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Mechanisms of Host Plant Infection by the Parasitic Angiosperm *Cuscuta*

Studies on Cell Wall Changes

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Stian Olsen

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CONTENTS

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Tromsø, March 2017

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SUMMARY

The thread-like plants of the genus *Cuscuta* lack proper leaves and roots and cannot sustain themselves by photosynthesis. Therefore, in order to survive and reproduce, these highly adapted parasites infect other plants to steal their water and nutrients. They do so by developing specialized infection organs called haustoria that, in a successful infection, grow into the tissue of the host plant establishing interspecies cell-to-cell connections through which the parasite feeds. Although it has long since been suggested that some manner of modification to host cell walls is necessary to allow the invasive growth of the infection organ, little is known about what specific wall components are altered and what actors facilitate these changes.

In this thesis, the process of infection was investigated from multiple angles to shed light on the mechanisms underlying host tissue penetration by the *Cuscuta* haustorium. The analysis of cell wall compositions and wall-localized enzyme activities in host and parasite revealed that the walls of both plants are changed during infection and further indicated that specific wall constituents in host cell walls could provide resistance against *Cuscuta*. A screen for *Cuscuta* genes whose expression levels were increased upon the onset of haustorium development identified several cell wall-related genes and some encoded products whose function could explain the wall changes detected in the previous study. In pursuing the putative activity of a specific class of cell wall-modifying enzymes, the xyloglucan endotransglucosylases/hydrolases, we found that these enzymes are secreted from the haustorium and that their activity is essential for host invasion. Intriguingly, the same enzyme activity was observed in host cell walls at the infection site during the defence response of a resistant host plant. This supports the idea that the cell wall is a crucial determinant for the outcome of an infection attempt. Genetic transformation is an indispensable tool to test the functions of candidate infection genes. To this end, the transient expression of transgenes in the parasitic plant was achieved and initial tests toward the regeneration of transgenic *Cuscuta* were performed. These accomplishments have increased our understanding of *Cuscuta* parasitization and will assist the development of new control strategies that are needed to efficiently combat parasitic plants in agricultural areas.

LIST OF PAPERS

Paper I

Johnsen HR, Striberny B, Olsen S, Vidal-Melgosa S, Fangel JU, Willats WG, Rose JK, Krause K. 2015.

Cell wall composition profiling of parasitic giant dodder (*Cuscuta reflexa*) and its hosts: *a priori* differences and induced changes.

New Phytologist 207(3), 805-816.

Paper II

Olsen S, Striberny B, Hollmann J, Schwacke R, Popper ZA, Krause K. 2016.

Getting ready for host invasion: elevated expression and action of xyloglucan endotransglucosylases/hydrolases in developing haustoria of the holoparasitic angiosperm *Cuscuta*.

Journal of Experimental Botany 67(3), 695-708.

Paper III

Olsen S, Popper ZA, Krause K. 2016.

Two sides of the same coin: Xyloglucan endotransglucosylases/hydrolases in host infection by the parasitic plant *Cuscuta*.

Plant Signaling and Behavior 11(3).

Paper IV

Olsen S, Krause K. 2017.

Are xyloglucan endotransglucosylases/hydrolases the keys to host invasion by the parasitic plant *Cuscuta reflexa*?

Manuscript submitted to *PLOS ONE* in February 2017.

(Thesis author is underlined)

ABBREVIATIONS

dsRed	*Discosoma* sp. red fluorescent protein
FR	Far-red (referring to light of ~740 nm wavelength)
XET	Xyloglucan endotransglucosylation
XTH	Xyloglucan endotransglucosylases/hydrolases

GLOSSARY

Haustoriogenesis	The growth and differentiation of the haustorium.
Haustorium	The multicellular infection organ of parasitic flowering plants that grows into the tissue of other plants and facilitates the uptake of resources.
Host-parasite interface	The cells immediately adjacent to the border between parasite and infected host.
Infection site	The location at which the parasite invades (or tries to invade) a potential host.
Parasitic plant	A plant that obtains resources from another plant via cell-to-cell connections.
Resistant host	An organism that actively or passively prevents parasitization. The term incompatible is used synonymously to resistant.
Susceptible host	An organism that is infected successfully and provides the parasite with resources. The term compatible is used synonymously to susceptible.

1. INTRODUCTION

Plants are highly regarded as primary producers, being photoautotrophic life forms that can sustain not only themselves but also make biomass to support others in their ecosystem. However, there are plants that have abandoned this generous demeanour in favour of a more selfish lifestyle. Some even have the nerve to steal from their not so distant relatives!

These are the parasitic plants.

1.1. Parasitic Plants

Like all forms of life, plants exist in a constant interplay with biotic and abiotic factors. After several hundred million years of diversifying, plants occupy many niches; from the living fossil *Welwitschia* in the desert to the tiny aquatic *Wolffia*, the insect-eating Venus flytrap or the research model *Arabidopsis*. Not even parasitism is untested by plants. Following the generally accepted nomenclature, parasitic plants will here be defined as plants that, through a direct physical connection, acquire resources from another plant (the host) at the expense of the latter (Poulin, 2011). Therefore, epiphytic plants that grow on the surface of other plants without feeding on them and myco-heterotrophic plants that interact with soil-borne fungi are not considered parasitic plants. Parasitism is apparently a very successful life strategy in angiosperms and, having evolved on at least 12 or 13 independent occasions, ~1 % of flowering plants (translating into approximately 4000 species) are estimated to be parasitic (Barkman *et al.*, 2007; Westwood *et al.*, 2010). The gymnosperm *Parasitaxus ustus* is also described as having a parasitic lifestyle (Feild and Brodribb, 2005). However, it lacks the defining feature of all parasitic flowering plants, the haustorium.

Haustoria are specialized infection organs that grow into the tissue of compatible host plants and establish the vascular connections needed for the parasite to obtain water and nutrients

from its host (Yoshida *et al.* (2016); Fig. 1). As the haustorium of parasitic plants is a multicellular organ that forms through an elaborate developmental process and consists of cells with specialized functions, it is not to be confused with the invasive unicellular hyphae of plant pathogenic fungi bearing the same name (Mayer, 2006). In addition to facilitating the uptake of the parasite's nutritional necessities from its host, the host-parasite bridge established by the haustorium allows for the trafficking of a large variety of molecules including nucleic acids, proteins and viruses between the two plants (Aly, 2013; Kim and Westwood, 2015). The interspecies flow of nucleic acids is reflected by the high rate of horizontal gene transfer between parasitic plants and their hosts (Davis and Xi, 2015).

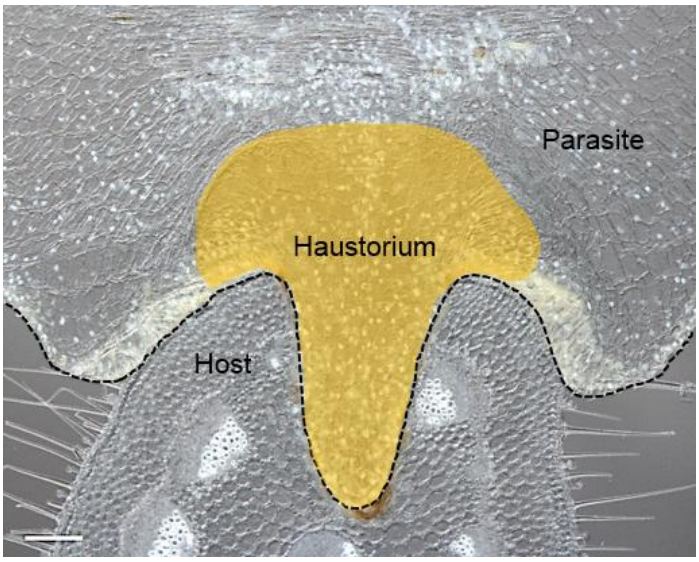

Fig. 1 The haustorium of parasitic plants. Cross-section of a parasitic plant infection site (the parasite *Cuscuta reflexa* on its compatible host *Pelargonium zonale*) in which the haustorium of the parasite (in orange) has penetrated the tissue of the host. The dotted line marks the surface of the parasite. Scale bar = 200 µm.

Parasitism can have major impacts on the general state and performance of hosts and, having to share their resources with the parasite, infected plants normally display reduced biomass. Thus, parasitic plants can help to maintain biodiversity in natural ecosystems by suppressing the growth of dominating species (Press and Phoenix, 2005). In agriculture, however, attacks by parasitic plants have devastating effects on crop yield. Moreover, the intimate connections created by the haustoria and the considerable similarity of host and parasite (both being flowering plants) makes the control of parasitic weeds especially difficult as conventional methods (e.g. herbicides and crop rotation) often are ineffective (Aly, 2007; Rispail *et al.*, 2007). As such, deeper knowledge about the mechanisms of haustorium development (or haustoriogenesis) and host infection is needed to support the development of new strategies for combating parasitic plants in agricultural areas.

1.1.1. Categorization of Parasitic Plants

Based on their degree of host dependence, parasitic plants can be divided into two groups: facultative and obligate parasites. Whereas obligate parasites cannot complete their life cycles without infecting a host plant, facultative parasites are fully functional photoautotrophs that possesses the ability to parasitize other plants (Yoshida and Shirasu, 2012). Holoparasitic plants are not capable of producing the reduced carbon they need by photosynthesis and must therefore obtain both sugars and water from their host plants. Hemiparasites on the other hand, are photosynthetically self-sufficient and parasitize other plants in order to acquire non-photosynthetic nutrients and water. Whereas all holoparasites are obligate, hemiparasitic plants can be either facultative or obligate (Westwood *et al.*, 2010). As it must locate, attach and invade a suitable host plant, the life of a parasitic plant in many ways stands in contrast to that of a "normal" photoautotrophic plant. Hence, many parasitic plants have evolved morphologies somewhat divergent from their self-sustaining relatives (Fig. 2).

In addition to being categorized according to the degree of their nutritional dependence, parasitic plants can be distinguished based on whether they attach to and infect their hosts above or below ground. Several lineages contain root parasitic plants that infect the below ground parts of other plants (Westwood *et al.*, 2010). Among them are species of the genera *Orobanche*, *Striga* and *Phelipanche* (all three of the Orobanchaceae family) that produce small seeds containing little food reserves. In order to assure host proximity, these obligate root parasites require host-derived stimulants to germinate (Cardoso *et al.*, 2011). The family Rafflesiaceae contains highly specialized root holoparasites (Fig. 2). Except for the flower, which is the world's largest (*Rafflesia arnoldii*), the body of these parasites live endophytically within the roots of their host (Nikolov *et al.*, 2014). Facultative root parasites (e.g. *Triphysaria* spp. and *Rhinanthus* spp.) on the other hand, are often indistinguishable from photoautotrophic plants except for their ability to form haustorial connections with host roots (Fig. 2). Shoot parasites infect the above ground parts of other plants. Mistletoes are well-known obligate shoot hemiparasites that infect branches of woody plants (Cocoletzi *et al.* (2016); Fig. 2). Species of the genera *Cassytha* and *Cuscuta* are often mistaken for one

another, as both encompass obligate shoot parasitic vines with reduced leaves (Fig. 2). However, whereas *Cassytha* seedlings develop roots and can survive using their own photosynthetic capacity for some time, *Cuscuta* must locate and infect a host plant shortly after germination (Dawson *et al.*, 1994; Furuhashi *et al.*, 2016).

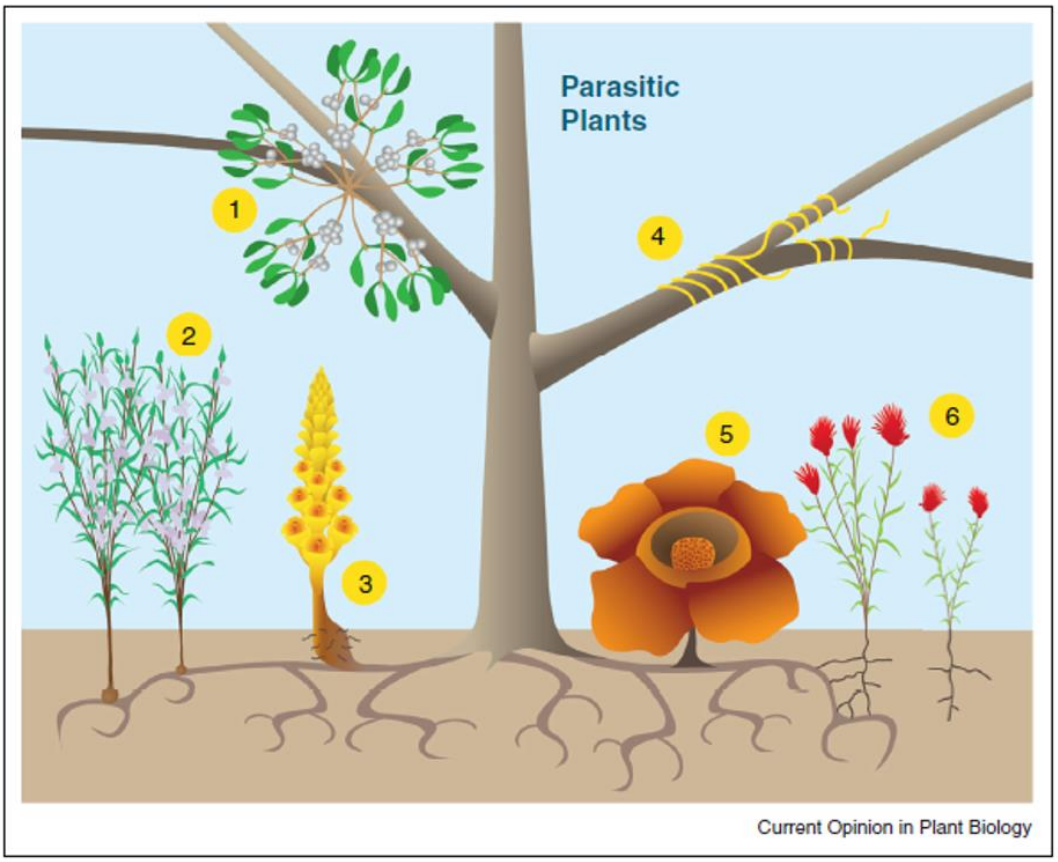

Fig. 2 Illustration of parasitic plant groups parasitizing the root or shoot of a host plant. (1) Obligate shoot hemiparasites commonly known as mistletoes (order Santalales). (2) Obligate root hemiparasites of the genus *Striga* (witchweeds). (3) Obligate root holoparasites of the Orobanchaceae family. (4) Obligate shoot holoparasites of the genus *Cuscuta* (dodders). (5) Obligate root-endophytic holoparasites of the genus *Rafflesia*. (6) Facultative root hemiparasites of the family Orobanchaceae. After Smith *et al*. (2013).

1.2. The Genus *Cuscuta*

As they are the subjects of this thesis, this chapter will introduce the parasitic plants of the genus *Cuscuta* in more detail. These thread-like parasitic plants commonly referred to as dodders show little resemblance to their photoautotrophic relatives (Fig. 2 and 3). The genus evolves within the Convolvulaceae (morning glory family) in the order Solanales and encompasses around 200 species of obligate shoot holoparasitic plants (Garcia *et al.*, 2014).

Cuscuta is divided into the three subgenera *Monogynella*, *Cuscuta* and *Grammica*. While species of *Monogynella* characteristically have thick stems (e.g. *Cuscuta reflexa*, Fig. 3), the other two subgenera include more delicate species (e.g. *Cuscuta campestris* of the subgenus *Grammica*) (Dawson *et al.*, 1994).

Fig. 3 The parasitic plant *C. reflexa* infecting its compatible host *P. zonale*. (a) Propagation of *C. reflexa (Cr)* on *P. zonale (Pz)* at the Phytotron at Holt, Tromsø, Norway. (b) Close up of infection site with arrows indicating where single haustoria grow into the host. (c) Cross-section of the parasite feeding on its host. The xylem bridge is seen as a line of white cells extending from the middle of the parasite stem, through the haustorium (h) and into the host where it fuses with the xylem bundle of the host plant (arrow). Scale bar = 200 µm.

Although of higher abundance in tropical and subtropical areas, dodders are considered to have a worldwide distribution and can be found on every continent except Antarctica. Species of *Cuscuta* attack important crops (e.g. alfalfa and tomato) and present serious problems to agriculture as they reduce crop yields (Aly, 2007). Upon germination, *Cuscuta* seedlings emerge without cotyledons or leaves and with a reduced root-like structure that rapidly degenerates (Sherman *et al.*, 2008). Some species of *Cuscuta* are clearly green (e.g. *C. reflexa*, Fig. 3), indicating photosynthetic activity. However, even in these relatively green species the rate of carbohydrate production is too low to sustain the parasite (Hibberd *et al.*, 1998; van der Kooij *et al.*, 2000). Therefore, in order to be able to complete its life cycle, newly

germinated seedlings must quickly locate, attach and infect a compatible host plant (Fig. 3b). In the later stages of infection, tip-growing cells called searching hyphae protrude from the main haustorium and grow in search of the vascular tissues of the host. When they make contact with xylem or phloem of the host, they differentiate into the respective cell types to allow the uptake of water and sugar from the host plant (Lee, 2009; Vaughn, 2006). In a fully developed feeding haustorium of *C. reflexa* on its compatible host *P. zonale*, the xylem bridge between the two species is clearly visible (Fig. 3c). Moreover, plasmodesmata have been observed between host cells and developing parasite hyphae (Birschwilks *et al.*, 2006; Birschwilks *et al.*, 2007; Dawson *et al.*, 1994; Lee, 2009; Vaughn, 2003), which proves that interspecific symplastic connections are established. When it comes to the range of host species parasitized, some *Cuscuta* species are generalists that can successfully infect a multitude of different plants, whereas others are restricted to only a single host species (Dawson *et al.*, 1994). The parasite seems to prefer dicotyledonous plants, as monocotyledonous plants are rarely infected. Grasses, especially, are in but very few cases, immune to *Cuscuta* (Dawson *et al.*, 1994). Nevertheless, there are also dicotyledonous species that display resistant mechanisms against *Cuscuta* attacks (Kaiser *et al*., 2015).

1.2.1. Plant Responses to Infection

Considering that plants possess systems to recognize and counteract attacks by other plant pathogens (Boller and Felix, 2009), it seems unlikely that the intrusive growth of the haustorium should go on unnoticed. As such, the fact that some plants, like *Pelargonium zonale*, display no visible defence reactions when infected by *C. reflexa* is quite remarkable. The haustoria of *C. reflexa* were reported to be able to invade the tropical liana *Ancistrocladus heyneanus*, but subsequently degenerated as a response to the production of pathogen repellents by the host (Bringmann *et al.*, 1999). Poinsettia (*Euphorbia pulcherrima*) responds to haustoria of *C. reflexa* and *Cuscuta japonica* by producing localized bark outgrowths that force out the infection organs (Christensen *et al.*, 2003). However, the host was described as only partially incompatible as the parasite could tolerate haustorium rejections by forming new infection sites. The cultivated tomato *Solanum lycopersicum* is resistant to infection by

C. reflexa whereas the wild tomato *Solanum pennellii* is susceptible (Hegenauer *et al.* (2016); Johnsen (2014)). These closely related species provide an excellent system for studying host-parasite interactions. During the early stages of haustorium development, *S. lycopersicum* cells at the infection site elongate and burst while a modified wound-like tissue is formed, preventing the parasite from entering the host (Kaiser *et al.* (2015); Fig. 4). The expression of genes encoding a cell wall enzyme and an aquaporin have been associated with the hypersensitive-type response of this tomato against *C. reflexa* (Albert *et al.*, 2004; Werner *et al.*, 2001). Although proposed to be involved in the expansion of host cells at the site of contact with the parasite, the exact roles of these genes in the defence response remain unknown (Kaiser *et al.*, 2015). The resistance of *S. lycopersicum* appears to be specific against *C. reflexa* as several other species including *Cuscuta pentagona* are able to successfully parasitize this plant species. Nevertheless, *S. lycopersicum* responds with strong inductions of the defence-related plant hormones jasmonic acid and salicylic acid upon being infected by *C. pentagona* (Runyon *et al.*, 2010). Both hormones are known to play essential roles in plant immunity (Pieterse *et al.*, 2012; Shigenaga and Argueso, 2016). Infection by *C. reflexa* also induces release of Ca^{2+}, a major actor in signal transduction pathways, in the resistant tomato (Albert *et al.*, 2010b). It remains to be investigated whether the hormones and the second messenger act through the same or different pathways and where the threshold for complete resistance lies (Albert *et al.*, 2010a; Kaiser *et al.*, 2015).

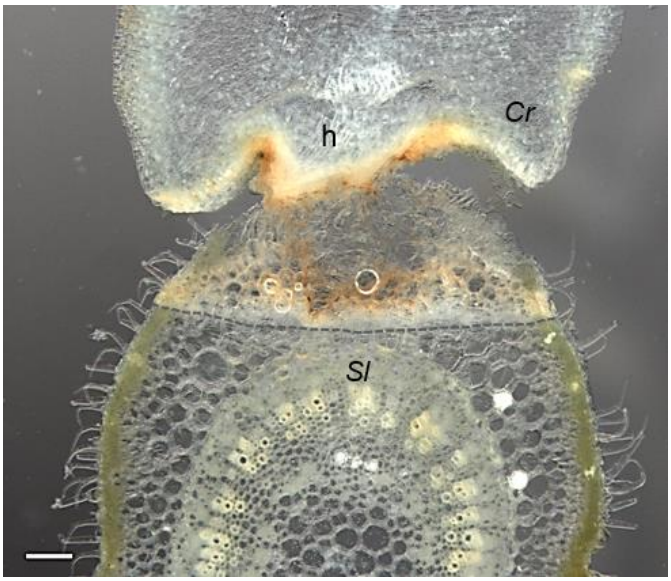

Fig. 4 Cross-section of *C. reflexa* trying to infect the resistant host *S. lycopersicum*. The dotted line shows where the wound-like tissue of *S. lycopersicum (Sl)* begins and h indicates the haustorium of *C. reflexa (Cr)*. Scale bar = 200 µm.

Recently, it was reported that the pattern recognition receptor CUSCUTA RECEPTOR 1 of *S. lycopersicum* detects a small peptide factor from *C. reflexa* and initiates the above mentioned defence responses (Hegenauer *et al.*, 2016). This shows that plants can sense intruding *Cuscuta* much in the same way as they do recognize other plant pathogens (Malinovsky *et al.*, 2014; Mitsumasu *et al.*, 2015).

1.2.2. Host Location and Onset of Haustoriogenesis

C. pentagona has been reported to perceive plant volatiles as a means to guide its growth towards compatible host plants (Runyon *et al.*, 2006). This sensing of chemical cues, however, has so far not been observed in other species of *Cuscuta*. For instance, *C. reflexa* is just as ready to commit to haustorium development on metal or plastic rods as it is on a green plant and even infects itself occasionally (Hong *et al.* (2011); Kaiser *et al.* (2015); own observations). In *Cuscuta planiflora* and *C. campestris*, far-red (FR) light induces positive phototropism (Benvenuti *et al.*, 2005; Orr *et al.*, 1996). As chlorophyll absorbs red light, the light passing through green foliage has a lower red:FR ratio than non-filtered light. Thus, shoot parasitic plants could be detecting light qualities in order to locate host plants. In fact, rates of host location and attachment by *Cuscuta* was shown to be reduced under light conditions with a high red:FR ratio (Johnson *et al.*, 2016). Further substantiating the significance of light in *Cuscuta* parasitization, the cooperative effect of FR light and tactile stimuli is sufficient to activate haustorium development in several *Cuscuta* species (Tada *et al.* (1996); Fig. 5). The effect of FR light was suggested to be mediated by phytochrome as it was reversible by red light (Furuhashi *et al.*, 1997).

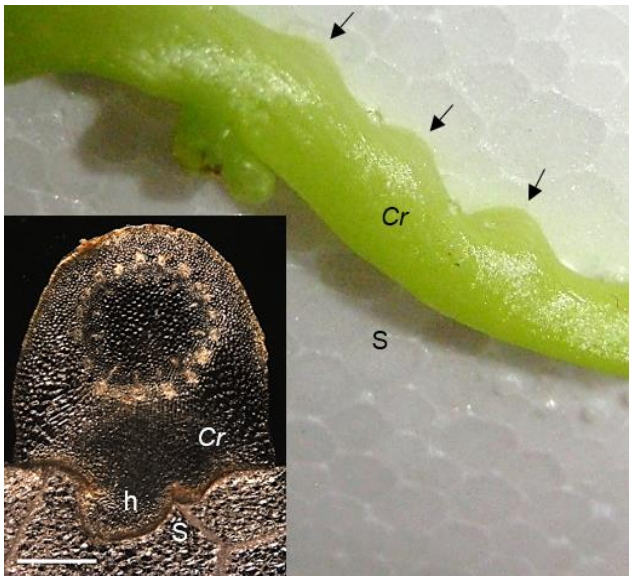

Fig. 5 Host-free induction of haustoriogenesis. Arrows indicate where *C. reflexa* (*Cr*) haustoria, whose development was activated by FR light and tactile stimuli, grow into Styrofoam (S). Cross-section of "infection site" with invading haustorium (h) is seen as smaller insert. Scale bar in insert = 1000 µm.

Once in contact with a host plant, the parasite begins to twine around stems and petioles before committing to host infection by developing the haustoria. Cytokinin has been reported to activate haustoriogenesis without FR light and is therefore anticipated to be involved in the signal transduction downstream of the initial light and contact signals (Furuhashi *et al.*, 2011; Haidar *et al.*, 1998; Ramasubramanian *et al.*, 1988). How these factors work in concert to govern whether the parasite commits to haustoriogenesis, remains unknown.

1.2.3. Invasion of Host Tissue

The unilateral swelling of the parasite's stem facing the host surface indicates the initiation of haustorium development. *Cuscuta* epidermal cells then secrete a cementing material that secures the attachment of parasite to host (Lee, 2008; Vaughn, 2002). Proper attachment to the host surface appears to be essential for invasion as the parasite also induces the host to produce substances that further increase the attachment force (Albert *et al.*, 2006). The activity of a cysteine protease from *C. reflexa* was shown to be required for successful host infection, proposedly by degrading host proteins (Bleischwitz *et al.*, 2010). Intriguingly, the inhibition of a similar cysteine protease in the obligate root parasite *Phelipanche aegyptiaca* also reduced infection rates (Rehker *et al.*, 2012). A SHOOT MERISTEMLESS-like protein is essential to host infection by *C. pentagona* as gene silencing by RNA interference disrupts the growth and development of the parasite haustorium (Alakonya *et al.*, 2012). Putatively involved in directing growth of searching hyphae, the exact function of this protein remains unknown. Recently, transcriptome analyses have correlated the elevated expression of genes encoding products related to transport activity and cell wall with haustorium development in both Orobanchaceae and *Cuscuta* (Convolvulaceae) (Ikeue *et al.*, 2015; Ranjan *et al.*, 2014; Yang *et al.*, 2015). The increased production of transporters is consistent with the parasite preparing to feed on its host. Cell wall modifiers, on the other hand, could promote host tissue penetration as the polysaccharide-rich wall of plant cells presents a major obstacle for any plant invader.

1.3. The Plant Cell Wall

Plant cells are surrounded by walls and, given the central role of this barrier in maintaining plant integrity, the structural components and dynamics of plant walls will be briefly presented in this chapter. One may identify three layers in a plant cell wall (Popper, 2008): (i) The middle lamella forms the adhesive boundary between cell walls of adjacent cells. (ii) The primary wall is a strong but extensible layer continuously synthesized and modified by growing cells. Some cell types (e.g. xylem cells) deposit a (iii) secondary wall between their primary wall and the plasma membrane to provide further strength and rigidity after growth cessation.

Cellulose is the principle structural component of plant cell walls. This polysaccharide is synthesized by large enzyme complexes at the plasma membrane and consists of long linear chains of β-(1→4)-linked glucose packed tightly into microfibrils (Doblin *et al.* (2002). Cellulose microfibrils are stiff rods of great mechanical strength that contribute structural stability to the cell wall when cross-linked by other wall polysaccharides (Fig. 6).

The term hemicellulose encompasses non-cellulosic polysaccharides with β-(1→4)-linked backbones that are often extensively branched. This includes xyloglucan, xylan, mannan and mixed-linkage glucan (which have β-(1→3)-linkages interspersed at regular intervals within their β-(1→4)-linked backbone) (Scheller and Ulvskov, 2010). Hemicelluloses are synthesized in the Golgi and secreted into the apoplast where their central role is to strengthen the cell wall by interacting with cellulose (Fig. 6).

Pectin is a heterogeneous group of complex cell wall polysaccharides that are rich in galacturonic acid. The three main types of pectin are homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II (Willats *et al.*, 2001). Pectic polysaccharides form a gel-like matrix between cellulose microfibrils that is associated with several different functions. In the middle lamellae of dicotyledonous plants, pectins are proposed to facilitate adhesion between adjacent cells (Jarvis *et al.*, 2003; Fig. 6). As with hemicelluloses, pectins are synthesized in the Golgi and delivered to the wall via vesicles.

The type of components that make up the cell wall networks can vary between different plant groups and species. Notably, the cell wall composition of grasses differs from that of

dicotyledonous plants. Whereas xyloglucan is the most abundant hemicellulose in the primary walls of dicotyledonous plants, cell walls of grasses contain more arabinoxylan (typically decorated with ferulic acid) and mixed-linkage glucan, but little xyloglucan (Scheller and Ulvskov, 2010). Also the pectic polysaccharides, which are major wall constituents of dicotyledonous plants, are found in only low concentrations in grass walls (Vogel, 2008).

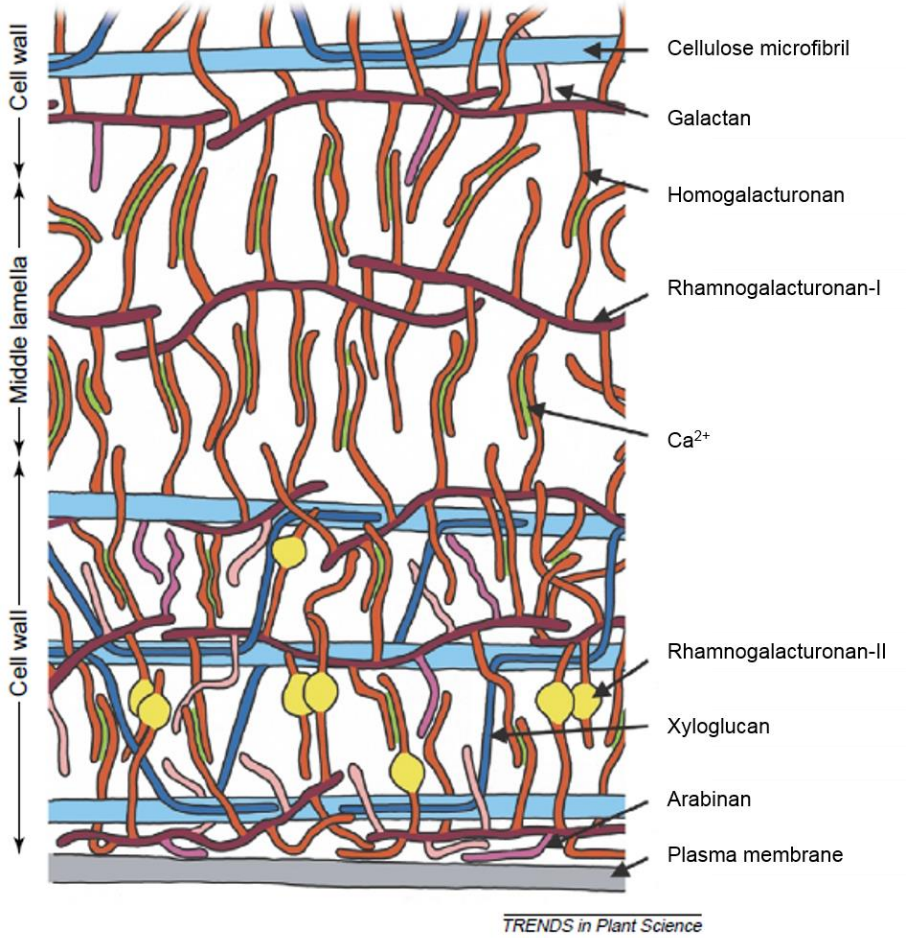

Fig. 6 Major polysaccharides of the primary cell wall and the middle lamella in dicotyledonous plants. Outside the plasma membrane, cellulose microfibrils, hemicelluloses (xyloglucan) and pectins (homogalacturonans, rhamnogalacturonan-I, rhamnogalacturonan-II, galactan and arabinan) interact to make up the cell wall. The pectic polysaccharides are also abundant in the middle lamella. Ca^{2+} forms additional cross-links between de-esterified homogalacturonans. Modified after Vorwerk *et al*. (2004).

Classically, the primary cell wall of dicotyledonous plants is described as a load-bearing network of xyloglucan-coated cellulose microfibrils embedded in a pectin matrix. However, increasing evidence argues that linkages exist between pectins and other cell wall components including cellulose and xyloglucan (Chebli and Geitmann, 2017; Popper and Fry,

2008; Thompson and Fry, 2000). These networks of polysaccharides make up a complex matrix that allows plant cells to maintain high turgor pressures and thus enable plants to stand tall to harvest sunlight efficiently (Fig. 6). However, as plant growth and development require cell division, expansion and differentiation, plant cells must be able to control and modify the mechanical properties of their walls.

1.3.1. Cell Wall Modifiers

In expanding cells, new wall polymers are secreted into the cell wall to prevent thinning as the cell surface increases. However, the structure, strength and flexibility of the primary cell wall is not only decided by what components are synthesized and deposited into the apoplast. Several proteins regulate plant growth and development by acting on the polysaccharides that make up the cell wall. Some of these are glycoside hydrolases that promote cell wall loosening by cleaving more or less specific polysaccharides. Others cleave one polysaccharide chain and graft it onto another (transglycosylation) and can thereby facilitate either loosening or the reversal strengthening of the cell wall (Frankova and Fry, 2013). The xyloglucan endotransglucosylases/hydrolases (XTHs) modify wall strength by transglycosylating or hydrolytically cleaving the hemicellulose xyloglucan, respectively termed xyloglucan endotransglucosylation (XET) and xyloglucan endohydrolysis (Rose *et al.*, 2002). Pectins are deposited into the cell wall in a highly methyl-esterified state. The de-esterification of pectins by pectin methyl esterases makes the polysaccharide more prone to degradation by pectate lyases and polygalacturonases (Wakabayashi *et al.*, 2003). However, as de-esterification of pectins also facilitates cross-linking and gel formation, pectin methyl esterases can be promoters of both cell wall loosening and strengthening (Chebli and Geitmann, 2017; Willats *et al.*, 2001). Expansins are proteins that induce wall loosening without exhibiting enzymatic activity, probably by disrupting hydrogen bonds between wall polysaccharides (Cosgrove, 2005). Whereas plants modify their own walls to regulate growth and development, plant tissue invaders have been shown to employ cell wall modifiers to achieve access across the cell wall.

1.3.2. Overcoming the Cell Wall Barrier

Similar to parasitic plants, plant pathogenic fungi, bacteria and nematodes are also dependent on breaching their host's cell wall. These have been shown to utilize a range of plant cell wall-modifying enzymes to facilitate plant cell penetration (King *et al.*, 2011; Malinovsky *et al.*, 2014; Vorwerk *et al.*, 2004). Structural and immunological studies on *Cuscuta* parasitization indicate that cell wall compositions of both host and parasite are altered through the process of infection. Moreover, host walls adjacent to parasite cells appear stretched and the chimeric wall of the two species display unique pectin compositions (Dawson *et al.*, 1994; Lee, 2009; Vaughn, 2003). There are also several reports on activities of cell wall enzymes in *Cuscuta*. Nagar *et al.* (1984) detected high activities of pectin esterase, polygalacturonase and xylanase in haustoria of *C. reflexa*. Supporting the significance of pectin-modification in host infection by *Cuscuta*, activities of pectin methyl esterases and polygalacturonases were also found in *C. campestris* (Bar Nun and Mayer, 1999; Bar Nun *et al.*, 1999). Johnsen and Krause (2014) reported higher cellulolytic activity in *C. reflexa* than in its compatible host *P. zonale*. Peroxidase activity during the invasion of coffee by *Cuscuta jalapensis* was suggested to promote modification of host cell wall components (Lopez-Curto *et al.*, 2006). Although the enzyme activities detected in *Cuscuta* can explain some of the reported cell wall changes occurring during host penetration, the specific executors in the parasite remain to be identified. A proteomic study by Li *et al.* (2010) revealed that the blue light-induced twining of *Cuscuta australis*, which is the step prior to haustoriogenesis onset, is accompanied by the increased abundance of two pectin esterases. Recent data on *Cuscuta* gene expression also associated the expression of pectin lyases, pectin methyl esterases, pectate lyases, polygalacturonases, cellulases and expansins with haustorium development (Ikeue *et al.*, 2015; Ranjan *et al.*, 2014). Nevertheless, as the development of the infection organ is a process involving plant growth, the identification of cell wall modifiers expressed by the parasite to promote penetration of host tissue requires comparisons with other growing tissues of the parasite as well as *in situ* localization of the cell wall-modifying activity. A more comprehensive picture of cell wall dynamics during infection is also needed to increase our knowledge on the role of cell walls in the parasitization strategy of *Cuscuta*.

2. AIMS AND OBJECTIVES

The main ambition of this project was to further our understanding of mechanisms ultimately leading to the establishment of cell-to-cell connections between the *Cuscuta* haustorium and the infected hosts. From a basic research perspective, *Cuscuta* parasitization offers a wealth of unsolved puzzles, as the mechanisms behind host plant penetration and parasite feeding remain largely uncharted. Moreover, this knowledge is needed to develop efficient strategies to control *Cuscuta* in agricultural areas. It became evident early on in the project that changes to both host and parasite cell walls take place during parasitization. Therefore, cell wall components and their modifiers became the focal points of the investigations on host infection by *Cuscuta* presented in this thesis.

The specific objectives and aims of this study were:

i. To determine cell wall compositions of host and parasite during infection to shed light on which components are altered and to examine if resistance against *Cuscuta* can be related to specific wall constituents (**Paper I**).

ii. To analyse *Cuscuta* gene expression during haustorium development to see what changes the parasite is orchestrating and to identify candidate infection genes (**Paper II**).

iii. To further investigate the putative functionalities of candidate infection genes to obtain a deeper understanding of the *Cuscuta* parasitization strategy (**Papers III**, **IV** and **Work in Progress**).

3. SUMMARY OF PAPERS

Paper I

Johnsen HR, Striberny B, Olsen S, Vidal-Melgosa S, Fangel JU, Willats WG, Rose JK, Krause K. 2015.

Cell wall composition profiling of parasitic giant dodder (*Cuscuta reflexa*) and its hosts: *a priori* differences and induced changes.

New Phytologist 207(3), 805-816.

Host penetration by the *Cuscuta* haustorium is hypothesized to be mediated by a combination of applied mechanical pressure and loosening of the host cell wall through the degradation of wall components. In this study, the latter was tested by performing an antibody-based profiling of wall constituents and enzymatic activities in the parasite *C. reflexa* and different hosts during infection. High pectinolytic activity was detected in haustoria of *C. reflexa* and infected tissue of the compatible host plant *Pelargonium zonale*. The de-esterification of homogalacturonans in host cell walls at the host-parasite interface and the expression of pectate lyase genes in haustoria of *C. reflexa* suggests that the parasite is inducing these cell wall changes. Lower abundances of the hemicelluloses xylan and mannan were detected in *P. zonale* than in the parasite. Intriguingly, these hemicelluloses were higher abundant in the *C. reflexa*-resistant *Solanum lycopersicum* than in five near-isogenic susceptible *Solanum pennellii* introgression lines. The results indicate that the infection strategy of the parasitic plant *Cuscuta* involves the modification of specific cell wall components and that host plants can resist infection by fortifying their walls with polysaccharides that the parasite cannot modify.

Paper II

Olsen S, Striberny B, Hollmann J, Schwacke R, Popper ZA, Krause K. 2016.

Getting ready for host invasion: elevated expression and action of xyloglucan endotransglucosylases/hydrolases in developing haustoria of the holoparasitic angiosperm *Cuscuta*.

Journal of Experimental Botany 67(3), 695-708.

As living organisms regulate gene expression to control their activities, the specific genes that are expressed during a certain biological process will tell something about the nature of that particular process. With the goal to identify haustorium-specific genes, suppression subtractive hybridization was used to isolate genes differentially expressed between early-developed haustoria and undifferentiated stem tissue of *Cuscuta reflexa*. Sequencing revealed that cell wall-related transcripts were of higher abundance in the haustorial tissue and several potential marker genes for haustorium initiation were identified. The expression levels of two genes encoding xyloglucan endotransglucosylases/hydrolases were much higher in young haustoria than in any other tissue of the parasite. Immunolabeling and activity assays verified that the enzymes were in fact present and active at the initial swelling stage of haustoriogenesis and further suggested that these cell wall-modifying enzymes could be involved in host plant penetration.

Paper III

Olsen S, Popper ZA, Krause K. 2016.

Two sides of the same coin: Xyloglucan endotransglucosylases/hydrolases in host infection by the parasitic plant *Cuscuta*.

Plant Signaling and Behavior 11(3).

In a successful infection, the *Cuscuta* haustorium grows through the host tissue and establishes the connections needed to mediate transport between the two plants. Although the establishment of this perfect graft has long intrigued researchers, the underlying mechanisms remain largely uncharted. In this hypothesis paper, the potential role of xyloglucan endotransglucosylases/hydrolases (XTHs) during host plant infection by *Cuscuta* is discussed in light of existing data. A model is presented, describing how XTHs could function both as promoters of cell wall loosening to aid the haustorium in its journey through the host tissue and as wall strengthening enzymes used by resistant host plants to counteract the parasite's enzymes.

This paper was published as an addendum to Paper II.

Paper IV

Olsen S, Krause K. 2017.

Are xyloglucan endotransglucosylases/hydrolases the keys to host invasion by the parasitic plant *Cuscuta reflexa*?

Manuscript submitted to *PLOS ONE* in February 2017.

Descriptive information is useful in order to get an overview of a biological process, but it cannot reveal the causal relationships that govern the mechanisms. In an effort to further pursue the putative function of xyloglucan endotransglucosylases/hydrolases (XTHs) in host plant penetration, the specific activity of these cell wall-modifying enzymes was examined during the host-invasive growth of the *Cuscuta* haustorium. During host infection, the level of xyloglucan endotransglucosylation (XET) activity was highest in penetrating haustoria of *C. reflexa*. Moreover, *in vivo* colocalization of XET activity and donor substrate indicated high activity at the border between host and parasite. *C. reflexa* was found to secrete XET-performing enzymes from their haustoria, indicating that the activity at the interface originated from the parasite-encoded enzymes. Coomassie Brilliant Blue R250 inhibited the XET activity of *C. reflexa in vitro* and coating of *Pelargonium zonale* petioles with this inhibitor compound suppressed the parasite's ability to invade this otherwise compatible host plant. The results signify that the activity of *Cuscuta* XTHs is essential for successful host infection and that XET-inhibitors are potential candidates for controlling *Cuscuta* in agriculture.

4. WORK IN PROGRESS

After four years of research on the parasitic plant Cuscuta*, several of the studies executed did not make it into this thesis as part of a paper. Many are minor detours that will never make it further than the lab journal, but some have and might yet provide knowledge on parasitic plants and as such deserve not to be forgotten.*

Here follows a small selection of such ongoing projects:

4.1. Function of XTHs in the Resistance Mechanism of Cultivated Tomato

Upon being infected by *C. reflexa*, the cultivated tomato (*S. lycopersicum*) undergoes a hypersensitive-type response preventing the parasite from reaching its vascular tissues (Kaiser *et al.*, 2015). Accompanying this defence reaction is the increased expression of a tomato-encoded XTH, *SlXTH1* (*LeXTH1* in Albert *et al.* (2004)). Although relatable to the expansion of host cells at the *Cuscuta* infection site, we wanted to investigate the possibility of a more active defence mechanism involving tomato XTHs. As discussed in Paper III, tomato XTHs could be secreted to counteract the wall loosening activity of *Cuscuta* XTHs. *In vivo* colocalization of XET activity and donor substrate (method described in Paper IV) in cross-sections of *C. reflexa* infecting *S. lycopersicum* L. cv. M82 demonstrated high XET activity in both parasite and host. The activity in *S. lycopersicum* appeared in cell walls of clearly enlarged cells forming a distinct band where the wound-like tissue appears to originate (Fig. 7a-b). No such wound tissue or high XET activity was observed in the susceptible tomato *S. pennellii* when infected by *C. reflexa* (Fig. 7c-d). The wound tissue of *S. lycopersicum* fluoresces under ultraviolet light (Kaiser *et al.*, 2015; own observations). However, under the green light used to excite the sulforhodamine-labelled xyloglucan, no autofluorescence was seen in unlabeled controls (data not shown). That the observed fluorescence originated from sulforhodamine was verified by the fact that the fluorescence emission spectrum was identical to the one presented in Paper IV (data not shown). The localization of XET activity in the parasite haustorium is comparable to that when *Cuscuta* infects *P. zonale* (Paper IV; Fig.

7). The activity in the *Cuscuta* haustorium appears to be higher when trying to invade *S. lycopersicum* than when infecting *S. pennellii* (Fig. 7b and d). A compelling explanation that remains to be tested is that the haustorium tries to compensate its inability to penetrate the host tissue by further increasing the level of xyloglucan endotransglucosylation.

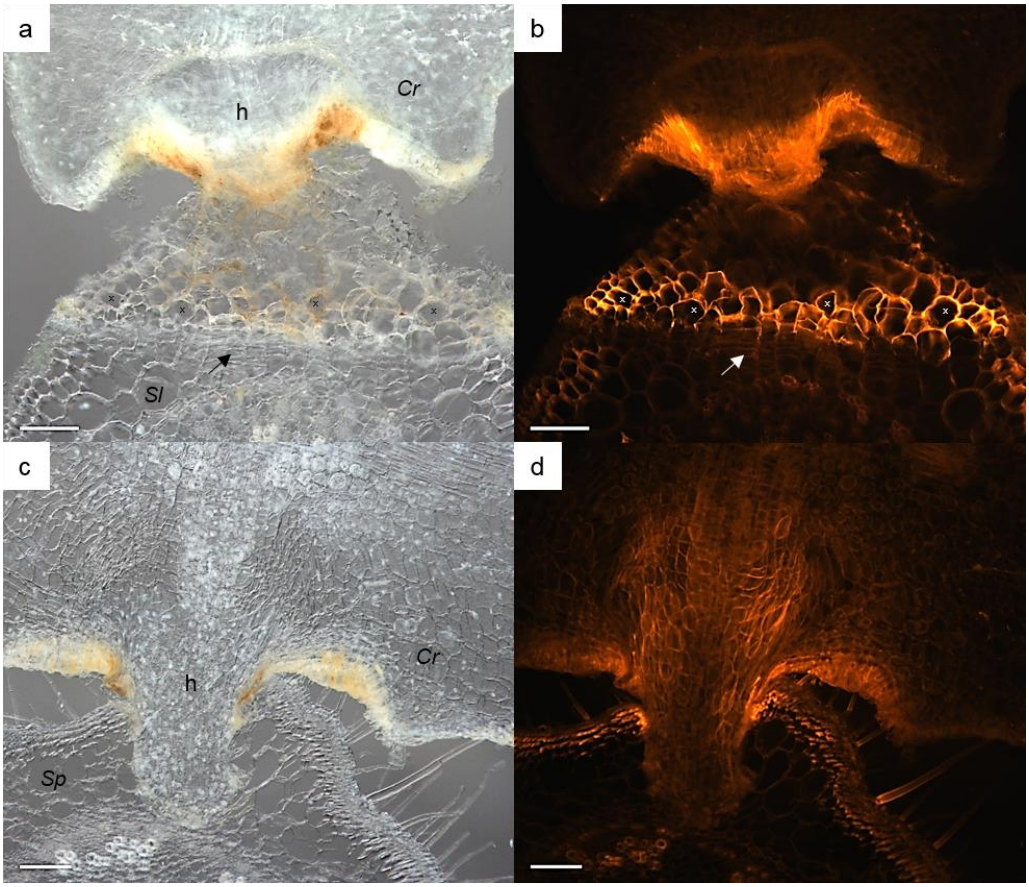

Fig. 7 *In situ* XET activity in infection sites of *C. reflexa* on *S. lycopersicum* and *S. pennellii*. (a, c) Brightfield images and (b, d) fluorescence images indicating colocalization of activity and donor substrate are shown for *C. reflexa* (*Cr*) attacking (a, b) *S. lycopersicum* (*Sl*) and (c, d) *S. pennellii* (*Sp*), respectively. In the brightfield images, h indicates the parasite haustorium. Arrows in (a) and (b) indicate area with small cells and low XET activity, while a selection of large cells with high XET activity in their walls are marked with x. Scale bars = 200 µm.

To address the significance of SlXTH1 in the tomato defence mechanism, *Cuscuta* infection trials were executed on tomato lines over-expressing or co-suppressing *SlXTH1* in the background of *S. lycopersicum* L. cv. Money Maker (Miedes *et al.*, 2011), namely OEX 13 and COS 33, respectively (provided kindly by Prof. Ester Pérez Lorences, University of Valencia). The wild type and both transgenic tomato lines displayed similar resistance responses to *C. reflexa* (Fig. 8), and the parasite was not able to establish itself on any of them.

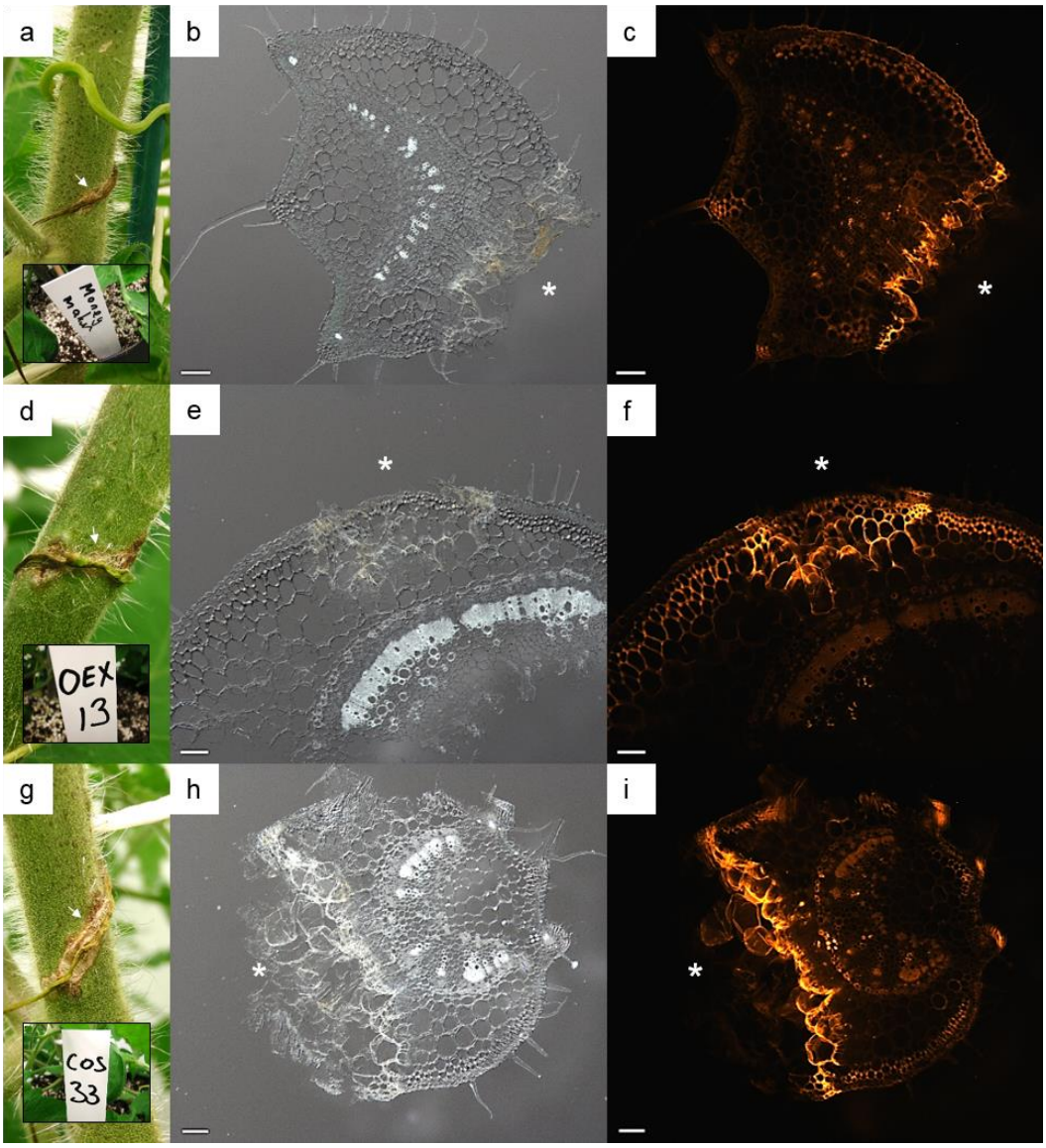

Fig. 8 Resistance response of different tomato lines against *C. reflexa*. The parasite was allowed to infect (a) wild type (Money Maker), (d) *SlXTH1* over-expressing (OEX 13) and (g) *SlXTH1* co-suppressing (COS 33) lines of *S. lycopersicum* cv. Money Maker. Arrows indicate the wound-like tissue formed by the host at the infection site. Brightfield images and fluorescence images revealing *in situ* XET activity in cross-sections of infection sites are shown for (b, c) the wild type, (e, f) the *SlXTH1* over-expression line and (h, i) the *SlXTH1* co-suppression line, respectively. As the parasite did not remain attached to the host during sectioning, asterisks indicate sites of contact with the parasite. Scale bars = 200 µm.

When analysing the colocalization of XET activity and donor substrate in cross-sections of infection sites of *C. reflexa* on the different tomato lines, the recognizable band of XET activity was present in all three lines (Fig. 8c, f and i). Therefore, as over-expression and co-suppression of *SlXTH1* in the respective mutant lines were confirmed by reverse transcription quantitative real-time polymerase chain reaction (Fig. 9), at least one redundant XTH must

be present to carry out the xyloglucan endotransglucosylation in the *SlXTH1* co-suppression line. Which one of the 25 XTHs identified in tomato (Saladie *et al.*, 2006) could be responsible for this redundancy has not been tested.

In summary, during the defence response of *S. lycopersicum*, XTHs are active in the walls of enlarged cells at the site of contact with *C. reflexa* (Fig. 7 and 8). This suggests that the xyloglucan-modifying enzymes are loosening cell walls to promote the cell expansion putatively required to form the wound-like tissue. However, whether the same enzymes are also re-strengthening these walls to oppose the activity of *Cuscuta* XTHs and whether tomato XTHs are essential to the resistance mechanism of *S. lycopersicum* are questions that remain to be resolved.

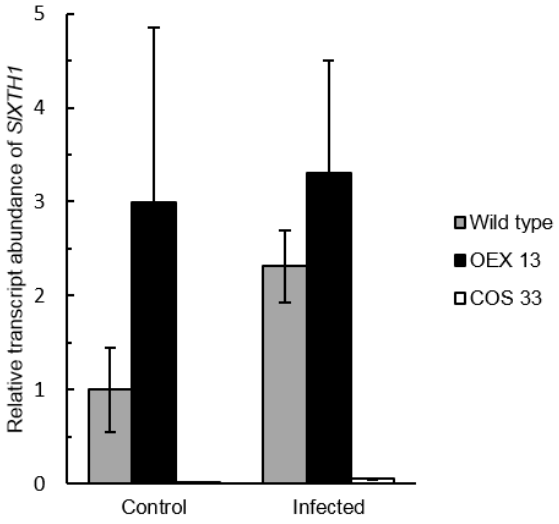

Fig. 9 Expression of *SlXTH1* in infected and control tissues of *S. lycopersicum* cv. Money Maker wild type, OEX 13 and COS 33. Transcript abundances are normalized to the abundances of eukaryotic initiation factor 4A-2-like (Gene ID: 101266405) and actin-7 (Gene ID: 101264601) transcripts and presented in relation to the normalized abundance of *SlXTH1* in control tissue of wild type (set to 1). Values are the means of two biological replicates (each analysed in technical duplicates) ± standard deviation.

4.2. Genetic Transformation of *Cuscuta*

The ability to express or knock out specific genes in an organism is a powerful tool for investigating gene function. We have established a protocol for the transient transformation of *C. reflexa* using a biolistic particle delivery system (Biolistic® PDS-1000/He Particle Delivery System from Bio-Rad). This system uses helium pressure to shoot DNA-coated microcarriers (e.g. gold) into cells where, if reaching any of the DNA-containing nucleus, mitochondria and plastids, the transgene may be stably incorporated into the respective genome or expressed transiently from a plasmid vector. As proof of concept, gold particles coated with an expression plasmid encoding a *Discosoma* sp. red fluorescent protein (dsRed) was bombarded onto the surface of *C. reflexa* stem pieces. The presence of polyphenol oxidases

in *Cuscuta* causes the production of brown pigments in tissues disrupted by the microcarriers (Bar Nun and Mayer (1999); Fig. 10a). The *dsRed* was transcribed from a ubiquitin promoter and appeared not to be specifically targeted to a cellular compartment, but rather to be distributed throughout the cytosol and in the nucleus (big arrows in Fig. 10b-c).

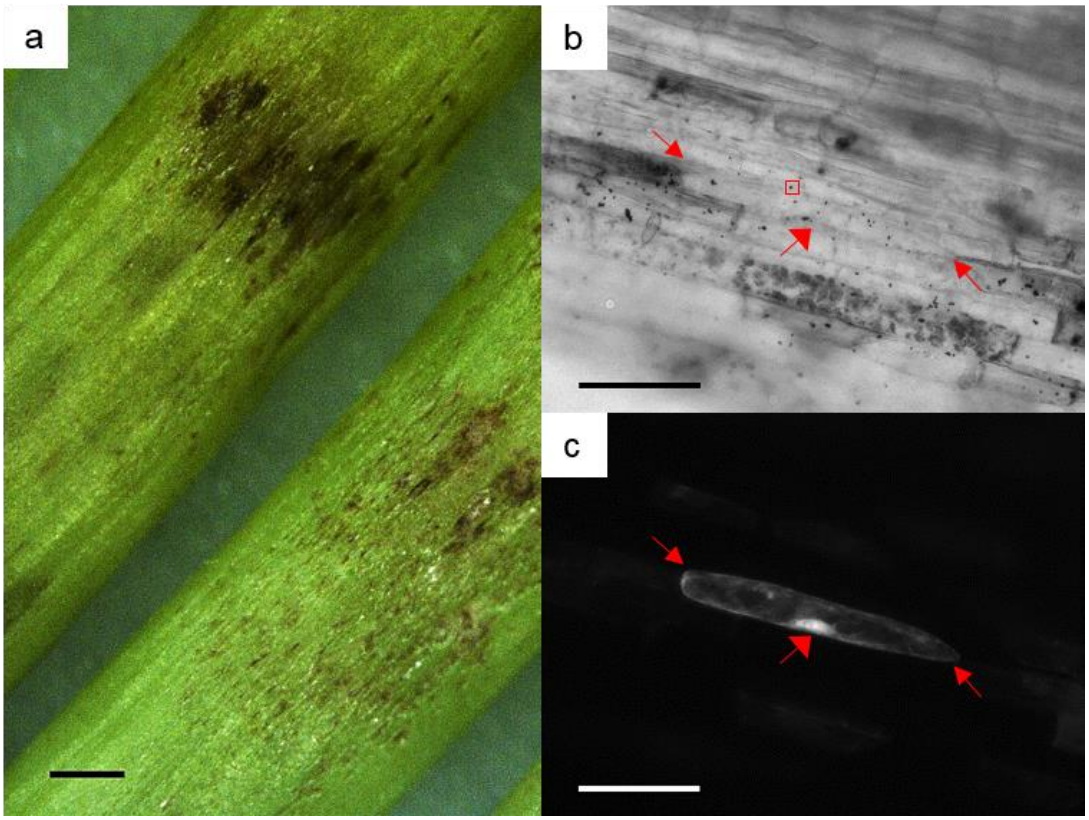

Fig. 10 Introduction and expression of transgenes in *C. reflexa*. (a) Browning indicate areas of *C. reflexa* stem pieces bombarded with plasmid-coated gold particles. (b) Brightfield image and (c) fluorescence image revealing the cellular localization of dsRed. Square in (b) indicates example gold particle. Arrows indicate points of reference between (b) and (c). Pictures in (b) and (c) were taken with a monochromal camera. Scale bars = 1000 µm in (a) and 100 µm in (b, c).

The cloning of *Cr-XTH-1* (Paper II) into the expression plasmid in front of *dsRed* to express the *C. reflexa* gene in fusion with the fluorescent protein enabled the cellular target of *Cr-XTH-1* to be determined. Cr-XTH-1-dsRed displayed a localization pattern that suggests targeting to vesicles (Fig. 11b). Moreover, strong fluorescence was also observed in what appear to be apoplastic regions (big arrows in Fig. 11). These observations correlate with the cell wall-modifying activities of these enzymes.

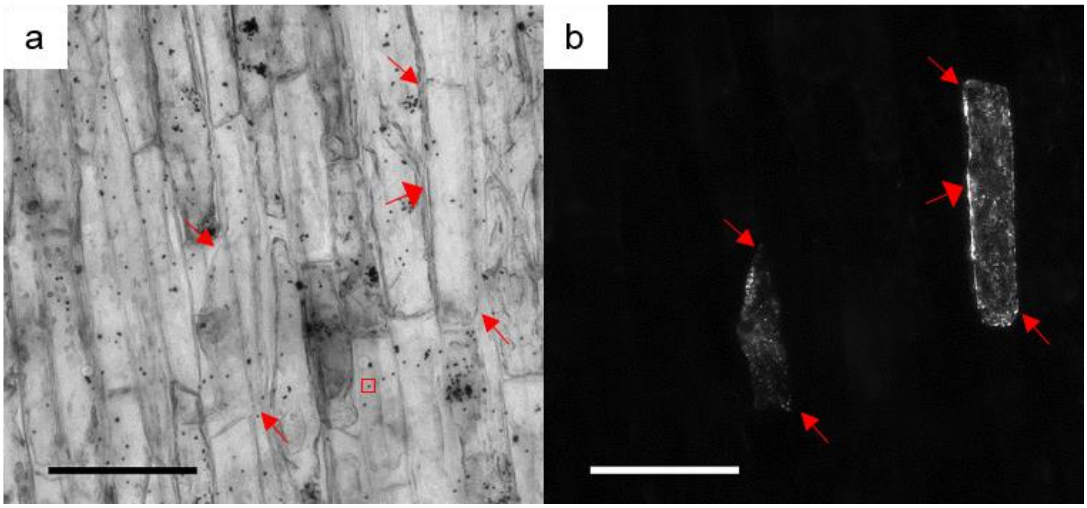

Fig. 11 Cellular localization of Cr-XTH-1-dsRed in *C. reflexa*. (a) Brightfield image and (b) fluorescence image showing localization of Cr-XTH-1-dsRed fusion protein. Square in (a) indicates example gold particle. Arrows indicate points of reference between (a) and (b). Pictures were taken with a monochromal camera. Scale bars = 100 µm.

Transient expression of fluorescence-tagged proteins is useful for studying subcellular localization. However, to be able to address the biological function of a gene product, stable incorporation of the transgene into the plant genome is necessary in order to pass on the trait to subsequent generations (Anami *et al.*, 2013). Protocols for *Agrobacterium*-mediated transformation of *Cuscuta* calli have been reported earlier (Borsics *et al.*, 2002; Svubova and Blehova, 2013), but none of these were able to regenerate genetically modified *Cuscuta* plants. An important aspect of stable transformation is the use of appropriate selective agents to only allow the proliferation of resistance-acquired transformed cells. We grow callus cultures of *C. reflexa* on Murashige and Skoog medium supplemented with sucrose (50 g/l), the cytokinin kinetin (3 mg/l), the auxin 1-Naphthaleneacetic acid (3 mg/l) and gibberellic acid (0.1 mg/l). A 5 µg/ml concentration of the protein synthesis-inhibiting hygromycin B killed *C. reflexa* calli within a week (data not shown). We expect that bombarded tissues of *Cuscuta* would require more time for treatment recovery and genomic integration to establish a stable expression of transgenes that enable survival and proliferation on the selective medium. Therefore, in order to find a suitable concentration that will allow transformed cells this recuperation time, the growth of callus on medium containing 0.2 and 1 µg/ml hygromycin B was tested (Fig. 12).

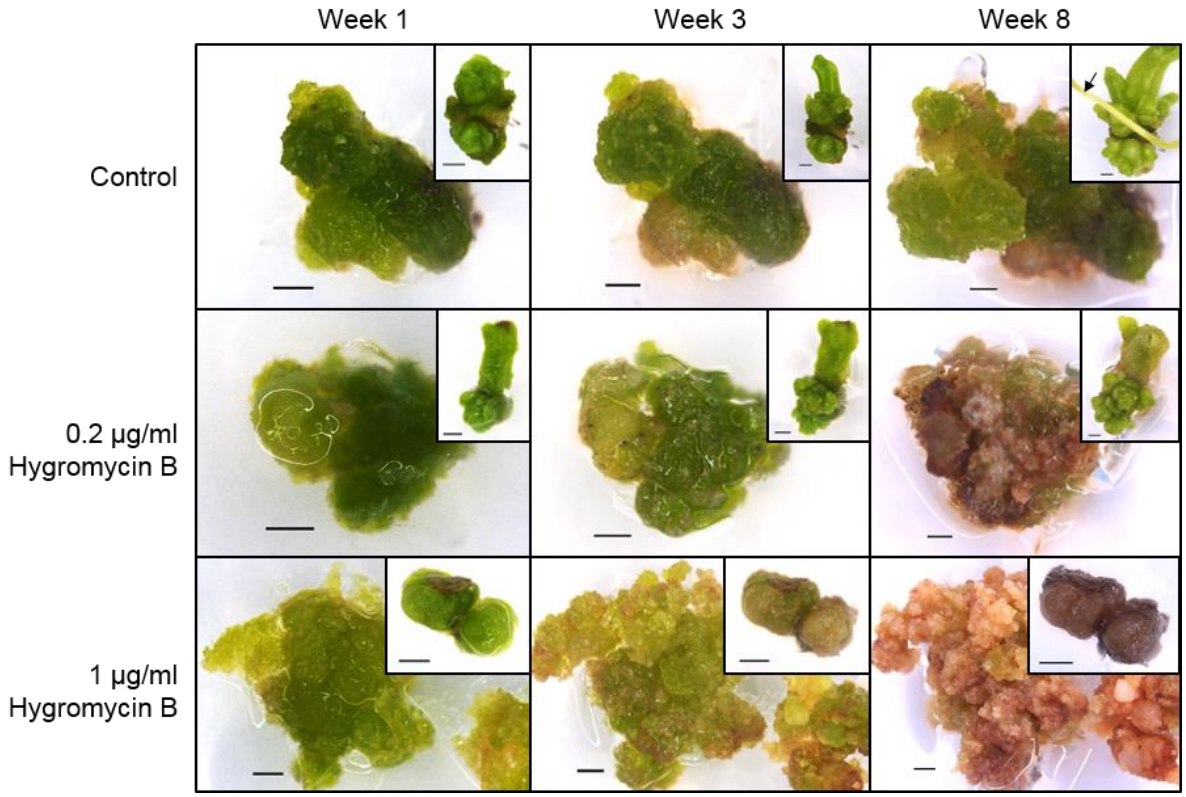

Fig. 12 Effect of hygromycin B on the growth of *C. reflexa* callus. Calli with shoot primordia are shown as smaller inserts. Arrow indicate shoot growth in the 8 weeks old control. Scale bars = 1000 µm.

Both 0.2 and 1 µg/ml hygromycin B reduced the growth of *C. reflexa* calli (Fig. 12). After 3 weeks, the callus on growth medium with 1 µg/ml hygromycin B showed signs of cell death, which were even more pronounced after 8 weeks. As reported earlier by Das *et al.* (2011), equal concentrations of auxin and cytokinin initiate regeneration of *C. reflexa* shoots from callus. Whereas no shooting was observed from any *C. reflexa* calli on media with hygromycin B, the shoot primordia-containing callus on control medium without the selective agent had started to produce stem tissue after 8 weeks (arrow in smaller insert of Control-Week 8 in Fig. 12). This demonstrates the possibility to selectively regenerate transgenic *Cuscuta* if transgenes encoding hygromycin-resistance can be stably expressed in the parasitic plant. Genomic integration and retention of transgenes are challenges left to tackle in the continuing efforts to establish a protocol for the stable transformation of the parasitic plant *Cuscuta*.

5. CONCLUSION AND FUTURE PERSPECTIVES

The ability of Cuscuta *to attach, invade and feed on other plants is an impressive feat of adaptation to the parasitic mode of life. In this thesis, the main body of research was on cell wall changes and modifiers during the host tissue penetration of the haustorium. This is reflected in the focus of the discussion on the cell wall aspect of host infection and should therefore not be interpreted as a complete picture of* Cuscuta parasitization. *By drawing upon data from the presented papers as well as from other studies, a model for the role of cell walls during host invasion by* Cuscuta *will here be presented. As scientific theories are valid only until new theories can explain the respective phenomenon better, it seems appropriate to begin this chapter with the following quote:*

"I do not know, but here is a reasonable guess" (May, 2001).

In the parasitic angiosperm *Cuscuta*, a swelling of the stem region where the infection organs will develop announces the onset of haustoriogenesis. The activity and immunolocalization of XTHs at this stage of haustorium development in *C. reflexa* proposes that these xyloglucan-modifying enzymes loosen parasite walls to allow the cell expansion that causes the swelling of the tissue (Fig. 8 and 9 in Paper II). Moreover, the coinciding expression of *Cr-XTH-1* and *Cr-XTH-2* suggests that these genes encode the specific actors (Fig. 6 in Paper II). After securely attaching to the host surface (Vaughn, 2002), the haustorium begins its host-invasive journey. The cell wall compositions of both host and parasite were found to be altered through the process of parasitization (Paper I). Gene expression data from Ranjan *et al.* (2014), Ikeue *et al.* (2015) and Paper II indicate that the expression levels of cell wall-related genes are increased in *Cuscuta* upon host infection, which suggests that parasite-encoded enzymes are causing these modifications. High degrees of pectin degradation in *C. reflexa* haustoria and infected *P. zonale* were the most conspicuous of the cell wall changes to occur during infection (Fig. 3 in Paper I). Several plant pathogens utilize pectin-degrading enzymes as a means to invade plants (Annis and Goodwin, 1997). Also in *Cuscuta*, the elevated

expression of genes encoding pectate lyases and polygalacturonases was associated with host plant infection (Ikeue *et al.* (2015); Ranjan *et al.* (2014); Fig. 5 in Paper I). As these enzymes preferably degrade pectins with low degree of esterification, the detection of de-esterified homogalacturonans in host cell walls adjacent to parasite cells and the increased expression of a pectin acetyl esterase in young haustoria of *C. reflexa* suggests that enzymes secreted from the parasite are conducting these modifications (Fig. 2 in Paper I; Fig. 6 in Paper II). These findings corroborate earlier reports on pectin-modifying activities in *Cuscuta* (Bar Nun and Mayer, 1999; Bar Nun *et al.*, 1999; Nagar *et al.*, 1984). Pectic polysaccharides make up the middle lamella that ensures adhesion between adjacent cells (Jarvis *et al.*, 2003). Therefore, a degradation of the middle lamella by the pectin-modifying activities just described could enable the *Cuscuta* haustorium to manoeuvre itself between the cells of the host tissue (Fig. 13). Earlier morphological observations that host middle lamellae are degraded or separated as *Cuscuta* hyphae expand by intercellular tissue penetration agree with this hypothesis (Lee, 2009; Vaughn, 2003). On the other hand, when hyphae grow through host cells, the chimeric cell wall made up of both host and parasite walls was reported to be enriched in pectins (Vaughn, 2003). As pectins control the sieving properties of the primary cell wall (Baron-Epel *et al.*, 1988), a decrease in the abundances of these polysaccharides may promote the degradation of other wall polymers by making them more accessible for enzymatic digestion. This idea is corroborated by the fact that the binding of antibodies to xyloglucan epitopes can be hindered by pectins (Marcus *et al.* (2008); Paper I). Indeed, pectins were not the only cell wall components found to be modified during host infection by *Cuscuta*. Activities reducing the abundances of xyloglucan were highest in haustoria of *C. reflexa* and in the infected tissue of *P. zonale* (Fig. 3 in Paper I). In Paper IV, the function of the haustorium-related expression and activity of *XTHs* in *C. reflexa* first discovered in Paper II was further investigated. The high XET activity at the host-parasite interface, the secretion of XTHs from *C. reflexa* haustoria and the prevention of host penetration by coating *P. zonale* petioles with an XET-inhibitor, strongly suggests that these xyloglucan-modifying enzymes are essential to the parasitization strategy of *Cuscuta* (Fig. 3, 4 and 6 in Paper IV). As the pectin-modifying enzymes pave the way by degrading the middle

lamella and making the primary cell wall more accessible, XTHs could further loosen the host cell walls by reducing the abundance of xyloglucan chains that cross-link cellulose microfibrils (Takeda *et al.* (2002); Paper III; Fig. 13). This could enable the earlier observed stretching of the host cell walls as the *Cuscuta* haustorium invades the tissue (Dawson *et al.* (1994); Lee (2009); Vaughn (2003)).

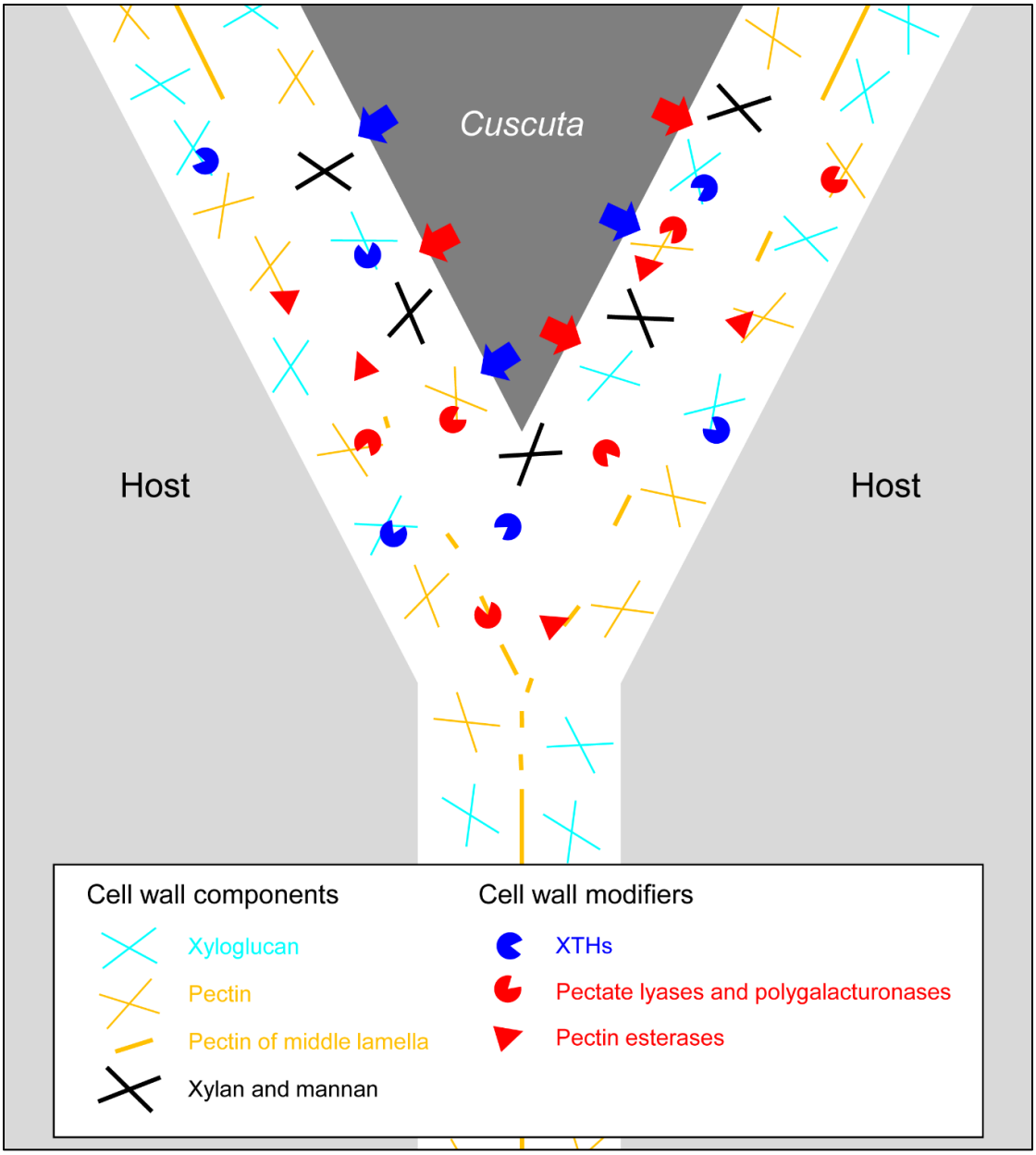

Fig. 13 Simplified model of hypothetical functions of cell wall components and modifiers during host infection by the parasitic plant *Cuscuta*. Cells of the *Cuscuta* haustorium secrete (indicated by arrows) pectin- and xyloglucan-modifying enzymes to loosen host cell walls and thereby promote tissue invasion. *Cuscuta* wall strength is maintained by fortification with the hemicelluloses xylan and mannan. Parasite enzymes acting on other wall components and putative host enzymes are not depicted. Sizes are not to scale.

Plant pathogenic microbes have been reported to utilize xylan-degrading enzymes to weaken plant cell walls during infection (Belien *et al.*, 2006). In the epitope deletion-based screen for carbohydrate active enzymes during infection of *P. zonale* by *C. reflexa*, no degradation of xylan was detected in the infected host tissue (Fig. 3 in Paper I). This indicates that xylan degradation is not part of the parasite's infection strategy, which does make sense considering that many *Cuscuta* species appear to have specialized on infecting dicotyledonous plants that contain low amounts of hemicelluloses other than xyloglucan in their primary cell walls (Scheller and Ulvskov, 2010). The general inability of *Cuscuta* to parasitize grasses could thus be explained by the high content of arabinoxylan in these plants. In fact, plant pathogenic fungi on monocotyledonous or dicotyledonous plants are better at degrading the hemicelluloses arabinoxylan or xyloglucan, respectively (King *et al.*, 2011), exemplifying that pathogens adapt their mode of attack to the cell wall composition of their host. Interestingly, the *C. reflexa*-resistant tomato, *S. lycopersicum*, possessed higher abundances of xylan and mannan than did the five susceptible *S. lycopersicum*/*S. pennellii* introgression lines (Fig. 6 in Paper I). Whether or not this difference in cell wall composition is the deciding factor between resistance and susceptibility remain unknown, but it is striking that in resemblance to the resistant host plant, the parasite itself contains relatively high amounts of xylan and mannan. It should here be mentioned that *Cuscuta* is also observed to self-parasitize, which means that xylan and mannan cannot present a completely impenetrable barrier for the parasitic plant. However, as the parasite is proposed to penetrate its own tissue by the use of mechanical force alone (Dawson *et al.*, 1994), additional studies are needed to resolve this issue.

Parasite cells must have a certain level of wall rigidity in order to accomplish the feat of pushing their way between host cells. Although some mannan and xylan appear to be degraded in haustoria of *C. reflexa* (Fig. 3 in Paper I), non-xyloglucan hemicelluloses could maintain the wall strength of parasite cells during infection when pectin and xyloglucan are being modified (Fig. 13). As many cell wall-modifying enzymes can promote both wall loosening and strengthening (e.g. pectin methyl esterases and XTHs), the enzymes could

loosen and strengthen host and parasite walls, respectively (Paper III). However, this option was not included in the model presented in Fig. 13.

Does the cell wall-modifying arsenal of a *Cuscuta* species determine its host range? In the studies presented in this thesis, nearly all experiments were carried out with *C. reflexa*. Although reports from different species of the genus generally coincide, little is known about how ubiquitous the host infection strategy of *Cuscuta* spp. is. As the range of suitable host plants is rather diverse across the genus *Cuscuta* (Dawson *et al.*, 1994), it would not be surprising if different species employ different invasion tactics. Therefore, it would be very interesting to compare the cell wall-degrading activities of *Cuscuta* species with different host preferences. For instance, Kaiser *et al.* (2015) reported that *C. australis* is able to infect grasses. The ability of *Cuscuta* to infect the model plant *Arabidopsis* presents an opportunity to further investigate the effect of differing cell wall compositions on host susceptibility. Infection trials on the xyloglucan-deficient *xxt1*/*xxt2* mutant (Cavalier *et al.*, 2008; Park and Cosgrove, 2012) could shed light on the putative significance of this hemicellulose in the parasitization strategy of *Cuscuta*.

As the development of the haustorium is a process involving plant cell growth, many of the detected wall-changing activities could be loosening the walls of the parasite cells to allow expansion and not walls of the host to allow invasion. Concerning the role of XTHs in haustorium development, high XET activity was detected in both the upper haustorium and at the host-parasite interface (Fig. 2 in Paper IV). While the latter can be associated with host wall-modifications, the former cannot. We plan to apply laser-assisted microdissection to harvest tissues from these specific areas. Differences in e.g. gene expression between host-adjacent and host-distal regions of the haustorium could help to identify specific *XTHs* (and other potential actors) at the host-parasite interface. This approach can also be applied to further examine the putative involvement of XTHs in the defence response of *S. lycopersicum*. Moreover, analysis of XET activity in normally compatible host plants that have acquired *C. reflexa*-resistance by expressing CUSCUTA RECEPTOR 1 (Hegenauer *et al.*, 2016), could reveal if this cell wall-modifying activity is part of the defence response initiated by this receptor. As they are regularly attacked by pathogens, plants have evolved mechanisms to identify threats

and to initiate appropriate defence responses. In a successful infection by a parasitic plant, the physiological similarity of host and parasite could possibly enable the latter to sneak in unnoticed. On the other hand, plants also monitor the integrity of their cell wall and products of pectin degradation have been shown to activate defence responses (Brutus *et al.*, 2010; Ferrari *et al.*, 2013; Hamann, 2015). Continued research on the signalling interplay between parasitic plants and their hosts and the role of cell wall therein, will be most exciting.

In this thesis, a variety of methods was applied to investigate the mechanisms of host plant infection by the parasitic plant *Cuscuta*. The combination of data on morphology, gene expression, enzyme activity and substrate dynamics enabled us to view the biological process from different perspectives. Taken together, the results indicate that the cell walls of host plants are modified during the invasive growth of the *Cuscuta* haustorium and that the parasite is secreting the enzymes that facilitate these changes. The cell wall polymers pectin and xyloglucan were further investigated in this thesis and found to be targets for parasite-encoded enzymes. However, additional cell wall components and *C. reflexa* genes with potential roles in the *Cuscuta* parasitization strategy were identified in Paper I and II, emphasizing that we have just seen a glimpse of the mechanisms of host plant infection by the parasitic angiosperm *Cuscuta*. Ultimately, our understanding of how *Cuscuta* is able to penetrate plant tissue and steal water and nutrients from its host would greatly benefit from the ability to produce transgenic *Cuscuta* that display different parasitic abilities.

> *"... you can catch phenomena in a logical box or in a mathematical box. The logical box is coarse but strong. The mathematical box is fine-grained but flimsy. The mathematical box is a beautiful way of wrapping up a problem, but it will not hold the phenomena unless they have been caught in a logical box to begin with."*
>
> Platt (1964)

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