1 Introduction

Land plants are sessile organisms that depend on their immediate surroundings throughout their lives. To survive, plants perceive and react to abiotic and biotic stimuli. Plants engaging with other organisms, such as symbiosis with mycorrhizal fungi or nitrogen-fixing bacteria, is an ancient concept (Parniske, 2008; Doyle, 2011). The symbionts provide the plant with nutrients and nitrogen, in return, the plant sends mostly sugars (Morell & Copeland, 1984; Bago *et al.*, 2003). However, plants are also susceptible to pathogenic organisms, like parasitic nematodes that colonize and feed on the plant root, thereby benefiting from the host without killing it (Davis *et al.*, 2000). Parasitism is a highly successful strategy, not only for nematodes but among all kingdoms of life (Poulin & Morand, 2000). Plants also evolved parasitism (Westwood *et al.*, 2010). Parasitic plants satisfy their nutritional needs by infecting and parasitizing their host through a multicellular invasive organ, the haustorium (Kuijt, 1969). Some parasitic plants infect crop plants, resulting in severe yield loss (Musselman, 1980). Parasitic weed management options, however, are limited (Runo & Kuria, 2018). Parasitism requires mobile signaling cues and their distribution within the parasite, as well as in-between parasite and the host (Shen *et al.*, 2020; Wakatake *et al.*, 2020; Ogawa *et al.*, 2022). Plant parasitism-related signaling pathways show parallels to other plant developmental programs, such as lateral root development (Yoshida *et al.*, 2019). This study aimed to uncover the biogenesis and function of mobile cues aiding parasitism of plants on host plants.

1.1 Parasitic Plants

1.1.1 Evolution and Classification of Parasitic Plants

Parasitism convergently evolved 12 times in angiosperms, creating approx. 4750 species (Westwood *et al.*, 2010; Nickrent, 2020). Parasitic plants may be divided by their ability to attach to other plants' stems or roots. For instance, the parasitic vine *Cuscuta* spp. belonging to the lineage Solanales (Convolvulaceae) or mistletoes attach to the host's stem (**Figure 1**). The latter belong to the order Santalales, which contains roughly half of all parasitic species (Nickrent, 2020). Furthermore, other species of the order Santalales parasitize host roots like the famous sandalwood, *Santalum album* (**Figure 1**) (Těšitel *et al.*, 2021). Almost all of the remaining half,

over 2100 exclusively root-parasitic species, fall into the family of Orobanchaceae (Laminales) (Nickrent, 2020).

Parasitic plants can be further divided into hemiparasites and holoparasites. Hemiparasites are photosynthetically active and develop a xylem connection to the host, the latter being a prerequisite to enable the withdrawal of water and nutrients (Neumann *et al.*, 1999; Wakatake *et al.*, 2018). Hemiparasites can be further grouped into the evolutionary older mode of facultative parasitism versus younger obligate parasitism (Westwood *et al.*, 2010). Facultative hemiparasites, like *Triphysaria versicolor* or *Phtheirospermum japonicum* (Pj), survive even without a host (**Figure 1**), but seek a connection under nitrogen-deficient conditions when a host is available (Albrecht *et al.*, 1999; Ishida *et al.*, 2011; Kokla *et al.*, 2022). On the contrary, obligate hemiparasites, such as *Alectra vogelii* or *Striga asiatica*, depend on the host to complete their lifecycle (**Figure 1**) (Dörr *et al.*, 1979; Yoshida & Shirasu, 2009). Holoparasites like *Cuscusta* spp., *Orobanche cumana*, or *Phelipanche ramosa* abandoned the ability to photosynthesize by drastic reductions in the plastid genomes, i.e., pseudogenization or loss of photosystem I and II genes (**Figure 1**) (McNeal *et al.*, 2007; Wicke *et al.*, 2013). Therefore, holoparasites are always obligate (Stewart & Press, 1990). In addition to a xylem connection, holoparasites may develop phloem connections to the host (Dörr & Kollmann, 1995; Ekawa & Aoki, 2017; Krupp *et al.*, 2019). The phloem connection allows holoparasites to receive photoassimilates and exchange macromolecules, like RNA or proteins, with the host (Aly *et al.*, 2011; Shahid *et al.*, 2018). However, we are only beginning to understand the function of these parasitism-related mobile cues.

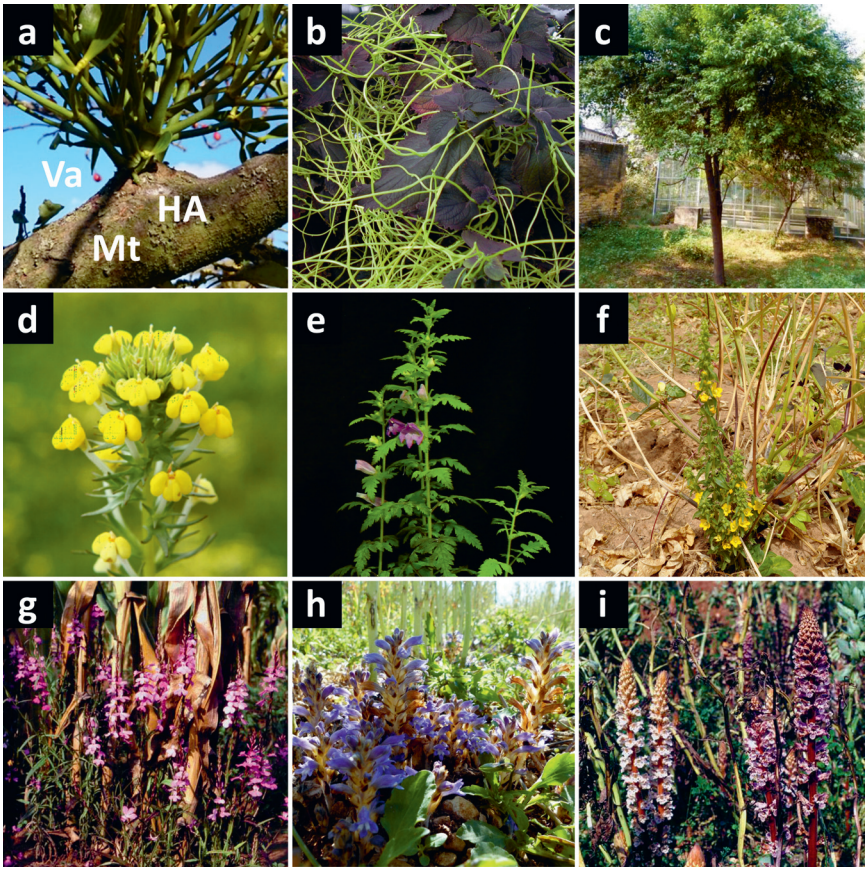

Figure 1: A selection of parasitic plants. Parasitic plant species repeatedly mentioned throughout this work are depicted: **a**, *Viscum album* (Va, Santalales) parasitizing crabapple (*Malus toringoides*, Mt) via the haustorium (HA) (Spallek *et al.*, 2017); **b**, *Cuscuta reflexa* (Solanales) overgrowing *Coleus blumei* (Hegenauer *et al.*, 2017); **c**, *Santalum album* (Santalales) (Bhargava *et al.*, 2018). **d-i**, Parasitic plants belonging to the Orobanchaceae family: **d**, *Triphysaria versicolor* (Hu *et al.*, 2020); **e**, *Phtheirospermum japonicum* (Spallek, 2017); **f**, *Alectra vogelii* parasitizing cowpea (*Vigna unguiculata*) (Anne Greifenhagen, unpublished); **g**, *Striga hermonthica* growing on maize (*Zea mays*) (Heide-Jørgensen, 2008); **h**, *Phelipanche ramosa* on winter rapeseed (*Brassica napus*) (Cartry *et al.*, 2021); **i**, *Orobanche crenata* on fabe bean (*Vicia faba*) (Heide-Jørgensen, 2008).

1.1.2 Agronomic Impact

Of the 292 genera of flowering parasitic plants, 25 genera impact agriculture and forestry (Nickrent, 2020). Among the pathogenic genera are dwarf mistletoes (*Arceuthobium* M. Bieb., (Hawksworth & Wiens, 1996)) and dodders (*Cuscuta* L., (Dawson *et al.*, 1994)), as well as some facultative, like *Rhamphicarpa* (Rodenburg *et al.*, 2016), and obligate Orobanchaceae including *Orobanche*, *Phelipanche*, and *Striga* spp. (Spallek *et al.*, 2013; Mwangangi *et al.*, 2021). *Striga* infestations are especially severe in sub-Saharan Africa (De Groote *et al.*, 2008), where the parasitic weed affects approx. 50 million hectares of arable land causing 20-100% yield losses in infected fields (Ejeta, 2007; Rodenburg *et al.*, 2016). The yield loss caused by *Striga,* amounts to an estimated annual economic loss of up to $117 million in rice alone (Rodenburg *et al.*, 2016). Parasitic weeds also impact crop production on the European continent (Westwood *et al.*, 2010), e.g., *Orobanche cumana* parasitizes sunflowers in several countries including France, Spain, and Russia (Fernández-Martínez *et al.*, 2015). Various strategies, such as 'suicidal germination', planting resistant crops, or simply hand weeding, are used in the field to manage parasitic weeds. However, most strategies exploit only one parasitic plant-specific trait for crop protection: the strigolactone-dependent germination (Ejeta & Gressel, 2007; Zwanenburg *et al.*, 2016; Li *et al.*, 2023). Strigolactones (SLs) induce germination in many parasitic plants, but in the absence of a host, the germinated obligate parasitic plants quickly die due to a lack of nutrients (Berner *et al.*, 1995). This 'suicidal germination' can be induced in *Orobanche ramosa* by sprinkling formulated synthetic SL analogs on fields prior to planting (Zwanenburg *et al.*, 2016). Another strategy to avoid parasite infestations is breeding or engineering resistant crops (Yoshida & Shirasu, 2009; Bari *et al.*, 2021). A recent study presents promising targets within the SL biosynthesis pathway for engineering maize resistance to *Striga* (Li *et al.*, 2023). However, control strategies are often cost-intensive and must be well-tailored to the respective parasite-host combination, farmers resources, and geographic region (Parker, 2009; Mallu *et al.*, 2021; Irafasha *et al.*, 2023). Furthermore, the presented strategies are most suitable for high-input agricultural systems (Ejeta & Gressel, 2007), while small-hold farmers have only limited options including intercropping and hand weeding that lead to only marginal improvements (Samaké *et al.*, 2006; Spallek *et al.*, 2013). Tackling the challenges and threats that parasitic plants pose to current agricultural systems and thus food security, would benefit from a deeper understanding of the intricate molecular parasitic plant–host plant relationship. In particular, other parasitic plant-specific traits,

such as haustorium formation, might be exploited to widen the range of applicable crop protection strategies.

1.2 The Haustorium

1.2.1 Stage I: Host Recognition and Protohaustorium Formation

In non-parasitic and facultative parasitic plants, seed dormancy is broken by favorable conditions such as appropriate temperatures, water, and oxygen availability (Brun *et al.*, 2021). In contrast, many obligate parasitic plants produce dust-like seeds, such as *Striga* with an average size of 200 µm containing only limited resources, forcing them to reach a host immediately after germination (Berner *et al.*, 1995; Joel, 2013). Hence, these parasite seeds only break dormancy upon perception of suitable host-derived germination stimulants (Stewart & Press, 1990). The best-characterized germination stimulants are SLs (Waters *et al.*, 2017). This group of plant hormones coordinates developmental processes like shoot branching, root architecture, cambial growth, and senescence within the host (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Ito *et al.*, 2022). But SLs are also exuded into the rhizosphere to recruit symbiotic arbuscular mycorrhiza fungi (Akiyama *et al.*, 2005). The first SL discovered was strigol, which stimulates the germination of *Striga lutea* (Cook *et al.*, 1966). Angiosperms perceive SLs via α/β hydrolases DWARF14 (D14) (Yao *et al.*, 2016), which subsequently interact with MORE AXILLARY GROWTH2 (MAX2) to mediate SL signaling (Hamiaux *et al.*, 2012). MAX2 is also involved in signaling by karrikins (Nelson *et al.*, 2011), compounds found in smoke (Flematti *et al.*, 2004). Karrikins are similar in structure to SLs and trigger germination of many plant species after fires, but not in parasitic plants (Flematti *et al.*, 2004; Nelson *et al.*, 2009). Karrikin, but not SL-signaling in *Arabidopsis thaliana* (Arabidopsis, At) requires *KARRIKIN-INSENSITIVE2 (KAI2)/ HYPOSENSITIVE TO LIGHT(HTL)*, a paralog of *D14* (Waters *et al.*, 2012). While *KAI2* is a single gene in Arabidopsis, Orobanchaceae parasites duplicated the gene evolving a uniquely conserved *KAI2 'divergent'-type* (*KAI2d*) gene family (Conn *et al.*, 2015). The duplication events likely resulted in neofunctionalization since parasitic Orobanchaceae, in contrast to non-parasitic species (Waters *et al.*, 2012), require KAI2d hydrolases for host-derived SL sensing (Zhang *et al.*, 2020b; Arellano-Saab *et al.*, 2023). Interestingly, host-SL perception by parasite KAI2ds not only controls parasite seed germination, but directs tropic responses of parasite towards host roots, thus acting as chemoattractants (Ogawa *et al.*, 2022).

Therefore, host dependency favored the loss of specific genes like photosystem I and II genes as part of the regressive evolution (Wicke *et al.*, 2013; Yoshida *et al.*, 2019), while others like the *KAI2d* gene family underwent duplication and parasitism-related neofunctionalization (Conn *et al.*, 2015).

Facultative parasitic plants like *P. japonicum* only form haustoria when nutrients (nitrate) are in very low abundance in the environment (Spallek *et al.*, 2017; Kokla *et al.*, 2022). Under these conditions, they develop lateral haustoria emerging from the root elongation zone of the continuously growing primary and lateral roots (Kuijt, 1969; Yoder, 1997; Ishida *et al.*, 2011). Following germination and facing low-nutrient conditions (Mwangangi *et al.*, 2023), obligate parasites like *Orobanche*, *Phelipanche*, *Alectra*, and *Striga*, on the other hand, form terminal haustoria by deforming the root apical meristem, thus terminating primary root growth (Musselman, 1980; Yoshida & Shirasu, 2009). In the Orobanchaceae, terminal haustoria emerged during the evolution of obligate from facultative parasitism (Westwood *et al.*, 2010). Despite these distinct haustorium types, both strategies of host infection are similar in their developmental and morphological features (Masumoto *et al.*, 2021).

Even under nitrogen starvation conditions, *P. japonicum* and *Striga* require additional signals to trigger the developmental processes leading to the transdifferentiation and proliferation of roots cells and the formation of proto-(or pre-)haustoria. These additional signals are collectively known as haustorium-inducing factors (HIFs; **Figure 2**): quinones, flavonoids, lignin units, H_2O_2, cyclohexene oxides, and cytokinins have been described as HIFs within the parasitic Orobanchaceae (Goyet *et al.*, 2019). These HIFs are host-derived small molecules, e.g., phenolic HIFs consist of an aromatic ring with hydroxyl groups and methoxy groups (Chang & Lynn, 1986; Cui *et al.*, 2018). Potent HIFs like 2,6-dimethoxy- 1,4-benzoquinone (DMBQ) were first identified in host root extracts (Chang & Lynn, 1986). Further research revealed that monomeric phenolics or quinones derived from lignin likely constitute active HIFs *in vivo* (Cui *et al.*, 2018; Wang *et al.*, 2020). Each parasitic plant species may respond differently to a certain HIF, if at all (**Figure 2**) (Cui *et al.*, 2018). For instance, cyclohexene oxides are only sensed as HIFs in *Orobanche cumana*, *Orobanche crenata*, and *Striga* (Fernández-Aparicio *et al.*, 2016), while DMBQ acts as a HIF across a broad range of species (Goyet *et al.*, 2019). Comparison of genome-wide transcriptomic changes after phenolic HIF (syringic acid) *versus* quinone HIF (DMBQ) application revealed distinct gene expression patterns, specifically in

the early stages after HIF perception (Aoki *et al.*, 2022). DMBQ is perceived by the leucine-rich repeat receptor-like kinase CANNOT RESPOND TO DMBQ 1 (CARD1) in Arabidopsis (Laohavisit *et al.*, 2020). CARD-like (CADL) receptors can complement the Arabidopsis *card1* mutant, suggesting that PjCADLs may also function in DMBQ perception in protohaustorium development (Laohavisit *et al.*, 2020). Responses to DMBQ perception in the parasite include Ca^{2+} elevation and mitogen-activated protein kinase (MAPK) activation (Laohavisit *et al.*, 2020), as well as ROS production (Wada *et al.*, 2019). Surprisingly, DMBQ is not detected in root exudates of the facultative parasite *Triphysaria versicolor*, explaining why *Triphysaria versicolor* and many other Orobanchaceae fail to self-induce haustoria (Westwood *et al.*, 2010; Wang *et al.*, 2020). Except for DMBQ and syringic acid, downstream signaling events of other HIFs have not been extensively studied.

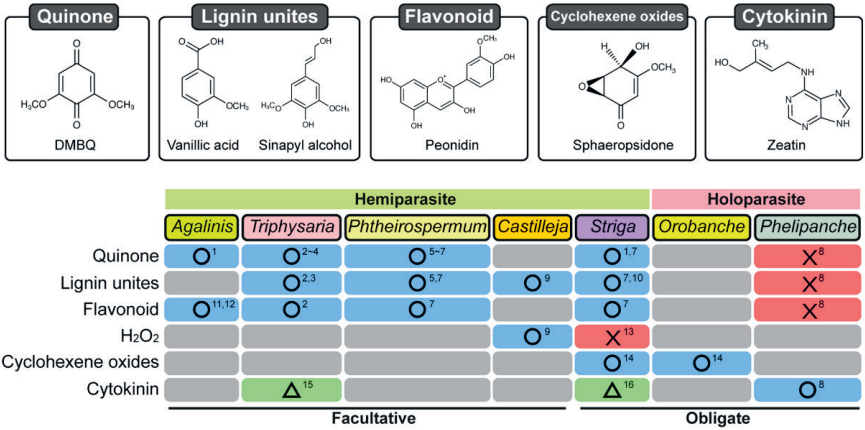

Figure 2: Haustorium-inducing factors (HIFs) in the Orobanchaceae. Major HIF classes and representative members are shown at the top and to the left of the table. The table depicts reported HIF activities. Blue box with circle: minimum one chemical reported; red box with cross: no haustorium induction with tested chemical(s); green box with triangle: haustoria-like structures reported; grey box: not reported. Numbers indicate references: (Chang & Lynn, 1986)[1]; (Albrecht *et al.*, 1999)[2]; (Bandaranayake *et al.*, 2010)[3]; (Bandaranayake *et al.*, 2012)[4]; (Cui *et al.*, 2016)[5]; (Ishida *et al.*, 2016)[6]; (Cui *et al.*, 2018)7; (Goyet *et al.*, 2017)[8]; (Salcedo-Morales *et al.*, 2014)[9]; (Lynn & Chang, 1990)[10]; (Lynn *et al.*, 1981)[11]; (Steffens *et al.*, 1982)[12]; (Wada *et al.*, 2019)[13]; (Fernández-Aparicio *et al.*, 2016)[14]; (Wrobel & Yoder, 2001)[15]; (Keyes *et al.*, 2000)[16]. Adapted from Goyet *et al.*, 2019.

Within 12h after contact with a suitable host and HIF perception, haustorium organogenesis begins with massive enlargement of inner cortex cells, leading to a lateral swelling along the root followed by anticlinal divisions of epidermal cells forming the haustorial apex (Baird & Riopel, 1984; Wakatake *et al.*, 2018). Subsequently, periclinal cell divisions progress from the

innermost cortex to other cortex, endodermis, pericycle, and stele layers, forming the dome-shaped protohaustorium (Baird & Riopel, 1984; Wakatake *et al.*, 2018). Protohaustorium initiation requires local auxin accumulation at the host-facing site of the root by epidermis-specific induction of the auxin biosynthesis enzyme YUCCA3 in *P. japonicum* (Ishida *et al.*, 2016). Auxin response maxima were detected at the haustorial apex, and ectopic *PjYUCCA3* expression in epidermal cells induced the formation of protohaustorium-like structures (Ishida *et al.*, 2016). Additionally, haustorial hairs develop from epidermal cells supporting the parasite's attachment to the host root (Baird & Riopel, 1984; Cui *et al.*, 2016). HIFs known to date, including DMBQ, are insufficient to initiate the transition from proto- to mature haustoria without a host, indicating the involvement of additional factors (Estabrook & Yoder, 1998).

1.2.2 Stage II: Development of Mature Haustoria

The characteristic feature of a mature haustorium is a xylem connection between parasite and host, the so-called xylem bridge, that enables the parasite to take up water and nutrients from its host (Yoshida *et al.*, 2016). Only some obligate holoparasites, including *Cuscuta* (Dawson *et al.*, 1994), *O. cumana* and *O. crenata* (Dörr & Kollmann, 1995; Krupp *et al.*, 2019), form additional phloem connections facilitating the exchange of RNAs or proteins with the host (Aly *et al.*, 2011; Shahid *et al.*, 2018). The molecular basis for phloem formation in parasitic plant haustoria, however, is poorly studied.

Upon direct contact with the host root, epidermal cells at the haustorium apex transdifferentiate into elongated intrusive cells that invade the host (Musselman & Dickison, 1975; Wakatake *et al.*, 2018). Disruption of ethylene signaling in the parasite or the host results in defects of haustorium growth termination and intrusive cell formation, highlighting the phytohormone's role during host invasion (Cui *et al.*, 2020). Endophytic growth of intrusive cells between host cortical cells towards the host xylem is facilitated by enzymatic activity (Neumann *et al.*, 1999). Pectin methylesterases (PMEs) active in intrusive cells control cell wall loosening relevant for tissue expansion and interaction with the host. Simultaneously, PME inhibitor (PMEI) activity stabilizes inner haustorial structures, thus supporting host intrusion (Leso *et al.*, 2023). Intrusive cells either insert between host xylem precursor cells and synchronously differentiate with them, as documented for *S. hermonthica*, or penetrate the host xylem and subsequently turn into tracheary elements (Dörr, 1997; Masumoto *et al.*, 2021). The first stages of xylem

bridge differentiation require PME activity to allow pectin degradation followed by lignification (Leso *et al.*, 2023). Tracheary elements at the haustorial apex and a mass of tracheary elements at the base of haustoria, called plate xylem, develop in parallel, eventually connecting in the center of the haustorium to form the mature xylem bridge (Ishida *et al.*, 2016; Wakatake *et al.*, 2018). *P. japonicum* mutants defective in ethylene signaling initiate differentiation of a single xylem strand without connection to the parasite vasculature upon treatment with DMBQ in absence of a host suggesting that ethylene signaling mediates xylem bridge formation (Cui *et al.*, 2020). Cooperative directed transport of auxin by PIN-FORMED (PIN) and AUXIN1/LIKE-AUX1 (AUX1/LAX) proteins directly controls proper plate xylem formation and xylem vessel connection, which can be disrupted by auxin transport inhibitors (Ishida *et al.*, 2016; Wakatake *et al.*, 2020). The participation of multiple hormonal pathways in various steps of haustorium development indicates a high level of complexity.

Several genome sequencing and transcriptomic analyses further highlight the complexity of haustorium development in the Orobanchaceae. Despite differences in host preferences, haustoria morphologies, and types of parasitism, studies in *Phelipanche aegyptiaca* (holo-, obligate), *Triphysaria versicolor* (hemi-, facultative) (Yang *et al.*, 2015), *Striga asiatica* (hemi-, obligate), *Striga hermonthica* (hemi-, obligate) (Yoshida *et al.*, 2019), and *P. japonicum* (hemi-, facultative) (Cui *et al.*, 2020) revealed a great level of conservation of the transcriptional programs associated with haustorium development (Wickett *et al.*, 2011; Yoshida & Kee, 2021). In addition to hormone signaling, the maturation of haustoria in all these species coincides with the strong induction of *subtilisin-like serine protease* (*subtilase*, *SBT*) genes (Yang *et al.*, 2015). The Arabidopsis genome contains 56 *SBT* genes (Rautengarten *et al.*, 2005), compared to 97 in *P. japonicum*, of which 43 *SBT* genes belong to Group-1, which is highly expanded compared to only 9 *SBTs* in Arabidopsis (Ogawa *et al.*, 2021). Similarly, Group-1 *SBT*s expanded in legumes engaging in symbiosis with nitrogen-fixing bacteria (Taylor & Qiu, 2017). In *P. japonicum*, *SBT1.1.1, SBT1.2.3, SBT1.7.2*, and *SBT1.7.3* genes are highly expressed in intrusive cells (Ishida *et al.*, 2016; Ogawa *et al.*, 2021). Tissue-specific inhibition of SBT activity by Extracellular Proteinase Inhibitor 10 (Epi10) (Schardon *et al.*, 2016), whose expression was driven by the promoter of *PjSBT1.2.3*, demonstrated that SBT activity in haustoria is required for intrusive cell and xylem bridge formation (Ogawa *et al.*, 2021). Even though *PjSBTs* genes display duplication and PjSBT proteins parasitism-related neofunctionalization (Ogawa *et al.*, 2021), substrates of parasitism-related SBTs remain unknown. SBTs posttranslationally process larger

proteins such as the abovementioned PMEs shaping cell wall structure (Sénéchal *et al.*, 2014), as well as plant peptide hormones such as INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) leading to floral organ abscission (Schardon et al., 2016), TWISTED SEED 1 (TWS1) involved in embryonic cuticle production (Doll et al., 2020), GOLVEN1 controlling cell elongation (Ghorbani et al., 2016), and CLAVATA3(CLV)/EMBRYO-SURROUNDING REGION-related (CLE) peptides like CLV3 or CLE40 involved in meristem regulation (Ni et al., 2011; Stührwohldt et al., 2020).

1.2.3 Stage III: Haustorium Regulation and Functions during Late Stages of Parasitism

Movement of RNAs or even large gene fragments via horizontal gene transfer between parasitic and host plants is well-documented (Shahid *et al.*, 2018; Yang *et al.*, 2019; Yoshida *et al.*, 2019; Park *et al.*, 2022). MircroRNAs from *C. campestris* and short interfering RNAs from the facultative *Triphysaria versicolor* were shown to target host messenger RNAs (Tomilov *et al.*, 2008; Shahid *et al.*, 2018). *Striga asiatica* obtained a ~30 kb monocot host gene fragment via horizontal gene transfer, potentially enabled by the direct haustorial connection (Yoshida *et al.*, 2019). However, the translocation of proteins, so far, has only been documented for a few parasitic plants developing a phloem-to-phloem connection with their hosts (Aly *et al.*, 2011; Liu *et al.*, 2020). *P. japonicum* lacks direct phloem connections to the host (Masumoto *et al.*, 2021), but when carboxyfluorescein diacetate (CFDA) is applied to Arabidopsis leaves, parasitizing *P. japonicum* rapidly takes up the fluorescent tracer through the xylem and inner region of the haustorium (Spallek *et al.*, 2017). *Phelipanche ramosa* also translocates CFDA, but directly through its haustorial phloem strands (Péron *et al.*, 2017). Green fluorescent protein (GFP) expressed under control of a companion cell-specific *AtSUC2* promoter (*pAtSUC2::GFP*) is only detected in the host during *P. japonicum* infection on Arabidopsis (Spallek *et al.*, 2017). In the same experimental setup, however, GFP is taken up by *Cuscuta reflexa* or *Phelipanche aegyptiaca* via the phloem at the haustorial interface and unloaded in parasite meristematic sink tissues (Haupt *et al.*, 2001; Ekawa & Aoki, 2017). A recent study demonstrates that *Cuscuta australis* receives the mobile protein signal FLOWERING LOCUS T (FT) from its host, thereby synchronizing its flowering time with that of the host (Shen *et al.*, 2020). However, functional studies on parasitism-related mobile proteins of Orobanchaceae parasites are still lacking.