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

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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

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
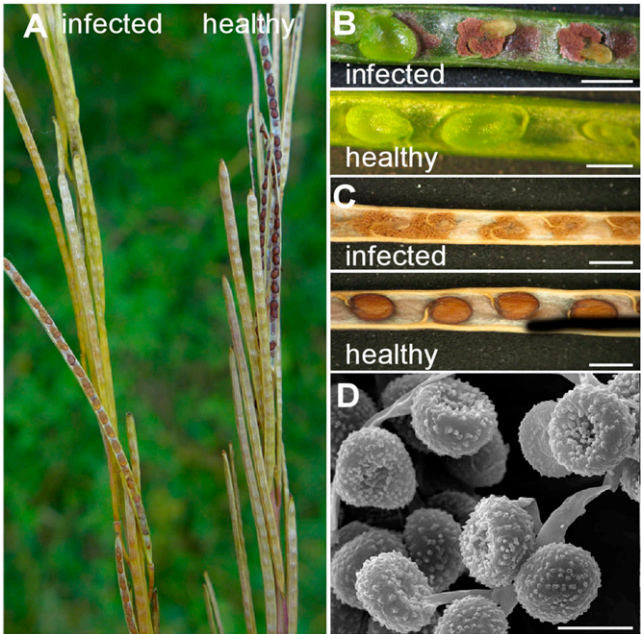

Fig. 1. Symptoms of *Thecaphora thlaspeos* infection on *Arabis hirsuta* and *Arabis ciliata*. **A,** Infected and healthy *Arabis hirsuta* plants collected in Germany appear macroscopically identical. Scale bar: 2 cm. **B,** In siliques of these infected plants, spores cover developing seeds. Scale 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
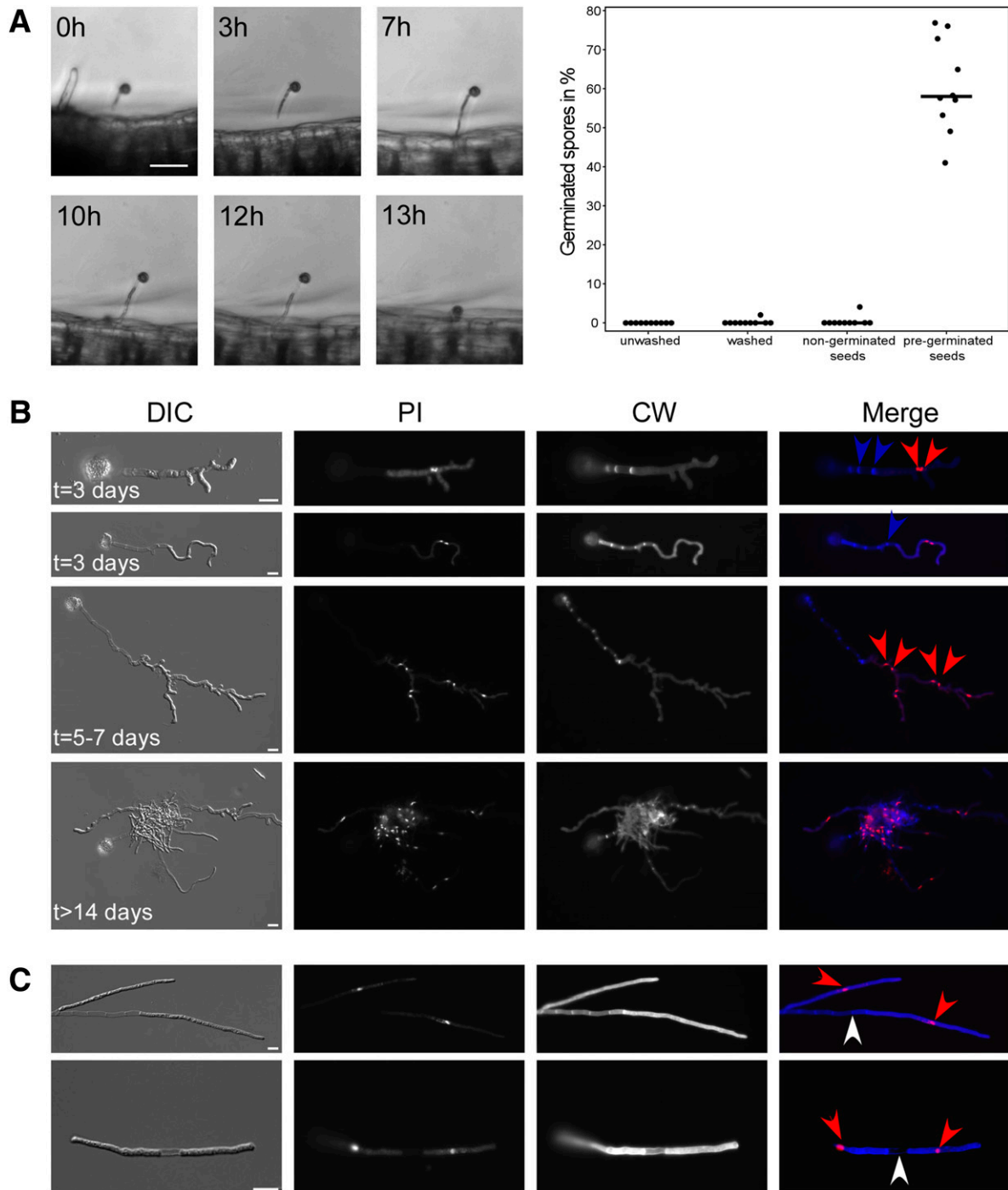

Fig. 2. Germination of *Thecaphora thlaspeos* teliospores. **A,** Teliospores germinate in the presence of seedlings (here *Arabidopsis thaliana* Col-0) and hyphae are growing toward the elongating root. Attachment to the root can be observed 12 to 13 h after spore germination and leads to drift of the 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 mono-karyotic (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, homo-dimerization 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
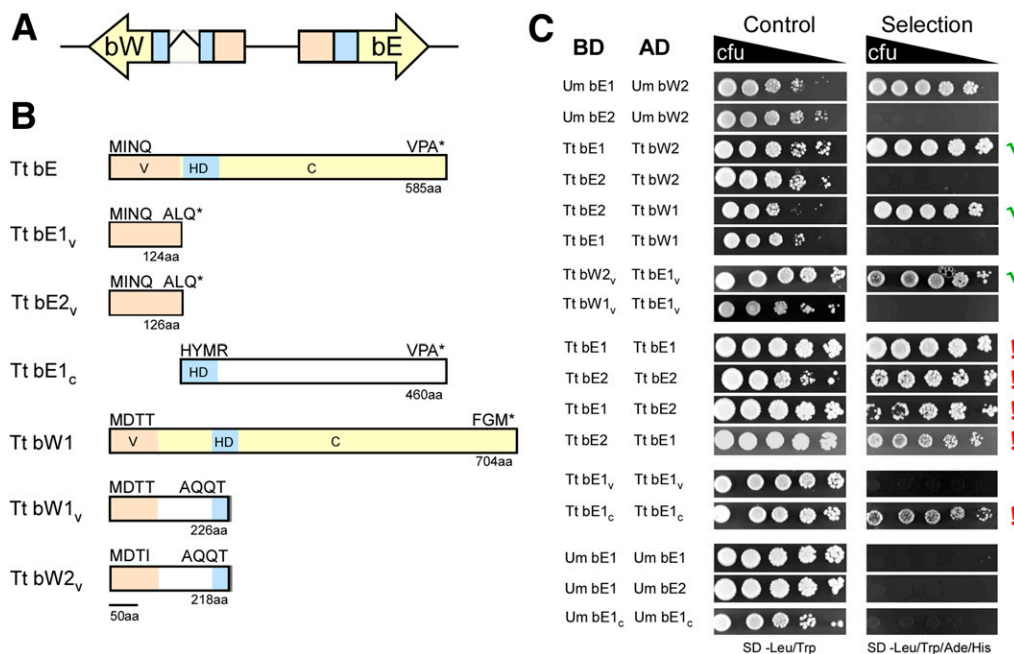

Fig. 4. The *b* mating locus of *Thecaphora thlaspeos* is conserved. The *b* locus was identified based on homology in sequence and organization of HD domains in the b proteins. Interaction of the b proteins was tested in a yeast two-hybrid assay. **A,** Schematic representation of the *b* mating locus 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
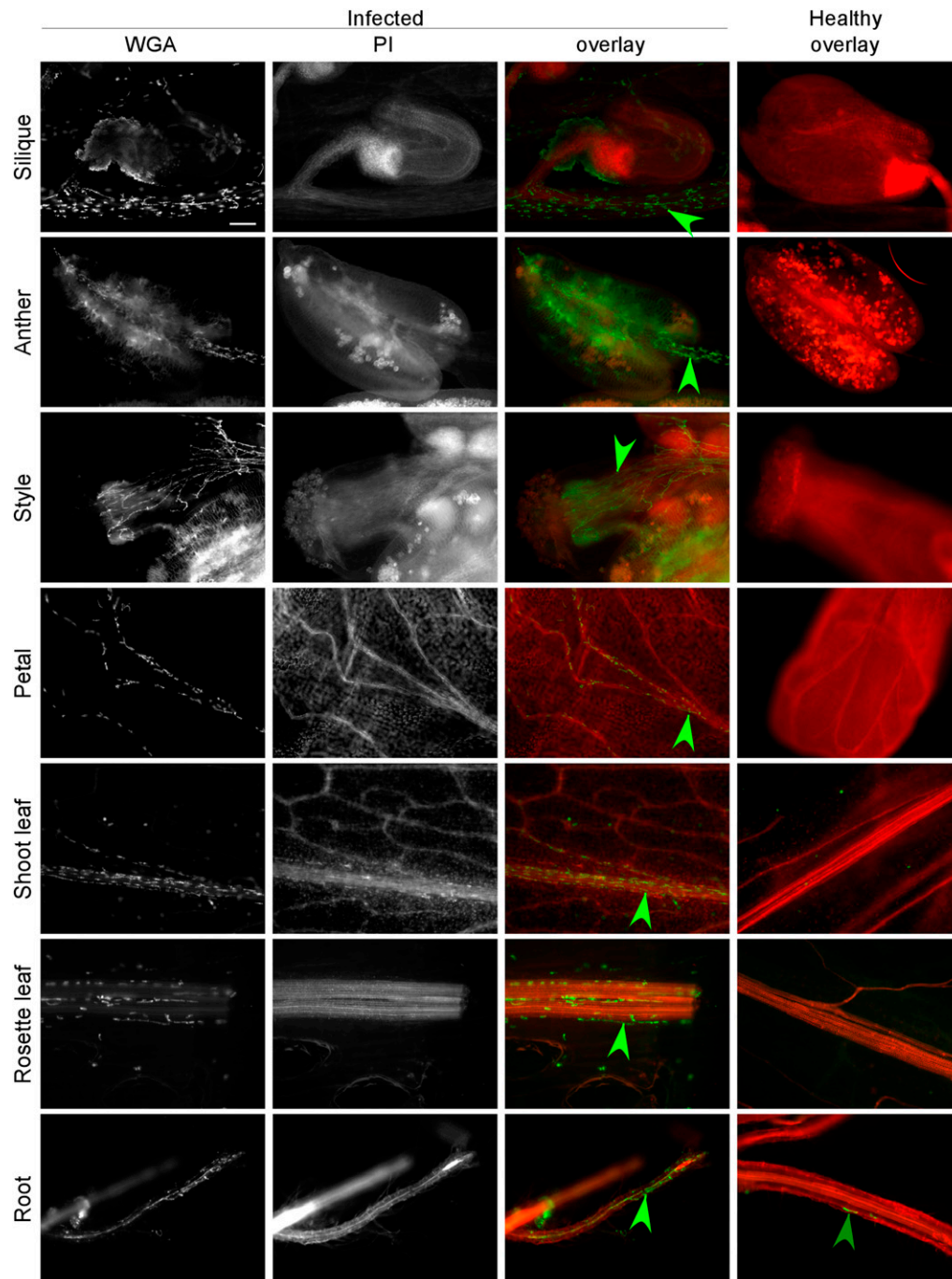
Fig. 5. *Thecaphora thlaspeos* systemically colonizes *Arabis hirsuta* in nature. In infected plants collected in Germany, fungal hyphae grow into all tissues, in which they are found predominantly along the main vasculature. Tissues were stained with wheat germ–agglutinin (WGA)-fluorescein isothiocyanate (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.
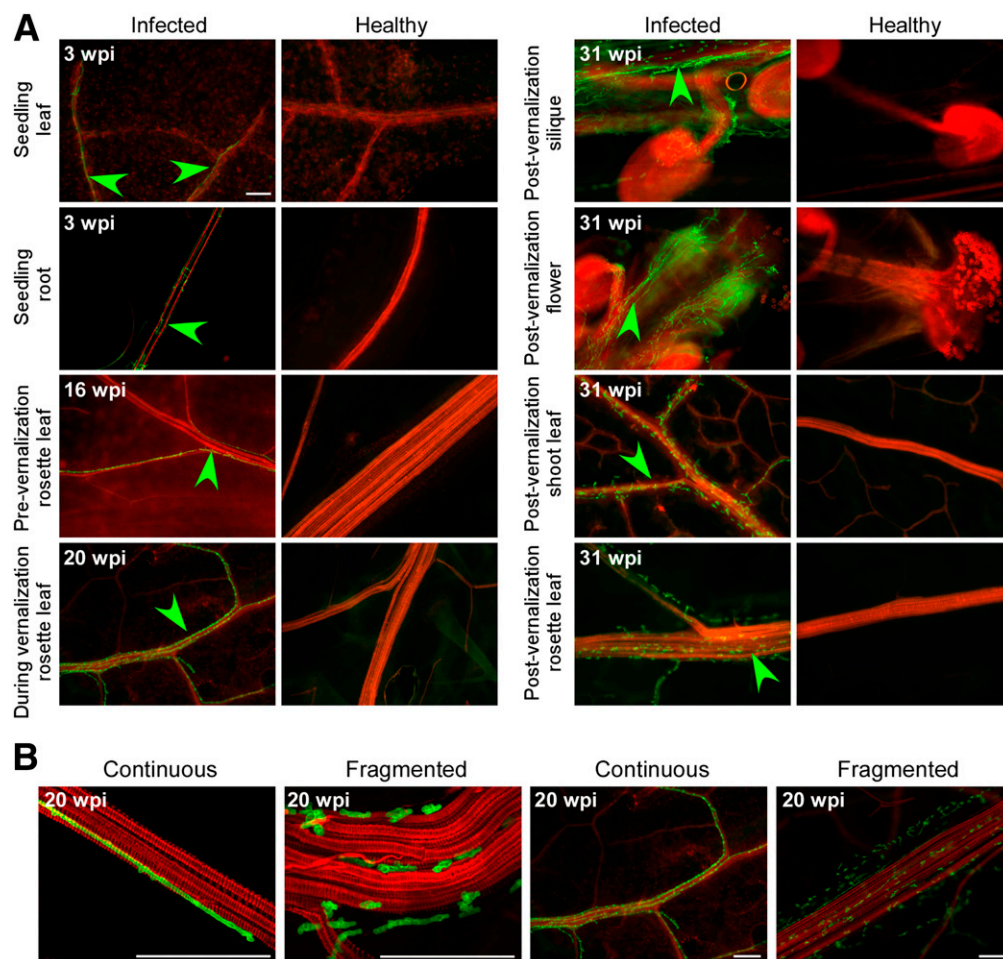

Fig. 6. *Thecaphora thlaspeos* systemically infects *Arabis hirsuta* under controlled conditions. **A,** Hyphal proliferation was observed in leaf and root tissues at the seedling stage and in adult rosette tissue prior to and during vernalization. Furthermore, during the flowering stage, spores were 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.

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,
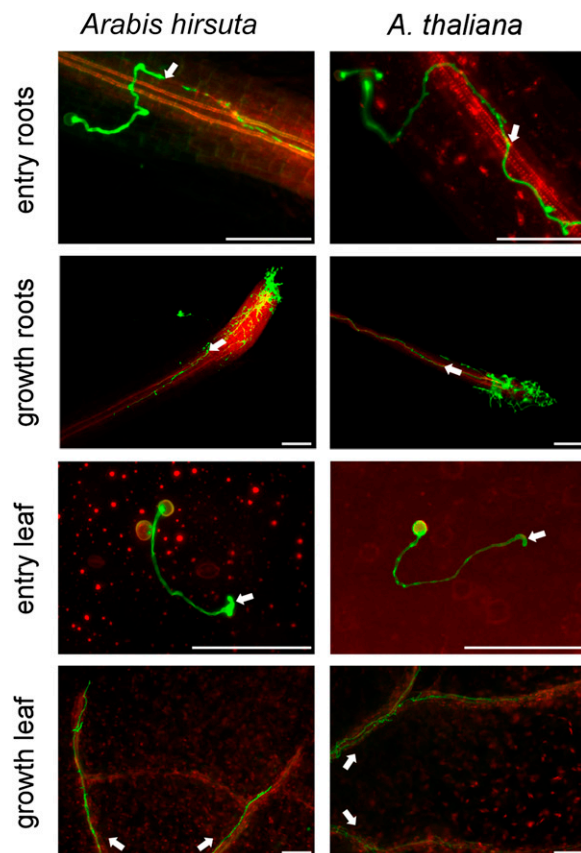
Fig. 7. Entry of *Thecaphora thlaspeos* into roots and leaves are identical in the host *Arabis hirsuta* and the model plant *Arabidopsis thaliana*. *T. thlaspeos* penetrates root and leaf tissues of seedlings, subsequently growing along the main vasculature in both species. No differences between the 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.
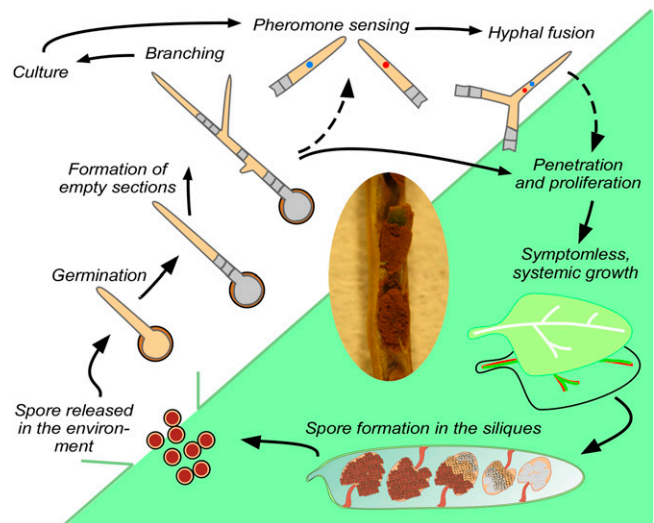
Fig. 8. Life cycle of *Thecaphora thlaspeos*. The macroscopic infection symptoms of *T. thlaspeos* are shown in the middle as seeds of the host covered in spores. Such teliospores are released into the environment or they propagate together with the seed. Upon perception of a to-date-unknown plant signal, 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. maydis* 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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