REVIEW

The mRNA mobileome: Challenges and opportunities for deciphering signals from the noise

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Abstract

Organismal communication entails encoding a message that is sent over space or time to a recipient cell, where that message is decoded to activate a downstream response. Defining what qualifies as a functional signal is essential for understanding intercellular communication. In this review, we delve into what is known and unknown in the field of long-distance messenger RNA (mRNA) movement and draw inspiration from the field of information theory to provide a perspective on what defines a functional signaling molecule. Although numerous studies support the long-distance movement of hundreds to thousands of mRNAs through the plant vascular system, only a small handful of these transcripts have been associated with signaling functions. Deciphering whether mobile mRNAs generally serve a role in plant communication has been challenging, due to our current lack of understanding regarding the factors that influence mRNA mobility. Further insight into unsolved questions regarding the nature of mobile mRNAs could provide an understanding of the signaling potential of these macromolecules.

Introduction

Multicellular organisms use long-distance signaling to communicate and coordinate responses to external biotic and abiotic stimuli (Segel et al., 2021; Takahashi and Shinozaki, 2019). Some possible vehicles for this long-distance communication include metabolites, hormones, proteins, peptides, electrical signals, and small RNAs (Choi et al., 2016; Parent et al., 2012; Durbak et al., 2012; Shi et al., 2018; Müller and Harrison, 2019; Kong et al., 2019). More recently, messenger RNAs (mRNAs) have been implicated in long-distance signaling. mRNAs were first detected in phloem sap in the 1970s (Kollmann et al., 1970); however, they were initially considered to be contaminants. It was not until decades later that consistent detection of mobile mRNAs in the phloem translocation stream led researchers to hypothesize that mRNAs may also function as non-cell autonomous information macromolecules—i.e., long-distance signals (Lucas et al., 2001; Kühn et al., 1997; Sasaki et al., 1998; Ruiz-Medrano et al., 2001; Wu et al., 2002; Ruiz-Medrano et al., 1999; Xoconostle-Cázares et al., 1999; Kim et al., 2001). Since this initial discovery, mRNAs have been found in the phloem sap of a broad range of families, including the Brassicaceae, Cucurbitaceae, and Euphorbiaceae (Deeken et al., 2008; Doering-Saad et al., 2002; Doering-Saad et al., 2006; Kanehira et al., 2010; Omid et al., 2007; Dinant and Kehr, 2013;

Ostendorp et al., 2017). These observations of mRNA in the phloem sap have subsequently been expanded upon, and now demonstrate that RNA movement is associated with diverse processes, including: gene silencing, development, abiotic responses, and plant-parasite interactions (Kim et al., 2001; Haywood et al., 2005; Melnyk et al., 2011; Pallas and Gómez, 2013; Kim et al., 2014; Molnar et al., 2010; Lewsey et al., 2016; Ghate et al., 2017; Banerjee et al., 2006; David-Schwartz et al., 2008; Shahid et al., 2018; Johnson et al., 2019; Thieme et al., 2016). Indeed, across a wide range of studies, there is a ubiquitous presence of long-distance mobile RNAs moving throughout the plant body. This review refers to the collective content of mobile macromolecules as the "mobileome," and focuses specifically on the role of mRNAs in the mobileome.

Investigations into the mRNA mobileome have uncovered hundreds to thousands of genes that produce mobile transcripts. While this profiling comprises a large database of identified mobile mRNAs, there is a general lack of evidence to support signaling functions for these transcripts (Kehr et al., 2022; Morris, 2018; Guan et al., 2020). Given the high proportion of genes that produce mobile mRNAs, current questions in the field center on the functional significance of this data (Oparka and Cruz, 2000; Morris, 2018; Kehr et al., 2022). It is challenging to determine the extent to which mobile transcripts function as signals due to our limited understanding of the factors influencing mRNA mobility. Insights into the transport, transcriptional origin and destination, and downstream impacts of the mRNA mobileome will increase our understanding of whether the mRNA mobileome functions in plant communication. The value of understanding the signaling capacity of mobile mRNAs is far-reaching. Beyond the basic implications for understanding this form of long-distance communication, there is a wide range of potential applications in commercial agriculture, including: engineering for disease resistance, increasing crop resilience to abiotic stresses, and enhancing yield in numerous herbaceous and woody plants (Gaion et al., 2018; Warschefsky et al., 2016; Williams et al., 2021).

Static methods to measure mobility

The two main approaches to profile the mRNA mobileome involve transcriptomic sequencing of either isolated phloem sap or grafted organ systems that comprise distinct genotypes. Phloem sap exudate collection has provided foundational insight into long-distance mobile proteins, RNAs, and metabolites (Silva et al., 2022; Hanhart et al., 2019; Ostendorp et al., 2017; Omid et al., 2007). Although this method can be difficult due to the low volume of phloem sap that is exuded by most species and the high potential for contamination from the surrounding tissue (Dinant and Kehr, 2013), experts in the field have identified methods to optimize this protocol (Pahlow et al., 2018). One method involves EDTA-facilitated exudation; however, in this approach the exudate is prone to RNA degradation and spatial contamination from other tissues bordering the vascular system (Deeken et al., 2008; for further detail see Kehr et al., 2022). Because of these limitations, most phloem profiling has been collected from plants that spontaneously exude their phloem sap, such as *Brassica napus* (Pahlow et al., 2018). In these studies, sampling location has been shown to influence exudate content. For example, researchers have uncovered different profiles, depending on whether they harvested phloem sap from petioles, cut stems, or small incisions into the stem (Gai et al., 2018a, 2018b; Omid et al., 2007; Yoo et al., 2004; Zhang et al., 2016b; Pant et al., 2009, 2008; Buhtz et al., 2010, 2008). Another method is aphid stylectomy, which involves stylet dissection from phloem-feeding aphids

followed by RNA profiling. This method can be used to overcome contamination from surrounding cells; however, it is highly technical, typically yielding low sample volumes, and can be impacted by insect-induced responses within the plant (Gaupels et al., 2008; Fisher and Frame, 1984).

Grafting offers an alternative approach for mobile mRNA discovery, and generally presents lower technical barriers. Grafting, which is widely used in agricultural crop production, takes advantage of the inherent regenerative ability of plants to reconnect their vascular tissue after wounding (Lee et al., 2010; Gaion et al., 2018; Melnyk and Meyerowitz, 2015). This allows researchers to combine rootstocks and scions from genetically distinct individuals and subsequently infer RNA mobility based on genotype-specific sequence differences. Until recently, this technique was exclusive to eudicots and gymnosperms. A new, innovative approach using embryonic grafting has successfully circumvented graft failure for monocots (Reeves et al., 2022), opening up a powerful approach for studying long-distance communication in this branch of angiosperms. When using this approach, some considerations must be taken into account to ensure the systemic transfer of water, nutrients, and macromolecules. First, researchers should confirm that the selected graft combination is compatible (i.e. can form reconnected vasculature between the graft partners) (Wang et al., 2017; Melnyk et al., 2015). Additional considerations include healing time, as sampling too soon after grafting will profile changes specific to graft formation, and that sample harvesting is performed at a sufficient distance from the junction itself to avoid tissue contamination from the graft site (Notaguchi et al., 2015). Since grafting can be applied to a wide range of species, this method has become the preferred approach for many researchers in the field of long-distance mRNA movement.

Biological methods are only half the story: the elucidation of putative mobile transcripts depends heavily on the bioinformatic pipelines that are used to process and classify reads. In the case of heterografting approaches, the origin of sequenced transcripts can be inferred using genotype-specific allele differences between the graft partners. These differences can be detected based on single-nucleotide polymorphism (SNP) calling or exclusive genomic alignment (Thieme et al., 2015; Kim et al., 2014). Differential alignment only works for grafts between species with sufficiently distinct genomes, while SNP calling works best when genomes are similar to one another such as between ecotypes or closely related species (Zhang et al., 2016b; Kim et al., 2014; Liu et al., 2020b; Wang et al., 2020; Xia et al., 2018; Li et al., 2021; Thieme et al., 2015). Based on these methods, sequences can be designated as non-mobile (i.e. produced within the harvested cells) or mobile (i.e. produced within cells from the reciprocal half of the graft) with a certain degree of accuracy (Thieme et al., 2015; Kim et al., 2014). RNAseq combined with bioinformatic pipelines have revolutionized our understanding of the mRNA mobileome. However, insufficiently stringent bioinformatic pipelines may overestimate the number of mobile transcripts (Kehr et al., 2022; Morris, 2018), and contribute to bioinformatic false positives, and thus obscure high-confidence identification of mobile mRNAs. Sequencing errors and transcript misalignment can also contribute to this noise, as they can both lead to false positives. One approach to reduce false positives is stringent quality trimming. A recent study provides another approach using Bayesian statistics to address misalignment in homografted samples and confidently infer RNA mobility in hetrografted samples (Tomkins et al., 2022). The widespread application of these improved bioinformatic methods will help refine a high confidence list of enriched mobile mRNAs across species.

Differences in existing data make it challenging to identify consistently mobile transcripts. These variables include species sampled, age of plants, developmental stage, apoplastic versus symplastic phloem loading, and timing of sample harvest (days post-grafting as well as circadian time), (Liu et al., 2020b; Xia et al., 2018; Kehr et al., 2022; Guan et al., 2020). Indeed, cross-species analyses have demonstrated that only a small fraction of mobile mRNAs identified within a given study overlap with orthologs from other published work (Xia et al., 2018; Liu et al., 2020b). It has also been noted that the population of identified mobile mRNAs correlates with the experimental approach employed and the number of biological replicates (Kehr et al., 2022; Morris, 2018). Another factor that may contribute to this noise across studies is the dynamics of transcription within the tissues of origin; gene expression is a process of sequential stochastic single-molecule events, making sporadic variations difficult to distinguish from intrinsic variations (Tutucci et al., 2018; Munsky et al., 2012; Sanchez and Golding, 2013). Despite these challenges, a select handful of enriched gene families have been identified across mRNA mobileomes from diverse species, which we will discuss below (Kehr et al., 2022; Guan et al., 2020).

Parasitism serves as a parallel to grafting. Plant parasites form haustoria with host species, connecting their vascular tissues, and thus allowing researchers to infer RNA mobility between the parasite and its host. Cuscuta has served as a particularly useful parasite model system for studying this process, as it infects diverse host plants, and is supported by a reference genome (Sun et al., 2018; Furuhashi et al., 2011). Bi-directional macromolecular movement has been detected between host-parasite interactions. From parasite-to-host, small regulatory RNAs, microRNAs (miRNA), proteins, and viruses have been shown to move across the haustorium (Bennett, 1940, 1944; Shahid et al., 2018; Liu et al., 2020a; Johnson et al., 2019). In the reciprocal direction, host plant RNAs and proteins have been shown to transmit back into the parasite (Liu et al., 2020a; Shen et al., 2020; Kim et al., 2014; Alakonya et al., 2012; David-Schwartz et al., 2008). This exchange of macromolecules between parasites and their hosts have been functionally connected to interspecies plant communication. Hosts are able to transmit FLOWERING LOCUS T (FT) into Cuscuta to trigger flowering, and in reverse direction, Cuscuta delivers miRNAs that are capable of silencing host mRNAs (Shen et al., 2020; Shahid et al., 2018).

Signals in the mRNA mobileome

RNA sequencing across graft junctions has led to the discovery of thousands of mobile transcripts (Kehr and Kragler, 2018). These studies have detected all major classes of RNA: mRNAs, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and various classes of small RNAs (Buhtz et al., 2010, 2008; Liu et al., 2013; Pant et al., 2009; Rodriguez-Medina et al., 2011; Varkonyi-Gasic et al., 2010; Yoo et al., 2004; Gai et al., 2018b). Roughly a quarter of the transcriptome has the potential to move according to both Arabidopsis-Cuscuta host-parasite interactions and Arabidopsis grafting experiments (Thieme et al., 2015; Kim et al., 2014).

In contrast to the huge number of mobile transcripts identified, only a handful of mRNAs are characterized as functional long-distance mobile signals— defined here as those which consistently move post-transcriptionally and have a phenotypic response associated with their movement. This list of functionally characterized mRNAs includes: *GA INSENSITIVE* 1 (*GAI1*) involved in leaf development (Haywood et al., 2005), *PYRO-PHOSPHATE-DEPENDENT*

PHOSPHOFRUCTOKINASE (PFP) and *LeT6* influencing leaf shape (Kim et al., 2001), *BEL1-RELATED HOMEOTIC PROTEIN 5* (*BEL5)* enhancing tuber production (Banerjee et al., 2009; Cho et al., 2012), *FLOWERING LOCUS T* (*FT*) for systemic floral signaling (Lu et al., 2012), *INDOLEACETIC ACID 18* and *INDOLEACETIC ACID 28* (*IAA18* and *IAA28)* inhibiting lateral root formation (Notaguchi et al., 2012), *CHOLINE KINASE1* (*CK1*) maintaining rosette leaf size (Zhang et al., 2016a), *CENTRORADIALIS homologue* (*ATC*) inhibiting floral initiation (Huang et al., 2012), and *TRANSLATIONALLY CONTROLLED TUMOR PROTEIN 1* (*TCTP1*) affecting root architecture (Yang et al., 2019; for further detail see Winter and Kragler, 2018). Interestingly, these characterized mobile mRNAs are all associated with key roles in organ growth and development. Beyond these demonstrated signaling mRNAs, there is also some enrichment, albeit low, for mobile mRNAs from certain gene families across diverse species, implying potential conservation of putative signaling functions; however, this remains to be tested (Gaupels et al., 2008; Walther and Kragler, 2016; Yang et al., 2015).

Pathways from transcriptional origin to vascular entry

Surprisingly little is known about where mobile transcripts are initially transcribed. While in specific cases reporter lines have informed their transcriptional origin, the vast majority of profiled mobile mRNAs are not understood at this level. Distance from transcriptional origin to the vasculature may influence whether a mobile transcript functions as a signal; as longer cellular pathways imply potential targeted intercellular trafficking (Figure 1). However, this hypothesis remains to be tested on a genomic-scale.

Situated within adjacent cell walls, plasmodesmata (PD) are membrane-lined pores forming a continuous symplast between neighboring cells that is essential for intercellular communication (Lee and Frank, 2018; Kitagawa and Jackson, 2017; Kirk and Benitez-Alfonso, 2022; Faulkner, 2018). PD dynamically range in their size exclusion limit and shape making it challenging to pinpoint precisely which molecules can feasibly move between cells (Sager and Lee, 2018). Factors that can influence the size of these pores are developmental stage, tissue connectivity, environmental stress, and as we will discuss later, movement proteins (Sager and Lee, 2018). Measurements of solute flux have further shown that small molecules permeate through PD at rates of only microns per second (Rutschow et al., 2011). Based on this rate, a molecule traveling intercellularly up a plant that is 30 cm high (~1 foot) at the rate of 10 microns per second would take approximately 35 days to go from the root to the shoot apex via PD. This implies that while PD may serve as conduits for local transport into the vascular transport stream, their transport rate is too slow to explain the long distance movement of mRNAs that have been observed in diverse species.

Specialized PD also play a direct role in molecular entry into the phloem transport stream. The phloem consists of companion cells and sieve tubes that are highly connected via specialized branched PD called pore-PD, which act as gates for the entry and exit of molecules moving through phloem sap (Oparka and Cruz, 2000; Lee and Frank, 2018). Pore-PD, unlike other PD, on average allow for the passage of larger macromolecules (permeable for molecules < ~70 kDa and < ~500 Da respectively) (Paultre et al., 2016; Lee and Frank, 2018; Oparka and Cruz, 2000;

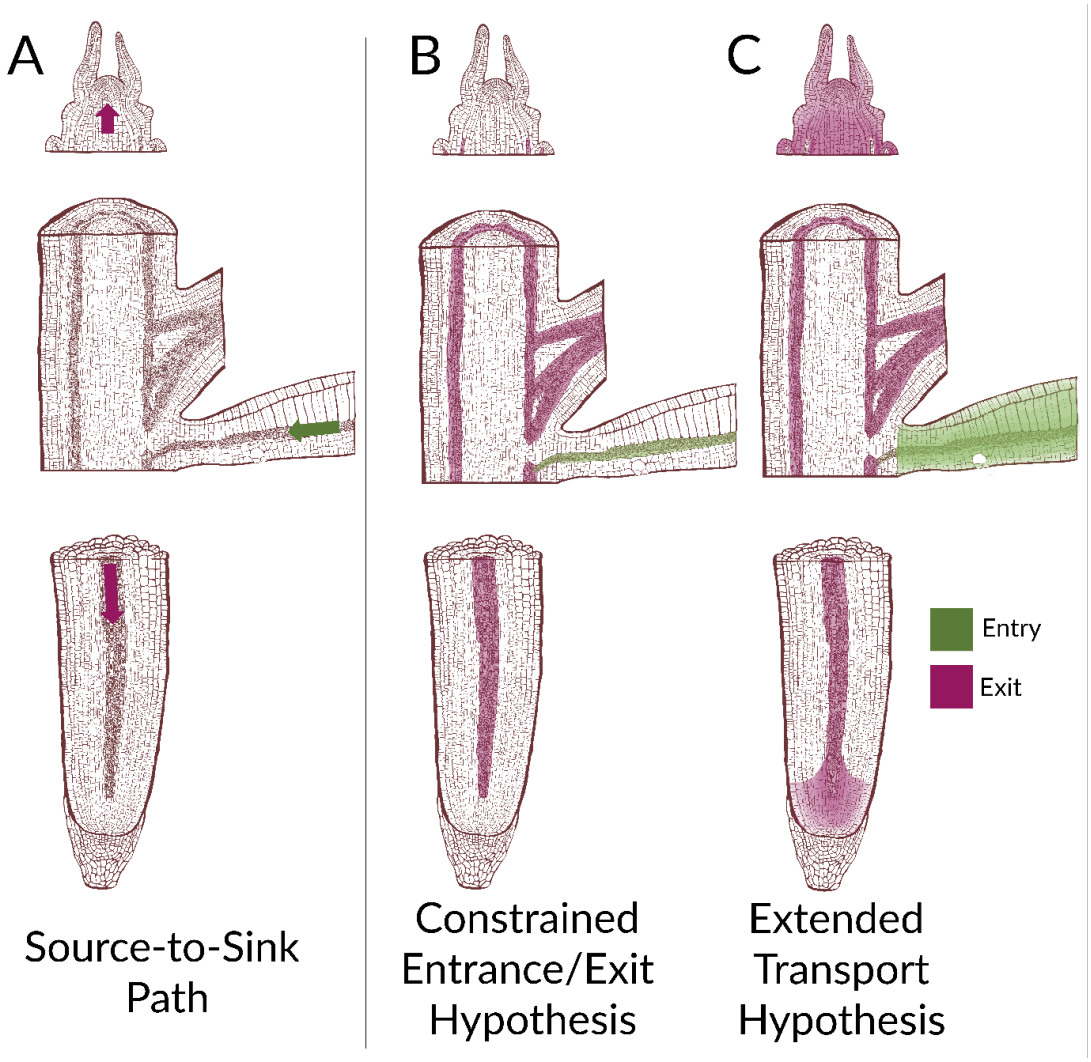

Figure 1. Anatomical drawing of a shoot apical meristem, stem with branch and subtending leaf, and root apical meristem. **A)** Source and sink flow is designated with arrows where green represents source and purple represents sink. **B-C)** Hypotheses on constraints of movement in transit: **B)** The hypothesis that mobile transcripts are unable to leave the constraints of the vascular stream. This would imply transcripts entering the stream are produced from companion cells and exiting would also be constrained cells immediately adjacent. **C)** The hypothesis that with active transport, mobile transcripts are able to leave the constraints of the vascular stream and be transported between cells with greatest concentration close to the vasculature.

for further detail see Peters et al., 2021). Thus, macromolecule movement may be restricted by other types of PD with lower permeability (Figure 1B). Transcriptional origin in companion cells, phloem cells sister to sieve elements, would be the shortest route into the phloem translocation stream and require the least PD based constraint for entry into the mobileome. There are particular instances, like that of FLOWERING LOCUS T (FT) mRNA, where transcripts have been shown to originate in a subset of companion cells (Chen et al., 2018). An independent modeling study further showed that transcripts expressed in high abundance in companion cells are often loaded into the phloem for transport, supporting the hypothesis that companion cell expression influences the content of the mRNA mobileome (Figure 1B) (Calderwood et al., 2016). However,

it has also been shown that high abundance alone is not sufficient for phloem loading (Huang and Yu, 2009), complicating a simple model in which abundance and close proximity directly correlates with phloem entry. Another factor that may influence vascular entry are fluctuating source-to-sink dynamics. The physiological dynamics of the transport stream shift over developmental and circadian time, changing the location of transient sources and sinks (Figure 2) (Knoblauch et al., 2016; López-Salmerón et al., 2019). These changes have the potential to influence the interface of transcript origin and mobility (Knoblauch et al., 2016; López-Salmerón et al., 2019). Overall, much remains unknown about the effect distance may have on transcript entry to the phloem, further research could provide insight into anatomical constraints on RNA entry into the plant mobileome.

Molecular superhighway: mRNA transit through the phloem

Phloem movement in the vascular system is controlled by high turgor pressure that drives phloem sap from source to sink, also known as the Münch hypothesis (Figure 2) (De Schepper et al., 2013; Turgeon and Wolf, 2009; Münch, 1930). The xylem and phloem can transport at much faster rates, 200–400 μm/s, than small molecules can permeate through PD transport paths, several μm/s (Peuke et al., 2001; Savage et al., 2013). Given that movement of mRNAs has been detected over substantial distances in woody and viny plants, such as grapes and beans, the relatively fast speed of vascular transport implicates this system as the most likely conduit for mRNA movement (Yang et al., 2015; Li et al., 2021). Both primary xylem and primary phloem are vascular tissues that originate from procambium; however, they are specialized for different functions. The xylem functions in long-distance apoplastic transport of water, primarily from root-to-shoot, while the phloem functions in long-distance symplastic transport of photosynthetic products from source-to-sink. One important distinction between these vascular tissues is that xylem cells are dead at maturity whereas the phloem is a living tissue system. A major factor that contributes to the content of the phloem sap is the maturation process of sieve elements, which are enucleate at maturity. As young sieve elements are connected to the phloem stream, macromolecules and complexes present within the cytoplasm prior to maturity are released into the transport stream (Knoblauch et al., 2018). The presence of ribosome and proteasome components in phloem exudate, which are too large to be transported into sieve elements, provides further evidence to support this model (Ostendorp et al., 2017; Knoblauch et al., 2018). Estimated macromolecule content from sieve element release into the transport stream can account for a significant proportion of RNAs and proteins detected in phloem exudate (Knoblauch et al., 2018). In other words, the inherent physiological and developmental processes underlying phloem maturation can contribute to the mobileome (Knoblauch et al., 2018).

There are three lines of evidence to support a model where mRNA moves via the phloem rather than the xylem. First, phloem sap has been shown to protect RNA from degradation (Doering-Saad et al., 2002; Gaupels et al., 2008; Sasaki et al., 1998), while xylem sap contains RNases (Bai et al., 2013; Alvarez et al., 2006). Second, most currently published studies profiling long-distance mRNA mobility show a strong source-to-sink relationship of movement (Zhang et al., 2016b; Notaguchi et al., 2015; Yang et al., 2015; Li et al., 2021; Ruiz-Medrano et al., 1999; Thieme et al., 2016). Third, modeling of factors that influence RNA entry into the phloem shows that proximity of transcript expression to sieve elements correlates with long-distance movement (Calderwood et al., 2016).

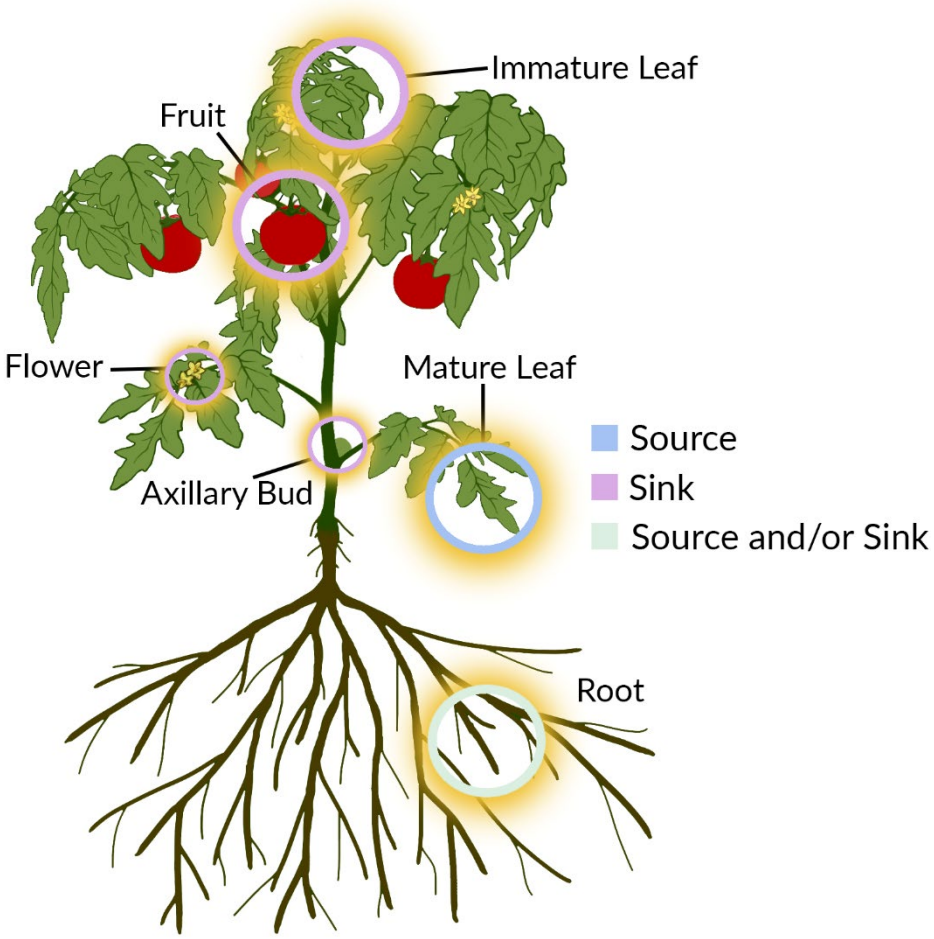

Figure 2. Schematic source-and-sink model of the plant body. Source-and-sink dynamics are driven by sucrose movement causing changes in vascular turgor pressure. The bulk of photosynthetic activity occurs in mature leaves, which serve as a source tissue. Meanwhile, the fruits, flowers, axillary buds, and immature leaves serve as transient sinks, while the shoot apical meristem serves as lifetime sink tissue. In annual plants, the root serves as sink tissue while in perennial plants roots can serve as a source or sink tissue depending on developmental needs.

Despite strong evidence in support of phloem-based movement, consistent evidence has shown that up to a quarter of identified mobile transcripts move against the source-to-sink gradient (from root-to-shoot in most cases). This observation conflicts with the assumption that the mRNA mobileome is transported via bulk phloem flow (Zhang et al., 2016b; Notaguchi et al., 2015; Yang et al., 2015; Li et al., 2021; Ruiz-Medrano et al., 1999; Thieme et al., 2016). These findings imply that mRNAs are likely moving against source-to-sink within the phloem, although no mechanism for this movement has been described (Thieme et al., 2015; Kehr and Kragler, 2018).

Intrinsically disordered proteins provide a potential route for movement against the source-to-sink gradient. Marked by an adaptable three-dimensional structure that can change when interacting with other molecules, intrinsically disordered proteins have recently emerged as a

mechanism for regulating dynamic cellular processes (Cuevas-Velazquez and Dinneny, 2018). Cells have been found to compartmentalize these intrinsically disordered protein-RNA aggregates via liquid-liquid phase separation, which could allow for insoluble, membraneless transport (Cuevas-Velazquez and Dinneny, 2018; Ostendorp et al., 2022; Fang et al., 2019). A striking example of liquid-liquid phase separation comes from the *PHLOEM-ASSOCIATED RNA CHAPERONE-LIKE* (*PARCL*) protein, which seems to bind to a broad spectrum of RNAs and act as an RNA chaperone (Ostendorp et al., 2022). Interestingly, it has been shown that the conformational flexibility of these disordered proteins can serve as 'molecular memory' that encodes external inputs, providing a potential conduit for connecting the environment with long-distance mRNA communication (Cuevas-Velazquez and Dinneny, 2018; Cuevas-Velazquez et al., 2021). Despite the potential for intrinsically disordered proteins to form these membraneless compartments, a mechanism for how these may move and shepherd mRNAs against bulk flow has yet to be investigated.

The xylem presents an alluring path for root-to-shoot movement. However, xylem transport represents an unlikely route for the mRNA mobileome given both the documented of RNAses and low abundance of RNA found in xylem sap profiling (Buhtz et al., 2008; Chukhchin et al., 2020; Bai et al., 2013; Alvarez et al., 2006). Protection from RNases in the apoplastic environment would be required for the xylem to act as a highway for long-distance mobile mRNAs. One potential route for xylem-based mRNA movement could take the form of extracellular vesicles (EVs), which are small lipoproteic membrane-derived structures that can contain or display on their surface: proteins, nucleic acids, and lipids (Urzì et al., 2022). Intriguingly,, RNA has been identified within and on the external surface of EVs (Cai et al., 2018; Doroshenk et al., 2010; Tian and Okita, 2014; Ruf et al., 2022; Baldrich et al., 2019; Zand Karimi et al., 2022). These vesicles can provide stabilization and protection from the surrounding environment (Kusuma et al., 2018). Experimental data has demonstrated that EVs extracted from the leaf apoplast encapsulate miRNAs, secondary small interfering RNAs (siRNAs), and a class of 'tiny RNAs,' in addition to protein-RNA complexes localized to the outer EV membrane (Cai et al., 2019; Baldrich et al., 2019; Cai et al., 2018, 2021; Zand Karimi et al., 2022). Although the prospect of EVs in the apoplast driving the movement of RNAs and proteins is exciting, only one group has documented the presence of EVs in plant vasculature (Chukhchin et al., 2020, 2021). Using atomic force microscope imaging this group has demonstrated the presence of exosomes in axial phloem and xylem parenchyma; however, the function of these EVs remains unconfirmed (Chukhchin et al., 2020; van Bel, 2021). This comparatively small body of research, paired with a lack of evidence showing larger mRNAs associating with EVs indicates that further study is required to determine whether EVs play a role in mRNA movement against source-to-sink gradients.

Are mobile mRNAs targeted to the correct destination?

Proteins are able to recognize RNA features, such as sequence motifs and structural motifs colloquially referred to as 'postal codes' or 'zipcodes', to traffic RNA to targeted subcellular compartments (Weis et al., 2013; Jeffery et al., 1983; Crofts et al., 2004; Lécuyer et al., 2007, 2009). Evidence to support that this zipcode concept extends to the intercellular, tissue-scale delivery of mRNAs is generally lacking; however, some studies provide intriguing evidence for

this model (Xoconostle-Cázares et al., 1999; Zhang et al., 2016a; Yang et al., 2019; Reagan et al., 2018).

Certain sequence and structural motifs have been shown to recruit localization proteins capable of mediating mRNA transport intercellularly (Xoconostle-Cázares et al., 1999; Lee et al., 2003). Studies of protein-RNA interactions have revealed around one hundred RNA-protein binding complexes in the phloem sap of various plant species (Pallas and Gómez, 2013; Gómez et al., 2004; Aki et al., 2008; Ham et al., 2009; Ostendorp et al., 2017; Pahlow et al., 2018; Yoo et al., 2004; Lin et al., 2009; Giavalisco et al., 2006). The role of many of these RNA-binding proteins remains unclear, however these proteins could potentially play roles in mRNA movement or assist in translation upon delivery (Lin et al., 2009; Rodriguez-Medina et al., 2011; Yoo et al., 2004).

Some RNAs have been documented to be actively targeted to and transported through PD (Gibbs, 1976; Fan et al., 2022; Wang and Dean, 2020). Movement proteins (MPs) are produced by RNA viruses, forming RNA–protein ribonucleoprotein (RNP) complexes that promote intercellular transport and thus the systemic spread of viruses (Lucas, 1995; Hipper et al., 2013; Gómez and Pallás, 2004; Ham et al., 2009; Itaya et al., 2002; Foster et al., 2002). MPs have been shown to increase the size exclusion limit of PD for larger molecules, allowing for permeability of otherwise impermeable molecules (Wolf et al., 1989; Oparka et al., 1997). Plants have proteins that may function in a similar fashion to MPs, these RNA binding proteins carry PD-localization signals that are sufficient to mark a transcript for intercellular trafficking. Compelling examples of this include PHLOEM PROTEIN 16 (PP16) RNA complex in *Cucurbita max* and the KNOTTED1 (KN1) TF mRNA-protein complex (Xoconostle-Cázares et al., 1999; Lee et al., 2003; Kitagawa et al., 2022a). The PP16 complex is able to bind both the sense and antisense sequences of its targets indicating that like several other phloem RNA-binding proteins, it may not bind in a sequence-specific manner (Xoconostle-Cázares et al., 1999; Ham et al., 2009). By interacting with PD and increasing their size exclusion limits, the PP16 RNP complex is able to increase the intercellular trafficking of bound RNAs (Xoconostle-Cázares et al., 1999; Ham et al., 2009). *KN1* mRNA and its related ortholog, *SHOOT MERISTEMLESS* (*STM*) is known to move from the L2 to the L1 layer of the shoot apical meristem (Kitagawa et al., 2022a; Lucas et al., 1995; Kim et al., 2002). It was first established that the KN1 complex targets PD and increases their permeability (Lucas et al., 1995) using the KNOX homeodomain of KN1 (Kim et al., 2005). However, it was recently shown that L2 to L1 movement is dependent upon the function of AtRRP44a, an RNA exosome subunit that both binds *KN1* and localizes to PD for intercellular transport (Kitagawa et al., 2022a). These two examples provide an alluring mechanism for RNA transport. However, the extent to which MP-like proteins facilitate mRNA movement more broadly within plants remains unclear (Ham and Lucas, 2017). MPs are not alone in using a selective PD RNA transport system, non-protein coding plant viroids exhibit selective trafficking of their RNA genomes using pseudoknot/hairpin motifs that are necessary for intercellular transport to specific cell types (Ding, 2009; Qi et al., 2004; Zhong et al., 2007; Zhu et al., 2002). Although MPs and viroids present an intriguing mechanism for PD targeting, in turn facilitating long-distance transport, the expansion of this targeting for long-distance mobile mRNA *in planta* is limited to a handful of examples.

tRNA-like structures are another potential line of evidence supporting the postal code hypothesis. These structures are enriched in phloem sap from grafted plants, and are sufficient

to mediate mRNA transport (Zhang et al., 2016a). Moreover, dicistronic mRNA:tRNA transcripts are enriched in the pool of mobile mRNAs. In a remarkable demonstration of the mobilizing function of tRNA-like sequences (TLS), the authors show that appending TLS to the dominant negative male-sterility gene, *DISRUPTION OF MEIOTIC CONTROL 1* (*DMC1*) is sufficient to mobilize *DMC1* from grafted stocks into the shoot apex where it induces male-sterility (Zhang et al., 2016a). Relatedly, CRISPR guide RNAs (gRNAs) can also be mobilized to the shoot apex by fusing the gRNA with FT transcript (Beernink et al., 2022; Ellison et al., 2020). A recent study went a step further, demonstrating that both Cas9 and its associated gRNAs can be mobilized across graft junctions by tagging these sequences with the previously characterized TLS motif (Zhang et al., 2016a; Yang et al., 2023). The authors show that this mobilized editing machinery can be delivered from transgenic rootstocks into recipient scions, and produce heritable, transgene-free edits in the scion (Yang et al., 2023). Another identified modulator of transport are m^5C epigenetic marks, which are deposited onto tRNA-like structures (Yang et al., 2019). Loss of the m^5C methyltransferases DNMT2/TRDMT1 and NSUN2B/TRM4B leads to mutants that have reduced methylation and exhibit decreased transcript mobility, indicating that this epigenetic mark plays an important role in regulating the mRNA mobileome (Burgess et al., 2015; Sibbritt et al., 2013; David et al., 2017; Cui et al., 2017; Yang et al., 2019). Strikingly, this study also showed that TCTP1, a known mobile mRNA, was immobilized in the methylation deficient mutant background, ultimately leading to a consistent phenotype of inhibited root growth (Yang et al., 2019).

Another intriguing example of mRNA movement comes from *GIBBERELIC ACID INSENSITIVE 1* (*GAI1*). This mRNA has been shown to move from grafted rootstocks into the shoot apex where it impacts the shape of emerging leaves (Haywood et al., 2005). This movement is not perturbed by changes in source-to-sink dynamics indicating that this process can be uncoupled from the bulk flow of the phloem (Haywood et al., 2005). RNA sequence of *GAI1* is both necessary and sufficient to facilitate *GREEN FLUORESCENT PROTEIN* (*GFP)* mRNA movement (Huang and Yu, 2009; Haywood et al., 2005). When researchers dissected the transcript to discover which motifs are necessary for mobility they identified that both the *GAI1* coding sequence and the 3' untranslated region are independently sufficient to facilitate movement (Huang and Yu, 2009; Haywood et al., 2005). As is the case for tRNA-like movement, this implies that RNA structure may be more critical than sequence for long-distance trafficking (Huang and Yu, 2009; Haywood et al., 2005; Zhang et al., 2016a). Not all functionally studied mRNAs are congruent with our understanding of the mobileome, underscoring the complexity of long-distance mRNA transport. For example the RNA binding protein RBP50 has been proposed to facilitate long-distance RNA transport by binding to its own mRNA via a polymer-pyrimidine CUCU domain (Cho et al., 2015; Ham et al., 2009). However, this domain is not significantly enriched in graft-mobile mRNA datasets from other species, indicating that this may be a species-specific mechanism (Yang et al., 2019).

The bulk flow hypothesis, as an alternative to the postal code hypothesis, proposes that contents of the phloem sap are passively transported down the pressure gradient (i.e., from source to sink) based on the physiological demands of the plant (Figure 2) (Leisner and Turgeon, 1993; Roberts et al., 1997). The role of bulk flow in long-distance macromolecular movement has primarily been examined in proteins. GFP expressed in companion cells moves non-selectively

into sieve tube elements and is then carried through the phloem transport stream (Imlau et al., 1999). Interestingly, in Arabidopsis roots, protein unloading from the sieve tube system into neighboring phloem-pole pericycle cells has been shown to occur in batch unloading, rather than as a continuous unloading process (Ross-Elliott et al., 2017). Moreover, this showed that macromolecules over 40 kDa are generally trapped within phloem-pore pericycle cells, where they are likely degraded or selectively transported into surrounding cells (Ross-Elliott et al., 2017; Lee and Frank, 2018). Though insights from mobileome proteins can shed light on biological constraints they cannot explain RNA mobility in its entirety.

Evidence for mRNA bulk flow primarily comes from literature showing that more mRNAs move from shoot-to-root (i.e., with phloem bulk flow) than root-to-shoot (Liu et al., 2020b; Thieme et al., 2015; Yang et al., 2015; Li et al., 2021). mRNA presents an intriguing outlier because it is capable of movement against the source-to-sink gradient while small RNAs, including miRNAs and phased secondary small interfering RNAs (phasiRNAs), are predominantly expressed in shoot systems and transported into root systems (Li et al., 2021; Thieme et al., 2015; Lewsey et al., 2016). It has been proposed that mobile mRNAs could serve as a recycling system, carrying carbon and nitrogen from source to sink tissues (Knoblauch et al., 2018; Ross-Elliott et al., 2017; Xia et al., 2018; Lee and Frank, 2018). Cycling of mobile mRNAs throughout the plant body, as seen in tripartite grafting experiments adds to the data supporting a model for the non-targeted recycling of phloem-mobile mRNAs (Xia et al., 2018). Taken together, these studies provide evidence for a large pool of non-targeted mRNAs traveling through the vascular transport stream. Given the strong evidence for selective moment of specific mRNA signals (KN1, GAI1, etc.) and substantial observations of untargeted movement, the vascular transport stream seems to comprise a mix of *bona fide* mRNA signals amid macromolecular noise. Deciphering the signal from the noise will clarify the full role of mRNAs in long-distance signaling.

An information theory perspective on mobile mRNA

Information theory broadly defines communication as having the following: a sending source of information, a medium for this information to pass through (often involving noise), and a receiver that attempts to reconstruct the meaning of the source message (Figure 3) (Shannon, 1948; Pierce, 2012). There is no agreement on what defines a signal for organismal, intra-organismal, or non-organic codified communication. However, the need to determine true signals from noise remains present across all of these systems. Information theory, once referred to as communication theory (Shannon, 1948), originally defined a signal as a codified message within a stochastic process. In the context of plant long-distance communication, we can think of the sending source as the cell of origin, the medium as the route of transport, and the receiver as the recipient cell (Figure 3). Noise can occur from the degradation of the signal and interference can affect the receiver's ability to perceive a signal from the other macromolecules flowing through the channel. As researchers listen in on this communication, biological and bioinformatic noise can occlude signal identity. Bioinformatic noise can come from RNA sequencing errors and transcript misalignment, while biological noise can come from RNA entry into the phloem sap from the close connection of companion cells to sieve elements and phloem tube maturation.

Biological signals convey information, or content, representing the world or instructing the receiver (Artiga et al., 2020). In the case of mRNA long-distance signaling, it has been proposed

that to consider an mRNA a ‘true’ signal, it must demonstrate that the transcript presence changes over time or space. That is, the transcript moves either spatially or temporally and is both received and decoded, causing a downstream effect in a reproducible manner (Morris, 2018; Kehr et al., 2022). We know from research that there are a few select miRNAs and mRNAs that move and function as true signals; however, for the majority of mobile transcripts, determining whether they are meaningful signals or noise from the bulk flow of the phloem remains unknown. Reciprocally, insights into how plants encode information in RNA signals could expand the field of information science, and be applied to in silico data compression.

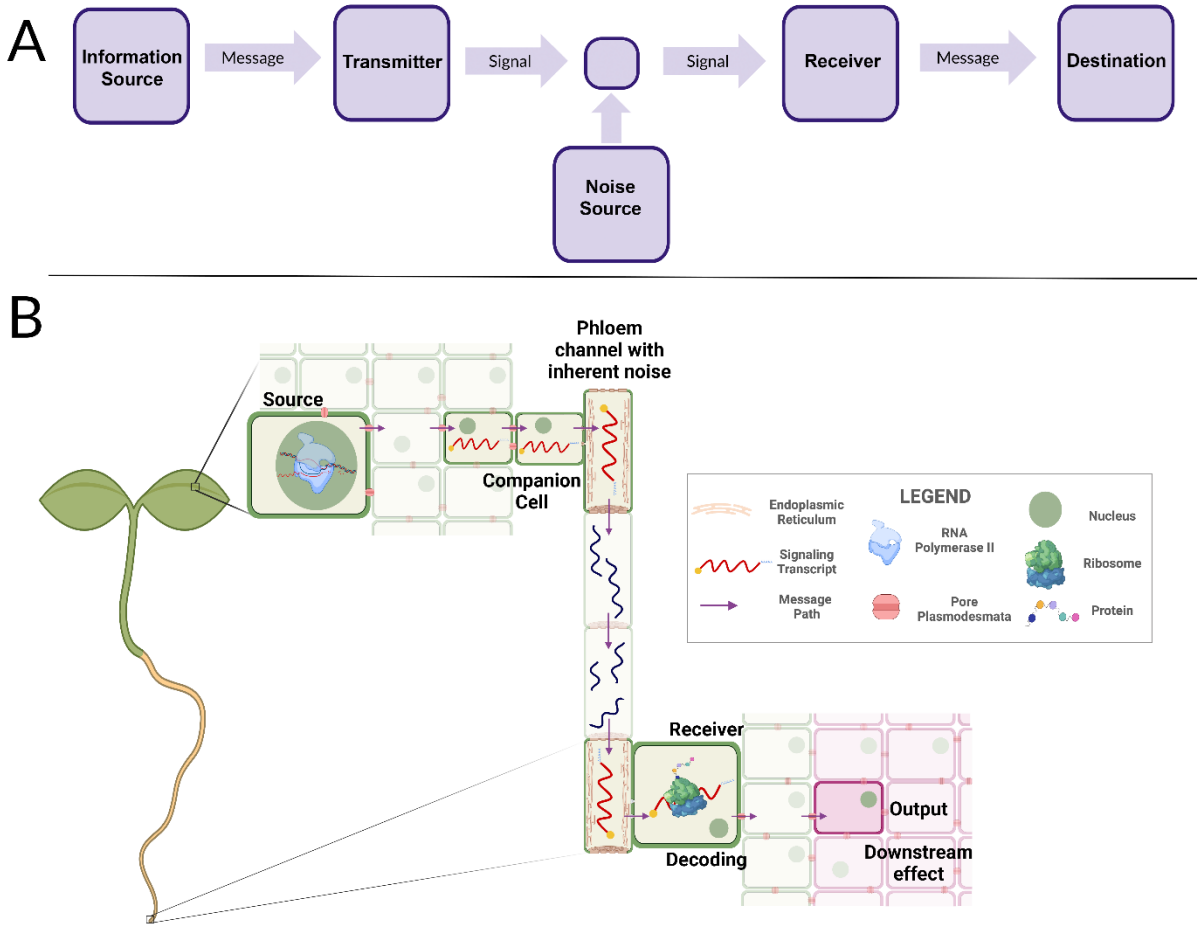

Figure 3. A general communication system. **A)** Schematic diagram adapted from Shannon (1948). This is a model of communication that is foundational to the field of communication theory. **B)** This system applied to the plant in the context of long distance mRNA communication. In this context, the source cell would produce the mobile transcript representing the signal. This transcript would travel to and through the phloem, our channel with inherent noise. The signaling transcript would exit the vascular stream to be decoded by a ribosome within the receiver and produce a downstream phenotype. This output could be potentially observed in tissue unique from the receiver. Created with BioRender.com.

Visualizing the gap: Leveraging RNA imaging for insight into the mobileome

One approach that could help separate signals from noise in the mRNA mobileome is to visualize the path of mRNA movement into and out of the vascular transport stream. While in vivo methods for tracking mRNA production and movement have rapidly advanced, none of these methods to date, have been applied to tracking long-distance mRNAs. Application of these methods have the potential to fundamentally change our understanding of the spatial and temporal factors that shape the plant mobileome.

The subcellular localization of mRNA in fixed and living cells has provided insights into transcriptional kinetics, mRNA export, and translation (Darzacq et al., 2007; Zenklusen et al., 2008; Corrigan et al., 2016; Alamos et al., 2021; Gould et al., 2018; George et al., 2018; Grünwald and Singer, 2010; Wu et al., 2016; Kitagawa et al., 2022a; Huang et al., 2020; Femino et al., 2003). Fixed tissue imaging techniques enable direct probing of native transcripts, however they limit temporal resolution. Spatio-temporal probing into mRNA dynamics will provide clarity on the production and movement of mobile mRNAs, helping resolve the extent to which these macromolecules may fill signaling functions. Questions surrounding the origin, receiver, and destination cells for mobile transcripts—recall the information theory model (Figure 3)—can be better understood using live imaging methods. This dynamic data can inform physiological constraints on the route connecting mRNAs to the phloem, and identify factors that influence RNA source and reception, illuminating the interconnectivity between dynamic gene expression and the likelihood of movement. Below we discuss some of the latest methods for in vivo and fixed tissue RNA imaging (Figure 4).

Bacteriophage-derived RNA tags help to visualize single-molecule RNA dynamics and movement in living cells (Bertrand et al., 1998; Golding et al., 2005; Tominaga et al., 2011). This system co-opts the MS2 bacteriophage coat protein PCP, which binds with high affinity to PP7 RNA stem-loop hairpins (Figure 4) (Peabody, 1993). An alternative system uses the MCP coat protein, which binds with high affinity to MS2 RNA stem-loop hairpins (Figure 4). MS2 and PP7 stem-loop hairpins are unique to bacteriophages (Figure 4) (George et al., 2018), making them an ideal candidate for directly targeting a native plant transcript of interest. To visualize mRNA, transcripts are typically fused to 6-24 stem-loop structures and co-expressed with the stem-loop binding coat protein (MCP or PCP) that is fused to a fluorescent reporter (Figure 4). To combat issues with background fluorescence, a split fluorophore approach has recently been developed (Huang et al., 2022). This method has provided in vivo insight into RNA transcription (Alamos et al., 2021), decay (Sheth and Parker, 2003), transport (Forrest and Gavis, 2003; Kitagawa et al., 2022a, 2022b), and translation (Morisaki et al., 2016).

Light-up aptamers are specialized RNA structures that can be appended to transcripts of interest, and specifically emit a signal when bound by a native fluorogenic dye (Figure 4) (Ouellet, 2016; Bouhedda et al., 2017). This approach is particularly appealing, as it has the potential to empower RNA imaging, much like how GFP and other fluorescent proteins have advanced in vivo protein visualization. New aptamers with high structural specificity and binding capacity for fluorogens can be generated through the systematic evolution of ligands by exponential enrichment (SELEX) (Stoltenburg et al., 2007). This method has led to an array of aptamers that are optimized for different cell systems (Bouhedda et al., 2017; Wu et al., 2019; Chen et al.,

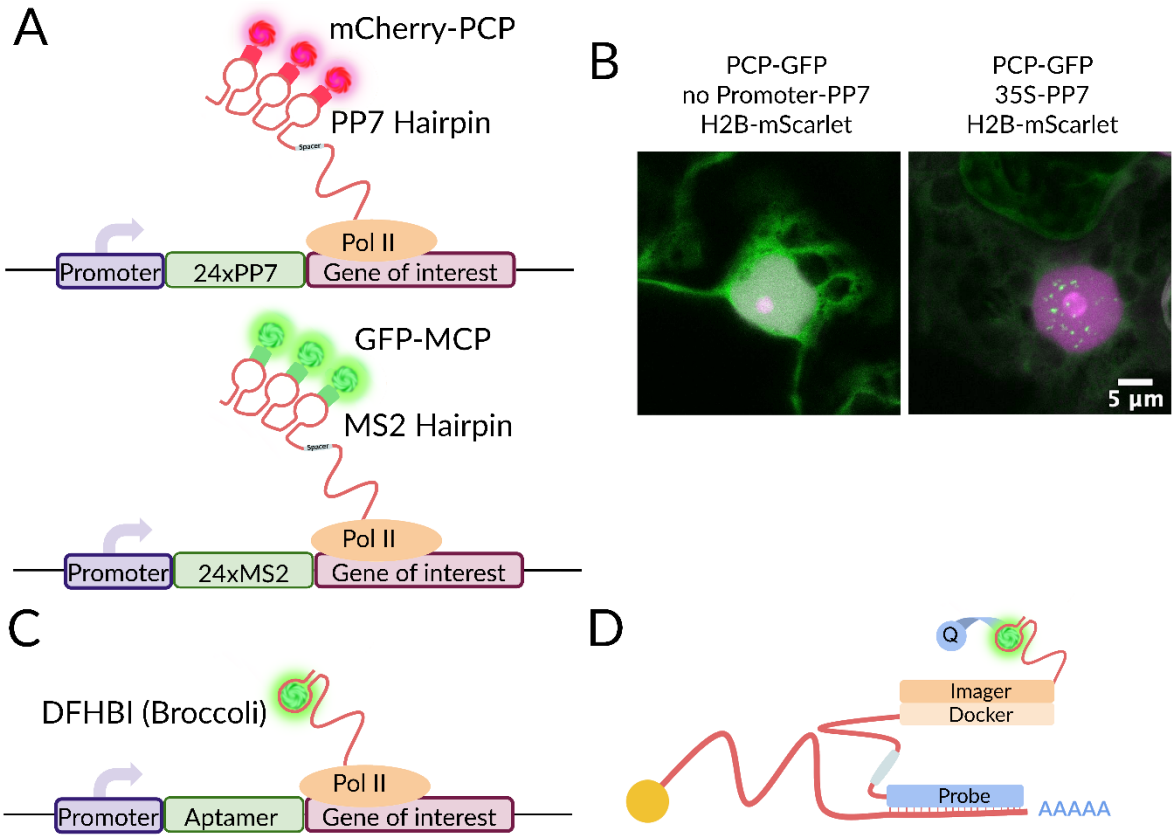

Figure 4. Visualizing RNA. **A)** Schematic of a live imaging system used to visualize RNA using encoded stem-loop repeats (MS2 or PP7) that are bound by the MS2-coat protein (MCP) fused to a fluorescent protein **B)** Cells expressing PCP–GFP, H2B-mScartlet, and the reporter construct with or without the constitutive 35S promoter driving expression of PP7. Foci represent labeled RNA molecules. Images were generated using the MCP-PP7 RNA tagging system described in Alamos et al. (2021) using transient expression of infiltrated *Agrobacterium tumefaciens* GV3101 (pMP90) in *Nicotiana benthemiana* leaves. The negative control on the left is from co-infiltration of three constructs: one driving ubiquitous MCP expression (pUBQ10-MCP-sfGFP cloned into a pCambia1300 - UMsfG), one that marks the nucleus with H2B-mScarlet (pUBQ10-H2B-mScarlet cloned into a pCambia3300 backbone), and one that contains promoterless 24 x PP7 loops (24xPP7-Gus-Luc - AL13Rb). The positive control on the right is from the same MCP and H2B constructs co-infiltrated with a construct that contains 24 x PP7 loops driven by the constituitive CAMV35S promoter (35S:24xPP7 - AL13Rb-35S). Both construct combinations were imaged 48 hours after infiltration on the LSM880 Confocal Microscope in Cornell's Biotechnology Resource Center using a 63x oil objective and 569 nm excitation/collecting in the 579-630nm emission range for the mScarlet fluorophore, and 488 nm excitation/collecting in the 498-559 nm emission range for sfGFP. The constructs used for this experiment are available on Addgene. **C)** Schematic of a live imaging system used to visualize RNA using an encoded aptamer. In the free state DFHBI (Broccoli) is lowly emissive, but upon binding to the RNA aptamer fluorescence is restored. **D)** RNA paint involves RNA hybridization on fixed tissue using an RNA probe, further addition of an RNA imager strand with an aptamer, and a transiently binding contact-quenched fluorophore–quencher. The signal is then detected upon binding and transient binding allows for an increased image acquisition time.

2019b). Since aptamers tend to have a relatively short sequence of 20–80 nucleotides they provide an ideal approach for tracking intercellular mRNA dynamics. While the technology has been around for several years, published examples of this application in plants are lacking. Given the potential of this system, it will be interesting to see whether it can be extended to in vivo plant studies.

Point accumulation for imaging in nanoscale topography (PAINT) uses transient fluorophores for single-molecule localization (Figure 4) (Sharonov and Hochstrasser, 2006). This transient binding allows for photobleached fluorescent molecules to be continuously replaced, enabling image acquisition beyond a photobleaching timescale which increases the imaging resolution (Schnitzbauer et al., 2017)**.** RNA PAINT uses base pair binding between an RNA probe and a target sequence to quantitatively visualize the subcellular localization and accumulation of a given RNA (Sunbul et al., 2021; Huang et al., 2020). When applied to the long-distance movement of mRNAs, these imaging technologies can be used to determine the transcriptional origin and route into and out of the vascular transport stream, resolving many of the knowledge gaps raised earlier in this review.

From destination to translation

Determining whether mobile transcripts make it into recipient tissue outside of the vascular transport stream, where they can be translated, has yet to be tested in vivo (Figure 3B). Plant-parasite interactions offer insight into the potential for mobile mRNAs to be translated upon delivery. Using host-parasite interactions between Arabidopsis and Cuscuta, researchers have found evidence for the transfer of GUS mRNA from the host plant into Cuscuta where GUS enzyme activity can be observed (Park et al., 2021). In grafted plants, proxy measures for translation have been observed, due to difficulties uncoupling mRNA translation from protein movement. One method is to connect mRNA movement with its downstream function (e.g. TCTP1 Yang et al., 2019; CK1 in Zhang et al., 2016a). Another approach uses mass spectrometry data coupled with RNA sequencing to correlate protein sequences with mobile mRNAs (Thieme et al., 2015). Using this method, 41 proteins had peptide sequences mapping to the reciprocal graft genome, and of these, 23 overlap with the mobile mRNAs identified in the study, thus inferring translation of the mobile transcripts (Thieme et al., 2015).

One promising technology for determining whether mobile mRNAs are translated is TRAP-seq (translating ribosome affinity purification followed by RNA sequencing). TRAP-seq utilizes epitope tagged ribosomal proteins that enable researchers to profile actively translated transcripts. Importantly, TRAP lines can be constructed to express tagged ribosomes in a cell type-specific manner (Dermit et al., 2017) enabling spatial profiling of active translation. While these tools were previously restricted to Arabidopsis (Fröschel et al., 2021; Jiao and Meyerowitz, 2010), they are now available in a range of evolutionarily diverse models (Traubenik et al., 2020; Reynoso et al., 2022; Zhao et al., 2017; Song et al., 2022; Kajala et al., 2021). Using the TRAP-seq approach in combination with genomically distinct hetrografting, researchers can address the extent to which mobile mRNAs are translated in recipient cell types.

Although data on translation upon delivery is limited, if mobile mRNAs are translated there are several potential outcomes. mRNA provides an alluring route for non-cell-autonomous

communication, as a single transcript has the potential to amplify its message into numerous proteins within recipient tissues. Within a grafted system, allelic variants can potentially provide alternative functions to the roles of endogenous mRNAs (Jiang et al., 2022). The diverse biological roles performed by the transcripts within the plant mobileome means that the conceivable impact of these mRNAs could be far-reaching.

Concluding remarks

The significance of the plant mRNA mobileome to long-distance communication remains unresolved, primarily due to the many unanswered questions regarding the dynamics of mRNA transit within the plant body. The effect of temporal dynamics such as developmental stage and circadian time on transcript production and propensity to enter the vascular transport stream has yet to be explored. Most studies to date have been static in nature, leaving the question of spatio-temporal dynamics open for future experimentation. Furthermore, there are discrepancies between the quantity of mobile mRNAs detected using heterografting and bioinformatic pipelines and the limited number of functionally characterized, *bona fide* mobile signals. More robust bioinformatic pipelines paired with experimental designs that capture developmental and circadian fluctuations in transcript production and mobility will help resolve this gap. Moreover, stringent signal-to-noise cutoffs in the mobile transcript identification pathway may create a stronger list of gene candidates for downstream analyses.

The path from RNA transcription to phloem entry, transport, unloading, and movement into surrounding cells remains unclear. If validated, the ‘postal code’ hypothesis which implies that plants can target molecular information to specific destinations within the body, has the potential to impact how we understand molecularly-encoded tissues and organ systems. Alternatively, if bulk phloem flow is predominantly responsible for dictating mRNA movement, this would imply that the mobileome is inherently a byproduct of phloem physiology, indicating that there is a high-level of noise in the mobileome. Imaging the pathway for mRNA movement will illuminate the extent to which these hypotheses feed into the mRNA mobileome. Further, this imaging will unlock our understanding of single molecule dynamics, effects, and regulation of the signals that confer disease resistance, stress responses, and yield increases in numerous herbaceous and woody crops.

If we could understand the factors controlling each of the steps involved in long-distance mRNA movement, that knowledge could be used to achieve powerful genetic engineering within plants. For example, scientists could design precise organ or tissue-specific delivery systems co-opting the transport of mRNAs that guide CRISPR editing machinery. In the age of precision agriculture (Chen et al., 2019a), decisions from a mobileome-informed perspective can also serve as another component of the plant breeding toolkit, whether this be predicting optimal combinations for grafting or fine-tuning expression in specific gene networks.

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Both authors contributed to writing the article.

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