Trans-kingdom RNAs and their fates in recipient cells: advances, utilization, and perspectives

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ABSTRACT

The phenomenon and potential mechanisms of trans-kingdom RNA silencing (or RNA interference, RNAi) are among the most exciting topics in science today. Based on trans-kingdom RNAi, host-induced gene silencing (HIGS) has been widely applied to create crops with resistance to various pests and pathogens, overcoming the limitations of resistant cultivars. However, a lack of transformation technology in many crops limits the application of HIGS. Here, we describe the various fates of trans-kingdom RNAs in recipient organisms. Based on the assumption that small RNAs can be transferred between the host and its microbiome or among microbiome members, we propose a possible alternative strategy for plant protection against pathogens without the need for crop genetic modification.

Keywords: trans-kingdom RNA, sRNA, HIGS, microbiome, RNAi

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INTRODUCTION

Small RNAs (sRNAs), including microRNAs (miRNAs) and short interfering RNAs (siRNAs), are the key molecular devices of RNA silencing (or RNA interference, RNAi), a conserved mechanism that regulates gene expression at either the posttranscriptional level (posttranscriptional gene silencing [PTGS]) or the transcriptional level (transcriptional gene silencing [TGS]) (Xie et al., 2004; Carthew and Sontheimer, 2009; Guo et al., 2019).

Trans-kingdom RNAi

After the discovery of RNAi, the first example of artificial trans-kingdom RNAi was the specific gene silencing in the nematode Caenorhabditis elegans fed with Escherichia coli that expressed exogenous double-stranded RNAs (dsRNAs) (Timmons and Fire, 1998). However, natural bidirectional transmission of RNAi signals between hosts and pathogens was not verified until recent years. Natural trans-kingdom RNAi was first observed in mammalian systems, although models involving RNAi had been proposed in plants prior to the reports from animal systems (Matzke et al., 1989; Napoli et al., 1990; Guo and Kemphues, 1995; Bots et al., 2006). Growing evidence has demonstrated that transferred RNAs can function in recipient organisms. The two mammalian miRNAs miR-451 and let-7i, which confer cell-intrinsic resistance to the malaria parasite Plasmodium falciparum on erythrocytes, were transferred to P. falciparum and integrated into parasite mRNAs to inhibit translation and impede parasite growth (LaMonte et al., 2012). Another study showed that the gastrointestinal nematode Heligmosomoides polygyrus secreted vesicles containing miRNAs and Argonaute (AGO) protein to suppress mouse type 2 innate responses and eosinophilia (Buck et al., 2014).

In plants, the expression of dsRNAs or RNAi constructs has been used to silence invading viral genes (Guo and Garcia, 1997; Qu et al., 2007; Duan et al., 2008). Proof of concept for host-induced gene silencing (HIGS) of nonviral pathogens was first demonstrated for the phytopathogenic filamentous fungus Blumeria graminis (Nowara et al., 2010) and then for Fusarium graminearum (Koch et al., 2013). HIGS was also successfully applied to protect cotton against the soil-borne fungus Verticillium dahliae, confirming that plant hosts can export heterologously expressed sRNAs to direct trans-kingdom RNAi against nonviral pathogens (Zhang et al., 2016a). However, the ability of endogenous plant sRNAs to direct trans-kingdom RNAi was not confirmed until a few years ago (Zhang et al., 2016b). In this study, many cotton sRNAs, including miR166 and miR159, were identified by sRNA sequencing in V. dahliae recovered from infected cotton plants. miR166 and miR159 were induced by V. dahliae infection and targeted the fungal virulence genes Clp-1 and HiC-15, respectively, for cleavage to impede hyphal growth and microsclerotia formation (Zhang et al., 2016b). This study provided the first evidence that trans-kingdom endogenous miRNAs could function in recipient fungal cells. On
the other hand, pathogen sRNAs are also transferred into plant cells and function as RNA effectors to suppress host innate immunity. For instance, Botrytis cinerea exports sRNA that associates with plant AGO1 to target host mitogen-activated protein kinase transcripts, thereby facilitating host colonization (Weiberg et al., 2013). These two studies provide direct evidence that natural bidirectional transmission of sRNA can occur in recipient organisms. Evidence for plant export of endogenous sRNAs that function in recipient organisms highlights the promise of HIGS for crop protection. Because the HIGS approach is independent of resistant cultivars, HIGS technology is considered an ideal strategy for crop breeding (Hua et al., 2018). Current applications of HIGS for crop protection against insects, fungi, nematodes, and mites, as well as the advantages and disadvantages of HIGS, have been summarized in detail (Baulcombe, 2015; Weiberg and Jin, 2015; Wang et al., 2017a; Wang and Jin, 2017; Hua et al., 2018; Zotti et al., 2018; Zhao and Guo, 2019; Zhu et al., 2019; Liu et al., 2020b; Das and Sherif, 2020; Hou and Ma, 2020; Islam and Sherif, 2020).

The discovery of natural trans-kingdom RNAi motivated researchers to explore the mechanisms of transferred sRNA delivery and sRNA function in recipient organisms. Recent research progress indicates that RNA motifs (Leblanc et al., 2012), RNA epigenetic modification (Yang et al., 2019), extracellular vesicles (Buck et al., 2014; Cai et al., 2018), and some specific RNA binding proteins (Leblanc et al., 2012; Wang et al., 2017b) influence sRNA selective transport. The latest research shows that the plasma membrane-localized protein SID1 plays a substantial role in mammalian absorption of sRNA from plants (Chen et al., 2020a). The mechanisms by which transferred sRNAs function in recipient organisms are also interesting. In principle, miRNAs negatively regulate gene expression by base pairing with specific miRNAs to induce cleavage or repress translation (Baulcombe, 2004; Brennecke et al., 2005). Intriguingly, miR-451 and let-7i impeded translation by integrating into parasite miRNAs, indicating that pairing requirements are not always necessary in trans-kingdom RNAi (Lamonte et al., 2012). Although plant miR166 and miR159 cleavage of fungal genes depends on base pairing, there is currently no direct evidence for fungal endogenous miRNA-mediated cutting of mRNA in fungal cells (Zhang et al., 2016b; Hua et al., 2018). These cases reveal the complexity and diversity of functional mechanisms in trans-kingdom RNAi.

### Suppression of trans-kingdom RNAi

In addition to protein-based immunity, trans-kingdom RNAi, another form of plant–pathogen interaction (Zhao and Guo, 2019), inevitably involves interplay among proteins. Recent studies have demonstrated that the plant pathogens Phytophthora sojae and Puccinia graminis f. sp. tritici (Pgt) secrete suppressor proteins to counteract disease resistance trans-kingdom RNAi by impairing host sRNA biogenesis (Qiao et al., 2013; Hou et al., 2019; Yin et al., 2019). P. sojae infection induces a class of plant secondary siRNAs through the action of plant RNA-dependent RNA polymerase (RDR) proteins, which play key roles in RNAi. Secondary siRNAs may function as antimicrobial agents by targeting pathogenic genes, and P. sojae encodes an RNAi suppressor (SR) to block the biogenesis of secondary siRNAs (Hou et al., 2019). The SR knockout mutant of P. sojae exhibits reduced virulence in plants (Qiao et al., 2013). Also, the Pgt SR1 protein promotes infection by multiple pathogens by altering the abundance of plant sRNAs involved in defense processes (Yin et al., 2019). The discovery of RNAi SRs encoded by nonviral pathogens suggests that trans-kingdom RNAi-based defense exists more widely than was originally believed.

In addition to the suppression of SR proteins, RNA decay pathways, which control RNA quality by eliminating endogenous aberrant RNAs (lsken and Maquat, 2007; Shoemaker and Green, 2012; Garcia et al., 2014; Liu and Chen, 2016), may also counteract trans-kingdom RNAi. In Arabidopsis, both 5'-3' and 3'-5' cytoplasmic RNA decay pathways repress the PTGS of transgenic and endogenous transcripts (Zhang et al., 2015). It is likely that plant RNA decay pathways prevent RNAi from inducing inappropriate gene regulation (Zhang et al., 2015; Liu and Chen, 2016; Szadeczky-Kardoss et al., 2018). Indeed, several components of RNA decay have been demonstrated to impede RNAi by competing for similar RNA substrates (Lange et al., 2014; Branscheid et al., 2015; Martinez de Alba et al., 2015; Zhang et al., 2015; Liu and Chen, 2016; Szadeczky-Kardoss et al., 2018). We suggest that the RNA decay pathway may impede the function or production of sRNA effectors by directly degrading or competing for their precursors.

### Trans-kingdom mRNA and long noncoding RNA

In addition to transferred sRNAs, RNA-sequencing analysis indicated that a large number of mRNAs were also transmitted between the parasitic plant Cuscuta and its host (Kim et al., 2014). It remains unknown whether trans-kingdom mRNAs can be processed into sRNAs to silence target genes or translated into proteins in the recipient organism (Zhao and Guo, 2019). A recent study showed that the majority of proteins transferred between Cuscuta and host plants, including Arabidopsis and soybean, were not translated from trans-kingdom mRNAs (Liu et al., 2020a). Another study reported that aphids translocated a long noncoding RNA (IncRNA) into plants that functioned as an IncRNA virulence factor by promoting aphid fecundity (Chen et al., 2020b). It would be fascinating to elucidate the roles and activity mechanisms of mRNAs and IncRNAs in plant–pathogen interactions.

Various aspects of trans-kingdom RNAs have been summarized in detail, including the challenges and triumphs of trans-kingdom RNA in agricultural applications (Liu et al., 2020b; Das and Sherif, 2020), the biosafety of plant-expressed dsRNA (Arpaia et al., 2020), and the potential for use of trans-kingdom RNA in the treatment of human diseases (Gorabi et al., 2020; Hu et al., 2020). Here, we raise the fascinating question of the fate of trans-kingdom RNAs in the recipient cell. We also discuss potential ways to promote crop disease resistance through the use of trans-kingdom RNAs without genetic modification. Based on the evidence that animals can acquire plant miRNAs through dietary intake, we investigate whether trans-kingdom RNAs can function as therapeutic agents to cure human disease. Answering these questions will advance our understanding and promote the further use of trans-kingdom RNAs.
Utilization of trans-kingdom RNAs and their fates in recipient cells

Cotton miR159 and miR166 were found to silence virulence genes in the fungus *V. dahliae*, providing strong evidence that natural trans-kingdom RNAi is an integral component of plant immunity (Hou and Ma, 2020; Zhang et al., 2016b). Both miR159 and miR166 were induced during *V. dahliae* infection. It is conceivable that these miRNAs are constantly exported into fungal hyphae during hyphal propagation inside cotton plants. However, after leaving the colonized plants, the transferred miR159 and miR166 could not be inherited by the next generation and were diluted by cell proliferation (Figure 1A). Indeed, miR159 and miR166 were detected only in *V. dahliae* freshly recovered from infected cotton and not in *V. dahliae* cultured in vitro (Zhang et al., 2016b). To date, endogenous miRNA-mediated cleavage of mRNA at a specific position and the generation of secondary siRNAs in fungal cells have not been reported, although RNAi components such as RDR proteins have been characterized in many fungal species (Chang et al., 2012; Jin et al., 2019). In *Fusarium asiaticum*, RDR-related secondary siRNA amplification appeared to be weak, as siRNAs were mapped only to the exogenous dsRNA but not to the target mRNA (Song et al., 2018). Moreover, exogenous dsRNA exhibited similar silencing efficiency in RDR mutants and wild-type *F. asiaticum* (Song et al., 2018). By contrast, pathogen-derived transferred sRNA effectors may trigger RNAi signaling in plants and probably have a different fate from plant-transformed siRNAs. In plants, sRNA-mediated PTGS often involves a signal amplification process mediated by RDR proteins in which primary siRNAs trigger the synthesis of secondary siRNAs, which in turn regulate target gene silencing in trans (Carthew and Sontheimer, 2009; Hou and Ma, 2020). In addition, a group of plant miRNAs can induce secondary sRNAs in a phased manner starting from the miRNA target site (Manavella et al., 2012; Deng et al., 2018; Guo et al., 2018; Cui et al., 2020). Therefore, we speculate that the trans-kingdom RNAi signal mediated by specific pathogen RNA effectors could be amplified by host RDR proteins, resulting in the silencing of multiple resistance genes during plant–pathogen interactions (Figure 1B).

Viral sRNA degradation is a special strategy to block antiviral RNAi (Csorba et al., 2015; Zhao et al., 2016). Thus, direct degradation of trans-kingdom sRNAs should not be ruled out. Additionally, in eukaryotic hosts, RNA decay and the RNAi system play vital roles in eliminating aberrant endogenous or exogenous RNAs to maintain proper development (Liu and Chen, 2016; Szadeczky-Kardoss et al., 2018). Trans-kingdom RNAs, including mRNAs, IncRNAs, and sRNAs, may activate the RNA decay pathway of recipient cells, resulting in degradation before functioning (Figure 1C). By contrast, some pathogen-derived IncRNAs can activate host PTGS and produce sRNAs to silence the plant host gene (Shimura
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et al., 2011; Smith et al., 2011). This suggests that at least a subset of mRNAs or IncRNAs may be cleaved to generate sRNAs and silence target genes by means of the recipient RNAi system (Figure 1D).

Most cases of horizontal gene transfer (HGT) in parasitic plants suggest that DNA is directly captured by the recipient organism (Zhang et al., 2014; Yang et al., 2016; Vogel et al., 2018; Yoshida et al., 2019). However, the fact that retroviruses transform their RNA genome into DNA and then integrate into the host genome provides evidence that cDNA synthesized from an exogenous RNA template can integrate into the host genome and be inherited (Jern and Coffin, 2008; Zhao and Guo, 2019). In the genome of the parasitic plant Striga hermonthica, the 3’ end of the HGT gene ShContig9483 contains 13 consecutive adenine nucleotides, suggesting that ShContig9483 was originally captured by S. hermonthica as mRNA or cDNA (Yoshida et al., 2010). This study suggests that trans-kingdom RNA can mediate HGT and may be heritable. The ortholog of ShContig9483, including an intron, was recently found in Striga asiatica, suggesting that this HGT event in S. asiatica was mediated by DNA (Yoshida et al., 2019) and differed from the capture of ShContig9483 by S. hermonthica. Nevertheless, HGT plays an important role in genome evolution and adaptation to certain specialized niches (Keeling and Palmer, 2008). Capturing specific DNAs or RNAs from organisms that are well adapted to the shared ecological niche is a simple and efficient mechanism for adapting to the environment (Zhao and Guo, 2019). Parasitic plants have been shown to act as vectors that mediate HGT among related species (Keeling and Palmer, 2008; Westwood et al., 2010; Yoshida et al., 2010). Based on the discovery of genome-scale bidirectional RNA transfer, we hypothesize that a subset of transferred RNAs capable of improving adaptation to the environment may be retained and fixed in the genome of recipient organisms under evolutionary selection pressure in an HGT process driven by trans-kingdom RNA (Figure 1E).

STRATEGIES FOR GENERATING DISEASE RESISTANT CROPS WITHOUT GENETIC MODIFICATION

The HIGS technique exploits the natural trans-kingdom RNA process for crop protection against parasites by expressing exogenous sRNAs homologous to genes that encode pathogenicity factors (Liu et al., 2020b). Although it is potentially effective, sustainable, and environmentally friendly, the application of HIGS may be limited by several factors: many crop species are difficult or impossible to transform, there is public concern about the biosafety of genetically modified crops, and artificial RNAi constructs may be unstable (Wang and Jin, 2017). Because of laws and regulations concerning genetically modified organisms, crops that express dsRNA require approval from various regulatory agencies. These factors complicate broader applications of HIGS worldwide, although the practicality and efficiency of HIGS strategies are beyond question (Liu et al., 2020b; Islam and Sherif, 2020). In addition, a lack of commercial applications restricts the full potential of HIGS research.

Spraying crude extracts of bacteria expressing dsRNA has been used to protect plants against viruses (Gan et al., 2010; Yin et al., 2010; Duan et al., 2012). Later, spray application of exogenous dsRNA or sRNA (spray-induced gene silencing [SIGS]) (Koch et al., 2016) initiated an era of RNAi-based fungicide strategies for the control of crop disease (Wang and Jin, 2017; Islam and Sherif, 2020). Compared with HIGS, SIGS avoids host genetic modification. Recent studies have reported that SIGS can be used against multiple pathogens such as F. graminearum (Koch et al., 2016) and B. cinerea (Wang et al., 2016). Furthermore, SIGS may be a feasible option for combating multiple pathogens at the same time by mixing dsRNAs or sRNAs. Under open field conditions, however, the stability and sustainability of dsRNAs and sRNAs acting as fungicides have been questioned. It has been reported that external RNAs can persist for 7 days on leaf surfaces (Koch et al., 2016). To improve the efficacy of SIGS, nanoparticles have been used as protectants for SIGS-based fungicides. A recent study demonstrated that dsRNA loaded onto nanoparticles could be detected on sprayed leaves 30 days after application (Mitter et al., 2017). Another report showed that nanoparticles facilitated the uptake of dsRNA through Arabidopsis root tips under laboratory conditions (Jiang et al., 2014). However, it remains unclear whether a SIGS-based fungicide would offer practical crop protection against soil-borne vascular pathogens such as V. dahliae and Fusarium oxysporum, which cause devastating yield losses in economically important crops and are a major threat to agriculture (Gao et al., 2019).

With the continued development of microbiome research, increasing evidence has shown that trans-kingdom RNAs and DNAs facilitate the adaptation of hosts and their associated microbiome members to the environment (Zhao and Guo, 2019). The associated microbiomes of the plant root and the vertebrate gut serve similar primary physiological functions in nutrient uptake (Haccquard et al., 2015). In addition, a recent study provided evidence that the bacterial root microbiota is essential for maintaining plant health by affecting the structure and diversity of the fungal and oomycete community (Duran et al., 2018). Several recent studies have also indicated that stressed plants may modify gene expression to favor beneficial microbes in their microbiomes, thereby increasing their resistance in what is termed the cry-for-help theory (Liu and Brettell, 2019; Liu et al., 2019). The idea of exploiting beneficial microbes to enhance crop resistance has existed for a long time (Wei and Jousset, 2017; de Vries et al., 2020). However, the difficulty of identifying which microbiomes maintain healthy ecosystems and the complex interactions between microbiome members make it challenging to use beneficial microbes for crop protection against pathogens (Wei and Jousset, 2017; Liu et al., 2019; de Vries et al., 2020). Nonetheless, important research advances in microbiome incorporation and host-microbiome feedback (Miller et al., 2018; Liu et al., 2019), microbiome-mediated plant disease resistance (Vannier et al., 2019), microbiome-shaping root exudates, and microbiome services (Arif et al., 2020; Tkacz et al., 2020) demonstrate the great potential of beneficial microbe application for crop protection. The crop protection effects of trans-kingdom RNAs shared among microbiome
members or between the host and its microbiome should not be ignored.

Based on the assumption that trans-kingdom RNAs can be transferred to different recipients and retained in the genomes of recipient organisms, we propose a strategy for using beneficial microbes to generate sRNAs for crop protection. This strategy avoids the production of genetically modified crops and the issues associated with sRNA degradation. The discovery of natural trans-kingdom RNA and HGT phenomena (Koonin et al., 2001; Keeling and Palmer, 2008; Weiberg et al., 2013; Zhang et al., 2016b) suggests that nucleotides can be transferred between related organisms. Therefore, we hypothesize that sRNAs can be transferred among host-associated microbiome members that share the same ecological niche. Presumably, sRNAs could be transferred from beneficial microbes to pathogens in the soil or on leaves, targeting virulence genes and causing a reduction in pathogenicity (Figure 2A). When beneficial microbes and pathogens colonize the same plant location (for instance, the plant vascular system), beneficial microbes could directly export sRNAs into pathogens (Figure 2B). It is worth investigating the possibility that hosts may mediate the exchange of RNA among different microbiome members; in this case, sRNAs would be transferred from beneficial microbes to host plants and finally into pathogens (Figure 2C). Nevertheless, it is worth exploring microbiome-mediated trans-kingdom RNAs for crop protection against pathogens.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The discovery of natural trans-kingdom RNAi represents an extraordinary milestone in RNA research history. Based on trans-kingdom RNAi, HIGS technology has been successfully used to control numerous pathogens and parasites (Hua et al., 2018). The lack of transformation technology in many crops and the instability of artificial RNAi constructs limit the application of HIGS (Wang and Jin, 2017). Alternatively, SIGS technology avoids the production of genetically modified crops, but researchers are concerned with the stability and sustainability of these RNA-based fungicides under open field conditions. Growing evidence indicates that trans-kingdom RNAs and DNAs facilitate the adaptation of microbiome members to the same ecological niche (Zhao and Guo, 2019). We propose an alternative strategy that utilizes functional sRNAs generated by beneficial microbes for crop protection, ensuring the stability and sustainability of sRNAs for trans-kingdom and/or trans-species applications and eliminating the need for genetic modification of crops.

Trans-kingdom sRNA has evoked strong debate on the effectiveness of dietary sRNAs as therapeutic agents. The first example of a food-derived miRNA functioning in mammalian cells was plant miR168, which was proposed to pass through the mouse gastrointestinal system and inhibit LDLRAP1 gene expression in the liver (Zhang et al., 2012). Following this discovery, studies have shown that miR2911 from honeysuckle decoction (Zhou et al., 2015), miR159 from different plants (Chin et al., 2016), and miR156a from green vegetables (Hou et al., 2018) can function in mammalian cells. The latest research suggests that plant miR2911, which is absorbed when humans consume honeysuckle decoction, can block the replication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhou et al., 2020). Both academic research institutes and
pharmaceutical companies are beginning to focus on the development and synthetic production of sRNA therapies for human diseases (Wang et al., 2020). However, the absorption and function of dietary sRNAs in mammals have been questioned. Several studies have been unable to find plant miRNA in animals (Dickinson et al., 2013; Snow et al., 2013; Witwer et al., 2013; Auerbach et al., 2016). Also, a comprehensive computational and experimental analysis of 824 public human data sets in which very-low-abundance plant sRNAs were present suggested that trans-kingdom RNAs may have resulted from technical artifacts rather than dietary intake (Kang et al., 2017). Current prospects for the use of dietary sRNAs as therapeutic agents are unclear, and more evidence is needed to prove their efficiency.

The uncertainty surrounding the regulation of mammalian gene expression by trans-kingdom RNAs and plant sRNAs has raised concerns among researchers regarding the effects of genetically modified dsRNA-expressing crops on human and animal health and environmental safety. Bioinformatics can help to minimize potential off-target effects and reveal potential risks. Nonetheless, the risk assessment process should be flexible, and decisions should be made on a case-by-case basis (Arpaia et al., 2020).

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