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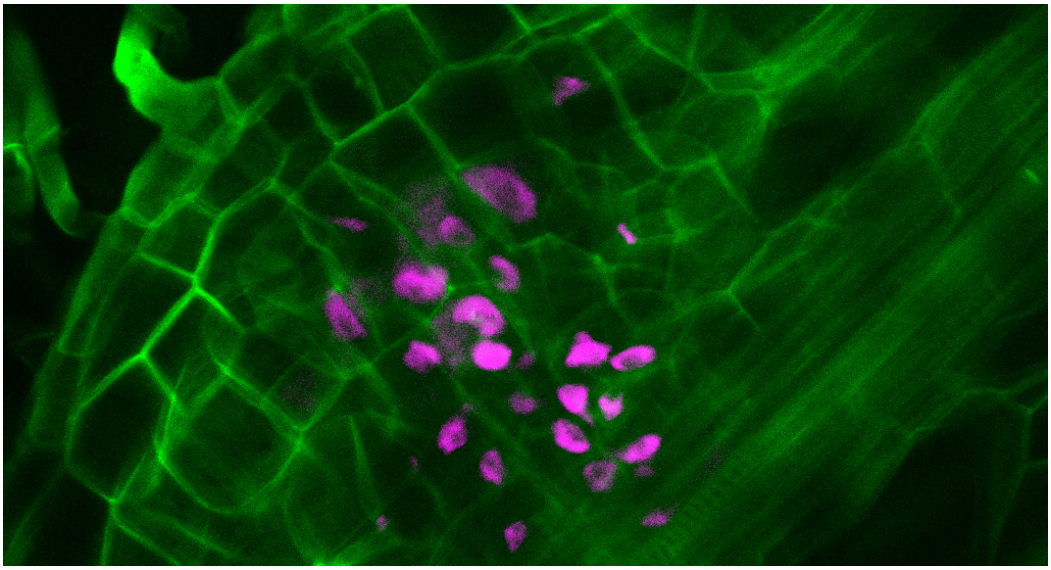
Roles of plant cell wall modifications and nutrient signalling in plant-plant interactions

Insights on plant parasitism and grafting

Martina Leso



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Cover: Developing *P. japonicum* haustorium expressing *pPMEI9:2xVenus* (purple), stained with calcofluor white (green)
(photo: M. Leso)

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Abstract

Parasitic plants and grafting are examples of plant-plant interactions and plant regeneration. In plant parasitism, one plant connects to another to steal its resources, while during grafting the reconnection allows the survival of both individuals as part of a new individual. Plant regeneration is linked to the availability of nutrients in the environment and involves the modification of the cell wall. In this thesis, we studied the roles of sucrose and nitrogen signalling and of cell wall modifications during *Arabidopsis thaliana* grafting and plant parasitism by the facultative parasite *Phtheirospermum japonicum*. We found that applying sucrose or nitrogen inhibited both plant parasitism and graft reconnection (papers I and IV). We showed that the inhibition of plant parasitism by nitrogen is mediated by abscisic acid signalling in *P. japonicum* (paper I). We also discovered a long-distance signalling mechanism in *P. japonicum* to balance the availability of nutrients with the number of parasitic organs (haustoria) (paper II). We termed this phenomenon "autoregulation of haustoria", and discovered it is mediated by cytokinin signalling. In paper III, we found that pectin methylesterification is modified during the development of *P. japonicum* haustoria in a tissue-specific manner, to allow host penetration and vascular connection to the host xylem. Finally, in paper IV we discovered that sucrose inhibits graft reconnection by activating cellulose biosynthesis, and that this phenotype can be rescued by applying abscisic acid. Overall, our results contribute to the understanding of the mechanisms regulating plant grafting and parasitism, and suggest interplay between cell wall, nutrients and hormone signalling.

Keywords: parasitic plants, grafting, cell wall, pectin, cellulose, sucrose signalling, nitrogen signalling, cytokinin, abscisic acid

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Funktioner för cellväggsmodifieringar och näringssignalering i växt-växt-interaktioner

Sammandrag

Parasitiska växter och ympning är exempel på växt-växt-interaktion och växtförnyelse. Vid växtparasitism ansluter en växt till en annan för att stjäla dess resurser, medan återkopplingen under ympningen tillåter överlevnaden för båda individerna som en del av en ny växt. Växtförnyelse är kopplat till tillgången på näringsämnen i miljön och innebär modifiering av cellväggen. I den här avhandlingen studerade vi sackaros- och kvävesignalering och cellväggsmodifieringar under *Arabidopsis thaliana* ympning och växtparasitism av den fakultativa parasiten *Phtheirospermum japonicum*. Vi fann att applicering av sackaros eller kväve hämmade både växtparasitism och ympning (kapitel I och IV). Vi visade att hämningen av växtparasitism av kväve förmedlas av abscisinsyra signalering i *P. japonicum* (kapitel I). Vi upptäckte också en långdistans signaleringsmekanism i *P. japonicum* för att balansera tillgången på näringsämnen med antalet parasitorgan (haustorier) (kapitel II). Vi kallade detta fenomen ”autoreglering av haustorier” och upptäckte att det förmedlas av cytokinin signalering. I kapitel III fann vi att pektinmetylesterifiering modifieras under utvecklingen av *P. japonicum* haustorier på ett vävnadsspecifikt sätt, för att möjliggöra värdpenetrering och vaskulär anslutning till värdxylemet. Slutligen, i kapitel IV upptäckte vi att sackaros hämmar ympning genom att aktivera cellulosabiosyntes, och att denna fenotyp kan räddas genom att applicera abscisinsyra. Sammantaget bidrar våra resultat till vår kunskap om mekanismerna som reglerar ympning och parasitism, och föreslår samspel mellan cellvägg, näringsämnen och hormonsignalering.

Nyckelord: parasitväxter, ympning, cellvägg, pektin, cellulosa, sackarossignalering, kvävesignalering, cytokinin, abscisinsyra

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Ruoli delle modificazioni della parete cellulare e della segnalazione dei nutrienti nelle interazioni pianta-pianta

Sommario

Le piante parassite e gli innesti sono esempi di interazione pianta-pianta e di rigenerazione vegetale. Nel parassitismo vegetale una pianta sfrutta un'altra pianta rubandone le risorse, mentre l'innesto consente la sopravvivenza post taglio di entrambe le piante in un nuovo individuo. In questa tesi, abbiamo studiato il ruolo di saccarosio e azoto e della parete cellulare durante l'innesto di *Arabidopsis thaliana* e il parassitismo da parte del parassita facoltativo *Phtheirospermum japonicum*. Abbiamo scoperto che l'applicazione di saccarosio o azoto inibisce sia il parassitismo vegetale che l'innesto (articoli I e IV) e che l'inibizione del parassitismo delle piante da parte dell'azoto è mediata dall'acido abscissico in *P. japonicum* (articolo I). Abbiamo anche scoperto un meccanismo di segnalazione a lunga distanza in *P. japonicum* che bilancia la disponibilità di nutrienti con il numero di organi parassiti (austori) (articolo II). Abbiamo chiamato questo fenomeno "autoregolazione degli austori" e abbiamo scoperto che è mediato dalle citochinine. Nell'articolo III, abbiamo scoperto che la pectina viene modificata durante lo sviluppo degli austori per consentire la penetrazione dell'ospite e la connessione vascolare al suo xilema. Infine, nell'articolo IV abbiamo scoperto che il saccarosio inibisce l'innesto attivando la biosintesi della cellulosa e che questo fenotipo può essere neutralizzato dall'acido abscissico. Nel complesso, i nostri risultati arricchiscono la nostra conoscenza di innesto e parassitismo vegetale, e suggeriscono un'interazione tra parete cellulare, nutrienti e segnalazione ormonale.

Parole chiave: piante parassite, innesto, parete cellulare, pectina, cellulosa, saccarosio, azoto, citochinina, acido abscissico

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Dedication

Alla mia famiglia, e agli amici che sono diventati famiglia.

To my family, and to the friends who became family.

Contents

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, Serivichyaswat P.T, Cui S, Ljung K, Yoshida S, Melnyk C. W.** (2022). Nitrogen represses haustoria formation through abscisic acid in the parasitic plant *Phtheirospermum japonicum*. *Nature Communications*, 13 (2976)

II. **Kokla A*, Leso M*, Simura J, Ljung K, Melnyk C. W**. Cytokinin mediates systemic auto-regulation of haustoria in the parasitic plant *Phtheirospermum japonicum*. (Submitted)

III. **Leso M, Kokla A, Feng M, Melnyk C. W.** (2023). Pectin modifications promote haustoria development in the parasitic plant *Phtheirospermum japonicum. Plant Physiology*, 194 (1)

IV. **Leso M, Cazzaniga F, Melnyk C. W.** Sucrose inhibits *Arabidopsis thaliana* grafting by activating *CELLULOSE SYNTHASE A* (*CESA*) genes (Manuscript)

Papers I and III are reproduced with the permission of the publishers.

The contribution of Martina Leso to the papers included in this thesis was as follows:

I. Performed and analysed part of the experiments; reviewed the manuscript.

II. Planned, performed and analysed the experiments, wrote the manuscript.

III. Conceived the project; planned, performed and analysed the experiments; wrote the manuscript.

IV. Planned, performed and analysed the experiments; wrote the manuscript.

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

1. **Serivichyaswat P.T*, Bartusch K*, Leso M, Musseau C, Iwase A, Chen Y, Sugimoto K, Quint M, Melnyk C. W.** (2022). High temperature perception in leaves promotes vascular regeneration and graft formation in distant tissues. *Development*, 149 (5).

List of figures

1. Introduction

One of the most striking differences between animals and plants resides in their overall structure. While (most) animals are unitary, with a predictable structure that is pre-determined at the embryo stage (e.g., the number of limbs), plants are notable examples of modularity, where new organs can develop throughout an organism's life in response to stimuli such as temperature, light, pathogens and presence or absence of nutrients.
To better adapt to their surroundings, plants have evolved complex and sophisticated signalling systems, which integrate the perception of factors from the environment with developmental responses. Many of the initial signals happen at the cell wall, the rigid outer layer of plant cells, and are then transmitted to the whole plant thanks to systemic signalling relying on small molecules known as hormones.

Part of plants interaction with the environment is "plant-to-plant" interaction, which can be either beneficial or harmful. Examples of these interaction are plant grafting, where two plants are joined together (either artificially by humans or naturally) to benefit both individuals, and plant parasitism, where one plant attacks another plant to steal its resources (Figure 1). Both these processes are highly regulated and complex, and they require changes in the plant cell wall structure and the cross-talk between several plant signalling pathways, including hormonal signalling.

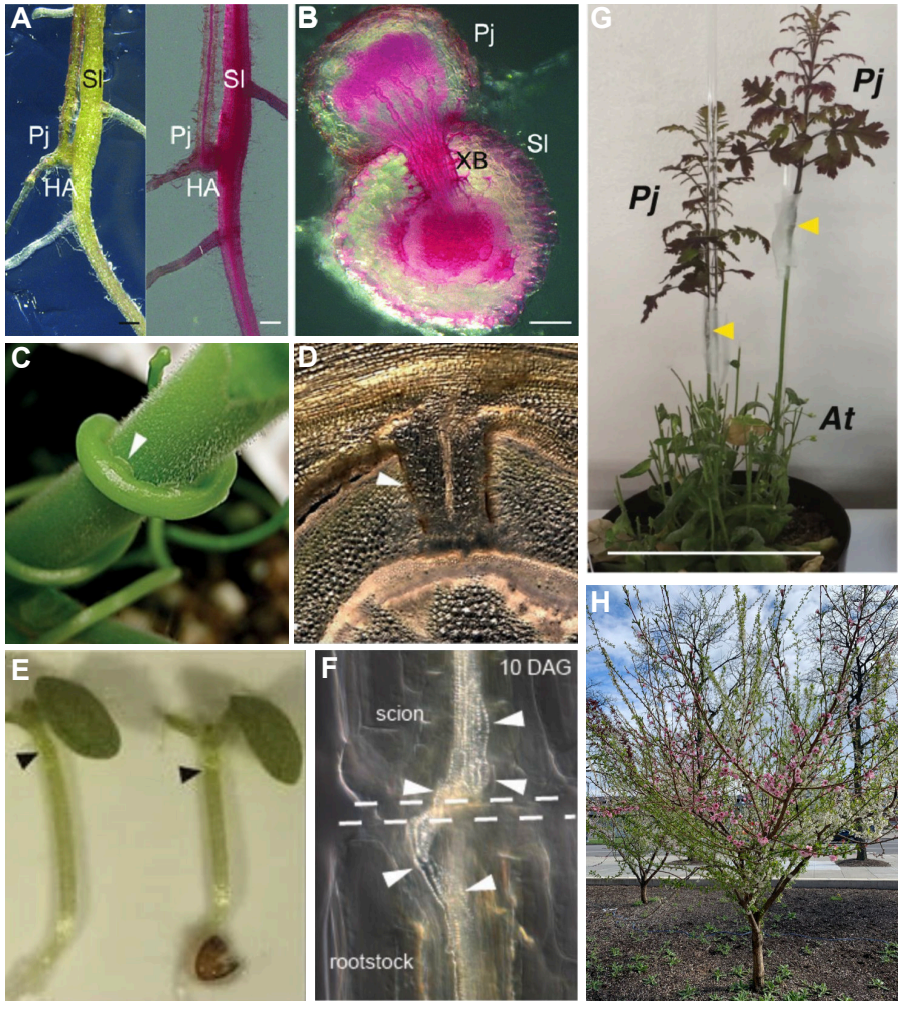

Figure 1: Examples of plant parasitism and grafting.

A-B) The facultative parasite *Phtheirospermum japonicum* (Pj) parasitizing tomato (Sl) and cross section of the haustorium. Adapted from Spallek et al. 2017. C-D) The shoot parasite *Cuscuta reflexa* parasitizing *Pelargonium zonale* and cross section of the haustorium. Adapted from Olsen et al. 2015. E-F) Grafted *Arabidopsis thaliana* hypocotyls and close-up of the reconnected graft junction. Adapted from Melnyk 2017. G) *P. japonicum* (*Pj*) scion grafted on *A. thaliana* (*At*). Adapted from Kurotani et al. 2020. H) Tree of Forty Fruit by Sam van Aken at Syracuse University, growing forty different stone fruits. Picture from https://www.samvanaken.com/.

1.1 The plant cell wall

1.1.1 Structure and function of the cell wall

The plant cell wall is one of the main characteristics differentiating plant cells from animal cells. The cell wall is a layer constituted mostly of polysaccharides, proteins and phenolics (Somerville et al. 2004; Cosgrove 2005). It is located outside the plasma membrane and functions as load-bearing, a cell-to-cell adhesion matrix, and counteracts the turgor pressure from the cell (Somerville et al. 2004; Cosgrove 2005). Most of the plant cell wall is constituted by polysaccharide chains, which are classified based on their monosaccharide composition and chemical bonds into cellulose, hemicellulose (xyloglucan and glucuronoarabinoxylan) or pectin (homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II). Finally, plant cell walls can be classified as either primary or secondary. While the primary cell wall is present around almost all plant cells, the secondary cell wall is a thicker layer enriched in cellulose and lignin that is mostly present in xylem tissues. As the cell wall is a highly complex structure, this thesis introduction will focus on cellulose and pectin, as they are most relevant to the findings presented in the later chapters.

1.1.2 Cellulose

Cellulose is the load-bearing component of plant cell walls. It is usually the most abundant polysaccharide in the cell wall, constituting about 30% of primary cell walls and 40-50% of the secondary cell walls (Cosgrove and Jarvis 2012; Zhong and Ye 2015). Cellulose is a linear polysaccharide constituted of glucose monomers linked via β-1,4 bonds. The synthesis and orientation of cellulose fibers determines the capability for plant cell expansion and plant growth. Cellulose fibers are synthesised at the plasma membrane by the action of cellulose synthase complexes (CSCs) (Mueller and Brown 1980). These complexes are formed by subunits of cellulose synthase (CESA) enzymes and their composition varies based on the type of cellulose to be synthesised. In *Arabidopsis thaliana*, 10 *CESA* genes have been identified (*CESA1-10*) (Richmond and Somerville 2000). While *CESA1*, *3* and *6* are mainly involved in the synthesis of primary cell walls, *CESA4*, *7* and *8* are involved in secondary cell wall biosynthesis (Desprez et al. 2007; Persson et al. 2007; Taylor et al. 2003). The directionality of cellulose filaments is determined by the structure of microfibrils, which

guide the movements of CSCs on the plasma membrane (Li et al. 2012; Endler et al. 2015).

As cellulose is a sugar polymer, cellulose biosynthesis is tightly linked to the availability of sugars in the plant. The main building block for cellulose biosynthesis is UDP-glucose, which plants generate from sucrose through the action of sucrose synthases (SuSys) or cytosolic invertases (CINVs). SuSys are plasma membrane associated enzymes that catalyse the direct conversion of sucrose to fructose and UDP-glucose, and this process is thought to be the major source of UDP-glucose in plants (Ruan 2014; Kleczkowski, Kunz, and Wilczynska 2010). The overexpression of SuSy in poplar was sufficient to increase wood cellulose content (Coleman, Yan, and Mansfield 2009), suggesting a direct link between sucrose metabolism and cellulose biosynthesis. However, Arabidopsis quadruple SuSy mutants could still synthesise cellulose, suggesting SuSy genes are not necessary for cellulose biosynthesis, although this hypothesis is still controversial (Barratt et al. 2009; Baroja-Fernández et al. 2012). The other source of UDP-glucose is through cytosolic invertases (CINVs), which hydrolyse sucrose into glucose and fructose. The glucose is then phosphorylated and converted to UDP-glucose by hexokinases (HXKs) and UDP-glucose pyrophosphorylases (UGPs) (Kleczkowski, Kunz, and Wilczynska 2010). Recently, a link between CINVs and cellulose deposition was hypothesised, as the Arabidopsis *cinv1,2* double mutant showed reduced cellulose content (Barnes and Anderson 2018). Therefore, a link might exist between sucrose availability and its metabolism and cellulose deposition. Surprisingly though, excess in exogenous sucrose enhanced cellulose deficiency in Arabidopsis *shv3svl1*, a double mutant in glycosylphosphatidylinositol (GPI)-anchored proteins (GAPs), involved in cellulose biosynthesis (Yeats et al. 2016). This phenotype was suggested to be linked to a shift in sugar use towards starch accumulation, which is antagonistic to cellulose biosynthesis (Yeats and Somerville 2016; Yeats et al. 2016). However, the mechanisms controlling this switch are still unknown. The relationship between sucrose metabolism and signalling and cellulose biosynthesis is therefore complex and still poorly understood.

1.1.3 Pectin

Pectin is a jelly-like substance present mainly in the primary cell wall and in the middle lamella, a layer in between the primary cell walls of neighbouring cells that facilitates cell-cell adhesion. Pectin is constituted mostly by three polysaccharides: rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II) and homogalacturonan (HG) (Mohnen 2008). These polysaccharides are synthesised in the Golgi apparatus and then secreted to the cell wall, where they can be further modified by different classes of enzymes (Daher and Braybrook 2015; Mouille et al. 2007). RG-I and RG-II are constituted by chains of the repeating disaccharide rhamnose-galacturonic acid. While RG-I harbours some galactan or arabinan side chains linked to the rhamnose residues, RG-II is characterised by more complex side chains (Mohnen 2008). HG is the main pectic polysaccharide, and it is constituted of a linear chain of galacturonic acid (GalA) residues. The synthesis of the HG chain is mediated by galacturonosyltransferases (GAUTs) (Sterling et al. 2006). The HG chain can then undergo further modifications like methylesterification and acetylation before being delivered to the plant cell wall (Sénéchal et al. 2014). HGs are secreted to the cell wall in a highly methylated form and can then be modified by removal of methyl and acetyl groups to control plant cell wall stiffness (Pelloux, Rustérucci, and Mellerowicz 2007). These modifications are regulated by large enzymatic families like pectin methylesterases (PMEs) and pectin acetylesterases (PAEs) (Sénéchal et al. 2014). De-methylesterified HGs can then be degraded by polygalacturonases (PGs) and pectate lyases (PLs), allowing for softening or the cell wall, or cross-linked through binding of calcium ions, forming a stiffer matrix (Yang et al. 2018; Grant et al. 1973). The loosening or stiffening of pectin is an important step in many different plant developmental processes, including response to biotic and abiotic stresses, organogenesis and organ elongation (Sénéchal et al. 2014; Daher and Braybrook 2015). Therefore, pectin stiffness needs to be tightly controlled by the plant. Both PMEs and PGs are post-translationally negatively regulated by PME inhibitors (PMEIs) and PG inhibitors (PGIPs), to restrict their function (Sénéchal et al. 2014). PME and PG activities are necessary to reduce pectin-mediated cell-cell adhesion, and downregulation of both enzyme families can prevent cell separation (Wen, Zhu, and Hawes 1999; Pose et al. 2013; Francis, Lam, and Copenhaver 2006). Due to its role in cell adhesion, pectin modification has a crucial role during response to wounding and plant parasitism. Plant parasites, including

parasitic plants, secrete pectin modifying enzymes at the invasion site to facilitate penetration into their host (Ben-Hod et al. 1993; Losner-Goshen et al. 1998; Veronesi et al. 2007). To counteract the activities of exogenous cell wall modifying hormones, hosts can upregulate PMEIs and PGIPs as a defence response following attack by parasites like bacteria, fungi and nematodes (Kalunke et al. 2015; Lionetti et al. 2017), and similar mechanisms might be employed following parasitic plant attacks. Furthermore, the degradation of pectin releases fragments called oligogalacturonides (OGs) which act as damage associated molecular patterns (DAMPs), possibly triggering defence responses in the host (Hou et al. 2019). The release of pectic components following damage has also been suggested as important for the early stages of adhesion and regeneration during graft healing (Sala et al. 2019; Frey et al. 2022). Therefore, similar mechanisms might regulate cell adhesion modification in processes that require tissue reconnection.

1.2 Plant hormones

Similar as in animals, every aspect of plant development is tightly controlled by the synthesis, signalling and interactions between several classes of hormones. Hormones are small organic signalling molecules that can be transported and act systemically to regulate an organism's development and response to the environment. In plants, most hormones can be synthesised by many different organs, and transported throughout the whole plant via the vasculature (composed of phloem and xylem) or via cell-to-cell diffusion. The first plant hormone (phytohormone) to be discovered was auxin, followed by gibberellic acid, cytokinin, abscisic acid and ethylene, and these compounds are now referred to as the five "classical" plant hormones (Kende and Zeevaart 1997). More recently, other compounds were described to act as hormones, including jasmonic acid, brassinosteroids and different types of peptides (Nambara and Van Wees 2021). As each of these hormones regulates many different developmental pathways, this thesis will focus on just a few of them, which are the most relevant for understanding plant parasitism and grafting in the context presented here.

1.2.1 Auxin

Auxin was the first phytohormone to be described and it is therefore the most well studied. Auxin is known to be involved in almost every stage of plant development from the seed to the mature plant, and its pathways interact at different levels with other hormonal pathways. Auxin is synthesised in the shoot apical meristem (SAM) and cotyledons, and transported basipetally (shoot to root) in the plant. The transport of auxin happens in different ways. The most well-known transporters are the PIN-FORMED transporters (PINs). PIN proteins allow for polar transport of auxin by localising polarly on plant cell membranes (Palme and Gälweiler 1999). Other known transporters are the AUXIN1/LIKE AUXIN1 (AUX/LAX) and ATP Binding Cassette subfamily B (ABCB) transporters (Geisler et al. 2017). Together, these proteins allow for precise auxin accumulation in tissues where auxin peaks are needed for plant growth and development. Once in the target tissues, auxin is perceived through the auxin binding proteins TIR1 and AFB in the SKP1-CUL1-F-box (SCF) ubiquitin ligase complex. When auxin is present, the negative regulators Aux/IAA are degraded, allowing the activation of auxin response factors (ARFs) to modify gene expression in response to auxin (Lavy and Estelle 2016; Leyser 2018).

Amongst its many functions, auxin signalling has been also implicated in cell wall modifications. A role for auxin in cell wall expansion was already proposed in 1971 by Achim Hager and Robert Cleland as the “acid growth hypothesis” (Cleland 1976; Arsuffi and Braybrook 2018). In the acid growth hypothesis, auxin induces the activation of H+ proton pumps, which release H+ into the extracellular matrix decreasing the pH of the apoplast. This acidification then leads to the activation of cell wall modifying enzymes including expansins, allowing for cell elongation (Arsuffi and Braybrook 2018). However, even though auxin induces cell wall acidification in the shoot, in plant roots auxin response leads to alkalinity and arrest of primary root elongation (Fendrych et al. 2018; Fendrych, Leung, and Friml 2016; Barbez et al. 2017; Lin et al. 2021), suggesting auxin might have different roles in regulating the cell wall in shoots versus roots. The pH of the cell wall is a key factor in regulating its structure, as many different cell wall modifying enzyme families have an optimal pH of activity. For example, PMEs, responsible for HG demethylesterification, have higher activity in slightly alkaline conditions, while their inhibitors PMEIs are more active in

slightly acidic environments (Sénéchal et al. 2015). Therefore, auxin might modify pectin structure by modulating pectin modifying enzymes through the modification of the cell wall or apoplastic pH. Furthermore, auxin signalling also induces the transcriptional activation of pectin remodelling genes, leading to cell growth and expansion (Kumpf et al. 2013). In addition to pectin, some studies have also suggested a link between cellulose deposition and auxin transport. Mutations in primary cell wall CESAs or treatment with the primary CESA-inhibitor isoxaben resulted in reduced PIN expression and altered PIN localisation (Feraru et al. 2011; Lehman and Sanguinet 2019). However, the links between auxin signalling and cellulose biosynthesis and modification remain to be explored.

Auxin also has a role in connection to carbon availability. Treating Arabidopsis with glucose induces the synthesis of auxin through increased expression of YUCCA genes, which are responsible for converting the auxin intermediary indole-3-pyruvic acid into the auxin indole-3-butyric acid (IAA) (Zhao et al. 2001; Sairanen et al. 2012). The sucrose-dependent promotion of plant growth is also dependent on auxin, as it can be mimicked by auxin treatment and blocked by chemically inhibiting polar auxin transport (Lilley et al. 2012).

1.2.2 Cytokinin

Cytokinins (CKs) are highly mobile hormones that can be transported both from shoot to root and from root to shoot (Hirose et al. 2008; Matsumoto-Kitano et al. 2008). Cytokinins are synthesised by enzymes including the LONELY GUY family (LOGs) and the ADENOSINE PHOSPHATE-ISOPENTENYLTRANSFERASE (IPT) family, and perceived by histidine kinase (HK) receptors localised at the endoplasmic reticulum and plasma membrane (Kubiasová et al. 2020). The signal is then conveyed through a chain of phosphorylations to the RESPONSE REGULATOR (RRs) proteins. In Arabidopsis, type A RRs act as negative regulators of cytokinin signalling, while type B RRs are positive regulators and required to initiate cytokinin signalling (D'Agostino, Deruère, and Kieber 2000; Argyros et al. 2008; To et al. 2007).

Cytokinins are involved in a multitude of plant developmental processes, including cell division, photosynthesis and nutrient allocation. In

Arabidopsis, the response to high availability of nitrate induces the expression of cytokinin synthesis and response genes, such as *IPT*s and *ARR*s (Takei et al. 2004; Miyawaki, Matsumoto-Kitano, and Kakimoto 2004; Brenner et al. 2005). The transport of the trans-zeatin (tZ) cytokinins, which are the main cytokinins in xylem sap (Hirose et al. 2008), is also increased following nitrogen application (Poitout et al. 2018). In addition to this, cytokinin application represses the expression of nitrate transporters (*NRT*s) and ammonium transporters (*AMTs*) in Arabidopsis (Brenner et al. 2005), suggesting a negative feedback loop mediated by cytokinin exists to control nitrogen uptake. Split-root experiments have also shown how cytokinins in the shoot can signal the nitrogen status to distant roots and regulate root growth and architecture (Poitout et al. 2018), further establishing cytokinin as a crucial hormone for systemic signalling of nitrogen availability. Finally, cytokinin biosynthesis in the roots is also increased following photosynthesis-enhancing treatments like exposure to high CO_2 (Kiba et al. 2019), suggesting cytokinin levels might act in balancing both nitrogen and carbon availability. Therefore, cytokinin might be a key hormone in regulating nutrient balance during plant development. Another major role of cytokinin is cell growth and division, suggesting a role for this hormone in cell wall synthesis and/or modification. However, the role of cytokinins in cell wall modification has not yet been described in depth. Treatment with isoxaben decreases the cytokinin content, possibly through increased transcription of the cytokinin-degrading *CYTOKININ OXIDASEs 2* and *3* (*CKX2/3*) (Gigli-Bisceglia et al. 2018). These findings suggest a link between cellulose and cytokinin biosynthesis. However, more studies will be needed to understand how these two pathways influence each other.

1.2.3 Abscisic acid

Abscisic acid (ABA) is commonly known as a stress hormone, as it is accumulated in response to abiotic stresses such as drought stress and salt stress (Zhu 2002). ABA also has important roles in plant development, such as the control of seed dormancy and germination, the deposition of cuticle and suberin, and xylem differentiation (Brookbank et al. 2021; Nambara et al. 2010) ABA is synthesised in both roots and shoots and transported systemically through the vascular tissues (Kumar et al. 2022). The primary receptors of ABA are PYRABACTIN RESISTANCE1 (PYR1), PYR1-LIKE (PYL) and REGULATORY COMPONENT OF ABA RECEPTORs

(RCAR) (Park et al. 2009; Zhao et al. 2013; Fuchs et al. 2014). When ABA is absent, clade A PROTEIN PHOSPATASE 2Cs (PP2Cs, including ABI1 and ABI2) bind to the SNF1-RELATED KINASE2s (SnRK2s) and phosphorylate them, blocking their activity. If ABA is present, it binds to its PYR1/PYL/RCAR receptors, which bind to PP2Cs and inactivate them (Melcher et al. 2009). SnRK2s are then free to activate downstream ABA signalling pathways (Fujii et al. 2009).

One of the targets of SnRK2s is the nitrate sensor and transporter NRT1.1 (Su et al. 2021). When NRT1.1 is phosphorylated by SnRK2s, nitrate uptake is decreased and root growth inhibited (Su et al. 2021), suggesting a negative crosstalk between ABA signalling and nitrogen uptake. The mechanisms of interaction between nitrogen and ABA signalling however have not been elucidated in depth yet. On the other hand, ABA signalling has been widely associated with carbon availability and sugar signalling. Many of the mutants identified during screens for sugar insensitivity, like the glucose insensitive (*gin*) mutants, are allelic to mutants identified in the ABA signalling pathway (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000; Rook et al. 2001). An increase in ABA signaling leads to the inhibition of TARGET OF RAPAMYCIN (TOR), a key transcription factor that promotes cell proliferation when nutrient conditions are optimal (Belda-Palazón et al. 2020; Belda-Palazón et al. 2022). This process is achieved by interactions between SnRK2s and SnRK1s, the kinases involved in starvation responses like polysaccharide hydrolysis and amino acid catabolism (Belda-Palazón et al. 2020; Belda-Palazón et al. 2022; Margalha, Valerio, and Baena-González 2016). Therefore, ABA has a fundamental role in nutrient homeostasis following plant stress response. As ABA is implicated in many stress responses and as cell wall modifications are crucial during response to stress, the existence of links between ABA signalling and cell wall modifications has been speculated. However, the evidence linking ABA and cell wall is still very limited, and mostly focused on the secondary cell wall. The Arabidopsis ABA biosynthesis mutant *aba2-1* and the signalling triple mutant *snrk2.2/3/6* have decreased expression of secondary cell wall related genes including genes involved in the biosynthesis of lignin, like *PAL1*, *CCoAOMT*, and *4CL1*, and cellulose, like *CESA4*, *7* and *8* (Liu et al. 2021*)*. The same mutants also showed a reduction in both cellulose and lignin content in stems, suggesting endogenous ABA levels and ABA signalling

have a role in secondary cell wall thickening. Furthermore, exogenous ABA treatment also increases the expression of secondary cell wall *CESA4*, *7* and *8* (Ramachandran et al. 2021). The Arabidopsis *irregular xylem 1* (*irx1*) mutant, which harbours a mutation in *CESA8*, shows increased ABA content and higher tolerance to both drought and salt stress (Chen et al. 2005), suggesting a link between secondary cell wall cellulose and ABA signalling. ABA responsive motifs have been reported in the promoter regions of several CESA genes, suggesting they might be downstream targets of ABA signalling (Heidari et al. 2019). However, their direct transcriptional activation by ABA signalling remains to be confirmed.

1.2.4 Brassinosteroids

Brassinosteroids (BRs) are steroidal hormones that regulate several processes in plant growth including cell elongation, cell division and cell wall homeostasis. When BR is absent, BRASSINOSTEROID INSENSITIVE 2 (BIN2) phosphorylates the transcription factors BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1) (Gampala et al. 2007; Ryu et al. 2007), leading to their degradation (Zhu et al. 2017) and preventing the activation of downstream brassinosteroid-related gene transcription (He et al. 2002). In the presence of BR, BR binds the leucine rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1). BRI1-bound BR is then recognized by the co-receptor BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) (Sun et al. 2013), triggering a signalling cascade that leads to the de-activation of the negative regulator BIN2 and allowing BZR1 and BES1 to initiate BR transcriptional responses.

BR signalling's role in controlling cell wall deposition has been known for over a decade. In Arabidopsis, BR signalling regulates the transcription of CESA genes involved in both primary and secondary cell wall cellulose biosynthesis, with CESA gene expression increasing following exogenous BR treatments (Xie, Yang, and Wang 2011), and both BR synthesis and BR signalling mutants exhibiting reduced cellulose content (Xie, Yang, and Wang 2011). Furthermore, treatments with the BIN2-specific chemical inhibitor bikinin increased cellulose accumulation (Sánchez-Rodríguez et al. 2017). These data suggest that BR signalling stimulates the synthesis and accumulation of cellulose as part of its role in cell elongation. In addition to

its function in cellulose deposition, BR signalling has also been suggested as part of a feedback loop controlling pectin loosening (Wolf et al. 2012). When pectin de-methylesterification is inhibited, the RECEPTOR-LIKE PROTEIN 44 (RLP44) triggers BR signalling, possibly by interacting with the BR receptor BAK1 (Wolf et al. 2014). Therefore, BR signalling is important to maintain cell wall homeostasis.

1.3 Plant parasitism

1.3.1 Classification and relevance

Parasitic plants are plants that rely totally or partially on the withdrawal of nutrients from another plant, defined as their host. Plant parasitism has evolved independently at least 12 times (Nickrent 2020), creating a very diverse variety of lifestyles, geographic distribution, and host compatibility. Parasitic plants that completely depend on their host for water and nutrients are called obligate parasites. These plants often lack photosynthetic abilities and require the presence of compounds from compatible hosts to induce seed germination (Heide-Jørgensen 2008; Spallek, Mutuku, and Shirasu 2013). Obligate parasitic plants include important agricultural pests like *Striga* and *Cuscuta*, which can destroy major portions of the crops they parasitize (Parker 2009; Spallek, Mutuku, and Shirasu 2013). Parasitic plants that can complete their life cycle even in the absence of a host are defined as facultative parasites. This group includes well-studied parasitic plant models like *Triphysaria versicolor* and *Phtheirospermum japonicum*.

Parasitic plants are a relevant agricultural and economic problem. Control strategies such as applying germination stimulants in the absence of a host or increasing field fertilisation have been developed (Mwakaboko and Zwanenburg 2011; Zwanenburg and Mwakaboko 2011; Mwangangi et al. 2021; Yoneyama, Xie, et al. 2007; Yoneyama, Yoneyama, et al. 2007). However, their implementation is still limited due to high production costs, impracticality and environmental impacts. Therefore, despite the advances made during the recent years in studying parasitic plants, it is still crucial to investigate the development of parasitic plants and their infection process in order to develop new and more efficient control methods. Although not a threat to agricultural production, *P. japonicum* has become a model species

for studies in parasitic plants thanks to its sequenced genome (Cui et al. 2020), the availability of a hairy-root transformation protocol (Ishida et al. 2011) and its broad range of hosts including the well-characterised *Arabidopsis thaliana*, rice and tomato (Ishida et al. 2016).

1.3.2 The haustorium: mechanisms of parasitism

Parasitism by parasitic plants relies on the development of a multicellular organ, called the haustorium, used for nutrient and water withdrawal from the host. Although all parasitic plants develop haustoria to infect, the exact mechanisms differ between different species due to their different lifestyles. As this thesis will focus mostly on *Phtheirospermum japonicum*, the following paragraphs will focus on haustorium development in this species (Figure 2).

Haustoria require the perception of a host to initiate formation. Root parasites like *P. japonicum* perceive compatible hosts via root exudates called haustoria inducing factors (HIFs). These compounds include phenolics, quinones, and cell wall components (Cui et al. 2018). An example is 2,6-dimethoxy-p-benzoquinone (DMBQ), which was isolated from infected *Sorghum* roots and was the first HIF to be identified and described (Chang et al. 1986). *P. japonicum* perceives HIFs through the CANNOT RESPOND TO DMBQ (CARD) leucine-rich-repeat receptor-like kinases (Laohavisit et al. 2020). The perception of HIFs is followed by an increase of calcium signalling in the cytosol and the accumulation of reactive oxygen species (ROS) at the site of haustorium initiation (Bandaranayake et al. 2010; Laohavisit et al. 2020). Perception of HIFs is followed by the expansion of cortical cells at 4-8 hours post infection (hpi), which causes the initial swelling of the pre-haustorium (Baird and Riopel 1984). At 12 hpi the epidermis, cortex, endodermis and pericycle root layers initiate cell division towards the host (Wakatake, Yoshida, and Shirasu 2018). At 18 hpi, the expression of the auxin biosynthesis gene *PjYUC3* is increased in epidermal cells, and auxin accumulates at the pre-haustorium site (Ishida et al. 2016). Inhibiting the expression of *PjYUC3* or chemically inhibiting auxin biosynthesis inhibited the formation of haustoria, suggesting auxin is necessary for haustoria development (Ishida et al. 2016). In addition to auxin, other hormones like cytokinin and ethylene increase their signalling during haustoria formation starting from early time points, suggesting a role for them during haustorium development (Cui et al. 2020; Spallek et al. 2017).

After the pre-haustorium reaches the host, it attaches to the host tissues. This process is helped by adhesive secretions from the haustorial hair, which develop from epidermal cells at the apex of the haustorium (Cui et al. 2016). Following attachment, the parasitic plant secretes cell wall modifying enzymes at the haustorium-host interface to initiate the invasion of host tissues. These enzymes include pectin modifying enzymes in *Orobanche* (Ben-Hod et al. 1993; Losner-Goshen et al. 1998), β-expansins in *Triphysaria versicolor* (Honaas et al. 2013), the hemicellulose-modifying xyloglucan endotransglucosylases in *Cuscuta* (Olsen and Krause 2017), and cellulose-degrading β-1,4-glucanases in *P. japonicum* (Kurotani et al. 2020). The invasion process is also facilitated by intrusive cells, enlarged epidermal cells at the haustorium apex that apply mechanical pressure to penetrate between the host cell walls (Heide-Jørgensen and Kuijt 1995; Ogawa et al. 2021). Finally, once the haustorium has penetrated the host endodermis, the differentiation of the vascular connection starts. In *P. japonicum*, the differentiation of the xylem connection is initiated at about 48-72 hpi with large numbers of tracheary elements forming from both the base and the apex of the haustorium (Wakatake et al. 2020). These xylem masses are defined as “plate xylem”. Once the plate xylem has formed, strands of xylem called “xylem bridges” differentiate from the haustoria inner cortical cells to connect the plate xylems. This process of vascular differentiation requires auxin transport to the central haustorial tissues, as chemically blocking auxin transport both from the shoot or locally results in partial or complete inhibition of xylem bridge development (Wakatake et al. 2020; Serivichyaswat et al. 2022).

Once fully developed, the haustoria allow the parasite to withdraw water and nutrients from the host. In addition to this, proteins, mRNAs, small RNAs and hormones are also transported from and to the parasite (Kim et al. 2014; Liu et al. 2020; Shahid et al. 2018). For example, *P. japonicum* transports cytokinins to the host *A. thaliana* through mature haustoria to induce host hypertrophy above the haustorium site, possibly to increase the parasite’s sink strength and improve the withdrawal of nutrients from the host (Spallek et al. 2017; Greifenhagen et al. 2021).

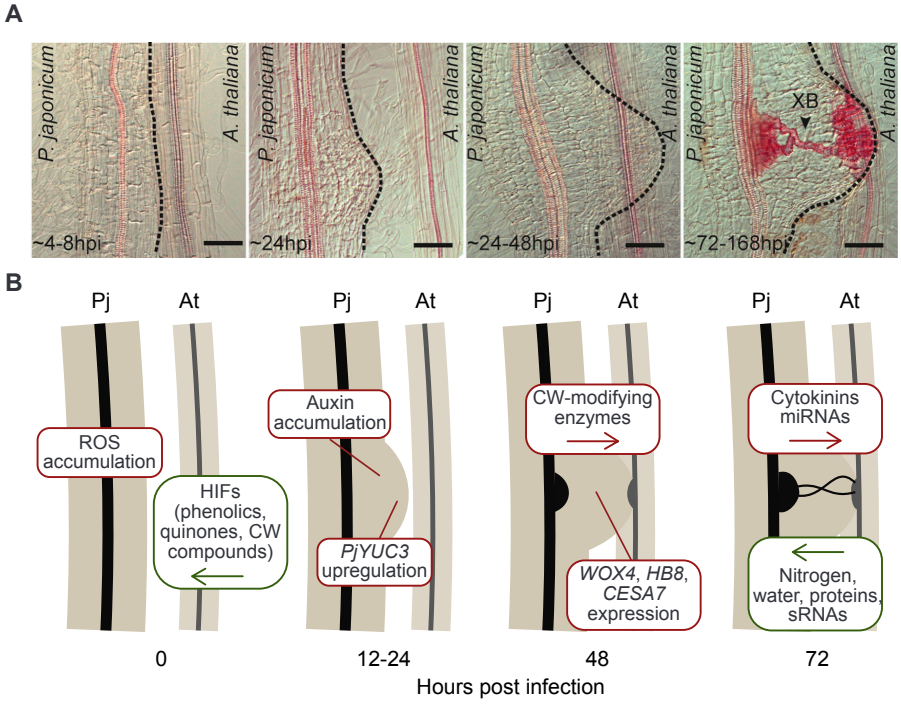

Figure 2: Development of a *P. japonicum* haustorium.

A) Close-ups of a *P. japonicum* haustorium infecting *A. thaliana* from 4 to 168 hours post infection (hpi). XB: xylem bridge. B) Schematics of the main processes involved in haustorium development at different time points. Adapted from Kokla and Melnyk 2018 and Leso et al. 2022.

1.4 Plant grafting

1.4.1 Relevance for agriculture and as a model system

Grafting is the practice of cutting and joining two different plants to create a chimeric individual with the lower part (rootstock) having a different genetic composition than the upper part (scion). Grafting has been used by humans for millennia and has several applications. For example, grafting can be used to vegetatively propagate plants that cannot be clonally propagated, to induce dwarfing in scions, to increase plant vigour or to increase resistance towards both biotic and abiotic stresses without compromising yield and other favourable produce qualities (Mudge et al. 2009). Despite its wide commercial application in many tree and crop species, the effectiveness of

grafting is limited by the relatively narrow range of compatibility between the two plants to be grafted. While intra-species grafting is usually successful, inter-species grafting can be incompatible even between very evolutionarily close species (Melnyk 2017c). Despite decades of research on the subject, the mechanisms of (in)compatibility are still very poorly understood.

In addition to its important role for agriculture, grafting has also become a popular research tool in plant science. In recent years many protocols have been optimized to graft *Arabidopsis thaliana* seedlings (micro-grafting) (Marsch-Martínez et al. 2013; Melnyk 2017b; Bartusch, Trenner, and Quint 2019; Turnbull, Booker, and Leyser 2002) allowing researchers to use the available Arabidopsis resources to study grafting mechanisms. Many studies on long distance signalling in plants have used grafting as a tool, and micrografting has proven useful to confirm the mobility of several classes of phytohormones, such as cytokinin and gibberellin (Matsumoto-Kitano et al. 2008; Ragni et al. 2011; Camut et al. 2019), as well as proteins and RNAs (Molnar et al. 2010; Yoo et al. 2013). Furthermore, since grafting requires tissue adhesion and regeneration at the junction, it can be also used as a model system to study the processes of plant tissue attachment, differentiation and regeneration.

1.4.2 Mechanisms of graft healing

Although the knowledge on the mechanisms of graft formation is still limited, mainly due to the complexity of the process, several advances were made in recent years thanks to Arabidopsis micrografting. The process of graft healing can be simplified to four main stages: tissue attachment, callus proliferation, phloem reconnection and xylem reconnection (Figure 3).
The attachment between scion and rootstock is mediated by oligosaccharides and cell wall components that are released by the wounded tissues at the graft junction (Melnyk 2017a). In particular, pectins are synthesised and deposited at the graft junction of Arabidopsis and tomato grafts, with low methylesterified homogalacturonans being prevalent at the junction (Sala et al. 2019; Frey et al. 2022; Frey et al. 2023). Xyloglucan, a hemicellulose component, is also increased at the graft junction (Frey et al. 2023). These substances are thought to act as a glue between grafted top and bottom, and likely also act as damage associated molecular patterns (DAMPs) to activate

wound response in these tissues (Hou et al. 2019). For example, the modification of cellulose and pectin during grafting induces the expression of DOF transcription factors (Zhang et al. 2022), which are key in activating regeneration processes (Ramirez-Parra et al. 2017). In addition to the release of cell wall components, cell wall modifying enzymes (CWMEs) are also involved in graft adhesion. Two hemicellulose modifying enzymes, *XTH19* and *XTH20*, increase their expression at the graft junction following realignment of the tissues and facilitate adhesion (Pitaksaringkarn et al. 2014). The cellulases β-1,4-glucanases are also secreted at the graft junction to facilitate tissue reconnection, and their transgenic overexpression in Arabidopsis increases tissue attachment rates (Notaguchi et al. 2020). Following initial attachment, a mass of undifferentiated tissue forms at the junction to fill the space between the scion and the rootstock. This tissue is referred to as callus, and has been speculated to be developmentally similar to wound-induced callus (Melnyk et al. 2015). The callus tissue is thought to differentiate from cortical cells and vascular tissues, where the AP2/ERF transcription factor *WOUND INDUCED DEDIFFERENTIATION1* (*WIND1*) is expressed following wounding (Iwase, Ohme-Takagi, and Sugimoto 2011). In petiole grafting, WIND1 activates WUSCHEL-RELATED HOMEOBOX 13 (WOX13), a promoter of callus cells differentiation and expansion (Ikeuchi et al. 2022). *wox13* mutants are defective in graft reconnection and WOX13 activates numerous cell wall related genes including cellulases, pectinases and hemicellulases (Ikeuchi et al. 2022), suggesting a role for these enzymes in graft formation.

The final step for successful grafting is the *de novo* development of functional vasculature between the scion and the rootstock. The phloem, transporting substances from the scion to the rootstock, is the first to reconnect in Arabidopsis hypocotyl grafting (3-4 days after grafting), while the xylem reconnects later (6-7 days after grafting) (Melnyk et al. 2015). The formation of the phloem connection relies on auxin signalling in the cambium and xylem pole pericycle at the graft junction, and cambial mutants show defects in phloem reconnection (Serivichyaswat et al. 2024). Finally, xylem reconnection is promoted by WIND1, which induces important xylem-related genes, including *VND6*, *VND7*, *LBD18* and *LBD30* (Iwase et al. 2021). In addition to studying the mechanisms of graft healing during standard conditions, the research focus in the field is slowly shifting to understanding the effects of different environmental conditions on grafting

efficiency. High environmental temperatures increase the rate of graft healing by increasing auxin production in the scion (Serivichyaswat et al. 2022). Nutrient availability is also a determining factor for graft efficiency. The presence of high levels of sucrose in the grafting medium has been reported to inhibit Arabidopsis hypocotyl grafting (Melnyk et al. 2018), while sugars have been suggested as beneficial for cucumber/pumpkin heterografts (Miao et al. 2021). The mechanisms through which exogenous nutrients affect graft healing remains to be investigated.

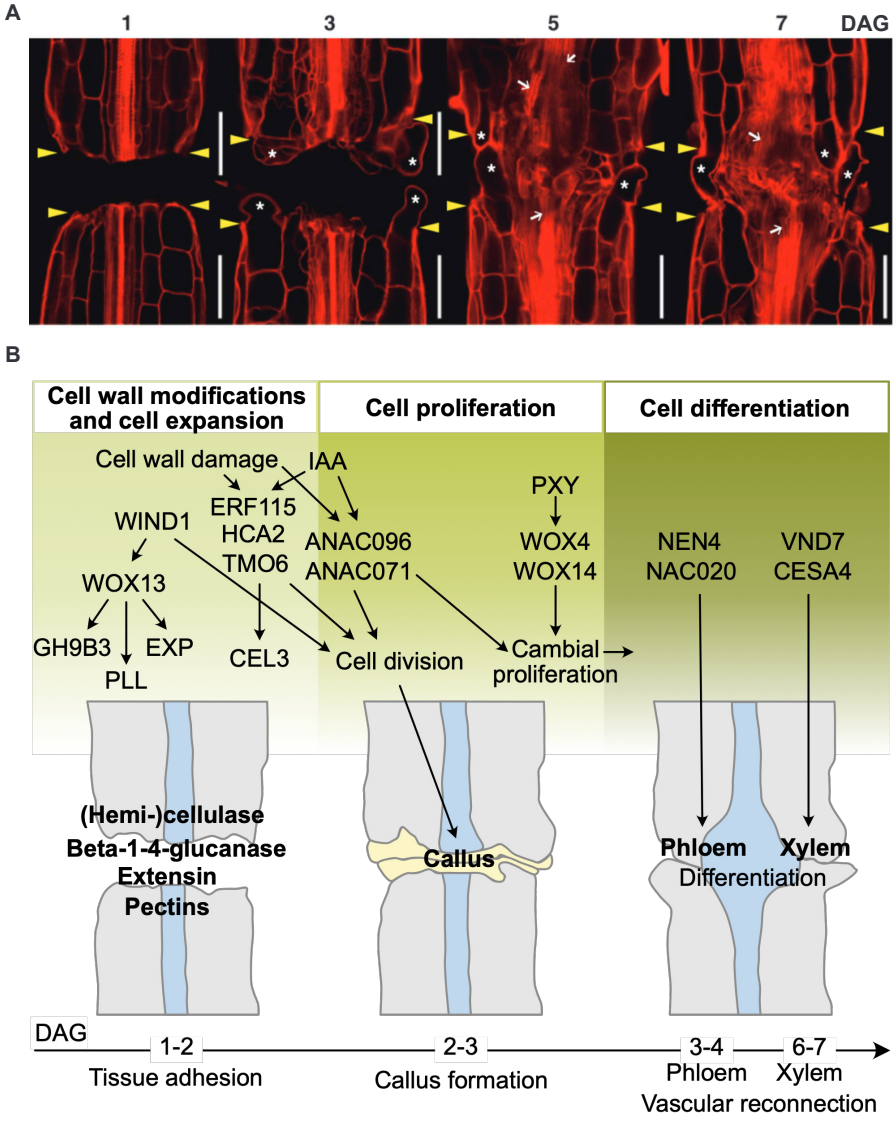

Figure 3: Healing of an Arabidopsis graft junction.

A) Close-ups of a propidium iodide-stained hypocotyl graft junction at different days after grafting (DAG). Arrowheads: position of the cut; asterisks: cortical cell expansion; white arrows: xylem strands. Adapted from (Matsuoka et al. 2016) B) Schematics of the main molecular pathways involved in graft reconnection. Reproduced from Feng et al. 2024.

1.5 Parallels between plant parasitism and plant grafting

Although plant parasitism and grafting might at a first glance look like very different processes, a lot of parallels exist in the mechanisms required to develop a haustorium or reconnect a graft junction. Trees can naturally graft in a process known as "inosculation" when stem or roots of neighbouring plants (often from the same species) come into contact and fuse together (Mudge et al. 2009). Inosculation has been suggested to improve trees ability to respond to environmental factors such as nutrient availability or mechanical stimuli by allowing trees to respond as a community instead of an individual plant (Quer et al. 2022). The molecular mechanisms allowing plants to graft naturally are likely similar to the mechanisms regulating human-induced graft reconnection, and the same pathways might have been adapted during evolution to allow plant-plant fusion during parasitism. Both plant parasitism and grafting require the recognition of a compatible host/grafting partner. However, the range of compatibility between the two processes is very different. Many parasites, like *Striga* and *P. japonicum* can infect a wide range of hosts including monocots and eudicots (Musselman 1980; Cui et al. 2016). Grafting on the other hand, is most often successful when two plants from the same species are grafted, and only sometimes successful between members of the same family, while grafting distantly related species almost always results in graft failure (with some notable exceptions, such as conifer or solanaceae grafting) (Melnyk 2017c; Feng, Zhang, et al. 2024). How compatibility is determined in either plant parasitism or grafting is still unknown, though it has been suggested that cell wall composition, as well as host defences, might have a role in host compatibility in plant parasitism (Cui et al. 2018; Johnsen et al. 2015). Once a compatible host/partner has been identified, both the haustorium and the graft junction require tissue adhesion to initiate the connection. In both processes, tissue adhesion seems to be mediated by the release of cell wall modifying enzymes like pectinases, cellulases, hemicellulases and expansins at the junction (Melnyk et al. 2015; Sala et al. 2019; Notaguchi et al. 2020; Pitaksaringkarn et al. 2014; Kokla and Melnyk 2018; Kurotani et al. 2020; Olsen et al. 2016), and genes related to cell wall modification are highly expressed already at early stages in both processes (Melnyk et al. 2015; Honaas et al. 2013; Ogawa et al. 2021). Following attachment, vasculature has to develop between parasite and host or between scion and rootstock. At

the graft junction both phloem and xylem *de novo* development is required, while only some parasitic plants develop both phloem and xylem connections to the host and other species like *Striga* and *P. japonicum* only develop xylem connections (Musselman 1980; Haupt et al. 2001). It is likely that vascular development follows similar pathways in both processes. For example, auxin channelling at the sites of xylem development is required in both processes, and the auxin transporter *PIN1* is activated in both processes (Wakatake et al. 2020; Melnyk et al. 2015; Kurotani et al. 2020). Furthermore, genes important for cambium development like *WUSCHEL-RELATED HOMEOBOX 4* (*WOX4*) and *HOMEOBOX PROTEIN 8* (*HB8*) or xylem development, like *CESA7* and *VND7*, are expressed in both processes at the time of vascular differentiation (Wakatake, Yoshida, and Shirasu 2018; Melnyk et al. 2018; Kurotani et al. 2020), suggesting the involvement of similar pathways. Studying the mechanisms regulating plant parasitism and grafting and applying plant parasitism knowledge on grafting and vice versa could offer a useful tool to better understand and overcome graft incompatibility in commercial species, and to find novel ways to control parasitic plants in agriculture.

2. Aims of the study

The aims of this thesis can be summarized as:

1. To investigate the effect of nutrient availability and resource balancing on plant parasitism and grafting (Papers I, II and IV)
2. To study the role of hormonal signalling on plant parasitism and grafting (Papers I, II & IV)
3. To investigate the role of cell wall modifications during plant parasitism and grafting (Papers III & IV)

3. Results and discussion

3.1 Nutrient availability effects on plant parasitism and grafting

Haustorium development and graft junction reconnection require many different steps to be successfully completed, ranging from attachment to *de novo* development of a vascular connection (Kokla and Melnyk 2018; Feng, Augstein, et al. 2024). Therefore, both plant parasitism and grafting are costly for the plant to complete in terms of resource usage such as carbon and nitrogen. The presence of elevated nitrogen in the environment is known to inhibit plant parasitism, and fertilizer application has been used as a control strategy in the field (Igbinnosa, Cardwell, and Okonkwo 1996). This phenotype has been linked to increased fitness of the host, increasing host defences against parasitic plants, and to lower exudation of haustoria inducing factors by the host in the presence of nutrients (Yoneyama, Xie, et al. 2007; Yoneyama, Yoneyama, et al. 2007; Mwangangi et al. 2021). However, no in-depth research has yet been done on the parasitic plant to understand if nitrogen availability might have an effect on its choice to parasitize.

In paper I we investigated how the availability of nutrients in the environment affects haustorium development in *Phtheirospermum japonicum*. *P. japonicum* normally requires nutrient-deprived conditions to infect (Spallek et al. 2017). We found that when *P. japonicum* was growing in high nitrogen conditions, either in soil or *in vitro*, the numbers of haustoria and xylem connections to the host were significantly reduced (Paper I, fig 1C-G), while other nutrients such as phosphorus and potassium did not have the same effect (Paper I, fig. 1D-E). Nitrogen also inhibited the induction of pre-haustoria by the haustorium inducing factor DMBQ (Paper I, fig. 1H), suggesting that this effect is dependent on the parasite and not only on the host as previously suggested. The application of nitrogen inhibited the expression of many genes that are normally upregulated during the early stages of haustorium development (Paper I, fig. 3A-B), and increased the expression of genes associated with increased ABA response (Paper I, fig. 5C). We also found that ABA levels were increased in *P. japonicum* plants

treated with nitrogen compared to untreated plants (Paper I, fig. 5A). In Arabidopsis, the ABA signalling pathway interacts with the nitrogen uptake pathway in a negative feedback loop to help regulate nitrogen uptake by the roots (Su et al. 2021). We therefore hypothesised that ABA might act to regulate haustoria numbers, and therefore nitrogen uptake from the host, in *P. japonicum* based on the availability of nitrogen in the environment, switching the behaviour of *P. japonicum* from parasitism to self-sufficient. Applying ABA in the medium reduced haustoria numbers, confirming ABA signalling is a negative regulator of haustoria formation (Paper I, fig. 6A). Furthermore, *P. japonicum* hairy roots transformed with the *Atabi1-1* construct, which blocks ABA signalling (Wu et al. 2003), did not show the nitrogen-induced inhibition of haustoria (Paper I, fig. 6D). Chemical treatment with fluridone, an ABA signalling inhibitor, could also partially rescue the nitrate phenotype (Paper I, fig. 6A). Taken together, our results confirm that ABA acts downstream of nitrogen and is necessary for inhibiting haustoria formation in response to high nitrogen availability.

We also confirmed that nitrogen inhibition of haustoria formation is not only specific to the facultative parasite *P. japonicum*. The obligate parasite *Striga hermonthica* also showed reduced parasitism efficiency under high nitrogen conditions (Paper I, fig. 7A-D), although ABA was not an haustorium inhibiting factor in *Striga* (Paper I, fig. 7E). We also found that nitrogen application inhibited haustoria formation in distant roots in *P. japonicum* (Paper II, fig. 4C), suggesting the existence of a long-distance signalling balancing resource availability and haustoria numbers. We found that existing infections systemically inhibited the formation of new haustoria on distant roots in *P. japonicum* (Paper II, fig. 4B). This phenotype was accompanied by an increase in cytokinin levels and signalling in the parasite's infecting roots and in the shoot (Paper II, fig. 1D; fig. 3; fig. 5A), suggesting that cytokinin, together with ABA, is a key hormone in controlling the balance between nutrients uptake and haustoria development.

Plant parasitism and grafting are conceptually similar processes (Melnyk 2017a). We found that exogenous nitrogen application was also inhibitory to graft reconnection already at the graft attachment stage (Paper IV, fig. 1B), suggesting that similar mechanisms might regulate pre-haustorium induction and graft attachment in the presence of nitrate. Previous work has shown that

phloem reconnection during *A. thaliana* hypocotyl grafting is impaired when sucrose is applied to the grafted plants (Melnyk et al. 2018). In paper IV we confirmed the previously published data, and showed that similarly to nitrate, exogenous sucrose application also acts as an inhibitor for grafting already at the attachment stage (Paper IV, fig. 1B-C). This phenotype was also observed with fructose and glucose, but not with the osmotic controls sorbitol and mannitol (Paper IV, fig. 2B). Applying sucrose to seedlings during grafting also increased both starch accumulation and callus formation at the scion and rootstock (Paper IV, fig. 2C-E). In standard grafting conditions (Melnyk et al. 2015), only a small amount of callus is formed at the graft junction, specifically at the scion, and primary root growth is arrested while the graft junction is healing. Sucrose treatment induces more callus formation at the graft junction (Paper IV, fig. 2C) and drives primary root elongation and lateral root patterning in Arabidopsis (Kircher and Schopfer 2023). It is possible that the sucrose-induced inhibition of graft reconnection is due to the activation of molecular pathways related to high nutrient availability and plant growth instead of the plant regeneration pathways required for healing. We found that applying the stress hormone ABA together with sucrose could partially rescue the sucrose effect on grafting (Paper IV, fig. 3B-C), although ABA alone was also inhibitory to graft healing (Paper IV, fig. B-C). An increase in ABA signalling is associated with starvation responses and to the inhibition of the plant growth and development pathways in favour of pathways to release carbon such as cell wall and polysaccharide hydrolysis (Margalha, Valerio, and Baena-González 2016). During grafting, as the phloem is severed by cutting, sugars accumulate at the scion, while the rootstock is starved until the phloem is reconnected again (Melnyk et al. 2018). This asymmetry in carbon availability at the graft junction leads to the accumulation of starch at the grafted top, and might induce pathways associated with high carbon availability such as cell wall biosynthesis (Verbančič et al. 2018). Furthermore, the accumulation of both auxin and sugars at the grafted top likely induces the proliferation of the callus tissue needed to reconnect the scion with the rootstock and the differentiation of phloem strands to reconnect vasculature at the graft junction (Melnyk 2017c). As ABA is inhibitory to callus proliferation (Ikeuchi et al. 2017), it is possible that exogenous ABA application reduces graft efficiency by blocking this process in the scion. Meanwhile, the grafted bottom shows upregulation of genes

related to sugar starvation until the phloem is reconnected (Melnyk et al. 2018). Carbon starvation induces processes such as cell wall catabolism (Lee et al. 2007), and the release of cell wall components at the graft junction is important for graft attachment. Exogenous sucrose application might therefore disrupt the attachment by inhibiting the cell wall catabolism triggered by starvation, and this response might be restored when sucrose is combined with ABA, which normally increases during starvation (Belda-Palazón et al. 2020; Belda-Palazón et al. 2022). Therefore, sucrose signalling might have both a positive role for graft reconnection in the scion, where endogenous sucrose levels are high, and a negative role in the rootstock, where endogenous sucrose levels are usually low. The tissue specificity of sugar signalling requirement might also explain why sugars have been reported as both inhibitory (paper IV, (Melnyk et al. 2018) and promoting (Miao et al. 2021) factors for grafting.

Interestingly, in our setup exogenous nutrients or ABA application are inhibitory to both plant parasitism and grafting (Paper I, fig. 6A; Paper IV, fig. B-C). However, the interaction between nutrients and ABA signalling might differ between the two processes, as nitrogen acts through ABA in controlling haustoria numbers, while sugar and ABA signalling are antagonistic during graft reconnection.

3.2 Hormonal regulation in plant parasitism and grafting

The development of haustoria and graft junctions requires *de novo* specification of different tissues, including the vasculature. Hormonal signalling and crosstalk between different hormones are therefore required for these processes to be successful. Auxin is the best studied plant hormone, and its role is also the most described in both plant parasitism and grafting. Auxin is synthesised and transported at the site of haustorium initiation and at the graft junction, and it is then channelled to the tissues that will develop into the vasculature (Serivichyaswat et al. 2024; Melnyk et al. 2015; Wakatake et al. 2020).

Many hormones increase their levels following haustorium development. ABA levels increase in *Rhinanthus minor* and *Cuscuta japonica* (Furuhashi et al. 2014; Jiang, Jeschke, and Hartung 2004) and we found that ABA levels are also increased in *P. japonicum* in the presence of high levels of nitrogen in the environment (Paper I, fig. 5A-B), similar to *A. thaliana* roots (Ondzighi-Assoume, Chakraborty, and Harris 2016). Even though ABA application was inhibitory to haustoria formation (Paper I, fig. 6A), we found that inhibiting ABA signalling through fluridone treatment reduced xylem bridge formation in haustoria (Paper I, fig. 6B). These results suggest that in addition to inhibiting haustoria formation when enough nitrogen is available in the environment, ABA signalling might have a role in the differentiation of haustoria xylem bridges. Since ABA promotes xylem differentiation in Arabidopsis roots (Ramachandran et al. 2018), the role of ABA signalling in vascular development might be conserved in *P. japonicum* haustoria development. Grafts treated with ABA showed reduced efficiency both at the attachment and phloem reconnection stages (Paper IV, fig. 3B-C). In *Medicago truncatula*, ABA signalling controls seed germination and radicle emergence by inhibiting enzymes involved in cell wall biosynthesis and modification (Gimeno-Gilles et al. 2009). As both the graft junction and haustoria require initial cell wall loosening and tissue expansion to bridge the gap between plants (Melnyk et al. 2015; Kokla and Melnyk 2018), it is possible that ABA treatment inhibits both processes through a similar mechanism leading to the inhibition of cell wall loosening. More research will however be needed to confirm this hypothesis.

Cytokinin levels also increase in *P. japonicum* following infection (Spallek et al. 2017, Paper I fig. 5A; Paper II fig. 1D; fig. 5A) and genes induced by cytokinin treatment are highly expressed in the early stages of infection (Paper II, fig. 2E). We found that in *P. japonicum* cytokinin treatment inhibited haustoria formation locally and systemically (Paper II, fig. 2A-C, 5B) and repressed the expression of genes that are normally expressed during haustoria development (Paper II, fig. 2D). *P. japonicum* roots treated with the cytokinin inhibiting chemical PI-55 or overexpressing the cytokinin degrading *AtCKX3* formed more haustoria than non-transgenic roots (Paper II, fig. 2A,F). Therefore, our data suggest that cytokinin acts as a local haustorium-inhibiting factor in *P. japonicum*. Furthermore, we found that cytokinin signalling has a role in the long-distance signalling controlling the

total numbers of haustoria. Cytokinin is increased in *P. japonicum* shoots following infection (Paper II, fig. 5; fig. S5A), and inhibiting cytokinin signalling in infecting roots through *AtCKX3* overexpression or chemically through PI-55 treatment allowed the formation of haustoria in distant roots (Paper II, fig. 5C-D). Therefore, our results point at cytokinin being a crucial haustorium inhibiting factor and a component of the mechanism regulating haustoria numbers both locally and systemically in *P. japonicum*. However, the role of cytokinin in plant parasitism is complex and cytokinins can also act as haustoria inducing factors in some species like the obligate root parasites *Phelipanche ramosa* and *Striga hermonthica*, but not in *P. japonicum* (Goyet et al. 2017; Aoki, Cui, and Yoshida 2022). Therefore, the role of cytokinin signalling is not conserved in all parasitic plant species. Obligate parasites develop terminal haustoria by differentiating their primary root tips, while facultative parasites develop lateral haustoria. As root tip differentiation is promoted by cytokinins (Dello Ioio et al. 2007), while lateral root initiation is inhibited by cytokinins (Laplaze et al. 2007), the presence of conserved mechanisms between haustoria formation and root development might explain the different roles for cytokinin in parasitic plants. Cytokinin is also transferred from *P. japonicum* to the host, where it induces hypertrophy (Spallek et al. 2017). This phenotype is dependent on cytokinins synthesised by the parasite, as hairy roots mutated in the *PjIPT1a* gene did not induce cytokinin signalling and hypertrophy in host roots, and haustoria on mutated roots did not show xylem bridges (Greifenhagen et al. 2021). A balance and interaction between cytokinin and auxin signalling is required for vascular differentiation (De Rybel et al. 2014). Therefore, it is possible that endogenous cytokinins in parasite and host, together with auxin signalling, might play a role in the differentiation of the xylem bridge. Cytokinin response is also activated at the graft junction, together with auxin response (Melnyk et al. 2015). Mutants in the cytokinin pathway do not show a grafting phenotype (Melnyk et al. 2015), but cytokinin signalling has been implicated in callus formation at the graft junction (Ikeuchi et al. 2017). Therefore, it is possible that cytokinin signalling has a positive role on grafting, but more studies will be required to confirm this hypothesis.

Finally, brassinosteroid signalling is also required for efficient graft healing, as Arabidopsis mutants in the BR pathway showed defects in both phloem and xylem reconnection (Mazumdar et al. 2023). We found that treating *P.*

japonicum with the brassinosteroid epibrassinolide reduced the total number of haustoria formed, and affected the expression of both PMEs and PMEIs (Paper III, fig. 5A, 5C). However, brassinosteroid treatment slightly inhibited the development of haustorial xylem bridges (Paper III, fig. 5B), suggesting brassinosteroid signalling might have a different role in vascular development in grafting and plant parasitism. As phloem and xylem development rely on previous attachment of the scion to the rootstock, another explanation could be that phloem and xylem reconnection is inhibited in brassinosteroid mutants due to defects in the attachment stage, therefore delaying vascular connection. Brassinosteroids have been associated with pectin modifications and maintenance of cell wall homeostasis in Arabidopsis by previous publications (Wolf et al. 2012; Wolf et al. 2014). Our data show that brassinosteroid treatment also affects pectin methylesterification levels in *P. japonicum* haustoria (Paper III, fig. 5D-E). It is possible that brassinosteroid signalling regulates pectin modification and cell wall loosening in both grafting and plant parasitism, therefore affecting both plant-plant attachment and vascular connection.

3.3 Cell wall modifications in plant parasitism and grafting

The processes of haustorium development and graft healing require modification of the cell wall from the early stages of tissue expansion and attachment, to the development of vascular connections. The modification of hemicellulose by xyloglucan endotransglucosylase/hydrolase (XTHs) has been reported as important in both parasitism by *Cuscuta* (Olsen and Krause 2017; Olsen et al. 2016) and plant regeneration and grafting (Pitaksaringkarn et al. 2014), suggesting similar molecular pathways might regulate cell wall changes during the two processes. We found that many genes belonging to cell wall metabolism pathways were upregulated during parasitism, especially at 72 hours post infection, when the xylem bridge is developing (Paper I, fig. S3). Among these genes, we also found the pectin methylesterase inhibitor *PjPMEI9* (Paper I, fig. 2C). These cell wall modifying genes were also downregulated when *P. japonicum* was treated with nitrogen, suggesting their importance during haustorium development

(Paper I, fig. 3B; fig. S4A). The modification of pectin has been suggested as important for host penetration in the parasitic plants *Orobanche* and *Cuscuta* (Losner-Goshen et al. 1998; Johnsen et al. 2015; Veronesi et al. 2007). We found that many *PjPME*s and *PjPMEI*s were expressed in *P. japonicum* haustorial tissues and in intrusive cells (Paper III, fig. 1). We found that pectin methylesterification levels were dynamic during haustorium development in *P. japonicum*, and that the changes were tissue specific (Paper III, fig. 2). Highly methylesterified pectin, associated with more rigid tissues (Sénéchal et al. 2014), was mostly found at the site of xylem development, while low methylesterified pectins were mostly localised at the host/parasite interface (Paper III, fig. 2C-E). Inhibiting PME activity using the global PME inhibitor EGCG (Lewis et al. 2008) delayed haustoria induction and xylem bridge formation (Paper III, fig. 4A-B). Furthermore, hairy roots overexpressing *PjPMEI* constructs showed reduced numbers of haustoria (Paper III, fig. 4E), suggesting PME activity is important already at the stage of haustorium emergence. Inhibiting xylem bridge formation by using the auxin transport inhibitor NPA or the XTH inhibitor Coomassie Brilliant Blue (Paper III, fig. 6B-D) inhibited the expression of *PjPME51* and *PjPMEI9* (Paper III, fig. 6G), suggesting that some PMEs and PMEIs might also be involved in xylem differentiation. The modification of pectins at the host-parasite interface might also be required for efficient attachment of *P. japonicum* to the host, as previously suggested for other parasitic plant species (Losner-Goshen et al. 1998; Johnsen et al. 2015; Veronesi et al. 2007). In a similar manner, a reduction in pectin methylesterification seems to be important for attachment between scion and rootstock during grafting (Sala et al. 2019).

In addition to pectin, similarities between cellulose modifications in plant parasitism and grafting have also been previously identified. The cellulase gene *GH9B3* was found to be important for tissue attachment during grafting, and improved graft compatibility between different species (Notaguchi et al. 2020). In *P. japonicum* a reduced expression of *PjGH9B3* caused defects in xylem bridge formation, suggesting that cellulase activity is required for efficient host penetration and establishment of a vascular connection (Kurotani et al. 2020). Therefore, cellulose degradation by cellulases might be a key step in initial plant-plant interactions during grafting and parasitism. On the other hand, genes like *CESA4*, involved in secondary cell wall

biosynthesis, were downregulated (Kurotani et al. 2020). Our transcriptome data show that most *AtCESA* genes slightly increase their expression in grafted tops, but have reduced expression in grafted bottoms, compatible with a sugar-induced expression pattern (Paper IV, fig. 4D, (Melnyk et al. 2018). The transcriptional reporters of *CESA1*, *CESA3* and *CESA6* showed stronger signals in the separated scion compared to the separated rootstock, confirming the transcriptome results (Paper IV, fig. 5A). Furthermore, *CESA1* and *CESA6* showed higher expression in scions treated with sucrose, confirming that *CESA* gene expression can be increased by sucrose treatment (Paper IV, fig. 5A). However, *CESA*s expression was not highly enhanced by sucrose treatment in the rootstocks, suggesting different factors might be required (Paper IV, fig. 5A). Auxin signalling is quickly re-established following realignment of scion and rootstock (Melnyk et al. 2015), but cannot be re-established in our callus assay setup, where scion and rootstock are left separated. The presence of auxin signalling might therefore be required for sucrose-induced *CESA*s activation. Treatment with cellulase or isoxaben (an inhibitor of primary cell wall CESAs) did not significantly affect graft attachment at 1 DAG (Paper IV, fig. 4A) but rescued the effect of sucrose on attachment (Paper IV, fig. 4A), suggesting sucrose might inhibit graft attachment by activating cellulose biosynthesis through *CESA* genes. During grafting, a trade-off between growth and regeneration might be necessary for efficient graft reconnection, as processes like primary root growth are arrested during graft healing until the vasculature is reconnected (Melnyk et al. 2015). The activation of cellulose biosynthesis pathways might lead to a switch from regeneration, including the activation of cell wall degrading enzymes that are required for initial graft attachment (Sala et al. 2019; Notaguchi et al. 2020), to growth, inhibiting graft healing. However, cellulase and isoxaben treatments inhibited phloem reconnection and did not rescue the sucrose effect on phloem (Paper IV, fig. 4B). These results suggest that cellulose biosynthesis might need to be arrested, at least in the rootstock, during attachment but is required for vascular reconnection during grafting, similar to the need for more loosened pectin during attachment to the host but stiffer pectin during vascular reconnection in parasitism (Paper III, fig. 2). Attachment and vascular reconnection are key steps in both grafting and parasitism, and depend on cell wall modifications. Furthermore, the presence of a "starving" rootstock seems to be required for inducing graft healing, similarly to the need for a "starving"/nitrogen-deprived parasitic plant to

induce haustoria development. As sucrose induces *CESA* gene expression in Arabidopsis grafts (paper IV) and nitrogen application inhibits cell wall modifying enzymes like laccases, *CESA*s and *PMEI*s (paper I), it is possible that both sucrose and nitrogen inhibit attachment and vascular connection in plant parasitism and grafting by acting through cell wall modifications. Studying the parallels between these processes more in depth might give insights into ways to improve compatibility during grafting and prevent infection by parasitic plants in the field (Figure 4).

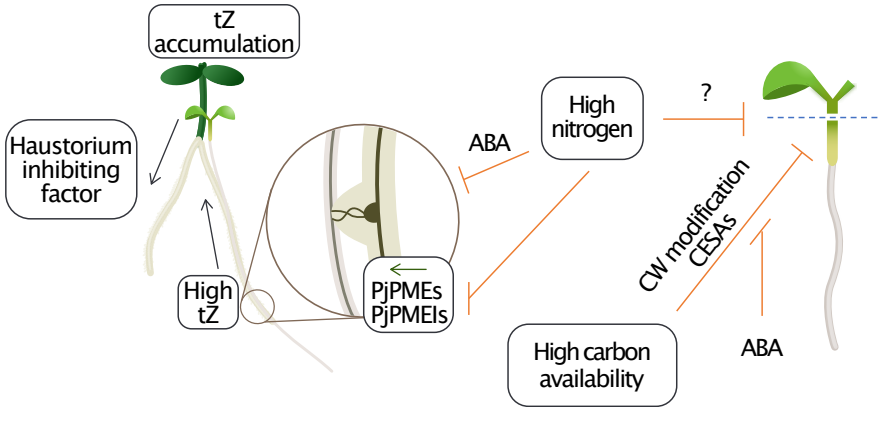

Figure 4: Schematic summary of the main findings of the thesis.

Haustorium formation in *P. japonicum* requires cell wall modification by PjPMEs and PjPMEIs. The formation of haustoria induces tZ accumulation in infecting roots and in the shoot. Cytokinin accumulation regulates haustoria numbers systemically, likely through a shoot to root haustorium inhibiting factor. High nitrogen availability inhibits haustoria through ABA signalling and graft healing through an unknown mechanism. High carbon availability also inhibits graft reconnection through cell wall modifications mediated by CESAs, and this effect is countered by ABA application.

4. Future perspectives

Grafting and plant parasitism share many common developmental pathways, such as the need to recognize a compatible host/grafting partner, tissue attachment and vascular reconnection. In this thesis I, with the help of my co-authors, tried to identify parallels and differences in the response to nutrients and in the modification of the cell wall during the processes of graft reconnection and haustorium development.

We found that the application of exogenous nutrients inhibits both plant parasitism and graft reconnection (papers I and IV). However, many molecular mechanisms underlying these phenotypes remain unresolved. The work in papers I and IV shows how abscisic acid (ABA) has an inhibitory role in both plant parasitism and grafting. However, while ABA acted downstream of nitrogen to inhibit haustoria formation, combining ABA with sucrose rescued the sucrose effect on grafting. These results suggest that sugar and ABA signalling are antagonistic, as suggested by previous work (Rodrigues et al. 2013). These results raise some additional questions. What is the mechanism behind ABA's inhibition of graft reconnection and haustorium development? Is this mechanism conserved between the two processes? How does sucrose signalling interact with ABA at the graft junction? Does sugar signalling have a role in parasitic plant infection too? More work will be required to answer these questions.
We found that the successful infection of a host can inhibit the formation of new haustoria in distant roots in *P. japonicum*, and therefore that a systemic signalling exists to control the number of infections in parasitic plants (paper II). Successful infections induce cytokinin signalling in the parasite and the host (Spallek et al. 2017), and we discovered that cytokinin is part of the *P. japonicum* haustoria regulation system (paper II). However, our results show that although cytokinin is likely travelling from infected roots to the shoot, it is not the signal travelling from the shoot to the distant roots to inhibit the development of new haustoria. What could this shoot to root signal be? In legumes, nodulation is a systemically regulated process that balances the uptake of sufficient nitrogen with the development of nodules (Mortier et al. 2012). This process is highly regulated, and the molecular pathways discovered so far include cytokinin signalling and C-TERMINALLY ENCODED PEPTIDE (CEP) transport and signalling (Mortier et al. 2012;

Sasaki et al. 2014; Taleski, Imin, and Djordjevic 2018). It is possible that plant parasitism uses similar mechanisms to nodulation and employs CEP or other peptides to regulate the amount of haustoria forming. More investigations are needed to confirm the possible involvement of peptides in haustoria development and to identify additional shoot to root mobile signals controlling haustoria numbers.

Cytokinin and nitrogen have also been linked in the control of root growth and architecture in *A. thaliana* (Hirose et al. 2008; Poitout et al. 2018), and therefore it is possible that these two signalling pathways might also interact during graft reconnection. However, more research is needed to confirm this possible parallel between plant parasitism and grafting. We have also seen in paper IV how exogenous sucrose inhibits *A. thaliana* grafting. Exogenous sucrose induces the formation of adventitious roots (Takahashi et al. 2003). It is possible that part of the reason behind grafting inhibition by sucrose is due to an increase in the formation of adventitious roots by the scion, therefore reducing the need for the scion to reconnect to a root system. More investigation will be required to explore this angle. It has also been reported that sucrose can increase grafting efficiency in other species, such as Cucurbitaceae (Miao et al. 2021). What is this difference due to? Are the molecular mechanisms regulating graft reconnection different between *A. thaliana* and Cucurbitaceae? Answering these questions might provide useful tools to improve grafting efficiency in economically relevant plants.

Previous publications have suggested that conserved cell wall modification mechanisms exist during parasitic plant attachment and graft attachment (Kurotani et al. 2020). In papers III and IV, we investigated the roles of pectin methylesterification and cellulose biosynthesis during plant parasitism and grafting respectively, and found that these processes need to be regulated to allow efficient tissue reconnection. The signals activating cell wall modifications during plant parasitism and grafting are still poorly understood. Our results suggest brassinosteroids might have a role in regulating pectin methylesterification during haustorium development. However, more investigations are needed to solidify this link. Previous literature has linked auxin signalling to the modification of cell wall to allow cell expansion and division (Arsuffi and Braybrook 2018; Barbez et al. 2017; Braybrook and Peaucelle 2013). Auxin signalling is key for the development of both the graft junction and haustoria, and therefore a link between cell wall modifications and auxin during these processes is likely. The cell wall

has also been previously linked to host compatibility in parasitic plants (Johnsen et al. 2015; Striberny and Krause 2015; Cui et al. 2018). Compatibility is perhaps the biggest and most puzzling difference between plant parasitism and grafting, as parasitic plants can parasitize even very distantly related species, while grafting tends to be successful only between very closely related species. Could the cell wall structure be a key player in determining compatibility in both plant parasitism and grafting? How do parasitic plants manage to overcome the incompatibility barriers, and is it through cell wall modifying enzymes? Could we improve graft efficiency and compatibility by applying cell wall degrading enzymes? Answering these questions will provide useful insights and tools to improve the use of grafting as a tool in horticulture and to breed for parasitic plant-resistant crops.

Finally, as my thesis used model systems like the facultative parasitic plant *P. japonicum* and *A. thaliana* micrografting, it will be important to test our discoveries also in other species to increase the applicability of our results. We have already confirmed that nitrogen can inhibit parasitism even in the agriculturally relevant obligate parasite *Striga hermonthica* (paper I), but the mechanisms behind this phenotype in this species are likely different from *P. japonicum*. Grafting with other species, as for example different tomato and pepper varieties, will also be helpful to confirm if our discoveries can be expanded to other species than Arabidopsis.

To conclude, this thesis has discovered important similarities and differences between plant parasitism and grafting. Future research building on this work is expected to lead to improved control of parasitic plants and to the improvement of commercial grafting efficiency.

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Popular science summary

Far from being “still life”, plants can sense their environments with incredible precision and adjust their growth to increase their chances of survival. Plants can also interact with each other. In some cases, their interaction is positive as plants can help each other heal. An example is grafting, a horticultural practice where the shoot of one plant is attached to the root system of another plant to create a plant with more desirable characteristics. However, plants can also damage other plants. This is the case with parasitic plants, which attack and feed on other plants using a special organ called “the haustorium”, causing huge agricultural losses every year. Both plant parasitism and grafting require different steps to be successful: the tissues from the two plants have to stick together, and the vasculature (the tissue transporting water and nutrients) of the two plants has to reconnect. In this thesis, I studied similarities and differences between plant grafting and plant parasitism. In particular, I focused on plants response to nutrients (sugars and nitrogen) and changes in the cell wall, the thick stiff layer surrounding plant cells. I and co-authors discovered that high levels of nitrogen inhibit both plant parasitism and grafting, and that an excess of sugars also delays graft reconnection. While in parasitic plants nitrogen acts through abscisic acid, the plant stress hormone, to reduce the numbers of haustoria and therefore infection, sugars and abscisic acid are antagonistic during graft healing. These results suggest that although plant parasitism and grafting are both inhibited by applying nutrients, the mechanisms behind these effects are different, and will require more investigation to be fully understood.

We also discovered that in parasitic plants, the number of haustoria is not only controlled by nutrients in the soil, but also by previous infections. We found that parasitic plants that had already infected other plants were less

likely to infect additional plants at a later time. This process is controlled, at least in part, by another plant hormone called cytokinin, suggesting that the formation of parasitic organs is a highly and complexly regulated process.
Finally, we found that modifying the plant cell wall is necessary for efficient plant parasitism and grafting. In particular, we discovered that the stiffness of pectin (the “glue” of the cell wall) has to be controlled by parasitic plants during invasion of their hosts, and that parasitic plants do so by regulating the activity of two families of enzymes: pectin methylesterases (PMEs) and their inhibitors (PMEIs). While pectin is assumed to be involved in the initial attachment during grafting, we found that cellulose also likely plays a role, as sugars seem to inhibit grafting by activating cellulose synthesis.
This thesis shows how many mechanisms are conserved in plant interactions between each other and with their environment. These results, together with the future research building on them, will allow us to better understand the molecular mechanisms behind plant-plant interactions, and help us improve plant grafting and reduce plant parasitism in agriculture.

Populärvetenskaplig sammanfattning

Växter kan känna av sina miljöer med otrolig precision och anpassa sin tillväxt för att öka sina chanser att överleva. Växter kan också interagera med varandra. I vissa fall är deras interaktion positiv eftersom växter kan hjälpa varandra att läka. Ett exempel är ympning, en trädgårdsodling där skottet från en växt fästs på en annan växts rotsystem för att skapa en ny växt med mer önskvärda egenskaper. Växter kan dock också skada andra växter. Detta är fallet med parasitiska växter, som attackerar och livnär sig på andra växter med hjälp av ett speciellt organ som kallas "haustorium", vilket orsakar enorma jordbruksförluster varje år. Både växtparasitism och ympning kräver olika steg för att lyckas: vävnaderna från de två växterna måste hålla ihop, och kärlsystemet (vävnaden som transporterar vatten och näringsämnen) hos de två växterna måste kopplas samman igen. I detta examensarbete studerade jag likheter och skillnader mellan växtympning och växtparasitism. Speciellt fokuserade jag på växternas respons på näringsämnen (socker och kväve) och förändringar i cellväggen, det tjocka styva lagret som omger växtceller. Jag (och medforskare) upptäckte att höga nivåer av kväve hämmar både växtparasitism och ympning, och att ett överskott av sockerarter också fördröjer återanslutningen av ympning. Medan kväve i parasitiska växter verkar genom abscisinsyra, växtens stresshormon, för att minska antalet haustorier och därför infektion, sockerarter och abscisinsyra verkar i motsatta riktningar under ympning. Dessa resultat tyder på att även om växtparasitism och ympning båda hämmas genom att applicera näringsämnen, är mekanismerna bakom dessa effekter annorlunda och kommer att kräva mer undersökning för att förstås fullt ut.

Vi upptäckte också att i parasitiska växter styrs antalet haustorier inte bara av näringsämnen i jorden, utan också av tidigare infektioner. Vi fann att parasitiska växter som redan hade infekterat andra växter var mindre

benägna att infektera ytterligare växter vid ett senare tillfälle. Denna process kontrolleras, åtminstone delvis, av ett annat växthormon som kallas cytokinin, vilket tyder på att bildningen av parasitiska organ är en mycket och komplext reglerad process.

Slutligen upptäckte vi att modifiering av växtcellväggen är nödvändig för effektiv växtparasitism och ympning. I synnerhet upptäckte vi att pektinets (cellväggens "lim") styvhet måste kontrolleras av parasitiska växter under invasion av deras värdar, och att parasitväxter gör det genom att reglera aktiviteten hos två familjer av enzymer: pektin metylesteraser (PME) och deras hämmare (PMEI). Medan pektin antas vara involverat i den initiala vidhäftningen under ympning, fann vi att cellulosa sannolikt också spelar en roll, eftersom socker verkar hämma ympning genom att aktivera cellulosasyntes.

Denna avhandling visar att många mekanismer bevaras i växtinteraktioner mellan varandra och med sin omgivning. Dessa resultat, tillsammans med den framtida forskningen som bygger på dem, kommer att tillåta oss att bättre förstå de molekylära mekanismerna bakom växt-växt-interaktioner och hjälpa oss att förbättra växtympningen och minska växtparasitism i jordbruket.

Riassunto per divulgazione scientifica

Lontane dall'essere "natura morta", le piante possono percepire l'ambiente circostante con incredibile precisione e regolare la crescita per aumentare le probabilità di sopravvivenza. Le piante possono anche interagire tra loro. In alcuni casi, la loro interazione è positiva dato che le piante possono aiutarsi a guarire a vicenda. Un esempio è l'innesto, una pratica agronomica in cui la parte superiore ("marza") di una pianta viene fusa al sistema radicale ("portinnesto") di un'altra pianta per creare un nuovo individuo con caratteristiche superiori. Tuttavia, le piante possono anche danneggiare altre piante. È il caso delle piante parassite, che attaccano e si nutrono di altre piante utilizzando un organo specializzato chiamato "austorio", causando ingenti perdite agricole. Sia il parassitismo che l'innesto richiedono varie fasi per essere completate con successo: i tessuti delle due piante devono fondersi e restare uniti e il sistema vascolare (il tessuto che trasporta acqua e sostanze nutritive) delle due piante deve riconnettersi. In questa tesi ho studiato somiglianze e differenze tra l'innesto e il parassitismo vegetale. In particolare, mi sono concentrata sulla risposta delle piante ai nutrienti (zuccheri e azoto) e sui cambiamenti nella struttura della parete cellulare, lo strato rigido che circonda le cellule vegetali. Io e coautori abbiamo scoperto che livelli alti di azoto inibiscono sia il parassitismo delle piante che gli innesti e che un eccesso di zuccheri può ritardare la riconnessione degli innesti. Mentre nelle piante parassite l'azoto riduce il numero di austori e quindi le infezioni agendo tramite l'acido abscissico (l'ormone dello stress nelle piante), gli zuccheri e l'acido abscissico agiscono in modo opposto durante la guarigione degli innesti. Questi risultati suggeriscono che, sebbene il parassitismo delle piante e l'innesto siano entrambi inibiti dall'aggiunta di nutrienti, i meccanismi alla base di questi processi sono diversi.

Abbiamo anche scoperto che nelle piante parassite il numero di austori non è controllato solo dalle sostanze nutritive presenti nel terreno, ma anche dalla presenza di infezioni precedenti. Infatti, le piante parassite che avevano già infettato altre piante avevano meno probabilità di infettare nuovamente in un secondo momento. Questo processo è controllato, almeno in parte, da un altro ormone vegetale chiamato citochinina, suggerendo che lo sviluppo degli austori è un processo complesso e finemente regolato.
Infine, abbiamo scoperto che la modifica della parete cellulare della pianta è necessaria per un parassitismo e un innesto efficienti. In particolare, la rigidità della pectina (il "collante" della parete cellulare) deve essere regolata dalle piante parassite durante l'invasione dell'ospite, e questo accade monitorando l'attività di due famiglie di enzimi: le pectina metilesterasi (PME) e i loro inibitori (PMEI). Pubblicazioni precedenti suggeriscono che la pectina sia coinvolta nell'iniziale fusione di tessuti durante l'innesto, ma abbiamo scoperto che anche la cellulosa potrebbe avere un ruolo in questo processo, dato che gli zuccheri sembrano inibire l'innesto attivando la sintesi di cellulosa.
Questa tesi mostra che molti dei meccanismi molecolari alla base delle interazioni delle piante fra loro e con il loro ambiente sono comuni in diversi processi. Questi risultati, insieme alla futura ricerca basata su di essi, ci consentiranno di comprendere meglio i meccanismi molecolari alla base delle interazioni tra piante e ci aiuteranno a migliorare l'innesto delle piante e a ridurre il parassitismo in agricoltura.

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I

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ARTICLE

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Nitrogen represses haustoria formation through abscisic acid in the parasitic plant *Phtheirospermum japonicum*

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Parasitic plants are globally prevalent pathogens that withdraw nutrients from their host plants using an organ known as the haustorium. The external environment including nutrient availability affects the extent of parasitism and to understand this phenomenon, we investigated the role of nutrients and found that nitrogen is sufficient to repress haustoria formation in the root parasite *Phtheirospermum japonicum*. Nitrogen increases levels of abscisic acid (ABA) in *P. japonicum* and prevents the activation of hundreds of genes including cell cycle and xylem development genes. Blocking ABA signaling overcomes nitrogen's inhibitory effects indicating that nitrogen represses haustoria formation by increasing ABA. The effect of nitrogen appears more widespread since nitrogen also inhibits haustoria in the obligate root parasite *Striga hermonthica*. Together, our data show that nitrogen acts as a haustoria repressing factor and suggests a mechanism whereby parasitic plants use nitrogen availability in the external environment to regulate the extent of parasitism.

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Parasitic plants make up ~1% of all angiosperm species, some of which are devastating agricultural weeds that cause major agricultural losses each year[1–3]. Parasitic plants range from obligate parasites that completely depend on their host for survival to facultative parasites that survive without a host but parasitize when conditions are suitable[2,4]. Despite differences in their lifestyle, all parasitic plants form an invasive organ termed the haustorium[5] through which they penetrate the host and uptake water, nutrients, RNA, proteins, and hormones[5–10].

Many parasitic plants, particularly the obligate parasites, require perception of host-exuded compounds such as strigolactones to initiate germination. Perception of a second host-derived compound, known as haustorium inducing factors (HIFs), initiates haustorium formation in both obligate and facultative parasites. The first identified HIF was 2,6-dimethoxy-1,4-benzoquinone (DMBQ), originally isolated from root extracts of infected sorghum plants[11]. DMBQ can induce early stages of haustoria formation even in the absence of a host[11] in a wide range of parasitic plant species. In the facultative parasitic plant *Phtheirospermum japonicum*, perception of a nearby host via HIFs is followed by cell expansion and cell division at the haustorium initiation site, forming the characteristic swelling of the prehaustorium. Downstream signaling of HIFs requires reactive oxygen species (ROS) that accumulate in the haustorium after HIF perception[12]. Later, the developing haustorium attaches to the host and starts penetrating to reach the vascular cylinder of the host. Once the haustorium has reached the host's vasculature, it starts forming a xylem connection between itself and the host known as the xylem bridge[13–16]. These series of events lead to the establishment of the mature haustorium.

Despite recent advances in our understanding of haustorium development, we know little about how environmental conditions affect plant parasitism. Nutrient availability is an important factor affecting plant parasitism. Infestations of the agriculturally devastating obligate parasite *Striga* are often associated with poor soil fertility[17]. Low soil fertility is thought to impede host defences and exacerbate the damaging effects of infection[17]. In addition, low nutrient levels in the soil, particularly phosphate, promotes host secretion of strigolactones which enhances *Striga* germination and infection levels. Improving soil fertility can reduce the production of germination stimulants while also improving host defences and host tolerance[17–21]. However, nutrients might also have effects on the parasite beyond germination. For instance, the application of certain nitrogen compounds reduces *Striga* shoot development[22] whereas *P. japonicum* requires nutrient starvation to efficiently infect its hosts in vitro[8,13,14] and high phosphorous inhibits *Rhinanthus minor* growth[23]. Together, these data suggest that nutrients might play a role beyond improving host fitness or reducing parasite development.

Nutrient availability affects many aspects of plant development including germination, root growth, shoot growth and flowering[24–26]. High nitrate levels generally promote shoot growth and repress root growth, in part, through the action of plant hormones. In *Arabidopsis thaliana*, rice, maize and barley, nitrates increase cytokinin levels which move to the shoot meristems to promote cell divisions and growth[27–31]. Nitrates also inhibit auxin transport and modify auxin response to promote root initiation but inhibit root elongation[32]. The hormone abscisic acid (ABA) too plays a role; nitrate treatments increase ABA levels in *Arabidopsis* root tips[33] whereas ABA signaling is required for the inhibitory effects of high nitrates on root growth[34]. However, the mechanisms through which nutrient availability affects plant parasitism remain unknown.

Here, we show that nutrient-rich soils greatly reduce both root size and haustorial density in *P. japonicum*, and this effect is dependent specifically on nitrogen concentrations. Nitrogen application reduced ROS levels, blocked gene expression changes associated with haustoria formation and modified xylem patterning in the root. Nitrogen increased ABA levels and activated ABA responsive gene expression. Treating with ABA reduced haustoria initiation whereas inhibiting ABA biosynthesis or signaling reduced the inhibitory effect of nitrogen. Finally, we investigated the effects of nutrients in *Striga hermonthica* and found that similar to *P. japonicum*, nutrients decreased haustoria formation rates and infection rates, and this effect was specific to nitrogen and could be overcome by modifying phytohormone levels.

Results

Nitrogen inhibits haustoria development. Previous work has demonstrated that nutrient-poor conditions are important for efficient *Striga* infestations and successful *P. japonicum* in vitro infections[8,13,35,36]. We tested whether successful *P. japonicum-Arabidopsis* infections in soil also required low nutrients by treating nutrient poor 50:50 soil:sand with or without fertilizer (51-10-43 N-P-K). *P. japonicum* shoot weights and heights were similar in both treatments, but root masses and haustorial density were higher under low nutrient conditions (Fig.1a–c; Supplementary Fig. 1a–c). To better understand the basis for reduced haustoria in high nutrient conditions, we grew 4–5-day old *P. japonicum* seedlings in vitro on water-agar or half-strength Murashige and Skoog medium (½MS)-agar (Supplementary Fig. 1d). Similar to fertilized soil, *P. japonicum-Arabidopsis* infections on ½MS-agar formed substantially fewer haustoria that also failed to form vascular connections with the host compared to those on water-agar (Fig.1d, e). To identify the compound(s) that caused haustoria arrest, we tested three of the major macroelements found in MS and tested one macroelement found in Gamborg's B5 medium at similar concentrations as those found in ½MS or Gamborg's medium. Agar media containing phosphate (KH_2PO_4 or NaH_2PO_4) or potassium (KH_2PO_4 or KCl) had little effect on haustoria formation, but agar media containing nitrogen including nitrates, ammonium or both (KNO_3, $NaNO_3$, NH_4Cl, NH_4NO_3) inhibited *P. japonicum-Arabidopsis* infections and xylem bridge formation similar to ½MS (Fig.1d, e, i). Infections on ½MS lacking nitrogen did not affect haustoria formation, xylem bridge formation or anatomy (Fig.1d, e, i; Supplementary Fig. 1d–g) indicating that nitrogen was sufficient and necessary to block haustoria formation. Nitrogen application led to a reduction of haustoria and xylem bridge formation in a 50 µM to 20.6 mM range of concentrations (Fig.1f, g; Supplementary Fig. 1e–g). However, plate xylem length, area and xylem bridge number were unaffected in haustoria that formed xylem bridges regardless of nitrogen treatment (Fig.1e, Supplementary Fig. 1e–g). To test whether nitrogen blocked infection by acting on the parasite or host, we applied NH_4NO_3 or ½MS to *P. japonicum* growing alone in the presence of the haustoria inducting factor DMBQ. Adding DMBQ to water or ½MS lacking nitrogen resulted in similar numbers of prehaustoria, whereas adding DMBQ to NH_4NO_3 or ½MS greatly reduced prehaustoria formation (Fig. 1h, i) indicating the effect of nitrogen on haustoria initiation did not depend on host infection.

Haustoria formation induces widespread transcriptional changes. To investigate how nitrogen availability affected haustoria formation in *P. japonicum*, we performed a time course RNAseq experiment of *P. japonicum* infecting *Arabidopsis* Col-0 in vitro on agar plates treated with water or 10.3 mM NH_4NO_3. We also included a treatment with 0.08 µM 6-benzylaminopurine (BA), a synthetic cytokinin, to test for similarities between NH_4NO_3 and BA transcriptional responses since previous studies

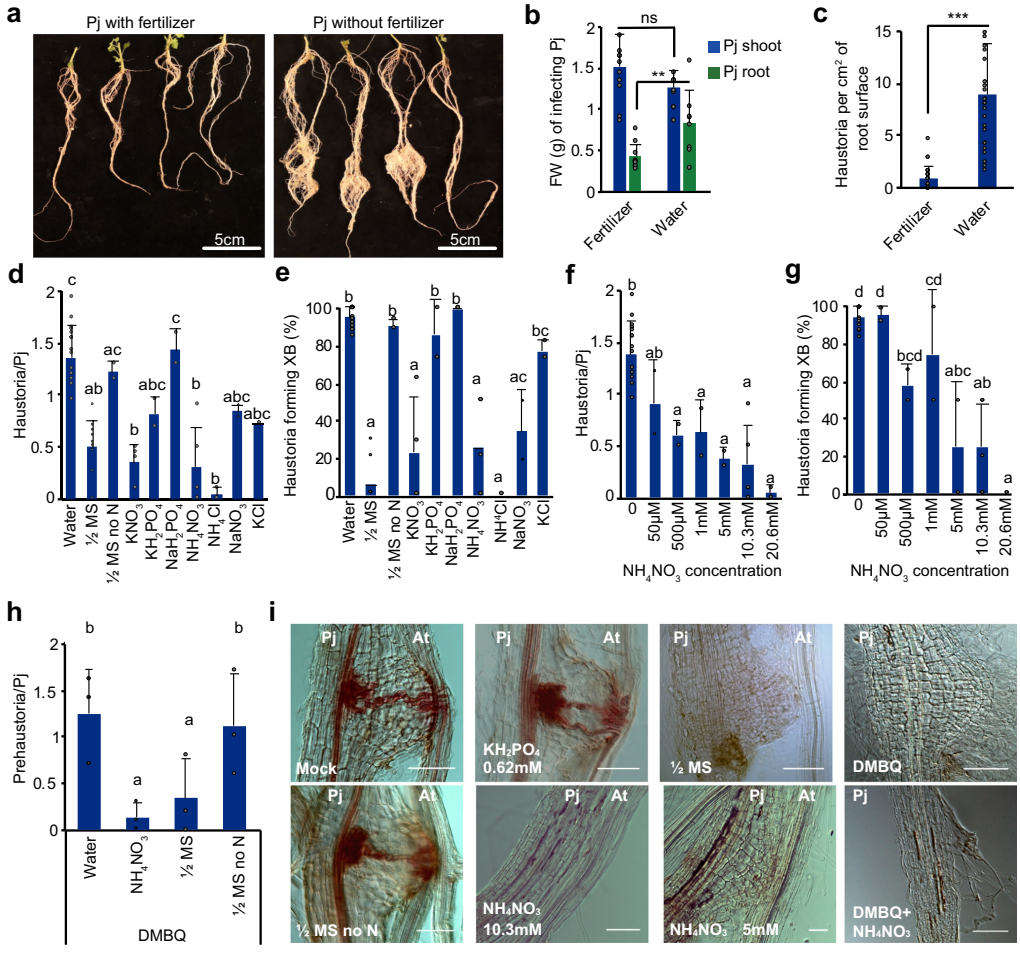

Fig. 1 Nitrogen inhibits *P. japonicum* haustoria formation. a, **b** Representative images and shoot and root fresh weight (FW) of *P. japonicum* during *Arabidopsis* infection with and without fertilizer (mean ± SD, n fertilizer= 11 plants, *n* water= 8 plants). **c** Haustoria numbers per cm^2 *P. japonicum* root during *Arabidopsis* infection with and without fertilizer (mean ± SD, *n* fertilizer= 24 images analyzed, *n* water= 37 images analyzed). **d**–**g** Haustoria numbers per *P. japonicum* seedling and xylem bridge formation percentage in in vitro *Arabidopsis* (Col-0) infections on ½MS (9 replicates), ½MS no *N* (2 replicates), 20.6 mM KNO_3 (4 replicates), 10.3 mM NH_4NO_3 (5 replicates), 0.62 mM KH_2PO_4 (2 replicates), 1.9 mM NaH_2PO_4 (2 replicates), 10.3 mM NH_4Cl (2 replicates), 10.3 mM $NaNO_3$ (2 replicates), 10.3 mM KCl (2 replicates) or various NH_4NO_3 (2 replicates) concentrations (mean ± SD, $n = 20$ plants per treatment per replicate). **h** Prehaustoria numbers per *P. japonicum* seedling with 10 µM DMBQ and half-strength MS, half-strength MS no N or 10.3 mM NH_4NO_3 (mean ± SD, $n = 10$ plants per treatment, 3 replicates). **i** Representative images of *P. japonicum* haustoria during *Arabidopsis* in vitro infections with various nutrient treatments (replicates as indicated in (**d**–**g**)). Scale bars 50 µm. **b**, **c** Asterisks represent $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$, Student's *t* test, two tailed. Comparisons to the water treatment. **d**–**h** Different letters represent $P < 0.05$, one-way ANOVA followed by Tukey's HSD test. Source data provided.

have found that nitrogen treatments increase cytokinin levels in *Arabidopsis*, rice, maize and barley[27–30]. *P. japonicum* and *Arabidopsis* were physically aligned at time 0 to synchronize infections (Supplementary Fig. 1d) and tissues surrounding the root tips where haustoria normally emerge were collected at 0,12, 24, 48, 72 h post-infection (hpi) for the water treatment and 0,12, 24 hpi for the NH_4NO_3 and BA treatments (Fig. 2a). Additionally, as a control to distinguish transcriptional changes specific to haustorium formation, we included *P. japonicum* that grew without a host on agar plates containing water, NH_4NO_3 or BA (Fig. 2a). As observed previously (Fig. 1), *P. japonicum* treated with NH_4NO_3 formed few to no prehaustoria whereas the water treatment resulted in successful haustoria formation. With the water treatment, we observed an increasing number of differentially expressed genes in infected samples compared to control samples as time progressed (Supplementary Fig. 2a). Co-expression analyses enabled us to classify genes into 8 clusters with distinct expression patterns during haustorium formation

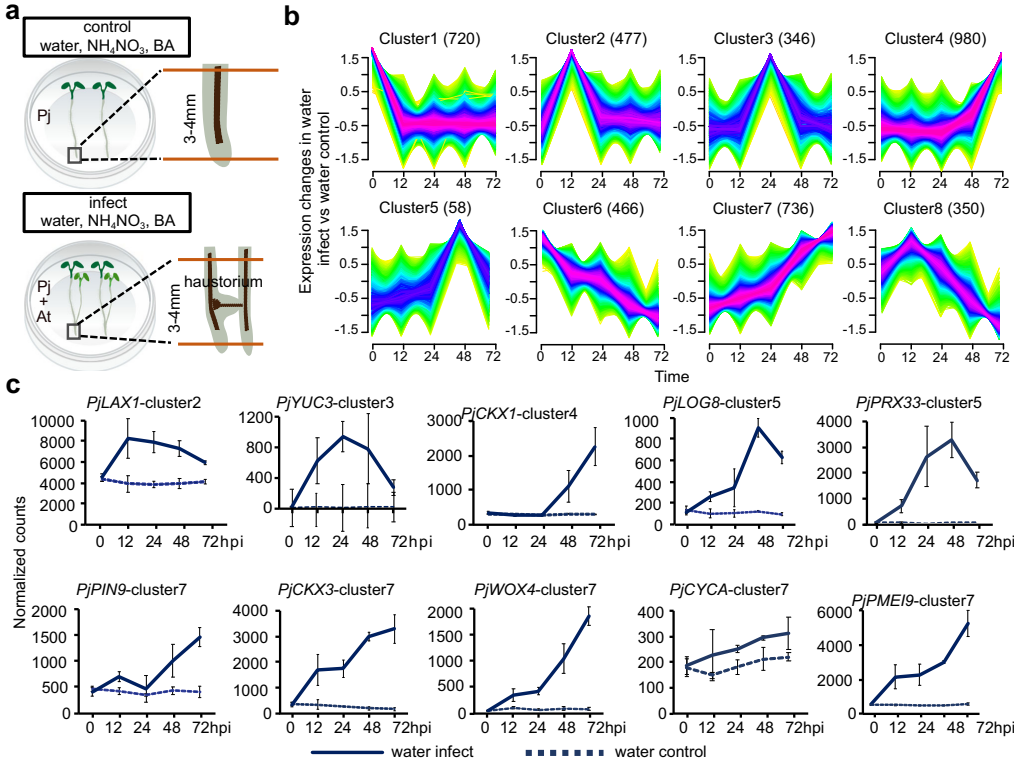

Fig. 2 Transcriptomic changes during haustorium formation. a Illustration describing the set-up for the RNAseq experiment. *P. japonicum* and *Arabidopsis* (infect) or *P. japonicum* alone (control) were placed on media containing water, 10.3 mM NH_4NO_3 or 0.08 µM BA; 3–4 mm of root tip or haustorium formation area was harvested at 0, 12, 24, 48, 72 hpi time points for the water treatment and 0, 12, 24 hpi time points for the NH_4NO_3 and BA treatments. **b** Clustering of differentially expressed genes in the water infect treatment based on their co-expression patterns over five time points; numbers in parentheses represent the number of genes in the cluster. **c** Normalized *P. japonicum* counts of *PjYUC3, PjLAX1, PjPIN9, PjWOX4, PjPMEI9, PjCKX1, PjCKX3, PjLOG8, PjCYCA, PjPRX33* over 5 time points in the water infect treatment (mean ± SD, $n = 3$ libraries).

(Fig. 2b, Supplementary Fig. 2b, Supplementary Data 1). Cluster 2, 3 and 8 whose gene expression peaked at early stages of haustoria formation (12 and 24hpi) had an over representation of genes that belong to Gene Ontology enrichment (GO) categories related to transcription, translation, signaling processes and cell expansion/replication (Supplementary Fig. 3). Cluster 4, 5, and 7 whose gene expression peaked at later time points in haustorium formation (48 and 72hpi) had an over representation of genes that belong to GO categories related to response to oxidative stress, cytokinin metabolic process, fatty acid biosynthetic process, lignin, sucrose and carbohydrate metabolism (Supplementary Fig. 3). We looked at the expression of individual genes in our transcriptome and identified an upregulation of *P. japonicum* auxin-related *YUCCA3* (*PjYUC3*), *LIKE AUXIN RESISTANT 1* (*PjLAX1*), *PIN-FORMED 9* (*PjPIN9*), and cambium-related *WUSCHEL RELATED HOMEOBOX 4* (*PjWOX4*), genes whose expression has been previously observed to increase during *P. japonicum* infections[14,15,37] (Fig. 2c). Genes associated with cytokinin metabolism such as *P. japonicum CYTOKININ OXIDASE 3* (*PjCKX3*), *CYTOKININ OXIDASE 1* (*PjCKX1*), *LONELY GUY 8* (*PjLOG8*), cell wall remodeling such as *PECTIN METHYLESTERASE INHIBITOR 9* (*PjPMEI9*), cell cycle such as *CYCLIN A* (*PjCYCA*) and ROS related such as *PEROXIDASE 33* (*PjPRX33*) were upregulated as well (Fig. 2c) indicating substantial transcriptional reprogramming as the haustoria formed.

Nitrogen inhibits genes associated with early haustorial development. Nitrogen prevented haustoria formation (Fig. 1) so we looked at when this block occurs transcriptionally. We compared the transcriptional differences between infections on water and infections on NH_4NO_3 and found between 4000 and 6000 genes were expressed differently between treatments at each time point (Supplementary Fig. 2c–h) including *PjYUC3*, *PjWOX4,* and *PjPMEI9* whose expression was upregulated during successful haustoria formation on the water treatment but were not activated in the NH_4NO_3 treatment (Fig. 3a, b). Moreover, NH_4NO_3 treatment reduced the expression levels of cell cycle and ROS related genes (Fig. 4a, b). We tested this observation further and found EdU staining for cell division decreased (Fig. 4c) whereas dihydroethidium (DHE) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) staining for ROS accumulation reduced at the haustorium formation site at 24 hpi of NH_4NO_3 treatment (Fig. 4d–g) consistent with nitrogen acting early to block haustoria induction (Fig.1). We next compared NH_4NO_3

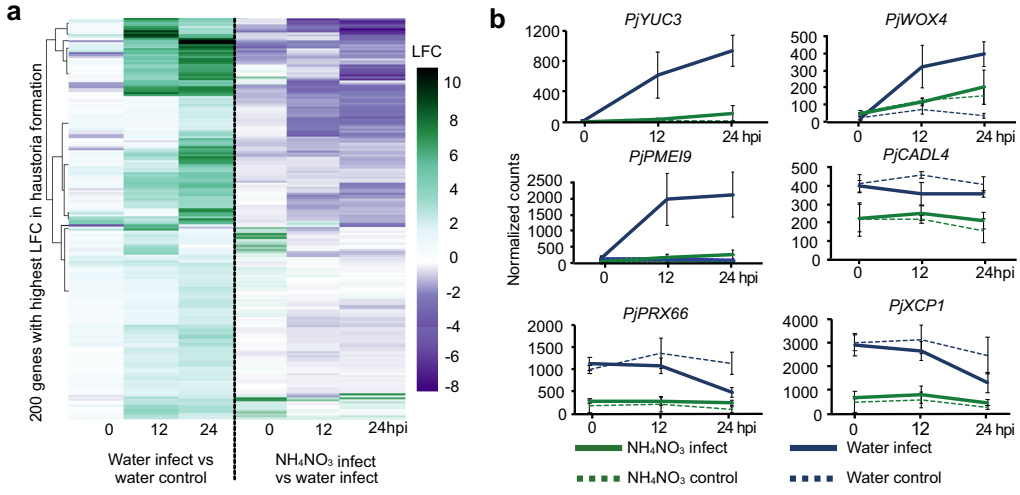

Fig. 3 NH_4NO_3 treatment modifies *P. japonicum* gene expression. **a** Heatmap of 300 *P. japonicum* genes with the highest log2 expression fold change during haustoria formation over three time points in the water infect vs water control and NH_4NO_3 infect vs water infect. **b** Normalized *P. japonicum* counts of *PjYUC3, PjWOX4, PjPMEI9, PjXCP1, PjCADL4, PjPRX66* over three time points shown for control and infect in the NH_4NO_3 and water treatments (mean ± SD, $n = 3$ libraries).

infect with NH_4NO_3 control and found fewer than 70 differentially expressed genes at any time point (Supplementary Fig. 2c) suggesting that very few infection-specific genes were upregulated in NH_4NO_3 infections. Consistent with this, less than 20 of these genes at each time point were also differentially expressed during water infections. Finally, we compared the NH_4NO_3 control to water control datasets to see which genes prior to infection might influence haustoria induction. We found a GO enrichment for cell wall and lignin-related genes downregulated in the NH_4NO_3 control compared to the water control (Supplementary Fig. 4a). Genes downregulated included xylem-related *P. japonicum XYLEM CYSTEINE PEPTIDASE 1* (*PjXCP1*), *LACCASE 11* (*PjLAC11*), *IRREGULAR XYLEM 3* (*PjIRX3*), *CELLULOSE SYNTHASE A4* (*PjCESA4*), *PEROXIDASE 66* (*PjPRX66*), quinone perception related genes *CANNOT RESPOND TO DMBQ LIKE 2* (*PjCADL2*) and *CANNOT RESPOND TO DMBQ LIKE 4* (*PjCADL4*)[38] and ROS related genes *PEROXIDASE 33* (*PjPRX33*) and *PEROXIDASE 25* (*PjPRX25*) (Fig. 3b, Supplementary Fig. 2i). Cytokinin-related GOs were also enriched in the genes downregulated by nitrogen (Supplementary Fig. 4a) and we found no substantial overlap between differentially expressed genes in the BA control and NH_4NO_3 control samples (Supplementary Fig. 5a). Together, these data suggested that nitrogen blocked the infection process at an early stage and nitrogen did not induce a substantial cytokinin response in *P. japonicum*.

Nitrogen increases ABA levels in *P. japonicum*. To further investigate how nitrogen arrests haustoria formation, we performed hormonal profiling on *P. japonicum* seedlings or mature roots infecting *Arabidopsis* with and without nitrogen treatment. In the parasite, levels of the active cytokinin trans-zeatin (tZ) and the cytokinin precursor trans-zeatin riboside (tZR) increased in successful infections, whereas in the host, levels of tZ and tZR increased in the presence of nitrates or successful infections, similar to previous studies[8,27–29,31] (Fig. 5a, Supplementary Fig. 6a, b). Neither jasmonic acid (JA), indole acetic acid (IAA), gibberellic acid (GA) A1, ABA nor salicylic acid (SA) were substantially induced in the parasite by infection (Fig. 5a, b, Supplementary Fig. 6a, b). However, ABA and SA were significantly increased by NH_4NO_3 treatments in 50-day-old *P. japonicum* control and infect roots compared to water alone (Fig. 5b). In 20-day-old *P. japonicum* whole seedlings, ABA levels also increased in both infect and control NH_4NO_3 treated *P. japonicum* seedlings compared to water treatments (Fig. 5a). In the *Arabidopsis* host, ABA levels were also increased both by nitrogen treatments and by *P. japonicum* infection (Fig. 5a). This increase was dependent on host ABA biosynthesis since the increase was blocked in the ABA biosynthesis mutant *aba deficient 2-1* (*aba2-1*) (Fig. 5a). Cytokinin moves from *P. japonicum* to *Arabidopsis* during infections[8] but we found no evidence that ABA moved from parasite to host since *Arabidopsis* ABA levels in *aba2-1* infections were similar to not infected *aba2-1* plants (Fig. 5a). However, *P. japonicum* ABA levels were reduced in *aba2-1* infections compared to Col-0 infections, perhaps from reduced movement of host-derived ABA to the parasite or from the host reducing parasite ABA signaling or biosynthesis. We looked at our transcriptome analysis and found that *P. japonicum* genes homologous to *Arabidopsis* ABA responsive genes had increased expression levels in the NH_4NO_3 treatment compared to the water treatment for both infect and control tissues (Fig. 5c, Supplementary Fig. 4b, Supplementary Fig. 5e). This expression pattern was not seen for the same genes when comparing the BA control to water control, BA infect to water infect, or water infect to water control samples (Supplementary Fig. 4c, Supplementary Fig. 5b, f, g) suggesting the increased ABA response was specific to NH_4NO_3 treatment. Most cytokinin-related genes were not differentially expressed in the NH_4NO_3 infect compared to the water infect or NH_4NO_3 control compared to water control treatments—with some exceptions—further supporting our finding that NH_4NO_3 treatment in *P. japonicum* induces an ABA response rather than a cytokinin response (Fig. 5d, Supplementary Fig. 4d, e). However, cytokinin-related genes were differentially expressed during later time points in water infect compared to water control samples

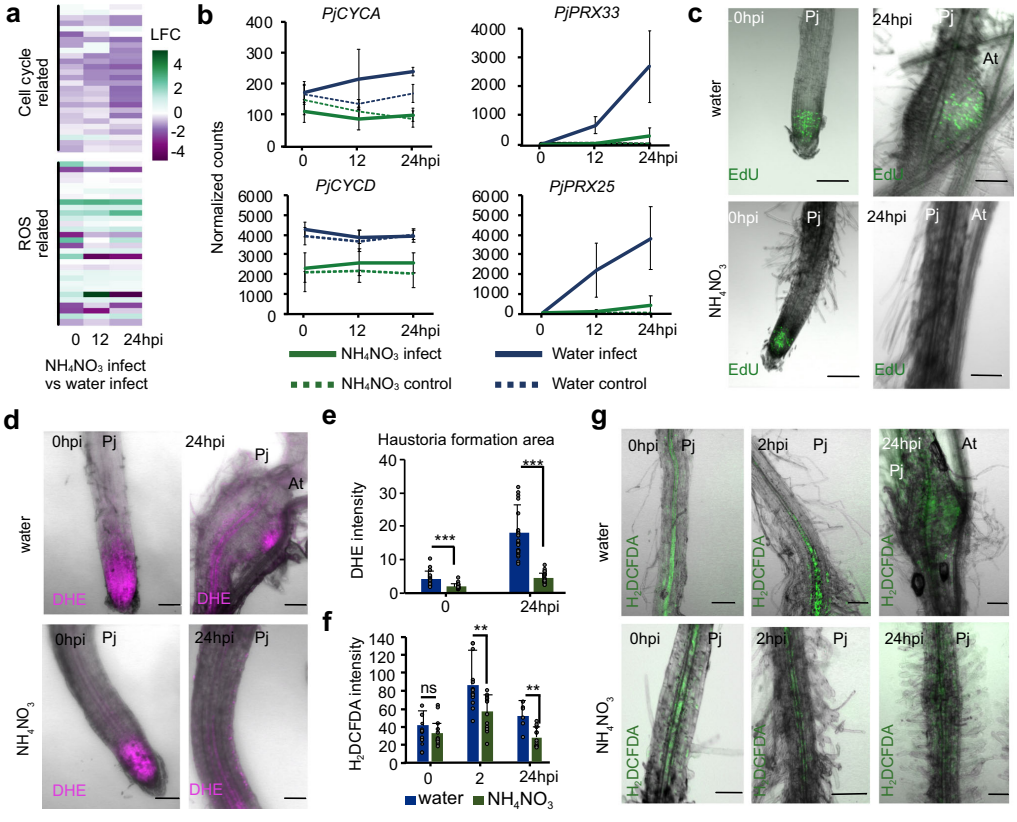

Fig. 4 NH_4NO_3 reduces ROS accumulation and cell division in *P. japonicum*. **a** Heatmap of 27 cell cycle and 30 ROS related *P. japonicum* genes in NH_4NO_3 infect compared to water infect RNAseq datasets. **b** Normalized *P. japonicum* counts of *PjCYCA, PjCYCD, PjPRX33, PjPRX25* over three time points shown for control and infect in the NH_4NO_3 and water treatment, bars represent mean ± SD. **c**, **d**, **g** Representative images of *P. japonicum* in vitro *Arabidopsis* Col-0 infections with water or 10.3 mM NH_4NO_3 at 0, 2 and 24 h post-infection (hpi) stained with EdU (c), dihydroethidium (DHE) (d) or 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) ($n = 20$ plants per treatment per replicate, 2 replicates). Scale bars 100 µm. **e**, **f** DHE at 0 and 24 hpi ($n = 22$, water 0 hpi $n = 17$) and H_2DCFDA at 0, 2, 24 hpi ($n = 12$, 24 hpi water treatment $n = 6$) intensity in the *P. japonicum* haustorium formation area after water infect or NH_4NO_3 infect (mean ± SD; Comparisons to water treatments. Asterisks represent $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$, Student's *t* test, two tailed). Source data provided.

(Supplementary Fig. 5h), implicating cytokinin response in later haustorium development. To see whether the *P. japonicum* genes homologous to *Arabidopsis* ABA responsive genes were ABA responsive, we selected four upregulated genes in our NH_4NO_3 control RNAseq dataset and found by qPCR that transcript levels of three of them were significantly increased by exogenous ABA (Supplementary Fig. 5c). We tested the expression levels of these genes and one ABA biosynthesis homolog, *PjABA2*, in *P. japonicum* grown on various soil:sand ratios and found that the expression levels of *FRUCTOSE-BISPHOSPHATE ALDOLASE 2* (*PjFBA2*), *ABA INSENSITIVE 1* (*PjABI*) and *PjABA2* were lower in *P. japonicum* roots in nutrient poor soils (Supplementary Fig. 5d), suggesting that some ABA responses and ABA biosynthesis were downregulated in plants grown in nutrient poor soils, and conversely, upregulated in nutrient-rich soils (Supplementary Fig. 5d). SA levels also increased during nitrogen treatment in the parasite (Fig. 5a,b) but most *P. japonicum* genes homologous to *Arabidopsis* SA-responsive genes were not differentially expressed in the NH_4NO_3 infect compared to the water infect treatment (Supplementary Fig. 5i). Together, these data suggest that nitrogen increased ABA levels and induced ABA responses in *P. japonicum*.

ABA affects haustoria formation. To investigate the role of ABA on haustorium formation in *P. japonicum*, we applied ABA exogenously using in vitro infection assays. ABA treated plants formed less haustoria than water treated plants (Fig. 6a, b). The application of fluridone, a chemical inhibitor of ABA biosynthesis, significantly reduced xylem bridge formation but did not affect haustoria formation (Fig. 6a–c). We reasoned that if nitrogen induced ABA to repress haustoria, we could overcome the inhibitory effects of nitrogen by blocking ABA biosynthesis with fluridone. Indeed, treating ½MS with fluridone increased haustoria numbers compared to ½MS alone but they remained intermediate to the water treatment (Fig. 6a–c). As a second

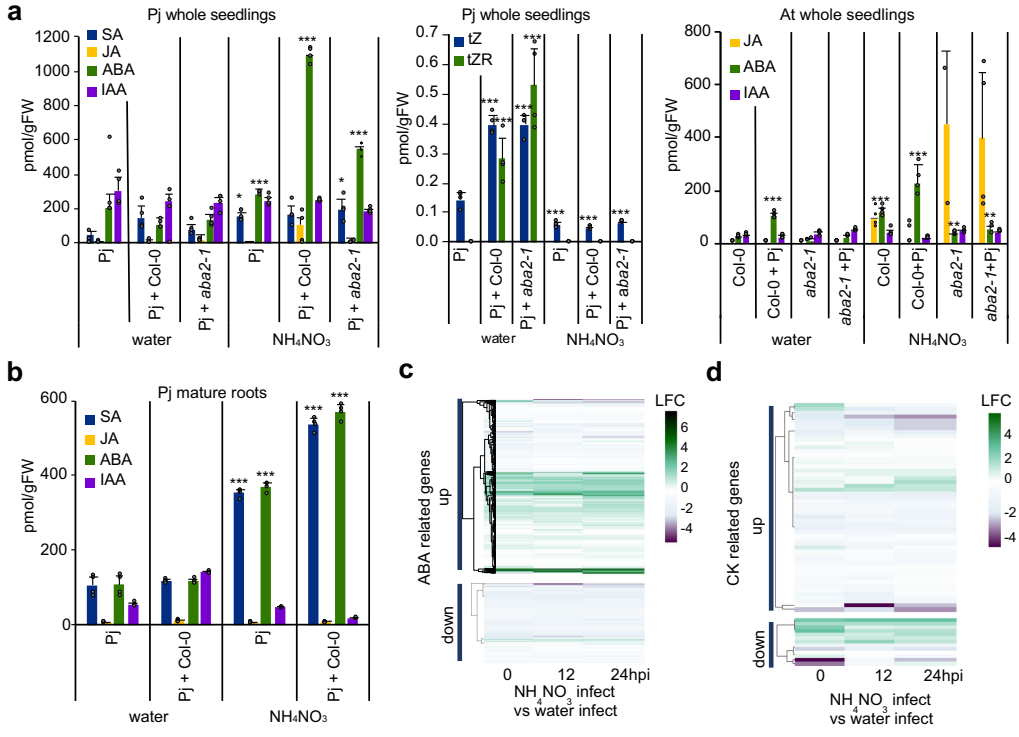

Fig. 5 ABA levels increase during nitrogen treatment. a Hormonal quantification of salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), indole acetic acid (IAA), trans-zeatin (tZ) and trans-zeatin riboside (tZR) in *P. japonicum* whole seedlings and *Arabidopsis* (Col-0, *aba2-1*) whole seedlings treated with 10.3 mM NH_4NO_3 (mean ± SD, $n = 4$ plants per treatment, 4 replicates). **b** Hormonal quantification of SA, JA, ABA, and IAA in *P. japonicum* mature roots treated with 10.3 mM NH_4NO_3 (mean ± SD, $n = 4$ roots per treatment, 4 replicates). **c** Heatmap of the log2 fold change of 629 genes homologous to *Arabidopsis* ABA responsive genes (up or downregulated) for three time points in the NH_4NO_3 infected vs the water infected RNAseq dataset in *P. japonicum*. **d** Heatmap of the log2 fold change of 67 genes homologous to *Arabidopsis* cytokinin responsive genes (up or downregulated) for three time points in the NH_4NO_3 infect vs the water infect RNAseq dataset in *P. japonicum*. **a**, **b** Comparisons to respective control samples. Asterisks represent $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$, Student's *t* test, two tailed. Source data provided.

approach, we used the *Arabidopsis abi1-1* dominant mutant that represses ABA signaling in *Arabidopsis* and when expressed in *Nicotiana*[39,40]. We found that haustoria formation in non-transgenic hairy roots was suppressed by nitrogen but when we overexpressed *Atabi1-1* in *P. japonicum*, haustoria formation was unaffected by the presence of nitrogen (Fig. 6d–f). These results demonstrated that blocking ABA signaling in *P. japonicum* roots was sufficient to rescue the suppressive effects of nitrogen. We investigated host ABA pathways but *P. japonicum* infecting *Arabidopsis aba2-1* or *aba1-1C* did not have differences in haustoria and xylem bridge formation, suggesting that altering host ABA biosynthesis or signaling did not affect parasitism (Supplementary Fig. 6c, d). SA levels were also induced by NH_4NO_3 in *P. japonicum* and *Arabidopsis* (Fig. 5a, b, Supplementary Fig. 6a) so we tested the exogenous application of SA and found it decreased haustorial numbers but did not affect xylem bridge formation (Supplementary Fig. 6e–g, j, k). Thus, SA might act as a second signal to regulate haustoria, however, SA related genes were not differentially expressed by NH_4NO_3 treatment (Supplementary Fig. 5i) suggesting nitrogen does not induce an SA response.

Since ABA is important for various developmental processes including xylem formation[41], we analyzed the *P. japonicum* transcriptome in water infect compared to water control treatments and found that some *P. japonicum* genes homologous to *Arabidopsis* ABA responsive genes increased expression late during infection indicating they might have a relevant role during later stages of haustoria formation such as xylem bridge formation (Supplementary Fig. 5g). Exogenous ABA treatments did not increase xylem bridge formation, numbers or size (Fig. 6b, c, Supplementary Fig. 6e–i) but treatment with fluridone blocked xylem bridge formation (Fig. 6b, Supplementary Fig. 6i). Exogenous ABA application to *P. japonicum* was previously shown to enhance the number of differentiating xylem strands in primary root tips[41]. We repeated this assay but used nitrogen treatments on *P. japonicum* seedlings. ABA, NH_4NO_3 and ½MS all had a similar phenotype of increased xylem strand differentiation compared to water-only treatments (Supplementary Fig. 6l). These data showed that ABA treatment could phenocopy nitrogen treatment and that ABA played additional roles in both xylem bridge formation and also in modulating xylem patterning in response to nitrogen levels.

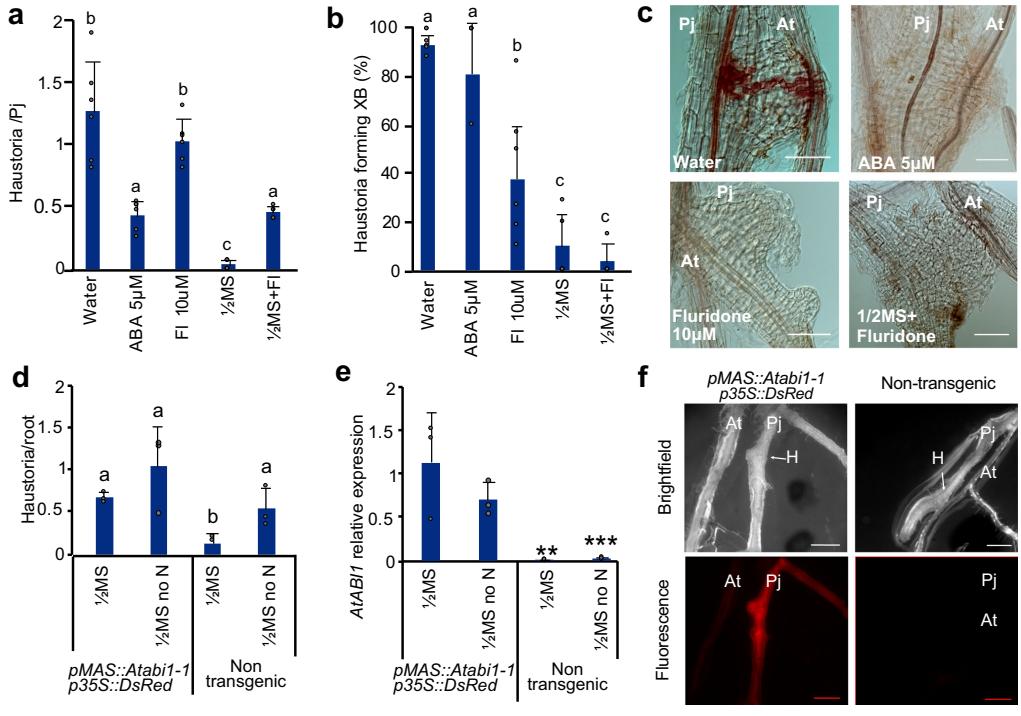

Fig. 6 ABA represses *P. japonicum* haustoria formation. a, **b** Haustoria number per *P. japonicum* seedling and xylem bridge formation percentage in in vitro *Arabidopsis* (Col-0) infections treated with ABA (6 replicates), fluridone (FI) (6 replicates), ½MS (6 replicates), ½MS no N (6 replicates) or ½MS + fluridone (5 replicates) (mean ± SD, $n = 20$ plants per treatment per replicate). **c** Representative images of *P. japonicum* haustoria during *Arabidopsis* in vitro infection with ABA, fluridone, ½MS or ½MS + fluridone. Scale bars 50 μm. 5 replicates. **d** Haustoria number per *P. japonicum* hairy roots overexpressing *Atabi1-1* in in vitro infection assay with *Arabidopsis* Col-0 and ½MS or ½MS no N (mean ± SD, *pMAS::Atabi1-1* $n = 19$ for ½MS and $n = 24$ for ½MS no N treatments, non-transgenic $n = 30$ for ½MS and $n = 29$ for ½MS no N treatments). **e** Expression levels of *Atabi1-1* in fluorescent and non-fluorescent hairy roots analyzed by qPCR (mean ± SD, $n = 3$ roots per treatment, 3 replicates). **f** Representative images of *P. japonicum* hairy roots in the ½MS no N treatment in brightfield and red fluorescence fields, the haustorium (H) is denoted by an arrow, scale bars 500 μm. 3 replicates. **a**, **b**, **d** Different letters represent $P < 0.05$, one-way ANOVA followed by Tukey's HSD test. **e** Asterisks represent $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ compared to ½MS transgenic roots, Student's *t* test, two tailed. Source data provided.

Nitrogen affects *Striga* infection rates. Previous field studies have shown that *Striga* infestation is decreased after nitrate application[18,42]. We investigated the effect of nutrients upon *S. hermonthica* using in vitro infection assays with rice as the host. In the presence of NH_4NO_3, KNO_3, KH_2PO_4, or NaH_2PO_4, *Striga* infection rates were not significantly decreased 2 weeks after infection (Fig. 7a). However, 4 weeks after infection nitrogen application lead to a significant decrease in the percentage of *Striga* that infected its rice host and formed more than three leaves (Fig. 7a, d). *Striga* development was also hindered in the presence of nitrogen where the appearance of plants with 3–5 leaf pairs and more than 6 leaf pairs were decreased compared to the water treatment (Supplementary Fig. 7a). We tested whether this effect was due to improved host fitness or reduced *Striga* infectivity by treating *Striga* with DMBQ in the presence of nitrogen. Prehaustoria formation by DMBQ was significantly reduced in the presence of NH_4NO_3, NH_4Cl, or KNO_3 (Fig. 7b, c, Supplementary Fig. 7b) demonstrating that, like *P. japonicum*, early *Striga* haustoria formation is inhibited by high nitrogen. *Striga* is highly ABA[43] resistant, but nonetheless we tested exogenous application of ABA or fluridone and found they did not have an effect on *Striga* haustoria formation in the presence or absence of nitrogen (Fig. 7e, Supplementary Fig. 7c, d). Auxin biosynthesis is important for haustoria formation[14] and we found that auxin-related genes were upregulated during *Striga*-rice infection indicating a possible role for auxin in promoting *Striga* haustoria formation (Fig. 7f). We applied exogenous auxin to nitrogen-grown *Striga* and found it could overcome the inhibitory effects of nitrogen (Fig. 7g), suggesting a role for auxin acting downstream of nitrogen. Exogenous auxin treatment did not affect haustoria formation in *P. japonicum* and did not rescue the haustoria inhibitory effect of ABA or ½MS (Supplementary Fig. 7e, f) consistent with *P. japonicum* and *Striga* using different hormone signaling pathways for nitrogen inhibition.

Discussion

Here, we describe a mechanism whereby external nitrogen levels regulate haustoria formation in the facultative root parasite *P. japonicum* (Fig. 7h). This effect did not occur with phosphate or potassium and instead appeared highly specific to nitrogen in micromolar concentrations (Fig. 1f, g). A previous study showed

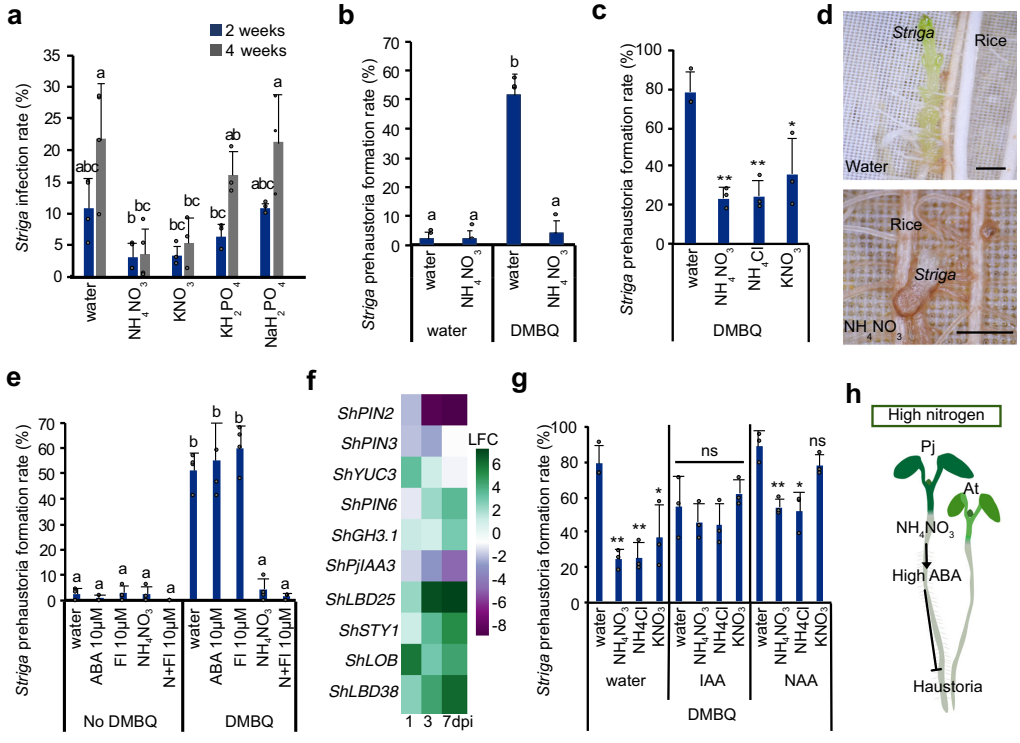

Fig. 7 Nitrogen inhibits *Striga* infection rates. a *Striga* infection rate of rice at 2 and 4 weeks with 20.6 mM KNO_3, 10.3 mM NH_4NO_3, 0.62 mM KH_2PO_4 or 1.9 mM NaH_2PO_4 (mean ± SD, $n = 8$ plants per treatment, 3 replicates). **b** Effect of 10.3 mM NH_4NO_3 on *Striga* prehaustorium formation induced by 10 µM DMBQ (mean ± SD, $n = 8$ per treatment, 3 replicates). **c** Effect of 10.3 mM NH_4NO_3, 10.3 mM NH_4Cl and 29.5 mM KNO_3 on *Striga* prehaustorium formation induced by 10 µM DMBQ (mean ± SD, $n = 8$ plants per treatment, 3 replicates). (d) Representative images of *Striga* infecting rice at 2 weeks after infection. Scale bars 1 mm. 3 replicates. **e**, **g** Effect of 10 µM ABA, 10 µM fluridone, 5 mM NH_4NO_3, 5 mM NH_4NO_3 + 10 µM fluridone, 500 nM NAA, 500 nM IAA, 10.3 mM NH_4NO_3, 10.3 mM NH_4Cl or 29.5 mM KNO_3 on *Striga* prehaustorium induction by 1 (**e**) or 10 (**g**) µM DMBQ (mean ± SD, $n = 8$ plants per treatment, 3 replicates). **f** Heatmap of *Striga* auxin-related gene expression during rice infection. **h** Graphical representation of a putative model of nitrate-ABA mediated haustoria regulation. **a**, **b**, **e** Different letters represent $P < 0.05$, one-way ANOVA followed by Tukey's HSD test. **c**, **g** Asterisks represent $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ compared to water treatments, Student's *t* test, two tailed. Source data provided.

that increased nitrogen supply to *Medicago sativa* also reduced *P. japonicum* parasitism[44], consistent with our results. We propose that local nitrogen supply at the site of infection has a suppressive effect upon the parasite in addition to nitrogen's proposed roles to stimulate host defenses and reduce host HIF production. *Striga* haustoria formation and infection rates were also inhibited by nitrogen (Fig. 7a–c) which is consistent with previous observations that external nitrogen reduces *Striga* growth[22,42]. However, our results point to an earlier additional role for nitrogen by preventing haustoria to develop beyond an initial swell (Fig.1h, Supplementary Fig. 7b). DHE and H_2DCFDA staining revealed nitrogen reduced ROS levels in *P. japonicum* roots prior to infection (Fig. 4), whereas quinone perception related genes *PjCADL2* and *PjCADL4* were downregulated by nitrogen which all could negatively affect HIF perception or downstream HIF signaling[12]. Alternatively, starvation could increase ROS and *PjCADL* levels to induce competency for HIF perception and haustoria elongation. As such, the observed reduction in *Striga* infestations in nutrient-rich fields[18] could be from a combination of reduced host germination stimulant production and our findings that nitrogen reduced haustoria formation and *Striga* growth. Our results also suggest a conserved role for nitrogen acting as a haustoria repressing factor in both facultative and obligate Orobanchaceae family members.

Beyond parasitism, nitrogen has strong effects upon plant root architecture and organogenesis. In *Arabidopsis*, mild nitrogen deficiency enhances lateral root elongation, whereas uniform high nitrogen levels repress lateral root development[45–47]. In nodulating plants, high nitrogen levels in the environment repress nodule formation in *Medicago truncatula*, soybean and alfalfa through a regulatory mechanism involving multiple hormones and peptides[48–52]. Our data and these previous findings suggest a common regulatory theme whereby low nitrogen levels promote organ growth to uptake additional nutrients, whereas high nitrogen levels repress organ growth to avoid unnecessary resources spent on nutrient acquisition.

In species like *Arabidopsis*, maize, rice and barley, high nitrates increase cytokinin levels and these are important for root development and shoot growth[27–30]. We found host and parasite cytokinin levels were strongly induced by infection (Fig. 5a,

Supplementary Fig. 6a), consistent with previous findings[8], but nitrogen itself did not increase tZ levels, tZR levels or induced a strong cytokinin response in young *P. japonicum* and had a mixed effect in mature *P. japonicum* where tZ levels increased but tZR levels decreased upon nitrate treatment (Supplementary Fig. 6b). Notably, a recent study found that nitrogen treatment of *Lotus japonicus* inhibited cytokinin biosynthesis, reduced cytokinin levels and reduced nodule formation[53]. We saw a similar situation in young *P. japonicum* since nitrogen treatment reduced trans-zeatin levels and did not induce a strong cytokinin response (Fig. 5a, d). Thus, both *P. japonicum* and *Lotus* appear to increase cytokinin levels in response to successful haustoria or nodule formation[8,54] yet do not necessarily increase cytokinin in response to high nitrogen. This situation differs from many other flowering plants and might be a convergent strategy to use cytokinin to signal successful symbiosis rather than nutrient abundance.

ABA plays an important role in parasitic plants and we observed that nitrogen increased ABA levels in *P. japonicum* independently of infection, while *P. japonicum* infection increased ABA levels in *Arabidopsis* (Fig. 5a). ABA levels are known to increase in both *Rhinanthus minor* and *Cuscuta japonica*, as well as their hosts, after infection[55,56]. *Striga* parasitism also increases host ABA levels in tomato and maize, and commonly induces symptoms in the host mimicking drought stress[57,58]. The increase in ABA we observed in the host *Arabidopsis* was likely due to a stress or defense response rather than movement from the parasite, however, increases in ABA levels in the parasite may have come in part from the host. Such ABA increases in the parasite appeared biologically relevant since treatments with exogenous ABA reduced haustoria numbers whereas perturbing ABA biosynthesis or ABA signaling in *P. japonicum* chemically or genetically overcame nitrogen inhibition (Fig. 6). These results suggest that nitrogen regulated haustoria formation in part via increasing ABA levels and ABA response which in turn repressed early stages of haustoria development including cell division. *Striga* and *Cuscuta* are highly insensitive to ABA[43,59] and *Striga* did not respond to ABA in our assays (Fig. 7, Supplementary Fig. 7), indicating that these species likely use additional mechanisms for nitrogen-induced haustoria repression such as modifying auxin response which differed from the situation in *P. japonicum* (Supplementary Fig. 7e, f). Other factors including SA or proteins known to affect lateral root or nodule formation likely also play a role in haustoria regulation.

Our assays revealed several developmental roles for ABA in *P. japonicum*. ABA was important for haustoria inhibition and ABA treatment produced haustoria that were underdeveloped or did not attach well, likely explaining the partial reduction in xylem bridge formation from ABA treatment (Fig. 6). ABA was also important for xylem development since chemical inhibition of ABA biosynthesis led to reduced xylem bridge formation whereas nitrogen and ABA treatments induced early xylem differentiation in the primary root tip (Fig. 6b, Supplementary Fig. 6l). However, nitrogen also reduced the expression of xylem-related genes in the haustoria and surrounding tissues (Fig. 3, Supplementary Fig. 2) which might relate to differing roles for ABA both promoting xylem differentiation but also inhibiting haustoria formation.

In nodulating plants, such as *Lotus japonicus*, *Trifolium repens* and *Medicago truncatula*, ABA acts as a negative regulator of nodules by repressing nod factor signaling and cytokinin responses[60–62]. Exogenous application of ABA blocks the early stages of infection in *Lotus japonicus*[61], similar to the situation we observe with haustoria in *P. japonicum*. We propose that at least some parasitic plants and legumes share another common regulatory theme whereby ABA inhibits symbiotic organ formation. However, more work will be required to investigate these parallels including whether nitrogen induces ABA in legumes and whether ABA inhibits HIF signaling in parasitic plants. Given that legumes and most parasitic plants are distantly related, it begs the question of whether such ABA and cytokinin regulatory features might be an important adaptation for symbiotic nutrient acquisition.

Methods

Plant materials and growth conditions. *P. japonicum* (Thunb.) Kanitz ecotype Okayama seeds harvested in Okayama and Karuizawa, Japan were used for our experiments[63]. *Arabidopsis* ecotype Columbia (Col-0) accession was used as *Arabidopsis* wild-type (WT). *Arabidopsis aba2-1* and *abi1-1C* were published previously[64,65]. For in vitro germination, seeds were surface sterilized with 70% (v/v) EtOH for 20 min followed by 95%(v/v) EtOH for 5 min then left to air-dry to remove remaining EtOH. The seeds were then sown on petri dishes containing ½MS medium (0.8% (w/v) plant agar, 1% (w/v) sucrose, pH 5.8). After overnight stratification in the dark and 4 °C, the plants were transferred to 25 °C long-day conditions (16-h light:8-h dark and light levels 100 µmol m^{-2} s^{-1}).

Striga hermonthica (Del.) Benth seeds were kind gifts provided by Dr A. G. T. Babiker (Environment and Natural Resources and Desertification Research Institute, Sudan). Rice seeds (*Oryza sativa L. subspecies japonica*, cvs Koshihikari) used in this study were originally obtained from National Institute of Biological Sciences (Tsukuba, Japan) and propagated in the Yoshida laboratory. The *Striga hermonthica* seeds were sterilized with a 20% (v/v) commercial bleach solution for 5 min and washed thoroughly with sterilized water on a clean bench. After that, these surface-sterilized *Striga* seed were placed in 9 cm petri dishes with moisturized glass fiber filter paper (Whatman GF/A) and conditioned at 25 °C in the dark for 7 days. The conditioned *Striga* seeds were treated with 10 nM Strigol[66] for 2 hours prior to rice-infection treatments. For haustorium induction assays, the conditioned *Striga* seeds were treated with 10 nM Strigol at 25 °C for 1 day in the dark before starting incubation in various nutrient media with or without DMBQ and hormones for 24 h in dark condition.

Rice seeds were de-husked and sterilized with a 20% (v/v) commercial bleach solution (Kao Ltd., Japan) for 30 min with gentle agitation. The rice seeds were then washed thoroughly with distilled water and placed on filter papers in 9 cm petri dishes filled with 15 mL sterilized water in a 16-h light/8-h dark cycle at 26 °C for 1 week.

In vitro infection assays with *P. japonicum*. Four to five days old *P. japonicum* seedlings were transferred for three days to nutrient-free 0.8% (w/v) agar medium or 0.8% (w/v) agar medium supplemented by nutrient or hormone treatment: ½MS, ½MS no N, 20.6 mM KNO_3, 50 µM-20.6 mM NH_4NO_3, 0.62 mM KH_2PO_4, 1.9 mM NaH_2PO_4, 10.3 mM NH_4Cl, 20.6 mM $NaNO_3$, 5 µM ABA, 10 µM Fluridone, 5 µM SA, 500 nM NAA, or 10.3 mM KCl. Five days old *Arabidopsis* seedling were aligned next to and roots place in contact with these pre-treated *P. japonicum* roots for infection assays. Haustorium formation and xylem bridge development were measured at seven days post infection using a Zeiss Axioscope A1 microscope. In vitro infection assays where a host was present were labeled as "infect" regardless of whether a successful infection occurred between host and parasite. Control assays where no host was added were labeled as "control". In these experiments 20 plants per sample were used and the experiments were replicated at least twice.

Haustorium induction assay. Four to five days old *P. japonicum* seedlings were transferred to nutrient-free 0.8% (w/v) agar medium or 0.8% (w/v) agar medium supplemented by nutrients (½MS, ½MS no N, NH_4NO_3) for a three days pre-treatment. Subsequently, seedlings were transferred to 0.8% (w/v) agar medium containing DMBQ (Sigma-Aldrich) or DMBQ with or without nutrient treatment and grown vertically for four to five days for haustorium induction. In these experiments 20 plants per sample were used and the experiments were replicated at least twice.

Greenhouse experiments. Ten days old *P. japonicum* seedlings were germinated in vitro as described above. The seedlings were then transferred to pots with 50:50 soil:sand ratio. *Arabidopsis* seeds were sprinkled around the *P. japonicum* seedling. The pots were placed at 25 °C and long-day conditions (16-h light:8-h dark and 100 µmol m^{-2} s^{-1}) and 60% humidity for 1.5 months. During this time the plants were given deionized water or water supplemented with fertilizer (commercial fertilizer Blömstra 51-10-43 N-P-K at 2 ml/L). Eight to fifteen plants per sample were used and this experiment was replicated three times.

Histological staining. Dissected *P. japonicum* roots or *P. japonicum* roots infecting *A. thaliana* were fixed in ethanol-acetic acid (75%/25%) solution under vacuum infiltration for 5 min. Then stained with Safranin-O solution (0.1%) at 90°C for 5 min. The root tissue was then cleared in chloral hydrate solution (chloral hydrate: glycerol: water 8:1:2) for two to three days before observation with a Zeiss Axioscope A1 microscope[13].

EdU and ROS staining. Five days old *P. japonicum* seedlings were treated with water or 10.3 mM NH_4NO_3 for three days before host addition (*Arabidopsis* Col-0). 20 plants per sample were collected at 0 and 24 hpi, these experiments were replicated twice. EdU (Click-iT™ EdU Cell Proliferation Kit, Invitrogen) staining was used for the estimation of cell division. Briefly, *P. japonicum* roots were incubated in 10 µM EdU for 30 min at 25 °C followed by tissue fixation and permeabilization following the manufacturer's instructions. EdU detection was performed with confocal microscopy (Zeiss LSM780).

For hydrogen peroxide staining 2′,7′-dichlorodihydrofluorescein diacetate CM-H_2DCFDA (excitation/emission 492 nm/517 nm; ThermoFisher™, C6827) was used and for superoxide staining dihydroethidium (DHE) (excitation/emission 510 nm/595 nm; Sigma-Aldrich, D7008) staining was used, *P. japonicum* roots were incubated in 10 µM CM-H_2DCFDA or 30 µM DHE solution in 50 mM PBS for 30 min in the dark with gentle shaking, followed by three times washing with 50 mM PBS. CM-H_2DCFDA or DHE detection was performed using confocal microscopy (Zeiss LSM780). Fluorescent intensity measurements were taken using ImageJ.

Xylem strand measurement. Five days old *P. japonicum* seedlings ($n = 19$) were treated with 1 µM ABA or 5 µM ABA, ½MS no N, ½MS or 5 mM NH_4NO_3 for three days. Afterwards, the number of xylem strands were measured at 2 mm from the root tip with a Zeiss Axioscope A1 microscope.

Sample preparation for RNAseq. 40 four to five days old *P. japonicum* seedlings were transferred to nutrient-free 0.8% (w/v) agar medium or 0.8% (w/v) agar medium supplemented with 10.3 mM NH_4NO_3 or 0.08 µM BA for 3 days prior to infection with *Arabidopsis* Col-0. As a control group, 40 *P. japonicum* seedlings per treatment (water, 10.3 mM NH_4NO_3 or 0.08 µM BA) remained without the *Arabidopsis* host. For the water treatment infect and control samples, five time points were prepared (0,12, 24, 48, 72 hpi). For the NH_4NO_3 and BA treatments, infect and control samples were prepared for three time points (0, 12, 24 hpi). One to two mm from *P. japonicum* and *Arabidopsis* root tips were harvested for the control plants and the 0 hpi infect plants. For the 12, 24, 48, 72 hpi time points, the haustorium, including 1–2 mm above and below tissue was collected together with the corresponding region of the *Arabidopsis* root. Three biological replicates were prepared for this experiment. RNA extraction was performed using the ROTI®Prep RNA MINI (Roth) kit following the manufacturer's instructions. The isolation of mRNA and library preparation were performed using NEBNext® Poly(A) mRNA Magnetic Isolation Module (#E7490), NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (# E7530L), NEBNext® Multiplex Oligos for Illumina® (#E7600) following the manufacturer's instructions. The libraries were then sequenced using paired end sequencing with an Illumina NovaSeq 6000.

Bioinformatic analysis. The adapter and low-quality sequences were removed using the fastp software with default parameters[67]. The quality-filtered reads were mapped to both the *P. japonicum*[68] and *Arabidopsis* genome (TAIR10) using STAR[69] and were separated based on mapping to *P. japonicum* and *Arabidopsis* reads. The separated reads were then re-mapped to their respective genomes. The read count was calculated using FeatureCounts[70]. The differential expression analysis was used to identify differentially expressed (DE) genes between treatments and time points and was performed using Deseq2 with the default settings and q-value < 0.05[71] (Supplementary Data 2–8). The gene expression clustering was performed using the Mfuzz software[72]. Custom annotations of the *P. japonicum* predicted proteins[68] were estimated using InterProScan[73], these were used for Gene ontology analysis that was performed using the topGO software[74]. ABA and cytokinin responsive genes in *P. japonicum* (Supplementary Data 9) were identified using the tBLASTp and tBLASTp algorithm of the *Arabidopsis* ABA and cytokinin responsive genes described by[75] or *Arabidopsis* genes responsive to SA described in[76] against the *P. japonicum* genome[68]. Cell cycle and ROS related genes (Supplementary Data 9) were identified using the tBLASTp and tBLASTn algorithm of the *Arabidopsis* cell cycle and ROS related genes described[77,78] in against the *P. japonicum* genome[68].

Statistics. Statistical analyses were performed using one-way ANOVA followed by Tukey's HSD post-hoc test. The results of this statistical analysis are represented by compact letter display; treatments with different letters are significantly different with p-value<0.05 whereas treatments with the same letter/letters are not significantly different. For haustoria per *P. japonicum* and xylem bridge formation percentage data, the statistical analyses were performed on the means of at least 2 biological replicates, where each biological replicate consisted of 20 plants. For single comparisons, two tailed student's t-tests was used.

qPCR. *P. japonicum* seedlings were grown for five days before transferring to nutrient-free 0.8% (w/v) agar medium or 0.8% (w/v) agar medium supplemented with ½MS, ½MS no N or 5 µM ABA for 5 days. Additionally, *P. japonicum* seedlings were placed on pots containing 100:0, 50:50, 33:66, 25:75 soil:sand ratios. The pots were placed at 25°C and long-day conditions (16 h light:8 h dark and 100 µmol m^{-2} s^{-1}) and 60% humidity for 1.5 months. During this time the plants were provided deionized water. The seedlings or the shoots and roots of the above described *P. japonicum* were then harvested and RNA extraction was performed using the ROTI®Prep RNA MINI (Roth) kit following the manufacturer's instructions. The extracted RNA was then treated with DNase I (Thermo Scientific™) following the manufacturer's instructions. cDNA synthesis was performed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific™) following the manufacturer's instructions. *PjPTB*[14] was used as an internal control. qPCR was performed with SYBR-Green master mix (Applied Biosystems™). The relative expression was calculated using the Pfaffl method[79]. All experiments were repeated at least three times with at least two technical replications each. For statistical analysis, the student's t-test was used. The primers used for this experiment are listed in Supplementary Data 10. 4 plants per sample were collected and these experiments were replicated three times.

Hormonal quantifications. *P. japonicum* seedlings were grown for four to five days before transferring to nutrient-free 0.8% (w/v) agar medium or 0.8% (w/v) agar medium supplemented with 10.3 mM NH_4NO_3 for three days. *Arabidopsis* Col-0 or *aba2-1* was placed next to the *P. japonicum* seedlings and left for 10 days. *P. japonicum* seedlings without a host were used as control. After 10 days with or without the presence of a host, four entire *P. japonicum* seedlings per sample and four to five entire *Arabidopsis* seedlings per sample were collected. For the mature *P. japonicum* root measurements, ~1-month-old *P. japonicum* was transferred to nutrient-free 0.8% (w/v) agar medium or 0.8% (w/v) agar medium supplemented with 10.3 mM NH_4NO_3 for seven days before *Arabidopsis* Col-0 addition. 4 roots per sample were collected at 10 dpi. The samples were crushed to powder using liquid N with mortar and pestle. Samples were extracted, purified and analyzed according to a previously published method[80]. Approximately 20 mg of frozen material per sample was homogenized and extracted in 1 mL of ice-cold 50% aqueous acetonitrile (v/v) with the mixture of ^{13}C- or deuterium-labeled internal standards using a bead mill (27 hz, 10 min, 4 °C; MixerMill, Retsch GmbH, Haan, Germany) and sonicator (3 min, 4 °C; Ultrasonic bath P 310 H, Elma, Germany). After centrifugation (20000 × g, 15 min, 4 °C), the supernatant was purified as following. A solid-phase extraction column Oasis HLB (30 mg 1 cc, Waters Inc., Milford, MA, USA) was conditioned with 1 ml of 100% methanol and 1 ml of deionized water (Milli-Q, Merck Millipore, Burlington, MA, USA). After the conditioning steps each sample was loaded on SPE column and flow-through fraction was collected together with the elution fraction 1 ml 30% aqueous acetonitrile (v/v). Samples were evaporated to dryness using speed vac (SpeedVac SPD111V, Thermo Scientific, Waltham, MA, USA). Prior LC-MS analysis, samples were dissolved in 40 µL of 30% acetonitrile (v/v) and transferred to insert-equipped vials. Mass spectrometry analysis of targeted compounds was performed by an UHPLC-ESI-MS/MS system comprising of a 1290 Infinity Binary LC System coupled to a 6490 Triple Quad LC/MS System with Jet Stream and Dual Ion Funnel technologies (Agilent Technologies, Santa Clara, CA, USA). The quantification was carried out in Agilent MassHunter Workstation Software Quantitative (Agilent Technologies, Santa Clara, CA, USA). These experiments were repeated 4 times.

Plasmid construction and *P. japonicum* transformation. Plasmid construction was done using modules of Greengate cloning[81] (Addgene). For the entry modules, the *pMAS* promoter, *terMAS* terminator, and *DsRed* reporter cassette were amplified from pAGM4723 using primers with the addition of *BsaI* restriction sites and respective overhangs on the 5'ends (Supplementary Data 10), then inserted into Greengate modules pGGA000, pGGE000, and pGGF000, to create pGGA-pMAS, pGGE-terMAS, and pGGF-DsRed modules, respectively. The CDS of *Atabi1-1* was amplified from the cDNA of *Arabidopsis abi1-1* mutant, then inserted into pGGC000 to create pGGC-*abi1*. All of the restriction and ligation reactions were done using BsaI-HF and T4 ligase (NEB), respectively. The resulted plasmids were transformed into *E. coli* using chemically competent cells (Subcloning Efficiency™ DH5α Competent Cells, ThermoFisher Scientific) according to the manufacturer's protocol. The transformed cells were cultured and selected on LB medium with 100 µg/mL ampicillin. The plasmids were extracted using Plasmid DNA Miniprep Kit (ThermoFisher), and the sequences were confirmed by sequencing of the ligation sites (Macrogen).

To create the final binary vector pGG-*abi1*, the Greengate reaction was performed using the previously described protocol[81] using the entry vectors pGGA-pMAS, pGGB003, pGGC-abi1, pGGD002, pGGE-terMAS, pGGF-DsRed, and the empty destination vector pGGZ001. The reaction product was used for *E. coli* transformation, then the cells were cultured on LB medium with 100 µg/mL spectinomycin. The sequence was initially confirmed by digestion analysis, then sequencing of the ligation site. The plasmid was then inserted in electrocompetent *Agrobacterium rhizogenes* strain AR1193 then the cells were cultured on LB medium with 100 µg/mL spectinomycin and 50 µg/mL rifampicin.

P. japonicum transformation was performed according to a previously published method[36]. Briefly, three to four-day-old *P. japonicum* seedlings were sonicated for 10 to 15 seconds followed by vacuum infiltration for 5 minutes with suspension of *Agrobacterium rhizogenes* strain AR1193 carrying the overexpressing *pMAS::Atabi1-1* construct. The seedlings were then transferred on co-cultivation media (Gamborg's B5 medium, 0.8% agar, 1% sucrose, 450 µM acetosyringone) at 22 °C for 2 days in the dark conditions. Later, plants were transferred on Gamborg's B5 medium supplemented with antibiotic (0.8% agar, 1% sucrose, 300 µg/ml cefotaxime) and incubated at 25 °C under long-day conditions for

~1 month. Hairy roots expressing the construct were identified by red fluorescence using a Leica M205 FA fluorescence stereo microscope. Roots were selected based on their florescent status before being placed on nutrient-free 0.8% (w/v) agar medium or 0.8% (w/v) agar medium supplemented by ½MS or ½MS no N for 3 days prior to *Arabidopsis* (Col-0) host application. Haustoria numbers were estimated at 7 dpi. This experiment was replicated three times. Construct expression levels were estimated by qPCR using *Atabi1-1* primers (Supplementary Data 10).

***Striga*-rice Infection in the rhizotron system**. The rice infection was performed in a rhizotron system[63]. 7-day-old rice seedlings were transferred to the rhizotron (10-cm × 14-cm-square petri dish with top and bottom perforation for shoot growth and water draining, filled with same size of rockwool [Nichiasu, Tokyo, Japan] onto which a 100 µm nylon mesh was placed) and fertilized with 25 mL half-strength Murashige & Skoog media per rhizotron. The root parts of the rhizotron were covered with aluminum foil and placed vertically in a growth chamber at 12-h light: 28 °C /12-h dark: 20 °C cycles for 2 weeks before *S. hermonthica* infection. Rice seedlings were inoculated with *S. hermonthica* seeds by placing Strigol-treated *S. hermonthica* carefully along rice roots with 5 mm intervals. Each rhizotron was inoculated by 20-60 *S. hermonthica* seeds. The rhizotron containing inoculated rice seedlings were incubated in the growth chamber described above, and developmental stages of *S. hermonthica* were categorized with a stereomicroscope (Zeiss Stemi 2000-C) after 2 and 4 weeks. Successful infection rates were calculated by the number of *S. hermonthica* with more than three leaf pairs divided by the total infected *S. hermonthica* seeds. Each rhizotron was watered with 25 mL of indicated nutrient or chemical containing solutions two times per week. The chemical concentrations used in this study were as following; 10.3 mM ammonium nitrate, 1.09 mM monosodium phosphate, 20.6 mM potassium nitrate, 0.62 mM monopotassium phosphate, 10 µM gibberellic acid, 0.08 nM 6-benzylaminopurine, 10 µM paclobutrazol, 10 or 100 µM fluridone, and 10 or 100 µM abscisic acid, 10.3 mM NH_4Cl, 19.69 mM KNO_3, 500 nM IAA or 500 nM NAA. 8 plants per sample were used and these experiments we replicated at least 3 times.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Sequence data are available at the Gene Expression Omnibus under accession numbers GSE177484. Sequence data of the *Phtheirospermum japonicum* genes studied in this article are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers provided in Supplementary Data 11. Other experimental data shown in this study are provided in the Source Data file.

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Author contributions

A.K. and C.W.M. conceived the experiments. A.K., M.L., X.Z., T.S., and J.S. performed the experiments. S.C., S.Y., K.L., and C.W.M. supervised the experiments. A.K. and C.W.M. wrote the paper. All authors edited and revised the final paper.

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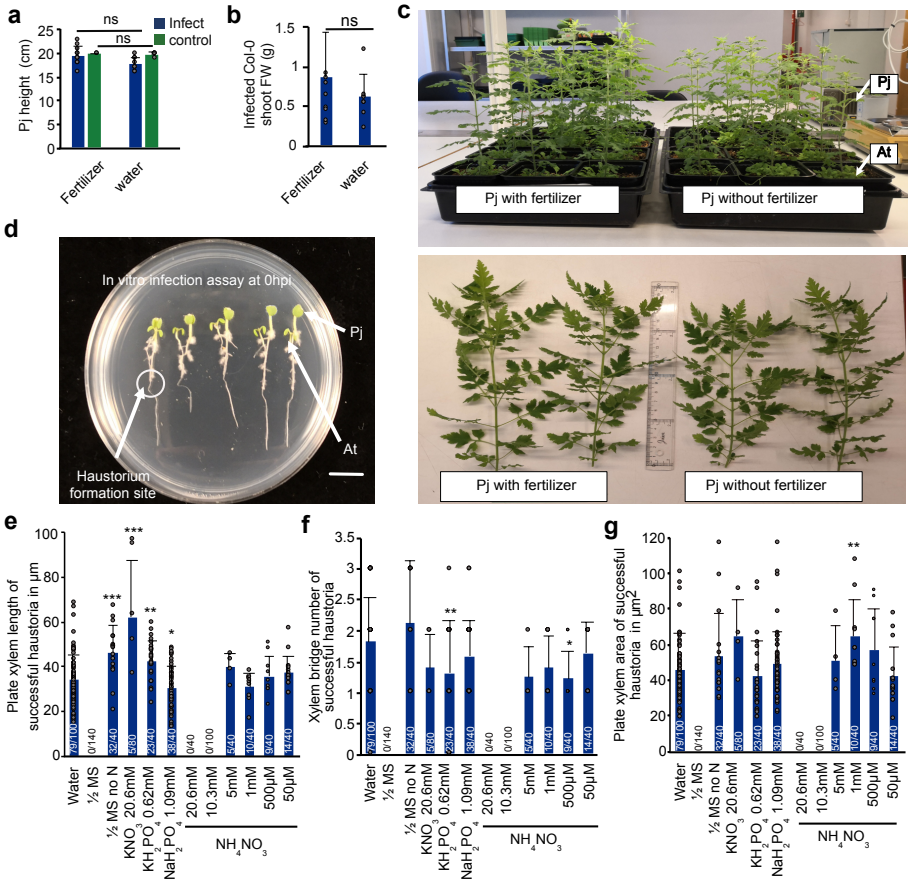
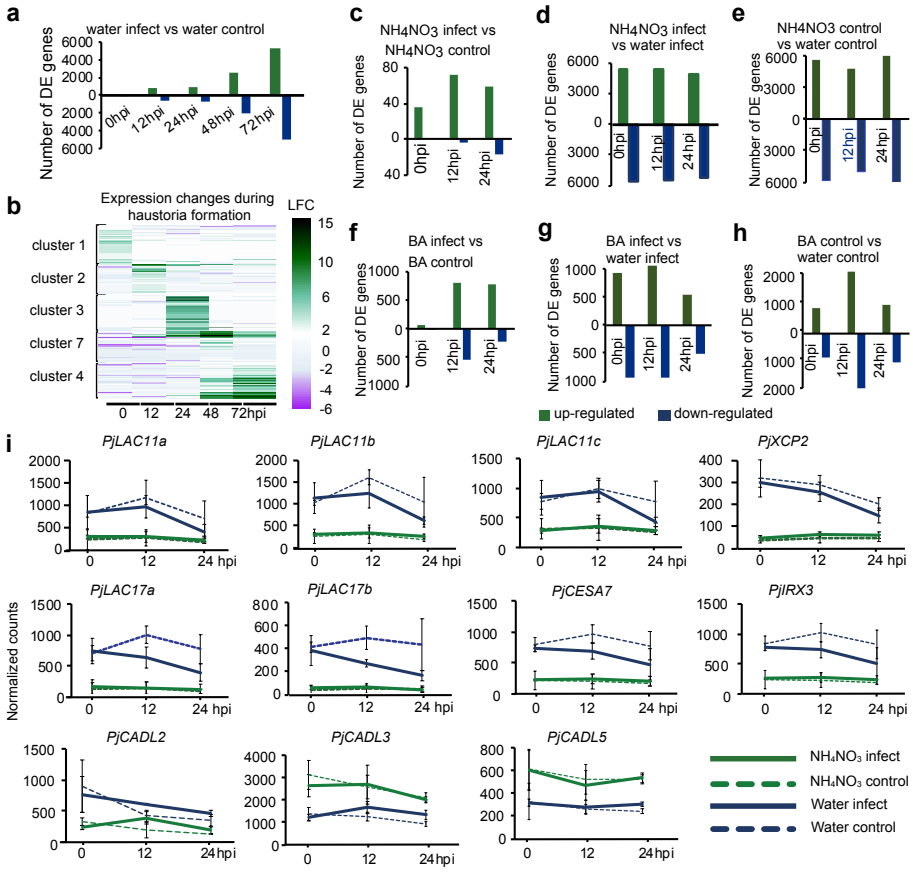


Supplementary Figure 1 Nutrient availability does not affect *P. japonicum* shoot growth and plate xylem size. (a-b) *P. japonicum* height and *Arabidopsis* Col-0 shoot fresh weight (FW) with or without fertilizer application during infection of *Arabidopsis* by *P. japonicum* (mean±SD, Fertilizer Pj infect n= 11 plants, Pj control n=3 plants, water infect n= 8 plants, control n= 4 plants, Col-0 fertilizer n= 11, water n= 8). (c) Photos of *P. japonicum* infecting with and without fertilizer application. (d) Photo of the *in vitro* infection assay set-up. Scale bar 1 cm. (e-g) Plate xylem length (µM), plate xylem area (µm^2) and xylem bridge number per haustorium under nutrient treatments (1/2MS, 1/2MS no N, KNO_3, NH_4NO_3, KH_2PO_4 or NaH_2PO_4), the numbers in the base of each column represent the number of measurements taken over the total number of plants, bars represent mean±SD. (a, b, e, f, g). Asterisks represent *P<0.05, **P<0.001, ***P<0.0001 compared to the water treatment, Student's t-test, two tailed. Source data provided.



Supplementary Figure 2 NH_4NO_3 affects gene expression and xylem genes. (a, c-h) Number of genes differentially expressed over three or five time points in the water only, NH_4NO_3 and BA RNAseq dataset between *P. japonicum* infect and control and in the NH_4NO_3 or BA vs the water RNAseq dataset in *P. japonicum*. (b) Heatmap of the log2 fold change of gene subsets that belong to five co-expression clusters over five time points in the water-only RNAseq dataset between *P. japonicum* water infect vs water control. (i) Normalized counts of *PjXCP2, PjLAC11a,b,c, PjLAC17a,b, PjIRX3, PjCESA7, PjCADL2*, *PjCADL3*, *PjCADL5* over three time points shown for *P. japonicum* control and infect in the NH_4NO_3 and water treatment (mean±SD, n=3 libraries).

cluster 1		p-value
GO:0016192	vesicle-mediated transport	3.10E-08
GO:0016567	protein ubiquitination	0.00023
GO:0034613	cellular protein localization	0.00269
GO:0023052	signalling	0.00093
GO:0015693	magnesium ion transport	0.01378
GO:0006808	regulation of nitrogen utilization	0.03685

cluster2		p-value
GO:0006260	DNA replication	2.50E-05
GO:0006397	mRNA processing	0.00045
GO:0008654	phospholipid biosynthetic process	0.00178
GO:0006281	DNA repair	0.00047
GO:0006413	translational initiation	0.00594
GO:0009611	response to wounding	0.00841
GO:1901657	glycosyl compound metabolic process	0.0073
GO:0046039	GTP metabolic process	0.01748
GO:0006396	RNA processing	1.60E-07
GO:0006366	transcription by RNA polymerase II	0.03476
GO:0030243	cellulose metabolic process	0.03904

cluster3		p-value
GO:0007018	microtubule-based movement	3.80E-13
GO:0045944	positive regulation of transcription	2.90E-05
GO:0000165	MAPK cascade	0.01294
GO:0033014	tetrapyrrole biosynthetic process	0.00602
GO:0007017	microtubule-based process	1.20E-12
GO:0044267	cellular protein metabolic process	0.00340

cluster4		p-value
GO:0006508	proteolysis	6.70E-07
GO:2001141	regulation of RNA biosynthetic process	7.50E-05
GO:0005975	carbohydrate metabolic process	1.40E-06
GO:0005985	sucrose metabolic process	0.00490
GO:0010411	xyloglucan metabolic process	0.00570
GO:0046274	lignin catabolic process	0.00710
GO:0007064	mitotic sister chromatid cohesion	0.01870
GO:0006952	defence response	0.03330

cluster 5		p-value
GO:0055114	oxidation-reduction process	2.30E-07
GO:0009060	aerobic respiration	3.80E-05
GO:0006979	response to oxidative stress	0.0024
GO:0006561	proline biosynthetic process	0.0029
GO:0042744	hydrogen peroxide catabolic process	0.0178
GO:0009073	aromatic amino acid family biosynthetic process	0.0238
GO:0030418	nicotianamine biosynthetic process	0.024
GO:0045944	positive regulation of transcription	0.0281
GO:0006633	fatty acid biosynthetic process	0.0293

cluster6		p-value
GO:0016192	vesicle-mediated transport	3.30E-17
GO:0046907	intracellular transport	1.10E-11
GO:0034613	cellular protein localization	1.70E-11
GO:0007264	small GTPase mediated signal transduction	1.80E-08
GO:0030163	protein catabolic process	0.0001
GO:0051225	spindle assembly	0.00017
GO:0015991	ATP hydrolysis coupled proton transport	0.0005
GO:0072330	monocarboxylic acid biosynthetic process	0.00015
GO:0016310	phosphorylation	0.0005
GO:0015833	peptide transport	2.40E-11
GO:0016052	carbohydrate catabolic process	0.01438

cluster 7		p-value
GO:0055114	oxidation-reduction process	0.00022
GO:0006779	porphyrin-containing compound biosynthesis	0.00046
GO:0030001	metal ion transport	0.00027
GO:0006749	glutathione metabolic process	0.00666
GO:0055085	transmembrane transport	0.00461
GO:0051252	regulation of RNA metabolic process	0.06845
GO:0009690	cytokinin metabolic process	0.02798
GO:0006979	response to oxidative stress	0.02835

cluster8		p-value
GO:0006412	translation	< 1e-30
GO:0022613	ribonucleoprotein complex biogenesis	7.80E-22
GO:0006396	RNA processing	2.30E-17
GO:0034660	ncRNA metabolic process	2.50E-14
GO:0009451	RNA modification	8.70E-09
GO:0006457	protein folding	1.10E-07
GO:0032259	methylation	1.50E-08
GO:0071826	ribonucleoprotein complex subunit	5.90E-08
GO:0016071	mRNA metabolic process	2.40E-07
GO:0042455	ribonucleoside biosynthetic process	1.00E-06
GO:0009089	lysine biosynthetic process	3.60E-05

Supplementary Figure 3 Gene ontology of the co-expression clusters. Gene ontology analysis for the differentially expressed genes assigned to each co-expression cluster. GO categories with the lowest p-values are shown (P<0.05 Fisher's exact test). Source data provided.

a

NH_4NO_3 control vs water control up-regulated

0hpi		p-value	12hpi		p-value	24hpi		p-value
GO:2000028	regulation of photoperiodism	0.0001	GO:0008272	sulfate transport	0.0006	GO:0009607	response to biotic stimulus	4.30E-07
GO:0051252	RNA metabolism regulation	0.0014	GO:0055114	oxidation-reduction process	0.0006	GO:0051252	RNA metabolism regulation	7.80E-06
GO:0006000	fructose metabolic process	0.0012	GO:0051252	RNA metabolism regulation	0.0040	GO:0055114	oxidation-reduction process	1.20E-04
GO:0005992	trehalose biosynthetic process	0.0021	GO:0005992	trehalose biosynthesis	0.0013	GO:0019344	cysteine biosynthetic process	0.0003
GO:0015698	inorganic anion transport	0.0006	GO:0009584	detection of visible light	0.0017	GO:0006952	defense response	0.0012
GO:0015969	guanosine tetraphosphate metabolism	0.0023	GO:0018298	protein-chromophore linkage	0.0017	GO:0006563	L-serine metabolic process	0.0013
GO:0055114	oxidation-reduction process	0.0035	GO:0009607	response to biotic stimulus	0.0040	GO:0019318	hexose metabolic process	7.20E-05
GO:0015995	chlorophyll biosynthesis	0.0086	GO:0006001	fructose catabolic process	0.0117	GO:0009785	blue light signaling pathway	0.0038
GO:0015979	photosynthesis	0.0028	GO:0034755	iron ion transmembrane transport	0.0117	GO:0006817	phosphate ion transport	0.0041
GO:0000160	phosphorelay signal transduction	0.0100	GO:0042128	nitrate assimilation	0.0117	GO:0015969	guanosine tetraphosphate metabolism	0.0041

NH_4NO_3 control vs water control down-regulated

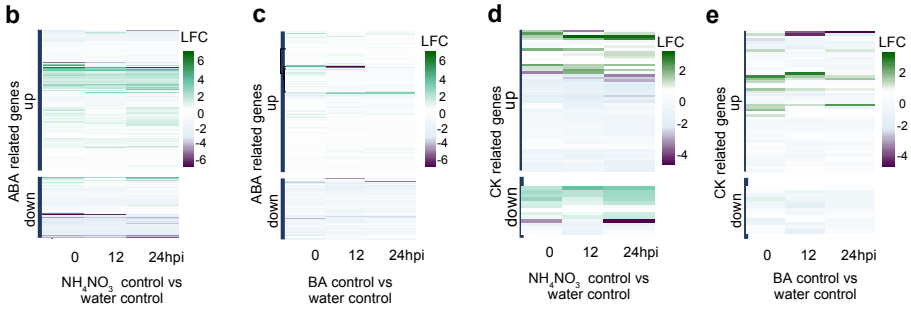
0hpi		p-value	12hpi		p-value	24hpi		p-value
GO:0042737	drug catabolic process	8.40E-06	GO:0046274	lignin catabolic process	5.60E-08	GO:0007017	microtubule-based process	1.30E-08
GO:0046274	lignin catabolic process	0.0001	GO:0042737	drug catabolic process	1.10E-06	GO:0046274	lignin catabolic process	9.10E-05
GO:0071554	cell wall organization/biogenesis	0.0001	GO:0055114	oxidation-reduction process	0.0009	GO:0008610	lipid biosynthetic process	6.20E-05
GO:0006857	oligopeptide transport	0.0031	GO:0006979	response to oxidative stress	0.0008	GO:0009690	cytokinin metabolic process	0.0011
GO:0006979	response to oxidative stress	0.0035	GO:0005975	carbohydrate metabolism	0.0003	GO:0006631	fatty acid metabolic process	0.0007
GO:0055085	transmembrane transport	0.0061	GO:0045492	xylan biosynthetic process	0.0057	GO:0009813	flavonoid biosynthesis	0.0026
GO:0005975	carbohydrate metabolism	0.0057	GO:0007017	microtubule-based process	0.0066	GO:0042737	drug catabolic process	0.0005
GO:0009269	response to desiccation	0.0269	GO:0000079	regulation of protein synthesis	0.0082	GO:0008202	steroid metabolic process	0.0040
GO:0044264	polysaccharide metabolism	0.0344	GO:0015743	malate transport	0.0086	GO:0055114	oxidation-reduction process	0.0040
GO:0042219	amino acid catabolism	0.0366	GO:0006542	glutamine biosynthesis	0.0139	GO:0006555	methionine metabolism	0.0084

BA control vs water control up-regulated

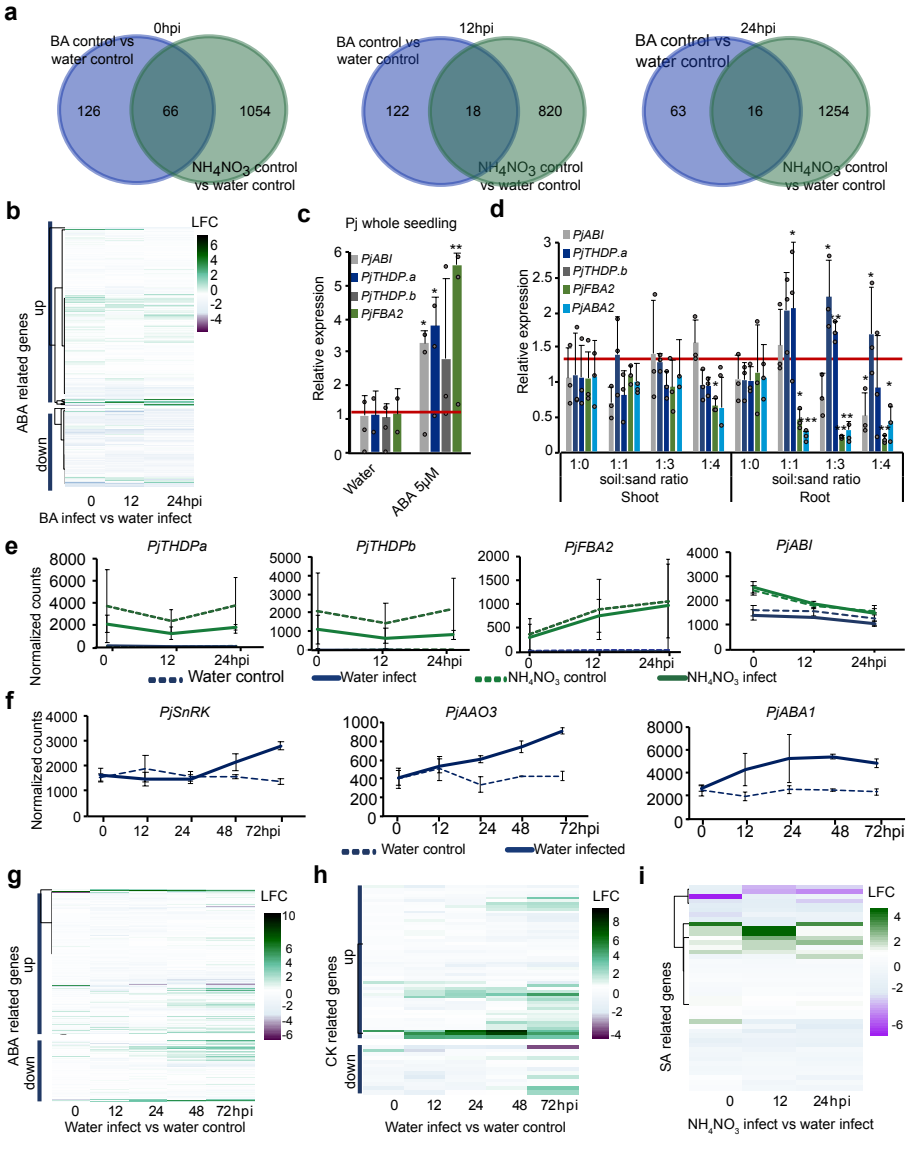
0hpi		p-value	12hpi		p-value	24hpi		p-value
GO:0009690	cytokinin metabolic process	1.50E-08	GO:0006260	DNA replication	1.10E-26	GO:0006412	translation	6.40E-07
GO:0000160	phosphorelay signal transduction	8.60E-08	GO:0006412	translation	1.40E-26	GO:0009690	cytokinin metabolic process	6.60E-05
GO:0055114	oxidation-reduction process	2.10E-06	GO:0006457	protein folding	6.00E-16	GO:0006457	protein folding	1.30E-04
GO:0006006	glucose metabolic process	0.0001	GO:0007018	microtubule-based movement	3.3E-13	GO:0009152	purine ribonucleotide synthesis	0.0004
GO:0006855	drug transmembrane transport	0.0004	GO:0006265	DNA topological change	10E-05	GO:0009168	ribonucleoside monophosphate synth	0.0004
GO:0009607	response to biotic stimulus	0.0027	GO:0006310	DNA recombination	5.50E-06	GO:0009206	ribonucleoside triphosphate synthesis	0.0005
GO:0042737	drug catabolic process	0.0018	GO:0009690	cytokinin metabolic process	0.0001	GO:0006855	drug transmembrane transp.	0.0008
GO:0008272	sulfate transport	0.0101	GO:0009082	amino acid biosynthesis	0.0003	GO:0006979	response to oxidative stress	0.0009
GO:0009664	plant-type cell wall organization	0.0177	GO:0006281	DNA repair	0.0004	GO:0042744	hydrogen peroxide catabolism	0.0027
GO:0006979	response to oxidative stress	0.0200	GO:0065004	protein-DNA complex assembly	0.0014	GO:0009664	cell wall organization	0.0048

BA control vs water control down-regulated

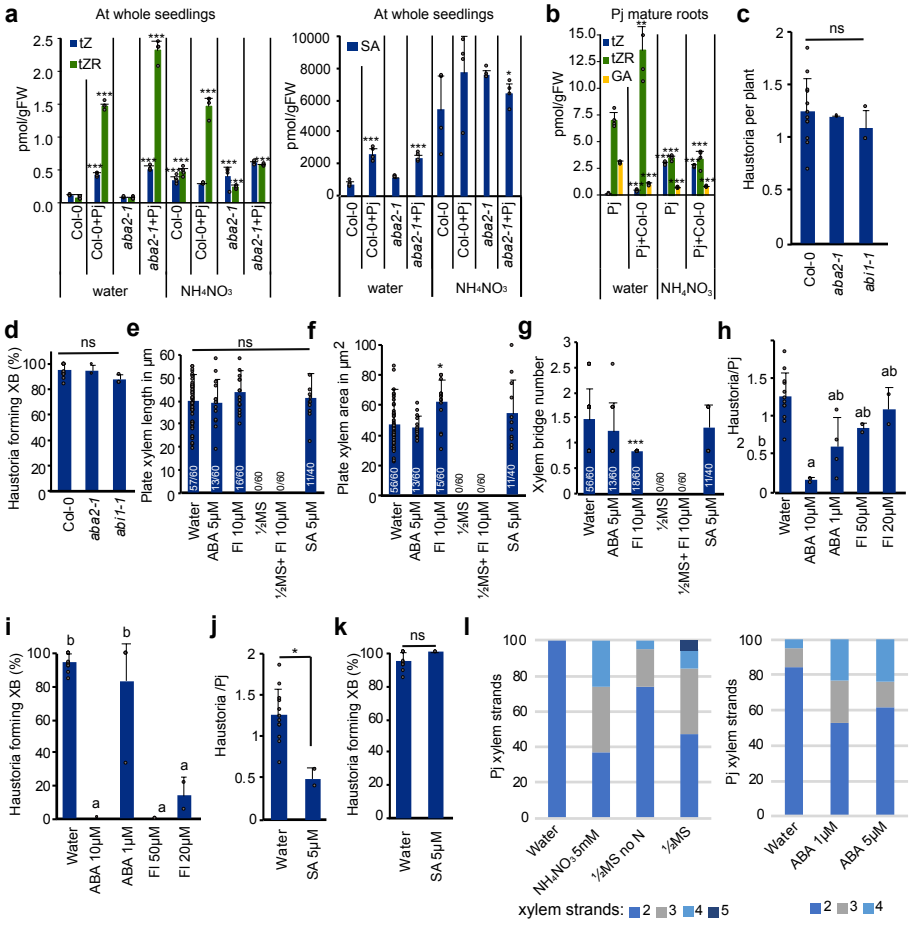
0hpi		p-value	12hpi		p-value	24hpi		p-value
GO:0019419	sulfate reduction	0.00076	GO:0055114	oxidation-reduction process	7.10E-05	GO:0006355	regulation of transcription	4.60E-04
GO:0071555	cell wall organization	0.00275	GO:0046274	lignin catabolic process	7.70E-05	GO:0006857	oligopeptide transport	5.90E-04
GO:0006857	oligopeptide transport	0.00332	GO:0019344	cysteine biosynthesis	0.0004	GO:0019419	sulfate reduction	9.60E-04
GO:0048544	recognition of pollen	0.00918	GO:0006468	protein phosphorylation	0.0002	GO:0005992	trehalose biosynthesis	0.0024
GO:0046274	lignin catabolic process	0.01246	GO:0009607	response to biotic stimulus	0.0002	GO:0019344	cysteine biosynthesis	0.0041
GO:0055085	transmembrane transport	0.05784	GO:0005992	trehalose biosynthesis	0.0009	GO:0055085	transmembrane transport	0.0205
GO:0015936	coenzyme A metabolism	0.01453	GO:0051179	localization	0.0033	GO:0048544	recognition of pollen	0.0147
GO:0006270	DNA replication initiation	0.02401	GO:0042737	drug catabolic process	0.0013	GO:0055114	oxidation-reduction process	0.0121
GO:0070588	Ca ion transmembrane transp.	0.02401	GO:0070588	Ca ion transmembrane transp.	0.0017	GO:0016567	protein ubiquitination	0.0286
GO:0055114	oxidation-reduction process	0.01327	GO:0008283	cell proliferation	0.0024	GO:0006558	L-phenylalanine metabolism	0.0299



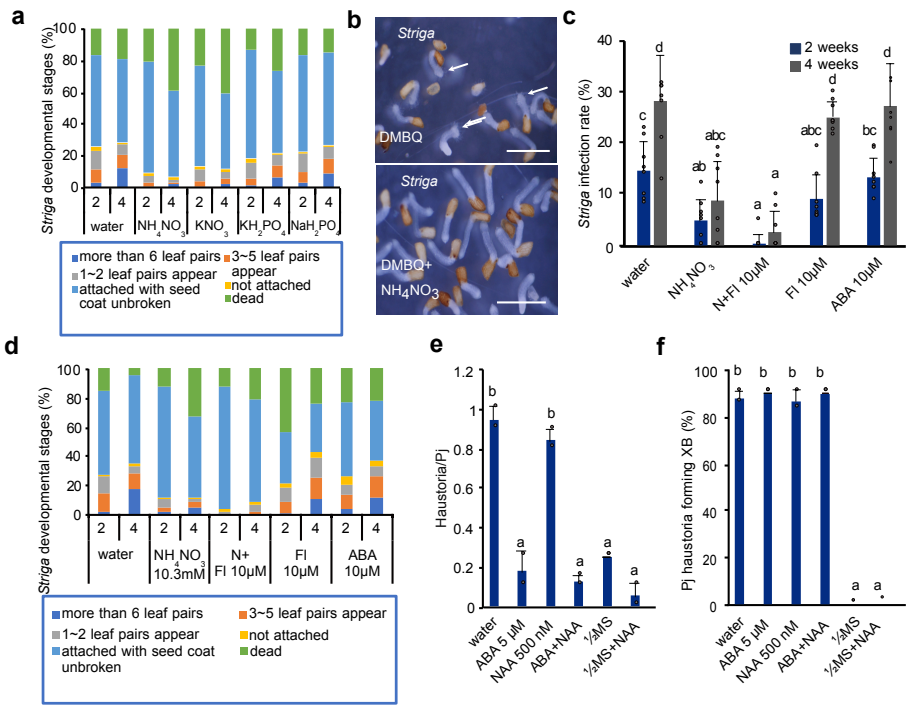
Supplementary Figure 4 Gene ontology analysis of the up and down regulated genes in NH_4NO_3 and BA control treatments. (a) Gene ontology analysis for genes differentially expressed during the three time points in the NH_4NO_3 control and BA control vs water control RNAseq datasets in *P. japonicum*, shown are the top 10 GO categories with P< 0.05 Fisher's exact test. (b-e) Heatmaps of the log2 fold change of 629 genes homologous to *Arabidopsis* ABA responsive genes (up or down regulated) and 67 genes homologous to *Arabidopsis* cytokinin responsive genes (up or down regulated) shown over three time points in the NH_4NO_3 control vs water control or in the BA control vs water control RNAseq datasets in *P. japonicum*.



Supplementary Figure 5 Expression changes of ABA related genes. (a) Venn diagrams of the DE genes in BA and NH_4NO_3 treatments over three time points in *P. japonicum*. (b) Heatmap of the log2 fold change of 170 genes homologous to *Arabidopsis* ABA responsive genes shown over three time points in the BA infected vs water infected RNAseq dataset in *P. japonicum*. (c-d) Expression levels of *PjABI, PjFBA2, PjABA2 and PjTHDPa,b* with water, 5 μM ABA and various soil:sand ratios analyzed by RT-qPCR (mean±SD, n=4 plants per treatment, 3 replicates). (e-f) Normalized counts of *PjSnRK, PjAAO3, PjABA1, PjFBA2, PjTHDPa,b, PjABI* over five time points shown for *P. japonicum* water and NH_4NO_3 treatments (mean±SD, n=3 libraries). (g-h-i) Heatmaps of the log2 fold change of 629 genes homologous to *Arabidopsis* ABA responsive genes (up or down regulated), 67 genes homologous to *Arabidopsis* cytokinin responsive genes (up or down regulated) and 45 SA related genes shown over five time points in the water infect vs water control or over three time points in the NH_4NO_3 infect vs water infect RNAseq datasets in *P. japonicum.* (c, d)Asterisks represent *P<0.05, **P<0.001, ***P<0.0001 compared to control treatments, Student's t-test, two tailed. Source data provided.



Supplementary Figure 6 Host ABA levels do not affect *P. japonicum* infection. (a-b) Hormonal quantification of salicylic acid (SA), gibberellic acid A1 (GA), trans-zeatin (tZ) and trans-zeatin riboside (tZR) in *Arabidopsis* (Col-0, *aba2*-1) whole seedlings and *P. japonicum* mature roots treated with 10.3 mM NH_4NO_3 (mean±SD, n= 4 plants per treatment, 4 replicates). (c-d) Average number of haustoria per *P. japonicum* seedling and xylem bridge formation percentage in *in vitro* infection assays with *Arabidopsis* Col-0, *aba2-1* and *abi1-1C* (*abi1-1*) as the host (mean±SD, n=20 plants per treatment, 3 replicates). (e-f-g) Plate xylem length (µM), plate xylem area (μm^2) and xylem bridge number per haustorium under ABA, fluridone, ½MS, ½MS + fluridone or SA treatments, the numbers in the base of each column represent the number of measurements taken over the total number of plants, bars represent mean±SD. (h-k) Average number of haustoria per *P. japonicum* seedling and xylem bridge formation percentage in *in vitro* infection assays with *Arabidopsis* Col-0 with ABA, fluridone, SA (mean±SD, n=20 plants per treatment per replicate, 2 replicates, ABA 1µM 4 replicates). (l) Number of lignified xylem strands at 2 mm from the root tip in *P. japonicum* seedlings treated with NH_4NO_3, ½MS, ½MS no N, ABA (n=19 roots per treatment). (c, d, h, i) Different letters represent one-way ANOVA followed by Tukey's HSD test P<0.05. (a, b, e, f, g, j, k) Asterisks represent *P<0.05, **P<0.001, ***P<0.0001 compared to Col-0 or water treatments, Student's t-test, two tailed. Source data provided.



Supplementary Figure 7 Effect of ABA on *Striga*. (a) Effect of 20.6 mM KNO_3, 10.3 mM NH_4NO_3, 0.62 mM KH_2PO_4, 1.9 mM NaH_2PO_4 on *Striga* development at two and four weeks after infection (mean±SD, n=8 plants per treatment, 3 replicates). (b) Images of *Striga* haustorium formation assay with 1 μM DMBQ or 1 μM DMBQ+ 10.3 mM NH_4NO_3 at 1 day after treatment. The arrows denote pre-haustoria. Scale bars 1 mm. 3 replicates. (c) *Striga* infection rates (*Striga* with more than 3 leaves after rice infection over the total *Striga* number) at two and four weeks after infection with rice as a host under ABA, fluridone, 5 mM NH_4NO_3 or 5 mM NH_4NO_3 + fluridone treatments (mean±SD, n=8 plants per treatment, 3 replicates). (d) Effect of ABA, fluridone, NH_4NO_3 or NH_4NO_3 + fluridone treatments on *Striga* development at two and four weeks after infection (mean±SD, n=8 plants per treatment, 3 replicates). (e-f) Average number of haustoria per *P. japonicum* seedling and xylem bridge formation percentage in *in vitro* infection assays with *Arabidopsis* Col-0 with 5 μM ABA, 500 nM NAA, ½MS, 5 μM ABA+500 nM NAA and ½MS+500 nM NAA (mean±SD, n=20 plants per treatment, 2 replicates). (c, e, f) Different letters represent one-way ANOVA followed by Tukey's HSD test $P<0.05$. Source data provided.

III

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Pectin modifications promote haustoria development in the parasitic plant *Phtheirospermum japonicum*

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Abstract

Parasitic plants are globally prevalent pathogens with important ecological functions but also potentially devastating agricultural consequences. Common to all parasites is the formation of the haustorium which requires parasite organ development and tissue invasion into the host. Both processes involve cell wall modifications. Here, we investigated a role for pectins during haustorium development in the facultative parasitic plant *Phtheirospermum japonicum*. Using transcriptomics data from infected Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), we identified genes for multiple *P. japonicum* pectin methylesterases (PMEs) and their inhibitors (PMEIs) whose expression was upregulated by haustoria formation. Changes in *PME* and *PMEI* expression were associated with tissue-specific modifications in pectin methylesterification. While de-methylesterified pectins were present in outer haustorial cells, highly methylesterified pectins were present in inner vascular tissues, including the xylem bridge that connects parasite to host. Specifically blocking xylem bridge formation in the haustoria inhibited several *PME* and *PMEI* genes from activating. Similarly, inhibiting PME activity using chemicals or by overexpressing *PMEI* genes delayed haustoria development. Our results suggest a dynamic and tissue-specific regulation of pectin contributes to haustoria initiation and to the establishment of xylem connections between parasite and host.

Introduction

Parasitic plants, which constitute around 1% of angiosperm species (Westwood et al. 2010), are important contributors to ecological systems but also include devastating pests that cause major agricultural losses each year (Rodenburg et al. 2016). Parasitism has evolved independently at least 12 times (Nickrent 2020), and despite these diverse origins, all parasitic plants form an invasive structure, the haustorium, which penetrates the host and allows the uptake of nutrients, hormones and signalling molecules (Birschwilks et al. 2006; Spallek et al. 2017; Shahid et al. 2018; Liu et al. 2020). The development of the haustorium starts with the perception of a suitable host through haustorium inducing factors (HIFs). Treatment with 2,6-Dimethoxybenzoquinone (DMBQ), the first discovered HIF, is sufficient to induce the formation of pre-haustoria in the parasitic plant family Orobanchaceae, even in the absence of a host (Chang et al. 1986). Other HIFs include hormones like cytokinin and lignin-related compounds (Goyet et al. 2017; Cui et al. 2018; Aoki et al. 2022). In the facultative root parasite *Phtheirospermum japonicum* the perception of HIFs is mediated by leucine-rich-repeat receptor-like kinases (Laohavisit et al. 2020) and increases auxin polar transport and auxin biosynthesis. The auxin signalling peak promotes cell expansion and division, leading to the formation of a swelling called the pre-haustorium (Ishida et al. 2016; Wakatake et al. 2020). Penetration of the host by the pre-haustorium is thought to depend on haustorium-secreted cell-wall-modifying enzymes such as expansins and peroxidases that loosen the host cell walls (Losner-Goshen et al. 1998; Veronesi et al. 2007; Honaas et al. 2013; Olsen et al. 2016). The invasion of the host tissues is then mediated by the intrusive cells, which differentiate from epidermal cells

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at the parasite-host interface and drive haustorial growth towards the host vasculature (Heide-Jørgensen and Kuijt 1995; Hood et al. 1998). Finally, a vascular connection develops between the parasite and the host. All parasitic plants form a xylem connection, which begins its differentiation from the cambium-like tissue at the centre of the haustorium (Wakatake et al. 2018). A mass of xylem tissue then develops close to the parasite vasculature (plate xylem) before strands of xylem (xylem bridges) differentiate to connect the xylem of the parasite to the xylem of the host.

Despite recent advances in our understanding of haustorium development, the mechanisms regulating haustoria initiation and host invasion remain largely unknown, but likely rely in part on cell wall modifications. In plants, lateral organ development relies on the fine tuning of cell wall modifications which are required for cell expansion, division and differentiation. These processes all require the modification of cell-to-cell adhesion. The main mediator of cell adhesion in plants is pectin, a jelly-like matrix composed of homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (Pelloux et al. 2007; Daher and Braybrook 2015). Homogalacturonan is secreted to the cell wall in a highly methylesterified state and is then modified in the cell wall by different families of pectin modifying enzymes including pectin methylesterases (PMEs), PME inhibitors (PMEIs), polygalacturonases (PGs) and pectate lyases (PLs). Highly methylesterified pectin forms a tight matrix with less elastic properties. During developmental processes such as tissue expansion and lateral organ emergence, homogalacturonans undergo de-methylesterification by PMEs to become looser (Pelloux et al. 2007; Daher and Braybrook 2015). In addition to the fundamental roles of PMEs and PMEIs in plant development, many plant pathogens hijack plant pectin modification mechanisms to allow tissue intrusion (Hewezi et al. 2008; Raiola et al. 2011). For example, the cyst nematode *Heterodera schachtii* secretes into Arabidopsis (*Arabidopsis thaliana*) a cellulose binding protein (CBP) that activates PME3, facilitating the entry of the nematode (Hewezi et al. 2008). *A. thaliana pme3* mutants are less susceptible to nematode infection (Hewezi et al. 2008). Furthermore, some nematodes and fungi can directly secrete PMEs that mimic plant PMEs and facilitate host tissue invasion (Valette-Collet et al. 2003; Vicente et al. 2019). Parasitic plants are thought to secrete cell-wall modifying enzymes (CWMEs) to promote growth of the haustorium, adhesion to the host and loosening of host tissues to allow for invasion (Veronesi et al. 2007; Honaas et al. 2013; Yang et al. 2015; Kurotani et al. 2020). The secretion of CWMEs has been partially investigated in some parasitic plant species. For example, different species from the *Orobanche* genus secrete PMEs, PGs, PLs and peroxidases close to the site of infection to modify the host's cell wall (Ben-Hod et al. 1993; Losner-Goshen et al. 1998; Veronesi et al. 2007), while the shoot parasite *Cuscuta* upregulates *PMEI* transcription important for host penetration and increases pectin degrading enzyme activity during infection (Nagar et al. 1984; Jhu et al. 2022).

Even though cell wall modifications are suggested to be crucial for haustorium development, this aspect remains largely unexplored. Here, we use a combination of transcriptomic and genetic approaches to identify PMEs and PMEIs relevant for haustoria formation. We go on to describe dynamic and tissue specific changes in pectin methylesterification and show the effects of perturbing host and parasite PME-related enzymes. Together, this study describes the role of pectin during parasitic plant infection and reveals the importance of pectin methylesterification-related genes for haustoria development.

Results

PjPMEs and *PjPMEIs* are differentially expressed during haustorium development

To study the role of pectin in *P. japonicum*, we focused on modifications of pectin by the pectin methylesterase (PME) and PME-inhibitor (PMEI) enzyme families. We performed a Hidden-Markov-Model search on the *P. japonicum* (Pj) proteome (Cui et al. 2020) and identified 73 putative PjPMEs and 62 putative PjPMEIs (Supplemental Table S1). We further filtered PjPMEs based on the presence of at least three of the five conserved catalytic amino acids (Johansson et al. 2002; Markovic and Janecek 2004) and retained 60 PjPMEs for downstream analyses (Supplemental Table S1). We aligned PjPMEs and PjPMEIs with *A. thaliana* PMEs and PMEIs, respectively, and built two Maximum-Likelihood phylogenetic trees. The trees showed co-clustering of *A. thaliana* and *P. japonicum* sequences, suggesting conservation in PMEs and PMEIs between parasite and host (Supplemental Fig. S1, A and B). We then looked at the expression of *PjPMEs* and *PjPMEIs* in two different published *P. japonicum* transcriptomic datasets. The first dataset sampled tissues at the site of haustorium development during a time-course infection of *A. thaliana* (Fig. 1A, Kokla et al. 2022), while the second dataset sampled intrusive cells (ICs) and non-ICs in mature haustoria infecting rice (*Oryza sativa*) (Fig. 1B, Ogawa et al. 2021). RNA levels of several *PjPME* and *PjPMEI* genes increased during haustorium formation, particularly at 48 and 72 h post infection (hpi), while very few genes showed decreased RNA levels (Fig. 1, C and D). Many *PjPME* and *PjPMEI* genes were also highly expressed during rice infection, including several in common with *A. thaliana* infection. We observed a greater number of highly expressed *PjPMEs* (>400 reads) in ICs compared to non-IC tissues, while several *PjPMEIs* were highly expressed in both IC and non-IC tissues (Fig. 1, C and D). In the *A. thaliana* host dataset, few *AtPMEs* and *AtPMEIs* changed expression during haustorium development with *AT1G23200* (*PME*) and *AT2G01610* (*PMEI*) showing the most consistent pattern of increased expression over multiple time points (Supplemental Fig. S1, C and D).

PME activity increases during haustorium development and is higher in intrusive cells

Since we observed differential expression of *PjPMEs* and *PjPMEIs* during haustorium development, we tested whether

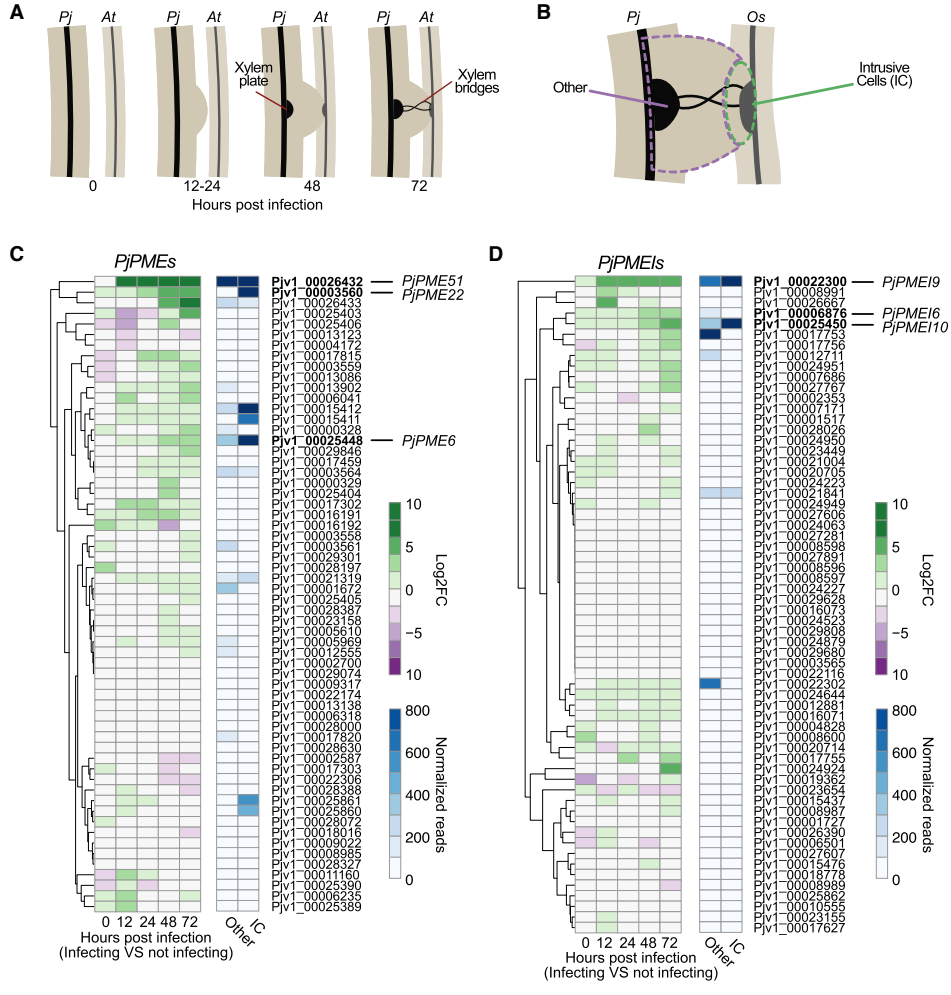

Figure 1. *PjPMEs* and *PjPMEIs* are differentially expressed during haustorium development. **A)** Illustration of *P. japonicum* (*Pj*) haustorium development during infection of *A. thaliana* (*At*) corresponding to the time points selected for the time course RNA-Seq (Kokla et al. 2022). **B)** Illustration of the sampling for the *P. japonicum* intrusive cells (IC) or rest of the haustorial tissues (other) during *O. sativa* (*Os*) infection for the RNA-Seq dataset presented in Ogawa et al. 2021. **C-D)** Heatmaps of the expression of candidate *P. japonicum PMEs* (*PjPMEs*) or *PMEIs* (*PjPMEIs*): log2 fold change (FC) between *P. japonicum* infecting and not infecting over five time points during infection, and normalized reads in intrusive cells and other haustorial tissue. The genes are clustered by expression according to the time-course dataset.

pectin methylesterification levels could also be affected. We first performed ruthenium red staining on whole roots during an infection time course (Fig. 2A). Ruthenium red binds with higher affinity to de-methylesterified homogalacturonan, and therefore a higher staining often corresponds with higher PME activity (Downie et al. 1998). We observed an increase in ruthenium red staining by 24 hpi at the interface between *P. japonicum* and *A. thaliana*. The staining intensity increased further in later stages corresponding to host invasion (48 hpi) and xylem differentiation (72 and 120 hpi) (Fig. 2, A and B, Supplemental Fig. S2A), but was not observed when inducing haustoria using DMBQ (Fig. 2B, Supplemental Fig. S2B), which does not induce xylem bridge formation. We also used LM19 and LM20 antibodies specific for de-methylesterified and highly methylesterified pectin (Verhertbruggen et al. 2009), respectively, to measure pectin modifications from 0 to 120

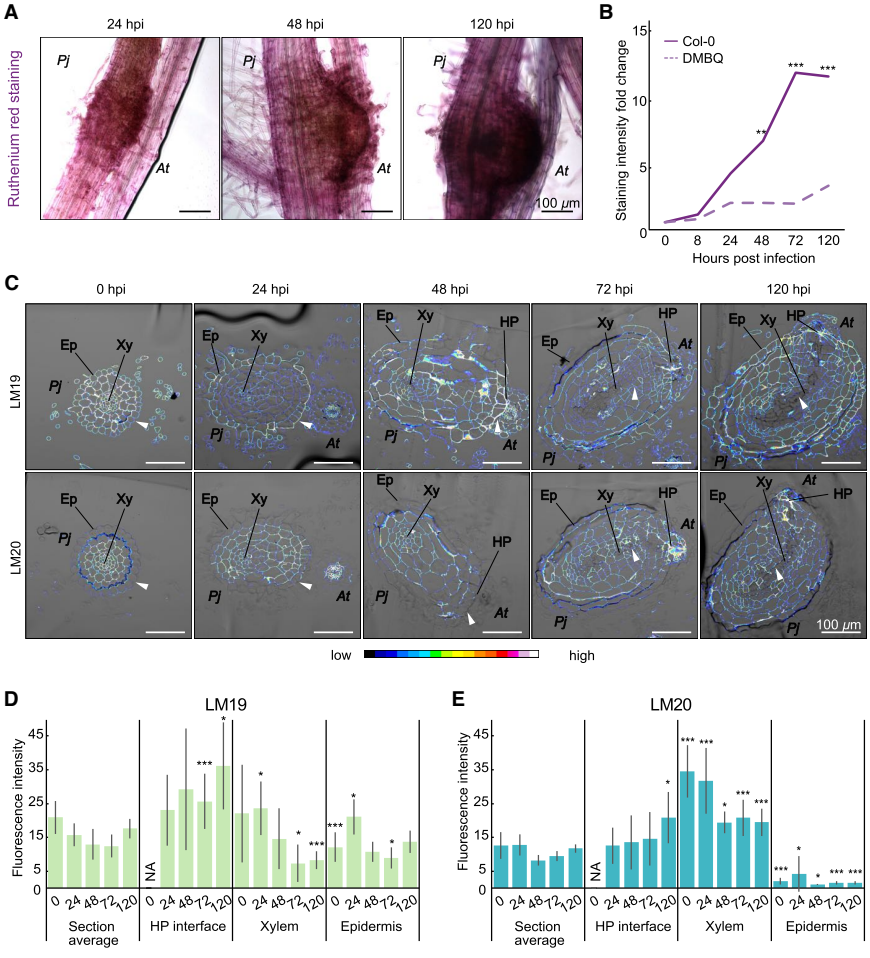

Figure 2. PME activity increases during haustorium development and is higher in intrusive cells. **A)** Ruthenium red staining of the developing haustoria at 24, 48 and 120 h post infection (hpi). Scale bars 100 μm. **B)** Quantification of staining intensity in infecting haustoria (solid line) or pre-haustoria formed on DMBQ (dashed line) during a time course from 0 to 120 hpi. Staining intensity normalized to 0 hpi. Asterisks indicate significant difference in staining intensity between haustoria and pre-haustoria at the same time point. (** $P < 0.01$, *** $P < 0.001$, Wilcoxon test, $n = 11$ to 20 roots, 1 replicate). **C)** Fluorescence images of antibody staining with LM19 (unmethylesterified homogalacturonan) and LM20 (highly methylesterified homogalacturonan) on *P. japonicum* (*Pj*) haustoria cross sections at 0, 24, 48, 72 and 120 hpi of *A. thaliana* (*At*). Arrowheads denote areas of differential staining between LM19 and LM20. Ep = epidermis, Xy = xylem, HP = host-parasite interface. **D, E)** Fluorescence quantification in whole *P. japonicum* sections (section average), host-parasite (HP) interface, xylem (root xylem and xylem bridge) and epidermis tissues for LM19 and LM20 antibodies. Asterisks indicate significant difference between a specific tissue and the section average at the same time point (Student's t-test, *P*-value corrected for multiple testing), bars represent standard deviation. Scale bars 100 μm; NA = tissue not present at the time point. * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$, $n = 3$ to 15 sections.

hpi (Fig. 2, C, D and E, Supplemental Fig. S2C). LM19 staining was mostly localised to the outer epidermis and at the host-parasite interface, especially in later time points (72 and 120 hpi), whereas staining intensity was reduced in xylem tissues during infection (Fig. 2, C and D). LM20 staining was nearly absent in epidermal tissues and staining was instead mainly focused to xylem tissues and at the host-parasite interface (Fig. 2, C and E). These results showed that pectin methylesterification was modified dynamically during haustorium development in a tissue-specific manner.

Differentially expressed *PjPMEs* and *PjPMEIs* activate in intrusive cells and cambium-like tissue

Since the pectin degree of methylesterification (DM) in the developing haustorium was different in specific tissues, we investigated candidate *PjPMEs* and *PjPMEIs* to understand if their expression pattern corresponded with the pattern of pectin DM. We chose three *PjPME* genes with increased expression in the time course dataset and renamed them based on the *A. thaliana* homolog (Fig. 1C, Fig. 3A). All three genes were also upregulated during *P. japonicum-O. sativa* infection. *PjPME6* (*Pjv1_00025448*) and *PjPME22* (*Pjv1_00003560*) expression levels increased in ICs compared to other tissues, while *PjPME51* (*Pjv1_00026432*) was highly expressed in both IC and non-IC tissues (Fig. 3B). We made and transformed transcriptional reporters in *P. japonicum* hairy roots and found *PjPME6* and *PjPME51* reporters showed signal mainly in ICs, while the *PjPME22* reporter was mostly expressed in *P. japonicum* vasculature (Fig. 3C). We then selected three *PjPMEI* genes upregulated during haustorium development in *A. thaliana* and *O. sativa* infections (Fig. 1D and 3D). In the *P. japonicum-O. sativa* dataset, *PjPMEI9* (*Pjv1_00022300)* and *PjPMEI10* (*Pjv1_00025450)* showed increased expression in ICs, while *PjPMEI6* (*Pjv1_00006876)* was more expressed in non-IC tissues (Fig. 3E). Our transcriptional reporters for these genes showed expression in ICs and cambium-like tissues for *PjPMEI9*, and plate xylem and ICs for *PjPMEI10* (Fig. 3F). The *PjPMEI6* reporter showed no fluorescence in 4 dpi haustoria (Fig. 3F). All the *PjPMEs* and *PjPMEIs* we investigated were not expressed in the primary root tip of the hairy roots, and showed little or no fluorescence at the lateral root emergence sites of the hairy roots, suggesting that upregulation of these genes was specific to haustorium development (Supplemental Fig. S3).

Inhibition of PME activity impairs haustoria induction and development

To determine if PME activity is necessary for haustorium development, we treated infecting *P. japonicum* with 50 μM or 100 μM of epigallocatechin gallate (EGCG), a chemical inhibitor of PME enzymes (Lewis et al. 2008). Treatment reduced the number of haustoria (Fig. 4A) and delayed the formation of xylem bridge connections to the host (Fig. 4B), although it did not substantially affect plate xylem area and number of xylem bridge connections at 7 dpi (Fig. 4C, Supplemental Fig. S4, A and B). EGCG treatment also reduced *PjPME22*, *PjPME51* and *PjPMEI10* expression at 72 hpi (Fig. 4D), and reduced *PjPMEI6* and *PjPMEI9* expression at both 0 and 72 hpi, suggesting that chemical inhibition of PMEs affected both haustoria development and the transcriptional regulation of endogenous *PMEs* and *PMEIs* (Fig. 4D, Supplemental Fig. S4C). We next overexpressed *PjPME6* and *PjPME51* in *P. japonicum* hairy roots (Supplemental Fig. S4, D and E) but did not observe defects in haustorium induction or development (Fig. 4E, Supplemental Fig. S4, F and G). However, overexpression of *PjPMEI6*, *PjPMEI9* and *PjPMEI10* significantly inhibited haustoria induction (Fig. 4E) but did not affect xylem connections (Supplemental Fig. 4, F and G). Finally, we tested whether modifying pectin status in the host could affect infection by using the *PMEI5*-overexpressing *A. thaliana* line *AtPMEI5OE*, which is characterised by highly methylesterified pectin (Wolf et al. 2012; Jonsson et al. 2021). Haustoria induction was not affected in the mutant compared to wild-type Col-0 (Fig. 4F), however, xylem bridge formation was delayed during infection of *AtPMEI5OE* (Fig. 4G). Taken together, these results suggest that parasitic PME activity is important for efficient induction and development of haustoria.

Brassinosteroid treatment reduces *PjPME* and *PjPMEI* expression and delays haustorium development

Brassinosteroid (BR) signalling mediates cell wall biosynthesis and remodelling, and has been implicated in feedback mechanisms with PME and PMEI activity and EGCG treatments (Wolf et al. 2012). To test the effect of BRs, we applied 100 nM or 200 nM of epibrassinolide (epiBL) during *P. japonicum* infection and found it reduced the number of haustoria per *P. japonicum* (Fig. 5, A and B, Supplemental Fig. S5A), similar to EGCG treatment (Fig. 4, A and , B). We also tested the expression of *PjPMEs* and *PjPMEIs* in haustoria following epiBL treatment. *PjPME51*, *PjPMEI6*, *PjPMEI9* and *PjPMEI10* were downregulated in haustoria treated with epiBL at 72 hpi but not at 0 hpi (Fig. 5C, Supplemental Fig. S5B), suggesting transcriptional control of pectin methylesterification by BR signalling during haustorium development. To determine if BR treatment could modify pectin methylesterification levels, we performed antibody staining using LM19 and LM20 on cross sections of 0 hpi and 72 hpi haustoria untreated or treated with epiBL (Fig. 5D, Supplemental Fig. S5C). The haustoria treated with epiBL had lower levels of both unmethylesterified pectin (LM19) and highly methylesterified pectin (LM20) compared to the control (Fig. 5E), suggesting overall pectin levels were reduced following epiBL treatment. In particular, staining for highly methylesterified pectin (LM20) was significantly lower in xylem and epidermis tissues treated with epiBL (Fig. 5E), corresponding with the reduced *PjPMEI* gene expression previously observed (Fig. 5C). Finally, we infected the *A. thaliana BRI1-EMS-SUPPRESSOR 1* mutants with modified BR signalling, *bes1-2* and *bes1-D*, to test the role of host BR signalling during infection. *P. japonicum* could efficiently infect both mutants and establish xylem connections (Supplemental Fig. S5, D and E), suggesting host BR signalling is not crucial for haustoria development and instead BR signalling might be important for parasite cell wall modifications during infection.

PjPMEs and *PjPMEIs* expression associates with xylem bridge development

Since some *PjPMEs* and *PjPMEIs* are expressed in the cambium and xylem-like tissues during haustorium development (Fig. 3C-F) and inhibiting PME activity delays xylem

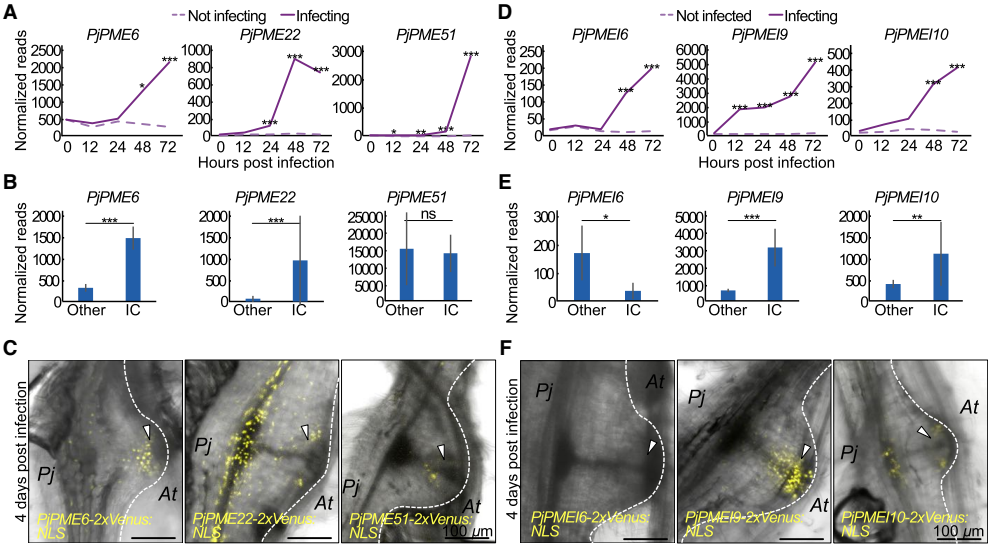

Figure 3. Upregulated *PjPMEs* and *PjPMEIs* are primarily expressed in intrusive cells and cambium-like tissue. **A, D)** Normalized reads of *PjPME6*, *PjPME22*, *PjPME51*, *PjPMEI6*, *PjPMEI9* and *PjPMEI10* over five time points during infection for *P. japonicum* infecting and not infecting. Asterisks indicate a significant difference between infecting and not infecting (Wald test with Benjamini-Hochberg correction for multiple testing, $n = 3$ libraries). **B, E)** Normalized reads of *PjPME6*, *PjPME22*, *PjPME51*, *PjPMEI6*, *PjPMEI9* and *PjPMEI10* in intrusive cells and non-IC (other) tissues. Asterisks indicate a significant difference between IC and other tissues (Student's t-test, $n = 3$ libraries). Bars represent standard deviation. **C, F)** Images of infecting transgenic hairy roots expressing *PjPME6*, *PjPME22*, *PjPME51*, *PjPMEI6*, *PjPMEI9* and *PjPMEI10* nuclear-localized (NLS) transcriptional reporters in fully developed haustoria (4 dpi). Arrowheads denote intrusive cells. Scale bars 100 μm. For all panels * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$.

formation (Fig. 4B), we investigated the role of *PjPMEs* and *PjPMEIs* during xylem-bridge formation. Looking at the expression pattern of cambium and xylem marker genes, we found the cambium marker *WUSCHEL RELATED HOMEOBOX 4* (*PjWOX4*) (Wakatake et al. 2018) co-expressed with *PjPME22*, *PjPME51* and *PjPMEI9* (Fig. 6A). The putative procambium marker *HOMEOBOX GENE 8* (*PjHB8*) co-expressed with *PjPME6*. The xylem-markers *CELLULOSE SYNTHASE A 7* (*PjCESA7*) (Wakatake et al. 2018), *VASCULAR RELATED NAC-DOMAIN PROTEIN 7* (*PjVND7*) (identified through BLAST using *AtVND7* as a query) and *XYLEM CYSTEINE PEPTIDASE 2* (*PjXCP2*) (Kokla et al. 2022) co-expressed with *PjPMEI6* and *PjPMEI10* (Fig. 6A, Supplemental Fig. 6A). To investigate if pectin methylesterification levels change in response to xylem-bridge development, we chemically inhibited xylem bridge formation by treatment with the auxin transport inhibitor N-1-naphthylphthalamidic acid (NPA). NPA treatment did not affect haustoria numbers, yet inhibited xylem bridge connection (Wakatake et al. 2020, Fig. 6, B and D). Treatment with the synthetic auxin 1-naphthaleneacetic acid (NAA) did not affect haustoria numbers or xylem bridge formation (Supplemental Fig. S6B, C and D). We also found that the commonly used dye Coomassie Brilliant Blue, an inhibitor of xyloglucan endotransglucosylase/hydrolase (XTH) activity (Olsen and Krause 2017), increased haustoria numbers, yet reduced xylem bridge formation by approximately 60% (Fig. 6B-D). LM19 and LM20 antibody staining of 72 hpi sections showed both de-methylesterified pectin and highly methylesterified pectin were reduced following NPA treatment, mostly in the host-parasite interface (Fig. 6, E and F, Supplemental Fig. S6E). Staining with LM20 was also reduced following Coomassie treatment (Fig. 6, E and F, Supplemental Fig. S6E). Expression levels of several PMEs and PMEIs were significantly affected by NPA and Coomassie treatments (Fig. 6G) including *PjPME51* and *PjPMEI9* that were decreased by both treatments at 72 hpi (Fig. 6g) but not at 0 hpi (Supplemental Fig. S6F). This decreased expression of *PjPME51* and *PjPMEI9* appeared specific to xylem bridge inhibition and suggested a role for these genes in xylem bridge formation. We then infected hairy roots expressing *PjPMEI9-2xVenus:NLS*, which showed fluorescence in cambium-like tissues (Fig. 3F, Supplemental Video S1). At 4 days post infection we observed a marked decrease in fluorescence when hairy roots expressing *PjPMEI9-2xVenus:NLS* were treated with NPA compared to the control, consistent with the RT-qPCR data (Fig. 6H) and demonstrating that xylem bridge formation is important for *PMEI9* expression.

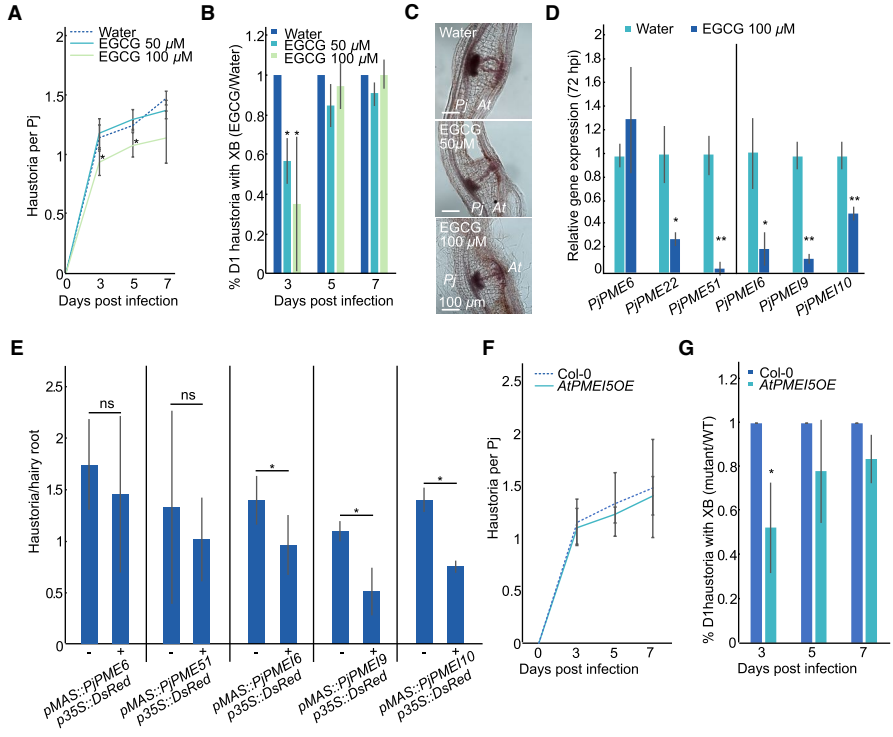

Figure 4. Inhibition of PME activity impairs haustorium induction and development. **A)** Number of haustoria per *P. japonicum* plant at four time points during treatment with 50 μM ECGC, 100 μM EGCG or water control. Asterisks indicate significance compared to control (Student's t-test, $n = 3$ replicates). **B)** Ratio of the percentage of day one (D1) haustoria with a xylem bridge (XB) formed during treatment with 50 or 100 μM EGCG over water at three time points. Asterisks indicate significance compared to control (Student's t-test, $n = 3$ replicates). **C)** Images of 7 dpi haustoria formed on water, 50 μM EGCG and 100 μM EGCG. **D)** Relative gene expression of selected *PjPMEs* and *PjPMEIs* at 72 hpi in *P. japonicum* haustoria treated with 100 μM EGCG, normalised to water. Asterisks indicate significance compared to control (Student's t-test, $n = 3$ replicates). **E)** Numbers of haustoria per hairy root transformed with *PjPME* and *PjPMEI* overexpression constructs. Non-transgenic hairy roots are marked as "-" and transgenic roots are marked as "+". Asterisks indicate significance compared to control (Fisher's exact test; $n = 2$ to 4 replicates, 12 to 53 total roots per sample). **F)** Number of haustoria per *P. japonicum* plant at four time points during infection of *AtPMEI5OE* mutant or Col-0 as control ($n = 3$ replicates). **G)** Ratio of the percentage of D1 haustoria with a XB formed during infection of *AtPMEI5OE* over Col-0 at three time points. Asterisks indicate significance compared to control (Student's t-test, $n = 3$ replicates). For all panels * for $P < 0.05$, ** for $P < 0.01$, bars represent standard deviation.

Discussion

Here, we investigated the role of PME-mediated pectin modifications during haustorium development in *P. japonicum* and identified multiple *PjPMEs* and *PjPMEIs* upregulated during *A. thaliana* and *O. sativa* infections. Induction dynamics varied with some genes highly activated during early stages of infection whereas others peaked late when xylem bridges formed (Fig. 1C-D and 3, A and D), suggesting they had different developmental roles as infection progressed. The reporters we generated showed PME-related gene expression in intrusive cells (Fig. 3B-C) and these outer tissues also showed low methylesterification (Fig. 2C) suggesting cell wall loosening was relevant for expansion of outer tissues and interaction with the host. Suppressing PME activity by overexpressing PMEIs or by chemical treatments with EGCG also reduced the ability for haustoria to form (Fig. 4) consistent with a role for PMEs and pectin loosening in organogenesis and haustoria expansion. Recently, it was found that pectin methylesterification levels are also crucial for lateral root initiation (Wachsman et al. 2020). Our findings suggested aspects of lateral root formation and haustoria emergence are conserved in *P. japonicum* as it has been previously suggested for *Cuscuta* and *Thesium* parasites (Ichihashi et al. 2018; Jhu et al. 2021).

We also observed strong induction of PMEI-related gene expression including in the vascular tissues of the haustoria

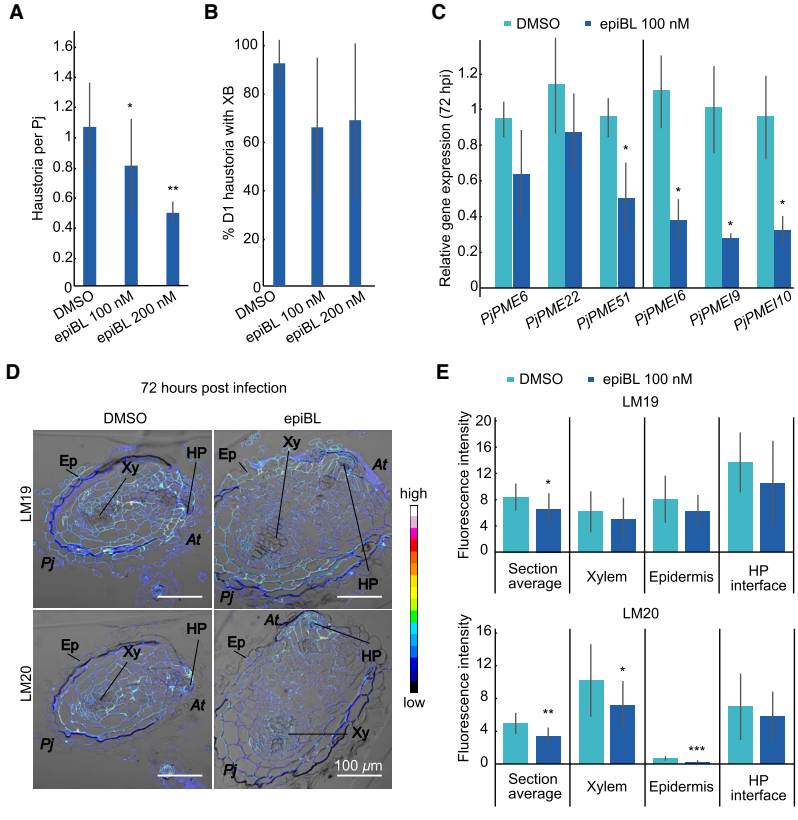

Figure 5. BR treatment reduces *PjPME* and *PjPMEI* expression and inhibits haustorium development. **A)** Number of haustoria per *P. japonicum* plant at 7 dpi during treatment with 100 nM epiBL, 200 nM epiBL or DMSO control ($n = 4$ to 6 replicates). **B)** Percentage of day one (D1) haustoria with a xylem bridge (XB) formed during treatment with 100 nM epiBL, 200 nM epiBL or DMSO at 7 dpi ($n = 4$ to 6 replicates). **C)** Relative gene expression of selected *PjPMEs* and *PjPMEIs* at 72 h post infection in *P. japonicum* haustoria treated with 100 nM epiBL, normalised to DMSO ($n = 3$ replicates). **D)** Fluorescence images of antibody staining using LM19 (unmethylesterified homogalacturonan) and LM20 (highly methylesterified homogalacturonan) on cross sections of 72 hpi haustoria formed on DMSO control or 100 nM epiBL. Scale bars 100 μm. Ep = epidermis, Xy = xylem, HP = host-parasite interface. *At* = *A. thaliana*, *Pj* = *P. japonicum*. **E)** Fluorescence quantification in whole *P. japonicum* sections (section average), host-parasite (HP) interface, xylem (root xylem and xylem bridge) and epidermis tissues for LM19 and LM20 antibodies ($n = 7$ to 14 sections). For all panels, asterisks indicate significance compared to DMSO (Student's t-test): * for $P < 0.05$, ** for $P < 0.01$, bars represent standard deviation.

(Fig. 1D and 3D-F). Antibody staining revealed high levels of methylesterified pectin in the inner haustoria tissues and xylem bridges (Fig. 2C-D) that could provide structural support to these tissues. Thus, the apparent co-expression of both esterase (PME) and inhibitor (PMEI) could be explained in part by differences in spatial expression and the degree of methylesterification in different tissues. Asymmetric pectin methylesterification is required in several plant developmental processes, including apical hook formation during seedling emergence and leaf patterning (Jonsson et al. 2021; Peng et al. 2022). Furthermore, differences in pectin methylesterification between lateral roots and its progenitor tissues might allow the emergence of the lateral root while preventing its own digestion (Laskowski et al. 2006). We propose that in haustoria high PME activity in intrusive cells drives host cell wall loosening and penetration, while high PMEI activity maintains inner haustorial tissue integrity and helps these structures push towards the host.

Our investigations also revealed a tight association between pectins and xylem bridge formation. DMBQ induces pre-haustoria that lack xylem bridges (Cui et al. 2016) and we observed little ruthenium red staining of DMBQ treatment samples (Fig. 2B, Supplemental Fig. S2B) suggesting that pectins were not highly de-methylesterified during pre-

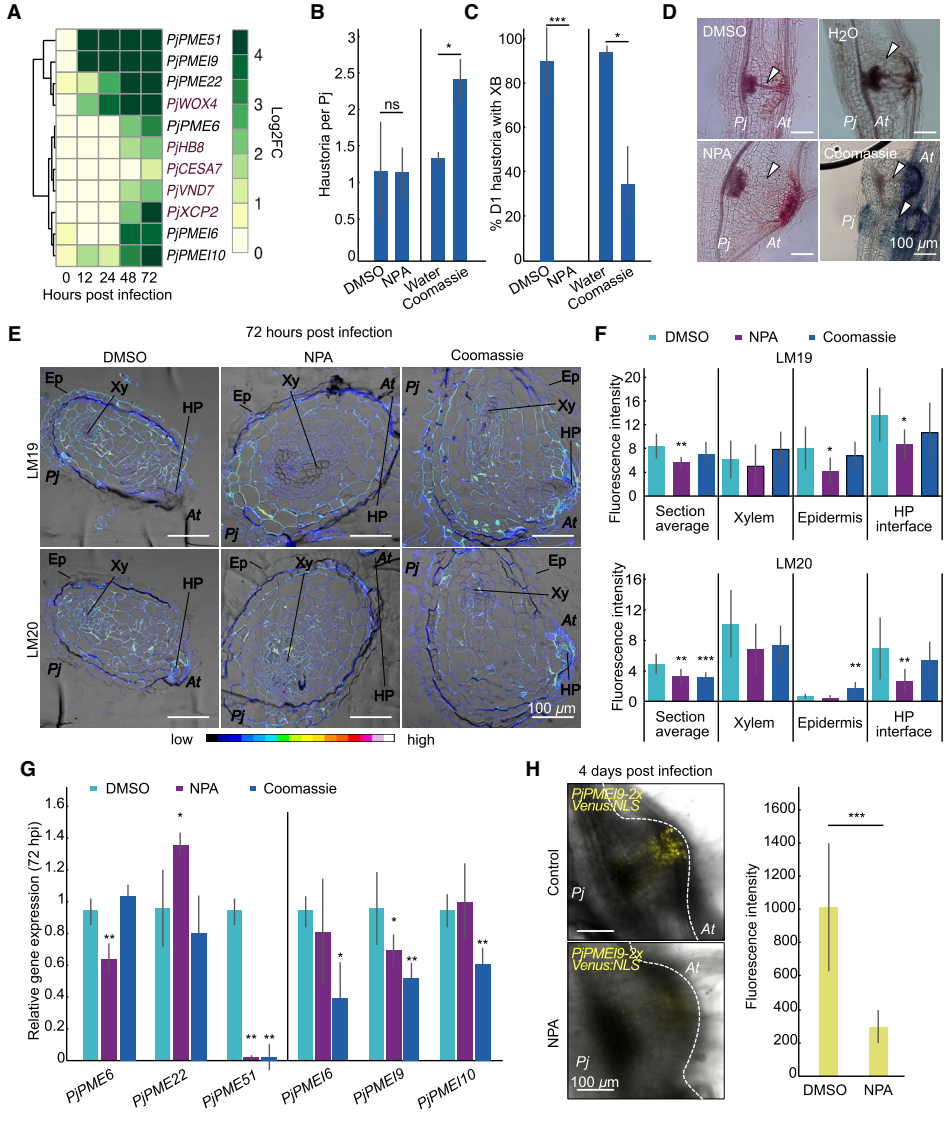

Figure 6. *PjPMEs* and *PjPMEIs* expression associates with xylem bridge development. **A)** Expression heatmap of *P. japonicum* cambium and xylem marker genes (red) and selected *PjPMEs* and *PjPMEIs*: log2 fold change between *P. japonicum* infecting and not infecting over five time points during infection, clustered by expression. **B)** Number of haustoria per *P. japonicum* plant at 7 dpi during treatment with DMSO, 5 μM NPA ($n = 2$ replicates), water or 0.05 mM Coomassie ($n = 3$ replicates). **C)** Percentage of day one (D1) haustoria with a xylem bridge (XB) formed during treatment with DMSO, 5 μM NPA ($n = 2$ replicates), water or 0.05 mM Coomassie ($n = 3$ replicates). **D)** Images of 7 dpi haustoria formed on DMSO, 5 μM NPA, water or 0.05 mM Coomassie. White arrowheads denote the location of XB development. **E)** Fluorescence images of antibody staining using LM19 and LM20 on cross sections of 72 hpi haustoria developed on DMSO, 5 μM NPA or 0.05 mM Coomassie + DMSO. Ep = epidermis, Xy = xylem, HP = host-parasite interface. *At* = *A. thaliana*, *Pj* = *P. japonicum*. **F)** Fluorescence quantification in whole *P. japonicum* sections (section average), host-parasite (HP) interface, xylem (root xylem and xylem bridge) and epidermis tissues for LM19 and LM20 antibodies. Asterisks indicate significance compared to DMSO (Student's t-test, *P*-value corrected for multiple testing, $n = 8$ to 14 sections). **G)** Relative gene expression of select *PjPMEs*

(continued)

haustoria formation. Similarly, EGCG, NPA and Coomassie Brilliant Blue treatments all delayed or inhibited xylem bridge formation (Fig. 4B and 6C) and reduced the expression of *PME51* and *PMEI9* (Fig. 4D and 6G) suggesting a close relation between xylem bridge formation and *PME51* and *PMEI9* activation. In alfalfa (*Medicago sativa*), xylem cell walls contain about 4% pectin, compared to 25% pectin in other tissues (Grabber et al. 2002), suggesting pectin might be degraded during xylem differentiation. In *A. thaliana*, five *PMEs* are expressed in xylem tissues (Pelloux et al. 2007), and the demethylesterification of pectin might be important for lignification (Lairez et al. 2005; Pelloux et al. 2007). PME activity including from *PjPME51* might therefore be required in the first stage of xylem bridge differentiation to allow pectin degradation, followed by lignification.

In the *A. thaliana* host, our data suggest the role of pectin modifications during infection is less important. Only *AT1G23200* (*PME*) and *AT2G01610* (*PMEI*) showed clear increases in expression during infection (Supplemental Fig. S1, C and D), suggesting these genes might either be involved in a defence response to the parasite or are activated by *P. japonicum* to facilitate parasitism. Notably, the *A. thaliana* overexpressor *AtPMEI5OE* (Wolf et al. 2012), which has high methylesterification levels (Wolf et al. 2012; Jonsson et al. 2021), delayed xylem bridge connections (Fig. 4, F and G) indicating that pectin modifications by the host could influence parasite development. Our EGCG treatments likely inhibited both parasite and host PMEs so our finding that EGCG reduces xylem bridge formation could be explained in part due to inhibition of host PMEs. Thus, cell wall modifications by both host and parasite appear relevant for successful parasitic plant infection and deserve further attention. By better understanding and modifying the host cell wall response, it may be possible to achieve durable resistance to parasites.

Materials and methods

Plant materials and growth conditions

Phtheirospermum japonicum and Arabidopsis (*Arabidopsis thaliana*) seeds were surface sterilized by washing with 70% v/v ethanol for 20 min, followed by 95% v/v ethanol for 5 min, and sown on ½ MS medium with 1% w/v sucrose and 0.8% w/v bactoagar. After stratification at 4 °C in darkness for 1 or 2 days for *P. japonicum* and *A. thaliana* respectively, the plates were moved to a growth cabinet at 25 °C in long day conditions (16 h light/8 h darkness), 100 μmol m^{-2} s^{-1} light. The *A. thaliana* Col-0 accession was used unless otherwise stated. The *AtPMEI5OE* line has been previously published (Wolf et al. 2012). The BR-signalling mutants *bes1-2* and *bes1-D* have been previously published (Yin et al. 2002; Lachowiec et al. 2013).

PjPMEs and PjPMEIs identification and phylogenetic analyses

A. thaliana PME (Louvet et al. 2006) and PMEI (Wang et al. 2013) sequences were downloaded from the Phytozome database (Goodstein et al. 2012). *P. japonicum* putative PMEs and PMEIs were identified by searching the HMM profiles (PF01095 and PF04043 respectively) on a *P. japonicum* proteome obtained from the published genome (Cui et al. 2020) using the HMMER3 software (Finn et al. 2011). The putative PjPMEs were aligned using Clustal W in MEGAX (Tamura Stecher and Kumar 2021), and the sequences lacking more than one of the five conserved catalytic amino acids (Johansson et al. 2002; Markovic and Janecek 2004) were removed from downstream analyses. *P. japonicum* and *A. thaliana* PME and PMEI sequences were aligned using ClustalW. Maximum-Likelihood phylogenetic trees were built using MEGAX with 100 bootstraps.

In vitro infection assays with *Phtheirospermum japonicum*

Infection assays were performed according to Kokla et al. 2022. Briefly, five days after germination *P. japonicum* seedlings were moved from nutrient medium to nutrient-free medium (water agar) for starvation. After three days, a six-day old *A. thaliana* seedling was aligned root-to-root to each *P. japonicum* seedling to allow infection. 50 μM or 100 μM EGCG, 100 nM or 200 nM epiBL, 5 μM NPA, 0.05 mM Coomassie Brilliant Blue and 0 to 500 nM NAA were applied directly in the nutrient-free medium and left until the end of the infection period. For measuring the plate xylem area and the number of xylem bridges, 7 days post infection (dpi) haustoria were stained with Safranin-O following the method in Spallek et al. 2017. Pictures were taken using an Axioscope A1 microscope and analysed in Fiji (Schindelin et al. 2012; Rueden et al. 2017).

Immunohistochemical staining of pectin residues

P. japonicum infecting *A. thaliana* was harvested at 0, 24, 48, 72 and 120 hpi for infections on water. For infections on DMSO, 100 nM epiBL (dissolved in DMSO), 0.05 mM Coomassie (plus DMSO) and 5 μM NPA (dissolved in DMSO) treated samples were harvested and at 0 and 72 hpi. The seedlings were fixed in a 1% v/v glutaraldehyde, 4% w/v formaldehyde, 0.05 M NaPi aqueous solution by vacuuming twice for 20 min, followed by overnight incubation at 4 °C. The samples were then dehydrated with an ethanol gradient (30 min in each of 10%, 30%, 50%, 70%, 96%, 100%,

Figure 6. (Continued)
and *PjPMEIs* at 72 hpi in *P. japonicum* haustoria treated with 5 μM NPA or 0.05 mM Coomassie + DMSO normalised to DMSO ($n = 3$ replicates). **H)** Images of haustoria developed on hairy roots expressing the *PjPMEI9* nuclear-localised (NLS) transcriptional reporter at 4 dpi on DMSO or 5 μM NPA, and quantification of fluorescence intensity ($n = 7$ to 10 haustoria). Scale bars 100 μm. For all panels, asterisks indicate significance compared to control (Student's t-test): * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$, bars represent standard deviation.

100% v/v ethanol) and incubated overnight in a 1:1 solution of 100% ethanol:Historesin solution (Leica). The solution was exchanged with Historesin and the samples incubated again overnight at 4 °C. The seedlings were then oriented in molds following the method in (Scheres et al. 1994), aligning the haustoria of different seedlings. The shoot was removed, and a 14:1 solution of Historesin and hardener was added to form a hard resin sheet. The haustoria were cross-sectioned at 8 μm thickness using a Microm HM355 S microtome. The sections were rehydrated in PBS, incubated in 0.05 M glycine in PBS for 20 min, and blocked in 2% w/v BSA in PBS (blocking buffer) for 30 min. Three consecutive slides were stained in 1:20 dilutions of LM19, LM20 in PBS or just PBS for the negative control and incubated for 2 h. After rinsing with blocking buffer three times, the sections were incubated for 1 h in a 1:100 dilution of Goat anti-Rat IgG Alexa Fluor 647 secondary antibody. After rinsing three times with PBS, the sections were mounted in PBS and immediately imaged on a Zeiss LSM-780 confocal microscope with 633 nm excitation, 0.5% laser power, 650 gain and 633 to 695 nm detection. Fluorescence was quantified in Fiji using the mean gray value measurement after selecting the area corresponding to the desired tissue on the brightfield channel. Three to fifteen different haustoria were imaged and quantified for each time point and treatment. The same DMSO control was used for epiBL presented in Fig. 5 and Coomassie and NPA presented in Fig. 6. Representative images were processed equally for each panel using the 16-colors LUT to allow easier visualization of fluorescence intensity.

Ruthenium red staining

Ruthenium red staining was performed by dipping infecting roots at 0, 24, 48, 72 and 120 hpi in 0.05% w/v ruthenium red in deionised water for 5 min, followed by rinsing 2 times with deionised water and mounting on 20% v/v glycerol. Pictures were taken using an Axioscope A1 microscope and analysed in Fiji (Schindelin et al. 2012; Rueden et al. 2017).

RNA-Seq datasets and gene accession numbers

The RNA-seq dataset used for gene expression analyses of the infection time course in *P. japonicum* and *A. thaliana* is presented in Kokla et al. 2022. The dataset used for gene expression analyses in intrusive and non-intrusive haustorial cells is presented in Ogawa et al. 2021. The heatmaps were generated using the "pheatmap" function in RStudio on the log2 fold change (time-course dataset) or normalised reads (IC vs non-IC dataset) of the genes indicated in each heatmap. The heatmaps were clustered by expression in the time course dataset. Genes IDs and accession numbers for the *P. japonicum* genes mentioned in the text are available in Supplemental Table S2.

Gene expression analyses

Forty 5-day-old *P. japonicum* seedlings per biological replicate per treatment were transferred to the starvation medium with 100 μM EGCG, 100 nM epiBL, 5 μM NPA, 0.05 mM Coomassie, or 1 μM NAA for three days or control DMSO. After infecting *A. thaliana*, 2 mm of root around the haustorium was collected at 0 or 72 h post infection. RNA was extracted using the ROTIPrep RNA MINI kit (Carl Roth, 8485) following the manufacturer's instructions. cDNA was synthesised with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher, K1642) following the manufacturer's instructions. RT-qPCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix 2x (Thermo Scientific, K022). *P. japonicum SERINE/THREONINE PROTEIN PHOSPHATASE 2A* (*PjPP2A*) was used as normalisation control (Serivichyaswat et al. 2022). For each experiment, three biological replicates and at least two technical replicates were used. The relative gene expression was calculated using the Pfaffl method. The primers used are available in Supplemental Table S3.

Cloning of *PjPMEs* and *PjPMEIs* and plasmid construction

All cloning was based on the Greengate cloning method following the standard protocols (Lampropoulos et al. 2013). Greengate plasmids used for cloning have been previously published (Lampropoulos et al. 2013). All primers used for GreenGate cloning are listed in Supplemental Table S3. Digestion and ligation reactions were performed using the BsaI-HFv2 (NEB #R3733) and T4 DNA Ligase (NEB M0202) enzymes respectively. For the overexpression constructs, the CDS of *PjPME6*, *PjPME51*, *PjPMEI9*, *PjPMEI6* and *PjPMEI10* were amplified using the CloneAmp HiFi PCR Premix (TakaraBio) from the cDNA of *P. japonicum* and inserted into the entry vector pGGC000 (Addgene plasmid # 48858) to yield pGGC-CDS vectors. The ligated plasmids were amplified in chemically competent *Escherichia coli* DH5α and confirmed by Sanger sequencing. The final binary vector assembly was performed using pGGA-pMAS, pGGB003 (Addgene plasmid # 48821), pGGC-CDS, pGGD002 (Addgene plasmid # 48834), pGGE-terMAS, pGGF-DsRed and pGGZ001 (Addgene plasmid # 48868). pGGA-pMAS, pGGE-terMAS and pGGF-DsRed were previously published (Kokla et al. 2022). For the reporter constructs, a sequence of ~3 kb upstream the starting codon was cloned as the promoter of the genes of interest (pGOI). *PjPME6* (3087 bp), *PjPME51* (2876 bp), *PjPME22* (3044 bp), *PjPMEI9* (3021 bp), *PjPMEI10* (3071 bp) and *PjPMEI6* (2988 bp) promoters were amplified using the CloneAmp HiFi PCR Premix (TakaraBio) from the gDNA of *P. japonicum* and inserted into the entry vector pGGA000 (Addgene plasmid # 48856) to yield pGGA-pGOI vectors. A 2xVenus-NLS sequence was cloned from a previously published GoldenGate vector backbone (Cui et al. 2016) and inserted in the pGGC000 entry vector to create pGGC-2xVenus-NLS. The ligated plasmids were amplified in chemically competent *E. coli* DH5α and confirmed by Sanger sequencing. The final binary vector assembly was performed using pGGA-pGOI, pGGB003, pGGC-2xVenus-NLS, pGGD002, pGGE001 (Addgene plasmid # 48839), pGGF-DsRed and pGGZ001. The final overexpression and

reporter plasmids were co-transformed in electrocompetent *Agrobacterium rhizogenes* AR1193 with the pSoup plasmid (Addgene plasmid # 165419), and the bacteria cultured in LB broth with 50 μg/ml spectinomycin and 50 μg/ml rifampicin.

P. japonicum hairy root transformation

P. japonicum transformation was performed according to Ishida et al. 2011. Seven-day-old *P. japonicum* seedlings were sonicated for 10 s and vacuum-infiltrated for 5 min in a solution of AR1193 carrying the construct of interest. The seedlings were then moved to solid B5 medium supplemented with 1% w/v sucrose and 450 μM acetosyringone and kept at 22 °C in the dark for 2 days. Seedlings were then moved to B5 medium containing 300 μg/ml cefotaxime and grown at 25 °C in long day conditions until formation of hairy roots. Transgenic hairy roots were identified through red fluorescence using a Leica M205 FA stereo microscope and placed on starvation medium for 4 days before addition of *A. thaliana*. Non-fluorescent hairy roots from the same transformation experiment were used as a control for each construct. Counting of haustoria and safranin-O staining were performed at 7 dpi for overexpression constructs. Imaging of transcriptional reporters was performed on 4 dpi haustoria using a Zeiss LSM780 confocal microscope with 514 nm excitation, 2.8% laser power, 950 gain and 519 to 550 nm detection.

Statistics

All experiments were replicated at least three times unless otherwise stated. For infection assays each biological replicate consisted of the average of results from at least 15 plants, and one-tailed Student's t-tests on means were used for single comparisons. For assays with transformed hairy roots overexpressing *PjPMEs* or *PjPMEIs*, the data from the biological replicates were pooled and divided in categories of 0, 1 or $\geq$2 haustoria per hairy root. A Fisher exact test was then used to calculate significance. For RT-qPCR data, one-tailed Student's t-tests on biological replicates were used for single comparisons of treatment vs control. For ruthenium red staining, one replicate was performed with 10 to 20 plants per time point and treatment. For antibody staining assays, two replicates were performed for each time point and treatment. The quantifications from each replicate were pooled together and one-tailed Student's t-tests were used for single or multiple comparisons. The p-values for multiple comparisons were adjusted using the Bonferroni correction.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers listed in Supplemental Table S2.

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Author contributions

ML and CWM conceived the experiments. ML, AK and MF performed the experiments. ML and CWM wrote the manuscript. All authors revised the final manuscript.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. *A. thaliana PMEs* and *PMEIs* are differentially expressed during haustorium development

Supplemental Figure S2. PME activity is increased during haustorium development

Supplemental Figure S3. *PjPMEs* and *PjPMEIs* are specific to haustoria

Supplemental Figure S4. *PjPME* and *PjPMEI* overexpression does not affect xylem connection to the host

Supplemental Figure S5. *A. thaliana* BR signalling mutants do not affect parasitism efficiency

Supplemental Figure S6. NAA treatment does not affect parasitism efficiency

Supplemental Table S1. List of identified PjPMEs and PjPMEIs

Supplemental Table S2. *P. japonicum* gene accession numbers and IDs

Supplemental Table S3. Primers used in the study

Supplemental Video S1. Confocal Z-stack of a haustorium expressing *PjPMEI9-2xVenus::NLS*

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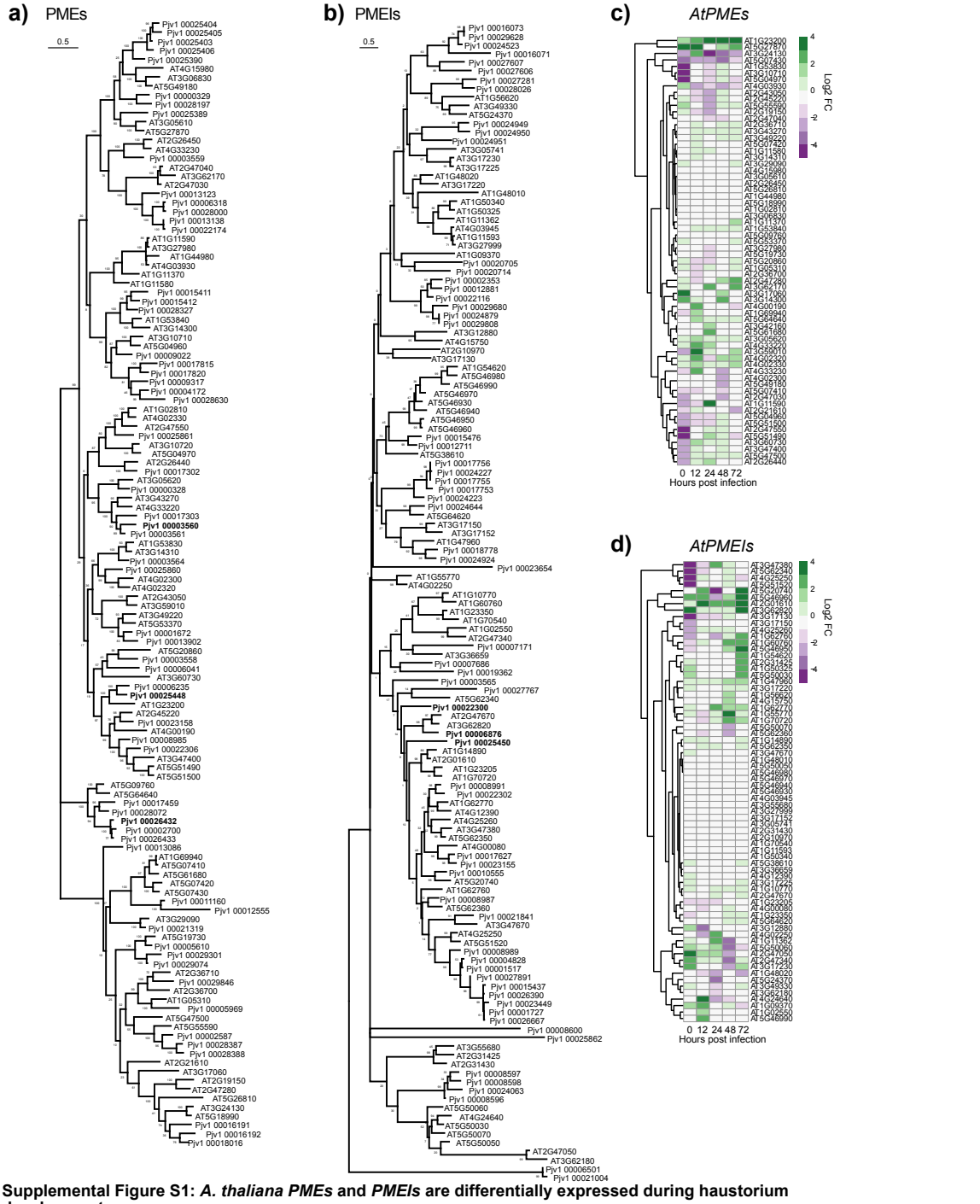
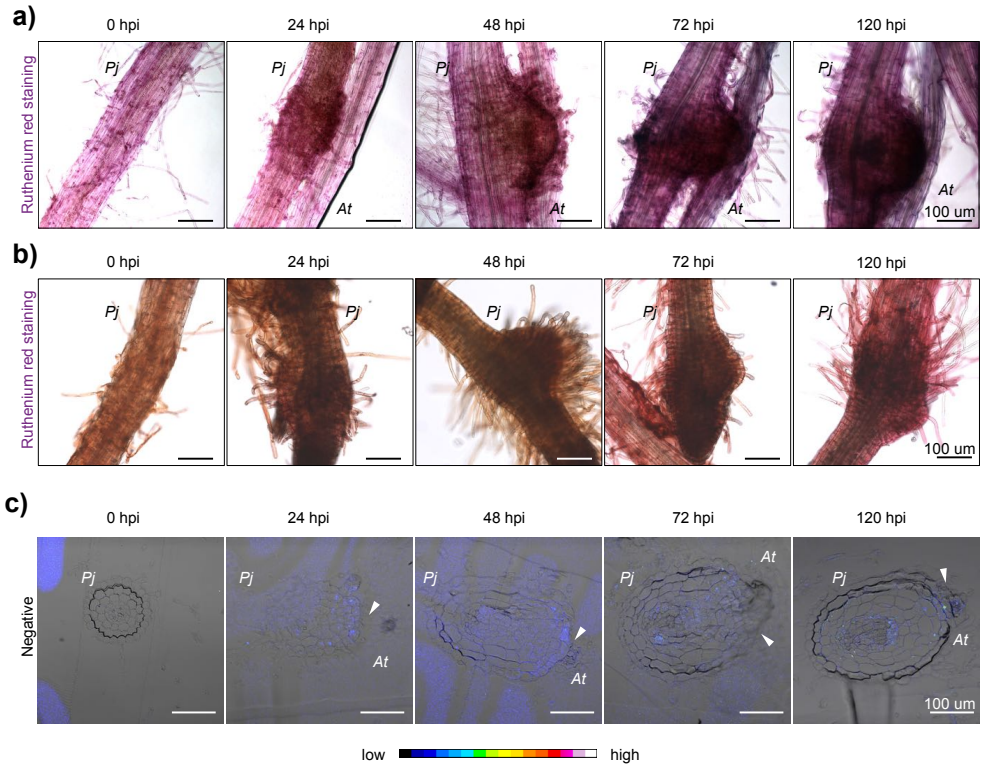
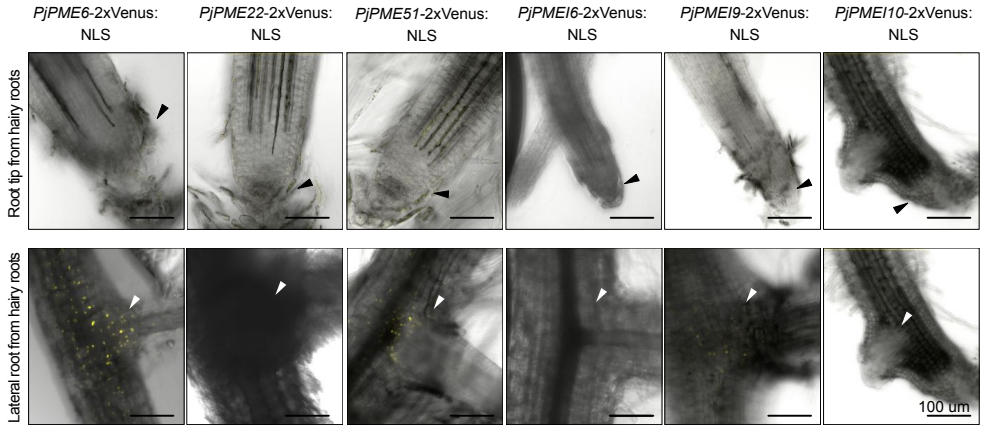


Supplemental Figure S1: *A. thaliana PMEs* and *PMEIs* are differentially expressed during haustorium development

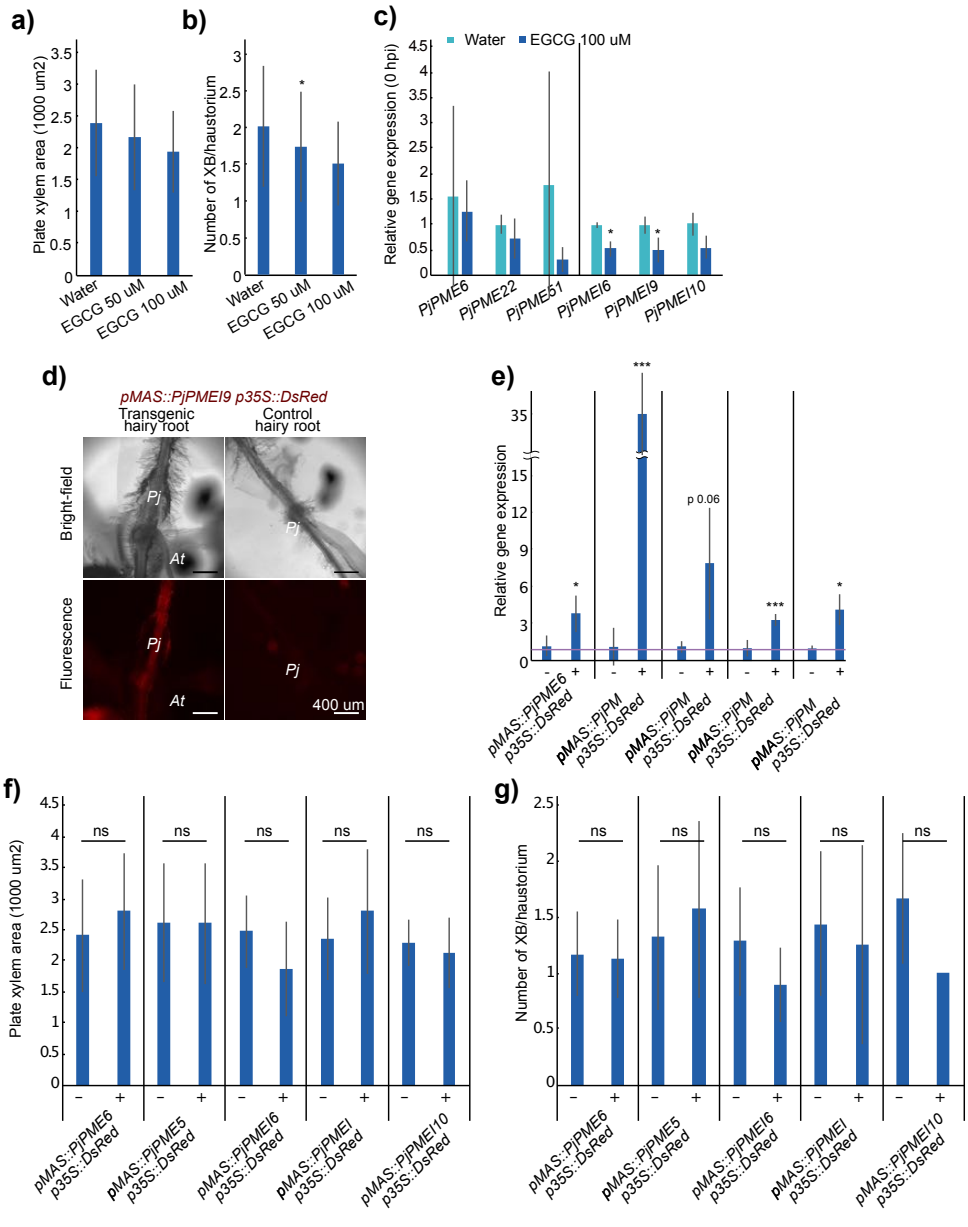
a-b) Maximum-Likelihood phylogenetic tree of PjPMEs and AtPMEs, or PjPMEIs and AtPMEIs. Branch lengths represent the number of substitutions per site, scale bars of 0.5. Bootstrapping values are presented at the nodes.
c-d) Heatmaps of the expression of *A. thaliana PMEs* and *PMEIs*: log2 fold change between infected and not infected *A. thaliana* over five time points during infection. The genes are clustered by expression profile.



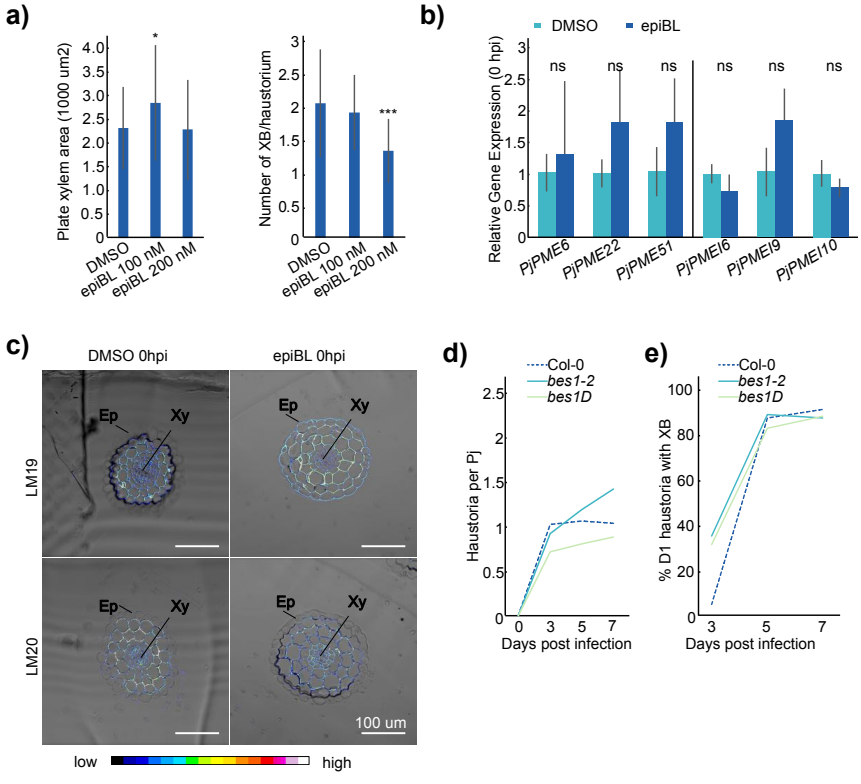
Supplemental Figure S2: PME activity is increased during haustorium development
a) Ruthenium red staining of the developing haustoria at 0, 24, 48, 72 and 120 hours post infection (hpi). 24, 48 and 120 hpi images are repeated from Fig. 2a b) Ruthenium red staining of the developing pre-haustorium at 0, 24, 48, 72 and 120 hours post exposure to DMBQ. c) Fluorescence images of the antibody staining negative control (PBS) on haustoria cross section at 0, 24, 48, 72 and 120 hpi. Scale bars 100 um; *Pj* = *P. japonicum*, *At* = *A. thaliana*; arrowheads point at the host-parasite interface.



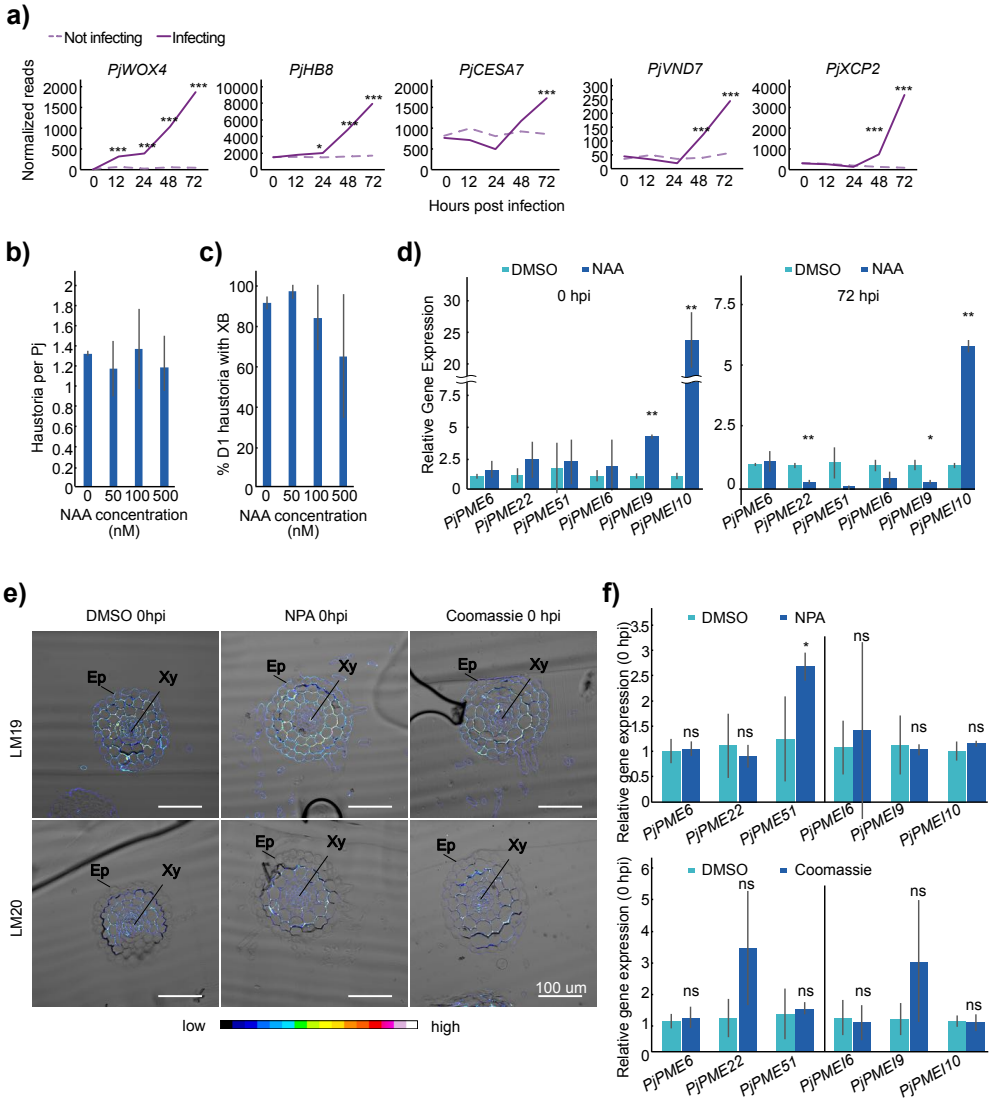
Supplemental Figure S3: *PjPMEs* and *PjPMEIs* are specific to haustoria
Images of transgenic hairy roots expressing *PjPME6*, *PjPME22, PjPME51, PjPMEI6*, *PjPMEI9* and *PjPMEI10* nuclear-localized transcriptional reporters: root tips and lateral roots. Scale bars 100 um. Black arrowheads point at root tips, white arrowheads point at lateral root emergence sites. The same image is presented for the root tip and lateral root of the *PjPMEI10*-2xVenus:NLS hairy root.



Supplemental Figure S4: *PjPME* and *PjPMEI* overexpression does not affect xylem connection to the host
a) Area of plate xylem in 7 dpi haustoria treated with 50 or 100 uM EGCG or water as control (n = 56-98 haustoria). b) Number of xylem bridges (XB) per haustorium in 7 dpi haustoria treated with 50 or 100 uM EGCG or water as control (n = 56-98 haustoria). c) Relative gene expression of selected *PjPMEs* and *PjPMEIs* at 0 hpi in *P. japonicum* haustoria treated with 100 uM EGCG, normalized to water (n = 3 biological replicates). d) Representative images of hairy roots transformed with gene overexpression constructs. Control hairy roots show no fluorescence, while transformed hairy roots have red fluorescence. *At* = *A. thaliana*, *Pj* = *P. japonicum*. Scale bar 400 um. e) Relative gene expression of the *PjPME* or *PjPMEI* of interest in hairy roots transformed with the indicated construct (+) and control roots (-) (n = 3 replicates). f) Area of plate xylem in 7 dpi haustoria formed on hairy roots transformed with the indicated construct (+) and control roots (-) (n = 3-29 haustoria). g) Number of XB per haustorium in 7 dpi haustoria formed on hairy roots transformed with the indicated construct (+) and control roots (-) (n = 3-29 haustoria). For all panels, asterisks indicate significance compared to control (Student's t-test)* for p<0.05, ** for p<0.01, *** for p<0.001, bars represent standard deviation. ns = not significant at p=0.05.



Supplemental Figure S5: *A. thaliana* BR signalling mutants do not affect parasitism efficiency
a) Area of plate xylem and number of xylem bridges (XB) per haustorium in 7 dpi haustoria treated with 100 nM and 200 nM epiBL or DMSO as control (n = 16-66 haustoria) b) Relative gene expression of selected *PjPMEs* and *PjPMEIs* at 0 hpi in *P. japonicum* haustoria treated with 100 nM epiBL, normalized to DMSO (n = 3 replicates). c) Fluorescence images of antibody staining using LM19 (unmethylated homogalacturonan) and LM20 (highly methylated homogalacturonan) on cross sections of haustoria developed on DMSO or 100 nM epiBL at 0 hpi. Scale bars 100 um. Ep=epidermis, Xy=xylem. d) Number of haustoria per *P. japonicum* plant at four time points during infection of *bes1-2* and *bes1-D* mutants or Col-0 as control (n = 2 replicates). e) Percentage of Day-1 (D1) haustoria with a XB formed during infection of *bes1-2* and *bes1-D* mutants or Col-0 at three time points (n = 2 replicates). For all panels, asterisks indicate significance compared to control (Student's t-test): * for p<0.05, ** for p<0.01, *** for p<0.001, bars represent standard deviation. ns = not significant at p=0.05.



Supplemental Figure S6: NAA treatment does not affect parasitism efficiency
a) Normalized reads of *PjWOX4*, *PjHB8*, *PjCESA7*, *PjVND7* and *PjXCP2* over five time points during infection for *P. japonicum* infecting and not infecting. Asterisks indicate significant difference between infecting and not infecting (Wald test with Benjamini-Hochberg correction for multiple testing, n = 3 libraries) * for p<0.05, *** for p<0.001. b) Number of haustoria per *P. japonicum* plant at 7 dpi during treatment with 0, 50, 100 or 500 nM NAA (n = 3 replicates). c) Percentage of Day-1 (D1) haustoria with a xylem bridge (XB) formed during treatment with 0, 50, 100 or 500 nM NAA (n = 3 replicates). d) Relative gene expression of selected *PjPMEs* and *PjPMEIs* at 0 and 72 hours post infection in *P. japonicum* haustoria treated with 1 uM NAA, normalized to DMSO (n = 3 replicates). e) Fluorescence images of antibody staining using LM19 (unmethylated homogalacturonan) and LM20 (highly methylated homogalacturonan) on cross sections of 0 hpi haustoria developed on DMSO, 5 uM NPA or 0.05 mM Coomassie + DMSO. Scale bars 100 um. Ep=epidermis, Xy=xylem. f) Relative gene expression of selected *PjPMEs* and *PjPMEIs* at 0 hpi in *P. japonicum* haustoria treated with 5 uM NPA or 0.05 mM Coomassie, normalized to DMSO (n = 3 biological replicates). For panels b to f, Asterisks indicate significance compared to control (Student's t-test) * for p<0.05, ** for p<0.01, bars represent standard deviation. ns = not significant at p=0.05.

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Parasitic plants and grafting are examples of plant-plant interactions that share many developmental processes. However, while grafting is used to improve plant traits in agriculture, plant parasitism leads to high agricultural yield losses. In this thesis, we studied the mechanisms regulating parasitic plant haustoria formation and graft reconnection, focusing on nutrient availability and cell wall modifications. Our results suggest interplay between cell wall, nutrients and hormone signalling, and contribute to the understanding of similarities and differences between these two processes.

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