A small *Ustilago maydis* effector acts as a novel adhesin for hyphal aggregation in plant tumors

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**Summary**

- The biotrophic basidiomycete fungus *Ustilago maydis* causes smut disease in maize. Hallmarks of the disease are characteristic large tumors in which dark pigmented spores are formed. Here, we functionally characterized a novel core effector *lep1* (*late effector protein 1*) which is highly expressed during tumor formation and contributes to virulence.

- We characterize *lep1* mutants, localize the protein, determine phenotypic consequences upon deletion as well as constitutive expression, and analyze relationships with the repellent protein Rep1 and hydrophobins.

- In tumors, *lep1* mutants show attenuated hyphal aggregation, fail to undergo massive late proliferation and produce only a few spores. Upon constitutive expression, cell aggregation is induced and the surface of filamentous colonies displays enhanced hydrophobicity. Lep1 is bound to the cell wall of biotrophic hyphae and associates with Rep1 when constitutively expressed in hyphae.

- We conclude that Lep1 acts as a novel kind of cell adhesin which functions together with other surface-active proteins to allow proliferation of diploid hyphae as well as for induction of the morphological changes associated with spore formation.

**Introduction**

The basidiomycete *Ustilago maydis* is a biotrophic pathogen causing corn smut disease (Banuett, 1995; Vollmeister *et al.*, 2012). The most conspicuous symptoms of the disease are tumors in which fungal hyphae form huge aggregates in the apoplastic space between enlarged plant cells. In these aggregates, hyphae become embedded in a polysaccharide matrix, their dikaryotic nuclei fuse, and massive proliferation ensues followed by hyphal fragmentation and spore formation (Vollmeister *et al.*, 2012; Redkar *et al.*, 2015; Matei & Doehlemann, 2016; Tollot *et al.*, 2016; Lanver *et al.*, 2017; Snetselaar & McCann, 2017). To promote colonization, *U. maydis* produces 467 putative secreted effector proteins whose expressions occur in discrete waves (Lanver *et al.*, 2018). Such effector proteins can either be secreted into the apoplastic space to protect fungal cells from the action of plant defenses or be translocated to directly modify processes inside plant cells (Lanver *et al.*, 2017). Most of the *U. maydis* effector proteins which have been characterized so far belong to the module associated with biotrophic development (Djamai *et al.*, 2011; Hemetsberger *et al.*, 2012; Mueller *et al.*, 2013; Redkar *et al.*, 2015; Ma *et al.*, 2018; Tanaka *et al.*, 2020). Candidate effector proteins which are induced after establishment of biotrophy reside in the so-called tumor module (Lanver *et al.*, 2018), but have not yet been characterized. The transcription factor Ros1 also resides in the tumor module (Lanver *et al.*, 2018). Ros1 regulates not only spore formation but also many effector genes as well as genes which might be responsible for the synthesis of the matrix in which hyphal aggregates and spores are embedded (Tollot *et al.*, 2016). Ros1 also upregulates the repellent gene rep1. In *U. maydis*, repellents are produced by Kex-2 cleavage of the repetitive Rep1 precursor protein, resulting in short peptides that form tightly bound amyloid-like fibrils on the hyphal surface. Rep1 peptides mediate attachment to hydrophobic surfaces and confer surface hydrophobicity but are not required for virulence (Wösten *et al.*, 1996; Teeftstra *et al.*, 2009). Repellents have largely functionally replaced the surface-active functions of hydrophobins in *U. maydis* (Teeftstra *et al.*, 2006). *Ustilago maydis* possesses two hydrophobin genes, of which *hum2* is expressed in aerial hyphae while the conditions under which *hum3* is expressed are unknown (Teeftstra *et al.*, 2006; Lanver *et al.*, 2018). Hum3 and the secreted protein Rsp1 both have repetitive domains which are predicted to be processed by Kex-2 (Muller *et al.*, 2008). Although double mutants lacking *hum3* and *rsp1* do not have defects in aerial hyphae formation or surface hydrophobicity, they have lost pathogenicity, suggesting an important role of these repetitive proteins during pathogenic development (Muller *et al.*, 2008).

In this study, we have analyzed eight putative effector genes from the tumor module. One of these genes, *lep1* (*late effector...*
protein 1; UMAG_11940), is a novel core effector contributing to tumor formation. The lep1 mutants fail to accumulate fungal biomass in late infection stages and show reduced aggregate formation. Lep1 protein is bound to hyphae and can associate with Rep1 peptides. We propose that Lep1 stimulates hyphal aggregation, a prerequisite for massive fungal proliferation in infected tissue and is required for the morphological changes associated with spore formation.

Materials and Methods

Strains and growth conditions

The *Escherichia coli* strain DH5α (Life Technology) was used for cloning purposes. *Ustilago maydis* strains were grown on a rotary shaker (200 rpm) at 28°C in liquid YEPSL medium (0.4% yeast extract, 0.4% peptone, 2% sucrose) or in liquid complete medium (CM) with 2% glucose (Holliday, 1974). Mating reactions and filamentation on PD-charcoal plates were performed as described by Krombach et al. (2018). To test aggregate formation, the respective strains were grown in YEPSL to an OD₆₀₀ of 1, harvested by centrifugation and resuspended in YEPSL to an OD₆₀₀ of 1. Of these cells, 5 or 10 ml was then transferred to glass vials or to 50 ml flasks and incubated with shaking (50 rpm) at 28°C for 48 h.

Plasmid and strain construction

All strains, plasmids and primers used in this study are listed in Supporting Information Tables S1–S3. PCRs were performed using the Phusion High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt, Germany). Restriction enzymes were all supplied by New England Biolabs. *Ustilago maydis* was transformed by protoplast-mediated transformation (Schulz et al., 1990).

Virulence assays

Infection assays were carried out as previously described (Tollot et al., 2016). The maize variety Early Golden Bantam (Urban Farmer, Westfield, IN, USA) was used for seedling infections and self-propagated Gaspé Flint corn was used for cob and tassel infections. The statistical analysis used two-tailed Student’s *t*-tests. The maize variety B73 and derived bx1 and bx3 mutants have been previously described (Frey et al., 1997; Schnable et al., 2009).

Expression analysis by quantitative PCR and fungal biomass analysis

Gene expression analysis from cells grown on PD-charcoal plates or from infected plant material was performed as described by Brefort et al. (2014), with some modifications. Briefly, filamentously growing *U. maydis* hyphae from PD-charcoal plates were scraped off and subjected to RNA extraction. From infected maize leaves, 2 cm long segments with the most prominent disease symptoms on the 3rd leaf were collected from 10 different plants. Samples were ground to powder in liquid nitrogen and total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Dreieich, Germany). Quantitative PCRs (qPCRs) were performed as previously described (Tanaka et al., 2014). Quantification of relative fungal biomass in infected maize leaves was performed as described by Tollot et al. (2016).

Immunolocalization

To visualize Lep1-HA protein in biotrophic hyphae, infected maize leaves at 8 d post-infection (dpi) were harvested and thin slices of tumors were prepared using a razor blade. Alternatively, fungal aggregates inside tumor tissue were removed with a fine tweezer. After washing with PBS, samples were fixed in 4% paraformaldehyde for 30 min at room temperature. Next, samples were incubated with 0.1 M glycine in PBS for 15 min, followed by immunostaining (Ma et al., 2018). Mouse anti-HA antibody (1 : 1500 dilution; Sigma-Aldrich) was used as a primary antibody and goat antimouse IgG conjugated with DyLight® 594 (1 : 500 dilution; Abcam, Cambridge, UK) was used as secondary antibody.

Microscopy

The proliferation of *U. maydis* in infected maize leaf tissue was visualized by confocal microscopy (TCS-SP8; Leica Microsystems) after staining with WGA-AF488 and propidium iodide as described by Tollot et al. (2016). DyLight® 594 signals were observed after excitation at 594 nm and detection at 608–628 nm. Spores inside tumor tissue were visualized with an Axioplan II microscope (Zeiss). To visualize amyloid fibrils, layers floating on the surface of cell cultures were harvested and stained by 7.5 μM Thioflavin T (ThT) for 10 min. After washing with PBS, samples were analyzed with DAPI filter sets (HC375/11BP, HC409BS and HC447/60BP; Semrock, NY, USA). To measure the intensity of ThT staining, the outlines of fungal cells were manually redrawn and size of the (μm²) and signal intensity of the circled area were determined by IMAGEJ software (https://imagej.nih.gov/ij/). The ThT signal intensity per μm² was calculated. To determine the size of fungal aggregates inside tumor tissue, they were removed with a fine tweezer and resuspended in PBS. Photographs were taken, the edges were manually redrawn and the dimension of the circled area (μm²) was determined by IMAGEJ. To measure the diameter of dikaryotic hyphae, colonies were removed from a PD-charcoal plate with tweezers and mounted. Photographs were taken and the diameter of dikaryotic hyphae at the edge of the colony was measured using IMAGEJ. To statistically analyze the measurements, one-way ANOVA followed by Duncan’s multiple range post-hoc test was used.

Protein extraction

To determine secretion, total protein fractions from cell pellets and culture supernatants of *U. maydis* were prepared as described previously (Djamei et al., 2011) with small modifications. Briefly,
**U. maydis** cells were grown in 100 ml of CM medium supplemented with 2% glucose to an OD$_{600}$ of 0.8–1.0. After centrifugation, Lep1-HA in the supernatant was immunoprecipitated with 40 µl of mouse monoclonal anti-HA-Agarose beads (Sigma-Aldrich) at 4°C overnight on a rotary shaker. Bound Lep1-HA was eluted from the beads with tricine sample buffer (200 mM Tris-HCl, pH 6.8, 2% SDS, 40% (v/v) glycerol, 2% β-mercaptoethanol, 0.04% bromophenol blue) by heating to 99°C for 5 min. The cell pellet was resuspended to an OD$_{600}$ of 20 with tricine sample buffer and glass beads were added before cell disruption using a FastPrep-24 homogenizer (MP Biochemicals, Eschwege, Germany). After centrifugation, the supernatant was directly used for analysis.

To investigate the extraction efficiency of Lep1-HA from strains grown on PD-charcoal plates, filamentously growing **U. maydis** hyphae were generated by spreading 500 µl of OD$_{600}$ = 1 cells on PD-charcoal plates. After incubation at 22°C for times given in each experiment, cells were scraped off and ground to powder in liquid nitrogen. Then, 400 mg of powder was mixed with 1.5 ml of radiolimunoprecipitation assay (RIPA) buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and Protease Inhibitor Cocktail (Roche)) or 2% SDS buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 2% SDS and Protease Inhibitor Cocktail). Samples extracted with RIPA buffer were mixed for 30 min at 4°C and glass beads, then centrifuged at maximum speed on a tabletop centrifuge for 20 min at 4°C. Supernatants were collected and immunoprecipitated with 40 µl of anti-HA-Agarose beads at 4°C for 90 min on a rotary shaker. For samples extracted with 2% SDS, the same steps were followed except that buffer extraction was at room temperature and supernatants were diluted with Tris buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl) to reduce the SDS concentration to <0.2%. Bound proteins were eluted from beads with tricine sample buffer by heating at 99°C for 5 min.

To identify Lep1-interacting proteins, filamentously growing SG200P$_{actin}$-lep1-HA and SG200P$_{orf}$-UMAG_11443-HA were grown on PD-charcoal plates at 22°C for 18 h. Extracts were prepared and Lep1-HA or UMAG_11443-HA and their interactors were precipitated with anti-HA magnetic beads as described above. The magnetic beads were washed three times with RIPA buffer followed by four washes with 100 mM ammonium bicarbonate. Subsequent peptide elution, acidification, desalination and LC-MS analysis were performed as described previously (Gómez-Santos et al., 2019). Label-free quantification of proteomics raw data was performed using MAXQUANT (Tyanova et al., 2016a) (v.1.6.11.0) using standard settings and the match between run option enabled. For the database search, the combined protein database for **U. maydis** and Zea mays downloaded from Uniprot (https://www.uniprot.org) was used. The following search parameters were used: full tryptic specificity required (cleavage after lysine or arginine residues); two missed cleavages allowed; carbamidomethylation (C) set as a fixed modification; and oxidation (M) and deamidation (N,Q) set as a variable modification. For statistical analysis the MAXQUANT output was loaded into PERSEUS (Tyanova et al., 2016b) (v.1.5.2.6).

**Accession numbers**

Amino acid sequences encoded by **U. maydis** and *Sporisorium reilianum* genes used in this study are available at NCBI under the following accession numbers: **U. maydis** lep1 (UMAG_11940), XM_011391264.1; UMAG_03138, XM_011391300.1; UMAG_03046, XM_011391117.1; UMAG_00466, XM_011388026.1;
Results

Identification of *lep1* as a late virulence-promoting effector

From the 467 genes predicted to encode secreted proteins in *U. maydis*, 104 belong to expression modules associated with tumor formation and could be candidate late effector genes (Lane et al., 2018). Of these, we selected eight genes (*UMAG_00466*, *UMAG_01778*, *UMAG_03046*, *UMAG_03138*, *UMAG_03154*, *UMAG_04033*, *UMAG_11940*, *UMAG_10972*) for functional analysis using the following criteria: proteins lack predicted functional domains; genes do not reside in gene families (Schuster, 2018); gene expression peaks at either 8 or 12 dpi; and genes are barely expressed in axenic culture while strongly upregulated at late time points (Fig. S1). Mutants of these eight late effector genes were generated by gene replacements in strains FB1 and FB2. Only deletion mutants of *UMAG_11940* (*lep1*) showed reduced virulence (Figs 1a, S2; Table S4). Most prominently reduced after infections with *lep1* mutants was the number of dead plants but the shape and distribution of leaf tumors were also altered (Fig. 1a,b). Tumors appeared restricted to the midribs and were not spreading to the leaf blade. Furthermore, leaf tumors induced by *lep1* mutants displayed red/brownish spots (Fig. 1b). The virulence phenotype of *lep1* mutants could be complemented by introducing single-copy *lep1-HA*, although complementation did not reach full wild-type levels (Fig. 1a; Table S5). As complementation constructs in *U. maydis* are usually inserted in the *ip* locus, gene regulation may be affected and this can affect complementation. The predicted mature Lep1 protein consists of only 62 amino acids and lacks cysteine residues (Fig. S3a). Genes related to *lep1* were detected in all sequenced Ustilaginiales and their average amino acid sequence identity is 46.6% (Table S6). To assess the functional relevance of conserved domains, we introduced five alanine substitutions in the N-terminal conserved domain (PIEDI) and substantial differences in expression of salicylic acid-responsive PR genes or genes from the phenylpropanoid pathway (Fig. S6a,c; Methods S3), significantly increased expression for *bx3* and *bx4* from the benzoxazinoid biosynthesis pathway was seen after infection with the *lep1* mutant (Fig. S6b,d; Methods S3). Benzoxazinoids represent defense compounds conferring protection against a wide range of herbivores, pathogenic fungi and bacteria (Frey et al., 2009). To investigate a possible connection between benzoxazinoid synthesis and reduced virulence of the *lep1* mutant, we analyzed virulence of the *lep1* mutant in *bx1* and *bx3* mutant maize lines which are deficient in producing benzoxazinoids (Frey et al., 1997). In the maize variety B73, *lep1* mutants formed fewer and restricted tumors compared to infections with FB1×FB2 (Fig. S7), similar to results in Early Golden Bantam (Fig. 1a). However, virulence of the *lep1* mutants did not increase in benzoxazinoid mutants (Fig. S7), suggesting that the virulence defect of *lep1* mutants is not caused by potentially increased benzoxazinoid levels.

*lep1* is important for hyphal aggregate formation

To investigate at which stage *lep1* is needed during late biotrophic development, we investigated the accumulation of fungal biomass in plants infected by FB1×FB2 and *lep1* deletion mutants by qPCR. In wild-type infected tissue, the ratio of *U. maydis*: maize biomass increased dramatically between 6 and 10 dpi as shown previously (Tollot et al., 2016; Lane et al., 2018) while the ratio remained at the 6 dpi level in plants infected with *lep1* deletion strains (Fig. 2a). To determine whether there is a specific developmental stage at which *lep1* mutants are blocked, fungal hyphae were stained with WGA-AF488 (wheat germ agglutinin-Alexa Fluor 488) and plant cell walls were stained with propidium iodide. From 6 dpi onwards, the wild-type developed hyphal aggregates, and between 8 and 10 dpi these aggregates expanded followed by gelatinization, fragmentation and differentiation into mature spores at 12 dpi (Fig. 2b) (Snetselaar & Mims, 1993; Banuett & Herskowitz, 1996; Tollot et al., 2016). By contrast, *lep1* mutants were severely attenuated in aggregation between 6 and 10 dpi and only small aggregates were observed at 12 dpi (Fig. 2b). After aggregates were enriched from plant tissue harvested at 8 dpi and their
contour was measured, it could be shown that lep1 mutants produced significantly smaller aggregates than the wild-type (Fig. 2c).

To see whether there is a connection between hyphal aggregates and nuclear fusion, we next analyzed whether lep1 mutants undergo karyogamy. To visualize the nuclear status, mCherry-tagged histone H1 was introduced as a nuclear marker into FB1, FB2 and corresponding lep1 mutant strains. Up to 4 dpi, biotrophic hyphae of both strain combinations were dikaryotic and afterwards only one nucleus was observed per cell compartment, indicating that there was no karyogamy defect in lep1 mutants (Fig. S8). This result suggests that lep1 is required for aggregate formation after successful karyogamy. To determine whether lep1 mutants have a defect in production of the polysaccharide matrix, aggregates of either FB1×FB2 or FB1Δlep1×FB2Δlep1 from tumor tissue were stained with concanavalin A (Methods S4). In wild-type as well as in lep1 mutant aggregates, fluorescence of concanavalin A-AF488 was detected in spot-like structures (Fig. S9), suggesting that lep1 mutants still produce extracellular polysaccharide matrix.

Constitutively expressed Lep1 changes the hydrophobicity of hyphae and induces cell aggregation

To determine the effect caused by constitutive expression of lep1, which is usually expressed only late during infection, we generated strains FB1P actin-lep1-HA and FB2P actin-lep1-HA.

Fig. 1 lep1 contributes to virulence and spore formation. (a) Pathogenicity assay of Ustilago maydis lep1 mutants and complementation strains. Plants were infected with the indicated strains and disease symptoms were scored at 12 d post-infection (dpi) using a previously developed scoring scheme (Kämper et al., 2006) plus a new disease category in which tumor development was restricted to the midribs of maize seedling leaves and tumors often developed brownish spots. Disease symptoms were quantified based on three biological replicates. The mean percentage of plants placed in a certain disease category is indicated. The total number of infected plants is indicated above the bar. Asterisks in the respective disease categories indicate significant differences (determined by a two-tailed Student’s t-test, $P \leq 0.05$) between the respective complementation strain and FB1Δlep1×FB2Δlep1. (b) Representative images of disease symptoms on maize leaves infected by the indicated strains after 12 dpi. The right panels depict enlargements of tumors. Midrib tumors induced by FB1Δlep1×FB2Δlep1 sometimes display red/brownish spots. (c) Spore formation in tumors induced by lep1 mutant strains. Spore production was analyzed after infection with the indicated strains by making transverse cuttings through infected stem areas in the region where the 3rd leaf emerged at 12 dpi (top panel). Lower panel: dispersed tumor tissue of the indicated strains was analyzed for the presence of dark pigmented spores by light microscopy.
that express lep1-HA under control of the actin gene promoter. Levels of lep1 in FB1P_\text{actin-lep1-HA} \times FB2P_\text{actin-lep1-HA} were approximately four times higher than in FB1 \times FB2 (Fig. S10). When FB1 \times FB2, FB1Δlep1 \times FB2Δlep1 and FB1P_\text{actin-lep1-HA} \times FB2P_\text{actin-lep1-HA} were mated on a PD-charcoal plate, all combinations produced comparably strong aerial hyphae and displayed comparable colony surface hydrophobicity after 24 h (Figs S11, S12a). After 48 h, however, patches of the FB1 \times FB2 and FB1Δlep1 \times FB2Δlep1 mixtures peeled off the plate while patches of FB1P_\text{actin-lep1-HA} \times FB2P_\text{actin-lep1-HA}
did not (Fig. S11), indicating differences in adherence to the plate.

When lep1-HA was constitutively expressed in the solopathogenic strain SG200 and plated on a PD plate, the colony surface of the strain expressing lep1-HA showed higher hydrophobicity than SG200 or hyphae expressing the Sta1 effector which is bound to fungal hyphae (Fig. 3a) (Tanaka et al., 2020). Another phenotype of the SG200P<sub>actin</sub>-lep1-HA strain became evident when cultures were grown in YEPLS in glass vials with little aeration for 2 d (Fig. 3b). Under these conditions, SG200P<sub>actin</sub>-lep1-HA formed a thick white layer of aggregated cells floating on the culture surface. Aggregated cells were also observed in cultures of FB2P<sub>actin</sub>-lep1-HA (Fig. S13). To explain the increased hydrophobicity as well as the increased aggregate formation, we considered a connection between lep1, the repellent gene rep1, and/or the two class I hydrophobin genes hum2 and hum3 because repellents and hydrophobins are important to provide surface hydrophobicity (Wösten et al., 1996; Teertstra et al., 2006). To test the possibility that Lep1 has a similar function as Rep1, we generated a strain expressing lep1 under the rep1 promoter in a rep1 deletion background. After mating on a PD-charcoal plate, rep1 mutant strains showed a dramatic reduction of aerial hyphae compared to FB1×FB2 (Fig. 4a) (Wösten et al., 1996). However, expressing lep1 in the rep1 mutant did not restore aerial hyphae formation but further reduced aerial hyphae formation. This is especially evident at the edge of the colony (Fig. 4a) and suggests that Lep1 suppresses aerial hyphae formation. To substantiate this, we introduced P<sub>actin</sub>-lep1-HA into rep1 mutant strains and checked aerial hyphae formation. Also here, we observed reduced aerial hyphae formation compared to rep1 mutants after 3 d of incubation (Fig. 4b). Next, we generated lep1,rep1 double mutants and tested aerial hyphae formation (Fig. 4b). Unexpectedly, these strains formed more aerial hyphae than the rep1 mutants and the colony surface became hydrophobic (Fig. S12b). Compared to the wild-type and lep1 mutants, the patch of lep1,rep1 double mutants did not peel off the plate after 3 d (Figs 4b, S11), probably because aerial hyphae formation of lep1,rep1 double mutants was still not as strong as the wild-type and lep1 mutants. However, the diameter of FB1Δrep1×FB2Δrep1 hyphae as well as of FB1Δrep1P<sub>actin</sub>-lep1-HA×FB2Δrep1P<sub>actin</sub>-lep1-HA hyphae was increased compared to FB1×FB2 hyphae, while the diameter of lep1,rep1 mutant hyphae was comparable to FB1×FB2 hyphae (Fig. 4c,d). This suggested that the increased hyphal diameter correlates with reduced aerial hyphae formation, and rep1 expression restricts the hyphal diameter. To explain why the lep1,rep1 double mutant produced more aerial hyphae with restricted hyphal diameter than the rep1 mutant (Fig. 4b–d), we considered gene compensation by either the hum2 or hum3 hydrophobin genes or by genes which have a potential function in cell wall modification and which are expressed during late infection stages (Fig. S14a). qPCR analysis was performed on the same strain mixtures plated in Fig. 4b after 3 d of incubation as well as on an FB1×FB2 cross as a control after 1 d of incubation (Fig. 4e). In FB1×FB2, hum2 expression increased significantly between 1 and 3 d of incubation. However, hum2 expression was not increased in the cross of FB1Δrep1P<sub>actin</sub>-lep1-HA×FB2Δrep1P<sub>actin</sub>-lep1-HA, while hum2 levels in all other strain combinations were comparable to the wild-type (Fig. 4e). Expression of hum3 could not be detected by qPCR (Fig. S15), consistent with a previous study.

**Fig. 3** Constitutively expressed Lep1-HA changes the hydrophobicity of hyphae and induces aggregation of filaments. (a) Colony hydrophobicity of strains constitutively expressing Ustilago maydis Lep1-HA. The indicated strains were spotted on a PD plate and incubated for 2 d at 28°C (top row). A droplet of water colored with 10 μl ml<sup>−1</sup> trypan blue was spotted on the colony surface for 1 h. Colonies were photographed from the side to show surface hydrophobicity. (b) Lep1-HA-induced cell aggregation. Cells of the indicated strains were grown in YEPLS liquid medium in glass vials with little aeration for 2 d at 28°C and photographed. Aggregated cells form a dense layer floating on top of the culture.
Fig. 4 *lep1* expression reduces aerial hyphae formation in *rep1* mutants. (a) Filamentous growth of *Ustilago maydis* mutants lacking *rep1* and expressing *lep1*. The indicated strain combinations were spotted on PD-charcoal plates for 3 d at 22°C and photographed. In the rightmost panels colony edges of the indicated strain combinations are enlarged. (b) Filamentous growth of *rep1* and *rep1,lep1* deletion mutants as well as *rep1* mutants expressing Lep1-HA. The indicated strain combinations were spotted on PD-charcoal plates, incubated for 1 d (upper panels) and 3 d (lower panels) at 22°C and photographed. (c) Filament formation of *rep1* and *rep1,lep1* mutant strains as well as *rep1* mutants expressing Lep1-HA. The indicated strain combinations were spotted on PD-charcoal plates and incubated for 2 d at 22°C. Hyphae at the edges of respective patches were studied by microscopy. (d) Hyphal diameter of strains lacking *rep1* and *lep1*, and *rep1* mutants expressing Lep1-HA. The diameter of hyphae of the strain combinations indicated in (c) was measured by ImageJ. Measurements are displayed by box and whisker plots. n indicates total numbers of hyphal diameters measured from three biological replicates. Asterisks indicate significant differences compared to the diameter of FB1 × FB2 (determined by one-way ANOVA followed by Duncan’s multiple range post-hoc test, *P* ≤ 0.001). (e) *hum2* expression in strains lacking *rep1* and *lep1*, and in *rep1* mutants expressing Lep1-HA. The indicated strain combinations were plated on PD-charcoal plates for 1 or 3 d at 22°C. After RNA extraction, the relative expression of *hum2* was measured by quantitative PCR (qPCR). Constitutively expressed *U. maydis* peptidylprolyl isomerase (*ppi*) was used for normalization. Three biological replicates were analyzed; error bars depict standard deviation. *hum2* expression in FB1 × FB2 at 3 d of incubation was set to 1.0. Asterisks indicate significant differences compared to *hum2* expression in FB1 × FB2 at 3 d of incubation (determined by a two-tailed Student’s t-test: *, *P* ≤ 0.05; **, *P* ≤ 0.01).
The five genes predicted to have a role in cell wall modification during late infection stages did not show significant expression differences among the strains (Fig. S14b). These results suggested a connection between lep1, hum2, and rep1.

To analyze whether constitutive expression or overexpression of lep1 affects virulence, plants were infected by strains expressing Pactin-lep1-HA as well as strains expressing lep1-HA from the UMAG_03046 promoter which drives about 14-times higher expression than the lep1 promoter at 8 dpi (Fig. S16a,c). Both of these strains showed reduced virulence compared to the wild-type (Fig. S16b,d; Table S8). This result suggests that both inappropriate timing of lep1 expression and elevated levels of Lep1 negatively impact virulence.

Lep1 is a secreted protein which attaches to the fungal cell wall and associates with Rep1.

To localize Lep1 protein we first determined whether Lep1-HA can be secreted. In the supernatant of SG200P actin-lep1-HA, a protein of the expected size of Lep1-HA without signal peptide (7.7 kDa) was detected by Western blot (Fig. 5a). However, in the cell pellet fraction, Lep1-HA did not migrate according to a protein with the signal peptide still attached (10.3 kDa), but had a higher molecular weight of about 12 kDa (Fig. 5a). In extracts from the same strains plated on a PD-charcoal plate and allowed to produce filaments, Lep1-HA also migrated at 12 kDa (Fig. 5a), suggesting a posttranscriptional modification. Lep1-HA from this sample did not show N- or O-glycosylation (Fig. S17) and a modification was not detectable by MS (Table S9). These results make it likely that Lep1 might exist in a soluble form of the expected size and a form bound to fungal cell walls with apparently higher molecular size, due to covalent attachment to cell wall compounds or due to an altered conformation. In plant tumors induced by FB1, lep1lep1-HA, Lep1-HA also largely migrated at 12 kDa (Fig. 5b). To determine the localization of Lep1-HA during plant colonization, we next performed immunostaining with the strains expressing Lep1-HA under its own promoter or the UMAG_03046 promoter (Fig. 5c). Nonpermeabilized fungal cells in infected tumor tissue at 8 dpi were subjected to anti-HA immunostaining using an anti-HA antibody followed by staining with a secondary antibody conjugated to DyLight 588 (magenta). The fungal cell wall was stained by wheat germ agglutinin-Alexa Fluor 488 (WGA-AF488; green). The right panel depicts an enlargement of the indicated section.

(16) Teertstra et al., 2006. The five genes predicted to have a role in cell wall modification during late infection stages did not show significant expression differences among the strains (Fig. S14b). These results suggested a connection between lep1, hum2 and rep1.
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suggests that the functions of Lep1 and Rep1 are distinct; that is, Lep1 does not contribute to amyloid fibril formation but promotes cell aggregation, while Rep1 contributes to formation of amyloid fibrils but is not important for cell aggregation.

Discussion
In this study we have characterized the lep1 core effector gene which is expressed late during U. maydis infection. The lep1...
deletion mutants showed attenuated fungal aggregate formation in tumor tissue, while \( P_{actin}\)-lep1-HA strains exhibited cell aggregation in liquid medium, stronger colony adhesion to the surface of an agar plate as well as increased surface hydrophobicity. These observations suggest that the function of Lep1 is to modulate surface hydrophobicity promoting cell–cell or cell–surface contacts (Fig. S20).

In \( U. maydis \) surface hydrophobicity has in the past been attributed to the repellent peptides which strongly attach to the hyphal cell wall and are considered to have replaced the function of hydrophobins (Wösthen et al., 1996; Teertstra et al., 2006). Besides providing for hyphal hydrophobicity, repellents in \( U. maydis \) are implicated in hyphal attachment to hydrophobic surfaces (Wösthen et al., 1996). \textit{Ustilago maydis} strains constitutively expressing \( lep1 \) showed stronger attachment to the surface of a PD-charcoal plate, suggesting that Lep1 enhances attachment. MS analysis indeed indicated that Lep1 can associate with Rep1 under these growth conditions. This is also supported by experiments which demonstrated that during aerial hyphae development when \( rep1 \) is also expressed, the extraction efficiency of Lep1-HA decreases rapidly. This suggests that Lep1 and Rep1 interact in aerial hyphae to form a strong hydrophobic layer. In line with this assertion, we observed that Lep1-HA is easier to extract when \( rep1 \) is absent. MS analysis also identified other cell wall-bound proteins as potential interaction partners of Lep1-HA. However, as these experiments were conducted with the SG2000\( P_{actin}\)-lep1-HA strain grown on PD-charcoal plates, we cannot formally conclude that these interactions are relevant during late biotrophic development. For example, \textit{UMAG}\_00330 encoding a putative carbohydrate-degrading enzyme is barely expressed in tumor tissue (Lanver et al., 2018). Unfortunately, we were unable to extract Lep1-HA protein efficiently from biotrophic hyphae because it either is shielded by other cell wall proteins or is inaccessible due to the mucilaginous extracellular matrix. Therefore, we were unable to identify interactors by MS from late developmental stages when Lep1 is naturally produced. For this reason, we conducted a set of experiments with cells grown in axenic culture to address whether Lep1 and Rep1 have related functions. Based on the overexpression phenotype of \( lep1 \) it is clear that Lep1 cannot complement the defect in aerial hyphae formation of the \( rep1 \) mutant. Furthermore, hyphal aggregation in liquid culture can be efficiently mediated by Lep1 but much less so by Rep1 and the propensity for amyloid formation is significantly higher for Rep1 than for Lep1. This shows that the two proteins have discrete functions. To further support this, we studied the effects of Lep1 in \( rep1 \) mutants which are strongly attenuated in aerial hyphae formation (Wösthen et al., 1996). It has been shown that in \( rep1 \) mutants a number of secreted proteins are upregulated and 80% of these are predicted to have regions prone to \( \beta \)-aggregation (Teertstra et al., 2011). This could indicate functional compensation and probably explains why some aerial hyphae are still formed in the \( rep1 \) mutant and why these hyphae are stained with ThT when exposed to air (Teertstra et al., 2009). The fact that \( P_{actin}\)-Lep1-HA reduces the residual filamentation of the \( rep1 \) mutant could suggest that \( P_{actin}\)-Lep1-HA suppresses the upregulation of genes encoding secreted proteins such as the \textit{hum2} hydrophobin gene. An additional possibility is that constitutive expression of Lep1-HA in the absence of Rep1 may facilitate stronger aggregation of dikaryotic hyphae. This possibility is supported by showing that aerial hyphae formation visible at the edge of \( rep1 \) mutant colonies is strongly reduced when \( lep1 \) is constitutively expressed and aggregated cell masses appear instead (Fig. 4a).

Assuming an involvement of Lep1 in adherence and aggregation, the strong aerial hyphae formation of \( lep1, rep1 \) double mutants is difficult to explain. As we excluded that \textit{hum2} is significantly upregulated in the \( lep1, rep1 \) double mutants, we have to assume that there might be compensation by other as yet unidentified genes encoding surface-active proteins. The thinner hyphal diameter and the higher surface hydrophobicity of \( lep1, rep1 \) mutants compared to filaments of the \( rep1 \) mutant suggests the existence of other surface-active proteins which restrict the extension of the cell wall and provide higher hydrophobicity – either because the respective genes are upregulated due to compensation or because these proteins can now attach to the cell wall when the rigid layer of Rep1 and Lep1 is absent.

Lep1 is a small secreted protein that shares a number of properties with repellents and hydrophobins, namely its small size, extractability with SDS, and providing for hydrophobicity and surface attachment. However, Lep1 also displays features not shared by either hydrophobins or repellents: Lep1 lacks cysteine residues which reside in characteristic patterns in hydrophobins (Wessels et al., 1991) and lacks the ability to induce amyloids, a property ascribed to repellents and class I hydrophobins (Ball et al., 2020). In addition, Lep1 probably needs to be modified or needs to adopt an altered conformation to be functional.

As Lep1 protein which is bound to hyphae is largely inaccessible to antibody staining in biotrophic hyphae, we cannot

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**Fig. 6** Lep1 attaches to the fungal cell and interacts with Rep1. (a) \textit{Ustilago maydis} Lep1-HA cannot be extracted efficiently from aerial hyphae. The indicated strain combinations were spotted on PD-charcoal plates to allow development of dikaryotic hyphae and were harvested at 1, 2, 3 and 4 days after incubation at 22°C. Total proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (left) or 2% SDS buffer (right) and Lep1-HA was immunoprecipitated by anti-HA magnetic beads. Bound protein was subjected to Western blot analysis. The Western blot was developed with anti-HA antibody. The gray triangles indicate increasing filament formation of the indicated strain combinations during the time course and the thickness of the triangles depicts the degree of aerial hyphae formation. (b) Lep1-HA binds to fungal hyphae. Enriched Lep1-HA and \textit{UMAG}\_11443-HA proteins were incubated with and without filamentous patches of \( FB1 \times FB2 \) grown on PD-charcoal. After incubation for 1 h, samples were centrifuged and the supernatants were subjected to Western blot. The Western blot was developed with anti-HA antibody. (c) Lep1-HA interacts with Rep1. Shown are results of the mass spectrometry analysis of the coimmunoprecipitation-MS (co-IP-MS) experiments using Lep1-HA and \textit{UMAG}\_11443-HA as a bait protein. Results are displayed by a volcano plot. Within the \textit{PERSEUS} software environment, \( P \) values and log\(_2\)-protein abundance differences are calculated between the two bait groups. Co-IP-MS was carried out in duplicated biological experiments. The significance cut-off was set to \( P \leq 0.05, S0 = 0.1 \). The significant area is highlighted for \( P \leq 0.01 \) (orange) and \( P < 0.05 \) (yellow) on the y-axis as well as by log\(_2\)-intensity difference of \textit{UMAG}\_11443 vs Lep1 > -4 on the x-axis.
formally conclude that Lep1 decorates the entire fungal cell wall late in infection. However, as we can detect Lep1-HA in newly synthesized cell wall material at the tips of biotrophic hyphae at late colonization stages when expression is driven by the UMAG_03046 promoter, we consider it likely that Lep1 forms a layer covering the cell wall. After cell wall maturation, Lep1-HA probably becomes undetectable due to an association with Rep1 or other surface-active proteins and/or the mucilaginous extracellular matrix. Moreover, the phenotype of mutants in which the hydrophobic region in Lep1 is substituted by amino acids rendering this region hydrophilic leads to weaker binding to hyphae and much reduced ability to complement the virulence defect of the lep1 mutants. Based on this we can conclude that strong cell wall binding via hydrophobic interactions is crucial for the biological activity of Lep1. The fact that Lep1-HA is difficult to extract even from rep1 mutants suggests that Lep1 is able

Fig. 7 Lep1 is involved in aggregation of cells exposing amyloid Rep1 fibrils. (a) The expression pattern of lep1, rep1 and two hydrophobin genes in Ustilagomaydis. Expression values of indicated genes during a plant infection time course with FB1×FB2 were retrieved from published RNA-sequencing data (Lanver et al., 2018). Error bars indicate ± SD. (b) Constitutive expression of rep1 does not induce cell aggregation. Cells of the indicated strains were grown in YEPSL liquid medium in glass vials with little aeration for 2 d at 28°C and photographed. (c) lep1 mutants show increased thioflavin T (ThT) staining. The indicated strains were grown in YEPSL liquid medium with little aeration for 2 d at 28°C. The cells floating on the surface of cell cultures were stained with 7.5 µM ThT for 10 min to analyze the formation of amyloid fibrils and observed by fluorescence microscopy. (d) Quantification of ThT staining in lep1 mutant cells. Fluorescent signal intensities shown in (c) were determined per µm². Total numbers of quantified fungal cells are indicated above the respective boxes. Asterisks indicate significant differences compared to the signal intensity of SG200 (determined by one-way ANOVA followed by Duncan’s multiple range post-hoc test, P ≤ 0.001).
to bind to fungal hyphae even in the absence of Rep1. However, under conditions when Rep1 is absent, other surface-active proteins are probably upregulated and this might occur also during biotrophic development. Therefore, our finding that lep1(rep1 double mutants do not show a stronger virulence phenotype than lep1 mutants might be caused by compensation through other surface-active proteins. If this assertion is correct, Rep1 would be the natural interaction partner of Lep1 during late biotrophic development and when absent, other surface-active proteins would substitute for Rep1. Fitting this proposition is the observation that rep1 expression is biphasic during colonization; that is, the gene is highly expressed very early during colonization (when lep1 is not expressed), decreases in expression during biotrophy establishment and is coinduced with lep1 late during infection. This could even indicate that the function of Lep1 is modulated when Rep1 (or other surface-active proteins) is expressed. To show an interaction of Lep1 with Rep1 (or with other surface-active proteins) one would need to analyze interaction partners for Lep1 during colonization in the presence or absence of Rep1. Unfortunately, we were unable to extract Lep1 protein efficiently from infected tissue, which precluded such analyses.

Why is Lep1 protein a virulence factor and is the reduced ability for aggregate formation seen after lep1 mutant infection connected to this? Or does the coating of fungal hyphae by Lep1 provide protection against plant defenses? When infecting plants with lep1 mutants, we detected no significant transcriptional changes in pathways restricting biotrophic fungi such as the salicylic acid pathway or the phenylpropanoid pathway and were unable to link elevated expression of two genes from the benzoxazinoid pathway with reduced virulence of the lep1 mutant. Investigations via RNA-sequence analysis are needed to determine whether plant defense genes not analyzed here are upregulated in lep1 mutant infected tissue. Without evidence for such a link we speculate that hyphal aggregation, which we also see after constitutive expression of Lep1 in culture, is the primary function of Lep1. Hyphal aggregation mediated by Lep1 might be a prerequisite for the strong proliferation of diploid cells and for induction of the morphological changes associated with spore formation. To establish whether hyphal aggregation in tumor tissue requires only Lep1 and presumably Rep1, or whether some kind of quorum sensing and extracellular matrix biosynthesis are involved in addition, needs to be studied and will be a true experimental future challenge because all of this occurs late during fungal development in tumor tissue.

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Author contributions

FF and RK conceived and designed the experiments. FF and KM performed experiments. NR generated strains. TG performed the MS and subsequent data analysis. FF and RK wrote the manuscript with input from all coauthors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The expression pattern of eight late effector genes during plant infection.

Fig. S2 Virulence of mutants lacking late effector genes.

Fig. S3 lep1 encodes a small conserved secreted protein.

Fig. S4 lep1 mutants are able to germinate.

Fig. S5 Virulence of lep1 mutants in maize cobs and tassels.

Fig. S6 qPCR analysis of defense-associated genes after infection with lep1 mutants.

Fig. S7 The virulence defect of lep1 mutants is not rescued in maize bx1 or bx3 mutants.

Fig. S8 lep1 mutants do not show the defects in karyogamy.

Fig. S9 lep1 mutants do not show defects in producing polysaccharide.

Fig. S10 lep1 expression in strains constitutively expressing Lep1-1 HA.

Fig. S11 Strains constitutively expressing Lep1-1 HA adhere to the PD-charcoal plate.
**Fig. S12** Hydrophobicity of the colonies constitutively expressing Lep1-HA.

**Fig. S13** Lep1-HA induced cell aggregation in strain FB2.

**Fig. S14** Expression of putative cell wall modification genes in strains lacking rep1 and lep1, and in rep1 mutants expressing Lep1-HA.

**Fig. S15** qPCR analysis of hum3 expression.

**Fig. S16** Virulence assay of strains constitutively expressing lep1 or overexpressing lep1 late during plant colonization.

**Fig. S17** Glycosylation analysis of Lep1-HA extracted from filamentous cells.

**Fig. S18** Detection of Lep1-HA expressed from its native promoter by immunostaining.

**Fig. S19** Virulence of lep1,rep1 double mutants.

**Fig. S20** Hypothetical model for the function of the *Ustilago maydis* late effector Lep1.

**Methods S1** Spore germination analysis.

**Methods S2** Infection assays in maize cobs and tassels.

**Methods S3** Accession numbers for Zea mays genes.

**Methods S4** Confocal microscopy for detecting mCherry signals and fungal polysaccharide matrix.

**Table S1** *Ustilago maydis* strains used in this study.

**Table S2** Plasmids used in this study.

**Table S3** Oligonucleotides used in this study.

**Table S4** Statistical analysis of disease ratings in Fig. S2.

**Table S5** Statistical analysis of disease ratings in Fig. 1(a).

**Table S6** Amino acid identity between Lep1 homologs in Ustilaginales.

**Table S7** Statistical analysis of disease ratings in Fig. S3.

**Table S8** Statistical analysis of disease ratings in Fig. S16.

**Table S9** Posttranslational modification analysis of Lep1-HA by MS.

**Table S10** Statistical analysis of disease ratings in Fig. S19.

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