Review

# Advances in imaging RNA in plants

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**Increasing evidence shows that many RNAs are targeted to specific locations within cells, and that RNA-processing pathways occur in association with specific subcellular structures. Compartmentation of mRNA translation and RNA processing helps to assemble large RNA–protein complexes, while RNA targeting allows local protein synthesis and the asymmetric distribution of transcripts during cell polarisation. In plants, intercellular RNA trafficking also plays an additional role in plant development and pathogen defence. Methods that allow the visualisation of RNA sequences within a cellular context, and preferably at subcellular resolution, can help to answer important questions in plant cell and developmental biology. Here, we summarise the approaches currently available for localising RNA *in vivo* and address the specific limitations inherent with plant systems.**

## RNA localisation in plants

A major paradigm in cell biology is that a protein's localisation is closely linked to its function [1]. By contrast, RNAs are often seen as simply diffusing through the 'cellular soup'. However, it is now clear that RNAs are usually bound to specific protein partners, and that the cellular processes they control occur at specific locations, often associated with particular organelles. An mRNA molecule can only exit the nucleus after it has been quality-controlled, spliced, capped and polyadenylated [2]. Some of the proteins it interacts with during these steps remain bound to it after nuclear export and play a role in guiding it to its final destination in the cytoplasm [2,3]. mRNAs that encode proteins targeted to the endomembrane system are recruited by the signal recognition particle (SRP) and translated on the surface of the endoplasmic reticulum (ER) [4]. However, some RNAs are also targeted to the ER surface independent of SRP [5], or even independent of translation [6]. An increasing number of mRNAs have been shown to be transported along the cytoskeleton to allow localised protein synthesis (reviewed in [7–10]), e.g. those for the seed storage proteins of cereals [5] and for actin-organising factor profilin in growing maize root hairs [11]. Some mRNAs are also transported to storage sites and activated later, for instance after cell division or germination: Tobacco (*Nicotiana tabacum*) pollen contains cytoskeleton-associated RNA storage granules [12], and in *Arabidopsis* (*Arabidopsis thaliana*) the *SHORT SUSPENSOR* mRNA is stored in pollen but only becomes activated for translation after delivery to the zygote [13]. A general link of the translation machinery with the cytoskeleton is emerging, where both non-targeted and targeted transcripts are associated with cytoskeletal elements [8,14–19]. mRNAs that are targeted for degradation by deadenylation are sorted into processing bodies (P bodies, see Glossary), and a block in translation can lead to their storage in stress granules, microscopically visible protein–RNA complexes that are characterised by their distinct protein constituents [2,20,21].

In addition to mRNAs, various types of small RNAs function in the regulation of gene expression and in pathogen defence through mechanisms collectively known as RNA silencing [22]. Several of these small RNAs originate in the nucleus [23], but their activities are required both in the nucleus and the cytoplasm, where they are associated with P bodies [21], and also with

### Glossary

**Aptamer**: a nucleic acid sequence evolved or selected to bind with high affinity to a ligand of choice.
**BiFC**: see Box 1.
**Fluorophore**: a molecule or group of atoms within a larger molecule that can absorb the energy from an excitation light source and re-emit it at a longer wavelength as fluorescence.
**FRET**: see Box 1.
**MicroRNA (miRNA)**: one of several species of small RNAs, miRNAs are generated by endonucleolytic processing of longer, non-protein-coding nuclear transcripts; they direct the sequence-specific silencing of complementary mRNAs [23].
**Molecular beacon**: a short oligonucleotide covalently linked to a fluorophore for detection of complementary sequences through Watson–Crick basepairing. Various different kinds of molecular beacons have been developed with the aim of restricting the emission of fluorescence to those beacons bound to their targets (see main text).
**MS2 coat protein**: the coat protein of the bacteriophage MS2 that binds to an RNA hairpin structure with high affinity and specificity.
**Nuclear localisation signal**: a sequence motif in a protein that directs its import into the nucleus.
**P body**: processing body. A microscopically visible ribonucleoprotein particle that contains mRNAs that have been marked for degradation by deadenylation [21].
**Pumilio**: a family of RNA-binding proteins that function as repressors of translation in eukaryotic cells. The Pumilio RNA-binding domain binds a short, nonstructured sequence motif and its sequence specificity can be modified in a defined way by site-directed mutagenesis (see main text).
**Quencher**: a photoreactive molecule with suitable characteristics to absorb the excitation energy from a nearby excited fluorophore by FRET (see Box 1), thereby preventing fluorescence emission from the fluorophore (i.e. 'quenching' it).
**Stress granule**: a microscopically visible ribonucleoprotein particle that contains mRNAs with associated polysomes that have been stalled during translation but can be reactivated for continued protein biosynthesis [21].
**UTR**: untranslated region. The noncoding RNA sequences at the 5′ and 3′ ends of protein-coding mRNAs, usually have functions in regulating RNA localisation, translation and turnover.
**$\lambda N_{22}$**: a short peptide motif from the N protein of the bacteriophage λ that binds an RNA hairpin (called '*boxB*') with high affinity and specificity.

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**Table 1. Techniques for RNA *in vivo* imaging and their advantages and disadvantages for plant application**

| Method | Alteration of target RNA | Knowledge of target RNA structure required | Mode of delivery | Fluorescence signal | Advantages | Disadvantages |
|---|---|---|---|---|---|---|
| FP-coupled RNA hairpin-binding peptides (Figure 1a) | Yes, insertion of stem loops. In some cases as many as 96 have been used. | No | Transgenic | Permanent. Nuclear sequestration to separate signal from unbound fluorescence (not essential) | RNA processed in the nucleus. Noninvasive delivery possible. | Large tag may interfere with RNA function. Comparatively low signal-to-noise ratio. Transgenic expression of tagged RNA may cause non-native transcript levels. |
| Pumilio-BiFC (Figure 1b) | No (can be used as a tag alternatively, then no introduction of secondary structure) | No (avoidance of target sites in secondary structures may be necessary) | Transgenic | Fluorescence switched on upon RNA binding, then remains permanently | Native transcript can be targeted. RNA processed in the nucleus. Noninvasive delivery possible. | Modification of Pumilio RNA-binding domain work-intensive and may reduce affinity. BiFC not background-free. Potential interference from endogenous Pumilio target RNAs (countered by BiFC approach). If used as a tag: transgenic expression of tagged RNA may cause non-native transcript levels. |
| Aptamer binding dyes (Figure 1c) | Yes, insertion of aptamers with secondary structure | No | Transgenic + dye infiltration | Fluorescence switched on upon binding | RNA processed in the nucleus. Potentially high signal-to-noise ratio. | Insertion of aptamers may interfere with function. Potential permeability or toxicity problems of dye not yet tested in plants. Transgenic expression of tagged RNA may cause non-native transcript levels. |
| Direct labelling (Figure 1d) | No | No | Microinjected | Permanent until labelled RNA degraded | No noise. No sequence alterations. Instant signal. | Delivered in large quantities. Invasive delivery. RNA not processed in the nucleus. |
| Molecular beacons (Figure 1e) | No | Yes, target sequences with secondary structure can prevent signal | Microinjected | Fluorescence (or FRET) upon binding to target | RNA processed in the nucleus. Native transcript targeted at native RNA level. Low noise level. | Invasive delivery. False positives due to partial degradation of the probe. Can be solved by using dual beacons. |

endosomal membrane compartments [24]. The subcellular pathways taken by small RNAs are particularly interesting as these molecules (and probably also mRNAs) can move and function non-cell autonomously [25,26] and move systemically throughout the plant [25,27,28] by as yet unexplained mechanisms.

As the functions of diverse RNAs depend on their association with various subcellular localisations during their lifetime, *in vivo* techniques that allow sequence-specific RNA visualisation, preferably with subcellular resolution, can provide important information in plant cell and developmental biology. RNA imaging is considerably more difficult than protein imaging, but a range of techniques are now available that are reviewed here. Despite successful use of *in situ* imaging techniques in a number of studies [11,13,29–32], subcellular resolution is difficult to achieve wherever sample sectioning is required, and *in situ* approaches can only give snapshots of RNA dynamics. We therefore focus on *in vivo* imaging techniques. Since many of these were developed for non-plant systems [33], we discuss their potential suitability for localising RNA in plant cells and describe some promising techniques that have yet to be used on plants (Table 1).

## Genetically encoded reporters for *in vivo* RNA imaging

### *MS2 and $\lambda N_{22}$ systems*

Many proteins can be imaged easily due to the widespread availability of fluorescent proteins (FPs). A straightforward approach to RNA imaging, therefore, is to fuse an RNA-binding protein (RBP) to an FP. Such a system must meet two requirements: (i) the RBP–RNA interaction must be highly specific and (ii) it must be possible to distinguish between the fluorescence of the unbound (noise) and RNA-bound RBP–FP (signal) fusion.

The first generally applicable system was based on the coat protein of the bacteriophage MS2 (MS2 CP), [34]. The MS2 CP binds to a 19-nucleotide RNA hairpin that is recognised with high specificity [35], and with an affinity sufficient for *in vivo* detection ($K_D$ = 6.2 nM) [36]. Additional advantages of this RBP are that no potential endogenous target sequences are known from eukaryotes, and a fusion of MS2 CP to GFP does not produce a specific

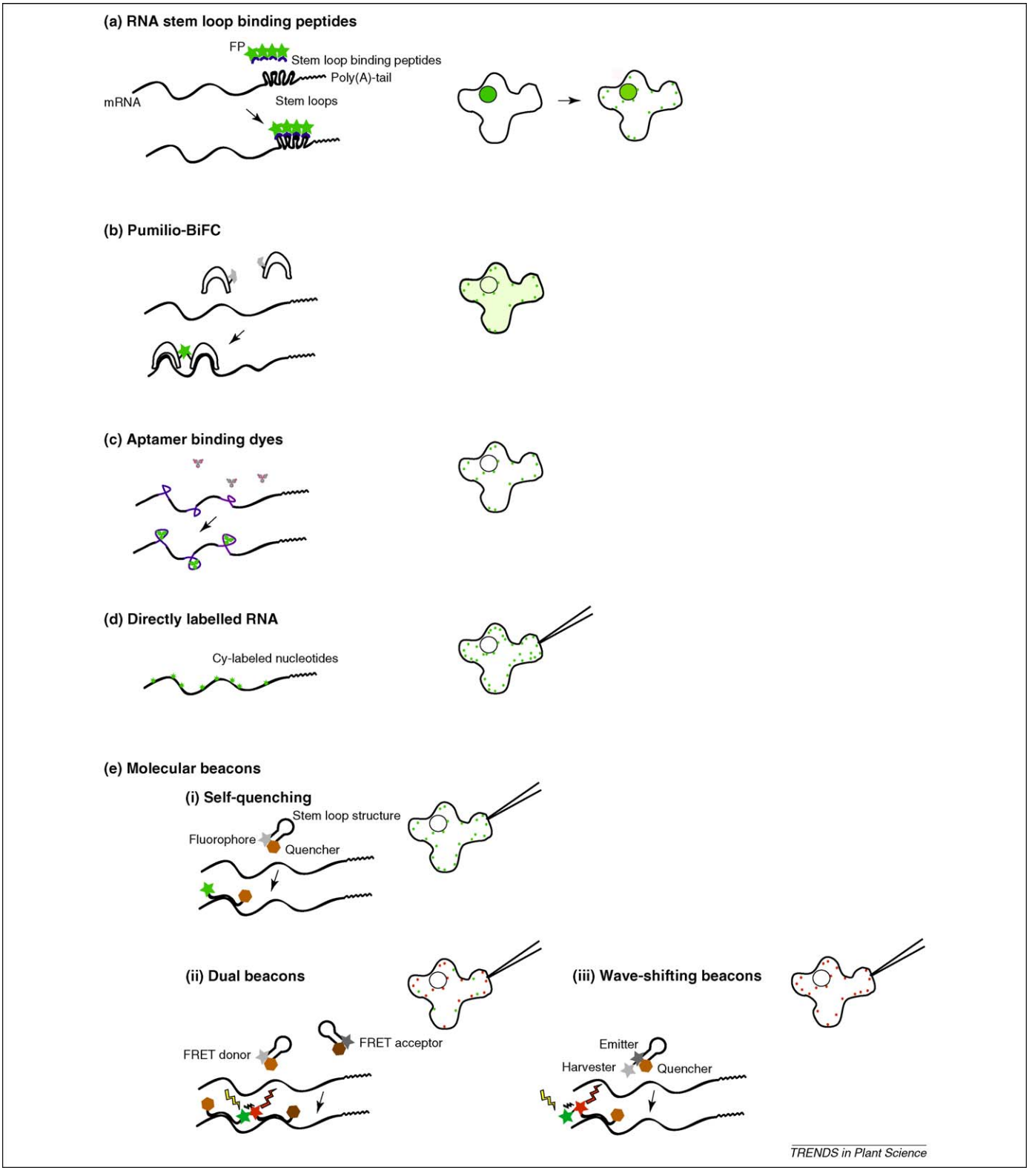


**Figure 1**. Principles of sequence-specific *in vivo* RNA imaging techniques. **(a)** RNA stem loop binding peptides: short bacteriophage-derived oligopeptides that bind specific RNA hairpins such as MS2 CP [34] or $\lambda N_{22}$ [46] are genetically fused to an intrinsically fluorescent protein (FP) and introduced into cells that express a modified RNA of interest, tagged with one or several copies of the hairpin. To distinguish unbound from bound fluorescent reporter, the FP fusion protein is targeted to the nucleus (left cell). Cytoplasmic fluorescence thus requires nuclear co-export with a bound RNA, or cytoplasmic trapping of the newly synthesised reporter by an mRNA (green granules in right cell). **(b)** Pumilio-BiFC: the RNA-binding domain of the Pumilio protein is engineered to bind a specific RNA of choice [54], requiring no introduction of additional sequences into the RNA. Two PUMHD variants targeting closely adjacent sequences on the same RNA are fused to the complementing halves of a split FP. Binding of both fusion proteins switches on fluorescence (bimolecular fluorescence complementation, BiFC, see Box 1), highlighting the RNA localisation [53] (green granules in the cell). **(c)** Aptamer-binding dyes: the RNA of interest is tagged with aptamers, short RNA sequences selected for specific binding of a cell-permeant dye through their secondary structure [61]. The dye is nonfluorescent unless bound to the aptamer, allowing specific visualisation of the RNA in the cell (green granules). **(d)** Direct RNA labelling: the RNA of interest is transcribed *in vitro* from a plasmid cDNA construct. Incorporation of nucleotides covalently linked to fluorescent molecules renders the RNA itself fluorescent so that it can be observed inside the cell after microinjection [17,63] (green granules). **(e)** Molecular beacons: molecular beacons are short oligonucleotides complementary to an RNA of interest, covalently coupled to a fluorophore [67]. After microinjection, they label the RNA by *in vivo* hybridisation. To eliminate background

subcellular localisation by itself. For RNA-imaging, the MS2 CP–FP fusion is co-introduced with a construct of the target RNA 'tagged' by introduction of MS2 binding sites. In order to distinguish unbound and RNA-bound MS2 CP–FP fusions, the reporter is generally targeted to the nucleus by a nuclear localisation signal (Figures 1a and 2a), from where it gets relocalised by binding to a tagged, cytoplasmic RNA. However, this is not essential if RNA binding leads to a significant redistribution, e.g. from a general nucleo–cytoplasmic signal to RNA granules. Untargeted MS2 CP–FP has been successfully used in the study of cytoplasmic and nuclear RNAs [37,38], and a nuclear-targeted reporter has been used in the study of subnuclear RNA distributions [39]. To increase the sensitivity of detection, usually 6–24 repeats of the MS2 binding site are introduced into target RNAs, though as many as 96 repeats have been used to enable single molecule-sensitivity [40]. For abundant RNAs such as viral genomes, fewer hairpins may be sufficient [41] and ideally, their number should be optimised according to target RNA levels.

A limited number of studies have used the MS2 system in plants to date. However, their diversity demonstrates its broad applicability. Uses include: (i) detection of turnip crinkle virus in *Arabidopsis* (without subcellular resolution) [41]; (ii) transport of prolamine mRNA to protein storage bodies in rice [42]; (iii) cytoplasmic redistribution of a nuclear RBP by the short open reading frame RNA *Enod40* in *Medicago truncatula* [37]; (iv) localisation of small RNA precursors in nuclear processing bodies [38,39]; and (v) co-localisation of a nuclear mRNA with its encoded viral protein [43].

A problem of the MS2 system lies in the introduction of novel sequences with extensive secondary structure into the RNA of interest, which may interfere with RNA–protein interactions, including factors that direct RNA localisation and function (Table 1). Additionally, the introduction of large numbers of hairpin-repeats represents a practical cloning challenge. In order to avoid such problems, careful selection of the tag position within the target RNA is necessary, and controls with alternative tag positions should be included. RNA sequences that determine subcellular localisation, so called 'ZIP codes', are often located within the 3′UTR [9,10]. In the few plant systems studied, ZIP codes have been found in the 5′ and 3′UTRs, as well as the coding region itself [31,32]. Furthermore, binding of MS2 CP close to the 5′cap represses translation in plants [44]. For mRNAs, a first choice for MS2 tagging is therefore the boundary of the open reading frame and the 3′UTR, directly downstream of the stop codon [45]. Another problem with nuclear targeted MS2 CP–FP is that the over-expressed reporter may act as a molecular 'trap' that redirects less abundant transcripts. To avoid such problems, the reporter system can be expressed under the control of an inducible promoter [39].

A second, similar detection system was described recently [46] based on the 22-amino-acid $\lambda N_{22}$ peptide, a fragment of the N protein from the bacteriophage λ. Similar to MS2 CP, $\lambda N_{22}$ binds to a 15-nucleotide hairpin structure called '*boxB*' [47], which is bound with slightly lower affinity ($K_D$ = 22 nM) [48] than MS2. The sensitivity of this reporter can be increased by fusing tandem copies of $\lambda N_{22}$ as well as GFP (4x$\lambda N_{22}$-3xGFP) [46]. A similar approach could also be used for MS2, although the larger tandem FP-fusions may interfere with nuclear import and export. The $\lambda N_{22}$ system has not been used in plants, but it has been demonstrated in yeast (*Saccharomyces cerevisiae*) that two different RNAs can be imaged simultaneously in the same cell using MS2 CP and $\lambda N_{22}$ in combination [45]. Similar studies will probably follow shortly in plant systems. Finally, both MS2 and $\lambda N_{22}$ are suitable for RNA particle tracking with fusions to photoswitchable FPs [49]. Considering the often highly dynamic nature of RNA localisations, photoswitchable fluorescent reporters [50,51] as well as photobleaching recovery studies [52] are particularly promising approaches that have yet to be fully exploited for RNA imaging.

### *Pumilio-BiFC*

An alternative to MS2 and $\lambda N_{22}$, based on a different type of RBP, was recently described [53]. This system is based on the RNA binding domain of the translational repressor human Pumilio1 (Pumilio homology domain, PUMHD). A promising feature is that the PUMHD can be engineered to bind to a target RNA of choice, eliminating the need to tag the RNA with foreign sequences [54] (Table 1). This is possible because of the PUMHD modular structure, which consists of eight imperfect repeats that each bind to one base of a non-structured eight-nucleotide target sequence. All protein–RNA contacts are mediated by amino acid side-chains and the RNA bases [55], allowing predictable alterations of the sequence specificity by genetic engineering [54]. For RNA imaging (Figure 1b), the authors of Ref. [53] engineered two PUMHD variants to bind to closely adjacent target sites of an mRNA, and fused them to the two halves of a split FP. Binding of both fusion proteins to the same RNA molecule brings the split FP parts into close proximity, allowing refolding of a complete FP, thus 'switching on' fluorescence (bimolecular fluorescence complementation, BiFC; see Box 1). In plants, Pumilio-BiFC has been used to show that the RNA genomes of tobacco mosaic virus and potato virus X have specific distributions within their respective replication sites in the cell (Figure 2b) [56] and to localise turnip mosaic virus RNA to invaginations of the chloroplast envelope [57].

The use of BiFC to generate an RNA-dependent fluorescent signal eliminates the need to sequester the PUMHD–FP fusion to the nucleus to distinguish unbound and bound RBP pools. This approach also increases the specificity of binding as simultaneous recognition of two

from unbound beacons, several different approaches have been developed, e.g. self-quenching **(i)** [67], or dual beacons **(ii)** [71]. Self-quenching beacons form a hairpin structure in their unbound state, bringing the fluorophore into close contact with a 'quencher', a fluorescence resonance energy transfer (FRET) acceptor (see Box 1) that can absorb the excitation energy from the fluorophore, preventing it from emitting fluorescence (green granules). Dual beacons hybridise to two adjacent sites on the same RNA and in the process bring a pair of fluorophores into close contact enabling FRET (Box 1) (red granules = FRET signal, green granules = false positives from degraded green beacons). Wavelength-shifting beacons **(iii)** use the three-way interaction between a FRET pair and a quencher to induce a change in the emission wavelength upon binding, enabling simultaneous use of multiple beacons excited by the same light source.

**Box 1. BiFC and FRET**

BiFC (reviewed in [79–81]) and FRET (reviewed in [79,80,82,83]) are techniques to detect protein–protein interactions in living cells by light microscopy.

**Bimolecular fluorescence complementation (BiFC)**
Green and red autofluorescent proteins and their derivatives have a barrel-shaped structure consisting of parallel β-sheets, which protect the fluorescence-emitting side chains (fluorophore) at its centre. They can be genetically split into a larger N-terminal half (FPN, containing most of the β-barrel and the unshielded fluorophore) and a smaller C-terminal half (FPC, containing the remainder of the β-barrel). By themselves, both split fluorescent-protein halves are nonfluorescent. However, a complete β-barrel can be reconstituted by refolding of the two halves together, resulting in activation of fluorescence. Refolding is a slow process thought to take tens of minutes. Therefore, it depends on prolonged close association of FPN and FPC. This can be used to detect protein–protein interactions: if the two halves of a fluorescent protein are fused to a pair of interacting proteins, this interaction will bring them into close proximity, allowing reconstitution of fluorescence. If the fused proteins do not interact, no fluorescence occurs. Once reconstituted, the FPN–FPC interaction is permanent, making BiFC unsuitable for the observation of temporary interactions.

**Fluorescence resonance energy transfer (FRET)**
FRET is the transmission of energy from one excited fluorescent molecule (FRET donor) to another which is not excited (FRET acceptor). FRET requires very close proximity (nanometers) between the two fluorophores and energy is transferred downhill, i.e. from a fluorophore excited by shorter wavelength (higher energy) light to one excited by longer wavelengths (lower energy). As a result of FRET, the donor fluorophore remains nonfluorescent whereas the acceptor can emit the excitation energy as fluorescence at a longer wavelength. FRET dims donor fluorescence and also shortens the duration of fluorescence emitted by a population of donor molecules after an ultra-short pulse from an excitation light source (fluorescence lifetime). By contrast, the acceptor fluoresces in response to excitation light suitable for donor excitation and absence of acceptor-specific excitation. All of these phenomena can be used to monitor FRET. In cell biology, FRET is often measured between pairs of fluorescent-protein fusions, to detect interactions between the proteins they are fused to, but FRET donors and acceptors can be any kind of fluorophore, not just fluorescent proteins. In contrast to BiFC, FRET relies on transient interactions and is therefore suitable for the monitoring of interaction dynamics.

target sites by different PUMHD variants is required. The requirement for adjacent binding sites should also suppress potential background signal from endogenous Pumilio target RNAs [58]. New structural evidence shows that Pumilio RNA binding domains are not as sequence-specific as originally believed, but can accommodate a certain degree of sequence promiscuity [59]. Again, use of dual-PUMHD binding should compensate for binding promiscuity as it is less likely that two adjacent alternative binding sites are present in other RNAs. Unfortunately, whilst the native PUMHD has a higher target affinity ($K_D$ = 0.48 nM, [54]) than both MS2 CP and $\lambda N_{22}$, changing its specificity may reduce affinity by one or two orders of magnitude [53,54]. RNA imaging using Pumilio domains may eventually take the approach of tagging RNAs with a generic recognition motif bound by a high-affinity PUMHD variant [54], rather than engineering new protein variants for each application. In such a case, a benefit of the Pumilio-BiFC system is that the PUMHD binding motif does not introduce additional secondary structures into the RNA [55].

The reconstitution of a complete, fluorescent FP during BiFC is permanent (see Box 1), i.e. there is no 'switch off', and eventually an RNA-independent fluorescence signal will accumulate. A further development for Pumilio-based RNA imaging could be the use of fluorescence resonance energy transfer (FRET, see Box 1). In FRET, close proximity of two proteins (e.g. two PUMHD variants bound to adjacent sites of the same RNA) is detected through a transient photochemical interaction between two FPs, eliminating the accumulation of the nonspecific signal. FRET is routinely used in RNA detection with molecular beacons (see below) and has also been applied to the detection of RNA–protein interactions *in vivo* [60]. However, FRET detection is technically more demanding than BiFC, in particular when observing dynamically changing localisations such as motile RNA granules.

## RNA labelling with cell-permeant dyes

Amongst the numerous fluorescent dyes that bind to nucleic acids by intercalation, some preferentially label RNA, but do not generally allow for sequence-specific RNA imaging. However, an exciting new approach is the development of non-fluorescent dyes that are 'switched on' by binding to an RNA aptamer (Figure 1c) [61]. This technique is still in its infancy, but could potentially provide a breakthrough in simplifying RNA imaging. As with the MS2 and $\lambda N_{22}$ systems, aptamer binding involves the introduction of new secondary structures into the RNA. For plant systems, this approach also requires permeation of the dye through the additional barrier of the cell wall (Table 1).

### *In vivo* RNA reporters that require invasive delivery

Some of the most specific RNA *in vivo* detection systems require the intracellular delivery of membrane-impermeant molecules. In animal systems this is achieved by electroporation, PEG precipitation or microinjection. Due to the presence of the plant cell wall, the former two techniques are only an option in protoplast systems [62]. Thus, microinjection is the only alternative for intact plant cells or tissues. Due to the cell's turgor pressure and the small volume of the cortical cytoplasm, microinjection into plant cells is a labour-intensive, low-throughput technique [17]. However, the option to introduce chemically 'aberrant' RNAs not normally found in plant cells may make this additional effort worthwhile.

#### *Direct RNA labelling*

For direct RNA labelling, the RNA of interest is covalently labelled by incorporation of nucleotides coupled to fluorescent moieties during *in vitro* transcription from a cDNA template, and then introduced into the cell [17,63] (Figure 1d). Provided that unincorporated fluorescent nucleotides are removed afterwards, this is the only technique that allows noise-free RNA imaging, at least immediately after injection. In plants, microinjection of directly labelled viral RNA has demonstrated the presence of motile RNA granules (Figure 2c) [17] similar to those found in animal systems [63,64], and has shown that the formation of these granules depends on the presence of the 5′cap [17].

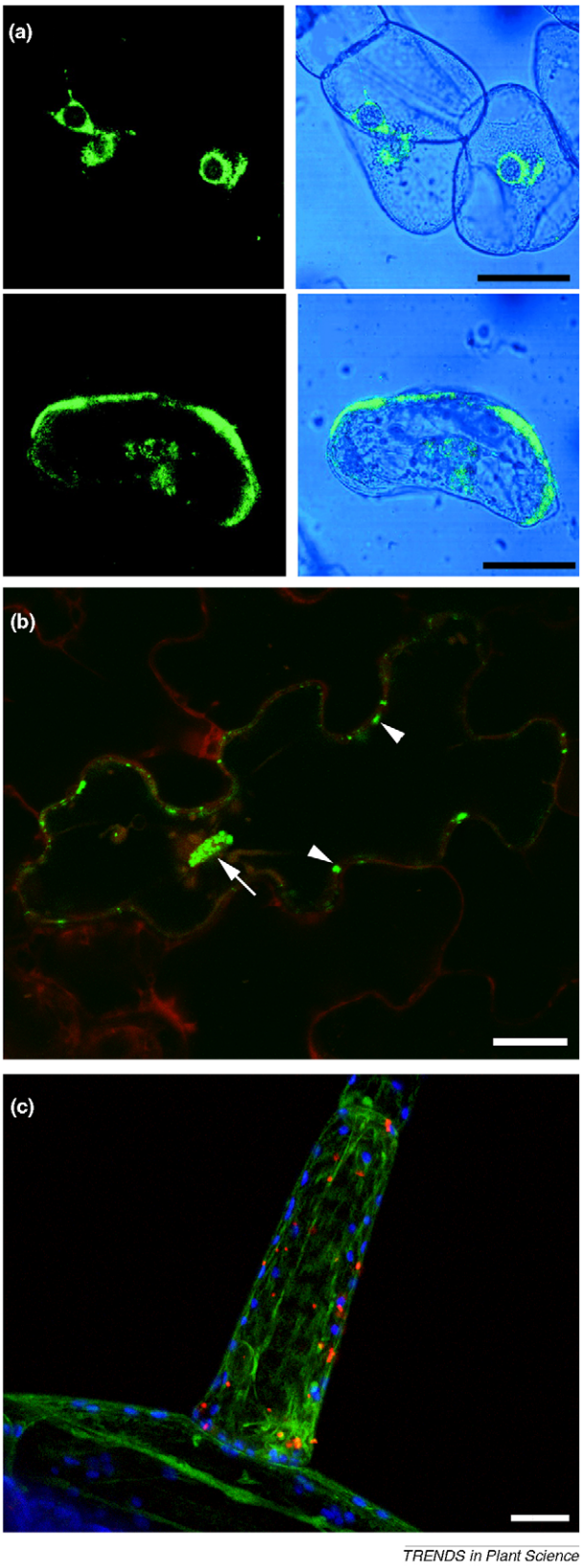


**Figure 2.** Examples of subcellular RNA imaging in plants. **(a)** MS2-system: an MS2 CP–GFP fusion protein with a nuclear targeting signal is present in the nucleus of tobacco BY-2 cells (upper panels). When a rice prolamine mRNA tagged with 6xMS2 hairpins is co-expressed in the same cell, the fluorescent reporter is redistributed to the cellular periphery, highlighting the RNA localisation (lower panels). The panels on the left show GFP fluorescence only, the panels on the right have a differential interference image overlaid to show the cell contours (reproduced with permission from [42]). Scale bar: 50 μm. **(b)** Pumilio-BiFC: genomic RNA of the plant virus, tobacco mosaic virus (TMV), is localised in a central replication site (arrow) next to the nucleus and smaller, peripheral replication sites (arrowheads) in a typical jigsaw-shaped tobacco leaf epidermal cell. The red signal derives from a nucleo–cytoplasmic red fluorescent protein expressed from the virus and used as an infection marker (reproduced with permission from [56]). Scale bar: 20 μm. **(c)** Direct RNA labelling: genomic RNA of TMV, transcribed *in vitro* and covalently labelled with the red fluorescent dye Cy3 (red), forms granules that attach to the actin–ER network immediately after introduction into a tobacco trichome by microinjection. Chloroplast autofluorescence is shown in blue and the endoplasmic reticulum (labelled with a GFP fusion protein) in green. (Christensen *et al.*, unpublished). Scale bar: 20 μm.

Other applications of direct RNA labelling in plants include the study of intercellular transport of viral RNAs [65], and the nuclear export of uncapped mRNAs in tobacco protoplasts [62]. Directly labelled RNA can also be microinjected into subcellular compartments such as the nucleus to investigate RNA splicing and nuclear export of mRNA [66], but such studies have not yet been conducted in plants.

Potential artefacts arising from microinjection of directly labelled RNA include overloading the cellular pool of RNA-binding proteins and lack of nuclear RNA processing (Table 1). Otherwise, this is a particularly exacting technique for *in vivo* RNA localisation that should see increasing use in plant systems.

### *In vivo hybridisation (molecular beacons)*

Molecular beacons are fluorescently labelled oligonucleotides (DNA or synthetic constructs with alternative backbones) that fluoresce only when hybridised to a complementary target sequence [67,68]. This is achieved by linking the oligonucleotide to a fluorophore and a quencher group that 'turns off' the fluorescence for as long as it remains close to the fluorophore. Originally, beacons were constructed as hairpin structures with three parts [see Figure 1e (i)]: a loop portion of generally 15–30 nucleotides complementary to the target molecule; and two complementary strands of 5–8 nucleotides that form a base-paired stem that keeps the fluorophore and quencher in proximity. Upon hybridisation of the loop portion with a target sequence, the stem structure of the beacon separates, ending the FRET contact between fluorophore and quencher (see Box 1), thus permitting the emission of fluorescence. Fluorescence intensity of molecular beacons increases over 200-fold upon binding to the RNA target [67].

Optimisation of molecular beacons requires sufficient knowledge of the secondary structure of the beacon and the target sequence because target sequences that have secondary structure themselves may be inaccessible [69] (Table 1). It is now recognised that the hairpin is not obligatory for the functioning of molecular beacons, and new 'stemless' constructs have been developed [70]. The specificity of molecular beacons can be increased by using dual beacons [68,69,71]. In this case, two beacons with quenched fluorophores are engineered to bind to adjacent sequences on the target RNA. Un-quenching upon binding enables FRET (Box 1) between the two fluorophores [Figure 1e (ii.)]. A further development is the introduction of wavelength-shifting molecular beacons [68,69,72] in which two fluorophores, a 'harvester' (FRET donor, Box 1) and an 'emitter' (FRET acceptor), are attached to one terminus of the oligonucleotide and the quencher to the

other. In the unbound beacon the efficiency of the quencher is far greater than the energy transfer from the 'harvester' to the 'emitter'. When the probe hybridises, FRET occurs and the emitted fluorescence is wavelength-shifted [Figure 1e (iii)]. Wavelength-shifting beacons allow for the simultaneous use of multiple beacons excited by the same wavelength [72].

Molecular beacons have allowed detection and localisation of RNA *in vivo* and provided insights into mRNA transport and translation [73–75], subnuclear RNA movement [76] and the transport and export of viral RNA [77]. Single-molecule sensitive RNA detection with molecular beacons has been achieved in living cells [76,78]. A great advantage of their use is that endogenous transcripts can be detected at their native levels and after correct mRNA processing. Potential problems in the use of molecular beacons for *in vivo* RNA imaging are the potential for self-quenching and the initial accumulation of the beacon in the nucleus by unexplained mechanisms, delaying the binding to cytosolic target RNAs [69] (Table 1). However, this has been prevented by linking the beacons to bulky proteins such as streptavidine [78].

So far, no applications of molecular beacons for RNA imaging have been reported for plant cells. Like directly labelled RNA, the technique requires microinjection unless protoplasts are used. The high sensitivity and signal-to-noise-ratio achievable with molecular beacons, the potential to image several RNA species simultaneously using wavelength-shifting beacons, and most importantly the possibility to image native RNA levels, may make this approach attractive enough to warrant the low-throughput delivery method required in plant systems.

## Conclusions and outlook

The field of RNA imaging is advancing rapidly, and many of the new techniques can be conducted on living cells at subcellular resolution or even single-molecule level [40,76,78]. Single-molecule imaging is hampered in plants by the strong autofluorescence of the tissue, but remains an important goal of further technical developments. As with most microtechniques, there is a clear trade-off between precision and throughput, and most of the emerging techniques will require careful optimisation and appropriate controls. The ever-increasing evidence for RNA dynamics in plant cells and the need to localise RNAs with precision to distinct subcellular compartments will require the continued development of localisation methodologies that keep pace with the rapid developments occurring in the fields of RNA processing, turnover and degradation.

## Disclosure

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