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Techniques for RNA in vivo imaging in plants

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Short Review

Techniques for RNA *in vivo* imaging in plants

Jens Tilsner

Biomedical Sciences Research Complex, University of St Andrews, BMS Building, North Haugh, St Andrews, Fife KY 16 9ST, Scotland, U.K.

Cell & Molecular Science, The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, Scotland, U.K.

Tel. +44 (0)1334 464829

Fax +44 (0)1334 462595

jt58@st-andrews.ac.uk

ABSTRACT

Since the discovery of small RNAs and RNA silencing, RNA biology has taken a centre stage in cell and developmental biology. Small RNAs, but also mRNAs and other types of cellular and viral RNAs are processed at specific subcellular localisations. To fully understand cellular RNA metabolism and the various processes influenced by it, techniques are required that permit the sequence-specific tracking of RNAs in living cells. A variety of methods for RNA visualisation have been developed since the 1990s, but plant cells pose particular challenges and not all approaches are applicable to them. On the other hand, plant RNA metabolism is particularly diverse and RNAs are even transported between cells, so RNA imaging can potentially provide many valuable insights into plant function at

the cellular and tissue level. This Short Review briefly introduces the currently available techniques

for plant RNA *in vivo* imaging and discusses their suitability for different biological questions.

INTRODUCTION

Many RNAs in cells exhibit dynamic, functional localisations related to processes such as nuclear

export, localised translation, RNA turnover within nuclear and cytoplasmic granules, and intercellular

communication. Fully understanding the functional significance of these localisations and the

mechanisms that underlie them requires the ability to track RNAs sequence-specifically in living cells.

A variety of RNA imaging techniques have been developed, but their use in plants, where RNA

localisations are still little understood, is limited by factors such as the impenetrability of the cell

wall, and sources of auto-fluorescence such as chloroplasts and phenolic cell wall compounds. This

Short Review compares live-cell RNA visualisation techniques that have been used in plants,

highlighting their advantages and limitations.

1. APPROACHES BASED ON FLUORESCENTLY LABELLED NUCLEIC ACIDS

RNA can be fluorescently labelled either by direct incorporation of fluorescent nucleotides, or by

hybridisation with a fluorescent probe. Both of these approaches are feasible *in vivo* but require

invasive delivery into cells. The permeabilisation and transfection techniques routinely used in

animal cell culture do not work on walled plant cells. *In planta*, this limits invasive delivery of

fluorescent nucleic acids to microprojectile bombardment or micro-injection, the former of which

causes significant damage to the cell whereas the latter is technically challenging and extremely low-

throughput. Electroporation or PEG-mediated transformation are only possible in protoplasts, i.e.

cells removed from their tissue context and significantly stressed. Nevertheless, these invasive

imaging techniques are valuable for plant research because they can enable visualisation of RNAs

that are unsuitable for genetically encoded sensors, e.g. small RNAs such as siRNAs and miRNAs,

which would become non-functional if tagged with additional sequences, or sequestered by RNA-

binding proteins.

1.1. Direct labelling

RNAs can be rendered fluorescent by incorporation of fluorescent nucleotide derivatives. To do this

in a sequence-specific manner, the fluorescent RNA has to be transcribed or synthesized *in vitro*. If

unincorporated fluorescent nucleotides are removed after synthesis, direct labelling allows for

essentially background-free RNA imaging.

The main disadvantage of direct labelling is that the invasive introduction of the labelled RNA into

the cell bypasses all nuclear and many cytoplasmic processing steps, resulting in potentially

significantly altered protein associations compared to endogenous transcripts. The amount of

labelled RNA introduced into the cell may also differ significantly from endogenous levels and thus

overload cellular processing and localization machineries.

Direct labelling has been used to study the behaviour of uncapped mRNAs and the early events of

RNA virus infections in plant cells. Fluorescent, non-capped mRNA PEF-transformed into protoplasts

became trapped in the nucleus when nuclear export was inhibited with leptomycin B (Stuger &

Forreiter, 2004). Directly labelled genomic RNA of *Tobacco mosaic virus* was recruited into

endomembrane/cytoskeleton-associated motile granules in a cap-dependent manner (Christensen

et al., 2009). Directly end-labelled 21 nucleotide siRNA duplexes were used in leaf bombardment

assays to demonstrate that they function as mobile silencing signals between cells (Figure 1A). The

subcellular localisations of these small RNAs were not further analysed in these experiments

(Dunoyer *et al.*, 2010).

1.2. Molecular beacons

Sequence-specific visualisation of RNAs by fluorescent *in situ* hybridisation (FISH) is a common technique in animal cell biology, though subcellular resolution is more difficult to achieve in plant samples. Hybridisation-based approaches can also be used *in vivo*, but because unlike FISH, unbound probe cannot easily be washed out, this requires probes that allow distinguishing between unbound and target-bound forms. Many different design variants have been developed for this purpose (Bao *et al.*, 2009), which are beyond the scope of this Short Review. The only hybridisation-based RNA probes used in live plant cells to date are molecular beacons (MBs). MBs are short hairpin-structured nucleic acids coupled to a fluorophore on one end, and a quencher molecule on the other. In the unbound form, the MB stem-loop brings the fluorophore and quencher into close proximity, preventing fluorescence. Binding of the MB to its target RNA separates the hairpin stems and thus leads to unquenching of the fluorophore.

The major benefit of MBs for live-cell imaging is that they permit visualising endogenously expressed and processed RNAs. Apart from the invasive delivery, their main drawback is that they require extensive optimisation, especially for *in vivo* uses. The target sequence has to be accessible and not hidden by secondary structures. The free energy of hybridisation to the target has to be sufficient to open the hairpin, but the stem-loop has to be stable enough not to open in the absence of target *in vivo*. Nonspecific fluorescence can also occur due to endonuclease processing. One way to overcome problems with nonspecific fluorescence is to include a MB with no cellular targets as an internal control for ratiometric imaging. In animal cells, nonspecific nuclear sequestration of beacons often needs to be prevented by linking them to large carriers such as PEG or streptavidin. In plants, 2'-O-methyl-RNA MBs accumulated in the nucleus whereas DNA beacons did not (Göhring *et al*., 2014) (Figure 1B).

Using MBs, Göhring *et al*. (2014) were able to distinguish different mRNA splice variants in electroporated Arabidopsis protoplasts. They found that intron-retaining transcripts that were

insensitive to nonsense-mediated decay (NMD) were retained in the nucleus, thereby evading the

cytoplasmic NMD machinery. These splice variants also showed reduced mobility in the nucleus,

possibly due to a different ribonucleoprotein complex composition. Subsequently, the authors

further optimised their MB protocol and achieved the first single molecule RNA detection in plants.

2. GENETICALLY ENCODED RNA REPORTERS

Invasive delivery of RNA probes can be avoided by using genetically encoded reporters, thereby

facilitating imaging in a large number of cells within the context of intact tissues. Sequence-specific

RNA detection is possible with sequence-specific RNA-binding proteins (RBPs) fused to fluorescent

proteins (FPs). Generally applicable methods that can be adapted to any RNA of interest require

either tagging of the RNA with an RBP target sequence, or RBPs whose specificity can be predictably

modified. Both types of reporters have been used in plants. It is also necessary to distinguish free

and RNA-bound RBP-FP fusions. Two different strategies have been employed: nuclear retention of

unbound reporter, or fluorescence complementation between two RBP-splitFP fusions binding the

same RNA.

2.1. MS2CP and λN_{22}

The capsid protein of bacteriophage *MS2* (MS2CP), and a 22-amino acid peptide of the N protein of

bacteriophage λ (λN_{22}) are both RBPs that recognise 19 and 15 Nt, stem-loop forming-sequences

(*MS2* and *boxB*) with dissociation constants of 6.2 and 22 nM, respectively, and have no targets

within plant genomes. For RNA imaging, either MS2CP or λN_{22} have been fused to an FP and a

nuclear localisation signal. Thus, the fusion proteins are sequestered in the nucleus in the absence of

a target RNA. The presence of the target RNA leads to a re-localization of the RNA-reporter into the

cytoplasm. (Similar systems were also developed using the BglG antiterminator protein and the

bacteriophage PP7 coat protein with their respective corresponding stem-loops, but neither of these systems has so far been applied in plants.)

A downside of the MS2CP or λN_{22} systems is that the RNA of interest has to be tagged with the *MS2* or *boxB* stem-loops, and thus needs to be expressed as a transgene. Preferably the RNA is transcribed from its native promoter, but due to the random insertion of T-DNAs into the genomes of higher plants, its expression level and nuclear processing may still differ from the native transcript, especially if the transgene does not include introns. Additionally, the secondary structure introduced by the *MS2* or *boxB* tags may disrupt function or location of modified RNAs. On the other hand, an advantage of these systems is that very high sensitivities can be achieved by using multiple tandem copies of the stem-loop tags (typically 6-24). In animal systems, a 96x*MS2* tag enabled single-molecule-sensitive RNA imaging, but obviously, increasingly large tags with extensive secondary structure exacerbate the risk of disrupting RNA processing and localisation.

MS2CP was the first genetically encoded RNA imaging system described (Bertrand *et al*. 1998) and is so far the one most extensively used in plants, where its applications have included tracking of storage protein-coding and other mRNAs, viral RNAs, and analysis of nuclear miRNA processing bodies. Between 2 and 24 copies of the *MS2* tag, and both nuclear-targeted and cytoplasmic MS2CP-GFP fusions were used in these studies. Recently, Schönberger *et al*. (2012) have developed Gateway-based, 35S promoter-driven plant expression vectors for tagging of target RNAs with 6x*MS2* or 16x*boxB* hairpins, respectively, at either the 5' or the 3' end. They also constructed expression constructs for multiple spectral variants of nuclear-targeted MS2CP-FP and λN_{22}-FP fusions. With these systems, they demonstrated the possibility of simultaneously imaging two different RNAs in the same plant cell. Both were full genomic transcripts including UTRs and introns. One RNA encoded a soluble protein, the other a membrane-targeted protein, so their translation should occur in the cytoplasm and on the ER, respectively. Two-colour imaging showed that both were targeted to different transport or processing granules (Figure 1C). Interestingly, in the absence

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of target RNA, both MS2CP-FP and λN_{22}–FP fusions accumulated in the RNA-rich environment of the

nucleolus despite the absence of natural binding sites in the endogenous transcriptome. The authors

also showed that in plants, insertion of *MS2* or *boxB* stem-loops directly upstream of an open

reading frame prevented its translation. Thus, tagging downstream of the stop codon is the

preferred choice in plants, although in yeast in some cases, 3' tags have disturbed mRNA

localisations whereas 5' tags did not.

2.2. Pumilio RNA binding domain

Pumilio/FBF family (PUF) proteins are sequence-specific RBPs found in all eukaryotes (26 in

Arabidopsis). Their RNA binding domain, the Pumilio homology domain (PUMHD) consists of eight

tandem repeat Puf motifs that each bind one nucleotide in an 8 Nt target sequence. Sequence-

specific interactions are mediated by the side chains of two amino acids per Puf repeat and the RNA

nucleobases (a third amino acid side chain in each repeat forms a non-specific stacking interaction

with the RNA). This makes it possible to re-engineer the specificity of the PUMHD with relatively few

modifications. Structural analysis of the native human Pumilio 1 and molecular evolution have

produced a complete code for recognition of the four RNA bases, and a GoldenGate pipeline for

rapid assembly of any PUMHD variant has been developed (Abil *et al.*, 2014). Within certain

limitations (which are beyond the scope of this Short Review), the PUMHD can thus be engineered

to bind any RNA of choice. When used for RNA imaging, this means that in contrast to MS2CP and

λN_{22} systems, untagged, native RNAs can be imaged. Alternatively, RNAs can be tagged with

recognition motifs of selected PUMHD variants. The benefit in this case is that these tags do not

introduce stable secondary structures. Wild-type PUMHD of human Pumilio 1 also has a very high

affinity to its target sequence (k_D = 0.48 nM), and variants with a k_D as low as 0.05 nM have been

described. This makes PUMHD-based RNA imaging potentially more sensitive than MS2CP- or λN_{22}–

FP fusions, and single molecule imaging has been achieved in animal cells. On the other hand, the

RNA affinity of PUMHD variants modified to bind novel RNA sequences is still unpredictable and can be an order of magnitude lower than the wild-type protein.

Disadvantages of the PUMHD compared with MS2CP and λN_{22} are the short length of the 8 Nt target sequence and the occurrence of natural binding sites in endogenous plant mRNAs. Additionally, the PUMHD shows degrees of binding preference for target sequence variants, rather than complete specificity. Because off-target binding in a complex eukaryotic cell *in vivo* is thus practically unavoidable, combinations of two PUMHDs are used to increase the specificity of RNA imaging. To distinguish RNA-bound and free reporter constructs, the two PUMHD variants are coupled to the two halves of a split FP for bimolecular fluorescence complementation (BiFC). The two modified PUMHDs bind closely adjacent (5-9 Nt) binding sites on the same RNA, so that fluorescence reconstitution occurs upon binding of both split FP fusion proteins to the same RNA. PUMHD-BiFC is unfortunately not background free, as the reconstituted FP is extremely stable and the fluorescent complex accumulates in plant cells. In animal cells, nuclear-targeted fusions of two PUMHDs coupled to a FP have also been used to distinguish free and RNA-bound fluorescence similar to the MS2CP and λN_{22} systems, but this approach has yet to be tested in plants.

In plants, PUMHD-BiFC imaging has been used to track vial RNAs (Tilsner *et al*., 2009). This has enabled the observations that potyvirus replication complexes develop from ER-associated to chloroplast-associated membrane sites, and that replication and cell-to-cell movement of a potexvirus are spatially coupled at plasmodesmata (Figure 1D).

CHOICE OF REPORTER

This Short Review has described RNA *in vivo* imaging systems that have been successfully used in plants, and compared their respective advantages and drawbacks (Table 1), as well as highlighting some insights obtained with each. No single technique suits every experimental system, and the

choice of RNA reporter must depend on the type of RNA that is being studied and the types of

questions that need to be addressed. For small RNAs direct labelling or molecular beacons are

probably the only suitable *in vivo* approaches. For mRNA imaging, considerations need to include

how abundant these are and which RNA processing pathways need to remain unaffected by the

imaging system, as well as if imaging can be performed in protoplasts or requires intact tissue. The

main consideration for imaging viral RNAs is how tags and RBPs affect their infectivity. As RNA

imaging systems are being developed further, the available toolbox will become even more diverse.

Some promising reporters, such as RNA aptamer tags like "Spinach" (and its improved derivatives

"Spinach2" and "BabySpinach"), "Mango" and IMAGE that selectively bind cell-permeant

fluorophores, have yet to be adapted to use in plants (Paige *et al.*, 2011). Thus, it is to be hoped that

in the near future, studying functional localisations of plant RNAs will become a routine approach.

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Figure legends

Fig. 1. Examples of RNA imaging in plant cells.

(A) Direct labelling. Fluorescently end-labelled, double-stranded siRNAs bombarded into an Arabidopsis epidermal leaf cell have moved into surrounding cells (1.) and there caused silencing of a GFP transgene (2.) (Dunoyer *et al*., 2010). (B) Molecular Beacons. A gene-specific (1.) and a control beacon with no cellular target (2.) were electroporated into an Arabidopsis protoplast (3.). A ratiometric image (4.) is generated representing the signal/noise ratio in each pixel. From this, a cell compartment-specific distribution of background-corrected signal intensities can be averaged for a population of cells (Göhring *et al*., 2014). (C) MS2CP-CFP and λN_{22}-mVenus. Nuclear-targeted MS2CP-CFP (1.) and λN_{22}-mVenus (2.) are both recruited into cytoplasmic granules by their co-expressed target mRNAs tagged with 6x*MS2*- and 16x*box*B, respectively. The MS2CP-imaged mRNA, encoding a cytoplasmic protein, and the λN_{22}-imaged RNA, encoding a membrane protein, localise to different granules (Schönberger *et al*., 2012). (D) PUMHD-BiFC. *Potato virus X* RNA (green) imaged by
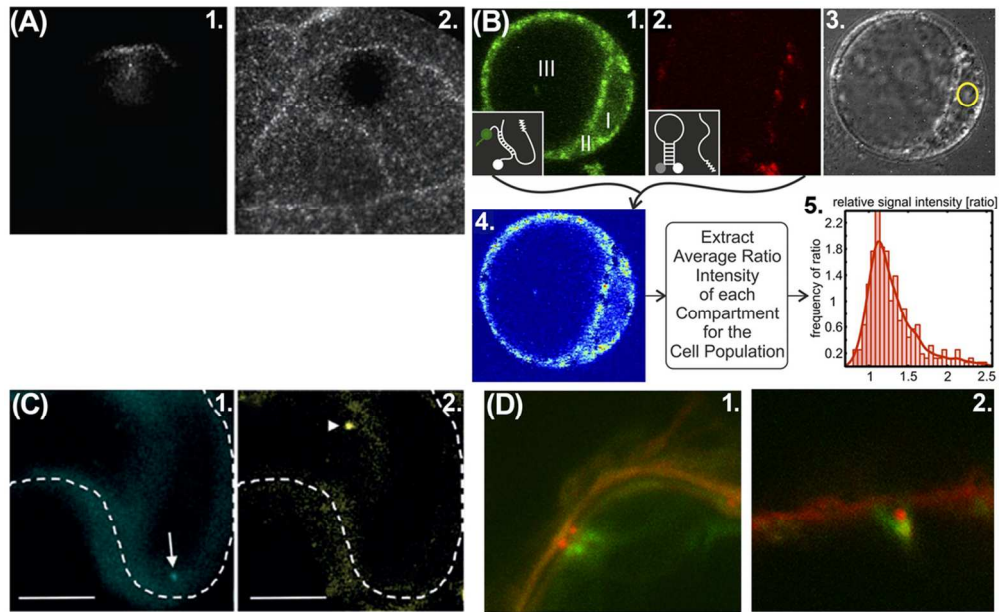
12

PUMHD-BiFC (Tilsner *et al*., 2009) localises to small membrane structures at the entrances of

plasmodesmata, which are labelled by the viral capsid protein (red) (Tilsner, unpublished images).

Table 1

Properties of RNA *in vivo* imaging systems used in plants.

Technique	Advantages	Disadvantages
Direct labelling	- No alterations to RNA sequence - Very high signal/noise ratio - Can use non-natural/modified RNAs - Can visualise small RNAs	- Invasive delivery - No endogenous processing - No native RNA levels
Molecular Beacons	- No alterations to RNA sequence - Native RNA levels and endogenous processing - Can visualise small RNAs	- Invasive delivery - Extensive optimisation
MS2CP-FP & λN$_{22}$-FP	- Non-invasive, easy to use - Can achieve very high sensitivity (single molecule)	- RNA tagged, hairpins may interfere with native localisation or processing
PUMHD-BiFC	- Non-invasive, easy to use - No alterations to RNA sequence required - Native RNA levels and endogenous processing possible - Can achieve very high sensitivity (single molecule)	- Problems with nonspecific BiFC signal

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modifications. Structural analysis of the native human Pumilio 1 and molecular evolution have

produced a complete code for recognition of the four RNA bases, and a GoldenGate pipeline for

rapid assembly of any PUMHD variant has been developed (Abil *et al.*, 2014). Within certain

limitations (which are beyond the scope of this Short Review), the PUMHD can thus be engineered

to bind any RNA of choice. When used for RNA imaging, this means that in contrast to MS2CP and

λN_{22} systems, untagged, native RNAs can be imaged. Alternatively, RNAs can be tagged with

recognition motifs of selected PUMHD variants. The benefit in this case is that these tags do not

introduce stable secondary structures. Wild-type PUMHD of human Pumilio 1 also has a very high

affinity to its target sequence (k_D = 0.48 nM), and variants with a k_D as low as 0.05 nM have been

described. This makes PUMHD-based RNA imaging potentially more sensitive than MS2CP- or λN_{22}–

FP fusions, and single molecule imaging has been achieved in animal cells. On the other hand, the

RNA affinity of PUMHD variants modified to bind novel RNA sequences is still unpredictable and can be an order of magnitude lower than the wild-type protein.

Disadvantages of the PUMHD compared with MS2CP and λN_{22} are the short length of the 8 Nt target sequence and the occurrence of natural binding sites in endogenous plant mRNAs. Additionally, the PUMHD shows degrees of binding preference for target sequence variants, rather than complete specificity. Because off-target binding in a complex eukaryotic cell *in vivo* is thus practically unavoidable, combinations of two PUMHDs are used to increase the specificity of RNA imaging. To distinguish RNA-bound and free reporter constructs, the two PUMHD variants are coupled to the two halves of a split FP for bimolecular fluorescence complementation (BiFC). The two modified PUMHDs bind closely adjacent (5-9 Nt) binding sites on the same RNA, so that fluorescence reconstitution occurs upon binding of both split FP fusion proteins to the same RNA. PUMHD-BiFC is unfortunately not background free, as the reconstituted FP is extremely stable and the fluorescent complex accumulates in plant cells. In animal cells, nuclear-targeted fusions of two PUMHDs coupled to a FP have also been used to distinguish free and RNA-bound fluorescence similar to the MS2CP and λN_{22} systems, but this approach has yet to be tested in plants.

In plants, PUMHD-BiFC imaging has been used to track vial RNAs (Tilsner *et al*., 2009). This has enabled the observations that potyvirus replication complexes develop from ER-associated to chloroplast-associated membrane sites, and that replication and cell-to-cell movement of a potexvirus are spatially coupled at plasmodesmata (Figure 1D).

CHOICE OF REPORTER

This Short Review has described RNA *in vivo* imaging systems that have been successfully used in plants, and compared their respective advantages and drawbacks (Table 1), as well as highlighting some insights obtained with each. No single technique suits every experimental system, and the

choice of RNA reporter must depend on the type of RNA that is being studied and the types of

questions that need to be addressed. For small RNAs direct labelling or molecular beacons are

probably the only suitable *in vivo* approaches. For mRNA imaging, considerations need to include

how abundant these are and which RNA processing pathways need to remain unaffected by the

imaging system, as well as if imaging can be performed in protoplasts or requires intact tissue. The

main consideration for imaging viral RNAs is how tags and RBPs affect their infectivity. As RNA

imaging systems are being developed further, the available toolbox will become even more diverse.

Some promising reporters, such as RNA aptamer tags like "Spinach" (and its improved derivatives

"Spinach2" and "BabySpinach"), "Mango" and IMAGE that selectively bind cell-permeant

fluorophores, have yet to be adapted to use in plants (Paige *et al.*, 2011). Thus,and it is to be hoped

that in the near future, studying functional localisations of plant RNAs localisations in plants will

become a routine approach in the near future.

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10

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Figure legends

Fig. 1. Examples of RNA imaging in plant cells.

(A) Direct labelling. Fluorescently end-labelled, double-stranded siRNAs bombarded into an

Arabidopsis epidermal leaf cell have moved into surrounding cells (1.) and there caused silencing of a

GFP transgene (2.) (Dunoyer *et al*., 2010). (B) Molecular Beacons. A gene-specific (1.) and a control

beacon with no cellular target (2.) were electroporated into an Arabidopsis protoplast (3.). A

ratiometric image (4.) is generated representing the signal/noise ratio in each pixel. From this, a cell

compartment-specific distribution of background-corrected signal intensities can be averaged for a

population of cells (Göhring *et al*., 2014). (C) MS2CP-CFP and λN_{22}-mVenus. Nuclear-targeted

MS2CP-CFP (1.) and λN_{22}-mVenus (2.) are both recruited into cytoplasmic granules by their co-

expressed target mRNAs tagged with 6x*MS2*- and 16x*box*B, respectively. The MS2CP-imaged mRNA,

encoding a cytoplasmic protein, and the λN_{22}-imaged RNA, encoding a membrane protein, localise to

different granules (Schönberger *et al*., 2012). (D) PUMHD-BiFC. *Potato virus X* RNA (green) imaged by

12

PUMHD-BiFC (Tilsner *et al.*, 2009) localises to small membrane structures at the entrances of

plasmodesmata, which are labelled by the viral capsid protein (red) (Tilsner, unpublished images).