figure 2A).
   e. Transfer the pollen spread to the solid medium by blotting the filter disc containing pollen suspension upside-down to the medium.
   f. Repeat the same procedure for the next samples.

⚠ CRITICAL: For our experience, a maximum of 6 samples can be prepared at once.

8. Particles bombardment procedure:
   a. Set up the PDS-1000/He helium gas driven gene gun particle delivery system (Bio-Rad) according to the manufacturer instructions (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000070900.pdf).
   b. Install the rupture disc (1100 psi, BIO-RAD) in the holder to create the vacuum and gently tight with screwdriver.
   c. Position the stopping screen and macrocarrier comprised of gold coated plasmids, in close proximity and insert them into the launch assembly at the uppermost position (L1 slot).
   d. Use the third free L3 slot to place the plate containing pollen spread.
   e. Vacuum the chamber to 25–28 psi by firmly holding and initiate the bombardment procedure by pressing the fire button (red color).
   f. After shooting, promptly release the vacuum and seal the plate with parafilm to prevent contamination and store the plate containing pollen in the dark at room temperature.
9. Visualization of RNP in transformed tobacco pollen tubes:
   This section describes the required time and conditions for pollen germination and observation of RNP foci in the growing pollen tube.
   Following particle bombardment transformation of pollen, keep the transformed pollen at room temperature or at 28°C incubator in the dark to facilitate pollen tube growth and enable the expression of the respective transformed plasmids. It is recommended to wait between 8–10 h before imaging the pollen tubes. For precise and detailed analysis of the RNPs dynamics, we recommend to use high resolution fast confocal microscope such as a Nikon Spinning disk or ZEISS LSM 900 equipped with an Airyscan module using a 63× oil immersion objective.
   Prepare a clean slide chamber (Thermo Fisher) for imaging or use a standard glass slide. However, since the phytagel with pollen tube is flipped for imaging, a slide chamber is a better option as it provides the optical advantages of imaging through the coverslip 1.4 NA numerical aperture in case high-resolution imaging is desired (Figure 2B).
   a. To start, excise approximately 15 × 15 mm piece of the phytagel containing transformed pollen tubes using a rounded medium size spatula.
   b. Place the excised phytagel by flipping the gel upside down with pollen tubes now in direct contact with the bottom of the slide chamber (Figure 2B).

c. To screen for transformed pollen tubes, we recommend to start with 10×–20× dry objectives. Once a desired area is located, it is advisable to switch to a higher objective of 63×/100× oil immersion for close-up imaging of the RNPs.
d. Capture at least three representative Z-stacks per construct that can later be reconstructed into 3D projection rendering if desired.

Note: The remaining bulk of images (minimum of 15–20 pollen tubes per experiment) can be captured as time lapse videos of 1 min long at a rate of 25 frames/sec or as still images.

e. To evaluate non-specific RNPs appearance independent of the PP7 tagged RNA, image the reporter alone (PCP-mCherry gene), PCP-mCherry with untagged RNA target or a combination of the reporter and the same RNA target but tagged with MS2 aptamer instead of the PP7 aptamer.

EXPECTED OUTCOMES

Compatible B1 RNA-PP7 aptamer-tagging exhibits efficient binding by the PCP reporter and induces cytoplasmic RNPs in pollen tubes

Once efficient expression of both modules is achieved in transformed pollen tubes after approximately 8–10 h, the compatible interaction between the PCP-reporter dimer with RNA-PP7 stem loops generates RNP foci that are localized at the subcellular location of the target RNA destination. In this study, to demonstrate the functionality of the RNA-PP7 tagged RNPs labeling and tracing in pollen tubes, we have tagged the B1-RNA motif found in RNA targets of LARP6C RNA binding protein in pollen tubes with 24× repeats of the PP7 aptamer sequences at the 3′ UTR or with 12× MS2 aptamer repeats as control (Figure 1). LARP6C is a La motif (LaM) containing RNA-binding protein from the La and related protein families (LARPs) with a bipartite RRM RNA recognition domain and a LAM domain that binds to B1 cis-element and functions to regulate pollen tube guidance and plant fertility in Arabidopsis.[17] When PP7 tagged B1-motif RNA was co-expressed with the PCP-mCherry reporter, we detected distinct B1 RNA-PP7 constituted RNPs foci throughout the pollen tube cytoplasm 8 h after pollen particle bombardment (Figure 3). These RNPs were distinctly structured as bright punctate of a near-uniform shape and size and distributed from the apex to the shank region of the pollen tube (Figure 3). Notably, these B1 RNA-associated RNP aggregates were only clearly visible when we transformed the target B1 RNA-PP7:PCP Reporter at a molar ratio of 3:1 respectively. At a lower molar ratio of 1:1, the B1-motif-PP7 RNPs were not as clearly distinguishable from the background fluorescence (Figure S3). Therefore, we recommend by default to start with a 3:1 tagged RNA:Reporter molar ratio that can later be optimized after the initial screen. A similar analysis using a promoter from a heat shock inducible *HSP101* gene driving the PP7 aptamers produced nuclear PCP-mCherry foci that were detectable upon heat shock induction at 42°C using the VAHEAT system (Figure S4).

Co-expression of B1-MS2 tag RNA with PCP reporter does not generate distinct RNP aggregates

To verify the specificity of the PCP reporter in recognizing the PP7 tagged RNA target, we co-expressed PCP:mCherry reporter with B1-motif:YFP without the PP7 aptamer RNA tagging in pollen tubes. No similar RNP foci were visible; instead, the PCP-mCherry reporter showed a uniform cytoplasmic localization indicating that the PCP binding is specific to PP7 stem-loop interaction (Figure 3).

To further assess the specificity of the PCP reporter on selectively binding PP7 RNA stem-loops, we co-expressed the PCP-mCherry reporter along with B1-motif RNA tagged with 12×MS2 aptamers instead of the 24×PP7 aptamers. MS2 aptamers are also bacteriophage derived RNA repeats that form RNA stem-loops recognized by the MS2 bacteriophage coat protein MCP dimers.[3] MCP shares only 15% similarity with the PCP coat protein, and although they exhibit high affinity binding (Kd ~1 nM) to their respective RNA aptamers stem-loops, they also show distinct RNA-stem loop recognition in the excess of 1000-fold less affinity in binding alternative stem-loops.[2,11,12] Confocal live cell imaging of the PCP-mCherry co-expression with B1-motif-MS2 tagged RNA revealed low

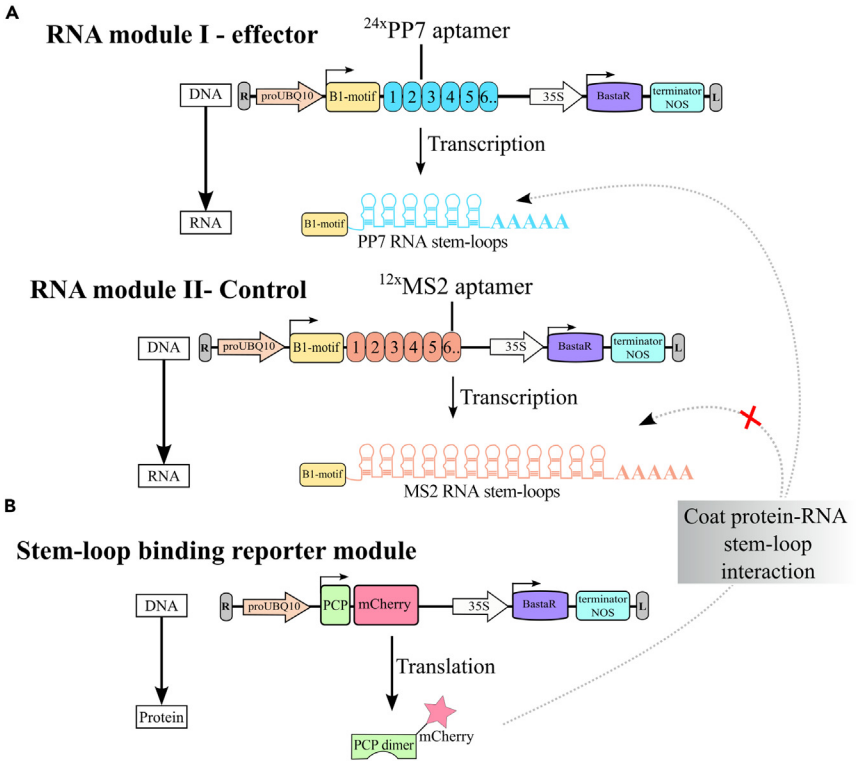

Figure 1. A setup for the aptamer-RNA fluorescent reporter system

(A) Schematic representation of the expression constructs exemplifying the B1-RNA motifs tagging with 24×PP7 or 12×MS2 aptamers under the expression of UBQ10 promoter.

(B) Illustration showing RNA binding bacteriophage coat protein PCP construct expressed under the same UBQ10 promoter with mCherry tag. The light gray arrows signifies the PCP reporter specific recognition of compatible PP7 RNA hairpin loops while distinguishing non-compatible MS2 hairpin loops.

density non-uniform RNP foci in the pollen tube cytoplasm (Figure 3). The MS2-derived RNPs were distinctively different from those induced by the PCP-mCherry binding to B1-motif-PP7 tag RNA (Figure 3). These observations emphasize the specificity of the PCP reporter in recognizing PP7 stem-loops and also serve as a precaution in the interpretation of nonspecific RNP foci in the presence of excess RNA and RNA-binding protein.

LIMITATIONS

The RNA-reporter assay is a highly suitable method for visualizing the formation of specific RNA-protein complexes in the form of ribonucleoprotein particles (RNPs). It allows gaining insights into RNP molecular dynamics and localization, particularly in pollen and pollen tubes where RNA storage and posttranscriptional regulation play a pivotal role in promoting tip growth and fertilization.[18,19] However, several limitations exist that must be carefully addressed. First, using the particle bombardment as a method of transformation, the occurrence of transformed cells (pollen tubes) that express both the PP7 tagged RNA candidate and reporter plasmids is relatively low (approximately 100 cells/5 mg pollen). In some instances, some cells may only express one type of the plasmid and not both. This limitation needs to be taken into consideration when analyzing the results (Troubleshooting problem 1). Second, the concentration and purity of the plasmids significantly influence achieving an adequate number of transformants (Troubleshooting problem 2). Third, it is essential to proceed with the particle bombardment immediately after spreading the pollen on the media. This is because tobacco pollen starts to germinate as soon as it comes into contact with the medium. Delaying the gene gun shooting may result in the bursting of germinated pollen, thereby impacting the efficiency of the pollen transformation (Troubleshooting problem 3). Fourth, very critical, one should first exploit placing the PP7 aptamer at the 5′ UTR

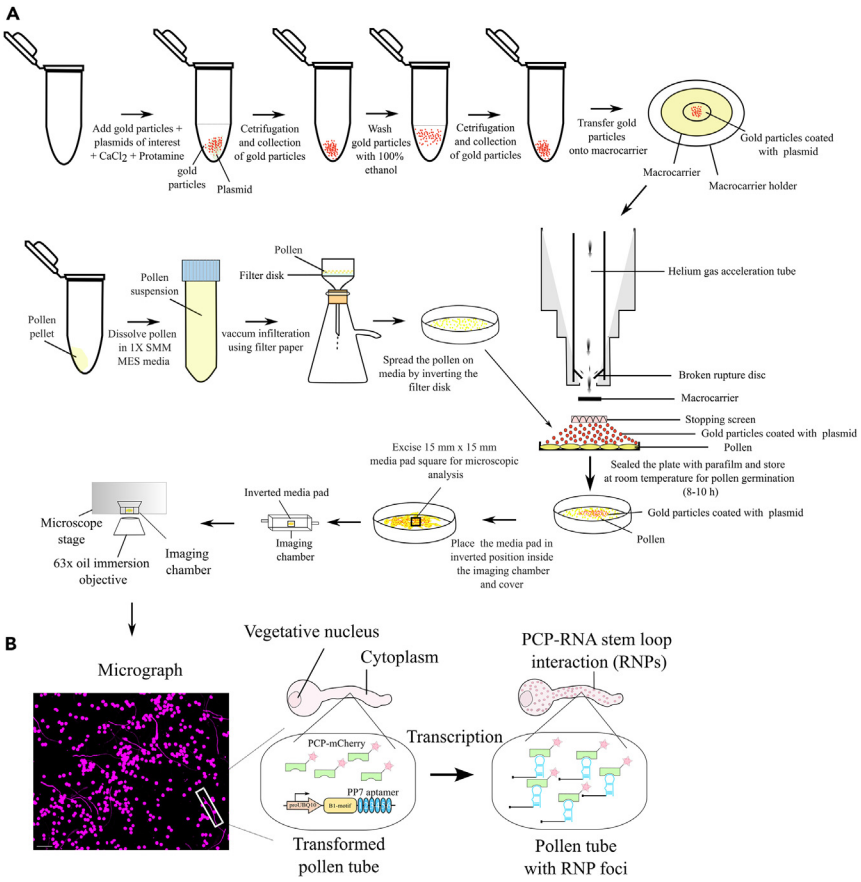

Figure 2. Experimental workflow for the biolistic gene gun transformation and screening of RNPs induction

Sequential illustration for the preparation of transient expression in pollen tube by a helium-driven biolistic gene gun particle delivery system (PDS-1000/He; BIO-RAD) as previously described.[14,15]

(A) Prior to the bombardment, the gold particles are coated with plasmid DNA carrying constructs of interest for each biolistic pollen transformation. In our case, a combination of 6 μg: 2 μg ratio (or 3:1 M ratio) for each plasmid DNA (proUBQ10-B1-PP7 or B1-MS2 constructs; PCP reporter:mCherry) was prepared before particle bombardment.

(B) Samples are then imaged after 8–10 h post-germination at room temperature. Images are acquired from the excised pollen gel media using either a Nikon Spinning disk or ZEISS LSM 900 equipped with an Airyscan module using 63× oil immersion objective. Right, illustration showing compatible interaction of PCP reporter with PP7 tagged B1-motif RNA stem-loops and formation of RNPs in pollen tubes.

and the 3′ UTR to obtain optimal results based on the characteristic of the nascent RNA folding (Troubleshooting problem 4). Fifth, this method has proved to be directly useful in studying RNA-protein interactions *in vivo* by comparing the co-localization of RNA binding protein (RBP) of interest tagged with different fluorophores to the RNA reporter (PCP) both targeting a common RNA target containing RBP motif and PP7 aptamer tag. This three-module combination would demonstrate a direct association of RBPs with their target RNA at respective subcellular locations in a cis-element dependent or independent manner. The limitation will be to obtain sufficient transformed pollen tubes expressing all three constructs. In conclusion, if the limitations are dealt with carefully, the RNA-reporter assay offers valuable insights into RNPs formation and molecular dynamics of posttranscriptional RNA regulation and fate in the pollen tube that is adaptable to other cell types.

TROUBLESHOOTING

Problem 1

First, using the particle bombardment as a method of transformation, the occurrence of transformed cells (pollen tubes) that express both the PP7 tagged RNA candidate and reporter plasmids is

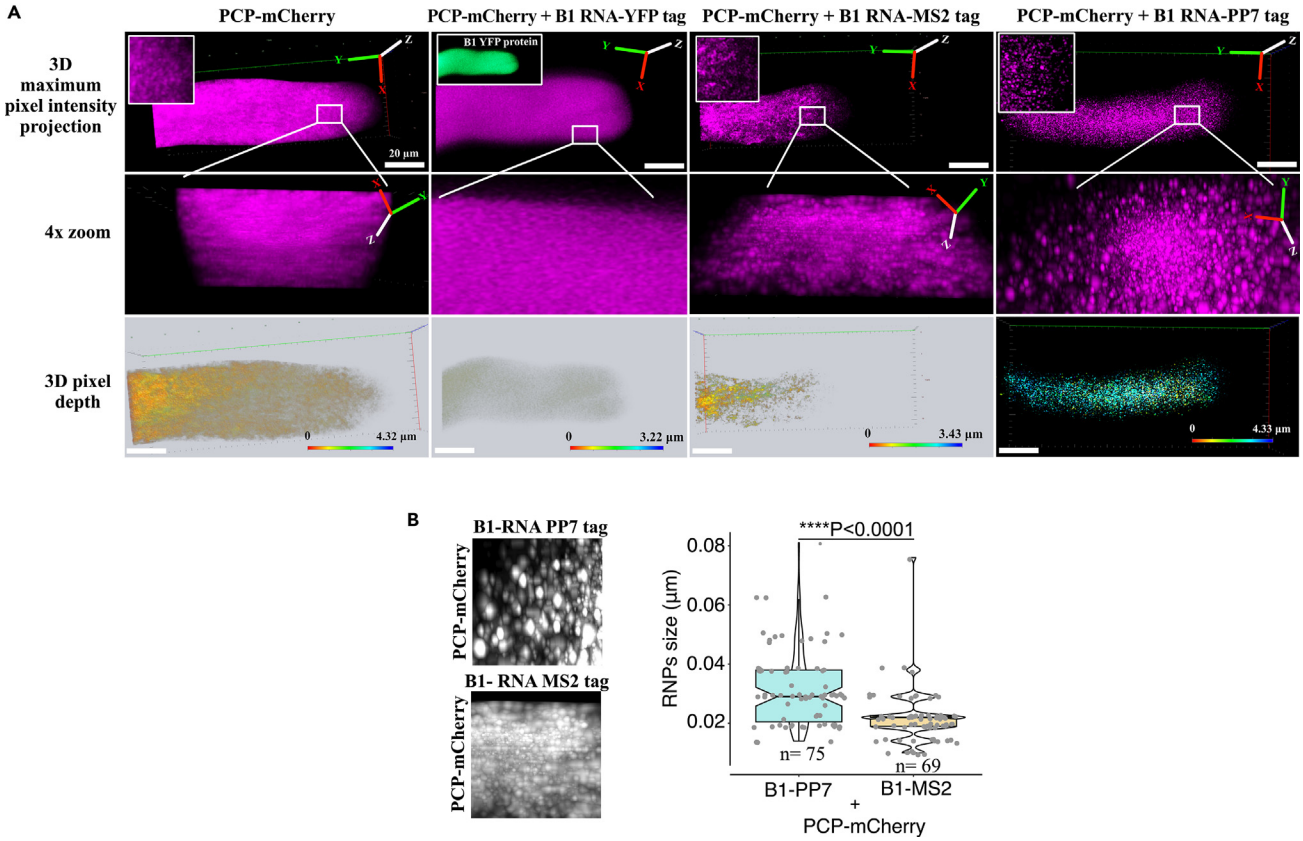

Figure 3. Expected RNPs visualization following PCP binding to PP7 tagged RNA stem-loops

(A) Maximum Z-projection reconstruction of the B1 RNA-PP7 tagged cytoplasmic RNPs foci that are detectable at a single focal plane however are best characterized as z-stack maximum projection in which non-PCP labeled foci are of low fluorescence intensity, smaller in size with variable morphology and show shallow pixel depth intensity compared to those from PCP-labeled PP7 tag RNA stem-loop interaction.

(B) Notch-Violin plot quantification showing distinctive features of PCP-mCherry labeled RNPs sizes from PCP-PP7 tagged B1 RNA interaction and control MS2-tagged B1 RNA. Image analysis was performed using ZEN blue software (ZEISS). Image processing and the area of the RNPs were measured using a segmented line tracing option in the ImageJ employed with analyze (toolbar menu) using the area option under set measurement. Statistics were performed with unpaired nonparametric t-test using Mann–Whitney *U* test analysis in GraphPad Prism 9.1.1 version (223) with *p*-value cut off of 0.05.

relatively low (approximately 100 cells/1 mg pollen). In some instances, some cells may only express one type of the plasmid and not both. This limitation needs to be taken into consideration when analyzing the results.

Potential solution

To achieve a good number of transformants, it is recommended to clone the respective gene of interest in the expression plasmid with a smaller size backbone and combining transcription units were applicable for co-expression. To overcome the problem of low transformation efficiency during co-transformation, it is important to use purified plasmid with 260/280 nm readout of ~1.8 and 230/260 nm readout of ~2.0–2.2. To further mitigate the drawback of low transformation efficiency, it's advisable to extend the incubation time during vortexing so that both plasmids can be efficiently coated onto the gold particles.

Problem 2

Second, the concentration and purity of the plasmids significantly influence achieving an adequate number of transformants.

Potential solution

Concentration of the plasmid is critical to facilitate the pipetting minimum volume of the plasmid to achieve effective drying and coating of the plasmids on gold particles. To obtain optimal concentration of the plasmid, try to increase the quantity of bacterial culture and combine multiple eluates into one final sample by concentrating them on column membrane or via NaOAc (pH 5.5) precipitation. Alternatively, use midipreps kits for isolation of highly concentrated plasmid such as GenElute HP Plasmid Midiprep Kit (MERCK, catalog number- NA0200–1KT).

Problem 3

Third, it is essential to proceed with the particle bombardment immediately after spreading the pollen on the media. This is because tobacco pollen starts to germinate as soon as it comes into contact with the medium. Delaying the gene gun shooting may result in the bursting of germinated pollen, thereby impacting the efficiency of the pollen transformation.

Potential solution

In order to minimize the low transformation efficiency, it is advised to first complete the preparation of all the material necessary for shooting and as a last step prepare the plates with pollen spread. This way you can proceed immediately with the gene gun pollen transformation (Figure 2).

Problem 4

Fourth, very critical, one should first exploit placing the PP7 aptamer at the 5′UTR and the 3′UTR to obtain optimal results based on the characteristic of the nascent RNA folding.

Potential solution

It is a good practice to design the construct for tagging the nascent RNA based on the objective of visualizing the RNPs. If the objective is to localize the RNA of interest, both 5′ UTR and 3′ UTR tagging can serve the purpose, however, one should study the structures of both 5′ and 3′ UTRs to decide how to proceed with tagging the candidate RNA. If the interest is to visualize approximate transcriptionally active loci location, labeling of transcription hot spot of the target gene of interest within the nuclei and measuring transcription rate, 5′ UTR tagging serve as a better strategy approach. If one is interested in visualizing splicing isoforms, for instance, the insertion of PP7 aptamer within a nascent RNA intron/exon of interest would be a design choice. Therefore, critically one should set the objective and understand the structure of the RNA to be traced, and by default for the novel target RNA, tagging both 5′ UTR and 3′ UTR for the initial screen would be a better strategy to follow.

RESOURCE AVAILABILITY

For any inquiries regarding supplementary material or information related to data or resources, please contact Dr. Said Hafidh (hafidh@ueb.cas.cz) or Vinod Kumar (vinod@ueb.cas.cz).

Lead contact

For all general enquiries, please contact Dr. Hafidh Said (hafidh@ueb.cas.cz).

Technical contact

The protocol primarily covers the technical inquiries; however, for additional details, individuals may seek guidance from Dr. Hafidh Said and Kumar Vinod (hafidh@ueb.cas.cz; vinod@ueb.cas.cz), who are available to provide more specific answers.

Materials availability

Biological material (*Nicotiana tabacum* pollen) and molecular material (plasmids) used in this study will be available based on the request to Dr. Hafidh Said (hafidh@ueb.cas.cz). We are also open for collaboration on this method if deemed preferable.

Data and code availability

The implementation of this research method did not provide any code or distinct dataset.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.H. and V.K.; methodology, S.H. and V.K.; investigation, S.H. and V.K.; writing – original draft, V.K.; writing – review and editing, S.H. and V.K.; funding acquisition, S.H.; resources, V.K. and S.H.; supervision, S.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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