RNAi-mediated plant protection: Identification and characterization of the molecular components of the HIGS and SIGS pathways

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Zusammenfassung

RNA-basierter Pflanzenschutz beruht hauptsächlich auf dem Mechanismus der RNA-Interferenz (RNAi), der verantwortlich ist für die Reduzierung der Genexpression, der Transposonregulation und der viralen Abwehr. RNAi ist in eukaryotischen Organismen evolutionär konserviert. Zu Beginn wird doppelsträngige (ds)RNA von Dicer-Enzymen in kleine interferierende (si)RNAs prozessiert. Diese werden anschließend in den RISC (RNA-induced silencing complex) aufgenommen und an Argonautenproteine (AGO) gebunden. Diese bilden die Kernenzyme des RISC. Bei der Bindung der siRNAs an AGOs wird der Leit- vom Folgestrang getrennt. Der verbleibende Strang unterstützt den RISC bei der Erkennung komplementärer Transkripte und führt somit zur Degradierung von messenger-RNA (mRNA), von der translationalen Inhibierung oder zur Remodellierung des Chromatins. Infolgedessen kommt es zu einem Funktionsverlusts des Zielgens.

Transgene, die dsRNA exprimieren, können als Initiator des RNAi-Mechanismus dienen, indem sie durch gentechnische Modifizierung in das Pflanzengenom eingebracht werden. Diese Transgene zielen durch ihre komplementäre Struktur auf essenzielle Gene pathogenen Ursprungs ab und können so die Pflanze resistent machen gegen schädliche Pilze, Nematoden, Insekten oder Viren. Beruht die Resistenz dabei auf dem Transgen, das für den dsRNA-Ursprung verantwortlich ist, spricht man von Wirts-vermittelter Genregulierung (host-induced gene silencing; kurz HIGS). Allerdings ist die Erstellung genmodifizierter Organismen (GMO) eine zeitintensive und kostspielige Methode und nur auf eine geringe Sortenwahl beschränkt, für die entsprechende Modifizierungsprotokolle verfügbar sind. Hinzu kommt die geringe Akzeptanz von GMOs auf dem europäischen Markt, sodass sich eine klassische oberflächliche Sprühapplikation von dsRNA auf Nutzpflanzen als Alternative anbietet. Dieses Verfahren wird Sprüh-vermittelte Genregulation (spray-induced gene silencing; kurz SIGS) genannt und vergrößert damit den Anwendbarkeitsbereich des RNA-basierten Pflanzenschutzes auf eine breitere Auswahl an Nutzpflanzen.

Der verschiedene Ursprung der RNA führt zu einer unterschiedlichen Beteiligung der Wirts- bzw. der pathogenen RNAi-Maschinerie. Während HIGS hauptsächlich auf den im Nukleus lokalisierten Dicer-Enzymen beruht, sind im SIGS-Ansatz die Dicer-Enzyme des Zielschaderregers beteiligt. Einen weiteren Unterschied stellt die verfügbare RNA-Menge von dsRNA in beiden Ansätzen dar. Im SIGS-Ansatz ist die dsRNA-Menge durch die aufgebrachte Sprühmenge limitiert, während im HIGS-Ansatz kontinuierlich RNA durch das Transgen im Wirt produziert wird. Daher sind RNA-Stabilität und RNA-Aufnahme in das Blatt bei SIGS von elementarer Bedeutsamkeit für den Erfolg der Sprühapplikation. Verschiedene Formulierungen verbessern die Haftfähigkeit der Sprühlösung, sodass möglicherweise eine „Stomataflutung“ mit einhergehender RNA-Aufnahme ins Blatt stattfinden kann. Die finale Aufnahme der RNA in die Pflanzenzelle ist ungeklärt, allerdings wird der Prozess der Endozytose als zellulärer Aufnahmemechanismus diskutiert. Unabhängig davon, ob die RNA über ein Transgen produziert oder durch einen Sprühvorgang appliziert wird, konnte gezeigt werden, dass RNA systemisch über das vaskuläre Gewebe in der Pflanze verteilt werden kann und so auch nicht

gesprühte Pflanzenorgane vor Befall geschützt werden können.

Von zentraler Bedeutsamkeit ist der Transport RNAi-vermittelter Faktoren von der Wirts- in die Schaderregerzelle. Am Beispiel des pilzlichen Schaderregers *Fusarium graminearum* konnte eine CYP3RNA-vermittelte Resistenz durch HIGS- und SIGS-vermittelte Ansätze gezeigt werden. Allerdings ist die Translokation CYP3RNA-assoziierter Faktoren zwischen Pflanzen und *Fusarium graminearum* unklar. Extrazelluläre Vesikel (EVs) sind sphärische Lipidkompartimente, die im apoplastischen Raum von der pflanzlichen Zelle abgesondert werden, sodass diese als potenzielle Transportvehikel zwischen Pflanze und *Fusarium graminearum* in Frage kommen können. Von EVs ist bekannt, dass sie eine hohe Diversität an Nukleinsäuren, Proteinen oder Lipiden spezifisch für ihr Ursprungsgewebe transportieren können. Um die EV-Beteiligung in der CYP3RNA-vermittelten Resistenz testen zu können, wurden EVs von CYP3RNA-exprimierenden *Arabidopsis thaliana*-Pflanzen und von CYP3RNA-besprühten Gerste Blättern isoliert. Ein Aufreinigungsprotokoll für EVs aus *Arabidopsis thaliana* ist bereits publiziert und konnte für die Studien herangezogen werden. Ein Isolationsprotokoll für Studien an EVs aus Gerste oder *Fusarium graminearum* wurde entwickelt (Verweis auf Sektion D**: Elucidating the role of extracellular vesicles in the Barley-Fusarium interaction**). Die Infiltration von pilzlichen EVs in Blätter zeigte wirtsspezifische Läsionen in Gerste, allerdings nicht in der Nichtwirtspflanze *Nicotiana benthamiana*. EVs, die aus Gersteblättern isoliert wurden, lösten nach mittiger Zugabe auf mit *Fusarium graminearum* bewachsenem PDA-Festmedium eine Verfärbung der pilzlichen Kolonien aus, was eine Stressreaktion auf pflanzliche EVs und ihren Inhalt darstellt. In weiteren Analysen der Gerste-EVs fanden sich kleine RNAs, die von der CYP3RNA abstammten. Dies deutet auf einen Transport CYP3RNA-abhängiger Komponenten zwischen pflanzlicher Wirtszelle und adressierter pilzlicher Zelle hin (Verweis auf Sektion **E: Isolation and characterization of barley (*Hordeum vulgare*) extracellular vesicles to assess their role in RNA spray-based crop protection**). Kokultivierungsuntersuchungen von pflanzlichen EVs, die aus CYP3RNA-exprimierenden Arabidopsis-Pflanzen oder CYP3RNA-gesprühten Gersteblättern stammten und in flüssiger Phase durchgeführt wurden, zeigten weder einen Effekt der pflanzlichen EVs noch der assoziierten CYP3RNAs auf das pilzliche Wachstum (Verweis auf Sektion **F: Extracellular vesicles isolated from dsRNA-sprayed barley plants exhibit no growth inhibition or gene silencing in *Fusarium graminearum***). Es ist daher fraglich, ob eine mangelhafte Aufnahme der EVs in *Fusarium graminearum* oder eine zu geringe Menge der EVs oder CYP3RNA-assoziierter siRNAs für die mangelnde Zielgenstilllegung verantwortlich ist, sodass die Beteiligung der pflanzlichen EVs im HIGS- und SIGS-vermittelten Pflanzenschutz unklar bleibt.

Abstract

RNA-based plant protection relies on the RNA interference (RNAi) mechanism responsible for gene silencing, regulation of transposable elements and viral defence. RNAi is evolutionarily conserved in eukaryotic organisms. At the beginning, double-stranded (ds)RNA is processed by Dicer enzymes into short interfering (si)RNAs. These, in turn, are incorporated into an RNA-induced silencing complex (RISC) with Argonaute (AGO) as the core component. AGO binds to siRNAs, separates the guide and follow strand and targets complementary transcripts, leading to the degradation of messenger (m)RNA, translational blocking or chromatin remodelling. dsRNA expressing transgenes as triggers of RNAi could be incorporated into hosts by modifying their genomes. These transgenes target essential genes of pathogenic origin and lead to resistance against fungi, nematodes, insects or viruses. If dsRNA is derived by transgenes RNAi-based silencing is termed host-induced gene silencing (HIGS) and relies on genetically modified organisms (GMOs). However, producing GMOs is a time-consuming and expensive process restricted to low numbers of varieties with existing transformation protocols. On the European market, GMOs are less accepted, so common foliar spray applications of dsRNA onto crops are more favourable. This approach is called spray-induced gene silencing (SIGS) and makes RNA-based pesticides applicable to a broader range of crops.

A different origin of initiating dsRNA results in different participation of host or pathogenic RNAi machinery. While HIGS relies on plant-endogenous Dicers located in the nucleus, SIGS relies on Dicers of the target pests. Another difference is caused by unequal amounts of initiating dsRNA. The RNA delivered by SIGS approaches is limited to the RNA applied to leaf surfaces, as opposed to rather unlimited amounts of dsRNA expressed by a host's transgene. Therefore, RNA stability and uptake into leaves are from special interest in SIGS approaches. Several formulations enhancing solutions' wetting capacity possibly allows stomatal flooding to occur in line with stomatal RNA uptake into leaf tissue. Finally, uptake into plant cells by endocytosis is speculated upon. However, if RNA is derived transgenically or by spray application, systemic spreading of RNA through plant tissue by vascular tissue has been shown in previous research and leads to systemic protection in unsprayed plant parts. Lastly, RNAi-associated factors must cross the borders between plant cell and pathogenic (i.e. fungal) cells. For *Fusarium graminearum,* CYP3RNA-dependant resistance was observed in HIGS and SIGS approaches, but translocation of CYP3RNA-associated factors between plant and *Fusarium graminearum* is still unclear. Extracellular vesicles (EVs), which are spherical lipid compartments, are released into the apoplast by plant cells and are possible transport vehicles of RNAi-mediating factors. EVs are known to contain a broad variety of nucleic acids, proteins and lipids specific to their source. To test EV dependency in CYP3RNA-mediated resistance, EVs were purified from CYP3RNA-expressing *Arabidopsis thaliana* plants or CYP3RNA-sprayed barley leaves. An EV isolation protocol from Arabidopsis plants has already been published. Isolation protocols to study barley or *Fusarium graminearum* EVs were developed (see section **D: Elucidating the role of extracellular vesicles in the Barley-Fusarium interaction**). Effects of fungal EVs on plants include host-specific lesions on barley but not in the non-host

plant *Nicotiana benthamiana* after the infiltration of EVs into leaves. Purified EVs of barley result after drop inoculation onto *Fusarium graminearum* plates in fungal colony discolouration visualising fungal stress reaction to plant EVs or content. Further characterization of barley EV content revealed CYP3RNA-derived small RNAs, indicating CYP3RNA transport between plant host and fungal recipient cell (see section **E: Isolation and characterization of barley (*Hordeum vulgare*) extracellular vesicles to assess their role in RNA spray-based crop protection**). Co-cultivation studies of plant-derived EVs from either CYP3RNA-expressing Arabidopsis plants or CYP3RNA-sprayed barley leaves in liquid phase have revealed no effect of plant EVs or associated CYP3RNAs on fungal growth (see section **F: Extracellular vesicles isolated from dsRNA-sprayed barley plants exhibit no growth inhibition or gene silencing in *Fusarium graminearum***). It is questionable whether an improper uptake of EVs or low number of either EVs or CYP3RNA-derived small RNAs are responsible for the lack of target gene silencing, leaving the responsibility of plant EVs in HIGS- or SIGS-mediated plant protection unclear.

Preamble

Agricultural producers of food and plant-based resources will face massive changes in the near future. The climate crisis is caused by humanity and their massive release of CO_2 into the atmosphere, which has already led to increasing average temperatures worldwide (Böhm et al., 2010; Hansen et al., 2010). Climate change results in acute events – such as hurricanes, floods, heavy rainfall and wildfires – long-term changes – such as drought and heat stress – and existential threats – such as higher temperatures, rising sea levels and altered environments. These phenomena also affect crops. The change of atmospheric composition, limited water resources or the effects of extreme events are hardly predictable for individual crop cultivars and regions (Challinor et al., 2009; Slingo et al., 2005), but, taken together, climate change impacts global food security (Wheeler and von Braun, 2013). Not only abiotic conditions will degrade cultivation conditions; climate change will also affect complete ecosystems and lead to habitat shifting of plants and pests, which includes the distribution of invasive pests by, for example, massive outbreaks of terrestrial insects (Harvey et al., 2020).

Chemical pesticides are still needed to prevent yield loss (North et al., 2019), but the intensive use of chemical pesticides leads to environmental pollution and the reduction of biodiversity caused by off-target effects on non-target organisms. It is rather unclear how crop and ecosystem productivity will behave as the loss of biodiversity continues. By now, strong efforts have been made by politicians to reduce and restrict the use of chemical pesticides and find alternative strategies to secure the production of plant-based resources and food. Pests are the major source of yield loss, illustrating the need for further improvement of plant protection strategies. Multiple strategies focus on plant secondary metabolites and plant immunity or microbial communities and pest antagonists to enhance resistance (Douglas, 2018).

Concurrently, the world population is growing. By 2100, the population will exceed 10 billion people (statista.com), which will necessitate higher food productivity but lead to the decrease in farmland due to the proliferation of infrastructure and neighbourhoods.

In late 2019, the outbreak of coronavirus (COVID-19) caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) (Hu et al., 2021) led the world into a dramatic and incomparable pandemic situation, with more than 100 million people infected within the first year. Vaccinations are predestined therapies to protect people from viral diseases. A new approach in drug development is using mRNA as an active ingredient (Sahin et al., 2014). In a project called “Lightspeed”, the companies BioNTech and Pfizer identified an effective mRNA against SARS-CoV-2 (Polack et al., 2020) and, by the end of 2020, released the first mRNA-based vaccine to the market after less than 12 months of development. This turned the focus of socioeconomic discussions to RNA-based technologies. mRNA-based vaccines deliver the blueprint of a pathogen’s relevant antigen, which is produced by host translation machinery, resulting in a viral non-reproducible protein (Anand and Stahel, 2021). Although the half-life of mRNA is restricted to a few days (Schlake et al., 2012), this is enough time for the host immune system to produce antibodies against the pathogen (Gergen and Petsch, 2020). The major advantage of

RNA-based agents is the programmability of RNA molecules. The mRNA sequence is interchangeable and adaptable to the antigen of the pathogen, making mRNA-based drugs highly specific. The specificity of RNA-based technologies is not only limited to vaccines, but can also extend into agronomically relevant solutions.

RNA interference

RNA-based plant protection mainly relies on the RNA interference (RNAi) mechanism, which was first characterized in 1998 in *Caenorhabditis elegans* (Fire et al., 1998). RNAi has been detected in many eukaryotic organisms. It is involved in defence mechanisms against transposable elements and viruses or in gene regulation on transcriptional and post-transcriptional levels (Cerutti and Casas-Mollano, 2006; Shabalina and Koonin, 2008). This wide spread of conserved RNAi components in eukaryotes leads to the hypothesis that RNAi components were already present in the last eukaryotic common ancestor. However, whether RNAi first evolved for antiviral defence or as a gene regulation mechanism is still a topic of debate (Torri et al., 2022).

During RNAi, double-stranded (ds)RNA is processed into small interfering (si)RNAs, which then guide a multi-enzyme complex called the RNA-induced silencing complex (RISC) to a corresponding target by base-pair homology. On the target site, RISC can cause DNA methylation on the DNA or histone level, leading to the suppression of gene expression or RISC-induced mRNA cleavage or initiation of translational blocking. In both cases, target genes are not expressed into functional proteins. Depending on which level RISC induces RNAi (DNA or mRNA/rRNA), RISC leads to transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS).

Dicer proteins catalyse the first step in RNAi. They relate to the RNaseIII family and process small RNAs from dsRNAs or dsRNA-forming elements of hairpin structures. Mammals, worms and yeast (*Schizosaccharomyces pompe*) only own one Dicer protein (Tomari and Zamore, 2005), while other species have evolved several Dicers with redundant or specialized functions (Catalanotto et al., 2004). For instance, *Arabidopsis thaliana* encodes four Dicer-like proteins (DCL), which are located in the nucleus and lead to small RNAs differentiating in their size (Hiraguri et al., 2005; Papp et al., 2003; Schauer et al., 2002). *At*DCL1 produces micro (mi)RNAs (Park et al., 2002), *At*DCL2 processes siRNAs responding to abiotic (Borsani et al., 2005) and viral triggers (Akbergenov, 2006; Xie et al., 2004) with a length of 22 nt (Fusaro et al., 2006), *At*DCL3 is involved in heterochromatin formation by releasing 24-nt-long siRNAs (Xie et al., 2004) and *At*DCL4 generates trans-acting siRNAs that regulate developmental timing or depend on RNAi (Dunoyer et al., 2005; Gasciolli et al., 2005; Xie et al., 2005). *At*DCL2 and *At*DCL4 are partially redundant to each other (Gasciolli et al., 2005).

To enhance sRNA half-life, siRNAs produced by DCL are methylated on the 2'-OH at both strands by HUA ENHANCER 1 (HEN1) to prevent degradation (Li et al., 2005; Yu et al., 2005). Afterwards, siRNAs are loaded into Argonaute (AGO) proteins.

In Arabidopsis, ten AGOs are encoded, defining three phylogenetic clades. The first clade is formed by *At*AGO1, *At*AGO5 and *At*AGO10; the second clade consists of *At*AGO2, *At*AGO3 and *At*AGO7; and the last clade includes *At*AGO4, *At*AGO6, *At*AGO8 and *At*AGO9 (Mallory and Vaucheret, 2010). AGOs contain three conserved domains which are responsible for correct sRNA loading: PAZ, MID and PIWI (Bologna and Voinnet, 2014). The MID domain binds the 5'nucleotide of the sRNA, while PAZ binds the 3' end (Frank et al., 2012). The PIWI domain forms the catalytic centre with an RNaseH-like endonuclease activity, leading to target degradation (Wei et al., 2012). Experimental studies have revealed

differentiated AGO sorting and loading dependant on sRNA length and the 5'nucleotide. *At*AGO1, *At*AGO2, *At*AGO5, *At*AGO7 and *At*AGO10 likewise bind 21–22-nt-long sRNAs, while *At*AGO4, *At*AGO6 and *At*AGO9 preferentially bind to 24-nt-long sRNAs (Bologna and Voinnet, 2014). *At*AGO1, *At*AGO2 and *At*AGO5 preferentially bind sRNAs with a uridine, adenosine or cytosine, respectively, at the 5' end (Mi et al., 2008). *At*AGO4, *At*AGO6 and *At*AGO9 preferentially bind to sRNAs with 5'adenosines. *At*AGO7 and *At*AGO10 are exclusively associated with miR390 and miR165/166 independent of the 5'nucleotide. Thus, most of the miR165/166 family members contain a 5'uridine which favours loading into *At*AGO1, but they are loaded into *At*AGO10 (Zhu et al., 2011). The same observation was made for miR390, which is loaded into *At*AGO7 instead of *At*AGO2 even though it contains 5'adenosine (Montgomery et al., 2008).

AGOs can be selected into two functional groups. The first group contains *At*AGO1, *At*AGO2, *At*AGO5, *At*AGO7 and *At*AGO10, which show slicer activity of their targets after binding 21- or 22-nt-long sRNAs generated from *At*DCL1, *At*DCL2 or *At*DCL4. The second group includes *At*AGO4, *At*AGO6 and *At*AGO9, which bind to 24-nt-long sRNAs processed by *At*DCL3 and mediate chromatin modifications (Waterhouse, 2016). *At*AGO8 is a duplication of *At*AGO9 and non-functional pseudogene, while *At*AGO3 is a duplication of *At*AGO2 and demonstrates high sequence identity. While *At*AGO2 is responsible for antiviral defence (Jaubert et al., 2011), the function of *At*AGO3 has long been discussed. Biochemical analysis has revealed the binding of 24-nt-long sRNAs to *At*AGO3 (Zhang et al., 2016), while mutant studies have shown functional replacement of *At*AGO4 in *ago4* mutants by *At*AGO3. Both findings indicate an AGO4-like function rather than an AGO2-like function, which was first predicted by sequence identity (Waterhouse, 2016).

Hordeum vulgare is a monocotyledonous plant of major importance to human and animal nutrition. The RNAi-based protection of barley against pathogens has been studied (Koch et al., 2016; Werner et al., 2020), but the RNAi core components DCL and AGO are rather less investigated. *In silico* analysis has revealed five *Hv*DCL and eleven *Hv*AGO proteins (Hamar et al., 2020). In comparison to dicots, monocots have evolved a DCL5 protein, whose PAZ domain is similar to DCL3 but shows a broad diversity in its dsRNA binding domain, causing the separation into a separate clade (Margis et al., 2006). Barley is part of the *Poaceae* family which covers the most cultivated crop plants worldwide and involves rice, maize and wheat. *Poaceae* are likely to have spontaneous gene or genome duplication, leading to expanding members of protein families that bring evolutionary advantages to a sessile lifestyle (Magadum et al., 2013). *Brachypodium distachyon* is a model organism for monocotyledonous plants (Scholthof et al., 2018). In Brachypodium, six *Bd*DCL and 16 *Bd*AGO proteins were identified (Šečić et al., 2019). In *Zea mays* and *Oryza sativa*, five *Zm*DCL and 18 *Zm*AGO (Qian et al., 2011) and eight *Os*DCL and 19 *Os*AGOs (Kapoor et al., 2008), respectively, were found, showing that AGO families are especially widespread in grasses.

Fusarium graminearum is a fungal pathogen that causes Fusarium head blight disease on barley and several other cereal crops (Goswami and Kistler, 2004). While infection might be symptomless or symptomatic (Brown et al., 2017; Urban et al., 2015), Fusarium species contaminate grains with the mycotoxins deoxynivalenol

(DON), nivalenol (NIV) or acetylated derivates (3A-DON, 15A-DON, 4A-NIV) (Desjardins et al., 1993; Ilgen et al., 2009; Jansen et al., 2005). In *Fusarium graminearum*, functional RNAi machinery relies on *Fg*DCL1, *Fg*DCL2, *Fg*AGO1 and *Fg*AGO2, which are responsible for conidiation, ascosporogenesis, virulence, deoxynivalenol production and fungal inhibition by exogenous dsRNA (Gaffar et al., 2019; Koch et al., 2016; Zeng et al., 2018). Targeting *Fg*DCL and *Fg*AGO by the RNAi approach leads to reduced virulence towards barley (Werner et al., 2020) by hijacking endogenous fungal RNAi machinery (Werner et al., 2021). According to the current knowledge, effective RNAi-based plant protection relies on a functional RNAi machinery in the target organism.

RNA origin, uptake and translocation

Plant protection via RNAi can be achieved by host-induced gene silencing (HIGS) or spray-induced gene silencing (SIGS), which have both been effectively proven to exist in several plant-pathogen systems (Koch et al., 2016, 2013; S. Liu et al., 2020; Qi et al., 2019; Rosa et al., 2018; Sang and Kim, 2020). The major difference between both approaches is the origin of dsRNA, which initiates the RNAi machinery and leads AGO to target specific gene silencing. Referring to plant-derived dsRNA origin in HIGS-based protection strategies, the uptake of RNA into plant tissue plays no important role. dsRNA is under the control of native (Fishilevich et al., 2019) or even viral (Koch et al., 2013) promotors for tissue-specific or constant expression. In contrast, when referring to exogenous RNA application methods such as spray-induced gene silencing, the amount of RNA is not limited to the RNA applied. Therefore, understanding the uptake mechanisms into plant tissue and the translocation inside plants and from plant to targeted recipient is the centre of focus, especially when dealing with SIGS.

Several physical barriers must be overcome before RNA reaches the target organism after its application onto plants. The first step is entering the plant tissue. Therefore, RNA must cross the plant surfaces, which are covered by a layered, waxy and hydrophobic cuticle and which protect plant tissues from environmental conditions (Becraft, 1999). Plant surfaces form the first barrier against biotic (Gorb and Gorb, 2017; Ziv et al., 2018) and abiotic stresses, such as heat, drought and cold stress and UV irradiation, and protect plants before the loss of water (Kane et al., 2020; Krauss et al., 1997; Liakoura et al., 1999; Schuster et al., 2017). In contrast, plant surfaces allow for the tightly regulated exchange of gases, such as oxygen and carbon dioxide. The landscape of plant surfaces is highly heterogenous and provides topographical differences that depend on macroscopic formations, such as trichomes, stomata or papillae, or microscopic roughness caused by cuticular formations or wax composition (Barthlott et al., 2017; Bediaf et al., 2015; Chowdhury et al., 2005; Koch et al., 2008; Koch and Barthlott, 2009). To summarize, these factors lead to specific interactions of leaf surfaces with topically applied solutions and determine the wettability of plant surfaces. Wettability can be classified as wettable ($\vartheta < 90°$), unwettable ($\vartheta \geq 90°$) or very unwettable ($\vartheta \geq 130°$), whereby ϑ represents the contact angle between leaf surface and an on-laying droplet (Fernández et al., 2021, 2017). A lower contact angle of droplets, which means high wettability, increases the liquid surface towards the leaf, resulting in potential increased absorption of liquid through the plant surface (Jura-Morawiec and Marcinkiewicz, 2020).

As naturally occurring openings, stomata are important. They are specialized cells of the epidermis and form openings in the epidermal layer by two guard cells, which regulate their opening state by the influx of osmotically active solutes, such as potassium ions (Eisenach and de Angeli, 2017), thus allowing for the interchange between plant tissue and the surrounding environment. Stomatal opening and closure are regulated by several complex signal cascades and can be induced by abiotic and biotic stimuli (Agurla et al., 2018). Beneath closure, stomata development and the resulting distribution across the epidermis are tightly regulated and important to aerial plant species, as they facilitate the optimal levels of gas exchange (Berger and Altmann, 2000; Geisler et al., 2000). Thus, the distribution of stomata is not random on the epidermal surface because at least one

epidermal cell lies in between two stomatal openings (Groll and Altmann, 2001; Larkin et al., 1996). Due to their specific architecture, capillary infiltration through stomatal openings is very unlikely (Schönherr and Bukovac, 1972), but stomatal flooding can be caused by reducing the surface tension by adding surfactants, such as organo-silicons (Field and Bishop, 1988; Zabkiewicz et al., 1993), which are favourable adjuvants of agrochemicals (Stevens, 1993). Nevertheless, even spraying unformulated (i.e. dissolved in water) RNA on barley leaves leads to efficient resistance towards *Fusarium graminearum* (Koch et al., 2016), indicating that RNA leaf uptake is also possible under unformulated conditions. The uptake of RNA through stomata might happen along liquid films along guard cells by diffusion (Eichert et al., 2008; Eichert and Goldbach, 2008) and has been previously shown for other solutes (Burkhardt et al., 2012; Eichert et al., 1998; Eichert and Goldbach, 2008; Schönherr and Bukovac, 1978). Stomatal architecture, distribution and pattering are highly different between monocotyledonous and dicotyledonous plants and from species to species (Hepworth et al., 2018), which must be taken into consideration when applying RNA onto plant leaves.

Testing different commonly available agrochemical formulations would be one strategy to improve RNA stability and leaf adherence. Additionally, several classes of nanoparticle approaches, referred to as possible cargo systems, arise (Yan et al., 2021) and have shown potential to improve RNAi efficiency (He et al., 2013; Parsons et al., 2018; Zhang et al., 2010). Nanoparticles, such as chitosan, liposomes and cationic dendrimers, could efficiently protect RNAs from degradational processes and translocate them across lipid membranes (Ahmadzada et al., 2018; Mahmoodi Chalbatani et al., 2019). Some of them have already been tested against insects by feeding assays (Kim et al., 2018; Q. Zhang et al., 2015; X. Zhang et al., 2015). So far, little is known as to whether these nanoparticles are translocated into plant tissue or whether they just cover the leaf surface after spray application. In the case of chewing insects, topically applied RNA might be sufficient for effective plant protection. However, to fully use the potential of RNAi-based plant protection, the uptake into plants must be guaranteed to also fight against pathogens with other lifestyles. Additionally, only uptake into the leaf and plant vascular tissue will cause the systemic spread of RNAi-related signals within the plant, leading to the protection of non-sprayed plant organs. At least for carbon dots, another type of nanoparticle, successful spray application of coupled siRNAs which target plant endogens or the transgene encoding for green fluorescent protein target gene silencing has been shown (Schwartz et al., 2020). This indicates sufficient uptake and activity of siRNAs inside plants, but it does not resolve the uptake of nanocarriers into apoplast or symplast.

After crossing the stomatal openings, RNA enters the apoplast. Mesophyll airspace in the leaf tissue plays a critical role in gas exchange rates (Baillie and Fleming, 2020), but whether a larger mesophyll lumen is beneficial or detrimental to RNA uptake into plant cells is unknown. It is speculative how RNA is further transported from stomatal openings to plant cells, but it is most likely the case that RNA spreads on the cell surfaces through a continuous liquid layer by diffusion.

Plant cells are surrounded by a cell wall, which seems to be a limiting barrier for bigger particles, such as carbon dots. The cell wall is built up by polysaccharides and has a size exclusion limit of 3–10 nm (Baron-Epel et al., 1988; Carpita et al.,

1979; Carpita and Gibeaut, 1993), while nanoparticles have a 100–200-fold greater diameter. While the release of RNA from nanoparticles such as carbon dots can be achieved artificially by adding detergents such as sodium dodecyl sulfate (Schwartz et al., 2020), release mechanisms inside plants are still elusive. Currently, changes in environmental pH or spontaneous disassociation of RNA from carbon dots are discussed as release mechanisms.

Nematodes possess systemic RNAi deficient-1 protein (SID-1), a channel responsible for the direct translocation of dsRNA from the environment into the cell (Shih and Hunter, 2011). So far, the presence of such a channel allowing for dsRNA uptake into plant cells is currently unknown. Therefore, endocytosis is discussed as a potential uptake mechanism into cytoplasm (Schwartz et al., 2020) whereby RNA could be released from endomembrane vesicles by proton influx followed by water uptake and the swelling of the vesicles – a phenomenon called the "proton-sponge effect" (Behr, 1997).

Systemic spreading of RNA has been observed for xylem and phloem. Furthermore, the delivery of miRNA via root-feeding, trunk injection or petiole absorption has revealed spreading through the xylem (Betti et al., 2021; Dalakouras et al., 2018; Liu et al., 2021). Short cell-to-cell communication of RNAs occurs through interconnections of plasmodesmata forming an intensive network between plant cells (Kehr and Buhtz, 2007). Plasmodesmata also allow for the loading of RNAs into phloem, causing systemic transport through the plants. Already in the 1970s, RNAs were found in phloem exudates but discussed as impurities (Kollmann et al., 1970). Since then, the systemic transport of several classes of RNA molecules have been shown (Citovsky and Zambryski, 2000; Mlotshwa et al., 2002; Voinnet and Baulcombe, 1997). Transport through phloem might also benefit from the lack of detectable RNase activity in the phloem sap (Doering-Saad et al., 2002; Sasaki et al., 1998). The accumulation of dsRNA in the vascular tissue after spraying onto barley leaves has been shown to lead to the systemic spreading of dsRNA but not siRNAs (Koch et al., 2016).

Host-induced gene silencing and spray-induced gene silencing

The most obvious difference between the HIGS and SIGS approaches is the origin of dsRNA, which initiates the RNAi defence mechanism against pathogenic invaders. In the HIGS approach, dsRNA is produced by an artificially introduced transgene responsible for dsRNA expression. Thus, HIGS includes the development of genetically modified organism (GMO). In contrast to that, SIGS was developed to avoid creating transgenic modified organisms. Creating GMOs is time consuming, very costly and limited to a small number of organisms or crops, and it requires specific expertise and long testing on positive and negative side effects due to strict regulations. Especially in Europe, the cultivation and marketing of GMO crops or GMO-based products is very restricted and of limited social acceptance (Hassani-Mehraban et al., 2009; Ishii and Araki, 2016). In the concept of SIGS, RNA is produced by *in vitro* transcription and later topically applied onto plant leaves by spray application. Thus, it avoids negative aspects of creating GMOs and makes RNA application available for more crops and RNAi-based plant protection products available for the European market. SIGS has long been limited by costly and inefficient RNA production methods resulting in low RNA amounts. This fact forces the discussion of whether SIGS approaches are transferable to field scales for economic reasons. Since COVID-19, at least two mRNA-based vaccines, BNT162b2 and mRNA-1273, were produced by the companies Pfizer-BioNTech and Moderna, respectively, showing that a highly upscaled *in vitro* transcription resulting in cheap RNA production is no longer limiting to SIGS approaches (Taning et al., 2020). However, questions about efficient upscaling of RNA production do not alone limit the applicability of SIGS-based products. Aspects of the sufficient storage of products or finding of suitable formulations must be forced. These aspects are omitted in the HIGS-based plant protection, which might contribute to why products have already entered the market (Zhang et al., 2017). However, it is much more likely that SIGS-based products will follow in the near future (Cagliari et al., 2019).

Apart from production costs of RNA, the spray application itself by farmers onto plants in every cultivation period involves the repeated investment of human and technical resources. Protecting the crops with HIGS omits these investments, but the farmer is restricted to licensed, possibly more expensive, seeds. Thus, the applicability of HIGS and SIGS is also an economic decision that must be made by individual farmers based on personally available resources and used crop systems.

RNA-silencing–based approaches for crop protection are promising for a wide range of organisms. As RNAi acts as natural antiviral defence, the closest link to that would be to induce new resistance against viral diseases by designing corresponding dsRNAs. RNA-silencing approaches against viruses have been intensively reviewed (Gaffar and Koch, 2019). Virus infections in plants rely on naturally occurring vectors, such as aphids, whiteflies, nematodes, plasmidiophorids and chytrids (Andika et al., 2016; Roger Hull, 2001), through wounding or seed transmission (Simmons et al., 2013, 2011). Some viral genomic sources consist of dsRNA or ssRNA (Roger Hull, 2001), which naturally trigger the RNAi defence mechanism, but some viruses also use ssDNA or dsDNA as genomic resources

which do not trigger RNAi. Independent of genomic structure, RNA-silencing strategies have been successfully tested for controlling RNA (Fahim et al., 2012, 2010) and DNA viruses (Vanderschuren et al., 2007) in monocots (Fahim et al., 2012, 2010) and dicots (Nahid et al., 2011). The replication of viruses relies on host transcription and translation machinery, making viral penetration into cytoplasm a precondition. Therefore, to affect viral target transcripts, complementary RNAi components must only be present in the plant cytoplasm, which is already the case.

Targeting insects or fungi, RNA delivery strategies must additionally rely on the targeted pest or pathogen. Plant-invading pathogens are derived by a wide range of organisms with different lifestyles and at different developmental stages. Therefore, RNA exposition or delivery tools play an important role in successful HIGS and SIGS approaches. For example, targeting insect eggs might be challenging for HIGS and SIGS approaches because of the scant interaction with the environment (Cooper et al., 2019). However, targeting phloem sap–sucking insects requires RNAi-related components in the phloem, which might be achieved by both HIGS and SIGS strategies whereby RNAi components spread through the vascular tissue. Instead, when referring to chewing insects, topically applied RNA might be sufficient for oral uptake. Traps or feeding spots containing attractants might additionally restrict RNA release to a minimum and avoid a spray application.

Fungi are less mobile than insects and classified into necrotrophic, hemi-biotrophic, biotrophic or saprophytic lifestyles. The saprophytic lifestyle is negligible because fungi feed from dead plant tissues. Necrotrophic fungi feed from nutrients which are released to the apoplast after fungal cells kill the host cells. The uptake of encapsulated or naked RNA might appear randomly by endocytosis in this case. It remains unclear whether the uptake of RNAi-mediating compounds is dependant on association with extracellular vesicles (EVs) (Schlemmer et al., 2022). Therefore, it is speculative if RNA uptake into plant cells is necessary, or if apoplastic appearance is sufficient for defence against necrotrophic fungi. Biotrophic fungi live from viable host cells, developing a feeding organ called the haustorium through which nutrients are taken up by the fungus. The haustorium forms an invagination of the host cell membrane while leaving host and fungal cell membranes intact. Following that, nutrient exchange is tightly regulated and appears through membrane transporters and endo- or exocytosis between both organisms. It is more likely that sufficient mediation of RNAi components relies on plant EVs in the case of biotrophic fungi than in the case of biotrophic (Micali et al., 2011). Therefore, sufficient uptake of externally applied RNAs into plant cells might be a precondition to fighting biotrophic fungi. So far, HIGS and SIGS have been efficiently tested for chewing (di Lelio et al., 2022) and sucking (Yoon et al., 2018) insects, as well as for necrotrophic (Koch et al., 2016, 2013; Tretiakova et al., 2022) and biotrophic fungi (Hu et al., 2020).

Under laborious conditions, SIGS was more efficient than HIGS in the barley-*Fusarium graminearum* pathosystem (Höfle et al., 2020). This difference in efficiency might rely on a different procession and delivery of dsRNA or sRNA. Direct procession of the dsRNA precursor by fungal DCL might lead to a more efficient RNAi response with higher potential for successful gene silencing. The participation of *Fg*DCL1 in SIGS has been previously shown (Koch et al., 2016).

Recent studies have provided information on not only dsRNA delivery by spray application on leaf tissue, but also other

exposure methods, such as trunk injection for woody plants, root exposure in hydroponic systems or directly soaking offshoots in RNA solutions, which have all proven to be successful delivery strategies. For example, dsRNA exposure to roots of white oak seedlings in a hydroponic system leads to RNA uptake (Bragg and Rieske, 2022). dsRNA was detectable over seven days after exposure via roots – not only in leaf tissue, but also in woody plant parts – suggesting that RNAi-based protection strategies are not limited to traditional field crops. In other studies, trunk injection into woody plants, such as *Malus domestica* and *Vitis vinifera*, has demonstrated the uptake of dsRNA into xylem and persistence of at least ten days (Dalakouras et al., 2018). However, no procession into siRNAs was visible in these experiments, which might be caused by insufficient uptake from apoplast into cytoplasm. Trunk injection of high amounts of RNA (2 g at a concentration of 133 mg/L) into citrus trees leads to the detection of dsRNA for up to seven weeks after injection (Hunter et al., 2012). dsRNA seems to be a very stable class of RNA molecules, which makes it very favourable for investigation in field conditions with challenging and changing environments.

RNAi is favourable for fighting against not only upcoming domestic pathogens, but also exotic and highly invasive pathogens which could spread their habitat due to human-related climate change. For example, the emerald ash borer from southeast Asia was first detected in North America in 2002 and has killed several million *Fraxinus* spp. in less than 20 years (Cappaert et al., 2005). This represents the devasting impact of invading foreign species, which threaten ecosystems, reduce biodiversity and cause unpredictable economic effects (Aukema et al., 2011). Designing dsRNA against three target proteins (heat shock 70 kDa protein, shibire and U1 small nuclear ribonucleoprotein) leads to sufficient mortality rates of emerald ash borer without any observable contemporaneous off-target effects on non-target organisms (Pampolini and Rieske, 2020). Further analysis has shown increased mortality after exposing emerald ash borer to dsRNA–pre-treated ash seedlings (Pampolini et al., 2020), offering hope to protecting trees against invading species.

Spray application or transgene-derived RNA will inevitably lead to RNA exposition to non-target organisms, such as herbivores, predators, detritivores, pollinators or parasitoids. dsRNAs or sRNAs are omnipresent molecules in nature, which makes risks caused by the RNA molecule itself very unlikely. Instead, risk assessment must focus on the effects caused by the specific investigated sequence, which might lead to downregulation of off targets by sequence complementarity. Software tools support the design process of dsRNAs or the finding of off-target effects by *in silico* analysis (Lück et al., 2019). RNAi approaches are thereby restricted to target organisms in which gene functions and appropriate sequences are known. On the other hand, to ensure a proper risk assessment by *in silico* analysis, genomes of all possible plant and pathogen interactors must be known. Studies have indicated that even >80% sequence identity is sufficient to trigger RNAi (Chen et al., 2021). Therefore, dsRNA's effects on target organisms and non-target organisms must be experimentally proven by bio or viability assays to ensure quality and to estimate the security of the tested dsRNA sequence during field application.

An ecological risk assessment of RNAi with plants was first discussed in 2009 (Auer and Frederick, 2009) and, apart from sequence-specific effects, the stability of dsRNA and sRNA in the environment is of special

interest. Here, following the hypothesis that a long life span of RNA molecules leads to an exposition to a broader spectrum of potential off-target organisms, the request to have RNA molecules of low stability to ensure low risk to the environment is, of course, contradictory to the request for high RNA stability for sufficient plant protection. The European Food Safety Authority has evaluated the classification of HIGS plants, supporting in principle the same guidelines for risk assessment as those used for other genetically modified plants (Papadopoulou et al., 2020). Additionally, *in silico* analysis to predict off-target effects caused by sequence similarity and field trials to verify *in silico* analysis must be performed to ensure the safety of HIGS plants (Papadopoulou et al., 2020).

Currently, reliable data are available for the MON87411 maize, which expresses the DvSnf7 RNA containing a 240–base pair–long dsRNA segment that leads to resistance against western corn rootworm (*Diabrotica virgifera virgifera*) (Bolognesi et al., 2012). Feeding assays using the 10-fold amount of the maximum expected environmental concentration of DvSnf7 RNA revealed no increased lethality in non-target organisms, including honeybees as pollinators, ladybird beetles as non-target arthropods, earthworms (*Eisenia andrei*) as representatives of the nutrient recycling soil biota, chickens as possible consumers of animal feed and catfish as representatives of aquatic residents (Bachman et al., 2016). Independent testing of dsRNAs in honeybees (*Apis mellifera*) using 400-nucleotide-long sequences targeting *Diabrotica virgifera virgifera* vATPase-A and *Apis mellifera* vATPase-A revealed only lethality using the bee-specific dsRNA (Vélez et al., 2016) and underlining only sequence-specific toxicity in non-target organisms. In studies investigating the soil persistence of dsRNA, DvSnf7-expressing maize tissue and *in vitro*–transcribed DvSnf7 dsRNA was mixed into different soil types. RNA was only detectable within the first 48 hours, indicating that long-term RNA persistence or even accumulation is very unlikely in soils (Dubelman et al., 2014). Non-essential, longer detection times of dsRNA were measured focusing on aquatic systems including aerobic sediments (Fischer et al., 2017). Finally, mammalian safety was tested by mice feeding experiments that resulted in no effects of DvSnf7 RNA on viability (Petrick et al., 2016). This led to the assumption that RNAi-based plant protection represents a very low-risk alternative to common chemical pesticides when sequence identity is tightly controlled and tested by *in silico* analysis combined with bio or feeding assays to non-target organisms.

CYP3RNA-derived resistance against *Fusarium graminearum*

CYP3RNA is a 791-nucleotide-long dsRNA which contains three complementary sequences of the fungal cytochrome P450 lanosterol C-14α-demethylase (*FgCYP51*) genes from *Fusarium graminearum*: *FgCYP51A*, *FgCYP51B* and *FgCYP51C*. Cytochrome P450 lanosterol C-14α-demethylase is essential in ergosterol biosynthesis and remains a favourable fungal target in medical and agricultural applications (Monk et al., 2020). Systemic fungicides, such as demethylation inhibitors (DMI), target the cytochrome P450 lanosterol C-14α-demethylase. Tebuconazole, triadimefon and prochloraz are DMIs and lead to the reduction of ergosterol amounts, disturb fungal membrane integrity (Yoshida, 1993). CYP3RNA was successfully tested in HIGS approaches in the plant model organism *Arabidopsis thaliana* and the agricultural relevant crop species *Hordeum vulgare* (Koch et al., 2013). CYP3RNA-expressing plants show less infection symptoms caused by *Fusarium* and significant transcript reduction of the target genes (Koch et al., 2013). In 2016, CYP3RNA was applied by spray application and led – similar to the results in Koch et al. (2013) – to reduced lesion symptoms and amounts of target transcripts, showing a clear resistance against Fusarium by SIGS (Koch et al., 2016). Application of fluorescent labelled dsRNA revealed that dsRNA is not only present in the apoplast, but can also be translocated into the symplast of phloem, parenchyma cells, companion cell, mesophyll cell, trichomes and stomata (A. Koch et al. 2016).

Further studies on dsRNA design have focused on the CYP3RNA elements. CYP-A, CYP-B and CYP-C single constructs, as well as CYP-AC, CYP-BC and CYP-AB double constructs, were tested in *Arabidopsis thaliana* and *Hordeum vulgare* plants. With the exception of CYP-C for Arabidopsis and CYP-A and CYP-C for barley, these studies have shown the same amount of symptom reduction as CYP3RNA against Fusarium (Koch et al., 2019). It is interesting to note that the gene silencing of single or double constructs was not restricted to the target transcripts. Instead, a certain degree of co-silencing of the other *FgCYP51* genes was measured and supported by an *in silico* analysis. Here, the individual sequences of the single and double constructs were split up into 21 k-mers representing possibly occurring siRNAs and mapped onto the reference sequence of the *FgCYP51A*, *FgCYP51B* and *FgCYP51C* genes. It becomes apparent, that base pair complementarity to also non-investigated CYP constructs have been found (Koch et al., 2019). This off-target silencing of the single and double constructs also strengthens the efficacy of SIGS-mediated resistance, resulting in resistance and transcript reduction by only applying single or double constructs onto barley leaves (Koch et al., 2019).

Testing the hypothesis that longer dsRNA precursors lead to higher rates of resistance and stronger reduction of target transcripts by generating more siRNAs from a longer precursor revealed no differences in the tested HIGS approaches (Höfle et al., 2020). All lengths showed roughly the same amount of infection and reduction of transcripts. It is interesting to note that, in SIGS approaches, increasing length correlates with increasing infection rates, and no significant reduction of the target transcript levels was observed for the longer constructs. These findings indicate that dsRNA length might not affect the

efficiency in HIGS-mediated plant protection, but it does in SIGS-mediated plant protection (Höfle et al., 2020). Increasing dsRNA length probably interferes with plant or fungal uptake mechanisms, leading to reduced uptake and lower amounts of RNA in plant tissue or fungal cells.

Extracellular vesicles in RNAi-based plant protection

As previously discussed, RNA pathways between HIGS- and SIGS-mediated plant protection might differ. Apart from all physical hurdles, EVs are discussed as potential carriers of RNAs in the bidirectional communication between plants and interacting fungi (Rodrigues and Casadevall, 2018; Woith et al., 2019; Zhou et al., 2022). Therefore, EVs are possibly shuttles of RNAi-related signals. EVs are spherically shaped droplets containing a bi-layered lipid membrane secreted by prokaryotic and eukaryotic cells. EVs can originate from different processes and may have variable sizes, forming subpopulations (Bobrie et al., 2012; Colombo et al., 2014). Concerning their diameter, apoptotic bodies define the largest EV population. They originate by programmed cell death and have a diameter of 1,000–5,000 nm. Based on diameter, the next smallest group is microvesicles (100–1,000 nm), which are produced by exocytosis. The smallest EVs are exosomes, which are released by the fusion of multivesicular bodies (MVB) with the plasma membrane. Therefore, EV formation depends on the endosomal sorting complex required for transport (ESCRT)–mediating membrane invagination and trafficking onto the plasma membrane (Juan and Fürthauer, 2018). Components of the plant ESCRT are involved in CYP3RNA-derived resistance towards *Fusarium graminearum*. Mutant studies have revealed increased susceptibility, although mutants were expressing the CYP3RNA. These findings indicate participation of plant ESCRT in HIGS-based plant protection (Schlemmer et al., 2021b).

EVs are of importance because of their diverse and tissue-specific cargo. Furthermore, EVs contain a wide variety of lipids, proteins, RNAs and secondary metabolites. Compared to plants, which have plasmodesmata as interconnections between individual cells, mammalian cell-to-cell communication relies on vesicular transport. Therefore, the specific packaging of EVs is essential for cell communication. EVs are also involved in cancer pathogenesis (Minciacchi et al., 2015), viral infection and pathogenesis (McNamara and Dittmer, 2020), cardiovascular diseases (Yang et al., 2021), fungal infections (de Toledo Martins et al., 2018), and they might act as antigen or drug delivery tool (Mehanny et al., 2021).

In contrast to mammalian cells, plant cells contain a cell wall. Therefore, the existence of plant EVs has long been speculated upon, though the fusion of MVBs and release of carrot EVs was identified by Halperin and Jensen in 1967 (Halperin and Jensen, 1967). It took more than forty years for isolation protocols for EVs of plant origin to be developed (Regente et al., 2017, 2009; Rutter et al., 2017). Since then, plant EV research has expanded and is not only limited to *Arabidopsis thaliana*. In addition, relevant crops come the focus of plant EV research and might contain RNAi signals (Regente et al., 2009; Schlemmer et al., 2022, 2021a, 2020). This is especially true because findings support the implication of EVs in naturally occurring bidirectional cross-kingdom interaction between plants and corresponding plant microbes (Cai et al., 2019; Cavaco et al., 2021; de Palma et al., 2020; N. J. Liu et al., 2020). The involvement of EVs in HIGS-based RNAi is likely because of the endogenous RNA origin, and the transport of corresponding plant RNAi machinery, *At*AGO1, was previously detected in plant EVs (He et al., 2021). Whether AGOs are necessary for sufficient sRNA transport remains elusive. Weiberg et al. demonstrated that fungal sRNAs are able to hijack endogenous plant

AGOs for cross-kingdom RNAi (Weiberg et al., 2013), indicating that sRNA transport with and without AGO stabilization occur.

The dependence of EVs on SIGS is rather speculative and might itself strongly depend on a pathogen's lifestyle (see also previous chapter **Host-induced gene silencing and spray-induced gene silencing**). Fungi are able to directly take up RNA (Gu et al., 2019; Koch et al., 2016; McLoughlin et al., 2018; Song et al., 2018; Wang et al., 2016). This possibly occurs by clathrin-mediated endocytosis (Kaksonen and Roux, 2018; Wytinck et al., 2020b), as in humans or insects (Saleh et al., 2006; Tatematsu et al., 2018). RNA uptake by SID-1, such as in *C. elegans* (Cappelle et al., 2016), still seems speculative because no SID-1 ortholog has yet been found in fungi (Wytinck et al., 2020a). Moreover, to the current knowledge, fungi lack dsRNA-degrading nucleases, and the presence of nucleases in apoplastic conditions derived by plants are rather unclear, making RNA stability in the apoplast inestimable. However, in the context of CYP3RNA-mediated resistance, CYP3RNA-derived sRNAs are co-purified with plant EVs in HIGS and SIGS approaches (Schlemmer et al., 2021a, 2021b). However, the localization of RNAs inside or attached to EVs is currently difficult to predict. Several pitfalls in plant EV research occur, including the lack of standardized isolation protocols (Rutter and Innes, 2020). Downstream RNA sequencing experiments for analysing EV content include RNaseA treatment before RNA isolation and complementary DNA library preparation. RNaseA treatment is used to eliminate extracellular and extravesicular RNAs, but whether RNaseA treatment alone is sufficient for complete degradation is still up for debate. RNAs might be protected before nucleolytic degradation by ribonuclear binding proteins. Taking this into account, the gathering of RNA sequencing data might be overinterpreted. Therefore, additional treatments combining RNases, proteases and detergents lead to more valid results (Schlemmer et al., 2021b; Zand Karimi et al., 2022). The International Society of Extracellular Vesicles (ISEV) composed some guidelines containing the minimal information for studies of extracellular vesicles (MISEV) (Théry et al., 2018). MISEV should ensure EV purity and identity to improve research quality on EVs. So far, these guidelines are hardly transferable from mammalian research to plant EV research, mostly because plant EV research lacks reliable EV markers. Mammalian EVs are enriched histocompatibility complexes I and II, tetraspanines (CD9, CD63, CD81, CD82) and heat shock proteins (HSC70 and HSP90) (Wubbolts et al., 2003). In Arabidopsis EVs, the syntaxin penetration 1 (PEN1) and the tetraspanin 8 (TET8) have been proposed as EV markers (Cai et al., 2018; Rutter and Innes, 2017). However, it remains to be seen whether they mark the same or different EV populations (Rutter and Innes, 2020).

Of even more interest is the RNA cargo of EVs. In Arabidopsis EVs, tiny (ty)RNAs and miRNAs are enriched (Baldrich et al., 2019) while siRNAs and circular RNAs are stabilized in the apoplastic fluid RNA-binding proteins (Zand Karimi et al., 2022). tyRNAs from Arabidopsis EVs are 10–17 nt in length and derived by transposable elements, coding sequences or intergenic regions. However, their function remains unknown. The RNA content of plant EVs regarding RNA-based plant protection was investigated in the Fusarium pathosystem. Plant EV isolation from HIGS and SIGS plants (Schlemmer et al., 2021b, 2020) and RNA content was assessed. CYP3RNA-derived sRNAs were found in samples of EVs derived by HIGS and SIGS plants (Schlemmer et al., 2021a, 2021b). So far, whether localization is inside or outside

remains unclear. Therefore, EV treatments were established (Schlemmer et al., 2021b). Differentially treated EVs were then tested in a co-cultivation assay in which EVs were mixed with Fusarium macroconidia. Taking EVs from HIGS and SIGS plants into account, neither EVs themselves nor associated RNAs impact fungal growth (Schlemmer et al., 2022). These findings leave the role of EVs in the CYP3RNA-mediated resistance open to interpretation.

Interesting to note, EVs from Fusarium lead to host-specific necrotic lesions in barley, while barley EVs cause stress-related discolouration of fungal colonies when incubating plant EVs in agar plates (Schlemmer et al., 2020). Similar observations were made of EVs of *Fusarium oxysporum* (Bleackley et al., 2020). These EVs also contain effectors, which are expressed during infection (Garcia-Ceron et al., 2021). It is probably the case that EV-related CYP3RNAs did not reach a critical threshold inducing RNAi-mediated silencing. Another possibility would be that the number of EVs was too low or that the uptake of EVs or sRNA either did not occur or was impaired in the liquid phase. Another possibility would be that EVs only contain stress-inducing components affecting fungal hyphae in sessile development and not during cultivation in the liquid phase.

In conclusion, the results indicate the participation of EVs in HIGS plants. Participation of ESCRT components, transported AGOs and a variety of RNAi-inducing RNAs of natural or artificial origin were at least associated with plant EVs. However, questions about plant EV uptake into fungal hyphea remain unanswered. Apart from HIGS, SIGS might not rely on plant EV–mediated RNA transport. The participation of fungal DCL in SIGS and the uptake of RNA in *in vitro* studies imply that RNA uptake or procession by plant RNAi compounds are not necessarily required. Instead, sufficient uptake into plant tissue and spreading within the apoplast might lead to sufficient protection of necrotrophic fungi. Nevertheless, further research is necessary to unravel open questions in both HIGS- and SIGS-mediated plant protection. In addition, EV-mediated transport seems not to be restricted to dsRNA or siRNA. Other types of RNAs will come into focus in the future. In particular, mRNA as a blueprint for protein production, or circular RNAs with their RNA sponge function, might lead to new strategies in RNA-based plant protection.

Specific aim of this work

The efficacy of the CYP3RNA-derived resistance in *Arabidopsis thaliana* caused by host-induced gene silencing and in *Hordeum vulgare* by spray-induced gene silencing against *Fusarium graminearum* has previously been shown. The transport mechanism for RNA translocation between host and recipient fungal hyphae remains unresolved. Potential transport may be possible by extracellular vesicles (EVs), which can be released by the plant plasma membrane into the apoplast. Inside EVs, potential RNA cargo can cross the apoplastic space without being affected by degradational processes. It has also been unclear whether EVs can be purified by not only Arabidopsis plants, but also by barley leaves. Therefore, a plant EV purification protocol was adapted to barley leaves, and EVs were purified from both plant species. EV sizes were measured, and RNA content was evaluated. In particular, CYP3RNA-derived small RNAs which could be involved in HIGS- and SIGS-mediated resistance were in focus. After identifying CYP3RNA-derived small RNAs in isolated EV samples, CYP3RNA-containing EVs from CYP3RNA-expressing *Arabidopsis thaliana* plants and CYP3RNA-sprayed barley leaves were tested in co-cultivation assays with *Fusarium graminearum* to identify the impact of these EVs on fungal growth behaviour.

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extracellular vesicles

Elucidating the role of extracellular vesicles in the Barley-Fusarium interaction

Fusarium graminearum (Fg) is a necrotrophic fungal pathogen that causes devastating diseases on its crop hosts barley and wheat. Recently, small RNAs (sRNAs) were identified as mobile communication signals between eukaryotes and their pathogens, symbionts or parasites. It has been shown that pathogens secrete sRNAs as effectors to suppress plant immunity and plants use endogenous sRNAs to resist infection, a phenomenon termed cross-kingdom RNAi; ckRNAi. However, little is known about the transport of fungus- or plant produced sRNAs to silence genes that contribute to immunity.

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Introduction

Fusarium graminearum (Fg) is one of the most important cereal killers worldwide that causes devastating diseases of crops with great economic and agronomic impact on global grain industry [1,2]. In addition to yield losses, food quality is affected by grain contamination with mycotoxins, which are produced by the fungi during plant infection, representing a serious threat to human and animal health [3,4]. Plant protection and toxin reduction strategies currently rely on the application of systemic fungicides [5]. However, many plant pathogenic fungi have become less sensitive or even resistant to chemical treatments. Thus, alternative control strategies have been developed that may lead to long-term reduction of pesticide use in modern agriculture.
RNA interference (RNAi; also known as RNA silencing) is a conserved mechanism for the regulation of gene expression in eukaryotes. RNAi is mediated by small RNAs (sRNAs) directing gene silencing at the transcriptional or post-transcriptional level. Transcriptional gene silencing results from epigenetic modifications, specifically DNA methylation and histone modifications regulating heterochromatin formation [6,7]. Post-transcriptional gene silencing starts with the cleavage of a precursor double-stranded (ds)RNA into short 21–24 nucleotide (nt) microRNAs (miRNAs) or small interfering RNAs (siRNAs) by type-III RNA endonuclease Dicer-like proteins (DCLs). Subsequent binding of siRNAs to an Argonaute protein (AGO) lead to the formation of the RNA-induced silencing complex (RISC) that mediates targeting of complementary mRNAs for degradation or translational inhibition [8,9]. Plants have evolved RNAi pathways/machineries that not only direct the epigenetic regulation of their own genome, but also provide protection from invasion by foreign nucleic acids, such as viruses [10]. Our laboratory and other groups previously demonstrated that this natural phenomenon can be used to control agronomically relevant plant diseases, based on the

demonstration that in planta expression of dsRNA (termed host-induced gene silencing; HIGS; [11]) as well as exogenous spray application of dsRNA (termed spray-induced gene silencing; SIGS; [12]) can signal post-transcriptional gene silencing of target genes in various plant pathogens and pests (for review see: [13–19]). Despite these findings, recent studies revealed RNAi between kingdoms as a new level of inter-species communication designated as cross-kingdom RNAi (ckRNAi). This phenomenon was first described by Arne Weiberg and Hailing Jin in 2013, demonstrating that the fungal pathogen Botrytis cinerea produces sRNAs that mimic plant sRNAs and bind to Arabidopsis AGO1 to antagonistically silence important plant immunity genes [20]. Since then, several studies have demonstrated bidirectional sRNA trafficking between plant hosts and their interacting pathogens [21,22]. However, the mechanisms underlying the transfer and uptake of ckRNAi-related sRNAs as well as transgene-derived artificial sRNAs (HIGS) remained unknown for a long time. It has been proposed that extracellular vesicles (EVs) playing a key role in the bidirectional transfer of sRNAs that mediate ckRNAi [13,21,23,24]. EVs are surrounded by a lipid bilayer and can be differentiated by size and their process, thus, classified in three major groups: The largest EVs are 5000-1000 nm in diameter known as apoptotic bodies, which are generated during cell lysis. Microvesicles are 1000-100 nm in size and produced by cells during exocytosis. The third class are exosomes which measure 150-30 nm and are released by fusion of multivesicular bodies with the cell membrane [25]. Plant EV research is an emerging field, so far only two reports described the isolation of plant EVs and the characterization of their protein/RNA content [26,27]. Therefore, lots of questions and methodological challenges need to be addressed to reach the quality standards in mammalian EV research. For example, identification of suitable plant EV markers as well as developing strategies that increase the purity of EV isolates, necessary "to distinguish bona fide EV cargo from merely co-purifying contaminants" (for more details see: [28]).

However, in plant EVs were firstly discovered in 1967 using electron microscopy [29]. Sixty years later Rutter and Innes successfully isolated the first EVs from apoplastic washes of the model plant Arabidopsis thaliana (Arabidopsis) [26]. The authors demonstrate that Arabidopsis EVs were enriched in proteins responsible for abiotic and biotic stress response which indicates an important role of EVs in plant immune responses. Moreover, in this study the first plant EV protein marker, the syntaxin PEN1 (PENETRATION 1) was described.
Recently, we showed that EVs purified from Arabidopsis leaf extracts and apoplastic fluids contain transgene/HIGS-derived sRNAs [27]. To our knowledge, this was the first report demonstrating sRNAs as plant EV cargo. In addition, we found that mutants of the ESCRT-III complex (ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT III) were impaired in HIGS further indicating that endosomal vesicle trafficking supports transfer of transgene-derived siRNAs between donor host cells and recipient fungal cells. Regarding the possibility of ckRNAi in Fg, we analyzed sRNA profiles of Fg axenic cultures and identified Fg sRNA target genes in barley and the monocot model plant Brachypodium distachyon. Subsequent expression analysis revealed that Fg-derived sRNAs caused significant downregulation of two host defense-related genes (unpublished data). Moreover, we recently demonstrated that targeting fungal DCL genes (FgDCL1 and FgDCL2) via SIGS could protect barley leaves from Fg infection [30]. Together, our recent results point to the direction that ckRNAi exists in the Fg – barley interaction. Supporting this notion and to further investigate whether Fg utilizes EVs for the transfer of sRNAs that manipulate and dampen immunity of its host barley, we isolated EVs from Fg liquid culture and performed Fg EV infiltration of barley leaves. In addition, we conducted an in vitro growth experiment, in which we isolated EVs from barley leaves to test their impact on fungal growth regarding the possibility of bidirectional ckRNAi in Fg-barley interaction.

Material and Methods

Plant cultivation and barley EV isolation

Barley (Hordeum vulgare cultivar Golden Promise) plants were grown for 14 days with 16 h light per day at a temperature rhythm of 18 °C/14 °C (day/night) and a humidity of 65 %. 80 leaves were taken for EV isolation. EVs were isolated as previously described [27]. EVs were resuspended in 190 µl phosphate buffered saline (PBS; 140 mM NaCl, 2,5 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7,4) for further analysis.

Co-incubation assays of barley EVs with Fg

400 µl Fg IFA65 macroconidia with a concentration of 30.000 macroconidia per millilitre were sprayed on 0.5 x PDA (potato dextrose agar) plates. One hour after spraying, 40 µl of barley EV suspension was dropped into the middle of each agar plate. Plates were incubated for six days at room temperature (RT) under constant illumination from one near-UV tube (Philips TLD 36 W/08) and one white-light tube (Phillips TLD 36 W/830HF).

EV isolation from Fg liquid cultures

For EV isolation, Fg was grown for four weeks in synthetic nutrient poor broth (SNP) containing 1.5 % carboxymethylcellulose (CMC) at 28 °C with 180 rpm shaking. Upon cultivation, fungal suspension was filtered through a miracloth mesh and centrifuged at 10,000 g for 20 minutes. The supernatant was filtered subsequently through a 0.45 µm and a 0.22 µm polyvinylidenfluorid (PVDF) membrane. EVs were concentrated ten times by tangential flow filtration (TFF) and centrifuged for 22 hours at 150,000 g at 4 °C. Afterwards, the pellet was resuspended in PBS buffer and loaded on the top of a discontinuous sucrose gradient with fractions of 8 %, 16 %, 35 %, 45 % and 60 % sucrose. The 30 %, 45 % and 60 % sucrose fractions were separated after centrifugation and resuspended

in PBS buffer and centrifuged again by 4 °C at 150,000 g. Finally, the pellet was resuspended in a small amount of PBS buffer for nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and plant infiltration assays.

Measuring vesicle size and concentration by nanoparticle trafficking analysis (NTA)

For NTA, purified barley and Fg EVs were diluted (1:50) with PBS buffer. Subsequently, 500 µl of vesicle suspension was loaded into Nanosight NS300 (Malvern Panalytical) and five measurements were performed at 25 °C. Size and concentration predictions as well as statistical analysis were performed using NTA 3.2 Dev Build 3.2.16 software.

Negative staining and transmission electron microscopy (TEM)

For TEM, five µl of purified EVs were loaded on copper formvar-coated 300-mesh electron microscopy grids, which were glow discharged prior to sample application for 40 sec. Redundant liquid was swabbed by using Whatman filter paper. Grids were incubated three times with 50 µl of 2 % uranyl acetate and washed with distilled water. Grids were air dried. Preparations were inspected at 120 kV under zero-loss conditions (ZEISS EM912a/b) and images were recorded at slight under focus using a cooled 2k × 2k slow-scan ccd camera (SharpEye / TRS) and the iTEM software package (Olympus-SIS).

Barley infiltration assays

For infiltration, the resuspended Fg EV pellet was adjusted to a final volume of 500 µl with PBS buffer. The downside of leaves from nine-day old barley (Hordeum vulgare cv. Golden Promise) and five-weeks old Nicotiana benthamiana plants was pressure-infiltrated with 500 µl of the Fg EV suspension/solution using a 1-ml syringe. After five days post infiltration leaves were photographed and harvested for further analysis.

Results and Discussion

Barley EVs affect Fg growth and pigmentation

In this study, barley EVs were purified from the second and third leaf of two-weeks old plants and were subjected to quality control using NTA and TEM analysis. NTA revealed 2.0 x 109 particles per millilitre that were isolated from 80 barley leaves with a mean size of 156 nm and a mode size of 165 nm (Tab. 1; Fig. 1A).

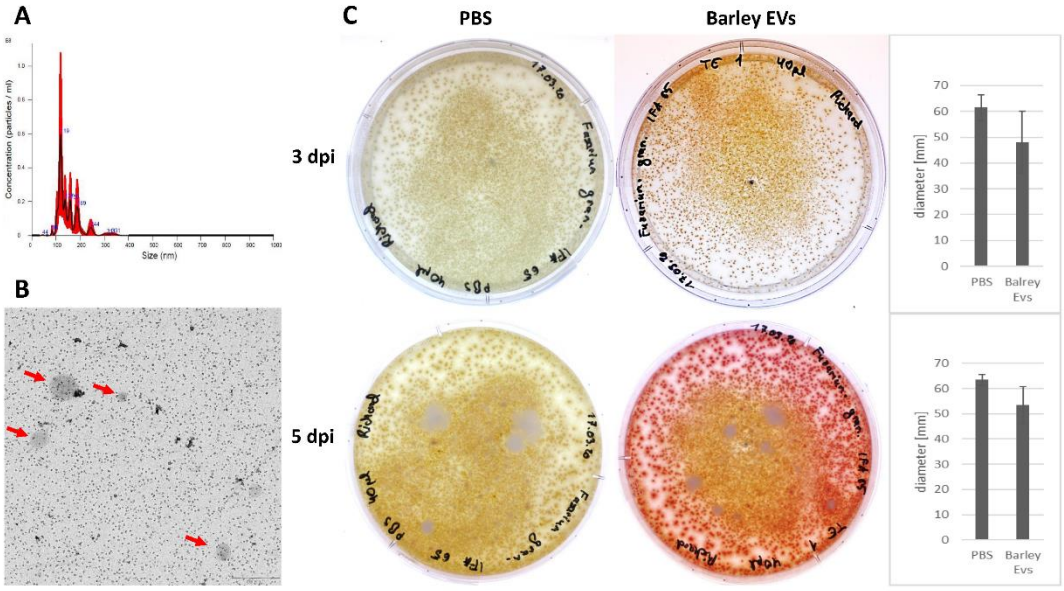

*Figure 1: Barley EVs affect Fg growth and culture pigmentation. Barley EVs were purified from apoplastic washes and resuspended in PBS. Size distribution was analysed by NTA (A) while morphological studies were performed by TEM (B). Barley EVs and PBS were drop inoculated on Fg macroconidia sprayed ½ PDA plates and pictures were taken three and six dpi. The diameter of the denser colony spots were measured for four biological replicates per treatment from PBS and EV inoculated plates three dpi and six dpi. The differences in colony density were statistically not significant (*P < 0.05; Student's t-test) (C).*

A clear peak was visible for particles with 119 nm in diameter, which agreed with the size range reported for EVs from mammalian cells (30–150 nm) [31] as well as plants (50–300 nm) [23]. Almost no particles

were measured above 350 nm and below 80 nm (Fig. 1A). In TEM analysis, particles negatively stained with uranyl acetate showed circular EV-like structures (Fig. 1B), as previously observed [27].
To test whether barley EVs have an impact on fungal growth, we drop-inoculated isolated EVs onto Fg culture plates. As mock control, PBS buffer was dropped on Fg plates. At six days post inoculation (dpi), different colony density at the drop-inoculated colony centre was found compared to the external area of the colony (Fig. 1C). We further observed that the colony density and pigmentation changes gradually, with the marginal colonies mainly produced white mycelium (Fig. 1C). The area of denser colonies was the same for both treatments (Fig. 1C), indicating that this effect was caused by the PBS buffer and not by the purified plant EVs. Besides this, PBS treated Fg colonies did not show any change in pigmentation (Fig. 1C). Barley EV-treated colonies underwent a change in pigmentation dependent from the distance to the EV inoculation spot. They turned from yellow (close to the inoculation) to purple the further away from the centre of the plate (5dpi; Fig. 1C). The change in pigmentation was independent from the amount of drop inoculated EVs. We suggested that barley EV treatment caused the change in pigmentation of Fg cultures. One possibility could be that the premature purple pigmentation was a stress response, which was induced by the EV treatment. Accumulation of blue-violet pigments is found in peridermal cells of Fg perithecia (fruiting bodies) [32], thus, we assumed an early switch into the reproductive/sexual lifecycle upon EV treatment. Further experiments would be necessary to test this hypothesis. Moreover, whether the changes in Fg pigmentation were caused by specific effects related to an unknown barley EV cargo or represented an unspecific effect due to the presence of plant EVs per se or co-purified contaminants needs to be assessed. Consistent with our results on Fg growth inhibition, a previous study demonstrated that EVs from sunflower seedlings affected the growth of Sclerotinia sclerotiorum spores, suggesting EV-mediated secretion of antifungal compounds

[33]. Supporting this, Arabidopsis EVs were also shown to contain defence-related cargo molecules, such as antimicrobial glucosinolates, proteins for oxidative stress response and reactive oxygen species (ROS) signalling [26,27]. However, further studies are needed to elucidate the role of plant EVs in plant-fungal interaction and the underlying molecular mechanism of EV loading and sorting in the donor plant cells as well as EV sensing, uptake into the recipient fungal cells.

EVs are produced by Fg

Whereas the isolation and characterization of mammalian EVs is well established, isolation of EVs from plants and plant-infecting pathogens is less advanced. To test whether Fg ckRNAi involves EVs as communication vehicles we conducted an experiment of infiltrating Fg EVs into barley leaves. We developed and optimized a protocol for EV isolation from Fg liquid cultures (see Material and Methods). To avoid EV contaminants from nutrient broth ingredients, we choose a synthetic nutrient-poor broth containing carboxymethylcellulose (CMC) as a solid compartment to allow fungal growth which only takes place attached on solid phases. The most particles counted by NTA were in the fraction (F) of 45 % sucrose with 1.97 x 1010 particles per millilitre (ml) compared to 1.18 x 1010 particles/ml for F 30 % and 1 x 1010 particles/ml for F 60 %(Tab. 1).

	Barley	Fg F-30%	Fg F-45%	Fg F-60%
Mean	156 +/- 12,2 nm	200 +/- 10,8 nm	123,8 +/- 4,0nm	232,9 +/- 9,5 nm
Mode	165 +/- 23,5 nm	93,6 +/- 9,2 nm	94,2 +/- 1,7 nm	115,2 +/- 14,5 nm
Concentration [particles/ml]	2,0 x 109 +/- 3,9 x 108	1,18 x 1010 +/- 4,11 x 108	1,97 x 10^{10} +/- 2,33 x 10^{9}	1,0 x 10^{10} +/- 5,81 x 10^{8}

Table 1 Mean, mode and concentration values measured during NTA analysis of EVs isolated from barley and Fg. Statistical values were calculated by NTA 3.2 Dev Build 3.2.16 software analysing one purification of two pooled Fg cultivations and one purification from 80 barley leaves.

The particle size distribution for F 45 % was more homogenous

compared to the F 30 % and F 60 % fractions (Fig. 2A).

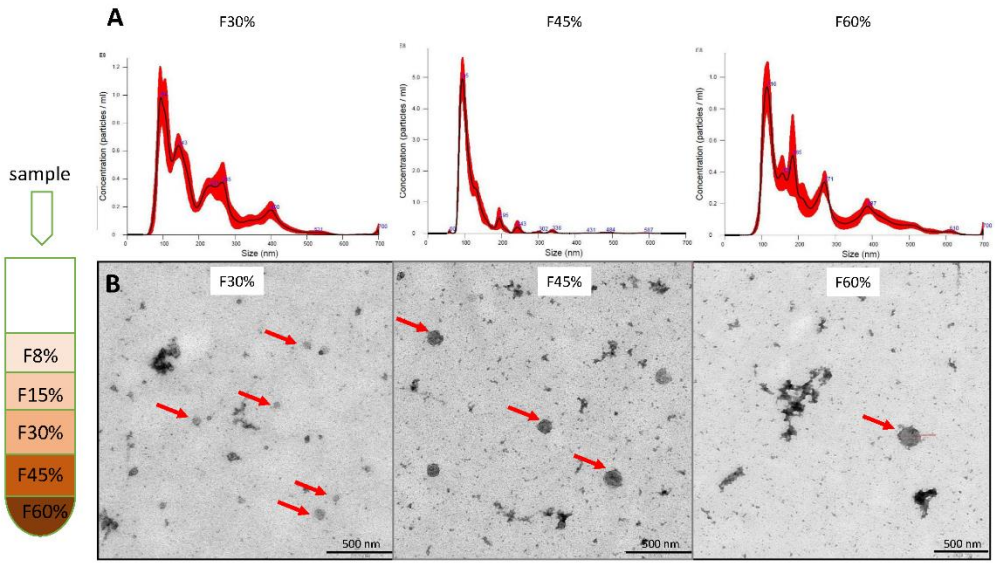

Figure 2: Fg EVs were purified by Sucrose density gradient separation. Scheme of the discontinuous sucrose density gradient used for Fg EV isolation with fractions (F) 8?% till 60?% sucrose per fraction. 10-fold concentrated Fg culture supernatant was filtered through a 200 µm PVDF membrane before density separation using a sucrose gradient. After density separation, fractions were washed with PBS and EVs were pelleted. All EV pellets were resuspended in 200 µl of PBS. 10 µl EV suspension of F 30?%, F 45?% and F 60?% were analysed by NTA (A) and 5 µl per fraction by TEM (B).

Moreover, F 45 % showed a distinct peak for particles with a size of 95 nm and only low abundance of particles of other sizes (Fig. 2A; Tab.1). The mean size of F 45 % was 123 nm and the mode size 94 nm, which was comparable with the size range reported for EVs from human fungal pathogens (~100 nm) and plant pathogenic fungi (150-155 nm) [34]. Notably, the F 30 % also contained a high number of particles with 95 nm in size, suggesting an overlap of the same EV population, however, particles with > 200 nm sizes were co-isolated in this fraction (calculated mean particle size of 200 nm and a mode size of 93 nm) (Tab. 1). F 60 % showed particles with a mean size of 232 nm and a mode size of 115 nm (Tab. 1). Consistent with our findings, previous studies also described a size range of EVs isolated from filamentous fungi, e. g. Alternaria infectoria (20-40 nm) [35] and Trichophyton rubrum (20-380 nm) [36].

Variation in EV size distribution was also described for the human fungal pathogens, Candida albicans and Candida neoformans [37], suggesting a heterogenous nature of fungal EV populations. Whether this correlates with a functional diversification of EVs in fungi per se remains to be elucidated. To confirm NTA data and to study fungal EV morphology, samples from F 30 %, F 45 % and F 60 % were fixed on a cupper mesh and negatively stained with uranyl acetate to perform TEM. Interestingly, TEM analysis revealed cup shaped EV structures in all three fractions. Consistent with NTA results, EVs from F 45 % were homogenous in size and appearance, while EVs from F 30 % and F 60 % varied in size and electron density indicating different EV composition and subpopulations (Fig. 2B).

Infiltration of Fg EVs caused phytotoxic effects in barley leaves

Our recent data pointed to the direction that Fg probably utilizes sRNAs as virulence factors ([30]; unpublished). To further support this finding and to study whether this ckRNAi was mediated by EVs, we conducted an experiment of infiltrating Fg EVs into barley leaves. Barley (host) as well as Nicotiana benthamiana (non-host) leaves were pressure-infiltrated with 500 µl of each EV fraction, F 30 %, F 45 % and F 60 %. To exclude unspecific effects caused by the infiltration procedure or the EV resuspension buffer, water and PBS were infiltrated as controls. In addition, Fg macroconidia were infiltrated to test whether barley leaves respond to foreign, pathogenic material itself. Finally, fungal culture supernatant after concentration by tangential flow filtration was infiltrated to assess whether the crude EV extract cause any damage on barley leaf tissue. The infiltration of water and fungal spores caused no effects on plants, whereas some barley leaves that were infiltrated with PBS showed small yellow spots restricted to the infiltration site. However, it was unclear, if this was due to PBS or resulted from the harsh infiltration procedure. Of note, barley leaves infiltrated with Fg EVs exhibited phytotoxic response observed as leaf bleaching and discoloration that

appeared around the infiltration area (Fig. 3A).

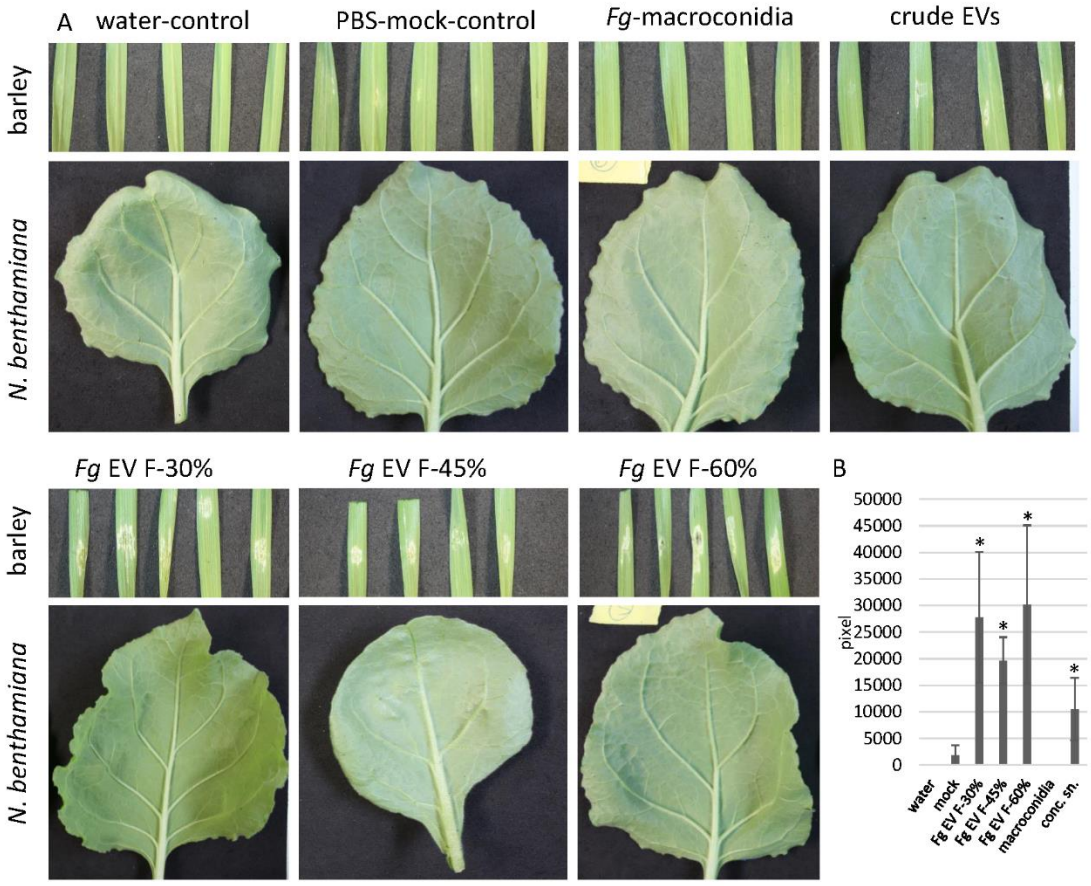

*Figure 3: Fg EVs caused host-specific phytotoxic effects in barley leaves. 500 µl Fg EV suspension of F-30?% (5,89 x 109 particles), F-45?% (9,82 x 109 particles) and F-60?% (4,99 x 109 particles) and, as controls, water, PBS, Fg macroconidia and concentrated Fg culture supernatant (crude EVs.) were pressure-infiltrated into barley (host) and tobacco (non-host) leaves. Pictures were taken and measured five dpi (A). Lesions site on barley leaves were measured by ImageJ and statistical differences were calculated compared to mock control (*P < 0.05; Student's t-test) (B).*

Importantly, we observed that these phytotoxic effects were independent of the Fg EV fraction that was used for infiltration (Fig. 3B). Remarkably, the infiltration of concentrated culture supernatant led to the formation of bleached spots as well, however, they were much smaller compared to those observed for the Fg EV fractions (Fig. 3B). This result may indicate a dose-dependent effect in barley, because the culture supernatant contained less EVs (diluted in a higher volume) compared to the concentrated Fg EV fractions. However, these results were consistent

with another study demonstrating phytotoxic effects of Fusarium oxysporum f. sp. vasinfectum EVs on cotton plants [34]. Interestingly and in contrast to our results the authors found that infiltration of resuspended spores and hyphae led to the formation of discoloured spots as well, even if they were smaller compared to the EV infiltration. Moreover, the authors demonstrated that phytotoxic effects were not host specific as EV infiltration of the non-host plant N. benthamiana caused similar phenotypes. We observed contrasting results for N. benthamiana, as neither the infiltration of water, spores, PBS, EV fractions nor concentrated culture supernatant caused any effect on the tobacco leaves (Fig. 3A), indicating species specificity of EV cargo in the genus Fusarium. Therefore, further analysis of Fg EV cargo (protein and RNA) should clarify the role of fungal EVs in the infection process and to unravel the molecular mechanisms of EVs underlying the ckRNAi phenomenon.

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Communication

Isolation and Characterization of Barley (*Hordeum vulgare*) Extracellular Vesicles to Assess Their Role in RNA Spray-Based Crop Protection

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Abstract: The demonstration that spray-induced gene silencing (SIGS) can confer strong disease resistance, bypassing the laborious and time-consuming transgenic expression of double-stranded (ds)RNA to induce the gene silencing of pathogenic targets, was ground-breaking. However, future field applications will require fundamental mechanistic knowledge of dsRNA uptake, processing, and transfer. There is increasing evidence that extracellular vesicles (EVs) mediate the transfer of transgene-derived small interfering (si)RNAs in host-induced gene silencing (HIGS) applications. In this study, we establish a protocol for barley EV isolation and assess the possibilities for EVs regarding the translocation of sprayed dsRNA from barley (*Hordeum vulgare*) to its interacting fungal pathogens. We found barley EVs that were 156 nm in size, containing predominantly 21 and 19 nucleotide (nts) siRNAs, starting with a 5′-terminal Adenine. Although a direct comparison of the RNA cargo between HIGS and SIGS EV isolates is improper given their underlying mechanistic differences, we identified sequence-identical siRNAs in both systems. Overall, the number of siRNAs isolated from the EVs of dsRNA-sprayed barley plants with sequence complementarity to the sprayed dsRNA precursor was low. However, whether these few siRNAs are sufficient to induce the SIGS of pathogenic target genes requires further research. Taken together, our results raise the possibility that EVs may not be mandatory for the spray-delivered siRNA uptake and induction of SIGS.

Keywords: plant EV; extracellular vesicles; RNA interference; RNAi; siRNA; dsRNA; RNA spray; barley; *Fusarium graminearum*

1. Introduction

RNAi-based plant protection strategies represent powerful tools to address the goals of the European "farm to fork" strategy in order to reduce the usage of pesticides by approximately 50% by 2030. As an alternative to conventional pesticides, RNAi-based plant protection holds enormous potential to prevent further drastic losses of biodiversity. Over the last two decades, more than 170 studies have demonstrated the feasibility of controlling agronomically and horticulturally relevant plant diseases by utilizing the

50–300 nm range for plant EVs [4,10]. Transmission electron microscopy (TEM) revealed no obvious differences in electron density for barley EVs compared to previously described *Arabidopsis* EVs [5] (Figure 1a), indicating the similar appearance of EVs isolated from the two different plant species. Notably, nanoparticle trafficking analysis (NTA) and TEM displayed a strong heterogenicity of size among barley EVs compared to *Arabidopsis* EVs. NTA revealed several peaks between 100 and 250 nm, which were confirmed by TEM-based size measurements (Figure 1a,b). However, further mechanistic research is required to confirm the differences in EV biogenesis between monocot and dicot plant species that might explain the heterogenicity of EV populations. To the best of our knowledge, this is the first report on EVs isolated from barley leaves. Thus, we currently lack an EV marker for immunodetection, which is necessary to prove the EVs' origin. For *Arabidopsis* EVs, syntaxin PENETRATION1 (PEN1) [9] and TETRASPANIN8 (TET8) [11] are the referenced EV markers. Currently, the limited information on EV markers in *Arabidopsis* as the plant model organism further impedes efforts to identify possible barley EV markers. Based on the amino acid similarity, we located 10 homologs for PEN1 and seven homologs for TET8 in barley (Figure 1c,d). However, whether the identified PEN1 and TET8 homologs represent valid barley EV markers requires further approval/analysis.

To assess the involvement of EVs in mediating the transport and uptake of SIGS-derived siRNA, barley leaves were sprayed with CYP3RNA, as previously described [6]. EVs were isolated from apoplastic fluids, and EV RNA cargos were analyzed by RNA-seq. We found that the overall amount of siRNAs that mapped to the sprayed CYP3RNA precursor was very low (Figure 1e). Comparing the RNA-seq data with existing EV-siRNA datasets from CYP3RNA-expressing *Arabidopsis* plants was less informative and reliable, because of the divergent dsRNA origins. While a CYP3RNA transgene leads to the constitutive expression and formation of endogenous dsRNA that can be easily incorporated into intracellular vesicles, exogenously applied dsRNA needs to overcome several cellular barriers before being loaded into EVs. Moreover, the amount of dsRNA continuously decreases after foliar spraying due to degradational and dilutional effects. Consequently, the siRNA quantities that reach the lower unsprayed leaf section for loading into EVs might be reduced compared to prerequisites in HIGS. Given these considerations, it was not surprising that the amount of CYP3RNA spray-derived siRNAs was low. In other words, we found less siRNA in barley EVs than in *Arabidopsis* EVs, which led to a low read coverage (number of reads that overlapped at a certain position of the sequence) compared to *Arabidopsis* EVs (Figure 1e) [5]. Importantly, EV biogenesis, as well as the loading and release mechanisms of EVs' RNA cargo, may also differ greatly between monocot and dicot plant species, which makes it even harder to compare HIGS and SIGS strategies among two different plant species.

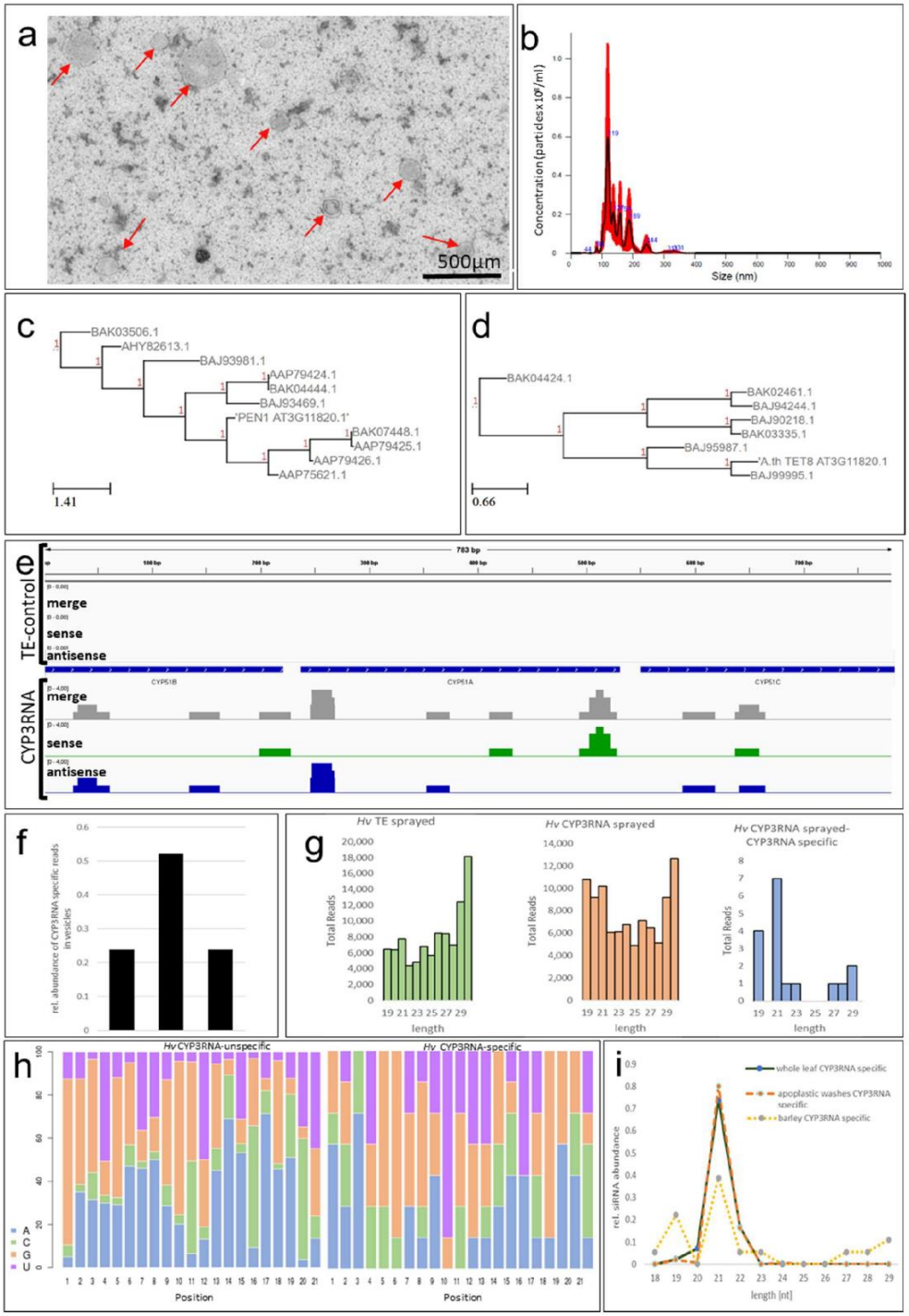

Figure 1. (**a**) Barley EVs were negatively stained onto copper formvar meshes using 2% uranyl acetate. (**b**) Next, 5 µL of purified EVs was diluted up to a volume of 500 µL. The vesicle suspension was loaded onto a Nanosight NS300 (Malvern Panalytical).

Five measurements were performed at 25 °C, and size, concentration prediction, and statistical analyses were performed using the NTA 3.2 Dev Build 3.2.16 software. (**c**,**d**) *Arabidopsis thaliana* PEN1 (AT3G11820) and TET8 (AT2G23810) paralogs of *Hordeum vulgare* subsp. vulgare (*Hv*) were predicted by the NCBI's protein BLAST service (Available online: https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 14 June 2021)) and visualized using the ete view tool. Available online: http://etetoolkit.org (accessed on 23 June 2021). (**e**) RNA was isolated from mock and dsRNA-treated barley leaves. Indexed sRNA libraries were pooled and sequenced on the Illumina MiSeq Platform (1 × 36 SE). The readings were then mapped onto the CYP3RNA sequence. (**f**) The relative abundance of reads aligned to each CYP3RNA fragment (*CYP51A*, *CYP51B*, *CYP51C*) were calculated and (**g**) reads were sorted based on their size. (**h**) The nucleotide distribution for every position was counted for the 21 nts long siRNAs of all barley siRNAs (left) and siRNAs with perfect complementarity towards the CYP3RNA precursor (right). (**i**) The relative abundances of siRNA with different lengths from barley EVs were compared with relative siRNA abundances from *Arabidopsis* EVs purified from apoplastic washes and *Arabidopsis* vesicles isolated by whole-leaf vesicle purification.

However, besides all of the aforementioned concerns regarding comparability, we found some convincing overlaps. For example, the siRNA pattern demonstrated a bias towards siRNAs that matched the CYP51A fragment in the middle of the CYP3RNA triple construct (Figure 1f), which was also observed for *Arabidopsis* [5]. Further analysis enabled the identification of several of the same siRNAs in both systems, *Arabidopsis*–HIGS and barley–SIGS. Our findings also indicate that the majority of siRNAs are 21 nts in length (Figure 1g) and preferentially begin with an A (Figure 1h), while siRNAs in EVs isolated from transgene-expressing (HIGS) *Arabidopsis* plants begin with an A or U [5]. Interestingly, siRNAs that are not derived from the CYP3RNA precursor preferentially begin with a G (Figure 1h). Based on sRNA-seq data revealing the 5′-identities and lengths of HIGS-derived siRNAs, we can speculate regarding the contributing RNA-binding proteins, insofar as they are known for their specific pathosystem. For barley, we observed a high abundance of siRNAs that were 19 nts in length (Figure 1g). We therefore analyzed the relative abundance of siRNAs of each length in comparison to all identified siRNAs, which we mapped to the precursor to compare the siRNA amounts between both species, and found that barley EVs revealed a second peak for 19 nts siRNAs, which we did not observe in EVs from *Arabidopsis* (Figure 1h,i). This finding—along with previously discovered differences in efficiencies between dsRNA originating from endogenous expression (HIGS) and dsRNA originating from exogenous spray [8]—underlines the mechanistic differences between HIGS and SIGS regarding dsRNA uptake, processing, and transfer. In summary, our current knowledge supports a model of HIGS that involves both plant Dicer-mediated processing of transgene-derived dsRNA into siRNAs and ESCRT-III component-mediated RNA transfer—possibly via EVs. Nevertheless, the process by which EVs traverse the plant–fungal interface remains unknown, while the question of whether *Fg* takes up EVs or siRNA/dsRNA released from EVs prior to uptake remains open. In contrast, sprayed dsRNA is only partially processed by plant Dicers, while unprocessed dsRNA was shown to be taken up by *Fg* [6,7]. Nevertheless, future research must determine whether the loading of unprocessed dsRNA into EVs contributes to SIGS.

Taken together, our data revealed CYP3RNA-derived siRNAs in barley EVs, indicating the uptake, transport and procession of exogenous spray-applied dsRNAs. However, whether the EV-mediated uptake of siRNAs is required to induce SIGS requires further verification regarding the fungal uptake ability of EVs containing siRNAs (and dsRNAs). Moreover, we assume that the fungal uptake of SIGS RNA triggers may depend on the lifestyle of the interacting fungal pathogen. Given this assumption, further research is required to unravel the routes of dsRNAs and siRNAs necessary to determine the strengths and limitations of the SIGS strategy in a pathosystem-specific manner.

3. Materials and Methods

3.1. Plant Cultivation and CYP3RNA Spray-Application

One hundred and sixty second leaves of barley cv. Golden Promise were harvested from plants grown for 3 weeks under long day conditions (16 h light, 22 °C, 60% humidity).

The leaves were transferred to square Petri dishes with 1% agar and divided into two groups. The upper part of the first group was sprayed with CYP3RNA diluted in TE buffer and the second group was sprayed with TE buffer as the mock control, as previously described [6], and incubated for 48 h before EV isolation was performed.

3.2. Negative Staining and Transmission Electron Microscopy (TEM)

For TEM, copper formvar-coated 300-mesh electron microscopy grids were glow discharged prior to sample application for 40s. Subsequently, 5 µL of the sample, resuspended in PBS, was applied to the grids. Samples were dabbed using Whatman filter paper and grids were washed three times in 50 µL of 2% uranyl acetate and once with distilled water. Needless staining or fixing solutions, buffers and water were removed using Whatman paper between each step. Finally, the grids were air-dried. Preparations were inspected at 120 kV under zero-loss conditions (ZEISS EM912a/b) and images were recorded at slight underfocus using a cooled 2 k × 2 k slow-scan ccd camera (SharpEye/TRS) and the iTEM software package (Olympus-SIS). Two replicates per sample were invested and at least ten meshes per grid were checked to avoid grid to grid or mesh to mesh variations.

3.3. Vesicle Size and Concentration Measurements by Nanoparticle Trafficking Analysis (NTA)

For size and concentration predictions, purified barley EVs were diluted (1:50) with PBS. Subsequently, 500 µL of the vesicle suspension was loaded into Nanosight NS300 (Malvern Panalytical). Five measurements were performed at 25 °C and size, concentration prediction and statistical analysis were performed by the NTA 3.2 Dev Build 3.2.16 software.

3.4. Identification of Arabidopsis PEN1 and TET8 Homologs in Barley

The *Arabidopsis thaliana* PEN1 (AT3G11820) and TET8 (AT2G23810) paralogs of *Hordeum vulgare* subsp. vulgare (*Hv*) were predicted based on their amino acid sequences. These were obtained from The *Arabidopsis* Information Resource (tair) (Available online: https://www.arabidopsis.org/ (accessed on 10 February 2021)). Paralogs were forecasted by the NCBI's protein BLAST service (Available online: https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 10 February 2021)) and the phylogenetic tree was built using ETE 3 [12].

3.5. Determine siRNAs Originating from CYP3RNA

Vesicle RNA was isolated using the Single Cell RNA Purification Kit (Norgen Biotek, Thorold, Ca) according to the manufacturer's instructions for cells growing in suspension. RNA concentrations were determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA was stored at −80 °C before samples were sent for RNA sequencing. Indexed sRNA libraries were constructed from RNA isolated from vesicles with the TruSeq® Small RNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Indexed sRNA libraries were pooled and sequenced on the Illumina MiSeq Platform (1 × 36 SE) and the sequences were sorted into individual datasets based on the unique indices of each sRNA library. The RNAseq libraries can be accessed on the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home (accessed on 11 February 2021); Accession ID: PRJEB45864). The quality of the datasets was examined with FastQC (version 0.11.9) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed on 11 February 2021)) before and after trimming. The adapters were trimmed using cutadapt (version 2.8) [13]. To filter out bacterial contaminations, kraken2 (version 2.1.1) [14] was used with a database obtained from the MGX metagenomics application [15]. All reads marked as unclassified were considered to be of non-bacterial origin and were used for the subsequent alignment. The trimmed and filtered reads were mapped to the CYP3RNA sequence using bowtie2 (version 2.3.2) [16] to identify siRNAs derived from the precursor dsRNA sequence. The mappings were first converted into bedgraph using bedtools (version 2.26.0) [17] and then to bigwig using bedGraphToBigWig [18]. These files were used for visualization with

IGV [19]. Read coverage is defined as the number of reads that match at a certain position in the sequence.

3.6. Determine Frequency of Different RNA Species

To determine the RNA species, the reference genome and annotation of *Hordeum vulgare* (IBSC v2—release 47) were downloaded from EnsemblPlants [20]. The adapter trimming of raw reads was carried out with TrimGalore (version 0.6.4) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/ (accessed on 12 February 2021)), which used cutadapt (version 2.8) [13]. In this process, all reads that became shorter than 18 nts were filtered out. Afterwards, nucleotides with a phred score below 20 and reads retaining less than 90% of their nucleotides in this process were removed using the FASTQ Quality Filter from the FASTX-toolkit (version 0.0.14) (https://github.com/agordon/fastx_toolkit (accessed on 12 February 2021)). The bacterial contaminations were filtered out as demonstrated in the previous section. The remaining reads were aligned to the reference genome using STAR (version 2.7.3a) [21]. The number of different RNA species was examined in R (version 4.0.2) (R Core Team, 2020) using featureCounts from the package Rsubread (version 2.2.5) [22]. featureCounts was run for each sample using the previously downloaded annotations of *Arabidopsis*. The following RNA types were examined: "lncRNA", "pre_miRNA", "mRNA", "ncRNA_gene", "rRNA", "snoRNA", "snRNA" and "tRNA". All alignments that could not be assigned to a feature were considered as "not assigned".

Author Contributions: A.K., T.S. and L.W. wrote the manuscript; A.K. and T.S. designed the study; T.S. and L.W., conducted the experiments; M.H. and A.M. conducted the microscopy; C.P. performed NTA measurements; T.S., L.W., A.K. and P.B. analyzed all data and drafted the figures. T.B. conducted the RNA-seq experiments and T.B. and P.B. performed the bioinformatics analysis. All authors have read and agreed to the published version of the manuscript.

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SHORT REPORT Open Access

Extracellular vesicles isolated from dsRNA-sprayed barley plants exhibit no growth inhibition or gene silencing in *Fusarium graminearum*

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Abstract

Numerous reports have shown that incorporating a double-stranded RNA (dsRNA)-expressing transgene into plants or applying dsRNA by spraying it onto their leaves successfully protects them against invading pathogens exploiting the mechanism of RNA interference (RNAi). How dsRNAs or siRNAs are transferred between donor host cells and recipient fungal cells is largely unknown. It is speculated that plant extracellular vesicles (EVs) function as RNA shuttles between plants and their pathogens. Recently, we found that EVs isolated from host-induced gene silencing (HIGS) or spray-induced gene silencing (SIGS) plants contained dsRNA-derived siRNAs. In this study, we evaluated whether isolated EVs from dsRNA-sprayed barley (*Hordeum vulgare*) plants affected the growth of the phytopathogenic ascomycete *Fusarium graminearum*. Encouraged by our previous finding that dropping barley-derived EVs on *F. graminearum* cultures caused fungal stress phenotypes, we conducted an in vitro growth experiment in microtiter plates where we co-cultivated *F. graminearum* with plant EVs isolated from dsRNA-sprayed barley leaves. We observed that co-cultivation of *F. graminearum* macroconidia with barley EVs did not affect fungal growth. Furthermore, plant EVs containing SIGS-derived siRNA appeared not to affect *F. graminearum* growth and showed no gene silencing activity on *F. graminearum CYP51* genes. Based on our findings, we concluded that either the amount of SIGS-derived siRNA was insufficient to induce target gene silencing in *F. graminearum*, indicating that the role of EVs in SIGS is minor, or that *F. graminearum* uptake of plant EVs from liquid cultures was inefficient or impossible.

Keywords: Extracellular vesicles, Plant EVs, Barley, *Fusarium graminearum*, RNAi, dsRNA-based pesticides, Spray-induced gene silencing, RNAi-based plant protection, dsRNA, siRNA

Background

Research on plant extracellular vesicles (EVs) is an emerging field that has undergone rapid progress in the last three years, with more than 260 studies published (PubMed). The published evidence that plant EVs and vesicle-like nanoparticles (VLNs) exhibit beneficial effects on human health [53, 60] encouraged scientists to isolate macro- and nanosized vesicles from diverse food sources, e.g., *Panax ginseng* [10], *Asparagus cochinchinensis* [63, 64], *Aloe vera* [24], garlic [38], bitter melon [61], grapefruit [50], strawberry [39], carrot [23] and honey [9]. This allowed them to study their anti-inflammatory, anticancer, antioxidative, and antisenescence properties. Notably, since EV-specific markers are not yet available for plant products such as fruits and vegetables, their extracellular origin needs to be verified. However, the strong bioactivity and biocompatibility of EVs and VLNs

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together with their efficient cellular internalization (e.g., penetration into glioma tissues by receptor-mediated transcytosis; [35] have raised the possibility of exploiting them as novel drug delivery vehicles [62].

Although plant EVs were first described in the apoplast in 1967 [18], it was almost half a century before they were separated from plant apoplastic fluids and then visualized with transmission electron microscopy (TEM) [42, 43, 45]. These pioneering works have laid the foundation for examining the role or contribution of EVs during plant-pathogen interactions [6, 8, 12, 32, 59]. These examples intensify the assumption that plant- or pathogen-derived EVs contribute bidirectionally to this highly specialized interspecies communication through the release of lipids, proteins, and small RNAs (sRNAs) that regulate and deregulate defensive and offensive responses [5, 51]. In particular, the identification of plant EV-derived sRNAs stimulated a debate about whether EVs function as shuttles in interspecies communication, directing plant antifungal defence responses [2, 7, 44, 47–49]. Conversely, fungal pathogens secrete sRNAs to dampen plant immunity [13, 29, 57, 58]. This sRNA-based crosstalk, also known as cross-species RNAi, was first described by Weiberg et al. [57], demonstrating that the fungal pathogen *Botrytis cinerea* produces sRNAs that mimic plant sRNAs and bind to *A. thaliana* AGO1 to antagonistically silence important plant immunity genes [57]. Similarly to the suggested plant EV-mediated sRNA transport, it is proposed that fungal sRNA delivery is also facilitated by EVs [31]. To prove this hypothesis, EVs isolated from different fungal pathogens, such as *Ustilago maydis* [30], *Zymoseptoria tritici* [20], *Fusarium oxysporum* [4, 16] and *F. graminearum* [17, 48], were established, which lay the foundation for further study of cross-species RNA transport in plant-fungus interactions in the near future.

In agriculture, RNAi technologies attract immense scientific and political interest as powerful substitutes for conventional chemical pesticides to reach the EU's and UN's [14, 55] sustainable development goals [52]. Currently, RNAi-based plant protection relies on two strategies that differ based on the origin of the dsRNA utilized. In the first strategy, endogenous dsRNA formation mediated by transgene expression is designated as host-induced gene silencing (HIGS). The second strategy is based on exogenous, foliar dsRNA application known as spray-induced gene silencing (SIGS). Notably, the principle of cross-species RNAi was biotechnologically used (HIGS) [36] before its naturally occurring equivalent was discovered [57].

We previously demonstrated that a transgene-derived CYP3RNA (a dsRNA designed to target *CYP51A*, *CYP51B* and *CYP51C* genes in *F. graminearum*), as well as foliar application of CYP3RNA, induced *CYP51* target gene silencing in *F. graminearum* [25, 27]. Remarkably, HIGS-or SIGS-mediated *F. graminearum CYP51* downregulation conferred strong *F. graminearum* disease resistance in *Arabidopsis thaliana* (HIGS) and *Hordeum vulgare* (HIGS and SIGS) [3, 21, 25–27] Despite many proof-of-concept studies demonstrating the efficacy of RNAi in pathogen and pest control (for review, see: Koch and Kogel [65–69] [32, 41], our mechanistic knowledge of HIGS and SIGS is still incomplete, although researchers hope to translate testing from the lab to the field soon [41]. Understanding the routes by which dsRNAs and siRNAs are delivered into fungal cells will be key to further improving cellular uptake and systemic distribution, and therefore increasing the stability and efficacy of exogenously applied dsRNA-based pesticides.

Studying the role or requirement of EVs in transferring HIGS- and SIGS-associated RNAs, we recently showed that EVs isolated from CYP3RNA-expressing *A. thaliana* plants contain CYP3RNA-derived siRNAs [47]. Notably, subsequent differential digestive treatments of EVs with RNase, protease, and a detergent revealed that the amount of intravesicular siRNA was low [47], more than 70% of the CYP3RNA-derived siRNAs were found to be extravesicular. EVs isolated from CYP3RNA-sprayed barley plants revealed CYP3RNA-derived siRNAs, too; however, their abundance was even lower compared with EVs isolated from HIGS *A. thaliana* plants [49]. This difference might be due to the various dsRNA origins in HIGS and SIGS approaches, whereby sprayed RNAs must be taken up by plant cells before being packed into plant EVs [28]. CYP3RNA uptake into plant cells and its systemic spread via the phloem have been previously reported, as well as its apoplastic transport in the xylem [3, 25]. However, since the amount of dsRNA-spray-derived siRNA in barley EVs was low, we asked whether EVs are required for the delivery and uptake of exogenously applied dsRNA to induce SIGS in *F. graminearum*.

To address this question, we assessed whether EVs isolated from SIGS plants can induce *F. graminearum CYP51* target gene silencing and fungal growth inhibition. For this, we performed in vitro treatments of *F. graminearum* with EVs isolated from CYP3RNA-sprayed barley plants. Remarkably, we found no effects on *F. graminearum* expression of *CYP51* or growth, further underlining the importance of clarifying whether EV-mediated sRNA transport is required during SIGS-barley–*F. graminearum* interaction.

Results

To test the possibility of plant EV uptake by *F. graminearum* in vitro, we isolated EVs from control [tris–EDTA (TE) buffer] and CYP3RNA-sprayed barley leaves using a protocol modified from those described by Rutter and Innes [45] and Schlemmer et al. [48]. In our recent studies, we observed that state-of-the-art EV purification from apoplastic fluids leads to impure EV isolates containing additional co-purified apoplastic substances [47]. This finding aligns with recent debates discussing the pitfalls of current plant EV research methods and the need for standardization, with different contamination risks reported for different plant EV separation and characterization methods [33, 40, 46]. To avoid such pitfalls that may lead to false conclusions, we performed a stringent digestive treatment of EV isolates to degrade extravesicular proteins and RNAs before in vitro treatment of *F. graminearum* with plant EVs. Each EV isolate was derived from 80 barley leaves and EVs were ultimately resuspended in 190 µl of phosphate-buffered saline (PBS). We reserved 40 µl for quality control measurements, TEM, and nanoparticle trafficking analysis (NTA). The remaining suspension was divided into three equal fractions (Fig. 1). To degrade extravesicular proteins, RNAs, and RNA–protein complexes, one fraction of EV isolates was treated with proteinase K and RNase A (PK + RA). In addition to PK + RA, the next fraction was treated with triton X-100 (TX + PK + RA), which is known to dissolve EVs [37, 45, 54] and denature extra- and intravesicular proteins, RNAs and RNA–protein complexes (Fig. 1). One fraction remained untreated to evaluate the effects of co-purified apoplastic fluid proteins or RNAs. Finally, EVs were co-inoculated with *F. graminearum* macroconidia and fungal growth was determined, after 20 h of pre-incubation, by optical density (OD) measurements every 20 min for a further 24 h.

To assess whether the effects depended on the investigated volumes, we used two different volumes of resuspended EV solution. We tested untreated EVs isolated from TE- or CYP3RNA-sprayed barley leaves and EVs treated with PK + RA and TX + PK + RA. We added 5 or 10 µl of each EV fraction to *F. graminearum* macroconidia. For each isolation, we investigated the same amount of barley leaves (80), which were previously sprayed either with TE or CYP3RNA; we then resuspended the EVs in the same volume of PBS before dividing them into the three fractions. Regardless of whether EVs were derived from CYP3RNA- or TE-sprayed barley leaves, or how EVs were treated after purification, no differences in *F. graminearum* growth were observed between treatment volumes (Fig. 2). At the beginning of the measurement period, 23 h post-inoculation (hpi), all samples showed an OD value of approximately 0.5. At 42 hpi, the OD for untreated and PK + RA-treated EVs had increased up to 0.9–1.1, while that for TX + PK + RA-treated EVs only rose to 0.7–0.9.

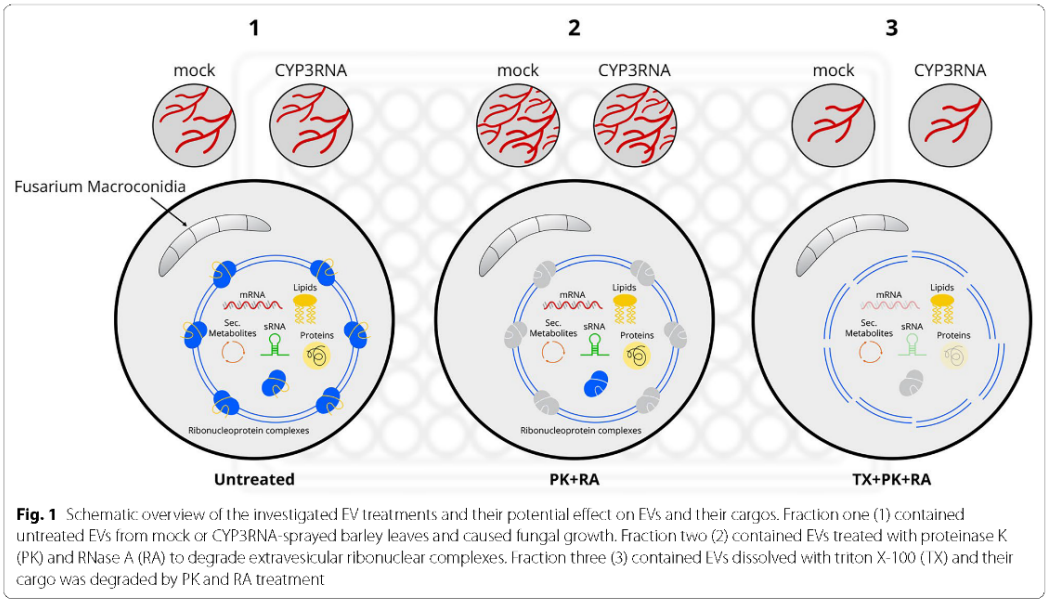

Fig. 1 Schematic overview of the investigated EV treatments and their potential effect on EVs and their cargos. Fraction one (1) contained untreated EVs from mock or CYP3RNA-sprayed barley leaves and caused fungal growth. Fraction two (2) contained EVs treated with proteinase K (PK) and RNase A (RA) to degrade extravesicular ribonuclear complexes. Fraction three (3) contained EVs dissolved with triton X-100 (TX) and their cargo was degraded by PK and RA treatment

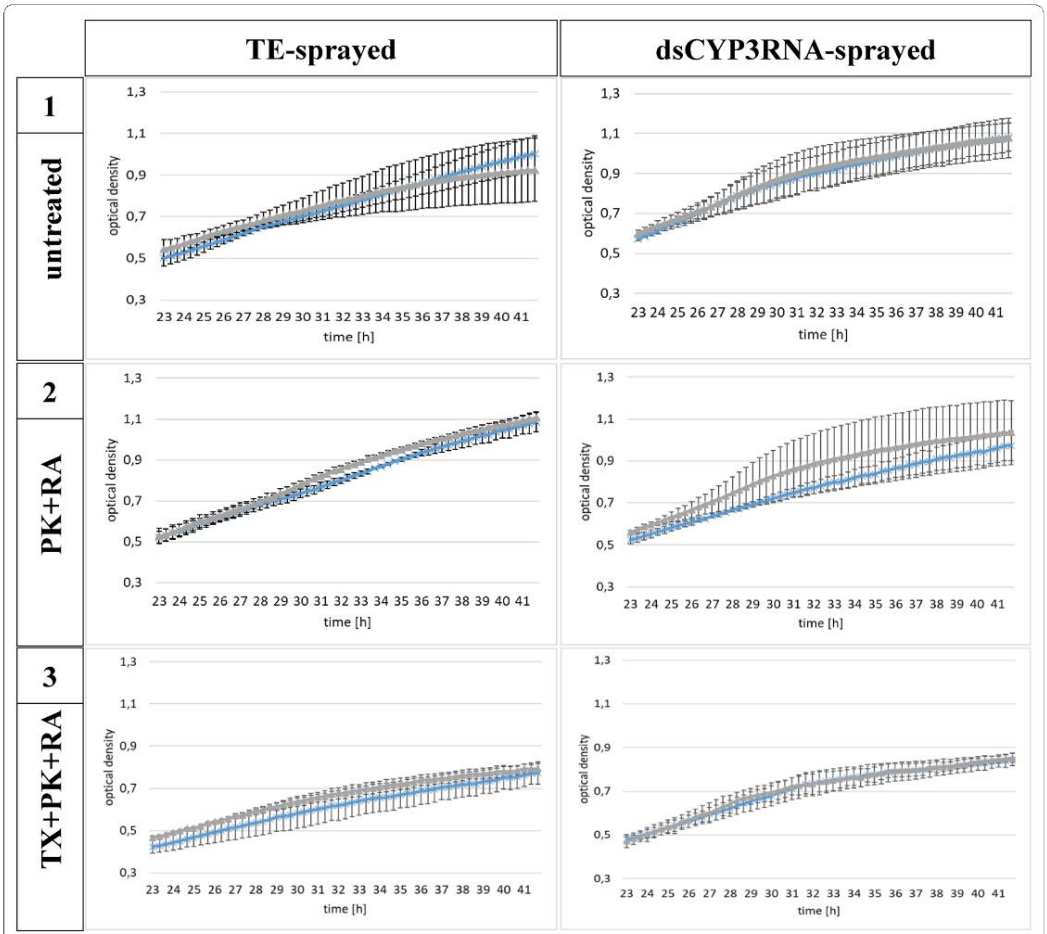

Fig. 2 5 µl (light blue cross) and 10 µl (gray triangle) of purified EVs from the control (tris–EDTA) and CYP3RNA-sprayed barley leaves were added to *F. graminearum* liquid culture. Growth for cultures treated with EVs from all three fractions was determined by optical density measurements between 23 and 42 h post-inoculation (hpi)

As we observed no difference in the effect on fungal growth induced by different EV volumes, we next assessed the effect of EV treatments on *F. graminearum* growth. As a control, we used EV-free PBS, which was used as a buffer for EV resuspension after isolation. We compared the fungal growth over the measured time between the different EV samples. Focusing on *F. graminearum* growth with EVs from TE-sprayed barley leaves, we observed that PK+RA-treated EVs increased *F. graminearum* growth compared to PBS-treated *F. graminearum* cultures (Fig. 3). This was possibly triggered by simplified nutrient uptake via the degraded proteins and RNAs the enzymatic treatment created, or by the destruction of proteins that usually inhibit *F. graminearum* growth. However, we did not observe growth promotion when *F. graminearum* was fed with untreated EVs. The same observation was made when we focused on EVs from CYP3RNA-sprayed barley leaves, where no difference in fungal growth was observed after treatment of *F. graminearum* with different EV samples. Regardless of whether EVs originated from TE- or CYP3RNA-sprayed barley leaves, or whether 5 or 10 µl was applied, *F. graminearum* co-cultivated with TX+PK+RA-treated EVs was more inhibited than *F.*

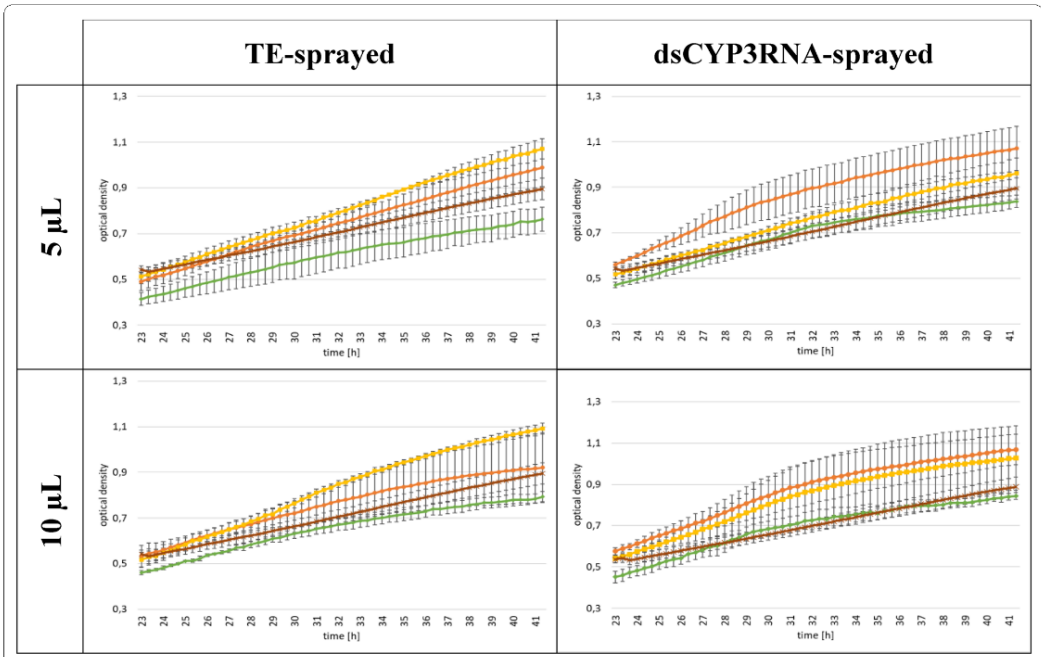

Fig. 3 Purified barley EVs were treated with RNase A and proteinase K (yellow square) or Triton X-100, RNase A, and Proteinase K (green rhombus) after isolation and co-inoculated with *F. graminearum*. Additionally, untreated (orange circle) and EV-free PBS (brown cross) were co-inoculated as positive and negative controls

graminearum co-cultivated with PBS, untreated EVs, or PK + RA-treated EVs (Fig. 3). Therefore, we tested the detergent's effect on *F. graminearum*. We mixed TX, PK, RA, PK + RA and TX + PK + RA with PBS, incubated them under the same conditions as the plant EVs and tested the mixtures in our growth assay. We observed no differences in the growth of *F. graminearum* treated with PK, RA, or a combination of both at the end of the growth assay (Fig. 4a–c). However, TX and TX + PK + RA reduced growth compared to the PBS control, indicating a clear effect of TX on fungal growth independent of plant EVs (Figs. 1; 4d). To avoid misinterpreting the effect of TX as that of CYP3RNA, we calculated the relative growth per EV treatment to compare the effects of TE- and CYP3RNA-sprayed EVs (Fig. 5). Remarkably, we found that CYP3RNA application did not inhibit growth, independently of how EVs were treated after isolation (Fig. 5). To verify this result and determine whether the unimpaired fungal growth could be explained by a lack of *F. graminearum CYP51* gene silencing, we isolated RNA from the *F. graminearum* cultures grown in microtiter plates and performed *F. graminearum CYP51* gene expression analysis. We found no downregulation of *CYP51* gene expression after co-cultivation with EVs isolated from CYP3RNA-sprayed barley leaves (Fig. 6). To test EV stability in PBS and the culture medium PDB, we added EVs resuspended in PBS to PBS or PDB in equal volumes. We froze samples in liquid nitrogen immediately after mixing and after 24 and 48 h of further incubation. The EVs were then analyzed using transmission electron microscopy (TEM) (Additional file 1: Fig. S1) and nanoparticle tracking analysis (NTA) (Additional file 2: Fig. S2). NTA measurements revealed low particle abundance, which was in line with the observations made by TEM. Therefore, we focused on close-up views of EVs and examined membrane integrity in the PBS and PDB medium at all three timepoints (Additional file 1: Fig. S1). Further, we harvested the cultured supernatant of *F. graminearum* after 24 h of pre-cultivation and added it to the EVs to test if they were degraded by secreted fungal enzymes such as lipases [56]. We measured particle concentration shortly after the fungal supernatant was administered to the EVs and 2 h post co-incubation. During this time, we saw no particle reduction, which suggests that the fungal culture supernatant did not cause any degradational processes in the EVs (Additional file 3:

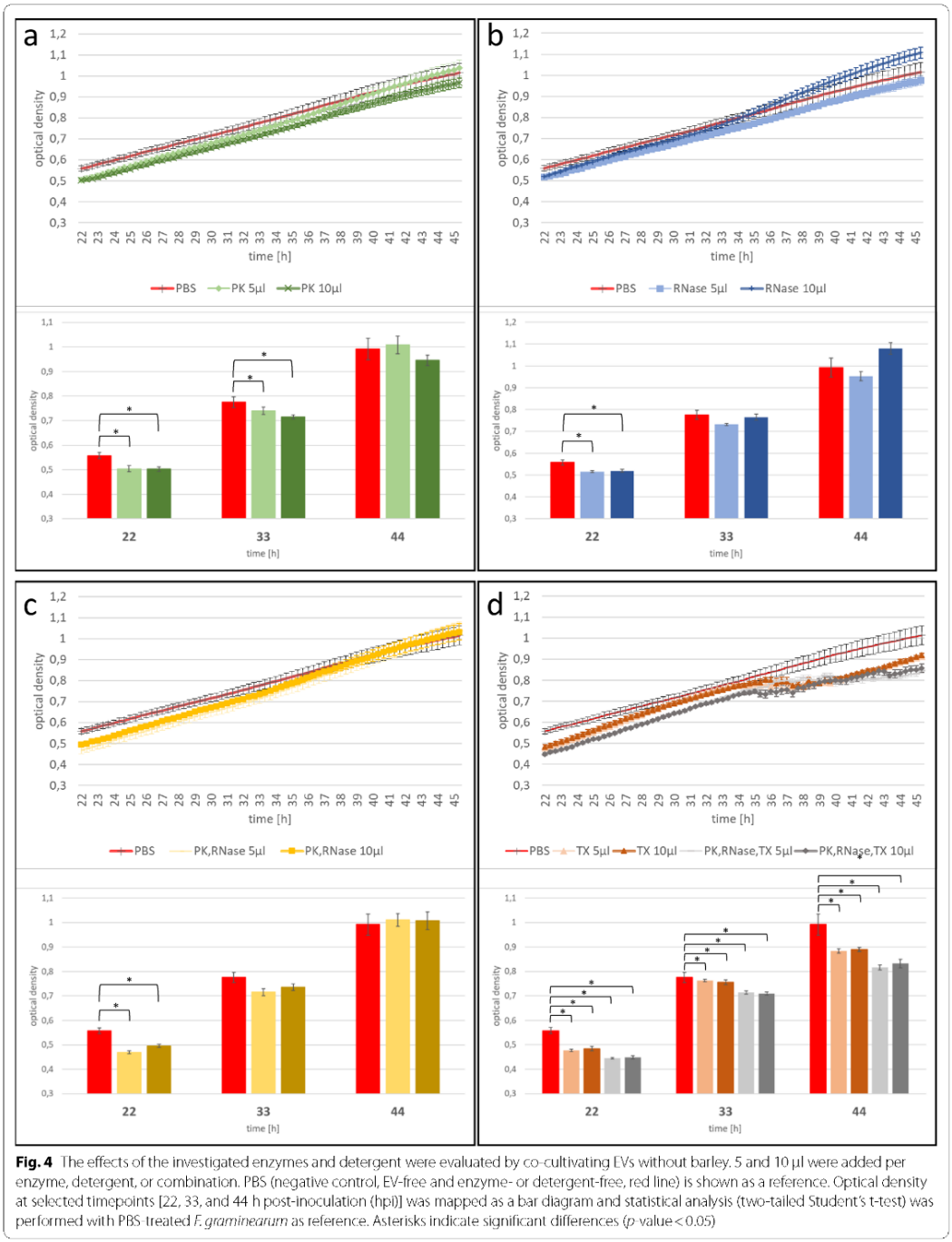

Fig. 4 The effects of the investigated enzymes and detergent were evaluated by co-cultivating EVs without barley. 5 and 10 µl were added per enzyme, detergent, or combination. PBS (negative control, EV-free and enzyme- or detergent-free, red line) is shown as a reference. Optical density at selected timepoints [22, 33, and 44 h post-inoculation (hpi)] was mapped as a bar diagram and statistical analysis (two-tailed Student's t-test) was performed with PBS-treated *F. graminearum* as reference. Asterisks indicate significant differences (p-value < 0.05)

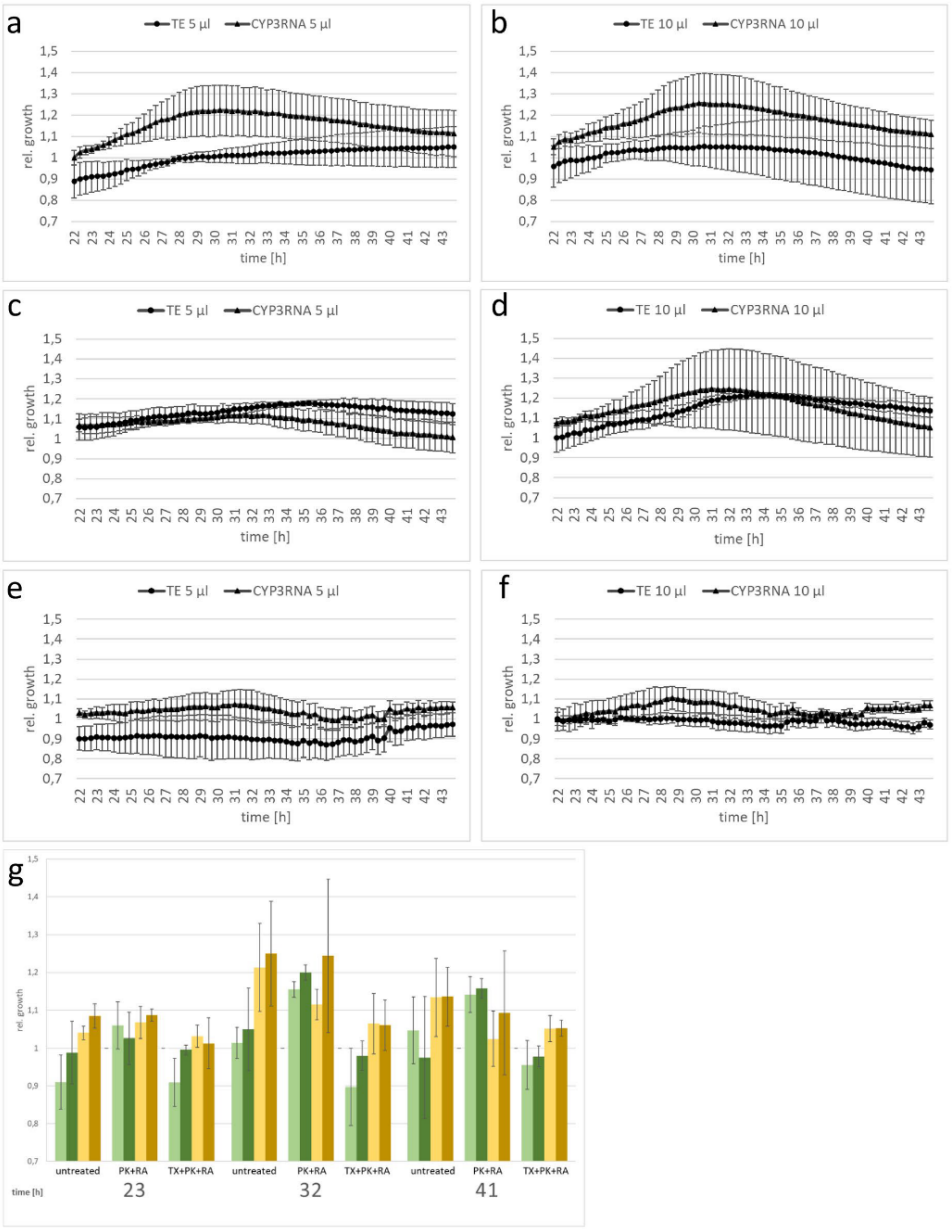

Fig. 5 The relative fungal growth in the co-culture was calculated using the EV-free cultivation conditions with enzymes and detergent only as a baseline. Control (tris–EDTA): circle; CYP3RNA: triangle (**a**–**f**). Selected timepoints were chosen for statistical analysis. Differences between TE- or CYP3RNA-sprayed barley leaves were calculated for each investigated volume and EV pre-treatment using a two-tailed Student's t-test (p-value < 0.05). 5 µl EVs of TE-sprayed barley leaves: light green; 5 µl EVs of CYP3RNA-sprayed barley leaves: dark green; 10 µl EVs of TE-sprayed barley leaves: light brown; 10 µl EVs of CYP3RNA-sprayed barley leaves: dark brown (g)

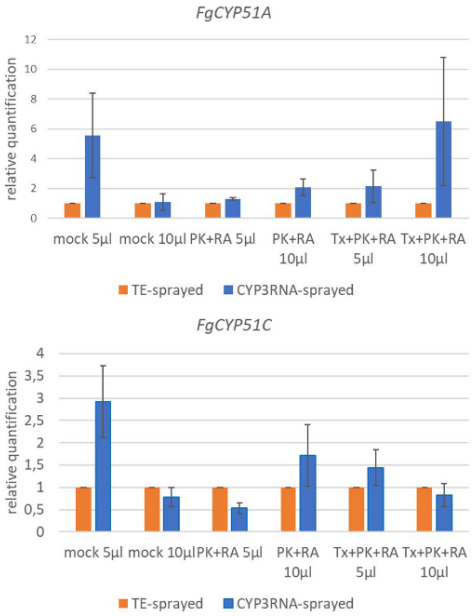

Fig. 6 42 h post-inoculation (hpi) EV–*F. graminearum* co-cultures were harvested and technical triplicates were combined before RNA isolation. Transcriptional analyses were performed and *FgCYP51A* and *FgCYP51C* expression was calculated with the ΔΔCt method using the elongation factor 1 α as the reference gene. ΔΔCt values were calculated by referring to ΔCt values of samples which were derived from *F. graminearum* cultures where EVs were equally treated after isolation but derived from control-sprayed (non-RNA) leaves

Fig. S3). Additionally, we repeated our fungal growth assay and investigated EVs from *A. thaliana* wildtype (wt) and double-stranded CYP3RNA-expressing transgenic plants. We observed that *A. thaliana* EVs had similar effects to those noted in barley EVs (Additional file 4: Fig. S4, Additional file 5: Fig. S5, Additional file 6: Fig. S6). No effects of the EV CYP3RNA cargo from HIGS plants were observed (Additional file 6: Fig. S6).

Discussion

More than 50 studies demonstrate RNAi-based control of fungal pathogens with an average plant disease resistance of about 60% [28]. These studies reflect the enormous potential of RNAi technologies to meet the socio-political demand to halve the use of chemical pesticides by 2030 in Europe which has been approved by the European Commission in their farm-to-fork strategy in 2021 European Commission [70]. To meet this challenge, we must gain more mechanistic insights regarding the uptake and transport of exogenously applied dsRNAs to ensure their integrity and stability when used as dsRNA-based pesticides in the field. For example, proper RNA uptake and transport essentially serve as effective protection against degradation under difficult environmental conditions. Inside plants, exogenously-originating RNA may be further stabilized by the formation of RNA–protein complexes and/or EVs that can encapsulate RNAs, thus sheltering them from RNases or degradation in general during short- (cell-to-cell) or long-distance (systemic) movement. Given the assumption that EVs may participate in or facilitate the transfer of sRNAs during plant-pathogen interaction, the question is whether they are required in the transfer of HIGS- and SIGS-derived RNAs as well. We previously found that EVs isolated from HIGS-*A. thaliana* and SIGS-barley plants principally contain transgene- and dsRNA-spray-derived siRNAs [47, 49]. However, since the amount of HIGS and SIGS-related siRNA inside EVs was low, we assessed here whether these siRNAs could induce the silencing of *F. graminearum CYP51* genes and thus fungal growth inhibition, despite their low abundance.

To address these questions, we treated *F. graminearum* with EVs isolated from dsRNA-sprayed barley plants as well as transgenic HIGS-*A. thaliana* in vitro. The impurity of plant EV isolates raised concerns about the reliability of findings and their interpretation [33, 46]; we thus performed rigorous digestive treatments of EV isolates before *F. graminearum* in vitro testing. Encouraged by our previous finding that drop inoculation of barley EVs on *F. graminearum* cultures grown on solid agar plates caused an increase in purple pigmentation, indicative of the stress-induced premature formation of fruiting bodies [48], we expected to observe similar effects in liquid cultures. Interestingly, another recent study demonstrated the antifungal activity of EVs derived from root exudates of tomato plants against *F. oxysporum*, *B. cinerea* and *Alternaria alternata* [12], supporting the validity of in vitro EV–fungal spore interaction tests.

Surprisingly, we found that neither wild-type barley EVs nor EVs isolated from SIGS and HIGS led to inhibition of *F. graminearum* growth (Fig. 5; Additional file 6: Fig. S6). In addition, even different EV volumes (5 or 10 µl EV suspension) did not affect fungal growth (Fig. 2). In our previous successful experiments on solid agar plates, 40 µl of EV suspension derived from 80 barley leaves was drop-inoculated onto *F. graminearum*, suggesting that the volumes of 5 and 10 µl used in the present experiments might be too low. Given our previous finding that barley EVs led to stress-related discoloration of *F. graminearum* colonies [48] we assume that *F. graminearum* may be unable to take up EVs in vitro. A second possibility is that the amount of spray-derived siRNA in EVs is insufficient to induce fungal target gene silencing and the expected

growth inhibition. To test the second possibility, we performed *F. graminearum CYP51* gene expression analysis on *F. graminearum* cultures after EV treatment, which was a more sensitive way to test CYP3RNA effects on *F. graminearum* than determining the OD of liquid fungal cultures. Underlining our results showing no growth inhibition in *F. graminearum*, we observed that EVs from CYP3RNA barley leaves did not show any gene-silencing activity (Fig. 6). However, this could still be explained by the inability of *F. graminearum* to take up plant EVs in vitro. Given this presumption, another study demonstrated sunflower-derived EV uptake by the ascomycete *Sclerotinia sclerotiorum* through reduced hyphae growth and spore germination [43], indicating that fungal uptake of plant EVs is possible in principle. In addition, recent studies indicated in vitro uptake of plant-derived (ginger, grapefruit, pineapple, and paprika) EVs and VLNs in human and rat cells [15, 22, 34], which is of great scientific interest due to their therapeutic potential in nanomedicine [11]. However, whether this holds for other fungal plant pathogens remains to be verified.

Notably, plant-derived EVs were shown to contain stress response-related proteins and lipids [8, 12, 32, 45, 47] and exhibit antifungal activity [12, 48]. It is therefore surprising that we did not observe any inhibitory effects of barley EVs on *F. graminearum*. This raises the question of whether EVs and their contents are stable in liquid media, and able to overcome the membrane or cellular barriers of *F. graminearum* and reach a defined threshold to activate the distinct RNAi machinery within its cells. To address concerns about EV stability in the resuspension buffer (PBS) and the cultivation media (PDB) we added barley EVs resuspended in PBS to PDB media or additional PBS (see detailed description in "Methods" section). At all three timepoints, which corresponded to the start of the experiment, the start of the OD measurements, and the end of the experiment, we observed EVs with intact membranes in both media (Additional file 1: Fig. S1). However, this assay does not provide any information on possible EV degradation by extracellular enzymes, e.g., fungal lipases secreted by *F. graminearum* Nguyen et al. [71]. Therefore, we tested the supernatant of 24-h-old *F. graminearum* cultures, which should have contained extracellular fungal enzymes together with barley EVs. There was no particle reduction measured after 2 h of co-incubation. Additionally, we previously isolated fungal EVs from *F. graminearum* which seemed to be resistant to lipases [48]. However, the lipid composition of plant and fungal EVs may be different.

Given our presumption that the low abundance of siRNA inside EVs may not be sufficient to induce a proper gene silencing response, the fundamental question about the relevance of EVs in transferring HIGS- and SIGS-associated RNAs remained. In addition to this, we already showed that more than 70% of HIGS-derived siRNAs were found to be extravesicular [47]. In support of this, the latest results from Roger Innes' group have demonstrated that treatment of purified EVs with the protease trypsin and subsequent treatment with RNase A sufficiently eliminates RNA–protein complexes adhering to the outside of EVs, leading to the conclusion that extravesicular RNAs mediate HIGS rather than RNAs inside the EVs Zand Karimi et al. [72]. It is important to note that previous reports only rely on RNase treatment of purified plant EVs [7, 19]. Thus, missing the protease treatment may leave RNAs stabilized and protected from nuclease by RNA-binding proteins, which makes it impossible to distinguish between intra- and extravesicular RNAs and proteins. Given this assumption, it remains to be assessed why we observed no effect when fungal spores were treated with undigested EVs (where extravesicular RNA–protein complexes were intact) (Fig. 3, Additional file 5: Fig. S5). Further research is required to determine if this supports our presumption that *F. graminearum* is not able to take up EVs from in vitro liquid cultures, or if it is correct that even EV-adhering RNAs may not lead/contribute to HIGS. Together, these latest findings suggest that EVs may only play a minor or indirect role in the delivery and uptake of HIGS- and SIGS-associated RNAs. At least in the case of SIGS, this seems reasonable, because *F. graminearum* was shown to take up unprocessed dsRNA from the apoplast [25], and thus did not require the uptake of EVs for SIGS. It would therefore be interesting to elucidate the role or necessity of EVs in SIGS targeting of biotrophic fungal pathogens.

In summary, we found no inhibition of *F. graminearum* growth after treatment of in vitro cultures with SIGS- and HIGS-derived plant EVs. Subsequently, we found no *F. graminearum CYP51* target gene silencing, raising the question of whether *F. graminearum* is unable to take up EVs from a liquid culture or whether EV-contained and -adhering RNAs are insufficient to induce a proper gene silencing response in the species. However, further research is required to differentiate between improper EV uptake and the possibility that EVs may not play an important role in the translocation and uptake of RNAs in HIGS and SIGS.

Conclusion

Mechanistic knowledge of RNA uptake and interspecies (plant–fungus) sRNA transfer is essential to the further development of RNAi technologies for plant protection. Here, we investigated the EV uptake ability of *F. graminearum* after in vitro treatment with SIGS-derived barley EVs. We found no growth inhibition or gene silencing in the species, indicating that either the fungus is unable to

take up EVs from liquid cultures or the amount of RNA inside and/or outside the EVs is not sufficient to induce gene silencing of the target fungal genes. Our findings illustrate the importance of developing experimental readouts that allow the dependency of EV-mediated bidirectional sRNA transport for cross-species RNAi to be studied. In this context, studies have begun to identify and characterize plant and fungal EV content, as well as the importance of further developing EV isolation and purification protocols to improve reliability and avoid false interpretation of results. However, using EVs as natural blueprints may lead to the development of nanocarrier-based technologies that facilitate the efficient delivery of CRISPR/Cas9 components in the future [1]. In addition, fungal uptake of plant-derived EVs may offer potential routes to cure fungal diseases in humans, based on emerging evidence that plant-derived EVs exhibit great potential for human health applications [11].

Methods

EV isolation

Arabidopsis thaliana EVs were isolated from the apoplastic washing fluids of 90 plants per genotype. The apoplastic washes were harvested from the leaf rosettes, and then filtered through a 0.22 µm filter, centrifuged at 10,000×*g* and 100,000×*g* and resuspended in 190 µl PBS (8 mM NaH_2PO_4, 150 mM NaCl, 3 mM KCl and 2 mM KH_2PO_4; pH 7.4) [47]. The barley EV isolation protocol was adapted from the *A. thaliana* EV isolation described by Schlemmer et al. [49]. Each isolation included 80 barley leaves sprayed with tris–EDTA or CYP3RNA. Plant cultivation was performed in triplicate for both plant species and was followed by EV isolation, digestive treatment, and fungal co-cultivation assay.

Differential EV treatments

Resuspended EVs were subdivided into three groups after isolation. The first group was untreated and served as a positive control (Table 1). The second group was treated with proteinase K and RNase A (PK + RA) and the third group with triton X-100, proteinase K, and RNase A (TX + PK + RA) (Table 1). All groups were incubated for 30 min at 37 °C and then added to *Fusarium graminearum* (*F. graminearum*) macroconidia.

Plant EV–*F. graminearum* co-culture assay

Plant EV–*F. graminearum* co-culture assays were performed in transparent 96-well plates with flat bottoms. PDB (potato dextrose broth, Formedium) was used as a carbon source. For macroconidia generation, *F. graminearum* strain IFA65 (IFA, Department of Agrobiotechnology, Tulln, Austria) was cultivated on synthetic nutrient-poor agar plates for 21 days at room temperature (RT) under constant illumination from one near-UV tube (Philips TLD 36 W/08) and one white-light tube (Phillips TLD 36 W/830HF). Macroconidia were washed off the plates with distilled water and filtered through sterile miracloth. 1 ml stock solutions were frozen in liquid nitrogen and stored at − 80 °C. All investigated stocks were derived from the same propagation event. One stock was thawed on ice per co-cultivation assay and macroconidia concentration (272,000 macroconidia/ml) was determined and adjusted to the investigated concentration by counting under a microscope in a Fuchs-Rosenthal counting chamber. Each well had 5440 macroconidia; 5 or 10 µl treated EV suspension and PBS were added (Table 2). 96-well plates were pre-incubated on the lab bench for 20 h before they were put into a plate reader (CLARIOstar, BMG Labtech) for another 24-h incubation at 25 °C with 60 rpm shaking. Optical density at 600 nm (OD600) was measured every 20 min in a 5 × 5 square pattern in each well. To exclude microbial contamination from EV isolates and prevent misinterpretation of optical density, one control (C) well contained no macroconidia (C1) (Table 3). Hygromycin was added to inhibit microbial growth and allow changes in optical density to be attributed to fungal growth (C2). C3 contained no PBS but rather an additional 0.5 × PDB.

Table 1 Components of the digestive EV treatments for eliminating intravesicular and apoplastic co-purified proteins and RNAs

Group	TE sprayed barley/wt *A. thaliana*			dsCYP3RNA sprayed barley/*A. thaliana* CYP3RNA		
	1	2	3	1	2	3
EV solution	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
RNase	–	1.2 µl	1.2 µl	–	1.2 µl	1.2 µl
PK	–	3 µl	3 µl	–	3 µl	3 µl
Triton X	–	–	5.8 µl	–	–	5.8 µl
PBS	10 µl	5.8 µl	–	10 µl	5.8 µl	–
Total	60 µl	60 µl	60 µl	60 µl	60 µl	60 µl

Investigated concentrations: Proteinase K (20 ng/µl) (Thermo Fisher Scientific); RNase A (20 ng/µl) (Thermo Fisher Scientific); 10% Triton X-100 (Sigma)

Table 2 Well composition for microtiter well co-cultivation of differentially treated plant EVs with *F. graminearum*

Group	1 (µl)	1 (µl)	2 (µl)	2 (µl)	3 (µl)	3 (µl)
Investigated vol	5	10	5	10	5	10
F. graminearum	20	20	20	20	20	20
0.5 PDB	125	125	125	125	125	125
PBS	10	5	10	5	10	5
Total	160	160	160	160	160	160

Table 3 Overview of tested controls and well composition

Controls	C1 (µl)	C2 (µl)	C3 (µl)	C4 (µl)
F. graminearum		20	20	20
0.5 PDB	160	125	140	125
PBS				15
Hygromycin		15		
Total	160	160	160	160

Table 4 Components of the digestive EV treatments used to measure the effects of treatment reagents on fungal growth

Controls	C5 (µl)	C6 (µl)	C7 (µl)	C8 (µl)	C9 (µl)
PBS	55.8	50	57	58.8	54.2
RNase	1.2	1.2		1.2	
PK	3	3	3		
Triton X		5.8			5.8
Total	60	60	60	60	60

Investigated concentrations: Proteinase K (20 ng/µl) (Thermo Fisher Scientific); RNase A (20 ng/µl) (Thermo Fisher Scientific); 10% Triton X-100 (Sigma)

C4 contained no EVs. C3 and C4 were used to estimate the effect of the PBS on the optical density and growth behaviour of *F. graminearum*. As a reference for different EV treatments during the co-culture assay, the effects of EV treatment detergent were determined by incubating EV-free PBS with PK + RA (C5), TX + PK + RA (C6), PK (C7), RA (C8) and TX (C9) (Table 4). PBS was added to compensate for volume differences resulting from differences in the added volume of EV suspension. The co-cultivation was then performed according to the plant EV–*F. graminearum* cultivation method described in Table 5. Each experiment was performed in three wells and the means were taken for further analysis.

F. graminearum CYP51 gene silencing analysis

After 44 h of incubation, the cultures were transferred into new tubes for RNA extraction. The three technical replicates in the microtiter plate were merged to increase RNA outcome. 1 ml of GENEzol™ (geneaid) was added and extraction was performed according to the manufacturer's instructions. cDNA synthesis was performed using QuantiTect ReverseTranscription kit (Qiagen). SYBER Green JumpStart Taq ReadyMix (Sigma-Aldrich) was used for qRT-PCR analysis of *F. graminearum CYP51A* and *CYP51C* genes as previously described [25, 27] (for primer sequences see [27]; Supplemental Table S2). Transcript levels of *CYP51* genes were determined via the $2^{-\Delta\Delta Ct}$ method by normalizing the amount of target transcript to the amount of translation elongation factor 1α. ΔCt values were calculated from three technical replicates. $2^{-\Delta\Delta Ct}$ values were calculated using three biological replicates.

EV stability assay

PBS resuspended barley EVs were diluted 1:1 with PBS or PDB. The suspension was then carefully mixed by pipetting up and down several times and subdivided equally into three tubes. One tube of EVs mixed with PBS and one mixed with PDB were immediately frozen in liquid nitrogen. One tube per medium was incubated at 25 °C for 24 h and one for 48 h. Afterwards, samples were frozen in liquid nitrogen and stored at − 80 °C until

Table 5 Well composition for microtiter well co-cultivation of EV-free detergent reagents to estimate treatment-dependent effects

Controls	C5 (µl)	C5 (µl)	C6 (µl)	C6 (µl)	C7 (µl)	C7 (µl)	C8 (µl)	C8 (µl)	C9 (µl)	C9 (µl)
Invest. vol	5	10	5	10	5	10	5	10	5	10
F. graminearum	20	20	20	20	20	20	20	20	20	20
0.5 PDB	125	125	125	125	125	125	125	125	125	125
PBS	10	5	10	5	10	5	10	5	10	5
Total	160	160	160	160	160	160	160	160	160	160

nanoparticle tracking analysis (NTA) or transmission electron microscopy (TEM) were performed. For NTA measurements, samples were diluted at 1:20 with PBS and 200 µl were loaded into a Nanosight NS300 (Malvern Panalytical). Five measurements were performed at 25 °C and concentration prediction and size and statistical analyses were performed by the NTA 3.2 Dev Build 3.2.16 software. For TEM, copper formvar-coated 300-mesh electron microscopy grids were glow discharged before sample application for 40 s. Subsequently, 5 µl of each sample were applied onto its own grid. Samples were dabbed using Whatman filter paper and grids were washed three times in 50 µl of 2% uranyl acetate and once with distilled water. Excess staining or fixing solutions, buffers, and water were removed using Whatman paper between each step. Finally, the grids were air-dried. Preparations were inspected at 120 kV under zero-loss conditions (ZEISS EM912a/b) and images were recorded at slight underfocus using a cooled 2×2 k slow-scan CCD camera (SharpEye/TRS) and the iTEM software package (Olympus-SIS). At least ten meshes per grid were analyzed to avoid grid-to-grid variations.

To test if degradational processes of EVs are dependent on fungal exudates or extracellular enzymes, *F. graminearum* macroconidia were cultivated in PDB medium for 24 h. Fungal cells were depleted by centrifugation with 16,000×*g* at 4 °C for 10 min. 10 µl of fungal supernatant was added to 10 µl of barley EVs. Concentrations were determined by NTA after mixing fungal supernatant and barley EVs or after 2 h of incubation at 25 °C. Therefore, 180 µl of PBS was added for NTA measurements.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40694-022-00143-w.

Additional file 1: Figure S1. EVs were mixed with PBS and PDB and quick frozen at the beginning [0 h (h)], 24 h and 48 h after incubation at 25 °C. After fixing onto formvar-layered cupper meshes, samples were visualized with TEM. EVs were highlighted with red arrows.

Additional file 2: Figure S2. EVs were mixed with PBS and PDB and quick frozen at the beginning [0 h (h)], 24 h and 48 h after incubation at 25 °C. Samples were thawed on ice and diluted with PBS (1:20). The final volume of 200 µl was then loaded into the Nanosight NS300 (Malvern Panalytical) and five measurements were performed at room temperature. Mean values are arranged as a black line, standard deviation is given as the red plot.

Additional file 3: Figure S3. EVs were mixed with supernatant of 24-h-old *F. graminearum* culture and incubated at 25 °C. Particle concentration was determined by NTA measurements.

Additional file 4: Figure S4. 5 µl (light blue cross) and 10 µl (gray triangle) of purified EVs from control (wt) and CYP3RNA-expressing *A. thaliana* plants were added to *F. graminearum* liquid culture. Growth was determined by optical density measurements between 23 and 42 h post-inoculation (hpi) for cultures treated with EVs out of all three fractions.

Additional file 5: Figure S5. Purified *A. thaliana* EVs were treated with RNase A and proteinase K (yellow square) or Triton X-100, RNase A, and Proteinase K (green rhombus) after isolation and co-inoculated with *F. graminearum*. Additionally, untreated (orange circle) and EV-free PBS (brown cross) were co-inoculated as positive and negative controls.

Additional file 6: Figure S6. The relative fungal growth in the co-culture was calculated using the EV-free cultivation conditions with enzymes and detergent only as a baseline. Control (wt): circle; CYP3RNA-expressing plants: triangle (a-f). Selected timepoints were chosen for statistical analysis. Differences between wt or CYP3RNA-expressing *A. thaliana* plants were calculated for each investigated volume and EV pre-treatment using a two-tailed Student's t-test (p-value < 0.05). 5 µl EVs of wt plants: light green; 5 µl EVs of CYP3RNA-expressing *A. thaliana* plants: dark green; 10 µl EVs of wt plants: light brown; 10 µl EVs of CYP3RNA-expressing *A. thaliana* plants: dark brown (g).

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Author contributions
Conceptualization, A.K. and T.S.; Methodology, T.S., R.L., L.W., K.E. and D.B.; Software, T.S. and R.L.; Validation, A.K., T.S., and R.L.; Formal Analysis, T.S., R.L., L.W., K.E and D.B.; Investigation, T.S. and R.L.; Data Curation, T.S., R.L., L.W. and K.E.; Writing – Original Draft Preparation, A.K. and T.S.; Writing – Review & Editing, A.K.; Visualization, T.S., R.L., L.W. and K.E. Supervision, A.K.; Project Administration, A.K.; Funding Acquisition, A.K. All authors read and approved the final manuscript.

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Availability of data and materials
All relevant data is contained within the article. The original contributions presented in the study are included in the article material, further inquiries can be directed to the corresponding author.

Declarations

Competing interests
The authors declare no competing interests. The authors declare no competing financial interests.

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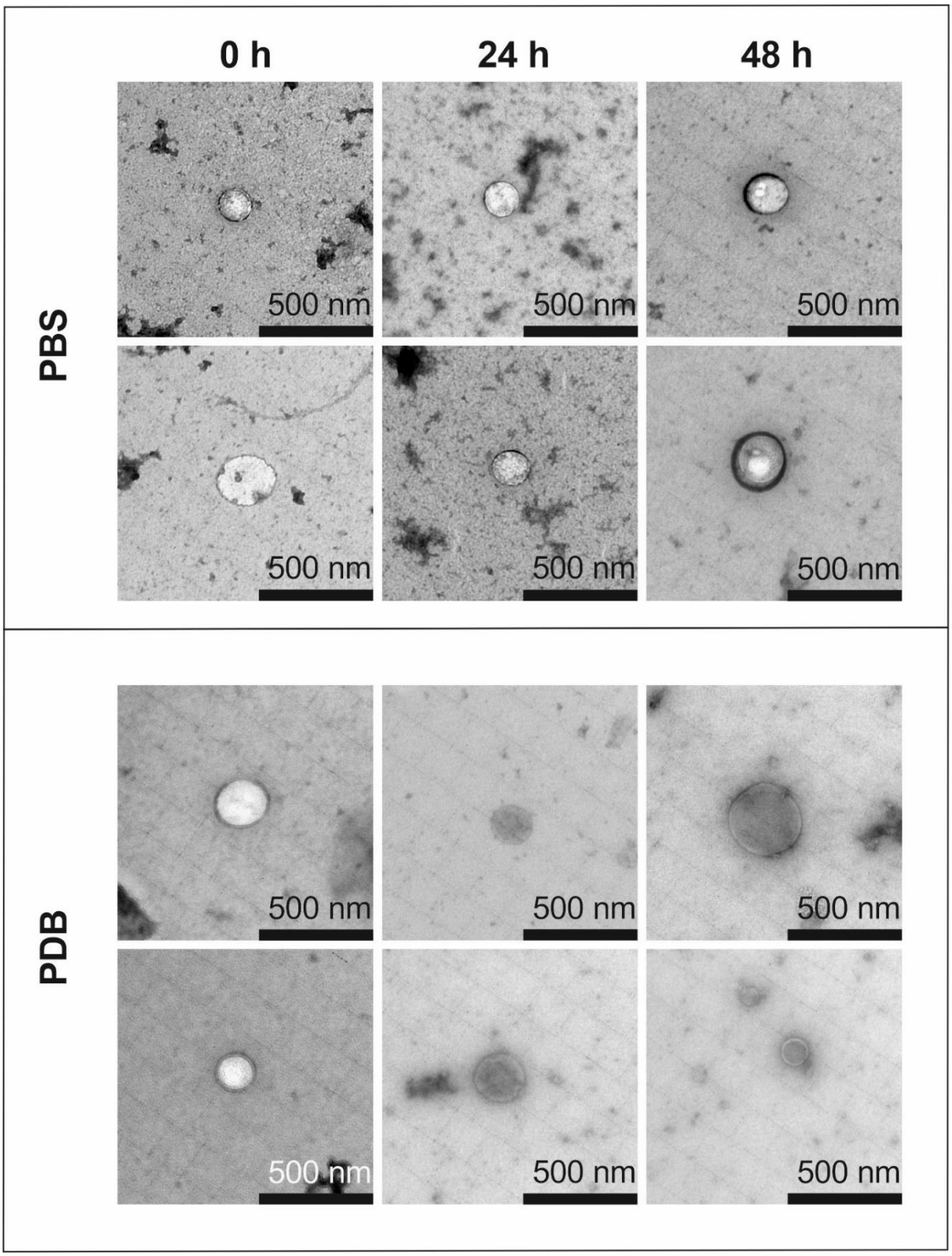

Figure S1.

EVs were mixed with PBS and PDB and quick frozen at the beginning [0 h (h)], 24 h and 48 h after incubation at 25 °C. After fixing onto formvar-layered cupper meshes, samples were visualized with TEM.

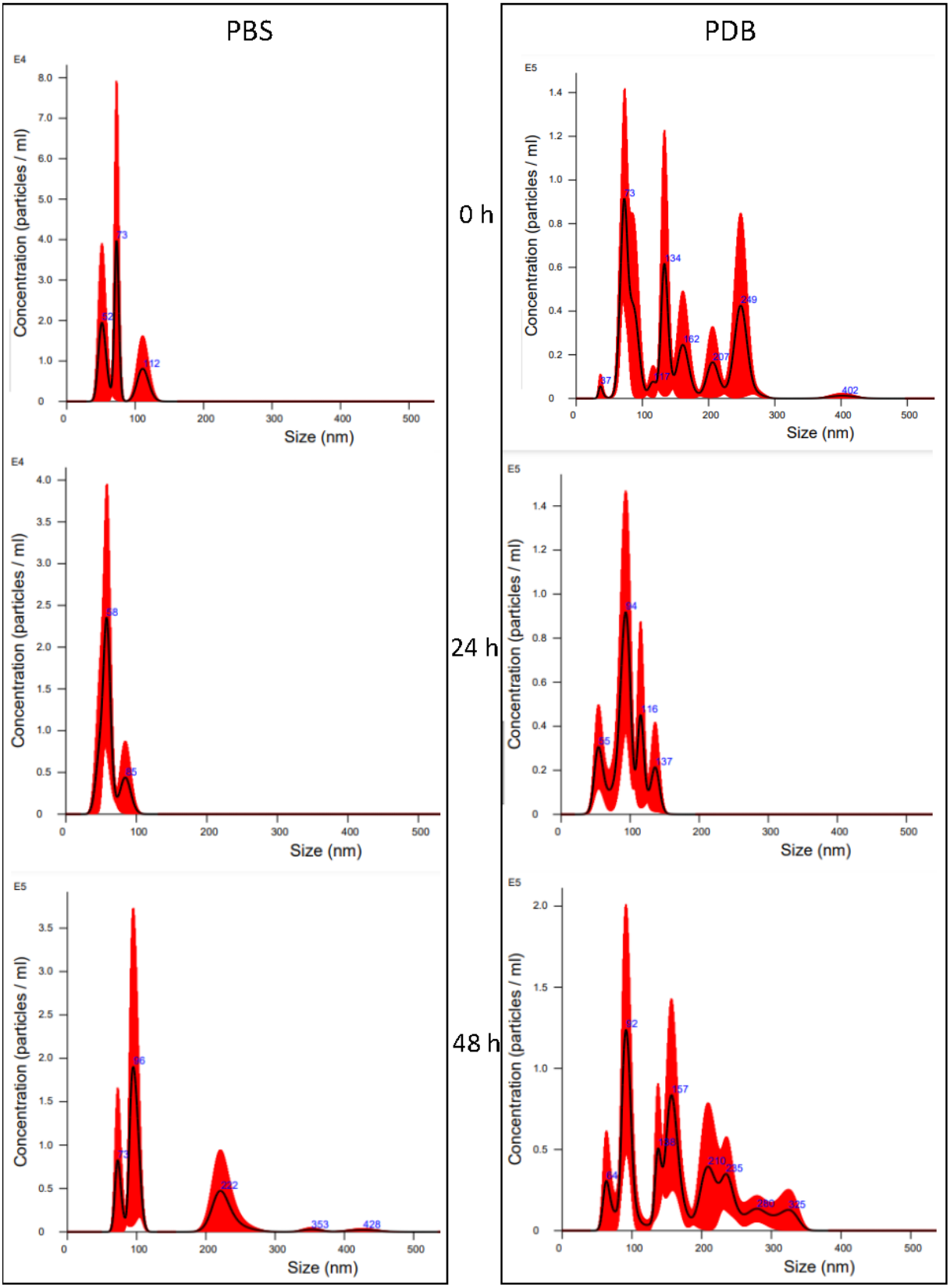
PBS
PDB
0 h
24 h
48 h
E4
E5
Concentration (particles / ml)
Size (nm)
73
82
112
134
249
162
207
187
402
68
85
84
116
55
137
96
73
232
353
428
92
157
188
210
235
64
289
325

Figure S2.

EVs were mixed with PBS and PDB and quick frozen at the beginning [0 h (h)], 24 h and 48 h after incubation at 25 °C. Samples were thawed on ice and diluted with PBS (1:20). The final volume of 200 µl was then loaded into the Nanosight NS300 (Malvern Panalytical) and five measurements were performed at room temperature. Mean values are arranged as a black line, standard deviation is given as the red plot.

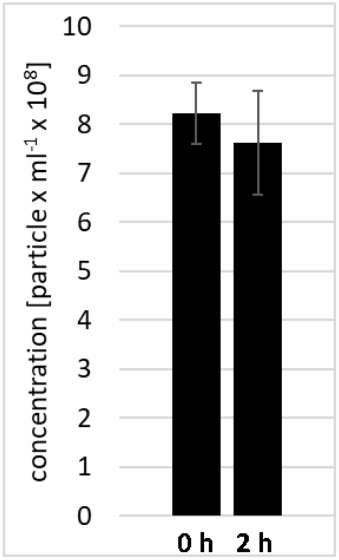

Figure S3.

EVs were mixed with supernatant of 24-h-old *F. graminearum* culture and incubated at 25 °C. Particle concentration was determined by NTA measurements.

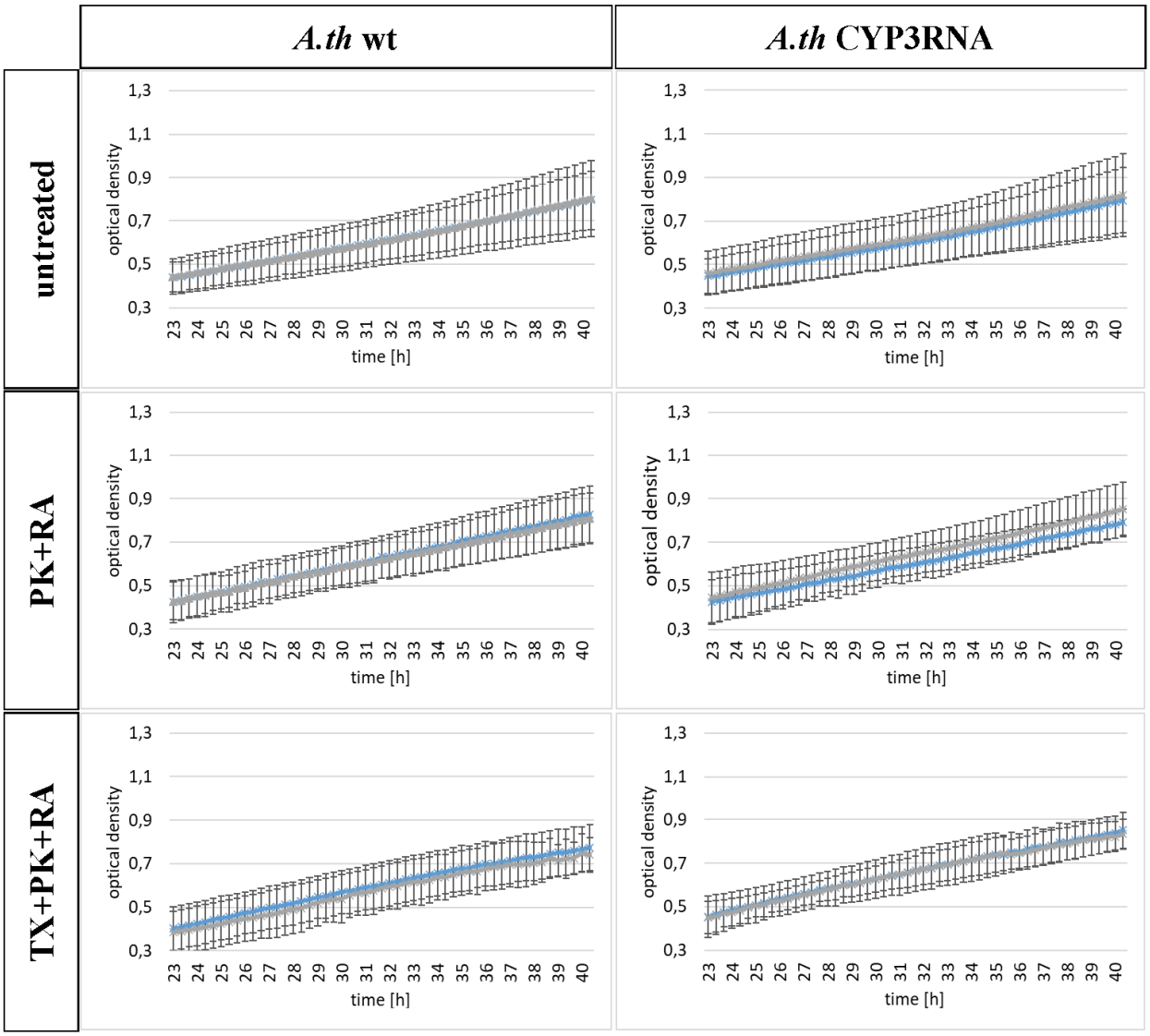

Figure S4.

5 µl (light blue cross) and 10 µl (gray triangle) of purified EVs from control (wt) and CYP3RNA-expressing *A. thaliana* plants were added to *F. graminearum* liquid culture. Growth was determined by optical density measurements between 23 and 42 h post-inoculation (hpi) for cultures treated with EVs out of all three fractions.

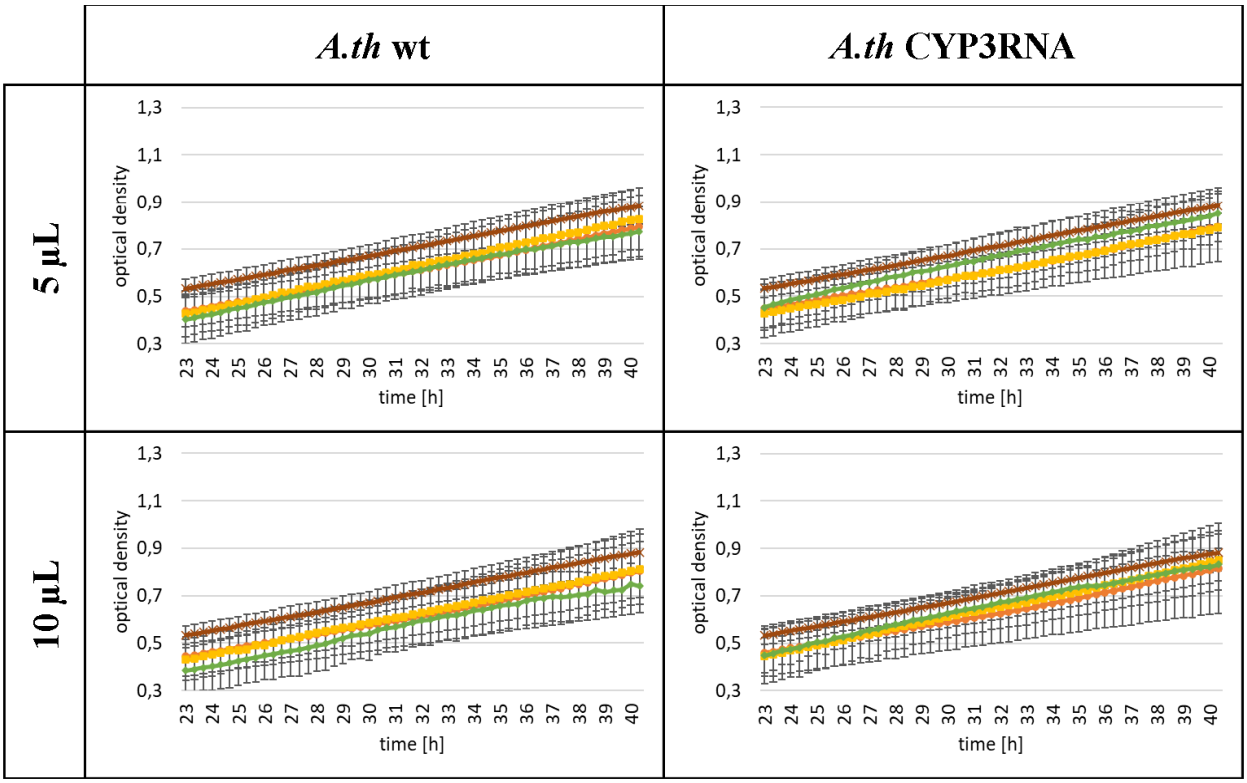

Figure S5.

Purified *A. thaliana* EVs were treated with RNase A and proteinase K (yellow square) or Triton X-100, RNase A, and Proteinase K (green rhombus) after isolation and co-inoculated with *F. graminearum*. Additionally, untreated (orange circle) and EV-free PBS (brown cross) were co-inoculated as positive and negative controls.

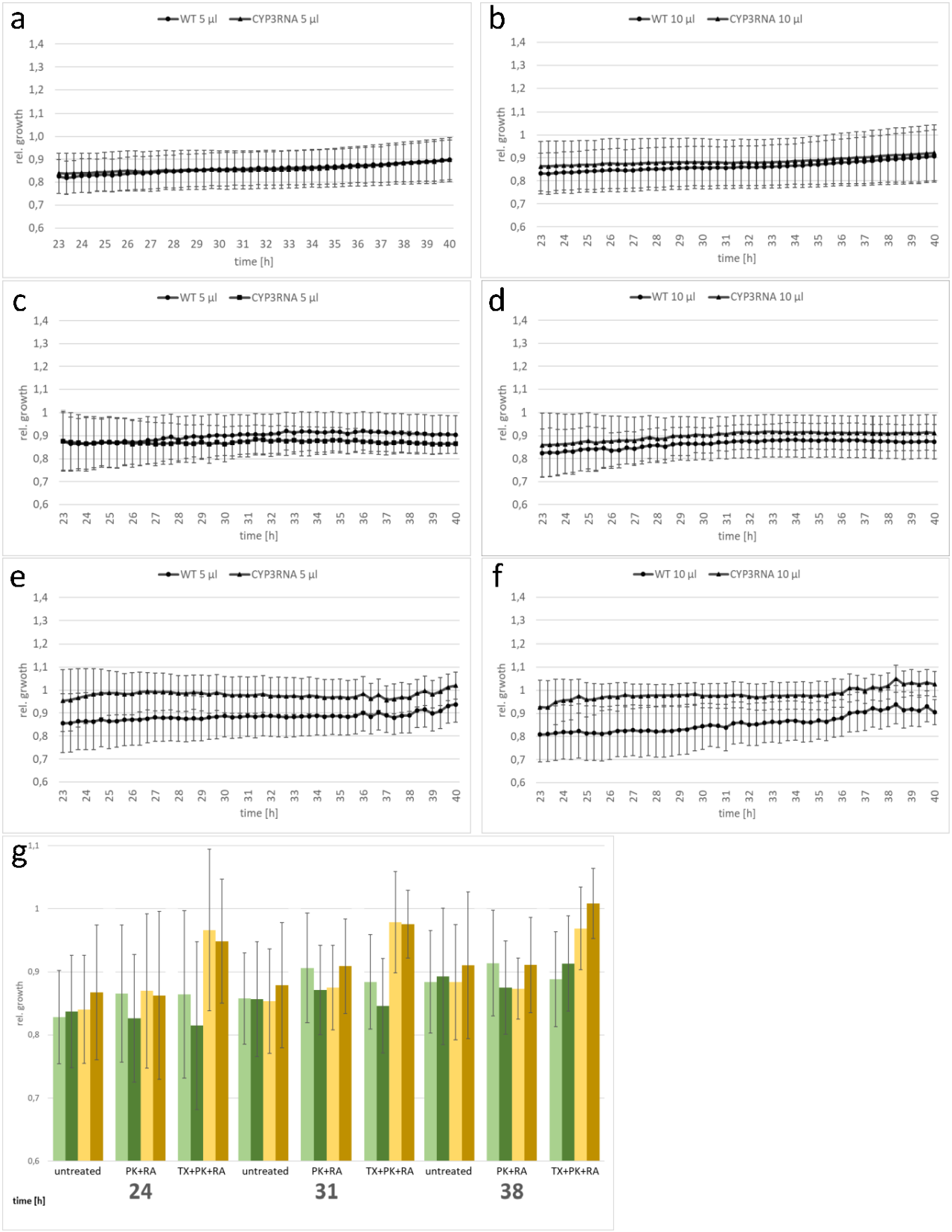
a
WT 5 µl
CYP3RNA 5 µl
rel. growth
time [h]
b
WT 10 µl
CYP3RNA 10 µl
rel. growth
time [h]
c
WT 5 µl
CYP3RNA 5 µl
rel. growth
time [h]
d
WT 10 µl
CYP3RNA 10 µl
rel. growth
time [h]
e
WT 5 µl
CYP3RNA 5 µl
rel. grwoth
time [h]
f
WT 10 µl
CYP3RNA 10 µl
rel. grwoth
time [h]
g
rel. growth
untreated
PK+RA
TX+PK+RA
24
31
38
time [h]

Figure S6.

The relative fungal growth in the co-culture was calculated using the EV-free cultivation conditions with enzymes and detergent only as a baseline. Control (wt): circle; CYP3RNA-expressing plants: triangle (a-f). Selected timepoints were chosen for statistical analysis. Differences between wt or CYP3RNA-expressing *A. thaliana* plants were calculated for each investigated volume and EV pre-treatment using a two-tailed Student's t-test (p-value < 0.05). 5 µl EVs of wt plants: light green; 5 µl EVs of CYP3RNA-expressing *A. thaliana* plants: dark green; 10 µl EVs of wt plants: light brown; 10 µl EVs of CYP3RNA-expressing *A. thaliana* plants: dark brown (g).

Danksagung

Unbezahlbar ist die Hand, die hilft, wenn man sie braucht.

Gleich vorweg möchte ich erwähnen, dass die Reihenfolge der Erwähnung in dieser Danksagung die Bedeutsamkeit für diese Arbeit nicht widerspiegelt. So gebe ich mich nicht der Illusion hin, dass diese Arbeit auch nur ohne eine, der hier erwähnten Personen in dieser Form zum Abschluss gekommen wäre.

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List of Publications

2020

- **Schlemmer, T., Lischka, R., & Koch, A**. (2020). Elucidating the role of extracellular vesicles in the Barley-Fusarium interaction. *Trillium Exctracellular Vesicles*, *2*(1), 28–35. https://doi.org/10.47184/tev.2020.01.03

2021

- **Schlemmer, T.*, Barth, P.*, Weipert, L., Preußer, C., Hardt, M., Möbus, A., Busche, T., & Koch, A**. (2021). Isolation and characterization of barley (*Hordeum vulgare*) extracellular vesicles to assess their role in RNA spray-based crop protection. *International Journal of Molecular Sciences*, *22*(13). https://doi.org/10.3390/ijms22137212

*these authors contributed equally to this work

2022

- **Schlemmer, T., Lischka, R., Wegner, L., Ehlers, K., Biedenkopf, D., & Koch, A.** (2022). Extracellular vesicles isolated from dsRNA-sprayed barley plants exhibit no growth inhibition or gene silencing in *Fusarium graminearum*. *Fungal Biology and Biotechnology*, *9*(1), 14. https://doi.org/10.1186/s40694-022-00143-w

Curriculum Vitae

Personal Data:

University Education: *2018 – 2022*

Ph.D thesis

RNAi-mediated plant protection: Identification and characterization of the molecular components of the HIGS and SIGS pathways

Institute of Phytomedicine, University Hohenheim, Stuttgart
Prof. Dr. Ralf Vögele

Institute of Phytopathology, Justus Liebig University, Gießen
Prof. Dr. Karl-Heinz Kogel

Master of Science (M.Sc.) Crop Science, Justus Liebig University, Gießen

2016 - 2018

Master Thesis

Charakterisierung der Mais Plasmamembran-H^+-ATPase (Isoform MHA4) nach heterologer Expression des Enzyms in Hefe

Institute of Plant Nutrition, Justus-Liebig-University, Gießen
Prof. Dr. Sven Schubert

2013 – 2016	Bachelor of Science (B.Sc.) Biology, Johann Wolfgang Goethe University, Frankfurt am Main
	Bachelor Thesis
	Aufreinigung und funktionelle Analysen der Retraktions-ATPasen der Typ-IV-Pili aus Thermus thermophilus
	Department of Molecular Microbiology and Bioenergetics, Johann Wolfgang Goethe University, Frankfurt am Main Prof. Dr. Beate Averhoff

Experience

2018-2022	International Giessen Graduate School for the Life Science (GGL)
2018-2021	Research Training Group 2355: Regulatory networks in the mRNA life cycle: from coding to noncoding RNAs

Teaching activities

Seminar supervision of the Bachelor Kernmodul BK25 Phytomedizin in WS 2018/2019 and WS 2019/2020, Justus-Liebig-University

Organization and leading of the masters' laboratory course Molecular Phytopathology (3601-410) in WS 2021/2022 and SS 2022

Supervision of students

Internships:

2019	Marina Grölz, Justus-Liebig-University

Humboldt Reloaded Program

2022	Vanessa Breckner, University Hohenheim

Forschungsprojekt:

2022	Lena-Marie Röhm, University Hohenheim
	Charlotte Nothelfer, University Hohenheim

Agrarbiologisches Projekt:

2020	Bastian Groß, University Hohenheim
2021	Yannick Ditton, University Hohenheim
2022	Paul Hanak, University Hohenheim

Bachelor Thesis‘	
2019/2020	Richard Lischka, Justus-Liebig-University
2021/2022	Julia Stauch, University Hohenheim
Master Thesis’	
2019/2020	Viviana Wotke, Justus-Liebig-University
2021/2022	Andreas Zimmermann, University Hohenheim

Conferences and Meetings

11-2018	RTG 2355, Retreat, Rauischholzhausen Talk: ***“Towards an RNAi-based control of plant diseases: Research into its mechanistic basis”***
09-2019	GGL, Annual Conference, Gießen Poster: **“*RNAi mediated plant protection: Identification of the molecular components of the HIGS pathway”***
09-2019	Global Conference on Plant Science and Molecular Biology, London **Poster: RNAi-mediated plant protection: Unraveling the molecular mechanisms underlying the Host-induced gene silencing technology**
11-2019	RTG 2355, Retreat, Rauischholzhausen Poster: “The ESCRT-III pathway in *Arabidopsis thaliana* regulates HIGS-mediated resistance against *Fusarium graminearum”*
03-2021	Deutsche Phytomedizinische Gesellschaft, Annual Conference of the Working Groups Mykology and Host-Parasite-Interactions Talk: ***Plant extracellular vesicles and their role in RNA-interference mediated plant protection***
09-2021	Deutsche Pflanzenschutztagung Talk: ***Plant extracellular vesicles and their role in RNA-interference mediated plant protection***
03-2022	Deutsche Phytomedizinische Gesellschaft, Annual Conference of the Working Groups Mykology and Host-Parasite-Interactions Talk: ***Identification and characterization of sRNA profiles derived from transgenically-expressed and exogenously applied dsRNA precursors***

Courses

05-2019	"Project and time management for first year GGL members" – Dr. Dirk Palm
05-2019	"Reference managers – Make your choice" – Florian Ruckelshausen
06-2019	„Publikation von Forschungsdaten im Zusammenhang mit wissenschaftlichen Artikeln" – Andreas Hübner
06-2019	„GGL abstract writing lab" – Laurence Henry
07-2019	"Exploring the company world" Part 1 and Part 2 – Dr. Gaby Schilling
09.2019	"Scientific writing course" by the RTG 2355 – Dr. Andrew Moore
10-2019	"Next generation multiplex confocal imaging with the new Zeiss LSM9 family" – Members of the personnel
11-2019	"Incomes for life science students" – Constanze Krätsch
02-2020	"Methods for protein analysis"- Prof. Dr. Günter Lochnit
03-2020	"BWL für Nicht-BWLer" – Gießen Graduate Centre for the Life Science
06-2020	"Processing and Analysis of scientific images" – Dr. Jan Brochner
02-2021	"Writing papers and theses in life science" – Dr. Martin Wild
03-2021	"Berufliche Strategieentwicklung" – Anja Kienitz
03-2021	"Biotech quality manager and regulatory affairs manager" – Christian Grote-Westrick
04-2021	"Conflict and negotiation management" – Dr. Dirk Palm
05-2021	"From Ph.D. to innovator" – Rebecca Peterson-Perry
06-2021	"Projektplanung" – Dr. Anja Vervoorts
01-2022	"Rapid and high-sensitivity analyses of cellular translation by ribosome profiling" – Dr. Jan Medenbach