Dodder and Tomato:
A plant-plant dialogue

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Zusammenfassung

Teufelszwirn oder **Seide** nennt man die Pflanzen der Gattung *Cuscuta*, die zu den **Windengewächsen** (Convolvulaceae) gehören. Alle Pflanzen dieser Gattung sind obligate Vollparasiten, sogenannte **Holoparasiten**. Nur wenige Pflanzen können sich erfolgreich gegen Teufelszwirn wehren. Die Kulturtomate (*Solanum lycopersicum*) gehört zu den wenigen Arten von resistenten Pfanzen. Sie kann sich erfolgreich gegen die **Großblütige Seide** (*Cuscuta reflexa*) behaupten. In dieser Arbeit werden das **Perzeptionssystem** der Tomate und das von ihm erkannte molekulare Muster (dem **Elicitor**) aus der Großblütigen Seide behandelt.

Die Kulturtomate hat viele Wildverwandte und ist mit den meisten von ihnen kreuzbar, deshalb eignet sie sich hervorragend für genetische Studien. Sie zeigt eine heftige Abwehrreaktion bei Teufelszwirn-Befall. Das Immunsystem der Tomate ist demnach in der Lage einen Elicitor des Tefelszwirns zu erkennen, während einige Wildverwandte dies nicht können und suszeptibel sind.

Dieses unbekannte molekulare Muster wurde charakterisiert und eine Identifizierung begonnen, auch der korrespondierende Mustererkennungsrezeptor aus der Tomate wurde gefunden. Dazu konnten noch Wege aufgezeigt werden, wie die aus der Interaktion zwischen der Tomate und dem Teufelszwirn gewonnenen Erkenntnisse dem Pflanzenschutz dienlich sein können.

Abstract

The plants of the genus *Cuscuta* or **dodder** belonging to the **morning glory family** (Convolvulaceae) are obligate parasitic plants, so-called **holoparasites**, meaning that they are fully host-dependent. Only few plants can successfully resist a dodder attack. The cultivated tomato (*Solanum lycopersicum*) however is among the resistant plants. It can successfully persist an attack of **giant dodder** (*Cuscuta reflexa*).In this work the **perception system** of tomato and its corresponding **elicitor** from giant dodder is investigated.

Cultivated tomato has many wild relatives and is with most of them intercrossable, which gives researchers an excellent opportunity for genetic investigations. It shows a violent reaction when attacked by giant dodder. Thus the immune system of tomato is able to perceive a

molecular pattern from giant dodder, whereas some wild tomatos cannot, which makes them susceptible.

This unknown molecular pattern was characterized and an identification process began, and in addition the corresponding receptor was found. It was furthermore revealed how the findings of the interaction between dodder and tomato can be used for plant protection.

1. Introduction

Little is known about the interactions of **parasitic plants** with their hosts compared to the knowledge about plant-microbe interactions. There are about 3000 parasitic angiosperm species from 17 families (Parker and Riches, 1993). Of agricultural importance among them are for example *Striga* and *Orobanche* from the **Orobanchiacea** family as well as *Cuscuta,* known as dodder, or devil's hair, witch's hair, love vine and amarbel from the morning glory family (**Convolvulaceae**). Plants of the genus *Cuscuta* comprise about 200 species, which all live as holoparasites. Hemiparasites are only partly dependent on their hosts and are still able to photosynthezise whereas holoparasites are completely host-dependent in terms of water, nutrients and assimilates. Some facultative parasitic plants such as *Rhinanthus minor* appear to have a functional photosynthetic apparatus and can grow without a host providing reduced carbon (Hibberd and Jeschke, 2001). Others cannot grow without the supply of photosynthate due to a very low photosynthetic capacity, e.g. *Cuscuta reflexa* (Hibbert et al., 1998) and are therefore obligate parasites.

Dodder is a nonspecific parasite that attacks, sometimes simultaneously a wide range of species including many cultivated species and dicotyledonous weeds, but not grasses or monocotyledonous weeds (Lanini and Kogan, 2005). *Cuscuta* spp. reduced their roots and leaves to optimize their parasitic lifestyle (Albert et al., 2008). The functional connection between the parasite and the host is called **haustorium**. *Cuscuta* haustoria penetrate the stem of their host plants to form direct connections to their vascular bundles. In early stages of development the searching hyphae on the tip of the haustorial cone form connections with the host tissue by interspecific plasmodesmata. Ten days after infection, the existence of a continuous connection between xylem and phloem of the host and parasite is demonstrated (Birschwilks et al., 2007). An example for a susceptible host plant to *Cuscuta* is the model plant *Arabidopsis thaliana* (Birschwilks et al., 2007). Right after a successful connection the parasite withdraws water, carbohydrates and solutes out of its plant victim. Under ideal conditions one *Cuscuta* stem can grow up to 20 cm per day and can proliferate massively (figure 1).

Figure 1: ***Cuscuta* infestation in the wild, Young'Cheon, South Korea.** The yellow vine *C. australis* grows on various hosts.

However, there are a few plants which exhibit an **active resistance** against an infestation by *Cuscuta* spp. For example tomato (*Solanum lycopersicum*) fends off *Cuscuta reflexa* and a few other *Cuscuta* species by a **hypersensitive-like response** (HLR). This thesis will also feature how Tomato can react to its plant attacker already in the early penetration phase. However the main focus will be on the parasite side dealing with the nature of the elicitor(s) which cause this violent reaction.

1.1 Introduction to parasitic plants

A parasitic plant can either attack the above ground part of the plant and is therefore called **shoot parasite** or the below ground part, and is then defined as a **root parasite**. According to their lifestyle parasitic plants are either **obligate** or **facultative**. The obligate parasites totally depend on their hosts in order to complete their life cycle, whereas a facultative parasite is able to survive on its own under favorable conditions. Functionally, parasitic plants can be divided into **holoparasites** (completely host-dependent) and **hemiparasites** (partially host-dependent and still able to photosythesize). Important root parasite genera are *Striga* and *Orobanche* from the Orobanchaceae family, whereas *Cuscuta* spp. of the *Convolvulaceae* family belong to the shoot parasites.

Striga is even named as one of the main biotic constraints of Africa's agriculture (Spallek, Mutuku and Shirasu, 2013) and *Cuscuta* spp. are **distributed worldwide** with a wide range of hosts making them one of the most damaging parasites (Parker and Riches, 1993). The approximately 170 *Cuscuta* species have their highest species diversity in the Americas, 7-8 species occur in Chile alone (Navas, 1979), whereas only 5 species are native in central Europe (Mabberley, 1997), with *C. europaea* as the most prominent one.

The unspecific **generalist** species *C. pentagona* (*C. campestris*) is the economically most important one with a wide distribution throughout the world affecting 25 crop species in 55 countries (Lanini, 2005).

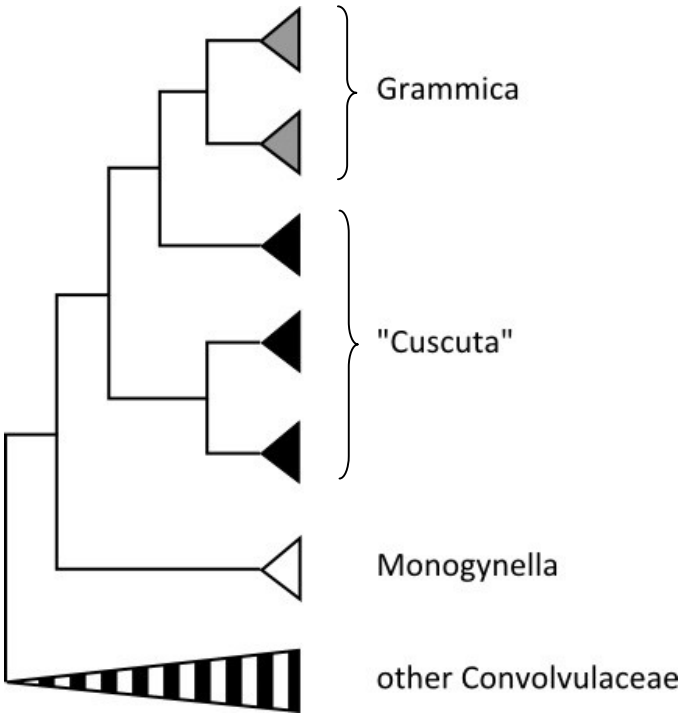

Figure 2: **Phylogeny of *Cuscuta*.** Simplified phylogenetic tree of the genus *Cuscuta* derived from separate *rbcL* (large subunit of the ribulose-bisphosphate carboxylase, plastid gene) and *nrLSU* (nuclear large ribosomal subunit gene) parsimony analyses. The genus *Cuscuta* has three subgenera, *Grammica*, *"Cuscuta"* and *Monogynella* (modified from Garcia, 2014).

As shown in figure 2, the genus *Cuscuta* includes the following three subgenera: the monophyletic group *Grammica*, its paraphyletic sister group "*Cuscuta*" and the outgroup *Monogynella* (Garcia et al., 2014), where *Cuscuta reflexa* belongs, the *Cuscuta* species this thesis is mainly focused on.

For the parasitic lifestyle chloroplasts for photosynthesis as well as organs like leaves and roots are reduced. Instead water and nutrients are taken up by various cell types of the haustorium depending on the degree and type of parasitism. Some parasites form direct links between their xylem and the host's one resulting in a continuous tube from host to parasite. The parasitic phloem is either linked to the host phloem by direct contacts to the sieve elements or just via apoplastic connections. Many parasites have evolved specialized transfer cells with an enlarged cell surface to optimize the uploading of host solutes. The protuberance formed by the parasite for solute transfer has been named **haustorium** (from Latin haurire, to drink) and defines a parasitic plant. The anatomical associations between cells and solute fluxes have been summarized by Hibberd and Jeschke in 2001, but it has been found as early as 1944 by Bennet,

that even viruses can travel between species and nowadays researchers evaluate the uses of this connection as also RNA has been found to travel between hosts and parasites (Westwood et al., 2009, Alakonya et al., 2012).

The formation, attachment and connection of the haustorium are the crucial stages of a parasite's **development**. Moreover the **germination** is also crucial: it must be timed after the emergence of potential host plants and in their vicinity. For *Striga* ssp. and *Orobanche* ssp. chemical cues have been reported, which trigger germination (Zwanenburg et al., 2009). *Cuscuta* ssp. on the other hand rely on a massive seed production in connection with a **dormant seed bank**. *Cuscuta* spp. seeds can be dormant for 10, 20 or more years (Lanini and Kogan, 2005) and continue to germinate throughout the warm season.

1.2 Cuscuta lifecycle

The annual *Cuscuta* ssp. **germinate** from small (~2mm), hard seeds. The dormancy of the seeds is broken through microbial activity, weathering or grazing and has a temperature optimum (30°C for *Cuscuta campestris*) but is independent on light (Benvenuti, Dinelli and Catizone, 2005). The seedling can grow up to 7 cm while performing a counter-clockwise rotational movement to find a host plant. A suitable host must be found within a few days for the seedling to survive. To navigate towards host plants *Cuscuta* ssp. seedlings perceive **plant volatiles** which chemically attract the young parasite (Runyon, Mescher and De Moraes, 2006). When an appropriate host stem is found, the seedling winds around it and a secondary meristem is formed in the bark of the parasite at the inner site of its coil (Heidejorgensen, 1991). The **attachment** is tightened by sticky substances secreted by the parasite (Vaughn, 2002) and also by the host (Albert, Belastegui-Macadam and Kaldenhoff, 2006). It is shown, that dodder induces the production of an arabinogalactan protein (attAGP) at the site of the attack in tomato, henceforth using the host's own machinery to attach itself (Albert, Belastegui-Macadam and Kaldenhoff, 2006). The invasion of the host is driven by mechanical pressure of the growing haustorium, helped by hydrolytic enzymes partly occurring in loosening particles (Vaughn, 2003).

Figure 3: **Pathway via which *Cuscuta* contacts its host and accesses host solutes.** The host sieve elements (HSE) of the phloem are lined by haustorial transfer cells (HauTC) of the parasite, which then allow unloading of host phloem solutes into the parasite haustorium. CC: companion cell, PAR: parenchyma (Hibberd and Jeschke, 2001).

The haustorium opens a fissure and grows towards the vascular bundle of the host; searching hyphae extent to the phloem and xylem cells and form chimeric cell walls with the host cells, which are connected through **plasmodesmata** (Vaughn, 2003). In the fully established connection, a host phloem cell is wrapped by a parasitic cell, which functions as a transfer cell and sieve element with a high cell surface and the xylem tubes of host and parasite are fused, forming a continuum (Bennet, 1944). Nevertheless *Cuscuta* spp. are **phloem feeders**, which take up almost 100% of their carbon from the phloem (Hibbert et al., 1999). The host sieve elements of the phloem are accompanied by haustorial transfer cells of the parasite allowing the upload of solutes into the parasite haustorium (figure 3, Hibbert and Jeschke, 2001). Only under developmental stress conditions *Cuscuta* ssp. are reported to develop xylem transfer cells (Christensen et al., 2003). The additional **sink** created by the parasite induces the host to increase its photosynthesis rate (Hibbert et al., 1999; Jeschke et al., 1994). *Cuscuta* spp.

themselves have no or only a reduced amount of **chlorophyll** and only a few species are capable of a limited amount of photosynthesis (Hibbert et al., 1998). These are designated 'cryptically photosynthetic' and it is suggested that only the fixation of CO_2 lost from respiration is enabled so that they are still holoparasites fully dependent on the host. All water, nutrients and carbohydrates are supplied by the host through the haustorium. Is the first connection successfully established, the parasite grows further, attaching itself to the same or neighbouring plants, with a growth rate of about 7 cm per day (Lanini and Kogan, 2005) and one plant can cover 3 m^2 in a growing season. If enough suitable hosts grow nearby, a dense vegetative mat can be formed. The **flowering** occurs from late spring to autumn, *Cuscuta pentagona* for example needs at least 60 days after the initial attachment to produce viable seeds (Lanini and Kogan, 2005). Seeds in the soil can remain dormant for many years, forming a seed bank and posing an enduring threat to agriculture.

1.3 *Cuscuta* spp. and their interaction with resistant plants

Even though many *Cuscuta* species cannot infect monocotyledons this seems to be due to anatomical reasons or undetectable signals (Dawson et al., 1994) rather than to an active defense.

Figure 4: **Unsuccessful infection of *S. lycopersicum* by *C. reflexa*.** After ~5 weeks of infection the *C. reflexa* vine dries out, necrotic spots are visible at the sites of haustorial formation.

Nevertheless a few plants exist which show a **specific defense response**: the *Malvaceaes Gossypium hirusutum* and *Hibiscus rosa-sinensis* block the access to the vascular bundles by a kind of wounding tissue (Capderon, Fer and Ozenda, 1985). Tomato (*Solanum lycopersicum)* shows an active defense at an even earlier stage (Ihl et al., 1988; Albert, Belastegui-Macadam and Kaldenhoff, 2006). 3-5 days after the initial contact, at the end of the attachment phase, the tomato cells in the contact area elongate and cells in the hypoderm and cortex divide. Hence the cell walls are strengthened by the incorporation of phenolic and aliphatic compounds reminding of wound suberin (Bernards, 2002). The parasite's haustoria are unable to penetrate this **barrier**, not even the epidermis is pierced. Macroscopically visible necrotic plaques appear at the tomato stem (Ihl et al., 1988; Sahm et al., 1995, see also figure 4). Around 14 days after the first contact, a *C. reflexa* vine begins to die off, probably due to starvation. As other *Cuscuta* species are able to infect tomato successfully, this reaction does not seem to be a simple wounding reaction, but a specific defense. This reaction is termed

hypersensitive-like response (HLR) and is similar to the hypersensitive response (HR) to pathogens, which is described for both monocotyledonous and dicotyledonous plants as a localized cell death, inhibiting the spread of biotrophic pathogens, but in some cases even facilitating the growth of necrotrophic pathogens (Ramachandran et al., 2017, Faris et al., 2010, Lorang, Sweat and Wolpert, 2007, Coll, Epple and Dangl, 2011). The difference of the HLR to the well-described HR however is a far more complex pattern of events, with cells that are first dividing, then ligninifying, forming a several cell deep, hardened layer of woody tissue and is quite unlike a simple necrosis (figure 5).

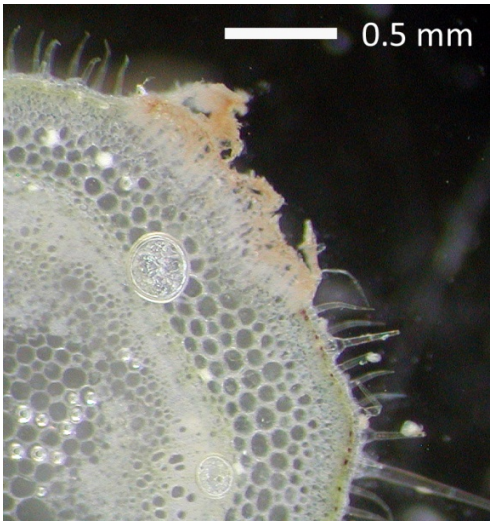

Figure 5: **HLR response of tomato**. This is a cross-section of a tomato stem after 15 days of unsuccessful infection with *Cuscuta reflexa*. The cells in the upper layer at the infection site have undergone cell division and ligninification to form a mechanical barrier.

As stated earlier, *Cuscuta pentagona* induces both SA and JA increase but without triggering an effective defense from the tomato side (Runyon, Mescher and De Moraes, 2010). It was also investigated, that the effectiveness of tomato's defense to *C. pentagona* varies with the age of the host and that also trichomes play a role in the plants' defense (Runyon, Mescher and De Moraes, 2010). Whether this is due to a more sophisticated perception mechanism for *C. reflexa* compounds or whether *C. pentagona* and other *Cuscuta* species have a suppressor for plant defense which lacks in *C. reflexa* is yet unknown. The perception of *Cuscuta* compounds by tomato will be investigated within this thesis.

1.4 Controlling Cuscuta spp. infections

Avoidance, control by mechanical means, hand removal, resistant varieties and herbicides are the methods currently used to control dodder growth and outspread. None of these strategies work for 100%, so that an **integrative approach** is needed to manage this pest. *Cuscuta* ssp. attack is unspecific and takes often place simultaneously on a wide range of hosts. Seeds are involuntarily spread by men in contaminated equipment, shoes, tires and machines as well as international seed commerce. Dodder seeds are known to be a **contaminant** of flax, alfalfa, linseed and niger seeds (Parker and Riches, 1993). Avoiding using seeds of contaminated fields or the use of seeds certified free of dodder can prevent new infestations. Also grazing animals can spread dodder seeds, as dodder seeds can survive the digestive system of many domestic animals (Gaertner, 1950).

The parasite rarely kills its host, but the infected plants are more susceptible towards other threats like fungi, bacteria and oomycetes. Furthermore the **yield** can be drastically **reduced** as *Cuscuta* ssp. poses a much stronger sink than fruits or other sink tissues than the infested host (Hibberd and Jeschke, 2001; Lanini, 2004).

So far, **avoidance** and **prevention** have been the most effective methods to reduce dodder infestations (Parker and Riches, 1993). Cleaning of equipment after working in an infested field and not permitting grazing animals to move from infested fields to dodder-free fields can prevent spreading. Weed control in and around fields can be useful to reduce the amount of suitable hosts. A delayed planting of crops after the emergence of *Cuscuta* seedlings can also reduce the infestation (Dawson, 1987).

A **rotation** of crops can effectively reduce *Cuscuta* spp. infestations. As *Cuscuta* ssp. are unable to persist on grasses, a period of 2 or more years of cereals and corn can reduce the *Cuscuta* infestation in the following years, if the field is properly weeded (Lanini, 2004).

Hand crews are a viable but expensive method to remove dodder from infested fields. Usually 10% of the dodder is missed (Lanini, 2004) and a crew needs to return 15-21 days to weed the remaining dodder and prevent seed shed. Also, this is only possible in minor infestations, when

infestations are detected early; in large-scale the costs of workers and the damage of crops become rampant.

Chemical controls with herbicides have been successful in certain crops, especially pre-emergence and on seedlings. Thiazopyr, for example, stunts dodder seedlings, the emergence is delayed and the circumnutation of the seedling is disabled, so that host attachment is prevented. Post-attachment the control is rather difficult. The host is usually also affected by herbicides in the concentrations which affect dodder. Nevertheless usage of contact herbicides which destroy both host and parasite or even burning is recommended to prevent seed set (Cudney, Orloff and Reints, 1992).

The use of **herbicide resistant crops** has not been shown to be promising, as the resistance protein PAT (phosphinothricin acetyl transferase) which confers the resistance against glufosinate can also travel from host to parasite (Jiang et al., 2013). More promising are transgenic plants, expressing specifically designed small RNAs. The phloem-specific **RNAi**-constructs for SHOOT MERISTEMLESS-like (STM), a gene of essential role in parasitic haustoria development has been expressed in host plants, moved to the attached parasite *C. pentagona* and inhibited haustorium development leading to reduced infection (Alakonya et al., 2012). The investigation of naturally resistant plants and the transfer of these resistances may open future prospects.

1.5 Introduction to innate immunity

Both animals and plants have an innate immune system in common. However, plants lack an adaptive immune system as humans have (Medzhitov and Janeway Jr., 2002; Abramovic, Anderson and Martin, 2006). How can they survive in an everlasting environmental change, even without means to move away from biotic and abiotic threats? They seem to be even more endangered than animals: with their sessile lifestyle, they cannot avoid abiotic stresses such as draught, floods, salt, a lack of nutrients, heat, cold, an excess of sun or the lack of it.

It seems that a plant is much dependent on luck: wherever a seed lands, it must take its chance. Moreover there are all the biotic stresses: herbivores such as grazers and insects, nematodes,

microbes (bacteria, fungi and oomycetes) and finally the stress that plants cause to each other: competition and parasitism. So a casual observer may ask the question: Why is the world still green (Wilkinson and Sherratt, 2016)?

Plants have evolved highly efficient mechanisms to counteract the microbial pathogens they are exposed to. In general, they are resistant through **passive protection** like waxy cuticular layers and pre-formed anti-microbial compounds.

To circumvent these mechanisms pathogenic microbes found entry through wounds, pores and stomata or by active penetration through the leaf or root surface. Yet the plant's arsenal is not at its end, even when the pathogen is already inside the host: Plant cell walls and antimicrobial secondary metabolites represent permanent barriers. Furthermore, an array of **inducible defense systems** is awaiting the penetrator when a threat is detected by pattern recognition receptors (**PRRs**) that recognize microbe/pathogen-associated molecular patterns (**MAMPs/PAMPs**). Most PRRs reside in the plasmamembrane and bind to MAMPs with their extracellular domains, but there are also some located to the cytosol (Spartz and Gray, 2008, Kakimoto, 2003, Benavente and Alonso, 2006, Chow and McCourt, 2006). The MAMP-binding leads to conformational changes in the receptor and an intracellular signaling to induce defense reactions.

These processes are defined as PAMP-triggered immunity (**PTI**) (Chisholm et al., 2006, Jones and Dangl, 2006).

The multiple cellular responses induced by MAMP/PAMP recognition include altered ion fluxes across the plasma membrane causing extracellular alkalinization and increased concentrations of the cytoplasmatic Ca^{2+} (Boller and Felix, 2009) and synthesis of the stress hormone ethylene caused by the fast activation of the ACC-synthase (Spanu et al., 1994). Additionally, the mitogen-activated protein kinase (MAPK) cascades are induced, causing the transcriptional activation of transcription factors (Nühse et al., 2000, Asai et al., 2002). Thus the transcription of defense related genes encoding defensins (antimicrobial proteins), lytic enzymes or enzymes for the synthesis of phytoalexins (anti-microbial secondary metabolites) is activated (Nürnberger et al., 2004).

Further typical plant defense reactions include the deposition of callose at the cell wall and the production of reactive oxygen species (ROS). This so-called oxidative burst has a direct toxic effect on pathogens and also causes cross-linking of the cell wall (Apel and Hirt, 2004).

Some pathogens have evolved **effectors** to counteract PTI, which suppress the defense response. This is called effector-triggered susceptibility (ETS). To circumvent this ETS some plants have specialized R (resistant) proteins which recognize these effectors. The recognition of these R proteins causes effector-triggered immunity (ETI), which generally leads to a hypersensitive response (HR) (Dangl and Holub, 1997) meaning that a cell death of the surrounding tissue of the infection site limits the growth of the pathogen (Chisholm et al., 2006, Jones and Dangl, 2006, Caplan, Padmanabhan and Dinesh-Kumar, 2008).

R proteins often belong to the NB-LRR protein family which consists of a nucleotide binding site (NB) and a leucine-rich repeat (LRR) domain. They interact directly with effectors via their LRR domains or indirectly, mediated by an additional protein (host factor) which binds the pathogen effector.

Defense responses towards parasitic plants are not as well characterized, although first studies show a sequential induction of jasmonic acid (JA) and salicylic acid (SA) resulting in a **HLR** (hypersensitive-like response) in tomato (Runyon, Mescher and De Moraes 2010) attacked by *Cuscuta pentagona*. As tomato is a compatible host for *C. pentagona* further studies with incompatible parasite-host interactions are of interest. The classical HR is a rapid programmed cell death, often microscopic and localized at the site of recognition limiting the process of pathogen spread and to be distinguished from disease-associated cell death which is macroscopic and simply a disease symptom (Abramovic, Anderson and Martin, 2006). The hypersensitive-like response of tomato to dodder goes beyond the HR, as cells are not simply dying, but first proliferating and then lignifying resulting in a mechanical barrier (Gertz, 1915).

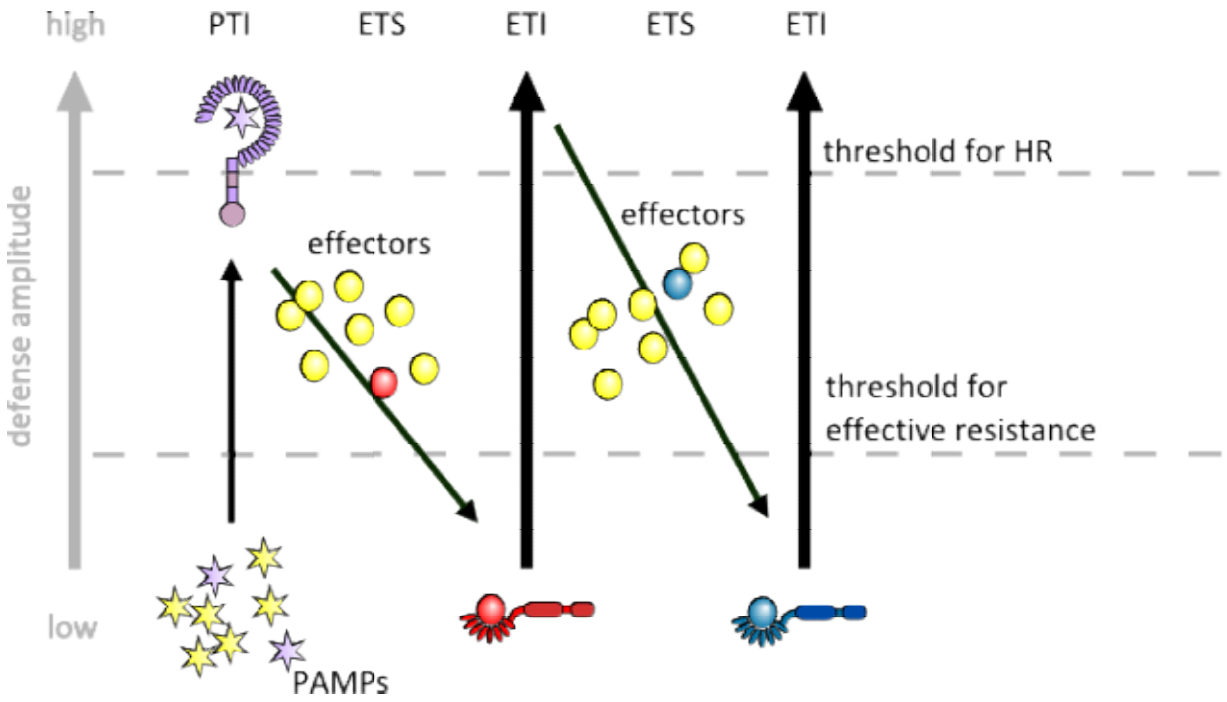

Figure 6: **Zig-zag model illustrating the quantitative output of the plant immune system.** In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI]. In the initial phase, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, stars) via PRRs which triggers PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favors new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI (modified from Jones and Dangl, 2006).

For the co-evolution of innate immunity in plants, MAMPs being recognized by PRRs, masked by pathogen derived effectors and their recognition by other receptors Jones and Dangl published in 2006 a well-known model, the zig-zag model (see figure 6). As microbes have little in common, or rather have easily distinguishable features from plants, their recognition by the plant immune system does not appear as miraculous as the recognition of other (parasitic) plants. Nevertheless, some plants have evolved mechanisms to counteract such threats. In this thesis is investigated, whether the recognition of parasitic plants is working in an analogous manner to the recognition of MAMPs. The postulated patterns derived from parasitic plants we named parAMPs, for parasite associated molecular patterns (see figure 7). So far, other yet uninvestigated signals like pressure changes or redox status of molecules like glutathione (Dron

et al., 1988, Wingate, Lawton and Lamb 1988) and NADPH (Noctor and Mhamdi, 2014) are not to be excluded.

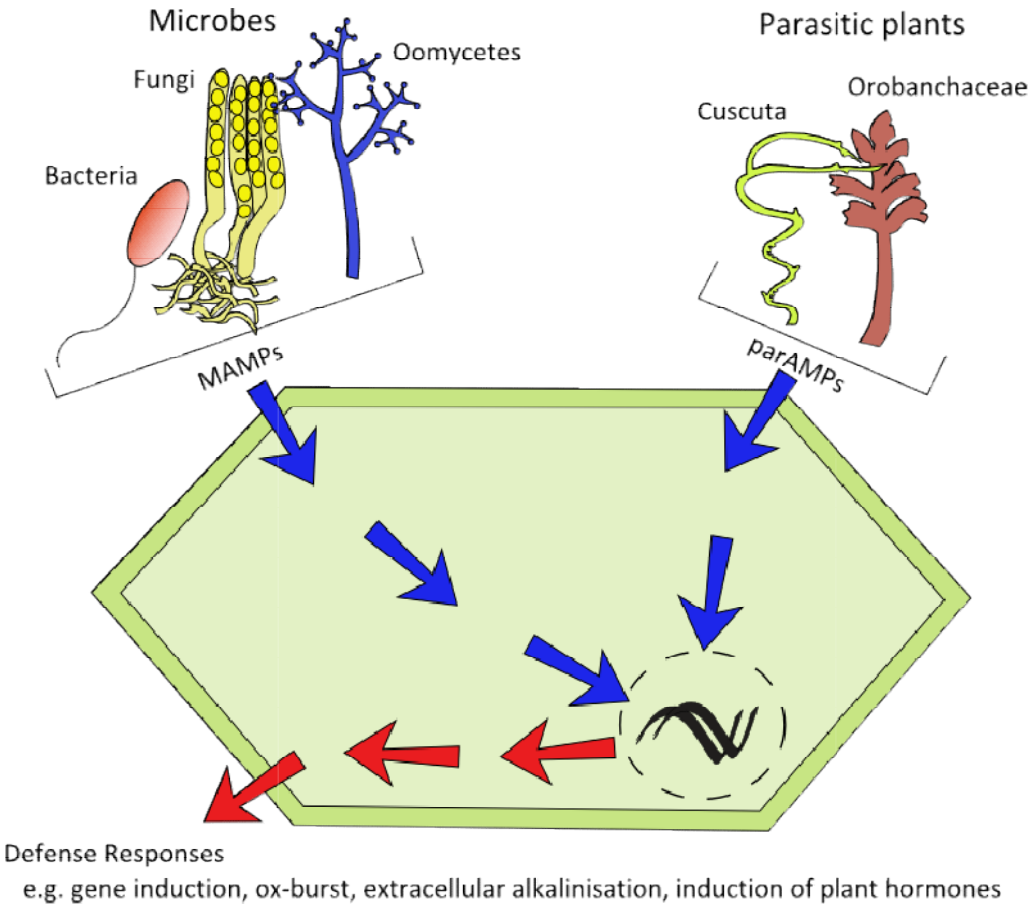

Figure 7: **Plant cell with stresses triggering defense responses.** Elicitors originating from microbes (MAMPs, microbe associated molecular patterns) or parasitic plants (parAMPs, parasite associated molecular patterns) are perceived and the signal is transduced into the nucleus, where defense responses are triggered.

1.6 Aims of this thesis

For further understanding and hence better control of parasitic plants it is necessary to study their molecular interaction with their hosts in detail.

Immune responses of plants attacked by other plants are not yet investigated as well as those to microbial attacks. There are a lot of questions still unaccounted for: Are they similar to responses against microbial threats? As plants are much more similar to other plants than to microbes, can there be such a thing, as a parasite specific pattern recognized by the plant? For microbes the term **MAMP** standing for microbe-associated molecular pattern is established, but is it possible, that parasitic plants are recognized through **parAMP**s – parasite associated molecular patterns? Or are they just perceived indirectly, for example through **DAMP**s (damage associated molecular patterns)? If molecules of parasites are perceived, what are their characteristics? Is this parAMP purifiable and identifiable by bioanalytical methods? And what kind of perception system is used? Are there receptors in tomato which specifically recognize molecular pattern specific for *Cuscuta reflexa* or *Cuscuta* species in general? If there is such a general *Cuscuta* motif, then why is tomato resistant only to *Cuscuta reflexa* but not to other *Cuscuta* species? If genes can be identified which are responsible for the resistance of tomato, is it possible to confer these to other plant species to render them more resistant against *Cuscuta reflexa* as well? Some of these questions will be answered throughout this thesis.

2. Material and Methods

2.1 Elicitors

Table 1: Names, amino acid sequence and origin of used elicitors

Name	AA sequence (if available)	Origin
flg22 (flagellin22)	QRLSTGSRINSAKDDAAGLQIA	Peptide from flagellum of *Pseudomonas* ssp.
PEN1 (pen1)		Extract from *penicillium crysogenum*
GP8 (glycopeptide)		Glycopeptide from yeast invertase
CuF		Extract from *Cuscuta reflexa,* also: Elicitor from *C. reflexa*
Cus-juice		Direct juice from *Cuscuta reflexa*

2.2 Organisms

Solanum lycopersicum cv. Moneymaker and cv. M82, and the wild tomato species ***S. cheesmaniae***, ***S. chilense***, ***S. chmielewskii***, ***S. lycopersicum var. cerasiforme***, ***S. hirsutum***, ***S. hirsutum f. glabratum***, ***S. parviflorum***, ***S. pennellii***, ***S. peruvianum***, ***S. peruvianum f. glandulosum***, ***S. pimpinellifolium***, ***S. juglandifolium***, ***S. lycopersicoides***, ***S. ochranthum*** and ***S. sitiens*** as well as **introgression lines** of ***S. lycopersicum cv. M82 x S. pennellii*** were grown in the greenhouse at 22°C and 60% relative humidity with the light rhythm of 16 h light, 8 h darkness. After germination, the seedlings were transferred to single pots of 10 cm diameter. For ethylene measurement, 4-5 weeks old plants were used. Older plants used for infection studies were transferred into bigger pots at the age of 5-6 weeks. Seeds for introgression lines, the M82 cultivar, the wild tomato relatives and the introgression lines were provided by the "Tomato Genetics Resource Center" (TGRC), UC Davis (USA).

The genome of the tomato has been sequenced by an international consortium, the sol genomics network (SGN) and is available online (http://solgenomics.net/).

Additionally, introgression lines, which are lines of cultivated tomato (in this case cv. M82) with introgressed wild-species segments in the genome (here from *S. pennellii*), were used. These

were obtained from the TGRC and developed by Esched and Zamir, 1995. Furthermore, fine mapping lines (e.g. IL 8-1-1) from the same source were used.

Cuscuta reflexa was cultivated vegetatively on *Coleus blumei* under the same conditions as tomato.

Nicotiana benthamiana, Nicotiana tabacum, Nicotiana plumbaginifolia, Solanum tuberosum and ***Capsucum annuum*** were grown under the same conditions as tomato.

Cuscuta europaea was harvested from plants growing in the wild in Germany.

Cuscuta australis was harvested from plants growing in the wild in Po'Cheon, South Korea.

Cuscuta parasitica was harvested from plants growing in the wild in Karaj, Iran.

Cuscuta monogyna was harvested from plants growing in the wild in Karaj, Iran.

Cuscuta pentagona was provided by the collaboration partners N. Sinha and M. Farhi from California, USA.

Rhinanthus alectorolophus was harvested in the wild in Tübingen, Germany.

2.3 Hardware

Table 2: Used hardware

hardware	**producer**
centrifuge 5415 R	Eppendorf
gas chromatograph C-R4AX	Shimadzu
HPLC Äkta micro 900	Äkta
FPLC Äkta explorer	Äkta
Shaker	Infors AG
shaker (overhead) INTELLI MIXER	neoLab
vacuum pump CVC 3000	vacuubrand
water bath 1083	GFL
Synapt G2 LC/MS system with elektrospray ionization	Waters Acquity UPLC
EASY-nLC II system	Proxeom Biosystems
LTQ Orbitrap Elite XL mass spectrometer	Thermo

Columns:

CEC polypropelene column, GE, SP Sepharose ff ~50ml bed volume, self-made

C18-precolumn, MACHERY-NAGEL, Chromabond C 18 ec

1st C18-column: Zorbax column, Zorbax Rx-C18 4.6mm x 25cm

2nd C18-column: PerkinElmer, AQUAPORE OD-300 µ220 x 4.66mm

LC-MS-column: Acquity HSS T3, 2.1 x 100 mm, 1.8 µm, C18-rp

2.4 Biological Assays

2.4.1 Ethylene-Assay

Leaves of 4-5 weeks old *S. lycopersicum* were cut in square pieces of about 4 x 4 mm and floated on water in petri dishes overnight. For bioassays, 3 leave pieces were carefully transferred into a glass reaction tube (6 ml volume) containing 0.5 ml water. Usually 3 replicates were made for each treatment. After addition of test substances and elicitors, the tubes were sealed with a rubber plug and incubated for 3 h on an orbitary shaker at room temperature. 1 ml samples of the gas phase were analyzed for ethylene content by gas chromatography (Felix et al.,1991).

2.4.2 Reactive-Oxygen-Species Assay

One of the strategies of plants for the defense against pathogens is the production of reactive oxygen species (ROS) (Lamb and Dixon, 1997). ROS include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxide radicals ($OH^{\bullet}$).

The induction of the fast defense responses can be observed in cell culture or leaf pieces within 5 min post treatments with elicitors. Released H_2O_2 oxidizes luminol, thus emitting chemical luminescence. This can be measured via photometer.

100 µl of watery solution with 20 µM luminol and ~10ng/ml peroxidase (from horseradish/ Aplichem™) were filled in each tube of a 96-well microtiter plate. One leave piece was placed on the surface. After application of elicitors/ controls, light emission in RLU (relative light units) was measured in a luminometer suitable for 96-well plates (Mithras, LB 940, Berthold Technologies Germany).

2.5 Purification of CuF (Cuscuta Factor)

2.5.1 Extraction

For all purification attempts *C. reflexa* plant material was used, that was aggrandized vegetatively on *Coleus blumei* host plants in the green house.

Grinding of fresh *Cuscuta* tissue resulted in a juice which showed activity as inducer of ethylene biosynthesis in tomato leaves. In initial experiments various extraction procedures and solvent conditions were tested to optimize extraction of activity and reduce complexity of the extract. A standard method was established to extract the heat stable *Cuscuta* Factor using acid extraction conditions.

Acidic CuF-extraction

1. 10 g of freeze-dried *Cuscuta*-material was filled into a 500 ml bottle.
2. 500 ml of 12 mM HCl was added.
3. The suspension was incubated at 60°C overnight with occasional shaking.
4. Thereafter it was vacuum-filtrated through a paper filter via a büchner funnel to remove big plant parts.
5. The extract was heated for 10 min at 95°C in a water bath.
6. MES-Powder was added to a final concentration of 50 mM and the pH was adjusted to 5.5 with KOH.
7. Vacuum-filtration through a 0.22 µm pore diameter nitrocellulose membrane was performed.

The resulting extract is henceforth called crude extract and was used as starting material for all purifications.

2.5.2 Cation exchange chromatography

The crude extract was then pre-purified with Cation Exchange Chromatography (CEC). Cation Exchange columns consist of a negatively charged resin which can bind positively charged molecules. The strength of the binding is according to the molecule's net surface charge. The higher the charge, the stronger is the binding. However, the net surface charge of a protein changes according to the pH. A protein has no charge when the pH is equal to the protein's isoelectric point or pI. Is the pH lower than the pI of the protein, the protein will be charged positive. If the buffer pH is higher than the protein's pI, the charge will be negative. The pI of a protein is determined by its amino acid structure. Therefore, various proteins bind to an ion exchange resin with different strength at a given pH. This can be used as criterion for their separation.

Is the working pH of the used buffer at pH 5.5 all proteins with pI >5.5 will be positively charged and bind to the negatively charged resin. When a salt gradient is applied, then the proteins with a pI close to 5.5 will elute with a low salt concentration whereas proteins with a higher pI will elute with high ionic strength.

Workflow

1. Buffer preparation

   All buffers were prepared and the pH was adjusted after the addition of salt, as the salt concentration is influencing the pH. The loading buffer MES with a concentration of 50 mM was used, which was adjusted to pH 5.5 with KOH. For the elution, 50 mM MES buffer with various salt concentrations of KCl was used. For washing and regeneration of the column 2 M KCl was prepared. All buffers were filtrated sterile with a 0.22 µm pore diameter nitrocellulose membrane.

2. Equilibration

   The column was first washed with deionized water then equilibrated with buffer A until the pH reached 5.5.

3. Sample loading

   The sample was loaded using at a flow rate of about 1 ml/ min.

4. Washing

   The column was washed with Buffer A for at least 2 column volumes.

5. Elution

   A step gradient of 100 mM steps from 100 mM to 600 mM was applied to elute the active components.

6. Regeneration and storage

   The column was regenerated and washed with 2 M KCl and stored in 20% Ethanol.

The CEC was used as a pre-purification step. 200 ml of *C. reflexa* crude extract was loaded in each run. In total 12 runs were performed. The Elution was collected in 10 ml fractions. The fractions were tested for their biological activity and conductivity. Under the assumption that similar components elute at a similar ionic strength and have therefore the same conductivity, fractions in a certain range of conductivity were pooled and further purified via C18 columns.

2.5.3 Reversed-Phase-Chromatography with C18 resin

Reversed-Phase-Chromatography is a powerful tool to separate biochemical compounds. The term reversed-phase has the historical background, that in the beginnings of liquid chromatography unmodified silica or alumina resins binding hydrophilic compounds were used, which was called 'normal phase chromatography'. In opposition to that the reversed phase uses a modified silica resin with a covalently bound alkyl chain binding hydrophobic compounds. While hydrophilic compounds pass together with the watery solvent through the resin, the bound hydrophobic compounds can be differentially eluded by decreasing the polarity of the solvent i.e. adding an organic solvent. Molecules of high polarity will elute first, then, with increasing concentration of the organic solvent the more unpolar molecules will elute.

The use of HPLC (high performance liquid chromatography) makes liquid chromatography highly reproducible and increases the resolution. Pumps are used to pass the solvents and the sample through the resin-filled column (figure 8).

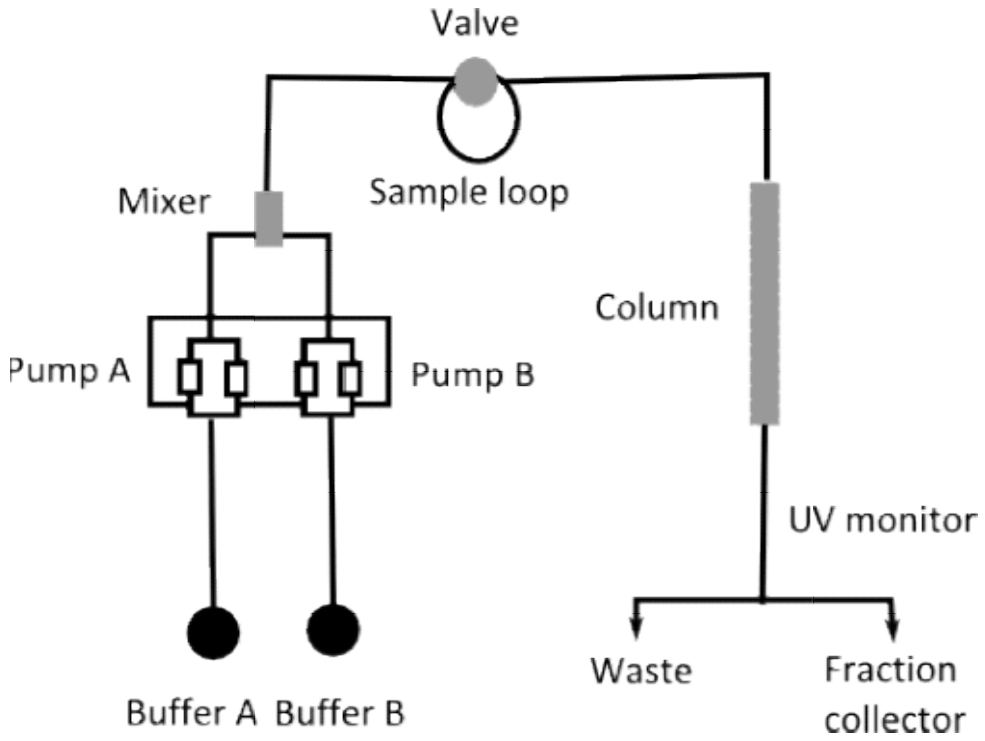

Figure 8: **Flow diagram of HPLC.** The computerized Äkta system which was used in this thesis runs via a program described in the Äkta System Manual. The ratio of the Buffers A and B are controlled by the velocity of the pumps A and B to achieve a gradient. Through the sample loop, the sample is loaded onto the column. The fraction size etc. of the fraction collector is also controlled via the program.

2.5.3.1 C18 Pre-column

For the concentration, desalting and further pre-purification, a bench-top C18 pre-column was used before applying HPLC. Here, components which are sticking irreversibly to the C18 resin can bind, in order to preserve the HPLC column. The C18ec column of Macherey-Nagel was performed with manual pressure and a flow rate of ~2 ml/ min. The column was pre-wet with 100 % acetonitrile, then equilibrated with 20 mM ammonium acetate. Up to 300 ml of the collected CEC fractions were applied as sample. After a washing step with 20 mM ammonium acetate, a stepwise elution was performed, first with 5 % acetonitrile, 20 mM ammonium acetate, then with 25 % acetonitrile, 20 mM ammonium acetate. The collected elution fractions were tested for their biological activity in the ethylene assay. Afterwards the most active fractions were concentrated by speed-vac. The dried pellets were dissolved in 1 ml 20 mM ammonium acetate and subjected to the HPLC.

2.5.3.2 HPLC 1st run

The column was equilibrated with 80 % acetonitrile (buffer B), then equilibrated with 20 mM ammonium acetate (buffer A). The OD (optical density) was measured by a UV-light detector at 215 and 280 nm. The sample was loaded into the sample loop then injected with 0.2 ml/ min. After a few minutes of washing with buffer A, a gradient from 0 % B to 31.25 % B (20% acetonitrile) in 60 min was performed. The flowthrough, the wash and the elutions were collected in 300 µl fractions for 60 min in a 96-well-plate. 1 µl of each fraction was tested in the ethylene assay. Instead of water, 5 mM MES was used for the glass tubes in the ethylene measurement, because acidity alone from the buffer can also induce ethylene production. The most active fractions were dried in the speed-vac, dissolved in 0.1 % formic acid, pooled and subjected to a 2^{nd} HPLC run under altered conditions. The column was washed with 80 % acetonitrile and stored in 50 % acetonitrile.

2.5.3.3 C18 HPLC 2nd run

The alteration of conditions can lead to a further purification, because the elicitor and the non-active components, which eluted at the same point in the 1^{st} HPLC run, may elute at different time points in a 2^{nd} run under different conditions. Therefore the active fraction 15 of HPLC 1st run (~40 mS CEC fractions) was loaded to another C18-column (PerkinElmer) and a different pH was used for further purification. The OD was measured with a UV-light detector at 195, 215 and 280 nm. The column was equilibrated with 80 % acetonitrile then equilibrated with 0.1 % formic acid. The sample was loaded onto the column via injection loop. Flowthrough, wash and elution were collected in a 96-well-plate by a fraction collector in 250 µl fractions. This time a shallower gradient from 0-20 % buffer B in 150 min at a flowrate of 0.1 ml/ min was used for a better resolution. The column was washed with 80 % acetonitrile and stored in 50 % acetonitrile. Afterwards 1 µl of all fractions were tested in the ethylene assay (single measurements).

2.5.3.4 LC-MS

In a mass spectrometer a sample is ionized and accelerated by electric or magnetic fields and deflected according to their mass-to-charge ratio. Ions with the same mass-to-charge ratio will be deflected to the same amount. After this process the ionized particles are detected by an electron multiplier. A result is a spectrum of the relative abundance of detected ions as a function of the mass-to-charge ratio. The atoms or molecules in the sample can be identified by correlating known masses (e.g. a specific amino acid) to the identified masses or through a characteristic fragmentation pattern if available.

A) Instead or in addition to the 2nd HPLC run, an LC-MS was performed under the same conditions as described in 2.5.3.3. The run, LC/MS with a Waters Acquitiy UPLC - Synapt G2 LC/MS system with electrospray ionization, was performed by Mark Stahl, ZMBP, central facilities. For separation a Waters Acquity HSST3 2.1 x 100mm, 1.8 µm column was used and the mass spectrometer was operated in ESI positive MS and MSE mode.

B) Also the Proteome Center Tübingen, analyzed samples by LTQ-Orbitrap XL mass spectrometer in the group of Prof. Boris Maček by Johannes Madlung with the following specifications:

60 minTop15HCD: NanoLC-ESI-MS/MS

An EASY-nLC II system (Proxeon Biosystems) coupled to an LTQ Orbitrap Elite (Thermo) was used for the LC-MS/MS measurements. Peptides were separated on a 15 cm PicoTip fused silica emitter ID 75 µm (New Objective) packed inhouse using reversed-phase beads ReproSil-Pur C18-AQ 3 µm (Dr. Maisch GmbH). Peptides were injected with Solvent A (0.5 % acetic acid) at a maximum pressure of 280 bar and separated at 200 nL/min. Separation was performed using a 57 min segmented gradient of 5-50 % solvent B (80 % ACN in 0.5 % acetic acid).

LTQ Orbitrap Elite was operated in the positive ion mode. Precursor ions were recorded in the Orbitrap mass analyzer at a resolution of 120,000 followed by MS/MS spectra acquisition. The 15 most intense precursor ions from the full scan were sequentially fragmented. High resolution HCD MS/MS spectra were acquired with a resolution of 15,000 and a target value of 40,000. The normalized collision energy was set to 35,

activation time to 0.1 ms and the first mass to 120 Th. Fragmented masses were excluded for 60 s after MS/MS. The target values were 1E6 charges for the MS scans in the Orbitrap and 5000 charges for the MS/MS scans with a maximum fill time of 100 ms and 150 ms respectively.

2.6 Characterization of Cuscuta Factor (CuF)

2.6.1 De-O-glycosylation

Purified *C. reflexa* parAMP was de-O-glycosylated using chemical deglycosylation as described by Rademaker et al., 1998. They showed that a β-Elimination with NH_4OH removed O-linked glycans, but not N-linked glycans, and the peptide backbone is degraded to a much lesser extent, so that it is still detectable after 16 h of treatment using an amount of 10 pmol starting material. When 100 pmol starting material was used, the obtained ESI-MS spectrum had an exellent quality. Therefore 20 µl of purified *C. reflexa* parAMP was incubated with 180 µl of 25% (w/v) NH_4OH, at 45°C for 16 h or MES buffer respectively. As a control a GP (glycopeptide) preparation from yeast invertase was used (Basse and Boller, 1992, Basse, Fath and Boller, 1993). As GP is an N-liked glycan, it should not be degraded by this method. The reaction was stopped by speed-vac. After the pellet was re-dissolved, the biological activity was measured via ethylene measurement. Both, the treated and the untreated sample were analyzed by mass spectrometry in the proteome center.

2.6.2 a. Tricine-SDS PAGE (polyacrylamide gel electrophoresis)

For the determination and visualization of the molecular size of CuF, tricine-SDS PAGE was used. Tricine-SDS PAGE allows a higher resolution in the low molecular weight range than normal SDS-PAGE. A 14% separating gel was casted and after polymerization, a 5% stacking gel was poured on top of it (using the ingredients below). For determination of the molecular size PageRuler PrestainedTM (Thermo Fisher, Fermentas) was used as a marker. The peptides were separated with an initial voltage of 30 V, then 200 V in the second step ending with a final voltage of 300 V.

Table 3: Composition of acrylamid gel

		5% stacking gel	**14% separating gel**
30 % Acryl/Bisacryl	(ml)	1.67	14
Gel buffer 3x	(ml)	2.5	10
Glycerol	(g)		3.1
Add water to final volume	(ml)	5.78	4.8
Polymerize by adding:			
APS (10%)	(µl)	50	100
TEMED	(µl)	20	40

On the gel, the CuF preparations utrafiltered below 3 kDa and above 3 kDa were applied twice in equal amounts right and left of the marker. After SDS PAGE two of these lanes were cut-off and only the remaining part of the gel was stained with silver nitrate (see fig. 17, left, in chapter 3.2.4). The other two lanes were cut into pieces of 1 mm width. They were then incubated separately in reaction tubes with 100 µl of water at 4°C overnight. During this incubation time some active components diffused into the water. Of each tube 1 µl was used for ethylene measurement on tomato leaf pieces (see fig. 17, right, in chapter 3.2.4).

2.6.2 b. Staining with silver nitrate

For staining SDS gels with silver nitrate, a protocol from Gromova et al., 2006 was used. After electrophoresis the gel was fixated overnight in fixation solution, then washed 3 times in 20%

ethanol for 20 minutes, then shook gently for 2 minutes in senzitizing solution. After washing two times for 1 minute with desalted water, the gel was rotated with cold silver staining solution for 20 minutes. After another two times of 1 minute washing with desalted water, the gels were incubated in developing solution until the bands became clearly visible ~2 minutes. Then the development was stopped by adding terminating solution. The ingredients of the solutions are noted below.

Fixation solution: 50% ethanol, 12% acetic acid, 0.05% formalin

Sensitizing solution: 0.02% (w/v) sodium thiosulfate ($Na_2S_2O_3$)

Staining: 0.2% (w/v) silver nitrate ($AgNO_3$), 0.076% formalin

Developing solution: 6% (w/v) sodium carbonate (Na_2CO_3), 0.0004% (w/v) sodium

Thiosulfate ($Na_2S_2O_3$), 0.05% formalin

Terminating solution: 12% acetic acid

2.6.3 Isoelectric focusing (IEF)

The isoelectric focussing was done in Immobiline™ DryStrips from GE Healthcare with an immobilized pH-gradient (IPG). The IPG DryStrips are rehydrated and focused in individual strip holders. A strip from pH 3 to pH 11 of 24 cm length was used. A strip holder is made up from a narrow thermally conductive ceramics tray with built-in platinum electrodes and a transparent lid. The sample is pipetted into the tray, the IPG strip is laid gel side down onto the solution and overlaid with 200 to 300 µl paraffin oil. The strip holders are placed onto the IPGphor platform, with the acidic side of the IPG strip on the anodal area, the basic side on the cathodal area. A scheme of IEF is displayed in figure 9.

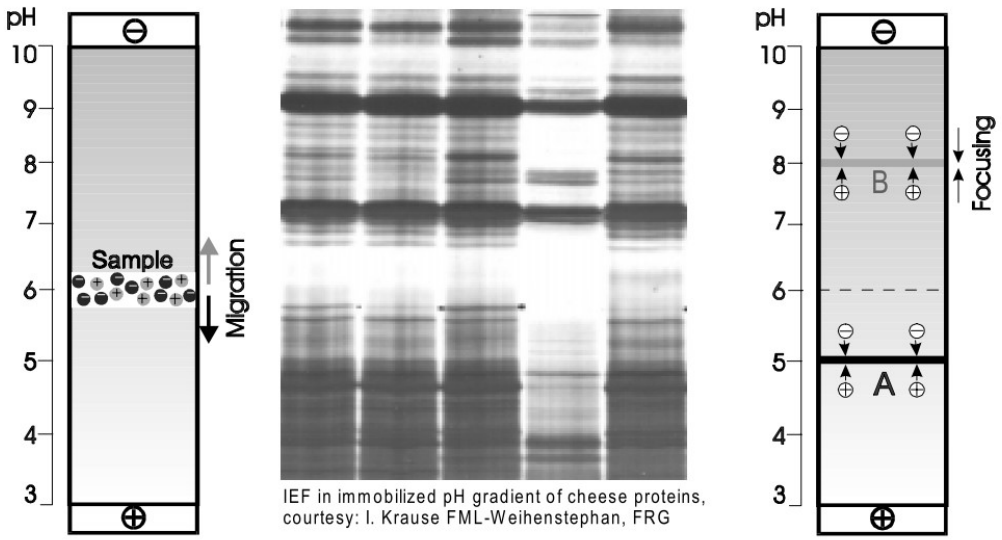

Figure 9: **Scheme of isoelectric focusing (IEF).** Left: starting point of the migration on the gel strip after application of the sample; right: focusing of the proteins after the run, according to their IP; middle: examples of IEF strips after isoelectric focusing.

The proteins are driven to their isoelectric points by the electric field. Through the pH gradient the sample components migrate towards the anode or the cathode to the pH values, where their net charges are zero: their isoelectric points (pI). Should a protein diffuse away from its pI, it would gain a charge and migrate back: this is the focusing effect. After the run the dry strip was cut into segments of 1 cm length. These segments were incubated over night at 4°C in plastic tubes with 100 µl of water, to elute the active components. The actual pH of the elutions was measured with pH paper (Macherey Nagel). One µl of each elution was used for the ethylene measurement.

2.7 Genetic determination of Cuscuta receptor 1 (CuRe1)

2.7.1 Screening of wild tomato species

A variety of wild tomato relatives were screened by infection and in the ethylene assay, as described (diploma thesis, Kaiser, 2010). For the infection assay, ~20 cm *C. reflexa* shoot pieces including the growing tip were wound around the tomato stems. After ~2-3 weeks, necrotic

spots or ingrowing *C. reflexa* haustoria were observed at the infection site. After 5-7 weeks successful infection was apparent through the growth of the *C. reflexa* shoots. Three species were found to be susceptible towards *C. reflexa* infection, and unresponsive in the ethylene assay. For one of these species (*S. pennellii*), introgression lines were available produced by Eshed, 1992 and provided by the tomato genetics resource centre (TGRC).

2.7.2 Screening of tomato introgression lines (S. lycopersicum cv. M82 with S. pennellii introgressions)

The introgression lines were screened in the same manner as the wild tomato species described above, to map genome regions relating to *C. reflexa* resistance (Kaiser, 2010).

2.7.3 DNA extraction and cloning of receptor candidates

Genomic DNA was extracted from frozen plant tissue using the Plant DNA Preparation Kit (Jena Biosciences, Germany), and a PCR was performed with gene specific primers for the candidate genes found on the respective chromosome region of *S. lycopersicum* on the Sol Genomics Network (SGN) homepage http://sgn.cornell.edu (Mueller 2005).

Solyc08g016270 (*CuRe1*): FW: ATGGGGAATATTAAGTTTTTG, REV: CCAACATTCTTGTACCATCTAC;

Solyc08g059730: FW: ATGGGGTCTTGGATTTCCC, REV: TCTTGGACCTGAGAGCCGAACAGC;
Solyc08g061560: FW: ATGGCATCATTTTTACTCCAAAG, REV: GCCACTATTCTGGGATATGACC;

Solyc08g016210: FW: ATGGGGAACGTTAAGTTTTTGTTG, REV: ATTAATCAACCTTCTACTCTTGATG;
Solyc08g016310: FW: ATGGGGAACATTAAGTTTTTGTTG, REV: ACCAACATTCCTGAACCAGCTAC).

The PCR products were cloned to the pENTR™/TEV/D-TOPO® vector (Invitrogen™). For directional cloning, a CACC tetra-nucleotide was added to the forward primers. Reverse primers without stop codon allowed for C-terminal fusion to tags of choice (e.g. GFP and myc tags) after recombining in a LR-reaction (LR-clonase® II Plus enzyme mix, Invitrogen™) into respective vectors (pB7FWG2.0, pK7FWG2.0, both with C-terminal GFP tag; plant systems biology, university of Gent). Total RNA was extracted from tomato plants using RNeasy Plant Mini Kit

(Quiagen), and cDNA was synthesized by reverse transcription (First-Strand cDNA Synthesis Kit, GE Healthcare Life Sciences).

2.7.4 Plant transformation

To test the receptor candidates *CuRe1* was transformed stably into *N. benthamiana* and *S. pennellii* by M. Smoker (Saintsbury Laboratory, Norwich, UK). The 35S::CuRe1:GFP constructs (in vectors pB7FWG2.0 and pK7FWG2.0) were transformed into *N. benthamiana* leaves using *Agrobacterium tumefaciens* (strain GV3101).

Stably transformed *S. pennellii* plants were regenerated by M. Smoker from either leaf disc or cotyledon-derived calli using *Agrobacterium tumefaciens* strain Ag11 as described by the ZMBP transformation uni (https://uni-tuebingen.de/fakultaeten/mathematisch-naturwissenschaftliche-fakultaet/fachbereiche/zentren/zmbp/res/cf/plant-transformation/) (TO generation).

For transient expression of 35S::CuRe1:GFP constructs, *A. tumefaciens* cultures OD_{600} = 0.1 in 10 mM $MgCl_2$) were infiltrated into leaves of 4 weeks old *N. benthamiana* plants, according to the described protocol (Albert et al., 2010). About 24 h post infiltration, leaves were cut into small pieces (3 x 4 mm), floated at room temperature overnight on water in a Petri dish (Albert et al., 2010) and used the following day in ethylene or ox-burst assays.

2.7.5 Cuscuta growth assay

Cuscuta reflexa shoots of ~15 cm length (diameter 0.2-0.3 cm), including the growing tip, were cut and wrapped around wooden sticks. One day later (before the formation of prehaustoria), the weight of each shoot was determined (~0.6 g ± 0.2 g), and the shoots were wrapped around the stems of the host plants. After 14 or 21 days, *C. reflexa* shoots were removed, and the fresh weight (FW) determined. Statistical analysis was performed using “R” (R 2015); boxplots were generated with the add-on “ggplot2” (R 2015).

2.7.6 Immunoprecipitation assay

For the immunoprecipitation assay, leaves of *N. benthamiana* transiently transformed with 35S::CuRe1:GFP, alone or in combination with 35S::SISSOBIR/SOBIR-like:myc (Liebrand et al., 2013) for ~48 h, were treated with *Cuscuta* Factor (1:100 in water) or water as control solution for 5 min, material was frozen in liquid nitrogen and ground to fine powder. Samples of 300 mg were solubilized and used for immunoprecipitation as reported (Chinchilla et al., 2007) using α-GFP trap sepharose beads (ChromoTek, IZB Martinsried, Germany). Samples were separated by SDS-PAGE (8% Acrylamide gels) and transferred to nitrocellulose membranes. Western blots were probed using α-GFP (Torrey Pines Biolabs) or α-myc (SIGMA) antibodies diluted according to the instructions of the suppliers, and developed with secondary antibodies conjugated to alkaline phosphatase as described (Albert et al., 2015, Albert et al., 2013).

2.7.7 CuRe1 binding assay

Leaves of *N. benthamiana* plants transiently expressing *CuRe1* or the control receptors AtRLP23 or EFR, respectively, were harvested, membrane-bound proteins were solubilized and immunoprecipitation was carried out as described above using 50 µl α-GFP trap beads/ sample. Beads were washed twice in solubilization buffer (150 mM NaCl, 25 mM Tris pH 8.0; 1% NP40, 0.5% DOC) and equilibrated by washing 2 x with binding buffer (25 mM MES pH 5.8, 50 mM NaCl, 2 mM $MgCl_2$). *Cuscuta* extract was added and samples were incubated on an over-head shaker (6 rpm) at 4°C. After 30 min, supernatant was discarded and samples were washed 5-6 x with 1 ml binding buffer (4°C). Receptor-bound ligand was eluted by boiling samples (95°C) in 100 µl binding buffer for 5 min; per treatment, 5 µl supernatant (eluted ligand) was used in the ethylene assay.

3. Results

When infected with *C. reflexa*, tomato responds with the generation of necrotic spots (see figure 10) at the site of the haustoria formation. The hypodermal host cells in this area are elongating then a hypersensitive like response (HLR) occurs, leading to the establishment of a **mechanical barrier** (Sahm et al., 1995, Ihl et al., 1988) which blocks the ingrowth of the haustoria.

Figure 10: ***C. reflexa* on a tomato plant.** Necrotic areas on tomato (*S. lycopersicum*) are visible at sites where *C. reflexa* haustoria attempted penetration. After ~two weeks of unsuccessful infection the *C. reflexa* shoot dries out at the rear end, while still growing at the tip.

This complex defense process begins with the recognition of *C. reflexa* as a foreign invader. Obviously, there is a way by which tomato can sense the attack by *C. reflexa.* To determine whether this occurs via a **factor** released from *Cuscuta* which is sensed by tomato, a crude extract of *C. reflexa* was produced and tested as a potential inducer of typical defense responses in tomato tissue.

3.1 Extracts of C. reflexa induce various defense responses in tomato

A juice made by grinding of fresh harvested *C. reflexa* material and subsequent centrifugation was active as inducer of an oxidative burst and ethylene (data not shown), but the yield was relatively low and the complexity of the mixture was high. Therefore, initial experiments aimed at improving the protocol for obtaining extracts with stable and high elicitor-type of activity, for details see section 2.5.1.

Most of the following experiments were done with this **crude standard extract** obtained according to the protocol described in 2.5.1, which performed better in **oxidative burst** (figure 11) and **ethylene biosynthesis** (figure 12). This crude extract was also the raw material for all purification attempts.

To test for the eventual presence of a defense-triggering molecule in *C. reflexa* extracts, the extract was investigated for biological activity with several common bio assays to measure plant innate immunity responses.

3.1.1 Oxidative burst

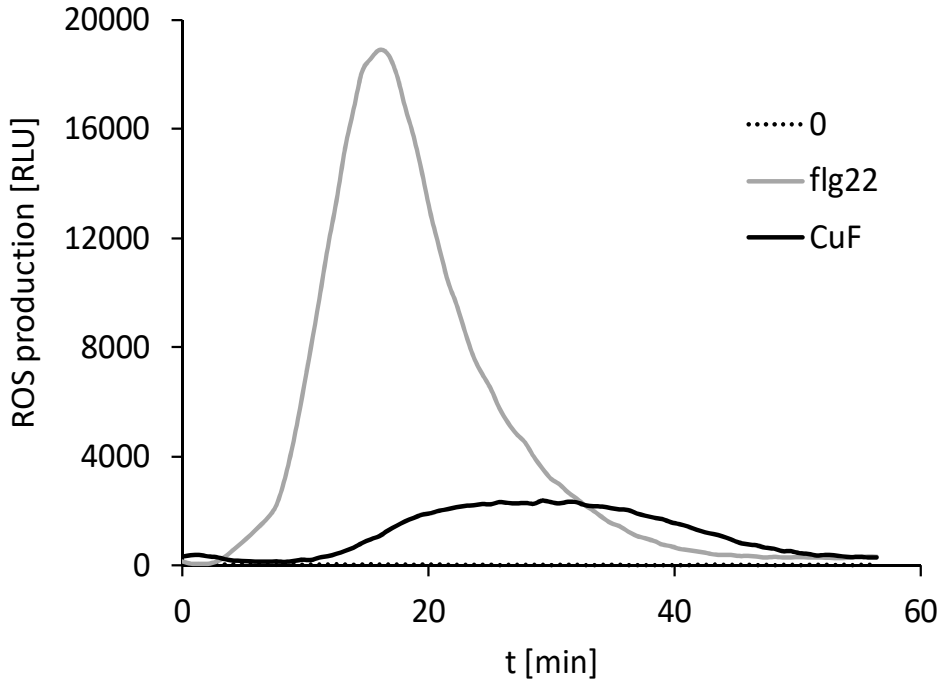

Figure 11: **Oxidative burst as a defense response to *C. reflexa*.** This diagram shows the ROS production in leaves of tomato after treatment with *C. reflexa* extract (CuF), 100 nM flg22 as positive control or 0.01 mg/ml BSA as mock control (0). ROS production was monitored using a luminol-based assay, and emitted light was detected as relative light units (RLU) by a luminometer.

The *Cuscuta* preparation induces a clear response in the oxidative burst assay. Compared to treatment with the known elicitor flg22 (Felix et al., 1999) the lag time is longer and also the maximum of the response was clearly lower (figure 11). Nevertheless, induced production of ROS occurred reproducibly after treatment of tomato leaves with the crude *Cuscuta* extract.

3.1.2 Ethylene production

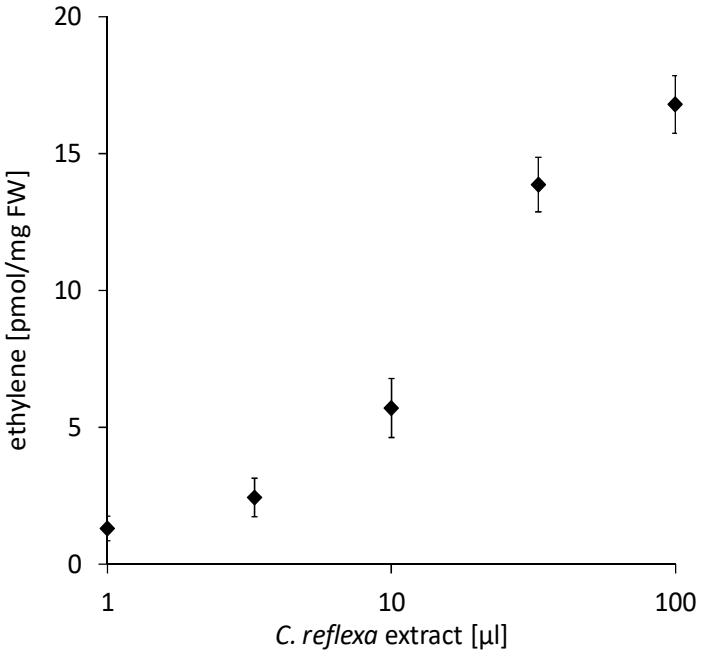

Figure 13: ***C. reflexa* extract triggers ethylene biosynthesis in *S. lycopersicum* in a dose-dependent manner.** Ethylene measurements show means of three technical replicates; error bars denote SD. This experiment was repeated more than three times.

Extract of *C. reflexa* actively induces the production of the phytohormone ethylene (figure 12). Even when diluted 1:500, the crude extract can significantly induce ethylene in tomato leaf pieces compared to mock-treated tomato leaf samples. The induction is **dose-dependent** (figure 13) and values indicate a highly sensitive detection of one or more inducing substances present in the crude CuF preparation. The extraction protocol includes a heating step (95°C, 10 min), indicating that the biologically active component(s) in the extract are heat stable.

Hence, the existence of a parasite associated molecular pattern (**parAMP**), originating directly from *C. reflexa* can be hypothesized. This parAMP, henceforth called *Cuscuta* Factor (CuF), could be purified as described in detail under 3.3. To support the purification and identification, certain characteristics had to be determined first.

3.2 Preliminary considerations

For the purification of the *Cuscuta* Factor, various preliminary theoretical considerations and practical characterizations needed to be done. It was of primary interest to find out, whether CuF is of **proteinaceous** origin and whether its native, tertiary structure is of relevance for recognition. Moreover, proteins are often **secondarily modified** e.g. hydroxylated, glycosylated, etc. which can also be an issue for the recognition. Furthermore criterions like charge, isoelectricity or the size of the factor can be investigated in simple experiments and are important to know for establishing protocols to isolate, enrich and purify the factor. The following chapter addresses these questions and shows a few characteristics of the *Cuscuta* factor.

3.2.1 Protease sensitivity of the Cuscuta Factor

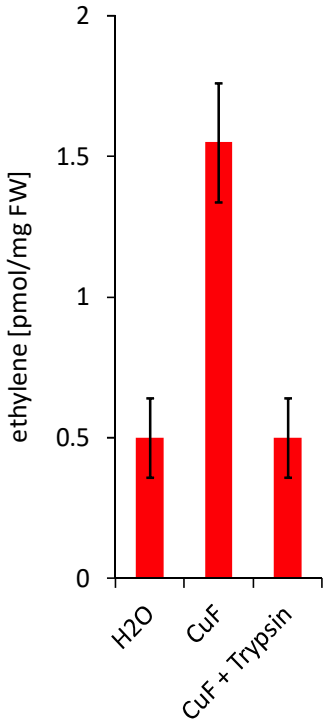

Figure 14: **Sensitivity of CuF to proteases.** Ethylene biosynthesis of tomato leaf pieces to *C. reflexa* extract, to boiled extract or to extract pretreated with the protease indicated. Water was used as a negative control. Ethylene measurements show means of three technical replicates; error bars denote SD. This experiment was repeated more than three times.

The first experiments with crude extracts from *C. reflexa* treated with proteases like Trypsin and Papain which cut unspecifically resulted in no reduction of bio-activity (data not shown). However, it was shown that in the crude *C. reflexa* extract effective protease inhibitors must be present (Kaiser, 2010). Therefore a pre-purified extract, in which many contents have been removed, was used in other experiments and the outcome did show clearer results (figure 14). In this experiment a treatment of *C. reflexa* extract with Trypsin over night led to a total loss of bioactivity, indicating a peptidic/ proteinaceous character for the *Cuscuta* Factor.

3.2.2 Heat stability of the Cuscuta Factor

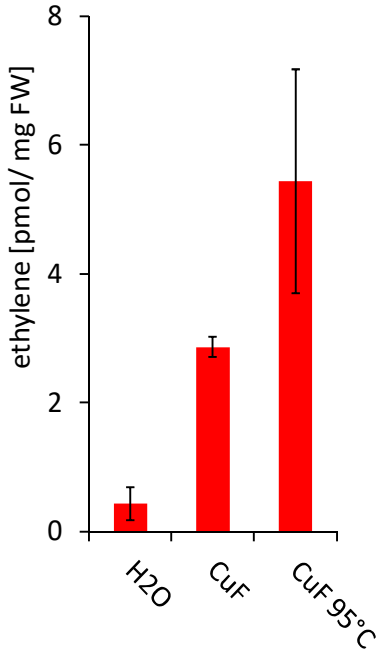

Figure 15: **Heat stability of CuF.** Ethylene measurement of tomato leaf pieces, untreated (H2O), treated with CuF unboiled and boiled for 10 minutes (CuF 95˚C). Ethylene measurements show means of three technical replicates; error bars denote SD. This experiment was repeated more than three times.

The **heat stability** of the *Cuscuta* Factor was shown by comparison of boiled and un-boiled extract (10 min 95°C). In figure 15 it can be seen, that the extract does not loose activity after boiling. On the contrary, it even seems to gain activity. Due to this discovery, all further experiments were done with boiled extract since boiling may destroy or reduce hydrolytic

enzymes that potentially could destroy the *Cusctua* factor. Thus, heating was a step to ensure that the extract remains stable throughout the long purification process and does not change its composition due to endogenous plant proteases or other hydrolases.

3.2.3 Base-treatment for deglycosilating the Cuscuta Factor

To check for eventually secondary modifications such as **glycosylations**, a sample from a C18 preparation and GP8, a glycopeptide preparation of fungi (Basse and Boller, 1992), were chemically deglycosylated after a protocol from Rademaker, 1998. After 16 h of treatment with NH_4OH the *C. reflexa* extract preparation was almost inactive in the ethylene assay (figure 15), while GP8 still showed similar activity as a mock-treated control of the sample. GP8 is an N-glycosylated peptide and dependent on its glycosylation for its activity. The used deglycosylation method has been successfully used to specifically remove O-glycosylations. Hence the *Cuscuta* Factor could be O-glycosylated or contain other modifications that are sensitive to the alkaline NH_4OH treatment.

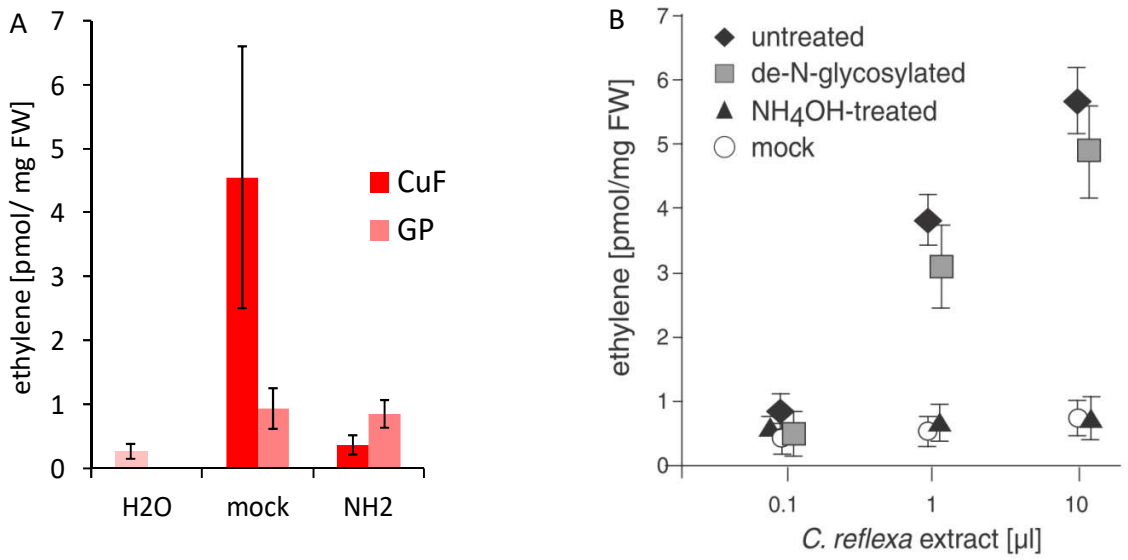

Figure 16: **De-O-glycosilation/ pH treatment of CuF.** A: Ethylene measurement of tomato leaf pieces treated with a *C. reflexa* preparation (CuF) and a N-glycopeptide preparation (GP) from yeast invertase as a control; after these preparations were mock treated 16 h with buffer (20 mM ammonium acetate) or NH_4OH. B: Ethylene response to different doses of the *Cuscuta* factor after enzymatic de-N-glycosylation or to *Cuscuta* factor treated with 20% NH 4 OH (45°C, 16 hours), respectively (modified from Hegenauer et al., 2016).

Enzymatic removal of N-glycosylations did not relate to any reduction of activity (figure 16 B). Samples treated with the enzyme Asp N, which removes N-glycosylations still show the same dose-dependent increase of ethylene production as untreated samples.

3.2.4 Size of the Cuscuta Factor

To determine the approximate size, the crude CuF preparation was separated on an **ultrafiltration** device with a 3 kDa cut-off. Activity was detected in the flow-through, indicating that the activity of CuF is smaller than **3 kDa**. To more precisely determine the size of CuF, high resoluting **tricine SDS gel electrophoresis** has been performed (figure 17).

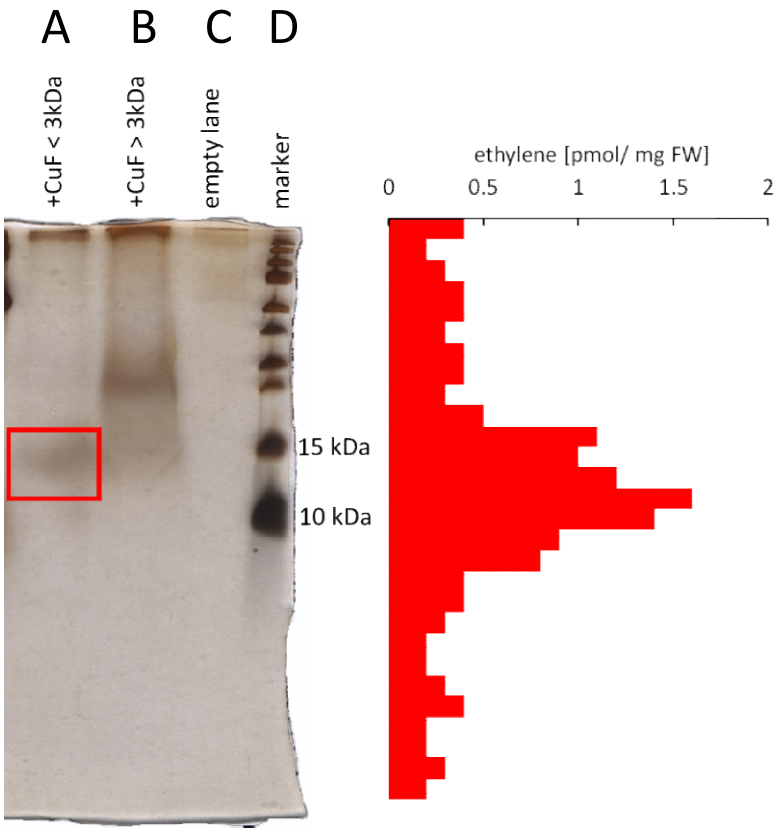

Figure 17: **Separation of CuF in an SDS gel.** Left: silver nitrate stained tricine SDS gel, on the gel are *C. reflexa* purified extract filtered by 3 kDa cut-off ultrafilter (A) the retentate bigger than 3 kDa (B), an empty lane (C) and the Marker (Page Ruler, Prestained, D). Right: ethylene measurement of a coomassie blue stained lane respecting B was cut into slices (2 mm); compounds/proteins were eluted overnight in 100 µl of water. 1 µl was used to induce ethylene biosynthesis in tomato leaf pieces.

The samples "smaller than 3 kDa" (CuF <3k) and "larger than 3 kDa" (CuF >3k) were applied twice to the tricine gel, once to visualize the proteins by silver nitrate staining (figure 16), and once for separation, elution and testing the compounds for activity in the ethylene bioassay. This was done due to an eventually disturbing/inactivating effect of silver nitrate in the ethylene measurement. The lane with CuF <3kDa of the un-stained part of the gel was cut into slices of 2 mm. These segments were incubated overnight in 100 µl of water and 1 µl aliquots of these elutions were tested for their capability of inducing ethylene biosynthesis in tomato leaf pieces (fig. 17 B). The activity seems to correlate to a diffuse smear that co-migrates with a cloudy area of stain in the silver stained gel. Peculiarly, the CuF migrates with an apparent size **between 10 and 15 kDa** according to the markers, a size significantly bigger than the size

estimated from ultrafiltration. This might be due to different effects such as unspecific interactions with the gel matrix, non-globular structure or a low charge/ size relationship.

3.2.5 Isoelectric point/ focusing of the Cuscuta Factor

To determine the isoelectric point of the CuF we performed isoelectric focusing by applying the CuF to an immobiline dry strip. After the electrophoresis the strip was cut in slices, activity was eluted from each part and tested for the capability to induce ethylene in tomato. Most activity was found in segment 22, which corresponded to a pH of 8.9, also in the more alkaline segments 23 and 24 (both pH 9.8) a considerable amount of activity could be found (figure 18).

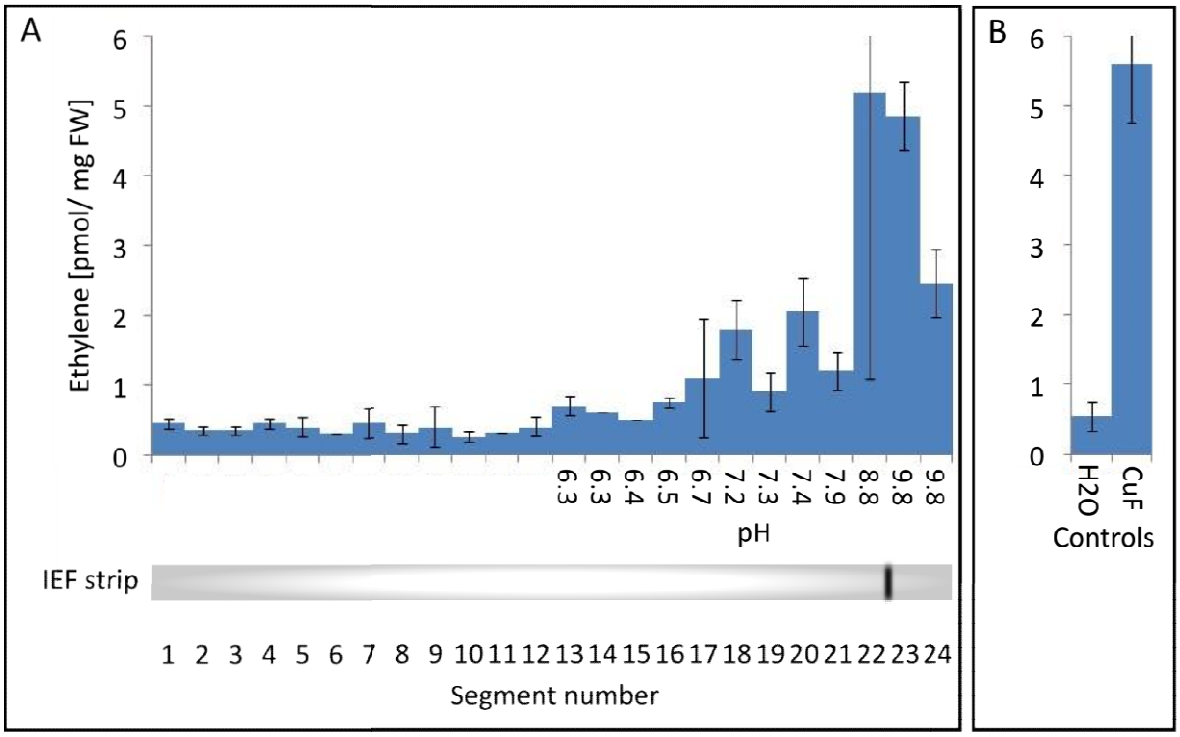

Figure 18: **Isoelectric focusing of the *Cuscuta* factor.** A. Ethylene measurement of *C. reflexa* extract eluded in 12 h from an immobiline dry strip (depicted below) after isoelectric focusing. Each bar represents the mean of three replicates. 1 µl of a 100 µl elution from each segment was used for the ethylene assay. B. Negative control (H2O) and 5 µl of the load of the strip (CuF).

This result suggests that the **pI** of CuF is around **9**.

3.3 Purification of Cuscuta Factor (CuF)

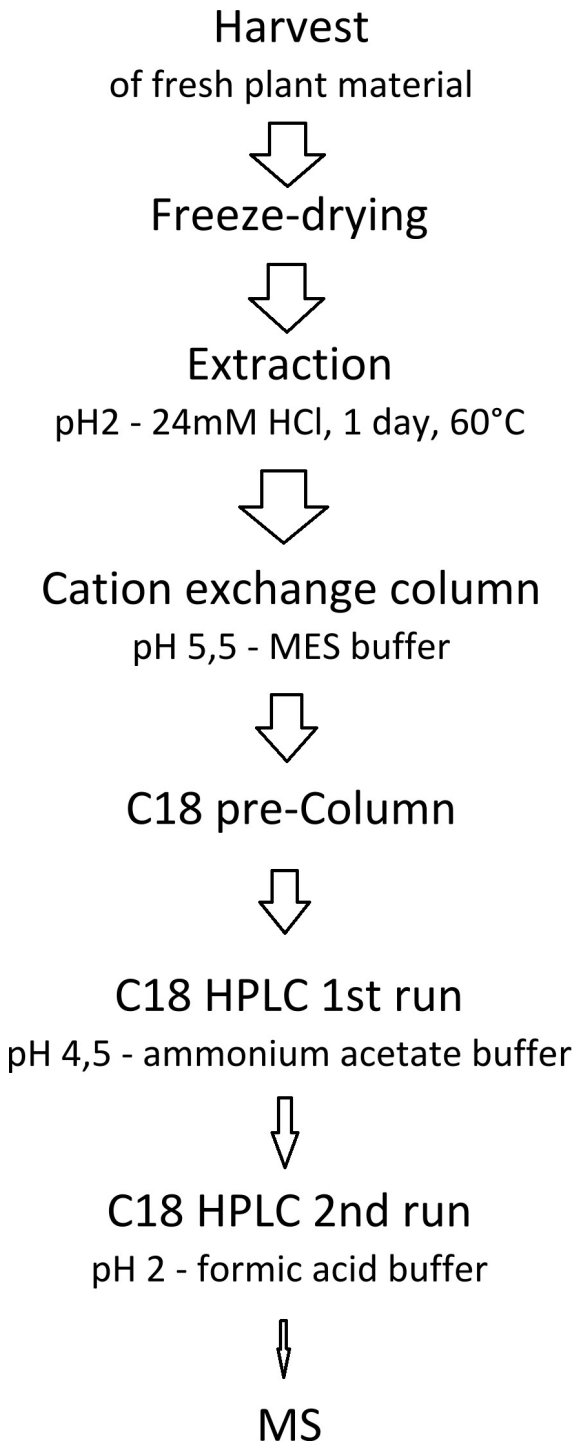

Figure 19: **Purification protocol of CuF.** After harvesting the fresh plant material and freeze-drying, an acidic extract was applied to a Cation Exchange column. The eluate was pre-purified by a C18 pre-column and loaded on C18 reversed phase HPLC columns under two different conditions. Active fractions were analyzed thereafter in a mass spectrometer.

To obtain sufficient amounts of CuF to be analyzed by LC-MS/MS it was indispensable not only to purify but also to enrich CuF. Throughout the purification process, the active CuF per ng dry material increased (figure 20) which indicated an **enrichment**.

3.3.1 Dose-Response Assay

It is difficult to determine the amount of CuF throughout the purification process. Nevertheless an approximation should be made to determine the loss of activity from one purification steps to the next and to estimate whether the sensitivity of the detection methods is sufficient for results of high quality. The CuF seems highly active and the receptor very sensitive for its perception. 1 CU (*Cuscuta* unit) was defined as the amount of CuF, which induces half-maximally in the ethylene assay. For comparison: 100 nM of the peptide elicitor flg22 may also be enough for half-maximal induction of ethylene production in tomato leaf pieces. In biological systems dose dependency is not linear, but follows an exponential curve followed by a saturation phase. To monitor this curve measurements must be undertaken logarithmically, for example 1 µl, 10 µl, 100 µl, not linearly (with the possible interstages of 3.3 µl and 33 µl).

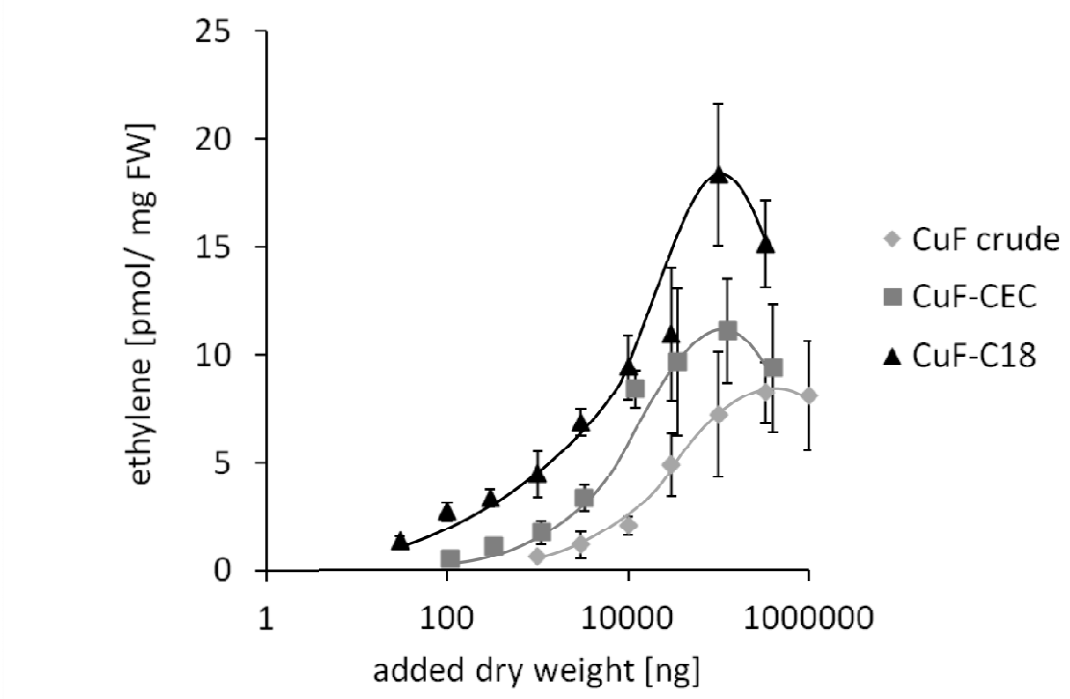

Figure 20: CuF activity of ***Cuscuta*** **extract after the different steps of purification.** Dose-response curves after treatment with *Cuscuta* crude extract (CuF crude), with extract eluted from the cation exchange chromatography (CuF-CEC) and extract eluted from the C18-reversed-phase pre-column (CuF-C18) show an increase of highest produced ethylene amount and sensitivity going along with the progression in purity. Values and error bars show means and standard deviations of three technical replicates. This experiment was repeated more than three times.

In this graph a smaller minimum dosage for ethylene induction as well as a higher maximum induction for CEC pre-purified extract and CEC/ C18-pre-purified extract is apparent by comparison with the crude *Cuscuta* extract. This results from a higher concentration of *Cuscuta* Factor as well as a reduction of disturbing substances. The dry weight of Cus-CEC is a calculated value, excluding the salt of the elution.

For the purification of the *Cuscuta* Factor, the extract as described in 2.5.1 was used as raw material. For application on CEC columns, 20 mM MES buffer was added and the pH was adjusted to 5.5, whereas 20 mM Tris buffer with HCl adjusted to pH 8.0 was used for application on the anion exchange column. However, purification attempts with Tris at pH 8.0 were not pursuit further as the *Cuscuta* activity decreased with pH 8.0 and higher (see also fig. 15, NH_4OH treatment).

3.3.1 Cation Exchange Chromatography

The *Cuscuta* Factor is positively charged at pH 5.5 (pI ~ 9) and can be expected to bind to a cation exchange column but not to positively charged resins of anion exchange columns. In the early purification attempts an anion exchange column was used that confirmed activity in the flow-through – a step which was later omitted. While not suitable for separation of CuF, the anion exchange chromatography may still remove disturbing (anionic) components. The resulting flow-through of the anion exchange column was subjected to cation exchange chromatography. All CuF activity sticked to this resin and was released henceforth by elution with an increasing salt gradient (figure 21). However, the active components eluted over a wide range of the salt gradient, suggesting several, overlapping peaks of activity, **peak 1**, **peak 2** and **peak 3** (figure 21).

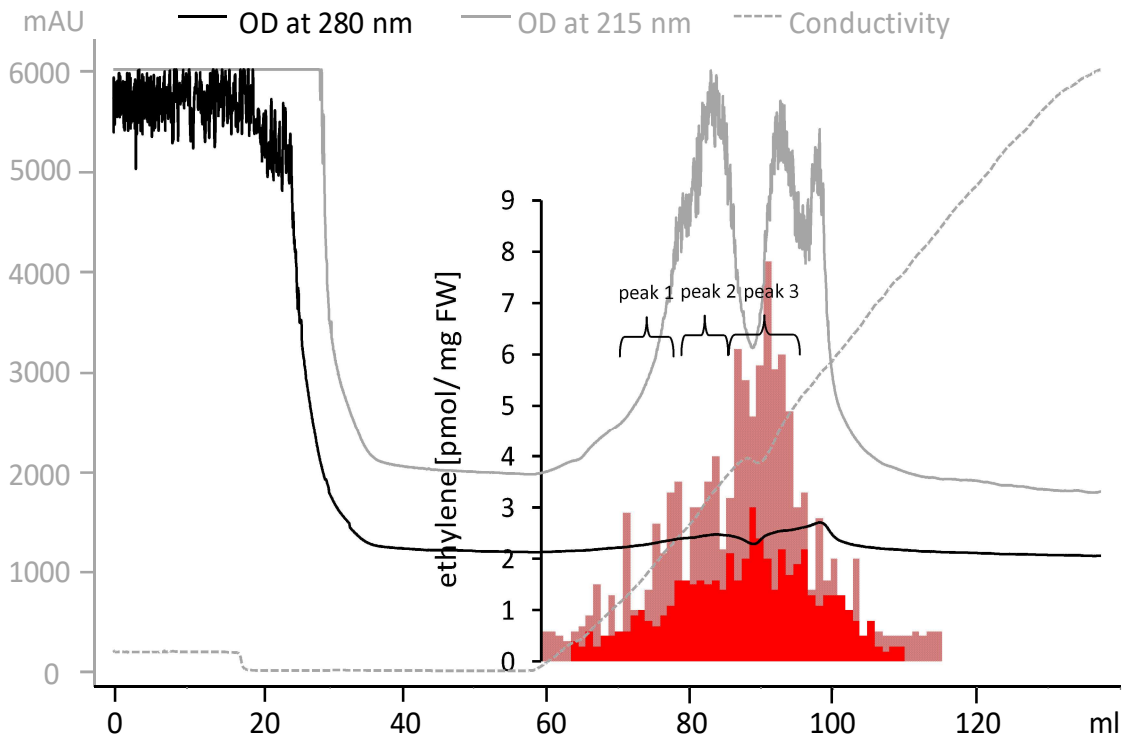

Figure 21: **Purification by cation-exchange chromatography.** Pre-purified *C. reflexa* extract was separated by cation-exchange chromatography (CEC) with SP Trisacryl M resin (10 ml) using a salt gradient (0–1 M NaCl) for elution. Fractions (1 ml) were tested in the ethylene bioassay with tomato leaf pieces at the dilutions 1:50000 (red) and 1:500000 (light red) displayed in the bar diagram. The optical density (OD) was measured by a UV detector in milli-absorbance unit (mAU).

For a scaled-up purification attempt a cation exchange column with a higher ion capacity was used containing about 100 ml of SP Sepharose fast flow as resin in a gravity flow setup. After loading 250 ml of *Cuscuta* extract and washing with the equilibration buffer, a step gradient (100 mM steps; from 100 to 600 mM KCl) was applied with 50 ml each and collected the elution in 10 ml fractions. The conductivity of these fractions and the biological activity in terms of ethylene production after treatment of tomato leaf pieces was measured. The activity of these runs showed a similar distribution as in figure 21 with several peaks. It was assumed that release from the column at different conductivities relates to different forms of elicitor, therefore the fractions of similar conductivity of several runs were pooled together and subjected to reversed phase chromatography on the HLPC.

3.3.2 Reversed Phase Chromatography with HPLC

As a next purification step the active eluate from the CEC was loaded to a **C18 pre-column** (Chromabond with 10 g of resin). In this step the CEC eluate is desalted and concentrated. The elution was performed in two steps with 20% acetonitrile, then with 80% acetonitrile. Within the 20% acetonitrile eluate no activity could be found, whereas the 80% eluate was highly active (data not shown). This eluate was concentrated in a speed-vac and re-dissolved in 20 mM ammonium acetate and loaded on the HPLC (figure 22).

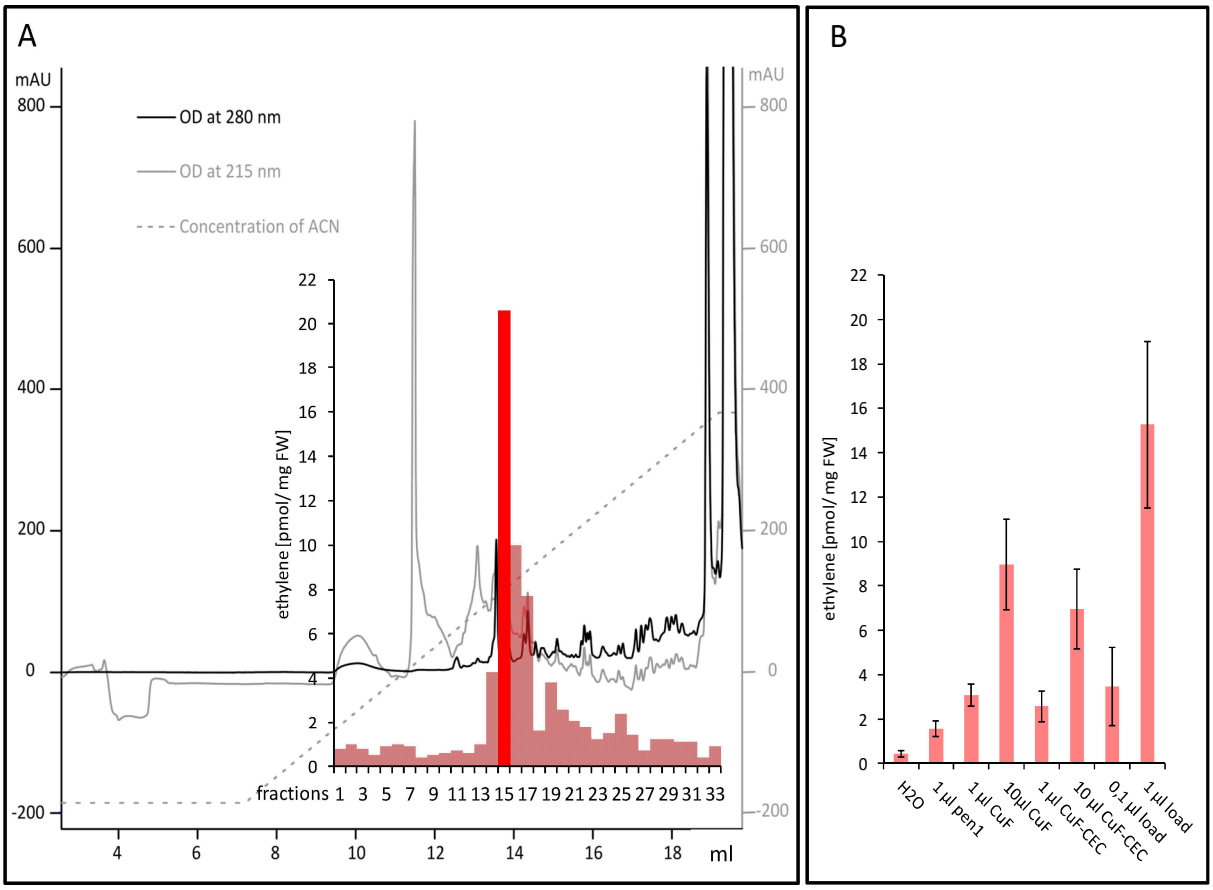

Figure 22: **2nd step of purification of *C. reflexa* extract by RPC.** In this run a C18 reversed phase column (Zorbax) was used under acidic conditions (20 mM ammonium acetate) and elution with an acetonitrile gradient of 0-25% demonstrated further separation of activity into fractions with clear activity but low absorbance at OD 280 and OD 215. Fractions (300 µl) were tested for induction of ethylene production (1 µl per 500 µl sample). Values and error bars show means and standard deviations of three replicates. B: Ethylene measurement of negative control (H2O), positive control (pen1) and 2 concentrations of *C. reflexa* crude extract (CuF), 2 concentrations of used CEC fractions and load of this run, all per 500 µl sample.

One fraction of this run showed very high activity in the ethylene measurement (fig. 22, dark red bar) and was therefore subjected to a second run on HPLC with altered conditions in expectancy of further purification. Instead of ammonium acetate (pH 4.5), formic acid (pH 2) was used. Additionally, a different C18 column was taken. Also, a **shallower gradient** was applied for a better resolution (figure 23).

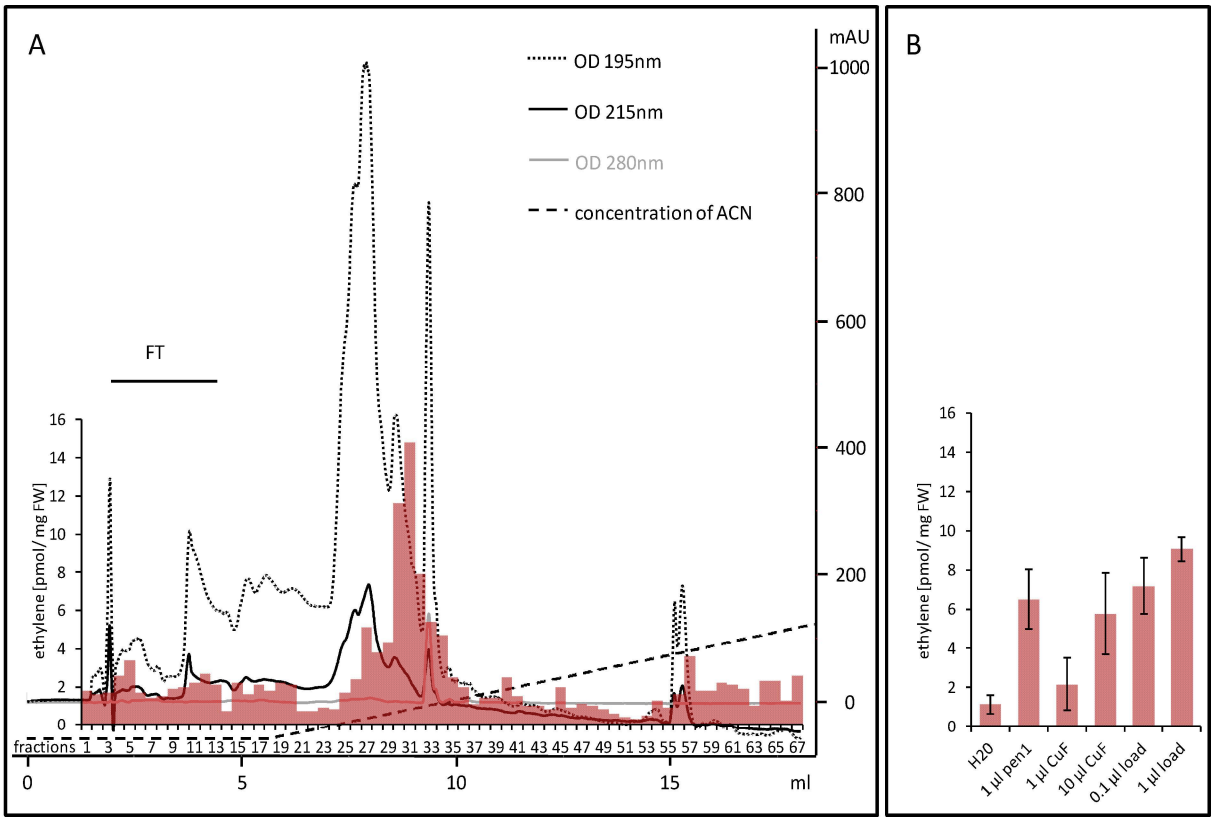

Figure 23: **3rd step of purification of *C. reflexa* extract by RPC at pH 2.A:** The active fraction of the first RPC run was re-run under different conditions. This time a C18 column (PerkinElmer) was used with 0.1% formic acid and elution with a shallower acetonitrile gradient resulted in a high resolution profile with few active fractions (250µl) demonstrated in the ethylene assay (red bars, 1 µl per 500 µl sample).B: Ethylene measurement of negative control (H2O), positive control (pen1) and 2 concentrations of *C. reflexa* crude extract (CuF), 2 concentrations of used CEC fractions and load of this run, all per 500 µl sample. Values and error bars show means and standard deviations of three replicates.

This second run revealed a main peak of activity, preceded by some factions that may represent a second, separable, form of activity. Tracing of optical density (OD195, OD215) shows several additional peaks separated from the active fractions, indicating that this second run was successful in removing substances unrelated to the CuF factors. The active fractions no. 30, 31 and 32 from this run were analyzed via mass spectrometry.

3.3.3 MS analysis

Using the protocol as described in 2.5.3.4 A), a peptide with a mass of **1015 Da** could be identified by MS in this fraction. MS/MS analysis revealed that it contained the sequence **NKGKVNKGNG**. Remarkably, the last 5 amino acids NKGNG were the same as in a second MS analysis with a slightly altered protocol (table 4). This peptide was synthesized, and tested for activity but did not show activity in the ethylene assay even when applied at micromolar concentrations.

As the elution profile of the preparative step-eluted C18 column showed multiple peaks, the pre-purification needed to be optimized. In the cation exchange chromatography the components elute in a large number of fractions. Elution pattern of activity in different runs also showed some variation, probably due to a heterogenous nature and somewhat different amounts of the active components in different *Cuscuta* preparations. Therefore, fractions of equal conductivity from several runs were pooled together to have higher amounts for further purification.

Four gravity flow CEC runs were performed and the fractions of ~40 mS were pooled to scale up the bioactive CuF. Each pool was separately pre-purified by a C18 pre-column to remove substances, which end up in the flow-through or are sticking irreversibly to the C18 resin. The active samples eluted from the C18-pre-column were dried in a speed-vac, re-dissolved in 20 mM ammonium acetate (pH 4.5) and subjected to the HPLC as previously described (figure 22). After a re-run with RPC (as in figure 23), the two most active fractions were subjected to LC-MS.

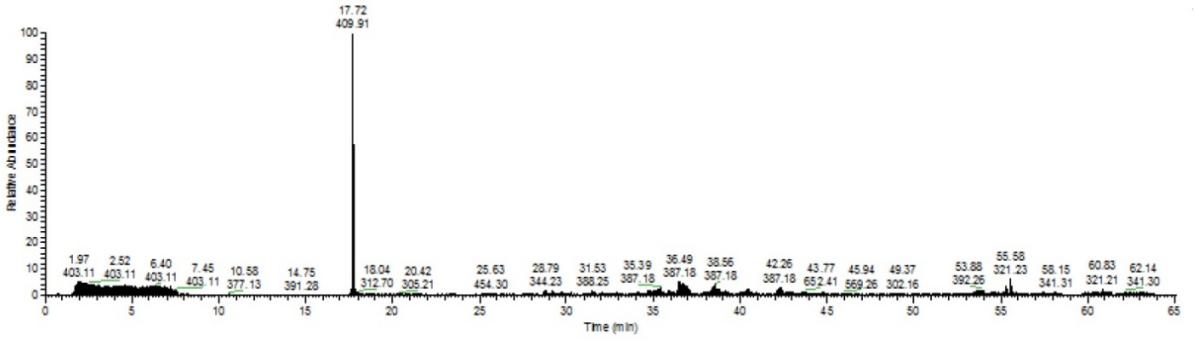

Figure 24: **TIC (total ion current) chromatogram** The chromatogram shows the sum of the ion current intensities across the scan range in a mass spectrum. Load: CuF sample from active fractions eluted from the C18 HPLC (2nd run). X-axis: retention time in minutes, y-axis: relative abundance (Maček and Madlung, Proteome Center, Uni Tübingen).

The chromatogram in figure 24 shows an example of an ESI-Oribitrap-LC-MS (Proteome Center, Uni Tübingen) obtained by the protocol as described in 2.5.3.4 B). The chromatogram shows a clear peak at 17.7 minutes. The MS spectrum of this peak is shown in figure 25.

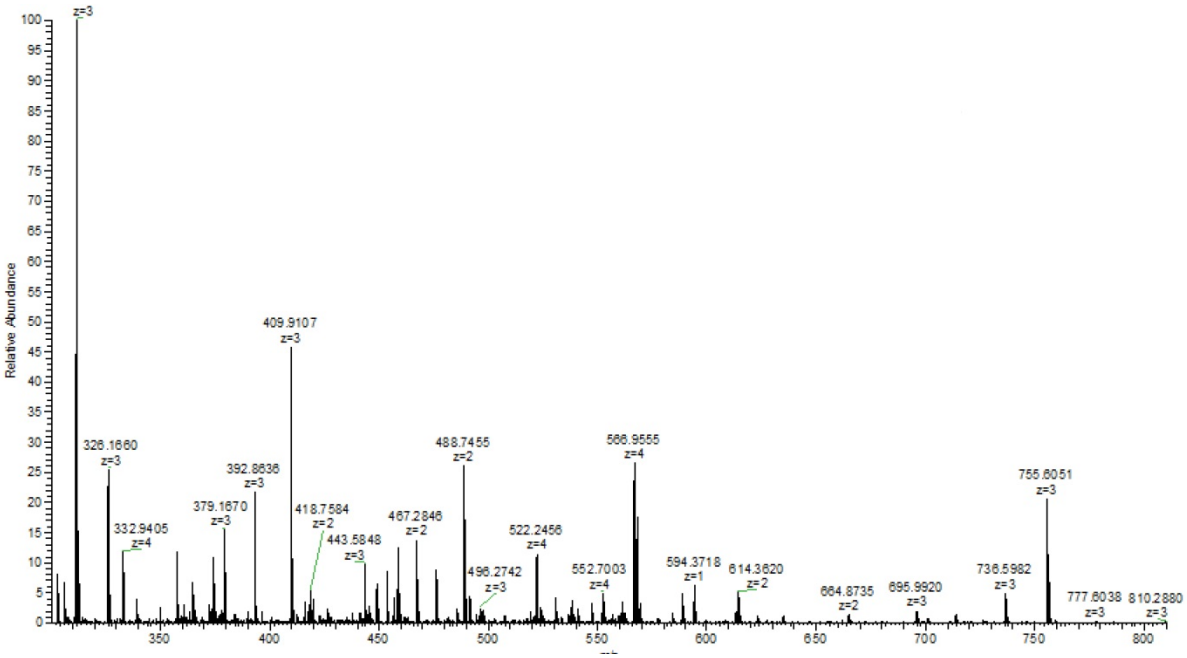

Figure 25: **MS spectrum at the retention time of 17.37-19.9 minutes of the LC-MS run shown in figure 24**. On the x-axis is given the mass/charge ratio; on the y-axis is given the relative abundance; z = charge state (Maček and Madlung, Proteome Center Uni Tübingen).

For two of the main masses in this peak, 2206 and 933, **MS/MS spectra** were obtained. The spectrum of 933 (related to the highest peak of 311 in the spectrum in figure 25) is shown in figure 26 with the amino acids fitting to the mass differences. Together with a repeated, similar analysis of mass 933, the sequence for this peptide was completed to EPRDPKN.

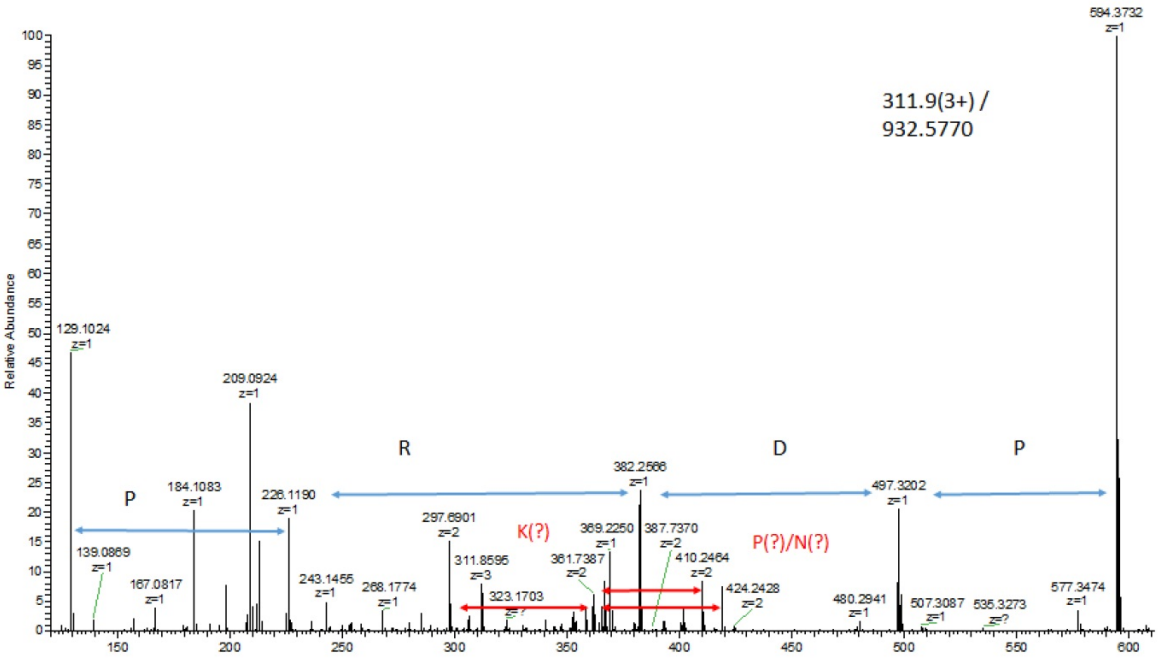

Figure 26: **MS/MS spectrum of the detected mass 933 Da that correlated with activity of CuF**. On the x-axis is given the mass/charge ratio; on the y-axis is given the relative abundance; z = charge state; the letters resemble the fitting amino acid (calculated by the mass difference) (Maček and Madlung, Proteome Center Uni Tübingen).

Various MS analysis were done, some with a slightly altered purification protocol, also the starting material for the RPC was varying, sometimes all active fractions were used, sometimes just selected fractions with a certain conductivity, then pooled with fractions of the same conductivity from different runs. Some MS analysis only resulted in one mass, but for others also short sequences could be obtained. Some masses were not reproducibly seen, others were confirmed several times, but in general the results of the MS/MS spectra were of bad quality, probably due to low amounts of substance and due to poor ion-fragmentation. A summary of the results is presented in table 4.

A protein BLAST for sequences detectable in the MS/MS spectra on databases (Nonnenmacher, P.) of "flowering plants" resulted in too many hits on various enzymes and other proteins (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The sequences were too short to yield unambiguous results. However, candidate proteins encoding for small and already known peptides such as the CLEs, IDA/IDL or CLE-likes were missing. A sequence comparison with a *Cuscuta* library (RNAseq) was not yet possible since the hitherto published database has a cut-

off for sequences shorter than 200 nucleotides (Ranjan et al., 2014). Therefore, the collaboration partners (Ranjan and Sinha) will try a sequence comparison as soon as possible.

Table 4: Summary of the CuF candidate sequences after MS/MS analysis of purified fractions.

Sequence	**Origin**	**Mass (Da)**
No	HPLC 2nd run, load: pooled fractions from 4 CEC runs, eluates from ~40mS, starting material ~1 l *Cuscuta* extract	2436
X-NKGNG	AEC(FT), HPLC 1st run, load: all active fractions from CEC, starting material: ~250 ml *Cuscuta* extract	1015
NKGKVNKGNG	HPLC 2nd run, load: pooled fractions of 5 CEC runs, eluates from ~40mS, starting material: ~ 1 l *Cuscuta* extract	1015
DKI/LVD	HPLC 2nd run, load: pooled fractions of 5 CEC runs, eluates from ~25mS, starting material: ~1 l *Cuscuta* extract	2207
EPRDPKN	HPLC 2nd run, load: pooled fractions of 5 CEC runs, eluates from ~25mS, starting material: ~1 l *Cuscuta* extract	933
No	HPLC 2nd run, load: pooled fractions of 5 CEC runs, eluates from ~25mS, starting material: ~1 l *Cuscuta* extract	2263

Unfortunately, no further evidence for the implication of any of these candidates as a CuF-type activity could be obtained. Also, with the exception of the sequence X-NKGNG, the sequence tags were only detected once in independent MS/MS analysis.

In Summary, six promising candidate masses between 933 and 2263 Da have been found, four of them could be also (partially) sequenced, but the sequences were all too short to find a corresponding gene sequence. For the longest sequence a peptide was synthesized, but it was biologically inactive.

To identify the CuF, further MS-analysis has to be done with higher amounts of active compounds. Therefore, a further scale-up is necessary, especially in the first steps of the purification scheme where total CuF activity separates into several, distinct fractions. The chemical heterogeneity of the CuF activity might originate from the same type of active molecular pattern being present on variable peptide backbones.

3.4 Identification of the tomato cell surface receptor Cuscuta receptor 1 (CuRe1)

In an initial screen of 15 wild tomato species the **distribution** of the *Cuscuta* recognition throughout the **tomato clade** was tested. The wild relatives of *S. lycopersicum* were tested both in the ethylene assay and in the infection assay. Three rather basal species, *S. pennellii*, *S. sities* and *S. juglandifolium*, were found which showed no response in the ethylene assay towards *Cuscuta* extracts. Interestingly, these three species also could be successfully infected by *C. reflexa* (Kaiser, 2010, diploma thesis).

Table 5: Ethylene response and susceptibility of tomato species towards *C. reflexa, S.* = *Solanum.* TGRC: Tomato Genetics Resource Center, UC Davis (Kaiser, 2010)

Species	**TGRC-No.**	**Ethylene response to *C. reflexa* extract**	**Resistance (HRL like response)**
S. cheesmaniae	LA0421	No	Yes
S. chilense	LA1968	Yes	Yes
S. chmielewskii	LA3661	Yes	Yes
S. lycopersicum var. cerasiforme	LA1226	Yes	Yes
S. hirsutum	LA1353	Weak	Yes
S. hirsutum f. glabratum	LA2103	Yes	Yes
S. parviflorum	LA1326	Yes	Yes
S. pennellii	LA0716	No	No
S. peruvianum	LA0107	Yes	Yes
S. peruvianum f. glandulosum	LA1292	Yes	Yes
S. pimpinellifolium	LA0722	Yes	Yes
S. juglandifolium	LA3325	Weak	No
S. lycopersicoides	LA2408	Weak	Yes
S. ochranthum	LA2160	Yes	Yes
S. sitiens	LA4105	No	No

The three species *Solanum sitiens*, *Solanum juglandifolium* and *Solanum pennellii* were **fully susceptible**, *C. reflexa* penetrated the stem via haustoria, connected to the vascular tissue (figure 28) and proliferated on these plants (figure 30). Although all three of these species are

basal in the phylogenetic tree of the tomatoes (figure 27), they do not represent an outgroup, but seem to have lost the ability to detect *Cuscuta* independently.

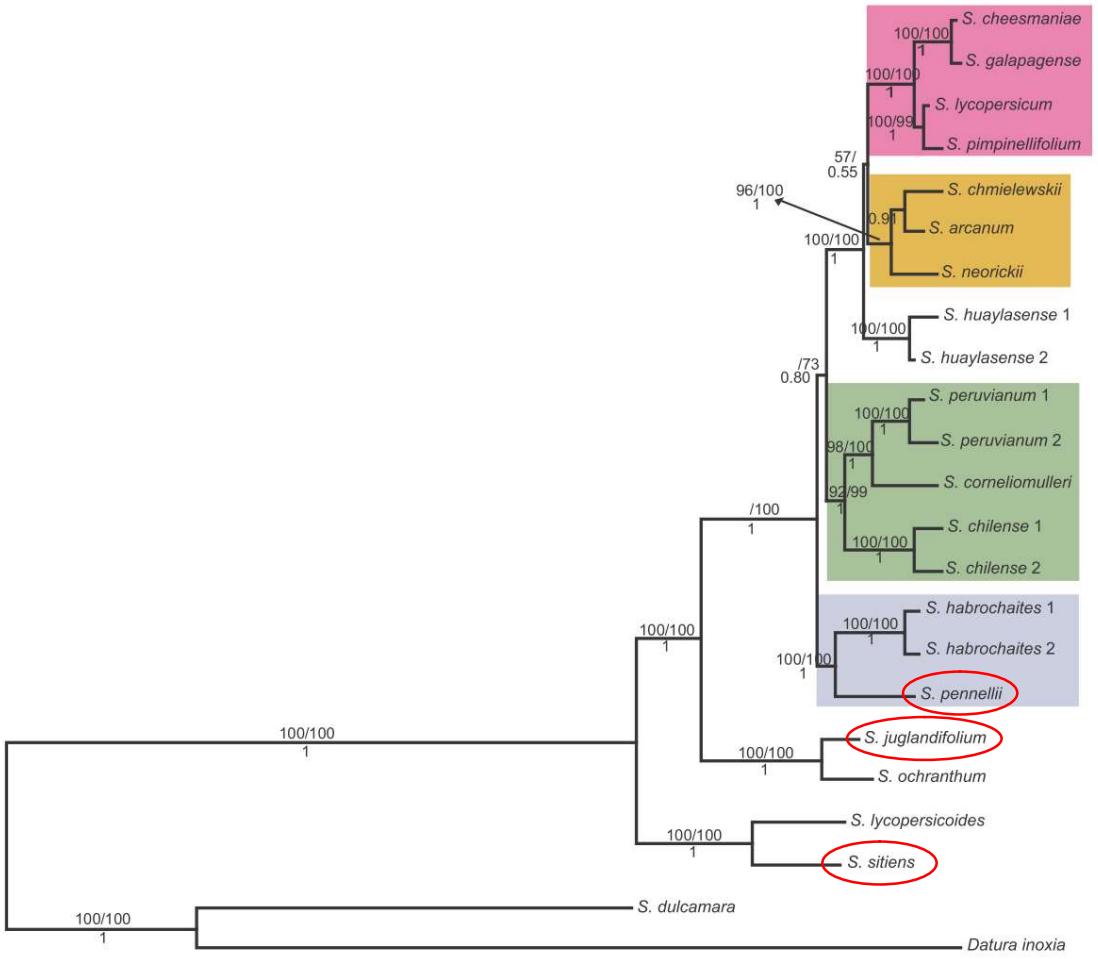

Figure 27: **Tomato Bayesian phylogram based on a combined analysis of 18 COSII (conserved orthologous set) sequences**. *Datura inoxia* and *Solanum dulcamara* were used as outgroups. *S.* = *Solanum*, respectively. Numbers after the species name indicate allelic variants. Branch length are drawn in proportion to the estimated number of substitutions per site and represent an average of the branch length of all trees sampled in the Markov chain that have that branch. Bootstrap values higher than 50% are indicated above branches, the first value refers to Maximum Parsimony and the second to Maximum Likelihood analysis; below branches are the posterior probability values. Clades with many species are shaded, *C. reflexa* susceptible species in ovals (modified from Rodriguez et al., 2009).

As previously summarized in the diploma thesis (Kaiser, 2010), there is no evolutionary pattern in the susceptibility/ resistance of the tomato species towards *C. reflexa*. The susceptible species *S. sitiens* is in an outgroup together with resistant *Solanum lycopersicoides*, susceptible *S. juglandifolium* is sister to the resistant *Solanum ochranthum* and susceptible *S. pennellii* is

sister to the resistant *Solanum hirsutum*. All tested **higher tomatoes** from the Lycopersicon[1] and Arcanum[2] section are **resistant**. Species not tested in the phylogenetic tree displayed above are *Solanum arcanum*, *Solanum neorickii*, *Solanum huaylasense* and *Solanum corneliomulleri* as well as the reference species *Solanum dulcamara* and *Datura inoxia*. Yet other solanaceous species were tested, namely *Solanum tuberosum*, *Nicotiana plumbaginifolia*, *Nicotiana benthamiana*, *Nicotiana tabacum* and *Capsicum anuum* and all were found not to respond to *C. reflexa* extract with increased ethylene production (data not shown).

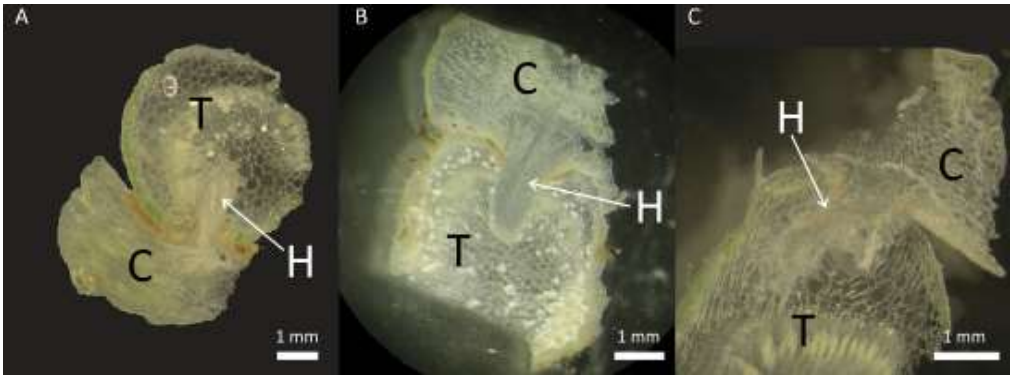

Figure 28: **Cross section of tomato (T) stems penetrated by a *C. reflexa* (C) haustorium (H).** The wild tomatoes *S. pennellii* (A), *S. juglandifolium* (B) and *S. sitiens* (C).

The susceptible species most closely related to *S. lycopersicum* is *S. pennellii*, which is a green fruited species originating from arid/ desert regions in Peru. *S. pennellii* is fertile with cultivated tomato and has a largely **co-linear genome** to *S. lycopersicum* (Kamenetzky et al., 2010, figure 29). The time of divergence is about 2.7 Mio. years ago, since then a genome expansion event might have happened in *S. pennellii*, as its genome is with 1.9 Gb much larger than the *S. lycopersicum* genome with 950 Mbs. In spite of being so closely related as to be intercrossable, many metabolic and developmental differences can be observed between the two species, so that they were subject to many studies concerning QTL (quantitative trait loci). *C. reflexa* can successfully infect and grow on this wild, round leafed tomato (figure 30).

[1] Species from the Lycopersicon section are *S. cheesmaniae*, *S. galapense*, *S. lycopersicum* and *S. pimpinellifolium*.
[2] Species from the Arcanum section are *S. arcanum*, *S. chmielewskii* and *S. neorickii*.

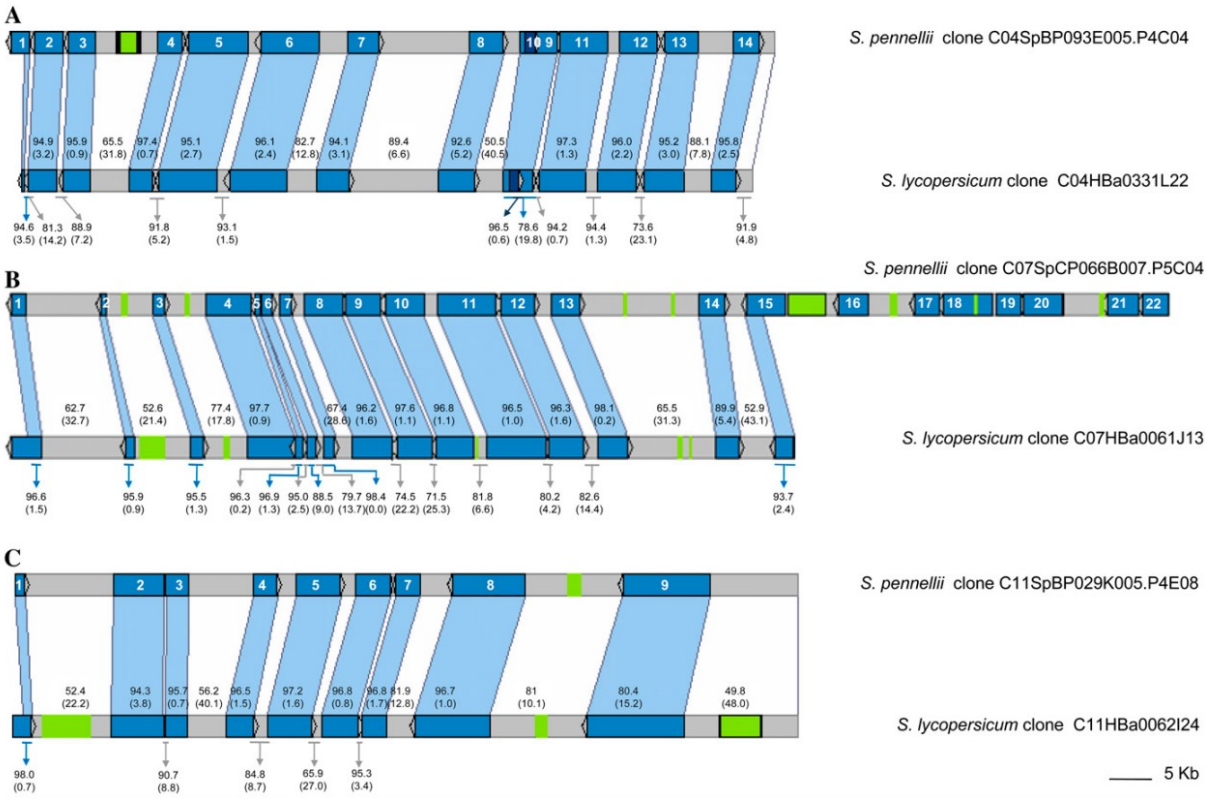

Figure 29: **Comparative analysis between *S. lycopersicum* and *S. pennellii* orthologous genomic regions.** Genes are indicated as blue arrows. Green blocks depict transposable elements. Green blocks squared in black correspond to retrotransposons, and their LTRs (long terminal repeats) are indicated as black bars at the ends of the elements. The percentage identity and InDels (insertion/ deletions, in parentheses) between genotypes along genic (including untranslated regions) and intergenic regions are indicated. Compared orthologous genomic regions of BIN 4I (A), 7H (B), and 11C (C) are presented (modified from Kamenetzky et al., 2010).

Figure 30: ***C. reflexa* twining around the stem of the green fruited tomato species *S. pennellii*.** After ~6 weeks of infection the parasitic plant *C. reflexa* has wrapped itself around the stem of *S. pennellii* and formed haustoria drawing water and nutrients from the host.

From crosses between *S. lycopersicum* and *S. pennellii* a collection of 50 **introgression lines** (ILs) was established by Zamir et al. in the 1990s (figure 31). Each of these lines contains one or more homozygous chromosome segments of *S. pennellii* replacing homologous segments in the *S. lycopersicum* genome (schematic overview, figure 31). These segments were mapped by restriction fragment length polymorphism (RFLP) and a series of molecular markers. Together, the 50 lines of the collection provide nearly complete coverage of the genome and are nearly isogenic to tomato cv. M82 (Esched and Zamir, 1995, Eshed et al., 1991, Eshed and Zamir, 1994).

First, an interspecific hybrid was backcrossed to *S. lycopersicum*, and a map of 981 cM, based on 146 molecular markers covering the entire genome, was produced. Then, the backcross 1 population was selfed for six generations, under selection for cultivated tomato phenotypes, to produce 120 introgression lines. The introgression lines were assayed for the above-mentioned molecular markers, providing a powerful tool for genetic studies, for example mapping of QTL.

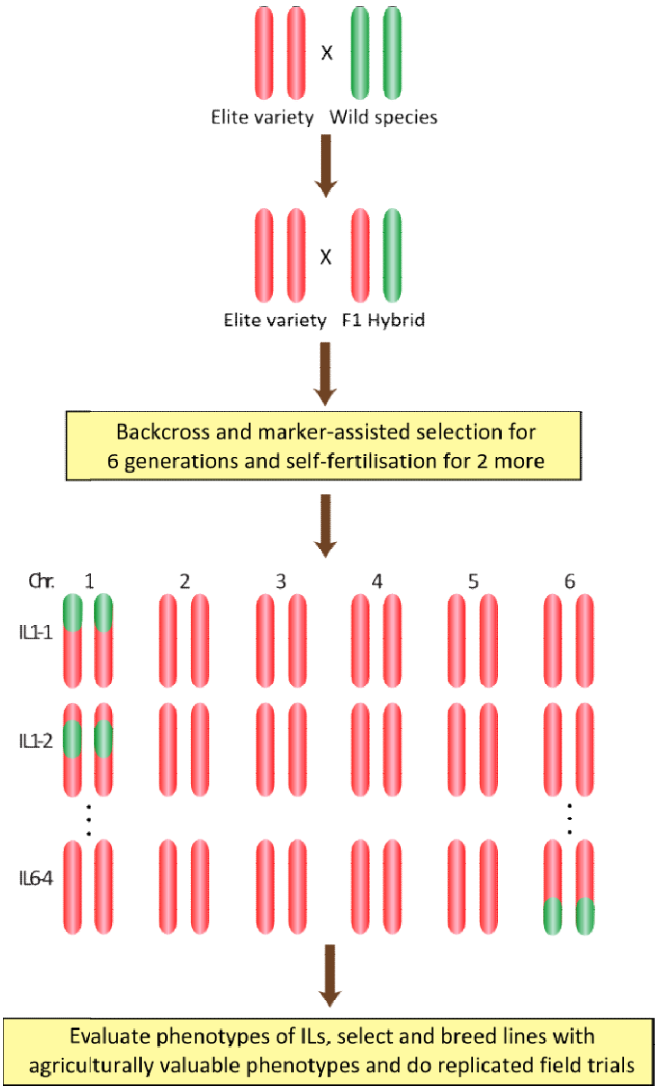

Figure 31: **Schematic display of the generation of introgression lines**. The figure shows a breeding scheme for generating and screening an exotic genetic library. The elite variety chromosomes are shown in red and the wild-species introgressions are shown in green. The haploid chromosome number of the plant is six, and backcross generations are shown for only a pair of homologues. The wild species (green) is crossed as a male parent to a leading cultivated variety (red), and the F1 hybrid is backcrossed to the elite parent. Through recurrent backcrosses, the average proportion of the wild species genome is reduced by 50% in each generation. Chromosome segments are traced through crosses by genotyping the lines with a genome-wide panel of polymorphic markers that can distinguish between parental alleles. By the sixth backcross generation, independent plants can be isolated, each heterozygous for a different segment of the wild-species genome. Further self-pollination and selection leads to homozygosity at targeted introgressed segments. The resulting exotic library shown for the first two lines (IL1-1 and IL1-2) and for the last line (IL6-4) can be used in many screens for different agriculturally important traits. Typically, three to five generations are required to identify wild-chromosome segments that improve the phenotype of the elite line and to eliminate the negative traits that are caused by linkage drag. Once a particular exotic library has been developed, it can be used to rapidly identify other traits of agricultural importance (modified from Zamir, 2001).

This introgression line population has recently been genotyped using RNA-Seq and RESCAN at ultra high density providing the exact gene content harbored by each line and a physical map (Chitwood et al., 2013). This revealed 7 ILs harboring multiple introgressions and the unique overlapping regions, termed “bins” can now be divided in smaller intervals, 112 instead of 107.

All 50 introgression lines of *Solanum lycopersicum* cv. M82 x *Solanum pennellii* representing the whole tomato genome were infected by *C. reflexa*. Several weeks after infection the *C. reflexa* shoots dried out and decreased in biomass, except in one line, the IL 12-2. Here, no necrotic spots appeared on the tomato stem and the *C. reflexa* shoot increased in biomass.

Additionally all lines were tested regarding the responsiveness to CuF. Only one line, **IL 8-1** showed decreased response in the ethylene assay when treated with *Cuscuta* extracts (see figure 32 A). Interestingly, the **IL12-2** which showed susceptibility was not affected in the ethylene response. Vice versa, the IL8-1 was not showing susceptibility or less resistance.

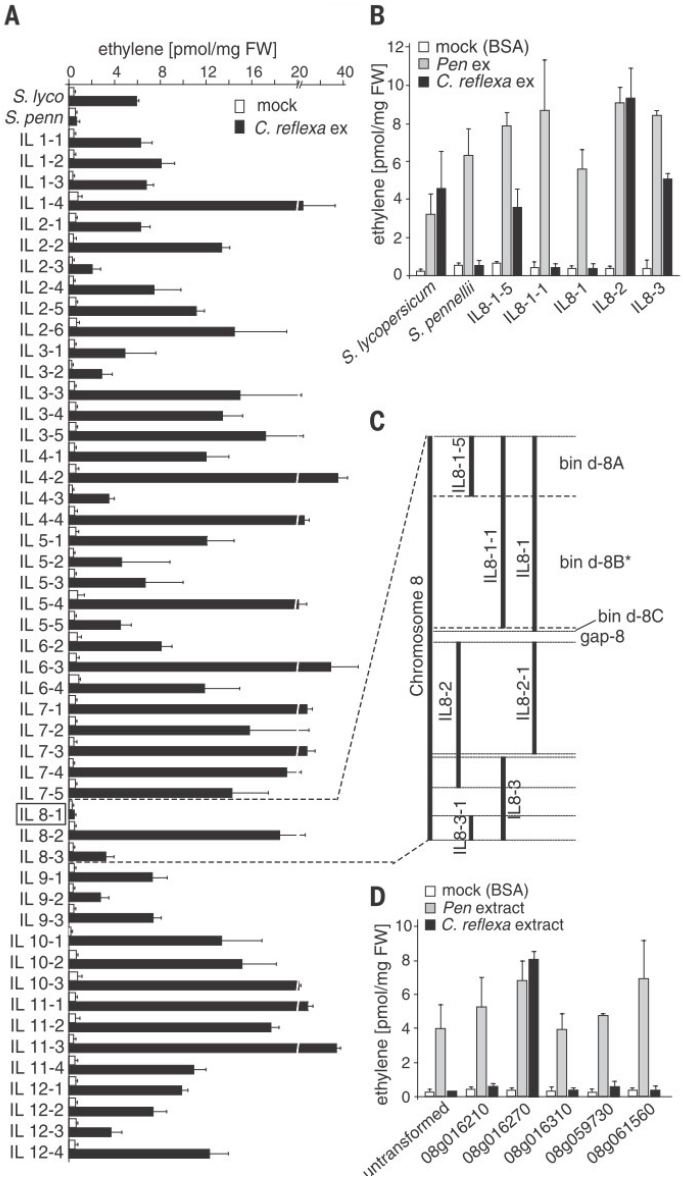

Figure 32: **Mapping the resistance/ susceptibility of tomato to *C. reflexa* by introgression lines and transiently expressed candidate genes in tobacco.** A. Ethylene production of *S. lycopersicum*, *S. pennellii* and the introgression lines without *C. reflexa* extract (mock, white bars) and with *C. reflexa* extract (black bars). B. Ethylene production of *S. lycopersicum*, *S. pennellii* and the lines in the region of interest without treatment, with pen1 as positive control and *C. reflexa* extract C. Map of chromosome 8 with ILs and IL bins. D. Ethylene production of *N. benthamiana* leaves untransformed and transformed with the receptor candidates (modified from Hegenauer et al., 2016).

The region of interest on chromosome 8 was narrowed down to the **IL bin d-8B** (see figure 32 B for the ethylene measurement of the relevant lines and Fig 32C for a graphic display of this chromosome region), as the neighboring line IL8-2, as well as the fine mapping lines IL 8-1-5

and IL8-2-1 showed responsiveness, whereas the fine mapping line 8-1-1 showed no responsiveness. The region comprises 822 annotated genes, but only five of them encode cell surface receptor-type proteins. We focused on these **receptor-type proteins** to investigate whether one of them is responsible for the recognition of CuF. These five genes were cloned and **transiently expressed** in *N. benthamiana*, a related solanaceous species which lacks *Cuscuta* perception and is susceptible towards *C. reflexa* infestation. Among these receptor candidates were three leucine-rich repeat receptor like proteins (LRR-RLPs) and two leucine-rich repeat receptor like kinases (LRR-RLKs). Four of these genes had no effect, but one gene (Solyc08g016270) enabled *N. benthamiana* leaf pieces to respond with ethylene production towards various *Cuscuta* preparations (figure 32 D). This LRR-RLP was henceforth termed CuRe1 for **Cu**scuta **Re**ceptor **1**.

CuRe1 is a **LRR-RLP** with an N-terminal signal peptide for export via the endoplasmatic reticulum, an LRR domain with 30-32 LRRs and 18 potential N-glycolsilation sites, a single transmembrane helix and a short cytoplasmatic tail (see figure 33). It can be found in *S. lycopersicum* genomic DNA and cDNA, but not in *S. pennellii* or IL8-1-1, which is also confirmed by the available sequence data, which show only truncated versions of *CuRe1* in *S. pennellii* (Hegenauer et al., 2016).

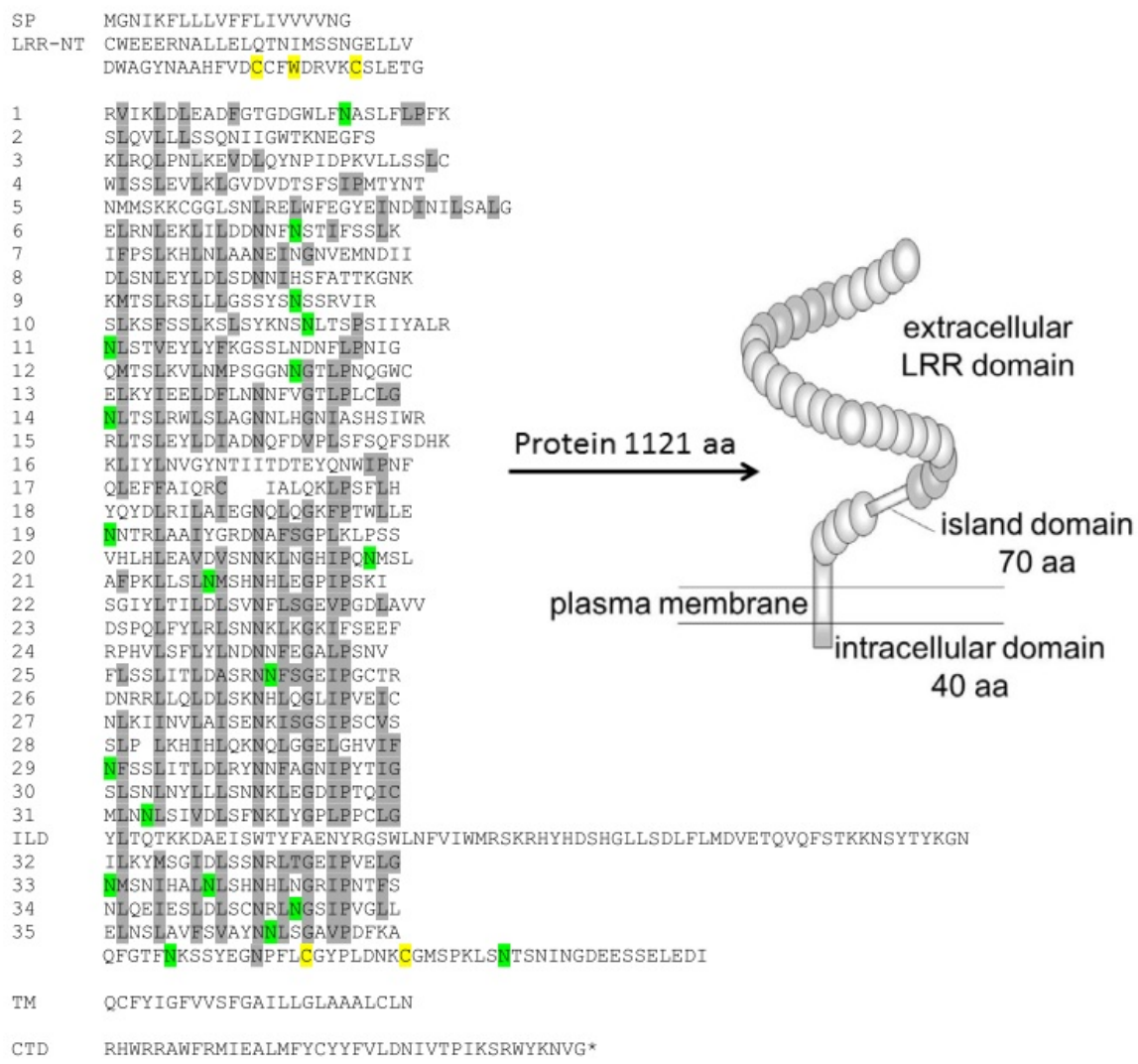

Figure 33: **CuRe1 protein amino acid sequence and model.** Left: amino acid sequence of the CuRe1 protein comprising a signal peptide (SP), a N-terminal domain (LRR-NT), tandemly arrayed LRRs (leucine-rich repeats, numbered, partially irregular LRRs #1, 2 and 5) interrupted by an island domain (ILD), a single pass transmembrane helix (TM) and a C-terminal cytosolic domain (CTD). Residues characteristic for the plant LRR consensus are highlighted in grey; potential N-glycosylation sites (NxT/S) are highlighted in green and residues characteristic for the N- and C-terminal parts of plant LRR-domains in yellow. Right: Model of the CuRe1 protein with LRRs indicated as ovals (Hegenauer et al., 2016).

Cuscuta preparations from the CEC column (peak1, peak2, peak3 in figure 21) were used to test whether all forms of CuF are able to act via CuRe1 (figure 34). As described in 3.3.1 the CuF has a heterogeneous structure, but since all forms can activate CuRe1, they appear to act on CuRe1 via a common molecular pattern as active principle.

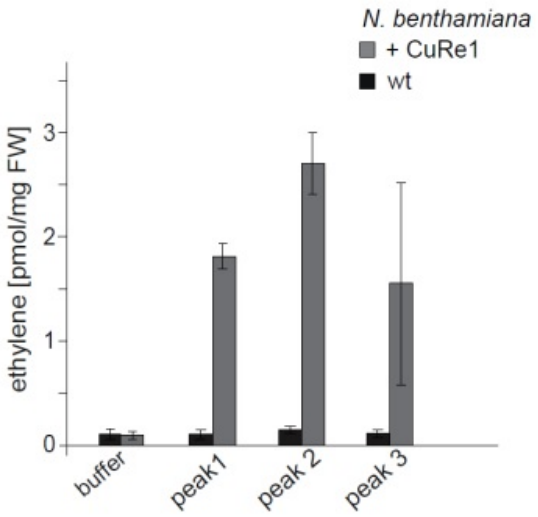

Figure 34: **CuRe1 expressed in *N. benthamiana* leaves is sufficient to confer responsiveness to the forms of *Cuscuta* factor present in eluate of CEC.** Ethylene response in non-transformed and in *N. benthamiana* leaves expressing *CuRe1*, an R-gene from IL bin 12-C, stimulated with buffer or various preparations of CuF (peak1, peak 2, peak3 from a CEC run, figure 21) (modified from Hegenauer et al., 2016).

Furthermore, to test whether the *Cuscuta* Factor is a common trait in all *Cuscuta* species, the extracts of **various *Cuscuta* species** were tested on *N. benthamiana* leaves expressing CuRe1 (figure 35). The transformed leaf pieces react with ethylene production towards all tested *Cuscuta* species, i.e. in addition to *C. reflexa* also *C. australis*, *C. parasitica*, *C. pentagona*, *C. europaea* and *C. monogyna*, but not the control plant *Rhinanthus* (Orobanciaceae), a root parasite.

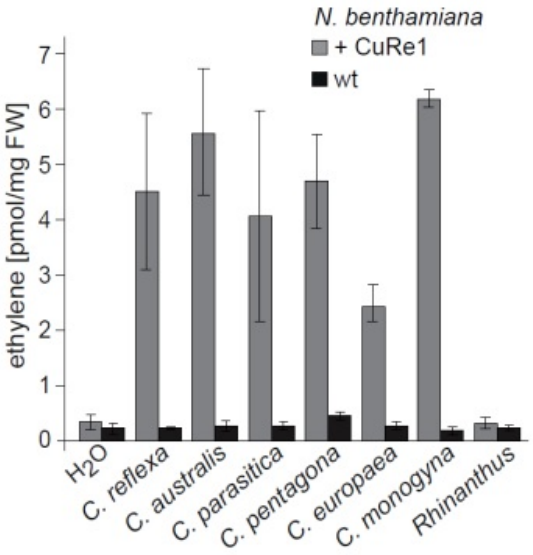

Figure 35: **Ethylene measurement of *N. benthamiana* leaf pieces transiently expressing CuRe1 with extracts of various *Cuscuta* species.** The Orobanciaceae *Rhinantus* was used as a control (modified from Hegenauer et al., 2016)

To test the ligand-receptor binding of CuRe1 with CuF an immunoprecipitation assay was performed with the receptors EFR and AtRLP23 from *Arabidopsis* and empty beads as controls as described, see 2.7.6. To corroborate its function as a genuine receptor that directly interacts with the *Cuscuta* factor as a ligand, it was tested whether **immunoprecipitates** of CuRe1 could specifically retain *Cuscuta* Factor when incubated with *Cuscuta* extract. *Cuscuta* Factor, assayed by the ethylene induction assay in tissue expressing CuRe1, was reproducibly detected in immuno-precipitates with CuRe1 but not with control receptors or empty beads (Hegenauer et al., 2016, figure 36).

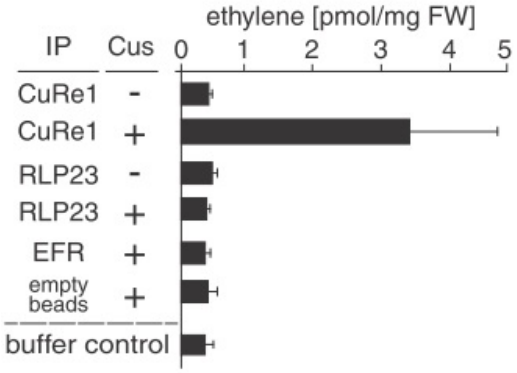

Figure 36: **CuRe1 exhibits properties as pattern recognition receptor for the *Cuscuta* factor (Binding)**. Binding of *Cuscuta* factor (Cus) to immunoprecipitates (IP) of CuRe1; EFR, AtRLP23, or empty beads were used as controls. Ethylene production of *N. benthamiana* leaf tissue expressing CuRe1 and treated with the elutions derived from the receptor IPs indicated; data are means ± SD of n = 3 replicates. The experiment was independently repeated >3 times (modified from Hegenauer et al., 2016).

For signalling output LRR-RLPs need an **interacting adaptor protein** with a kinase domain of the SOBIR1 (supressor of BAK1 interacting kinase)-type since they lack a cytoplasmic kinase. To confirm this interaction of CuRe1 with such kinase type in tomato, we made a coimmunoprecipitation with both tomato proteins, SlSOBIR1 ans SlSOBIR1-like. A constitutive interaction was shown, irrespective of the presence or absence of the CuF as stimulus (figure 37).

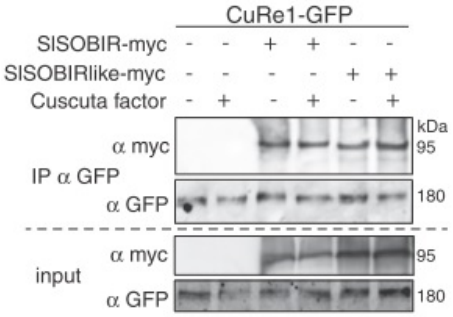

Figure 37: **CuRe1 exhibits properties as pattern recognition receptor for the *Cuscuta* factor (Co-Receptor)**. CuRe1 forms a complex with SOBIR1-type adaptor kinases. Immunoblots of SlSOBIR1-myc and SlSOBIR1-like-myc, coimmunoprecipitated with CuRe1; pulldown against the C-terminal green fluorescent protein tag present at CuRe1. Proteins were coexpressed in *N. benthamiana*, and samples were treated with CuF (+); 1:100 diluted in water or water alone (-) as control (modified from Hegenauer et al., 2016).

N. benthamiana and *S. pennellii* plants were stably transformed with *CuRe1* to investigate its biological function for resistance. Both plant species are otherwise completely susceptible towards *C. reflexa* attacks. The plants transformed with *CuRe1* gained responsiveness towards CuF in the ethylene assay as well as increased resistance. A hypersensitive response of the host, however, was not visible at the outer stem surface but only visible at the site of *C. reflexa* haustoria penetration after cutting the penetrated stem. Hence increased resistance can be conferred by the transformation of the tomato receptor in both, the closely related *S. pennellii* species as well as the more distant related *N. benthamiana* (figure 38). To confer this increased resistance to species outside the nightshade family a co-transformation with *CuRe1* and *SOBIR1* would be necessary. Additionally the resistance trait located on chromosome 12 needs to be taken into consideration to restore full resistance.

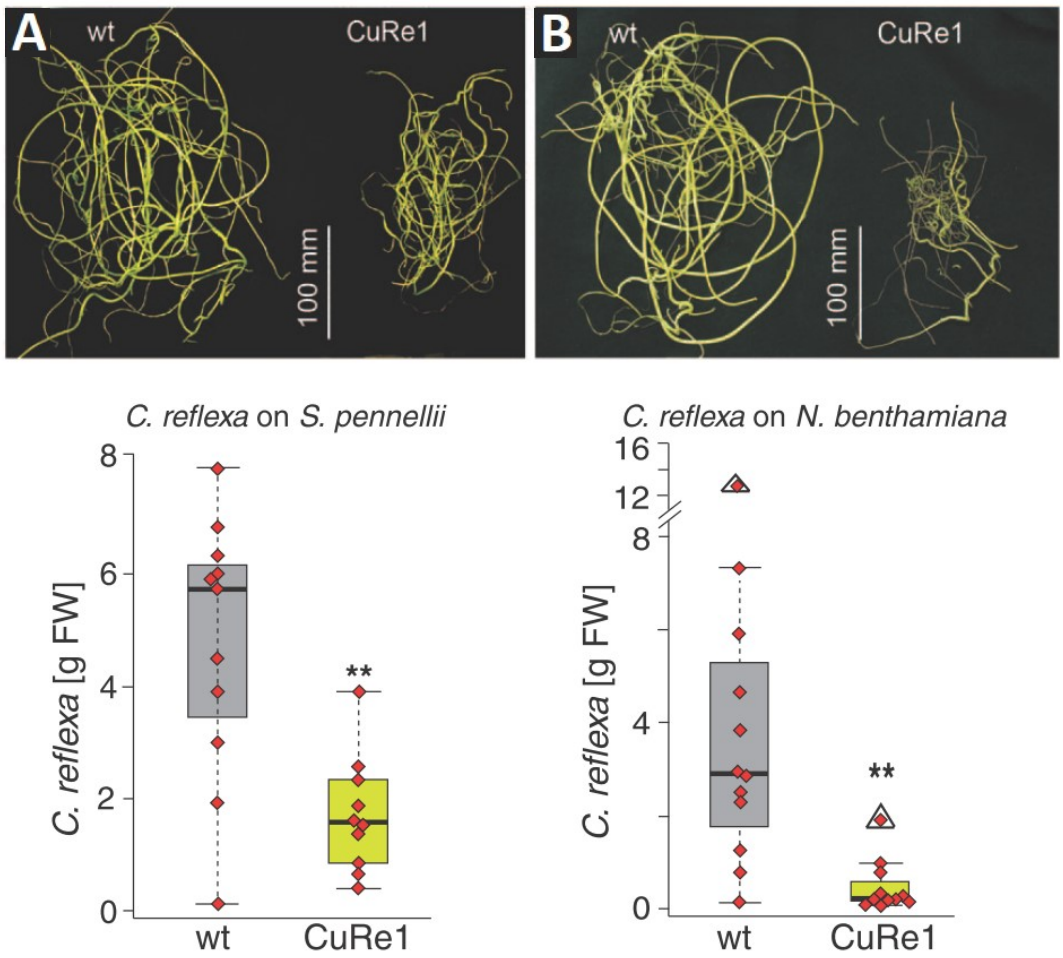

Figure 38: **Growth assay of *C. reflexa* on CuRe1-transformed and untransformed plants.** (A) Growth of *C. reflexa* shoots on *S. pennellii* plants transformed with *CuRe1* (T 1 generation) or nontransformed wild-type (wt) controls during 14 days of infestation with one *C. reflexa* shoot [15 cm in length, ~0.6 g FW] per host plant. Red diamonds represent weight of individual *C. reflexa* shoots. Boxplots show median values of n = 12 replicates. ** P adj = 0.0015 (Tukey honestly significant difference test). (B) Growth of *C. reflexa* shoots on *N. benthamiana* plants stably transformed with *CuRe1* (homozygous T 2 generation) or non-transformed wild-type controls during 21 days of *C. reflexa* infestation. Experimental conditions and data evaluation were as in (E). Triangles mark outliers not included in analysis. ** P ≤ 0.005 (Student t test). Data presented in (E) and (F) are representative of three independent repetitions, each with different lines of transformants (modified from Hegenauer et al., 2016).

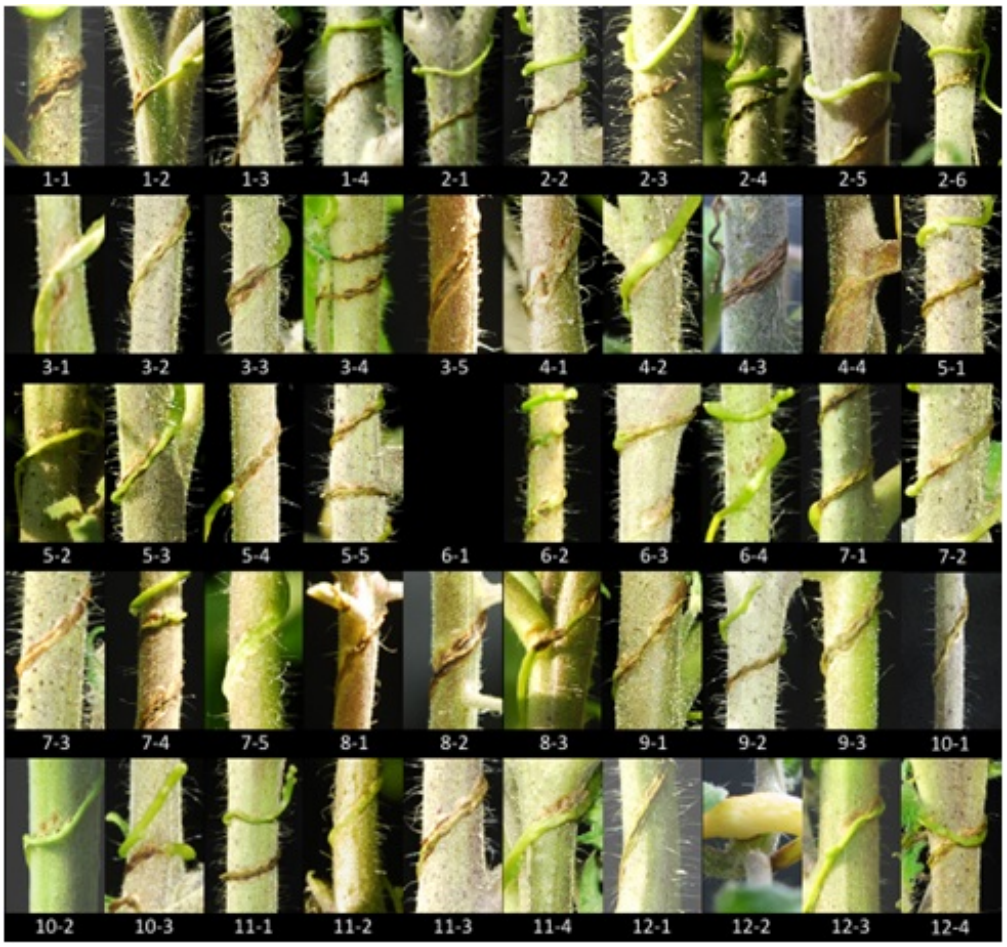

Figure 39: **Infection assay of the introgression lines.** The pictures show the *S. lycopersicum* x *S. pennellii* introgression lines after ~ 5 weeks of infection with *C reflexa*. The parasite is starting to dry out in all but one line (12-2) and necrotic spots are visible at the tomato stems (Kaiser, 2010).

To establish full resistance towards *C. reflexa* in plants other than *S. lycopersicum*, more than CuRe1 perception is needed. Evidence for this is that the IL8-1-1 and IL8-1 are lacking CuRe1 but are still fully resistant towards *C. reflexa* attacks. The key to full resistance is likely to be found on tomato chromosome 12, in the IL bin 12-B, as only the line 12-2 showed decreased resistance in the infection assay (figure 39).

Figure 40: **Infection assay of IL12-3-1**. *C. reflexa* is able to penetrate the stem of IL12-3-1 successfully and to grow on it. Tomato shows a reduced defense reaction (left picture taken by B. Kaiser, right picture taken by V. Hegenauer).

For fine mapping also the line 12-3-1 was tested in the infection assay. This fine mapping line reduces the region of interest to **bin 12-D** (**d-12E** in the new designation by Chitwood et al., 2013) and comprises much less genes (~300) than the 12-2 line, where nearly all of chromosome 12 is *S. pennellii* genome material (see for details: figure 41, Chitwood et al., 2013). For a list of candidate genes see supplementary table 1.

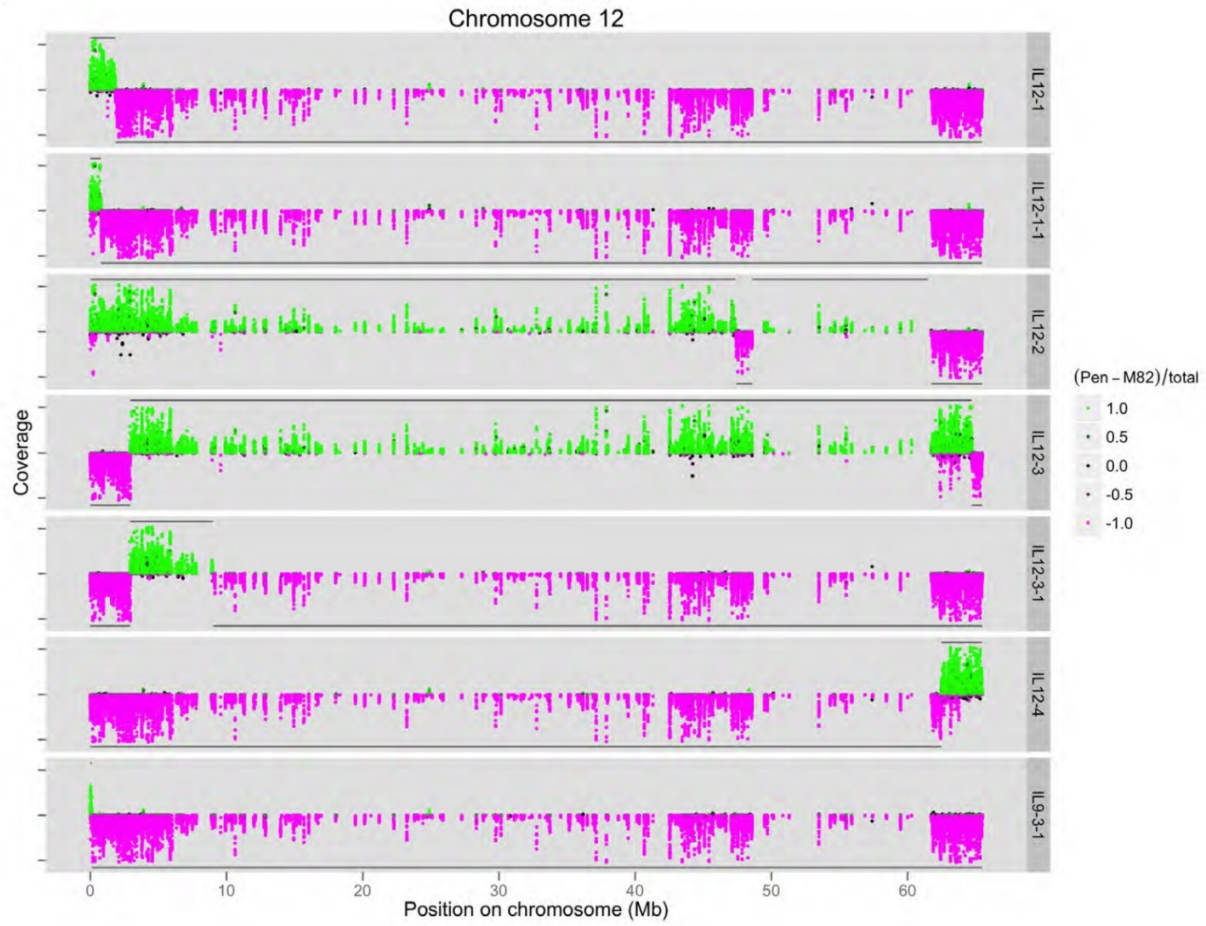

Figure 41: **RNAseq-based genotyping of chromosome 12.** Shown are the *S. pennellii* introgression regions for ILs covering chromosome 12 as determined by RNA-Seq. The depth of coverage (distance from midpoint on y-axis) and genotype (color and direction on y-axis) of each SNP/ indel is plotted against chromosomal position (x-axis). Polymorphisms that match *S. pennellii* are colored green and plotted on the top half of each IL panel, while polymorphisms matching cv. M82 are plotted in magenta in the bottom halves. The coloring is on a continuum such that the color approaches black as a position's genotype approaches heterozygosity. The y-axis tick marks indicate depths of coverage ranging from 0 to 100. Subsequent to genotyping, introgression boundaries consistent between the RNA-Seq and RESCAN analyses were delineated. Using these breakpoints, *S. pennellii* and cv. M82 regions are summarized by horizontal lines at the top and bottom of each IL panel, respectively (Chitwood et al., 2013).

4. Discussion

4.1 The Cuscuta Factor (CuF)

The results confirmed the assumption, that the recognition of parasitic plants works analogous to the recognition of microbes. A parAMP, i.e. a parasite associated molecular pattern from *C. reflexa* is recognized in the resistant host plant *S. lycopersicum* and other related plants in the genus of *Solanum*. This parAMP named CuF for *Cuscuta* Factor is of proteinaceous origin and can be **enriched** and **purified** from watery extracts of *C. reflexa*.

The purification and identification of CuF is a still ongoing process. A promising protocol has been established, starting with an acidic, watery extraction, a pre-purification by cation exchange chromatography, followed by several runs of reversed phase chromatography for enriching and purifying the CuF and finally a mass spectrometry analysis. Limiting factors, so far, include the amount of starting material, i.e. freeze dried *C. reflexa* shoots. Also the extraction method could be improved to increase the initial yield of bioactive CuF, defined as *Cuscuta* Units (CU). It could be shown by Hegenauer et al. that a lower pH in the extraction buffer resulted in a 10 x higher amount of extracted CuF. Furthermore it is unclear whether a more rapid handling could improve the quality of the purification and keep extracted CuF more stable.

To establish an appropriate purification protocol, CuF has been characterized in more detail. While the *Cuscuta* factor is of **proteinaceous** character, the 3D structure seems less important, because boiling does not result in loss of activity. Binding to **cation exchangers** (but not to anion exchangers) demonstrates that CuF has a positive charge. CuF is not localized to any special organ of the plant, but can be found everywhere in the *C. reflexa* shoot. Additionally, CuF can be extracted with the same protocol from other *Cuscuta* species.

CuF is most likely a **heterogenous** mixture of compounds, as the active components spread over the various columns no matter which principle is used for separation (size/ charge/ polarity). Similar problems have been encountered before in MAMP purification e.g. in the purification of the elicitor pen1 (here often used as positive control) from *Penicillium chrysogenum*, which turned out impossible to purify. Pen1, when applied to any kind of column separating by size, charge or polarity, no sharp peak occurred, but the elicitor activity spread

over a wide number of fractions (Thuerig et al., 2006). An elution fraction of pen1 eluted at the same point of the gradient, when repeatedly separated under the same conditions. This shows that the outcome of the first chromatographic step is no artefact, but is probably due to the characteristics of the molecules.

A spreading during column chromatography could also occur when the protein or peptide has a secondary modification, such as sugar residues of diverse lengths. Proteins from plants are often secondarily modified. Examples for such modifications are glycosyl residues, lipid chains, sulfates or phosphor residues. Purification and identification of such post-translationally modified proteins is often complex and involves specialized equipment such as gas chromatography coupled to mass spectrometry or nuclear magnetic resonance (NMR) spectrometry. Especially for NMR, a high amount of pure protein is needed. However, a few simple assays exist, to exclude possible modifications. For example, to test whether CuF is N-glycosylated, it was treated with de-glycosylating enzymes. This approach, however, turned out to be negative since CuF samples were not destroyed by such enzymes. To test if O-glycosylations are relevant for activity, a chemical assay was used in which the glycan side chains are removed by β-elimination with NH_4OH (Rademaker et al., 1998). The inactivation of CuF by such treatments, would indicate an O-glycosylation. However, later it was discovered that any exposure to high pH is detrimental for the *Cuscuta* activity, even the addition of Tris buffer with pH8, and must be avoided throughout the whole purification process. This means, that another assay to check for O-glycosylations needs to be found, which does not involve base treatment.

In general, we collected a lot of evidence for the presence of a **parAMP** in *Cuscuta* species. There is a soluble peptidic factor present in the crude extract of *C. reflexa* capable of inducing ethylene and other plant defense responses in tomato plants with Cure1. As this ability ceases with protease treatment, the assumption is favored that the factor responsible requires a peptide backbone. Moreover, the identification of a receptor in tomato, which confers ethylene production and enhanced resistance to plants otherwise susceptible to *C. reflexa* is the most convincing argument for the presence of a parAMP in *Cuscuta* species.

But what might be the sense of such a “Cuscuta Factor”? Was it an evolutionary invention for being detected by tomato and for initiating defense? Rather not. But, maybe, it is something specific to and necessary for *Cuscuta* species, something that is helpful for their parasitic lifestyle, or something which is used for communication with the host plants, which can be investigated when the sequence is found.

4.2 CuRe1 and downstream signaling

In spite of being yet unidentified, the *Cuscuta* Factor is now characterized as a small molecule with a peptide backbone. The corresponding receptor is a leucine-rich repeat receptor-like protein (**LRR-RLP**) which is named **CuRe1** (*Cuscuta* Receptor1). As LRR-RLPs are already known to mediate resistance in plants, this is similar to the perception of microbe associated molecular patterns (MAMPs). Examples are the perception of eMax from the bacteria *Xanthomonas* spp. by the Arabidopsis RLP ReMAX (Jehle, 2013a; Jehle, 2013b) or the perception of fungal Xylanase (EIX) by LeEIX of tobacco and tomato cultivars (Leibman-Markus, Schuster and Avni, 2017, Hanania and Avni, 1997). Since RLPs do not work singularly, but as a dimer with a helper protein possessing a kinase domain to transfer the incoming signal through the plasma membrane, an interaction partner is crucial and could be identified as **SOBIR1** (Hegenauer et al., 2016), which is also known to interact with other LRR-RLPs (Liebrand et al., 2013) . Also known, is a ligand-dependent heteromer formation of RLP-SOBIR1 with co-receptors of the SERK family (Albert et al., 2015, Böhm et al., 2014).

If transferred to other usually susceptible *Solanaceae* species such as *S. pennellii* and *N. benthamiana*, CuRe1 mediates partial resistance (Hegenauer et al., 2016). On transgenic lines of *S. pennellii* and *N. bethamiana* expressing the tomato *CuRe1* gene, the growth rate of *Cuscuta reflexa* was reduced, but infection was not totally abolished as usually observed in cultivated tomato. Therefore, the recognition of CuF by CuRe1 contributes to the resistance of cultivated tomato towards *C. reflexa*, but is not solely responsible as parasite growth was not completely blocked. Also important, the strong HR-symptoms at the tomato stem could not be observed in transgenic *N. benthamiana*. Instead, HR-symptoms occurred at the interface of ingrowing haustoria tips. A further hint that CuRe1 is not the inducer of HR is given by the fact

that IL8-1-1, the IL lacking CuRe1 still shows resistance and a HR similar to infected wild type tomato.

4.3 Other R-genes related to Cuscuta in tomato

Screening for susceptible tomato ILs, which indicate for other resistance related genes seeming to exist, especially as a probable region is already mapped on tomato chromosome 12 (figure 39, chapter 3.4). After mapping the **Bin d-12E**, a region of ~5Mbps comprising 431 genes, we obtained a list of potential LRR-receptor proteins and other resistance related gene candidates. Since the number is limited to ~15 potential resistance receptors, it will be one goal to clone and stably transform them into *N. benthamiana* plants which are usually susceptible for a *C. reflexa* infection (supplementary table 1). Testing those transgenic plants for susceptibility or resistance to *C. reflexa* infestation will clearly indentify relevant resistance receptors.

Finding an elicitor corresponding to this putative receptor will be rather complicated as the only substantial finding is the non-resistance of *S. lycopersicum* x *S. pennellii* introgression lines 12-2 and 12-3-1. There is no suitable fast bio-assay such as the measurement of ethylene, which can be used as a convenient output system. Both ILs are still responding with ethylene production as well as the production of reactive oxygen species (ROS) towards treatments with *C. reflexa* extracts. Other output systems should be tested and established as bioassays, for example the induction of resistance genes known from PAMP-activated model systems (like FRK), which can be monitored in transiently transformed protoplasts. (Asai et al., 2002) This output system is not as convenient as the ethylene production assay: The production of tomato protoplasts is rather time consuming and in the required amount not always successful. For the ROS assay, only extracts with no quenching effects can be used, which makes it unsuitable for initial screening. Another potential output system could be extracellular medium alkalinization of cell suspension cell cultures. For this bioassay extracts must be prepared which have no effect (e.g. as buffer) on the extracellular pH by themselves. As a control, a cell culture line of the tomato introgression line 12-3-1 would be useful, but the establishment of cell culture lines is a long and complicated process. Otherwise a screening may be done on the basis of infection or to

screen for active HR-inducing compounds of *C. reflexa* haustoria extract an infiltration assay into tomato leaves may be feasible. Moreover it must be taken under consideration, that the resistance gene in the IL-bin 12-C could be no pattern recognition receptor at all, but a downstream component of the signaling pathway or a more general factor. This was already tested by applying various MAMPs on leaf pieces of IL 12-2. As line 12-2 shows normal ethylene responses towards other MAMPs, it is assumed that the resistance locus on IL-bin 12-C is indeed a **receptor protein**, and further investigations will focus on receptors in this region, for a list of candidates, see also supplementary table 1. A putative **model for *Cuscuta* resistance** adapting the zig-zag model (Jones and Dangl, 2006) is displayed in figure 42. Importantly, if R-genes are involved and a kind of ETI is displayed, typical MAMP responses as mentioned above, like ethylene production and ROS-burst commission, could not be detected. In turn, HR-responses such as observed in *C. reflexa* – tomato interaction, are typically controlled by R-genes and encoded NBS-LRR proteins. Indeed, in bin 12-d-E, we can also find three genes encoding NBS-LRR proteins (supplementary table 1, supplementary material).

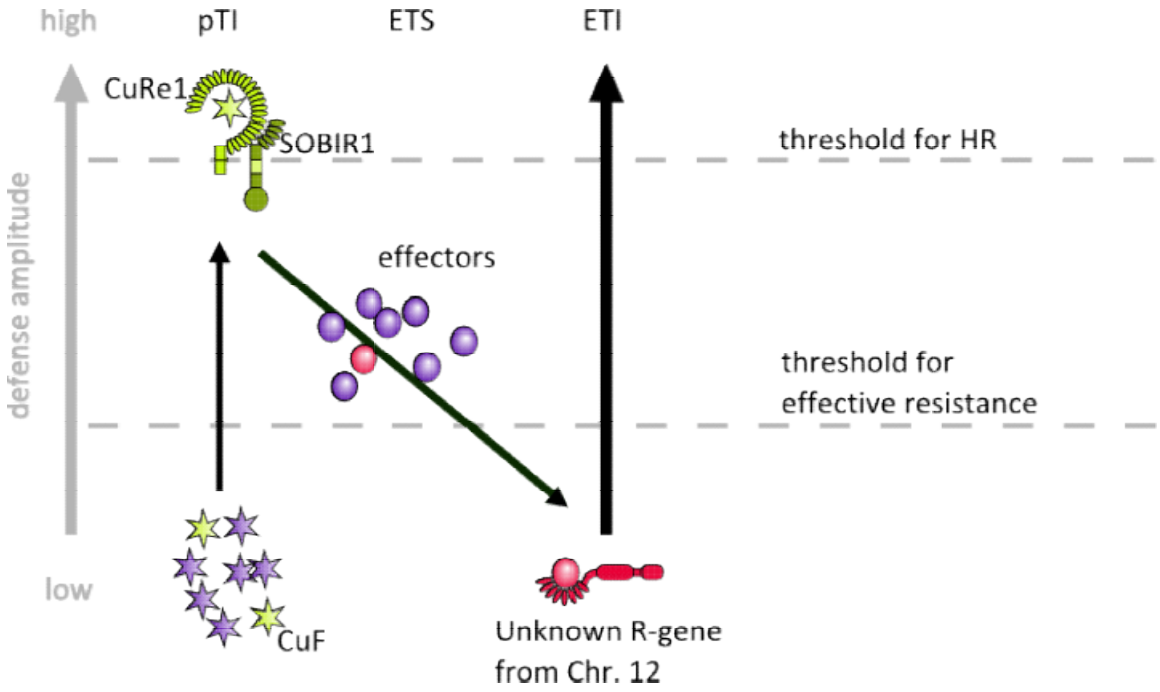

Figure 42: **Model for *Cuscuta* resistance:** The recognition of CuF by the CuRe1 receptor and the signal transduction via its co-receptor SOBIR1 leads to parAMP triggered immunity (pTI), effectors of *Cuscuta* lead to effector triggered susceptibility (ETS). Some of these effectors are recognized by other receptors, especially the putative receptor on chromosome 12. This leads to effector triggered immunity (ETI).

Tomato's resistance against *C. reflexa* is rather specific. Other *Cuscuta* species, e.g. *C. campestris* and *C. pentagona* native to North America can successfully infect tomato with yield losses in the US and India of 50-75% (Lanini and Kogan 2005, Mishra, 2009). Interestingly, *C. reflexa* is indigenous in Asia, whereas tomato's origin is in South America and has no history of co-evolution with *C. reflexa*.

It can be speculated, that the *Cuscuta* species native to South America, which undergo a co-evolution with tomato, have evolved effectors to circumvent the tomato defense, as there are no resistances of tomato towards any South American species reported so far.

Moreover, the relatedness of the North American species, which are known to aggressively attack tomato, to the South American species, would be worth investigating. Unfortunately, phylogenetic data and knowledge about ecological importance as well as geographical distribution of *Cuscuta* species is insufficient. Sometimes species are known only by single specimens in herbarium material (Costea and Stefanovic, 2009).

4.4 Chances, prospects and considerations of the results

Invasive species are a growing problem in many ecosystems all around the world. Dodder is a parasitic plant which has all features of a successful invader: fast vegetative growth, massive proliferation and broad host specificity. Modern agriculture with its vast monocultures and damaged ecosystems are setting the scene for the ongoing rise of **invasive weeds**. Worldwide markets, seed exports, climate change and the increasing amount of travelers give new chances for the so-called neobiota. This challenge will only be mastered by humans with a combination of traditional containment strategies and modern technology. An approach is to use green biotechnology to transfer genes from resistant species to susceptible crops.

It was shown in this thesis that the biotech approach is feasible, although the path to a commercial product is still far, and under the current climate of skepticism towards genetically modified crops, it may not come to actually selling *Cuscuta* resistant crops at all, at least not here in central Europe.

Nevertheless an important milestone was reached, as the identification of the tomato receptor CuRe1 enabled a better understanding of the incompatible host plant's perception mechanism. Before, the mechanisms of perception were completely unknown. It was clarified, that the molecular detection of the invading plant parasite is functionally similar to the detection of microbes. There is at least one (if not more) parasite associated molecular pattern (parAMP) in *Cuscuta* ssp., which we named *Cuscuta* Factor (CuF) with a corresponding perception mechanism in *Solanum lycopersicum* and other *Solanum* species.

4.5 Outlook

This thesis opens many perspectives for future research and applications. So far, CuRe1 has only been transferred to agriculturally unimportant species. But it can also be transferred to crop species especially growing in Asia, where *C. reflexa* is native and most devastating. Plant species of **commercial interest** for transformation are for example chili, green gram/ black gram, lentils, chickpea and alfalfa. Yield losses due to *Cuscuta* infestation in India in these plants vary from 31-34% in greengram/ blackgram and 87% in lentils (Mishra, 2009).

To get further insights about how the resistance mechanism evolved in tomato, the CuF needs to be identified. After identification CuF could be produced synthetically and binding studies could be performed. This can also be investigated with the CuF sequence: has it homologues in tomato, and what are their functions? Or has CuRe1 any other functions, maybe it recognizes microbe associated molecular patterns that structurally mimick CuF and induce immunity against microbes? Important though, recognition of CuF by Cure1, while inducing defense responses known to be induced by microbial patterns, does result in a significantly induced resistance of host plants to the infestation by *Cuscuta*. To find out more about this, it may be also feasible to look whether *Cuscuta* or other organism exhibiting CuF-type of elicitors is/was a selective trait in tomato species in their native habitats.

With the sequence of the gene encoding the CuF precursor protein, and DNA from various *Cuscuta* species, the ones we already found to elicit ethylene via CuRe1, as well as the South American *Cuscuta* species and *C. campestris*[3], it could be further confirmed, whether CuF is indeed a common trait of all *Cuscuta* species. It would corroborate its designation as parAMP.

Also, to mediate full resistance, the other locus on chromosome 12 must be investigated to ultimately answer the question why tomato is resistant towards *C. reflexa* and not to *C. campestris*? Are there effectors suppressing the innate immunity originating from *C. campestris* comparable to effectors known from bacteria (Espinosa and Alfano, 2004)? A first hint for this theory is that *C .campestris* extract can trigger ethylene production, but not HR in tomato. In the PTI/ETI theory, a MAMP alone is often not strong enough to trigger HR, whereas detection of an effector by a corresponding R-gene often mediates full response including HR-based resistance (Jones and Dangl, 2006). PTI and ETI induction pathways for defense responses overlap, as shown in microarray experiments (Tao et al., 2003). Hence it can be stated, that the CuF parAMP is an effective trigger of defense against *Cuscuta*. If the binding and function of CuRe1 are further investigated, a genetically engineered CuRe1 protein could be produced, which can also induce an effective HR towards *C. campestris*. It should also be investigated,

[3] The *Cuscuta* species *C. pentagona* (fiveangled dodder) and *C. campestris* (field dodder) are both native to North America and closely related. Unlike *C. pentagona*, *C. campestris* has become established on other continents and is for example also a major pest in India (Mishra, 2009).

whether there is another CuF-binding protein present in tomato, which modifies and attenuates CuF defense responses, similar to the LeEix1 receptor which has this function in Eix binding (Bar et al., 2010). Thus, a tomato (and other crop plants) could be produced, which fends off *C. campestris* as effectively as *C. reflexa*. This could be feasible as *C. campestris* poses a threat to tomato yield as well as to other important crop plants. Native to North America *C. campestris* is introduced to 55 countries around the world and is the most important weed, as it has a broad host range (reported in 25 crops) (Lanini and Kogan, 2005).

For the completion of the picture, also the wild tomato species should be taken under consideration. As mentioned before, there are still important species in the tomato clade, which should be tested for resistance. Furthermore, it would be interesting to sequence the different versions of CuRe1 in the various tomato species and compare their ability for binding CuF. Maybe even insights of the molecular binding mechanism can be gained thus. A whole array of differing CuRe1 receptors from the various wild tomato species is supposable, which could also differ slightly in their binding affinity. From this data a 3-D model could be made picturing how CuF exactly binds to CuRe1 and how changes in the amino acid structure of the receptor can affect the binding. This could be a step towards engineered receptors modified to bind the ligands of the current threats to agriculture. This way green biotechnology would empower the crops to keep the biotic attacks to their growth and yield in check.

5. Thanks to…

To complete this work, many people have contributed, but I will name here only a few.

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5. Supplementary material

Supplementary table 1: *C. reflexa* resistance candidate genes on tomato chromosome 12, bin d-12E

Leucine rich repeat-receptor like proteins		
1	LRR-RLP	Solyc12g009720
2	LRR-RLP	Solyc12g009730
3	LRR-RLP	Solyc12g009740
4	LRR-RLP	Solyc12g009750
5	LRR-RLP	Solyc12g009770
6	LRR-RLP	Solyc12g009780
7	LRR-RLP	Solyc12g013680
8	LRR-RLP	Solyc12g013730
9	LRR-RLP	Solyc12g013740
10	LRR-RLP	Solyc12g013680
Leucine rich repeat-receptor like kinases		
1	LRR-RLK	Solyc12g010740
2	LRR-RLK	Solyc12g014350
Coiled-coil-nucleotide-binding site-leucine-rich repeat proteins		
1	CC-NBS-LRR	Solyc12g016220
2	CC-NBS-LRR	Solyc12g017800
Other resistance proteins		
1	AHRD V1	Solyc12g013970
2	AHRD V1	Solyc12g016130
3	LRR resistance protein fragment	Solyc12g017480
4	AHRD V1	Solyc12g017810
5	AHRD V1	Solyc12g017550
6	AHRD V1	Solyc12g009870
7	NBS protein fragment	Solyc12g010660

Supplementary table 2: Abbreviations.

Abbreviation	Description
ACC	1-Aminocyclopropane-1-carboxylic acid
AEC	Anion exchange chromatography
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
BIR1	BAK1-interacting receptor-like kinase 1
CC-NBS-LRR	Coiled-coil nucleotide binding site leucine-rich repeat protein
CEC	Cation exchange chromatography
cDNA	Complementary desoxyribonucleic acid
cM	Centi Morgan
CuF	Cuscuta factor

CuRe1	Cuscuta receptor 1
DAMP	Damage associated molecular pattern
Da	Dalton
ESI	Electrospray ionization
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
FW	Fresh weight
GFP	Green fluorescent protein
HR	Hypersensitive response
HLR	Hypersensitive like response
HPLC	High Performance Liquid Chromatography
IEF	Isoelectric focusing
IP	Isoelectric point
IL	Introgression line
JA	Jasmonic acid
LC	Liquid chromatography
LRR-RLK	Leucine-rich repeat receptor like kinase
LRR-RLP	Leucine-rich repeat receptor like protein
MAMP	Microbe associated molecular pattern
MAPK	Mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
mS	Milli Siemens
MS	Mass spectrometry
OD	Optical density
PAMP	Pathogen associated molecular pattern
parAMP	Parasite associated molecular pattern
PRR	Pattern recognition receptor
pTI	parAMP triggered immunity
PTI	PAMP triggered immunity
QTL	Quantitative trait locus
RFLP	Restriction fragment length polymorphism
Rpm	Rounds per minute
RNAi	Interference ribonucleic acid
RPC	Reversed Phase Chromatography
ROS	Reactive oxygen species
SA	Salicylic acid
SGN	Sol Genomics Network
SOBIR1	Suppressor of BIR1
TIC	Total ion current
UPLC	Ultra Performance Liquid Chromatography

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