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Short distance spreading of non-coding RNAs: Vesicle trafficking and exosome secretion

Koch A, Höfle L, Jelonek L, Busche T, Möbus A, Hardt M

Institute for Phytopathology, Centre for BioSystems, Land Use and Nutrition, Justus Liebig University, Giessen, Germany

Email: Aline.Koch@agrar.uni-giessen.de

IMPROVEMENT OF PLANT IMMUNITY USING RNA SILENCING-BASED PLANT PROTECTION STRATEGIES

RNA silencing (also known as RNA interference, RNAi) is a conserved and integral aspect of gene regulation mediated by small RNAs (sRNAs) that direct gene-silencing at the level of transcription but also post-transcriptionally. At the transcriptional level, gene expression is inhibited via RNA-directed DNA methylation (RdDM) while at the post-transcriptional level (PTGS) direct mRNA interference causes inhibition of translation. Originally, RNA silencing is associated with protection against viral infection, control of epigenetic modifications, regulation of genome stability, curbing of transposon movement and regulation of heterochromatin formation (e.g. Koch *et al*. 2017). Besides its natural function, RNA silencing has emerged as a powerful genetic tool for scientific research over the past several years. It has been utilized not only in fundamental research for the assessment of gene function, but also in various fields of applied research, such as agriculture. In plants, RNA silencing strategies have the potential to protect host plants against predation or infection by pathogens and pests mediated by lethal RNA silencing signals generated *in planta* (Host-induced gene silencing, HIGS; e.g. Nowara *et al*. 2010; Koch *et al*. 2013; Abdellatef *et al*. 2015). In addition to the generation of RNA silencing signals *in planta*, plants can be protected from pathogens and pests by exogenously applied RNA biopesticides (Spray-induced gene silencing, SIGS; e.g. Koch *et al*. 2016; Wang *et al*. 2016; Mitter *et al*. 2017). Regardless of how target-specific inhibitory RNAs are applied (i.e. by endogenously or exogenously), the use of target-specific inhibitory RNAs (iRNAs) for plant protection is a potential alternative to conventional pesticides because iRNAs are i) highly specific and easy to produce and ii) can be developed against an unlimited range of pathogens possessing an RNA silencing machinery.

VESICLE-MEDIATED TRANSFER AND UPTAKE OF HIGS-DERIVED RNAS

Despite the striking efficiency of HIGS-based technology holds for agriculture, the mechanisms underlying the transport of RNAs from the plant host to the interacting microbial pathogen are inadequately understood. While in insects and nematodes a transmembrane channel-mediated RNA uptake mechanism based on the *Caenorhabditis elegans* SID-1 (Systemic RNA

interference deficient-1) protein has been described (Huvenne and Smagghe 2010), fungi seem to lack this protein (own observations). Alternatively, it has been hypothesized that small RNAs could be associated with the cargo of extracellular vesicles (EVs) (Cai *et al*. 2018; Rutter & Innes 2017; Rutter & Innes 2018). In line with this hypothesis, the plant-fungal interface, which is comprised of the fungal haustorial plasma membrane (HPM), the fungal cell wall (HCW), the extrahaustorial matrix (EHMx), the extrahaustorial membrane (EHM) and the plant plasma membrane (PPM), is the primary site for plant-fungal recognition (Fig. 1). Uptake of nutrients into the pathogen, delivery of enzymes and toxins into plant cells, secretion of fungal effector proteins and plant antimicrobial molecules (e.g. PR proteins) and biogenesis of cell surface sensors for mutual detection also occur at this interface (Fig. 1). Because most plant pathogens are extracellular, the plant possesses a specialized secretion system, which enables delivery of defense molecules to the site of attack. This secretion pathway mediates the transport of low molecular weight compounds required for immune responses, for example cell surface proteins, which reach their cellular destination via exocytosis from the host cell and is involved in synthesizing the EHM. This latter function indicates that the plant is actively exporting a variety of molecules to the plant-fungal interface, possibly including siRNAs.

Several types of EVs, defined according to their size and origin, have been identified in eukaryotic cells. Exosomes originating from the endosome are only 30-150 nm in diameter and are released through fusion of multivesicular bodies (MVBs) with the plasma membrane (PM). While most of the research on exosomes has been carried out using mammalian cell cultures and animal models such as *Caenorhabditis elegans* and Drosophila, Halperin & Jensen (1967) speculated already more than 50 years ago that a fusion of plant MVBs with the PM may result in the release of small vesicles into the extracellular space. In fact, biogenesis of MVBs and the release of their cargo via exosomes also is inherent to the plant secretory pathway that is activated upon pathogen attack as part of the immune response (An *et al*. 2006a; 2006b; Rutter & Innes 2017). Based on transmission electron microscopy (TEM) studies in barley, MVBs have been identified to proliferate next to cell wall papillae during attack by the powdery mildew fungus, and it was argued that these MVBs release their small vesicles into the paramural space thus leading to the assumption that exosomes exist in plants (An *et al*. 2006a; 2006b). In the latter case, the plant EVs may carry defense compounds to strengthen the plant cell wall at the site of fungal attack. Supporting this notion, proteins, hydrogen peroxide and callose could be identified inside MVBs next to the PM (Xu & Mendgen 1994; An *et al*. 2006b). EVs were also identified in the extrahaustorial matrix of powdery mildew fungus (Micali *et al*. 2011), though it could not be determined in this study whether these vesicles were of plant or fungal origin. Recently, Rutter & Innes (2017) isolated EVs of endosomal origin with a size range of 50-300 nm from the apoplast of *Arabidopsis* leaves, and thus found direct proof that exosomes exist in plants for the first time. These recent findings are in line with our earlier hypothesis that the endosomal vesicle trafficking pathway is the route that transgene-derived siRNAs take to be transferred between individual cells of host and parasite (Koch & Kogel 2014) (Fig. 1).

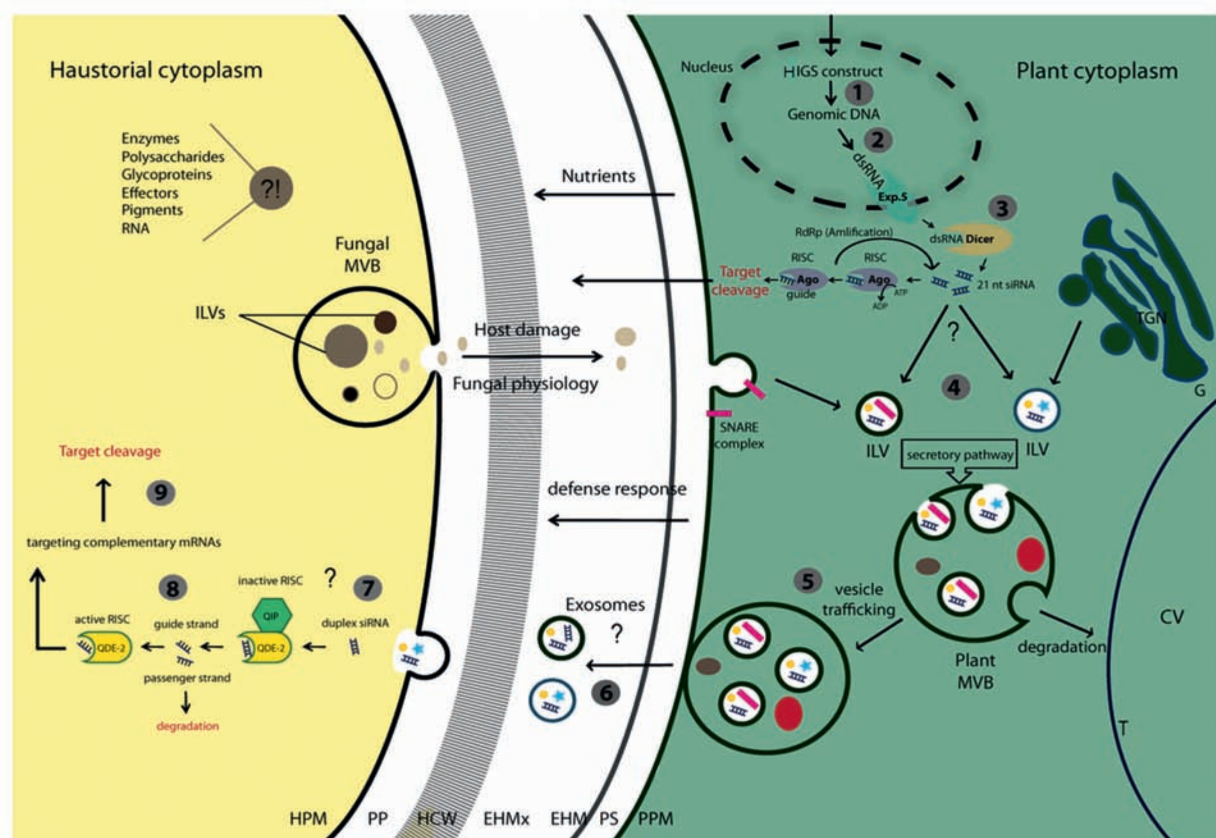

Figure 1 Vesicle-mediated transfer of HIGS-derived RNAs. A potential siRNA translocation pathway is indicated (1-9) (Koch & Kogel 2014).

A potential siRNA translocation pathway involves the integration of a HIGS construct, its transcription into dsRNA and the translocation into the cytoplasm, where it is loaded and processed by DCL enzymes (Fig 1, 1-3). The resulting siRNA duplexes are either delivered to the plant's RNA silencing machinery or are incorporated as duplexes into intraluminal vesicles (ILVs) that originate either from the Golgi body (G) via the trans-Golgi network (TGN) or from endocytosis at the cell membrane, respectively (Fig 1, 4). The ILVs, consisting of several cargos, are internalized by MVBs that enter the secretory pathway (Fig 1, 5). MVBs fuse to the PPM followed by subsequent release of ILVs (now called exosomes) (Fig 1, 6). Exosomes cross the cellular interface, entering the fungal cell and release their cargo, possibly including plants siRNAs (process unknown) (Fig 1, 7). The siRNAs may subsequently enter the fungal RNAi machinery resulting in target gene silencing where they are wrenched by the AGO protein QDE-2 (quelling deficient-2), while the passenger strand is removed by the exonuclease QIP (Fig 1, 8). The guide strand remains in the RNA-induced silencing complex (RISC) which is activated and targets complementary mRNAs, resulting in degradation and gene silencing, respectively (Fig 1, 9). Translocation of sRNAs via exosomes might require membrane associated receptors for attachment at the fungal cell layers and for further entry into the fungal RNA silencing machinery. However, alternative routes for siRNA delivery also may exist, including (i) converted bidirectional trafficking of RNA species across the plant-fungal cellular interface via several exocytic/endocytic receptors; (ii) uptake during fungal nutrient acquisition via specific

transporters; or (iii) passive crossing via trans-cell wall diffusion or through various transmembrane channels or pores. The latter could be a reasonable possibility if the siRNA size is sufficiently small.

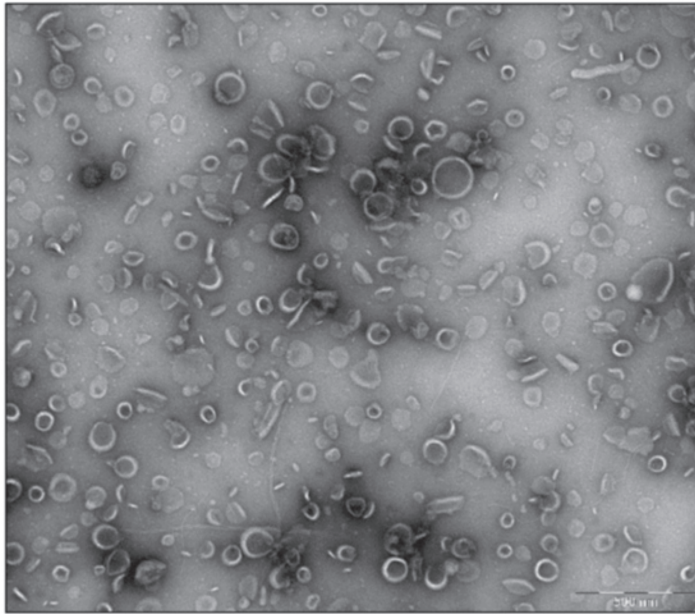

Figure 2 Isolation of EVs from the apoplast of CYP3RNA-expressing *Arabidopsis* leaves. Vesicles were analysed by negative staining and TEM (unpublished).

To test the hypothesis that sRNA transfer during HIGS requires the secretion of EVs, we adjusted/adapted recently developed protocols (Rutter & Innes 2017) for the isolation of EVs and their cargo from *Arabidopsis* leaves. Vesicles isolated by these methods were around 100 nm in diameter (Fig. 2), which is in good agreement with the size range reported for exosomes from mammalian cells (30-150 nm, Raposo & Stoorvogel 2013) as well as plants (50-300 nm, Rutter & Innes 2017). The physical appearance of vesicles in the TEM analysis was comparable to typical exosome preparations from cell culture supernatants (Li *et al*. 2017), especially as they were surrounded by a characteristic lipid bilayer which has an average thickness of around 5 nm in diameter (Fig. 2). Next, we assessed the RNA cargo of EVs isolated from the apoplastic fluid of transgenic *Arabidopsis* plants expressing a 791 nt dsRNA. Therefore, RNA was isolated and subjected to RNA sequencing. Mapping of siRNA reads to the dsRNA precursor identified dsRNA-precursor-specific siRNAs, while no specific reads were detected in the EVs from the controls (unpublished). In conclusion, our findings further corroborate that EVs of transgenic dsRNA producing HIGS plants contain siRNAs derived from the transgene. However, a better understanding the mechanistic basis (i.e. processing, translocation and uptake) of RNA silencing-based plant protection strategies such as HIGS and SIGS is critical for the successful implementation of these technologies for future field application. While we found strong indication for vesicle-mediated transport during HIGS-mediated plant protection, questions that remain open are:

- How are siRNAs (or dsRNAs) packed into vesicles?
- What is the nature of the transported RNA?
- How can these vesicles cross the plant-fungus interface?
- How are they released into fungal cells?

Thus, the overall scientific goal of our research aims to clarify central mechanisms concerning the uptake and translocation of non-coding RNAs, necessary for enhancing the efficacy and specificity of RNA silencing-based plant protection technologies.

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