Analysis of processes at the haustorial interfaces between *Cuscuta reflexa* and its hosts

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1 Acknowledgements

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2   Thesis abstract

The genus *Cuscuta* comprises a group of holoparasitic dicotyledonous angiosperms that cause damage to many economically important crops. Parasitic plants form physical and physiological connections (haustoria) with the parenchyma and vascular vessels of compatible host plants. These connections provide them with water, mineral nutrients and organic compounds. Over time, this life style has led to evolutionary adaptations that include a reduction or loss of leaves and roots and the inability to live photoautotrophically. The penetration of host plants by *Cuscuta* is believed to rely on mechanical pressure applied to the cells of the host and on the secretion of hydrolytic, cell-wall degrading enzymes.

Despite the ecological and economic significance of the genus *Cuscuta*, the mechanisms behind its attack and the corresponding reactions observed in susceptible and resistant host plants remain largely unknown. The goal of this thesis has been to broaden the understanding of the processes unfolding during the interaction between this parasite and its hosts. This knowledge is, among others, crucial to evaluate the biotechnological potential of *Cuscuta*–derived enzymes. To reach this goal, established biochemical, immunohistological and genetic approaches were exploited, where possible. However, in some cases the established techniques failed for *Cuscuta* so that new approaches had to be devised.

The work focused on one hand on the proteins involved in the infection process and on the other hand on the cell wall polysaccharides that are substrates to the hydrolytic secreted enzymes. An improved method for the reliable detection of cellulase activity in plant extracts and in tissue prints was developed and confirmed the high cellulolytic activity exhibited by the parasite. With a combination of high throughput and high resolution approaches, we succeeded furthermore in profiling cell wall components as well as CAZyme activities *in situ*. Finally, the investigation of a tomato introgression line population which exhibited differences in susceptibility to *C. reflexa* will give new impulses in the quest to elucidate the genetic and molecular background for susceptibility to *C. reflexa* in compatible species.

3 List of papers

Paper I

Cellulase Activity Screening Using Pure Carboxymethylcellulose: Application to Soluble Cellulolytic Samples and to Plant Tissue Prints

Hanne Risan Johnsen[1], Kirsten Krause[1], 2014

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Paper II

Comprehensive microarray profiling of cell wall polymers and enzymes in the parasitic plant *Cuscuta reflexa* and the host *Pelargonium zonale*

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Paper III

From susceptibility to resistance against parasitic dodder (genus *Cuscuta*): What can we learn from a wild tomato introgression line population?

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4   Abbreviations

AGP	Arabinogalactan protein
CAPS	Cleaved amplified polymorphic site
CAZymes	Carbohydrate-active enzymes
CBMs	Carbohydrate binding modules
cM	Centimorgan
CMC	Carboxymethyl cellulose
GalA	Galacturonic acid
HG	Homogalacturonan
HGT	Horizontal gene transfer
IL	Introgression line
JA	Jasmonic acid
mAb	Monoclonal antibody
MLG	Mixed-linkage (1→3) (1→4)-β-D-glucan
MS	Mass spectrometry
PG	Polygalacturonase
PL	Pectate lyase
PME	Pectin methylesterase
QTL	Quantitative trait loci
RG	Rhamnogalacturonan
SA	Salicylic acid
SP	Signal peptide
YST	Yeast secretion trap

5   Introduction

5.1 Parasitic plants

Plants are predominantly photoautotrophic organisms with the capability of producing their own food from inorganic substances using light as an energy source. However, approximately 1 % of angiosperms display heterotrophic behavior, deriving their nutrients and water from other plants. The parasitic lifestyle has originated independently a number of times during the evolution of angiosperms (Barkman *et al.* 2007; Westwood *et al.* 2010), which has resulted in approximately 4500 species, distributed in 19 families, that are parasitic on other plants (Kuijt 1969; Nickrent *et al.* 1998). The parasitic way of living in higher plants is in principal restricted to the dicotyledons, as there are no known parasitic monocots and only one very rare parasitic gymnosperm (Feild and Brodribb 2005). Facultative hemiparasites comprise the earliest stages of the parasitic lifestyle whereas obligate hemiparasites and holoparasites evolved as the dependence on host resources increased (Yoder et al. 2009). The parasitic angiosperms are found in a range of ecosystems, from the subarctic tundra to tropical forests (Press 1998).

A feature that distinguishes parasitic plants is the invasive haustorium (Kuijt 1969). The appearance of haustoria is considered the key evolutionary step to plant parasitism (Westwood *et al.* 2010) as they facilitate the attachment of the parasite to the host, penetration of the host, and the establishment of vascular connections between the conductive systems of the two plants (Albert *et al.* 2008; Heide-Jørgensen 2008). Through the haustorial connections parasites withdraw water, assimilates and nutrients sufficient for their own growth and reproduction. The origin of the haustorial genes is still unclear; it has been hypothesized that they evolved from duplication of genes already present in non-parasitic plants, or were introduced from non-plants by either endosymbiosis or horizontal gene transfer (Yoder *et al.* 2009). A xylem bridge connecting the xylem of the parasite to host xylem that takes up water and minerals from their hosts is commonly found in all parasitic species (Kuijt 1969). Phloem connections with sieve elements are mainly found in holoparasites for withdrawal of carbon in addition to water and minerals. The presence of phloem with fully differentiated sieve tubes in the shoot parasite *Cuscuta* close to the host sieve tubes may explain the fast growth of the parasite (Heide-Jørgensen 2013).

Carbon and nitrogen are the main building blocks of nucleic acids, proteins, metabolites and other cellular components that are essential for plant growth. Autotrophic plants acquire inorganic carbon through photosynthetic activity, and nitrogen is commonly taken up in the form of nitrate and ammonium. In a number of parasitic plants, genes associated with photosynthesis are thought to be of less importance. In some cases this has resulted in deletion of photosynthetic genes and a reduction of the plastid genome (Krause 2011). In which form carbon is taken up from the host is unknown because the parasites immediately convert it into parasite metabolites. Sugars are commonly converted into storage products, and accumulation of starch is often observed in parasite tissue.
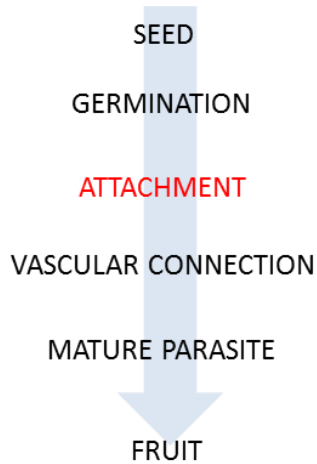
Parasitic plants impact not only their host plants but entire plant communities as well as other species, including herbivores and pollinators (Press and Phoenix 2005). They are represented in practically all terrestrial ecosystems, suggesting that they are of considerable ecological importance. The greatest impact on plant communities appears to be a general reduction in host plant biomass. Reduction in host performance is generally significant, causing yield losses and, in extreme cases, death of the host. However, as parasitic plants often favor the most dominant species, the host biomass reduction can again lead to an overall increase in plant diversity by redistribution of resources (Niemela *et al.* 2008). High plant diversity reduces the effects that are observed on monocultured species.

Only a small percentage of parasitic plants are considered agricultural weeds, but those few species are causing severe yield losses and limiting crop production with great economic impact in many parts of the world (Nickrent *et al.* 1998). The most economically damaging parasitic species are the root parasites *Striga* spp. (witchweeds), *Orobanche* spp. and *Phelipanche* spp. (both broomrapes), and the shoot parasite *Cuscuta* spp. (dodders) (Qasem 2006; Rubiales *et al.* 2009). Parasitic weeds are hard to control because of the tight relation between the host and parasite. A possible solution to the problem can be found in natural host resistance occurring at different stages of the parasitic lifecycle; pre-attachment, parasite establishment and post-establishment (Yoder and Scholes 2010). However, breeding for host plant resistance is challenging because of the complexity and low heritability of the resistant traits (Rispail *et al.* 2007). Also, for some species no crop cultivars or wild relatives with full resistance have been found to date (Ejeta and Gressel 2007). This could potentially be solved by genetic engineering of the host to induce resistance or the parasite to prevent parasitic behavior (Yoder *et al.* 2009; Yoder and Scholes 2010), but public concerns regarding genetically modified organisms imposes limitations on the use of this technology.

5.1.1 Hemiparasites versus holoparasites

The majority (90 %) of all parasites are hemiparasites (Heide-Jørgensen 2013). Hemiparasites were the first plants to develop a parasitic lifestyle and they represent the earliest evolutionary stage in the transition from autotrophy to heterotrophy. All hemiparasites are capable of photosynthetic activity and fixation of carbon, but the efficiency of photosynthesis varies considerably among different species. A few hemiparasites are facultative parasites and can, on cost of performance, complete their life cycle without attaching to any host (Mutikainen *et al.* 2000). This might be the main discrepancy to holoparasites, which in contrast always are obligate parasites and thus reliant on a host plant to complete their life cycle. Holoparasitic plants have completed the transition from an autotrophic to a heterotrophic way of life. Most holoparasites are incapable of photosynthesis due to reduced chlorophyll content and low affinity to CO_2, and therefore completely rely on host plants both for maturation and reproduction (Fig.1). However, some holoparasites still retain photosynthetic activity, but this activity is generally not sufficient to cover their carbon need (van der Kooij *et al.* 2000).

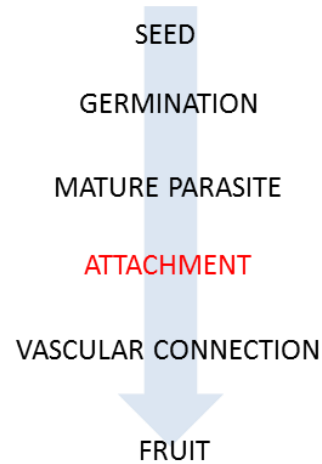OBLIGATE HOLOPARASITE   FACULTATIVE HEMIPARASITE

SEED   SEED

GERMINATION   GERMINATION

ATTACHMENT   MATURE PARASITE

VASCULAR CONNECTION   ATTACHMENT

MATURE PARASITE   VASCULAR CONNECTION

FRUIT   FRUIT

Figure 1. Obligate holoparasitic and facultative hemiparasitic Orobanchaceae. Life cycles illustrating differences in length of the independent phase during parasite development in holoparasitic and hemiparasitic species of the family Orobanchaceae, but also commonly observed among holoparasites and hemiparasites from other families. Whereas some hemiparasites establish autotrophically and mature in the absence of a host plant, most holoparasites depend on resources from a host immediately after germination. However, the obligate hemiparasites, similar to holoparasites, attach to a host early in their life cycle but then become autotrophic at a later developmental stage. Modified from (Joel 2013).

5.1.2 Root versus shoot parasites

Parasites are further classified as either root or shoot parasites depending on which part of the host they attach to; the above ground or below ground parts (Bell and Adams 2011). Shoot parasites include mistletoes (e.g., *Viscum* and *Arceuthobium*) and *Cuscuta* spp. Root parasites represent 60 % of all parasitic plants, and include broomrapes (*Orobanche* and *Phelipanche* spp.) and witchweeds (*Striga* spp.). The morphology of shoot parasites are often adapted to the parasitic lifestyle with reduced leaves and little chlorophyll, whereas the areal parts of root parasites commonly share morphology with non-parasitic angiosperms and are not easily distinguished. Root parasites spend most of their life cycle underground where they acquire and store resources from the host. This makes them particularly hard to control as they cause damage on host plants before they even emerge from the soil. Several classes of plant secondary metabolites are known to induce seed germination of root parasites. Strigolactones are chemicals that are exuded by the host plant to stimulate mycorrhizal fungi colonization and root branching, but is also recognized by root parasites and used as germination stimulants. Due to their role in regulation of plant architecture and important symbiotic interactions, elimination of strigolactones from crops is not suitable as control strategy of root parasites (Cardoso *et al.* 2011; Fernandez-Aparicio *et al.* 2011).

5.1.3 The parasitic genus *Cuscuta*

The obligate shoot parasite, *Cuscuta* spp. (commonly known as dodder), is the only parasitic genus within in the family Convolvulaceae. The genus consists of about 200 parasitic species (Dawson *et al.* 1994), which is further divided into 3 subgenera based on morphology of style and stigma; *Monogyna*, *Cuscuta* and *Grammica* (McNeal *et al.* 2007). As an adaptation to their parasitic lifestyle, *Cuscuta* species have no roots and their leaves are reduced to minute scales (Fig.2a). The genus has a worldwide distribution and *Cuscuta* species are found on every continent with the exception of Antarctica. However, the greatest species diversity is in subtropical and tropical regions. Four species are native to Europe, but only two species (*C. europaea* and *C. epithymum*) have presently been found in Southern Norway (Fig.3).
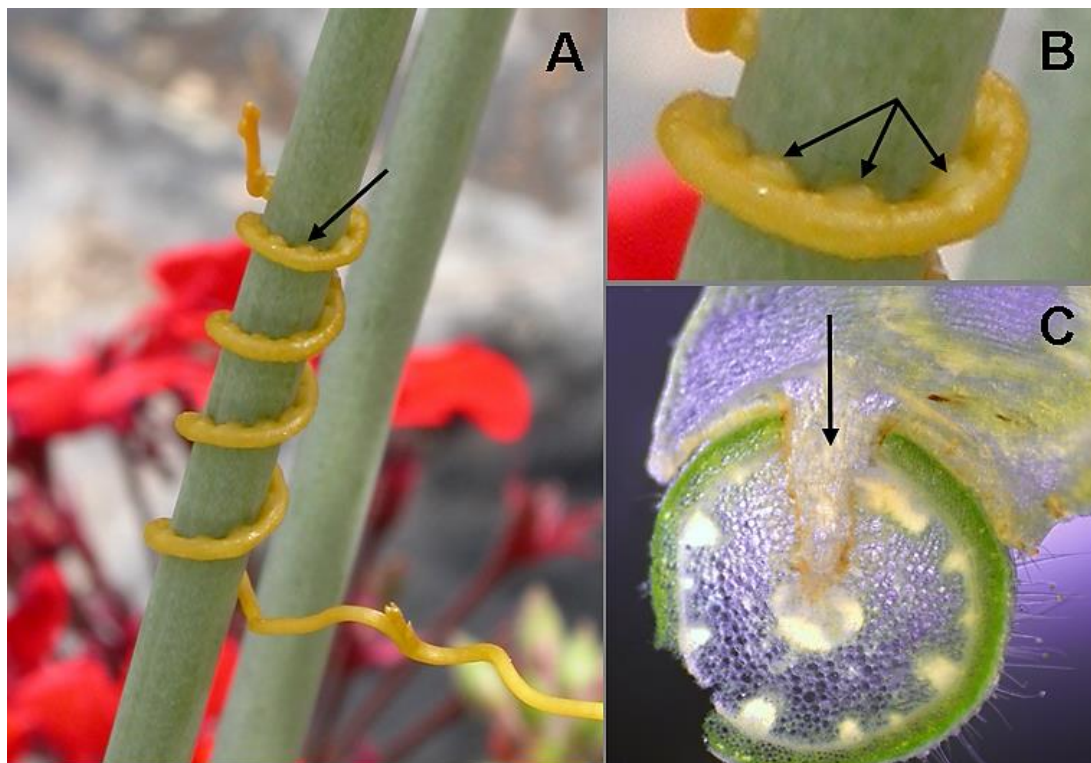
Figure 2. Stem of *Cuscuta gronovii* parasitizing a susceptible host, *P. zonale*. a) *Cuscuta* winding around its host plant. b) Detailed view of (a) showing the penetrating organs (arrows). c) Cross section showing penetration of host tissue by haustorium (arrow) (Pictures: Kirsten Krause)

Most species within the genus *Cuscuta* spp. still retain chloroplasts, but the chlorophyll content and photosynthetic capacity vary significantly (van der Kooij *et al.* 2000; Krause 2008). The chlorophyll containing species *Cuscuta reflexa* has a relatively high photosynthetic activity with only minor changes observed mainly in the non-coding regions of the plastid genome, whereas the species *C. odorata* and *C. grandiflora* completely lack thylakoids and chlorophyll, and are thus incapable of fixing CO_2 (van der Kooij *et al.* 2000). Although plastids of *C. reflexa* have fewer thylakoids and less chlorophyll than non-parasites, the photosynthetic activity in *C. reflexa* is evidently contributing to the parasite`s carbon balance (Machado and Zetsche 1990; Hibberd *et al.* 1998). However, the genus is classified

as holoparasitic and all species are reliant on a host plant to complete their life cycle (Hibberd *et al.* 1998; van der Kooij *et al.* 2000). A transcriptomic study of *C. pentagona* showed reduced chlorophyll biosynthesis and photosynthesis upon successful parasitism. Furthermore, genes associated with transport of both nutrients and sugars were up-regulated in the haustorial stage. Taken together, these results indicate that *Cuscuta* relies on transfer of nutrients and solutes from its host and thus reduce photosynthesis to a minimal level (Ranjan *et al.* 2014).
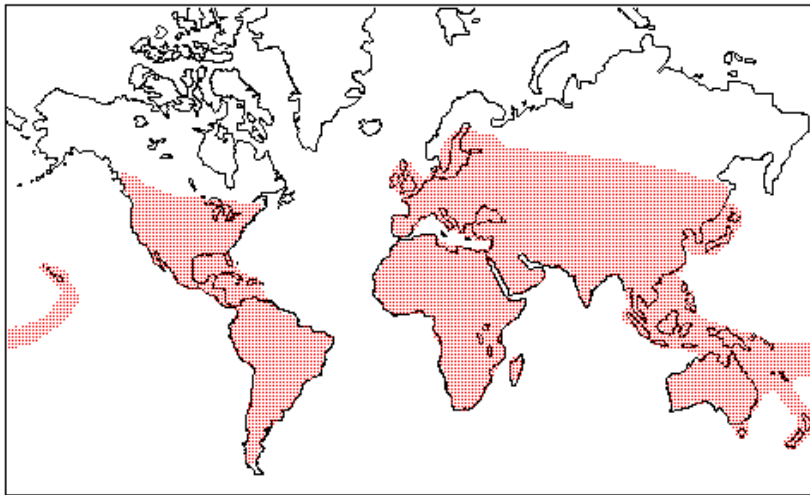

Figure 3. Distribution map of the parasitic genus *Cuscuta* spp. (www.parasiticplants.siu.edu/Cuscutaceae/)

As weeds, *Cuscuta* spp. is economically one of the most important groups of parasitic plants. The parasite causes damage to a number of crop plants (e.g. alfalfa, clover, tomato, tobacco, sugarbeet and carrot), with yield losses of up to 90 %. However, the vast majority of *Cuscuta* species are not considered weeds, as less than 20 species worldwide are known to cause agricultural damage (Dawson *et al.* 1994). Still, the main attention of *Cuscuta* research concerns strategies on how to control the parasite. Common approaches include crop rotation, flooding or flaming, tilling, and use of selective herbicides. However, the use of herbicide resistant crops is not always successful as the intimate association between *Cuscuta* and its host allows the parasite to benefit from the host`s herbicide resistance. The control of the parasite is complex and highly crop dependent and so far there are no strategies that are both effective and sustainable (Rispail *et al.* 2007; Alakonya *et al.* 2012).

In contrast to other parasitic plant species, the germination of *Cuscuta* seeds is seemingly independent of chemical signals from host plants. Alternatively, they rely on chemical cues that have not yet been detected. As root parasites commonly produce a large number of small seeds that germinate only in response to chemical cues from a host, *Cuscuta* produce few large seeds that have sufficient resources to search for nearby hosts after germination (Mescher *et al.* 2009). A germinated *Cuscuta* seedling emerges as a cotyledonless shoot, with only a short-lived root-like structure that possibly supports the growing tip with necessary carbon by degeneration (Sherman *et al.* 2008). The growing seedling contains sufficient nutrients for only a few days growth as the root becomes senescent, during which it must establish contact with a host or die. In search for host

plants, the seedling rotates in a circular, anti-clockwise motion until it finds a point of attachment. It has been suggested that *Cuscuta* require volatile chemical cues to detect a host plant (Runyon *et al.* 2006), but as pointed out by Furuhashi et al. (2011) this does not corroborate with the ability to self-parasitize or grow towards and attach to any rod-like structure (Fig.4) without chemical cues (Furuhashi *et al.* 2011). Self-parasitism with establishment of functional haustoria is frequently observed in *Cuscuta* species (Fig.4a-b).
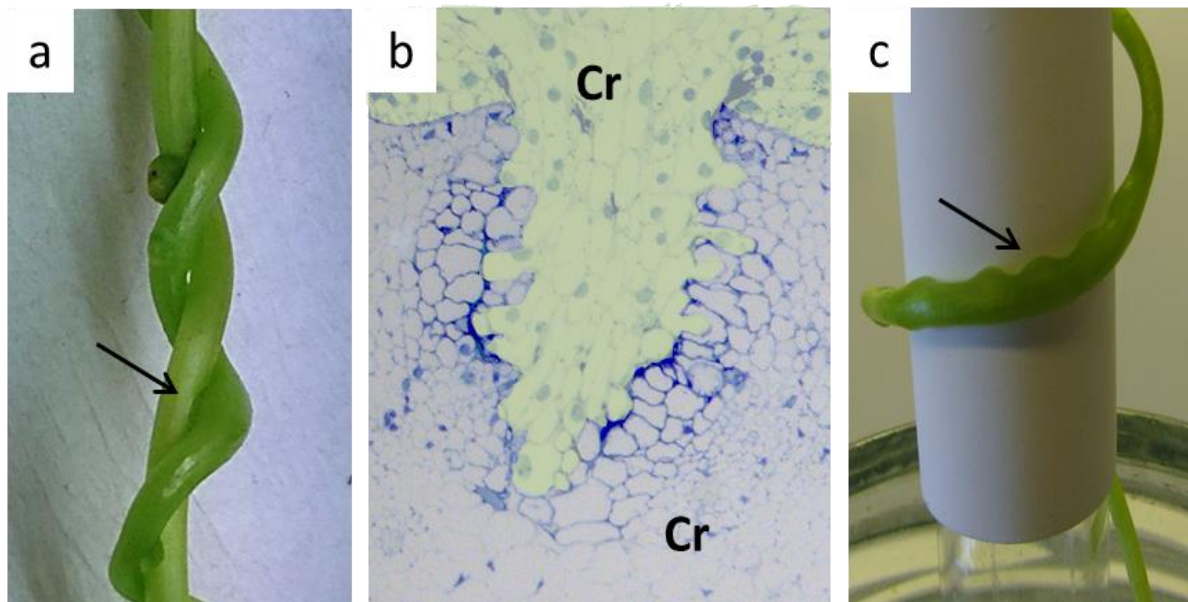

Figure 4. Unspecific host recognition. a) Self-parasitation between two stems of *C. reflexa*. One attachment site is indicated by arrow. **b)** Micrograph of cross section from the interface between two *Cuscuta* stems (Cr). **c)** Stem of *C. reflexa* twining around and attaching to (arrow), a glass rod covered with nitrocellulose membrane.

Further, it was demonstrated that *Cuscuta* seedlings tend to grow toward light sources with low red/far red ratio, which may help the parasite to localize potential host plants (Orr *et al.* 1996). In addition, far-red and blue lights are evidently important for the induction of twining response, which, together with tactile pressure induce haustorial formation (Tada *et al.* 1996; Li *et al.* 2010; Furuhashi *et al.* 2011). Once contact with a host is established, vines twine tightly around the host stem or petiole and induce haustoria formation. When a connection to the host`s vascular system is established, the seedling loses its connection to the soil (Albert *et al.* 2008).

Some *Cuscuta* species are considered generalists that occasionally parasitize different host species simultaneously (Dawson *et al.* 1994); thus potentially facilitating transmission of phloem-mobile viruses from an infected to a healthy plant, both within and between host species. This has been reported for a number of viruses, chiefly without any apparent disease symptoms in the transmitting *Cuscuta* plant (Johnson 1941; Bennett 1944; Hosford 1967). Also, successful transmission of phytoplasmas from naturally infected host species to healthy plants using *Cuscuta* as a bridge between botanically unrelated hosts has been demonstrated (Marcone *et al.* 1997).

5.2 Parasite-host interactions

Plants have evolved sophisticated systems for defense, consisting of several layers of constitutive and inducible responses. Constitutive defense involve physical barriers such as cuticle, thorns, trichomes, lignified cell walls and secondary metabolites. Induced host resistance to pathogens is typically determined by recognition of pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) such as bacterial flagellin, lipopolysaccharides, peptidoglycans and fungal chitin. Pattern recognition receptors (PRRs) in the plant cell walls recognize the molecular patterns, and further induce pattern-triggered immunity (PTI) (Dangl and Jones 2001). Upon infection the plant immune system triggers a variety of defense mechanisms, including the hypersensitive response (Coll *et al.* 2011), production of phytoalexins (Bednarek *et al.* 2009) and plant cell wall modifications (Aist 1976). Also, plants produce hydrolytic enzymes such as β-1,3-glucanases and chitinases, targeted to decompose pathogen cell walls (van Loon *et al.* 2006). Certain pathogens can counteract the PTI by introducing virulence effectors into the plant which inhibit subsequent downstream signaling processes. As a response, plants have in turn developed a second layer of immunity termed effector-triggered immunity (ETI). Whereas PTI interactions are commonly mediated in a non-host manner, ETI interactions are often highly specific between a particular plant and a pathogen race (Dangl and Jones 2001). Additionally, damage to the plant cell wall releases cell wall fragments that function as damage-associated molecular patterns (DAMPs) perceived by the plant. Involvement in defense reactions and defense signaling has been assigned to several cell wall polysaccharides. Pectin-derived oligosaccharides are known to elicit defense responses in plant cells and tissues as part of a signaling cascade induced upon cell wall degradation by pathogen attacks (Ridley *et al.* 2001; Pelloux *et al.* 2007; Lionetti *et al.* 2012).

In comparison to pathogen-plant interactions, little is known about the molecular and genetic factors of parasitic plant-plant interactions. The outcome of an attempt of parasitation is determined by the virulence of the parasite and the resistance of the host. Resistance is defined by the ability of the host to prevent parasite establishment and growth (Timko and Scholes 2013). The plant cell wall is the site of initial contact and early host responses upon a parasite infection. Mechanical pressure and cell wall degrading enzymes involved in host penetration by microbes, fungi and nematodes have been thoroughly studied (Mendgen *et al.* 1996; Mayer 2006; Gibson *et al.* 2011; Bohlmann and Sobczak 2014). Despite the obvious differences concerning host invasion (Mayer 2006), it is reasonable to believe that parasitic plants share some of the same strategies. However, compared to the single celled fungal hyphae and the needle-like stylet of nematodes, the size and nature of the multicellular penetrating haustorium of parasitic plants is strikingly different. Whereas pathogenic fungi can utilize small natural openings such as stomata or lesions to enter the host (Dean 1997), the parasitic plant haustorium requires significantly larger openings in the host tissue to access.

Fungi penetrating host cell walls typically secrete pectolytic enzymes at an early stage of infection to weaken the host cell wall, followed by cellulases and hemicellulases (Walton 1994; Lionetti *et al.* 2010). Pectin methylesterase (PME) has previously been identified and purified from seedlings of the root parasite *Orobanche*, where it later also was detected at the penetration site during host invasion (Ben-Hod *et al.* 1993; Losner-Goshen *et al.* 1998). Immunolocalization studies demonstrated that PME of parasite origin is present in *Orobanche* haustorial cells and is detectable in the apoplast of adjacent host tissue. Their results suggested that a combination of pectolytic activity and mechanical pressure was involved in the dissociation of the middle lamella which allows the intrusive cells to grow between the host cells, rather than penetrating the cells (Losner-Goshen *et al.* 1998).

Evidence for mechanical pressure applied to the cells of the host by parasitic plants comes from the appearance of crushed host cells commonly observed at the interface between haustoria and hosts tissue (Heide-Jørgensen and Kuijt 1995; Reiss and Bailey 1998; Neumann *et al.* 1999). Expansins are novel non-enzymatic plant cell wall-loosening proteins that function by loosening or weakening the non-covalent bonds between cellulose microfibrils and associated hemicelluloses, thus making the cell wall more accessible for cell wall degrading enzymes, and more susceptible to mechanical pressure (McQueen-Mason and Cosgrove 1994; Cosgrove 2005). A recent study showed increased expression of certain expansins genes in the pre-haustorium of *C. pentagona*, suggesting that cell wall-loosening proteins are involved in penetration of host tissue and, additionally the rapid expansion of the haustorial tissue (Ranjan *et al.* 2014).

5.2.1 Host susceptibility to *Cuscuta*

The mechanisms by which *Cuscuta* attaches to the host have been previously described (Vaughn 2002). It starts with development of a pre-haustorium (Lee 2007; 2008), followed by haustoria penetration with subsequent establishment of vascular connections (Vaughn 2003; Lee 2009). Contact between parasite and host is supported by a cementing substance enriched in de-esterified pectin which is secreted by the pre-haustoria (Vaughn 2002). Also, increased expression of one arabinogalactan protein (*att*AGP) in tomato at the contact site in an early stage of infection has been reported. The up-regulation of the host AGP was induced by pre-haustoria formation and secretion of the cementing substance, presumably to enhance attachment (Albert *et al.* 2006).

In response to tactile stimuli upon host contact (Tada *et al.* 1996; Furuhashi *et al.* 2011), a haustorium initial originates from dedifferentiation of cortical parenchyma in the middle of the parasite stem towards the side of the host. The cortical cells as well as the epidermal cells facing the host produce denser cytoplasm, larger nuclei and accumulate starch grains. The endophyte starts developing from meristematic cells derived from the haustorial initial (Lee 2007; 2008), and penetrates the host tissue by making a fissure in the host by either mechanical pressure or enzymatically modifications (Vaughn 2003). Following penetration, the parasite extends searching hyphae that are individual epidermal cells of the haustorium, in order to establish contact with the vascular system of the host (Dawson *et al.*

1994). The searching hyphae can increase in length up to 800 µm before connecting to the host`s vascular system. Different invasive tactics have been suggested for the searching hyphae; there is evidence for hyphal growth in the middle lamella, but also through the host cell walls supported by an expansion of the host cell wall (chimeric wall) coating the invasive hyphae. Both mechanisms are thought to reduce stress responses in the host plant by preventing destruction of host cell integrity (Dawson *et al.* 1994; Vaughn 2003).
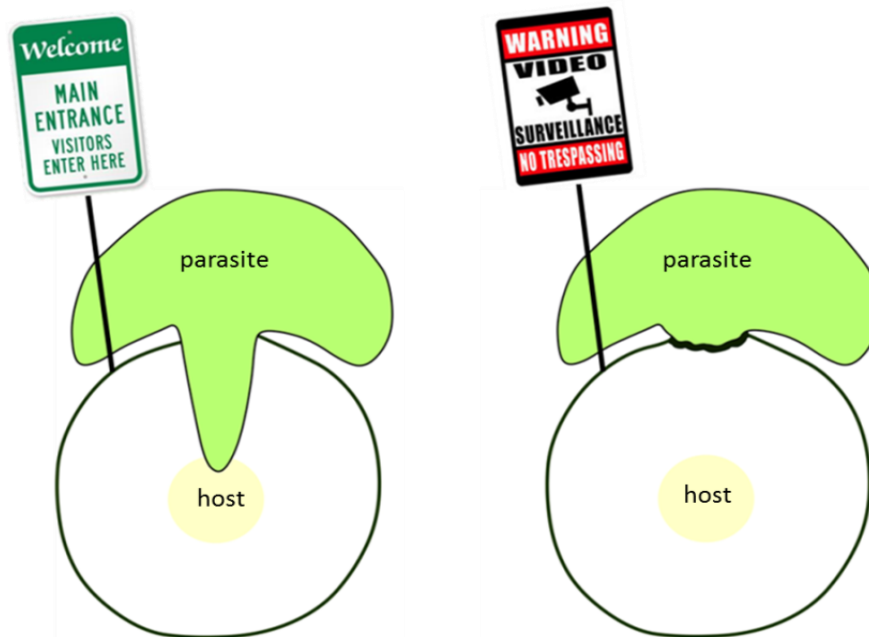
Figure 5. Illustration of compatible and incompatible host interactions. In a successful infection a functional haustorium with physiological connections to the host vascular tissue is established without host responses inhibiting the process. An incompatible infection on the other hand induces responses in the host that prevent growth of the haustorium into the host tissue, and further establishment of a functional contact between the parasite and the host.

Due to the lack of roots and an effective photosynthetic system, water, mineral nutrients and organic compounds are withdrawn from the host facilitated by xylem and phloem connections. The searching hyphae of *Cuscuta* differentiate into either xylic or phloic cells depending on which vascular tissue they come across (Vaughn 2006). Phloem connections were verified by the translocation of green fluorescent protein (GFP) and the phloem-specific dye carboxyfluorescein, from transgenic tobacco plants into the phloem of *Cuscuta* (Birschwilks *et al.* 2006). More recent detection of host plant mRNA in *Cuscuta* demonstrates that mobile genetic material also is transferred, and further confirm the exchange of macromolecules between the host and parasite (Westwood *et al.* 2009; LeBlanc *et al.* 2012). In addition to direct vascular connections, there is evidence for cytoplasmic contact between host parenchyma and parasite through plasmodesmata localized on the searching hyphae (Vaughn 2003; Birschwilks *et al.* 2006).

5.2.2 Host resistance to *Cuscuta*

Stems of *Cuscuta* are indiscriminate in their selection of hosts, and independent of host susceptibility they twine around and attach to plants as well as non-plants (Fig.2). In cases where it attaches to incompatible hosts, the haustorium is either prevented from entering the host tissue (Fig.5), or is at a later stage prevented from connecting to the vascular system of the host (Dawson *et al.* 1994). This is often achieved by local necrosis or reinforcement of host cell walls, and typically includes accumulation of lignin or callose. In the less obvious cases it is necessary to section apparent attachment sites to confirm presence of xylem and phloem continuity between the parasite and the host to distinguish between compatible and incompatible hosts.

Figure 6. *Cuscuta reflexa* on the resistant tomato variety *Solanum lycopersicum,* **M82.** The tomato (Sl) reacts with necrosis (arrow) following attachment of *C. reflexa* (Cr).

The interaction between *Cuscuta* and resistant tomato varieties has been studied to some extent. Incompatible interactions between 30 tomato varieties and *C. reflexa* was previously described (Sahm *et al.* 1995). This was followed by a study including 22 tomato varieties and four wild tomato species. In all cases, the tomatoes displayed a hypersensitive response and the formation of functional haustoria was prevented (Ihl and Miersch 1996). Incompatible tomatoes typically display early defense reactions by necrotic tissue developing around the prehaustoria (Fig.6). On the molecular level, tomatoes respond to *C. pentagona* by a sequential increase in the plant hormones jasmonic acid (JA) and salicylic acid (SA) (Runyon *et al.* 2010a). SA signaling is known to be activated as a response to pathogens, where it is involved in the regulation of hypersensitive response and the synthesis of phytoalexins and pathogenesis-related proteins, whereas the JA pathway is generally induced by herbivores (Runyon *et al.* 2010b). A hypersensitive-like response and phytoalexin production have also been detected in a resistant host plant upon *C. reflexa* infection (Bringmann *et al.* 1999).

Elevated levels of Ca^{2+} upon infection by *C. reflexa* has been detected in tomato plants where it might contribute to signaling involved in expression of genes related to the hypersensitive response in the host. The increase of Ca^{2+} is most likely chemically induced

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rather than by local touch responses triggered by the attachment of the parasite, suggested by the duration of the signal and the delayed start (30 hours) after the initial contact. The Ca^{2+} signal activation was exclusively induced by *C. reflexa* haustoria, indicating that the response is species and tissue specific (Albert *et al.* 2010a; Albert *et al.* 2010b). Recent molecular studies have shown expression of two aquaporin genes (*LeAqp2* and TRAMP) and a cell wall-modifying xyloglucan endotransglycosylase/hydrolase (LeXTH1) in incompatible tomatoes upon *C. reflexa* infection, but their roles in tomato resistance remain unclear (Werner *et al.* 2001; Albert *et al.* 2004).

5.2.3   Tomato breeding - a tool to decipher resistance against *Cuscuta*?

Plant breeding is used to genetically improve plants for human benefit. Two plants with favorable traits are crossed to produce genetic variation, and the progeny with most desirable characteristics are selected. Whereas the early domestication processes were based on human selection of cultivars possessing rare mutations associated with favorable traits, such as large fruit size or sweet taste, modern plant breeding takes advantage of molecular markers and biotechnology (Zamir 2001). Essentially all cultivated varieties of tomato available today originate from the cherry tomato *Solanum lycopersicum*, native to South America. *S. lycopersicum* was domesticated by native Americans before the tomato was introduced to Europe (Jenkins 1948), but details around early tomato domestication remains unknown. However, the domestication process is correlated with an increase in fruit size compared to wild tomatoes, indicating that they were selected for mutations associated with larger fruits. Cultivated tomatoes have a narrow genetic basis as a consequence of the domestication process, which leads to increased susceptibility to biotic and abiotic stresses (Tanksley and McCouch 1997; Tanksley 2004). Thus, wild tomato relatives have been a valuable source of resistance genes, and current tomato varieties possess several wild-derived resistance genes which provide resistant cultivars (Zamir 2001).

Introgression lines (ILs) are a set of nearly isogenic lines produced by repeated backcrossing and marker-assisted selection (Fig. 7). Each line contains a single genetically defined chromosome segment introgressed from a donor parent (the wild relative accession of interest) into the background of a recurrent parent (the cultivated tomato accession) (Zamir 2001). Due to transgressive variation, the progeny phenotypes cannot be predicted based on the phenotypes of their parents (Fig.8). This could be explained either by complementary action of genes from the two parental lines, or unmasking of recessive genes that are normally heterozygous in the wild species (Devicente and Tanksley 1993).

IL populations are effective tools to identify and stabilize quantitative trait loci (QTLs), because any phenotypic difference between an IL and the recurrent parent is associated solely to one or more genes from the introgressed chromosomal segment (Eshed and Zamir 1994a; 1995). Each IL carries numerous genes and often several phenotypes are connected to a single introgression. Genetic variations underlying quantitative traits are hard to dissect due to the possible involvement of several genes or QTLs, each explaining only a small portion of the total variation (Eshed and Zamir 1996). Since only a small part of the donor

genome is represented in each line, IL populations provide limited ability to study epistatic interactions between multiple, unlinked genes or loci.

Unique overlapping regions, "bins", between different ILs covering the same chromosome define smaller intervals than the ILs, and can thus be used to further dissect QTLs. If a significant phenotype is observed in one IL but not in an overlapping IL, the QTL interval can be narrowed by exclusion. Similarly, a sheared region between two ILs with similar phenotypes can be used to further define the involved region of the introgressed segments (Chitwood *et al.* 2013).
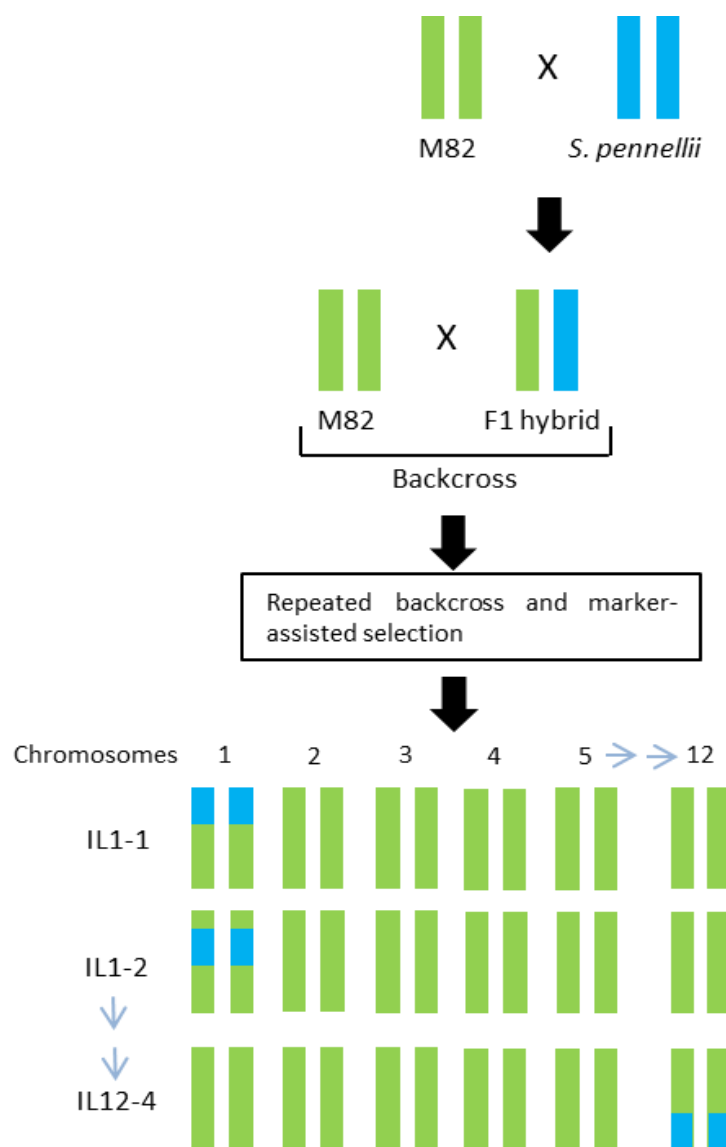
Figure 7. Breeding scheme for generating an IL population between the cultivated variety M82 and the wild species *S. pennellii*. The cultivated variety chromosomes are shown in green and *S. pennellii* introgressions are shown in blue. The wild species (male) is crossed to M82 (female), and the F1 hybrid is repeatedly backcrossed to M82 to reduce the portion of the wild species genome. Each line is homozygous for a single chromosome segment introgressed *from S. pennellii*. Modified from (Zamir 2001).

An IL population obtained from the self-compatible *Solanum pennellii* (LA0716) covers the complete genome of the wild tomato in the genetic background of *S. lycopersicum* cultivar M82 (Eshed *et al.* 1992; Eshed and Zamir 1994b). The population presently consists of 76 ILs with overlapping segments that are connected to a high-resolution F2 chromosome map, comprising more than 4000 markers (Lippman *et al.* 2007). The *S. pennellii* x *S. lycopersicum* IL population was originally divided into 107 marker-defined mapping bins with an average size of 12 cM, but a recent more exact re-definition of the IL boundaries revealed 112 bins (Chitwood *et al.* 2013). In the framework of a currently running EU project (EU-SOL), additional 500 sub-ILs are being generated from the current ILs to further improve the mapping resolution. So far, 285 smaller ILs have been produced that break up the 37 largest ILs of the initial population (Alseekh *et al.* 2013).

The introgressed segments of *S. pennellii* are defined by flanking molecular markers. Marker-assisted selection enables to select specific segments of DNA that are associated with different measurable differences and effects on a complex trait. Molecular markers have been used extensively in tomato breeding, mainly for marker-assisted selection, map-based cloning of genes or QTLs and the construction of high-density maps and mapping populations. Several molecular markers are associated with the genetic map of tomato. Restriction fragment length polymorphisms (RFLPs) were the first DNA markers developed in tomato plants (Bernatzky and Tanksley 1986), but they were later replaced by the more efficient and PCR-based cleaved amplified polymorphic sequence (CAPS) markers (Bombarely *et al.* 2011). Additionally, DNA fingerprinting techniques such as amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RADP) have later been used to develop DNA markers in tomato (Saliba-Colombani *et al.* 2000).

Figure. 8. Fruit phenotypes. a) Green fruits of the wild species *S. pennellii*, b) red fruits of the cultivated variety M82, c) three ILs were backcrossed to the recurrent parent (M82). All backcrosses were conducted onto the same parental plant. However, presumably due to transgressive variation the progeny phenotypes differ from each other and the parental lines in both size and color (modified from Zamir (2001)).

Altogether, a total of 3069 QTLs have been identified using the *S. pennellii* x *S. lycopersicum* IL population (Lippman *et al.* 2007; Alseekh *et al.* 2013), including fruit weight and size (Alpert and Tanksley 1996; Frary *et al.* 2000), color (Ronen *et al.* 2000), fruit metabolism and yield (Schauer *et al.* 2006). Additionally, a number of QTLs responsible for resistance in tomato have been determined from the *S. pennellii* ILs; late blight caused by *Phytophthora infestans* (Smart *et al.* 2007), bacterial spot disease (Sharlach *et al.* 2013) and the herbivore thrips (Romero-González *et al.* 2011). However, there are no previous reports on research involving the use of ILs to study host resistance to any *Cuscuta* species.

5.3 Enzymes that aid the haustorial penetration: potential candidates for biofuels production?

An increasing demand for transportation fuels together with a growing concern about climate changes caused by the use of fossil resources, requires alternative renewable sources for fuel production (Himmel *et al.* 2007; Jorgensen *et al.* 2007). As an attempt to reduce the use of fossil fuels, biomass is utilized for biofuels production. Biofuels can contribute to addressing some of the problems connected to climate change. Hydrolysis of the plant cell wall polysaccharides cellulose and hemicellulose to fermentable sugar monomers is a critical step in the conversion of lignocellulosic material to ethanol. Therefore, fermentation of food crops and extraction of burnable oil from oil-rich plants have been the two main approaches for production of biofuels until recently. However, the conversion of edible plant material into biofuels is highly controversial in a world experiencing famine. Agricultural residues, including perennial energy plants, crop residues and forest residues, are good alternatives to food-sources due to their abundance, low cost, renewability, and biodegradability. Nonetheless, the utilization of non-food sources is limited by the recalcitrance of lignocellulosic plant material, requiring pretreatment involving high temperatures or toxic chemicals.

A great variety of plant pathogens possess unique enzymes that could complement commercial enzyme preparations, resulting in faster and more complete hydrolysis without extensive pretreatment being necessary. To date, most of the commercially available enzymes for that purpose are secreted by the filamentous fungus *Trichoderma reesei*. Other rich sources of cell wall degrading enzymes are microorganisms that live in the guts or rumen of termites or ruminants, respectively, where they digest the lignocellulosic biomass their hosts feed on (Kudo 2009; Banerjee *et al.* 2010; Cai *et al.* 2010). The sequencing of *Arabidopsis thaliana* as the first plant genome revealed a seemingly higher abundance of carbohydrate-active enzymes (CAZymes) in plants than any other organisms previously sequenced (Henrissat *et al.* 2001). This is not surprising, as plants need cell wall hydrolyzing enzymes in order to maintain developmental processes including cell elongation, abscission of ripe fruits and shedding of leaves (Coutinho *et al.* 2003b).

In addition to enzymes involved in modification of its own cell walls, parasitic plants possess enzymes that penetrate lignified and unlignified plant tissue in order to withdraw nutrients from their host plants. Compared to the vast knowledge about hydrolytic enzymes secreted by plant parasitizing fungi and microbes, little is known about the mechanisms behind host penetration by parasitic plants. Previously, there have been some reports on elevated levels of pectin methylesterases (PMEs), polygalacturonases (PGs), cellulases and peroxidases in *Cuscuta* spp. (Nagar *et al.* 1984; Srivastava *et al.* 1994; Bar Nun and Mayer 1999a; Bar Nun *et al.* 1999b; Lopez-Curto *et al.* 2006). More recently, a transcriptome study of *C. pentagona* showed increased expression of enzymes involved in cell wall modifications in the infective stages of the parasite, resembling those previously identified by biochemical methods (Ranjan *et al.* 2014). Although very promising candidates for bioprospecting of novel cell wall degrading enzyme activities, parasitic plants have so far not been included in respective screening attempts and therefore represent an entirely unexploited resource for the biofuels industry (Lopez-Casado *et al.* 2008).

5.3.1 The plant cell wall

The plant cell wall is a general property of all plants which provides the cell with support and shape, but also represents the first line of defense against pathogens and insects. In addition to the mechanical and impenetrable properties, the cell wall is metabolically active and allows exchange of materials and signals between cells. Despite its rigidity, the cell wall is dynamic and capable of expansion (Scheller and Ulvskov 2010). The most abundant structural component of plant cell walls is cellulose, whereas pectins and hemicelluloses contribute to the biochemical diversity (Park and Cosgrove 2012). Two types of cell walls can be distinguished in a plant cell; the primary wall, which is deposited during cell growth, and the secondary cell wall, which is deposited inside the primary wall of some cells when the cell has reached its final size. The primary cell walls need to be both mechanically stable and extensible to permit cell expansion. They consist mainly of cellulose, hemicelluloses, pectins and structural proteins. In contrast, secondary cell walls are much thicker and further strengthened by incorporation of lignin that covalently binds to hemicelluloses (Vanholme *et al.* 2008).

Cellulose is a long, linear homopolymer composed of β-(1-4)-linked glucose molecules. Individual cellulose chains bind together through hydrogen bonds and van der Waals forces to form microfibrils (Nishiyama *et al.* 2003). In the cell wall cellulose occurs in two forms; a highly organized and rigid crystalline structure and an amorphous form, which gives the wall viscoelastic properties (Cosgrove 1997; Mazeau and Heux 2003). Crystalline cellulose microfibrils are interconnected covalently and non-covalently in a matrix of branched hemicelluloses, pectins and lignin. Together, these components ensure that the cell wall is robust on one hand but stays flexible and extensible on the other (Cosgrove 2005; Hématy *et al.* 2009). However, this cross-linking to other cell wall components impacts the accessibility of cellulolytic enzymes, which is one of the current bottlenecks for the utilization of cellulosic biomass for biotechnology applications.

Hemicellulose is a heterogeneous family of polysaccharides with a highly branched β-(1-4)-linked backbone. The backbone can be composed of glucose (in xyloglucans and β – glucans), xylose (in xylans and arabinoxylans) mannose (in mannans and galactomannans) or mannose and glucose (in galactoglucomannans). Xyloglucan is structurally related to cellulose, but has numerous side branches composed primarily of xylose, galactose, arabinose and fucose. The decoration of xyloglucan dramatically changes the physical properties of the polymer, and differs between species, and even tissues (Fry 1989). Hemicelluloses are water soluble and more flexible than cellulose due to its highly branched backbone and presence of acetyl groups. However, the main role of hemicelluloses are to contribute to strengthening the cell wall by interactions with cellulose and, in some walls, lignin. In cellulosic biofuel production, hemicelluloses affect the saccharification of biomass, and the released sugars, mainly pentoses (xylose, arabinose), are less suitable for fermentation than hexoses (Scheller and Ulvskov 2010).

Pectins are major components of the primary cell wall and middle lamella of dicotyledonous species with numerous roles in plant growth and development. They represent a complex family that all contain (1-4)-linked-α-D-galacturonic acid (GalA) residues which can be acetylated or methyl esterified. Pectic polysaccharides are subdivided into three major polymers with two primary backbones: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and the substituted rhamnogalacturonan II (RG-II). Some plant cell walls contain additional substituted galacturonans known as apiogalacturonan (AGA) and xylogalacturonan (XGA), but their role is still unclear. However, there is some evidence for involvement of substituted galacturonans in areas of cell detachment related to root cap cells (Willats *et al.* 2004). The different pectic polysaccharides are not separate molecules but covalently linked domains that form a macromolecule in the cell wall (Willats *et al.* 2001a), contributing to cell wall strength and adhesion. HG is the most abundant pectin in plant cell walls and the one with the simplest structure, consisting of a linear homopolymer of GalA (Mohnen 2008). It is polymerized in the Golgi apparatus by glycosyl transferases (Caffall and Mohnen 2009) and secreted to the cell wall in a highly (70 – 80%) methyl-esterified state where it is subsequently de-esterified by the action of PMEs (Pelloux *et al.* 2007). Whereas de-esterified pectin is mainly detected in the middle lamella and cell corners, the esterified form is partaking in the cellulose-hemicellulose network of the cell walls (Knox *et al.* 1990). The de-esterification of pectins by PMEs affects the interaction of pectin with celluloses and xyloglucan (Caffall and Mohnen 2009) and enables it to form calcium cross-links between pectin fibers, resulting in a strengthening of cell walls (Willats *et al.* 2001b). On the other hand, de-esterification also allows subsequent degradation by PGs and PLs and can thereby be involved in softening and degradation of the cell wall (Wakabayashi *et al.* 2003), which plays an important role in the invasion of plant tissues by bacterial and fungal pathogens (Zhang and Staehelin 1992; Orfila *et al.* 2001). A subsequent degradation of de-esterified pectins has been shown to facilitate further breakdown of cellulose and hemicelluloses (Lionetti *et al.* 2010). RGs are complex and highly branched polymers. The RG-I backbone consist of alternating residues of GalA and rhamnose. The

backbone is unesterified at the GalA residues but the rhamnosyl residues are highly substituted with side chains of mainly arabinose and galactose. RG-II is a highly conserved and complex modified HG with at least 12 different glycosyl residues. Together with HG, RG-II is known to participate in the strengthening of the cell wall by forming dimers through borate ester-bonds, whereas the function of RG-I is still unclear. However, there are indications of involvement of RG-I side chains in the physical properties of the cell wall, where they might bind specifically to cellulose and serve as plasticizers (Harholt *et al.* 2010).

Lignin and associated phenolic acids are found in certain differentiated cell types such as sclerenchyma, xylem vessels and tracheids. Lignin biosynthesis can be induced upon various biotic and abiotic stress conditions, such as wounding or pathogen and parasite infections (Vance *et al.* 1980). The chemical structure of lignin is very complex, consisting of three-dimensional cross-linked aromatic polymers made up from phenylpropane units (Vanholme *et al.* 2010). Due to its hydrophobicity, lignin plays an important role in cell wall recalcitrance, and the removal of lignin from plant biomass is a costly process that limits the conversion into biofuels (Himmel *et al.* 2007). Another phenolic compound worth mentioning is ferulic acid, which is esterified to pectins (Fry 1983) and hemicelluloses (Ishii and Hiroi 1990), and might be involved in covalent cross-linking.

In addition to polysaccharides, the cell wall consists of a diverse array of structural and soluble **proteins**. The most abundant structural proteins include extensins, glycine-rich proteins (GRPs) and proline-rich proteins (PRPs). Extensins are structural glycoproteins that contribute to cell wall extensibility and rigidity. They typically have one hydrophilic and one hydrophobic repetitive peptide motif with the potential for cross-linking (Smallwood *et al.* 1994). In response to pathogen attacks they accumulate and covalently cross-link to provide additional strength to the cell wall (Mazau and Esquerretugaye 1986). GRPs are located to the vascular tissue, mainly xylem cells, where they are associated with xylem development (Keller *et al.* 1989). Similar to extensins, GRPs are up-regulated in response to pathogen attacks where they contribute to strengthening of the cell wall (Brady *et al.* 1993). PRPs contribute to a range of cellular processes, including cell elongation (Dvorakova *et al.* 2012) and maintenance of structural integrity (Ye *et al.* 1991). Additionally, PRPs are like the other structural proteins involved in perception of pathogen attacks.

Arabinogalactan proteins (AGPs) are soluble, structurally complex proteoglycans. The polysaccharide portion of AGPs account for more than 90% of the molecule, and the high diversity among the carbohydrate side chains decorating the protein backbone makes it difficult to assign them a specific function. However, AGPs are known to be involved in cell expansion, cell division, pollen tube growth and guidance, resistance to infection, signaling, cell death, and linking the plasma membrane to the cytoskeleton, but the exact mode of action is still unknown (Ellis *et al.* 2010). Involved in cell-cell communication and adhesion, AGPs are important during early stages of pathogen attacks (Albert *et al.* 2006; Cannesan *et al.* 2012). Some AGPs are wall bound, whereas the rest is present in the apoplast in a highly soluble state (Baldwin *et al.* 1993; Svetek *et al.* 1999). Besides AGPs, soluble proteins present in the cell wall include transport proteins, defense proteins, lectins and enzymes.

5.3.2 Carbohydrate-active enzymes

Carbohydrate-active enzymes (CAZymes) are involved in the assembly and modifications or degradation of mono-, oligo- and polysaccharides. Plant cell walls have the largest diversity of CAZymes known to date (Henrissat and Davies 1997; Coutinho *et al.* 2003a), and plant genome projects have revealed that plants carry genes that encode enzymes required to degrade all polysaccharide components in their own cell walls (Albersheim *et al.* 2011). The CAZymes have traditionally been classified by substrate specificity and reaction products. However, recent extensive genome sequencing and protein crystallization have exposed limitations to the old classification system. Consequently, CAZymes have been grouped into distinct phylogenetic families according to their sequence and structural similarities, which can be found in the CAZy database (www.cazy.org) (Cantarel *et al.* 2009). The different families of CAZy have been clustered into four major classes of enzymes based on the type of reaction catalyzed: glycoside hydrolases, glycosyltransferases, polysaccharide lyases, and carbohydrate esterases. Glycoside hydrolases are responsible for the hydrolysis of glycosidic bonds between monosaccharides. Glycosyltransferases generally function in polysaccharide synthesis, catalyzing the formation of new glycosidic bonds. Polysaccharide lyases catalyze the β-elimination on uronic acid-containing polysaccharides, such as pectins, whereas carbohydrate esterases remove ester-based modifications of mono-, oligo- and polysaccharides, like methylesters, acetyl groups and feruloyl groups, which allows further breakdown by glycoside hydrolases (Cantarel *et al.* 2009). The recent discovery of lytic polysaccharide monooxygenases (LPMO) which is a new family of oxidative enzymes with the capacity to degrade recalcitrant crystalline cellulose (Vaaje-Kolstad *et al.* 2010), added a new class to the CAZy database; auxiliary activities. This class further includes enzymes involved in lignin degradation (Levasseur *et al.* 2013). In addition to the five enzyme classes mentioned, there is one class of associated modules, comprising the carbohydrate binding modules (CBMs). CBMs are non-catalytic proteins with ability to bind carbohydrates, thus they are often associated with other CAZymes (Boraston *et al.* 2004).

Due to its crystalline structure and insolubility cellulose is very resistant to enzymatic hydrolysis, and only a small number of bacteria and fungi have so far been acknowledged as being capable of degrading native cellulose (Goyal *et al.* 1991). Parasitic plants are, however, promising candidates, as they, too, depend on degrading host cell walls to establish feeding connections. Cellulose degradation requires sequential action of at least three enzymes; endoglucanases cleave the internal bonds of the cellulose chain and increase the number of accessible ends, exoglucanases cleaving cellulose chains into oligosaccharides from the exposed ends, and β-glucosidases further hydrolyzing oligosaccharides into monosaccharides (Ward and Mooyoung 1989).

Hemicelluloses and pectins are complex heterogeneous structures that require groups of enzymes working in concert for their deconstruction. In addition to enzymes that cleave the various backbones, both hemicelluloses and pectins are heavily decorated by side chains that need to be removed prior to further degradation (Williamson *et al.* 1998). PLs, PGs and rhamogalacturonases are responsible for the degradation of the pectin backbone, whereas

PMEs and pectin acetylesterases remove methylesters and acetylesters from esterified GalA residues, respectively. Depending on cell wall properties such as pH and degree of pectin methylesterification, PMEs can either act randomly or linearly. A randomized de-methylation promotes further degradation of pectin by PGs and PLs that are specific for unmethylated substrates. A subsequent degradation of de-esterified pectins has been shown to facilitate further breakdown of cellulose and hemicelluloses (Lionetti *et al.* 2010). A linear de-methylation of pectin induces cross linking of pectins by Ca^{2+} and consequently strengthens the cell wall and make HG unavailable for further degradation (Micheli 2001). Pectin lyases degrade methylated forms of pectin, but little is known about their role during pathogenicity.

In lignified cell walls, removal of the complex and insoluble lignin is necessary to access other cell wall polysaccharides. Two major classes of enzymes are involved in lignin degradation; peroxidases and laccases (Martinez *et al.* 2009). Interestingly, peroxidases have been localized in cell walls of *Cuscuta jalapensis* associated with haustorial development by facilitating the restructuring of host cell walls (Lopez-Curto *et al.* 2006). Other enzymes with a proposed function in the host penetration by *Cuscuta* include pectinases, cellulases and proteases (Nagar *et al.* 1984; Srivastava *et al.* 1994; Bar Nun and Mayer 1999a; Bar Nun *et al.* 1999b; Lopez-Curto *et al.* 2006; Li *et al.* 2010; Johnsen and Krause 2014).

6 Aims of the study

Despite the ecological and economic significance of the genus *Cuscuta*, the mechanisms behind its attack and the corresponding reactions observed in susceptible and resistant host plants remain largely unknown. The goal of this thesis was to shed light on the interaction between this parasite and its hosts. This knowledge is crucial to evaluate if and how *Cuscuta* can be exploited biotechnologically.

The initial focus on *C. reflexa* as a source for novel enzymes valuable for the biofuels industry expanded into a more comprehensive study aiming at increasing the understanding of the mechanisms going on at the interface between the parasite and its hosts. One of the main questions that are yet to be answered is how the parasites manage to direct their presumed arsenal of CAZymes exclusively towards the host without degrading their own haustorial cell walls. Furthermore, we have observed variations in compatibility to *Cuscuta reflexa* among wild and cultivated tomato varieties that potentially offer a unique system to approach the elucidation of defense responses from a genetic viewpoint.

The specific aims were:

1. To characterize carbohydrate-active enzymes involved in modification or degradation of host cell walls upon *Cuscuta* infection (**Papers I, II** and **work in progress**)

2. To generate an overview of the cell wall compositions of both host and parasite as an attempt to explain the host specific cell wall degradation (**Paper II**)

3. To evaluate the potential of a tomato introgression population in the quest to decipher host plant resistance towards *Cuscuta* (**Paper III**)

7 Summary of papers

Paper I

Cellulase Activity Screening Using Pure Carboxymethylcellulose: Application to Soluble Cellulolytic Samples and to Plant Tissue Prints

Hanne Risan Johnsen, Kirsten Krause.

One important aim of this study was to quantify and characterize CAZyme activities from the parasitic plant genus *Cuscuta*. Our initial attempts to quantify cellulase production in *Cuscuta* species using published methods revealed that one assay was not specific for cellulase activity and the other was incompatible with extracts from *Cuscuta* due to a high background of naturally occurring sugars. The findings after initial experiments with a widely used agar-based method with carboxymethylcellulose (CMC) as substrate corroborated previous establishment of cellulolytic activity in *Cuscuta* (Nagar *et al.* 1984), but additional control experiments showed formation of clearance zones in the absence of CMC as well as with other enzymes than cellulases. There was also no detectable difference between the color of plates with and without CMC after staining with Congo red or Gram's iodine, indicating unspecific binding of both dyes. We have, hence, developed a method that is free of these artifacts by omitting all gelling agents other than CMC. The establishment of this method in conjunction with 96-well microtiter plates gives the additional advantage of allowing small sample volumes to be assayed and photometrically quantified by a microplate reader. Our new protocol was further modified to allow detection of cellulase activity in tissue prints. As expected, we could detect high cellulase activity in the parasite, whereas two non-parasitic species (*Pelargonium zonale* and *Solanum lycopersicum*) studied did not display any detectable activity in either the plate assay or tissue prints. This could however be due to limited sensitivity of the methods.

Paper II

Comprehensive microarray profiling of cell wall polymers and enzymes in the parasitic plant *Cuscuta reflexa* and the host *Pelargonium zonale*

Hanne Risan Johnsen[1], Bernd Ketelsen[1], Stian Olsen[1], Silvia Vidal-Melgosa[2], Jonathan U. Fangel[2], William G.T. Willats[2] and Kirsten Krause[1]

The mechanisms behind cell wall modifications during host infection by the holoparasitic plant genus *Cuscuta* spp. remains unclear. However, it has been suggested that a combination of mechanical pressure applied to the host tissue and enzymatic modification or degradation of host cell walls by a cocktail of secreted carbohydrate-active enzymes is involved. One of the main questions that remain unanswered is how these parasites manage to direct the action of the assumed enzymatic cell wall breakdown exclusively towards the host and how they protect their own haustorial cell walls from self-degradation. In this paper we investigated the cell wall composition and CAZyme activities of *Cuscuta reflexa* and the compatible host *Pelargonium zonale* by comprehensive glycan microarray techniques and immunohistolabeling. Our results showed that esterified pectins dominate in the cell walls of uninfected *Pelargonium zonale*, while adjacent to the infection site esterified pectins are obviously de-esterified. Epitope depletion assays revealed high activity against methylated pectins in *C. reflexa* extracts as well as some activity in infected host tissue. Furthermore, unmasking of xyloglucan epitopes in host cells close to the intruding haustorium as well as qRT-PCR measurements indicate high pectate lyase activities in *C. reflexa*'s haustoria and point to active pectin degradation at the infection site. The pectin methylation status and cross-linking with other molecules in middle lamella of the parasite's haustorium might bear the secret to why the haustorium is not degraded by its own lytic arsenal. Our results corroborate the notion that enzymatic degradation softens the host tissue for an infection and paves the way for the intrusion of the haustorium.

Paper III

From susceptibility to resistance against parasitic dodder (genus *Cuscuta*): What can we learn from a wild tomato introgression line population?

Hanne Risan Johnsen[1], Anna Pielach[1], Karsten Fischer[1], Leidulf Lund[1], Jocelyn K.C. Rose[2] and Kirsten Krause[1]

In this study, we phenotyped an introgression population of tomato for resistance against *Cuscuta reflexa* based on differences in susceptibility that were observed in the wild tomato species *Solanum pennellii* and the domesticated tomato *Solanum lycopersicum*, cultivar M82. The cultivated variety is resistant to *C. reflexa*, whereas the wild species *S. pennellii* is susceptible as host. *S. pennellii* is sexually compatible with *S. lycopersicum* and produces fertile offspring which has been exploited to generate a comprehensive set of introgression lines (ILs). The introgression population between these two species, where a portion of the *S. lycopersicum* genome has been substituted with the corresponding portion of the genome of *S. pennellii*, provides a potential tool to reveal the genetic background of resistance in tomato. Differences in resistance to *C. reflexa* across 48 *S. pennellii* ILs were evaluated by the ability of the parasite to form functional haustoria upon infection. A local hypersensitive reaction typical for the resistant cultivated parent was conserved in most ILs, but a small number of lines displayed either cell death beyond the infection site or susceptibility to *C. reflexa*. We are further aiming to select recombinant plants originating from a backcross between the cultivated tomato and the compatible ILs identified in our study, to obtain sub-ILs with shorter introgressions in order to reduce the size of the introgressed fragment and increase the resolution of candidate genes responsible for resistance in some tomato varieties.

8   Work in progress

8.1 Approaches to study the *Cuscuta* secretome

Carbohydrate microarray techniques provide comprehensive overviews of cell wall compositions and CAZyme activities, but they do not provide any information of which enzymes, if any, are secreted by the parasite upon host infection. Evidence for secretion of cell wall modifying enzymes at the interface between *Cuscuta* and the host would be a great step forward in understanding the mechanisms involved in parasitism.

Secreted proteins or secretomes play important roles in development and communication within a plant, but are also involved in plant-pathogen interactions (Krause *et al.* 2013). The plant secretome refers to the set of proteins that are secreted into the extracellular space of a cell, tissue, organ or organism (Agrawal *et al.* 2010). Typically, secreted proteins contain an N-terminal signal peptide (SP) which targets them to the endoplasmic reticulum (ER), but several proteins lack this peptide and are secreted by alternative secretion mechanisms.   Commonly used methods for studying plant secretomes include vacuum infiltration and centrifugation but are sensitive to intracellular contamination. Subsequent proteomic analyses are routinely done by gel-based methods to separate proteins, followed by mass spectrometry (MS). MS can also be applied directly to crude extracts without a pre-separation of the proteins. We are currently using two different approaches to investigate the proteins that are secreted by *Cuscuta reflexa*.

8.1.1   Yeast Secretion Trap (YST)

A yeast secretion trap (YST) functional screen was used to characterize proteins secreted externally by the cells of *C. reflexa*. The technique involves ligation of heterologous cDNAs derived from infective plant tissue (haustoria) to a yeast invertase gene (suc2), followed by transformation of the resulting library into an invertase-deficient yeast mutant. Invertase is necessary to hydrolyze sucrose (to glucose and fructose) and allow cell growth on a sucrose medium, but is not produced and secreted when its signal peptide is deleted, resulting in yeast cells that are unable to grow on a sucrose selection medium. Yeast transformants containing heterologous cDNAs with SPs that direct secretion of the recombinant invertase fusion protein are able to grow on a medium containing sucrose as the only carbon source (Lee and Rose 2012). The main disadvantage with this method is that secreted proteins lacking SPs are not identified.

The YST screen did not yield the expected results as we identified only glycine-rich proteins and plant defensins. Plant defensins are small, secreted, cysteine rich proteins with antimicrobial activity against bacteria and fungi (Albersheim *et al.* 2011). It was recently demonstrated that basic pathogenesis related proteins increased in pre-haustoria and haustoria of *C. pentagona* upon host contact. Thus, it can be hypothesized that parasitic plants recruit defense related genes for host recognition. Alternatively, the parasite is under biotic stress due to defense responses in the host and recruits its own defense mechanisms to overcome the counter-attack (Ranjan *et al.* 2014).

8.1.2 Far-red induction of haustoria

The previous method provides information on the entire secretome of the plant, including proteins secreted within the plant but lacks spatio-temporal information. Therefore, we have developed a setup (Fig. 9) that allows us to collect secreted proteins at the surface of developing haustoria. A combination of far-red treatment and tactile stimuli induce haustoria in stems of *C. reflexa* independent of the presence of a suitable host (Furuhashi *et al.* 1997). Stems of *C. reflexa* are fixed between two plastic plates, one of which is covered with filter paper. Haustoria are induced by far-red treatment and tactile stimuli, a process during which they secrete their lytic proteins. These can be subsequently eluted from the filter paper and analyzed by SDS-PAGE and MS. This system provides a tool that has overcome the apparent problems with investigating proteins that are secreted into another plant, mainly without the disadvantage of contamination from the host.

Interestingly, peptide annotations from preliminary results include proteins that are associated with cell wall modifications and breakdown of cell walls. There are several overlaps between the MS-data and previously identified proteins from the carbohydrate microarrays. A number of pectin related enzymes, as well as cellulases and xyloglucanases were among the identified secreted proteins.
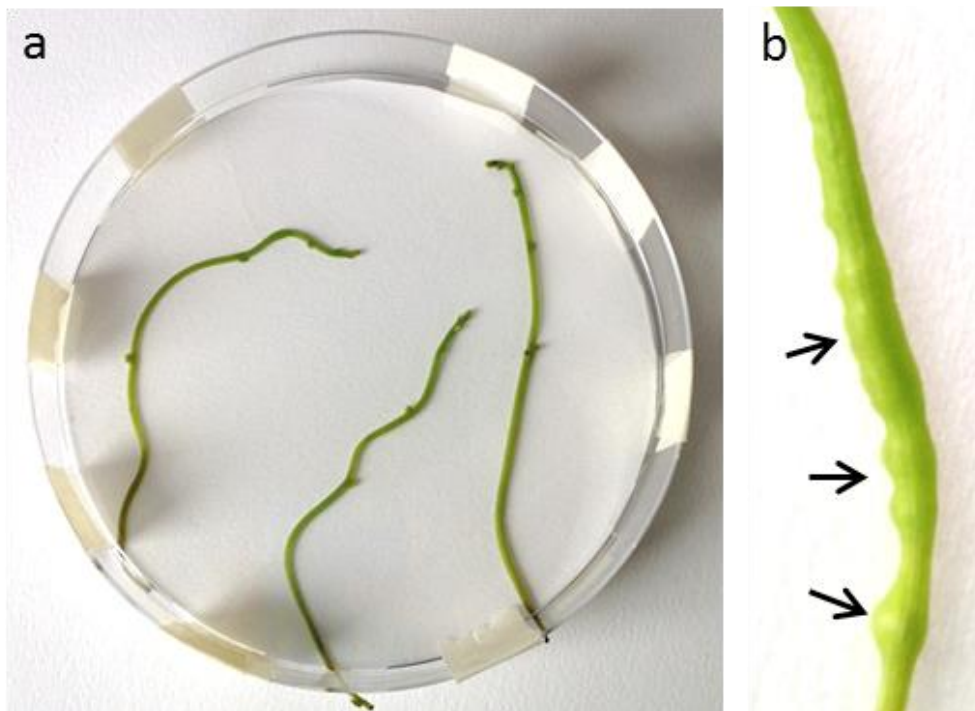
Figure 9. A system to study secreted proteins at the surface of developing haustoria. a) Setup for far-red induction of haustoria in stems of *C. reflexa* fixed between to plastic plates to apply tactile stimuli. Filter paper covers one side in order to recover secreted proteins during the haustorial development. The plates are incubated in dark at room temperature. b) Haustoria (arrows) forming in the direction of the filter paper three days after far-red induction.

9   General discussion and outlook

Although *Cuscuta* spp. is one of the best studied families of parasitic plants, the molecular and genetic basis of parasitism remains largely unknown. The mechanisms by which *Cuscuta* attaches to the host and enters through the host cell wall have been thoroughly studied, but mainly on a morphological and immunocytochemical level (Vaughn 2002; 2003; 2006; Lee 2007; 2008; 2009; Hong *et al.* 2011). Consequently, little is known about the proposed cocktail of cell wall degrading enzymes and cell wall loosening complexes that ostensibly are involved in the modifications of host cell walls upon establishment of functional haustoria. Necrotrophic pathogens cause extensive tissue maceration by secretion of abundant CAZymes. Similar to biotrophic pathogens, *Cuscuta* spp. rely on keeping their hosts alive and unaware of their presence. However, both biotrophs and parasitic plants secrete enzymes to facilitate host entry, but less abundant than necrotrophs.

Prior to this study, little work was published focusing on cell wall degrading enzymes in, or secreted by parasitic plants. Nagar *et al.* (1984) have demonstrated elevated activity of several CAZymes in the haustoria of *Cuscuta reflexa*. Nevertheless, the enzyme activities were at low levels compared to CAZymes identified in necrotrophic parasites. Early light microscopic studies of *Cuscuta australis* revealed an apparent growth of the searching hyphae through the host tissue (Lee and Lee 1989). Later, electron microscopic studies showed that the hyphae apparently do not grow through the host cells but are coated with a layer of expanded host cell wall (Dawson *et al.* 1994; Vaughn 2003). In either case, the progression of infective haustoria is reliant of modifications of host cell walls in order to pave their way into the host tissue.

Improved molecular and genetic tools are currently being exploited to dissect the mechanisms involved in parasitic plant–host plant interactions, and a number of genes with apparent cell wall associated functions have been identified. Honaas *et al.* (2013) studied transcriptomes generated from laser microdissected tissues at the interface of the parasitic plant *Triphysaria versicolor* parasitizing *Zea mays*. Genes that were highly expressed by *T. versicolor* at the parasite-host interface included pathogenesis-related proteins and expansin, which is a cell wall loosening protein. Furthermore, Ranjan *et al.* (2014) recently reported increased expression of several cell wall modifying enzymes in the infective stages of *Cuscuta pentagona*. Differentially expressed transcripts in pre-haustoria and haustoria were compared to reference tissues, stem and seedlings. Up-regulated transcripts interestingly included cell wall modifying enzymes such as PMEs, PLs and cellulases. While the field of genomics and transcriptomics is expanding and provides a chance to understand the genetic basis of cellular events, this information alone is not sufficient to explain all processes and interactions in the plant cell wall. Polysaccharides are not directly encoded by genes as they are synthesized by a huge diversity of enzymes, thus they cannot be readily sequenced (Fangel *et al.* 2012). Current approaches to get sequence information from polysaccharides therefore require chemical or enzymatic cleavage with subsequent sequencing of released oligosaccharides (Albersheim *et al.* 2011).

Research presented in this thesis implemented new tools together with a suitable combination of existing methods for studying the mechanisms at the interface between *C. reflexa* and different host species. With these approaches, we succeeded in profiling cell wall components as well as CAZyme activities *in situ* (Paper I, II and work in progress). Furthermore, investigation of a tomato IL population with apparent differences in susceptibility to *C. reflexa* was shown to be an applicable tool for studying the genetic and molecular background for susceptibility to *C. reflexa* in a wild tomato species (Paper III).

9.1 Detection of cellulolytic activity in *Cuscuta reflexa*: challenges with existing methods and implementation of crucial improvements

A number of pathogens are known to produce cellulases which are either continuously secreted or induced upon contact with a host plant where they facilitate penetration of host cell walls (Lebeda *et al.* 2001). Interestingly, cellulases have been detected in the parasitic plant species *Orobanche aegyptiaca* (Shomer-Ilan 1994) and *Striga hermonthica* (Olivier *et al.* 1991) where they apparently reduce the cellulose content of host plants. Cellulolytic activity in *C. reflexa* has been previously described (Nagar *et al.* 1984; Chatterjee and Sanwal 1999), but there are no studies that confirm direct involvement of cellulases in the establishment of functional haustoria.

The initial objective of this thesis was to investigate whether *C. reflexa* employs cellulases for the penetration of host plants. Parasitic plants represent an entirely unexploited potential resource for the biofuels industry despite their ability to efficiently modify and degrade host cell walls (Lopez-Casado *et al.* 2008). The major cell wall component, and hence the most abundant natural source of organic carbon, is cellulose. The conversion of lignocellulosic biomass into biofuels or chemicals requires efficient cellulolytic enzymes in order to break down the cellulose fibers. The difficulty of processing or degrading cellulosic biomass to fermentable substances has so far prevented the development of a successful cellulosic biofuel industry.

Preliminary studies included detection and quantification of cellulolytic activity in *C. reflexa* by two published screening assays specific for cellulases. Qualitative studies of cellulases were obtained by plate clearing assays (Kasana *et al.* 2008), and further quantifications of activities were measured colorimetrically based on the amount of reducing sugars released from the hydrolysis of CMC (Miller 1959). Screening for cellulase production is routinely done on CMC-containing agar plates where cellulolytic activity is detected using Congo red or Gram's iodine. Both dyes form a colored complex with cellulose but not with hydrolyzed cellulose, resulting in a distinct zone where CMC has been degraded (Kasana *et al.* 2008). Initial plate assays detected high cellulolytic activity in *C. reflexa*, but further control experiments without any cellulosic substrate showed that the clearance zone formed after incubation with plant extracts was not due to cellulolytic degradation of CMC (Paper I). Additionally, cellulolytic activity in extracts from *C. reflexa* and *P. zonale* was

determined by a colorimetric method using 3,5-dinitrosalicylic acid (DNSA) with CMC as substrate (Miller 1959). This assay is based on increase in reducing ends of carbohydrates. Total cellulolytic activity appeared to be significantly higher in the parasite than in its host, but further control experiments (boiling and trypsin digestion of samples to inactivate enzymes) gave the same results as untreated samples. This indicates that the sugar-background in *C. reflexa* is incompatible with the colorimetric detection of reducing sugars. Consequently, reliable and rapid detection methods for cellulolytic enzymes that would be used for biological and environmental samples were missing. Because of the initial problems with established methods, it became necessary to develop a method for qualitative and semi-quantitative measurements of cellulase activity which is compatible with the high natural sugar content in plant material and does not yield unwanted artifacts (Paper I).

We utilized the gelling capacity of pure CMC to make a stainable gel, degradable by commercial cellulases as well as crude plant and soil extracts. The same detection method was further employed to tissue prints for detection and localization of cellulase activity in sections of plant tissues. The CMC assay with plant tissue prints was used to detect cellulolytic activity in *C. reflexa*, and compare it with the two non-parasitic plants, *P. zonale* and *S. lycopersicum*. Cellulase activity was strongly detected in longitudinal sections of non-infectious stems of *C. reflexa*, whereas both non-parasitic plants did not show any detectable activity against CMC. Cross-sections of the interface between *C. reflexa* and *P. zonale* further confined the activity to the parasite (Paper I). Corroborating the findings in Paper I, microarray data presented in Paper II show activity against cellulose (recognized by CBM30) in extracts from *C. reflexa* haustoria and the infected host.

9.2 A comprehensive study of cell wall polymers and CAZymes

The complexity and heterogeneity of the plant cell wall make the study of its functions challenging. Although most plant cell wall polysaccharides have been well characterized, it is still not properly understood how they dynamically interact with each other and what biological functions they confer to the cell wall biology (Cosgrove 2005; Caffall and Mohnen 2009). The localization of individual polymers is crucial to understand their interactions with other components in the cell wall. To get the full picture, an integrated approach combining several methods is essential. Our attempts at investigating cell wall composition and CAZyme activities in *C. reflexa* include high throughput carbohydrate microarrays and high resolution immunolocalization. Together these approaches have contributed to an extensive antibody screen of the parasitic plant *C. reflexa* (Paper II).

9.2.1 Monoclonal antibodies as tools to study cell wall composition

Techniques based on monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) with defined specificities are powerful tools to identify and localize polysaccharides *in muro* (Knox 1997; Willats *et al.* 2001a). A range of mAbs and CBMs have been generated against a large number of cell wall epitopes, which allow us to study structures and cellular processes of intact cell walls. However, the full complexity of the cell wall is yet to be understood as the coverage of existing antibodies only gives a minor insight to the full picture.

Carbohydrate microarrays provide tools for plant research that combine the high throughput capacity of microarrays with the specificity of mAbs and CBMs (Obro *et al.* 2009). The comprehensive microarray polymer profiling (CoMPP) technique provides semi-quantitative information about the relative abundance of cell wall polysaccharides, and gives an overview of polymer occurrence via epitope frequency (Moller *et al.* 2007). The CoMPP analysis further provides information on interactions between cell wall polymers based on extractability or co-extraction using various solvents. However, we can only gain limited information from the microarray data as the extractability can be influenced by several factors. The solvents used for extraction may not be equally effective across different tissues, and epitopes can be destroyed, modified or masked during extraction. Likewise, these factors might alter the amount of cell wall components that are extracted, compared to the absolute levels present *in muro*. Different mAbs and CBMs have different binding affinity to their epitopes, thus relative levels of different polymers cannot be compared. The technique is therefore most suitable for studying variations in relative abundance of one particular epitope either within plants or between plants. In addition to the CoMPP technique, carbohydrate microarrays can be used to produce defined glycan arrays to study CAZymes activities. This technique is based on diminished binding of mAbs or CBMs due to degradation or modification of defined polysaccharides that are immobilized non-covalently by absorption onto nitrocellulose membranes (Willats *et al.* 2002; Obro *et al.* 2009).

Esterified pectins in the cell walls of *C. reflexa* display reduced extractability when compared to esterified pectin in the host cell wall (Paper II, Fig. 2) and other plants previously studied by the CoMPP technique (Nguema-Ona *et al.* 2012; Moore *et al.* 2014). A recent study showed evidence for covalent cross-links between pectin and AGPs in plant cell walls. Together with xylan, pectin and AGP form a complex called Arabinoxylan Pectin Arabinogalactan Protein1 (APAP1), and *apap1* mutants displayed increased extractability of pectin and xylan (Tan *et al.* 2013). We detected high levels of AGPs in the cell walls of the haustorium (Paper II, Fig. 2). Hence, the abundance of AGPs in the haustoria of *C. reflexa* might be involved in the reduced extractability of pectins by forming insoluble cross-linked networks. The next step will be to investigate whether these polymers are interacting *in muro* to further determine their potential role in the parasite cell walls. A recent paper described epitope detection chromatography as a tool to study interactions and co-appearance of cell wall polymers (Cornuault *et al.* 2014). This method will be ideal to further investigate the limited extractability of esterified pectins in *Cuscuta* cell walls.

Mixed-linkage (1→3) (1→4)-β-D-glucan (MLG) recognized by mAb BS-400-3 was detected in the NaOH extracts from *C. reflexa* (Paper II, Fig.2). This was surprising as MLGs are rare in the plant kingdom and their occurrence in higher plants appeared restricted to cell walls of grasses and *Equisetum* (Smith and Harris 1999; Fry et al. 2008; Sorensen et al. 2008), while in most other species studied the epitope has been either absent or detected at very low levels (Popper and Fry 2003; Sorensen et al. 2008). MLGs have been assigned a potential function as template for silica deposition in cell walls (Fry *et al.* 2008). Deposition of silica may function as a physical barrier against enzymatic degradation by insects and fungal pathogens (Currie and Perry 2007). Thus the occurrence of MLG in *C. reflexa* could potentially be involved in the elusive self-protection mechanisms of *Cuscuta*. However, the presence of MLGs in the cell walls of *C. reflexa* has to be further confirmed by immunocytochemical studies, and additional experiments to determine the silica content of the cell walls of *C. reflexa* will be necessary. The unexpected detection of MLG in *C. reflexa* can possibly be explained by horizontal gene transfer (HGT) from host plants. Previous studies have revealed several cases of HGT events in plants, most of them involving parasitic plants. Parasitic systems allow the occurrence of HGT due to the long-term physical connections to their host plants, and two recent studies have shown evidence for genomic integration of host-derived nuclear protein coding sequences (Yoshida *et al.* 2010; Xi *et al.* 2012). HGT can possibly contribute to adaptation of species to new ecological niches, but the long-term evolutionary impact of HGT in higher plants remains largely unknown (Zhang *et al.* 2013). A potential uptake of host derived MLG by *C. reflexa* has to be confirmed by phylogenetic analysis to determine the origin of the responsible genes.

As mentioned earlier, pectin degradation is an important preparative step for subsequent degradation of hemicelluloses and cellulose. Thus, pectinases have been considered the most important enzymes involved in infective growth and are discussed in more detail in the next chapter (9.2.2). However, recent work has shown that enzymes degrading the hemicellulose-cellulose network, specifically the xylans and xyloglucans contribute to the success of some pathogens (Rajeshwari *et al.* 2005; Brito *et al.* 2006). We show activity against both xyloglucan and xylan in haustorial extracts (Paper II; Fig.4), indicating involvement of these enzymes in host cell wall modifications.

Previous studies have demonstrated that cell wall modifications by the host itself can contribute to susceptibility (Cantu *et al.* 2008). Our results obtained with the carbohydrate microarray techniques revealed elevated activities of several enzymes in the infected host when compared to the uninfected (Paper II; Fig.2-4). Further studies will be necessary to investigate the origin of these enzymes, as the previous methods do not distinguish whether the specific enzymes are up-regulated in the host or secreted by the parasite. Studies of transcript levels of the respective genes could possibly aid in determining whether increased activities in the host is induced upon infection.

Immunocytochemical techniques are useful for investigating interactions between haustoria and host tissues as haustoria of many plants are relatively small structures, thus obtaining sufficient material for extraction or separating tissues of interest is often difficult. Also, the carbohydrate microarray techniques which are based on extraction of plant tissues provide no information about the spatial localization of molecules in the tissues or individual cell walls. Importantly, polysaccharides are not always readily accessible to mAbs or CBMs *in muro*, but enzymatic removal of pectin has shown to unmask other cell wall components (Knox *et al.* 1990). This was also demonstrated by us in Paper II by PL removal of pectin to enhance the binding to xyloglucan epitopes. Due to the complexity of the pectic network, the number of antibodies recognizing the different domains of pectin is high. In most cases the exact epitope structure is not known, but the development of a series of model pectins with different degrees and patterns of methylation has made it possible to thoroughly characterize the specificity of probes recognizing HG (Ridley *et al.* 2001; Willats *et al.* 2001b).

In addition to the incomplete coverage of mAbs and CBMs already mentioned, further development of the defined glycan microarray technique is currently limited by the access of well-defined oligosaccharides and polysaccharides used as substrates in epitope depletion studies. Some plants provide relatively pure polysaccharides, however the extraction process is laborious and the purity never exceed 90 %. Thus, most oligo- and polysaccharides are currently synthesized chemically (Fangel *et al.* 2012).

9.2.2 The role of pectinolytic enzymes in *Cuscuta* parasitism

Pectins envelope the cellulose-hemicellulose network and are therefore the first to be digested in order to increase the accessibility of CAZymes to other polymers (Collmer and Keen 1986; Hématy *et al.* 2009). Methyl esters and acetyl groups commonly decorating the HG backbone prevent pectins from further degradation. Hence, pectin esterases cleave off the branches of acetyl or methyl groups, which make the backbone more susceptible to subsequent attacks by PLs and PGs (Lionetti *et al.* 2012). Pectinolytic enzymes are produced by a variety of plant pathogens to reduce cell wall rigidity. Evidence for involvement of pectinolytic enzymes in haustoria establishment have been previously reported for the parasitic plant *Orobanche.* Abundance of low esterified pectins in cell walls adjacent to the penetrating haustoria, was later confirmed by the presence of PMEs both in cell walls of the parasite and the host close to the infection site (Losner-Goshen *et al.* 1998). A number of investigations suggest involvement of pectinolytic enzymes in parasitism by *Cuscuta* (Nagar *et al.* 1984; Bar Nun and Mayer 1999a; Bar Nun *et al.* 1999b). Additionally, the green tea polyphenol catechin which is suggested to inhibit PME (Sagi and Lewis 2009), prevented *Cuscuta pentagona* penetration but did not affect the pre-haustoria formation when applied to compatible host plants (Lewis *et al.* 2010). Thus, PME seems to be important for invasion of host plants. Furthermore, a proteomic study of *Cuscuta australis* detected two pectin methylesterase proteins that were up-regulated upon twining around the host stem (Li *et al.* 2010).

In Paper II we show evidence for involvement of pectinolytic enzymes in the establishment of *C. reflexa* haustoria on the compatible host *Pelargonium zonale*. Our results show that esterified pectins dominate in the cell walls of the host, but upon infection by *C. reflexa*, pectins adjacent to the infection site are de-esterified, probably as a preparative step for subsequent pectin breakdown. Epitope deletion assays further revealed high de-esterification activities in infected host tissue that most likely originates from the parasite. Natural unmasking of xyloglucan epitopes in host cells close to the intruding haustorium additionally point to active pectin degradation at the infection site. Further, we show that the transcript abundances of one PL is higher in haustorial attachment sites than in stem tissue, indicating that the transcription of this gene is up-regulated at some point in the haustorial development and that PL might be involved in the parasitation of the host.

Earlier studies of the invasive process of *C. reflexa* have revealed evidence for hyphal growth in the middle lamella of the host (Vaughn 2003), possessing similar growth patterns as previously described for *Orobanche* (Losner-Goshen *et al.* 1998). Based on high pectinolytic activity detected in the infective tissue of *C. reflexa* our results agree with the common notion that parasites grow between host cells rather than through them (Losner-Goshen *et al.* 1998; Vaughn 2003; Heide-Jørgensen 2008). However, histological micrographs of the parasite-host interface clearly show that the hyphae occasionally do penetrate host cells, and we can therefore not exclude a combination of extra- and intracellular growth of *C. reflexa* within host tissues. Vaughn (2003) demonstrated that the intracellular hyphae do not grow directly through the host cells, but are coated with a thin layer of cell wall material. Based on immunocytochemical labeling, this chimeric wall is mainly composed of pectins and differs in composition from both the host and the parasite. It remains unclear whether the progressing hyphae induce synthesis of a new wall or if a part of the existing host cell wall is extended. Such extension of the host cell wall would necessarily involve loosening or degradation of wall components. In contrast to extracellular growing hyphae, intracellular growth promotes formation of abundant inter-specific plasmodesmata connections at the tip of the developing hyphae (Vaughn 2003). Further analysis of the chimeric cell walls is necessary to understand the progressive growth of the searching hyphae and the proposed plasmodesmatal connections between the parasite and host.

9.2.3   The secretome of *Cuscuta reflexa*

One striking difference between parasitic plants and non-plant pathogens is that plants not only possess enzymes for the purpose of parasitism, but also for maintaining own vital processes. Thus, detection of CAZyme activities in extracts and tissue sections from *C. reflexa* (Paper I and II) is not alone evidence for enzyme facilitated penetration of host plants. To verify the involvement of previously detected enzymes in the infective growth of the parasite, we are implementing methods for investigating the secretome of *C. reflexa* (work in progress). Preliminary results from our secretome approach indicate an overlap in secreted proteins with findings from the carbohydrate microarrays presented in Paper I and II. In addition to pectinases, cellulases and hemicellulases, we also identified lipolytic enzymes. Lipases are important in fungal adhesion and penetration of plant cell walls during the early stages of infection, where they are postulated a function in cuticle degradation (Voigt *et al.* 2005; Kikot *et al.* 2009). The potential involvement of lipases in cuticle degradation to facilitate *Cuscuta* infection has to be further investigated, but to my knowledge there are no previous reports on how *Cuscuta* deals with the cuticle of host stems.

The artificial induction of haustoria with growth onto a filter paper covered plastic surface cannot resemble the mechanisms involved in natural infections of host plants. Consequently, the set of secreted proteins obtained from this system might not be fully representable, but contributes to a first indication of which proteins are involved in host cell wall modifications. Another interesting aspect that should be further studied is whether *Cuscuta* alters the set of secreted proteins with regard to the cell wall composition of host plants. Nagar *et al.* (1984) studied cell wall degrading enzymes of *C. reflexa* parasitizing different hosts, and detected the same set of enzymes but variable levels of activity dependent on host species and the time needed for haustoria establishment. Earlier studies involving a phytopathogenic fungus demonstrated that it is possible to identify fungal proteins inside the infected host plant (Paper *et al.* 2007), but this would necessitate genome sequences of both *Cuscuta* and the infected host to distinguish between host and parasite derived proteins.

9.3 Resistance to *Cuscuta reflexa* across a tomato IL population

Despite the evident importance of cell walls in plant defense, there is little information on the molecular and genetic changes related to modifications of the cell wall upon parasite attacks, both in compatible and incompatible plant species. Host susceptibility to *Cuscuta* infections has been previously described through microscopic examination of incompatible interactions (Sahm *et al.* 1995; Ihl and Miersch 1996; Farah 2007). Defense responses observed in tomatoes resistant to *C. reflexa* represent one of the best studied incompatible host-parasite interactions (Sahm *et al.* 1995; Ihl and Miersch 1996). The growth of *C. reflexa* haustoria into the host is prevented by a mechanical barrier resulting from elongation of hypodermal host cells, hypersensitive response, and accumulation of phenolic compounds and peroxidases at the site of attachment (Bringmann *et al.* 1999; Runyon *et al.* 2010a). Tomato is an ideal host for several reasons: (i) its genome is sequenced; (ii) it is amenable to genetic modification and (iii) the germplasm resources from decades of breeding approaches are unsurpassed in other agriculturally relevant dicots.

After making the important observation that *C. reflexa* can successfully infect the wild tomato species *S. pennellii*, the collection of introgression lines between *S. pennellii* and *S. lycopersicum* cv. M82 (see chapter 5.2.3) deserved some attention. Alas, we have commenced the phenotypic characterization of 48 tomato ILs in which marker-defined genomic regions of the compatible wild species *S. pennellii* are replaced with homologous regions of the resistant cultivated variety M82. The *S. pennellii* x *S. lycopersicum* IL population represent a unique system for identifying genomic regions associated with differences in compatibility to the parasitic plant *C. reflexa*, this opens a door to potentially determining the genetic basis for incompatibility in domesticated species.

Our results indicate that introgressions on chromosomes 1, 2 and 6 are associated with host resistance to *C. reflexa*. Interestingly, a number of genes and QTLs responsible for disease resistance in tomato have been previously mapped to chromosome 6 in response to infections by fungi, nematodes and viruses (Zamir *et al.* 1994; Dixon *et al.* 1996; Ammiraju *et al.* 2003; Bai *et al.* 2005). The apparent co-localization of genomic positions of genes/QTLs involved in resistance might be an indication of shared mechanisms that provide immunity against a variety of plant pathogens and parasites.

9.3.1 Susceptible IL candidates

Differences in resistance to *C. reflexa* across a population of 48 *S. pennellii* ILs were evaluated by the ability of the parasite to form functional haustoria upon infection (Paper III). A local hypersensitive reaction typical for the resistant cultivated parent was conserved in most ILs, but a small number of lines displayed either cell death beyond the infection site or susceptibility to *C. reflexa* (Paper III; Fig. 5 and Table 1). We identified 6 ILs that were apparently compatible to *C. reflexa*. This phenotypic effect is mapped to bin 1-H (IL1-3 and IL1-4) on chromosome 1, and an overlapping region in bin 2H comprising the three lines IL2-4, IL2-5 and IL2-6 on chromosome 2. Additionally, we identified one line (IL6-2) on

chromosome 6 (Fig.10). The presence of each one of these loci coming from the susceptible host *S. pennellii* seems to be enough to partially abolish the resistance displayed by the cultivated parental line. However, it may be that several QTLs or genes will need to be combined to obtain a sufficient level of susceptibility, and this might be the reason why the apparently compatible ILs showed beginning signs of a defense reaction at some occasions.

We are further aiming to select recombinant plants originating from a backcross between the cultivated tomato and the compatible ILs identified in our study, to obtain sub-ILs with shorter introgressions in order to reduce the size of the introgressed fragment. The recombinant frequency of introgressed segments is decided by the genetic distance (cM) between two flanking markers, which is relatively low. Thus, from an initial screen of approximately 200 F2 population seedlings we so far only identified two plants with a recombination in the inserted fragment from *S. pennellii*.
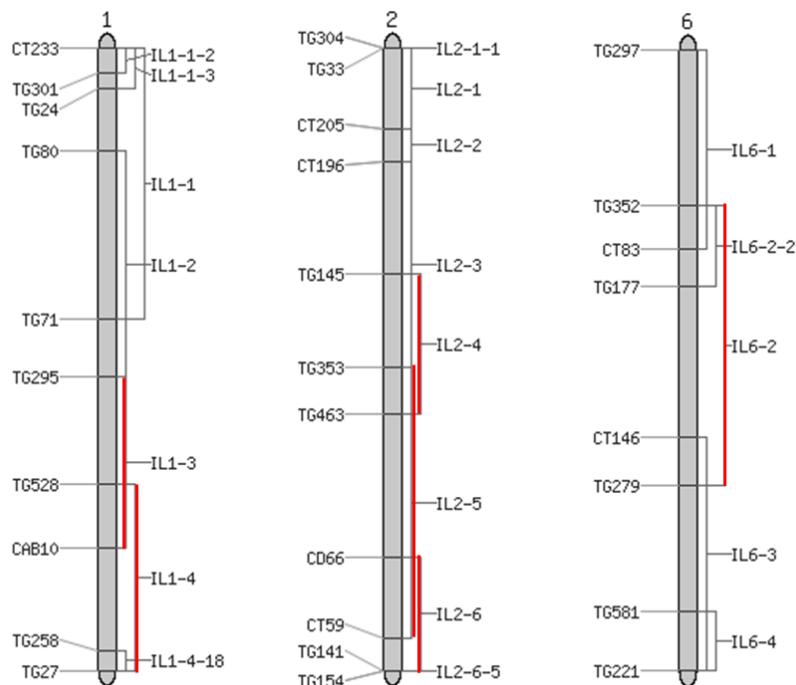

Figure 10. Genetic map of *S. pennellii* introgression lines on chromosome 1, 2 and 6. Red bars matching introgressed fragments of the compatible ILs identified in our study (Figure modified from Bombarely *et al.* (2011)).

The identified recombinant plants from our study have been self-pollinated and future work will include genotyping and phenotyping of offspring from the selfed recombinant lines to check whether the ILs still retain the phenotypic effect after reduction in the introgressed fragment. If the phenotype is maintained, repeated backcrossing with the sublines can further decrease the size of the introgressed fragments and hence increase the potential to reveal the genetic background for resistance. In a previous study, the *S. pennellii* x *S. lycopersicum* IL population was used to resolve the QTL for fruit pericarp firmness, and 7500 lines from a backcross population were screened for recombinants to nominate candidate

genes. From this, a total of 124 informative recombinant individuals were identified (Chapman *et al.* 2012). The capacity to conduct large enough screens for recombinant lines from the backcrosses will be our limitation. The marker assisted selection of recombinant lines is tedious work, which requires several years of tomato growth, phenotyping and genotyping and will not be possible to conclude within the time frame of a single PhD thesis.

The availability of the genome sequence for domesticated tomato has been important for genetic mapping and genotyping in tomato (Van Schalkwyk *et al.* 2012). Genome sequences of the M82 cultivar of *S. lycopersicum* and the wild species *S. pennellii* will soon be released, and this will further assist in defining the introgressed fragments of the *S. pennellii* ILs.

9.3.2 Cell wall composition in susceptible and resistant species

In addition to induced defense responses, plant resistance can possibly be explained by differences in cell wall composition. To date, little information is available on the involvement of plant cell wall polysaccharides in the outcome of plant–pathogen interactions. The lack of genetic data on differences in cell wall composition within plant species limits the ability to associate cell wall composition with host compatibility (Vorwerk *et al.* 2004). Recent progress in the field of plant genetics has revealed correlations between altered cell wall compositions and altered susceptibility to pathogens (Underwood 2012).
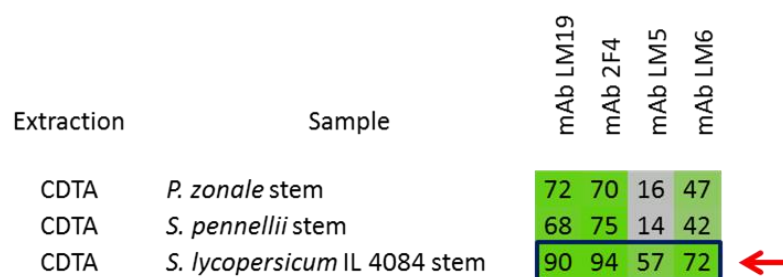
Extraction	Sample	mAb LM19	mAb 2F4	mAb LM5	mAb LM6
CDTA	*P. zonale* stem	72	70	16	47
CDTA	*S. pennellii* stem	68	75	14	42
CDTA	*S. lycopersicum* IL 4084 stem	90	94	57	72

Figure 11. Differences in cell wall composition between compatible and resistant hosts. Heatmap showing the abundance of low esterified HG (LM19), Ca^{2+} cross linked HG (2F4) and the RG-I backbones arabinan (LM6) and galactan (LM5) in two susceptible hosts *P. zonale* and *S. pennellii*, and the resistant IL4084.

Implementing the CoMPP technique used in Paper II in an initial screen of the compatible wild tomato *S. pennellii* and one resistant tomato IL (4084) we detected some differences in cell wall compositions. Interestingly, a generally higher content of unesterified and Ca^{2+} cross linked pectins (recognized by mAbs LM19 and 2F4) was detected in the resistant variety when compared to the compatible host. If compared also with the compatible host *P. zonale* investigated in Paper II, which displayed a similar cell wall composition to the compatible tomato (Fig.11), a pattern may begin to emerge that needs to be investigated with further compatible and resistant lines. Corroborating these results, a decrease in pectin methyl esterification in *Arabidopsis thaliana* mutants has been shown to cause resistance to powdery mildew (Vogel *et al.* 2002). More studies are needed to understand the implications of cell wall composition on host resistance.

10 References

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