The infection biology of the smut fungus *Thecaphora thlaspeos* and its molecular cross-talk with its Brassicaceae hosts

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Statement of authorship

I hereby declare that this dissertation is the result of my own work. No other person's work has been used without due acknowledgement. This dissertation has not been submitted in the same or similar form to other institutions. I have not previously failed a doctoral examination procedure.

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The research detailed in this thesis was conducted from March 2014 until February 2018 in Düsseldorf at the Heinrich Heine University Düsseldorf in the institute for Microbiology under the supervision of Prof. Dr. Michael Feldbrügge.

Table of contents

1 Summary

Modern agriculture faces challenges to increase overall crop yield in order to feed a growing population. A key factor contributing to these challenges include the impact of biotic stress on crops, of which fungal pathogens instigate a large portion and are therefore crucial to study in order to develop agricultural practices that can mitigate their impact. A prominent order of plant pathogenic fungi within the Basidiomycete phylum is the Ustilaginales, commonly known as "smut fungi", which mostly infect grasses. Despite the vast information and tools that exist to functionally study the model smut fungus that infects maize, *Ustilago maydis*, especially regarding pathogenicity, the genetics of its maize host remain complex. To date, an R-gene that confers resistance to *U. maydis* remains to be identified, cloned, and ultimately introduced into maize. Furthermore, the genetic complexity of the host has also made it difficult to functionally characterize host responses to smut fungal infection. *Thecaphora thlaspeos* is the only described smut fungus to infect within the Brassicaceae family. Here, I present the infection process of *T. thlaspeos*, whose spores I have collected annually in the field, which displayed that germ tubes arising from germinating spores can directly enter the roots and leaves of the host. *T. thlaspeos* eventually proliferates throughout the entire plant in an endophyte-like fashion and infection studies showed that *T. thlaspeos* infects several *Arabis spps*. as well as the model plant *Arabidopsis thaliana.* Extensive confocal microscopy indicated that *T. thlaspeos* colonization occurs along but outside the host vasculature tissues, contrary to other systemic-infecting smut fungi. The establishment of this infection system in the lab successfully led to the sporulation of *T. thlaspeos* in silique tissue of *Ar. hirsuta*, these host plants otherwise lacking obvious infection symptoms. Transcriptome analysis on *T. thlaspeos*-infected *Ar. hirsuta* revealed that *in planta* induced fungal genes are enriched for unique, secreted proteins, from which I demonstrated that two have virulence functions in *A. thaliana*, *Tt*Nlp1 potentially suppressing PTI and *Tt*Tue1 potentially activating host immune responses. Typical for biotroph infection, my data strongly suggest that biotic stress is enriched during *T. thlaspeos* infection. Interestingly, the expression of several key genes involved in salicylic acid-dependent defense signaling is not significantly altered while a key gene involved in ethylene-dependant defense signaling is induced, potentially indicating how *T. thlaspeos* behaves as a "minor" pathogen. This work lays the foundation for studying how *T. thlaspeos*-specific effectors likely play a role in the overall endophytic nature of this fungal pathogen and ultimately provides a more genetically accessible platform for the identification and functional characterization of R-genes that confer resistance to smut fungal pathogens.

2 Zusammenfassung

Die moderne Landwirtschaft steht vor der Herausforderung, den gesamten Ernteertrag zu erhöhen, um eine wachsende Bevölkerung zu ernähren. Ein wichtiger Faktor, der zu diesen Herausforderungen beiträgt, sind die Auswirkungen von biotischem Stress auf Nutzpflanzen, von denen pilzliche Krankheitserreger einen großen Anteil haben und daher für die Entwicklung landwirtschaftlicher Praktiken, die ihre Auswirkungen mildern können, von entscheidender Bedeutung sind. Eine prominente Ordnung von pflanzenpathogenen Pilzen innerhalb der Basidiomyceten sind die Ustilaginales, allgemein bekannt als "Brandpilze", die hauptsächlich Gräser infizieren. Trotz der umfangreichen Informationen und Werkzeuge, die existieren, um den Modellbrandpilz *Ustilago maydis*, der Mais infiziert, insbesondere in Bezug auf Pathogenität funktionell zu untersuchen, bleibt die Untersuchung der Pflanzenantwort aufgrund der komplexen Genetik seines Maiswirts schwierig. Bis heute gibt es kein bekanntes R-Gen, das eine Resistenz gegen *U. maydis* verleiht. Darüber hinaus erschwert die genetische Komplexität des Wirtes Wirtsantworten gegen Pilzbefall zu charakterisieren. *Thecaphora thlaspeos* ist der einzige beschriebene Brandpilz, der Pflanzen der Familie der Brassicaceae befällt. Hier stelle ich den Infektionsprozess von *T. thlaspeos* vor, dessen Sporen ich jährlich auf dem Feld gesammelt habe. Dabei konnte ich zeigen, dass Keimschläuche, die von keimenden Sporen stammen, direkt in die Wurzeln und Blätter des Wirtes eindringen können. *T. thlaspeos* wächst endophytisch durch die gesamte Pflanze. *T. thlaspeos* infiziert mehrere *Arabis*-Arten und insbesondere auch die Modellpflanze *Arabidopsis thaliana*. Umfangreiche konfokale Mikroskopie zeigte, dass *T. thlaspeos*-Kolonisierung im Gegensatz zu anderen systemisch-infizierenden Brandpilzen entlang, aber außerhalb des Wirtsgefäßgewebes auftritt. Die Etablierung dieses Infektionssystems im Labor führte erfolgreich zur symptomfreien Infektion bis hin zur Sporulation von *T. thlaspeos* im Schotengewebe von *Ar. hirsuta*. Eine Transkriptomanalyse von *T. thlaspeos*-infizierten *Ar. hirsuta* zeigte, dass *in planta* induzierte Pilzgeneangereichert sind, die für einzigartige, sekretierte Proteine kodieren. Ich konnte zeigen, dass zwei dieser Proteine Virulenzfunktionen in *A. thaliana* haben, wobei *Tt*Nlp1 möglicherweise PTI supprimiert und *Tt*Tue1 potentiell Wirtsimmunreaktionen aktiviert. Wie typisch für biotrophe Infektionen, legen meine Daten nahe, dass biotischer Stress während der *T. thlaspeos*-Infektion erhöht ist. Interessanterweise wird die Expression mehrerer Schlüsselgene, die an Salicylsäure-abhängigen Abwehrsignalen beteiligt sind, nicht signifikant verändert. Im Gegensatz dazu ist ein Schlüsselgen induziert, das am Ethylen-abhängigen Abwehrsignalweg beteiligt ist, , was möglicherweise darauf hinweist, dass sich *T. thlaspeos* wie ein "schwacher" Krankheitserreger verhält. Diese Arbeit legt den Grundstein um zu verstehen, welche Rolle *T. thlaspeos*-spezifische Effektoren wahrscheinlich bei der gesamten endophytischen Natur dieses Pilzpathogens spielen und bietet

schließlich eine genetisch besser zugängliche Plattform für die Identifizierung und funktionelle Charakterisierung von R-Genen, die eine Resistenz gegen Brandpilze verleihen.

3 Introduction

3.1 Plant pathogens and modern agriculture

It is well established that the greatest challenge facing modern agriculture is filling the crop yield gap (Godfray *et al.*, 2010), much of which is widened by the devastating effects of biotic stress (Savary *et al.*, 2012; Suzuki *et al.*, 2014; Pandey *et al.*, 2017). Modern agriculture heavily relies on a variety of practices to combat the constant challenge that pathogens present. From the early twentieth century, fungicides were developed as a key tool to keep crop loss to a minimum (Brent & Hollomon, 2007; Ellis *et al.*, 2014). However, over time, fungicide resistance observed especially for the gray mold fungus *Botrytis cinerea* (Hahn, 2014), powdery mildew fungus *Blumeria graminis* (Brent & Hollomon, 2007), and rust pathogens (Oliver, 2014) has become an ever worsening issue. Similarly, as plant resistance genes (R-genes) were being used in breeding programs beginning in the early and mid-twentieth century to generate crops resistant to select pathogens, the risk of new virulent strains emerging that could overcome a single R-gene within in a few years of deployment has become apparent (Ellis *et al.*, 2014). Efforts in developing crops that are not only quantitatively resistant to select pathogens but provide "broad spectrum" resistance has risen in recent years especially for wheat (Ellis *et al.*, 2014), soybean (Kawashima *et al.*, 2016), and potato (Zhu *et al.*, 2012).

The study of the molecular interactions between pathogenic microbes and their respective hosts is crucial for the advancement of modern agriculture and provides a broader understanding of the biological principles by which eukaryotic organisms function. As new diseases emerge and climate change persists to threaten crop yield (Godfray *et al.*, 2010), the potential that translating fundamental knowledge in the plant-microbe interactions field into sustainable solutions has for agricultural problems cannot be underestimated (Hogenhout *et al.*, 2009; Białas *et al.*, 2017).

3.2 The *Ustilago maydis* – maize pathosystem

Smut fungi are a prevalent group of plant-pathogenic fungi that affect agriculturally important cereal crops (Christensen, 1963; Banuett, 1995). These biotrophic basidiomycetes specifically interfere with seed development by producing large amounts of brown, dust-like teliospores, ultimately interfering with the inflorescences of their host plants (Bölker, 2001; Vanky, 2012). Some of the most commonly studied smut fungi include *Sporisorium reilianum* which infects maize (head smut) and sorghum, *Ustilago hordei* which infects barley, as well as *U. maydis* which infects maize. Not only can *U. maydis* be cultured, contrary to rusts and mildews, but its efficient homologous recombination system has made it possible to functionally

characterize individual effector proteins (Brachmann *et al.*, 2004). Furthermore, transcriptomic data of *U. maydis*-infected plant tissue also provided further information regarding the molecular mechanisms of infection. These data revealed that maize genes associated with biotic stress are significantly enriched during infection at early time points, predominantly those encoding pathogenesis-related (PR)-like proteins, indicating the fungus was recognized upon entry while at later time points of infection, these plant defense responses are attenuated (Doehlemann *et al.*, 2008). Doehlemann and colleagues also revealed that *U. maydis* infection impairs C_4 photosynthesis and tumor induction ultimately results in increased transcript levels of enzymes involved in several primary and secondary metabolic pathways (Doehlemann *et al.*, 2008).

Despite the tools developed and knowledge gained regarding the pathogenicity of *U. maydis* on both macroscopic and molecular levels, the maize response to *U. maydis* infection is not functionally well-described. Given that maize has a large genome and genetic manipulation is not trivial (Schnable *et al.*, 2009), we sought to identify a smut fungus that infects the model plant *A. thaliana*. This strategy would still allow for the use of tools developed for *U. maydis* but would additionally offer a plant host with a relatively small genome (The Arabidopsis Genome Initiative, 2000). *A. thaliana* is also genetically amendable, its immune system and defense responses to biotic stress are well described, and vast arrays of mutant and marker lines are available (Thomma *et al.*, 1998; Glazebrook, 2005; Jones & Dangl, 2006).

3.3 Infection biology of plant pathogens

In order to dissect the molecular dialogue between microbes and their hosts, first a basic understanding of the microbial infection biology and lifecycle needs to be gained. Plant pathogens not only vary in the degree of disease severity, but they also have various lifestyles and are therefore categorized into the following groups: necrotrophs, hemi-biotrophs, and biotrophs. Necrotrophs are the most aggressive of the three classifications, killing their host plants and feeding off the dead tissue. A classic example of a necrotroph is *B. cinerea*, which infects many fruit and vegetable varieties as well as *A. thaliana* (Williamson *et al.*, 2007). Biotrophs, for example, smut fungi such as *U. maydis* infecting maize (Banuett, 1995) and rust fungi such as *Phakopsora pachyrhizi* infecting soybean (Kawashima *et al.*, 2016), are less destructive and require a living host plant for nourishment and survival. Lastly, hemi-biotrophs are in between necrotrophs and biotrophs. They begin their lifecycle as biotrophs feeding off living tissue, later killing their host plant and feeding off the dead tissue as necrotrophs.

Plant pathogenic fungi may have overall different lifestyles but their lifecycles all have key components that are crucial for the fungus to germinate, sense its host, enter, proliferate, feed, and reproduce. Beginning at the germination stage, in well-described smut fungi such as *U. maydis* and *U. hordei*, their teliospores can germinate in soil under favorable, nutrient-rich conditions (Banuett, 1995; Laurie *et al.*, 2012). It has also been shown in the lab that spores of *U. maydis* readily germinate in H_20, indicating that they do not require a plant signal in order to germinate. To sense the host plant, soil fungi such as arbuscular mycorrhiza can detect the proximity of host plant roots by the presence of root-secreted compounds such as strigolactones (Akiyama & Hayashi, 2006). Recently, the vascular wilt pathogen *Fusarium oxysporum* was reported to sense active plant peroxidases resulting from an unrelated wounding event in tomato and even re-orient its growth towards this opening (Turrà *et al.*, 2015; van der Does & Rep, 2017).

Once a fungal plant pathogen has reached a morphological stage where it is able to infect its host plant and sensed it if necessary, it requires a means by which to enter. *U. maydis* and maize head smut *S. reilianum* form appressoria-like structures that secrete cell-wall degrading enzymes, allowing the fungi to enter the plant (Mendoza-Mendoza *et al.*, 2009; Ghareeb *et al.*, 2011). Others such as *Colletotrichum higginsianum* and *Magnaportha oryza* form melanized appressoria using turgor pressure to enter the plant (Shimada *et al.*, 2006). Additionally, other fungi can enter via natural openings of the plant such as stomata and proliferate in an extracellular fashion, such as *Cladosporium fulvum*, the biotrophic ascomycete tomato leaf mold fungus (Giraldo & Valent, 2013).

Infection symptoms that plant-pathogenic fungi cause to their host plants can range from hardly visible to quite drastic. *U. maydis* infects locally and forms large tumors on all aerial tissues while both *S. reilianum* and *U. hordei* infect systemically and sporulate in the spikelets of the inflorescences of their host plants (Bölker, 2001; Ghareeb *et al.*, 2011; Laurie *et al.*, 2012). Like *U. maydis*, *C. higginsianum* infects locally but instead of forming tumors, it causes leaf anthracnose symptoms caused when the fungus switches to its necrotropic state (O'Connell *et al.*, 2012). Endophytes, on the other hand, grow symptomless within their host plants such as the symbiotic root fungus *Piriformspora indica* (Zuccaro *et al.*, 2011) and several Epichlöe species (Scott, 2001).

Understanding the infection biology of a plant pathogen sets the foundation for deciphering the molecular dialogue occurring between this pathogen and its host. Since effectors are among the key governing

factors that determine host infection and colonization, knowledge of a pathogen's lifestyle and lifecycle can provide important insight its effector repertoire (Selin *et al.*, 2016).

3.4 Effectors of plant pathogens

To detect invading microbes, plants utilize two major strategies, Patterned-Triggered Immunity (PTI) and Effector-triggered Immunity (ETI), the latter often including the induction of a hypersensitive response (HR) and systemic acquired resistance (Jones & Dangl, 2006; Göhre & Robatzek, 2008). PTI is the first line of plant defense in which conserved microbial elicitors or pattern-associated molecular patterns (PAMPs), such as chitin, are recognized by pattern recognition receptors (PRRs) (Zipfel, 2014). ETI serves as the second line of defense for the plant in which intracellular host nucleotide-binding leucine-rich repeat (NB-LRR) immune receptors recognize pathogen effectors (Cui *et al.*, 2015). If a specific effector is recognized by the plant, fungal proliferation is restricted via a hypersensitive response (HR) induced by the host, leading to programmed cell death (Giraldo & Valent, 2013; Cui *et al.*, 2015).

Large-scale data approaches have heavily assisted in the detection of effector candidate genes in fungal pathogens in recent years, especially those sets of effectors unique to each pathogen. Considering fungal genes that are highly up-regulated during infection conditions, whose encoded proteins contain a predicted secretion signal and do not have transmembrane domains has emerged as useful strategy to uncover the effector repertoires of fungal pathogens (De Jonge, 2012; Lo Presti *et al.*, 2015). Although these techniques have proven quite useful in the identification of candidates, the functional characterization of fungal effectors remains difficult due to a lack of transformation capability in many fungi such as the rust clade within the Basidiomycetes. Extensive work has been done to characterize the effectors of the model smut *U. maydis* utilizing its efficient homologous recombination system to generate targeted gene-deletion mutants (Brachmann *et al.*, 2004).

The first molecularly characterized effector of *U. maydis* is Pep1 and it is required by the fungus to enter its maize host (Doehlemann *et al.*, 2009). Pep1 is secreted into the apoplastic space of its host and interacts with maize peroxidase POX12, preventing the plant oxidative burst, hence suppressing PTI (Hemetsberger *et al.*, 2012). Pep1 orthologs from related smut fungi such as *S. reilianum*, *U. hordei*, Persicaria gall smut *Melanopsichium pennsylvanicum*, and Brachypodium smut *U. bromivora* functionally complement the *U. maydis* infection phenotype of the deletion mutant (Hemetsberger *et al.*, 2015; Rabe *et al.*, 2016). Considering that Pep1 is present and functionally conserved in smut fungi whose hosts range from monocot grasses to dicot weeds, it is deemed an important core effector of smut fungi

(Hemetsberger *et al.*, 2015). Additional effectors of *U. maydis* such as chorismate mutase Cmu1 (Djamei *et al.*, 2011), See1 (Seedling efficient effector) that is required for plant DNA synthesis reactivation (Redkar *et al.*, 2015), and the cysteine-protease inhibitor Pit2 (Protein important for tumor induction) (Doehlemann *et al.*, 2011), have also been functionally well-characterized.

Although rust fungi are not genetically tractable, significant work has been conducted to understand the functions of candidate effectors using heterologous systems. Using genome (Duplessis *et al.*, 2011) and infection transcriptome sequencing technologies (Petre *et al.*, 2012) with the poplar rust *Melampsora larici-populina*, Petre *et al.*, 2015 identified effector candidates using a prediction pipeline and characterized these candidates via an 'effectoromics' approach. To this end, they cloned individual effector candidate genes tagged with green fluorescent protein (GFP) and transiently expressed them in *Nicotiana benthamiana* via *Agrobacterium tumefaciens*-mediated transformation to determine their subcellular localization. This method can indicate what the potential localization of individual effector proteins might be and subsequent co-immunoprecipitation (co-IP) assays allow the identification of potential plant target proteins *in vivo*. The majority of the effector candidates localized to the nucleus, indicating that they could function as transcription factors. Although a high rate of protein promiscuity resulted from the co-IP screen i.e. several plant target genes for each fungal effector candidate, these data provide a sound foundation for more targeted characterization of a few select effector candidates. One such effector candidate, MLP124017, was confirmed to interact with transcriptional co-repressor TOPLESS-related protein 4 from poplar (Petre *et al.*, 2015), a potential negative regulator of jasmonate responses (Pauwels *et al.*, 2010).

A second heterologous system that can be utilized to characterize effectors is the model plant *A. thaliana*. To further characterize effectors of *M. larici-populina,* Germain *et al.*, 2017 generated stable transgenic *A. thaliana* lines expressing individual effector candidates and plants expressing effector candidate MLP124499 displayed delayed senescence compared to Col-0. Similarly, necrosis and ethylene-like inducing proteins (Nlps) in the downy mildew *Hyaloperonospora arabidopsidis* were investigated by generating transgenic *A. thaliana* lines constitutively expressing these effectors. Several of these lines resulted in severe growth reduction (Oome *et al.*, 2014). Strikingly, when Oome and colleagues infected their effector-expressing *A. thaliana* lines with *H. peronospora*, the same lines that showed a growth reduction displayed a severe reduction in susceptibility to this pathogen, while typically, the expression of an effector in a host plant enhances susceptibility to the pathogen (Oome *et al*., 2014). Since the growth

phenotype resembles those of *Arabidopsis* auto-immune mutants (Bowling *et al.*, 1994, 1997) and resistance is enhanced, this might indicate that the reduction in growth is caused by an over-activation of plant immunity (Bolton *et al*., 2009).

In a third heterologous system using a luminescent bacterial pathogen of *A. thaliana*, *Pseudomonas syringae* pv. *tomato* DC3000 LUX (*Pst-LUX*), Fabro *et al.*, 2011 introduced several *H. peronospora* effectors into the plant cell by fusing each effector to the leader sequence of the *Pst* effector AvrRPS4 (Sohn *et al.*, 2007). To determine whether the effector candidates had virulence functions, they monitored bacterial proliferation by measuring luminescence. Surprisingly, 70% of their effector candidates contributed to a significant increase in bacterial proliferation (Fabro *et al.*, 2011), suggesting that many of their effectors could function in suppressing PTI.

The use of genome and infection transcriptome data have clearly enabled the identification of effector candidates in recent years and the characterization of these candidates from a variety of pathogens has been made possible due to the availability of heterologous systems. That being said, however, the most targeted and revealing approach in investigating specific effector candidates to date is still via reverse genetics, extensively exemplified for the model smut fungus *U. maydis* (Doehlemann *et al.*, 2009, 2011; Djamei *et al.*, 2011; Redkar *et al.*, 2015).

3.5 The Brassicaceae smut fungus *Thecaphora thlaspeos*

Although grass smuts are used to study the molecular basis of a variety of topics including mating type determination, signaling pathways, and pathogenicity (Bölker, 2001; Basse & Steinberg, 2004), the response of the host plant to such fungal infection is difficult to study because grasses such as maize are complex and difficult to genetically modify (Schnable *et al.*, 2009). Therefore, efforts in mapping resistance loci and the subsequent breeding of maize, wheat, and barley have shown to be quite laborious and to date, besides ZmWAK, no locus that confers resistance to smut fungi has been molecularly characterized (Baumgarten *et al.*, 2007; Grewal *et al.*, 2008; Knox *et al.*, 2014). With such difficulty in accessing the molecular mechanisms of the host plant upon fungal infection, there exists a need for a new smut pathosystem with a host that can be more readily investigated.

Thecaphora thlaspeos is the only known smut fungus to infect members of the Brassicaceae family (Vánky *et al.*, 2007), to which the genetically amendable perennial and annual model plants, *Ar. alpina* and *A. thaliana*, respectively, belong (Koch *et al.*, 1999). *T. thlaspeos* does not cause obvious infection symptoms

on its hosts and infected plants can only be identified in nature once silique tissue is opened and brown, dust-like teliospores are detected (Fig. 1)(Frantzeskakis *et al.*, 2017). As a vast amount of information exists regarding the plant defense responses of *A. thaliana* (Jones & Dangl, 2006; Nishimura & Dangl, 2010), it would be advantageous to utilize such resources in order to better elucidate and experimentally verify plant responses to smut infection. Since a *T. thlaspeos* - Brassicaceae pathosystem would allow for genetic manipulation on both fungal and plant sides, more targeted investigation could be conducted into the roles of both fungal effectors and plant immune responses to infection. Additionally, agriculturally important crops such as potato and peanut are infected by members of the *Thecaphora* clade, *T. solani* and *T. frezi*, respectively, thus deeming the study of this pathogen-host molecular cross-talk even more relevant (Andrade *et al.*, 2004; Conforto *et al.*, 2012).

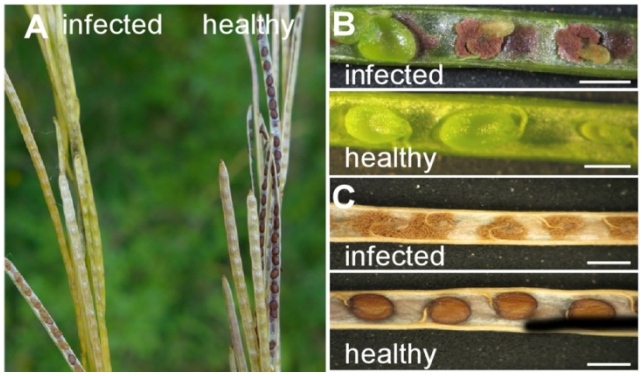

Figure 1. *T. thlaspeos* infection symptoms on *Arabis spps*. (A) No obvious infection symptoms appear in infected *Ar. hirsuta* when compared to healthy plants collected in Germany. (B) The siliques of infected *Ar. hirsuta* show brown teliospores on seeds. (C) Siliques of infected *Ar. ciliata* collected in Slovenia in 2013 also have fungal teliospores. Scale bars indicate 1 mm. (Modified from Frantzeskakis *et al*., 2017).

4 Aims

Describe the lifecycle and infection process of *T. thlaspeos*

1) The lifecycle of a smut fungus that infects Brassicaceae was not yet described. I studied the lifecycle of *T. thlaspeos* in its Brassicaceae hosts in order to describe the infection process at all plant growth stages. This knowledge was crucial in order to successfully infect plants in the lab that will be used to ultimately uncover the underlying molecular mechanisms of infection.

Identify unique fungal effectors and unravel host responses during infection

2) The molecular basis of how smuts infect Brassicaceae is not known. Via transcriptomics, I intend to predict unique effectors of *T. thlaspeos* that are required for infection of Brassicaceae. This will provide insight into how smut fungi can manipulate and finally fully adopt different hosts depending on their individual effector repertoires.

3) The molecular basis of Brassicaceae immune responses to the presence of smuts is not functionally described. Transcriptomics can additionally reveal how plants respond to smut infection but in a genetically tractable pathosystem.

5 Results

5.1 List of publications included in this dissertation

1) Frantzeskakis L.[*], **Courville K.J.**[*], Plücker L., Kellner R., Kruse J., Brachmann A., Feldbrügge M., Göhre V. 2017. The plant-dependent lifecycle of *Thecaphora thlaspeos*: a smut fungus adapted to Brassicaceae. *Molecular Plant-Microbe Interactions* 30(4): https://doi.org/10.1094/MPMI-08-16-0164-R

2) **K.J. Courville**[*], L. Frantzeskakis[*], N. Haeger, R. Kellner, B. Day, B. Usadel, Y.K. Gupta, H.P. van Esse, Brachmann A., E. Kemen, M. Feldbrügge, V. Göhre. 2018. The genome and transcriptome of the Brassicaceae smut fungus *Thecaphora thlaspeos* reveal novel insights into systemic and sustained virulence. *New Phytologist*. In review.

[*] *equal contribution*

5.2 Publication I: The plant-dependent lifecycle of *Thecaphora thlaspeos*: a smut fungus adapted to Brassicaceae.

Authors: Lamprinos Frantzeskakis*, **Kaitlyn J. Courville***, Lesley Plücker, Ronny Kellner, Julia Kruse, Andreas Brachmann, Michael Feldbrügge, Vera Göhre.

* *equal contribution*

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https://apsjournals.apsnet.org/doi/suppl/10.1094/MPMI-08-16-0164-R

MPMI Vol. 30, No. 4, 2017, pp. 271–282. http://dx.doi.org/10.1094/MPMI-08-16-0164-R

e-Xtra*

The Plant-Dependent Life Cycle of *Thecaphora thlaspeos*: A Smut Fungus Adapted to Brassicaceae

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Smut fungi are globally distributed plant pathogens that infect agriculturally important crop plants such as maize or potato. To date, molecular studies on plant responses to smut fungi are challenging due to the genetic complexity of their host plants. Therefore, we set out to investigate the known smut fungus of Brassicaceae hosts, *Thecaphora thlaspeos*. *T. thlaspeos* infects different Brassicaceae plant species throughout Europe, including the perennial model plant *Arabis alpina*. In contrast to characterized smut fungi, mature and dry *T. thlaspeos* teliospores germinated only in the presence of a plant signal. An infectious filament emerges from the teliospore, which can proliferate as haploid filamentous cultures. Haploid filaments from opposite mating types mate, similar to sporidia of the model smut fungus *Ustilago maydis*. Consistently, the *a* and *b* mating locus genes are conserved. Infectious filaments can penetrate roots and aerial tissues of host plants, causing systemic colonization along the vasculature. Notably, we could show that *T. thlaspeos* also infects *Arabidopsis thaliana*. Exploiting the genetic resources of *A. thaliana* and *Arabis alpina* will allow us to characterize plant responses to smut infection in a comparative manner and, thereby, characterize factors for endophytic growth as well as smut fungi virulence in dicot plants.

Fungal diseases are a major threat to modern agriculture (Fisher et al. 2012). Despite vast efforts undertaken to control disease, e.g., chemical treatments, resistance breeding, and improved agricultural practices, significant economic losses still befall every year (Oerke 2006). The smut fungi are a group of biotrophic plant pathogens that infect important crops such as barley, wheat, maize, and potato and can cause substantial yield losses and grain quality reduction (Kronstad 1996). Typical symptoms of smut-infected crop plants are formation of melanized, dark-pigmented teliospores in inflorescences, seeds, or leaves, leaf tissue rupture, and stunted growth (Vánky 2012).

Many smut fungi can grow as nonpathogenic, saprotrophic sporidia until they find a mating partner with which they fuse to form a filamentous, pathogenic dikaryon (Brefort et al. 2009). This dimorphic life cycle is best characterized in *Ustilago maydis*, the causal agent of corn smut (Banuett 1992; Vollmeister et al. 2012). The switch from saprophytic growth to pathogenic development starts with mating (Bölker et al. 1992), and filament formation is controlled by a heterodimerizing transcription factor (Feldbrügge et al. 2004; Kämper et al. 1995). In these filaments, plant signals from the leaf surface induce appressorium formation and penetration of the plant tissue (Lanver et al. 2014; Mendoza-Mendoza et al. 2009). Upon successful penetration, fungal hyphae rapidly proliferate in infected tissue and induce tumor formation for the deposition of diploid teliospores. In corn, these can be released within four weeks after initial infection (Pataky and Chandler 2003) to start a new infection cycle (Feldbrügge et al. 2004). In contrast to the rapid life cycle of *Ustilago maydis*, which allows several infections per season, the head smut fungus *Sporisorium reilianum* infects maize via the roots and grows slowly, like an endophyte, through the plant until sporogenesis occurs in the floral meristem (Martinez et al. 2002). Similarly, several small grain smuts, such as *U. hordei* infecting barley, follow the plant life cycle. Their teliospores overwinter inside seeds or in the soil and infect seedlings during early development in the season (Hu et al. 2002).

Although there is detailed molecular understanding of morphological changes and infection strategies in smut fungi (Vollmeister et al. 2012), the investigation of molecular mechanisms underlying resistance remains difficult, due to the genetic complexity of polyploid host grasses. Despite extensive breeding and mapping efforts that led to the identification of resistance genes in maize, barley, and wheat (Baumgarten et al. 2007; Grewal et al. 2008; Knox et al. 2014), the molecular mechanisms of resistance remain largely elusive. By contrast, a wealth of information on the plant immune system and plant responses to fungal infection comes from the model plant *Arabidopsis thaliana* (Asai and Shirasu 2015; Cook et al. 2015), which has led to translational approaches for plant protection (Brewer and Hammond-Kosack 2015; Lacombe et al. 2010). Therefore, it would be highly useful to combine the sophisticated molecular tools and resources of smut fungi and *A. thaliana*.

T. thlaspeos is a smut fungus that adapted to infect Brassicaceae hosts. It is described in at least 15 host species, including

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*The *e*-Xtra logo stands for "electronic extra" and indicates that six supplementary figures, three supplementary tables, and two supplementary movies are published online.

Arabis hirsuta as well as the perennial model plants *Arabis alpina* and *Arabidopsis lyrata* and also *Cardamine* spp. (Vánky et al. 2008), a plant genus that is used to study the evolution of plant development (Hay et al. 2014). In infected host plants, *T. thlaspeos* produces single spores in the siliques, replacing seeds with spores. Previously, rDNA sequencing provided molecular data for clear identification of *T. thlaspeos* in *Arabis hirsuta* (Vánky et al. 2008). Despite morphological descriptions of teliospores and their germination, the infection process of *T. thlaspeos* in Brassicaceae is still unknown. Here, we describe the plant-dependent life cycle of *T. thlaspeos* and demonstrate that this smut is able to systemically infect *A. thaliana*.

RESULTS

Collection and identification of the Brassicaceae smut fungus *T. thlaspeos*.

In the last five years, we identified *T. thlaspeos* in Germany, Slovenia, and Greece (Table 1) on three different host species, i.e., *Arabis hirsuta*, *Arabis ciliata*, and *Arabis sagittata*. Repeated collections at four sites in Germany during four consecutive years indicate that these populations are stable. *T. thlaspeos*–infected plants showed no macroscopic symptoms such as growth defects, distorted leaf growth, abnormal root, shoot, or flower development (Fig. 1A). Infected plants were identified by the presence of spores in place of developing seeds within the siliques (Fig. 1B and C). Further sequencing of large subunit ribosomal DNA from genomic DNA of these spores confirmed *T. thlaspeos* as the causal agent of plant disease (Table 1; Supplementary Fig. S1B). Spores inside siliques differentiated from young hyaline, white-colored spores into a pale brown spore mass (Fig. 1B and C). Characteristic warts were visible on mature teliospores (Fig. 1D). In contrast to previous observations (Vánky 1999), we observed infected plants with siliques that contained both spores and viable seeds. Cogermination of seeds and spores from sporulating siliques resulted in infected plants (Supplementary Fig. S2). This suggests vertical transmission as one propagation route for *T. thlaspeos*.

Overall, the Brassicaceae smut *T. thlaspeos* is dispersed throughout Europe (Vánky 1994) and populations are stable over years. Teliospores that are typical for smut fungi specifically develop in siliques of the host plant.

Teliospores germinate in response to a dormancy-breaking plant signal.

Teliospores of various smut fungi germinate readily in minimal media such as 0.2% malt agar or water agar without any requirements for specific temperature or humidity (Ingold 1992). Surprisingly, mature *T. thlaspeos* teliospores remained dormant in standard nutrient-rich and nutrient-poor media commonly used for fungi (Andrade et al. 2004; Holliday 1961; Ingold 1988; Kent et al. 2008; Murashige and Skoog 1962). However, in the presence of germinating host seeds, teliospores readily germinated at rates up to 76% (Fig. 2A; Supplementary Movie S1). This suggests that, in contrast to all characterized smut fungi, a plant signal might be essential to break dormancy and initiate growth in *T. thlaspeos* teliospores. The plant signal is not host-specific, as spores also germinated in the presence of nonhost plants such as *Arabis montbretiana* and *Brassica napus* (Brassicaceae), *Cleome hasseleriana* (Cleomaceae), as well as *Oxalis stricta* (Oxalidaceae). By contrast, dormancy was not broken in the presence of *Calystegia sepium* (Convolvulaceae) (Supplementary Table S1). Furthermore, autoclaved exudates of plant leaves still induced germination, suggesting that the signal is heat-stable.

Teliospores germinate with infectious filaments.

After germination, the germ tubes proliferated without development of yeast-like sporidia as described for *T. saponariae* or *T. schwarzmaniana* (Vánky and Lutz 2007; Vasighzadeh et al. 2014). In contrast to infectious filaments formed by *U. maydis*, *T. thlaspeos* filaments were not arrested in cell cycle

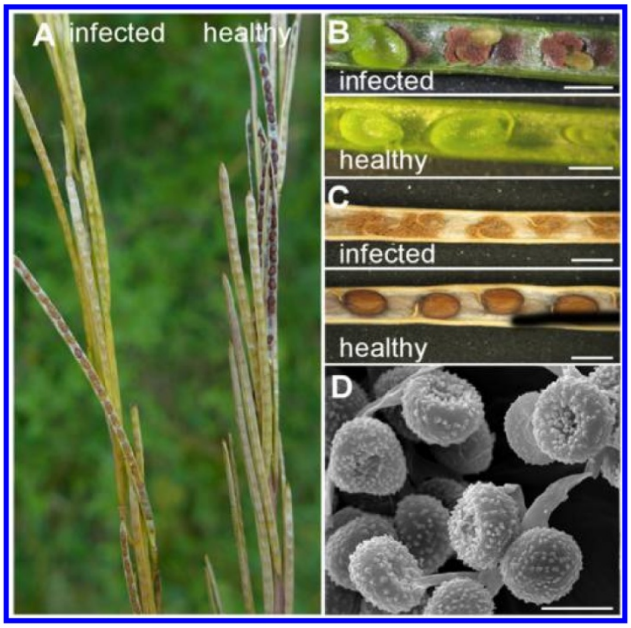

Fig. 1. Symptoms of *Thecaphora thlaspeos* infection on *Arabis hirsuta* and *Arabis ciliata*. **A,** Infected and healthy *Arabis hirsuta* plants collected in Germany appear macroscopically identical. Scale bar: 2 cm. **B,** In siliques of these infected plants, spores cover developing seeds. Scale bar: 1 mm. **C,** Similarly, infected *Arabis ciliata* siliques found in Slovenia in 2013 contained fungal teliospores. Scale bar: 1 mm. **D,** Scanning electron microscopy of *T. thlaspeos* spores shows the typical dense wart-like surface decoration. Scale bar, 10 µm.

Table 1. Isolates of *Thecaphora thlaspeos*[a]

Host	Location	Identifier	Citation
Arabis hirsuta	Romania	LSU (EF647754), ITS (KJ579178.1)	Vánky and Lutz 2007
Arabis hirsuta	Germany, Ronheim	LSU (KX686748)/ITS	This study
Arabis hirsuta	Germany, Bad Berneck	LSU (KX686747)	This study
Arabis hirsuta	Germany, Eselsburg	LSU (KX686746)	This study
Arabis hirsuta	Germany, Hohe Leite	LSU (KX686745)	This study
Arabis ciliata[b]	Slovenia	Spores in siliques	This study
Arabis sagittata[b]	Greece	Spores in siliques	This study

[a] Spores were collected from infected plants from 2011 to 2015 in Germany. *T. thlaspeos* occurred in the described host *Arabis hirsuta.* Sequencing of the large subunit rDNA (LSU) confirmed the macroscopic classification as *T. thlaspeos*. ITS = intergenic transcribed spacer.
[b] Two additional hosts, *Arabis ciliata* and *Arabis sagittata*, were observed in Slovenia and Greece.

but continued growth in the absence of the host plant. The growing germ tubes inserted empty sections at the basal pole of every cell, attached to the plant surface, and directly infected the cocultured seedlings (Fig. 2A). During this proliferation, we detected neither clamp cells nor coiled structures, as described in the potato smut *T. solani* (Andrade et al. 2004), nor intratetrad mating between compatible compartments of the germ tubes, as reported for some Ustilaginaceae species (Ingold 1988, 1989). Whereas most *T. thlaspeos* germ tubes initially contained two nuclei (Fig. 2B), they seem to distribute into daughter filaments during further cell divisions (Fig. 2B). Prolonged incubation of germinated spores for 4 to 6 weeks

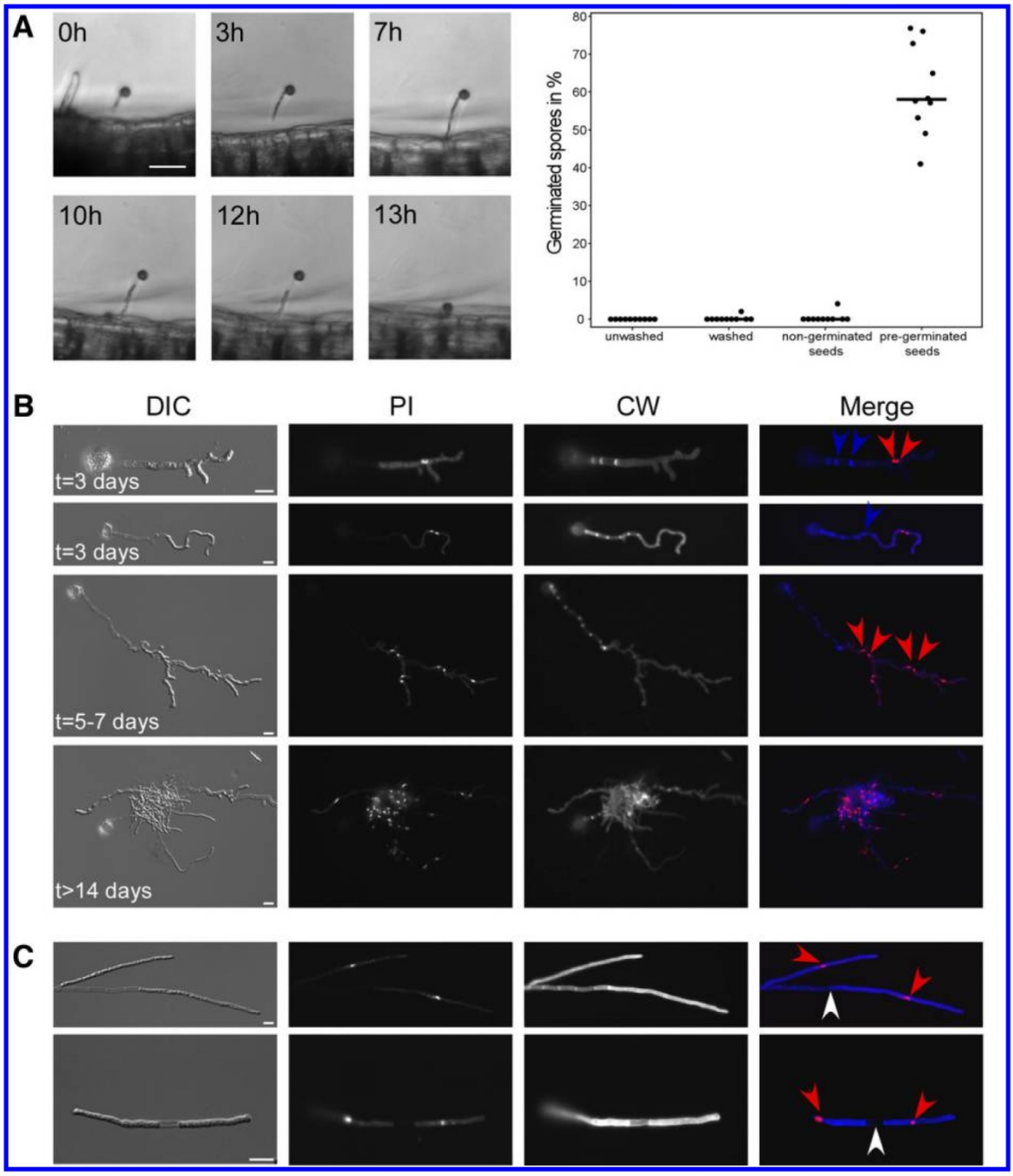

Fig. 2. Germination of *Thecaphora thlaspeos* teliospores. **A,** Teliospores germinate in the presence of seedlings (here *Arabidopsis thaliana* Col-0) and hyphae are growing toward the elongating root. Attachment to the root can be observed 12 to 13 h after spore germination and leads to drift of the spore away from its initial location (13 h). Scale bar: 50 µm. Quantification of the germination rate is shown on the right. Unwashed and washed teliospores were inoculated for 4 days in water, with nongerminating seeds or with pregerminated seeds; 1,000 spores per individual and treatment from 10 spore samples were surveyed. Bars indicate average rates of germination estimated. **B,** Early stages of *T. thlaspeos* spore germination and nuclear content after propidium iodine (PI) (red arrowheads) staining. Filaments emerging from the teliospore contain 1 or 2 nuclei and grow by inserting septa at the basal pole (shown by Calcofluor white staining [CW], blue arrowheads). Prolonged incubation (>14 days) in the absence of a host plant results in distorted growth of the filaments and the formation of clumps. **C,** Vigorous shaking of such clumps leads to isolation of haploid filaments, which divide mitotically and separate, leaving behind empty sections (white arrowheads). These filaments form compact colonies when plated on solid media. Scale bar, 10 µm.

resulted in compact colonies that were exclusively monokaryotic (Fig. 2C; Supplementary Movie S2).

In summary, *T. thlaspeos* teliospores develop infectious filaments that are not arrested in cell cycle and that can differentiate into monokaryotic mycelial cultures.

T. thlaspeos mating genes are conserved.

Shotgun genome sequencing of *T. thlaspeos* teliospores revealed 5 Gb of primary sequence information that was assembled into contigs. Fungal contigs contained homologs of the mating genes: the pheromone receptor genes *pra1* and *pra2*, lipopeptide pheromone-encoding genes *mfa1* (Bölker et al. 1992), as well as the HD transcription factor genes *bW* and *bE* from *U. maydis* (Kämper et al. 1995). All sequences were confirmed by amplification from genomic DNA of fungal cultures.

The predicted *T. thlaspeos* pheromone receptor genes (*Ttpra*) contain three (*Ttpra1*) and two (*Ttpra2*) introns. TtPra1 and TtPra2 both span 388 amino acids (Fig. 3A). Maximum likelihood phylogeny placed TtPra1 in the Pra1 clade (46% identity, 65% similarity between *T. thlaspeos* and *U. maydis*) and TtPra2 in the Pra2 clade (43% identity, 64% similarity) of pheromone receptors (Supplementary Fig. S3A). Polymerase chain reaction (PCR) screening for *pra1* and *pra2* genes in spore samples from geographically distant populations revealed the presence of both genes in all tested spore samples (Fig. 3B). Interestingly, monokaryotic filamentous cultures that were isolated from these spore samples were either *a1* or *a2* mating types (Fig. 3B), suggesting that our axenic cultures are haploid for the *a* locus. Although the tested spore samples contained the same two alleles of *pra*, the presence of additional, yet-unidentified *pra* genes in *T. thlaspeos*, as observed in many other smut fungi (Kellner et al. 2011), cannot be excluded.

The predicted pheromone genes *Ttmfa1* and *Ttmfa2* are 126 and 117 bp in length, with no introns (Fig. 3C). Both proteins, TtMfa1 and TtMfa2, have a C-terminal CaaX motif (Spellig et al. 1994) and a potential Kex2 cleavage site (Bader et al. 2008; Brown and Casselton 2001). With 12 and 9 amino acids, the predicted mature pheromones Tta1 and Tta2 are of comparable size to *U. maydis* homologs Uma1 and Uma2. Of the four functionally important amino acid residues in *U. maydis* pheromones (Szabo et al. 2002), only proline (P7) and glycine (G9) are conserved (Fig. 3C).

The presence of homologs for the mating genes of smut fungi suggested the occurrence of mating also in *T. thlaspeos*. Indeed, a mating assay showed that filaments of opposite mating types grew toward each other, fused at their tips, and continued growth with a single filament (Fig. 3D). Furthermore, pheromones and receptors are expressed in axenic culture (Fig. 3E).

TtbE and *TtbW* were identified based on their conserved homeobox sequence and homologous gene orientation (Fig. 4A). Both are expressed in axenic culture (Fig. 3E). The HD DNA-binding sequences in TtbE and TtbW are well-conserved compared with *U. maydis* (82% amino acid similarity). In addition to the N-terminal variable domain similarity also dropped below 50% in parts of the more conserved C-terminus. In particular, the activation domain of UmbW1 (Kämper et al. 1995) and the predicted nuclear localization sequence in bE proteins are more variable in *T. thlaspeos* (Supplementary Fig. S4).

Overall, the presence of mating genes typical for smut fungi suggests a conservation of genetic exchange despite the morphological differences.

T. thlaspeos HD transcription factors form hetero- and homodimers.

To investigate the interaction of bE and bW homologs in *T. thlaspeos* in comparison with HD transcription factors of *U. maydis* (Kämper et al. 1995), a yeast two-hybrid approach was performed. As N-terminal fusions of the Gal4-DNA-binding (BD) domain to full-length *Tt*bW1 and *Tt*bW2 were auto-active (Supplementary Fig. S5C), we used N-terminal fusions of the GAL4-activation domain (AD) in our yeast two-hybrid screens. In the test for dimer formation, full-length BD-TtbE and AD-TtbW interacted only when the two proteins originated from different alleles (Fig. 4C). Combining the N-termini of TtbE1 and TtbW2 or TtbE2 with TtbW1 showed that interallele-specific interaction is mediated by the variable domain of the proteins (Fig. 4B and C), which is typical for basidiomycetes (Casselton and Olesnicky 1998; Kämper et al. 1995).

Unexpectedly, besides the conserved interallelic heterodimer-formation between TtbE and TtbW, TtbE proteins are also able to form homodimers. Homodimerization is independent of the allele, as TtbE1 can interact with itself and with TtbE2. This is not the case for UmbE1 and UmbE2 (Fig. 4C). In contrast to the bE-bW interaction mediated by the variable domain, homodimerization of TtbE1 proteins takes place in the conserved domain, which includes the HD (amino acids 125 to 460) (Fig. 4B and C).

Taken together, the *b* locus controlling the dimorphic switch in grass smuts seems to be functionally conserved in *T. thlaspeos*. However, TtbE proteins can form homodimers raising questions about the nature of the complex, for example, as a tetramer or additional functions of TtbE in the fungal lifecycle. Further studies await genetic manipulation of *T. thlaspeos* to test for deletion phenotypes.

T. thlaspeos establishes a systemic infection along the vasculature.

To characterize the growth of *T. thlaspeos* within its host plants, we harvested a variety of tissues from infected plants during annual collections and visualized the presence of fungal hyphae via wheat germ agglutinin fluorescein isothiocyanate (WGA-FITC)/propidium iodide (PI) staining. All tested tissues, such as roots, rosette leaves, shoot leaves, flowers, and siliques, were colonized (Fig. 5) and *T. thlaspeos* grew systemically along the vasculature. To exclude that the stained fungal structures are part of the microbiome (De Coninck et al. 2015; Horton et al. 2014), we additionally confirmed their identity as *T. thlaspeos* by PCR, using *T. thlaspeos*-specific intergenic transcribed spacer primers.

We further characterized the colonization process in infection experiments with teliospores. Upon coincubation of fungal spores and *Arabis hirsuta* seeds, *T. thlaspeos* hyphae were detectable in seedlings grown under sterile conditions after three weeks as well as in adult, soil-grown plants (Fig. 6). As in the collected samples (Fig. 5), infection was restricted to the vasculature in all growth conditions (Fig. 6) and no macroscopic symptoms appeared. Interestingly, the hyphae in adult plants were fragmented near the base of the leaf and continuous near the fungal growth apexes (Fig. 6B). To induce flowering, *Arabis hirsuta* required a vernalization period of 12 weeks. During this time, hyphae remained in the leaves and showed both fragmented and continuous hyphae (Fig. 6A). After vernalization, inflorescences emerged, in which fungal structures were detected. Similar to the plants collected from nature, hyphae grew into flowers and siliques, in which they differentiated into viable spores (Fig. 6A), which again germinated to infect a new generation of plants. Hence, the life cycle can be completed under controlled conditions.

In our infection experiments, the seedling is entirely exposed to fungal spores, allowing the fungus to enter into any tissue. At early stages of cogermination, fungal hyphae grew toward the root tip, attached to the surface to penetrate the outer layers, and grew toward the vasculature (Fig. 7A). At later stages,

T. thlaspeos proliferated from the root throughout the whole seedling along the vasculature. In some cases, fungal hyphae also wrapped around the root tip prior to penetration but, unlike *S. reilianum*, *T. thlaspeos* did not proliferate into a thick fungal layer upon penetration (Martinez et al. 2000) nor form pseudohyphae composed of yeast-like cells (Martinez et al. 2000). To test for the entry of *T. thlaspeos* into aerial tissues, spores were dropped on cotyledons of one-week-old *Arabis hirsuta* seedlings. After 4 days, spores had germinated and appressoria-like structures were formed at the apical pole of the germ tubes (Fig. 7A; Supplementary Fig. S6).

T. thlaspeos establishes a systemic infection along the vasculature of both roots and shoots of *Arabis hirsuta*. Further, a robust method for infection under controlled conditions based on cogermination of spores and seeds was developed that reflects the natural infection process and allows completion of the life cycle.

T. thlaspeos infects the model plant *A. thaliana*.

Important insights into the plant immune system come from studies of the model plant *A. thaliana* (Asai and Shirasu 2015). While *T. thlaspeos* teliospores have not been reported in

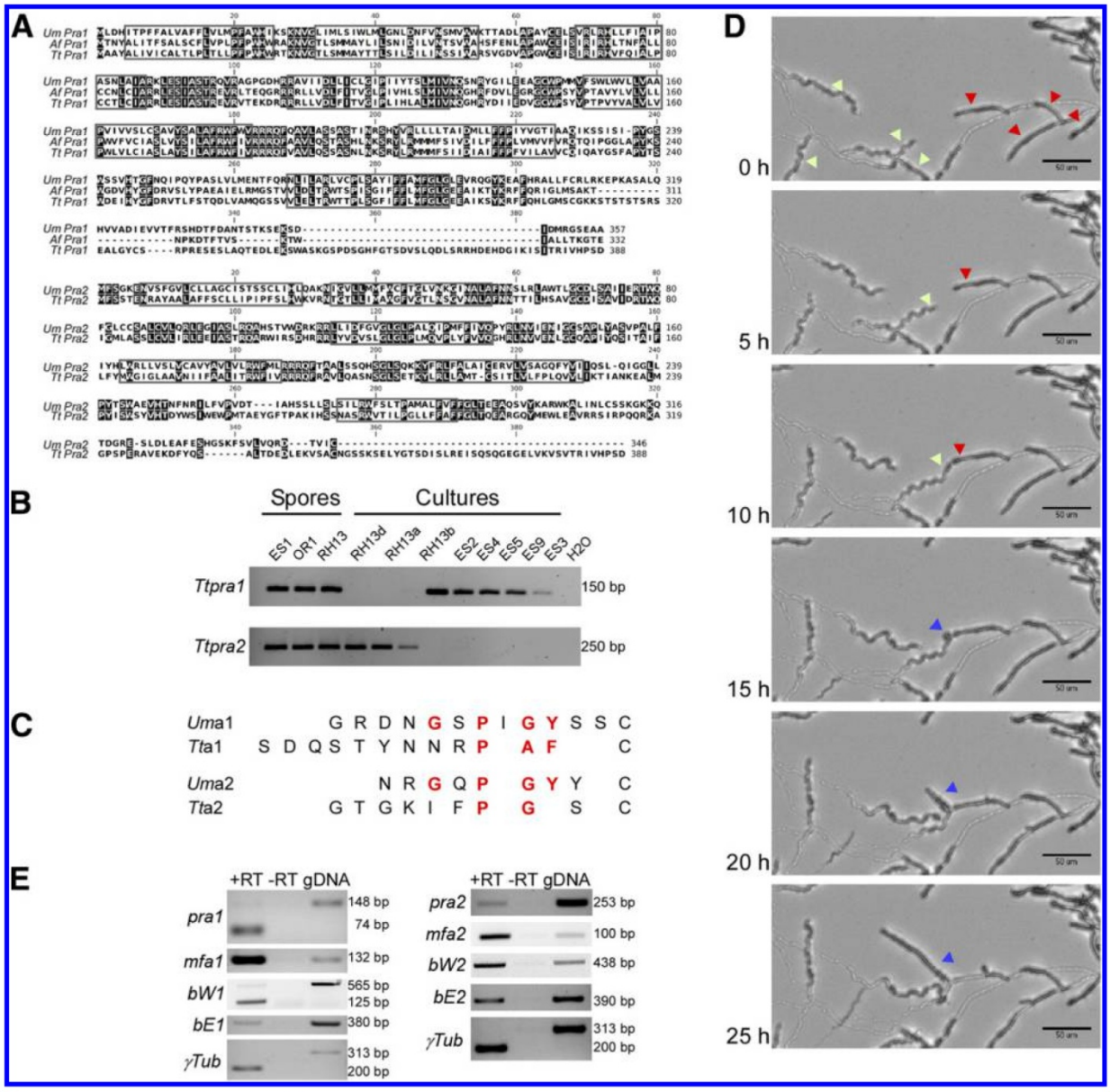

Fig. 3. The *a* mating locus of *Thecaphora thlaspeos*. Shotgun genome sequencing of *T. thlaspeos* spores revealed homologs of the genes encoded in the a loci in *Ustilago maydis*: *pra1*, *pra2*, and *mfa1*. **A,** Amino acid alignments of the predicted *T. thlaspeos* TtPra1 with the corresponding *U. maydis* (UMAG_02383) and *Anthracocystis flocculosa* (PFL1_01711) homologs and *T. thlaspeos* TtPra2 with the corresponding *U. maydis* homolog (AAA99768.1). Gray boxes show the transmembrane domains as predicted in the *U. maydis* sequences (TMHMM Server v 2.0). Highlighted squares show conserved amino acids. **B,** Polymerase chain reaction (PCR) with *Ttpra1*/*Ttpra2* specific markers reveals that spores from different strains contain both mating types. Cultures of isolated filaments derived from these spores are haploid. **C,** The predicted *T. thlaspeos* mature pheromones contain two out of the four functionally important amino acids (red). **D,** For the mating assay, cultures of the *a1b1* strain LF1 (green arrowheads) and the *a2b2* strain LF2 (red arrowheads) were dropped in close proximity on plates and were monitored for interaction over 73 h. Filaments fused and continued growing in a single hybrid filament (blue arrowheads). **E,** Reverse transcription PCR on the cultures shows expression of the *a* and *b* mating genes in the haploid cultures LF1 and LF2. γTubulin (γTub) was used as a control.

A. thaliana siliques in more than a century of collections, in cogermination assays, *T. thlaspeos* infected the ecotype Col-0. Colonization resembled systemic infection of the natural host *Arabis hirsuta*, in that *T. thlaspeos* entered via roots, formed appressoria on leaves, and fungal hyphae systemically spread along the vasculature (Fig. 7B). Interestingly, we could not observe sporulation in *A. thaliana* siliques. Thus, early events in the infection process of *A. thaliana* are identical to the natural host *Arabis hirsuta*, making this model plant an ideal experimental host.

DISCUSSION

Smut fungi are important pathogens of crop plants that were investigated for decades (Kronstad 1996). The corn smut fungus *U. maydis* has developed into a model system for fungal infection biology (Martinez-Espinoza et al. 2002) and was selected among the top ten fungal pathogens (Dean et al. 2012). Its host plant, *Zea mays*, is agriculturally highly relevant with an annual corn production of >300,000 metric tons in the United States and >50,000 metric tons in Europe (IndexMundi website). However, its complex 2.5-Gb tetraploid genome (Wei et al. 2007) still makes genetic studies of host responses challenging. Therefore, we set out to describe a new smut infection system for Brassicaceae, using *T. thlaspeos* as the fungal partner.

The life cycle of *T. thlaspeos*.

To gain insight into the biology of *T. thlaspeos*, we initially characterized its life cycle (Fig. 8). Teliospores develop in the siliques of the host plant, either replacing or covering the seeds. Interestingly, mature teliospores depend on a plant signal to induce germination, while spores of all characterized smut fungi germinate readily in water. Stimulation of germination or presymbiotic development by plant-derived compounds is known in other fungi, e.g., monoterpenes stimulate spore germination in *Verticillium longisporum* (Roos et al. 2015), strigolactones induce hyphal branching (Akiyama et al. 2005), and flavonoids stimulate hyphal growth (Becard et al. 1992) in arbuscular mycorrhizal fungi. However, these compounds are heat-labile, while the signal inducing *T. thlaspeos* germination is heat-stable, indicating that a novel perception pathway might be involved in this species. In the future, we will identify the plant signal and the fungal signal perception pathways, making use of the qualitative nature of teliospore germination in *T. thlaspeos*.

Upon successful germination, filaments emerge from the *T. thlaspeos* teliospore (Fig. 8) that can directly penetrate the host plant via leaves and roots. Similarly, teliospores from the pearl millet smut *Moesziomyces penicillariae* germinate in a solo-pathogenic diploid form that, most likely, is an adaptation to the rapid life cycle of its host (Diagne-Leye et al. 2013). In contrast to dikaryotic filaments of dimorphic model smut fungi (Begerow et al. 2014; Perez-Martin et al. 2006), *T. thlaspeos* infectious filaments are not arrested in cell cycle but can differentiate into monokaryotic mycelial cultures of opposite mating types (Fig. 2). Importantly, only cultures of opposite mating types fuse to develop a potentially infectious, dikaryotic filament (Fig. 3). The capability to perform both, either direct infection from a single spore or outcrossing before infection, might be an adaptation of *T. thlaspeos* to its specific ecological niche. In particular, spatiotemporal dynamics of host resistances and pathogen virulences in rather disconnected host populations might have selected for the persistence of outcrossing, while the occasional spread of *T. thlaspeos* spores together with seeds facilitates infection without outcrossing (Jousimo et al. 2014; Karl and Koch 2014; Morran et al. 2011). In addition, hyphae persist in the vegetative tissue of the perennial host and repeatedly deposit spores when the plant flowers in consecutive years. Hence, *T. thlaspeos* adapted its

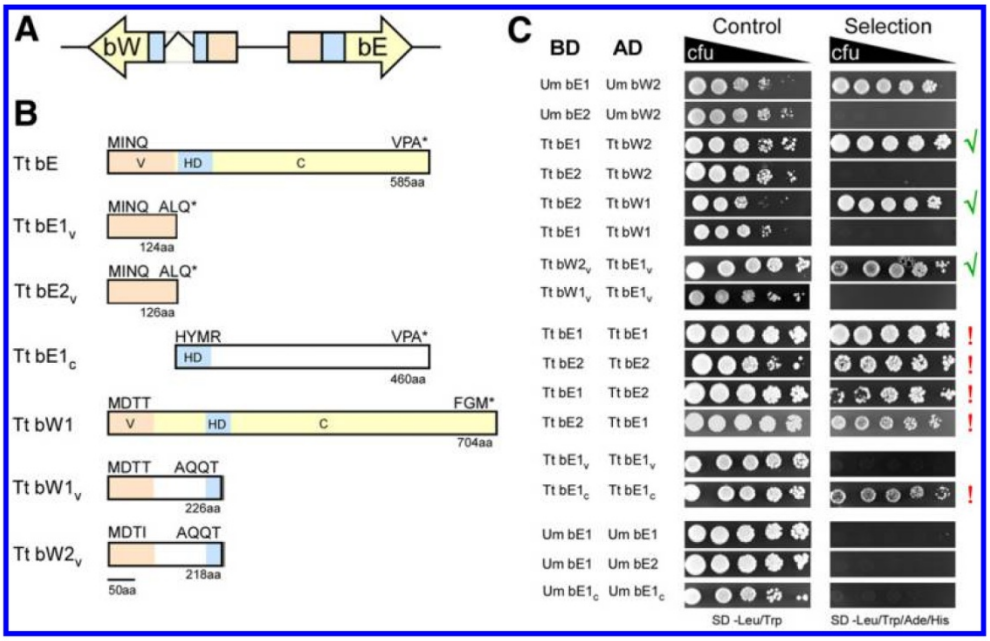

Fig. 4. The *b* mating locus of *Thecaphora thlaspeos* is conserved. The *b* locus was identified based on homology in sequence and organization of HD domains in the b proteins. Interaction of the b proteins was tested in a yeast two-hybrid assay. **A,** Schematic representation of the *b* mating locus of *T. thlaspeos*: red, variable regions; yellow, conserved regions; blue, homeobox; and white box, intron of TtbW. **B,** Schematic representation of the constructs used in the yeast two-hybrid assay. **C,** *T. thlaspeos* bE and bW present the same heterodimer formation behavior as described in *Ustilago maydis* (green). However, bE in *T. thlaspeos* can form homodimers with itself as well as with other bE alleles (red). The heterodimer formation in *T. thlaspeos* is governed by the variable domains of bE and bW, as demonstrated in *U. maydis*, while, for the *T. thlaspeos* bE homodimer formation, the conserved part of the protein might have a role.

biotrophic interaction to sustain extreme seasonal abiotic changes and to overwinter together with its perennial host plant.

Mating genetics of *T. thlaspeos*.

For most plant-pathogenic smut fungi, sexual fusion of compatible haploid mating types is mandatory to initiate pathogenic growth (Feldbrügge et al. 2004; Fisher 1957; Kellner et al. 2011). Consistently, following spore germination, *T. thlaspeos* can be cultured in haploid mycelial cultures of two opposite mating types that grew toward each other, fused, and further proliferated as filaments, indicative of sexual fusion events (Fig. 3B and C). However, unlike the model species *U. maydis* and *S. reilianum*, *T. thlaspeos* did not develop yeast-like sporidia from germinated spores.

Crucial genetic components are the pheromone/pheromone receptor system, which regulates sensing of compatible mating partners, and heterodimerizing transcription factors, which initiate pathogenic growth after fusion in smut fungi (Casselton and Feldbrügge 2010). *T. thlaspeos* encodes and expresses homologs of these genes (Figs. 3 and 4). Initial screening of teliospores from different populations (Fig. 3C) showed the presence of two *pra* alleles that are more distantly related as well as two *b* alleles. Further analysis of mating genes in other fungi of the *Thecaphora* clade will contribute to characterizing

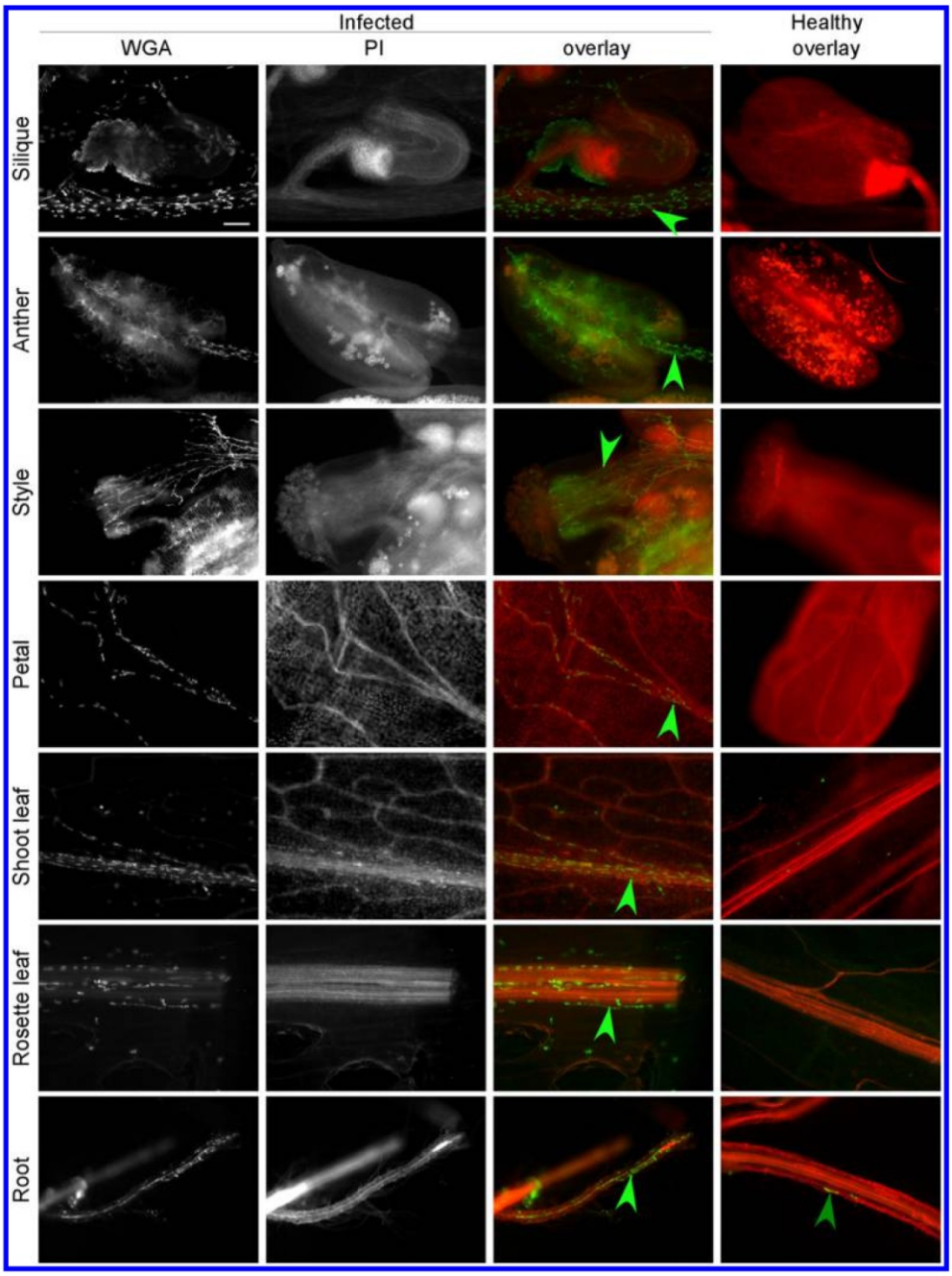

Fig. 5. *Thecaphora thlaspeos* systemically colonizes *Arabis hirsuta* in nature. In infected plants collected in Germany, fungal hyphae grow into all tissues, in which they are found predominantly along the main vasculature. Tissues were stained with wheat germ–agglutinin (WGA)-fluorescein isothiocyanate (green) and propidium iodide (PI) (red). Green arrowheads indicate fungal hyphae. Scale bar, 100 µm.

the ancestral state of the mating locus. Interaction studies of the heterodimerizing transcription factors showed smut-typical cross-compatibilities between bE and bW from opposite mating types (Fig. 3). Interestingly, bE1 and bE2 of *T. thlaspeos* additionally formed homodimers with themselves and each other (Fig. 4). This might indicate that they have a function outside of mating that is not known from other smuts. Similarly, the pheromone receptor Ste2 of *Fusarium oxysporum* gained an additional function to pheromone sensing by also sensing elusive plant signals that trigger directed hyphal growth toward plant roots (Turrà et al. 2015). Alternatively, the active bE/bW complex in *T. thlaspeos* might be multimeric, containing multiple bE proteins.

In summary, our findings suggest that *T. thlaspeos* has an active mating system that would allow exchange of genetic material.

T. thlaspeos: a future model pathogen?

Our aim was to develop a smut pathosystem with a genetically tractable host, such as *A. thaliana* (Provart et al. 2016), to utilize the genetic resources for molecular characterization of infection and plant responses. *A. thaliana* is an excellent experimental host for the investigation of molecular interactions of several pathogenic fungi, e.g., *Fusarium* spp. (Urban et al. 2002), *Verticillium longisporum* (Roos et al. 2015), *Ustilaginoidea virens* (Andargie and Li 2016), *Botrytis cinerea* (Thomma et al. 1999), and *Magnaporthe oryzae* (Park et al. 2009). *T. thlaspeos* is the only known smut fungus of Brassicaceae that can infect, among others, the sequenced perennial species *Arabis alpina* and *A. lyrata* subsp. *petraea* (Vánky 1994, 2012). Interestingly, under lab conditions, *T. thlaspeos* colonized *A. thaliana*, enabling use of the genetic tools of this model plant for molecular characterization. Together, the recent genome resources from the 1,001 genomes project in *A. thaliana* and novel genome editing techniques used in natural Brassicaceae hosts (Koenig and Weigel 2015) will enable us to compare the dynamics of infection in the different genetically tractable host plants.

In contrast to many other biotrophic pathogens, *T. thlaspeos* can be grown in haploid axenic cultures (Fig. 2). This cultivation of single-cell descendent isolates is the first step toward genetic manipulation. Transformation protocols have been successfully transferred between smut fungi, in which homologous recombination is highly efficient (Cervantes-Chavez et al. 2011; Schirawski et al. 2005; Yu et al. 2015). Recently, transformation of *U. bromivora*, the grass model *Brachypodium distachyon*, also was achieved (Rabe et al. 2016) yielding another interesting monocot model species in a smut pathosystem for comparative research.

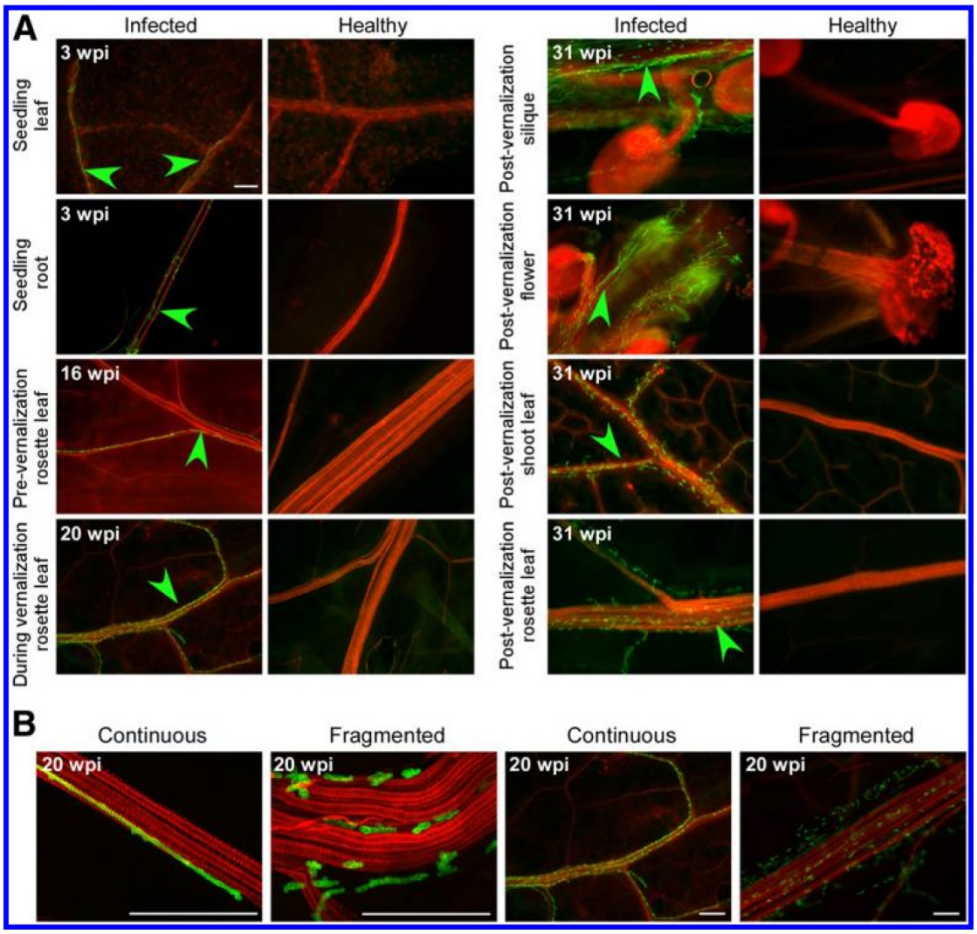

Fig. 6. *Thecaphora thlaspeos* systemically infects *Arabis hirsuta* under controlled conditions. **A,** Hyphal proliferation was observed in leaf and root tissues at the seedling stage and in adult rosette tissue prior to and during vernalization. Furthermore, during the flowering stage, spores were present in silique tissue and hyphal proliferation was observed in flower, shoot leaf, and rosette leaf tissues. **B,** Infection (here in rosette tissue) appears as both continuous and fragmented hyphae. Fungal growth in all images is predominantly along the main vasculature. Tissues were stained with wheat germ–agglutinin (WGA)-fluorescein isothiocyanate (green) and propidium iodide (PI) (red). Green arrowheads indicate fungal hyphae. Scale bar, 100 µm.

T. thlaspeos is the first member of the *Thecaphora* clade that is analyzed in molecular detail. Fungi of this clade are of great agronomic relevance, e.g., the potato pathogen *T. solani* causes yield losses of up to 80% in South America and the peanut pathogen *T. frezii* causes yield losses of up to 51% (Conforto et al. 2013). To prevent its spread, *T. solani* is strictly quarantined by the European and Mediterranean Plant Protection Organization (OEPP/EPPO 1979). Markedly, *T. solani* teliospores also germinate with filaments (Andrade et al. 2004) and our research on the infection biology of the closely related *T. thlaspeos* could stimulate the development of novel strategies aiming to protect potato production in Europe.

T. thlaspeos has evolved a long-lasting, tight interaction with its perennial host plants, in which it is capable of overwintering. We speculate that this requires special adaptations in order to balance virulence, a characteristic typical of microbial plant endophytes (Schulz and Boyle 2005). The molecular characterization of such determinants could improve, e.g., production of Jiaobai, swollen stems caused by growth of *U. esculenta* in the wild rice *Zizania latifolia* (Terrell and Batra 1982). To date, the fungus has to be maintained in the plant by agricultural practice, but selection has already shaped the genome of the host plant toward reduced defense (Guo et al. 2015).

Beneficial effects of endophytes on their host plants are of particular interest for crop improvement (Aly et al. 2011). For example, systemic colonization by the ascomycete genus *Epichloë* improves resistance of Pooideae grasses to biotic and abiotic stresses. Therefore, a number of strains are already used as biocontrol agents in agriculture (Johnson et al. 2013). Investigating potentially beneficial effects of *T. thlaspeos* in its Brassicaceae host might lead to agricultural applications in Brassicaceae crops in the future. Further, *T. thlaspeos* and the variety of its genetically tractable Brassicaceae host plants, including both annuals and perennials, serve as a promising model to disentangle the molecular determinants of long-lived systemic interactions as well as factors involved in specific resistances of one or both annuals and perennials. Examples like the *Silene*-infecting anther smut fungus *Microbotryum violaceum* show that closely related annual and perennial plant species differ in susceptibility to the same pathogen in the field but can both be infected under natural conditions (Gibson et al. 2013; Hood et al. 2010).

In conclusion, *T. thlaspeos* is a valuable, novel pathogen to study the molecular communication of smut fungi with their hosts. It also will give insight into the establishment and maintenance of prolonged endophytic growth as well as differences in colonization between annual and perennial plants. Such detailed knowledge of smut infection strategies will ultimately contribute to the development of novel plant protection strategies.

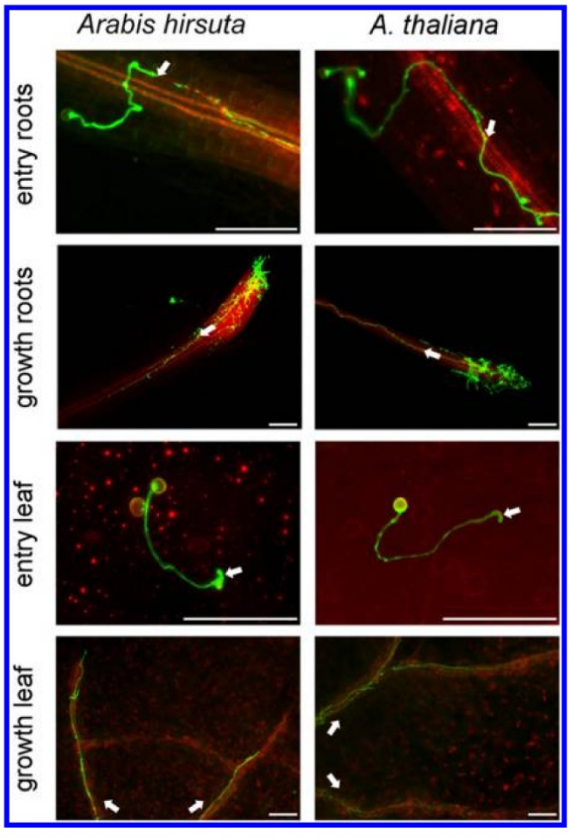

Fig. 7. Entry of *Thecaphora thlaspeos* into roots and leaves are identical in the host *Arabis hirsuta* and the model plant *Arabidopsis thaliana*. *T. thlaspeos* penetrates root and leaf tissues of seedlings, subsequently growing along the main vasculature in both species. No differences between the natural host plant *Arabis hirsuta* and the experimental host *A. thaliana* are detectable. Tissues were stained with wheat germ–agglutinin (WGA)-fluorescein isothiocyanate (green) and propidium iodide (PI) (red). White arrows indicate penetration sites or direction of hyphal growth. Scale bar, 100 µm.

MATERIALS AND METHODS

Spore germination, infection, and cultures.

T. thlaspeos teliospores from infected siliques were treated with ampicillin (100 µg/ml) for 24 h at room temperature. Sterilized seeds were mixed with these teliospores in half-strength MSN medium (Duchefa) containing 1% sucrose or in water, for quantification, and were incubated for 3 to 7 days (12 h,

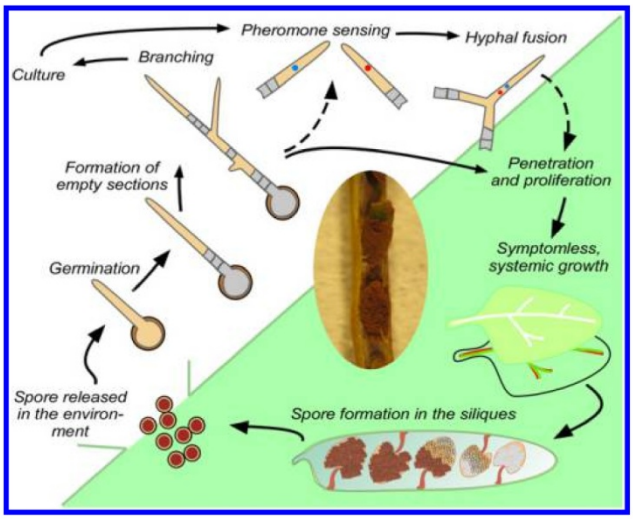

Fig. 8. Life cycle of *Thecaphora thlaspeos*. The macroscopic infection symptoms of *T. thlaspeos* are shown in the middle as seeds of the host covered in spores. Such teliospores are released into the environment or they propagate together with the seed. Upon perception of a to-date-unknown plant signal, the spores germinate in a filamentous form. In this stage, the resulting hyphae insert retraction septa, form empty sections, and hyphal branching occurs. Filaments can directly infect the plant or proliferate into haploid cultures of opposite mating type that can be grown in axenic culture. When two hyphae of compatible mating types are close in proximity, they sense each other, most likely via their pheromone-receptor system, and fuse at the tips, resulting again in a filament. Based on similarity to other smut fungi, these fusion filaments presumably are infectious (dashed arrow). Infectious fungal filaments can enter the plant directly via the root or form appressoria-like structures on leaves. *T. thlaspeos* systemically proliferates inside the plant tissue along the vasculature. Macroscopic infection symptoms are absent until the host produces seeds that are either coated or completely replaced by fungal teliospores.

100 µE), until seeds and spores germinated, or for 3 weeks, until the seedlings were infected. To obtain cultures, hyphal clumps were transferred to complete medium (CM) + 1% glucose (Holliday 1974) and were incubated on a shaker (200 rpm) at 16°C. The medium was renewed every 5 days. After 1 month, 200 µl of culture was plated on CM + 1% glucose plates. Single colonies were reisolated at least five times. Cultures resulting from these colonies were screened by PCR and were sequenced for *a* and *b* locus alleles, using primer pairs DD207 to DD210 and pair DD175 and DD15, respectively. Cultures that had a single allele for each mating type were kept (LF1: *pra1*, *b1*; LF2: *pra2*, *b2*). For leaf infection, approximately 100 spores in 1 µl of H_2O were pipetted onto the cotyledons of 7-day-old seedlings, were grown on MSN with sucrose, as described above, and were incubated for 4 to 12 days (12 h, 100 µE). For soil infection, untreated seeds and spores were mixed. The spore-covered seeds were sown on soil, were stratified for 5 days, and were incubated in long day conditions (16 h, 125 µE). After approximately 6 weeks, plants were transferred to 4°C for vernalization for 12 weeks and returned to long day until flowering.

Extraction of RNA and genomic DNA.

To extract genomic DNA, the phenol-based method established for *U. maydi*s was followed (Bösch et al. 2016). RNA was extracted from *T. thlaspeos* using the Qiagen RNAeasy kit (Qiagen) and cDNA was synthesized using the Protoscript kit (New England Biolabs), following manufacturers' instructions.

Genomic sequencing and bioinformatics analysis.

For the shotgun sequencing of *T. thlaspeos*, genomic DNA from teliospores of three infected siliques was extracted. The genomic sequencing library was constructed with the Nextera DNA sample preparation kit (Illumina), according to manufacturer's instructions. Quality control by analysis on an Agilent 2000 Bioanalyzer with Agilent high sensitivity DNA kit (Agilent Technologies) showed fragment sizes of 200 to 450 bp. Sequencing on a MiSeq sequencer (Illumina) (2× 250 bp) was performed in the Genomics Service Unit (LMU Biocenter, Martinsried, Germany), yielding about 29 Mio paired reads and 5 Gb of primary sequence. The reads were assembled into 96,471 contigs, using CLC Genomics Server 5.0.2 (Qiagen), with the following parameters: word size, 23; bubble size, 172; mismatch cost, 2; insertion cost, 3; deletion cost, 3; length fraction, 0.5; similarity fraction, 0.8; minimum contig length, 200. Approximately 5% of the sequences corresponded to fungi (MG-RAST [Meyer et al. 2008]). The majority was bacterial and plant contamination. Alignments were performed using MUSCLE 3.8 via CLC Main Workbench 7.6.2 (Qiagen), and maximum likelihood phylogeny trees were performed, using CLC Main Workbench 7.6.2 with default parameter (Qiagen). Branches with bootstrap support lower than 60% were collapsed.

Yeast two-hybrid assay.

Yeast two-hybrid analysis was carried out using the Clontech MatchMaker system as described (Kämper et al. 1995; Pohlmann et al. 2015). Sequences for the clones were amplified from cDNAs of cultures containing the *b1* or the *b2* locus (Supplementary Tables S2 and S3).

Staining and microscopy.

Nuclear staining of filaments was carried out as described (Sabbagh et al. 2010). Staining of infected material by WGA-FITC (1 µg/ml) and PI (10 µg/ml) was modified from Doehlemann et al. (2008), by shortening the boiling step to 3 min. Staining by trypan blue was carried out as described by Kemen et al. (2005). Chitin of fungal cells on cotyledon tissue was stained by immersing plantlets in Calcofluor white (CW) at 1 µg/ml for 1 min. For scanning electron microscopy, seedlings with spores on the cotelydons were fixed with 2.5% glutaraldehyde. Samples were dried via the critical point drying (CPD) technique, were mounted, and were coated with gold, using a sputter coater.

Fluorescence microscopy was performed as described by Langner et al. (2015) on a Zeiss Axio Immager M1. The movies were recorded on the same microscope. All parts of the microscope system were controlled by the software package MetaMorph (version 7; Molecular Devices), which was also used for image processing. Confocal microscopy was performed on the Zeiss LSM 780, using laser lines 488 and 561 for WGA-FITC and PI, respectively, and image processing was conducted using the ZEN 2012 (black version) software package. Scanning electron microscopy was performed on the Leo 1430 VP.

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5.3 Additional results to Publication I

In order to get an overview of *T. thlaspeos*-infected *Ar. hirsuta* leaves ten weeks post infection, the tile scan application on the confocal microscope was used to compose an image of whole infected *Ar. hirsuta* leaves. As shown below in the bottom half of an infected leaf in **Figure 2**, the hyphae colonize and grow alongside the vasculature, potentially branching into the mesophyll on occasion. These results further illustrate the endophyte-like growth of *T. thlaspeos* within *Ar. hirsuta*.

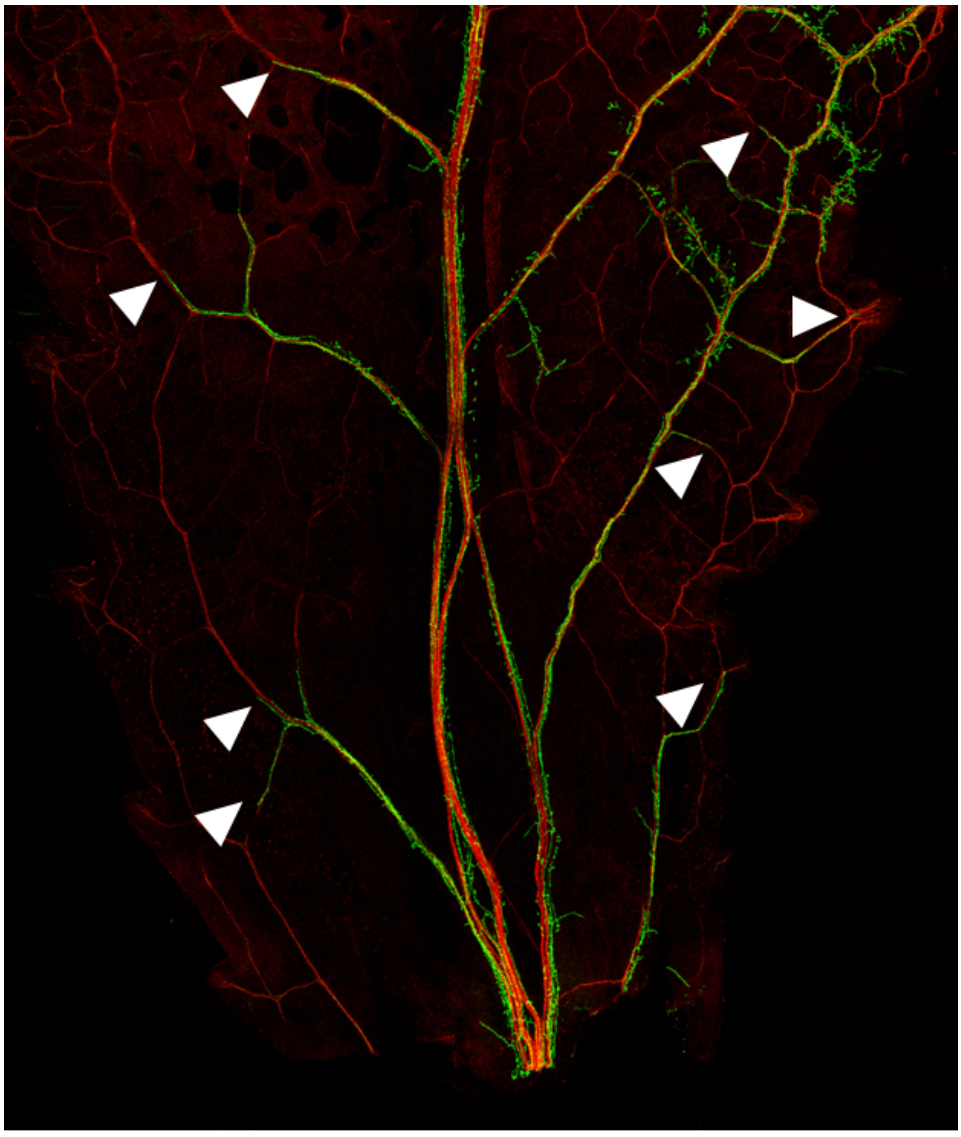

Figure 2. *T. thlaspeos* hyphae colonize throughout *Ar. hirsuta* leaf tissue along the vasculature
The fungal material was stained with WGA-FITC and visualized through the GFP channel (green), and the plant tissue was stained with PI and visualized through the mCherry channel (red) to generate these merged images on the confocal microscope. White arrows indicate growth apexes. Portion of leaf shown is approx. 0.5 cm in length. Modified from my original image that was featured on the cover of *Molecular Plant-Microbe Interactions* in April 2017.

Since the collection of *T. thlaspeos*-infected *Ar. hirsuta* plants occurred during the fungal sporulation phase in nature, the infection biology of *T. thlaspeos* as well as potential infection symptoms could not be observed during the prolonged biotrophic stage that occurs prior to sporulation. Therefore, I infected *Ar. hirsuta* plants with *T. thlaspeos* spores in the lab and described fungal growth throughout the entire plant-dependant portion of the *T. thlaspeos* lifecycle (**Publication I: Figure 6**). After *Ar. hirsuta* plants were analyzed for infection at the rosette stage as shown above in **Figure 2**, they were placed in the 4°C room for a 12-week vernalization phase. Rosette tissues were sampled during this phase to check whether the fungus remained and the fungus did still systemically colonize these tissues along the main vasculature. Once the plants completed the vernalization phase and were placed back into the growth chamber, inflorescences began to emerge and flower after 6 weeks. The rosette tissue, shoot leaves, flowers (including petals, anthers, and styles), and siliques were checked for infection via WGA-FITC/PI staining. The fungal pattern in these plants is comparable to infected *Ar. hirsuta* plants collected from nature (**Publication I: Figure 5**). As observed in natural infection of *Ar. hirsuta*, there were no obvious symptoms of infection in the plants infected in the lab except for the presence of spores in the siliques, as shown in **Figure 3**. These results show that *T. thlaspeos* systemically colonizes *Ar. hirsuta* throughout the entire lifecycle without causing obvious infection symptoms during the prolonged biotrophic phase, further illustrating its endophyte-like behavior.

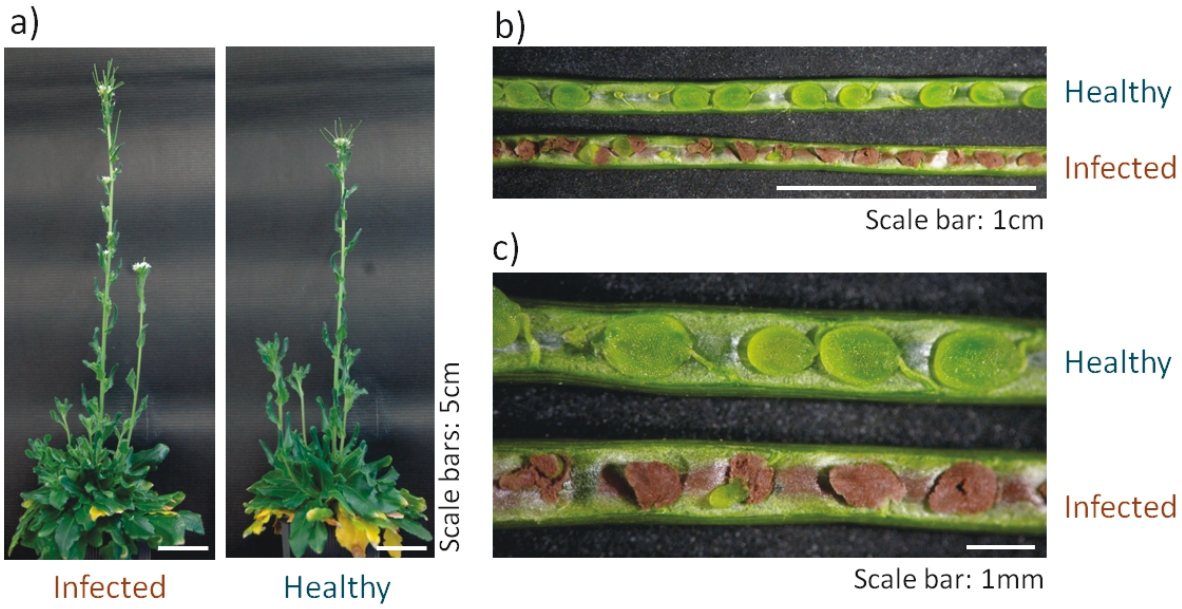

Figure 3. *T. thlaspeos* sporulates in laboratory and shows no obvious symptoms of infection
(a) Healthy and infected *Ar. hirsuta* plants look very similar in general. (b-c) Comparison of healthy and infected siliques shows healthy seeds without spores and infected seeds covered with spores and possibly reduced in size.

5.4 Summary of Publication I

In order to study the host responses to smut infection, we collected *Thecaphora thlaspeos*, the only known smut fungus infecting Brassicaceae hosts (Vánky *et al.*, 2007) in perennial *Arabis spps*. from several locations across Germany (**Publication I: Figure 1 and Table 1**). The aim of this publication was to describe the entire lifecycle of *T. thlaspeos* and provide a thorough description of its infection biology to not only understand the pathogen itself and identify its unique characteristics among smut fungi, but to also develop a foundation from which the molecular biology of this pathogen and its hosts could be later explored.

Contrary to the model smut *U. maydis* whose teliospores germinate under favorable conditions and produce haploid sporidia (Banuett, 1995), *T. thlaspeos* spores require a signal from a plant in order to germinate and do so in a filamentous fashion (**Publication I: Figure 2**) . These haploid filaments can also be cultured and we have observed hyphal fusion of those having opposing mating types. To characterize the biotrophic phase of *T. thlaspeos*, I established the infection system in the lab by developing infection protocols both under sterile and unsterile conditions. By co-incubating seeds and spores together in sterile plates or directly on soil and then utilizing several staining techniques and microscopy platforms, I determined that filamentous *T. thlaspeos* germ tubes arising from teliospores can infect its host plants via root and leaf tissues (**Publication I: Figure 6**) as these plants mature, predominately proliferating along the vasculature (**Figure 2**). *T. thlaspeos* colonizes its entire host plants and I have achieved to complete this infection process until sporulation in our lab in natural described host *Arabis hirsuta* (**Figure 3**). This lab infection process fully mirrors my observations of *T. thlaspeos*-infected *Ar. hirsuta* in nature. Strikingly, we observed that *T. thlaspeos* can overwinter within perennial hosts *A. hirsuta* and *A. ciliata*, a unique characteristic of the smut fungi. In addition, I was able to infect *A. thaliana* with *T. thlaspeos* (**Publication I: Figure 7**), which shed light on a novel avenue to investigate the molecular mechanisms of infection between the fungus and its model host.

5.5 Publication II: The genome and transcriptome of the Brassicaceae smut fungus *Thecaphora thlaspeos* reveal novel insights into systemic and sustained virulence.

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[*] *equal contribution*

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The genome and transcriptome of the Brassicaceae smut fungus *Thecaphora thlaspeos* reveal novel insights into systemic and sustained virulence

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Contains seven figures (all in color), three tables, and supporting information (six figures and 18 tables).

Summary

- Biotrophic fungal plant pathogens can balance their virulence and form intricate relationships with their hosts. Sometimes, this leads to systemic host colonization over long timescales without macroscopic symptoms e.g. major induction of plant defense or interference with plant development. However, how plant-pathogenic endophytes manage to establish and maintain their sustained systemic infection and what determines these types of interactions remains largely unknown.
- Here, we present a genomic and transcriptomic analysis of *Thecaphora thlaspeos*. This relative of well-studied grass smut *Ustilago maydis* is the only known smut fungus adapted to Brassicaceae hosts including the model species *Arabis alpina*. Its capability to overwinter with perennial hosts and its systemic plant infection including roots are unique characteristics among smut fungi.
- *T. thlaspeos* has a typical smut genome with a set of candidate effector genes that comprises common smut and unique members. For three candidates, we have functionally proven effector activity. Studying transcriptional responses of *Ar. hirsuta* towards *T. thlaspeos* infection, we reveal evidence for an immune response that lacks activation of key salicylic acid-dependent signaling-related genes.
- Our findings suggest that *T. thlaspeos* distinctly balances its virulence during biotrophic growth ultimately allowing for long-lived infection of its perennial hosts.

Key words: infection, effector, endophytes, plant immune responses, RNA-seq

Introduction

The *Thecaphora thlaspeos*-Brassicaceae pathosystem is a remarkable example of a sustained systemic plant-microbe interaction. *T. thlaspeos* establishes an infection of the entire plant, which can be maintained over several years (Vanky *et al.*, 2008; Frantzeskakis *et al.*, 2017). After penetration, intercellular hyphae of *T. thlaspeos* grow towards and proliferate along the vasculature throughout the entire plant without visible interference with plant development. When the host plant develops siliques each year, fungal hyphae differentiate into spores that in most cases entirely replace all developing seeds. In addition, fungal hyphae keep proliferating in the newly growing vegetative tissue. The capability of *T. thlaspeos* to overwinter with its perennial hosts and sustain the systemic infection within the entire plant is a unique characteristic among smut fungi studied to date.

T. thlaspeos is a distant relative of the well-studied grass smut *Ustilago maydis,* and it is adapted to Brassicaceae hosts including the perennial model plant *Arabis alpina* (Vanky *et al.*, 2008; Frantzeskakis *et al.*, 2017). Closely related sister species of *T. thlaspeos* comprise devastating crop pathogens such as *T. solani* (up to 85% losses) on potato or *T. frezii* on peanut (Andrade *et al.*, 2004; Conforto *et al.*, 2013). In addition to its *Arabis* hosts, *T. thlaspeos* can colonize the model plant *Arabidopsis thaliana*. Therefore, the *T. thlaspeos*-Brassicaceae pathosystem benefits from the well-developed resources of *A. thaliana* and *Ar. alpina* research that overcome experimental constraints of grass smuts due to the genetic complexity of their hosts (Frantzeskakis *et al.*, 2017). While plant-fungus interactions of pathogens and symbionts are well-studied (Gutjahr & Parniske, 2013; Lo Presti *et al.*, 2015), the molecular mechanisms that enable *T. thlaspeos* to establish and maintain its remarkably long biotrophic interaction with Brassicaceae over years are completely unknown. A deeper understanding of the *T. thlaspeos*-Brassicaceae pathosystem therefore might unveil molecular processes related to the endophytic phase of fungal infections.

Biotrophic pathogens evolved distinct mechanisms to evade plant immunity and establish molecular interaction with their host (Brefort *et al.*, 2009). During invasion, plant cell wall-degrading enzymes are secreted that allow fungal penetration of the plant cell (Choi *et al.*, 2013). Subsequently, fungal hyphae proliferate inside the apoplast and/or grow through host cells, establishing an intimate contact zone for the exchange of nutrients and proteins. Genome and molecular studies of the grass smut fungi *U. maydis*, *Sporisorium reilianum* and *U. hordei* greatly contributed to our understanding of smut infection (Kämper *et al.*, 2006; Brefort *et al.*, 2009;

Ghareeb *et al.*, 2015; Lanver *et al.*, 2017). This revealed different repertoires of conserved and host-adapted effector proteins (Okmen & Doehlemann, 2014; Lanver *et al.*, 2017). In *U. maydis* and other monocot grass smuts, effector-encoding genes are clustered (Kämper *et al.*, 2006; Schirawski *et al.*, 2010) although clustering of effector genes is not always conserved as exemplified for *U. bromivora*, the false brome (*Brachypodium* sp.) smut (Rabe *et al.*, 2016). Of particular importance to the colonization process of grasses by smut fungi is a locus of 26 genes named “Cluster 19A” (Kämper *et al.*, 2006; Skibbe *et al.*, 2010). When the entire cluster is deleted, tumor formation in maize is impaired and ultimately spore formation is defective, indicating an important virulence function for the members of this cluster (Kämper *et al.*, 2006). Functional analyses in *U. maydis* confirmed the contribution of single effector proteins to fungal virulence (reviewed in Lanver *et al.*, 2017). For example, Pep1, a protein essential for penetration, was identified outside the effector clusters and was characterized as an apoplastic peroxidase inhibitor (Doehlemann *et al.*, 2009; Hemetsberger *et al.*, 2012), which is conserved in several grass smut species as well as the dicot-infecting smut *Melanopsichium pennsylvanicum* (Hemetsberger *et al.*, 2015).

In response to fungal colonization, plants evolved mechanisms to inhibit pathogen attack (Dodds & Rathjen, 2010). To detect microbes, plants deploy two major strategies. First, plasma membrane-located pattern recognition receptors recognize conserved microbial elicitors, called pathogen-associated molecular patterns (PAMPs), and induce PAMP-triggered immunity (PTI, Zipfel, 2014). Second, pathogen effector molecules are recognized by intracellular host nucleotide-binding leucine-rich repeat (NLR) immune receptors that induce effector-triggered immunity (ETI, Gassmann & Bhattacharjee, 2012; Białas *et al.*, 2017). PTI and ETI involve similar immune responses including the activation of signaling cascades, massive transcriptional reprogramming and the accumulation of the two defense hormones, salicylic acid and jasmonic acid (Thomma *et al.*, 1998; Tsuda & Somssich, 2015). In addition, ETI induces local programmed cell death called hypersensitive response as well as systemic acquired resistance (Giraldo & Valent, 2013; Lo Presti *et al.*, 2015).

While factors conferring resistance to smut infection are of agronomic importance, to date, the only known resistance gene is the maize wall-associated kinase *Zm*WAK which protects maize against the head smut *S. reilianum* (Zuo *et al.*, 2014). In addition, the barley smut *U. hordei* encodes three dominant avirulence genes, but the corresponding resistance genes remain undiscovered (Linning *et al.*, 2004).

Here, we study a systemic and long-lasting smut infection in Brassicaceae. We combined genome and RNA sequencing of the recently described smut fungus *T. thlaspeos* with functional characterization of effector candidates to elucidate how *T. thlaspeos* balances its virulence during biotrophic growth.

Materials and Methods

Cloning of expression vectors

Standard USER cloning procedures (NEB) were followed to generate the AvrRPS4-*Tt*Nlp1 construct. *Ttnlp1* was amplified from cDNA from start codon to stop codon excluding the signal peptide and inserted in frame after the *AvrRPS4* leader sequence in the pEDV3 expression vector (Sohn *et al.*, 2007). The empty control was generated in the same manner but without *Ttnlp1*. Standard Golden Gate cloning (Engler *et al.*, 2014; Patron *et al*., 2015) procedures were followed to generate binary expression vectors used to obtain effector-expressing transgenic *A. thaliana* Col-0 lines. From left boarder to right boarder, expression cassettes contained kanamycin resistance, an olesin *At*OLE1-RFP protein fusion (Shimada *et al.*, 2010), and then each *T. thlaspeos* effector gene whose expression was driven and terminated by the CaMV 35S promoter and g7 terminator, respectively. Standard Gateway cloning procedures were used to generate pEarley Gate 103_*Tt*Nlp1-Gfp and pEarley Gate 103_*Psoj*NIP-Gfp expression vectors (Karimi *et al.*, 2002; Qutob *et al.*, 2002; Earley *et al.*, 2006). Cloning of *Ttpep1* into plasmid p123-pep1 (Aichinger *et al.*, 2003) and transformation of the resulting plasmid into solo-pathogenic strain SG200Δpep1 was carried out according to Hemetsberger *et al*., 2015.

Strains, transgenic *A. thaliana* lines, and infection assays

Pseudomonas syringae pv. *tomato* DC3000-LUX (*Pst-LUX*) was transformed with pEDV3_*Ttnlp1* and pEDV3_empty (Katagiri *et al.*, 2002). Four-week old *A. thaliana* Col-0 plants were spray-inoculated as described in Fabro *et al.*, 2011. At 3 days post-infection (DPI), the total photon counts were measured and normalized to the foliar area as determined using ImageJ.

Transgenic *A. thaliana* Col-0 effector-expressing lines were generated via the floral dipping method using the *A. tumefaciens* AGL1 strain expressing the effector constructs (Koncz & Schell, 1986). Primary *A. thaliana* transformants (T1) for two independently transformed lines per effector were selected based on RFP-marker-fluorescence of the seeds (Shimada *et al*., 2010) and rosettes were measured and calculated using ImageJ four-weeks post sowing. One leaf per plant was harvested and stored at -80°C for subsequent confirmation of effector mRNA accumulation.

Transient expression of *Tt*Nlp1-Gfp and *Psoj*NIP-Gfp in *Nicotiana benthamiana* was assessed on the Zeiss LSM780 confocal microscope (Bleckmann *et al.*, 2010) two days after infiltration of *A. tumefaciens* strain GV3101 (pMP90 RK) containing these respective effector expression cassettes. All bacterial strains in this study were grown overnight at 28°C in Luria-Bertani (LB) medium.

U. maydis growth, infection of maize and microscopy of maize infection was performed as described in Hemetsberger *et al*., 2015.

T. thlaspeos genome assembly, annotation and comparative genomics

For genomic sequencing of the *T. thlaspeos,* high molecular weight genomic DNA (gDNA) was prepared from pure cultures using a phenol-extraction (Bösch *et al.*, 2016). LF1 gDNA was sequenced by PacBio long read sequencing (P6-C4, Max Planck Genome Centre, Cologne) and by Ilumina short read sequencing (2x300bp, Illumina Mi-Seq, v3 chemistry, Genomics Service Unit at the Biocenter of Ludwig-Maximilians University, Munich). Long reads were assembled with Canu v1.3 (Koren *et al.*, 2017) and short reads trimmed with Trimmomatic v0.32 (Bolger *et al.*, 2014) were used with Pilon (Walker *et al.*, 2014) for error correction. LF2 gDNA was sequence by short read sequencing (2x150bp, Illumina HiSeq, Biomedical Research Center, HHU). The LF2 short reads were assembled using SPAdes v3.8.0 (Bankevich *et al.*, 2012). RepeatMasker v4.0.5 was subsequently used to report and mask repetitive regions in the genome (Jurka *et al.*, 2005; Tempel, 2012).

Annotation of both genomes was performed using MAKER2 (Holt & Yandell, 2011) as previously described (Campbell *et al.*, 2014). Briefly, for the LF1 genome, an annotation was generated providing as evidence to MAKER assembled transcripts of LF1 in nutrient rich culture conditions (Complete Medium; Holliday, 1961), proteomes of several Ustilaginales species (Supporting Information Table S18), and data from the UniProt protein reference database. After two iterations, 397 gene models were manually curated and used to train AUGUSTUS v 3.0.3 (Stanke & Morgenstern, 2005) and SNAP v 2006-07-28 (Korf, 2004). For assessing the completeness of the datasets BUSCO v1.1b1, was used (Simão *et al.*, 2015).

Functional annotation was carried out using InterProScan 5.19 (Jones *et al.*, 2014), in addition dbCAN (Yin *et al.*, 2012) and AntiSmash v4.0 (Weber *et al.*, 2015) were used respectively to mine the genome for CAZymes and secondary metabolism related genes.

Genome to genome alignments were performed using MUMmer v3.23 (Delcher *et al.*, 2003) using default options and the results were processed using auxiliary scripts provided with the package (show-coords, dnadiff). Search for orthologs between the Ustilaginales genomes used here (Supporting Information Table S18 and the generation of a multi-locus based phylogeny tree was done utilizing OrthoFinder v1.1.2 (Emms & Kelly, 2015).

Data availability: The data generated during this study are deposited in ENA (PRJEB24478).

Quantitative RNA-sequencing

Samples from LF2 culture, *T. thlaspeos* spore-infected *Ar. hirsuta*, and healthy *Ar. hirsuta* were snap-frozen and total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) including a DNaseI treatment (NEB). cDNA for RT-PCRs was generated using the Protoscript II First Strand cDNA Synthesis kit (NEB) and cDNA libraries were generated using the TruSeq RNA Library Prep kit v2 (Illumina) and sequenced on a Illumina Hi-seq 3000 platform (Biomedical Research Center, HHU).

RNA-seq data was assembled using Trinity (Grabherr *et al.*, 2011) and the resulting transcript models were added to the ones derived from the genome release. Subsequently, *Ar. hirsuta* RNA-seq data from infected plants was mapped against this combined transcriptome set using bowtie (Langmead *et al.*, 2009). Reads that could not be mapped to *T. thlaspeos* were retained and merged with RNA-seq data from healthy Arabis control plants. This combined set was then used to generate a transcriptome using Trinity either using a relatively standard pipeline or correcting errors in the reads using rcorrector (Song & Florea, 2015) and assembling the data using minimal coverage of 2. Both assemblies were than filtered and analyzed using transrate (Smith-Unna *et al.*, 2016). As the standard approach yielded better transrate values, the resulting transrate filtered standard trinity assembly was used in the subsequent analysis. These *Ar. hirsuta* gene models were then pooled with those from the *T. thlaspeos* genome assembly and all RNA-seq data from healthy and infected *Ar. hirsuta* plants was mapped against this combined set using subread. The data was summarized using eXpress (Roberts *et al.*, 2011) but only uniquely mapped reads were extracted. Data was split and separately analyzed for the plants using edgeR (Robinson *et al.*, 2009). Gene ontology enrichment assessment was carried out using Gorilla (Eden *et al.*, 2009) and visualizations were generated with REVIGO (Supek *et al.*, 2011).

For the analysis of the fungal transcriptome short reads were mapped to the genome using STAR 2.5.2 (Dobin *et al.*, 2013), tables with raw read counts were parsed and further analyzed with DESeq2 (Love *et al.*, 2014).

Results

Assembly and annotation of *T. thlaspeos* LF1 and LF2 genomes

To assemble the reference genome for *T. thlaspeos*, genomic DNA from the haploid strain LF1 of the mating type *a1b1* (Frantzeskakis *et al.*, 2017) was sequenced using both long-read (PacBio, ~40x coverage) and short-read (Illumina MiSeq, ~53x coverage) platforms. The two approaches resulted in 332,950 single long reads and 5,433,377 paired short reads, respectively. PacBio long reads were assembled into 33 scaffolds and further polished using short Illumina reads. The resulting assembly is of high continuity, reaching chromosome level. The mitochondrial genome was fully assembled in a single scaffold of 108.2 kb (Supporting Information Fig. S1).19 out the 32 nuclear scaffolds have telomeric repeats (TTAGGG) at both ends, 5 have repeats at one end (Table 1, Supporting Information Table S1). Hence, *T. thlaspeos* has at least 22 chromosomes, similar to its distantly related sister species *U. maydis* and other grass smuts featuring 23 chromosomes (Kämper *et al.*, 2006; Schirawski *et al.*, 2010; Rabe *et al.*, 2016). In parallel, a draft genome of the compatible mating type LF2 (*a2b2)* was assembled from short read data (7,795,622 paired reads, ~107x coverage; Table 1).

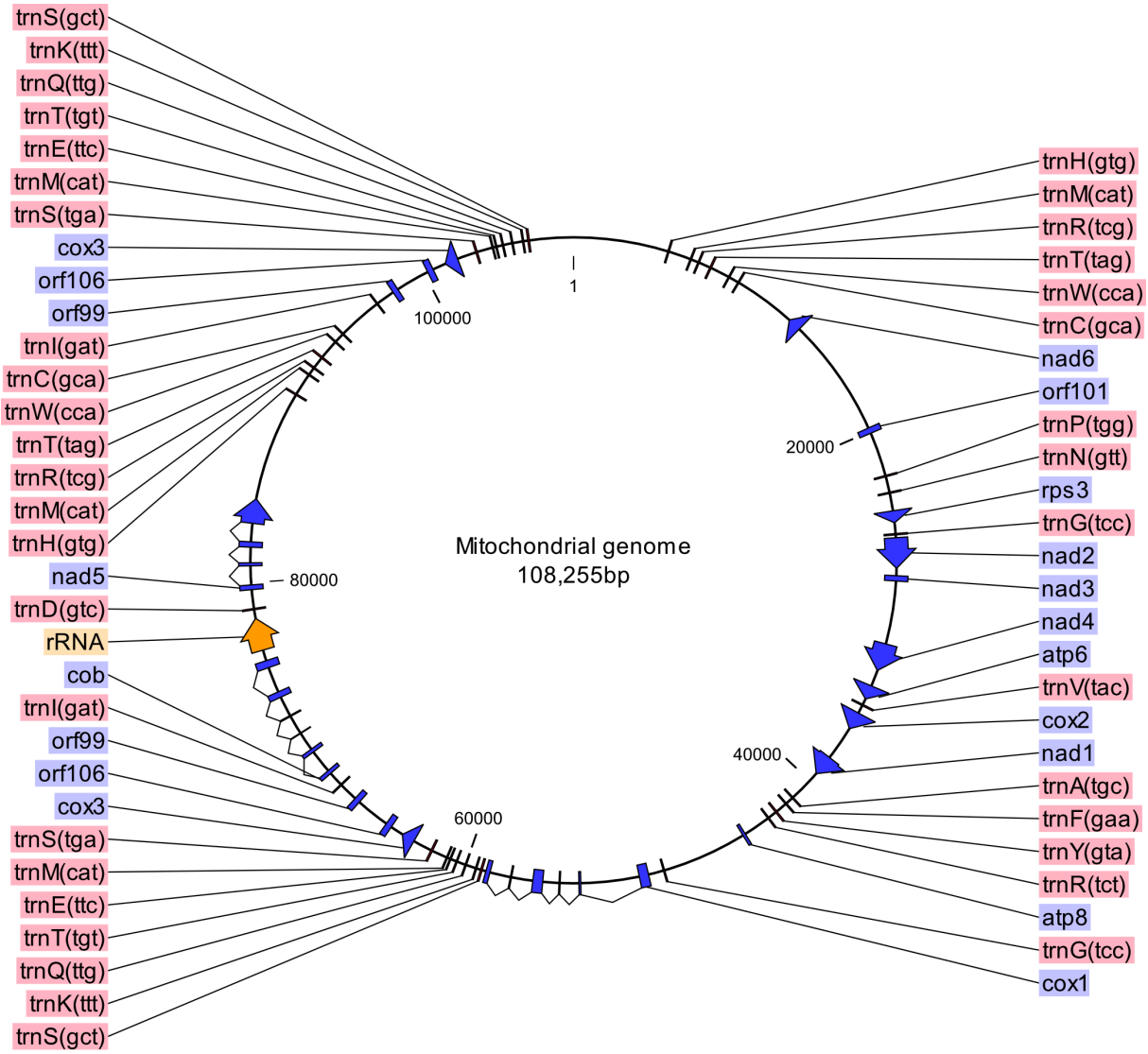


Figure S1. Map of the mitochondrial genome of the *T. thlaspeos* strain LF1. Mitochondrial genome map was generated with CLC Sequence Viewer, depicting annotations of genes (blue), tRNAs (red) and rRNA (yellow) generated by MFannot and RNAwasel.

Table 1. Assembly statistics

	LF1	LF2
Number of scaffolds	32	537
Minimum size (bp)	17,833	82
1st quartile (bp)	259,599	135
Median (bp)	594,082	354
Mean (bp)	643,487	38,053
3rd quartile (bp)	863,537	480
Max (bp)	1,714,324	1,222,775
Total (bp)	20,591,595	20,434,990
N50 (bp)	863,537	347,457
N90 (bp)	456,084	93,318
N95 (bp)	382,946	51,399
GC content	61 %	61%

For the gene annotation of strain LF1, we combined *ab initio* prediction, homology-based modeling using 21 smut fungal proteomes, and transcriptomic data from *T. thlaspeos* in the MAKER2 pipeline (Holt & Yandell, 2011). The resulting 6,509 gene models were manually curated using Apollo (Lee *et al.*, 2013) removing unsupported gene calls (absence of expression or protein homology evidence), giving a final dataset of 6,239 high-confidence gene models. We then used these curated models to annotate the second strain LF2 with the MAKER2 pipeline, and generated 6,504 gene models. This number is slightly lower than for the sequenced grass smuts (Rabe *et al.*, 2016). Verification of completeness in both LF1 and LF2 using BUSCO (Simão *et al.*, 2015) showed that the genomes contain ~93 % and the annotations ~97 % complete single copy BUSCOs (Supporting Information Table S2). Hence, despite the fragmentation of the LF2 dataset, high gene space completeness was achieved (Supporting Information Table S2).

Subsequent functional annotation focused on the high confidence gene models predicted from LF1. 5,093 of the 6,239 protein models (81%) contain known domains (Supporting Information Table S3), and 355 genes were found to encode putative secreted proteins (Supporting Information Table S4). Interestingly, this is 1/3 less than predicted for some of the grass smut fungi (Supporting Information Table S5) and only 135 of these secreted candidate proteins have at least one ortholog in species of the genus *Ustilago*, *Sporisorium* or *Melanopsichium*, while 42 are shared between all of them (Supporting Information Table S11). We found one conserved effector (THTG_03661), *pep1*, among these secreted proteins, while other well described effectors, such as the chorismate dismutase Cmu1 (Djamei *et al.*, 2011), the Seedling efficient effector required for tumor induction See1 (Redkar *et al.*, 2015), and the cysteine protease inhibitor Pit2 (Doehlemann *et al.*, 2011) are missing in *T. thlaspeos*, suggesting a reduced overlap between *T. thlaspeos* and grass smut effectors.

As expected for a biotrophic smut fungus, the repertoire of carbohydrate-active enzymes (CAZymes; Huang *et al.*, 2017) is small (Supporting Information Table S6). *T. thlaspeos* carries several genes encoding for pectin degradation enzymes (GH53, PL3, PL4), which are absent from grass smut fungi (Supporting Information Table S6). These might reflect an adaptation to the pectin-rich cell wall of dicot host plants. Furthermore, the genome of *T. thlaspeos* lacks the secondary metabolite clusters known from *U. maydis* important for the production of ustilagic acid, itaconic acid or MELs, as well as the flocculosin gene cluster encoded in the closest relative *Anthracocystis flocculosa* (syn. *Pseudozyma flocculosa*). Despite the overall agreement in whole genome alignments between these species (see next paragraphs; Fig. 1a), in this specific locus synteny is lost (Fig. 1b; Teichmann *et al.*, 2007, 2011) and AntiSmash (Weber *et al.*, 2015) predictions did not reveal any novel clusters for secondary metabolites (Supporting Information Table S7).

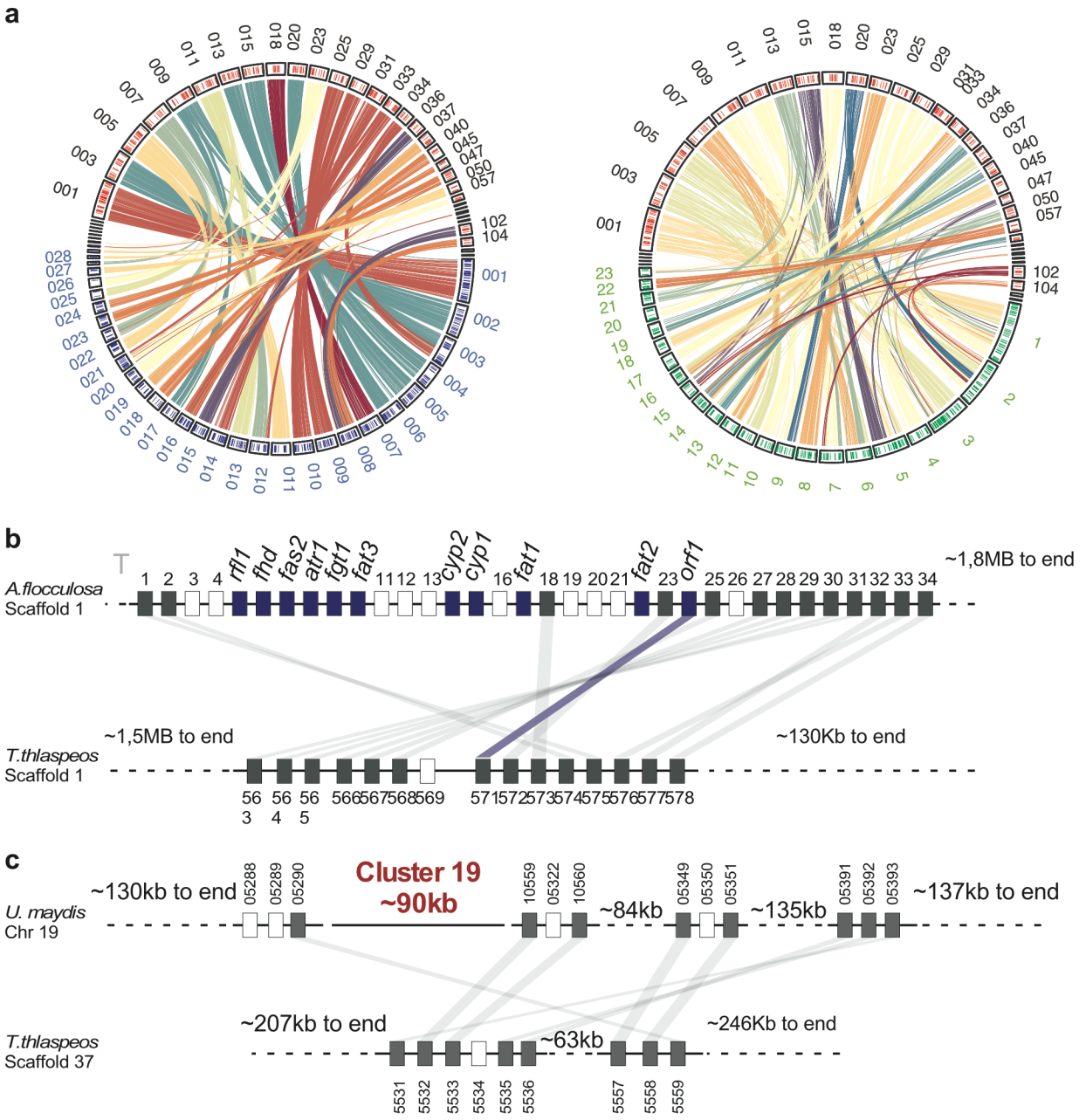


Figure 1. **Synteny is higher between *T. thlaspeos* and *A. flocculosa* than *T. thlaspeos* and *U. maydis***. (a) Circos plots between *T. thlaspeos* and *A. flocculosa* (left) or *U. maydis* (right). Colored lines depict syntenic blocks larger than 2kb. Outer ring depicts the location of secreted proteins in the corresponding scaffold or chromosome. Scaffolds of *A. flocculosa* are in blue, while scaffold of *U. maydis* are in green (b) Synteny of the flocculosin secondary metabolite cluster in *A. flocculosa* and *T. thlaspeos*. Blue boxes depict genes involved in flocculosin production, white boxes depict genes with no orthologs in the compared genome. (c) Synteny of the effector cluster 19A between *U. maydis* and *T. thlaspeos*. White boxes depict genes with no orthologs in the compared genome.

Taken together, assembly and annotation delivered a high-quality dataset comparable to the well-established genomes of *U. maydis* and *S. reilianum.* Based on our results, *T. thlaspeos* has a typical smut genome, which is small in size (~20 Mbp), is organized in 22-24 chromosomes and has a low repeat content mostly comprised of dinucleotide repeats (Supporting Information Table S8).

Mating in *T. thlaspeos* relies on conserved molecular mechanisms

Previously, we described the presence of a mating system in *T. thlaspeos* (Frantzeskakis *et al.*, 2017). Now, the mating type loci *a1* and *a2* of LF1 and LF2 are assembled revealing massive rearrangements between *T. thlaspeos* (Supporting Information Fig. S3) and other smut fungi (Kellner *et al.*, 2011). In contrast to *U. maydis*, in *T. thlaspeos* the pheromone receptors *pra1* and *pra2* are not flanked by the border genes *lba* and *rba*. This rearrangement is conserved in *A. flocculosa* suggesting divergent evolution in the *Thecaphora*-clade from the grass smuts. In grass smut fungi, the *a2* allele of the mating locus harbors the locus-specific genes *rga2* and *lga2*, which are important for uniparental inheritance of mitochondrial DNA (Fedler *et al.*, 2009). While *rga2* is present in the *a2* locus of *T. thlaspeos*, *lga2* is absent from the genome, similar to *Ustanciosporium gigantosporum*, the white beak-sedge smut (Kellner *et al.*, 2011).

Besides the mating locus, pheromone response elements (Urban *et al.*, 1996) (Supporting Information Fig. S3) and downstream signaling components of mating such as the transcription factor *prf1*, and genes involved in signaling via the cAMP pathway and the MAPK cascade are conserved in *T. thlaspeos* (Supporting Information Table S15) (Feldbrügge *et al.*, 2004). Hence, the mating process of *T. thlaspeos* (Frantzeskakis *et al.*, 2017) appears to rely on the same molecular processes that are conserved in smut fungi.

Intra- and inter-species comparison between *T. thlaspeos*, commensal and grass smut fungi

To scan genomic assemblies of 13 smut fungi species (<100 scaffolds or N50 >500kb) for conserved synteny with *T. thlaspeos* we used whole genome alignments (Supporting Information Fig. S2). Overall, *T. thlaspeos* scaffolds align best to the epiphytic biocontrol yeast *A. flocculosa* with an average alignment rate of 51.4% and an average similarity ranging from 74.2% to 78% (Fig. 1a, Supporting Information Table S9). In contrast, alignment rate and sequence similarity between *T. thlaspeos* and the model smut fungus *U. maydis* drops to averages of 32.4% and 73.4%, respectively (Fig. 1a, Supporting Information Table S9).

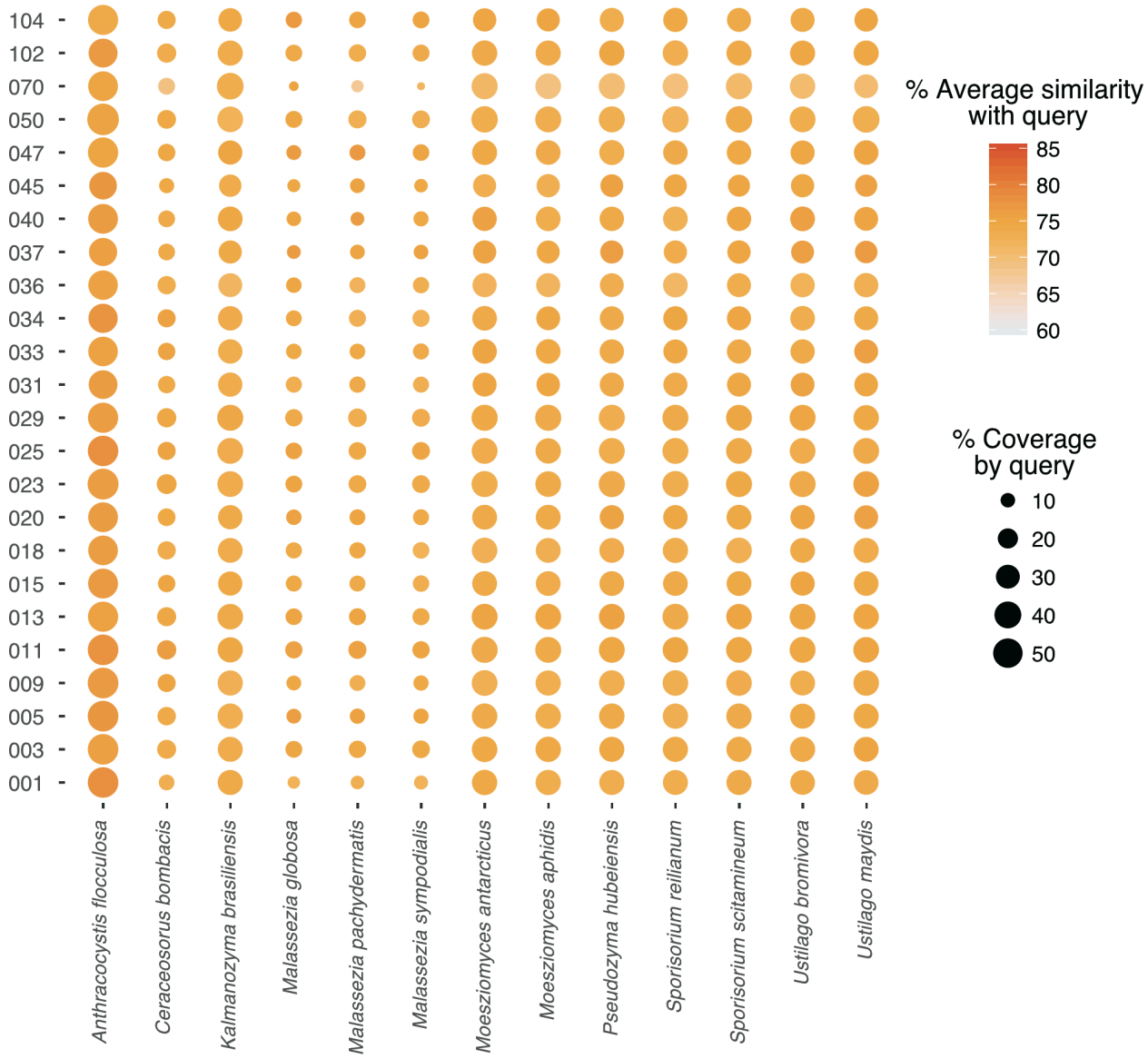


Figure S2. Genome to genome alignments between *T. thlaspeos* and other Ustilaginomycotina species. Genome sequences were obtained as detailed in Supporting Information Table S18. Alignments were conducted with MUMmer and % coverage and % similarity was calculated using MUMmer show-diff (Delcher *et al.*, 2003). On the y-axis are *T. thlaspeos* scaffolds with telomeric repeats at their ends and on the x-axis are Ustilagomycotina species. Average similarity ranges between 67-78 %, but differences clearly occur in % coverage (range 5-59%).

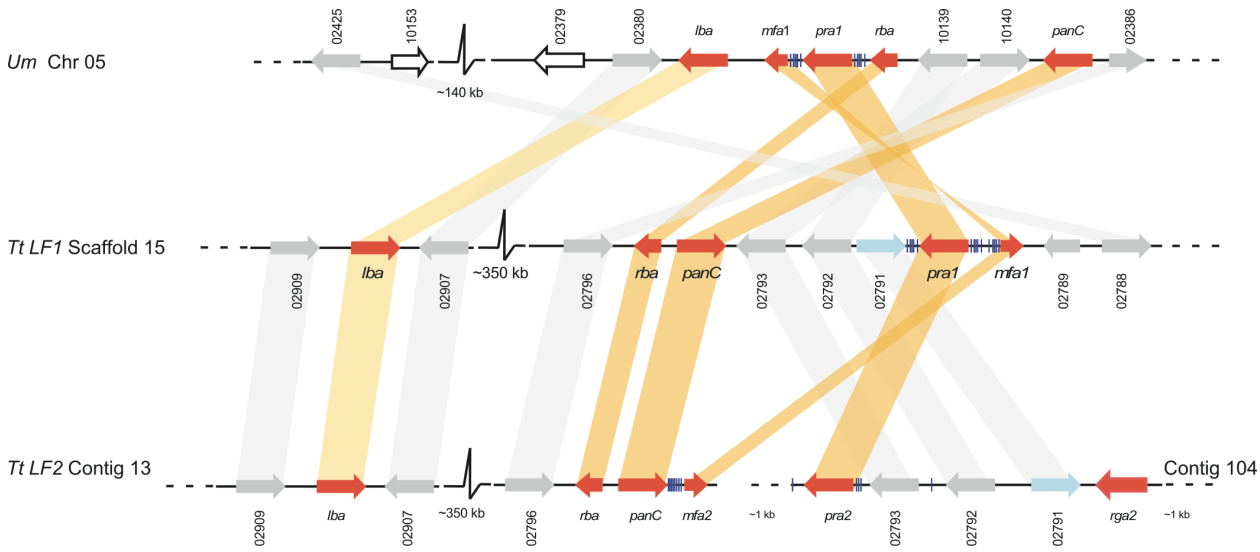
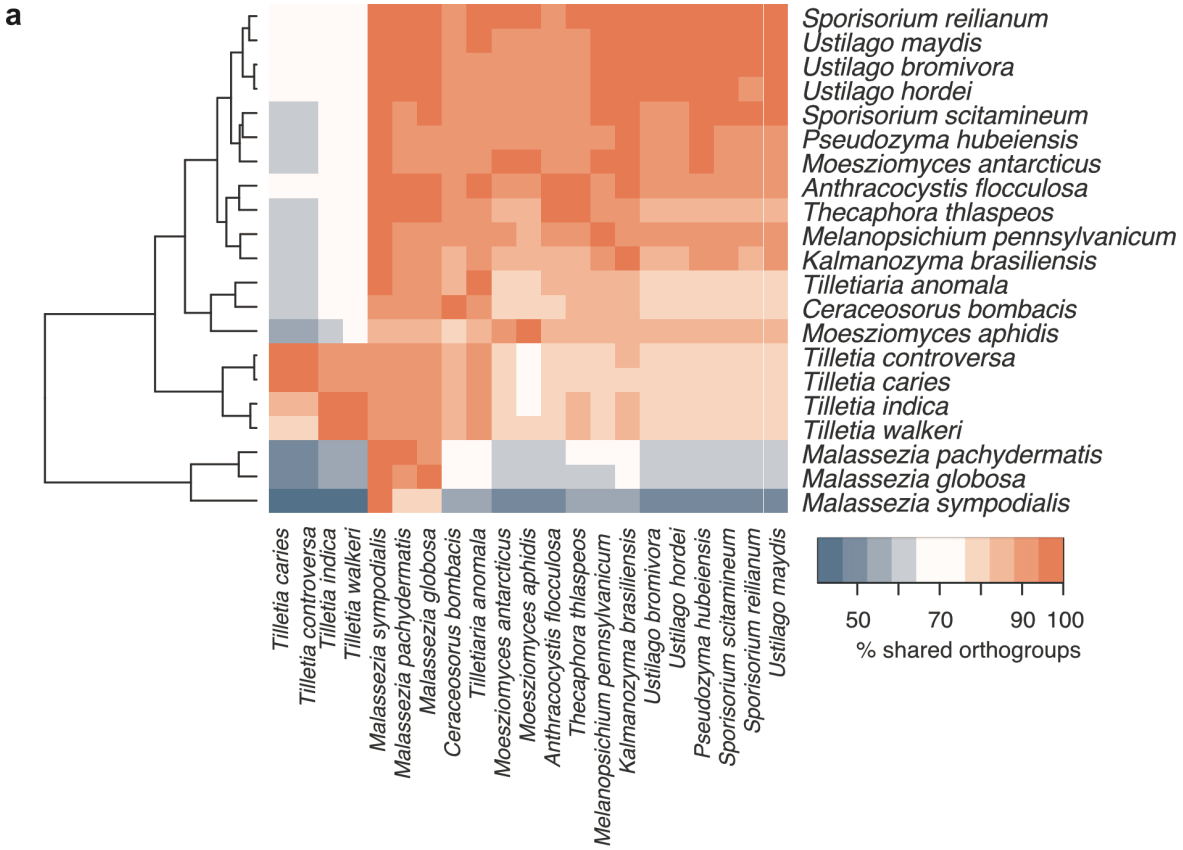


Figure S3. The mating type locus a of *T. thlaspeos*. Conformation of the mating type alleles *a*1 in the isolate LF1 and *a*2 in the isolate LF2. Red arrows indicate genes involved in mating or typically associated with the mating locus *a* in smut fungi. Grey arrows indicate conserved genes, while white arrows indicate *T. thlaspeos* genes with no corresponding orthologs. Blue arrows indicate a new gene that could be associated with the mating locus a in *T. thlaspeos*. Blue lines indicate pheromone response elements (sequence ACAAAGGGA; Urban *et al.*, 1996) detected by simple sequence search in both genomes.

Loss of synteny between genomes of fungal plant pathogens has been shown to increase with genetic distance and involves genomic regions that often are enriched for virulence-related genes (Raffaele & Kamoun, 2012). In the grass smuts, synteny breaks are almost exclusively found in so-called virulence gene clusters that encode effector genes with partly crucial virulence function (Schirawski *et al.*, 2010; Rabe *et al.*, 2016). In *T. thlaspeos*, except for Stp1 (UMAG_02475) in cluster 5B (Schipper, 2009) and the non-virulence related cluster 9A, we did not find any of the *U. maydis* virulence clusters (Supporting Information Table S10). In some cases, cluster-flanking genes were partially present and rearranged in *T. thlaspeos* as exemplarily shown for “Cluster 19A” in Fig. 1c.

In order to identify unique genes of *T. thlaspeos* which might functionally replace the missing effector clusters in plant colonization, we searched for shared orthologues using OrthoFinder (Emms & Kelly, 2015). Out of the 145,061 genes included in the analysis, 93.4% (135,570) could be placed in 10,059 orthogroups. *T. thlaspeos* shares most orthogroups with *A. flocculosa* (Fig. 2a, Supporting Information Table S11). This close relationship is further supported by multi-type locus phylogeny generated from 1,307 single-copy orthologs (Fig. 2b), which clearly places *T. thlaspeos* and *A. flocculosa* separate from the grass smuts.


a
Sporisorium reilianum
Ustilago maydis
Ustilago bromivora
Ustilago hordei
Sporisorium scitamineum
Pseudozyma hubeiensis
Moesziomyces antarcticus
Anthracocystis flocculosa
Thecaphora thlaspeos
Melanopsichium pennsylvanicum
Kalmanozyma brasiliensis
Tilletiaria anomala
Ceraceosorus bombacis
Moesziomyces aphidis
Tilletia controversa
Tilletia caries
Tilletia indica
Tilletia walkeri
Malassezia pachydermatis
Malassezia globosa
Malassezia sympodialis
Tilletia caries
Tilletia controversa
Tilletia indica
Tilletia walkeri
Malassezia sympodialis
Malassezia pachydermatis
Malassezia globosa
Ceraceosorus bombacis
Tilletiaria anomala
Moesziomyces antarcticus
Moesziomyces aphidis
Anthracocystis flocculosa
Thecaphora thlaspeos
Melanopsichium pennsylvanicum
Kalmanozyma brasiliensis
Ustilago bromivora
Ustilago hordei
Pseudozyma hubeiensis
Sporisorium scitamineum
Sporisorium reilianum
Ustilago maydis
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90
100
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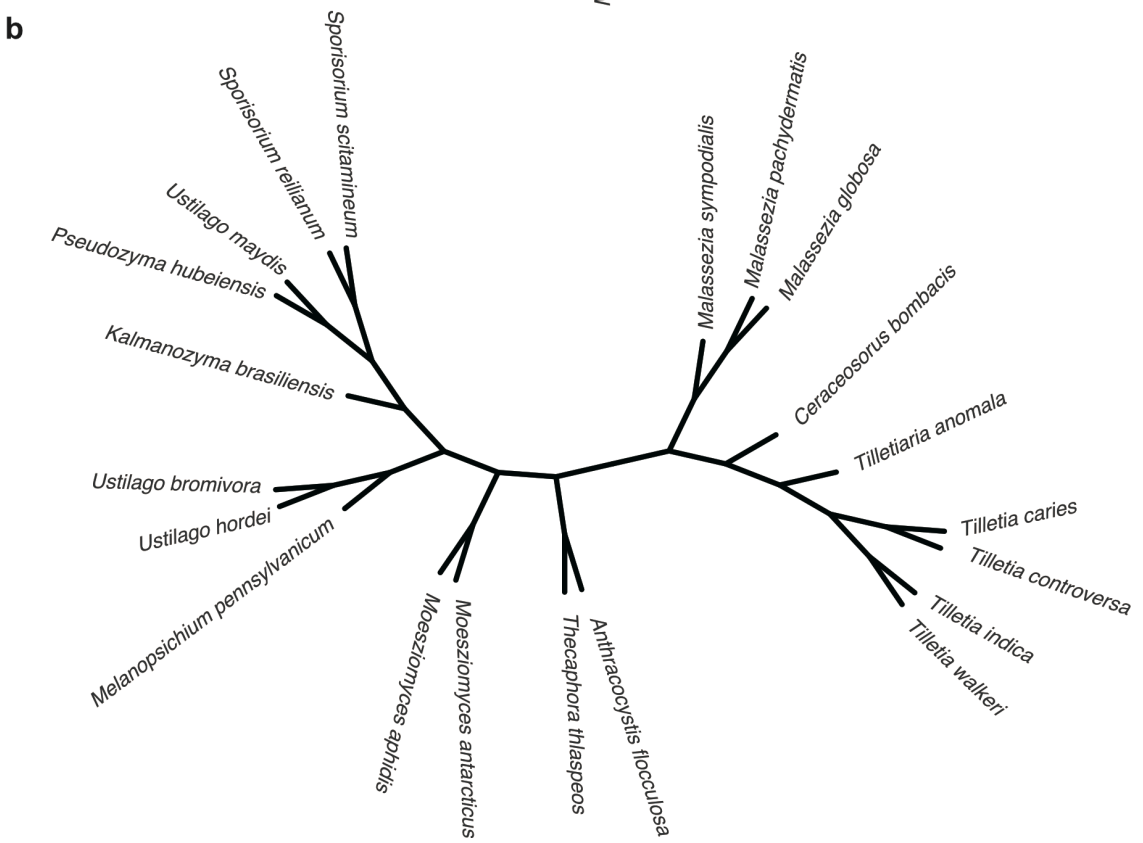
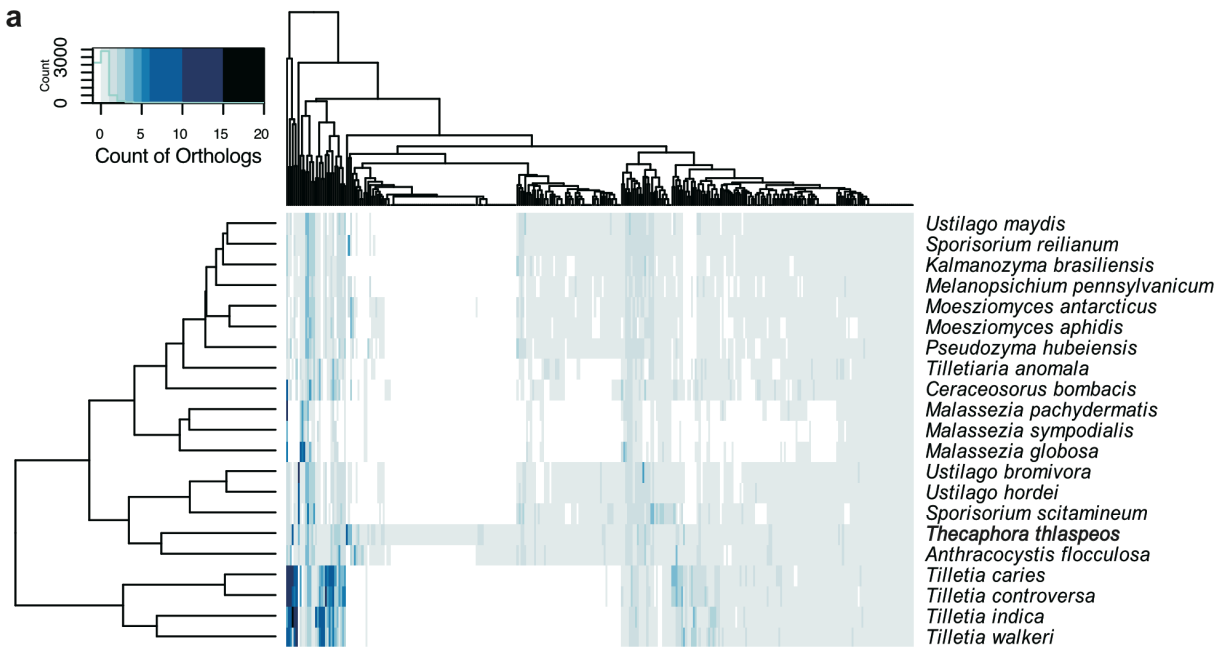

b
Sporisorium scitamineum
Sporisorium reilianum
Ustilago maydis
Pseudozyma hubeiensis
Kalmanozyma brasiliensis
Ustilago bromivora
Ustilago hordei
Melanopsichium pennsylvanicum
Moesziomyces aphidis
Moesziomyces antarcticus
Thecaphora thlaspeos
Anthracocystis flocculosa
Malassezia sympodialis
Malassezia pachydermatis
Malassezia globosa
Ceraceosorus bombacis
Tilletiaria anomala
Tilletia caries
Tilletia controversa
Tilletia indica
Tilletia walkeri

Figure 2. *T. thlaspeos* and *A. flocculosa* are genetically separate from the grass smuts. (a) Orthology analysis of all *T. thlaspeos* predicted genes in comparison to the predicted genes of other Ustilaginales species. The heatmap depicts the % overlap of orthologous groups. Cladogram on the left is based on hierarchical clustering (Euclidean method). (b) Multi-locus phylogeny of the Ustilaginales species used for the analysis based on 1,307 single copy orthologs.

Out of the 6,239 *T. thlaspeos* predicted proteins, 233 have no orthologs in smut fungi. The majority of these genes (205 out of 233) encode proteins of unknown function (Supporting Information Table S12). Notably, 44 of the unique proteins contain a predicted signal peptide (Fig. 3a), indicating that they might be involved in the interaction between *T. thlaspeos* and its host. Hence, we have generated unique repertoire of *T. thlaspeos* specific candidate virulence related genes. Indeed, two of the unique and secreted proteins carry a necrosis-inducing protein (NPP) domain, which is a ubiquitous effector protein of dicot plant pathogens (Oome & Van den Ackerveken, 2014).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Count
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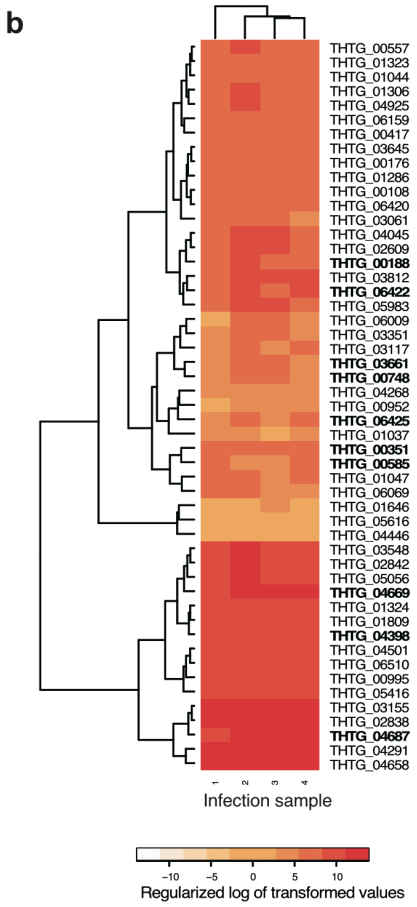
Count of Orthologs
Ustilago maydis
Sporisorium reilianum
Kalmanozyma brasiliensis
Melanopsichium pennsylvanicum
Moesziomyces antarcticus
Moesziomyces aphidis
Pseudozyma hubeiensis
Tilletiaria anomala
Ceraceosorus bombacis
Malassezia pachydermatis
Malassezia sympodialis
Malassezia globosa
Ustilago bromivora
Ustilago hordei
Sporisorium scitamineum
Thecaphora thlaspeos
Anthracocystis flocculosa
Tilletia caries
Tilletia controversa
Tilletia indica
Tilletia walkeri
Secreted protein coding genes


b
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THTG_02842
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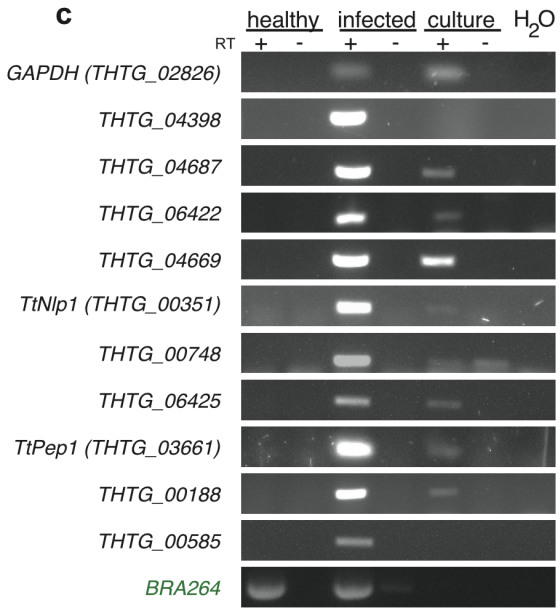
THTG_04669
THTG_01324
THTG_01809
THTG_04398
THTG_04501
THTG_06510
THTG_00995
THTG_05416
THTG_03155
THTG_02838
THTG_04687
THTG_04291
THTG_04658
1 2 3 4
Infection sample
−10 −5 0 5 10
Regularized log of transformed values


c
healthy
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culture
H2O
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GAPDH (THTG_02826)
THTG_04398
THTG_04687
THTG_06422
THTG_04669
TtNlp1 (THTG_00351)
THTG_00748
THTG_06425
TtPep1 (THTG_03661)
THTG_00188
THTG_00585
BRA264

Figure 3. Candidate effectors of *T. thlaspeos* are identified via differential expression analysis during infection and confirmed by RT-PCR. (a) Orthology analysis of the *T. thlaspeos* predicted secretome in comparison to the predicted secretomes of other Ustilaginales species. Cladogram on the left and on top is based on hierarchical clustering (Euclidean method). Color-coding depicts the amount of orthologs in other species for every *T. thlaspeos* secreted protein-coding gene. (b) Expression values of 51 differentially expressed secreted protein coding genes during the infection of *A. hirsuta*. Each column represents a biological replicate. Cladogram on the left is based on hierarchical clustering (Euclidean method) and effector candidates verified by RT-PCR are highlighted in bold. Color code represents regularized log transformed values derived from the DESeq2 analysis. (c) Effector candidates have visibly higher mRNA accumulation during infection compared to in culture. Effector mRNA accumulation is normalized by that of *gapdh*. Plant marker *BRA264* (Stockenhuber *et al.*, 2015) was used to verify samples containing plant tissue cDNA. RT: Reverse Transcriptase.

Finally, genome comparison of the two *T. thlaspeos* strains LF1 and LF2 as expected showed overall a high degree of synteny, as well as 11,509 single nucleotide polymorphisms (SNPs). In total, we obtained 280 syntenic blocks with on average 75.3 kb and 99.8% identity. We only detected very few structural variations in the one-to-one alignments with insertions or gaps that are short in length (Supporting Information Table S13). One example is the mating type locus *a* (Supporting Information Fig. S3). In addition, 31 genes were found to have no orthologous sequences in one or the other isolate (Supporting Information Table S14). In particular, an effector candidate presented in this study, THTG_04398, was identified in LF1 and not in LF2 (Supporting Information Fig. S4a). Interestingly, THTG_04398 is also absent in isolates of *T. thlaspeos* collected in Hohe Leite, Germany suggesting that there might be population differences in the effector distribution (Supporting Information Fig. S4b). Additionally, THTG_01646 is specific to LF1 and matches effector criteria (secreted, no functional annotation and orthology to other smut fungi), indicating that these candidate effectors could be isolate and/or mating-type specific.

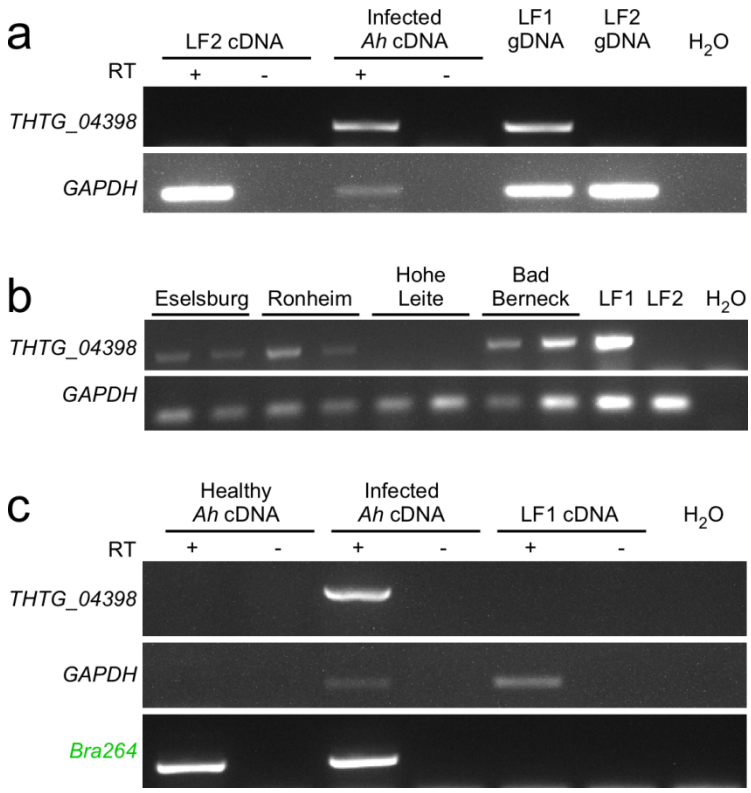

Figure S4. *T. thlaspeos* unique effector THTG_04398 is absent in strain LF2 and in isolates from Hohe Leite, Germany, while present in LF1. (a) THTG_04398 is present in the LF1 strain (originating from Eselsburg, Germany) and in the RNA-seq data from infected *Ar. hirsuta* tissue (with spores from Ronheim, Germany). mRNA accumulation of THTG_04398 is not detectable and the gene is not amplified in the LF2 strain (originating from Ronheim, Germany). (b) THTG_04398 is present in both isolates from each collection site except for those from Hohe Leite. Four infected siliques were pooled from each isolate. The fungal marker GAPDH is amplified in all samples containing fungal cDNA or gDNA. (c) THTG_04398 is up-regulated during infection compared to in LF1 culture confirming it remains an effector candidate. Expression was normalized to GAPDH. Plant marker gene Bra264 (Stockenhuben *et al.*, 2015) was used as a control for both healthy and infected tissues. RT: Reverse Transcriptase.

In planta-induced genes are enriched for unique, small, secreted proteins

To gain insight into the fully established biotrophic phase of *T. thlaspeos*, we conducted a whole transcriptome sequence experiment (RNA-seq) comparing *T. thlaspeos*-infected *Ar. hirsuta* rosette leaf tissue with axenic *T. thlaspeos* cultures and healthy *Ar. hirsuta*. RNA of rosette leaves from ten-week old teliospore-infected and healthy plants as well as fungal culture was sequenced, resulting in approximately 30 million reads for each of the in total twelve samples. Although the abundance of *T. thlaspeos* reads in infected samples was low (0.18% - 0.28%), we captured 988 genes expressed during infection (>5 raw read count, averaged between 4 infection samples). As expected, infected samples showed higher variation compared to culture samples (Supporting Information Fig. S5), due to less homogeneous *in planta* growth conditions compared to axenic culture.

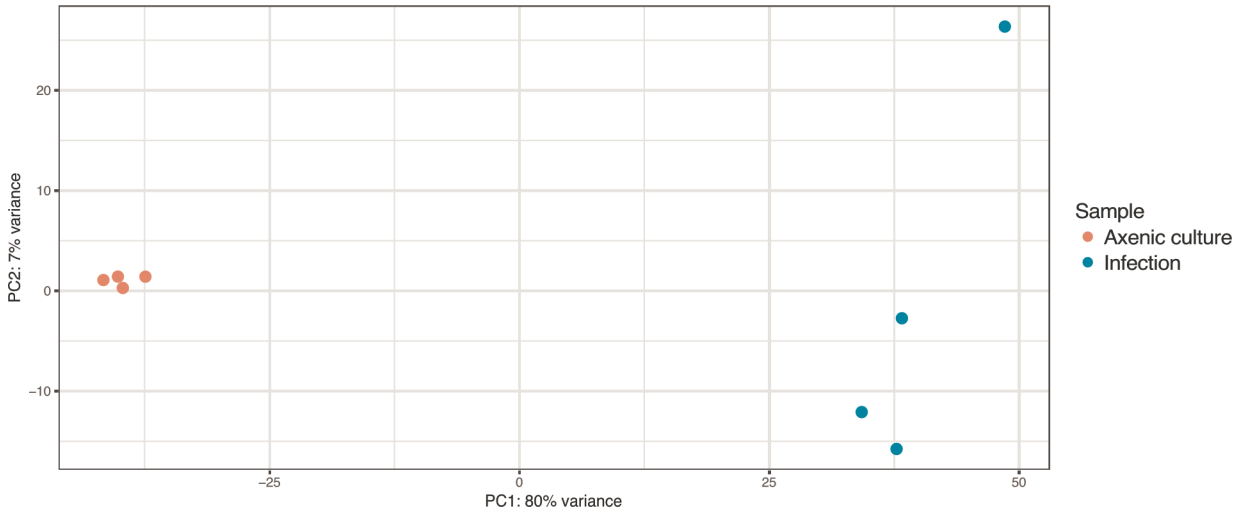

Figure S5. Principal component analysis (PCA) for the fungal RNA samples collected during axenic culture and infection of *A. hirsuta*. PCA plot for the RNAseq datasets derived by the *T. thlaspeos-A.hirsuta* interaction was generated using DESEQ2 in R.

132 genes are differentially expressed during infection (log2 fold change > +/- 2, adjusted p-value < 0.05; Supporting Information Table S16). Among the induced genes, there are several infection related factors such as a necrosis and ethylene-inducing like protein (Nlp1, THTG_00351), an ammonium transporter (THTG_03538), a sugar transporter (THTG_00350) as well as several hydrolases and cytochrome p450s. However, more than half of the differentially expressed genes have no functional annotation. Thereof, 51 are predicted to be secreted (Fig. 3b) and 29 are unique to *T. thlaspeos*. Out of these 29 protein-coding genes, we confirmed the top 10 candidates based on up-regulation during infection, including *Ttpep1* and *Ttnlp1* (Fig. 3c, Supporting Information Fig. S4c, Table 1). Hence, these genes are Thecaphora-unique effector candidates (Tues) and were further investigated together with *Ttpep1* and *Ttnlp1* by heterologous expression for their virulence activity.

The *T. thlaspeos* ortholog of the conserved smut effector Pep1 is active in *U. maydis*

Pep1 is a peroxidase inhibitor that allows penetration by inhibiting apoplastic plant defense peroxidases (Doehlemann *et al.*, 2009; Hemetsberger *et al.*, 2012). As the function of this effector is conserved among grass smuts (Hemetsberger *et al.*, 2015; Rabe *et al.*, 2016), we tested whether also the *T. thlaspeos* ortholog can rescue virulence in *U. maydis*. Integration of *Ttpep1* into the *U. maydis* deletion strain SG200*Δpep1* partially complemented the infection phenotype in that tumors were formed in the leaves during seedling infection (Fig. 4a, b). Furthermore,

*Tt*Pep1-mCherry is secreted into the apoplast (Fig. 4c) similar to *Um*Pep1-mCherry (Doehlemann *et al.*, 2009). This suggests that TtPep1 potentially targets an *Arabis* peroxidase related to POX12 of *Z. mays* and thereby inhibits the apoplastic ROS burst also in Brassicaceae hosts.

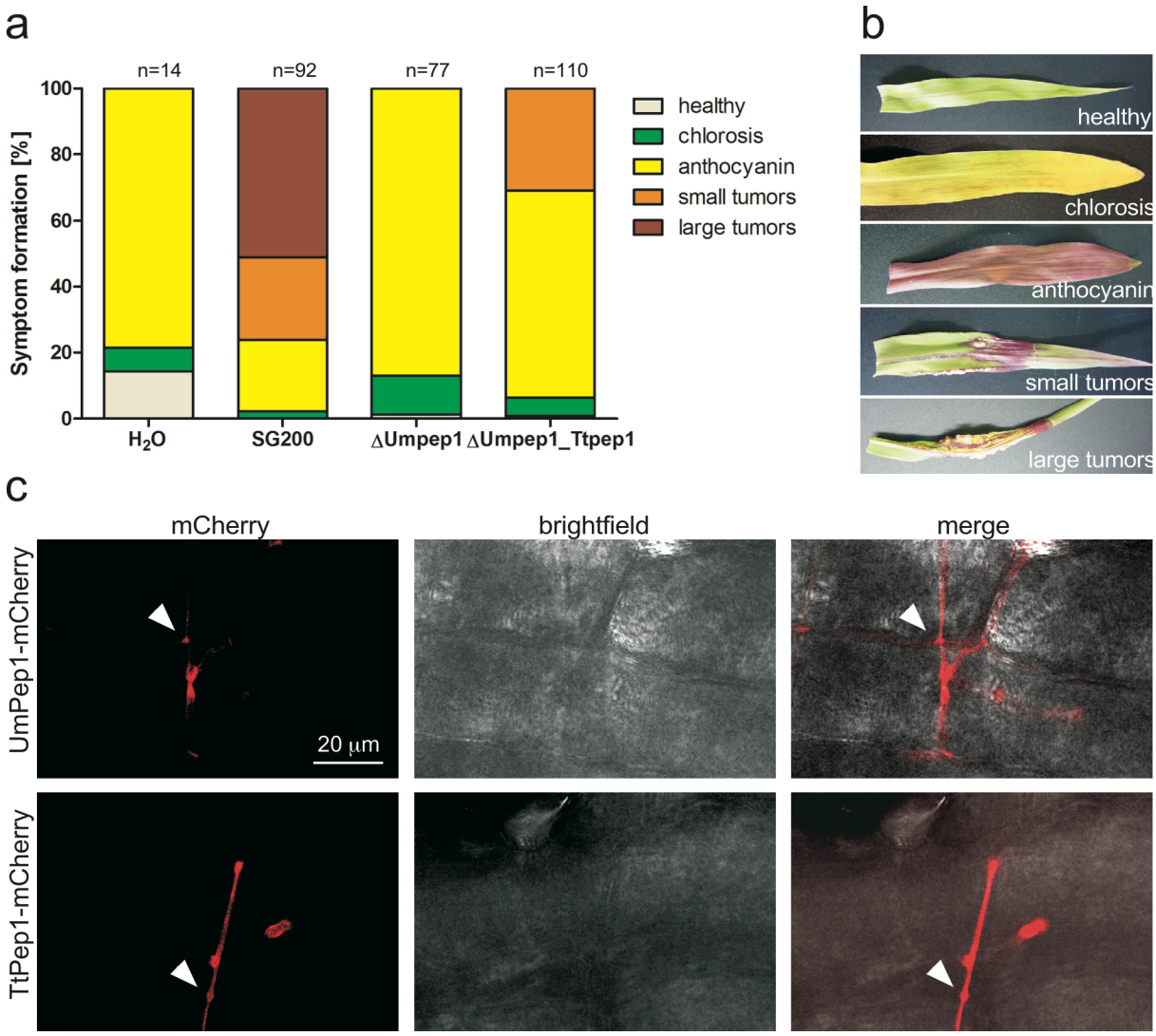

Figure 4. *Ttpep1* partially complements deletion of *Umpep1* in maize infection. (a) Disease rating of one week-old Early Golden Bantam maize plants 3 dpi with H_2O (mock) and *U. maydis* strains SG200_*Δpep1* (ΔUmpep1), SG200, and SG200_*Δpep1*_Ttpep1 (ΔUmpep1_Ttpep1). The values indicate the total number of plants infected in three independent experiments. (b) A representation of each disease category. (c) Confocal imaging of maize leaves infected with *Um*Pep1-mCherry (SG200_*Δpep1*_*Um*Pep1-mCherry), top, and *Tt*Pep1-mCherry (SG200_*Δpep1*_*Tt*Pep1-mCherry), bottom. Arrows indicate apoplastic regions into which *Um*Pep1 is secreted, as shown by Doehlemann *et al.*, 2009, and therefore where *Tt*Pep1 is also likely secreted.

The *Tt*Nlp1 is a non-cytotoxic effector of *T. thlaspeos*

Nlp effectors primarily occur in genomes of pathogens infecting dicot plants and the known grass smut fungi do not use such effectors. Their activity was mapped to a region comprising a highly conserved heptapeptide inducing necrosis (GHRHDWE; Schouten *et al.*, 2008; Ottmann *et al.*, 2009) and a 20 amino acid domain (nlp20) that induces immune responses such as ethylene production or ROS burst (Böhm *et al.*, 2014). A second class of Nlp proteins are non-cytotoxic and induce immune responses, but do not elicit HR-related cell death (Santhanam *et al.*, 2012; Oome *et al.*, 2014). To date, the role of the non-cytotoxic class of Nlps during infection remains elusive.

In addition to the induced *Ttnlp1*, the genome of *T. thlaspeos* encodes two *nlp* genes with predicted NPP1-domains (Pfam accession PF05630): THTG_00343=*nlp2*, and THTG_04815=*nlp3*. *Ttnlp1* and *Ttnlp2* are located on scaffold 1 and have predicted signal peptides. This *nlp*-locus contains additional genes with predicted signal peptides and, hence, might comprise the first *T. thlaspeos* effector gene cluster (Fig. 5a). *Ttnlp3*, located on scaffold 33, is substantially shortened at the N-terminus and does not have a signal peptide. Amino acids in the necrosis-inducing heptapeptide are not conserved in the three *T. thlaspeos* proteins suggesting they are non-cytotoxic (Fig. 5b). In line with these findings, transient expression assays in *N. benthamiana* confirmed that *Tt*Nlp1 fails to cause necrosis (Fig. 5c). To test whether *Tt*Nlp1 plays a role in virulence, we utilized *Pseudomonas syringae* pv. *tomato* DC3000-LUX (*Pst-LUX*) for pathogen-mediated delivery of *Tt*Nlp1 into *A. thaliana*. As expected, this non-cytotoxic protein does not cause HR. However, bacterial growth significantly increased in the presence of *Tt*Nlp1, suggesting a virulence function for this candidate effector (Fig. 5d, e). In the future, it will be important to confirm this virulence function by generating deletion mutant strains *T. thlaspeos*.

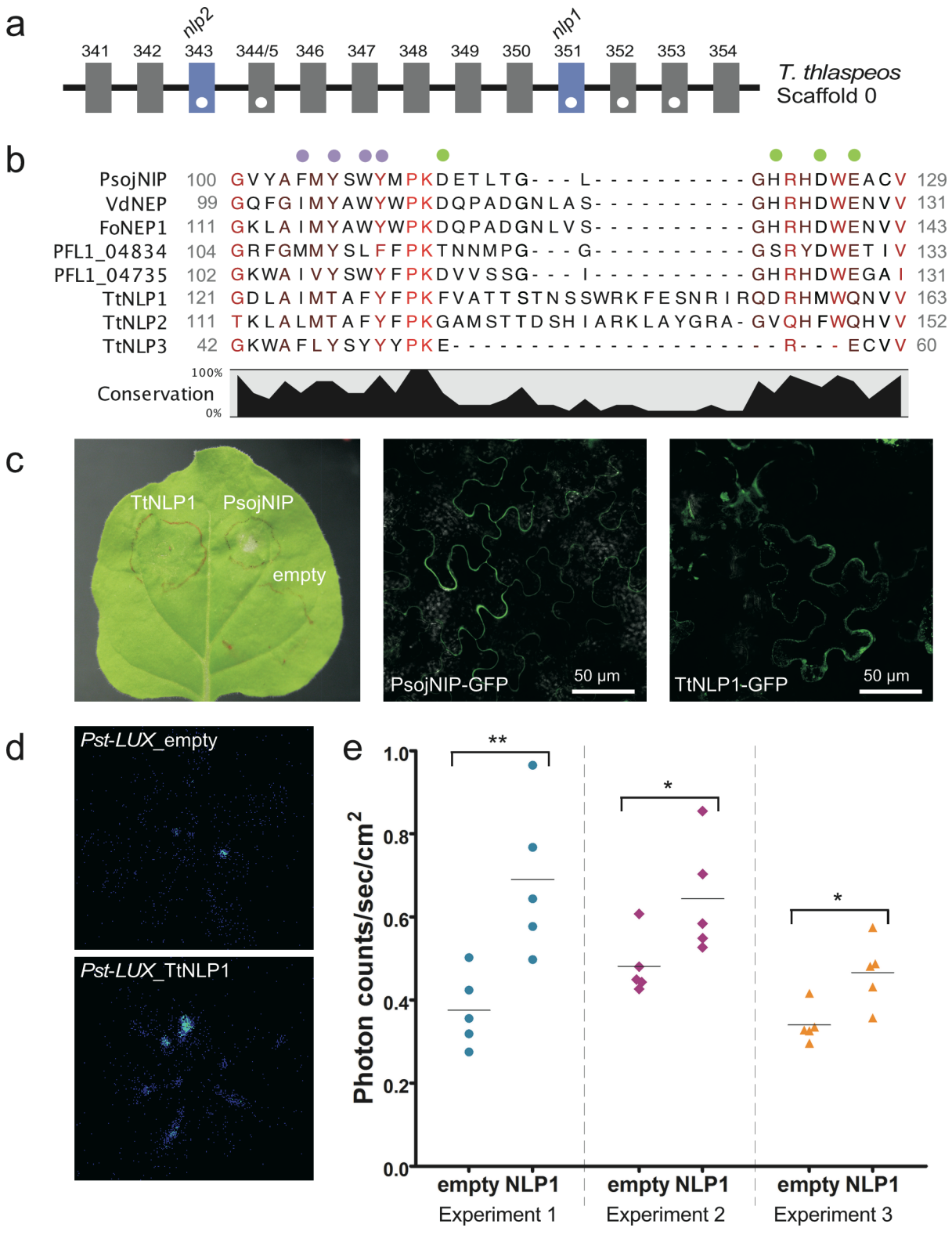
a
nlp2
nlp1
341 342 343 344/5 346 347 348 349 350 351 352 353 354
T. thlaspeos
Scaffold 0
b
PsojNIP 100 GVYAFMYSWYMPKDETLTG---L----------GHRHDWEACV 129
VdNEP 99 GQFGIMYAWYWPKDQPADGNLAS----------GHRHDWENVV 131
FoNEP1 111 GKLAIMYAWYWPKDQPADGNLVS----------GHRHDWENVV 143
PFL1_04834 104 GRFGMMYSLFFPKTNNMPG---G----------GSRYDWETIV 133
PFL1_04735 102 GKWAIVYSWYFPKDVVSSG---I----------GHRHDWEGAI 131
TtNLP1 121 GDLAIMTAFYFPKFVATTSTNSSWRKFESNRIRQDRHMWQNVV 163
TtNLP2 111 TKLALMTAFYFPKGAMSTTDSHIARKLAYGRA-GVQHFWQHVV 152
TtNLP3 42 GKWAFLYSYYYPKE----------R---ECVV 60
100%
Conservation
0%
c
TtNLP1
PsojNIP
empty
PsojNIP-GFP
50 µm
TtNLP1-GFP
50 µm
d
Pst-LUX_empty
Pst-LUX_TtNLP1
e
Photon counts/sec/cm2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
*
empty NLP1
Experiment 1
empty NLP1
Experiment 2
empty NLP1
Experiment 3

Figure 5. *Tt*Nlp1 does not induce a hypersensitive response and increases bacterial luminescence on Col-0. (a) The Nlp locus in Scaffold 1. Genes encoding proteins with a signal peptide are marked with a white dot. (b) Alignment generated with CLC Main (Qiagen) of the region including the ethylene-inducing domain and the heptapeptide sequence of *T. thlaspeos* Nlps and homologs from other plant pathogens (*Psoj*NIP: *Phytophthora sojae* AAK01636.1, *Vd*NEP: *Verticillium dahliae* AAS45247.1, *Fo*NEP1: *Fusarium oxysporum* AAY88967.2, PFL1_0434: *A. flocculosa* XP_007880553.1, PFL1_04735: *A. flocculosa* XP_007880454.1). Amino acids indispensable for ethylene induction are marked with a purple dot and amino acids indispensable for necrosis are marked with a green dot. (c) *A. tumefaciens*-mediated transient expression of *Tt*Nlp1-Gfp in *N. benthamiana* along with the positive control *Psoj*NIP and the empty vector negative control pEG-103. Agro-infiltration of Gfp-tagged *Tt*Nlp1 and *Psoj*NIP results in heterologous protein expression as detected by the Gfp signal in *N. benthamiana*. Necrosis is only visible upon infiltration of *Psoj*NIP but not with *Tt*Nlp1. (d) Col-0 sprayed with *Pseudomonas syringae* pv. *tomato* DC3000-LUX (*Pst-LUX*) containing *Tt*Nlp1 display increased luminescence compared to Col-0 sprayed with *Pst-LUX* containing the empty vector control. (e) *Tt*Nlp1-containing *Pst-LUX* significantly increases luminescence in Col-0 compared to the empty vector control strain.

Thecaphora thlaspeos unique effector 1 (Tue1) is a novel virulence factor

To investigate effector function, stable expression in *A. thaliana* offers several advantages in that growth phenotypes and morphological alterations can be detected *in planta* (Germain *et al.*, 2017), and plant targets can be identified by interaction studies. Therefore, we successfully generated transgenic *A. thaliana* Col-0 lines for 6 of the top 10 effector candidates as well as the bona-fide effector *Tt*Nlp1 and monitored rosette size and color. Four week-old rosettes of transgenic plants expressing the *T. thlaspeos* unique effector *Tt*Tue1 (THTG_04687) were significantly smaller than the control plants and displayed minor chlorosis (Fig. 6a, b). The other candidate effector lines showed larger variations in size than the control plants, but overall resembled the control plants, even though the fungal effectors were expressed (Fig. 6a, b, Supporting Information Fig. S6). In addition, we did not observe growth or morphological alterations caused by the effector *Tt*Nlp1 This phenotype is similar to the non-cytotoxic *H. arabidopsidis Hp*Nlp1 (Oome *et al.*, 2014). In summary, we could confirm *Tt*Tue1 as the first novel virulence factor specific to *T. thlaspeos*, which we will functionally characterize in the future.

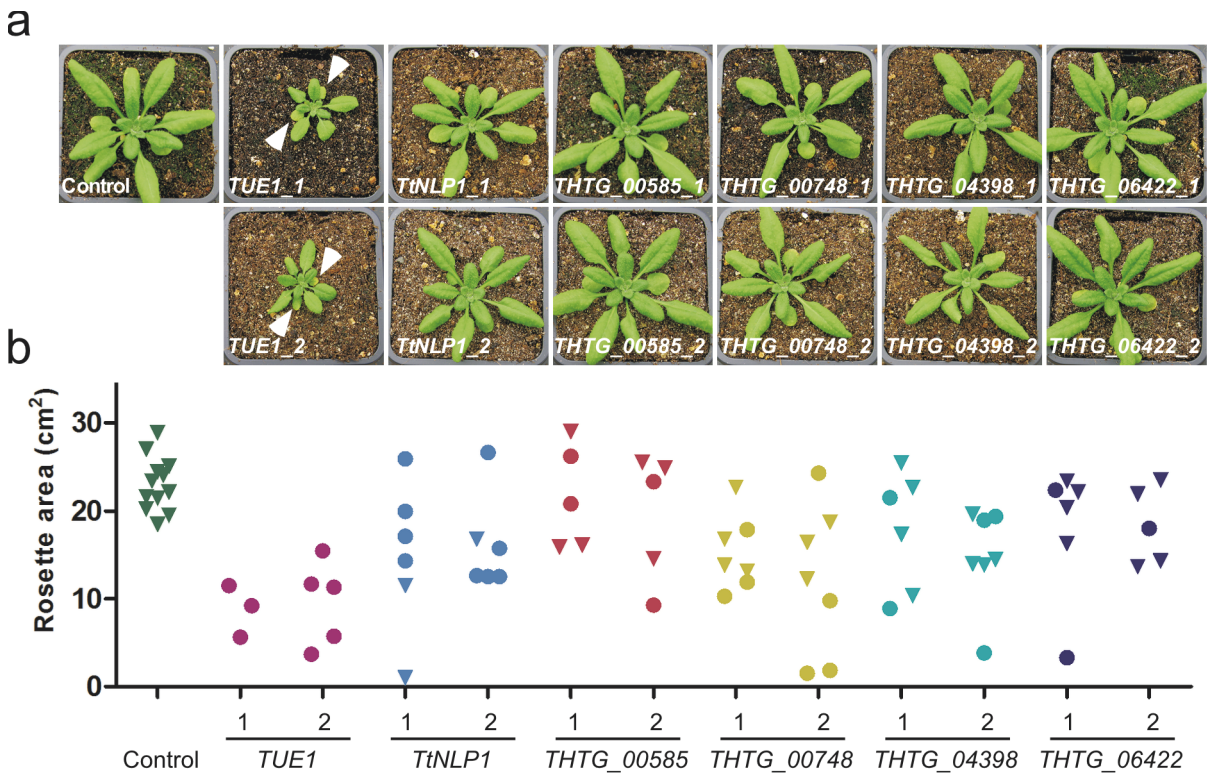

Figure 6. Transgenic *A. thaliana* accumulating mRNA of *Tttue1* are small with slightly chlorotic first true leaves. (a) Representative images of two independent plant lines (1 and 2) expressing the up-regulated effector candidate. *Tt*Tue1 expressing lines are clearly smaller and show signs of chlorosis (arrowheads) (b) Quantification of rosette area confirms that *Tt*Tue1 plants are significantly smaller than the controls (p-values are 6.48E-06 and 4.25E-06, respectively, based on student s t-test) and no individuals accumulating *Tttue1* mRNA resemble the control plants in size, while variation is high in lines expressing different effector candidates. Circles indicate plants whose effector mRNA accumulation was confirmed via RT-PCR, triangles indicate additional individuals.

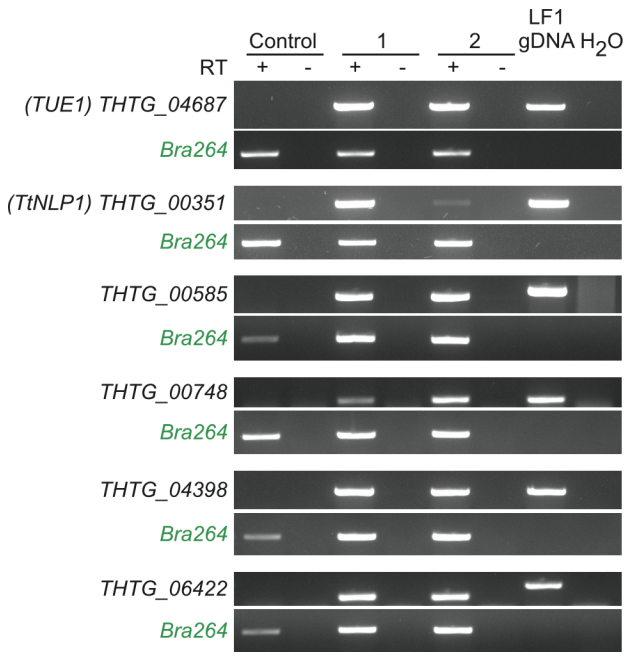

Figure S6. Transgenic *A. thaliana* lines of this study accumulate mRNA of each respective effector. Representative images of effector and plant marker Bra264 mRNA accumulation confirmation via PCR on cDNA in all studied *A. thaliana* lines. Independent transformants are indicated by (1) and (2). RT: Reverse Transcriptase.

Table 2. Features of *T. thlaspeos* effector candidates investigated in *A. thaliana*. Phenotype refers to macroscopically detectable changes in growth or morphology 4 weeks post sowing. Candidate effectors are listed from most up-regulated during infection to least and those in gray were not further analyzed in phenotype screen.

Candidate effector protein	Protein length (aa)	Signal peptide length (aa)	Up-regulation in infection (RT-PCR)	Phenotype when expressed in *A. thaliana*
THTG_04398	235	27	Yes	-
THTG_04687 (*Tt*Tue1)	294	22	Yes	Small rosettes
THTG_06422	162	18	Yes	-
THTG_04669	269	22	Yes	Few transformants in *A. thaliana*
THTG_00351 (*Tt*Nlp1)	292	19	Yes	-
THTG_00748	137	25	Yes	-
THTG_06425	271	18	Yes	No effector mRNA accumulation
THTG_03661 (*Tt*Pep1)	142	22	Yes	Few transformants in *A. thaliana*
THTG_00188	252	23	Yes	No *A. tumefaciens* strains
THTG_00585	449	22	Yes	-

T. thlaspeos infection mildly induces plant defense responses

The lack of macroscopic infection symptoms during the biotrophic growth of *T. thlaspeos* suggests that host gene expression might only be mildly affected. Our expression dataset provides a snapshot of plant responses at the time of infection when biotrophy of the fungus is fully established and sporulation has not started. This status is representative for the major part of the interaction because *T. thlaspeos* remains in the vegetative tissues for several months and overwinters in perennial host species. *De novo* transcript assembly of healthy and infected *Ar. hirsuta* plants resulted in 170,196 transcripts, out of which 103,288 have a homologous transcript in *A. thaliana*.

Analysis of differentially expressed plant genes revealed that infected samples are enriched for functional categories related to biotic stress and defense (Fig. 7, Supporting Information Table S17). This includes receptor-like proteins and kinases, peroxidases, chitinases and NLR domain carrying proteins. Among them are also wall-associated kinases (WAK), which have been shown recently to be involved in the interaction between smut fungi and their respective hosts (Zuo *et al.*, 2014). Salicylic acid-dependent signaling is a response associated to defense against biotrophic pathogens (Glazebrook, 2005; Huot *et al.*, 2014). *EDS5,* the pathogen responsive SA-extrusion exporter (Serrano *et al.*, 2013) as well as *NIM1*, involved in the signal transduction cascade leading to SAR (Ryals *et al.*, 1997), are up-regulated in infected compared to healthy plant tissue, while the expression of the essential regulator of plant systemic acquired resistance *NPR1*, an essential component of *R* gene-mediated disease resistance *EDS1*, and host defense regulatory gene phytoalexin deficient 4, *PAD4* was unchanged upon infection (Table 3). In addition, the expression of *NDR1* and *PR2*, genes also involved in disease resistance, was unchanged upon infection (Table 3).

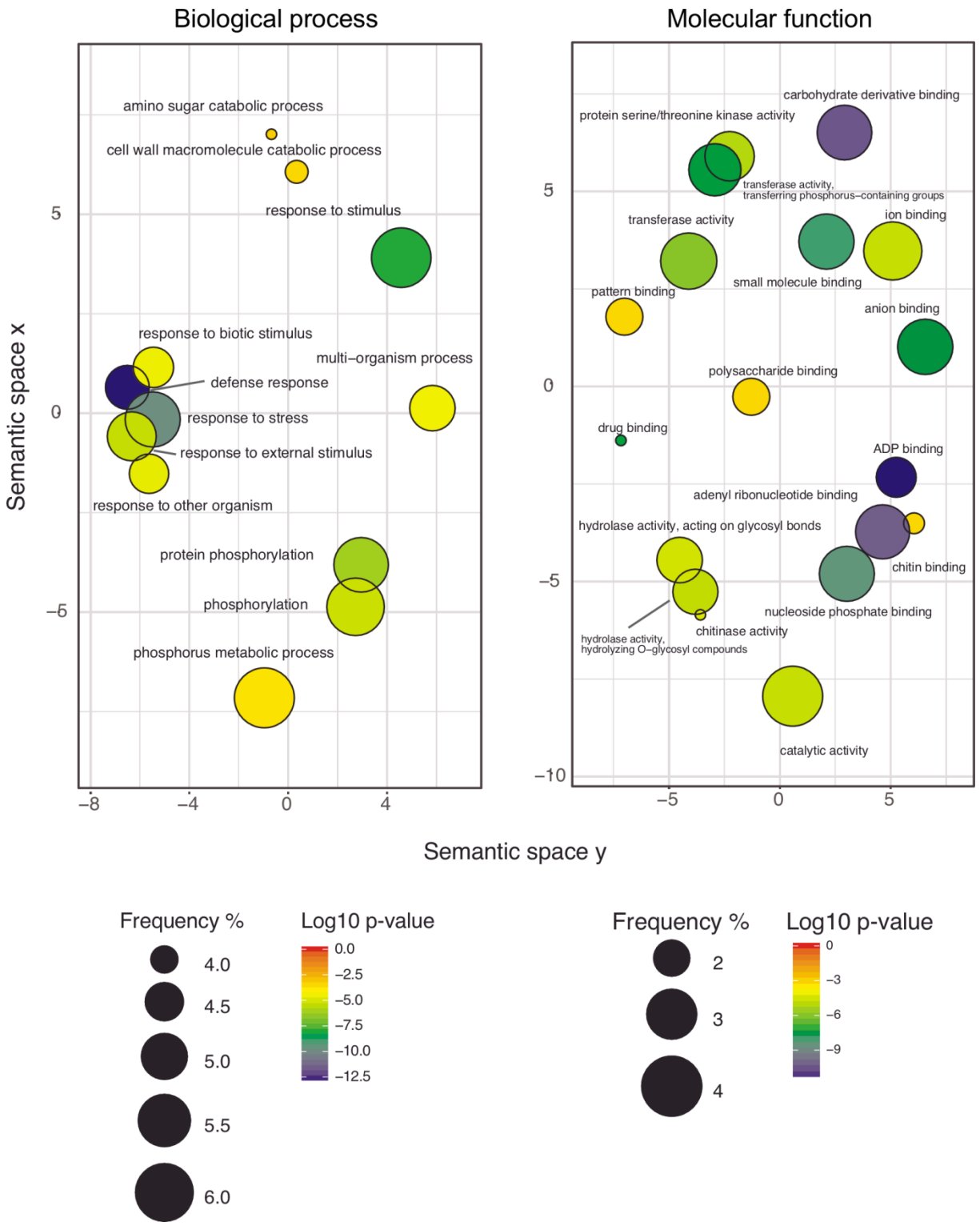

Figure 7. Over-represented gene ontology (GO) terms during the infection of *A. hirsuta* by *T. thlaspeos* show prevalence of biotic stress. Two-dimensional semantic space scatterplots to describe over-represent GO-terms were generated with Gorilla and REVIGO (p-value < 0,001). Circle sizes represent the frequency of the GO term in the Arabidopsis genome while the color indicates the p-value for the enrichment.

Table 3. Expression of SA-dependent defense signaling genes in healthy and infected *A. hirsuta* plants. Genes with a role in SA-signaling were selected based on literature (see text). Reads mapping to these assembled transcripts were identified to compare expression. For some genes, more than one transcript was assembled and only those with both a p-value and FDR value less than 0.05 are listed. Significant logFC values are shown in bold. For *A. thaliana* SAG101, assembled transcripts all had FDR values exceeding the cutoff. No transcripts were assembled for *At*PR1.

***A. thaliana* homolog**	**Trinity transcript ID**	**logFC**	**logCPM**	**p-value**	**FDR**
EDS5/SID1	DN21698_c3_g2_i1	**2.14**	1.93	3.59E-08	9.52E-06
	DN23650_c0_g2_i9	**2.25**	2.60	3.20E-09	1.05E-06
PAD4	DN24048_c0_g1_i4	1.23	5.20	3.75E-08	9.85E-06
	DN44396_c0_g1_i1	0.78	4.62	8.09E-05	8.21E-03
NDR1	DN26713_c0_g1_i1	1.52	5.05	2.54E-09	8.42E-07
	DN17709_c0_g1_i3	1.48	4.38	1.04E-10	4.51E-08
NPR1	DN18824_c0_g2_i2	1.07	3.78	3.06E-05	3.57E-03
PR2	DN21822_c0_g2_i5	1.10	6.33	5.19E-07	1.04E-04
NIM1	DN35844_c0_g1_i1	**2.73**	3.63	9.68E-21	2.47E-17
EDS1	DN23682_c0_g6_i2	0.99	3.67	5.33E-06	7.95E-04
	DN23791_c1_g3_i3	0.97	6.12	1.74E-06	3.04E-04
	DN20445_c0_g1_i9	0.75	5.06	1.67E-04	1.48E-02

Taken together, we find that in line with the infection biology of *T. thlaspeos* resembling that of an endophyte, the apparently mild impact of *T. thlaspeos* on host defense salicylic acid-dependent signaling provides further evidence of its endophyte-like behavior during the biotrophic growth phase in vegetative tissues.

Discussion

T. thlaspeos has a typical smut genome with unique features that suggest adaptation to dicot hosts

With a size of approximately 20 Mb, a low repeat content and 6,239 predicted gene models, the genome of *T. thlaspeos* has the typical characteristics of most sequenced smut fungi. Despite the adaptation to a dicot host, its absolute gene content and predicted functional categories largely overlap with grass-infecting smut fungi (Fig. 2a, Sharma *et al.*, 2015; Dutheil *et al.*, 2016). However, two unique features stand out from the genome assembly and annotation. First, synteny between *T. thlaspeos* and the grass smuts is low (Supporting Information Fig. S2) and second, *T. thlaspeos* shares only few known effector candidate genes with its grass smut relatives. Hence, *T. thlaspeos* seems to deploy a different repertoire of effectors to establish and maintain its biotrophic lifestyle. Remarkably, *M. pennsylvanicum*, the only example of grass smuts that underwent a host jump from grasses to the dicot genus *Persicaria*, has maintained its typical grass smut effector repertoire and accordingly has a very low number of *T. thlaspeos* orthologs suggesting independent dicot adaptation in *Thecaphora* and *M. pennsylvanicum* (Sharma *et al.*, 2014, Fig. 2a, Supporting Information Table S11). For example, the Nlps are well-known effectors that distinguish *T. thlaspeos* from other smut fungi. Notably, these are also absent in earlier diverging species of the Ustilaginales such as *Ceraceosorus bombacis* (Sharma *et al.*, 2015), suggesting independent acquisition e.g. by horizontal gene transfer.

More closely related to *T. thlaspeos* is the epiphytic biocontrol agent *A. flocculosa* which has a significantly higher degree of synteny and a larger overlap in gene content including 17 candidate effector genes (Supporting Information Table S11, Supporting Information Table S4). Interestingly, *A. flocculosa* also carries Nlp domain encoding genes (Lefebvre *et al.*, 2013), yet these are non-orthologous to the *T. thlaspeos* Nlps. The close genetic distance to *T. thlaspeos,* along with the presence of these candidate effectors, supports the previously raised hypothesis that *A. flocculosa* besides being a mycoparasite of powdery mildews (Laur *et al.*, 2017), could also be a yeast anamorph of a dicot infecting smut species (Begerow *et al.*, 2014).

Comparing the two *T. thlaspeos* isolates LF1 and LF2 revealed the first isolate-specific smut effectors (Supporting Information Table S14). This is particularly interesting as the infectious form of smut fungi is a dikaryon. Considering that *T. thlaspeos* genetically contains the capacity for mating (Supporting Information Fig. S3, Supporting Information Table S15) and that haploid isolates of opposite mating types form fusion hyphae (Frantzeskakis *et al.*, 2017), genetic

exchange during mating in *T. thlaspeos* provides the potential to bring together certain virulence-related genes of the single strains. Hence, the combination of different mates could result in distinct fitness levels of the fungus due to alterations in effector dosage and/or content or due to complementation of effector gene losses. In the future, population genetics approaches can reveal distribution of effectors throughout populations and the stability of such populations over the years.

T. thlaspeos infection strategy enables perennial biotrophy

In addition to pathogens, also fungal endophytes possess an astonishing diversity of host colonization strategies that independently evolved in several taxonomic groups (Rodriguez *et al.*, 2009; Brader *et al.*, 2017). Endophytes are microorganisms that colonize the inner plant tissues of macroscopically healthy host plants (Schulz & Boyle, 2005). Some fungal endophytes establish long-lasting interactions such as the generalist root endophyte *Piriformospora indica* or members of the grass endophyte genus *Epichloë* that remain inside their host throughout the growing season (Rodriguez *et al.*, 2009; Franken, 2012). As for biotrophic pathogens, successful colonization requires complex molecular mechanisms. For example, the basidiomycete *P. indica* establishes biotrophy in *A. thaliana* and barley in a host species-dependent manner with distinct transcriptional responses (Lahrmann *et al.*, 2013). Despite a few well-studied examples, it remains largely unknown how plant-pathogenic endophytes manage to establish and maintain such sustained systemic infections and what determines the type of interaction and host specificity.

Here, we characterized the biotrophic phase of *T. thlaspeos*. To maintain this “hidden” growth without macroscopic symptoms, it employs typical effector proteins such as Pep1 and Nlps, but also unique effectors such as *Tt*Tue1. The latter, when overexpressed in *A. thaliana*, causes a growth defect (Fig. 6a) reminiscent of the phenotypes observed in autoimmune mutants (Bowling *et al.*, 1994, 1997; Oome *et al.*, 2014), suggesting that *Tt*Tue1 might activate host immune responses or distort developmental processes. Overall, the identification of these novel smut effectors opens the door to study the specific activity of the *Thecaphora*-clade effectorome and how, or when, it is utilized to manipulate the host s responses.

On the plant side, transcriptional changes reflect a response to a biotic interaction. The expression of several key genes involved in SA-dependant signaling remains unchanged during the infection

of *Ar. hirsuta* with *T. thlaspeos* (Table 3, Supporting Information Table S17). Therefore, although colonization by *T. thlaspeos* registers as a biotic stress event in the plant s transcriptome (Fig. 7), specific modules of the plant s immune system remain dormant. This resembles the majority of endophytes, which do not have any known effects on their host plants resulting from colonization and therefore can possibly act commensally with their host (Brader *et al.*, 2017). This observation also agrees with previous studies on smut fungi where despite an overall up-regulation of stress-related gene expression during infection, fungal effectors are suggested to keep defense hormonal triggers at low levels in the respective host (Doehlemann *et al.*, 2008; Djamei & Kahmann, 2012). This effective balance and continuous interaction between *T. thlaspeos* and the host s immune system may therefore limit excessive fungal proliferation which could undermine plant fitness.

In summary, *T. thlaspeos* colonizes Brassicaceae hosts using a unique set of secreted proteins, different from both monocot infecting species but also the dicot infecting *M. pennsylvanicum*. Excitingly, we find smut-typical effectors such as Pep1, dicot-typical effector genes such as the Nlps but also novel effector candidates such as *Tt*Tue1. In addition, we show that the effector repertoire could differ between *T. thlaspeos* isolates. Further studies on *T. thlaspeos* could elucidate whether the secreted protein coding genes identified here present different expression patterns in various tissues or at different points during its long-term biotrophic stage. Finally, using the information and the resources provided here, more extensive studies could address the pathogen-endophyte continuum using *T. thlaspeos* as a model organism.

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Author contributions

VG and MF planned and designed the research. LF, KC and NH designed and performed the experiments. AB contributed genomic sequences. LF and BU performed the bioinformatic analysis. EK, YKG, HPvE, and BD contributed materials and supported the experimental design. KC, LF, RK, and VG wrote the manuscript.

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Supporting Information

Additional supporting information can be found on the CD accompanying this dissertation.

Supporting Information Table S1. Scaffolds with telomeric repeats
Supporting Information Table S2. BUSCO analysis of the genomic assembly and annotation for the genomes of LF1 and LF2
Supporting Information Table S3. Functional annotation of the LF1 predicted proteome
Supporting Information Table S4. List of secreted proteins of *T. thlaspeos LF1*
Supporting Information Table S5. Count of predicted secreted proteins of other smut fungi species
Supporting Information Table S6. Number of carbohydrate-active enzyme coding genes in several smut fungi genomes including *T. thlaspeos*
Supporting Information Table S7. Genes related to secondary metabolism in *T. thlaspeos*, *U. maydis* and *A. flocculosa* as predicted by AntiSMASH 4.0.2
Supporting Information Table S8. Repetitive content of the *T. thlaspeos* LF1 genome
Supporting Information Table S9. Whole genome alignments between *T. thlaspeos* LF1 and other Ustilaginomycotina species
Supporting Information Table S10. Ortholog genes to *U. maydis* known effector clusters and effectors
Supporting Information Table S11. Orthology analysis between the *T. thlaspeos* LF1 and 20 Ustilaginomycotina proteomes
Supporting Information Table S12. *T. thlaspeos* LF1 genes with no homologs to other smut fungi
Supporting Information Table S13. Summary of differences between the genomes of *T. thlaspeos* LF1 and LF2
Supporting Information Table S14. *T. thlaspeos* gene models with no hits in the genomic assembly of the opposite isolate
Supporting Information Table S15. *U. maydis* genes involved in mating and their putative orthologs in *T. thlaspeos*
Supporting Information Table S16. DESeq2 analysis of the fungal transcriptome during infection
Supporting Information Table S17. Transcriptome analysis of healthy and infected *A. hirsuta* plants
Supporting Information Table S18. Datasets used in this study

5.6 Additional results to Publication II

In order to gain insight into whether *T. thlaspeos*-infected *Ar. hirsuta* plants display immune responses compared to the healthy control plants, I searched for expression values of *Ar. hirsuta* transcripts orthologous to *AtPR1* in **Publication II Table S17** for both conditions**.** The activation of *AtPR1* in *A. thaliana* is pathogen-induced and known to be SA-dependant (Thomma *et al.*, 1998), therefore it serves as an indicator of plant immune response. As no *Ar. hirsuta* transcripts were identified to be homologous to *AtPR1*, I attempted to amplify *AhPR1* via RT-PCR with primers designed for *AtPR1*. For this RT-PCR, I used cDNA that I generated from the RNA samples used in my infection RNA-seq experiment to determine whether a homolog of *AtPR1* could be identified and if so, whether its expression was altered in *T. thlaspeos*-infected *Ar. hirsuta* compared to the healthy control plants. Interestingly, as shown below in **Figure 4**, *AhPR1* was amplified and is clearly up-regulated in all three replicates compared to the healthy control plants. This suggests that *T. thlaspeos* activates SA-dependant immune responses, despite the fact that the expression of several other key genes involved SA-dependant defense signaling is unchanged during this infection (**Publication II: Table 3**). This also illustrates the difficulty in using a tetraploid host for which there is no genome available and reinforces the advantages that using the diploid, sequenced *A. thaliana* as the host plant would offer.

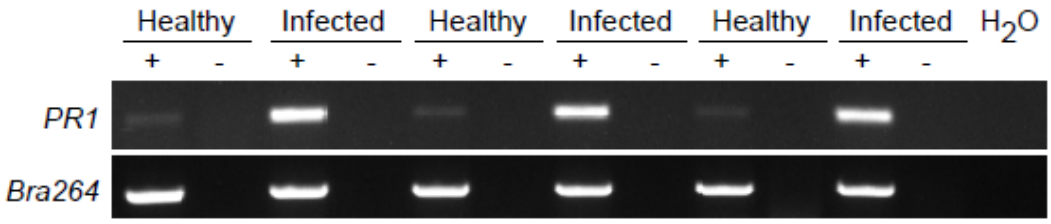

Figure 4. *Ar. hirsuta* rosette leaf tissue infected with *T. thlaspeos* have a visibly higher level of *PR1* mRNA accumulation compared to the basal level in healthy leaves. mRNA accumulation of the plant marker gene *Bra264* (Stockenhuber *et al.*, 2015) was consistent for both healthy and infected tissues. Symbols (+) and (-) indicate the use of cDNA template generated with and without reverse transcriptase, respectively. Semi-quantitative PCRs both performed with 25 cycles.

In addition to SA-dependant defense signaling, JA/ET-dependant defense signaling also plays an important role in for the plant immune system and these three pathways interact extensively (Glazebrook, 2005). Given that the expression of key SA-dependant defense signaling genes was unchanged during infection (**Publication II: Table 3**) but *PR1* is activated (**Figure 4**), I expected that key JA/ET-dependant defense signaling related genes such as *COI1* (Xie *et al.*, 1998) and *PDF1.2* (Manners *et al.*, 1998) would be down-regulated during infection due to SA-JA antagonism (Glazebrook, 2005). Furthermore, since ET

signaling also plays a role in maintaining microbial growth (Plett & Martin, 2017), I was particularly interested in determining whether the expression of ET-inducible plant genes was altered during infection, potentially as a result of fungal effector manipulation. Therefore, I investigated the expression of four key transcription factors of a subfamily of the Ethylene Responsive Factor (ERF) family that regulate genes responsive to biotic stress, *ERF1*, *ERF6*, *ERF104* and *ORA59* (reviewed in Tsuda & Somssich, 2015). My expression data (**Publication II: Table S17**) show, as summarized in **Table 1** below, that a *Ar. hirsuta* transcript orthologous to a key *A. thaliana* gene involved in JA/ET-dependant signaling defense responses, ETHYLENE RESPONSIVE FACTOR 6 (*ERF6*), is significantly up-regulated during infection *T. thlaspeos*. *ERF6* induction targets the defense gene PLANT DEFENSIN 1.2 (*PDF1.2*) (Meng *et al.*, 2013). As a majority of the investigated *Ar. hirsuta* transcripts orthologous to these *A. thaliana* genes either did not have reliable expression values or were not present in my dataset, further JA/ET-related genes would have to be investigated to determine the overall effect that *T. thlaspeos* infection has on JA/ET-dependant defense signaling.

Table 1. Expression of key JA/ET dependant signaling defense-related genes during *T. thlaspeos* infection of *Ar. hirsuta* ten weeks post infection. No *Ar. hirsuta* transcripts homologous to *AtPDF1.2* were recovered. Transcripts marked in gray indicate those that do not meet the standard cut-off (p-value < 0.05, false discovery rate < 0.05) and were therefore not considered.

***At* homolog**	***Ah* transcripts**	**Log fold change**	**P-value**	**False discovery rate**
COI1	TRINITY_DN19174_c0_g1_i4	0.275849	0.307281	1
	TRINITY_DN42705_c0_g1_i1	0	1	1
	TRINITY_DN25308_c0_g1_i1	0	1	1
	TRINITY_DN26860_c2_g10_i12	-0.37795	0.147412	1
	TRINITY_DN24181_c1_g1_i4	-0.57577	0.0200357	0.482549
	TRINITY_DN19217_c1_g1_i3	-1.53317	1	1
ERF1	TRINITY_DN19174_c0_g1_i4	0.275849	0.307281	1
	TRINITY_DN42705_c0_g1_i1	0	1	1
	TRINITY_DN25308_c0_g1_i1	0	1	1
	TRINITY_DN26860_c2_g10_i12	-0.37795	0.147412	1
	TRINITY_DN24181_c1_g1_i4	-0.57577	0.0200357	0.482549
	TRINITY_DN19217_c1_g1_i3	-1.53317	1	1
ORA59	TRINITY_DN22238_c1_g4_i2	3.038414	0.0017813	0.094148
	TRINITY_DN27421_c1_g1_i2	1.263891	0.2915705	1
	TRINITY_DN15273_c0_g1_i5	0	1	1
ERF6	TRINITY_DN23708_c1_g6_i1	3.409436	2.59E-06	0.000426
	TRINITY_DN24045_c1_g1_i4	2.327114	0.0035088	0.154548
ERF104	TRINITY_DN24031_c2_g2_i6	1.32127	0.1551918	1
	TRINITY_DN26301_c0_g1_i2	0.931796	0.4654564	1

5.7 Summary of Publication II

Several studies on smut infection in grass hosts have been conducted in recent years (Doehlemann *et al.*, 2008; Taniguti *et al.*, 2015; Rabe *et al.*, 2016). Although a majority of these studies have reported on fungal genes involved in pathogenicity and/or the host response to infection, the host responses during infection have yet to be functionally verified. In addition, the effectors of perennial and dicot-infecting smuts (Sharma *et al.*, 2014) have also not been studied in extensive detail. Using the tools established in **Publication I**, we began to address these open questions first by sequencing, assembling, and analyzing the genome of the Brassicaceae smut *T. thlaspeos*. These efforts revealed that the genome of *T. thlaspeos* largely resembles those of well-described smut fungi including *U. maydis*, *U. hordei*, and *S. reilianum* in completeness, mating-type genes, and is approximately 20 Mb in size (Kämper *et al.*, 2006; Schirawski *et al.*, 2010; Laurie *et al.*, 2012). Interestingly, the closest sequenced genome to *T. thlaspeos* in synteny is that of the fungal bio-control agent *Anthracocystis flocculosa* (syn. *Pseudozyma flocculosa*). We sequenced *T. thlaspeos* cultures originating from two locations in Germany and identified the core smut effector Pep1 as well as the dicot effector *Tt*Nlp1 in both strains. Using heterologous expression systems, we demonstrated that both *Tt*Pep1 and *Tt*Nlp1 are functional in virulence and *Tt*Nlp1 is not cytotoxic (**Publication II: Figure 4, 5C,D,E**).

The lab infection system that I previously established allowed me to obtain a genome-wide expression dataset using RNA-seq from *T. thlaspeos*-infected *Ar. hirsuta* tissues during the biotrophic phase. This led to the identification of several *T. thlaspeos* unique effector candidates, which I characterized in *A. thaliana*. Notably, I observed that one effector candidate was only present in the LF1 isolate and absent in LF2 (**Publication II: Figure S4**). To investigate whether the unique effector candidates have a potential role in virulence, I generated transgenic *A. thaliana* lines expressing six individual candidates and monitored for growth alterations and morphological differences compared to the control plants. In plants expressing *Thecaphora thlaspeos* unique effector 1 (*Tt*Tue1), the rosette sizes were significantly smaller than those of the control plants, while the transgenic lines expressing remaining studied candidates resembled the control plants in size (**Publication II: Figure 6**). Notably, although the infection of *T. thlaspeos* overall evoked biotic stress in the host plant, the expression of many plant genes involved in salicylic acid-dependent signaling did not change (**Publication II: Table 3**), potentially revealing an infection profile resembling that of a “minor” pathogen (Conn *et al.*, 2008), one that is only lowly detected by the host plant.

Taken together, further investigation of *T. thlaspeos* effectors and their functions could elucidate how this fungus maintains its long-term growth without causing visible infection symptoms and ultimately where it lies on the endophyte continuum.

6 My contributions to the manuscripts

6.1 Contributions to Publication I

- **General**
  - Location: Heinrich Heine University Düsseldorf, Germany
    - Led and organized *T. thlaspeos* spore-collection trips in Germany in 2014 and 2015
    - Participated in total collection trips in 2013, 2014, 2015, and 2017
    - Established infection protocols with *T. thlaspeos* spores in the lab resulting in infected seedlings and fully infected adult *Ar. hirsuta* plants containing spores in silique tissue (as outlined in materials and methods of **Publication I**).

- **Figures**
  - I generated the following figures with my data and wrote the figure legends:
    - **Figure 5**: *Thecaphora thlaspeos* systemically colonizes *Arabis hirsuta* in nature.
    - **Figure 6**: *Thecaphora thlaspeos* systemically infects *Arabis hirsuta* under controlled conditions.
    - **Figure 7**: Entry of *Thecaphora thlaspeos* into roots and leaves are identical in the host *Arabis hirsuta* and the model plant *Arabidopsis thaliana*.
    - **Supplementary Figure S1A**: LSU and ITS sequencing confirms that the fungus is *T. thlaspeos*.
    - **Supplementary Figure 6**: *T. thlaspeos* forms appressoria on cotyledons.

  - My data allowed for the plant-dependant part of the lifecycle to be graphically represented in:
    - **Figure 8**: The lifecycle of *T. thlaspeos*

- **Text**
  - Wrote materials and methods sections
    - " Spore germination, infection, and cultures"
    - "Staining and microscopy"
  - Drafted results sections
    - **"***T. thlaspeos* establishes a systemic infection along the vasculature"
    - "*T. thlaspeos* infects the model plant *A. thaliana*"
  - Contributed to Abstract, Introduction, Discussion

- **Cover image**
  - **MPMI** (April 2017, Volume 30, Number 4)

6.2 Contributions to Publication II

- **General**
  - Location: Heinrich Heine University Düsseldorf, Germany
    - Prepared and infected all plant material used in infection RNA-seq experiment
    - Prepared all RNA and cDNA for RT-PCRs
    - Prepared all RNA and cDNA libraries for RNA-seq experiment
    - Selected unique effector candidates of *T. thlaspeos* within the smut fungi from my fungal RNA-seq dataset to experimentally study
    - Performed confocal microscopy of *U. maydis*-infected maize tissues
    - Designed and performed phenotyping experiments of *A. thaliana* lines expressing individual *T. thlaspeos* effector candidates
    - Performed *A. tumefaciens*-mediated transformation of *Tt*Nlp1 in *N. benthamiana* and performed confocal microscopy

  - Location: The Sainsbury Laboratory, Norwich Research Park, United Kingdom
    - Cloned *Tt*Nlp1 and all *T. thlaspeos* unique effector candidates for subsequent expression assays in *A. thaliana*
    - Modified and performed spray infection of *Pst-LUX* on *A. thaliana*

- **Figures**
  - I generated the following figures with my data and wrote the figure legends:
    - **Table 2**: Features of *T. thlaspeos* effector candidates investigated in *A. thaliana.*
    - **Table 3**: Expression of SA-dependent defense signaling genes in healthy and infected *A. hirsuta* plants.
    - **Figure 3C:** Candidate effectors of *T. thlaspeos* are identified via differential expression analysis during infection and confirmed by RT-PCR.
    - **Figure 5C** (reproduced image of infiltrated leaf (Frantzeskakis, 2016) and produced confocal images)**, D, E:** *Tt*Nlp1 does not induce a hypersensitive response and increases bacterial luminescence on Col-0.
    - **Figure 6:** Transgenic *A. thaliana* accumulating mRNA of *Tttue1* are small with slightly chlorotic first true leaves.
    - **Figure S4:** *T. thlaspeos* unique effector THTG_04398 is absent in strain LF2 and in isolates from Hohe Leite, Germany, while present in LF1.
    - **Figure S6:** Transgenic *A. thaliana* lines accumulate mRNA of each respective effector.

  - I generated the following figure based on data obtained by Natalie Haeger in her Bachelor´s thesis and wrote the figure legend:
    - **Figure 4C:** *Ttpep1* partially complements deletion of *Umpep1* in maize infection.

- **Text**
  - Wrote results sections
    - "*In planta*-induced genes are enriched for unique, small, secreted proteins"
    - "The *T. thlaspeos* ortholog of the conserved smut effector Pep1 is active in *U. maydis*"
    - "The *Tt*Nlp1 is a non-cytotoxic effector of *T. thlaspeos*"
    - "*Thecaphora thlaspeos* unique effector 1 (Tue1) is a novel virulence factor"
    - "*T. thlaspeos* infection mildly induces plant defense responses"
  - Drafted discussion section "*T. thlaspeos* infection strategy enables perennial biotrophy" and led scientific discussion regarding effector characterization and plant responses for the discussion outline.
  - Wrote materials and methods sections
    - "Cloning of expression vectors"
    - "Strains, transgenic *A. thaliana* lines, and infection assays"
  - Drafted summary and introduction

7 General Discussion

The infection biology of *T. thlaspeos*

Plant-pathogenic fungi from necrotrophic to biotrophic lifestyles persist as a threat to agriculture despite years of research advancements. In order to combat their infection, it is crucial to understand how they infect their hosts. While necrotrophs destroy plant tissue, biotrophs depend on living plant tissues and therefore do not have drastic infection symptoms in their interaction with their host plants. Examples of such biotrophic fungi include powdery mildews of wheat and barley, an array of rust fungi that infect crops such as soybean, wheat, or barley, and smut fungi that infect crops such as maize, sugarcane, oat, or barley. Interestingly, endophytes are an additional classification of plant-colonizing microbes that do not cause visible infection symptoms on their host plants (Schulz & Boyle, 2005). Endophytes can be classified as pathogenic or non-pathogenic, those of the latter group behaving commensally or beneficially to their host plant (Hardoim *et al.*, 2015; Brader *et al.*, 2017).

A classic example of a biotrophic pathogen that has been well studied for decades and whose infection biology in maize is well-understood is the model smut fungus *U. maydis.* Our group identified taxonomical and mycological descriptions of the smut fungus *T. thlaspeos* infecting members of the Brassicaceae family such as *Arabis spps.* (Vánky *et al.*, 2007) and we hypothesized that *T. thlaspeos* could also infect *A. thaliana* despite no reports of this infection existing in nature. Successfully, our group identified *T. thlaspeos* in nature and brought infected samples back to the lab, thus initiating our work with the *T. thlaspeos* – Brassicaceae pathosystem and annual collection trips.

A unique aspect of the infection biology of *T. thlaspeos* compared to other smut fungi is the lack of obvious infection symptoms that it has on its perennial Brassicaceae hosts in nature and in the lab despite its long-lived endophytic growth phase (**Figure 2**). This is in stark contrast to the model smut fungus *U. maydis*, whose whole infection process takes approximately only one week (Brefort *et al.*, 2009) and results in large tumors on maize (Christensen, 1963; Banuett, 1995; Bölker, 2001). Similarly, sugarcane smut *Sporisorium scitamineum* infection also causes obvious infection symptoms on its host by forming whip-shaped sori at the top of the plant stalk as well as sori on the leaves (Trione, 1990; Marques *et al.*, 2017). The emergence of whip-shaped sori has been observed to form on infected sugarcane plants up to 270 days post infection (Marques *et al.*, 2017). The maize head smut fungus *S. reilianum* on the other hand

does not form tumors or sori but changes the morphology of maize flowers, which becomes obvious at flowering time (Ghareeb *et al.*, 2011). The entire infection process of *S. reilianum* in maize lasts approximately eight weeks (Schirawski *et al.*, 2010). Therefore, *T. thlaspeos* offers the opportunity to study prolonged biotrophy that lasts for years in its hosts without visible infection symptoms, suggesting that it somehow controls its sustained growth within its hosts.

The spore deposition of these three grass smuts, *U. maydis*, *S. scitamineum, and S. reilianum*, on or in their host plants is indicative of horizontal transmission, as spores can be carried by the wind to neighboring plants and thereby infect. As *T. thlaspeos* sporulates within the silique tissue and coats the host seeds (Vánky *et al.*, 2007; **Publication I**), it is highly likely that *T. thlaspeos* predominantly spreads via vertical transmission as it is typical of endophytes such as *Epichlöe* spps. and *Claviceps* spps. (Saikkonen *et al.*, 2002; Hardoim *et al.*, 2015). Sometimes spores could be carried by the wind once spore-filled siliques break open and hence spread horizontally. Here, vertical transmission refers to the direct passing of a microbe from host to offspring (Saikkonen *et al.*, 2002). I have also observed in nature that infected and healthy *Arabis* plants can grow in close proximity to one another, further indicating that horizontal transmission is uncommon for *T. thlaspeos* and it rather spreads mainly via vertical transmission.

Another unique aspect of the infection biology of *T. thlaspeos* compared to other smuts is that the infection appears to be strictly restricted to the vascular tissue of the host (**Figure 2**). Confocal microscopy of *T. thlaspeos* leaf tissue revealed that this vascular growth appears to be outside of the vascular bundles, indicating that *T. thlaspeos* grows intercellularly along the vascular tissues of its hosts. In addition, I have not observed clear intracellular growth events as known e.g. for *U. maydis* (Banuett 1995) and *S. reilianum* (Ghareeb *et al.*, 2011). Here, I refer to intercellular growth as fungal growth between plant cells i.e. in the apoplastic space and intracellular growth is defined as the ability of a fungus to pass through plant cell walls, therefore the fungal plasma membrane coming into close contact with the plasma membranes of host plant cells.

The current staining methods used to identify and describe *T. thlaspeos* infection in its host tissues are limiting in that they cannot elucidate exactly where the fungus is growing in the host plant. Tools that are currently being developed in our group could further address the exact location of *T. thlaspeos* growth along the vasculature as well as whether events of intracellular growth occur. Significant progress recently made in developing a transformation protocol for *T. thlaspeos* led to the generation of a GFP-expressing

strain (L. Plücker and K. Bösch, unpublished data). Furthermore, using culture infection protocols developed recently which show that *T. thlaspeos* culture can infect roots and penetrate leaves (T. Schoen, master´s thesis), the GFP-expressing *T. thlaspeos* strain could be used to infect *A. thaliana* lines expressing multiple markers such as for the plasma membrane and microtubules to clearly indicate intracellular growth events (Ghareeb *et al.*, 2016).

In contrast to *T. thlaspeos*, *S. reilianum* grows both intercellularly as well as intracellularly (Ghareeb *et al.*, 2011) as do *U. maydis* (Banuett, 1995) and *S. scitamineum* (Marques *et al.*, 2017). Interestingly, *S. reilianum* and *S. scitameum* both grow within the tracheary elements of the maize (Maytac, 1985) and the sugarcane (Marques *et al.*, 2017) xylem tissue, respectively. It has been shown for the vascular wilt pathogen *Fusarium oxysporum* that pathogenic strains infect intracellularly by entering the host xylem tissue and further proliferate using this vascular compartment while non-pathogenic strains do not display such intracellular growth (Aimé *et al.*, 2013; Brader *et al.*, 2017). Additionally, beneficial endophytic *Epichlöe* species are also known to predominately grow throughout their grass hosts via intercellular spaces (Saikkonen *et al.*, 2002). If there is a general trend that pathogens which colonize their host vasculature do so intracellularly while non-pathogens do so intercellularly, this raises interesting questions: How does *T. thlaspeos* achieve and maintain pathogenicity on a molecular level? How does it transfer its effectors via two cell walls into the host plant cell? How do such fungi gain access to nutrients in absence of feeding structures?

The effectors of *T. thlaspeos* and their potential roles in virulence

T. thlaspeos is a unique smut fungus in terms of its infection process.This distinctiveness can also be reflected in its effector repertoire and might provide insight into the general potential effector functions in manipulating host metabolism, preventing host recognition, or inhibiting host defense responses (Rodriguez-Moreno et al., 2018).

As the infection biology of *T. thlaspeos* observed to date largely resembles that of an endophyte on a macroscopic level, what does this indicate about its effector repertoire, if it would have/require one at all? Interestingly, even mutualistic fungi such as arbuscular mycorriza have effectors that promote their own growth by modulating the defenses of their host (Kloppholz *et al.*, 2011; Plett *et al.*, 2011; Martin *et al.*, 2017). Symbionts such as *P. indica* also have effectors that are required for infection of barley (Zuccaro *et al.*, 2011), and also pathogenic endophytes as well as non-pathogenic endophytes have effectors that

enable their infection and interaction with their hosts (Brader *et al.*, 2017). Therefore, expectedly *T. thlaspeos* has an effector repertoire, yet the main question is whether we can find commonalities with other more aggressive smut fungi or mildly infecting endophytes.

As its growth has only been observed along vasculature tissue within its host plants, does this indicate that *T. thlaspeos* would only secrete effectors that are localized in the apoplastic space? This is unlikely to be the case, since examples drawn from other vascular pathogens indicate that they carry effectors which are translocated into the host cells. The vascular wilt fungus *Verticillium dahliae* in particular carries *Vd*Isc1, which is required for full pathogenicity, and it is secreted in the host cell and specifically suppresses salicylate-mediated immunity in cotton (Liu *et al.*, 2014). Additionally, Avr2 of the soil-borne fungus that causes vascular wilt disease on tomato, *Fusarium oxysporum* f. sp. *lycopersici* (Fol), is secreted into the xylem and then translocated to the plant cell via an unknown mechanism, where it exerts its virulence function (Di *et al.*, 2016).

Aiding the search of smut fungal effector candidates, the genomes of *U. maydis, S. reilianum*, and *U. hordei*, have served as essential tools in understanding how smuts infect their grass hosts on a molecular level (Kämper *et al.*, 2006; Schirawski *et al.*, 2010; Laurie *et al.*, 2012). In mining for effectors of *U. maydis* from the genome, Pep1, a protein essential for penetration, was identified and characterized (Doehlemann *et al.*, 2009; Hemetsberger *et al.*, 2012). As *Um*Pep1 interacts with apoplastic maize peroxidase POX12 to suppress the ROS burst that results from PAMP perception and thus PTI, what would *Tt*Pep1 target in *Ar. hirsuta* to promote *T. thlaspeos* proliferation? Specifically, *Tt*Pep1 could interact with an *Ar. hirsuta* ortholog of apoplastic *A. thaliana* peroxidase33 (PR33) or peroxidase34 (PR34) (**Figure 5**), whose diminished expression in T-DNA knockdown lines resulted in a blocked oxidative burst in response to a fungal elicitor (Daudi *et al.*, 2012).

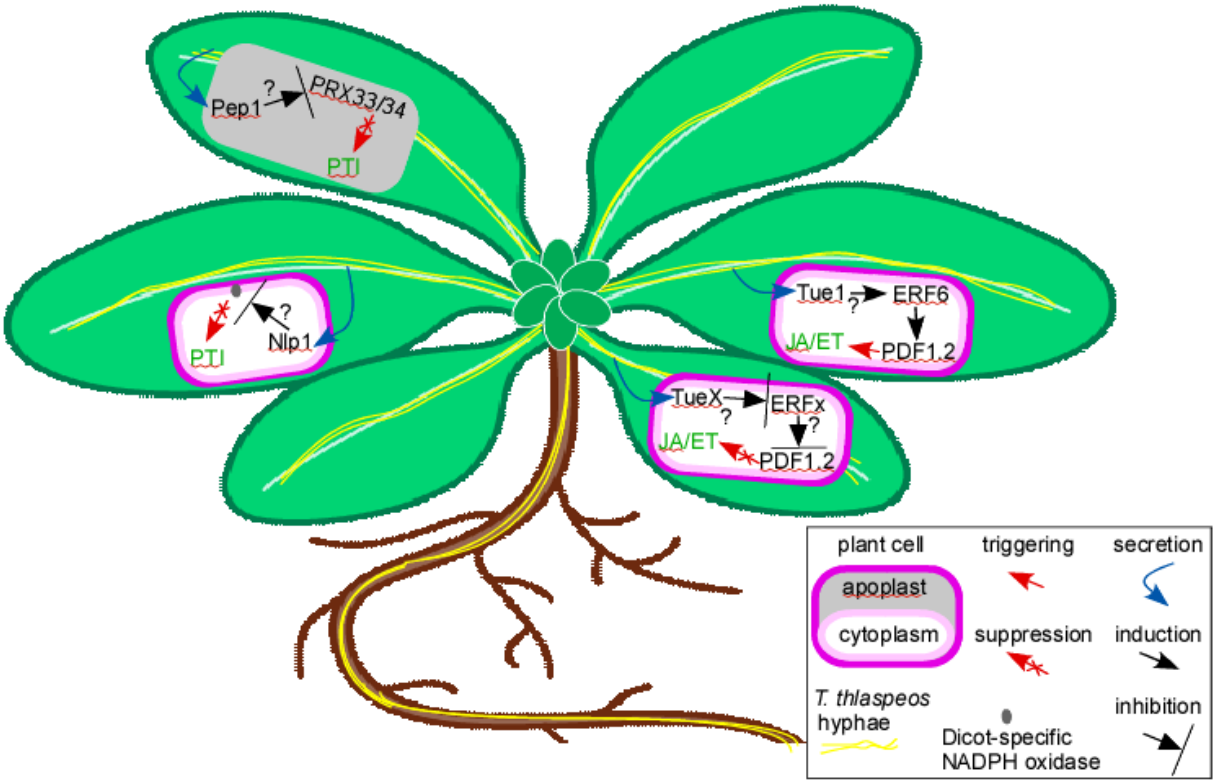

Figure 5. A proposed model for potential virulence functions of *Tt*Pep1, *Tt*Nlp1, *Tt*Tue1 and *Tt*TueX in *T. thlaspeos*-infected *Ar. hirsuta* rosette leaves 10-weeks post infection
*Tt*Pep1 is likely localized to apoplastic tissue and could potentially inhibits peroxidase33/34 (PRX33/34) function thereby suppressing PTI. *Tt*Nlp1 is likely localized to the cytoplasm of the plant cell and I hypothesize that it could potentially inhibit the function of a dicot-specific NADPH oxidase. I hypothesize it thereby could suppress PTI. *Tt*Tue1 is likely localized to the cytoplasm of the plant cell and potentially induces expression of ETHYLENE RESPONSIVE FACTOR 6 (*ERF6*). ERF6 induces *PDF1.2* gene expression (Meng *et al*., 2013), therefore triggering JA/ET-dependant defense responses. *Tt*TueX could be localized to the cytoplasm of the plant cell to potentially inhibit an unknown ERF (ERFx). ERFx could inhibit PDF1.2 gene expression, therefore suppressing JA/ET-dependant defense responses. Plant immune responses are marked with green text.

In addition to Pep1, Pit2 (protein important for tumor 2) and Stp1 (stop after penetration 1) are crucial for the establishment of biotrophy (Schipper, 2009; Doehlemann *et al.*, 2011; Mueller *et al.*, 2013; Lanver *et al.*, 2018). Most probably due to the fact that *T. thlaspeos* does not cause tumors on its host while *U. maydis* does on maize, a *pit2* ortholog does not exist in the genome of *T. thlaspeos* (**Publication II**). An *stp1* ortholog was identified and *Ttstp1* was one of the most highly up-regulated fungal genes in my infection RNA-seq dataset, indicating that it is also important for the penetration of *T. thlaspeos*. Work currently being conducted in our group by S. Gul addresses whether *Ttstp1* can functionally complement the *U. maydis* mutant phenotype. Since our dataset is from the biotrophic phase in which *T. thlaspeos* grows along the vasculature of its host plant, our data on *Ttpep1* and *Ttstp1* indicate the presence of intracellular growth events of the fungus during which these effectors would be required.

A classic example of a cytoplasmic effector of *U. maydis* is the chorismate mutase Cmu1, which spreads to neighboring plant cells to suppress salicylic acid levels, thus promoting the proliferation of the fungus (Djamei *et al.*, 2011). Cmu1 is one of the most up-regulated effectors during *U. maydis* infection of maize and is important for virulence by suppressing SA-signaling (Djamei *et al.*, 2011). *T. thlaspeos* does not encode a Cmu1 homolog, but based on the plant responses to *T. thlaspeos* infection, *T. thlaspeos* seems to suppress SA albeit by a different means. Therefore, it will be interesting to investigate more *T. thlaspeos*-unique effectors for such activity.

As effectors of the *Thecaphora* genus have not been previously explored, we also focused on characterizing effector candidates that are predicted to be unique to *T. thlaspeos* within the smut fungi. *T. thlaspeos* contains three genes coding for non-cytotoxic necrosis and ethylene-inducing like proteins (Nlp, Oome *et al*., 2014). Since *Ttnlp1* was highly up-regulated in my infection RNA-seq dataset while *Ttnlp2* and *Ttnlp3* were not, I focused on characterizing *Ttnlp1* in heterologous systems to determine whether it has a virulence function. As described in **Publication II**, *Tt*Nlp1 increased infection of *Pst-LUX* on *A. thaliana* Col-0. This likely indicates that it could function in suppressing PTI, thereby promoting the infection of *Pst-LUX*. As *Tt*Nlp1 is a fungal effector which increases bacterial growth in its host, this suggests that its potential function may be a more common one to plant pathogens in general, such as suppressing PTI, rather than being important in tumor formation, as Tin2, See1, or Pit2 do for *U. maydis* during maize infection. Since Nlps are typically dicot-specific effectors (Oome *et al.*, 2014) and cytotoxic Nlps are known to affect plant responses in dicots but not monocots (Böhm *et al.*, 2014), perhaps *Tt*Nlp1 could function in suppressing PTI in a way only effective in dicot hosts.

Cytotoxic Nlps are known to trigger cell death and elicit strong immune responses (Qutob *et al*., 2006), which requires key residues of a GHRHDWE heptapeptide motif (Ottmann *et al*., 2009). *Tt*Nlp1 does not contain these key residues required for necrosis-inducing activity (**Publication II: Figure 5B**) and is non-cytotoxic. Cytotoxic Nlps also have a second means by which they can evoke plant immunity. They contain a stretch of twenty amino acids (nlp20) of which key residues are necessary for ethylene induction (Böhm *et al*., 2014). Böhm and collegues showed that the nlp20 peptides of fungi such as *F. oxysporum*, when in contact with *A. thaliana* seedlings, results in ethylene and reactive oxygen species production. Since *Tt*Nlp1 harbors two out of four of these key residues present in the nlp20 region of *Fo*Nlp1 (**Publication II: Figure 5B**), it could be possible that *Tt*Nlp1 displays immunogenic activity, despite not being cytotoxic. On the other hand, I observed that *A. thaliana* plants constitutively expressing *Tt*Nlp1 resembled the

control plants and did not appear immunocompromised (**Publication II: Figure 6A**). In any case, the role that non-cytotoxic Nlps play in virulence remains elusive. My data suggest three general parameters regarding the potential function of *Tt*Nlp1: 1) *Tt*Nlp1 may suppress PTI since it increased the growth of *Pst-LUX* on *A. thaliana*. 2) *Tt*Nlp1 likely functions inside the plant cell since it is delivered there by *Pst-LUX* in this infection experiment. 3) *Tt*Nlp1 functions in a dicot-specific manner since Nlps are dicot-specific (Oome *et al*., 2014). One hypothesis that fits these three parameters is that *Tt*Nlp1 could interact with a plasma membrane bound NADPH-oxidase, which in general play a role in PTI (Kadota *et al.*, 2015). Strikingly, it was recently shown there are several monocot and dicot-specific NADPH oxidases (Kaur *et al.*, 2017). Therefore, it could make sense that *Tt*Nlp1 functions inside the plant cell to somehow disrupt the function of a dicot-specific NADPH oxidase, thus aiding in the suppression of PTI (**Figure 5**).

In efforts to further elucidate the potential molecular function of *Tt*Nlp1, I observed its subcellular localization in *Nicotiana benthamiana* via *Agrobacterium tumefaciens*-mediated transformation, however the resulting localization patterns were inconclusive. Therefore, to determine the target of *Tt*Nlp1 by proceeding with co-immunoprecipitation experiments in *N. benthamiana* would not be the logical next step, but rather using a yeast two-hybrid (Y2H) approach in which an *A. thaliana* Y2H cDNA library (Lu, 2012) preferably from biotic stress conditions would be used as prey. This approach would take advantage of *A. thaliana* as a tool and would allow for the detection of the target of *Tt*Nlp1 in a described (**Publication I**) host plant.

Plant-pathogenic fungi have specific effector repertoires that enable for their diverse lifestyles and colonization strategy (Selin *et al.*, 2016). *Thecaphora* unique effector 1 *Tttue1* has a virulence function, which was demonstrated when constitutively expressed in *A. thaliana* (**Publication II**). These plants show severe growth reduction, similar to those observed in auto-immune mutants (Bowling et al., 1997; Bowling et al., 1999), suggesting that *Tt*Tue1 may be activating host immune responses by direct or indirect recognition from the host's immune system.

Considering that *Tttue1* is highly up-regulated during *T. thlaspeos* infection of ten-week old *Ar. hirsuta* leaves, it is possible that it could be involved maintaining the overall endophytic-like growth during the long-lived biotropic stage. As it has been proposed that ethylene signaling and transcription factors targeted by ethylene are necessary to balance the beneficial and non-beneficial effects of *P. indica* in *A. thaliana* (Camehl *et al.*, 2010), I hypothesize that *Tt*Tue1 induces ethylene-responsive defense genes

(**Figure 5**). Ethylene (ET) signaling in plants is also important in balancing microbial growth in order to keep a low defense strategy (Plett & Martin, 2017). Furthermore, effectors such as AvrPto and AvrPtoB from *Pseudomonas syringae* induce ET signaling in tomato (Cohn & Martin, 2005). On the other hand, *Pseudomonas syringae* also contains effector HopAF1 which represses ET signaling in *A. thaliana* (Washington *et al.*, 2016), illustrating that pathogens do secrete effectors with seemingly antagonistic functions which could overall keep a balance on ethylene. Similarly, *Tt*Tue1 could be up-regulating certain ET defense-related genes to prevent overgrowth of *T. thlaspeos* within the plant, therefore potentially causing a local immune response which would be kept at low detection due to additional unknown effectors. These to-date unknown effectors could then down-regulate ET signaling (**Figure 5**), therefore altogether providing an overall balance for *T. thlaspeos* to continue to grow but keeping the growth from over running the plant.

In order to test whether *Tt*Tue1 could be inducing ET signaling as shown in **Table 1** from my RNA-seq data, I would utilize plant tissues from the *Tttue1*-expressing *A. thaliana* lines to determine whether individual ET defense-related genes are induced using qRT-PCR. I would first check for the expression of the ETHYLENE RESPONSIVE FACTOR (ERF) genes, especially *At*ERF1, 6, and 104, as these transcription factors are particularly important for the regulation of the host immune responses to biotic stress (Tsuda & Somssich, 2015). In parallel, I would express *Tt*Tue1 in *N. benthamiana* via *A. tumefaciens*-mediated transformation, perform a co-IP, and ultimately use mass spectrometry to identify the plant target of *Tt*Tue1.

Lastly, the continued development of the current transformation protocol for *T. thlaspeos* will ultimately allow for the targeted deletion of individual effector candidates to explore the roles they play in virulence. Infection of *A. thaliana* with these mutant strains will substantially enhance our understanding of the effectorome of *T. thlaspeos*. Additionally, access to the many *A. thaliana* plant defense mutants or over-expression lines available would assist in determining how *T. thlaspeos* effectors could be involved in targeting specific defense-related genes.

Plant responses to *T. thlaspeos* infection

As *T. thlaspeos* effectors were identified during the prolonged biotrophic infection stage that could potentially interfere with defense-related processes, I also studied the plant response at this stage and expected to find induced immune responses. As reported in **Publication II**, *T. thlaspeos* infection induces

biotic stress in *Ar. hirsuta* while the expression of many SA-dependant signaling related genes is unchanged. Although a homolog of *A. thaliana PR1* could not be detected in our dataset of *Ar. hirsuta* transcripts (**Publication II**), *PR1*, a key indicator of SA-mediated induction of defense genes (Thomma *et al.*, 1998; Glazebrook, 2005; Dobon *et al.*, 2016), is up-regulated during infection as shown by RT-PCR (**Figure 4**).

During *U. maydis* infection of maize, it was shown by global expression profiling that *U. maydis* also overall induces biotic stress in its host (Doehlemann *et al.*, 2008). During early infection stages, *U. maydis* invading hyphae are recognized and defense responses are triggered. At later stages, these genes were suppressed during the biotrophic colonization phase two days post infection (dpi). Specifically, a germin-like protein known to be SA-induced was induced at twelve hours post infection (hpi) but was subsequently repressed at later infection stages while *PR1* was not detected during early stages of infection but showed low expression at later time points (Doehlemann *et al.*, 2008). This two days post infection stage is most comparable to the time point of *T. thlaspeos* infection used in my RNA-seq experiment and these results are comparable to my observation of overall low but present SA-dependant defense signaling-related gene expression. Jasmonic acid (JA)-responsive genes are up-regulated at twelve hpi and this expression is overall consistent from then on (Doehlemann *et al.*, 2008). Interestingly, in *T. thlaspeos* I also observed that the key transcription factor ERF6, which is important in regulating host JA/ET responses to biotic stress (Sewelam *et al.*, 2013), is significantly up-regulated during infection (**Table 1**). These observed plant responses to *T. thlaspeos* and *U. maydis* are overall expected responses to biotroph infection. Biotrophic pathogens greatly elicit SA-dependant signaling defense responses in effective defense of the host plant against the invading pathogen (Glazebrook, 2005), while during compatible interactions, biotrophic pathogens elicit JA/ET responses (Brader *et al.*, 2001). Additionally, biotrophs such as *Phakopsora pachyrhizi*, causal agent of Asian soybean rust, was also shown to elicit host responses during the colonization stage of soybean (24 -48 hpi) (Schneider *et al.*, 2012). The potential that genome-wide expression studies have in dissecting the time course of biotrophic infection cannot be underestimated and would allow for a significantly more dynamic view of how the Brassicaceae hosts of *T. thlaspeos* respond to its infection.

Beneficial effects of microbes on plant health

It is clear that *T. thlaspeos* is pathogenic and clearly induces biotic stress, but it would be exciting to explore whether *T. thlaspeos* infection could be potentially beneficial to its hosts under growth conditions yet to be investigated. Often times, the pathogenicity of microbes is not only dependent on the biotic interactions with its host but also on environmental parameters (Brader *et al.*, 2017). Several examples are known: (1) Fluorescent bacteria of the *Pseudomonads* genus are able to cause disease on leatherleaf ferns under select conditions but its colonization usually confers beneficial effects to the plant (Kloepper *et al.*, 2013). (2) *Verticillium longisorum*, a soil-borne vascular pathogen, typically triggers hyperplastic xylem formation in *A. thaliana* under standard growth conditions that result in early senescence (Reusche *et al.*, 2014). Strikingly, Reusche and collegues (2014) showed that under drought conditions, *V. longisporum* has a conditional mutualistic interaction with its host by providing enhanced tolerance to drought stress. (3) Beneficial endophytes of the *Epichlöe* genus colonize temperate grasses and improve their nutrient acquisition thereby promoting growth (Scott, 2001; Schardl *et al.*, 2004). *Epichlöe*-colonized plants are additionally more resistant to drought as well as to diseases (Scott, 2001; Schardl *et al.*, 2004). (4), When colonized by the endophytic root fungus *Piriformospora indica*, barley benefits from enhanced nitrate assimilation (Sherameti *et al.*, 2005; Waller *et al.*, 2005; Zuccaro *et al.*, 2011). Barley infected with the endophytic root fungus *Piriformospora indica* also has enhanced resistance to necrotrophic root parasites and to the biotrophic leaf pathogen *Blumeria graminis* (Waller *et al.*, 2005; Kogel *et al.*, 2006). (5) Leaf endophytic *Curvularia* species have aided the grass *Dichanthelium* to adapt to extreme heat (Redman *et al.*, 2002). Therefore, challenging *T. thlaspeos*-infected plants to various growth environments and abiotic stresses such as drought conditions, heat stress, and nutrient-depleted soil is a very interesting avenue that research on the *T. thlaspeos* – Brassicaceae pathosystem could take to explore how the host plant responds to *T. thlaspeos* in a more ecological context. Additionally, since it is unclear whether *T. thlaspeos* infection would confer resistance to other pathogens, challenging *T. thlaspeos*-infected plants with pathogens whose biomasses are easily measured such as *Pst-LUX* (luminescence) (Fabro *et al.*, 2011) or *B. cinerea* (lesion size) (van Esse *et al.*, 2008) could also reveal potentially beneficial aspects of *T. thlaspeos*.

In addition to the abiotic environmental factors that plant-colonizing microbes face, they are also in contact with the billions of microbes that make up the microbiome of their host plant (Plett & Martin, 2017). Recently, a beneficial fungus of the Helotiales order was identified in the *Arabis alpina* root microbiome to promote plant growth and supply the plant with phosphorous (P) in the low-P soil

conditions of its natural habitat (Almario *et al.*, 2017). As the pioneering work regarding the root microbiome of *A. thaliana* was released only six years ago (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012), there is still much to explore regarding how pathogens interact with the microbiomes of their host plants, both of root tissues and leaf tissues. To this end, studies investigating the root and leaf microbiomes of *Ar. hirsuta* plants collected from nature in connection to *T. thlaspeos* infection are currently under way (Kellner, R.; pers. comm.).

Taken together, *T. thlaspeos* is clearly detected by the plant during infection but due to the long-lived, endophytic-like infection biology of this pathogen, studying the potential benefits that *T. thlaspeos* could confer to its Brassicaceae hosts would offer a new, interesting avenue in exploring this pathosystem.

Conclusion and future perspectives

The efforts exemplified in this dissertation resulted in significant accomplishments for the development of the *T. thlaspeos* – Brassicaceae pathosystem. The methods developed, sequencing data generated and analyzed, and the first work in identifying and functionally characterizing effectors unique to *T. thlaspeos* within the smut clade aid in the use of this pathosystem to address novel questions of the plant-microbe interactions field.

The original goal in developing this model pathosystem was to have two genetically tractable partners, *T. thlaspeos* and *A. thaliana*. Transferring the tools from the model smut *U. maydis* and utilizing the model plant *A. thaliana*, we aimed to describe and experimentally verify the plant immune responses to smut infection. Assuming the culture infection will be established in *A. thaliana* and *T. thlaspeos* will be transformable, our model pathosystem could not only achieve our original aim of a complete investigation of the plant responses to smut infection in *A. thaliana* but also offers an especially interesting and new avenue of research into the mycology and effector biology of plant-pathogenic endophytes.

It is clear that *T. thlaspeos* fits the broad definition of an endophyte in regards to its infection biology but it is unclear whether it is closer to the pathogen or non-pathogen (commensal or mutualistic) side of the endophyte continuum (Brader *et al.*, 2017). A deeper dissection of the effectorome of *T. thlaspeos* could address this open question, especially considering the knowledge gained in this work regarding its infection strategy. Exploring the intercellular spaces near to the host plant vasculature via cell-type specific RNA-seq could identify effectors important in maintaining the prolonged infection of *T. thlaspeos*,

as this method was recently used to identify which plant cells serve as the origin for *U. maydis* hyperplastic tumor cells (Matei *et al.*, 2018). This method could also assist in determining whether *T. thlaspeos* periodically grows intracellularly by isolating tracheary elements of the xylem tissue or mesophyll cells. If *T. thlaspeos* were present, potential cell-type specific effectors could be ultimately identified and functionally characterized. Furthermore, the characterization of effector candidates unique between *T. thlaspeos* and closest relative biocontrol agent *Pseudozyma flocculosa* could reveal novel insights into the tight regulation of growth between endophytes and their hosts.

In concluding my work with *T. thlaspeos* and considering the knowledge that has been gained and the tools that have been developed, I believe that this pathosystem has the potential to be further developed into a model pathosystem for the study of effectors functioning on the endophyte continuum. This is a highly interesting avenue of potential research resulting from my work as effectors play a major role in orchestrating endophytic plant-fungal interactions. Nevertheless, this field overall remains largely unexplored and *T. thlaspeos* offers plenty of opportunities to explore the responses of different hosts with annual and perennial lifecycles.

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