



DOCTORAL THESIS No. 2022:5
FACULTY OF NATURAL RESOURCES AND AGRICULTURAL SCIENCES

Haustoria regulation in the facultative parasitic plant *Phtheirospermum* *japonicum*

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Hauatoria regulation in the facultative
parasitic plant *Phtheirospermum*
japonicum

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SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS

Uppsala 2022

Acta Universitatis Agriculturae Sueciae
2022:05

Cover: Graphical representation of *Phtheiospermum japonicum* infecting *Arabidopsis thaliana* and microscopy image of the haustorium (Anna Kokla).

ISSN 1652-6880

ISBN (print version) 978-91-7760-885-1

ISBN (electronic version) 978-91-7760-886-8

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Uppsala

Print: SLU Grafisk Service, Uppsala 2022

Haustoria regulation in the facultative parasite *Phtheirospermum japonicum*

Abstract

Parasitic plants are important agricultural pests that lead to considerable yield losses annually. Parasitic plants that completely rely on their hosts for their survival are known as obligatory parasites, whereas those independent of their hosts but parasitize under the right conditions are known as facultative parasitic plants. All parasitic plants form a multicellular organ termed the haustorium via which they invade their host, establish vascular connections and uptake water and nutrients. Despite recent advances in our understanding of parasitic plants, the mechanisms of haustoria regulation remain largely unknown. Here we aimed to identify how the environmental nutrient status affects haustoria formation in the facultative parasitic plant *Phtheirospermum japonicum*. We showed that *P. japonicum* inhibits haustoria formation in response to the macronutrient nitrogen and that this nitrogen-mediated haustoria inhibition is mediated by the hormone abscisic acid (Paper I). Further investigation of haustorium regulatory mechanisms demonstrated that *P. japonicum* harbors a haustoria autoregulation mechanism (AOH) that utilizes a long-distance signalling mechanism that involves small mobile peptides (Paper II). Lastly, we investigated the role of cell wall modifying enzymes in haustoria and xylem bridge formation. We identified two genes encoding cell wall modifying enzymes, one pectin methylesterase and one pectin methylesterase inhibitor, that are involved in both haustoria and xylem bridge formation in *P. japonicum* (Paper III). Overall this thesis contributes to our understanding of haustoria regulation in response to nitrogen availability or pre-existing established haustoria.

Keywords: parasitism, haustorium, nitrogen, hormones, cytokinin, ABA, peptides

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Haustoria-reglering i den fakultativa parasitväxten *Phtheirospermum japonicum*

Abstrakt

Parasitiska växter är jordbruksskadegörare som leder till betydande skördeföruster årligen. Parasitiska växter som helt förlitar sig på sina värdar för sin överlevnad är obligatoriska parasiter, medan de som är oberoende av sina värdar men som parasiterar under de rätta förhållandena är fakultativa parasitväxter. Alla parasitiska växter bildar ett flercelligt organ som kallas haustorium, via vilket de invaderar sin värd, etablerar kärlförbindelser och tar upp vatten och näringsämnen från värden. Trots de senaste framstegen i vår kunskap om parasitiska växter, är mekanismerna för hur haustorier regleras i stort sett okända. Här avsåg vi att undersöka hur miljöns näringsstatus påverkar bildning av haustorier hos den fakultativa parasitväxten *Phtheirospermum japonicum*. Vi visade att den hämmas av makronäringsämnet kväve och att denna kvävedierade haustorium-hämning förmedlas av växthormonet abskissinsyra (Paper I). Våra studier visade också att *P. japonicum* använder en autoregleringsmekanism (AOH) för kontroll av haustorium-bildning och att denna baseras på långdistanssignalering med hjälp av små mobila peptider (Paper II). Slutligen undersökte vi vilken roll cellväggsmodifierande enzymer spelar vid bildning av haustorier och xylembryggor. Vi identifierade två gener som kodar för cellväggsmodifierande enzymer, ett pektinmetylesteras och en pektinmetylesterashämmare, som är involverade i både haustorium- och xylembrygg-bildning i *P. japonicum* (Paper III). Sammantaget bidrar denna avhandling till vår förståelse av hur bildning av haustorier regleras som svar på kvävetillgång eller redan etablerade haustorier.

Keywords: *Orobanchaceae*, haustorium, nitrogen, hormones, cytokinin, ABA, peptides, parasitism

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Ρύθμιση των μυζητήρων στο προαιρετικό παρασιτικό φυτό *Phtheirospermum japonicum*

Περίληψη

Τα παρασιτικά φυτά είναι σημαντικά γεωργικά παράσιτα που οδηγούν σε σημαντικές απώλειες γεωργικής παραγωγής ετησίως. Τα παρασιτικά φυτά που βασίζονται πλήρως στους ξενιστές τους για την επιβίωσή τους είναι γνωστά ως υποχρεωτικά παράσιτα, ενώ όσα επιβιώνουν ανεξάρτητα από τους ξενιστές τους αλλά παρασιτούν υπό τις κατάλληλες συνθήκες είναι γνωστά ως προαιρετικά παρασιτικά φυτά. Όλα τα παρασιτικά φυτά σχηματίζουν ένα όργανο που ονομάζεται μυζητήρας (haustorium) μέσω του οποίου διεισδύουν στον ξενιστή τους, δημιουργούν αγγειακές συνδέσεις και στη συνέχεια εξάγουν νερό και θρεπτικά συστατικά. Παρά την ερευνητική πρόοδο σχετικά με τον σχηματισμό των μυζητήρων, οι μηχανισμοί ρύθμισης τους παραμένουν σε μεγάλο βαθμό άγνωστοι. Στην παρούσα διατριβή στοχεύσαμε να προσδιορίσουμε πώς η διαθεσιμότητα θρεπτικών συστατικών επηρεάζει τον σχηματισμό των μυζητήρων στο προαιρετικό παρασιτικό φυτό *Phtheirospermum japonicum*. Δείξαμε ότι το *P. japonicum* αναστέλλει τον σχηματισμό των μυζητήρων ως απόκριση στα επίπεδα άζωτου διαμέσου της φυτοορμόνης αμψισικό οξύ (άρθρο I). Περαιτέρω έρευνα έδειξε ότι το *P. japonicum* διαθέτει έναν συστημικό μηχανισμό αυτορρύθμισης του αριθμού των μυζητήρων (AOH) που περιλαμβάνει μικρά σηματοδοτικά πεπτίδια (άρθρο II). Τέλος, διερευνήσαμε τον ρόλο ενζύμων τροποποίησης του κυτταρικού τοιχώματος στον σχηματισμό των αγγειακών συνδέσεων μεταξύ παρασίτου και ξενιστή. Προσδιορίσαμε δύο γονίδια που κωδικοποιούν ένζυμα τροποποίησης του κυτταρικού τοιχώματος, που εμπλέκονται τόσο στον σχηματισμό των μυζητήρων όσο και στο σχηματισμό της αγγειακής σύνδεσης μεταξύ παρασίτου και ξενιστή στο *P. japonicum* (άρθρο III). Εν κατακλείδι, αυτή η διατριβή περιγράφει τους μηχανισμούς ρύθμισης του σχηματισμού των μυζητήρων ως απόκριση στη διαθεσιμότητα αζώτου ή στους προϋπάρχοντες μυζητήρες.

Keywords: *Orobanchaceae*, haustorium, nitrogen, hormones, cytokinin, ABA, peptides, parasitism

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I. **Kokla, A., Leso, M., Zhang, X., Simura, J., Phanu T. Serivichyaswat, Cui, S., Ljung, K., Yoshida, S., and Melnyk, C.W.** (2021). Nitrogen regulates haustoria formation in the parasitic plant *Phtheirospermum japonicum* through abscisic acid. (under revision).
- II. **Kokla, A., Leso, M., Zhang, X., Simura, J., Cui, S., Ljung, K., Yoshida, S., and Melnyk, C.W.** (2021). Auto-regulation of haustoria in the parasitic plant *Phtheirospermum japonicum* is mediated by CEP signaling. (manuscript)
- III. **Leso, M., Kokla, A., Melnyk, C.W.** (2021). PME-mediated cell wall modification is important for efficient haustorium development in the parasitic plant *Phtheirospermum japonicum*. (manuscript)

The following paper was written during the course of my doctoral studies but are not part of the present dissertation:

1. **Kokla, A., Melnyk, C.W.** (2018). Developing a thief: Haustoria formation in parasitic plants. *Developmental Biology*, 442, 53-59

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1. Introduction

Plants interact with a wide variety of organisms both above and below ground throughout their life. Many of these interactions are mutualistic, meaning that both species benefit from their interaction. For instance, leguminous plants form mutualistic symbiosis with nitrogen fixing bacteria where the plant is supplied nitrogen and the bacteria is supplied carbohydrates. In other occasions two organisms interact with one organism benefiting while the other is neither benefited or harmed, such relationship is termed commensalism. Epiphytes are an example of commensalism where one plant gains structural support while the other is not harmed. In other cases, two organisms form a parasitic relationship where one of the organisms is harmed (host) where the other benefits (parasite). Parasites usually take nutrients from their hosts but they do not always kill their hosts. For example, parasitic nematodes can invade plants to feed from them causing extensive agricultural damages. Plants have also evolved parasitic lifestyles that allows them to parasitize other plants causing minor to extensive damages to their hosts (Taiz and Zeiger, 2014).



Figure 1. Examples of parasitic plants. (A) *Striga hermonthica* (Orobanchaceae). (B) *Viscum album* (Santalaceae). (C) *Thesium chinense* (Santalaceae). (D) *Psittacanthus calyculatus* (Loranthaceae). (E) *Phtheirospermum japonicum* (Orobanchaceae). (F) *Rafflesia keithii* (Rafflesiaceae). (G) *Hydnora africana* (Hydnoraceae). (H) *Comandra umbellata* (Santalaceae). Images adapted from Yoshida et al. (2016), Heide-Jørgensen (2013) and Spallek et al. (2017).

1.1 Parasitic plants

Plants that invade other plants to take advantage of their resources are known as parasitic plants. Plant parasitism has independently evolved more than 11 times (Barkman et al., 2007) resulting in thousands of species (Heide-Jørgensen, 2013) with a wide distribution, host range and various lifestyles. Parasitic plants can range from complete dependence on the host to completing their life cycle even in the absence of a host. Some parasites have complete lack of photosynthetic ability while others retain all or part of it. Additionally, parasitic plants can vary on the type of host tissue they infect with most parasites in *Orobanchaceae* infecting the root while other parasitic plants infect the shoot of their host (Heide-Jørgensen, 2013).

1.1.1 Classification

Almost half of all parasitic plants belong to the *Orobanchaceae* family. It is the largest and more widespread parasitic plant family with more than 2000 species that are found in all continents except Antarctica (Heide-Jørgensen, 2008).

Parasitic plants can be classified based on their lifestyle. Some parasitic plants such as the well-known agricultural pests *Striga* and *Cuscuta*, completely depend on their host for their survival and thus are known as obligate parasites. Obligate parasites often have reduced or no photosynthetic ability and completely rely on their host for water, nutrients and sugar supply. Many obligate parasites require perception of a nearby host for seed germination (Heide-Jørgensen, 2008; Spallek et al., 2013). Other parasitic plants, known as facultative parasites, retain their photosynthetic ability and can complete their life cycle in the absence of a host. Well studied parasites such as the model parasitic plant *Phtheirospermum japonicum* and *Triphysaria versicolor*, are facultative parasites. *Phtheirospermum japonicum* is native to east Asia and Japan and infects a wide host range including tomato, rice, corn and the model plant *Arabidopsis thaliana*. Although *Phtheirospermum japonicum* can photosynthesize, it invades the roots of nearby hosts in nutrient deprived conditions. *Phtheirospermum japonicum* is considered a model parasitic plant due its wide range of well-studied hosts, short life cycle, ease of growth and availability of genomic data (Ishida et al., 2011; Cui et al., 2016).

1.1.2 Importance

Many *Orobanchaceae* species, such as *Striga*, *Orobanche* and *Phelipanche spp.*, are devastating agricultural pests destroying up to 80% of the crops they infect (De Groot et al., 2007; Hassan et al., 1995; Parker, 2009; Spallek et al., 2013). Despite the extensive agricultural damages parasitic plants cause, there is no effective control method to date. Parasitic plants such as *Striga* produce small long-lived seeds that can remain dormant more than 10 years (Spallek et al., 2013). Herbicides damage the host plants along side with the parasite. Other techniques such as “suicidal germination”, utilize the application of germination stimulants in the field in absence of host plants. This forces the parasitic plants to germinate and soon after die due to the lack of resources provided by host plants. Although these techniques have been proven potentially effective, the large-scale and cost-effective production of such stimulants that retain their chemical stability has not yet been achieved (Sugimoto et al., 1998; Johnson et al., 1981; Zwanenburg and Mwakaboko, 2011). Increased field fertilization has been shown to decrease the production of germination stimulants by host plants and thus decrease parasitic plant infection (Yoneyama et al., 2007a, 2007b; Sun et al., 2014; Mwangangi et al., 2021; Jamil et al., 2012). Although this seems promising, increased fertilization can have a great economical cost and lead to environmental damages. Lastly, due to the complexity of the parasitic plant- host interaction and the low heritability of such traits, resistant crops have been identified for only a few parasitic plant species (Hausmann et al., 2000; Pérez-de-Luque et al., 2008). To tackle the problems and damages parasitic plants cause in agricultural production, further research is required to understand the complex parasite-host-environment interactions as well as the biology and mechanisms that regulate parasitic plant infectivity.

1.2 The haustorium

The haustorium is a multicellular organ that all parasitic plants develop to invade the host's plant tissue and take up water and nutrients. Some parasites, like *Cuscuta*, form haustoria from their shoots, but most parasitic plants form root haustoria. Depending on their formation site, haustoria can be characterized as lateral or terminal (Kuijt, 1969).

Terminal haustoria form at the root tip and terminate root growth (Joel and Losner-Goshen, 1994), whereas lateral haustoria form at the root elongation zone allowing the root to continue growing and potentially form multiple haustoria (Ishida et al., 2011; Matvienko et al., 2001). Despite some differences, both terminal and lateral haustoria require the perception of a nearby host to initiate their formation. Upon host perception, the formation of the pre-haustorium starts which will later mature to a haustorium that has formed a vascular connection with the host (Fig.2).

1.2.1 Seed germination

Obligate parasites, such as *Striga*, *Orobanche spp.* and *Phelipanche spp.*, are known to produce numerous, small and long lived seeds (Yoneyama et al., 2010; Spallek et al., 2013). These parasitic plants heavily rely on their host and they only germinate after the perception of germination stimulants produced by nearby hosts. Due to the limited nutrient amount stored in the seeds, failure to parasitize a host soon after germination leads to death (Parker, 2009). These germination stimulants are known as strigolactones or strigolactone-like and can lead to seed germination even in the absence of a host (Yoneyama et al., 2010; Brun et al., 2018). Strigolactones are produced and secreted by host plants to attract arbuscular mycorrhizal symbionts. Obligate parasites have “highjacked” this system to identify hosts in their rhizosphere before germination (Yoneyama et al., 2010; Brun et al., 2018). Facultative parasites, such as *Phtheirospermum japonicum*, lacks germination stimulants and can germinate and grow independently of a host.

1.2.2 Host perception

Perception of a nearby host is required for the haustorium to start forming. Parasitic plants recognise proximal hosts by sensing host root exudates known as haustoria inducing factors (HIFs). HIFs include various host root exudates such as strigolactones, flavonoids, quinones and cytokinins (Chang and Lynn, 1986; Goyet et al., 2017; Albrecht et al., 1999; Joel, 2013) (Table 1, Figure 2). HIFs are perceived by leucine-rich-repeat receptor-like kinases, called CANNOT RESPOND TO DMBQ (CARDs) in *Phtheirospermum japonicum*,

which leads to increased cytosolic Ca^{2+} signalling (Laohavisit et al., 2020), this is followed by reactive oxygen species (ROS) accumulation in the parasitic root and the initiation of haustoria formation (Bandaranayake et al., 2010) (Figure 2). 2,6-dimethoxy-p-benzoquinone (DMBQ) isolated from infected sorghum roots was the first identified HIF and was sufficient to initiate haustorium formation in the absence of a host in both obligate and facultative parasites (Chang and Lynn, 1986). DMBQ is produced when host derived phenolic acids are oxidized by parasite derived hydrogen peroxide (Keyes et al., 2000). Oxidization of the cell wall phenolic precursor syringic acid by hydrogen peroxide resulted in DMBQ that successfully initiated haustoria formation in *Striga*. Furthermore, hydrogen peroxide depletion inhibited haustoria formation which was restored by the addition of DMBQ (Kim et al., 1998). Except of DMBQ, substances with similar structure and reduction potential can also act as HIFs (Smith et al., 1996). Lignin and similar phenolic compounds can induce haustoria formation in *Striga hermonthica* and *Phtheirospermum japonicum* (Cui et al., 2018). Cytokinins and flavonoids have also been shown to induce haustoria formation in *Phelipanche ramosa* and *Triphysaria versicolor* (Albrecht et al., 1999; Goyet et al., 2017), suggesting that a variety of root exudates can act as HIFs (Table 1, Figure 2). Some parasites like *Cuscuta* self-parasitize (Furuhashi et al., 2011) whereas others like *Phtheirospermum japonicum* and *Triphysaria versicolor* do not. This self-avoidance in *Phtheirospermum japonicum* and *Triphysaria versicolor* parasitism opens the question of how these parasites differentiate between self and non-self. A recent study showed that the facultative parasite *Triphysaria versicolor* restricts the expression of oxidases that could oxidize its own root exudates to active HIFs to differentiate self from host (Wang et al., 2020). The mechanism via which *Phtheirospermum japonicum* avoids self-parasitism remains unknown.

Table 1. Summary of the characteristics of four commonly studied parasitic plants (adapted from (Kokla and Melyk, 2018).

Species/Family	Parasitic category	Haustoria type	HIFs	Vascular connection	Reference
<i>Phtheirospermum japonicum</i> (<i>Orobanchaceae</i>)	Facultative hemiparasite	Lateral	DMBQ, lignin, polymers	Xylem	(Ishida et al., 2011; Cui et al., 2018)
<i>Triphysaria versicolor</i> (<i>Orobanchaceae</i>)	Facultative hemiparasite	Lateral	DMBQ, flavonoids	Xylem	(Matvienko et al., 2001; Albrecht et al., 1999)
<i>Striga hermonthica</i> (<i>Orobanchaceae</i>)	Obligate hemiparasite	Terminal	DMBQ, cell wall phenolics, lignin polymers	Xylem	(Spallek et al., 2013; Dörr and Dorr, 1997; Cui et al., 2018; Mitsumasa et al., 2015)
<i>Cuscuta reflexa</i> (<i>Convolvulaceae</i>)	Obligate holoparasite	Lateral	Volatile compounds, far red light, tactile pressure	Xylem & phloem	(Kim et al., 2014; Haupt et al., 2001; Runyon et al., 2006; Tada et al., 1996)
<i>Phelipanche ramosa</i> (<i>Orobanchaceae</i>)	Obligate holoparasite	Terminal	cytokinin	Xylem & phloem like	(Goldwasser et al., 2001; Ekawa and Aoki, 2017; Yoshida and Shirasu, 2012; Goyet et al., 2017)

1.2.3 Pre-haustorium development

After HIF perception, the pre-haustorium starts forming. At 4-8 hours post infection (hpi) the cortical cells of the haustorium initiation site expand and form the pre-haustorium swelling (Baird and Riopel, 1984; Bandaranayake and Yoder, 2013) (Figure 2). In *Striga*, DMBQ induces the expression of expansin encoding genes, *SaExp1* and *SaExp2*, that potentially contribute to cell wall loosening and cell expansion (O'Malley et al., 2000). In the facultative parasite *Phtheirospermum japonicum* cell divisions start at 12 hpi in the

epidermis, cortex, endodermis and pericycle of the root facing the host (Wakatake et al., 2018) (Figure 2). Among the hundreds of genes that respond transcriptionally to DMBQ in *Phtheirospermum japonicum*, an auxin biosynthesis gene, *YUC3*, increases in activity in the epidermal cells of the haustorium initiation site at around 18 hpi, which correlates spatiotemporally with the observed auxin response maximum at the apex of the pre-haustorium (Ishida et al., 2016). The importance of *YUC3* expression during haustoria formation is highlighted by the reduction of haustoria numbers when *YUC3* expression was reduced by RNAi. Also, ectopic expression of *YUC3* in *Phtheirospermum japonicum* roots increased auxin accumulation and haustoria like structures (Ishida et al., 2016). Moreover, auxin biosynthesis inhibition by L-Kynurenine and Yucasin completely blocked haustoria formation in *Phtheirospermum japonicum* (Wakatake et al., 2020). These data suggest that *YUC3* expression and auxin accumulation are important for cell expansion and division in the early haustoria formation stages (Ishida et al., 2016; Wakatake et al., 2020) (Figure 2). In addition to auxin biosynthesis, auxin transport is also important during early haustorium formation (Figure 2). Inhibition of polar auxin transport in the facultative parasite *Triphysaria versicolor* reduced haustoria formation, which was restored by auxin application. Furthermore, for DMBQ induced haustoria to form in *Triphysaria versicolor*, an intact root was required suggesting that auxin transport is needed for haustoria initiation (Tomilov et al., 2005). On the other hand, chemical inhibition of polar auxin transport in *Phtheirospermum japonicum* lead to failed xylem bridge formation but did not affect haustoria numbers (Wakatake et al., 2020). Phytohormones are important regulators of plant growth and development and thus involved in haustorium formation. Hormones such as ethylene, gibberellins, abscisic acid, auxin and cytokinin increase in parasitic plants during infection or chemical haustoria induction (Spallek et al., 2017; Tomilov et al., 2005; Zhang et al., 2012). Plant hormones, such as ethylene and auxin, can also increase the activity of HIFs (Tomilov et al., 2005), further supporting the involvement of hormones in early haustorium development. It was recently discovered that the accumulation and biosynthesis of very long chain fatty acids (VLCFA) increase during infection in the hemiparasite *Thesium chinense*. This increase correlates with

expression changes in auxin responsive genes as well as lateral root-related genes (Ichihashi et al., 2017). VLCFAs are components of the cell membrane but also act as signalling molecules to regulate the regeneration ability of plants (Shang et al., 2016). Despite this little is known about their role in haustorium formation.

1.2.4 Host attachment

After haustorium initiation, the haustorium develops towards and attaches to the host's root (Figure 2). Haustorial hairs that derive from epidermal cells at the haustorial apex assist in the attachment of the haustorium to the host's root by secreting adhesive substances (Hood et al., 1998; Pérez-de-Luque, 2013; Baird and Riopel, 1984). However useful, haustorial hairs do not appear necessary for haustoria formation. *Phtheirospermum japonicum* with defective haustorial hair development can form successful haustoria although in lesser numbers than parasites with haustorial hairs (Cui et al., 2016).

1.2.5 Host invasion

After attachment to the host, the parasite starts penetrating the host (Figure 2). This invasion is facilitated by the intrusive cells that are enlarged epidermal cells at the haustorium apex (Figure 2). The intrusive cells are mechanically supported by the penetration peg formed by the division of haustorial cortex and pericycle cells (Heide-Jorgensen and Kuijt, 1995; Bandaranayake and Yoder, 2013; Pérez-de-Luque, 2013). Additionally, cell wall degrading enzymes are utilized by the parasite to loosen the host's cell walls and allow the haustorium to invade the host's tissues. Pectin degrading enzymes known as pectin methylesterases have been detected at the penetration site of *Orobancha cumana* and *Phelipanche aegyptiaca* (Losner-Goshen et al., 1998) while in *Triphysaria versicolor* cell wall modifying enzymes known as β -expansins are upregulated during infection (Honaas et al., 2013). Obligate parasites such as *Cuscuta reflexa* secrete cell wall modifying xyloglucan endotransglucosylation enzymes during penetration. Inhibition of the activity of these enzymes reduced the number of penetrated haustoria (Olsen and Krause, 2017) further underlining the importance of cell wall modifying enzymes during host invasion. Another well studied

obligate parasite, *Striga*, utilizes cell wall digestion to dissolve the casparian strip of its host and with the mechanical force from the penetration peg penetrates between the host cells with minimal damage (Neumann et al., 1999; Pérez-de-Luque, 2013; Heide-Jørgensen, 2013; Hood et al., 1998). In *Phtheirospermum japonicum* the intrusive cells penetrate the host tissues by degrading cellulose, a cell wall component, via the secretion of type β -1,4-glucanases (Kurotani et al., 2020). To summarize, most *Orobanchaceae* parasites utilize cell wall degrading enzymes to loosen the host cell walls and penetrate between the host cells causing minimal damage (Ben-Hod et al., 1993; Pérez-de-Luque, 2013). Some other parasites such as *Agalinis aphylla* follow a different strategy where it breaks through and damages the host's cells during penetration (Musselman and Dickison, 1975). Phytohormones have a role during host invasion. Impaired ethylene signalling in *Phtheirospermum japonicum* results in failed host invasion probably by blocking the differentiation of haustorium apex cells to intrusive cells (Cui et al., 2020). Another study showed that subtilase genes, involved in the maturation of plant peptide hormones, are specifically expressed at the intrusive cells of the haustorium and inhibition of their activity affects intrusive cells development and the subsequent vascular connection (Ogawa et al., 2021).

1.2.6 Vascular connection

Once the haustorium breaches the endodermis of the host, vascular connections between parasite and host start to form to allow for water and nutrient uptake (Heide-Jørgensen and Kuijt, 1995) (Figure 2). In *Striga*, intrusive cells differentiate to tube like vessel elements, named oscula, whereas cells at the centre of haustorium differentiate to xylem, forming what is known as the xylem bridge (Dörr and Dorr, 1997). In *Phtheirospermum japonicum*, xylem bridge formation starts at around 72 hpi both at the haustorial base and at the distal tip, where bulks of tracheary elements form before joining in the middle (Wakatake et al., 2018) (Figure 2, Figure 3). For the xylem bridge to complete, haustorial inner cortical cells differentiate to procambial cells that then differentiate to xylem cells (Wakatake et al., 2018) (Figure 2, Figure 3). Auxin that is transported to the central haustorial cells regulates the differentiation of cortical to xylem cells (Wakatake

et al., 2020). Perturbation of auxin transport by chemical treatment with the auxin transport inhibitor NPA leads to failure of xylem bridge formation underlining the importance of auxin transport during xylem bridge connection (Wakatake et al., 2020). While all known parasitic plants form a xylem bridge, phloem connections have only been observed in obligate parasites potentially to uptake photosynthetic products (Heide-Jørgensen, 2008; Dörr and Dorr, 1997; Musselman, 1980). In *Orobanche crenata* and *Cuscuta reflexa* the phloem connection between host and parasite is facilitated by continuation of the symplasmic sieve elements (Dörr and Kollmann, 1995; Haupt et al., 2001). On the other hand, the parasite *Phelipanche aegyptiaca* transports phloem expressed proteins but via cells that have nuclei and lack sieve plates unlike conventional sieve elements (Ekawa and Aoki, 2017).

Successful xylem bridge formation signifies the beginning of water and nutrient up take from the host. In addition to water and nutrients, proteins, mRNAs, small RNAs and viruses are also transported to and from the parasite via the haustorium (Kim and Westwood, 2015; Smith et al., 2013) (Figure 2). The obligate parasite *Cuscuta* exchanges more than 1000 functioning proteins with its hosts *Arabidopsis* and soybean via the haustorium (Liu et al., 2020). RNAs and hormones can also be transported from the parasite to the host. *Cuscuta* introduces miRNAs in its host that targets host defence-related genes (Shahid et al., 2018) and more than 8000 host mRNAs have been detected in *Cuscuta* (Kim et al., 2014). *Cuscuta*, among other obligate parasitic plants, forms both xylem and phloem connections with their hosts which allows for this bi-directional transport of RNAs and proteins. Despite this, functional miRNAs can be transported by a lettuce host to the facultative parasite *Triphysaria versicolor* although it lacks phloem connections (Tomilov et al., 2008). The plant hormone cytokinin is produced by the haustorium intrusive cells and transported via the haustorium from the facultative parasite *Phtheirospermum japonicum* to the host *Arabidopsis* where it induces the formation of hypertrophic host tissues above the haustorium site (Spallek et al., 2017; Greifenhagen et al., 2021). Although, hypertrophy is commonly seen in parasitic infections as for example mistletoe infection on trees (Figure 1), the exact function of these hypertrophic tissues remains unknown. These findings show that obligate and facultative parasitic

plants facilitate bidirectional transport of various substances via the haustorium with various and complex functions. Such exchange of substances is also seen in nematode and microbial plant infections which uptake nutrients from their plant host and introduce cytokinins to increase their virulence (Akiyoshi et al., 1984; Siddique et al., 2015), suggesting a common strategy for parasites.

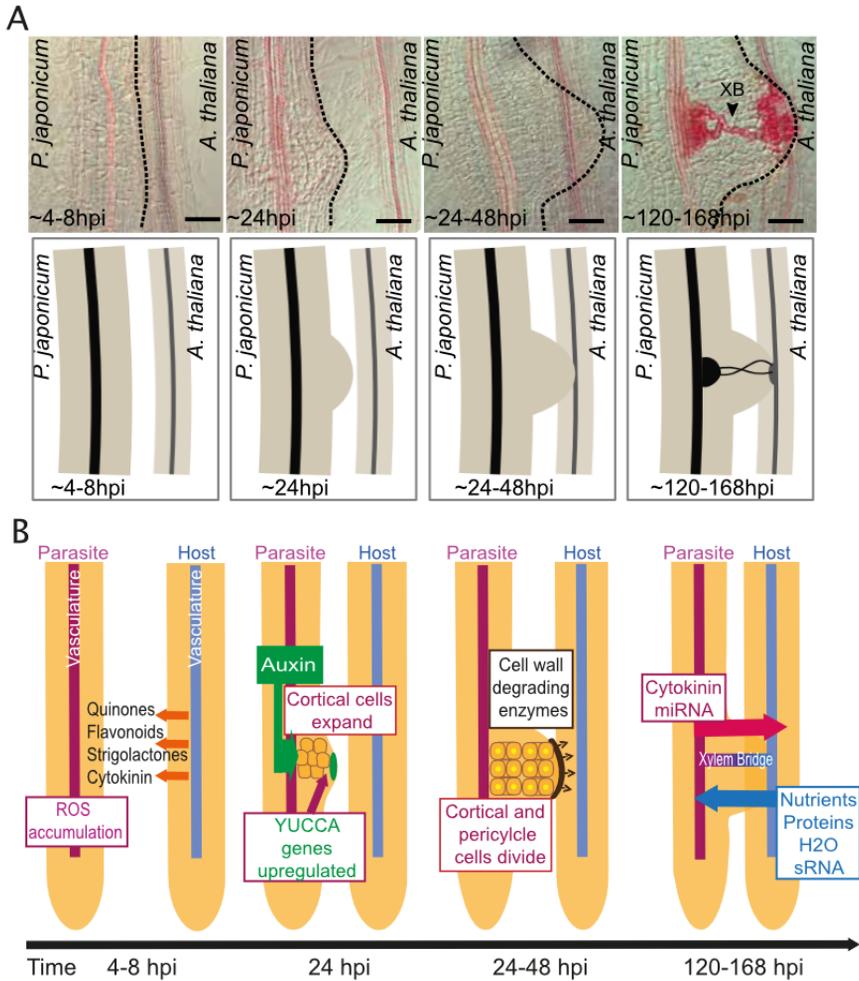


Figure 2. Representation of root haustoria development. (A) Microscopic images and corresponding cartoon from haustorium developmental stages. The parasite *Phtheirospermum japonicum* is depicted on the left and the host *Arabidopsis thaliana* is depicted on the right of each image. The dashed line outlines the epidermis of the parasite and the xylem bridge (XB) is denoted. Scale bar is 100 μm . (B) A cartoon of generalized haustorial development. The vasculature of the parasitic plant is depicted in magenta whereas the vasculature of the host plant is depicted in blue. The time scale is approximate and the figure incorporates events observed in various species (adapted from (Kokla and Melnyk, 2018), cartoon in A was designed by Martina Leso).

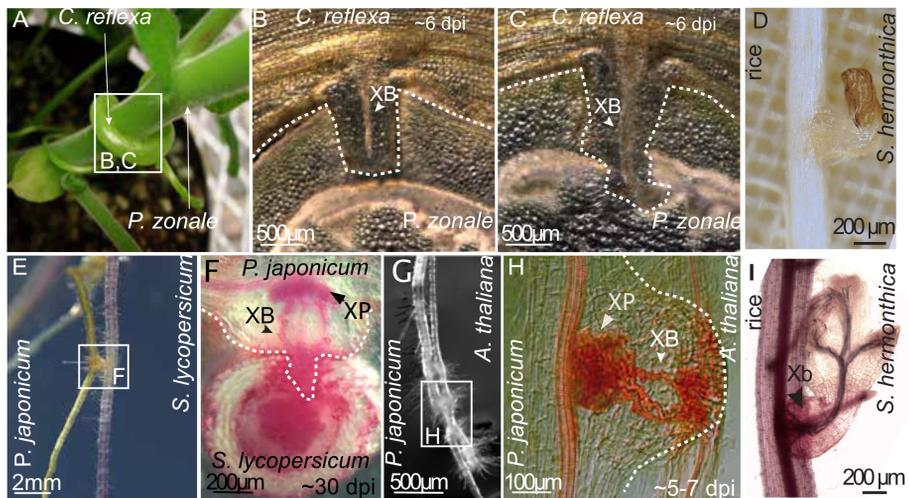


Figure 3. Examples of xylem bridge formation in the haustorium. (A-C) *Cuscuta reflexa* infecting *Pelargonium zonale* approximately 6 days post infection (dpi); images reprinted from Olsen et al. (2016) (E-H) *Phtheirospermum japonicum* infecting *Solanum lycopersicum* (30 dpi) or *Arabidopsis thaliana* (5–7 dpi). (D & I) *Striga hermonthica* infecting rice; images reprinted from (Yoshida et al., 2016). Overview and cross sections of each infection site are shown; the box indicates the approximate area from which cross sections are taken. The dashed line outlines the parasite; xylem bridges (XB) and *Phtheirospermum japonicum* xylem plate (XP) are noted by a triangle (adapted from (Kokla and Melnyk, 2018)).

1.3 Nutrients and plant parasitism

Plants use their root system to uptake the necessary nutrients from their environment for optimal growth. Thus, nutrient availability can affect many aspects of plant growth and development such as root and shoot growth and flowering (Zhang and Forde, 2000; Alboresi et al., 2005; Castro Marin et al., 2011). Parasitic plants are also affected by nutrient availability in their environment. In soil with poor nutrient levels, *Striga* infestations are usually more severe due to impaired host defence mechanisms and an increase in strigolactones production by the host (Mwangangi et al., 2021; Yoneyama et al., 2007a, 2007b). Phosphorous deficiency in sorghum and red clover increases the production of strigolactones to attract mycorrhizal symbionts, which in turn stimulate the germination of parasitic plant seeds that often leads to increased infection rates (Yoneyama et al., 2007a, 2007b). Nitrogen is another important macronutrient and its deficiency also leads to increased levels of germination stimulants from the host (Yoneyama et al., 2007a). On the other hand, optimum nutrient levels improve the host's fitness and thus improve host tolerance and reduce the production of strigolactones germination stimulants (Jamil et al., 2012; Mwangangi et al., 2021). Nutrient availability also affects the parasite's infectivity and growth. Nitrate application reduces *Striga* shoot development as well as the number of parasites successfully attaching to the host (Cechin and Press, 1993; Igbinnosa et al., 1996). The facultative parasite *Rhinanthus minor* exhibits reduced growth in phosphorus rich environments while *Phtheirospermum japonicum* requires nutrient poor conditions to successfully infect its hosts *in vitro* (Davies and Graves, 2000; Cui et al., 2016; Spallek et al., 2017; Ishida et al., 2016). These data suggest that nutrient availability effect on parasitic plants is not only via reducing host root exudates and fitness but also affects the parasite's physiology and ability for host invasion. However, it remains unknown how nutrients affect parasite growth.

1.4 Parallels between plant parasitism and other plant developmental processes

Plant parasitism has independently evolved at least 11 times (Barkman et al., 2007) although much remains unknown regarding how this

evolution occurred (Westwood et al., 2010). Given there are several parallels between haustorium formation and other developmental processes, one possibility is that parasitic plants adapted an already existing developmental process and used this to be able to parasitize, such as via the formation of lateral roots.

1.4.1 Lateral root formation

A developmental process that shares similarities with haustorium development is lateral root development. Haustoria formation and root development have similar transcriptomic profiles (Yang et al., 2015). In *Arabidopsis*, *LBD25* and *KNAT6* have opposing roles during lateral root formation with *LBD25* promoting auxin and thus lateral root formation whereas *KNAT6* represses lateral root formation (Dean et al., 2004; Mangeon et al., 2011). During infection by *Thesium chinense*, *LBD25* and *KNAT6* homologs increase and decrease respectively (Ichihashi et al., 2017). Moreover, an extensive study of the *Striga asiatica* genome and transcriptome revealed that haustoria appeared to have evolved from co-opting the pre-existing lateral root formation mechanisms (Yoshida et al., 2019). In addition to transcriptomic profiles, transcription of the auxin biosynthesis gene *PjYUC3* increases during haustoria formation, this increase corresponds with an auxin response maxima at the haustorium apex in *Phtheirospermum japonicum* (Ishida et al., 2016) (Figure 2). In roots, on the other hand, auxin response maxima defines lateral root founder cells and are important for primary root growth (Overvoorde et al., 2010). Moreover, *YUC* genes are induced in *Arabidopsis* root tips during root growth (Chen et al., 2014). Intriguingly, *Phtheirospermum japonicum* impaired in haustorial hair formation also failed in root hair formation suggesting a common mechanism for the formation of both structures (Cui et al., 2016). Lastly, cell wall degrading enzymes are utilized to remodel cell walls and facilitate both haustorium penetration of host root tissues (Figure 2) and the lateral root emergence through the tissues of the parental root (Honaas et al., 2013; Olsen and Krause, 2017; Swarup et al., 2008). Despite similarities in auxin responses, gene expression and cell wall remodelling, *Phtheirospermum japonicum* haustoria emerge from epidermal, cortex, endodermal and pericycle cells whereas lateral root form from pericycle cells (Wakatake et al., 2018). Considering that auxin

signalling and biosynthesis regulation as well as cell wall remodelling are common in plant organ development, more research is required to better understand the similarities and differences between haustoria and lateral root development.

1.4.2 Nodulation

Leguminous plants form mutualistic symbiotic relationships with rhizobia bacteria in order to assimilate nitrogen. Bacteria enter the root via the root hairs and then reside in specialized organs known as nodules (Suzaki et al., 2015). On the other hand, parasitic plants form haustoria via which they invade their hosts to uptake water and nutrients. Both nodulating and parasitic plants have evolved to form multicellular organs specialized to help them cover their nutritional needs. Although nodulation and plant parasitism have major differences in the type of organisms involved (plant-plant versus plant-bacteria) and the type of interaction between them (parasitic versus mutualistic) there are some parallels in the development of nodules and haustoria that are worth mentioning. Haustoria and nodules serve a common purpose of nutrient uptake (Suzaki et al., 2015). Similar as for parasitic plants, rhizobia sense the presence of a host via their root exudates (Peck et al., 2006). Another common characteristic is that early stages of both nodule and haustorium formation are dictated by cortical cell divisions (Bandaranayake and Yoder, 2013; Suzaki et al., 2012) (Figure 2). Furthermore, in both the haustorium and during early stages of nodulation subtilases involved in the maturation of signalling peptides are expressed and are necessary for successful infections (Ogawa et al., 2021; Taylor and Qiu, 2017). Hormones also play an important role in both processes. In leguminous plants such as *Medicago truncatula* and *Lotus japonicus* auxin accumulates and forms a maximum at the nodule primordium (van Noorden et al., 2007; Takanashi et al., 2011; Suzaki et al., 2012); auxin also accumulates at the pre-haustorium apex and is necessary for haustoria formation (Ishida et al., 2016) (Figure 2). Cytokinin signalling activation is important for nodule organogenesis (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007). Cytokinins increase during successful infections (Spallek et al., 2017) but their exact role in haustorium formation remains unknown. Lastly, ethylene is important in both haustorium and nodule formation. Ethylene affects haustoria

numbers and is essential for successful host invasion in the parasitic plant *Phtheirospermum japonicum* (Cui et al., 2020). Ethylene is also important for nodulation since via *EIN2*, ethylene negatively regulates nodule formation in the leguminous plant *Lotus japonicus* (Miyata et al., 2013). To conclude, despite fundamental differences between haustoria and nodules, there are some notable similarities during their developmental process that could suggest that these processes are more related than we have previously considered.

2. The aims of the study

The objectives of this study can be summarized as follows:

1. To investigate how nutrient availability affects plant parasitism (Paper I).
2. To investigate haustoria regulation by environmental cues and hormones (Paper I & II).
3. To investigate the role of cell wall remodelling during parasitic plant infection (Paper III).

3. Results and discussion

3.1 Nutrient effect on haustoria formation

Parasitic plants acquire all or part of the nutrients they require from their hosts via a specialized organ, known as the haustorium, through which they invade their hosts and uptake water and nutrients. In Paper I we aimed to identify how the environmental nutrient status affects parasitic plant infections and haustoria formation. To date little is known on this subject. *Phtheirospermum japonicum* requires nutrient deprived conditions to infect (Spallek et al., 2017) and the obligate parasite *Striga* has lower infection rates in fields with increased nitrogen fertilization (Igbinnosa et al., 1996). To date, it is theorized that the increased host fitness and decreased germination stimulant production is the reason for reduced parasitic plant infection in nutrient rich environments (Yoneyama et al., 2007a, 2007b; Jamil et al., 2012; Mwangangi et al., 2021). Greenhouse and *in vitro* experiments on the facultative parasitic model plant *Phtheirospermum japonicum* showed that nutrient rich environments inhibited haustoria formation (Paper I Fig.1). Our experiments showed that of the three main macronutrients potassium, phosphorous and nitrogen (K, P, N), nitrogen application in the form of ammonium or nitrate was sufficient to reduce haustoria formation in *Phtheirospermum japonicum* and *Striga* (Paper I Fig.1, Fig.7). Moreover, ammonium nitrate application negatively regulated haustoria numbers in distant non-treated roots (Paper II Fig.1) suggesting not only local but also systemic haustoria inhibition. In addition to haustoria formation inhibition, ammonium nitrate application led to wide transcriptional changes in *Phtheirospermum japonicum* and specifically reduced the expression levels of genes

associated with successful haustoria formation as well as ROS and cell cycle-related genes (Paper I Fig.2, Fig.3, Fig.4). Moreover, ROS accumulation and cell divisions were reduced at the haustorium formation site in the presence of ammonium nitrate (Paper I, Fig.4). These findings are consistent with nitrogen blocking haustoria formation at an early stage and before host invasion (Paper I, Fig.1, Fig.2, Fig.3, Fig.4). To further investigate the mechanism via which nitrogen inhibits haustoria formation, we measured the levels of various phytohormones in *Phtheirospermum japonicum* infecting *Arabidopsis* with and without ammonium nitrate application. Our results showed an increase of abscisic acid and salicylic acid upon nitrate treatment regardless of the presence of a host (Paper I Fig.5). Of these two hormones, only abscisic acid increase was accompanied by an upregulation of abscisic acid responsive genes after ammonium nitrate treatment (Paper I Fig.5). Exogenous application of abscisic acid reduced haustoria formation in *Phtheirospermum japonicum* (Paper I Fig.6), while chemically blocking abscisic acid biosynthesis partly rescued the reduced haustoria phenotype caused by ½ MS nutrient application (Paper I Fig.6). Additionally, we infected *Arabidopsis* plants with *Phtheirospermum japonicum* roots transformed with a construct that blocks abscisic acid signalling. The roots expressing the construct were able to form haustoria even in the presence of nitrogen whereas the roots not expressing the construct formed few to no haustoria (Paper I Fig.6). This was not the case in *Striga* where abscisic acid application did not affect haustoria formation and chemically blocking abscisic acid biosynthesis did not rescue the nitrogen-mediated haustoria inhibition (Paper I, Fig.7). Instead, auxin application was able to overcome the haustoria inhibitory effect of nitrogen in *Striga* suggesting different mechanisms via which nitrogen blocks haustoria in *Phtheirospermum japonicum* and *Striga* (Paper I, Fig.7).

In various plants such as *Arabidopsis*, rice, maize and barley, nitrate leads to an increase in cytokinin that in turn regulates further development of the plant (Samuelson and Larsson, 1994; Takei et al., 2001, 2004; Kamada-Nobusada et al., 2013; Landrein et al., 2018). In *Phtheirospermum japonicum* cytokinin levels did not increase in response to nitrogen and the transcription of cytokinin-related genes remained unaffected (Paper I, Fig.5) suggesting that this parasitic plant

uses different mechanisms to regulate its development in response to nitrogen. A similar effect has been observed in the leguminous plant *Lotus japonicus* where nitrate treatment reduced cytokinin levels and nodule formation (Lin et al., 2021). In *Arabidopsis*, abscisic acid increases in root tips treated with ammonium nitrate and abscisic acid signalling is involved in root growth arrest in high nitrate conditions (Ondzighi-Assoume et al., 2016; Signora et al., 2001). Although, abscisic acid levels increase upon infection in other parasites such as *Rhinanthus minor* and *Cuscuta japonica* (Jiang et al., 2004; Furuhashi et al., 2014), the relation between nitrogen and abscisic acid in parasitic plants is described in Paper I for the first time. These findings show that in *Phtheirospermum japonicum* nitrogen availability negatively regulates haustoria formation via the phytohormone abscisic acid (Fig.4).

Host invasion and xylem bridge formation are necessary haustoria development steps for successful infections. Although exogenous application of nitrogen and cytokinin led to reduced xylem bridge formation (Paper I, Paper II Fig.2), haustoria development under these treatments arrested at the prehaustorium stage (Paper I, Paper II Fig.2) meaning that most haustoria failed to invade the host and thus failed to form vascular connections with the host. These findings suggest that nitrogen and cytokinin primarily affect early haustoria formation but not xylem bridge formation. Chemical inhibition of abscisic acid biosynthesis lead to a reduction in xylem bridge formation suggesting a dual role of abscisic acid during haustoria formation. In early stages abscisic acid negatively regulates haustoria formation in response to elevated nitrogen levels whereas in late stages it is involved in successful xylem bridge formation (Paper I Fig.6).

3.2 The role of cell wall modifications on haustoria formation

Parasitic plants modify their cell walls throughout haustoria development and specifically during pre-haustorium expansion, host attachment and penetration (paragraph 1.2), moreover the cell wall modifying enzymes xyloglucan endotransglucosylases/hydrolases (XTHs) have been shown to be involved in haustoria development in *Cuscuta* (Olsen and Krause, 2017). In Paper III we aimed to identify

cell wall modifying genes and processes that are necessary for successful haustoria development. Looking at our RNAseq analysis of haustoria formation (Paper I) we found that genes that encode cell wall remodelling proteins such as the pectin methylesterases (*PMEs*) and their regulators *PMEIs* were differentially expressed during time points in haustoria development that correspond to host penetration and xylem bridge formation (Paper III, Fig.1). In addition, chemical staining for pectins showed increased pectin activity during host penetration and xylem bridge formation suggesting a role for these cell wall modifications in successful haustoria development in *Phtheirospermum japonicum* (Paper III, Fig.1). These results lead us to investigate if the host *PMEs* and *PMEIs* can affect haustoria development. Several *Arabidopsis* *PMEs* and *PMEIs* were upregulated during haustoria formation (Paper III, Fig.2). Overexpression of *AtPMEI5* delayed xylem bridge formation and reduced the size and number of xylem bridges (Paper III, Fig.2) suggesting the involvement of host cell wall modifying enzymes during vascular connection between *Phtheirospermum japonicum* and *Arabidopsis*. *Phtheirospermum japonicum* cell wall alteration via chemical treatments that inhibited the action of *PMEs* delayed xylem bridge formation and decreased the size and number of xylem bridges. Additionally, the inhibition of XTHs decreased xylem bridge formation and the expression levels of *PMEs* and *PMEIs*, further supporting the involvement of *PMEs* and *PMEIs* in xylem bridge formation (Paper III, Fig.3). To conclude, pectin methylesterases are involved in both haustoria development and xylem bridge formation with pectin-related *Phtheirospermum japonicum* genes being involved in the formation of vascular connection between parasite and host (Fig.4).

3.3 Effect of hormones on haustoria formation

Hormones are important regulators of plant development with various hormones being implicated in haustoria development and regulation (Paper I & II) (Spallek et al., 2017; Cui et al., 2020). In *Phtheirospermum japonicum* cytokinin doesn't increase in response to nitrogen but cytokinin levels increased during infection (Paper I Fig.4). This is in agreement with previous findings showing cytokinin

increases in late infection time points and is introduced in the host via the haustorium to induce host hypertrophy (Spallek et al., 2017). Moreover, the expression of cytokinin related genes increased during infection suggesting a role for cytokinin during haustoria formation. Exogenous cytokinin application blocked haustoria formation in the pre-haustorium stage (Paper II Fig.2) similar to nitrogen (Paper I Fig.1), and *Phtheirospermum japonicum* roots expressing a cytokinin degrading construct formed more haustoria than non-transgenic roots (Paper II Fig.2). Exogenous cytokinin application also repressed the expression of genes involved in haustoria development (Paper II Fig.2). Despite similarities in the effect of nitrogen and cytokinin exogenous application, cytokinin levels did not respond to nitrogen in *Phtheirospermum japonicum* and cytokinin responsive genes were downregulated in nitrogen compared to water treatments (Paper I, Fig.5). These findings suggest that nitrogen haustoria inhibition is abscisic acid-mediated (Paper I) and is independent of cytokinin but cytokinin is involved in haustoria formation. Cytokinin levels could increase in successful haustoria not only to induce host hypertrophy but to block haustoria formation in proximal tissues to another established haustorium.

Another hormone we investigated was gibberellic acid. Gibberellic acid levels decrease in *Phtheirospermum japonicum* infecting roots (Paper I, Fig.5, Paper II, Fig.3). Exogenous application of gibberellic acid increased haustoria numbers whereas chemical inhibition of gibberellic acid biosynthesis blocked haustoria formation (Paper II, Fig.2). Moreover, gibberellic acid-related genes were upregulated during late time points in haustoria formation (Paper II, Fig.2), suggesting that optimal gibberellic acid levels are necessary for haustoria development. On the other hand, gibberellic acid application resulted in a similar transcriptional profile to that of untreated plants (Paper II, Fig.2). Although, gibberellic acid application did not lead to major transcriptional changes in *Phtheirospermum japonicum* we observed an upregulation of *YUC* auxin biosynthesis genes that are known to be involved in and necessary for successful haustoria development (Ishida et al., 2016) (Paper II Fig.S2). These findings suggest that possibly gibberellic acid increased haustoria numbers via an increase in auxin biosynthesis. Auxin biosynthesis is crucial at early haustorium development stages (Ishida et al., 2016) and in our

experiments, application of higher concentrations of auxin decreased xylem bridge formation in late infection stages and increased the expression of *PMEIs* (Paper III, Fig.4). On the other hand, chemical inhibition of auxin transport blocked xylem bridge connection and decreased expression levels of both *PME* and *PMEIs* implicating auxin in both early and late haustoria developmental stages (Paper III, Fig.4) (Wakatake et al., 2020). Although both exogenous auxin applications and chemical inhibition of auxin transport resulted in less xylem bridges, haustoria that formed under exogenous auxin application seemed to fail to penetrate the host and thus fail to form xylem bridge. This suggests that exogenous auxin application affected earlier haustoria development stages and subsequently lead to decreased xylem bridge. In leguminous plants and *Arabidopsis*, spatiotemporal auxin accumulation is necessary for lateral root formation as well as for nodule formation in leguminous plants (Demina et al., 2019; Benková et al., 2003; Ng and Mathesius, 2018). Perturbation of the delicate auxin balance during the development of these organs can affect their formation. Auxin accumulation is necessary for successful haustoria formation (Ishida et al., 2016), although, constant high auxin amount, such as when exogenously applied, might hinder late haustoria formation stages such as host penetration. On the other hand, chemical inhibition of auxin transport directly affected xylem bridge formation by inhibiting the xylem connection of plate xylem and haustorium apex.

Lastly, brassinosteroids regulate the expression of *PMEs* in *Arabidopsis* (Qu et al., 2011; Wolf et al., 2012) and since *PMEs* and *PMEIs* are involved in haustoria and xylem bridge formation we investigated if brassinosteroids also affect haustoria formation in *Phtheirospermum japonicum*. Application of brassinosteroids on *Phtheirospermum japonicum* decreased haustoria numbers, vascular connection and the expression levels of both *PME* and *PMEIs* (Paper III, Fig.4) suggesting a role for brassinosteroids during haustoria formation and *PME/PMEI* expression regulation. To conclude, various phytohormones are involved in the formation and regulation of haustoria formation in *Phtheirospermum japonicum*, with many of them being implicated in both early and late haustoria developmental stages.

3.4 Local and systemic haustoria regulation

In Paper I we demonstrate that *Phtheirospermum japonicum* regulates haustoria numbers in response to environmental nitrogen levels. Thus, we aimed to further investigate haustoria regulation mechanisms in *Phtheirospermum japonicum*. We discovered that previously formed haustoria or nitrogen application negatively regulated haustoria numbers in distant roots (Paper II Fig.1). In these experiments the root was split in two parts with no contact with each other suggesting the existence of a long distance haustoria regulation mechanism in *Phtheirospermum japonicum* that includes a signal transmitted from the infecting roots to the distant roots via the shoot (Paper II Fig.1).

Cytokinin and gibberellic acid affect haustoria numbers (Paper I & II) and are able to move throughout the plant to regulate growth and development (Ragni et al., 2011; Sakakibara, 2006). Hormone profiling revealed that cytokinin metabolites were increased in infecting *Phtheirospermum japonicum* roots but were not increased in the shoot or distant roots while gibberellic acid levels were reduced in infecting and distant *Phtheirospermum japonicum* roots (Paper II, Fig.3). Furthermore, neither cytokinin nor gibberellic acid affected haustoria numbers in distant roots (Paper II, Fig.3), suggesting that these hormones do not act themselves as the long-distance regulation signal but regulate haustoria numbers locally (Paper II Fig.2, 3). Additionally, gibberellic acid levels were reduced in both infected and distant roots (Paper II, Fig.3), this suggests the possibility that gibberellic acid levels are under the control of regulation mechanisms to result in optimal haustoria numbers.

Small mobile peptides are known for their involvement in regulating various developmental processes. We identified five CEP peptide and ten CLE peptides in *Phtheirospermum japonicum* with one CEP (*PjCEP5*) and two CLE (*PjCLE1*, *PjCLE3*) peptides that were significantly differentially expressed during haustorium formation (Paper II Fig.4). In *Arabidopsis* CEP peptides are perceived by the CEPR1 receptor in the shoot. After CEP shoot perception, another peptide, named CEPD peptide, acts as shoot-to-root signal to regulate root growth (Taleski et al., 2018). Our experiments showed that the expression levels of *PjCEP5* were increased in infected *Phtheirospermum japonicum* roots and shoots (Paper II Fig.5). Moreover, *PjCEPR1*, the putative CEP peptide receptor encoding

gene, and *PjCEPD*, the putative shoot-to-root signalling peptide encoding gene, were upregulated in *Phtheirospermum japonicum* infected shoots. Furthermore, exogenous application of PjCEP5 peptide decreased haustoria numbers in distant *Phtheirospermum japonicum* roots suggesting that CEP peptides are involved in the long-distance signalling mechanism of haustoria regulation in *Phtheirospermum japonicum* (Paper II Fig.5). PjCLE3 peptide negatively affected haustoria numbers locally and in distant roots. Although *PjCLE3* was upregulated in infected roots, neither *PjCLE3* nor the potential CLE receptor *PjBAM3* was upregulated in the shoot of infected *Phtheirospermum japonicum* (Paper II, Fig.4, Fig.5). On the other hand, PjCLE1 positively affected haustoria numbers locally and did not affect haustoria in distant roots (Paper II, Fig.4, Fig.5). Overall, our data show that *Phtheirospermum japonicum* harbors local and systemic mechanisms for haustoria regulation that involves phytohormones (Paper I & II) and small mobile peptides such as CEP and CLE peptides (Paper II) (Fig.4). It remains to be shown if nitrogen, cytokinin and abscisic acid are involved in CEP regulation in *Phtheirospermum japonicum*.

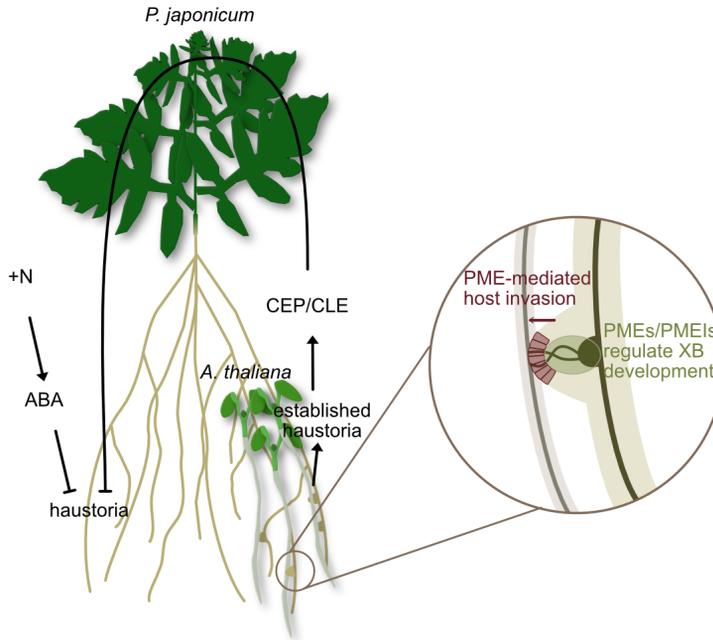


Figure 4. Illustration summarizing the main findings of the three papers included in this thesis. Illustration of *Phtheirospermum japonicum* demonstrating that in the presence of nitrogen (N), haustoria formation is inhibited via the phytohormone abscisic acid (ABA). In addition, previously established haustoria inhibit haustoria formation in distant roots via a systemic mechanism that involves CEP and CLE small mobile peptides. Lastly, pectin methylesterases (PMEs) and pectin methylesterase inhibitors (PMEIs) are involved in host invasion and xylem bridge (XB) formation regulation.

4. Future perspectives

In this work we aimed to identify how nutrient availability and hormones can affect and regulate haustoria formation in the facultative parasitic plant *Phtheirospermum japonicum*. In paper I we described how nitrogen inhibits haustoria formation via the phytohormone abscisic acid. Our results demonstrated that from the three main macronutrients (K-P-N) only nitrogen inhibited haustoria formation in *Phtheirospermum japonicum*. In Paper I we focused on the effect of nutrients on plant parasitism but water availability could be another important factor for plant parasitism. It is currently unknown how drought affects parasitism by *Phtheirospermum japonicum*. Further research could reveal if haustorium formation is affected by water deprivation. Nitrogen suppresses haustoria development via an increase in abscisic acid. What are the molecular mechanisms via which haustoria development is hindered by abscisic acid? We investigated the transcriptomic changes during ammonium nitrate application during haustoria formation in *Phtheirospermum japonicum*. Further experimentation could help to identify the genes whose transcription is specifically affected by abscisic acid during haustorium formation. Furthermore, genetic alteration of *Phtheirospermum japonicum* abscisic acid biosynthesis or signalling genes could provide answers to these questions.

In paper II we focused on how hormones and peptides are involved in haustoria number regulation in *Phtheirospermum japonicum*. We discovered that cytokinin and gibberellic acid were involved in the local regulation of haustoria number with opposite effects. It is possible that cytokinin and gibberellic acid interact to regulate haustoria formation. Gene expression studies on *Phtheirospermum japonicum* plants that over/under produce gibberellic acid and

cytokinin could reveal if there is such interaction and, in addition, could reveal if these hormones interact with abscisic acid. PjCEP and PjCLE peptides are involved in haustoria number regulation and our results suggested their involvement in long distance haustoria regulation. We also demonstrated that *Phtheirospermum japonicum* harbors a haustorium regulation mechanism with potential similarities to nodule autoregulation. Proteomics could help to further identify the levels of these peptides in *Phtheirospermum japonicum* roots and shoots. Although the lack of stable transformation in *Phtheirospermum japonicum* hinders genetic analysis of the peptide role in long-distance haustoria regulation, over expression of these peptides in *Phtheirospermum japonicum* roots could further reveal the pathways and mechanisms that are involved in long-distance haustorium regulation and the role of these peptides. Split root experiments and gene expression analysis could also reveal if nitrogen and abscisic acid act to suppress haustoria via this long-distance haustorium regulation mechanism or via an CEP/CLE independent pathway of systemic haustoria regulation.

In the last chapter of this thesis we investigated cell wall modifications during haustorium development. We showed that cell wall modifying enzymes were crucial for successful haustoria and xylem bridge formation with a focus on two genes that were differentially expressed by various treatments and that affected xylem bridge formation. Moreover, we demonstrated that brassinosteroids and auxin were involved in the regulation of these genes. Further studies using reporter lines and over/under expression of these genes coupled with gene expression studies could reveal more information on their role in xylem bridge formation and conditions or processes that regulate the expression and activity. Are genes encoding for cell wall modifying enzymes directly regulated by brassinosteroids or auxin? Are other hormones or environmental conditions involved in the regulation of these genes? Is the host connection part of this regulatory mechanism? Answering these questions could bring us further in understanding haustoria development.

To conclude, in this thesis we showed that *Phtheirospermum japonicum* regulates its haustoria numbers in response to environmental nitrogen levels via the phytohormone abscisic acid. This work is in agreement with previous reports that infection rates are

lower in fertilized fields (Yoneyama et al., 2007a; Jamil et al., 2012). Moreover, *Phtheirospermum japonicum* regulates its haustoria numbers in response to already established haustoria via a systemic haustoria regulation mechanism that involves small mobile peptides. Last, we showed that cell wall modifying enzymes are important during xylem bridge formation in *Phtheirospermum japonicum*. Although more research is needed to fully unravel these mechanisms, this work is a step further in our understanding of the haustoria formation and regulation in parasitic plants. Our findings in *Phtheirospermum japonicum* and *Striga* suggest that the negative haustoria regulation in response to nitrogen is conserved among parasitic plants. This could potentially lead to the development of novel ways to suppress haustoria formation in the field by “hacking” the haustoria regulation mechanism by either applying optimal nutrient levels or using chemical or genetic means to induce the haustoria regulation mechanisms to block haustoria formation. Blocking haustoria formation in the parasite would leave the host plants unharmed while it would reduce the well-being of the parasites and even lead to their death in case of obligate parasites.

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Popular science summary

Plants that parasitize other plants are known as parasitic plants. All parasitic plants form an organ known as the haustorium through which they penetrate their host plant to uptake water and nutrients. Many parasitic plants are damaging pests for agriculture leading to important economical losses every year. Despite this importance and recent research advancements, we still lack effective methods to combat parasitic plant infestations. In this thesis we aimed to expand our current knowledge regarding how haustoria are regulated depending on external nutrient availability using the model parasitic plant *Phtheirospermum japonicum*. We discovered that when *Phtheirospermum japonicum* is given sufficient nitrogen amounts then it fails to form haustoria and thus fails to infect available hosts suggesting that *Phtheirospermum japonicum* can regulate its infectivity depending on environmental nitrogen levels. We further showed that this haustoria inhibition is due to the increased levels of the phytohormone abscisic acid. These findings lead us to further investigate how phytohormones are involved in haustoria regulation. We found that two more phytohormones, cytokinin and gibberellic acid, are involved in the local haustoria regulation. Further work revealed that *Phtheirospermum japonicum* harbors a systemic mechanism to self-regulate the number of haustoria forming based on existing haustoria numbers. Mobile signaling peptides are involved and possibly mediate this mechanism. In the last chapter of this thesis we investigated how cell wall alterations at the haustorium formation site are needed for successful haustorium formation. We identified two *Phtheirospermum japonicum* genes that encode for cell wall modifying enzymes that are involved in vascular connections between the parasite and the host. These genes are regulated by plant phytohormones but further experiments are needed to uncover if nitrogen availability can affect those

genes. To conclude this thesis demonstrates how both environmental and endogenous factors alter the infectivity of a parasitic plant via utilizing phytohormones and signaling peptides.

Populärvetenskaplig sammanfattning

Växter som parasiterar andra växter kallas parasitväxter. Alla parasitiska växter bildar ett organ som kallas haustorium genom vilket de penetrerar sin värdväxt för att ta upp vatten och näringsämnen. Många parasitiska växter orsakar stora skador inom växtodlingen, vilket leder till betydande ekonomiska förluster för jordbrukare varje år. Detta till trots saknar vi fortfarande effektiva metoder för att bekämpa angrepp av parasitiska växter. I detta avhandlingsarbete syftade vi till att utöka vår nuvarande kunskap om hur bildning av haustorier regleras av extern tillgång på näringsämnen med hjälp av modellparasitväxten *Phtheirospermum japonicum*. Vi upptäckte att när *P. japonicum* ges tillräckliga mängder kväve så misslyckas den med att bilda haustorier och därmed också med att infektera tillgängliga värdar. Det tyder på att *Phtheirospermum japonicum* kan reglera sin parasitism i förhållande till hur tillgängligt kväve är i dess växtmiljö. Vi visade vidare att kväve-relaterad hämning av haustorium-bildning förmedlas via ökade nivåer av växthormonet abskisinsyra. Dessa fynd föranledde oss att undersöka om fler växthormoner är involverade i reglering av haustorier. Vi fann att två till växthormoner, cytokinin och gibberellinsyra, är involverade i den lokala regleringen av haustorier. Ytterligare arbete avslöjade att *Phtheirospermum japonicum* använder en systemisk mekanism för att självreglera antalet haustorier som behöver nybildas baserat på hur många haustorier som individen redan producerat. Mobila signalpeptider är involverade och förmedlar möjligen denna information. I det sista kapitlet av avhandlingen undersökte vi hur cellväggsförändringar vid platsen för initiering av ett haustorium behövs för framgångsrik haustorium-bildning. Vi identifierade två *Phtheirospermum japonicum*-gener som kodar för

cellväggsmodifierande enzymer som har betydelse för den vaskulära sammankopplingen mellan parasiten och värden. Dessa gener regleras av växthormoner, men ytterligare experiment behövs för att avslöja om kvävetillgänglighet också kan påverka deras aktivitet. Avslutningsvis visar denna avhandling hur både miljöfaktorer och endogena faktorer via växthormoner och signalpeptider kan påverka en parasitisk växts parasitiska aktivitet.

Εκλαϊκευμένη περίληψη

Τα φυτά που παρασιτούν άλλα φυτά είναι γνωστά ως παρασιτικά φυτά. Όλα τα παρασιτικά φυτά αναπτύσσουν όργανα γνωστά ως μυζητήρες (haustoria) μέσω των οποίων διεισδύουν τους ιστούς του φυτού ξενιστή για να εξάγουν νερό και θρεπτικά συστατικά. Πολλά παρασιτικά φυτά είναι επιβλαβή παράσιτα για τη γεωργική παραγωγή προκαλώντας σημαντικές οικονομικές ζημιές κάθε χρόνο. Παρά την οικονομική τους σημασία και την πρόσφατη ερευνητική πρόοδο, εξακολουθούμε να έχουμε έλλειψη αποτελεσματικών μεθόδων για την καταπολέμηση των παρασιτικών φυτών. Σε αυτή τη διατριβή στοχεύσαμε στην επέκταση του γνωστικού μας πεδίου σχετικά με τη ρύθμιση των μυζητήρων ανάλογα με την διαθεσιμότητα θρεπτικών συστατικών στο μοντέλο παρασιτικό φυτό *Phtheirospermum japonicum*. Ανακαλύψαμε ότι όταν το *Phtheirospermum japonicum* λαμβάνει επαρκείς ποσότητες αζώτου, τότε αποτυγχάνει να σχηματίσει μυζητήρες και επομένως αποτυγχάνει να παρασιτήσει διαθέσιμους ξενιστές. Αυτό υποδηλώνει ότι το *Phtheirospermum japonicum* μπορεί να ρυθμίσει τη παρασιτικότητά του ανάλογα με τα περιβαλλοντικά επίπεδα αζώτου. Περαιτέρω δείξαμε ότι η αποτυχία σχηματισμού μυζητήρων οφείλεται στα αυξημένα επίπεδα της φυτοορμόνης αμψισικό οξύ. Αυτά τα ευρήματα μας οδηγούν στην περαιτέρω διερεύνηση του τρόπου με τον οποίο οι φυτοορμόνες εμπλέκονται στη ρύθμιση του σχηματισμού των μυζητήρων. Βρήκαμε ότι δύο ακόμη φυτοορμόνες, η κυτοκίνη και οι γιβερελλίνες, εμπλέκονται στην τοπική ρύθμιση του σχηματισμού των μυζητήρων. Περαιτέρω πειράματα αποκάλυψαν ότι το *Phtheirospermum japonicum* διαθέτει έναν συστημικό μηχανισμό για την αυτορρύθμιση του αριθμού των μυζητήρων με βάση τους προϋπάρχοντες μυζητήρες. Σε αυτόν τον μηχανισμό εμπλέκονται μικρά σηματοδοτικά πεπτίδια. Στο τελευταίο κεφάλαιο αυτής της διατριβής διερευνήσαμε πώς οι τροποποιήσεις του κυτταρικού τοιχώματος στη θέση σχηματισμού των μυζητήρων εμπλέκονται στον επιτυχή σχηματισμό τους. Εντοπίσαμε δύο γονίδια που στο *Phtheirospermum japonicum* κωδικοποιούν ένζυμα τροποποίησης του

κυτταρικού τοιχώματος και που εμπλέκονται στις αγγειακές συνδέσεις μεταξύ του παρασίτου και του ξενιστή. Αυτά τα γονίδια ρυθμίζονται από φυτοορμόνες, αλλά απαιτείται περαιτέρω έρευνα για να αποκαλυφθεί εάν η διαθεσιμότητα αζώτου μπορεί να επηρεάσει την έκφραση τους. Εν κατακλείδι, αυτή η διατριβή υποδεικνύει πώς τόσο οι περιβαλλοντικοί όσο και οι ενδογενείς παράγοντες μεταβάλλουν τη παρασιτικότητα ενός παρασιτικού φυτού μέσω της χρήσης φυτοορμονών και μικρών σηματοδοτικών πεπτιδίων.

Acknowledgements

Throughout my PhD many colleagues, friends and family have supported me and helped me reach the goal of completing this thesis. I would like to express my deep gratitude to all of you.

Ευχαριστώ
Thank you
Tack

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DOCTORAL THESIS NO. 2022:5

Parasitic plants are important agricultural pests that lead to considerable yield losses annually. Here, we describe the mechanisms via which *Phtheirospermum japonicum* regulates haustoria numbers in response to nitrogen availability. Moreover, we show the existence of a systemic haustoria regulation mechanism in *Phtheirospermum japonicum*. Last, we analyzed the role of cell wall modifying enzymes during haustoria formation. This thesis contributes in the understanding of haustoria numbers regulation in response to nutrient availability or pre-existing established haustoria in *Phtheirospermum japonicum*.

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ISSN 1652-6880

ISBN (print version) 978-91-7760-885-1

ISBN (electronic version) 978-91-7760-886-8