UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics

How to tame a parasite – Developing biotechnological pipelines for gene function studies in *Cuscuta*

Lena Anna-Maria Lachner

A dissertation for the degree of Philosophiae Doctor

March 2022

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Cover image: *Cuscuta campestris* flowering on a *Pelargonium zonale* host in Klimalab Holt, Tromsø.

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Acknowledgements

I would like to thank everybody who has done their part in helping me to make this thesis possible. Special thanks to my supervisor Kirsten Krause for her enthusiasm, uncountable creative ideas and her fierce faith in *Cucuta's* claim to fame, as well as her faith in me. Thank you for always having an open door, taking the time to help, and encouraging me to see my "failures" less critical and more positive. Thank you for your support and supervision!

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Summary

Cuscuta is a genus of parasitic plants that form cross-species bridges and connect to the xylem and phloem of a wide variety of host plants. Because of their unique lifestyle, research into processes like the formation of connections in between plant cells and plant immunity would benefit from *Cuscuta* as a model system. However, a method to study gene functions in a *Cuscuta* species is still missing. Its development was therefore chosen as the goal of this thesis.

In vitro culture techniques for *Cuscuta campestris* were developed and *Agrobacterium* transformation of *Cuscuta* tissue was successfully performed in two different systems, the adhesive disk area close to the parasitic infection organ, and seedlings of *C. campestris*. As an alternative method for gene function studies in *Cuscuta*, virus induced gene silencing was tested in *C. campestris*. Mineral transport across the host-parasite border was studied and the potential use of interspecies plasmodesmata between *Cuscuta* and its hosts was investigated.

The results presented in this thesis allow for first gene function studies in *Cuscuta* and are good foundations for the development of pipelines for transgenic plant production and gene silencing techniques. Challenges and opportunities with this system are outlined. The conclusions of this thesis will help to “tame” *Cuscuta* and use its unique physique and strategies for host plant infection to establish a model species for studying plant-cell connections, transport processes and plant immunity.

List of Papers

Paper 1

A highly efficient protocol for transforming *Cuscuta reflexa* based on artificially induced infection sites

Lena Anna-Maria Lachner, Levon Galstyan, Kirsten Krause (2020)

Plant Direct, DOI: 10.1002/pld3.254

Paper 2

Cuscuta campestris* tissue culture and seedling transformation via *Agrobacterium rhizogenes

Lena Anna-Maria Lachner, Zahra Zangishei, Kirsten Krause

Manuscript Research paper prepared for submission to Plant Direct

Paper 3

Selective mineral transport barriers at *Cuscuta*-host infection sites

Frank Förste, Ioanna Mantouvalou, Birgit Kanngießer, Hagen Stosnach, Lena Anna-Maria Lachner, Karsten Fischer and Kirsten Krause (2019)

Physiologia Plantarum, DOI: 10.1111/ppl.13035

Paper 4

The enigma of interspecific plasmodesmata: insight from parasitic plants

Karsten Fischer, Lena Anna-Maria Lachner, Stian Olsen, Maria Mulisch and Kirsten Krause (2021)

Frontiers in Plant Science, DOI: 10.3389/fpls.2021.641924

Abbreviations

AGP	Arabinogalactan protein
CFDA	5-Carboxyfluorescein di-acetate
dsRed	Discosoma Red (Red fluorescent protein)
dsRNA	double-stranded ribonucleic acid
FIB-SEM	Focused ion beam scanning electron microscopy
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GOI	Gene of interest
HGT	Horizontal gene transfer
iPD	Interspecies plasmodesmata
MCS	multiple cloning site
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
qPCR	quantitative polymerase chain reaction
PD	Plasmodesmata
PDS	Phytoene desaturase
PTGS	Post-transcriptional gene silencing
RNAi	Ribonucleic acid interference
RISC	RNA-induced silencing complex
siRNA	Small interfering ribonucleic acid
ssRNA	Single-stranded ribonucleic acid
TRV	Tobacco rattle virus
TEM	Transmission electron microscopy
Ti-plasmid	Tumor inducing plasmid
UTR	Untranslated region
VIGS	Virus induced gene silencing
XRF	X-ray fluorescence spectroscopy
XTH	Xyloglucan endotransglucosylase/hydrolase

Explanatory remarks

Up front, a few explanations of the used terminology and some words, whose meaning might not be completely clear:

The term “gene function studies” is used in this thesis though discussions around this term exist, as it is argued that it is not really the “gene function” but rather the “protein function” that is evaluated in those studies.

The expressions symplasm/symplasmic and apoplasm/apoplasmic are used for transport matters as suggested by Erickson (1986), who recommended to reserve the word symplastic/ apoplastic for “growth deformation” in contrast to symplasmic/apoplasmic for “transport processes”.

In this work, “*Agrobacterium rhizogenes”* is used. The same bacterium is sometimes also referred to as “*Rhizobium rhizogenes”* in other literature.

The expressions “plant tissue culture” and “plant *in vitro* culture” are both used concurrently for plant tissue that is grown under sterile conditions on nutrient medium and influenced by hormones in its development.

Part 1

Thesis

1 Introduction

1.1 Parasitism in plants

Parasitic plants differ in many aspects from photoautotrophic plants. While the latter have developed photosynthesis to convert sunlight to chemical energy and use their roots to take up water and minerals from the soil, parasitic plants have turned their attention on exploitation of their photoautotrophic relatives. Well-known examples for parasitic plants are dodders (*Cuscuta*), broomrapes (*Orobanche*) and witchweed (*Striga*). Figure 1 gives an overview over the variety of appearances of parasitic plants.

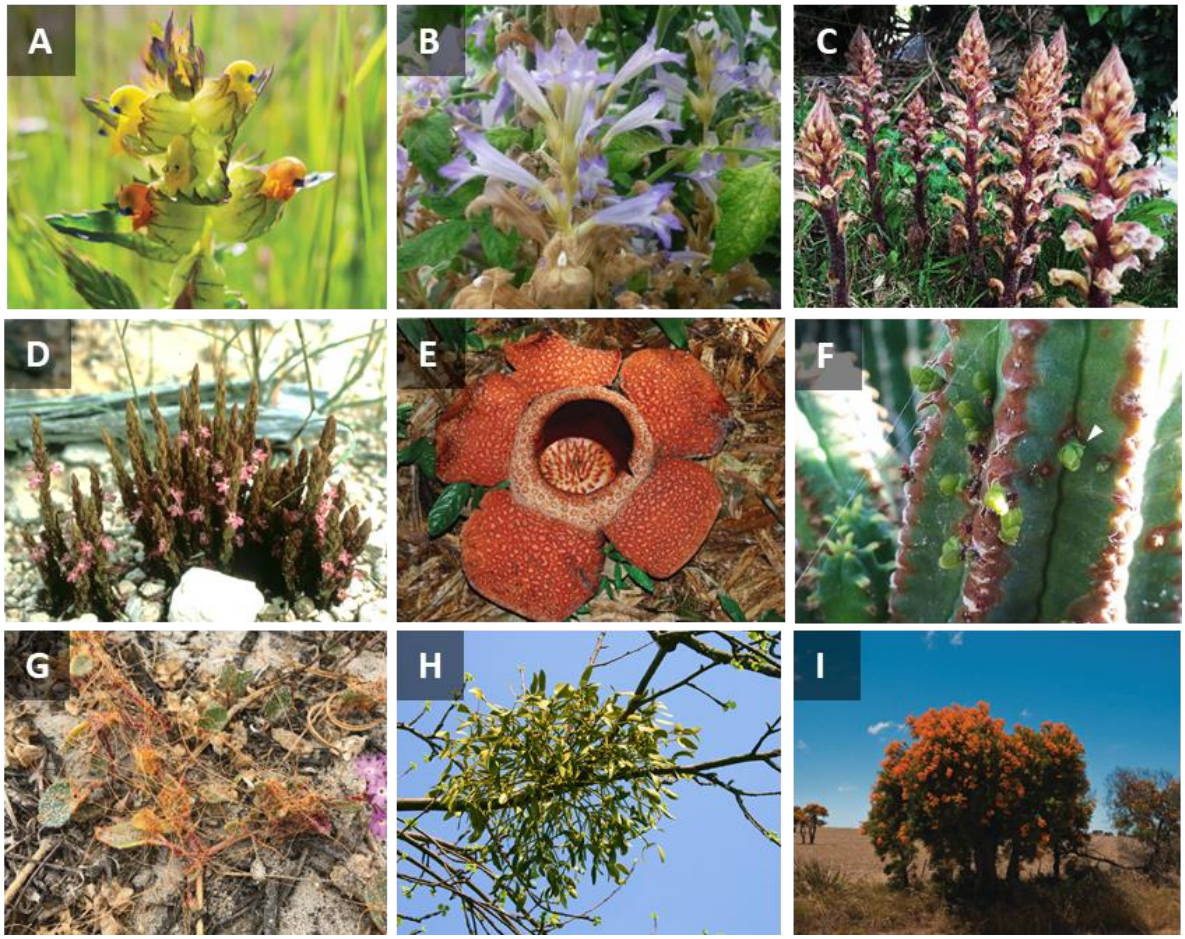

Figure 1: Examples for the variety of parasitic plants. Typical views of the parasites' parts that grow on the surface. Yellow rattle (Rhinanthus minor) (A), Orobanche aegyptiaca (B), Orobanche hederae (C), Striga gesneroides (D), Rafflesia (E), Viscum minimum (indicated by a white arrowhead) (F), Cuscuta salina (G), Viscum album (H), Australian mistletoe (I). Some images modified from Twyford (2018) (A, C, E, G, I), some images (B, D, F, H) taken by Kirsten Krause.

Parasitic plants tap other plants' nutrient streams via the haustorium, an organ that grows into the host tissue and creates an infection site (Fig. 2) serving as a feeding connection between the two plants (Clarke et al., 2019). The term for this organ "*haustorium*" is derived from the Latin word "*haurire*", which means to draw out, drink or swallow.

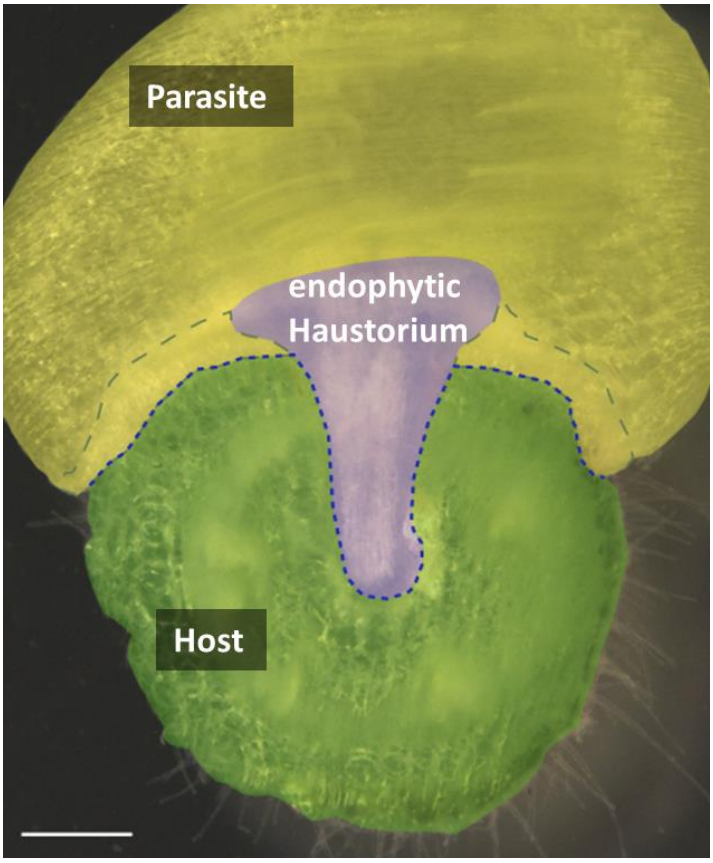

Figure 2: The haustorium of a parasitic plant. Cross-section of an infection site of the parasitic plant Cuscuta reflexa (top, yellow) on the host Solanum pennellii (bottom, green). The endophytic part of the haustorium (purple) has grown into the host tissue. The attachment ring (green dashed line) is sometimes also counted as part of the haustorium. The dark blue line marks the border between parasite and host. Scale bar = 500 µm

For the evolutionary origin of haustoria in parasitic plants there are two theories. They suggest that either the genes involved in haustoria formation have an exogenous origin and were introduced via horizontal gene transfer or pathogen infection, or genes related to haustoria formation fulfilled other tasks in the plant before several mutations resulted in altered functions used for parasitic purposes (Clarke et al., 2019).

1.1.1 The impact of parasitic plants

Parasitic plants are generally important players in their natural environments, influencing competition and biodiversity, as well as nutrient distribution (Těšitel, 2016). They play key roles in defining their ecosystem and have a major effect on population dynamics of plants and even mammals, birds and insects in the system (Press & Phoenix, 2005). Performance of non-host species can be enhanced by suppression of host plants and they influence above ground as well as below ground processes by regulating the biomass (Bardgett et al., 2006). Thus parasitic plants can even be used to control the spread of invasive species (Yu et al., 2008). However, some of them can also present a serious problem if introduced in agriculture. The problematic parasites found to disturb agriculture are foremost *Cuscuta* (devil's thread, dodder), *Orobanche* (broomrape) and *Striga*, also known as witchweed (Nickrent, 2020). Plant parasites successfully spread around the world, contained in crop seeds. Once attached to their hosts, separating them is difficult to impossible and often the only chance of getting rid of the parasite is to destroy the crops along with it (Fernández-Aparicio et al., 2020). Common crop plants like tomato, alfalfa, coffee, tea and others belong to their host spectrum, and an infection of fields with parasitic plants results in heavy losses in yield or might even ruin the complete harvest. This problem is predicted to affect especially African countries in an increased manner in the near future, as *Cuscuta* species are on the rise there (Kagezi et al., 2021; Masanga et al., 2021; Fig 3). In contrast to other plant pathogens, parasitic plants have long been neglected in research, and thus knowledge on how interaction with their host plants is regulated still needs to be revealed. Recent availability of transcriptomic and genomic data is speeding up the rate of discovery (Clarke et al., 2019).

Figure 3: Cuscuta species threatening to invade crop fields. C. reflexa infecting a coffee plant with infection sites marked by red arrowheads (A). A windbreaker bush in a tea plantation infected by Cuscuta reflexa with vines threatening to infect the plantation (white arrowheads) (B). C. reflexa infecting a tea plant with infection sites marked by red arrowheads (C). Images modified from Masanga et al (2021)

1.1.2 Parasitic plant classification

Within the range of parasitic plant species, a variety of different grades and specializations can be distinguished and used for classification. One differentiation criterion is the type of connection the parasite establishes with its host plant. While all parasitic plants connect to the xylem stream of their hosts, only some of them also tap the host phloem (Hibberd & Dieter Jeschke, 2001). According to these features, parasitic plants are distinguished as xylem feeders and phloem feeders (Fernández-Aparicio et al., 2020; Irving & Cameron, 2009). As phloem is living tissue in contrast to xylem, forming a connection to phloem is a bigger challenge for the parasite. On the other hand, the reward of being able to not only acquire water and minerals, but also sufficient nutrients from the host is apparently a worthwhile one. Another method of classification is by the ability of the parasitic plant to photosynthesize. Hemiparasites are able to photosynthesize and produce their own sugars, while holoparasites have completely lost the ability of photosynthetic carbon fixation (Clarke et al., 2019; Fernández-Aparicio et al., 2020; Fig. 4). It seems advantageous for parasitic plants to uphold at least a low, rudimentary ability to photosynthesize, as this provides resources for their sexual reproduction (Těšitel, 2016). Efficient photosynthesis is mandatory for xylem feeders, as they withdraw only a small amount of carbon but can satisfy their needs for water and minerals via the host xylem (Bell et al., 2011; Těšitel et al., 2010). There are facultative and obligate hemiparasites – those that opportunistically live with a host but can theoretically live without it and those that cannot complete their life cycle on their own (Clarke et al., 2019). A third criterion for classifying parasitic plants is the location of the haustoria on the host, which splits them in stem or root parasites depending on where they parasitize their host (Nickrent, 2020; Fig. 4). Parasitic plants, especially holoparasites, are no longer dependent on photosynthesis, thus they can appear quite different from photoautotrophic plants, with reduced or absent leaves and roots (*Cuscuta*), or even completely living underground (*Rhizantella*) or within another plant (*Rafflesia*) during most of their life cycle (Clarke et al., 2019; Fig. 4). The success of parasitic lifestyle among plants is obvious since according to the current knowledge, parasitism has evolved 12 times independently in different orders of the angiosperm lineage and parasites comprise 2.2% of the genera and 1.6% of the species of angiosperms. It seems that once parasitism is achieved, reverting to photoautotrophy does not occur in nature – also, hemiparasites seem to evolutionally be pushed towards holoparasitism (Nickrent, 2020).

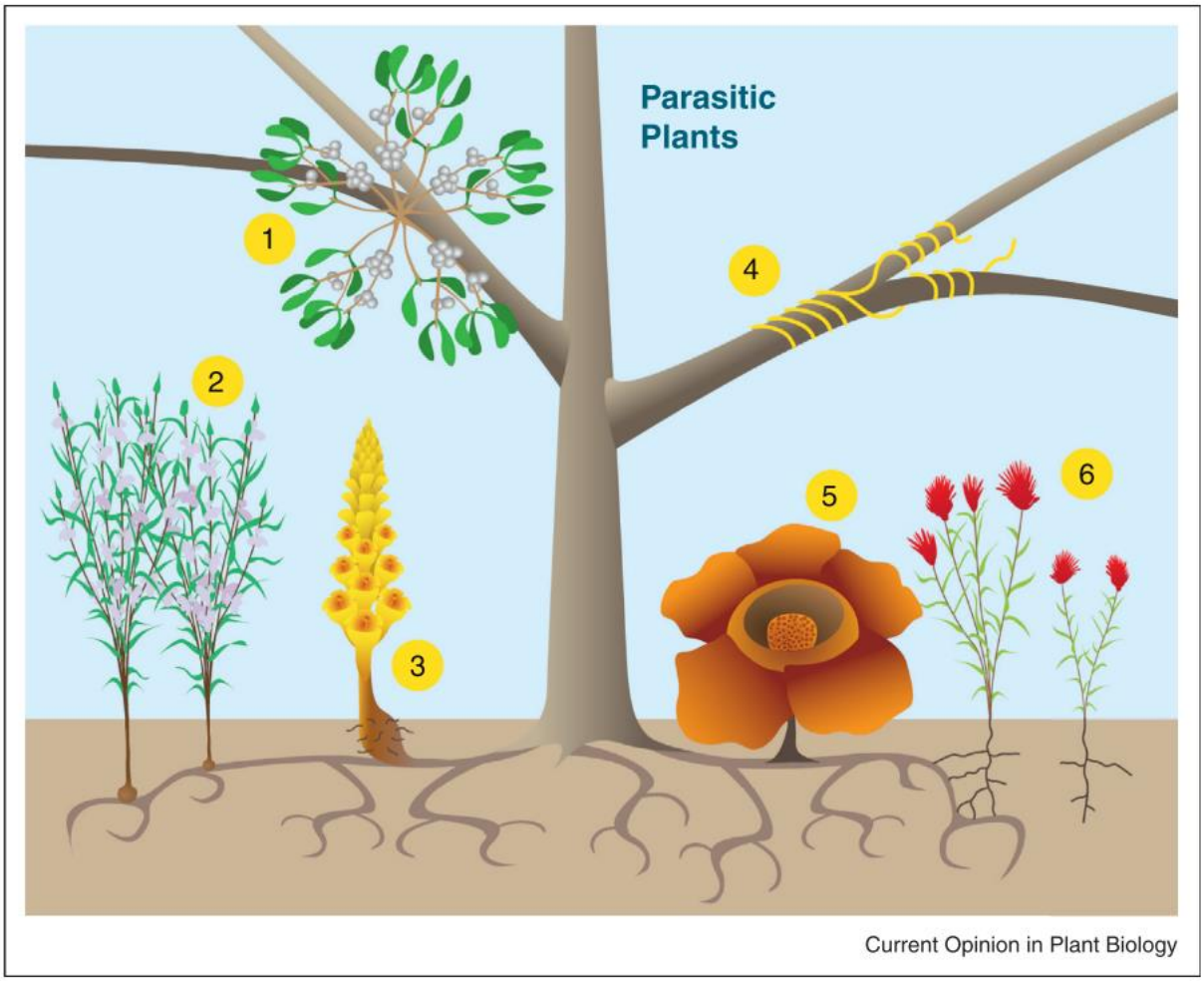

Figure 4: Overview over parasitic plants. Different locations on a plant to attach for parasitic plants are either shoots (1,4) or roots (2, 3, 5, 6). Mistletoe (Viscum) (1), witchweeds (Striga) (2) and figworts (Scrophularia) (6) are hemiparasites, while broomrapes (Orobanche) (3), dodders (Cuscuta) (4) and corpse flowers (Rafflesia) (5) do not have leaves and are generally regarded as holoparasites. Figure from Smith et al., (2013)

1.2 *Cuscuta*

1.2.1 Classification and life cycle of *Cuscuta*

The parasitic genus *Cuscuta* grows all over the world and consists of about 200 different species grouped in 3 subgroups: *Cuscuta*, *Grammica* and *Monogynella* (Costea et al., 2015; Fig. 5). Only some 15-20 species, like, for example, *Cuscuta campestris*, are serious threats to crops and agriculture (Costea & Tardif, 2006; Nickrent, 2020) , while others might be endangered or even threatened with extinction (Costea & Stefanović, 2009).

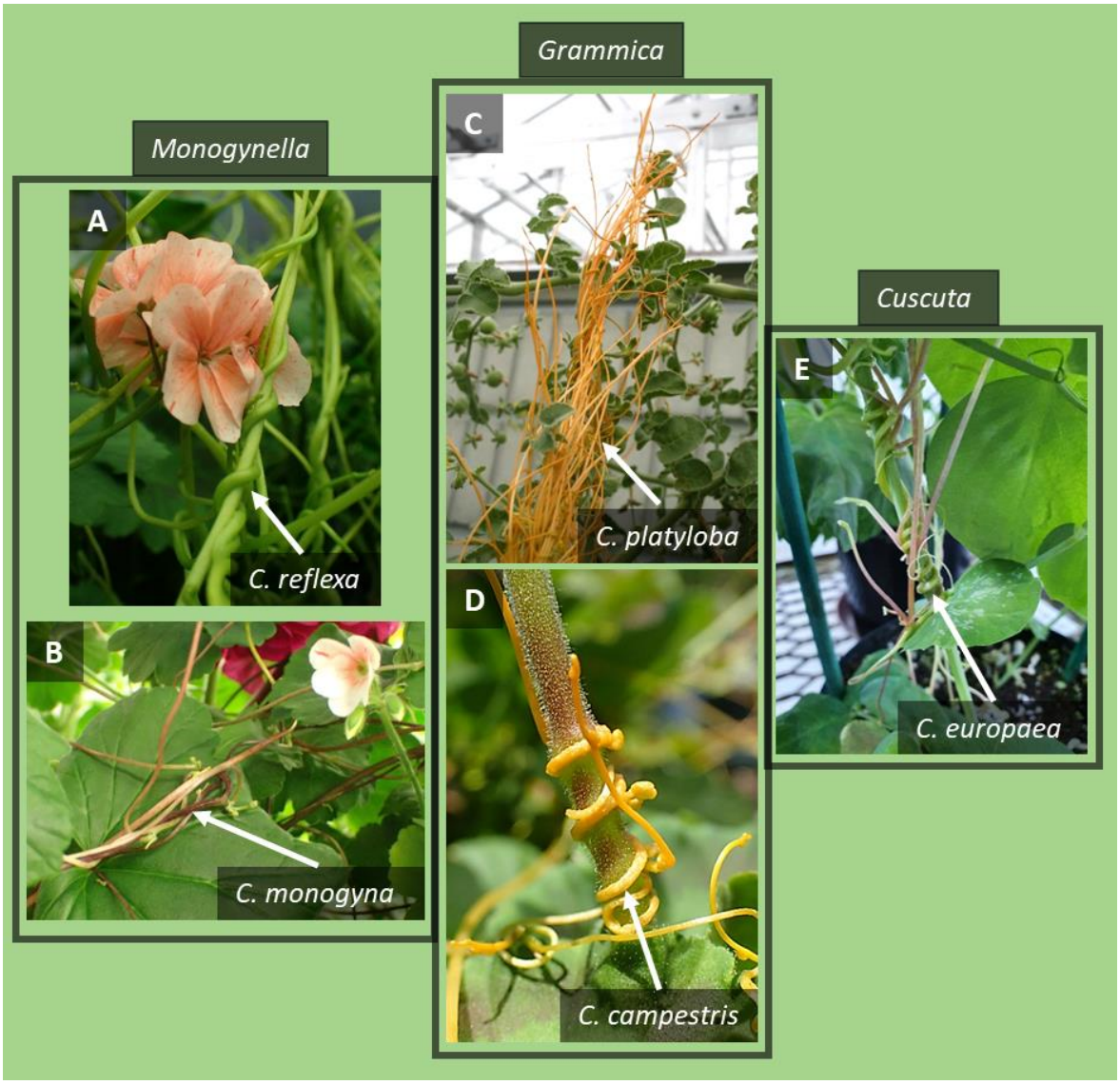

Figure 5: Overview over different Cuscuta species. C. reflexa (green) and C. monogyna (purple) parasitizing on Pelargonium zonale with flowers from the host (A, B); C. platyloba (orange) on Solanum pennellii (C); C. campestris on P. zonale (D); C. europea on Pisum sativum (E). C. reflexa and C. monogyna belong to the subgroup Monogynella, while C. campestris and C. platyloba belong to the subgroup Grammica and C. europaea to the subgroup Cuscuta.

Cuscuta is a phloem feeder and stem parasite that wraps around its host as a vine and connects to xylem and phloem. These plants are members of the family *Convolvulaceae* (García et al., 2014), all of which are vines. However, *Cuscuta* species are completely missing roots, and leaves are reduced to few small scales. Photosynthesis rates seem to vary greatly between *Cuscuta* species, with some completely lacking chlorophyll a and b as well as thylakoids, while others show low rates of photosynthesis (Machado & Zetsche, 1990; van der Kooij et al., 2000). This means that while the whole genus *Cuscuta* is often described as (functionally) holoparasitic because of their sugar uptake from the host, some *Cuscuta* species still possess the ability to photosynthesize, what is thought to be important for production of lipids for seeds (Nickrent, 2020; Těšitel, 2016). The lack of chlorophyll becomes apparent in the color of the plant, as many *Cuscuta* species are not green but rather exhibit an orange, purple, white, or very light green color, depending on the presence of other pigments (Fig. 5).

When *Cuscuta* seedlings germinate, they first form a root-like organ that degenerates after a few days (Costea & Tardif, 2006; Sherman et al., 2008). It is crucial for the seedlings to attach to a host as soon as possible, as they cannot survive on their own for more than a couple of days (Dawson et al., 1994). *Cuscuta* seedlings follow light cues and tactile stimuli to find a host and both are also crucial signals in the development of the haustorium (Furuhashi et al., 2011; Tada et al., 1996; Fig. 6). When attaching to a host, the *Cuscuta* seedling wraps around it in a counterclockwise manner (Costea & Tardif, 2006; Dawson et al., 1994). *Cuscuta* is a fast-growing plant that can branch many times and that has been reported to grow up to 8 cm in 24 hours and to a total length of almost 750 m in certain species (Costea & Tardif, 2006). It can reach far and enclose whole trees and meadows in net-like covers. It reproduces vegetatively and is known to often, but not always, flower simultaneously with its host (Shen et al., 2020). *Cuscuta* seeds typically enter a dormant stage by drying out after being shed and need to go through scarification before regaining the ability to germinate (Hutchison & Ashton, 1980; Hutchison & Ashton, 1979).

1.2.2 The *Cuscuta* haustorium

After wrapping around its host, *Cuscuta* starts to develop its infection organs induced by tactile and light stimuli (Tada et al., 1996). The process of haustoria formation can be seen as a local swelling of the *Cuscuta* stem that occurs at the respective site. During host penetration, the infection organ invades the host plant tissue using a combination of mechanical pressure, enzymatic digestion, and an adhesive substance (Johnsen et al., 2015; Kaiser et al., 2015; Vaughn, 2002). After penetration, the haustorium develops its own xylem and phloem, although both seem to be slightly different from regular vascular tissue in that their cells remain in an immature state (Israel et al., 1980; Shimizu & Aoki, 2018; Fig. 6). Starting from the haustorium, long, fingerlike cells called the searching hyphae grow into the host tissue (Vaughn, 2003). In the first stage, those searching hyphae grow in between host cells. Upon contact with one of the two types of vasculature, xylem or phloem, they start a differentiation process into xylic or phloic transfer hyphae (Kaga et al., 2020; Vaughn, 2006; Yoshida et al., 2016). The xylic

hyphae connect to the host xylem by forming an open connection. Phloic hyphae have been observed to wrap around the host phloem (Dörr, 1972; Fig. 7). There they form invaginations that increase surface contact in between parasite and host cells (Dörr, 1972; Vaughn, 2006).

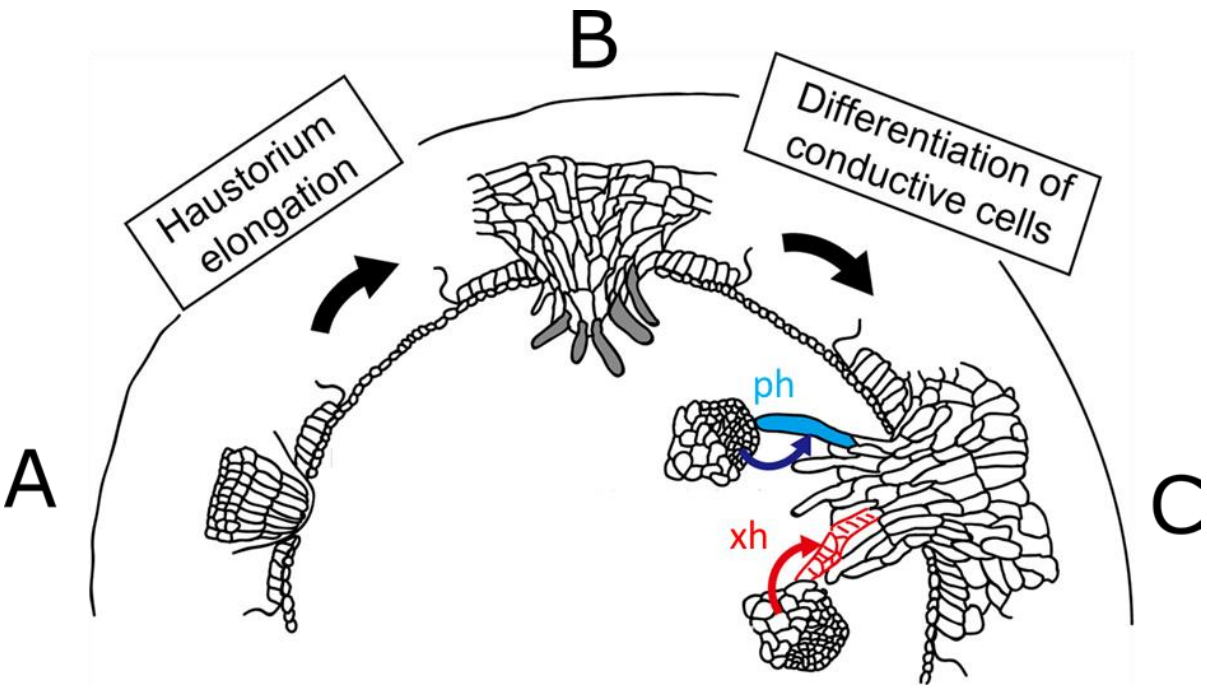

Figure 6: Schematic representation of Cuscuta haustorium development. After the haustorium penetrates the host (A), finger-like searching hyphae grow into the host tissue (B) and develop into xylic (red, xh) and phloic (blue, ph) hyphae upon contacting the suitable host-vascular elements (C). Image modified from Shimizu & Aoki (2019)

1.2.3 Transport between *Cuscuta* and its host

Long-distance transport in plants is mediated via the bulk flow of solutions through xylem and phloem. Short distances can be bridged by other transport modes like, for example, diffusion, transporters, vesicles, or plasmodesmata (PD). PD are channels across the plant cell wall lined with endoplasmic reticulum (ER) that connect plant cells and tissue and thus form a continuous symplasm among the connected cells. In parasitic plants, PD can form connections between cells of two different organisms. Those connections are called interspecies plasmodesmata (iPD). IPD have been observed to occur on *Cuscuta* searching hyphae connecting them to host parenchyma cells (Dörr, 1969; Vaughn, 2003). In general, it is known that *Cuscuta* connects to host xylem and phloem streams (Fig. 6, 7). The substances *Cuscuta* takes up from its host are transported within the haustorial xylem and phloem streams into the parasite stem (Birschwilks et al., 2006). However, while open interspecies xylem connections have been detected via the xylic hyphae of the parasite connecting to the host xylem, it is unclear how exactly the phloem content from the host is transported into the parasite haustorial phloem. No direct interspecies sieve plate connections have been observed, but scattered PD have been found to connect *Cuscuta* searching hyphae to host cells and apoplasmic features have been described at the cells

connecting *Cuscuta* phloic hyphae to host phloem. These have been interpreted as a sign that the transport at this parasite-host border is of apoplasmic nature (Dörr, 1968, 1972; Vaughn, 2003, 2006; Fig. 7).

Transport rates for *Cuscuta* from their hosts have been found to be massive and the transported goods diverse (Liu et al., 2020). While the transport of pathogens, like viruses, viroids and phytoplasma between *Cuscuta* and its host was shown a long time ago (Hosford, 1967; Kamińska & Korbin, 1999), during the last years also the transport of other substances has been studied extensively. Secondary metabolites like alkaloids that are used for defense (Flores-Sánchez & Garza-Ortiz, 2019; Zhao et al., 2018), as well as glucosinolates (Hettenhausen et al., 2017; Smith et al., 2016; Zhuang et al., 2018) that can protect both *Cuscuta* and the host plant from insect feeding are frequently transported to and from *Cuscuta.* Herbicide resistance of the host has been shown to increase the tolerance of a parasitizing *Cuscuta* plant against that same herbicide (Jiang et al., 2013). RNA transport – mRNA, siRNA and miRNA – has been shown to occur (Johnson & Axtell, 2019; Kim et al., 2014; Liu et al., 2020; Shahid et al., 2018; Westwood & Kim, 2017) and though it was initially unknown if RNAs are functional after their transport, this has now been observed (Park et al., 2021). Proteins seem to be taken up in high numbers according to abundance, subcellular location and molecular weight (Liu et al., 2020). Even flowering in *Cuscuta australis* has been accredited to the host plants' mobile protein signal (Shen et al., 2020).

RNA movement between *Cuscuta* and its hosts is a topic of active research (Westwood & Kim, 2017). The bulk movement of RNAs seems to be bi-directional, while some RNAs only or mostly move in one direction. Though the total abundance of RNA in the host seems to be a factor of how much RNA moves to the parasite, there are also different mechanisms at work and it appears that RNA transport does not necessarily follow the usual source-sink mechanism: Some RNAs move in greater or less abundance than would be expected due to their presence in the host (Kim et al., 2014).

The intimate connections that *Cuscuta* develops to its host as a parasite and the transfer of RNA seem to have promoted horizontal gene transfer (HGT) (Gao et al., 2014; Yang et al., 2019). HGT is known to be an important driving force in the evolution of plants with the most famous example being the transfer and integration of *Agrobacterium* tumor-inducing genes. In *Cuscuta* several genes have been found to be acquired from host plant species like for example *Daucus carota* (carrot) (Vogel et al., 2018). The evolutionary events of HGT in *Cuscuta* species suggest that *Cuscuta* is a plant species that is prone to what one might call "natural transformation". It is possible that by taking advantage of the parasitic characteristics of this plant, one might be able to use them for developing a specialized transformation protocol.

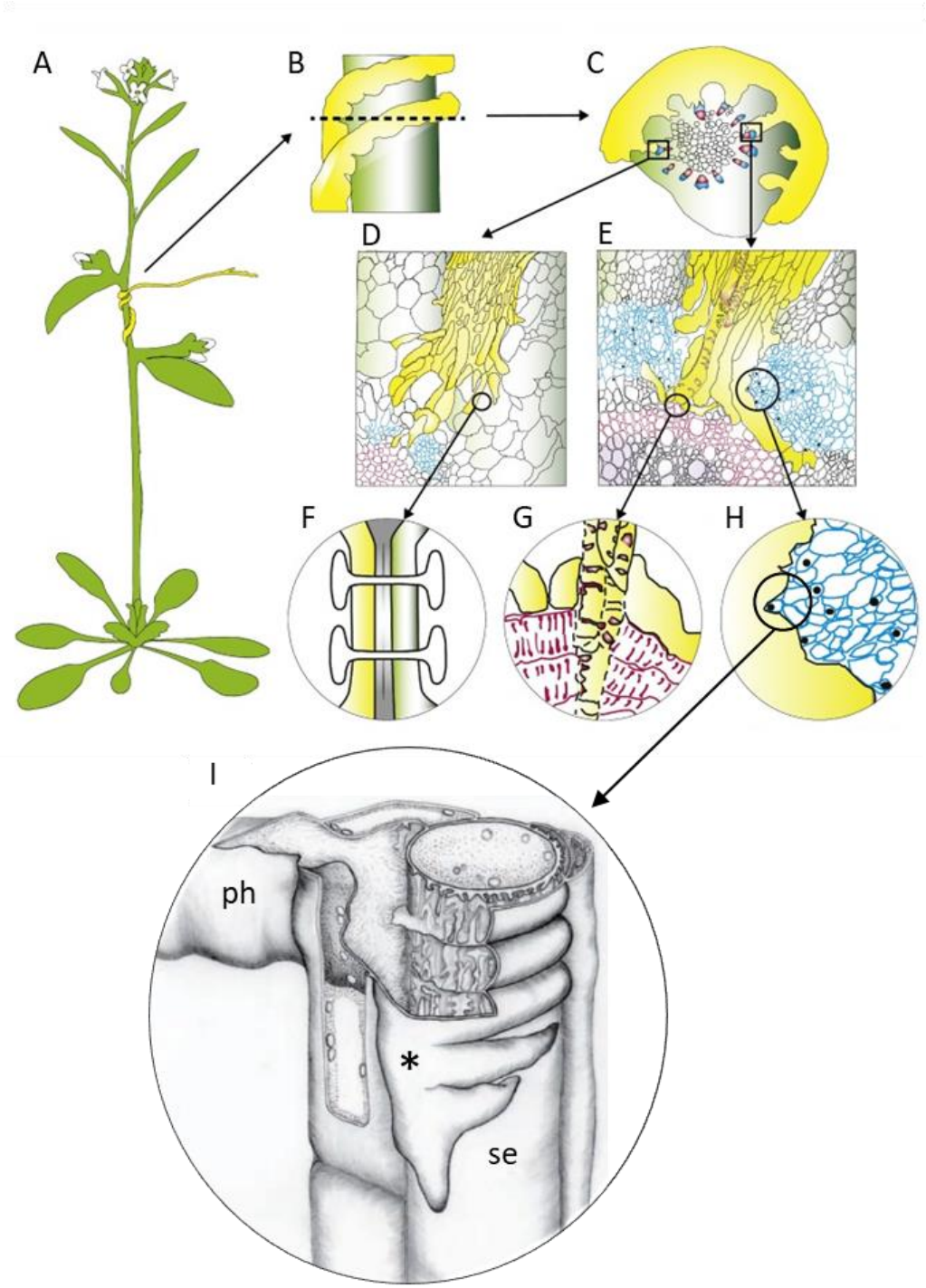

Figure 7: Connections formed between Cuscuta and its host. Overview of Cuscuta (yellow) growing on Arabidopsis thaliana (green) (A), close-up view (B) and schematic cross section (C) of infection sites. Magnified views of an invading haustorium (D) and a feeding haustorium (E) show the searching hyphae grow in between the host cells to establish symplasmic connections via iPD (F). In the mature state (E), transfer hyphae have established connections with the host xylem (G) and phloem (H, I). A schematic drawing of a Cuscuta phloic hyphae (ph) shows its contact to the host phloem element (se) and the formed cell wall invaginations (*) to increase the surface (I). Illustration modified from Kim & Westwood (2015) (A-H) and Dörr (1972) (I)

1.3 On the brink of becoming a model species

1.3.1 Transformation and regeneration

The idea of creating *Cuscuta* transgenic plants is not new and has already been evaluated when studies about *Cuscuta trifolii* and *Cuscuta reflexa* tissue culture and transformation methods were published (Borsics et al., 2002; Švubová & Blehová, 2013). However, a few years ago the publication of the first genomes of *Cuscuta* species, *Cuscuta campestris* (Vogel et al., 2018) and *Cuscuta australis* (Sun et al., 2018) , renewed the interest in this topic immensely.

To create a transgenic plant, the "traditional" way is the development of a tissue culture system of that plant, which allows for transformation of plant cells that can then be regenerated into a whole plant via one of two regeneration pathways – somatic embryogenesis or somatic organogenesis (Fig. 8). Somatic embryogenesis is, in short, the formation of an embryo that can develop into an entire new plant, from somatic and thus differentiated tissue. Somatic organogenesis, accordingly, the development of organs from somatic tissue. Both can either be induced directly from a plant part brought in tissue culture (explant) or indirectly from callus. Callus is originally the name for wound tissue but is in plant *in vitro* culture commonly used for an intermediately formed and unorganized looking group of cells that usually possesses the ability to differentiate into various kinds of tissues. The development of the plant tissue is guided by exposing it to different hormone concentrations that can, depending on the internal hormone conditions of the explant itself, induce, for example, callus, embryo, shoot or root formation.

For *Cuscuta trifolii*, formation of callus from young seedlings and regeneration via somatic embryogenesis, as well as subsequent maintenance of the formed *C. trifolii* shoots in culture without the need for a host has been achieved previously (Bakos et al., 1995, 2000). However, regeneration could no longer be induced after transformation of the tissue using *Agrobacterium tumefaciens* (Borsics et al., 2002). For *Cuscuta reflexa*, only tissue culturing (Srivastava & Dwivedi, 2001), but no transformation and attempt for subsequent plant regeneration has been reported so far. *Cuscuta europaea* has been transformed using *A. tumefaciens* carrying a green fluorescent protein (GFP) for detection (Kaštier et al., 2017; Švubová & Blehová, 2013) but a transgenic plant has likewise not been regenerated. From these studies it can be concluded that *Cuscuta* species can successfully be brought into tissue culture and be transformed by *Agrobacterium* but tend to lose the condition to realize their regenerative potential after transformation. Additionally, none of these transformation techniques has been reported to be reproducible by other research groups so far. Genetic modifications, meaning the addition or deletion of genes or introductions of mutations, are important for functional analysis of the gene products. However, the creation of transgenic plants by tissue culture, transformation and regeneration, is not the only way to study the function of genes.

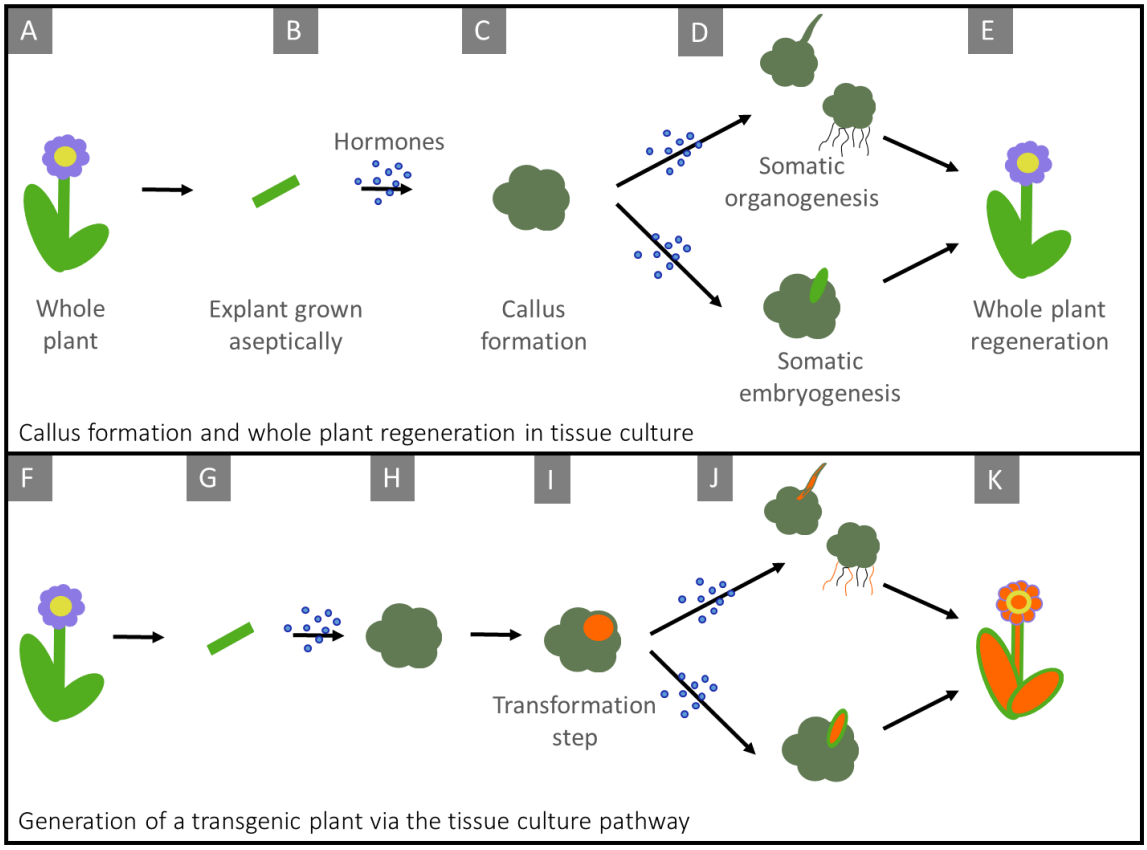

Figure 8: Stylized concept for creation of transgenic plants. A piece is cut from a mother plant (A) to be grown in vitro as an explant (B). Callus formation is induced via a specific hormone concentration in in vitro culture (C). By changing that hormone concentration, somatic organogenesis or somatic embryogenesis can be induced (D) that ultimately leads to the regeneration of a new plant (E). The same concept can be used for creating a transgenic plant (F-K) when a transformation step is included (I) and the transformed tissue is used for regeneration of a new plant (J, K). Transformed tissue is depicted as orange with originally colored outline in (I-K) compared to (C-E) that depicts the process without a transformation event.

1.3.2 Gene silencing techniques

Alternative options for gene function studies are gene silencing approaches. RNA interference (RNAi) using transgenic host plants that express small interfering RNAs (siRNAs) has been studied on *Cuscuta pentagona* (Alakonya et al., 2012) and *Cuscuta campestris* (Jhu et al., 2021). RNAi is part of each plants' defense system against viruses (Szittya & Burgyán, 2013). Upon recognizing the double stranded RNA of the virus, the protein Dicer cuts it into small 23-nucleotides long pieces, siRNAs. These are then recruited to the RNA inducing complex (RISC) and unwound to single stranded RNA (ssRNA). The antisense strands integrate within the complex and serve as a template to recognize and degrade homologous RNA. Performing RNAi, however, includes a high amount of work, as for every single gene to be tested a new transgenic host plant must be created. Virus induced gene silencing (VIGS), a plant functional genomics tool based on the post-transcriptional gene silencing response of RNAi, is an easier

and faster way to test genes for their phenotypes and also utilizes the RNAi pathway for silencing.

During VIGS, a viral vector is included in a strain of *Agrobacterium tumefaciens* for delivery to a host plant. Different virus vectors can be used for VIGS and depending on the characteristic spread of the virus, silencing occurs in the respective tissues. Tobacco rattle virus (TRV) is the one most frequently applied, as it causes silencing in the sink tissues of a plant. This means that it spreads through the entire plant, including meristem tissue, that is often unaffected when using other viruses. TRV is a bipartite virus, which means that it consists of two plasmids that each contain information necessary for the creation of a functional virus. TRV1 carries the replication and movement viral functions, while TRV2 includes the coat protein and the target sequence for silencing. Each of them is cloned into one strain of *Agrobacterium* that are mixed to deliver their viral load to a plant and enable the virus to spread within the plant. By taking advantage of the defense mechanism for virus induced RNAi, the plant is coerced into silencing its own genes (Fig. 9). In the affected tissues, the gene silencing phenotype becomes visible. This technique does not change a plant genetically but instead results in the sequence-specific degradation of endogenous mRNAs and subsequent silencing of specific genes via RNAi (Burch-Smith et al., 2004; Senthil-Kumar & Mysore, 2014) to create an epigenetic effect. As a positive control for VIGS, phytoene desaturase (PDS) silencing is commonly used. It causes a defect in carotene synthesis and subsequent photobleaching of chlorophyll due to the missing carotenoids that usually act photoprotective for chlorophyll (Kumagai et al., 1995; Velásquez et al., 2009). The result is a white phenotype in the affected tissue. The development of at least one of those techniques, genetic manipulation or gene silencing, would mean substantial progress in enabling further studies of the genus *Cuscuta*.

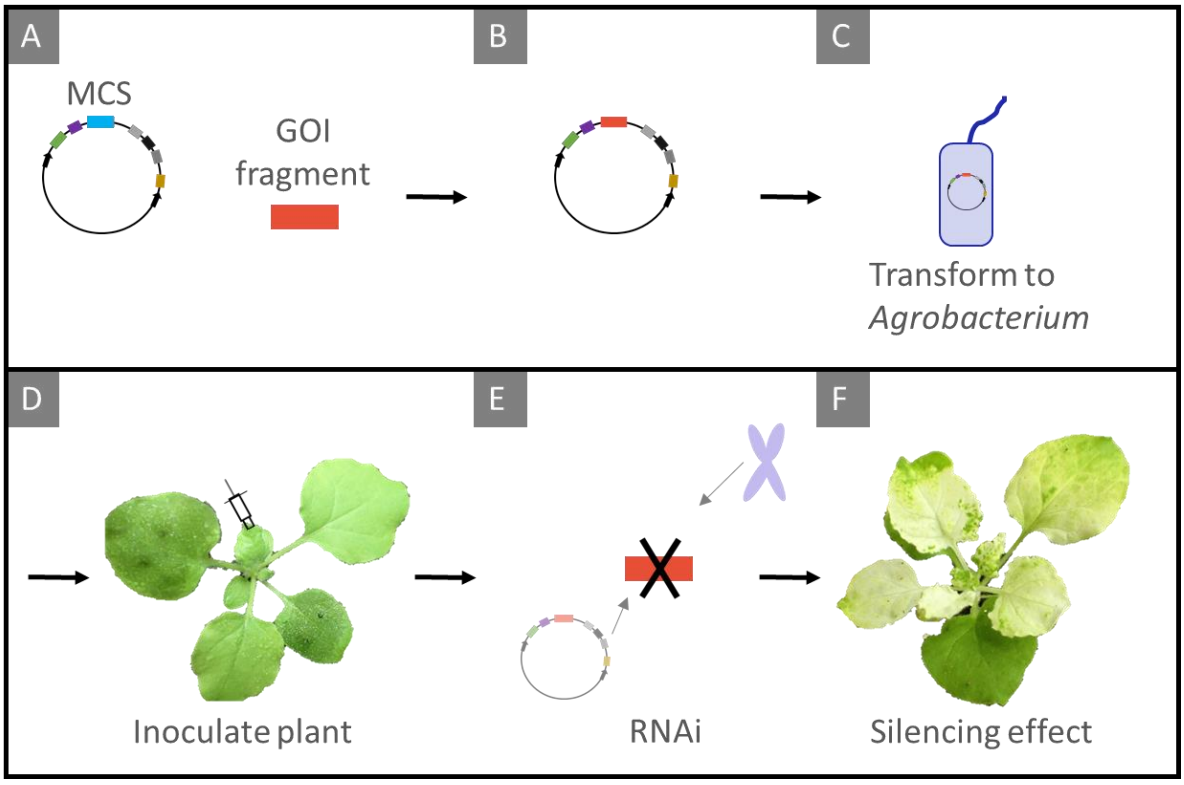

Figure 9: The stylized concept of VIGS. A fragment of a gene of interest (GOI) is cloned into the multiple cloning site (MCS) of a virus vector (A, B), which is cloned into Agrobacterium (C). A host plant is inoculated with Agrobacterium carrying the viral vector (D). When the virus spreads throughout the plant, the RNAi pathway is activated as a virus defense mechanism and the product of the candidate gene is silenced from the viral vector and from the plant genome alike (E). Silencing occurs and the silencing phenotype becomes visible (F)

1.3.3 Possibilities as a research model

As a plant that can connect two or more plants via a living bridge (Haupt et al., 2001), *Cuscuta* provides unique possibilities as a research system. While there are a bunch of early studies published in the 20th century about the *Cuscuta* system (Bennett, 1944; Dörr, 1968), parasitic plants in general and especially *Cuscuta* are only recently coming closer to developing into possible model systems for studying plant-cell connections, plant immune responses and transport processes. They feature closely connected but genetically different cells in the interface region with their hosts, which permits to use them to evaluate processes between cells while being able to distinguish between them because of their distinct nature. Additionally, they can circumvent the immune system of a variety of host plants, seemingly without alerting them. Understanding how it does that could provide valuable new insights in plant immunity, while finding the mechanisms via which resistant host plants avoid infection by *Cuscuta* could help develop strategies to strengthen crop resistance to *Cuscuta* and other foes.

This is especially attractive as, apart from providing interesting opportunities for research parasitic plants also account for rising agricultural crop losses. *Cuscuta*, especially the species

Cuscuta campestris, is one of the main scourges. The inability to study the parasite without the host, as well as missing genetic information, have long been an issue. Recently, host-free systems to grow *Cuscuta* independently are emerging (Bernal-Galeano & Westwood, 2021) and *C. campestris* was the first parasitic species whose genome was published in 2018 (Vogel et al., 2018), followed shortly after by *Cuscuta australis* (Sun et al., 2018). These new systems and datasets bring researchers closer to be able to identify the genes important for the parasitic lifestyle in *Cuscuta* and to find out which genes have been lost in comparison to photoautotrophic plants and which genes might have been gained or changed their function. While before that point, only the information from some transcriptomes was available, the hurdle of missing genetic information has now been removed. Thus, studies for gene functions in *Cuscuta* to unravel the parasites' secrets about its successful mode of infection are now just lacking the appropriate protocols to be carried out. The ability to combine a protocol for gene function studies on *Cuscuta* with the available genetic information would now allow us to systematically test the influence of genes on the parasite's infection capability. This could help to find a measure to contain this agricultural pest by knowing which genes need to be targeted to restrict infection of host plants. At the same time, knowledge about the mechanism of infection could also, in the long term, allow to turn aspects of parasitic life into something positive and use them to help agriculture. An example would be adjustments that could be used to improve grafting processes. *Cuscuta* species connect to unrelated plants without problem, while grafting techniques are restricted to combining traits of closely related plants. If knowledge on how *Cuscuta* manages to connect successfully to so many different plants can be used to improve grafting possibilities, this would immensely further the possibilities in growing crops that are well-adjusted to environmental conditions and thus help secure food for the world. With two published genomes for *Cuscuta* species in place, the element that is lacking in order to catch up with knowledge on other plant-pathogen systems and enable us to proceed in evaluating this plants' special connection to its host, is now a protocol that allows for gene function studies (Clarke et al., 2019).

2 Aims

As outlined above, no reproducible protocols for genetic transformation exist for any species of the genus *Cuscuta*, despite efforts by different research groups. This limits the use of the genomic information currently accumulating for *Cuscuta* and makes this project an aim with high merit and of global interest. For the development of transgenic *Cuscuta* plants, approaches of tissue culture, transformation and whole plant regeneration were targeted. Additionally, VIGS, a promising gene silencing technique that provides an easy and fast way to observe phenotypes, was tested as a tool for analysis of gene function in *Cuscuta*.

In contrast to other plants, *Cuscuta* is not autonomous but depends on its host plants, with which it exchanges all kinds of nutrients and other compounds. This is an important aspect of a parasitic lifestyle and needs to be taken into consideration for the experimental design of both types of techniques mentioned above, genetic manipulation and gene silencing. To judge possibilities to integrate the parasitic mode of life to design suitable protocols for this plant, the literature on reciprocal interspecies transport between host and parasite was reviewed. During this task, substantial gaps in our knowledge on the pathways of interspecies transport became evident so that the investigation of transport processes became a second focus of this thesis. It was mostly addressed by the investigation of transport at the host-parasite border, including mineral distribution and plasmodesmata between the two plants. Figure 10 is a graphical overview that connects the main and sub goals of the project and also indicates the affiliation of the results, papers and projects of work in progress that will be presented in the next section.

The specific aims of this study were:

1. To develop a pipeline that enables gene function studies in a *Cuscuta* species

2. To investigate the modes of transport and nature of contact at the *Cuscuta*-host interface, including the role of plasmodesmata in this area

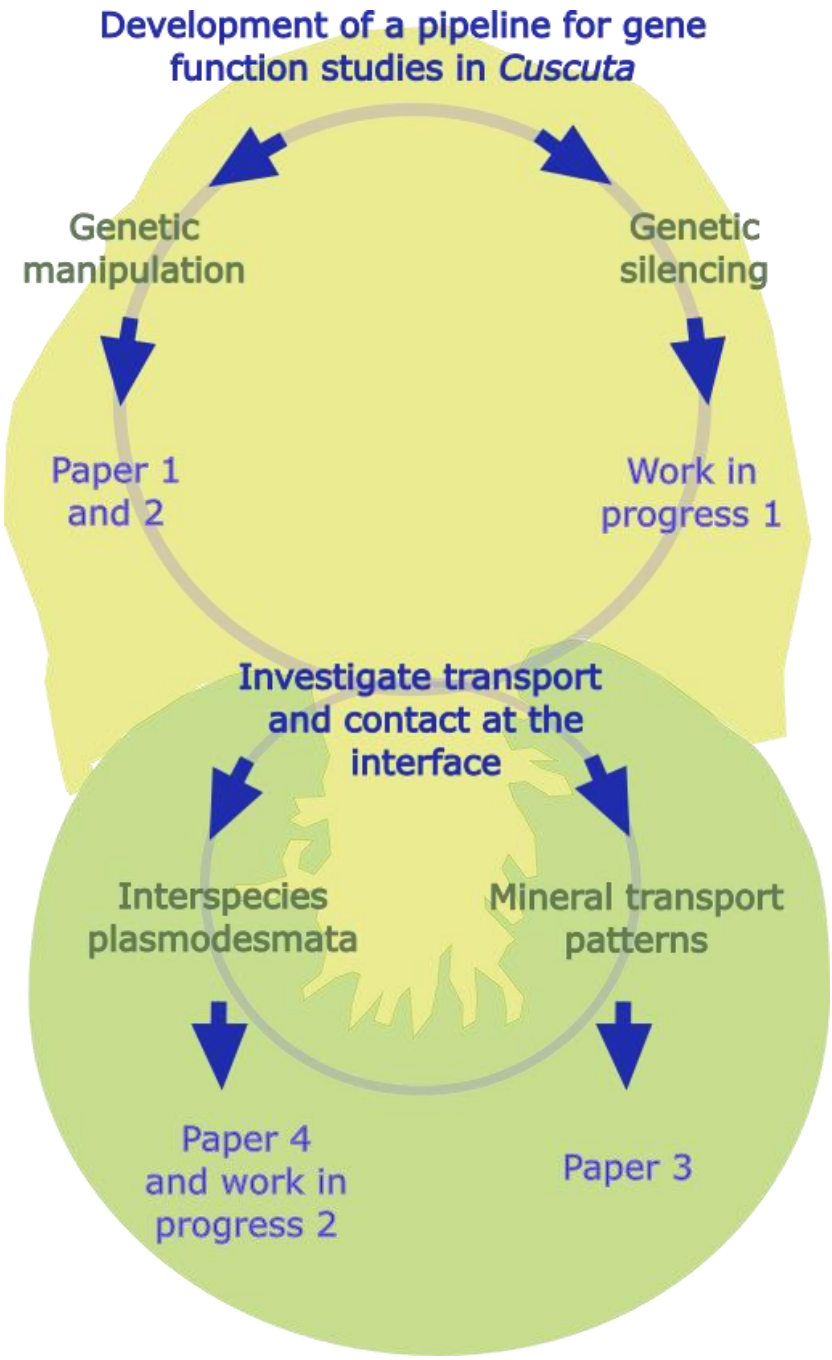

Figure 10: Project overview showing the two main goals of the project. The development of a pipeline for gene function studies (top) was addressed by genetic manipulation and gene silencing and is represented in papers 1 and 2 as well as the "work in progress" chapter 1. The second main goal of investigation of transport and contact at the interface (middle) is addressed by the evaluation of interspecies plasmodesmata and mineral transport patterns and represented in the results of papers 3 and 4 as well as "work in progress" 2. The background shows a schematic image of a typical cross-section of Cuscuta (yellow) parasitizing on a host plant (green) and helps locate where in the Cuscuta-host system the projects were set up. While the development of gene function studies is focused on Cuscuta itself, the project addressing transport and contact is centered around the interface region between the two plants, where contact and exchange occur.

3 Results

3.1 Summary of Papers

3.1.1 Paper 1: A highly efficient protocol for transforming *Cuscuta reflexa* based on artificially induced infection sites

Lena Anna-Maria Lachner, Levon Galstyan, Kirsten Krause

Research article published in Plant Direct 2020

Several attempts have been made to develop a transformation protocol for the creation of transgenic *Cuscuta* plants. None of them has been successful in producing transformed plants so far. In this study, a simple, fast, and efficient method to transform *Cuscuta* cells is presented. Host-free induction of haustoria was initiated by a setup that applies far-red light and mechanical pressure to *Cuscuta* stems. At the same time, this setup permits the exposure of the developing parasitic structures to *Agrobacteria* carrying a fluorescence marker gene for transformation. After 7 days, when plenty of haustoria had developed, fluorescent cells could be detected in at least half of them. They were mostly located in a cell layer below the epidermis in the region of the adhesive disk, around the parasitic organ. Cells in the haustorial disk were reliably transformed, developing in this host-free haustoria induction setup. It is notable that *Agrobacterium rhizogenes* exposure did not induce the typical growth of hairy roots in *Cuscuta*. This can probably be attributed to missing genes for root-formation in the rootless parasitic plant. Fluorescence signals that indicated transformation of cells could be observed for several weeks when the respective explants were sustained on plates containing nutrient medium. However, the transformed cells could not be propagated, and the fluorescent tissue ultimately died without dividing or regenerating into callus or shoots.

While mainly the species *Cuscuta reflexa* was used to carry out the experiments, it could be shown that the technique is also applicable on other *Cuscuta* species, for example, *Cuscuta campestris*. Formation of haustoria in this system did however not work as optimal as for *C. reflexa* and the amount of necrotic tissue and auto fluorescence was higher and transformation efficiency lower compared to the *C. reflexa* system. *Agrobacterium rhizogenes* carrying a dsRed construct and *Agrobacterium tumefaciens* carrying a GFP construct both showed a similar efficiency and could be co-transformed frequently. This study also showed that *Agrobacterium* transformation took place only after haustorium formation had begun. The stem tissue itself seemed to not be accessible to *Agrobacterium* and the same was true for the uptake of the life-stain 5-carboxyfluorescein di-acetate (CFDA).

3.1.2 Paper 2: *Cuscuta campestris* tissue culture and seedling transformation via *Agrobacterium rhizogenes*

Lena Anna-Maria Lachner, Zahra Zangishei, Kirsten Krause

Manuscript Research paper prepared for submission in Plant Direct

Cuscuta campestris, a parasitic plant, is both an agricultural pest as well as an interesting research plant. During the last years it has become a focus species in *Cuscuta* research. With a published genome and plenty of other information accumulating, the tool for transformation and gene function studies is clearly the missing part for decoding the secrets of infection and its parasitic lifestyle. Since the transformation of adhesive disks presented previously targets a unique tissue and did not work as efficiently for *C. campestris* as for *C. reflexa*, in this study more suitable approaches for *C. campestris* were tested. A method for growing callus tissue of *C. campestris* seedlings was established and, subsequently the development of shoots that were able to infect a host plant and thus grow back into a whole plant was demonstrated. Whole seedlings were grown in a liquid culture or cut seedlings on solid plates with a ½ MS culture medium supplemented with kinetin at 5-25 µg/mL supporting growth best. Addition of 1-Naphthaleneacetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D) influenced growth negatively. During the culturing process or directly after germination and cutting of seedlings, it was possible to transform cells within the seedlings using a dsRed marker and *Agrobacterium rhizogenes*. While it has so far not been possible to regenerate whole plants with this technique, it is the optimal base for the creation of an efficient regeneration protocol. In addition, this protocol can be used together with existing transformation techniques, for example the protocol for transformation of cells in the adhesive disc of developing haustoria presented earlier, to conduct protein studies comparing the effect of transformation in different locations of the parasite.

3.1.3 Paper 3: Selective mineral transport barriers at *Cuscuta*-host infection sites

Frank Förste, Ioanna Mantouvalou, Birgit Kanngießer, Hagen Stosnach, Lena Anna-Maria Lachner, Karsten Fischer and Kirsten Krause

Research article published in Physiologia Plantarum 2019

Cuscuta reflexa is a parasitic plant known to establish xylem as well as phloem connections to its host plant. Xylem tissue consists of dead cells that form pipes for long-distance bulk transport of water and minerals. In this study, the uptake of inorganic nutrients (minerals) by the parasitic plant *C. reflexa* from its host *Pelargonium zonale* was charted by using X-ray fluorescence spectroscopy (XRF). This was combined with 2-D and 3-D (confocal) microscopy imaging. Although open xylem connections between the parasite and its host are known to exist, this study found that not all minerals are entering *Cuscuta* evenly. They seem to be taken up selectively with some showing lower amounts in the parasitic *Cuscuta* compared to its host. Calcium levels showed the highest difference with around 90% average decrease from host to parasite. Bromine and strontium levels were about 80-90% lower in the parasite, manganese about 75% and chlorine about 50%. This in turn led to a changed proportion of elements in the parasite compared to the host plant. While calcium and strontium levels were found to be lower on the parasite side of the interface compared to the levels in the host, manganese accumulated on the host side around the haustorium. Chlorine levels were found to be lower further along within the parasite tissue at the transition point from haustorium to stem, compared to the host and the parasite haustorium. Contrary to the open xylem connections believed to exist so far, these results point to selective barriers that regulate uptake via the xylem at the host-parasite border as well as within the parasite at the tissue border from the endophytic haustorium to the stem of the plant. These borders have, however, not been detected or identified until now. The extremely low calcium levels detected in *Cuscuta* are only comparable to *Poales* that exhibit a low pectin content in their cell walls, which is compromising their cell wall stability (Broadley et al., 2003). However, missing calcium-crosslinked pectin in cell walls could not be shown in *C. reflexa*. Another possibility is to associate low calcium levels in *C. reflexa* with the low calcium concentration found in sink tissues, like fruits and seeds, of other plants. Those seem to plug or even reverse xylem flow at a certain point of their development and are instead only provided by the phloem. As calcium cannot be transported via the phloem, this comparison might be able to explain the low calcium level in *Cuscuta*. Together with the fact that *Cuscuta* exhibits a very low transpiration activity due to the lack of stomata and can thus not get rid of surplus water the way other parasites do, led to the tentative proposition of a “flux reverse model” that proposes that not only could phloem transport via species borders be bidirectional, but also xylem transport.

3.1.4 Paper 4: The enigma of interspecific plasmodesmata: Insight from parasitic plants

Karsten Fischer, Lena Anna-Maria Lachner, Stian Olsen, Maria Mulisch and Kirsten Krause

Opinion article published in Frontiers in Plant Science 2021

The details of contact, transport, and signal exchange between parasitic plants and their hosts are still widely unknown. Regular plasmodesmata (PD) are channels lined by endoplasmic reticulum (ER) that form symplasmic connections between genetically identical cells of an individual plant and thus form a symplasm – a system of cells in contact by open channels through their cell walls that can be used for macromolecular exchange. In contrast, "interspecific" plasmodesmata (iPD) connect cells of two genetically different plants and even completely unrelated organisms. This occurs for example in the plant parasitic connection between *Cuscuta* and its hosts, where iPD can be observed between the searching hyphae of the parasitic organ and host parenchyma cells. Currently, it is not known what these connections are used for, but signal exchange to inhibit immune responses from the host as well as their direct involvement in the exchange of nutrients and other substances are tempting options. While water and minerals are transported via apoplasmic and symplasmic routes, other compounds, such as proteins and nucleic acids, rely on symplasmic transport via for example PD or the sieve pores or sieve plates of direct phloem connections to travel within a plant.

In this paper, the evidence that has been found on connections in the *Cuscuta*-host interface was collected with a focus on iPD. The type of connections observed, as well as the type of experimental methods that were used to assemble the data, whether they had been observed directly by electron microscopical methods or confirmed indirectly by molecular approaches were described. How PD formation in general is regulated, if interspecies plasmodesmata appear and act the same as intraspecies plasmodesmata and their potential role in parasitism and or transport between the two plants, is still unclear. Therefore, some theories on the regulation of iPD formation were proposed. It has been suggested that the formation of PD is controlled by both cells that are to be connected. At a host-parasite border, this would mean a cooperation of cells from two different organisms and thus communication across the species border. In this paper, several hypotheses are presented as to how this might be achieved generally and the advantage that can be taken of this system that provides two genetically and physiologically different organisms on either end of the iPD is elucidated. This should make it possible to investigate PD initiation, formation, and control of their positioning in a much more convincing and efficient way than it would be possible in systems with two genetically identical cells.

3.2 Work in progress (WIP)

In addition to the published work and the manuscript, some ongoing collaborative projects are included in the results and discussion in this thesis and denoted as “Work in progress” (WIP). Herein, some background information, methods and current results of these projects are described. They might or might not be continued in the future but have, at this point, not reached the potential of being published. However, they are relevant for the topics discussed in this work and should thus not be left out.

3.2.1 WIP1: Virus induced gene silencing (VIGS) adaptation for gene studies on *Cuscuta campestris*

Lena Anna-Maria Lachner, Kirsten Krause and Katja Karppinen (UiT) in collaboration with Brandon Reagan and Tessa Burch Smith at the University of Tennessee Knoxville, USA

Transformation and the establishment of a transgenic plant is a time consuming and not very efficient process. Virus induced gene silencing (VIGS) is a much easier and faster technique to study the function of genes in a plant. While this technique is well established for some plants like *Nicotiana benthamiana*, its use for parasitic plants like *Cuscuta* needs optimization and adjustment. Application of *Agrobacterium* directly to the *Cuscuta* stem had not been very successful in previous trials, so it was decided to settle on an approach that takes advantage of *Cuscuta' s* parasitic nature.

Several *C. campestris* genes, among them phytoene desaturase (PDS) and xyloglucan endotransglucosylase/hydrolase (XTH) were chosen for a trial. To design VIGS silencing constructs, sequences of a length of approximately 300 base pairs in the untranslated regions (UTR) were chosen using the available genome from Vogel et al. (2018) and taking into account the principles described in Burch-Smith et al. (2004). Each of these sequences was inserted into a TRV 2 vector (described in Liu et al. (2002)) and cloned into *Agrobacterium tumefaciens* GV3101. Infiltration using a needless syringe of 3-4 weeks old *N. benthamiana* plants was performed according to the protocol presented in Senthil-Kumar & Mysore (2014).

N. benthamiana was infected with *Cuscuta campestris* at different time points right before and after *Agrobacterium* infiltration of the host to deduce the optimal timing of infection to refer the silencing effect to *C. campestris*. As *Cuscuta* is known to take up and pass on viruses from its host plant (Bennett, 1944; Hosford, 1967) as well as a wide range of molecules, amongst others RNAs (Clarke et al., 2019), it was expected that the *C. campestris* RNA silencing response should be triggered as a response to the virus entering from the host plant. *N. benthamiana* plants experienced visible photobleaching in response to PDS silencing from the *C. campestris* PDS silencing construct. *C. campestris* exhibited a distinct green phenotype at the infection site, in contrast to the usually observed orange color in plants that had not been exposed to virus including the PDS silencing construct (Fig. 11). However, it was not possible to observe the typical white, photobleached phenotype of other PDS silenced plants anywhere in *C. campestris*. No color change could be observed in apical shoot parts or any visual spread of silencing effect (photobleaching) into untreated *N. benthamiana* plants that were connected to a silenced host via a "*Cuscuta* bridge". Furthermore, no significant silencing effect was detected when performing qPCR analysis on the newly developed *C. campestris* tissue (growing shoot tips) at different time points within 3 weeks after infection of the silenced *N. benthamiana* plants. Due to serious growth inhibition of PDS silenced *N. benthamiana* host plants, the growth success of *C. campestris* itself could not be compared between treated and untreated plants.

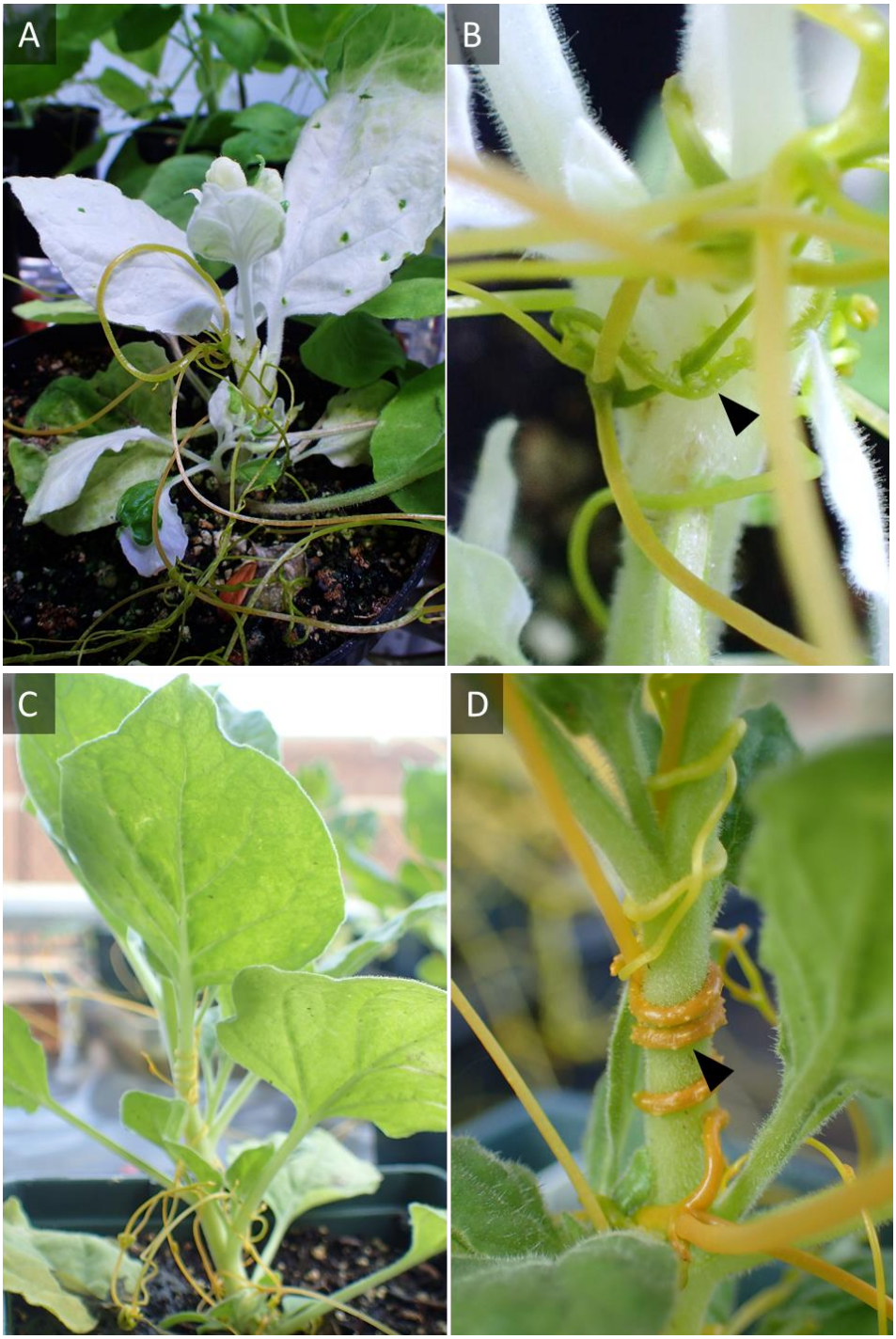

Figure 11: Virus induced gene silencing with C. campestris parasitizing on N. benthamiana. N. benthamiana plant showing a photobleached phenotype after silencing of phytoene desaturase (PDS) by the construct developed for C. campestris (A). Green phenotype of C. campestris at the infection site indicated by a black arrowhead (B). C. campestris parasitizing a control plant (N. benthamiana) exposed to TRV carrying no specific silencing construct (C) and close-up on a typically observed orange phenotype of C. campestris at the infection site (D).

3.2.2 WIP2: Investigation of the connection in between *C. campestris* and its host via electron microscopy

Lena Anna-Maria Lachner and Kirsten Krause (UiT) in collaboration with Brandon Reagan, John Dunlap and Tessa Burch-Smith from the University of Tennessee Knoxville, USA

It is known that plasmodesmata (PD) exist between *Cuscuta* hyphae and host parenchyma cells. The aim of this project was to capture one of these interspecies plasmodesmata (iPD) connections using focused ion beam scanning electron microscopy (FIB-SEM) and establish a 3D-model of it to compare its ultrastructure to conventional (intraspecies) PD. For this purpose, *C. campestris* was grown on *N. benthamiana* and infection sites harvested and flash-frozen using high-pressure freezing (HPF) and freeze substitution according to Bobik et al. (2014). Sections were investigated using a transmission electron microscope (TEM) to distinguish the morphology of parasite and host cells. Several special features and morphological differences could be distinguished in the parasitical haustoria and hyphae cells to set them apart from host cells.

The differences in *N. benthamiana* and *C. campestris* plastids are clearly visible with *C. campestris* plastids mainly accumulating starch (Fig. 12A, B, C). Haustorial cells were small and dense, full of organelles and had accumulated an especially high number of mitochondria (Fig. 12D). *Cuscuta* ER was stained using 1% osmium tetroxide and 0.1% uranyl acetate in sample preparation, while *N. benthamiana* ER was not stained and thus not visible under the same conditions. Furthermore, *Cuscuta* cells often showed a curious blotched pattern with electron transparent spots of about 1-2 µm (Fig. 12E) that might be related to difficulties in fixation. However, it might also be a special feature of *Cuscuta* infection, especially as cell death is to be expected around the interface, where the haustorium ruptures the host stem and grows in between its cells.

For FIB-SEM several regions depicting the interface between a *Cuscuta* cell and a host cell were chosen. The program Imod was chosen to assemble a 3-D model (Fig. 12F). However, no PD could be found neither on the TEM nor FIB-SEM images in early investigations. It was concluded that better knowledge on when and where in the haustorial development PD occurred was necessary to continue with the project. Due to following covid-19 restrictions the collaboration and follow up on this project was postponed.

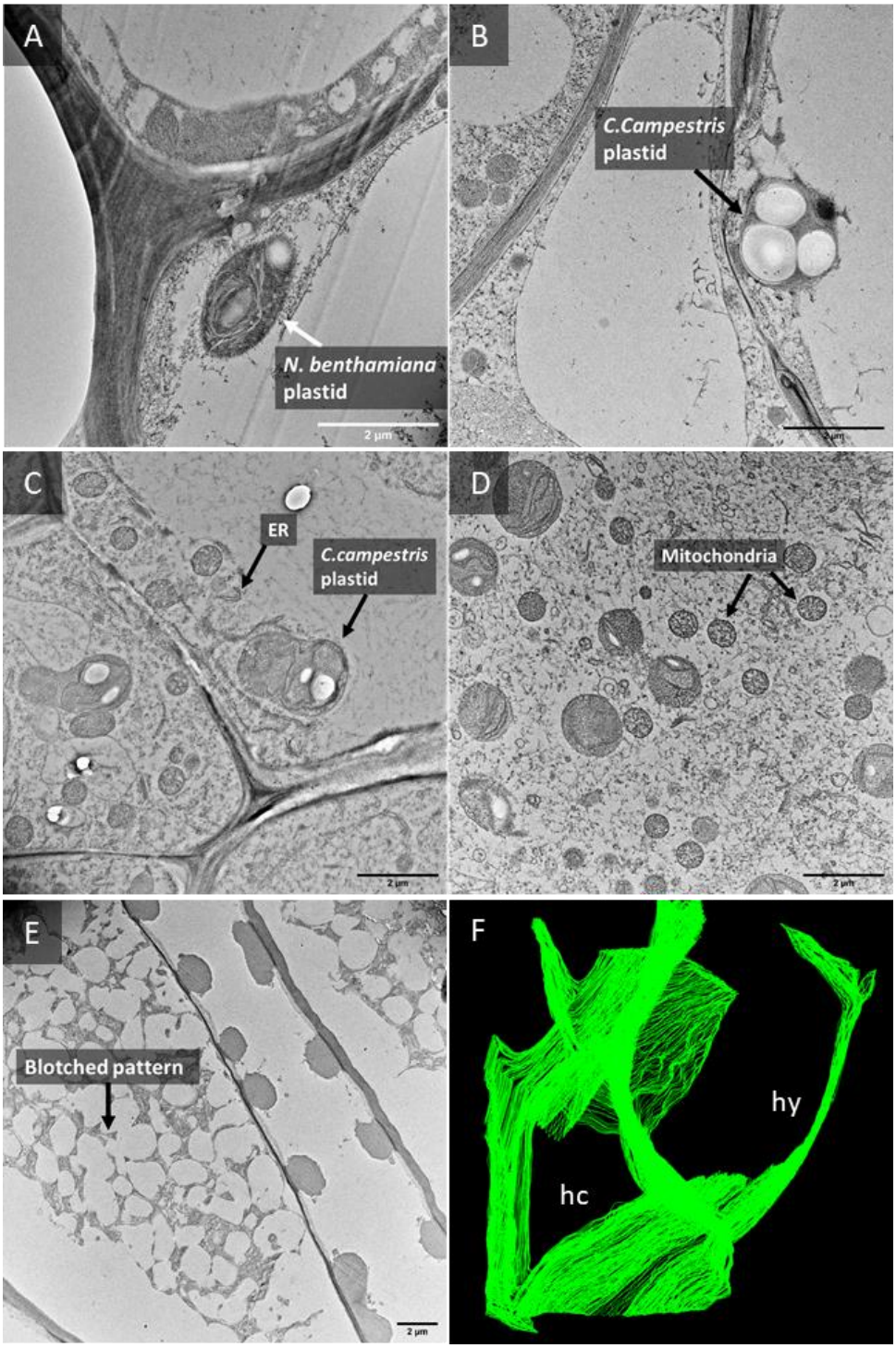

Figure 12: TEM images of C. campestris parasitizing on N. benthamiana (B, C, D, E). Plastids of the two organisms can easily be distinguished: N. benthamiana: (A), C. campestris: (B). C. campestris plastids are full of starch and do not show the typical pattern of thylakoids stacked in grana (B, C). ER of C. campestris stains visibly (C) while ER of N. benthamiana stays invisible. C. campestris haustorial cells densely packed with mitochondria and other organelles (D). Blotchy patterns about 1-2 µm of size often observed in Cuscuta tissue (E). Half-finished 3D model of a C. campestris hyphae (hy) contacting a host cell (hc) based on FIB-SEM data (F).

4 Discussion

This thesis focused on developing *Cuscuta* as a research model plant for open questions in plant-plant and plant-cell interactions by exploring possibilities for its transformation (Paper 1 and 2), as well as introducing applications for a *Cuscuta* system with its unique properties of interspecies connections for the research community (Paper 4). During this work, transport between *Cuscuta* and its host was found to play a key role in both endeavors. Two protocols for transformation of *Cuscuta* cells using *Agrobacterium* species were presented, one of them possibly closely connected to the topic of interspecies transport, as the area around developing haustoria was found to be especially suitable as a target tissue for *Agrobacterium* transformation (Paper 1). In addition, mineral transport was investigated and shown to be selective and restricted from a *Pelargonium* host to a *Cuscuta* species (Paper 3). The formation of PD between *Cuscuta* and its host was presented as a possibility to involve *Cuscuta* as a model in general plant research to expand possibilities by taking advantage of the cell-to-cell connection that forms among a parasitic plant and its host (Paper 4). Figure 13 presents a graphical overview over the main remaining questions ensuing the results of this thesis, and describes, in which chapters they are examined in the discussion.

4.1 Transport modes in *Cuscuta* are not fully understood

While a lot is known about transported substances between *Cuscuta* and its host, information about how the transport is performed and regulated are still scarce or missing. Open xylem connections can clearly be observed (Vaughn, 2006), but these might feature selective barriers when it comes to influx of mineral elements or alternatively efficient selective efflux pathways (Paper 3). It is also possible that mineral transport rates might be connected to other modes of transport, like, for example, phloem connections, that can be more easily regulated. Open phloem connections have been hypothesized and seem to be proven by molecular data (Birschwilks et al., 2006; Haupt et al., 2001). Ultrastructural studies, however, have up to date not been able to find them. On the contrary, they have revealed structures suggesting an apoplasmic mode of transport at the interface of phloic hyphae and host phloem elements (Dörr, 1972; Vaughn, 2006; Fig. 7). Since the connection in question is only being established in between very few specialized cells at the host-parasite interface, studies investigating these transport mechanisms are hard to conduct. The special cell-to-cell connections between the two organisms can, as far as is known, not be established outside of the *in vivo* system and hardly be separated from the rest of the system when investigating the interface area. Cell wall invaginations that have been described at the phloic hyphae-sieve element interface would promote uptake of compounds from the apoplasm into the haustorial cells at the very border to the host rather than suggesting a free flow through the connected apoplasm in the infection sites. However, this is speculative, and the specific mode of transport cannot be deduced from the observations that have been made so far between *Cuscuta* and its host.

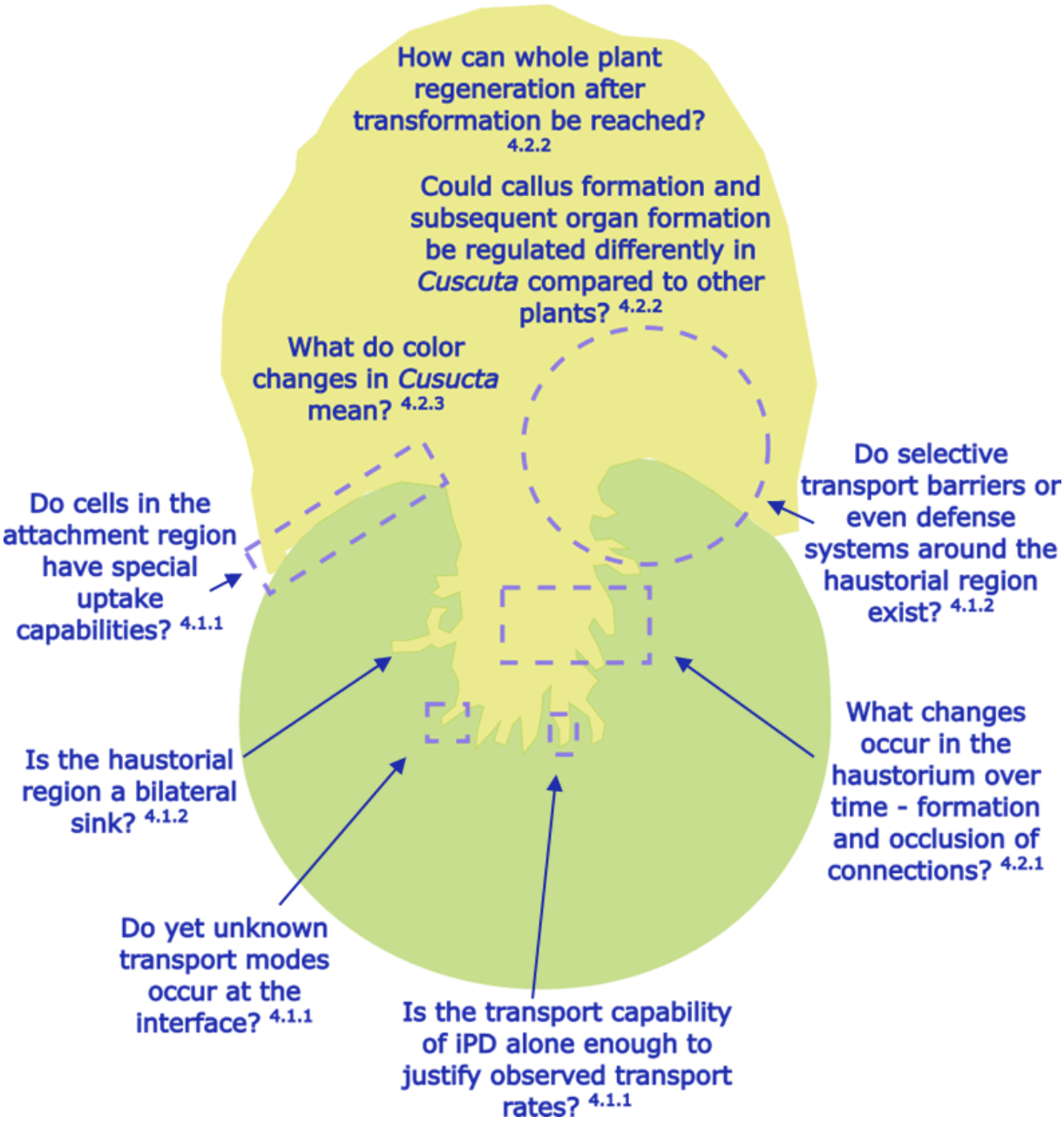

Figure 13: Summary of remaining open questions discussed in this thesis. Questions referring to Cuscuta alone are included in the parasite part of the figure (yellow, top), while questions referring to the connection between parasite and host are shown together with their approximate regions of impact (blue arrows and purple outlines). The chapters that discuss the respective questions are indicated in superscripted numbers.

4.1.1 Interspecies plasmodesmata and transport between *Cuscuta* and its host

Massive observed transport rates (Liu et al., 2020) as well as experiments that used fluorescent phloem tracers (Birschwilks et al., 2006; Haupt et al., 2001) would suggest an open phloem connection in the form of direct sieve tube connections between *Cuscuta* and host sieve tubes. Such direct connections have been observed in other parasitic plants like *Orobanche* (Dörr & Kollmann, 1994; Krupp et al., 2019), but not in *Cuscuta*. In contrast to sieve tubes, PD have frequently been observed in between *Cuscuta* and host cells (Paper 4) (Fig. 7). However, these connections have been interpreted to only exist in the searching hyphae stadium, and to be closed up later, when searching hyphae differentiate (Dörr, 1968; Vaughn, 2006). While PD can potentially develop into sieve pores (Kalmbach & Helariutta, 2019), the latter ones have never been reliably observed at the host-parasite interface by ultrastructural studies. Though the transport capability of plasmodesmata is unknown, the number of observed iPD connections between parasite and host cells thus seem to not be enough to account for the nutrient uptake (Fig. 13). This might suggest that those PD serve another purpose or, they are not the only way for the feeding of *Cuscuta* and there is yet another mechanism involved. PDs are known to be an important factor in symplasmic communication between cells as well as the coordination of the immune response of plants (Kim, 2018; Lee, 2015). Signals from host to parasite could thus be transferred via the established iPD at the stage of the searching hyphae and make it possible for it to recognize, which cells they are in contact with to start the respective differentiation when in contact with host xylem or phloem cells. Another possibility is that those iPD transfer signals from parasite to host to silence the latter's immune system and enable *Cuscuta* to stay undetected by its host.

While the claim for open phloem connections still lacks its full validation, other transport modes at the host-parasite interface should be taken into consideration (Fig. 13). Extracellular vesicular transport, for example, is a newly re-discovered mode of transport in plants (Rutter & Innes, 2018). It has been proposed as a possible mode for small RNA transport for plants to silence pathogen genes (Cai et al., 2018), as well as parasitic plants to prevent host immune reactions (Clarke et al., 2019). While this mode of transport has been known in animals for some time, in plants it has only recently been taken up for investigation and found to be mainly involved in plant stress and immune reactions (Rutter & Innes, 2017). Up to now, results suggest that extracellular vesicular transport plays an important role in mediating defence responses (Cai et al., 2018; Rutter & Innes, 2017). Whether it might be a suitable candidate for high-throughput transport in the *Cuscuta*-host system will have to be investigated.

In the investigations of the exchange of substances between *Cuscuta* and its host the focus usually lies on the haustoria and haustorial hyphae. It has not been evaluated yet if other regions of the parasite-host border could contribute as well (Fig. 13). When working with *Agrobacterium* for transformation of the haustorial regions of *C. reflexa*, a persistent pattern of localization of transformed cells within the haustorial adhesive disk was distinguishable (Paper 1). A similar pattern could be observed using the live-stain CFDA. This could be

interpreted as a heightened capability for uptake into the cells surrounding the haustorial region. It is known that plants can sense their environment by taking up volatiles through stomata or adsorption on the leaf surface, to compile information about their surroundings (Sugimoto et al., 2016). In the same way, it cannot be ruled out that *Cuscuta* might be able to take up molecules from the surface of its host plants to collect information about them before starting the infection.

4.1.2 The existence of a strong sink and a specialized selection system at the haustorial site can be hypothesized

Transport processes between *Cuscuta* and its hosts seem to be largely unregulated, or the regulation mechanisms have at least not been identified yet. Xylem flow, protein and RNA transport appear to happen bidirectionally (Kim & Westwood, 2015; Liu et al., 2020; Yoshida et al., 2016). Considering this extensive network *Cuscuta* forms with its host plants, there must be a way the parasite is able to protect itself from unwanted substances that are entering via its hosts, like siRNAs, viral pathogens or mycoplasma. The idea of *Cuscuta* possessing a special immunity against pathogens, especially viruses coming from the host, is not new. During the 20th century, virus studies in *Cuscuta* were very popular and it was found that transmission of viruses to and from *Cuscuta* as well as their movement and illness symptoms in *Cuscuta* varied with the virus type, the *Cuscuta* species, the host plants, and environmental stresses (Hosford, 1967). Virus inhibitors were found in several *Cuscuta* species (Awasthi, 1981; Hosford, 1967), however, TRV, the virus used for the VIGS assay was not studied back then and its effect on and interaction with *Cuscuta* is thus fairly unknown.

The effort of developing a VIGS approach for *C. campestris* has so far not yielded the desired results that would make it possible to use this technique as a tool to analyze gene function in *C. campestris.* This is because silencing does not occur in the growing apical part of the shoot but close to the haustorial sites (WIP1; Dyer et al., 2021), where it cannot be used to evaluate if silencing of specific genes might show an effect on the infection process which, in turn, is the most interesting topic up for investigation. When using a PDS silencing construct in combination with TRV in a VIGS experiment, it is usually expected to observe a white, photobleached phenotype in newly forming tissue of the silenced plant. This is due to TRV spreading to the (strongest) sink of the system, which usually is the developing tissue. In the experiments conducted on *C. campestris*, this usual photobleaching effect could not be observed. qPCR results of the region around the growing shoot tips did not show any significant degree of silencing either (WIP1; Dyer et al., 2021). When evaluating the possibilities, it is known from before hat viruses move freely in between *Cuscuta* and its hosts (Bennett, 1944) and from one host to another via *Cuscuta* (Hosford, 1967). TRV has been identified in *Cuscuta* species (Dikova, 2006) and RNA-mediated gene silencing has been shown to be functional (Alakonya et al., 2012). Additionally, RNAs have been shown to freely move between parasitic plants and their hosts (David-Schwartz et al., 2008; Roney et al., 2007; Tomilov et al., 2008). Thus, it was concluded that TRV should be able to spread normally from *N. benthamiana* to

C. campestris and induce the RNA silencing pathway there, while an additional possibility of how silencing could be induced in the parasite was concluded to be the direct transport of siRNAs produced by the host to *C. campestris.* Nevertheless, no white *Cuscuta* tissue at all and no silencing response in apical shoot parts could be observed. It seems that an RNAi response in *C. campestris* does, in fact, occur in reaction to TRV. However, it appears to be contained around the infection site in *C. campestris* instead of the newly growing tissues in the apical shoot, where TRV would be expected to move and thus silencing to occur, like in other plants.

It is possible that apoplastic and plasmodesmal connections but no direct sieve elements in between *Cuscuta* and host cells inhibit a fast and systemic distribution of the virus and additionally contain it to the proximal region around the infection site. The study of Shahid et al. (2018) found that host mRNAs were targeted by *Cuscuta* miRNAs that were accumulated in the haustorium. Chlorine distribution showed a border in between haustorial site and the rest of the tissue (Paper 3). It can thus not be ruled out that there is a selection or even defense system in place around the haustorial site where substances cross from host to parasite (Fig. 13). In case the *Cuscuta* immune system in general and specifically RNAi response was especially active around this site, it could also not be ruled out that the major silencing response to TRV occurs in this area because of that. However, that the majority of entering TRV could be prevented from further spread into distant *Cuscuta* tissue by being "silenced on arrival" or otherwise contained to the surrounding tissue of the haustorium, disagrees with observations in other viruses that show that these can be transferred from one *Cuscuta* host to another and require that viruses are mobile within *Cuscuta*. Consequently, this theory would have to be investigated very closely, otherwise it could not explain why no silencing can be observed in the sink tissue of the growing apical shoot parts. The most tempting explanation for the observed phenomenon might be that as a parasitic plant, *Cuscuta* possesses different, even stronger sinks than the region around its growing shoot tip. If the proposed "flux reversal" hypothesis (Paper 3) proves right, this could not just make *Cuscuta* a sink to the host system, but also, to a certain degree, the host to the *Cuscuta* system (Fig. 13). The strongest sink within the system might thus be centered around the infection site and this might also be where the TRV virus spread is highest. This in turn could explain the results of Dyer et al. (2021), who found that silencing effect in VIGS occurred up to around 70% in the proximal regions around the haustorium but not significantly in the distal regions of the apical shoot. However, this would also mean that *Cuscuta* could be inhibited in its growth by the host sink. This has not been observed so far and would thus have to be investigated closely together with mode and regulation of transport at the host-parasite interface.

4.2 Adjustments to parasitism in *Cuscuta* might influence the development of standard techniques

It is logical to suspect that *Cuscuta* as a parasitic plant, having evolved under different evolutionary pressures than photoautotrophic plants, also has different priorities. While *Cuscuta* does not have real roots at any point in its life cycle and leaves are strongly reduced, it features an additional and unparalleled organ that no photoautotrophic plant possesses – the haustorium. It needs to develop a possibility to grow this organ newly from differentiated stem cells and penetrate the stem of a host plant. Then, searching hyphae must find xylem and phloem streams of the host and develop into suitable cell types to form a feeding connection. Finally, all this needs to happen while evading the host immune system. Via the established feeding organ, it then faces the challenge of being exposed to all kinds of transported substances via its host.

As high-quality genetic information about *Cuscuta* species is only recently emerging, not much is known about the regulation of infection and transport mechanisms, and which exact genes have been lost, gained, or repurposed to enable this parasitic mode of living. However, it can be assumed that all of the above has happened as the differences it has caused can be observed. It has been evaluated that a large amount of genes related to root and leaf development, photosynthesis, flowering, mediation of environmental responses and defense are missing (Sun et al., 2018; Vogel et al., 2018) in *Cuscuta*. Equally a high number of HGT events has been asserted (Vogel et al., 2018; Yang et al., 2019). It must be considered that the changes in *Cuscuta* compared to photoautotrophic plants can influence efforts to develop standard techniques for this plant like, for example, tissue culture and regeneration techniques, as well as gene silencing approaches like VIGS. Efforts and standard protocols might be hampered because of the differences in *Cuscuta*, but also new possibilities of using its special way of life, its reduced form and this unique infection organ should arise.

4.2.1 Haustorium and interface regions

The *Cuscuta* parasitic haustorium consists of several specialized types of cells. A lot of details about what is happening in the haustorial region during infection are already known from previous ultrastructural studies (Dörr, 1968, 1969, 1972; Vaughn, 2002, 2003, 2006) and could be confirmed in the WIP 2 within this thesis. One observation was that several features made it possible to differentiate *C. campestris* cells from *N. benthamiana* like, for example, hyphae packed with organelles (Dörr, 1969) and plastids full of starch in the parasite (Machado & Zetsche, 1990; van der Kooij et al., 2000). Cells packed with organelles like mitochondria and ribosomes can be expected to provide for the energy needs and components of the fast-growing haustorial cells and infection and transport processes in the haustorium, while starch in the plastids points towards a role of storage rather than photosynthetic activity (Machado & Zetsche, 1990). Though iPD are known to exist at the border between searching hyphae and host parenchyma cells (Paper 4), it proved difficult to find them during this thesis. Since iPD

have been hypothesized to be closed up in later stages and only exist during a certain time (Vaughn, 2003), studies to more closely evaluate when and where iPD appear in this system would be necessary and could give an indication about their role during host infection. In general, the time aspect of haustorial development and the fact that the appearance and function of the whole haustorium as well as its cells and connections to the host might significantly change over time has not been elucidated and studies usually do not pay attention to this and rather use the whole "feeding stage" as one and the same. One reason for that is probably that it is difficult to specify the age and or state of a haustorium after the initial feeding connection has been established. Additionally, not much is known about the processes within the haustorium after that point, like, for example, if further growth of searching hyphae and their differentiation into feeding hyphae still occurs, or if other processes might occlude or reverse the flux, as proposed in paper 3 of this thesis.

While it is not known how exactly the transport from host phloem to haustorial phloem is regulated and achieved, it has been found that haustorial phloem in *Cuscuta* and its development, differs from conventional phloem. Companion cells could not be identified in *Cuscuta*, and sieve elements seem to not fully differentiate and remain in an immature state, as they were found to retain nuclei, which are commonly missing in fully differentiated phloem and the compartmentalization of regions where xylem and phloem marker genes were expressed was found to be incomplete (Shimizu & Aoki, 2019; Shimizu et al., 2018). When mapping the mineral composition of a *Cuscuta*-host system it was found that chlorine concentration in the haustorium was similar to the host level but lower than that in the *Cuscuta* stem (Paper 3). This led to the hypothesis that this division could be related to maintaining the capability of taking up nutrients from its host by actively retaining chlorine in the haustorium region. If this is correct or whether it has any other unknown purpose in the parasitic connection remains to be investigated. It is clear that the haustorium is a complexly built and regulated organ that is far from being understood completely. Additionally, theories of *Cuscuta* species depending on host factors for certain developmental stages like flowering (Shen et al., 2020) have been brought forward and many factors about the regulation of processes in the connection and transport between the organisms connected to *Cuscuta* are still unknown or unresolved.

In summary, the natural dependency of *Cuscuta* on a host and many yet unknown factors in the regulation of processes within the parasitic organ, especially the transport of substances and regulation of processes in the haustorium, leads to a generally difficult situation when developing standard protocols for *Cuscuta* species. Even the development of host-free systems (Bernal-Galeano & Westwood, 2021) that are recently emerging does not completely resolve this problem as long as so many factors of what happens in the interaction between parasite and host and their influence on the whole system remain in the dark. These organisms have a complex lifestyle that might change a lot of what we know about plants so far. They can hardly be investigated on their own, outside their parasitic connection, whose details are barely understood.

4.2.2 Loss of root-related genes

While the haustorium is the biggest addition to *Cuscuta*, enabling it to live parasitically, the loss of roots, leaves, and the ability to fully thrive on photosynthesis are the greatest reductions its parasitic lifestyle has caused. The loss of roots specifically, as *Cuscuta* species are often still able to use photosynthesis to sustain themselves or at least boost their energy balance but none of them seem to be able to form roots any longer (Machado & Zetsche, 1990). This deficit might also influence callus formation and thus tissue culture performed on *Cuscuta* species. Atta et al. (2009) and Sugimoto et al. (2010) reported that callus formation is guided by the lateral root-initiation program and callus has the identity of a root-meristem in *A. thaliana*, no matter what kind of tissue was used as explant material. As a result, root formation from callus requires no further reprogramming in this system, while shoot-formation is more complex. Lateral root meristem has been hypothesized to be totipotent and ready for differentiation into different cell lineages, among them roots, shoots and leaves (Sugimoto et al., 2019). This could prove interesting during further investigation of the origin of haustoria also, as it has been indicated before that root parasitic plants co-opted this mechanism for haustorium development (Ichihashi et al., 2018). As a result of the lack of some root development genes in *Cuscuta*, specifically PLETHORA (PLT) 1, 2 and 5 that are involved in the specification of the root stem cell niche (Sun et al., 2018), the possibility that *Cuscuta* callus is regulated differently, for example, like a shoot meristem, should be considered. In contrast to the root hemiparasitic plant *Phteirospermum japonicum*, whose haustorial cells develop from root epidermal cells, *Cuscuta* haustorial cells are originated from the inner stem cortex and give rise to a meristem before invading the host (Lee, 2007; Lee & Lee, 1989; Shimizu & Aoki, 2019). It has not been specified if the originating meristem shows signs of a lateral root meristem but it has been found that in *Cuscuta pentagona*, SHOOT MERISTEMLESS (STM), a gene known to be involved in shoot formation in other species, is highly expressed in haustorial development (Alakonya et al., 2012). If *Cuscuta* callus tissue really exhibits signs of a shoot meristem, shoot formation from callus might consequently be easy and inducible even without the addition of external hormones. In studies presenting shoot regeneration from *Cuscuta* callus tissue, sometimes this indeed occurs very easily and without addition of hormones (Paper 2; Bakos et al., 1995). Studies developing tissue culture systems for different *Cuscuta* species however, contradicted each other in this aspect. In *C. reflexa*, a reversed proportion of cytokinin to auxin, meaning high auxin to cytokinin as opposed to high cytokinin to auxin in normal plants, was necessary to regenerate shoots for Srivastava & Dwivedi (2001), while Das et al. (2011) reported that equal concentrations of auxin and cytokinin worked best for shoot regeneration. In *C. trifolii*, Bakos et al. (1995) regenerated shoots like mentioned above without adding any hormones to the medium or, by addition of cytokinin. The development of a tissue culture is still very much based on trial and error and general guidelines about what hormone combinations cause a desired development exist but have to be optimized for each plant and might vary greatly depending on many factors. Thus, it cannot be ruled out that optimal conditions for tissue culture of at least some *Cuscuta species* are different from other plants' (Fig. 13) and that this

is caused by a different expression pattern of *Cuscuta* callus. However, no conclusion can be drawn at the current point. What is certain at this point is that after transformation, shoot formation of *Cuscuta* callus, especially of transformed cells, is inhibited (Paper 1 and 2; Borsics et al., 2002). Recalcitrance, the inability of callus lines to respond to hormone treatment, is a common problem in tissue culture and for whole plant regeneration. It is up to date not known what exactly the underlying cause for recalcitrance is, but it is known that the chance for recalcitrance is growing, the longer plant material is kept in tissue culture. Additionally, transformation of cells often causes them to become recalcitrant. All studies that report successful regeneration of *Cuscuta* after tissue culture achieve their goal only if no prior transformation event was involved. After transformation, *Cuscuta* became recalcitrant to whole plant regeneration. Thus, *Cuscuta* is no exception to the rule that transformation can cause recalcitrance of tissue and might, for unknown reasons, even be especially prone to this process. This brings up the question of how this recalcitrance could be overcome to achieve whole plant regeneration after transformation and produce a fully transgenic *Cuscuta* plant (Fig 13).

4.2.3 Reduction of photosynthesis

In contrast to the complete loss of roots, photosynthesis can still be performed by most *Cuscuta* species. It is, for example, known that while genes necessary for photosynthesis at higher light intensities were lost in *C. campestris*, basic genes for photosynthesis are preserved (Vogel et al., 2018). Chloroplasts of some *Cuscuta* species have visible thylakoids (for example *C. reflexa* and *C. campestris*), even if their number is lower than in photosynthetic plants and grana could not be observed. Those same species also contain chlorophyll and can perform at least some photosynthesis. On the other hand, there are also some *Cuscuta* species where no thylakoids could be found (for example: *C. europaea*). For those, as well as for some species that have reduced and strangely arranged thylakoids (for example *C. campestris*) and low photosynthesis efficiency, the plastids are mostly comprised of starch (Machado & Zetsche, 1990; van der Kooij et al., 2000), which fits well with observations in *C. campestris* in this thesis (WIP2). Chlorophyll as a photopigments and carotenoids as accessory photopigments are both present in *C. campestris* (van der Kooij et al., 2000). Carotenoids do not only act as accessory photopigments but also as photoprotectants for chlorophyll. In photoautotroph plants, chlorophyll is photobleached in absence of the protecting carotenoids, and a white phenotype is the result after silencing of PDS that is involved in carotenoid synthesis (Kumagai et al., 1995). This is the standard control for the VIGS assay. Nevertheless, so far, no white phenotype in *C. campestris* could be observed after introducing TRV containing the PDS silencing construct in *C. campestris* (WIP1). Dyer et al. (2021) who recently reported to have developed a functioning VIGS protocol for *C. campestris* on *A. thaliana* did not analyze this, though they mentioned that the *A. thaliana* stems *C. campestris* attached to, exhibited a white phenotype in response to VIGS with the *C. campestris* PDS silencing construct. A white phenotype was also observed on *N. benthamiana* plants in reaction to the silencing construct for *C. campestris* during the experiments in this thesis. In contrast to the photobleached phenotype normally observed in PDS silenced photoautotrophic plants, *C. campestris* exhibited a clear green

phenotype in the proximal area around the infection site, where silencing was found to be strongest in the study of Dyer et al. (2021). It could be observed that on untreated *N. benthamiana* plants, the region around the infection site appeared mostly yellow or orange after a successful infection. However, greenish phenotypes could also be observed in *C. campestris* shoots that did not infect properly or were flowering and had thus reached the end of their life cycle. It is likely that under normal conditions, carotenoids mask chlorophyll in *C. campestris* and result in an orange phenotype (Smith et al., 2001). The loss of carotenoids after silencing of PDS could then potentially lead to the visibility of the underlying green color of chlorophyll. In photoautotrophic plants, sunlight does not just serve the photosynthesis but also leads to energizing of the transported electrons that can then give rise to reactive oxygen molecules that in turn damage chlorophyll if no carotenoid or other protection is present (Zulfiqar et al., 2021). *Cuscuta* might simply possess alternative photoprotective molecules, like tocochromanols (van der Kooij et al., 2005) that act as scavengers in place of the missing carotenoids and thus prevent photobleaching of chlorophyll upon loss of its carotenoids. It is also possible that due to the low photosynthesis activity in *C. campestris* (van der Kooij et al., 2000), reactive oxygen molecules might not develop at all or, in very low amounts, and thus even unprotected chlorophyll could remain intact and unbleached. Why greening also seems to occur in times when *C. campestris* seems to not be growing well, and how the production, regulation and functions of chlorophyll and carotenoids in *C. campestris* are connected will need to be elucidated in the future (Fig. 13).

5 Outlook and future directions

Parasitic plants are an interesting research system as they can be considered both a nuisance or even threat to food production, but also a chance for revolutionary findings in agriculture and biotechnology alike. The two transformation systems introduced in this thesis can be employed to compare seedlings and haustorial sites when investigating gene functions. This in turn can be used to discover new candidate genes that have an important function in the infection mechanism. The methods are fast, easy, efficient and have the potential to be developed further into biotechnological pipelines to evaluate *Cuscuta* gene functions in a high-throughput manner in the future. At the same time, new knowledge and methods are emerging that enable us to focus on new, exciting techniques that could be combined with them. The traditional approach for creating transgenic plants consists of using tissue culture, transformation, and regeneration, three steps with a high potential need for optimization that are also slow. Recently, new possibilities of high-efficiency protocols that enlist the help of transcription factors to enhance regeneration and transformation in recalcitrant plants emerged and are being developed (Niazian et al., 2017). One specific possibility that has been employed more frequently in recent years is the use of developmental regulators that pattern the meristem to induce new meristems directly on plant parts. It has been found that when combinations of developmental regulators are expressed ectopically in somatic tissue, *de novo* organs can be formed. While these techniques were initially only developed for *A. thaliana*, they have recently been adjusted for other plants as well. Maher et al. (2020) reported the modification of a protocol developed for transient transformation of *A. thaliana* seedlings by *A. tumefaciens* for use on *N. benthamiana* seedlings and called it Fast-TrACC (fast-treated *Agrobacterium* co-culture). The technique combines developmental regulators and gene-editing agents to create transgenic and gene-edited plants by inducing *de novo* growth of shoot-like structures directly on seedlings. Developing this technique for *Cuscuta* as a parasitic plant would pose a unique chance and allow for the resolution of many open questions.

The same would be true for the development of a successfully applicable VIGS technique for *Cuscuta*. With the current knowledge that silencing only occurs around the haustorial site but not in growing apical shoot parts (Dyer et al., 2021), VIGS does not seem to be a very useful technique for *Cuscuta campestris*. However, the silencing response might be different for other *Cuscuta* species or could be overcome by using a different viral vector. Thus, further exploration of this versatile method should not be neglected. The possibilities of having a tool for gene function studies in *Cuscuta* would be numerous and could also help to study for example the role of vesicle transport as a transport mode between parasite and host, which could be a promising candidate for elucidating the riddle of how substances are transported from host phloem to the haustorial vasculature.

In the meantime, however, a lot of other topics that could be explored even without such tools are left to be illuminated. It would, for example, be of interest to investigate the expression of

shoot- and root-meristem genes in *Cuscuta* callus as well as during haustorium development. This could help to shed light on possible differences in regulation of *Cuscuta* callus compared to plants that possess roots, as well as elucidating the origin and regulation of haustorium formation. Additionally, to come closer to an understanding of how *Cuscuta* might react to and tolerate being exposed to so many viruses and pathogens from its host plant, it would be interesting to chart the virus and pathogen distribution within *Cuscuta* after their transfer from a host plant, to investigate if there might be a difference in pathogen occurrence between the haustorial region and the rest of the plant. Techniques like electron microscopy or fluorescent tags and dyes could be used for that, while phenotyping techniques like hyperspectral imaging should be tested as well as a possible easy and fast way to detect changes in *Cuscuta* after virus infection. Immune responses of *Cuscuta* should furthermore be studied in detail. In case it really possesses a higher tolerance towards viruses or pathogens, this might lead to discoveries that could help make crop plants more resistant to them as well.

As a general indicator of *Cuscuta* plant health and a detail that seems to have been neglected so far, the color changes that can be observed in several *Cuscuta* species and often seem to indicate the status of the plant, should be evaluated closer. What the color changes might mean, which patterns they follow and how they are regulated can only be speculated about at this point. However, solving this riddle could give a useful new indicator to measure the quality of the host-parasite connection by indicating plant health, good growth, and host compatibility, as well as it might point to regulation options *Cuscuta* possesses in respect to pigment production. This could be evaluated closer, for example, by comparison of growth and color development of different *Cuscuta* species on good and bad hosts or, under known favorable and unfavorable growth conditions combined with the application of transcriptomics and proteomics techniques.

The results presented in this thesis will help to further develop *Cuscuta* as a model species by contributing new techniques for transformation and examining distinct and possible characteristics that discriminate this system from other plant systems. With this knowledge, it is possible to understand the challenges and opportunities the work with this system includes and learn more about where the focus of attention has to be placed on in the future. All of this is necessary for the goal to fully “tame” this parasite and make it a versatile research model.

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Part 2

Papers

List of papers and contributions (co-author statements)

Name of candidate: Lena Anna-Maria Lachner

Papers
The following papers are included in my PhD thesis:

Paper 1
A highly efficient protocol for transforming *Cuscuta reflexa* based on artificially induced infection sites
Lena Anna-Maria Lachner, Levon Galstyan, Kirsten Krause (2020)
Plant Direct, DOI: 10.1002/pld3.254

Paper 2
Cuscuta campestris* tissue culture and seedling transformation via *Agrobacterium rhizogenes
Lena Anna-Maria Lachner, Zahra Zangishei, Kirsten Krause
Manuscript Research paper prepared for submission to Plant Direct

Paper 3
Selective mineral transport barriers at *Cuscuta*-host infection sites
Frank Förste, Ioanna Mantouvalou, Birgit Kanngießer, Hagen Stosnach, Lena Anna-Maria Lachner, Karsten Fischer and Kirsten Krause (2019)
Physiologia Plantarum, DOI: 10.1111/ppl.13035

Paper 4
The enigma of interspecific plasmodesmata: insight from parasitic plants
Karsten Fischer, Lena Anna-Maria Lachner, Stian Olsen, Maria Mulisch and Kirsten Krause (2021)
Frontiers in Plant Science, DOI: 10.3389/fpls.2021.641924

Contributions

	Paper 1	**Paper 2**	**Paper 3**	**Paper 4**
Concept and idea	KK	LL	IM, KF, KK	KF, KK
Study design and methods	LL, KK	LL, ZZ, KK	FF, IM, BK, KF, KK	KF, LL, SO, KK
Data gathering and interpretation	LL, LG, KK,	LL, ZZ	FF, HS, LL, KF, KK	KF, LL, MM, SO, KK
Manuscript preparation	LL, LG, KK	LL, ZZ, KK	FF, IM, HS, LL, KF, KK	KF, LL, MM, SO, KK

With my signature I consent that the above listed articles where I am a co-author can be a part of the PhD thesis of the PhD candidate.

KF: Karsten Fischer

FF: Frank Förste

LG: Levon Galstyan

BK: Birgit Kanngießer

KK: Kirsten Krause

IM: Ioanna Mantouvalou

MM: Maria Mulisch

SO: Stian Olsen

HS: Hagen Stosnach

ZZ: Zahra Zangishei

Paper 1

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ORIGINAL RESEARCH

A highly efficient protocol for transforming *Cuscuta reflexa* based on artificially induced infection sites

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Abstract

The parasitic plant genus *Cuscuta* is notoriously difficult to transform and to propagate or regenerate in vitro. With it being a substantial threat to many agroecosystems, techniques allowing functional analysis of gene products involved in host interaction and infection mechanisms are, however, in high demand. We set out to explore whether *Agrobacterium*-mediated transformation of different plant parts can provide efficient alternatives to the currently scarce and inefficient protocols for transgene expression in *Cuscuta*. We used fluorescent protein genes on the T-DNA as markers for transformation efficiency and transformation stability. As a result, we present a novel highly efficient transformation protocol for *Cuscuta reflexa* cells that exploits the propensity of the infection organ to take up and express transgenes with the T-DNA. Both, *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* carrying binary transformation vectors with reporter fluorochromes yielded high numbers of transformation events. An overwhelming majority of transformed cells were observed in the cell layer below the adhesive disk's epidermis, suggesting that these cells are particularly susceptible to infection. Cotransformation of these cells happens frequently when *Agrobacterium* strains carrying different constructs are applied together. Explants containing transformed tissue expressed the fluorescent markers in in vitro culture for several weeks, offering a future possibility for development of transformed cells into callus. These results are discussed with respect to the future potential of this technique and with respect to the special characteristics of the infection organ that may explain its competence to take up the foreign DNA.

KEYWORDS

adhesive disk, *Agrobacterium* transformation, *Cuscuta*, haustorium, parasitic plants

1 | INTRODUCTION

Parasitic plants account for considerable agricultural losses of almost all food and fodder crops around the world. Many plant lineages contain one or more genera that live a parasitic live. Some are highly specialized and are only found in certain biotopes while others are widely distributed generalists. The obligate parasitic plant genus *Cuscuta*, commonly known as "dodder" is found almost worldwide and infects

Link to the manuscript on the biorxiv preprint server: https://doi.org/10.1101/2020.04.06.028191

a broad range of host plants and, thus, belongs to the latter group. The parasitic attack by all *Cuscuta* species starts by winding around the host stem. A multicellular feeding structure termed the haustorium, which can reach 2–3 mm in size in some species and originates from a secondary meristem in the cortex close to the vascular bundles, develops within a few days and breaches the host tissue integrity using mechanical pressure and enzymatic digestion of cell walls (Nagar et al., 1984; Johnsen et al., 2015; Kaiser et al., 2015). To make the penetration possible, a sucker-shaped attachment organ provides the necessary counterforce. The development of this organ, termed "adhesive disk" or "upper haustorium" (Vaughn, 2002; Lee, 2007; Kaiser et al., 2015), is easily visible as a swelling of the host-facing side of the parasite's stem. A bio-adhesive substance, secreted by the epidermal cells of the adhesive disk, anchors the parasite to the host (Vaughn, 2002; Galloway et al., 2020). Morphologically, the development of the adhesive disk is marked by major local growth processes and shape changes of the involved cells: cells in the parasite's cortex around the developing haustorium elongate significantly and epidermal cells that are normally rectangular and stretched in a longitudinal direction reshape into digitate cells that appear slightly rounded on the surface (Shimizu and Aoki, 2019).

In light of the detrimental effect to important crops exerted by *Cuscuta* worldwide, it is imperative that mechanisms guiding the parasitic attack are better understood. One prerequisite for achieving a better understanding of the molecular processes and mechanisms of a *Cuscuta* attack is the knowledge of the genomic disposition. Recently, complete genome sequences have been published for two *Cuscuta* species, *C. campestris* and *C. australis* (Sun et al., 2018; Vogel et al., 2018), covering this need. However, two significant bottlenecks to perform in situ protein studies or even empower targeted genome manipulations do remain. These are low transformation efficiencies on one hand and recalcitrance of callus propagation and shoot regeneration in vitro on the other hand. Few approaches to transform *C. trifolii* (Borsics et al., 2002) and *C. europaea* (Svubova and Blehova, 2013) have been reported. Explants from seedlings were transformed using *Agrobacterium tumefaciens*, and expression of the transgenes was demonstrated in the calli. However, a lack in efficiency of these approaches have so far precluded the use of transient or stable transformation for functional studies of the proteins and cells involved in establishing the parasitic interaction.

Besides *A. tumefaciens*, *Agrobacterium rhizogenes* has become a popular agent for transforming plant genomes (Ozyigit et al., 2013). *A. rhizogenes* is a soil-borne bacterium infecting many angiosperms and causing them to produce a copious number of roots which became known as the "hairy root syndrome" (Bahramnejad et al., 2019). Like *A. tumefaciens*, *A. rhizogenes* transfers a segment of DNA known as T-DNA into its hosts. The transfer process is controlled by virulence (*vir*) genes that are induced by phenolic signal molecules (Gelvin, 2003). The *A. rhizogenes* T-DNA is stably integrated into the plant nuclear genome where it expresses the *rol* (*rooting locus*) genes required for excessive adventitious root growth (Ozyigit et al., 2013). What has made these hairy roots popular for plant biotechnology is that they can be propagated in the absence of exogenous plant hormones. Very recently, it was shown that an *A. rhizogenes* gene coding for the mikimopine synthase was horizontally transferred into several *Cuscuta* species (Zhang et al., 2020), including *Cuscuta campestris* (Vogel et al., 2018) and *Cuscuta australis* (Sun et al., 2018), suggesting that *Cuscuta* species may be susceptible to infection by this *Agrobacterium* species despite their lack of roots.

With this study, we set out to test the applicability of the hairy root transformation protocol in *Cuscuta*. Although hairy roots as such were not obtained, we were able to obtain high numbers of transformed cells in the species *Cuscuta reflexa*, particularly in its adhesive disks. We describe here the simple and highly efficient protocol that can yield hundreds of transformed cells within a week based on the use of *A. rhizogenes*. We further show that the protocol is applicable to use with *A. tumefaciens* with equally high success, which widens possibilities for single or cotransformation of different constructs, thus allowing functional studies of gene products like protein localization and interaction studies or expression of heterologous or synthetic transgenes within these cells that play a decisive role for *Cuscuta*'s pathogenicity.

2 | MATERIALS AND METHODS

2.1 | Plant material and *Agrobacterium* strains

Cuscuta reflexa, *Cuscuta campestris*, and *Cuscuta platyloba* were grown in a greenhouse on the host *Pelargonium zonale* under continuous illumination and a constant temperature of 21°C (Förste et al., 2020). The bacteria and binary T-DNA-containing vectors pRedRoot and XM82, respectively, were kindly contributed by Prof. Harro Bouwmeester (University of Amsterdam, Netherlands) (*A. rhizogenes*) and Prof. Tessa Burch-Smith (University of Tennessee, USA) (*A. tumefaciens*) and are described in more detail in other studies (Limpens et al., 2004; Libiakova et al., 2018; Bobik et al., 2019). Cultures of *A. rhizogenes* MSU440 and *A. tumefaciens* GV3101 without binary plasmids were grown on LB medium (tryptone 10 g/L, NaCl 10 g/L, yeast extract 5 g/L, agar 7,5 g/L) supplemented with 100 mg/L Spectinomycin or 50 mg/L Rifampicin plus 50 mg/L Gentamycin, respectively. For bacteria containing the respective binary vectors, Kanamycin at 50 mg/L was added to the growth medium.

2.2 | Induction of infection structure formation by far-red light

For induction of infection structures, *Cuscuta* apical shoots of approximately 12 cm were harvested from the stock plant and exposed to far-red light (740 nm) in an otherwise dark room for 90–120 min as described before (Olsen et al., 2016) with modifications. The steps were conducted in far-red light to not reverse the induction (Tada et al., 1996). To provide a tactile stimulus, four shoots of roughly equal diameter were placed next to each other between two layers of bench paper with one-sided plastic coating (Whatman® Benchkote® surface protector; Cat. # 2300731) that was moistened

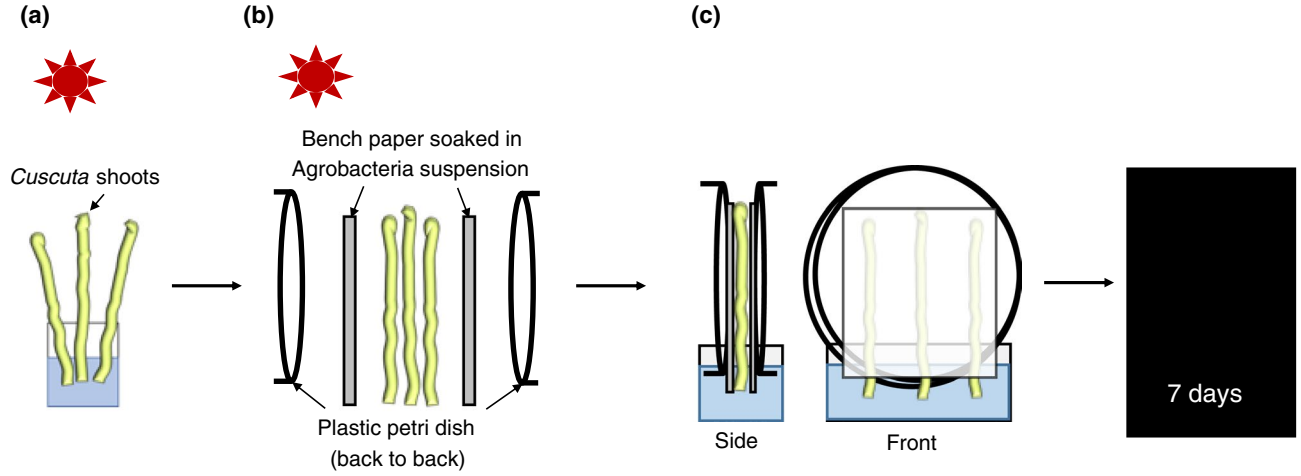

FIGURE 1 Overview of the experimental setup. (a) Exposure of *Cuscuta* shoots to far-red light (740 nm) for 90–120 min. (b) Placement of *Cuscuta* shoots between *Agrobacterium*-soaked bench paper sheets and two petri dish halves (back to back). (c) Incubation in darkness with shoots and bench paper placed in a water reservoir

with tap water (filter-paper side facing the shoots). This set-up was carefully placed between two back-to-back facing petri dish halves (with the filter paper and the cut ends both protruding into a container with tap water [see Figure 1]). Moderate pressure was applied by taping the two petri dish plates together. When kept at room temperature in darkness, infection structures started to develop after about 3 days (Olsen et al., 2016).

2.3 | Transformation of *Cuscuta* cells

An *Agrobacterium* culture was grown overnight in selective media (see above) and adjusted to an OD (600 nm) of 1–1.6, before using 2 ml of this suspension to soak the paper side of the bench paper (approximately 8 × 8 cm area). For mock controls, agrobacteria lacking a T-DNA-encoding binary vector were used. The assembly with far-red light-treated *Cuscuta* stems was done as described above and the whole setup was then incubated in a dark incubator set to room temperature for 7 days. After disassembling the setup, shoots were briefly rinsed under tap water, remnants of filter paper sticking to the adhesive disks were carefully removed without damaging the plant tissue, and stems were kept for up to 2 days in water or wrapped in wet paper towels before being subjected to microscopical analysis.

For exchange of water to *Agrobacterium* culture and *vice versa* (see Tables S1 and S2), the setup was disassembled on day 3 under far-red light (740 nm) and the bench paper layer exchanged before the setup was re-assembled and subjected to further incubation in the dark for another 5 days.

2.4 | Microscopical imaging

Fluorescence localization in the *Cuscuta* stems exposed to agrobacteria and in the corresponding controls was documented using a StereoLumar V12 stereo microscope or an Axiovert M200 inverted microscope (both from Zeiss) using Zeiss filter sets 43 (for dsRed) and 38 (for GFP). Images were taken using the Axiovision Software (Version 4.8.2). The same exposure times were used for the different fluorescence filter sets for a given sample or magnification, unless specified otherwise. FIJI/ImageJ (V 2.0.0) was used to analyze the pictures, add scale bars, and produce overlays. When adjusting brightness, contrast, minimum and maximum intensities, all fluorescence images of one set were treated alike.

Fluorescence intensity scanning was performed on the marked area containing the region of interest (see Figure S5) using the histogram function of FIJI/ImageJ. Intensity counts were exported to Microsoft Excel for visualization in one joint colored graph.

2.5 | Vibratome sectioning

Transformed infection sites were cross-sectioned using a Vibratome (Leica VT1000 E vibrating blade microtome). Section thickness was 100 µm. Sections were viewed and documented using a StereoLumar V12 stereo microscope or an Axiovert M200 inverted microscope (Zeiss) using Zeiss filter sets 43 (for dsRed) and 38 (for GFP).

2.6 | Life cell staining with 5-carboxyfluorescein di-acetate (CFDA)

To evaluate the vitality of the transformed cells, the vital stain CFDA (50 mM in DMSO) was diluted immediately prior to use to a final working concentration of 1 mM in water. Stems were covered with a thin layer of CFDA by spreading small drops of a few µl each evenly over the *Cuscuta* stem and infection sites (adjusted from "Drop-And-See assay" [Cui et al., 2015]). After incubation for 10 minutes in the dark, the CFDA was removed by briefly rinsing with tap water, gently dried with paper, and viewed under a StereoLumar V12 stereo microscope using Zeiss filter set 38.

2.7 | Cultivation of explants

Cuscuta reflexa stems with infection sites and confirmed transformation events were sterilized for 2–5 min in 70% Ethanol and during this time gently cleaned using a brush. After a subsequent 15 min incubation step in 1.2% Sodium hypochloride, the stems were incubated on a shaker in sterile tap water containing 400 mg/L Cefotaxime overnight, then cut into pieces that contained one or two infection structures each and transferred to MS (Murashige and Skoog) Basal Medium supplemented with 0.8% Agar, 3% Sucrose, MS Vitamin solution, and 400 mg/L Cefotaxime. Plates were covered with aluminum foil to avoid photobleaching and were kept at 23°C. Explants were transferred to fresh medium approximately every 4 weeks.

3 | RESULTS

3.1 | *Agrobacterium rhizogenes* does not induce hairy roots in *Cuscuta* species

A. rhizogenes is typically applied to the roots (Ho-Plagaro et al., 2018), hypocotyl (Alagarsamy et al., 2018) or cotyledons (Ron et al., 2014) of dicotyledonous angiosperms by cutting, puncturing, or otherwise wounding these tissues. In the absence of proper roots, cotyledons, or other leaves, we first tested hairy root induction on young germinating seedlings of *C. campestris* and on different parts of the shoots from different *Cuscuta* species using *A. rhizogenes* with and without the pRedRoot T-DNA. Occasionally, transformed cells exhibiting an intense orange fluorescence from expression of the reporter protein dsRed have indeed been observed, particularly in shoot tips (Figure S1). However, no root development could be observed in any of our trials with the strain MSU440, independent of whether it carried the pRedRoot T-DNA or not and transformation success reliability was poor.

3.2 | *A. rhizogenes* transforms adhesive disk cells of *C. reflexa* with high efficiency

We next decided to expose developing infection structures to a pRedRoot-containing *A. rhizogenes* culture. For this we used the method for induction of haustoriogenesis in *C. reflexa* described by Olsen et al. (2016) that uses a combination of far-red light illumination and tactile stimuli to synchronize haustorial development (Tada et al., 1996). The stems on which the infection sites developed where exposed to *A. rhizogenes* for as long as it took for haustoria to emerge (7–8 days) (Figure 1). Upon microscopical analysis, a large number of intensely orange-fluorescing cells expressing dsRed were revealed that were almost exclusively located in the adhesive disks around the protruding haustorium (Figure 2a–f). The dsRed fluorescence was visible in distinct spots often consisting of 5–15 clustered cells, but single transformed cells and bigger clusters were also observed (Figure 2). Cross sections through sites where transformation had occurred revealed that the cells expressing the dsRed were mostly not located at the very surface but rather in a cell layer directly below the elongated epidermal cells (Figure 2g–l). Fifty-two percent of the adhesive disks exhibited one or more spots with dsRed fluorescence (based on 52 infected shoots with 426 infection structures) (Table 1), but there was considerable variation between individual shoots. While some green and blue autofluorescence was observed in the central haustorial tissue (Figure S2), the adhesive disk of *C. reflexa* exhibited little to no autofluorescence, as demonstrated by mock transformations with *A. rhizogenes* cells that lack the pRedRoot T-DNA (Figure 2m,n). Experiments where *A. rhizogenes* was removed or added after 2 days, showed that the uptake of the T-DNA in the first 2 days is minimal to absent, and seems to happen only once the development of the infection sites has commenced (Tables S1 and S2).

3.3 | Adhesive disk cells maintain their integrity and viability during the transformation process

Life cell stains like CFDA are membrane permeable and are hydrolyzed in the cytoplasm to the green fluorescent carboxyfluorescein. In the present case, CFDA fluorescence in the adhesive disks would be evidence that the transformed cells maintain their integrity and viability during the treatment and transformation. When shoots that showed dsRed fluorescence in the adhesive disks where exposed to a CFDA solution, the adhesive disks and often also the haustoria exhibited green fluorescence (Figure 3). CFDA fluorescence was also observed regularly in young side-shoot buds but only very rarely in the intact stems (Figure S1). The fluorescence was observed within 1 minute in the adhesive disks (Figure S3). This indicates that the cuticle that protects *Cuscuta* stems and that hinders the uptake of the stain into stems is most likely "leaky" or absent in the adhesive disks and haustoria, thus permitting the stain to enter the respective tissue with such efficiency.

3.4 | Application of the transformation protocol is not limited to *A. rhizogenes*

In order to reveal whether the high transformation rates were a result of a specific susceptibility of *C. reflexa* to *A. rhizogenes*, we exposed far-red light induced stems to *Agrobacterium tumefaciens* carrying a GFP gene in the T-DNA of a binary plasmid (Bobik et al., 2019) using the same setup. Only very weak background fluorescence was seen in this case in the orange channel and in the blue channel, while the high intensity of green fluorescence in a ring corresponding to the adhesive disk indicated that the GFP was expressed in this tissue as a result of the transformation (Figure 4A–I). As with the dsRed, GFP was expressed in elongated cells beneath the layer of epidermal cells in the adhesive disk (Figure S4). The infection frequency was on average 43% for

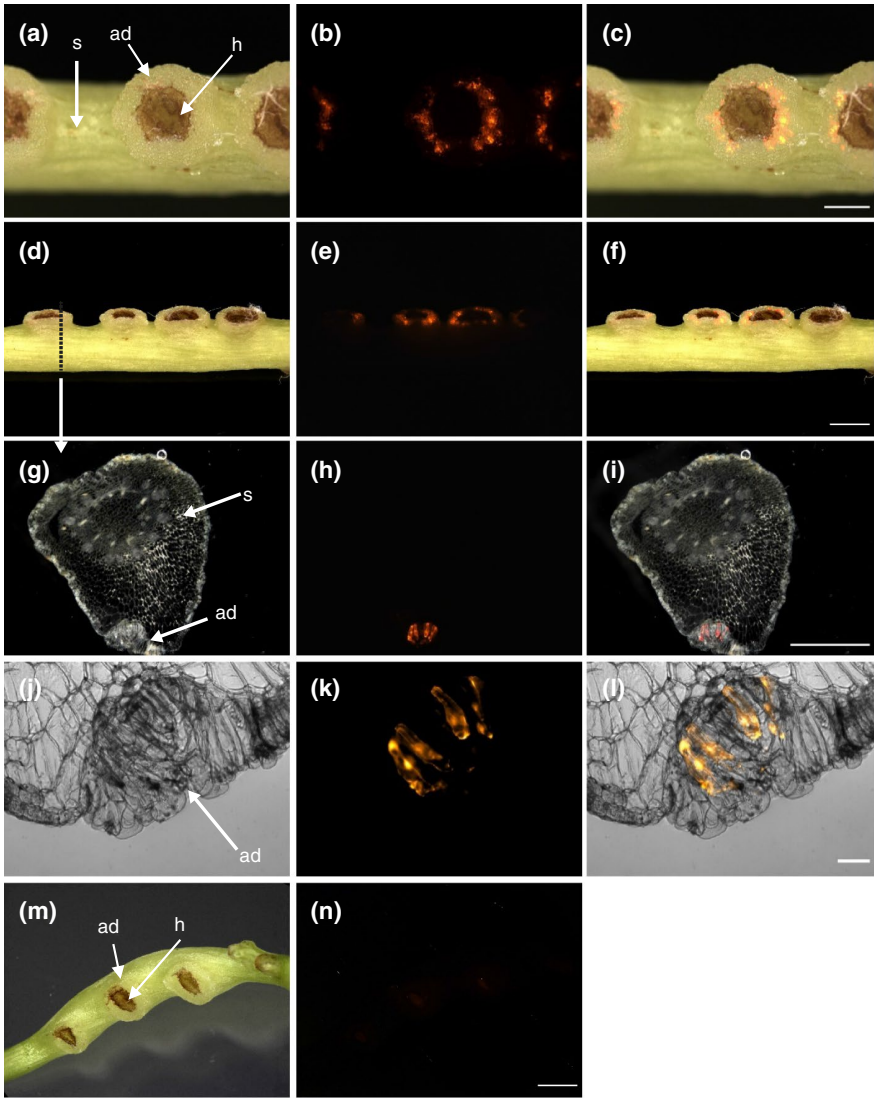

FIGURE 2 Transformation of *C. reflexa* adhesive disk cells by *A. rhizogenes* containing the binary vector pRedRoot. (a-f) Intact infection sites after transformation. Topview (a-c) and sideview (d-f) of transformed adhesive disks are shown. (g-l) Semi-thin vibratome sections of transformed adhesive disk tissue in the region of the stippled line (in d) show subepidermal localization of transformed cells in the adhesive disk (ad). (m, n) Mock transformation with *A. rhizogenes* lacking the binary pRedRoot plasmid. Darkfield or brightfield pictures (first column) are shown alongside the fluorescence images taken with a Cy3 filter (middle column). Overlays of both are shown in the right column. Adhesive disks (ad), haustoria (h), and stems (s) are indicated by arrows. Scale bars are 1,000 μm (c and i), 2,000 μm (f and n), and 100 μm (l)

TABLE 1 Transformation efficiency overview

Number of shoots	# infection sites total	# adhesive disks transformed	*Agrobacterium* strain	Reporter fluorochrome
6	61	46	*A. rhizogenes* MSU440	dsRed
4	16	13	*A. rhizogenes* MSU440	dsRed
6	37	33	*A. rhizogenes* MSU440	dsRed
4	77	37	*A. rhizogenes* MSU440	dsRed
8	41	11	*A. rhizogenes* MSU440	dsRed
12	57	38	*A. rhizogenes* MSU440	dsRed
12	137	43	*A. rhizogenes* MSU440	dsRed
3	21	11	*A. tumefaciens* GV3101	GFP
3	25	8	*A. tumefaciens* GV3101	GFP
8	63	15	*A. tumefaciens* GV3101	GFP
8	52	15	*A. tumefaciens* GV3101	GFP
8	113	74	*A. tumefaciens* GV3101	GFP
8	93	49	*A. tumefaciens* GV3101	GFP
5	38	0	*A. rhizogenes* MSU440	None
4	28	0	*A. rhizogenes* MSU440	None
4	50	0	*A. rhizogenes* MSU440	None

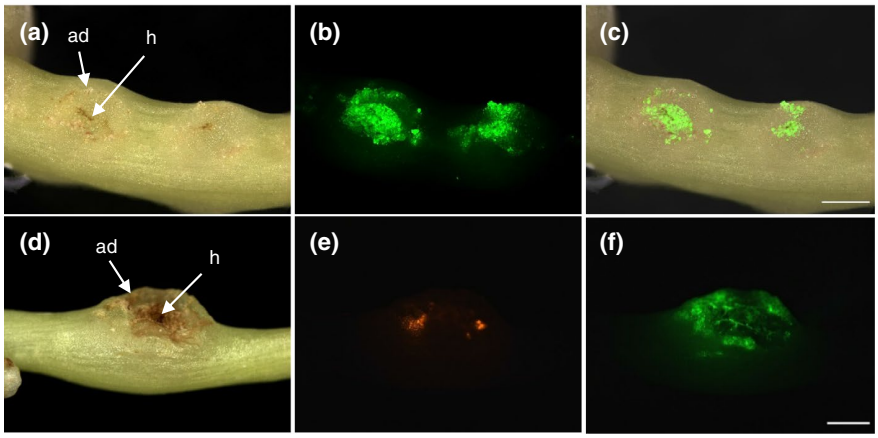

FIGURE 3 Uptake of 5-Carboxyfluorescein-diacetate (CFDA) into adhesive disks of *C. reflexa*. (a–c) Green CFDA fluorescence is shown in a shoot segment containing two developing infection sites. A superimposition of the images from (a) and (b) is shown in (c). (d–f) CFDA uptake into an adhesive disk containing dsRed-expressing cells. Darkfield images (left column) and fluorescence images (middle column and lower right image) are shown. Adhesive disks (ad) and haustoria (h) are indicated. Scale bars are 1,000 μm

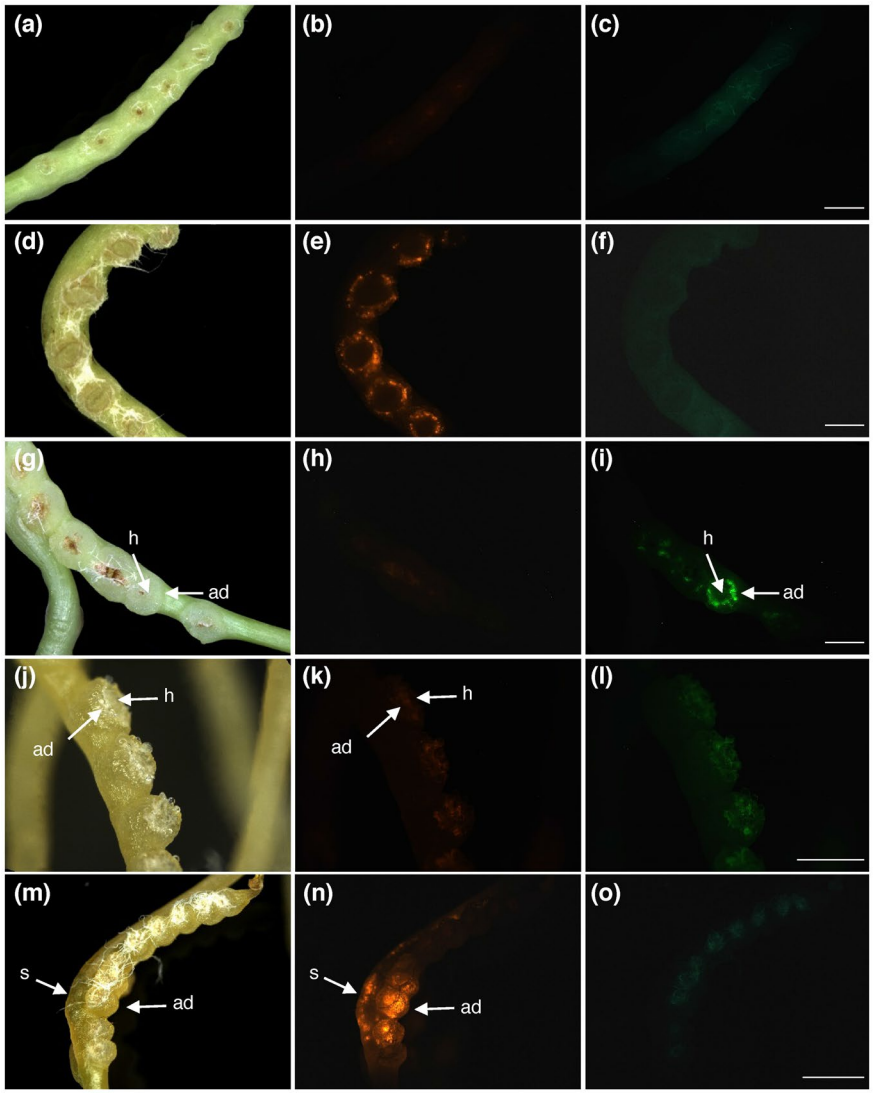

FIGURE 4 Extension of the protocol to *A. tumefaciens* and *C. campestris*. (a–f) Negative (a–c) and positive (d–f) controls using the combination of *C. reflexa* and *A. rhizogenes* (see also Figure 2). (g–i) Transformation after combining *C. reflexa* with *A. tumefaciens* containing a binary GFP-expressing vector. (j–o) Negative control (j–l) and pRedRoot transformation (m–o) using the combination of *C. campestris* and *A. rhizogenes*. Scale bars represent 2,000 μm (c, f, i and o) and 1,000 μm (l), respectively. White fibers of the bench paper from the experimental setup can be seen adhering strongly to the adhesive disks in some darkfield images (d, g, j and m)

A. tumefaciens (based on 38 infected shoots with 367 infection structures) (Table 1).

3.5 | Transformation of other *Cuscuta* species

Within the genus *Cuscuta*, three subgenera are distinguished: subgenus *Monogyna*, which includes *C. reflexa*, subgenus *Grammica*, and subgenus *Cuscuta* (Yuncker, 1932). To test whether our protocol is applicable to *C. campestris* whose sequenced genome (Vogel et al., 2018) would make it a very interesting target for genome modifications, we repeated the same transformation setup with this species (Figure 4) and a third *Cuscuta* species, *C. platyloba* (Figure S1), both belonging to the subgenus *Grammica*. With both *Agrobacterium* species, a higher degree of necrotic tissue was observed in these two species as a result of this treatment, which, in turn, created a higher amount of unspecific autofluorescence. While adhesive disk transformation could be observed in *C. campestris* (Figure 4K–L), it was by far not as frequent as in *C. reflexa* and was often weaker than in stem tissue adjacent haustoria-forming sites.

3.6 | Simultaneous exposure to both *Agrobacterium* strains yields a high number of cotransformation events

A desired feature of transformation protocols is the possibility to express multiple transgenes in the same cell. This can be achieved by time-consuming sequential transformation or the cloning of suites of genes into the T-DNA of one vector, often yielding large unwieldy constructs. However, the high susceptibility of the same *C. reflexa* tissue to both, *A. rhizogenes* and *A. tumefaciens*, opens for the possibility of introducing multiple constructs into the same cell by co-infection. To achieve this, both species of *Agrobacterium* carrying each their respective fluorescent reporter construct (dsRed in *A. rhizogenes* and GFP in *A. tumefaciens*) were mixed in a 1:1 ratio (based on their ODs at 595 nm) prior to exposing the *C. reflexa* stems to them in our transformation setup. Fluorescence microscopy revealed that both, dsRed and GFP, were visible with similar yields in the adhesive disks as in single transformation experiments. A considerable amount of overlapping fluorescence indicated that cotransformation did in fact occur at a high rate (Figure 5A–D). In order to see whether the same cells (and not just cells in the same area) indeed expressed both fluorescent proteins, we prepared semi-thin cross sections through transformed regions and documented the fluorescence location with microphotography (Figure 5E–H) and by densitometry scanning of fluorescence intensities over an area containing several transformed clusters (Figure 5I, Figure S5). Both revealed an exact coincidence of the two fluorophores in several cells, suggesting that there are hot spots of susceptible tissue that is frequently co-transformed. At the same time, the occurrence of cells transformed with only one fluorochrome shows that each fluorescence signal is specific.

3.7 | Transgene expression after a transformation event is preserved over several weeks

The frequent occurrence of transformed cell clusters raised the question whether these arose through cell division and propagation of single transformed cells, indicating not only a stable insertion of the transgenes but, moreover, also the possibility to regenerate vegetative or reproductive tissue by in vitro propagation from the transformation events. To test this, we sterilized explants containing transformed tissue and maintained them in in vitro cultures. The explants showed slight growth of cells at the edges of the adhesive disks, including the transformed regions, but significant propagation was not observed over a period of up to 8 weeks. The fluorescence was consistently high for at least 4 weeks (Figure 6) but started to decrease during longer cultivation times.

4 | DISCUSSION

In the last few years, research on *Cuscuta* has seen a rapid increase and with the publication of the first two genome sequences of *C. campestris* (Vogel et al., 2018) and *C. australis* (Sun et al., 2018), our knowledge on these parasites has experienced a significant leap forward. Nevertheless, there are still several obstacles that need to be overcome before the possibilities offered by these genomes can be fully exploited. The main bottleneck is the lack of an efficient and reproducible protocol with which transgene expression or even genetic manipulations can be performed. Previous attempts to transform *Cuscuta* have shown that *A. tumefaciens* is able to transform callus cells (Borsics et al., 2002; Svubova and Blehova, 2013). However, in our hands these transformation events were very scarce, explaining maybe why this approach has not yielded greater success.

With the work presented here, we show that transformation of *Cuscuta reflexa* works very efficiently when developing infection sites are targeted. As our experiments show, the protocol works with both *A. rhizogenes* and *A. tumefaciens*, and allows the expression of reporter proteins from different binary vectors and under the control of different promoters. The T-DNA of pRedRoot, a binary vector developed for *A. rhizogenes*, contains the dsRed protein under control of the Ubi10 promoter (Libiakova et al., 2018), while the *A. tumefaciens* line used by Bobik et al. (2019) contains a GFP gene controlled by the 35S promoter. Both promoters allow strong constitutive expression of transgenes. It remains to be shown if other – in particular inducible – promoters work in *Cuscuta* as well as the standard constitutive promoters do, or if adaptations are required for use with the parasite. The simplicity of the method (low-tech, cheap, and easy-to-implement) (Figure 1) and the high reproducibility make it suitable for larger-scale screening approaches or to multiplex functional studies. The high numbers of transformed cells and the longevity of transgene expression, furthermore, permit investigations of transgene product behavior, protein localization or secretion over time, and in situ interaction assays between molecules or cells.

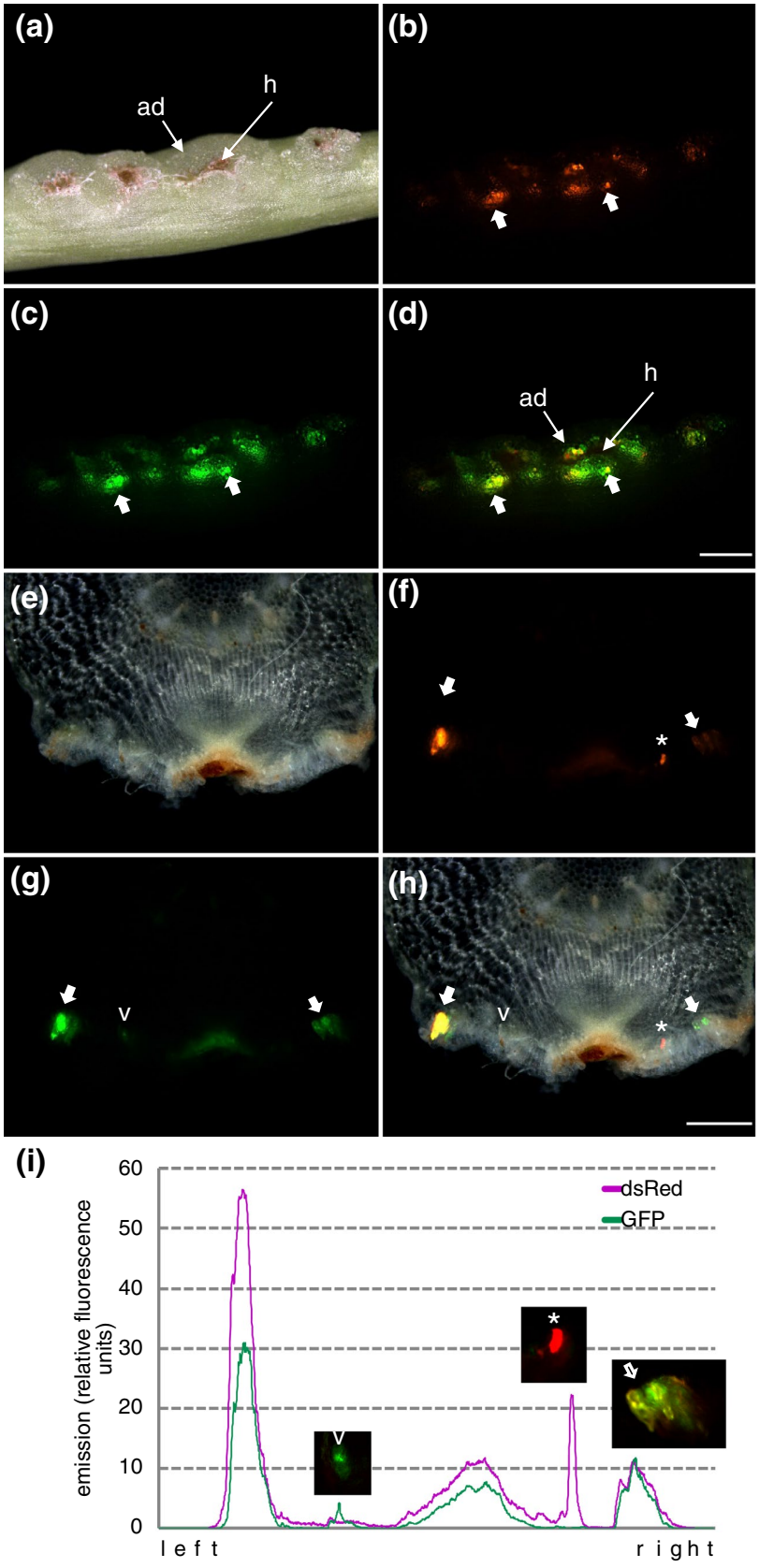

FIGURE 5 Cotransformation of different reporter proteins in *C. reflexa* adhesive disks. DsRed was introduced with *A. rhizogenes* while GFP was introduced with *A. tumefaciens*. (a–d) Side view of intact stem with darkfield (a), red fluorescence (b), and green fluorescence (c) images. Figure (d) shows a superimposed image of the two fluorescence images with thick arrows pointing at spots where both reporter proteins coincide (yellow color due to overlay). The scale bar measures 1,000 µm. (e–h) Cross section through a transformed infection site with darkfield (e), red fluorescence (f), and green fluorescence (g) images. Figure (h) shows a superimposed image of all three images. The scale bar measures 500 µm. The asterisk and the arrowhead indicate cells that are transformed with only one reporter protein. The thick arrow indicates cells were both reporter proteins coincide. (i) Intensity scan performed on the two single fluorescent images with the purple line representing the dsRed fluorescence and the green line representing the GFP fluorescence

The high transformation rates specifically of adhesive disk cells are certainly noteworthy. These cells are naturally in close contact with the host and may thus exhibit a higher propensity to take up different types of substances, but the same is true for the haustorium cells which did not show the same aptness for transformation. It is not yet clear what structural or metabolic characteristics are

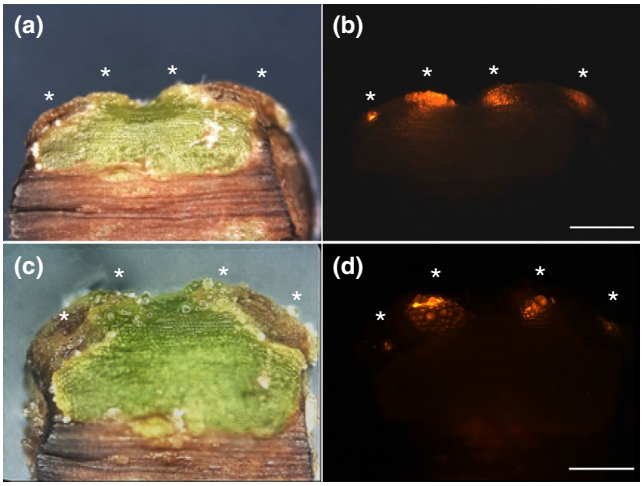

FIGURE 6 Cultivation of pRedRoot-expressing explants from *C. reflexa*. The shown explant contains two infection structures with transformed adhesive disks. Asterisks indicate clusters of transformed cells. Images were taken after 5 days (a and b) and 4 weeks (c–d) in sterile culture. Brightfield images are shown next to the red fluorescence. The scale bars represent 1,000 μm

responsible for this behavior, but some speculative scenarios are more likely than others and shall be discussed here.

Agrobacteria are known to be attracted to polyphenolic substances exuded by plant roots (Ozyigit et al., 2013). Haustorial sites are also rich in hydroxycinnamic acid derivatives, caffeic acid depsides and other polyphenols (Löffler et al., 1995), which is evident by the rapid browning of infection sites upon their exposure to air (Johnsen et al., 2015). Comparably high concentrations of phenolic substances, albeit in a slightly different composition, are interestingly also found in the meristematic apex of *Cuscuta* shoots (Löffler et al., 1995). After adhesive disks, shoot tips were the second tissue that showed a heightened susceptibility toward agrobacterial infection tentatively suggesting that infection success can in part be explained by a metabolically driven attraction of the agrobacteria to these sites. However, attraction by polyphenols does not explain why only the adhesive disks and not the haustoria are transformed as the latter exhibit high phenolic substance production as well (Löffler et al., 1995). Both, haustoria as well as the surrounding adhesive disks, are characterized in general by a high metabolic activity, so the differential transgene expression is likely not caused by differences in transcription and translation activities. The fluorescent cell integrity indicator CFDA stains adhesive disk cells when applied externally to the surface (Figure 3 and Figure S3), indicating their viability throughout the transformation process. The uptake of the CFDA happens with such high speed (Figure S3) that it is reasonable to conclude that the protective cuticular layer that ostensibly slows down its uptake in stems is altered or missing. Although CFDA follows a different uptake route than the T-DNA, which is delivered by agrobacterial cells through a type IV secretion system, the similarities in staining and transformation patterns suggest that the nature of the surface and its permeability may influence the ability of agrobacteria to infect the cells beneath. This as well as the presence of potentially other mechanisms in *Cuscuta* that can regulate agrobacterial infection success should be investigated further in the future.

None of the species that belong to the genus *Cuscuta* possesses roots and there are, therefore, no obvious natural targets of the plant pathogenic bacterium *Agrobacterium rhizogenes*. Nevertheless, some *Cuscuta* species were recently shown to have acquired a gene coding for the Mikimopine synthase (*mis* gene) (Zhang et al., 2020) that is typically transferred to plants during *A. rhizogenes* infection in order to supply the bacterium with opines. Plant homologues of the *mis* gene are found only in a handful of plant species belonging to the genera *Nicotiana* and *Linnaria* where they are believed to have originated by three independent horizontal gene transfer (HGT) events (Kovacova et al., 2014). These species do not display the hairy root syndrome, which hypothetically could be attributed to the HGT-derived *mis* gene. By way of small interfering RNAs from the HGT-derived *mis* that may degrade T-DNA-borne *mis* transcripts during an infection, these acquired genes could potentially prevent *A. rhizogenes* growth. Their evolution under selective pressure and the coverage of *mis*-derived siRNAs, at least, seem to corroborate this possibility for *Nicotiana* (Kovacova et al., 2014). It can be debated whether this could also be the case in *Cuscuta*. However, the *mis* gene was so far only found in species belonging to the subgenus *Grammica* (Zhang et al., 2020), but was not detected in a transcriptome database (Olsen et al., 2016) of *C. reflexa*. Therefore, it is more likely that the loss of key genes involved in root development is responsible for the failure to produce hairy roots in *A. rhizogenes*-infected *Cuscuta* tissue.

5 | CONCLUSIONS

Using fluorescent reporter proteins and far-red light mediated infection structure induction, we have shown that the adhesive disk of *C. reflexa* is highly susceptible to *Agrobacterium*-mediated transformation. With the high number of transformation events that were observed using our protocol and with the stability of transgene expression, it will be possible to perform transformations with a high number of constructs. Our trials with other *Cuscuta* species showed some transformation success but species-specific adaptations may be required to apply the technique to other dodder species. Our protocol is very useful to provide further functional insight on *Cuscuta* and its enigmatic infection structure because it is cheap, low-tech, easily scalable, and suitable for transformations of genes at low or high throughput. Moreover, if the transformed cells can be induced to produce callus and ultimately whole regenerated plants, it will enable plant scientists to harness the genome sequence information and create *Cuscuta* mutants.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

L.G. and K.K. designed the transformation setup; L.L. and L.G. performed the transformation experiments; L.L. performed the life cell staining experiments, evaluated all data, and performed image analyses; K.K. conceived the project and wrote the article with contributions of all the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Paper 2

Cuscuta campestris tissue culture and seedling transformation via *Agrobacterium rhizogenes*

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Keywords: *Agrobacterium rhizogenes*, *Cuscuta*, plant tissue culture, parasitic plants, transformation

Keymessage: Seedlings of the parasitic plant *Cuscuta campestris*, a notorious agricultural pest and interesting research model, can be cultivated best by addition of kinetin. Cells in their stem tip can be transiently transformed by cultivation and co-cultivation with *Agrobacterium rhizogenes*.

Abbreviations:

2,4-D - 2,4-Dichlorophenoxyacetic acid, BAP – benzylaminopurine, dsRed - Discosoma Red (Red fluorescent protein), NAA - 1-Naphthaleneacetic acid, UBQ - Ubiquitin 10 promoter, virus induced gene silencing (VIGS)

Structured abstract:

- In order to develop the parasitic plant *Cuscuta campestris* into a research model, we are lacking a tool for functional in situ protein studies.
- A new method is presented for cultivation of young seedlings of *C. campestris* and transgene introduction that uses *Agrobacterium rhizogenes* for vector delivery.
- This method allows for easy transformation of cells in *C. campestris* seedling shoot tips.
- It can be used alone or in conjunction with other methods to conduct studies on protein localization, interaction and movement and has the potential to be developed into a pipeline for creating mutant *Cuscuta* plants.

Abstract

Cuscuta campestris is a parasitic plant with a broad host spectrum that feeds off its host by establishing ties to its vasculature system. It is also one of two *Cuscuta* species whose genome has been published. Knowledge about the functional processes behind its parasitic connection to the host plant would be the key to unravel its secrets and pave the way to crop protection against this agricultural pest and, at the same time, allowing for development of this plant as a versatile research model. Tissue culture techniques for *C. campestris* have not been published so far and whole plant regeneration of different *Cuscuta* species has repeatedly failed after transformation of cells. Thus, this study addresses the development of callus development and shoot regeneration as well as a transformation protocol for *Cuscuta campestris* seedling cells to allow functional in situ protein studies at a very early developmental stage of the parasite. Cells of cultivated seedlings were successfully transformed using *Agrobacterium rhizogenes*. Seedlings with transgenic cell patches were cultivated for several weeks. In simplicity, this approach matches the protocol for transformation of haustoria published earlier and will be a useful tool for comparative studies between the two processes of germination and infection that are crucial to the parasite's success. With both tissues having meristems of different origin, the protocol also allows for investigative comparisons of regulatory processes. Furthermore, seedlings are an excellent material for attempts to extend the protocol and develop it into a pipeline for creation of mutant *Cuscuta campestris* plants by regenerating the transformed cells into whole plants.

Introduction

Cuscuta campestris is a parasitic plant that is reduced to a vine without roots or leaves (Fig. 2A) and one of the *Cuscuta* species that count as an agricultural pest (Dawson et al., 1994). It lives off water and nutrients that it draws from its host by connecting its own vasculature system to the host phloem and xylem (see Shimizu & Aoki, 2019 as a review on the topic) and it is one of two *Cuscuta* species, whose genome has been published (Vogel et al., 2018). Additionally, extensive transcriptomic studies have been performed on it (Bawin et al., 2022; Jhu et al., 2021; Ranjan et al., 2014). Thus, *C. campestris* is a natural choice as a research model system for further exploration of general plant biological questions as well as in the development of a strategy to defend crops against this parasite. However, there is up to date no protocol that describes callus culture and regeneration methods in *C. campestris*. Equally, no method exists that allows for genetic modification with subsequent shoot or whole plant regeneration for any *Cuscuta* species despite a considerable amount of work on that topic in several laboratories around the world. Regeneration of shoots from callus derived from *Cuscuta* seedlings has so far only been described for *Cuscuta trifolii* (Bakos et al., 2000; Bakos et al., 1995). However, after applying an *Agrobacterium tumefaciens* based transformation protocol, regeneration could no longer be achieved (Borsics et al., 2002). A novel approach for transforming *C. reflexa* haustorial cells by *Agrobacterium rhizogenes* and or *Agrobacterium tumefaciens* has been shown recently (Lachner et al., 2020). That approach likewise failed to regenerate stable transgenic plants but showed that the fluorescent transgenes were expressed over weeks and in

several adjacent cells in the region located next to the parasitic organ, called the adhesive disk. An alternative to the creation of a transgenic plant could be the use of a gene silencing approach on *C. campestris*, like virus induced gene silencing (VIGS), which has recently been reported by Dyer et al. (2021). However, gene silencing in *C. campestris* could so far only be achieved in close vicinity around the infection site, not in growing shoot tips where it would be needed to best assess an effect on the infection mechanism. Overall, until the bottleneck of creating stable, truly transgenic *Cuscuta* plants or other possibilities for carrying out gene function studies has been overcome, sets of complementary approaches of expressing foreign genes is our only chance to use the genomic information of *Cuscuta* for functional investigations. In this spirit, the current study presents a new method to transform *C. campestris* cells in seedlings via *Agrobacterium rhizogenes*.

Methods

Plant material and *Agrobacterium* strains

Cuscuta campestris plants were grown at the Holt Climate Laboratory in Tromsø under continuous illumination and a constant temperature of 21°C (Lachner et al., 2020). *Pelargonium zonale* was used as host plant and the *C. campestris* culture was regularly transferred to a fresh host plant to be maintained. *C. campestris* seeds were collected when ripened and stored in a dry place at room temperature for at least half a year until use (Hutchison & Ashton, 1980). *Agrobacterium rhizogenes* MSU 440 transformed with pRedRoot, a binary vector containing a dsRed fluorescence marker under the control of the *Arabidopsis* ubiquitin 10 promotor (UBQ) was kindly contributed by Prof. Harro Bouwmeester (University of Amsterdam, Netherlands) and is described in more detail in other studies (Libiaková et al., 2018; Limpens, 2004). It was grown on LB medium with the respective selective antibiotics (Kanamycin at 50 mg/L).

Seed germination and explant sterilization

C. campestris seeds were incubated in 98% Sulfuric acid for 25 min to scarify and sterilize them (Benvenuti et al., 2005; James M. Hutchison & Ashton, 1979), then rinsed with autoclaved tap water 5-6 times to remove the sulphuric acid and poured onto water agar for germination with the remaining rest of water. Plates containing seeds were stored at 28 °C in darkness for 4-5 days until germination had started before seedlings were either cut in pieces of 0.5-1 cm length or added to culture medium as a whole. *Cuscuta campestris* shoots were sterilized for 1 min in 70% Ethanol and for 3-5 min in 1.2% Sodium hypochlorite before being thoroughly washed in autoclaved tap water. Shoot tips were cut in pieces of about 0.5-1 cm length and incubated on solid culture plates or added to liquid culture flasks.

Tissue culture of *C. campestris*

Half strength Murashige and Skoog (½ MS) (Sigma) was used as a basal medium, 3% sugar, with equal amounts (1.5% each) of sorbitol and maltose and MS vitamin solution (Sigma) according to the manufacturer's specifications applied at a dilution of 1:1000, was added. The tissue culture medium was used as either liquid solution or solidified to plates by adding 0.8% agar. The plant hormones kinetin (Kin), benzylaminopurine (BAP), 1-Naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) were added to the medium after

autoclaving at varying concentrations. For a standard medium, 5 mg/mL kinetin without the addition of other hormones was supplemented. Explants (seedlings or shoot tips) were transferred to either a 100 mL culture flask containing 15 mL of liquid medium, or solid plates with the cut ends pushed slightly into the medium. Liquid cultures were incubated in the dark at 27 °C at 200 rpm and the medium exchanged weekly, solid plates were incubated at 23 °C, at low light conditions and plantlets were transferred to fresh medium every 4 weeks.

Agrobacterium Transformation

Liquid cultures of *A. rhizogenes* were grown overnight at 28°C, the OD600 adjusted to 1, and 200 µM of Acetosyringone was added. Before co-cultivation, *C. campestris* plantlets in liquid culture were transferred to solid medium. Co-cultivation of *C. campestris* and *A. rhizogenes* was performed on ½ MS plates without hormones at 23°C in the dark for 48 h. After co-cultivation, plates were rinsed with water containing either 150 µg/mL timentin or cefotaxime or carbenicillin at 400 µg/mL each, for 20 minutes. Plantlets were then transferred to fresh plates containing standard medium (½ MS, vitamins, sorbitol and maltose (1.5% each), 0.8% agar and kinetin at 5 mg/mL) with 150 µg/mL timentin or cefotaxime or carbenicillin at 400 µg/mL each added. Plantlets were transferred to fresh plates every 2 weeks when the medium contained antibiotics.

Microsopy and imaging

For imaging of cultures and detection of dsRed fluorescence indicating successful transformation, a StereoLumar V12 stereo microscope (Zeiss) with Zeiss filter sets 43 (for dsRed), 38 (GFP) and 01 (autofluorescence) was used. Microscopic Images were taken using the Axiovision Software (Version 4.8.2). A standard camera (Olympus stylus tough TG4) was used for imaging whole plates, plants, and cultures in liquid containers. Colour, growth, and formation of callus tissue of the explants were evaluated to rate success of culturing (0, +, ++) (Fig. 1).

Results

Effect of different media and hormone compositions on callus and shoot growth

Figure 1 shows a schematic of initial trials that were used to determine suitable conditions for C. *campestris* culture. Seeds and shoots are abundantly produced in greenhouse-grown parasite cultures on a host. (Fig. 2A). We found that *C. campestris* tolerated ½ MS medium better than full strength MS and the use of seedlings (Fig. 2B) as explants was more successful than shoot tips. For growth in liquid solution, seedlings needed to be used whole while cutting the seedlings was necessary for growth on solid plates. Hormone trials on solid plates were successful with Kinetin only at 5-25 mg/L and BAP at 12 mg/L. Combinations of BAP and NAA in different concentrations and relations from 0.5 to 24 mg/L for BAP and 1-24 mg/L for NAA, 2-4D applied at concentrations from 1-3 mg/L as well as a combination of kinetin, NAA and 2,4-D at concentrations from 1-3 mg/L did not lead to successful callus growth in solid cultures. Kinetin and NAA combined at 5 mg/L each, Kinetin at 5 mg/L and 2,4-D at 0.75 mg/L combined and NAA only at 5 mg/L were tested in liquid cultures. The best results overall were achieved when adding Kinetin only at 5 mg/mL to the liquid medium or Kinetin up to 25 mg/L

on solid plates. A combination of callus growth and shoot aggregate growth was observed as a result. Kinetin in combination with NAA or 2,4-D did result in reduced callus growth, while the addition of only NAA to the medium yielded no growth at all. Too high concentrations of kinetin were reached and inhibited any kind of growth, when applying 50 mg/L on solid plates. The same was true for BAP at concentrations of 25 mg/L. When seedlings were cut and kept on solid plates, callus structures developed after about 1-2 months (Fig. 2C), transitioned into shoot aggregates after about 2-3 months (Fig. 2D) and were able to infect sterile grown *Solanum pennellii and Solanum lycopersicum* plants, and feed off them successfully for at least 5 weeks (Fig. 2F). Cultures that were kept in liquid medium developed callus growth after about 3-4 weeks. Whenever parts of the plant material were not immersed in medium any longer or, when cultures were transferred from liquid culture to solid plates of the same medium type, shoot growth was initiated after 2-3 weeks (Fig. 2E).

Agrobacterium rhizogenes transforms cells in *Cuscuta campestris* seedlings

Whole seedlings grown for at least 3 weeks in tissue culture or cut seedlings on solid plates directly after cutting could be co-cultured successfully with *Agrobacterium*, yielding transformed cells in the seedling structures that showed dsRed fluorescence after 10-12 days (Fig. 3). No growth of hairy roots could be observed. Fluorescence indicating transformation was visible until at least 5 weeks after the transformation event, but no propagation of transformed cells could be observed. No significant effect of the use of the different antibiotics timentin at 150 µg/mL, carbenicillin at 400 µg/mL cefotaxime at 400 µg/mL, or a combination of carbenicillin and cefotaxime on the plant tissue and tissue regeneration could be observed. Timentin was effective for *Agrobacterium* removal at 150 µg/mL while Cefotaxime and Carbenicillin had to be applied at concentrations of 400 µg/mL each to prevent growth of *Agrobacterium* on culture plates.

Discussion and Outlook

Optimization of tissue culture for *Cuscuta campestris*

In this work, we targeted *C. campestris* seedlings as material for tissue culture and transformation. Compared to *C. campestris* shoot tips, the use of seedlings as explants proved to be more successful in our tissue culture studies. Additionally, immersion of seedlings in liquid culture medium seemed to help speed up callus formation compared to growth on solid plates by a few weeks. It is noteworthy that the standard method of using equal amounts of auxin and cytokinin for callus formation was not effective in the case of *Cuscuta campestris*. Instead, applying only cytokinin (kinetin or BAP) yielded the best results of callus growth with subsequent shoot formation. Whether this might be attributable to a very high internal auxin concentration in the seedlings that leads to an equilibrium with the added cytokinin, is currently unknown. However, cytokinin seems to also play an important role in the growth of the parasite and development of the parasitic organ and connection. In *Cuscuta japonica* it has been shown that while addition of cytokinin enhanced the growth of novel protuberances, auxin had a negative effect on them (Furuhashi, 1991). In *C. reflexa*, cytokinin treatment was able to increase the chlorophyll level of the plant (Das et al., 2011). Bernal-Galeano & Westwood

(2021) found that kinetin in the culture medium of an artificial host system enhances holdfast growth and haustoria development in *C. campestris*. They observed a higher biomass as well as a greater length of the plants when cytokinin was added. Thus, cytokinin might either promote the growth of *Cuscuta* in several ways or might not be produced by the parasite in sufficient amounts, making at least some *Cuscuta* species dependent on an external supply that is taken up from its host or the supplied growth medium.

In the past the use of the antibiotics timentin, carbenicillin and cefotaxime has been found to be efficient for *Agrobacterium* removal in different concentrations depending on bacterial strain and culture conditions and to sometimes also enhance or inhibit plant growth depending on concentration and plant type (Kumar et al., 2017; Li et al., 2007; Maher et al., 2020; Nauerby et al., 1997). In our studies timentin could be applied for *Agrobacterium* removal at lower concentrations than cefotaxime and carbenicillin, but no effect on the plant material could be confirmed.

Agrobacterium rhizogenes does not induce roots in *C. campestris* seedlings but transforms cells of its tissue

Agrobacterium rhizogenes did not induce fully transformed hairy roots in *C. campestris* seedlings. The same was observed when applying *A. rhizogenes* on callus of in root-parasitic plants (Fernández-Aparicio et al., 2011; Libiaková et al., 2018) and in a previous study where *Cuscuta reflexa* and *C. campestris* adhesive disks were transformed with *Agrobacterium rhizogenes* (Lachner et al., 2020). As discussed in that paper, the reason might be a loss of genes that are necessary for root development, and that cannot be circumvented by the regulatory gene suite that the *Agrobacterium* dispatches during transformation. In studies on other plants, *A. rhizogenes* was usually applied to specifically suited tissue for root development, like the root itself (Ho-Plágaro et al., 2018), hypocotyl (Alagarsamy et al., 2018), or cotyledons (Ron et al., 2014) to enable hairy root development. Thus, the seedling and developing adhesive disk tissues we applied *A. rhizogenes* to in those two studies might simply not be suitable for hairy root development.

In *C. campestris* seedlings, *Agrobacterium rhizogenes* was able to transform what appeared to be strings of adjacent cells. Moreover, dsRed fluorescence was expressed over longer periods of time and in multiple cells, as shown in Lachner et al., 2020 and the present study. This indicates that there is a basic high susceptibility of *Cuscuta* for genetic manipulation. The critical aspect seems to be that not all tissues and developmental stages have an equally high capacity for uptake of DNA from *Agrobacterium*. The very reduced anatomy of *Cuscuta*, unfortunately does not leave a lot of choices, and the only distinctly different target tissue that has to our knowledge not yet been tested are the flowers. Independent of this, the setup presented here can be used in combination with the earlier published *Agrobacterium* transformation protocol in adhesive disks (Lachner et al., 2020) to compare this two crucial stages and sites in the development of this plant parasite.

The future of transformation in *Cuscuta*

In a natural environment *Cuscuta* has the ability to regenerate a whole plant from only a single shoot tip or even remnants of haustorial tissue left in a host plant (Costea & Tardif, 2006), as long as it can draw nutrients from its host. As *Cuscuta* does not have roots or leaves, whole plant regeneration in principle resembles simple shoot regeneration and should thus be easy to achieve. In our own trials, the most common development of *Cuscuta campestris* grown on a nutrient medium containing kinetin was the formation of callus like structures that reverted to shoot aggregates. Thus, it seemed like the plant itself reverted back to growing shoots as soon as possible, which corroborates the findings in *C. trifolii* (Bakos et al., 1995) that also formed shoots as soon as grown on a hormone-free medium or medium with added kinetin at 2 mg/L. Despite the unproblematic regeneration of *Cuscuta* under natural conditions and in culture however, it has so far not been possible to induce that kind of regeneration in transformed *Cuscuta* tissue. The problem of regenerating transformed tissue is not uncommon in other plants and one of the main reasons why creation of mutant plants via the tissue culture and regeneration pipeline is so hard to achieve and often fails. A possible solution might be the addition of growth genes in the transformation vector, a technique that has recently been refined even to the point of the possibility to transform and regenerate a mutant plant without the need for tissue culture techniques (Maher et al., 2020). Our study provides methods for culturing and transformation of *C. campestris* seedlings that could be used as a basis to develop a resembling protocol for *C. campestris* in the future. Alternatively and until the creation of mutant *Cuscuta* plants is possible, this protocol can, in conjunction with our previously published protocol for transformation of cells in the adhesive disk (Lachner et al., 2020), be employed as a valuable tool for functional in situ protein studies on protein localization, interaction and movement.

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Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

L.L. conceived the project; L.L and K.K. designed and performed the tissue culture trials and designed the transformation experiments; L.L. and Z.Z. performed them; Data was evaluated by L.L and Z.Z.; L.L. wrote the article with contributions of all the authors.

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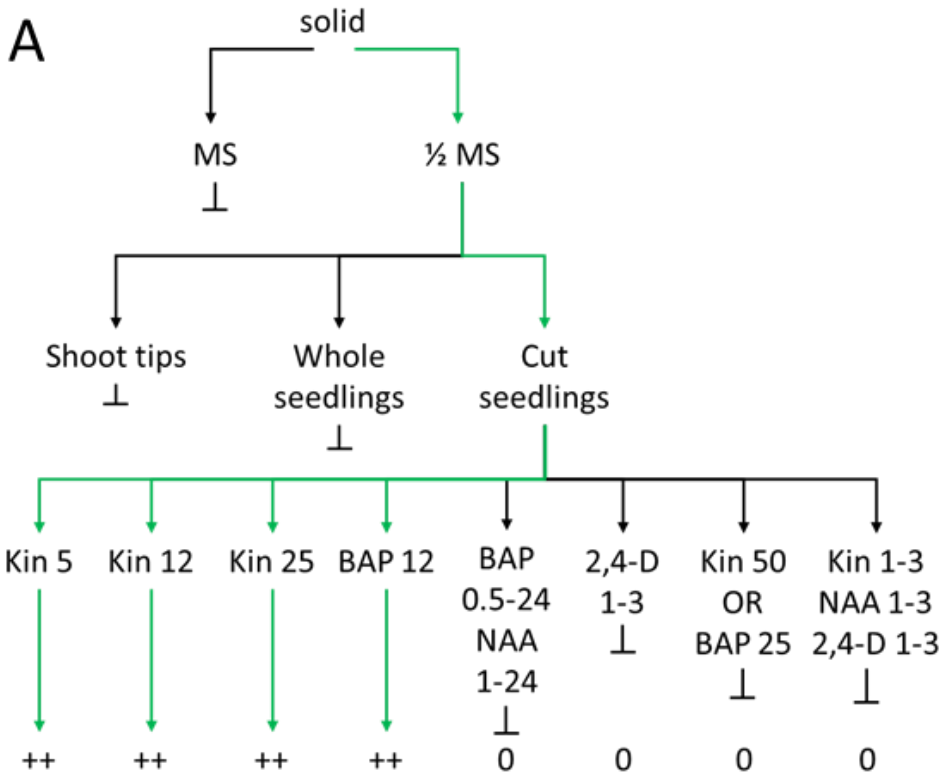
Figures


A
solid
MS
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Shoot tips
Whole seedlings
Cut seedlings
Kin 5
Kin 12
Kin 25
BAP 12
BAP 0.5-24 NAA 1-24
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Kin 50 OR BAP 25
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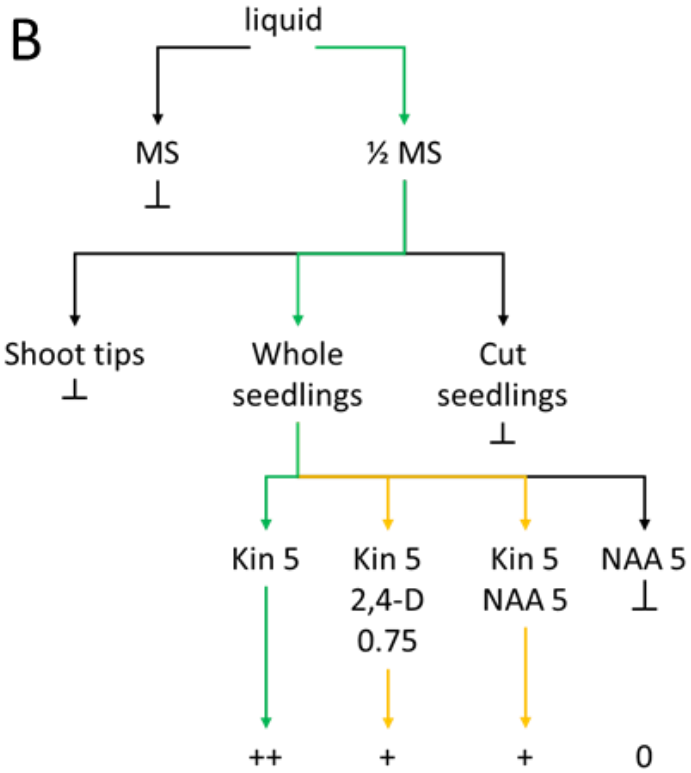
B
liquid
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½ MS
Shoot tips
Whole seedlings
Cut seedlings
Kin 5
Kin 5 2,4-D 0.75
Kin 5 NAA 5
NAA 5
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Figure 1: **Comparison of tested growth conditions for *C. campestris* culture.** Shown are tested culture type; solid (A) or liquid (B). The used medium type is either MS or ½ MS as indicate in the second row of both flow charts. The third row indicates explant type (shoot tips, whole seedlings and cut seedlings) and the fourth row the different hormones and hormone combinations that were added with their respective concentrations as a number given in mg/L. Green arrows show successful culture conditions (++), while black arrows and T ends show conditions that did not result in the desired growth (0). Yellow arrows show culture conditions that resulted in reduced growth (+).

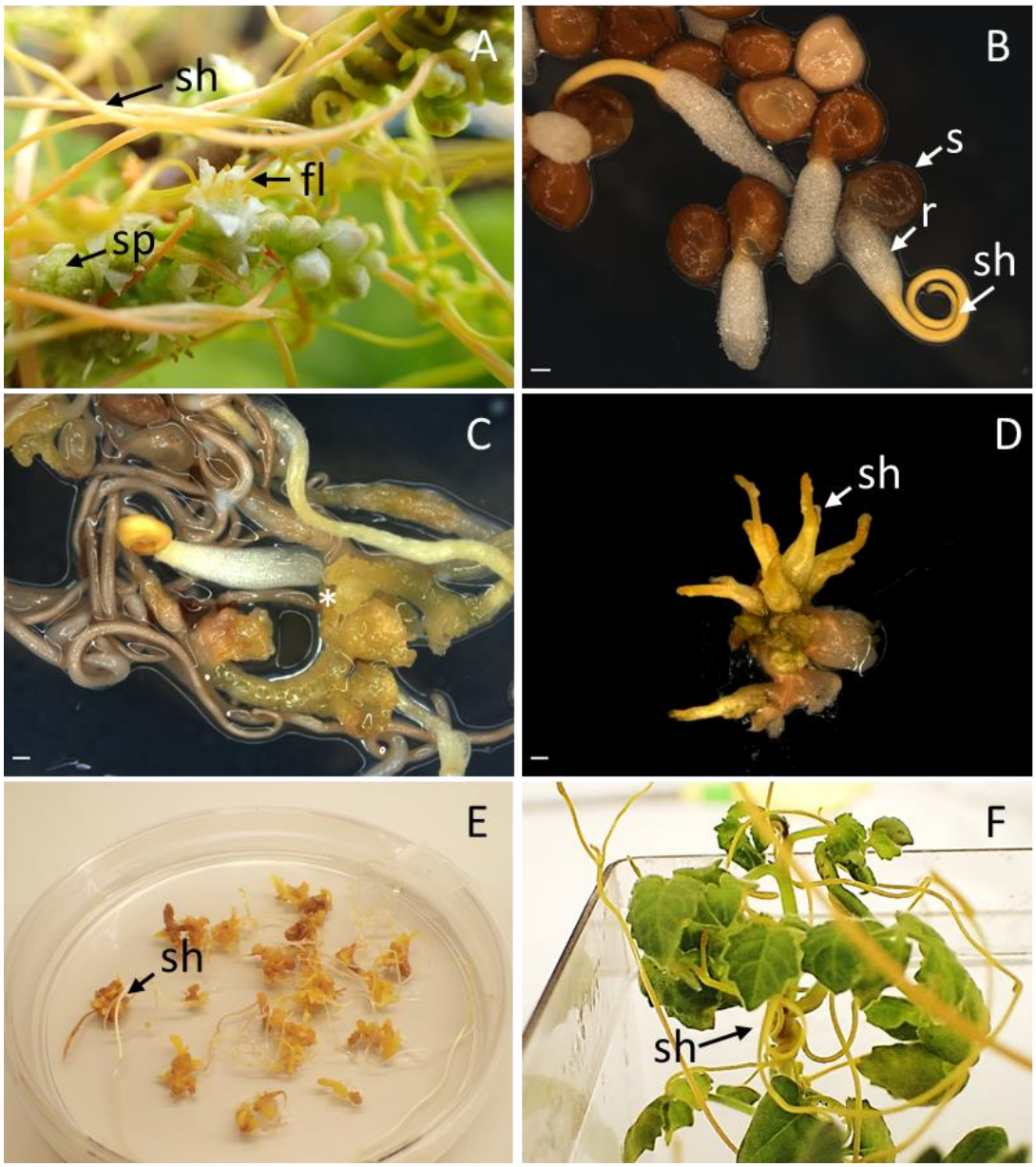

Figure 2: **Steps of *Cuscuta campestris* culture.** *Cuscuta campestris* growing and flowering on host plant *P. zonale*. Indicated are shoots (sh), flowers (fl) and developing seed pots (sp) (A). Germinating seedlings with seed shells (s), "root-like" organ formed directly after germination for a short time and the shoot (sh) (B). Callus growth (*) on seedlings after 5 weeks of solid culture containing 5mg/L kinetin (C). Aggregates with shoot growth (sh) growing in culture of cut seedlings on solid plates, 3 months after culture start (D). Callus cultures grown in liquid culture and transferred to solid medium for shoot formation (sh) on plates containing 5mg/L kinetin (E). *C. campestris* tissue-culture-produced-shoot (sh) infecting a host plant (*S. pennellii*) under sterile conditions (F). Scalebars are 500 µm where indicated.

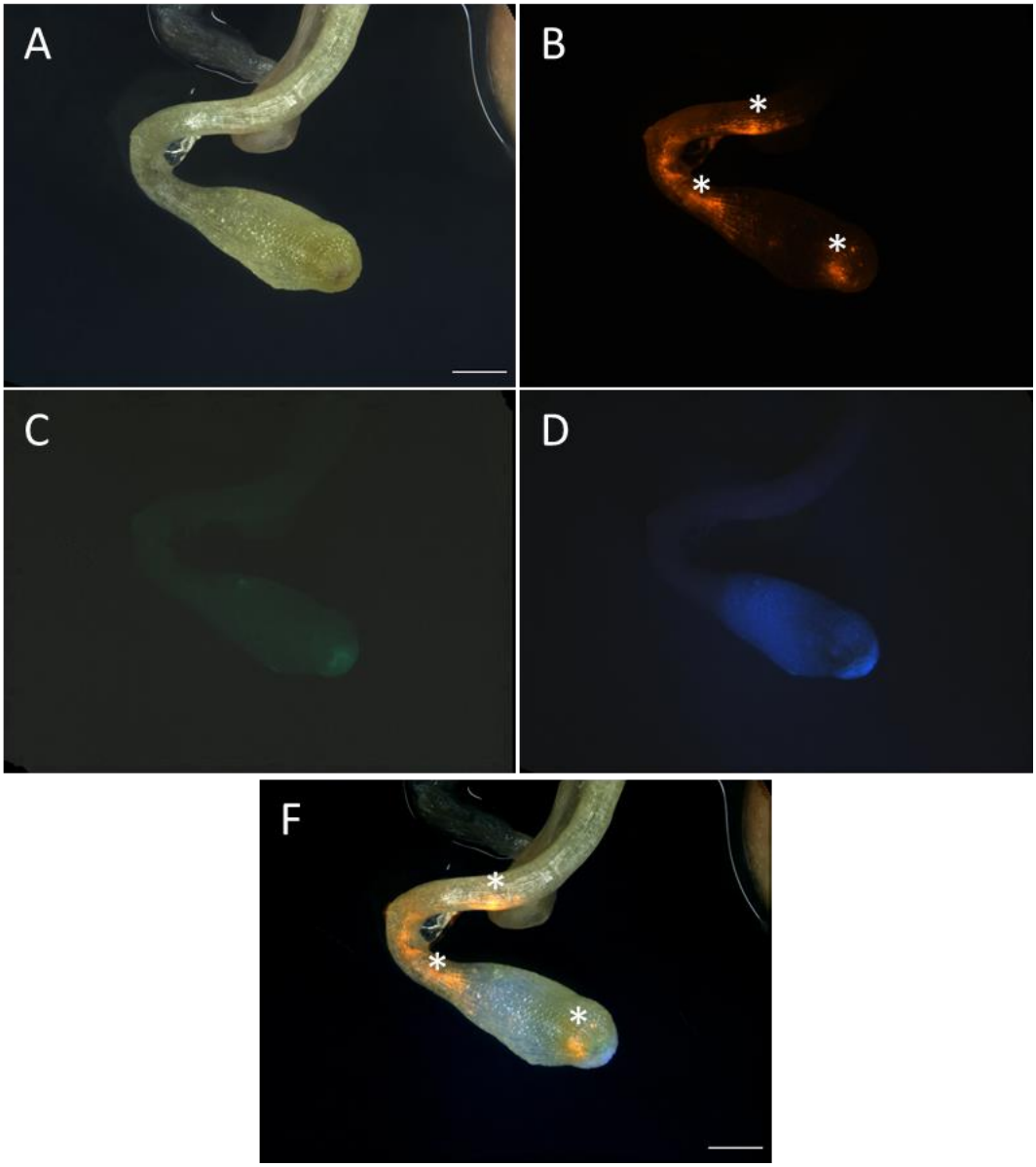

Figure 3: **DsRed fluorescence in *C. campestris* cut seedlings after co-cultivation and transformation with A. rhizogenes pRedRoot.** Natural colours of the seedling under bright light conditions (A), dsRed fluorescence as seen under Cy3 filter (B), control images taken under GFP filter (C) and autofluorescence filter (D), as well as an overlay of (A-D) indicating that the signal seen in the Cy3 filter is not overlapping with signals in other channels (F). Images were taken 3 weeks after the initial fluorescence had occurred. Scalebars are 500 µm where indicated.

Paper 3

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Selective mineral transport barriers at *Cuscuta*-host infection sites

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The uptake of inorganic nutrients by rootless parasitic plants, which depend on host connections for all nutrient supplies, is largely uncharted. Using X-ray fluorescence spectroscopy (XRF), we analyzed the element composition of macro- and micronutrients at infection sites of the parasitic angiosperm *Cuscuta reflexa* growing on hosts of the genus *Pelargonium*. Imaging methods combining XRF with 2-D or 3-D (confocal) microscopy show that most of the measured elements are present at similar concentrations in the parasite compared to the host. However, calcium and strontium levels drop pronouncedly at the host/parasite interface, and manganese appears to accumulate in the host tissue surrounding the interface. Chlorine is present in the haustorium at similar levels as in the host tissue but is decreased in the stem of the parasite. Thus, our observations indicate a restricted uptake of calcium, strontium, manganese and chlorine by the parasite. Xylem-mobile dyes, which can probe for xylem connectivity between host and parasite, provided evidence for an interspecies xylem flow, which in theory would be expected to carry all of the elements indiscriminately. We thus conclude that inorganic nutrient uptake by the parasite *Cuscuta* is regulated by specific selective barriers whose existence has evaded detection until now.

Introduction

Photosynthetic plants need many essential elements for their growth. These elements are taken up by the roots from the soil in the form of metal cations, anions or oxyanions. The macronutrients nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P) and sulfur (S) are present in plant tissues in large amounts (>50 μmol g^{-1} dry weight [DW]; Maathuis 2009). The micronutrients boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn) are required in much smaller amounts and are therefore found at much lower concentrations in plant tissues (< 5 μmol g^{-1} DW, with the exception of Cl; Hänsch and Mendel 2009). All plants have to acquire appropriate amounts of each nutrient. Deficiencies in essential nutrients lead to inhibition of growth and to specific disorders. To avoid this, plants accumulate essential nutrients by active transport processes to much higher levels than those found in the surrounding soil. However, several nutritional minerals are toxic at elevated concentrations, entailing a duality of beneficial vs detrimental effects that necessitate the development of precisely tuned homeostatic networks for the uptake,

Abbreviations – DW, dry weight; MXRF, micro-X-ray fluorescence spectrometry; TXRF, total reflection X-ray fluorescence spectrometry; XRF, X-ray fluorescence spectrometry.

Frank Förste and Ioanna Mantouvalou contributed equally to this work.

transport and storage of nutrients (Hänsch and Mendel 2009). Long-distance transport from roots to other plant parts is mainly achieved through the transpiration stream in the xylem, while a re-distribution of many, but not all, elements between the different plant organs occurs via phloem transport (Conn and Gilliham 2010, Etienne et al. 2018). Within the organs, distribution is achieved via tissue- or cell-specific membrane transport processes and by membrane-lined intercellular tunnels called plasmodesmata.

Parasitic vines that belong to the angiosperm genus *Cuscuta* are rootless and consequently have no direct access to soil nutrients. The threadlike *Cuscuta* vines infect stems, petioles and pedicels of other plants (Fig. 1A–C) to cover their nutritional requirements. The connection between the two partners is formed by specialized, shoot-borne infection organs termed haustoria (Kuijt 1969, Dawson et al. 1994). These haustoria functionally replace the roots, which have been lost in the course of the parasite's evolution. Microscopic and electron microscopic studies as well as fluorescent tracers support the notion that there is a host-to-parasite continuum of the long-distance vascular system between both plants through the haustoria. This continuum is secured by different types of specialized cells that are summarized under the term 'feeding hyphae' and that develop at the tip of the haustorium (Doerr 1969, Vaughn 2003). The differentiation of these feeding hyphae depends on which host cell type they have established contact with. For example, xylem vessels of the host, which are comprised of dead tube-like cells, were observed to be intercepted by xylem-like hyphae that play a role in the redirection of water and minerals to the parasite (Christensen et al. 2003). On the other hand, amino acids, sugars, nucleotides and other organic molecules in the phloem sap are believed to be channeled to finger-like hyphae from phloem cells for long distance transport or from parenchymatous cells for short distance transport (Doerr 1972). Plasmodesmata that establish a symplastic continuum between neighboring cells were shown to exist between the *Cuscuta* hyphae and the host cells (Doerr 1969, Birschwilks et al. 2006, Vaughn 2006). Many *Cuscuta* species are fast-growing and form hundreds to thousands of haustoria on a single host (Fig. 1D).

Because of the enormous importance of nutrients for plant growth and also for human nutrition, the 'ionome', which corresponds to the total concentration (i.e. all forms) of elements in a sample (Conn and Gilliham 2010), has been studied extensively in crop plants. Many proteins involved in nutrient transport and storage, e.g. transcription factors, transporters and ion channels for membrane transport and metal binding

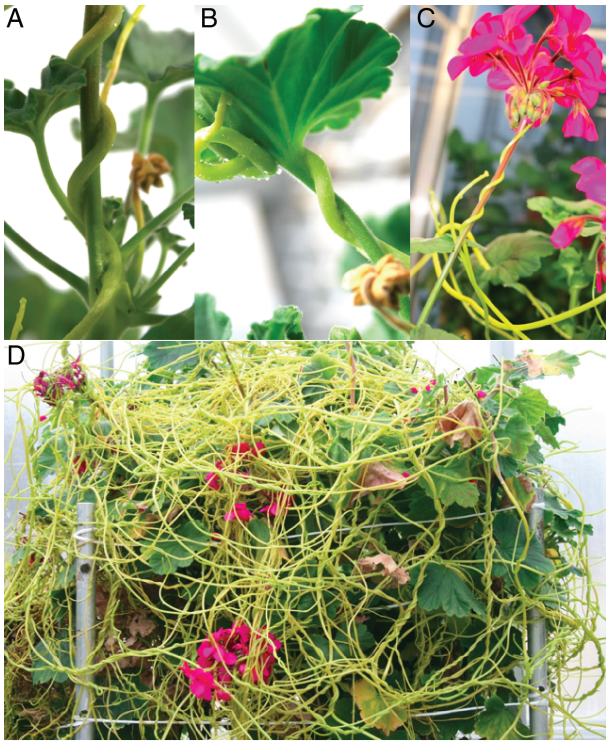

Fig. 1. Host–parasite interactions. (A–C) *Cuscuta reflexa* (giant dodder) infecting stems (A), petioles (B) and pedicels (C) of the ornamental plant *Pelargonium zonale*. (D) *C. reflexa* overgrowing *P. zonale* in a greenhouse.

proteins, have been identified and their physiological function has been determined in model plants (White and Broadley 2009, Etienne et al. 2018). Increasing the mineral concentrations in edible crops, e.g. is a big task in the strive for food security (White and Broadley 2009, Mantouvalou et al. 2017). An infection by *Cuscuta* species often causes severe crop yield losses (Costea and Tardif 2005), which in part seems to be related to the depletion of photosynthates in its hosts. A few studies have been published showing, surprisingly though, that *Cuscuta* does not deplete the host noticeably of its mineral elements (Wallace et al. 1978, Saric et al. 1991). The parasite was even shown to have much lower calcium levels than the plant supporting it. Sadly, three to four decades later, the knowledge around the transport fluxes of different compound classes from host to parasite and the uptake and distribution of inorganic nutrients at the *Cuscuta*-host border has not been refined despite a significant advancement of the technical possibilities to study macro- and microelement content and distribution.

Ionomics (the simultaneous measurement of the elemental composition by high-throughput elemental analysis technologies; Salt et al. 2008) is performed by X-ray

fluorescence spectroscopy (XRF). This is an elemental detection technique, which relies on the principle that atoms are excited with ionizing X-ray radiation and the resulting X-ray fluorescence is detected with an energy-dispersive detector (Beckhoff et al. 2007). Owing to the distinctive energy levels in atoms, the X-ray fluorescence spectrum is characteristic for each element, thus, elemental information can be obtained. Quantification of elemental composition is possible by linking the measured X-ray fluorescence intensity to the concentration of the elements either through the measurement of similar reference samples or through the use of the fundamental parameter approach (van Grieken and Markowicz 2001). By using X-ray optics, the exciting radiation can be focused onto a micrometer-sized spot, thereby resolving the elemental distribution within a sample (Haschke and Haller 2003, Havrilla and Miller 2004). Due to the penetrating nature of X-rays, three-dimensionally resolved elemental imaging can be achieved when utilizing two X-ray optics in a confocal arrangement (Mantouvalou et al. 2012). For biological specimens, the main matrix elements (C, H, O) are not typically accessible with XRF, thus elemental imaging is especially suited for the distribution analysis of minerals and metallic trace elements. Generally, the low density of the material and the low concentrations of the fluorescence elements lead to a low fluorescence yield.

We present here the first space-resolved study of the element distribution in a system that involves two interacting plants, i.e. *Cuscuta reflexa – Pelargonium zonale* infection sites and the surrounding stem sections. With XRF, we show that the parasite does not indiscriminately absorb all of the hosts' nutrients but, instead, appears to specifically regulate the uptake of calcium, strontium, chlorine and manganese.

Materials and methods

Plant material and growth conditions

The parasite *Cuscuta reflexa* ROXB. and the host *Pelargonium zonale* 'Kardinal' were originally obtained from the Botanical Garden of the Christian-Albrechts-University in Kiel. Host and parasite were cultivated together under continuous illumination in daylight chambers supplemented with additional neon lights at the Phytotron of the University of Tromsø, Norway. Growth rooms had a constant temperature of 21°C. Host plants were propagated by taking cuttings from uninfected plants. The parasite was propagated by placing apical shoot segments of approximately 20 cm in water-filled tubes in the pots of the host and allowed to infect the hosts as described (Krause et al. 2018). All experiments were performed on hosts that were infected at least 4 weeks prior to sampling.

Sample collection and preparation

Infection sites closest to the apical shoot tips of *C. reflexa* were used for analysis. The selection of these sites was guided by the tell-tale signs that a feeding connection was established (resumed growth of the shoot above the infection site and side branch production [Olsen et al. 2016]). The distance between infection sites and shoot tips of the parasite was kept within a range of 15 to 25 cm. To avoid contamination from the equipment, samples were handled only with plastic forceps and cut only with ceramic knives or ceramic razor blades. For total reflection XRF (TXRF) measurements with the Picofox instrument, 3–6 mm shoot sections of parasite and host, above and below the infection site, were carefully cut with a ceramic knife, weighed and placed in plastic containers. For TXRF measurements with the S4 T-Star, shoot tips with 2–3 branching nodes (10–12 mm) were collected. For micro X-ray fluorescence spectrometry (MXRF), 2 mm sections (+/– 0.5 mm) of an infection site were cut with a ceramic knife and placed on a piece of carbon tape inside a plastic tube. The plant material was weighed, flash-frozen in liquid nitrogen and subsequently freeze-dried for 24 h under 5 mTorr vacuum. The freeze-dried samples were weighed again and kept protected from moisture at room temperature until they were scanned.

S2 Picofox measurements

Five milliter ultrapure water, 10 µl of a gallium standard solution (1 $g\,l^{-1}$) and 10 yttrium-coated ceramic balls (diameter 2 mm) were added to the sample tubes containing fresh pre-weighed plant samples. The samples were placed in an automatic homogenizer (Omni Bead Ruptor 24, Omnilab International Inc.) and homogenized with the following settings: speed (6 $m\,s^{-1}$), number of cycles (2), homogenization time (35 s), dwell time between cycles (10 s). 10 µl of the homogenate was transferred onto siliconised quartz glass sample carriers and dried in vacuum. The instrument (S2 Picofox, Bruker Nano GmbH) used for the measurements was equipped with a 50 W Mo-tube, operated at 50 kV/600 µA, and a 30-mm^2 silicon drift detector. Measurement times were 1000 s. As this technique is nearly free from any matrix effects, the concentration of all detected elements is directly calculated based on the measured fluorescence intensities and the concentration of the internal standard element. To convert the concentrations to milligram per kilogram dry weight, an average ratio of fresh weight to

dry weight of 13.3:1 (as determined by weighing before and after freeze-drying) was applied.

S4 T-Star measurements

Pre-weighed freeze-dried plant material was homogenized in 0.5 ml ultrapure water supplemented with 10 µl of a gallium standard solution (10 mg l^{-1}) and 3 yttrium-coated ceramic balls (diameter 3 mm) first in an ultrasonic bath (3 min) and then in an automatic benchtop mill (Retsch MM400; 3 min at 30 Hz). 10 µl of the homogenate was transferred onto siliconised quartz glass sample carriers and dried in vacuum. The instrument (S4 T-Star, Bruker Nano GmbH) was operated with Mo-K excitation at 50 kV/1000 µA for 1000 s.

MXRF and confocal MXRF

The commercial MXRF spectrometer (Bruker M4 tornado) was equipped with a Rh microfocus X-ray tube, a polycapillary lens to focus the radiation and a SDD detector (Lachmann et al. 2016). In front of a second SDD a second polycapillary lens was mounted perpendicular to the first, thus creating a confocal MXRF setup which enabled three-dimensional-resolved measurements with a resolution of 30 µm at Fe Kα (Mantouvalou et al. 2017). A fisheye camera with two magnification stages (10× and 100×) was used for positioning of the sample. The sample stage could be moved in three directions with a maximal speed of 100 mm s^{-1}. In the normal configuration, the sample environment can be evacuated to pre-vacuum conditions (20 mbar). For the analysis of cryogenic samples, the spectrometer was equipped with a Cryojet from Oxford instruments. The snout of the jet is inserted through an unoccupied flange at the measuring head and positioned for a local freezing of a sample at the measuring position. The sample temperature could be monitored. The whole sample environment was flushed with nitrogen to prevent icing of the samples during transfer. All measurements were performed with a tube voltage and current of 50 kV and 600 µA, respectively. Measurement times and pixel sizes are described in the figure legends or in the main text.

For the high-resolution confocal scan, a voxel (3D pixel) size of 50 µm and a dwelling time of 130 s per voxel was used.

Visualization and quantification of MXRF data

The Esprit software from Bruker Nano was used for the visualization of the elemental images in Figs. 3, 4A, 5, S3 and S4. For this purpose, the fast deconvolution setting was used, yielding images of the distribution of the net peak intensities of the fluorescence peaks of the respective elements. For an adapted visualization, the contrast was set either to maximal contrast or to an optimal differentiation between host, haustorium and parasite.

The element distributions of the confocal measurement in Fig. 4B and video supplement were generated through deconvolution and absorption-correction (Mantouvalou et al. 2017) by in-house software. The compound visualization in Fig. 4B was created with the software Tomviz using the contour visualization method.

For the charts shown in Fig. 5, sum spectra of the circular regions of the transect consisting of approximately 16 pixels were generated with the Esprit software. The spectra were normalized to the elastically scattered Rh Kα peak originating from the excitation spectrum in order to render a comparison feasible. Subsequently, the spectra were deconvolved and the net peak intensities of the respective fluorescence lines plotted against the number of the region with OriginPro 8.5.1. Similarly, the graphs in Fig. S2A,B were prepared by normalizing the sum spectra of the complete samples.

Xylem transport analysis

Infected pedicels were cut 6–8 cm below the infection site and placed immediately in a ink:tap water (1:10) mix. Meanwhile, the infecting shoot of the parasite was not severed and remained connected to the rest of the *C. reflexa* plant. The infection site was hand-cut after 24 h and inspected with a stereomicroscope equipped with a color camera.

Immunolabeling of Ca-crosslinked pectin

Infection sites were fixed in Farmer's fixative (Ethanol: Glacial acetic acid in a ratio of 3:1 [v:v]) under vacuum for 2 × 15 min and an additional 6-24 h under ambient pressure at 4°C, dehydrated in an ethanol series (75, 85, 95 and 100% [v/v]) with transfer every 2 h. Half of the volume of 100% EtOH was removed, the sample incubated at 38°C for 10 min and molten Steedman's wax added to achieve a 1:1 mixture. After a 12-h incubation at 38°C, the mixture was exchanged with 100% molten wax 3 times with 2 h intervals keeping it at 38°C. After letting the wax solidify at RT (for 24 h or more before cutting) cross-sections of 10–12 µm thickness were cut with a microtome, mounted on poly-L-lysine-coated microscopy slides and dried over night at 32°C. Prior to immunolabeling, slides were dewaxed by adding 100% EtOH and warming the slides to 32°C. Sections were blocked with 5% milk powder dissolved in T/Ca/S buffer (Tris-HCl 20 mM pH8.2, $CaCl_2$ 0.5 mM, NaCl 150 mM)

for at least 30 min at RT and then incubated for 90 min with the 2F4 Anti-homogalacturonan antibody (PPG1-2F4, Plant Probes) (Liners et al. 1989) diluted 1:25 in T/Ca/S buffer as specified by the producer.

After three 5-min washes with T/Ca/S buffer, followed by a 1-h incubation in the dark in a 1:1000 dilution of the secondary antibody (Alexa Fluor 555 goat anti-mouse) in T/Ca/S buffer and three further 5-min washes, the sections were mounted on microscopy slides using a drop of citifluor AF1 mounting medium (Glycerol/PBS) and stored in the dark at 4°C until viewing. The immunolabeling was investigated using fluorescence microscopy. The primary antibody was omitted in controls for autofluorescence background and unspecific binding of the secondary antibody.

Results

Calcium, manganese and chlorine are strongly reduced in *C. reflexa* compared to its host *P. zonale*

It has been an unsolved issue whether the diversion of nutrients from the host to the parasite might lead to a change in element composition around the infection site in either of the partners. To investigate this, we first measured the total content of major mineral nutrients and trace elements in stems of both interaction partners, above and below infection sites, using TXRF (Klockenkämper and van Bohlen 2015). With this particularly sensitive variant of XRF, elemental concentrations of homogenized specimens down to the parts-per-billion level can be derived. This analysis revealed that the sites above and below the host–parasite connection in each interaction partner were very similar with regard to almost all elemental concentrations (Fig. 2) and that they matched the concentration range measured in other plants (Watanabe et al. 2007). The same is true for most nutrients when comparing the tissue of the host and the parasite. Most of the macroelements (P, S, K) and microelements (Fe, Cu, Zn) showed the same concentration in both plants or, as in case of the non-essential rubidium, they were slightly more abundant in *C. reflexa* (10–20%). None of the elements, however, accumulated to significantly higher values (increase of more than 50%) in the parasite compared to the host plant. However, several elements showed a clear decrease in the tissue of the parasite. These include Cl (~50% average decrease), Ca (>90% average decrease), Mn (~75% average decrease) as well as the non-essential or toxic trace elements bromine (Br) and strontium (Sr) (80–90% average decrease; Fig. 2). These differences lead to different relative ratios of the elements in the host and the parasite. While in *P. zonale*, the macroelement concentrations decreased in the order $K > Ca > P \cong Cl > S$, the order in *C. reflexa* was $K > P > S \cong Cl > Ca$. In apical shoot tips, the absolute quantities were in the same range and the relative order differed only slightly due to a shift of the chloride abundance ($K > P > S > Cl \cong Ca$). No indication was obtained for an accumulation of any element except Mn in the apical tips of growing shoots (Fig. S1). This suggested that selective exclusion mechanisms of minerals might exist at the host–parasite border, calling for a spatially resolved analysis of the host–parasite interfaces at the infection sites themselves.

Spatially resolved element patterns in situ can be achieved by MXRF

MXRF is able to capture changes in element distribution non-invasively with a high lateral spatial accuracy and was chosen for this task. To obtain the desired resolution, measuring times of 3 h and more had to be applied, necessitating cryofixation of the samples to avoid dehydration and migration of mobile elements (Gianoncelli et al. 2015, de Carvalho et al. 2018). Samples with a thickness of 2 mm proved to be optimal for our measurements since they provided sufficient fluorescence intensities while simultaneously keeping low the occurrence of artifacts due to tissue inhomogeneity.

Initially, MXRF images of infection site cross-sections were compared between samples that were either flash-frozen and kept frozen under a stream of nitrogen gas applied with a CryoJet (Oxford Instruments) during the measurements or that were freeze-dried prior to the measurements. It was found that the distribution of the elements was not influenced by the preparation method, implying that in the probed lateral dimension scale and for the investigated samples no significant movement of mobile elements during the freeze-drying process took place. However, with the exception of chlorine, the intensities of the elemental spectra were often higher in freeze-dried samples (Fig. S2) due to the elimination of scattering from water or ice. All following MXRF data were therefore obtained on freeze-dried cross-sections.

Element content differences between host and parasite are independent of the type of host stem and the species

C. reflexa indiscriminately infects the main stems, petioles and pedicels of its hosts. While petioles connect source tissue (the leaves) to the main shoot, pedicels supply a sink tissue (the flowers), so the relative amounts of the elements could deviate in these tissues. To determine whether the tissue type that is infected has an impact

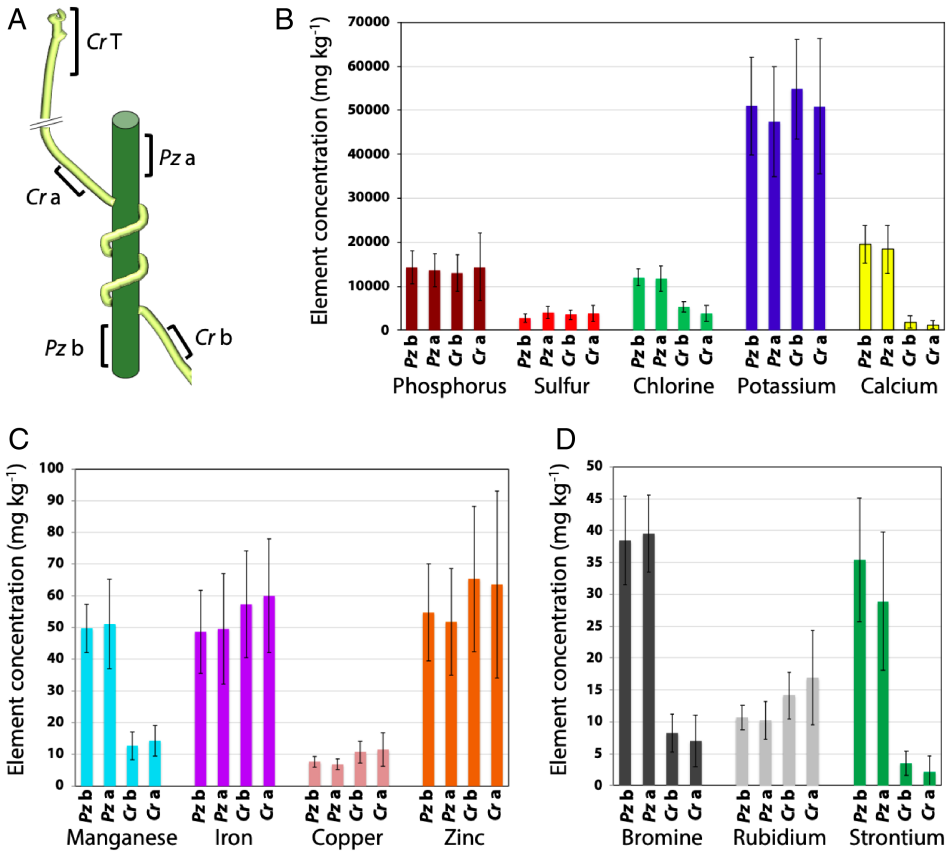

Fig. 2. Element composition of host and parasite stem extracts below and above the infection site. (A) Schematic representation of the sample sites. *Cr* a and *Cr* b refer to sample sites in the stems of the parasite, *Cuscuta reflexa*, above and below the infection sites, respectively. *Pz* a and *Pz* b refer to sites in the stems of the host, *Pelargonium zonale*, above and below the infection sites, respectively. Apical tips of *C. reflexa* (*Cr* T) were between 15 and 25 cm away from the infection site. The average element composition of shoot tips is shown in Fig. S1. (B–D) Macronutrient (B), micronutrient (C) and non-essential (D) element concentrations are given in mg/kg dry weight (mean ± SE; n = 15). Samples were taken in groups of three on five different days over a period of 2 weeks. All samples are derived from a set of four infected *P. zonale* plants.

on the elemental distribution patterns within the infection sites, we compared cross-sections through infection sites on each of them. This survey revealed that the intensity of all elemental spectra was similar in the three stem types (Fig. S2). Likewise, relative ratios of the elements between host and parasite were not influenced by the stem type, and only small differences were observed in the distribution within each partner (Figs 3 and S3). These differences are mostly connected to the organization of the vascular tissue in the host and are most evident in the pedicels. The distribution between host and parasite showed the same tendential differences when another *Pelargonium* species (*P. citriodorum*, lemon-scented *Pelargonium*) was used as host (Fig. S2), with the notable exception of Cl, which was found to be higher in *C. reflexa* when attached to *P. citriodorum*.

Discrimination against calcium, manganese and chlorine between *C. reflexa* and *P. zonale* occurs at two different barriers

MXRF-scanned cross-sections of *C. reflexa*-*P. zonale* petiole infection sites confirmed that the macronutrients P and K and the micronutrient Zn were present at similar concentrations in host and parasite, although local fluctuations were recorded. Cl, Ca and Sr were clearly reduced in *C. reflexa* (Fig. 4A), confirming the TXRF measurements. The measured concentrations of sulfur were more inconsistent and showed a decrease in parasite shoots infecting *P. zonale* petioles (Fig. 4A), and an increase in stem- and pedicel-infecting *C. reflexa* shoots (Figs S3 and S4). For the element Mn, a diffuse signal throughout the host stem was observed that was more intensive than the signals in the parasite. Additionally, striking accumulations around the haustorium were visible, which were not observed with any of the other elements (Fig. 4A). To get a more accurate picture of the distribution of the elements in the different regions of an infection site, a high-resolution confocal scan of the endophytic haustorium and its surrounding tissue (volume of 1550 µm × 450 µm × 1000 µm) was performed (Fig. 4B and Movie S1). This confocal approach clearly showed that K, Ca and Mn each have a very distinct distribution pattern: while the haustorium is depleted for Ca and Mn, K is slightly enriched here. A distinct thin layer of Mn engulfing the haustorium is also visible (Movie S1).

In addition, for the 2D scans of two separate infection sites, 22 and 24 punctate sum spectra, respectively,

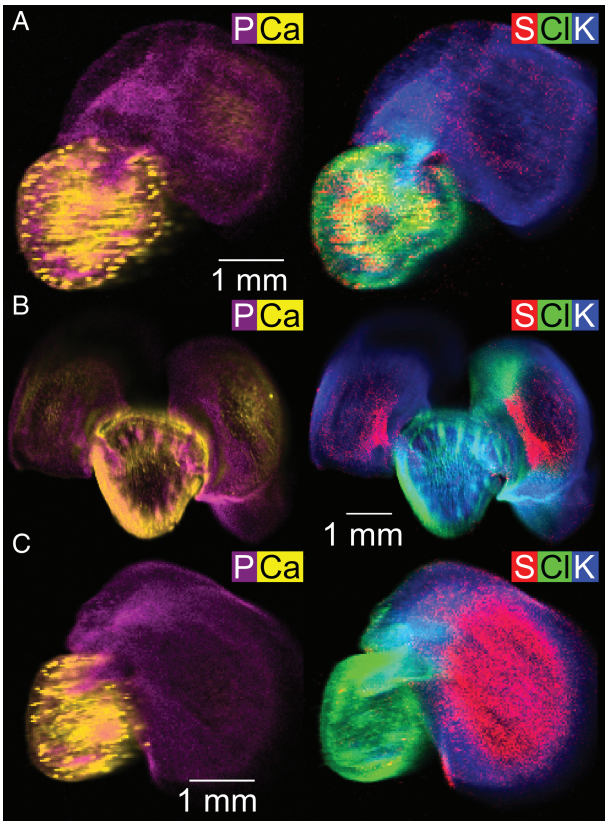

Fig. 3. Elemental distribution in infection sites. (A–C) Overlays of elemental patterns at infection sites on stems (A), pedicles (B) and petioles (C). The intensities for the P (purple), Ca (yellow), S (red), Cl (green) and K (blue) fluorescence peaks of the XRF spectra were derived with integration times of 3 s (for A), 3.85 s (for B) and 5.6 s (for C) per pixel, respectively, and a pixel size of 30 μm. The scaling of the intensity was chosen for an optimal visual differentiation of the different tissues. The intensities for each element are shown in numbers in Fig. S2. Individual pictures for each element are shown in Fig. S3. The relevant part of the sum spectra of the three measurements is shown in Fig. S2C.

were obtained (see Fig. S2C for an example) for spots placed along a transect from host epidermis through the middle of the haustorium into the parasite to its epidermis (Fig. 5A,B). The plotted intensities in Fig. 5C–F show that P shows on average the most stable distribution with similar intensities in all tissues. K showed an intensity peak at the top of the haustorium, where the signals were up to twice as high as in the remaining tissue, while fluorescence peaks from Ca and Sr, on the other hand, decreased sharply at the border between host and parasite, continuing to be low in the parasite stem with only a minor accumulation in the pith of *C. reflexa* (Fig. 5C,D). Although the values for S were on average a bit lower in the parasite (including its haustorium), the differences were by far not as drastic as for Ca and Sr Signals specific for Cl, in stark contrast, stayed at host levels throughout the haustorium and only dropped sharply in the *C. reflexa* stem, where they leveled out at ~10% of the values seen in the host (Fig. 5E,F).

Reduced Ca levels in *C. reflexa* are not due to a lack in xylem connections

The long-distance transport of Ca in plants is unidirectional from roots to shoots and leaves and only mediated by the xylem stream and there is overwhelming evidence showing that calcium is not translocated in the sieve tubes of the phloem (e.g., White and Broadley 2009, Conn and Gilliham 2010, Gilliham et al. 2011, Etienne et al. 2018). Although there is good microscopic evidence for the formation of xylem bridges between *C. reflexa* and *P. zonale*, we wanted to investigate whether the drop in Ca concentrations in *C. reflexa* could nevertheless reflect an inability to take up substances from the xylem. To this end, we placed infected host pedicels into tap water supplemented with blue ink for 24–36 h and hand-sectioned infection sites for subsequent microscopical analysis. The dark blue ink could be easily observed in the host xylem and was clearly transferred into the *C. reflexa* haustorium (Fig. 6). This simple experiment confirms that functional xylem connections do exist between *P. zonale* and *C. reflexa* and that the exclusion of Ca at the border of the haustorium must be due to some type of selective barrier.

Reduced Ca levels in *C. reflexa* do not appear to impact the parasites' ability to cross-link pectin

To analyze whether differences of calcium ions cross-linked in the cell walls of *C. reflexa* might explain its low Ca level, we used the monoclonal antibody 2F4 which binds to Ca^{2+}-crosslinked pectin (Rydahl et al. 2018). Immunolabeling of thin sections of fixed and embedded infection sites of *C. reflexa* on *P. zonale* showed no significant difference in the labelling intensity of cell walls between host and parasite (Fig. S5).

Discussion

A few studies on elemental composition in *Cuscuta* were performed already several decades ago. They reported on the levels of five elements (N, P, K, Ca and Mg) in *C. reflexa* infecting five different genotypes of *P. zonale* (Saric et al. 1991) as well as in *C. nevadensis* grown on eight different host species (Wallace et al. 1978). Owing to limitations of the measuring techniques used back then, only one element at a time could be measured and the tissue was used up in the process, aggravating

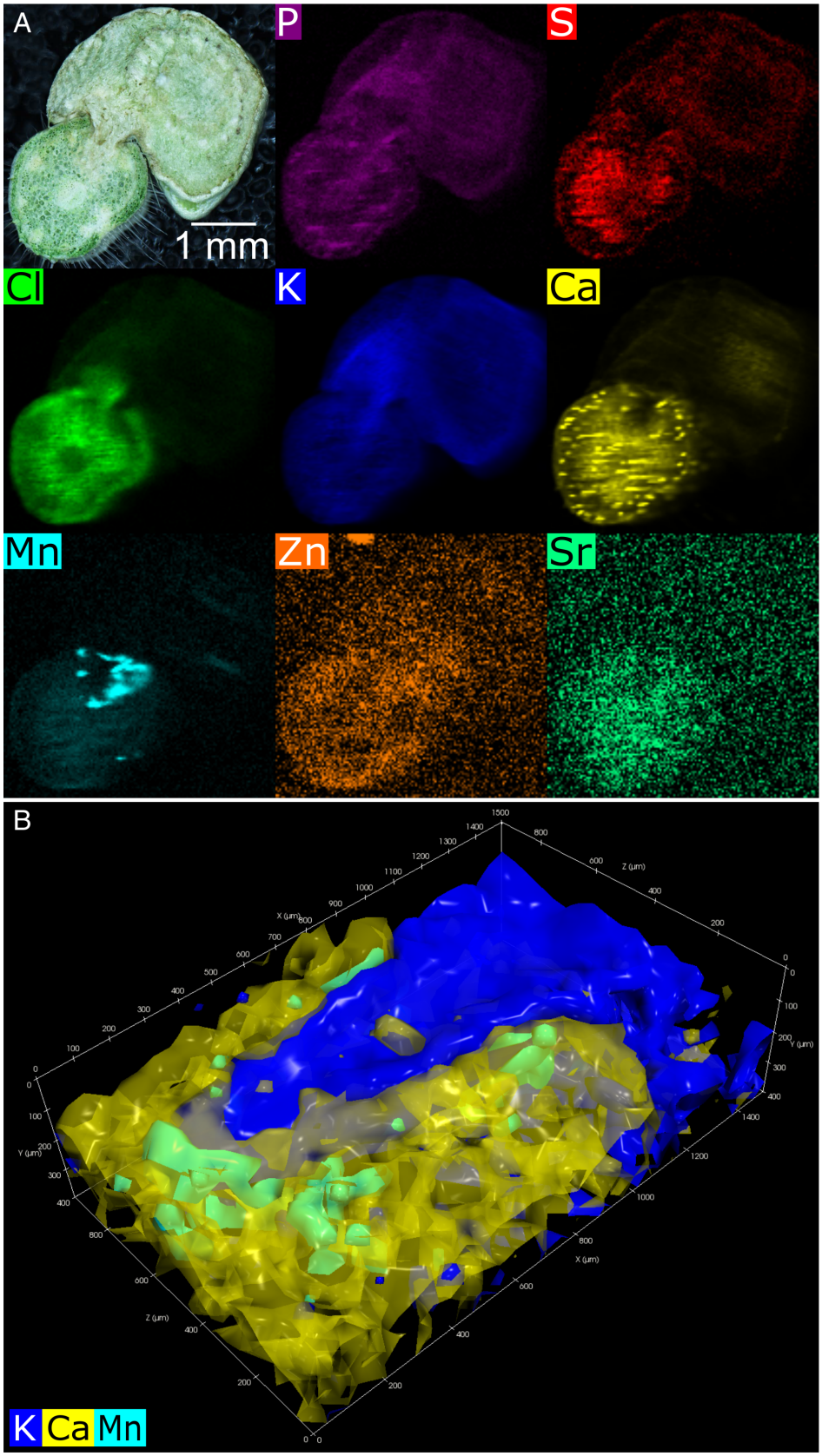

Fig. 4. Elemental distribution maps of a *Cuscuta reflexa* infection site on a representative *Pelargonium zonale* petiole. (A) Two-dimensional scan conducted with 30 µm pixel size and 3 s measuring time per pixel. Intensity distributions are scaled to maximal contrast. Further scans for petioles of the same and another species are shown in Fig. S4. (B) Confocal (three-dimensional) scan of the haustorium. The measurements were conducted in a volume of 1550 × 450 × 1000 µm using scanning steps of 50 µm and a measuring time of 130 s per voxel. *Cr*, *Cuscuta reflexa*; *Pz*, *Pelargonium zonale*. A video with rotating views of the scanned area is provided as Movie S1.

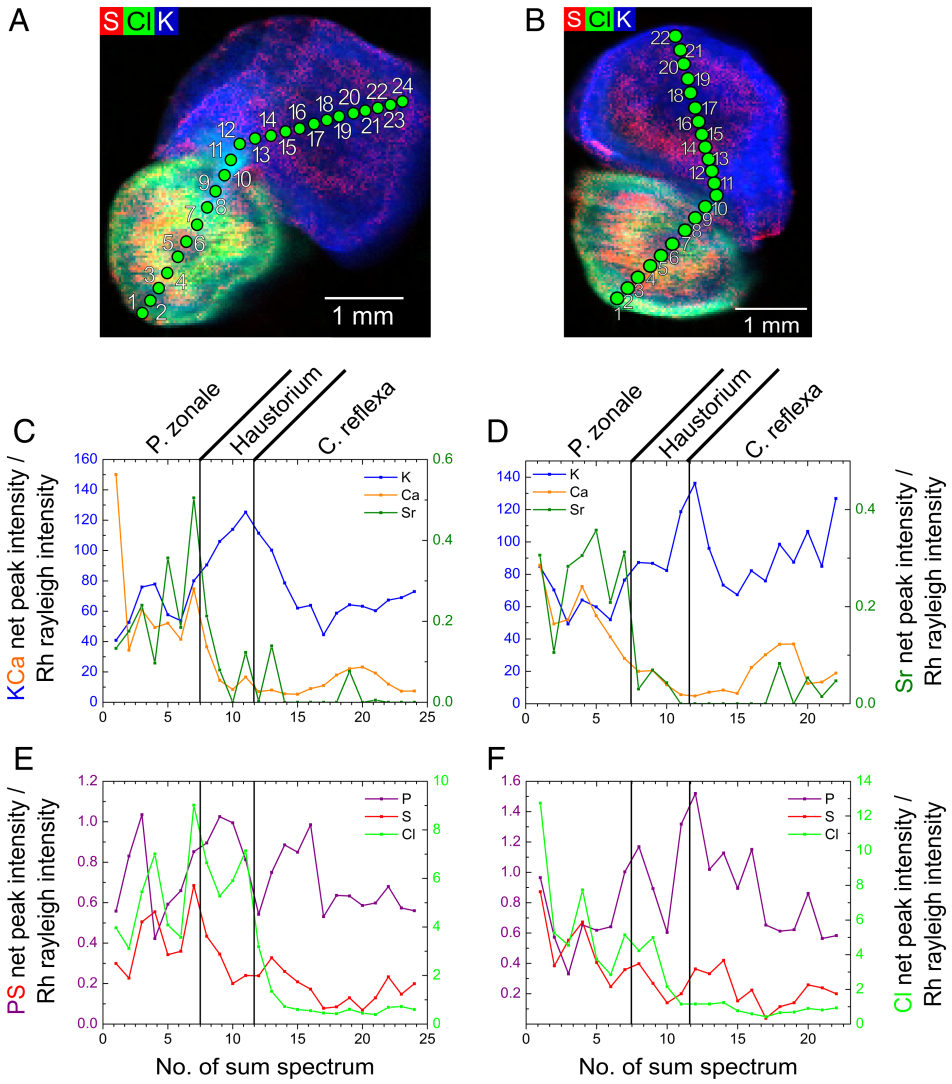

Fig. 5. Elemental distribution along a median transect in two independent infection sites on *Pelargonium zonale* petioles. (A-B) In the elemental images 24 (A) and 22 spectra (B), respectively, were generated by summing the intensity of approximately 16 pixels for each spectrum at the positions marked with the green circles. (C) Net peak intensity values for the K fluorescence lines of K, Ca and Sr normalized to the Rh Rayleigh scattering signal for each green circle shown in (A). (D) Same as (C) but representing green circles in (B). (E) Net peak intensity values for the K fluorescence lines of P, S and Cl normalized to the Rh Rayleigh scattering signal for each green circle shown in (A). (F) Same as (E) but representing green circles in (B).

accurate ratio comparisons. Also, the needed sample volumes limited the spatial resolution of these studies significantly. Modern XRF techniques, such as TXRF or MXRF, allow to do parallel measurements with the same sample for a wide range of elements, including those present in minute amounts (Andresen et al. 2018), which increases the accuracy of pattern comparisons also in the micronutrient range of elements. These techniques have been applied to many single plants, but not yet to a system where two plants interact with each other. By presenting XRF-based data on the *Cuscuta-Pelargonium* interaction system, our study is the first to show a detailed spatial analysis of mineral nutrient uptake at the interface between a shoot parasitic plant species and its compatible host.

While, surprisingly, we failed to find evidence for a pronounced accumulation of any macro- or microelement in the parasite compared to the host plant, we did observe sharp reductions in the Cl, Mn and Ca contents, in addition to 80–90% reductions of non-essential or toxic elements such as Br and Sr. The relative patterns of all elements resembled each other in all parts of the stems, whether measured around the infection sites or in the shoot tips. They were also not changed when the parasite was connected to a stem supporting a sink tissue (flowers), as opposed to a source tissue (leaf). The observed concentrations of all elements in *C. reflexa* are generally in accordance with the earlier mentioned studies (Wallace et al. 1978, Saric et al. 1991) and reveal that the parasite does not invest much energy into their (pronounced) accumulation or storage. It underlines the strict dependence of the parasite on its hosts despite the parasite's ability to photosynthesize (van der Kooij et al. 2000), and

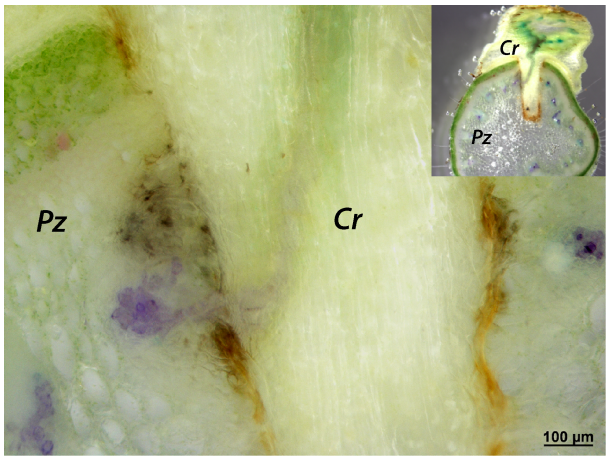

Fig. 6. Xylem connections between *Cuscuta reflexa* (*Cr*) and *Pelargonium zonale* (*Pz*). Hand-sectioned cross-section showing the host/haustorium interface of an infected host pedicel watered with blue Chinese ink (diluted 1:10 in water). The inset in the top right corner shows the entire infection site with the parasite on top of the host.

explains the limited survival time of *C. reflexa* shoots in the absence of a host. The strong depletion of some elements suggested the existence of one or more filtering mechanism(s) that hinder the import of these nutrients into the parasite. For Ca, Mn and Sr, MXRF provided evidence for a tentative selectivity barrier at the host–parasite interface and for a second tentative gateway that seems to control the uptake of Cl between the endophytic and exophytic tissue of *C. reflexa*. The identical distribution of Ca^{2+} and Sr^{2+} ions at the infection sites can be explained by the lack of discrimination of many Ca transport systems between these two ions and is corroborated by the fact that Sr can be used as analog for Ca to label the routes it travels through plants (Storey and Leigh 2004). In the following, the physiological role of the three depleted nutritional elements, Cl, Mn and Ca, and the putative implications of their lower abundance in *Cuscuta* shall be discussed.

Chlorine

Chloride (Cl^-), the anion of the element chlorine, is required, among others, in the oxygen-evolving complex of photosystem II (PSII) (Wege et al. 2017) and is thus essential for photosynthetic plants. However, only micromolar concentrations are required to avoid Cl-deficiency symptoms like chlorosis. The levels found in *C. reflexa* are several times higher than what is necessary for photosystem II function so that the very low photosynthesis rates in *C. reflexa* (van der Kooij et al. 2000) cannot be the reason for the low Cl accumulation rates.

It was reported that the macronutrient concentrations at which the Cl^- anion is present can act as a charge balance of essential cations such as K^+ and Ca^{2+} and as an osmoticum to regulate cell turgor (Franco-Navarro et al. 2016, Raven 2017, Wege et al. 2017). It also regulates stomatal conductance, which has an impact on water-use efficiency (Franco-Navarro et al. 2016). These attributes make Cl a beneficial macronutrient (Geilfus 2018) and may explain its observed distribution pattern in *C. reflexa*. On one hand, the haustorium with its rapid and invasive growth requires a high turgor pressure, which again calls for high Cl concentrations. On the other hand, *C. reflexa* stems lack leaves for transpiration and their stems have very few stomata, reducing transpiration and therefore the need of Cl to maintain their water balance. How the differential depletion of Cl is achieved in the parasite remains to be answered.

Manganese

Mn was the only element found to accumulate moderately in shoot tips, so that its depletion in tissue close to the infection site may be a result of low uptake rates paired with a relocation within the parasite. Mn is bound to more than 30 proteins as a catalytically active metal, among others in the water-splitting apparatus of photosystem II (Hänsch and Mendel 2009). It is required for photosynthesis, carbohydrate metabolism, and lipid and lignin biosynthesis. Photosynthetic activity was slightly higher in the shoot tips (van der Kooij et al. 2000), which could explain the moderate accumulation of Mn there. Whether *Cuscuta* restricts the uptake of Mn as part of a control mechanism to avoid Mn phytotoxicity symptoms that are known from crops (Fernando and Lynch 2015) is currently unknown.

Calcium

Ca is a macronutrient whose levels can differ by a factor of 50 between different species (from 1 to 50 mg Ca g^{-1} dry matter; Watanabe et al. 2007, Neugebauer et al. 2018, White et al. 2018) and in different tissues of the same plant. Ca participates in numerous processes involving nearly all aspects of plant development (Hepler 2005). The extremely low concentrations at which Ca is needed in the cytoplasm (≤ 0.1 µM) are reached even in *C. reflexa*, but the situation is different in the apoplast, where more than 50% of the total cellular Ca are present in its charged form (as the Ca^{2+} ion). Ca is particularly found in the primary cell wall, middle lamella and at the outer face of the plasma membrane. Its removal rapidly compromises membrane integrity (Hepler and

Winship 2010, de Freitas et al. 2012) and impacts the cross-linking of pectic homogalacturonan (HG), thereby jeopardizing the structure and the rigidity of the pectic cell wall fraction (Willats et al. 2001, Bosch and Hepler 2005, Hongo et al. 2012).

With less than 2 mg g^{-1} dry matter, the Ca levels in *Cuscuta* tissues proximal to an infection site and in distal shoot tips are decidedly at the lowest end of this spectrum and are only matched by Poales (2–10 mg g^{-1} dry matter). Here, the low Ca levels are a result of the low pectin content of their cell walls (Watanabe et al. 2007, Neugebauer et al. 2018, White et al. 2018). Immunological analyses of the cell wall composition of *C. reflexa*, however, revealed no apparent differences in Ca-crosslinked pectins (Johnsen et al. 2015 and Fig. S5), but this tentative conclusion should be viewed with caution since the antibody used to detect Ca-crosslinked pectins requires Ca in the binding buffer in order to work, and this could mask a Ca-deficiency in the tissue. Future work will have to find answers to imposing question like whether Ca was replaced by other ions in the apoplast and in other Ca-rich organelles in *Cuscuta* or how Ca deficiency symptoms at these far-below-average Ca levels are avoided.

Transport processes as the basis for element uptake selectivity

Long distance transport of mineral nutrients in the xylem of plants is driven strongly by the transpiration stream but many, though not all, elements can also be remobilized and redistributed by transport in the phloem, for example from leaves to roots, fruits (Etienne et al. 2018) or to a parasite. Cl^- is highly mobile both in xylem and phloem and should in theory experience no restrictions in its uptake, and yet the parasite shows lower levels of Cl. With the Cl concentrations in the haustorium matching that of the surrounding host tissue, one explanation is that the element is retained actively in the haustorium to maintain turgor pressure (see above) by reducing transfer from there to the *Cuscuta* stem. This is assumed to be particularly important in the young growing haustoria that were analyzed in our study. A theoretical alternative to explain the uneven Cl distribution is that it is depleted from the stems close to infection sites through rapid transport into other parts of the parasite. That the growing shoot tip could be a sink for Cl was not substantiated in this study, but it is still possible that older parts of the parasite could accumulate Cl. A search in the sequence databases for *Cuscuta* revealed that the parasite contains homologs for almost all Cl^- transporters and channels found in autotrophic plants (data not shown). However, it should be pointed out that these transporters belong to gene families for which the specific functions have not been dissected in detail. Functional studies, protein localization and spatially-resolved expression patterns of all putative Cl^- transporter candidates should be investigated before the basis for the differential Cl distribution can be explained.

Strikingly, the two essential elements Mn and Ca, which show the largest reduction in their concentration in *C. reflexa* compared to the host, have low (Mn) or no (Ca) mobility in the phloem. These elements can therefore enter *Cuscuta* mainly, or only, via the xylem bridge between the two species. Seeds and fruits that are mainly phloem-fed sink tissues have low Ca-values (Dayod et al. 2010, Hocking et al. 2016). Like parasitic plants, fruits are strong sinks for metabolites and nutrients and the analogous Ca-deficiencies in both tissues are therefore noteworthy. With the ripening process, many fruits (e.g. tomato, apple, kiwifruit and grape) exhibit a dramatic shift in the proportion of xylem and phloem transport, as the contribution of the xylem gradually becomes negligible (Choat et al. 2009, Song et al. 2018). This shift is often accompanied by a sharp drop in Ca uptake (Hocking et al. 2016) and is generally believed to be based on a physical disruption of the fruit xylem or the development of pectin plugs, which reduce the direct hydraulic link of fruit water status to that of the plant (Choat et al. 2009, Hocking et al. 2016). However, new evidence from grapes suggests that ceasing transpiration in ripe fruits leads to a diminishing water potential gradient and this leads to a stop of xylem transport (Chatelet et al. 2008, Choat et al. 2009). Recent work by Keller et al. 2015 even showed that water export, as part of a recycling process to remove surplus water from phloem import, is mediated by a reversal of water flux in the xylem of grape berries (Zhang and Keller 2017). This is an attractive model for the *Cuscuta*-host interaction that may help explain some of the observed mysteries. Adopting this model, we would have to assume that young haustoria, i.e. directly after fusion with the vascular system of the host, would import water and nutrients by both phloem and xylem, which is corroborated by published data (Christensen et al. 2003) and by our ink transport analysis (Fig. 6). In older haustoria, water import by the xylem would be expected to stop or even reverse to remove surplus phloem water that cannot be lost by transpiration in *Cuscuta* due to its low stomata count and lack of leaves. Support for this tentative 'flux reverse model' comes from the observation that proteins (Haupt et al. 2001) or RNA (Kim et al. 2014) can move in both directions over the host/parasite border.

The holoparasitic root parasite *Aeginella indica* (Orobanchaceae), like *C. reflexa*, also exhibits very low Ca levels (2.4 mg g^{-1} dry matter) and low Cl and Mn

concentrations (Watanabe et al. 2007). Interestingly, and in contrast to these two holoparasitic species, the hemiparasite *Odontites verna* (Rozema et al. 1986) that sequesters only inorganic nutrients via xylem connections with their hosts boosts a high accumulation of Ca. Unfortunately, no transpiration rates have been reported for this species, but the high transpiration rates observed in the hemiparasite *Striga*, as a result of the incapacitation of stomatal closure (Fujioka et al. 2019), may hint at the importance of transpiration for the parasite and thus for the Ca-balance and make more detailed comparative studies among parasites imperative.

Author contributions

Conceptualization: I.M., K.F. and K.K. Methodology: F.F., I.M., H.S., K.F. and K.K. Investigation: F.F., I.M., H.S., L.A-M.L., K.F. and K.K. Visualization: F.F., I.M., L.A-M.L., K.F. and K.K.; Figure design, writing and editing: F.F., I.M., B.K., H.S., L.A-M.L., K.F. and K.K. Funding acquisition: B.K. and K.K.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Element composition of extracts from apical shoot tips of *Cuscuta reflexa*.

Fig. S2. Impact of sample preparation, stem age and stem type on element spectrum intensity and relative amounts.

Fig. S3. Element distribution in *Cuscuta reflexa* infection sites.

Fig. S4. Elemental distributions in infection sites of *Cuscuta reflexa* on two different hosts, *Pelargonium zonale* and *Pelargonium citriodorum*.

Fig. S5. Immunological detection of calcium-crosslinked pectin.

Movie S1. Video file showing a rotating confocal 3-D representation of the scanned area shown in Fig. 4B.

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Paper 4

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The Enigma of Interspecific Plasmodesmata: Insight From Parasitic Plants

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Parasitic plants live in intimate physical connection with other plants serving as their hosts. These host plants provide the inorganic and organic compounds that the parasites need for their propagation. The uptake of the macromolecular compounds happens through symplasmic connections in the form of plasmodesmata. In contrast to regular plasmodesmata, which connect genetically identical cells of an individual plant, the plasmodesmata that connect the cells of host and parasite join separate individuals belonging to different species and are therefore termed "interspecific". The existence of such interspecific plasmodesmata was deduced either indirectly using molecular approaches or observed directly by ultrastructural analyses. Most of this evidence concerns shoot parasitic *Cuscuta* species and root parasitic Orobanchaceae, which can both infect a large range of phylogenetically distant hosts. The existence of an interspecific chimeric symplast is both striking and unique and, with exceptions being observed in closely related grafted plants, exist only in these parasitic relationships. Considering the recent technical advances and upcoming tools for analyzing parasitic plants, interspecific plasmodesmata in parasite/host connections are a promising system for studying secondary plasmodesmata. For open questions like how their formation is induced, how their positioning is controlled and if they are initiated by one or both bordering cells simultaneously, the parasite/host interface with two adjacent distinguishable genetic systems provides valuable advantages. We summarize here what is known about interspecific plasmodesmata between parasitic plants and their hosts and discuss the potential of the intriguing parasite/host system for deepening our insight into plasmodesmatal structure, function, and development.

Keywords: *Cuscuta*, feeding hyphae, haustorium, interspecific plasmodesmata, parasitic plants, secondary plasmodesmata, symplasm

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INTRODUCTION

Symplasmic domains are operational units which are formed by joining the protoplasts of cells by way of plasmodesmata (PD) that form complex structures across the plant cell walls (Ehlers and Kollmann, 2001) or by sieve pores that originate from PD (Kalmbach and Helariutta, 2019) but are limited to the sieve elements of the phloem. Based on when and where they originate, two different types of PD are distinguished: primary PD originate during cell division, while secondary PD are

formed across already existing cell walls. Despite their different origin, no structural differences can be discerned between them (Burch-Smith et al., 2011). Studies of secondary PD have, therefore, focused on non-division walls, which are of ontogenetically different origin and contain exclusively secondary PD (Ehlers and Kollmann, 2001). While this is a convenient system for structural analyses, a challenge that remains is to delineate the chain of molecular events that regulates secondary PD formation. To this end, the study of PD formed between genetically different plants promises a possibility to distinguish the molecular steps in each of the two cells that contribute to their establishment. Such interspecific PD (iPD) are by definition secondary as they are inserted in principle into existing cell walls of two unrelated individuals. Such a situation occurs either in graft unions (Kollmann and Glockmann, 1985, 1991) or at the interface between parasitic plant haustoria and the invaded tissue of their hosts (Dörr, 1969; Lee, 2009). While grafting is limited to closely related species of a few angiosperm families, some parasitic plants infect a wide range of distantly related host plant species encompassing both monocots and dicots (Westwood et al., 2010).

Parasitic plants, by definition, procure part or all of their nutrients from autotrophic plants, which serve as their hosts. Having initially evolved from fully photoautotrophic ancestors, they now occupy a narrow and specialized but apparently lucrative niche – given that the evolution of parasitic lineages has taken place many times independently within the angiosperms (Nickrent, 2020). The specialized lifestyle has led to various adaptations of which the invention of an infection organ, termed haustorium, was the primary key to their success (Yoshida et al., 2016). The term haustorium refers to the tissue of the parasite that develops endophytically within the infected host plant and is a morphological trait that is common to all parasitic plants (Smith et al., 2013). Unlike their fungal counterparts, parasitic plant haustoria are complex multicellular organs. With them, parasites can invade either the shoots (e.g., dodders, mistletoes) or the roots (e.g., broomrapes) of their hosts and withdraw either only water and inorganic nutrients through xylem connections (hemiparasites) or inorganic plus organic compounds via connections to host xylem, phloem, and parenchyma cells (holoparasites).

One parasitic plant genus that has been classified as a noxious weed in many countries is *Cuscuta* (dodder) (**Figure 1A**). *Cuscuta* species are destructive shoot parasites due to their broad host spectrum that includes annual plants and perennial shrubs and trees from most orders within the angiosperm lineage (Vogel et al., 2018). The endophytic haustorium of *Cuscuta* species protrudes from the center of a suction cup-like ring, the adhesive disk, which anchors the parasite to the host surface (Vaughn, 2002; Lee, 2007). At an early stage of infection, the haustorium penetrates the host plant surface by applying mechanical pressure and releasing cell wall degrading enzymes that weaken the host tissue cohesion (Vaughn, 2003; Johnsen et al., 2015). Following this initial invasion, the haustorium expands and grows through the cortex and often the sclerenchymal ring in search of the vascular tissue of the host. At the final stages of the infection, elongated cells (so-called searching and feeding hyphae) emerge from the tips and flanks of a haustorium (**Figure 1B**). The active feeding stage usually only lasts for a limited time, and the process of nutrient acquisition is taken over by younger haustoria as the parasite grows and finds new hosts.

The haustorial hyphae form physical and physiological bridges between host and parasite (**Figures 1C–E**) and facilitate the nutrient and water transfer. The hyphae appear to recognize which host cell type they approach, and they differentiate into a matching cell type (Vaughn, 2006). Thus, xylem vessels of the host, which are comprised of tube-like dead cells are intercepted by xylem-like (xylic) hyphae that re-direct water and minerals to the parasite (Christensen et al., 2003). On the other hand, amino acids, sugars, and other organic molecules in the phloem sap are channeled to the parasite through phloic hyphae that surround the host sieve elements (Dörr, 1972; Hibberd and Jeschke, 2001; Birschwilks et al., 2006; Vaughn, 2006). Hyphae connecting to parenchymal host cells show fewer morphological changes but are characterized by an electron-dense and organelle rich cytoplasm (Dörr, 1969). Chimeric cell walls and symplasmic connections between the different hyphae and the host tissue provide cohesion between the partners and it is tempting to also assume that they ensure the efficiency in nutrient uptake that the parasite depends on.

EVIDENCE FOR SYMPLASMIC CONNECTIONS BETWEEN HOLOPARASITIC PLANTS AND THEIR HOSTS

Although investigations of cytoplasmic contacts between parasitic plant haustoria and the infected host tissue are not exceptionally abundant, indirect and direct evidence for iPD at the host/parasite interface has accumulated over the past half century (**Table 1**).

Physiological and Molecular Evidence From the Genus *Cuscuta*

In contrast to mineral nutrients and small organic compounds that in plants take both apoplastic and symplastic transport routes (Offler et al., 2003; Zhang and Turgeon, 2018), macromolecules (proteins or nucleic acids) require symplasmic connections, either in the form of PD between neighboring cells or through sieve pores or sieve plates between sieve elements (Kalmbach and Helariutta, 2019). A nice demonstration of macromolecular transport between host plants and *Cuscuta* and, with it, unequivocal proof for a continuous and efficient connection between parasite and host vascular bundles was provided using the green fluorescent protein (GFP) (Haupt et al., 2001; Birschwilks et al., 2007). That the exchange of proteins in fact occurs at a large scale was recently shown through a proteomics approach (Liu et al., 2020). Several 100 host proteins were identified in *C. australis* growing on *A. thaliana* or soybean and, surprisingly, hundreds of *Cuscuta* proteins were found in the two host plants, indicating a massive bidirectional protein movement. Furthermore, mRNAs were found to move from host to parasite (Roney et al., 2007; David-Schwartz et al.,

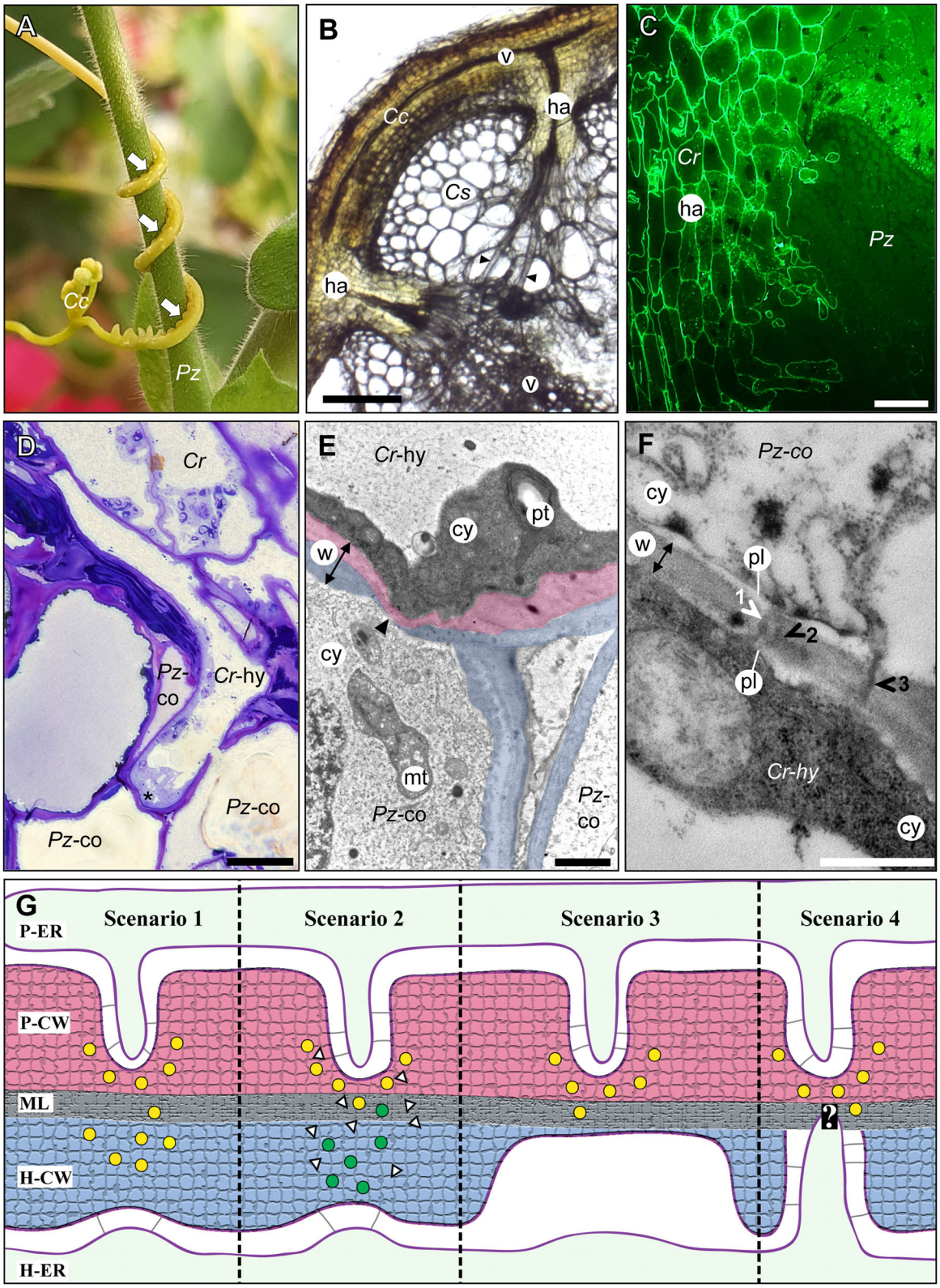

FIGURE 1 | The host/parasite feeding interface. **(A)** The yellow vine *C. campestris* (*Cc*) twines around its host *Pelargonium zonale* (Pz) making infection sites (arrows) where parasitic haustoria penetrate the host tissue. **(B)** Light micrograph of a transverse vibratome section of *C. campestris* (*Cc*) infecting *Cucumis sativus* (*Cs*)

(Continued)

FIGURE 1 | revealing the endophytic haustoria (ha) with their protruding hyphae (black arrowheads) that connect both plants' vascular elements (v). Scale bar: 300 μm. **(C)** Fluorograph of an immunolabeled microtome cross section of a parasite/host boundary. A monoclonal antibody (JIM8) against arabinogalactan proteins selectively labels *C. reflexa* (*Cr*) cell walls but not cell walls of the host *P. zonale* (*Pz*) and enables the precise identification of the haustorium (ha) interface. Scale bar: 100 μm. **(D)** Light micrograph of a toluidine blue-stained section showing a hypha (*Cr*-hy) of the parasite *C. reflexa* (*Cr*). The hypha has grown through one host cortex cell (*Pz*-co) and is in the process of penetrating another (site marked with an asterisk, *). Scale bar: 20 μm. **(E)** Electron micrograph of the hypha (*Cr*-hy) shown in **(D)** penetrating a host cortex cell (co). The thinned or ruptured host cell wall is marked with an arrowhead. Parasite (*Cr*) and host (*Pz*) cell walls are highlighted with red and blue shading, respectively. The cell wall (w, with double-sided arrow indicating its width), cytoplasm (cy), host cell mitochondrion (mt), and parasite plastid (pt) are labeled. Scale bar: 2 μm. **(F)** Electron micrograph of a cell wall (w) between a *C. reflexa* hypha (*Cr*-hy) and a penetrated *P. zonale* cortex cell (*Pz*-co). Three plasmodesmata (1, 2, and 3) are marked with arrowheads that are colored either white where they connect to both cells' plasmalemma (pl) and black where they appear to cross the wall only partially. PD 2 appears to be branched, while the others are seemingly unbranched PD. Scale bar: 0.5 μm. cy = cytoplasm. **(G)** Schematic illustration of four hypothetical scenarios (Scenarios 1–4) how PD formation at the parasite/host interface could be coordinated. Cell walls are shaded with red (parasite) and blue color (host) like in **(E)**. Cell wall enzymes secreted to thin/loosen the cell walls are represented by yellow (from parasite) or green (from host) dots. In Scenario 1, the parasite-secreted enzymes are moving across the middle lamellae (ML) to act on the host cell wall (H-CW). In Scenario 2, unknown signals (white triangles) from the parasite induce the release of host cell wall enzymes (green dots) to autodecompose their cell walls locally. In Scenario 3, the parasite cell wall enzymes are secreted in a location where the host wall is already thin [see situation at hyphal tips in **(E)**]. In Scenario 4, the parasite cell wall enzymes are secreted in a location where a pre-infection host PD is present. The white question mark indicates that this scenario is the most speculative because it assumes that the parasite is able to locate the host PD. The association of parasite ER (P-ER) and host ER (H-ER) with their respective plasma membranes is indicated by gray lines. The methods used to generate the microscopy images are described in the **Supplementary Materials** file.

2008; LeBlanc et al., 2013) and this happens at a genomic scale involving transcripts of thousands of genes (Kim et al., 2014). MicroRNAs are also shuttled from the parasite to the host to target host gene expression (Shahid et al., 2018; Johnson and Axtell, 2019). Last but not least, plant viruses have for 75 years been known to move between *Cuscuta* and its host plants (Bennett, 1944; Mikona and Jelkmann, 2010), a transmission also depending on PD. Collectively, these data point to a massive flow of substances in both directions that cannot be explained by apoplastic translocation alone but necessitates open symplasmic connections between *Cuscuta* and its hosts. There are to date no molecular studies that explain how this massive flow could be regulated or to what degree it is selective.

Ultrastructural Evidence From the Genera *Cuscuta* and *Orobanche*

Despite the molecular data discussed above, there is only limited ultrastructural evidence for symplasmic connections between parasite and host vascular tissues (**Table 1**). In the root parasitic genus *Orobanche* a connection between parasite and host via sieve elements has been convincingly shown for *O. crenata* connecting to *Vicia narbonensis* (Dörr and Kollmann, 1995) and for *O. cumana* parasitizing *Helianthus annuus* (Krupp et al., 2019). In both cases, interspecific sieve plates were observed. For the shoot parasite *Cuscuta*, in contrast, compelling evidence for sieve plates at the parasite/host border is still lacking. Claims regarding sieve pores between phloic hyphae of *Cuscuta japonica* and sieve elements of *Impatiens* (Lee, 2009) were not supported by visual evidence and have not been confirmed when the same host was infected with *Cuscuta pentagona* (Vaughn, 2003, 2006). However, several accounts of plasmodesmata between host parenchyma cells and *Cuscuta* searching hyphae have been published (**Table 1**). Such investigations revealing iPD have used five different *Cuscuta* species infecting an even larger range of different hosts from genera like *Pelargonium*, *Vicia, Impatiens, Nicotiana* or *Arabidopsis* (Dörr and Kollmann, 1995; Vaughn, 2003, 2006; Birschwilks et al., 2006, 2007). The reports differ with respect to the abundance of iPD and it was proposed that they may be relatively short-lived and present only in hyphae from the younger parts of the haustorium while they seem to degenerate later (Dörr, 1969; Vaughn, 2003). Both authors provided very detailed descriptions of the versatile iPD structures with unbranched and complex branched forms with visible desmotubules occurring side by side. Vaughn (2003) also described collars and fibrillar spokes radiating out from the desmotubule, suggesting that their ultrastructure could be very similar to that of PD that connect cells from the same organism. Later stages were observed to contain occlusions or appear to fuse and form hairpin loops running back to the same cell, but it should be kept in mind that the reports show 2-dimensional snapshots of a complex system and both the spatial and temporal dimensions have not been investigated. Therefore, caution should be exercised when interpreting findings of incomplete iPD (see **Figure 1F** and literature cited in **Table 1**). This notion, together with the still unexplained sustained transport activities, calls for higher temporal resolution of haustorial development and additional modern technologies in future studies of the host/parasite connections.

ESTABLISHMENT OF INTERSPECIFIC SECONDARY PD

iPD are a special case of secondary PD as they span the cells of different individuals, species and even higher order phylogenetic lineages. So far, very little is known about this type of PD.

Control of Secondary PD Formation

Some evidence suggests that PD do not develop from one side only, but that they are formed in a coordinated process by the two opposing cells (Kollmann and Glockmann, 1991; Ehlers and Kollmann, 2001). The process is believed to start with a local thinning of the cell wall on both sides followed by the trapping of ER cisternae which develop into plasmodesmal desmotubules, the fusion of the two plasma membranes and finally the reconstruction of the cell wall (Ehlers and Kollmann, 2001; Burch-Smith et al., 2011). If both cells contribute to the formation of complete secondary PD, some kind of communication across the cell borders is needed. Potential scenarios how this could

TABLE 1 | Summary of studies investigating cell-to-cell connections between parasitic plants and their hosts.

	Species		Host cell type		Interspecific symplasmic connection	Experimental method						References (chronologically sorted for each category)
	Parasite	Host	Parenchyma	Phloem		EM	IL[a]	FP[b]	FS[c]	RT[d]	V[e]	
Ultrastructural studies	*O. cumana*	*Helianthus annuus*		●	SP	●						Krupp et al., 2019
	C. japonica	*Impatiens balsaminea*		●	PD, SP	●						Lee, 2009
	C. reflexa	*Arabidopsis thaliana*	●		PD	●						Birschwilks et al., 2007
	C. platyloba	*Arabidopsis thaliana*	●		PD	●						Birschwilks et al., 2007
	C. odorata	*Arabidopsis thaliana*	●		PD	●						Birschwilks et al., 2007
	C. reflexa	*Vicia faba*	●		PD	●						Birschwilks et al., 2006
	C. platyloba	*Nicotiana tabacum*	●		PD	●						Birschwilks et al., 2006
	C. odorata	*Nicotiana tabacum*	●		PD	●						Birschwilks et al., 2006
	C. pentagona	*Impatiens balsaminea*	●		PD	●	●					Vaughn, 2006
	C. pentagona	*Impatiens sultanii*	●		PD	●	●					Vaughn, 2003
	O. crenata	*Vicia narbonensis L.*	●	●	PD, SP	●						Dörr and Kollmann, 1995
	C. odorata	*Pelargonium zonale*	●		PD	●						Dörr, 1969
Macromolecular transport	*P. ramosa*	*Brassica napus*		●	SP				●			Peron et al., 2016
	P. aegyptiaca	*Solanum lycopersicum*		●	SP		●	●	●			Ekawa and Aoki, 2017
	P. aegyptiaca	*Solanum lycopersicum*		●	SP			●				Aly et al., 2011
	C. reflexa	*Arabidopsis thaliana*		●	SP			●	●	●		Birschwilks et al., 2007
	C. odorata	*Arabidopsis thaliana*		●	SP			●	●	●		Birschwilks et al., 2007
	C. platyloba	*Arabidopsis thaliana*		●	SP			●	●	●		Birschwilks et al., 2007
	C. reflexa	*Vicia faba*		●	SP			●	●	●	●	Birschwilks et al., 2006
	C. odorata	*Nicotiana tabacum*		●	SP			●	●	●	●	Birschwilks et al., 2006
	C. platyloba	*Nicotiana tabacum*		●	SP			●	●	●	●	Birschwilks et al., 2006
	C. reflexa	*Nicotiana tabacum*		●	SP			●	●			Haupt et al., 2001

Ultrastructural studies provided direct evidence for the presence of interspecific symplasmic connections, while molecular studies provided indirect evidence for their existence based on macromolecular transport analysis. The main experimental approaches in each study (EM, electron microscopy; IL, immunolabeling; FP, fluorescent protein transport; FS, fluorescent stain; RT, radioactive tracer labeling; V, virus movement) are indicated. PD, plasmodesmata; SP, Sieve pores.

[a] *Callose antibody.*

[b] *AtSUC2-GFP, Tobacco mosaic virus movement protein-GFP, ER-targeted GFP.*

[c] *5,6-carboxyfluorescin diacetate (CFDA) for transport studies or aniline blue for callose staining.*

[d] *14C or 3H.*

[e] *potato virus Y isolate N.*

happen are depicted in **Figure 1G**. It should be noted that these are hypothetical alternatives and experimental insight regarding the regulation of secondary PD formation and the molecules involved in signaling is lacking. Whether PD initiation happens unilaterally by one cell in a given tissue or starts simultaneously in two neighboring cells, is also unresolved. While in the parasite/host system it is presumably the parasite that initiates PD formation as this connection appears to be vital for the parasite's survival, it is likewise still unclear how and how much the host contributes (**Figure 1G**).

Cell Wall Degradation and Rebuilding

Cell wall breakdown and rebuilding are thought to be important steps of secondary PD formation (Ehlers and Kollmann, 2001; Burch-Smith et al., 2011). In the case of intraspecific PD the two parts of the common cell wall and the enzymatic machinery for the cell wall remodeling are in principle identical. The cell walls of the host and parasite, on the other hand, do differ to some degree (Johnsen et al., 2015) (**Figure 1C**) and accordingly the enzymes involved in remodeling the cell walls are also expected to differ. It is well-known that during invasion of the host the parasite secretes a cocktail of enzymes which degrade the cell walls of the host but not their own (Nagar et al., 1984; Losner-Goshen et al., 1998; Olsen et al., 2016). Host cell walls abutting haustorial cells were observed to have a lower degree of pectin esterification than walls that were not in contact with the haustorium (Johnsen et al., 2015). Young hyphae were also often found to be surrounded by host cell walls that were stretched extremely thin [**Figures 1D,E** and Vaughn (2003)]. This provided evidence for extensive deconstruction and loosening of the host cell walls at the site of contact, but it remains speculative whether this thinning is mediated by host or parasite enzymes (see **Figure 1G**, scenarios 1 and 2). With cell wall degradation products being discussed as potential signaling molecules for cell wall integrity (Ferrari et al., 2013), the parasite's enzymes could tentatively contribute to the coordination of PD formation between parasite and host by inducing host enzyme secretion in corresponding places (**Figure 1G,** scenario 2). Alternatively, similar signals may help the parasite identify regions with thinned host walls and

induce PD formation in these regions (**Figure 1G**, scenarios 3 and 4).

iPD in Graft Unions

Besides parasitic/host interfaces, graft unions are sites where interspecific symplasmic connections can potentially be formed. Already Jeffree and Yeoman (1983) observed cell wall thinning and formation of plasmodesmata in opposing cells of autografted tomato (*Solanum lycopersicum*) plants. More pertinent, Kollmann and colleagues were able to show iPD in heterografts between different species (Kollmann et al., 1985) and different orders (Kollmann and Glockmann, 1985, 1991). Anatomically, both full and partial unbranched connections as well as complex branched PD were described, thus resembling closely what has been found at the parasite/host interface. Cell wall thinning seemed to precede the PD formation in the described cases. Using serial sections, Kollmann and Glockmann (1985) could show that apparently incomplete "half" iPD were in fact continuous structures connecting both adjacent cells. However, this seems to depend on the cell types that align with each other and "half PD" that end at the middle lamella were found where the alignment was not perfect (Kollmann et al., 1985). Diffusion through graft interface iPD was demonstrated using fluorescein in grafts between different Prunus species (Pina et al., 2009), demonstrating the functionality of these structures in transport.

PARASITIC PLANTS AS TOOLS FOR THE ANALYSIS OF SECONDARY PD

Secondary iPD at the host/parasite border are an excellent system to overcome limitations of current PD research for several reasons. First, the symplasmically connected partners have different genotypes, and form many more different combinations than grafting currently offers. This facilitates the identification of the origin of the genes and proteins involved in the establishment of secondary PD, which could finally answer the question whether the PD are initiated uni- or bilaterally. Moreover, the searching and feeding hyphae of the parasite can be faithfully distinguished based on their characteristic ultrastructure (Dörr, 1969, 1972; Vaughn, 2003, 2006) or on unique epitopes in their cell walls (Vaughn, 2003; Johnsen et al., 2015) (**Figure 1C**). Thus, the border between parasite and host tissues and thereby the location of heterospecific cell walls can be precisely mapped. The parasite/host system therefore allows detailed analyses of the roles that each of the two symplasmically connected cells have in this process. In contrast, in successful grafts the two partners are often very closely related, making such differentiation more challenging, if not impossible.

Second, quite many parasitic plants, including the well-researched *Orobanche* and *Cuscuta*, can infect many different hosts (Yoshida et al., 2016; Shimizu and Aoki, 2019). Their host range includes popular model plants like *A. thaliana,* tobacco or tomato and thus offers the opportunity to harness all molecular genetic tools developed for those. Among them, a plethora of transgenic and mutant lines (overexpressing lines, knock-out lines, introgression lines) are available and have already been used to dissect parasite/host interactions (Hegenauer et al., 2016; Krause et al., 2018). Classical transgenic technology, RNA interference (Mansoor et al., 2006) and genome editing technologies like CRISPR-Cas9 (Doudna and Charpentier, 2014) are available for many compatible hosts. Furthermore, whole genome sequences and large-scale transcriptomic datasets are available for hundreds if not soon thousands of plants (Wong et al., 2020). On the parasite side, the first genome sequences have been published for *Cuscuta* (Sun et al., 2018; Vogel et al., 2018). Although transgenic parasitic plants cannot yet be produced efficiently, recent progress gives reason to believe that genetic manipulation of these parasites will soon be a standard (Lachner et al., 2020).

With the development of new methodology for tracing symplasmic transport via non-invasive approaches and suitable biotracers, the origin and fate of enzymes and structural components and maybe even of signaling molecules might in the future be traceable or even manipulated unilaterally using interspecific interfaces in parasites, but also in grafts.

WHAT CAN WE LEARN ABOUT PD USING THE PARASITE/HOST SYSTEM?

The basic structure of primary and secondary PD is very similar (Brunkard and Zambryski, 2017; Sager and Lee, 2018). The ER membranes and the plasma membranes of the two cells are fused and span the PD to provide a symplasmic connection. However, it is unclear whether the fusion resembles well-described membrane fusion processes, e.g., those between vesicles and the plasma membrane, or whether it is completely different. In the parasite/host system the protein composition and most likely also the lipid composition of the membranes of the two cells are sufficiently different to be of benefit for more detailed analyses of the fusion process.

Proteins also contribute to the structure of PD (Sager and Lee, 2018). Although in the last decades many proteins localized in PD have been identified (Han et al., 2019), their physiological and molecular functions are mostly unknown. It is not even known if the proteins are contributed by one or both cells. The different genotypes of the host and parasite cells provide an optimal instrument to answer such developmental questions.

Transport through PD changes during the course of plant development and in response to stress, and is therefore tightly controlled through the size exclusion limit (SEL) or pore size, or by closure of the PD (Brunkard and Zambryski, 2017). Although some factors regulating transport through PD such as light, the circadian clock (Brunkard and Zambryski, 2019) or sugars (Brunkard et al., 2020) have been identified recently, there is limited knowledge about PD regulation at the physiological and molecular level. Only a few molecules regulating SEL have been characterized. Among them are virus movement proteins which increase SEL to allow movement of viruses in a process called gating. In the parasite/host system similar processes are assumed to take place and it is tempting to speculate that this is achieved by

"gating molecules" produced by the parasite to prevent closing of the PD by the host. Indeed, it has been proposed that the control of the common host/parasite symplast is the key characteristic of compatible interactions (Cheval and Faulkner, 2017), a claim that could be tested by investigating the iPD.

CONCLUSION

iPD established between parasitic plants and their hosts offer a unique perspective on symplasmic domains and secondary PD in general. They promise to be an advantageous system to address and answer open questions regarding their formation and regulation. In particular, the respective contribution of neighboring cells can be analyzed and discriminated. Considering that adequate molecular tools for the parasites are only now beginning to emerge, we will hopefully see many new pieces of valuable information generated in this highly contemporary field in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.641924/full#supplementary-material

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