The *Ustilago maydis* repetitive effector Rsp3 blocks the antifungal activity of mannose-binding maize proteins

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To cause disease in maize, the biotrophic fungus *Ustilago maydis* secretes a large arsenal of effector proteins. Here, we functionally characterize the repetitive effector Rsp3 (repetitive secreted protein 3), which shows length polymorphisms in field isolates and is highly expressed during biotrophic stages. Rsp3 is required for virulence and anthocyanin accumulation. During biotrophic growth, Rsp3 decorates the hyphal surface and interacts with at least two secreted maize DUF26-domain family proteins (designated AFP1 and AFP2). AFP1 binds mannose and displays antifungal activity against the *rsp3* mutant but not against a strain constitutively expressing *rsp3*. Maize plants silenced for AFP1 and AFP2 partially rescue the virulence defect of *rsp3* mutants, suggesting that blocking the antifungal activity of AFP1 and AFP2 by the Rsp3 effector is an important virulence function. Rsp3 orthologs are present in all sequenced smut fungi, and the ortholog from *Sporisorium reilianum* can complement the *rsp3* mutant of *U. maydis*, suggesting a novel widespread fungal protection mechanism.
The dimorphic fungus *Ustilago maydis* causes smut disease in maize. Prominent symptoms of the disease are anthocyanin induction and large tumors in which the fungus proliferates and produces spores. The disease cycle is initiated by the dikaryotic hyphae. Upon the perception of surface cues, these hyphae form infection structures that penetrate epidermal cells. During penetration, the host plasma membrane invaginates and surrounds the invading hypha, thus establishing an extended interaction zone characteristic for a biotrophic interaction. *U. maydis* secretes 467 putative effector proteins that contribute to the establishment of the biotrophic stage and tumor formation. In *Saccharomyces cerevisiae* allows adaptation to environmental changes or escape from host intragenic tandem repeats, and these also show length polymorphisms, which is considered to result from homologous recombination or slippage during replication. Variations in length polymorphisms were caused by deletion or expansion of repeats in the C-terminal domain (Fig. 1a; Supplementary Fig. 1).

Repeat-containing effectors have been identified in several filamentous microbes. They can either associate with microbial surfaces, reside in the interface between microbe and host, or translocate into specific compartments of the host cells.

*U. maydis* encodes fifteen effector proteins containing internal repeats. In eight of these proteins, the repeats are shown or anchors and their repeat domains are often highly variable in properties. Their repeat domains are predicted to be separated by Kex2-like cleavage sites and thus likely processed while the other seven repeat-containing proteins are not predicted to be processed by Kex2. Of these, only the membrane-bound signaling mucin-like protein Msb2 was characterized and shown to regulate appressorium development. In other fungi, many of the non-processed repetitive proteins are attached to the cell wall by glycosylphosphatidylinositol-anchors. Their repeat domains are often highly variable in length, which is considered to result from homologous recombination or slippage during replication. Variations in repeat numbers are thought to provide functional diversity and allow adaptation to environmental changes or escape from host immunity.

*Saccharomyces cerevisiae* encodes 44 proteins containing intragenic tandem repeats, and these also show length polymorphisms. In the Flo1p protein, the repeat number is positively correlated with an increase in cell–cell adhesion properties. *Mycosphaerella graminicola* harbors 23 genes predicted to encode surface-associated proteins possessing internal tandem repeats, and isolates vary in intragenic repeat numbers. The genome of *Aspergillus fumigatus* contains 292 genes with internal repeats, and size variation in repeat-containing regions was detected in clinical isolates.

In addition, there are repeat-containing effectors that translocate into specific compartments of host cells where they elicit their virulence function. For example, *Colletotrichum graminicola* CgEP1 was detected in the plant nucleus and could bind DNA nonspecifically. *Rhizopus irregularis* SP7 is presumed to enter the host nucleus and target the transcription factor MIERF150. *Phytophthora infestans* PexRD54 binds the host ATG8C protein and triggers autophagosome formation. Whether the tandem repeats contribute to these functions is still unknown.

In this study, we functionally analyze the repetitive effector protein Rsp3 (UMAG_03274) of *U. maydis* and provide evidence that this effector has a conserved virulence-promoting function conferred by its ability to shield fungal hyphae from the action of maize antifungal proteins.

**Results**

**rsp3 shows length polymorphisms.** Rsp3 belongs to the *U. maydis* effectors without predicted functional or structural domains, and represents a core effector that is present in five sequenced smut fungi. Rsp3 is predicted to be a non-processed repeat-containing protein lacking a GPI anchor. Downstream of the signal peptide, a glutamine and proline-rich region (QP) is followed by a cysteine-rich region (Cys). The long C-terminal domain comprising more than half of the molecule consists of a complex array of several different repetitive units (Fig. 1a).

As the *rsp3* genes in the compatible haploid strains FB1 and FB2 showed significant length differences of 2.61 and 2.37 kbp, respectively (Fig. 1a; Supplementary Fig. 1a, b), we analyzed length polymorphisms also in field isolates from different locations in Mexico. The *rsp3* alleles amplified from these strains varied in length between 1.8 and 2.6 kbp. Sequencing revealed that these polymorphisms were caused by deletion or expansion of repeats in the C-terminal domain (Fig. 1a; Supplementary Fig. 1).

A qRT-PCR analysis showed that *rsp3* was not expressed in axenic culture but was specifically and highly induced during the biotrophic interaction (Fig. 1b). Induction was already observed when appressoria develop between 0.5 and 1 day post infection (dpi). *rsp3* expression peaked at 2 dpi and then gradually decreased (Fig. 1b). A recent time-resolve transcriptome analysis placed *rsp3* into an effector-enriched module associated with the establishment of biotrophy, i.e., the early stages of fungal development inside the plant.

**Rsp3 is an important virulence factor in *U. maydis*.** To investigate a contribution to virulence, *rsp3* was deleted in the solopathogenic haploid strain SG200 and its derivative SG200AN1 expressing a *UMAG_01779* promoter-GFP fusion protein in cells developing appressoria. To study early infection-related development, appressorium formation and penetration were analyzed microscopically and quantified 18 h post infection. Appressorium formation as well as penetration of SG200AN1rsp3 occurred with comparable efficiency to SG200AN1 (Supplementary Fig. 2a, b).

SG200rsp3 strains showed a strong attenuation of virulence and both tumor numbers as well as tumor size were significantly reduced compared to SG200 (Fig. 2a, b). In addition, the mutant failed to induce anthocyanin (Fig. 2b). These phenotypes could be rescued by complementation with a single copy of the *rps3* allele of FB1 or the shortest *rps3* allele from Toluca-6 (*T*; Fig. 2a, b). *rps3* alleles carrying HA-tags at either the N terminus downstream of *rsp3* occurred with comparable efficiency to SG200AN1rsp3 (Fig. 2a, b). However, a *rps3* allele carrying substitution of all nine cysteine residues to alanine (*rps3*<sub>Cys</sub>) in the Cys-rich domain (Fig. 1a) or truncated alleles lacking the Cys-rich domain (*rps3*<sub>Cys</sub>; Δ244–333 amino acids) could not complement the virulence phenotype of the *Δrps3* mutant (Fig. 2a, b) while anthocyanin production was observed (Fig. 2b). Orthologs of *Rps3* are found in all sequenced smut species and display a relatively high conservation in their N-terminal domains (35–55% identity) while the repetitive domains show only 4–21% identity (Supplementary Fig. 3). The *S. reiliani* ortholog SrRps3, which has only 34% sequence identity to *U. maydis* Rps3 (FB2), could fully complement the virulence phenotype of the *U. maydis* rps3 mutant (Fig. 2c).

Next, we investigated plant colonization by SG200rsp3 by confocal microscopy and compared this to colonization by SG200. Reduced proliferation of *Δrps3* strains was observed at all stages of the infection and mutant hyphae remained intracellularly and did not proliferate along the veins where wild type hyphae accumulated and were found to spread from 4 dpi
onwards (Fig. 2d; Supplementary Fig. 2c). Compared to the \( \text{rsp3} \) mutant and SG200, SG200AN1\( \Delta \text{rsp3-rsp3} \)\( \Delta \text{Cys} \) showed an intermediate phenotype characterized by a higher degree of colonization and some hyphal growth along the veins (Supplementary Fig. 2d). These results illustrate that Rsp3 is an important virulence factor.

The N terminus of Rsp3 is processed. The first 23 amino acids of Rsp3 are predicted by SignalP 4.1\( ^{26} \) to be a signal peptide. To visualize secretion, we generated SG200\( \Delta \text{rsp3} \) strains, which constitutively expressed the biologically active Rsp3-HA or a protein with an additional Myc-tag inserted between amino acid 46 and 47, Myc\( ^{47} \)-Rsp3-HA (Fig. 3a). In supernatants of these strains, Rsp3-HA and Myc\( ^{47} \)-Rsp3-HA could be detected by western blot developed with an anti-HA antibody (Fig. 3b). Surprisingly, we failed to detect Myc\( ^{47} \)-Rsp3-HA in the supernatant fraction with an anti c-Myc antibody (Fig. 3b), despite being able to detect the protein with this antibody in the cell pellet fraction (Fig. 3b). This indicates that Rsp3 is processed at the N terminus.
terminus. We also noticed that Rsp3-HA did not migrate according to its expected size (88 kD) but had an apparent molecular weight of about 150 kD (Fig. 3b). As Rsp3-HA did not show N- or O-glycosylation (Supplementary Fig. 2e), we assume that the anomalous migration of Rsp3 is due to intrinsic properties of the protein. Using the D2P2 database (http://d2p2.pro/search) and the ProtParam tool (http://web.expasy.org/protparam/), Rsp3 is predicted to be negatively charged and intrinsically unstructured with two disordered regions located between amino acids 23–265 and amino acids 330–869. Negatively charged amino acids and disordered structures have been reported to contribute to anomalous migration behavior 27–29, making it likely that these features explain the anomalous migration of Rsp3.

To determine the processing site in Rsp3, Rsp3-HA purified from the supernatant of SG200Δrsp3-P Def-rsp3-HA was subjected to N-terminal sequencing by Edman degradation. This allowed to identify DGGA as the four N-terminal amino acids of the secreted protein suggesting that processing occurs between amino acids 60 and 61 (Fig. 3a). To determine whether the region between signal peptide and N terminus of the processed Rsp3 is relevant for function, we produced a truncated version of Rsp3-HA lacking the region between amino acids 24 and 60 (Rsp3Δ24–60-HA; Fig. 3a) and examined its secretion. Surprisingly, this
deletion abolished secretion and Rsp3Δ24–60-HA accumulated inside the cells (Fig. 3c, upper panel). It could be that cleavage of the signal peptide does not occur when the signal peptide is fused differently to the disordered N-terminal domain, although other possibilities are not excluded. Next, we attempted to abolish processing by generating alanine substitution of amino acids in the vicinity of the cleavage site (Fig. 3a; Myc47-Rsp3(RRDG*)-HA and Myc47-Rsp3(FFRRD*)-HA). Both mutant proteins were still secreted (Fig. 3c, lower panel) and could complement the virulence phenotype of the Δrsp3 strain (Supplementary Fig. 4a, b). However, compared to Rsp3-HA protein, the molecular weights of both mutant proteins was higher and two discrete protein species were detected in the supernatant fraction (Fig. 3c, lower panel). This indicates that the introduced substitutions have eliminated the identified cleavage site between amino acids 60 and 61, but retained additional cleavage sites further upstream. These results suggest that N-terminal processing of Rsp3 is linked to its secretion and most likely to its function.

Rsp3 decorates the surface of fungal hyphae. To assess how Rsp3 contributes to virulence of U. maydis, the effector was localized. Plant samples infected with either SG200 or SG200Δrsp3-rsp3-HA, which produces biologically active Rsp3-HA under its native promoter, were subjected to immunogold labeling and the distribution of gold particles was quantified (Supplementary Table 1). While labeling of SG200-infected tissue revealed low and non-specific background in most compartments (Fig. 4a, top panel), Rsp3-HA was primarily detected inside the fungal cytosol and in the biotrophic interface but rarely inside plant cells (Fig. 4a, lower panel; Supplementary Table 1).
As an accumulation in the biotrophic interface could be a sign of attachment to fungal hyphae, we next investigated this possibility using immunofluorescence microscopy. SG200Δrsp3-HA derived strains constitutively expressing either Rsp3-HA, non-secreted Rsp3Δ24–60-HA or Cmu1-HA, an effector that is translocated to plant cells7, were stimulated with hydroxy-fatty acids and sprayed on Parafilm M to induce filamentation. Non-permeabilized cells were subjected to anti-HA immunostaining using an anti-HA antibody followed by a secondary antibody conjugated to Alexa Fluor 488. We detected fluorescence signals around hyphae of SG200Δrsp3-P_cmu1-rsp3-HA but not around hyphae, which failed to secrete Rsp3Δ24–60-HA or hyphae secreting Cmu1-HA (Fig. 4b). In areas on Parafilm M, where the SG200Δrsp3-P_cmu1-rsp3-HA strain had not differentiated filaments but grew by budding, strong fluorescence was detected on the surface (Supplementary Fig. 4c). This demonstrates that constitutively expressed Rsp3-HA can attach to the surface of budding as well as filamentous cells of U. maydis.

To investigate whether Rsp3 when expressed from its native promoter attaches to fungal hyphae during colonization, leaf
samples infected by SG200Δrsp3-rsp3-HA or SG200 Pcmul-mCherry-AvitagHA expressing cytotoxic mCherry-AvitagHA from the cmul promoter were subjected to anti-HA immunostaining after partially macerating the infected plant tissue. Rsp3-HA was detected around the outside of fungal hyphae (Fig. 4c, upper panel) while non-secreted mCherry-AvitagHA could not be detected (Fig. 4c, lower panel).

To determine whether Rsp3 might also reside as non-attached form in the biotrophic interface, we analyzed apoplastic fluid from maize leaves infected with SG200Δrsp3-rsp3-HA and SG200-cmul-AvitagHA at 3 dpi. Although Cmul-AvitagHA was detected by western blot in TCA-precipitated apoplastic fluid samples, we failed to detect soluble Rsp3-HA (Supplementary Fig. 4d).

As Rsp3-HA can bind to the surface of budding cells and filamentous hyphae of *U. maydis* when it is constitutively expressed, it is conceivable that Rsp3 might bind to cell wall components also when added externally. To test this, we purified Rsp3-HA from the supernatant fraction of SG200Δrsp3-PoteF-rsp3-HA. When incubated with budding cells of SG200 grown in liquid medium and hyphae of SG200 generated on paraffin after spraying, i.e., cells which do not express rsp3 at this stage, fluorescence signals were mostly restricted to tips and septa, and were sometimes surrounding filaments (Supplementary Fig. 5).

To test whether Rsp3 binds to the specific cell wall components of *U. maydis*, we tested binding to *Colletotrichum graminicola* (CmG2) conidia and budding cells of *S. cerevisiae* AH109. Binding of added Rsp3-HA could not be detected (Supplementary Fig. 6a, b). This suggests that Rsp3 might attach to cell wall components, which are *U. maydis* specific, and efficient cell wall association may require that the protein is synthesized by the fungus. Alternatively, cell wall components required for the binding of Rsp3 may not be present or accessible in conidia of *C. graminicola* or budding cells of *S. cerevisiae*. In fungal pathogens, infection-related development and plant colonization have been shown to involve coordinated changes in cell wall composition to escape recognition by the immune system and such alterations might be needed to allow binding of Rsp3.

**Rsp3 interacts with secreted maize DUF26-domain proteins.** Having shown that Rsp3 is attached to biotrophic hyphae of *U. maydis*, we expected putative interaction partners from maize to be secreted apoplastic proteins. To identify interaction partners of Rsp3, we purified Rsp3-HA from culture supernatants of strain SG200Δrsp3-P eag-rsp3-HA. The purified protein was mixed with apoplastic fluid from SG200 or mock inoculated plants. After affinity purification on HA agarose beads, bound proteins were eluted and analyzed by SDS-PAGE. A band with an approximate molecular weight of 25 kD was more intense in the sample of Rsp3-HA incubated with apoplastic fluid of SG200-infected plants than in the sample of mock inoculated plants (Supplementary Fig. 7a). By mass spectrometry analysis from two independent experiments, the bands were shown to contain maize proteins, GRMZM2G043878 and GRMZM2G334181, while these proteins also when added externally. To test this, we purified Rsp3-HA from the supernatant fraction of SG200Δrsp3-rsp3-HA. By mass spectrometry analysis from two independent experiments, the bands were shown to contain maize proteins, GRMZM2G043878 and GRMZM2G334181, while these proteins also when added externally. To test whether Rsp3 binds to the specific cell wall components of *U. maydis*, we tested binding to *Colletotrichum graminicola* (CmG2) conidia and budding cells of *S. cerevisiae* AH109. Binding of added Rsp3-HA could not be detected (Supplementary Fig. 6a, b). This suggests that Rsp3 might attach to cell wall components, which are *U. maydis* specific, and efficient cell wall association may require that the protein is synthesized by the fungus. Alternatively, cell wall components required for the binding of Rsp3 may not be present or accessible in conidia of *C. graminicola* or budding cells of *S. cerevisiae*. In fungal pathogens, infection-related development and plant colonization have been shown to involve coordinated changes in cell wall composition to escape recognition by the immune system and such alterations might be needed to allow binding of Rsp3.

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Maize AFP1 protein binds mannose. BLASTP searches revealed that maize AFP1 and AFP2 proteins have sequence similarity to Gn2, a secreted antifungal mannose binding protein from *Ginkgo biloba* seeds containing a DUF26 domain (C-X₈-C-X₂-C motif) (stress-antifungal domain PF01657; http://pfam.sanger.ac.uk/family/PF01657) (Fig. 5a). Maize B73 contains 45 DUF26 family members, which form 14 classes based on their predicted domain architecture (Supplementary Fig. 8a). Ten of the DUF26 family genes are predicted to encode secreted proteins. AFP1 and AFP2 proteins belong to class 5 whose members contain a signal peptide and two DUF26 domains but lack other domains (Supplementary Fig. 8a). By qRT-PCR analysis, AFP1 was shown to be highly induced during infection by *U. maydis* with induction being highest after 12 h and then returning to a lower level (Supplementary Fig. 9a). On the basis of a time-resolved RNAseq data set of *U. maydis* infected plants, AFP1 was the most highly induced DUF26 gene of maize followed by AFP2 (Supplementary Fig. 9b, c). Of the eight DUF26 genes predicted to encode secreted proteins (beside AFP1 and AFP2), GRMZM2G365282 was not expressed under our infection conditions and the remaining seven genes were either not induced or induced weakly (Supplementary Fig. 9d).

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Rsp3 blocks antifungal activity of maize AFP1. As Rsp3 is bound to the surface of fungal hyphae and interacts with maize
AFP proteins, we speculated that Rsp3 might act as a shield for fungal hyphae and protect them against a potential antifungal activity of AFP1. To test whether AFP1 has antifungal activity, we monitored the survival of SG200Δrsp3 after incubation with AFP1-His and AFP1**-His. A cell suspension (OD<sub>600</sub> = 0.001) was incubated with AFP1 proteins for 3h and plated on PD-

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**Fig. 5** Rsp3 interacts with maize secreted AFP1 protein. **a** Amino acid sequence alignment of maize AFP1, APF2, and G. biloba Gnk2. The signal peptide is underlined with a blue line. Conserved amino acids are highlighted in black. The DUF26 domains (C-X<sub>8</sub>-C-X<sub>2</sub>-C) are indicated by red dashed boxes. The red and green arrows indicate residues putatively involved in mannose binding in the N- or C-terminal domains of AFP1 and APF2, respectively. **b** Secreted Rsp3 interacts with purified AFP1. Leaf tissues of *N. benthamiana* infiltrated with *A. tumefaciens* carrying either a AFP1-His expressing plasmid or an empty vector (EV) as negative control were subjected to NTA-affinity purification. Prior to protein elution the NTA-agarose beads were mixed with culture supernatants of SG200Δrsp3 expressing the indicated Rsp3-HA variants under control of the otek promoter. The input and bound proteins were detected by western blot using anti-His-HRP and anti-HA antibodies. The asterisk (*) indicates a truncated form of the Rsp3<sub>Δ412-869</sub>-HA. **c** Secreted Rsp3<sub>Um-Sr</sub> hybrid protein interacts with purified AFP1. Culture supernatants of SG200Δrsp3 expressing the indicated Rsp3-HA variants were incubated with AFP1-His as described in **b** and interaction was shown by western blot as in **b**. The experiments in **b** and **c** were repeated three times and one representative experiment is shown. Full blots are shown in Supplementary Fig. 12.
plates. SG200Δrsp3 showed lower survival after treatment with AFP1-His than with AFP1**-His (Fig. 6b). A graphical presentation of results from three biological repeats revealed a significant difference in titer between the rsp3 mutant treated with AFP1-His and AFP1**-His (Fig. 6c). This suggests that AFP1 has antifungal activity and mannose binding is required for this activity. To elucidate whether Rsp3 interferes with the presumed antifungal activity of AFP1, we compared the survival of SG200Δrsp3 and SG200Δrsp3-P_{ef}Δ-rsp3-HA, a strain constitutively expressing Rsp3-HA and expressing the protein on its surface (Supplementary Fig. 4c). When incubated with AFP1, we observed a significantly higher plating efficiency of the strain constitutively expressing Rsp3-HA compared to the rsp3 mutant (Fig. 6b, c), suggesting that Rsp3 can protect hyphae against the antifungal activity of maize AFP1. We further tested the presumed antifungal activity of AFP1 by staining the treated cells with the cell death stain SYTOX Orange33. In SG200Δrsp3, the percentage of dead cells was about 83% in presence of AFP1-His versus 19% in the presence of AFP1**-His (Fig. 6d, e), confirming the antifungal activity of AFP1.

Silencing of maize AFP1 and AFP2 genes enhances virulence. Having shown that Rsp3 protects hyphae against AFP1, we investigated the biological relevance of this protective effect. To this end, we employed the Foxtail mosaic virus (FoMV) system34 to downregulate the expression of maize AFP1 and AFP2 genes simultaneously. In two independent experiments, plants silenced for both AFP1 and AFP2 were significantly more susceptible to SG200Δrsp3 infection than plants that had received the empty vector (Fig. 7a). qRT-PCR analysis of individual plants revealed that silencing of AFP1 had occurred in the majority of FoMV-infected plants that had received the silencing constructs (Supplementary Fig. 10a). However, expression of AFP2 was highly upregulated in some of the FoMV-infected plants carrying the silencing constructs (Supplementary Fig. 10b), uncovering some

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kind of crosstalk between AFP1 and AFP2 genes. This might explain why rescue of the virulence phenotype of the rsp3 mutant was only partial. Plants silenced for AFP1 and AFP2 were also significantly more susceptible to U. maydis SG200 (Fig. 7b) and C. graminicola (Fig. 7c, d), than plants that had received the empty vector illustrating that AFP1 and AFP2 target several plant pathogenic fungi.

Discussion

In this work, we demonstrate that the repetitive secreted effector Rsp3 of U. maydis strongly contributes to virulence. Rsp3 counteracts plant defense responses by shielding hyphae from antifungal maize proteins.

Rsp3 protein is not predicted to possess a GPI anchor but is nevertheless associated with the surface of hyphae through its N-terminal domain. A mutant Rsp3 protein with cysteine to alanine substitutions in the cysteine-rich domain of Rsp3. It is therefore likely that the cysteine-rich region contributes to structural integrity of Rsp3, which is needed for its shielding function.

Although uniform association with hyphae or budding cells was seen when the proteins were expressed by U. maydis, added purified Rsp3-HA associated primarily with actively growing regions, i.e., hyphal tips and cell poles as well as septa. This most likely indicates that cell wall components bound by Rsp3 are accessible during growth or when septation occurs but may be otherwise shielded by other surface-associated proteins like repellents and hydrophobins. We speculate that when Rsp3-HA is expressed by U. maydis, the protein could have a better chance to become incorporated in the cell wall already during secretion leading to the more uniform distribution. The fact that we did not detect binding to cells of C. graminicola S. cerevisiae could either suggest that these fungi lack the substrate bound by Rsp3. Alternatively, the substrate may not be accessible. Future studies need to identify the substrate of Rsp3 and elucidate the mechanism of cell wall attachment.

Maize plants respond to U. maydis infection by upregulating the expression of a large set of defense-associated genes. We now show that this set includes the genes for the two DUF26-domain proteins AFP1 and AFP2, which are predicted to be secreted and detected in apoplastic fluid of infected tissue. For both proteins, we were able to demonstrate that they interact with Rsp3. In the subsequent more detailed analysis of AFP1, we have shown that AFP1 is mannose binding and displays antifungal activity that requires its mannose-binding property. This is similar to the G. biloba protein Gnk2, which has a single DUF26 domain and has antifungal activity depending on mannose-binding. Whether ginkgo trees use the antifungal activity of Gnk2 to defend themselves against fungal attackers is unknown. However, the finding that Gnk2 is expressed in seeds might point to such a function. Genes encoding secreted DUF26 domain-containing proteins were also upregulated upon M. oryzae
infection in rice\textsuperscript{38}. Our finding that AFP1 and AFP2 are strongly induced when \textit{U. maydis} grows on the leaf surface of maize, could suggest that they are PAMP induced. As mannosne binding and antifungal activity appear coupled, we consider that AFP1-binding to mannoside in the fungal cell wall might affect the integrity of the fungal cell wall and thus lead to cell death. Alternatively, AFP1 and AFP2 might bind to mannosylated proteins, which are important for virulence. It has been demonstrated that the O-mannosylation pathway of \textit{U. maydis} has a critical role in virulence and mutants lacking the mannosyltransferase Pmt4 are defective in early infection-related development\textsuperscript{39}. In addition, to explain the requirement of Pmt4 also at later stages of biotrophic development, it has been hypothesized that mannosylated effectors required at a later stage of the infection, might exist\textsuperscript{40}. The mechanism of AFP activity is unknown and will require the identification of binding partners in the fungal cell wall. At present, we do not know which properties allow Rsp3 to interact with AFP1 and AFP2 and speculate that electrostatic interactions might be involved. Rsp3 has an isoelectric point of 3.8 while AFP1 has an isoelectric points of 8.0. Length of the repetitive domain and charge of Rsp3 might thus correlate with the amount of trapped AFP. The highly divergent C-terminal domains of UmRsp3 and SrRsp3 both contain several copies of the sequence motifs PG[T/Q][P/N]G, P/N[K/GD][D/N][S/D][E/A], [G/A][D/P][E/P/S][V/I][PYG/D/A], and G/[D/K][K/P][D/S/N][S/G][D/K/N]. As \textit{S. reilianum} rsp3 can complement the rsp3 mutant of \textit{U. maydis}, these motifs could constitute specific binding sites for AFP proteins. However, it is also apparent that UmRsp3 and SrRsp3 have evolved differently despite the fact that \textit{U. maydis} and \textit{S. reilianum} parazitize the same host. Repeat structures are extremely unstable and are prone to repeat polymorphisms\textsuperscript{41}, which we also observe in the natural \textit{U. maydis} rsp3 alleles. In addition, point mutations that may have occurred in an individual repeats could have spread throughout the repetitive C terminus via gene conversion and/or recombination events leading to scrambling and re-assembly of certain repeats. The fact that \textit{U. maydis} and \textit{S. reilianum} do not productively cross, may have led to fixation of the distinctively different repeat arrangements in the two species that allows the detection of only four degenerate conserved motifs, which are interspersed with motifs unique to each species (Fig. 1a). A closer look at the all unique motifs shows that short submotifs like GDA, QQP, and PYG (Fig. 1a) are actually also found in the longer degenerate motifs detected in both smut species. It will be a future task to define the actual AFP1 and AFP2 binding sites in the C-terminal domain of Rsp3.

Rsp3\textsubscript{4,12-869}, lacking the most C-terminal repetitive domain but retaining the domain consisting of the degenerate [D/N]NN and PG[T/Q][P/N]G repeats present in \textit{U. maydis} and \textit{S. reilianum} interacts poorly with AFP1 in vitro but partially complements the virulence phenotype of the rsp3 mutant. This truncated protein still binds to fungal hyphae. To explain the partial biological activity of this truncated protein, we speculate that the attachment of this protein to the hyphal surface might hinder access of AFP1 to mannoside residues in the cell wall and provide some degree of protection that is independent from the proposed specific interaction with the C-terminal domain. The finding of strong length variations in natural rsp3 alleles could reflect evolutionary pressure to adjust to specific maize varieties that can all be colonized by \textit{U. maydis} but which may differ with respect to AFP proteins and how much they express. In this context, it will be interesting to analyze whether \textit{U. maydis} strains harboring long and short rsp3 alleles vary in virulence on different races of maize or teosinte, the only other host of \textit{U. maydis}.

It is also possible that Rsp3 has additional functions and might bind to extracellular domains of membrane-bound DUF26 members known as cysteine-rich RLKs (CRKs), which have a role in plant defense responses\textsuperscript{42-44}. Rsp3 could specifically recognize structural features of DUF26 proteins also present in the extracellular domains of such CRKs and via this block downstream defense signaling. This might additionally contribute to a successful colonization. On the basis of the results obtained in this study, our hypothesis on how the Rsp3-AFP interaction could affect \textit{U. maydis} virulence is depicted in a schematic model (Fig. 8).

By transient silencing of AFP1 and AFP2, the virulence phenotype of the rsp3 mutant is partially rescued. This provides a direct link between Rsp3 and AFP. As we were unable to restore virulence fully in the silenced lines, this could indicate that Rsp3 has additional functions. Alternatively, the outcome may be influenced by the crosstalk between AFP1 and AFP2, i.e., the observation that AFP2 was upregulated in some plants, which should have been silenced for AFP1 as well as AFP2. At this point, we cannot exclude that other DUF26 encoding proteins with antifungal activity may be upregulated in the silenced plants, and this could prevent full rescue of the rps3 virulence phenotype.

The finding that AFP1 and AFP2 silenced plants show stronger disease symptoms also of wild type \textit{U. maydis} strains suggests that Rsp3 provides only partial protection against these antifungal maize proteins. Plants silenced for AFP1 and AFP2 also become more susceptible to the anthracnose fungus \textit{C. graminicola} suggesting that the antifungal activity of AFP1 (and likely of AFP2 and other secreted DUF26-domain proteins) is directed against several fungal pathogens of maize.

In future, it will be important to investigate whether proteins like Rsp3 that provide protection from antifungal proteins...
targeting mannose also exist in other fungi. Protection of fungal hyphae from plant defense responses has so far been ascribed mainly to LysM domain proteins, which bind chitin and protect hyphae from plant chitinases. Plant pathogenic fungi can possess several LysM domain effectors, which can reside either in the apoplast or be associated with fungal hyphae. We could envisage that fungi have developed a similarly broad defense spectrum against mannose-binding antifungal proteins. So far, Rsp3-related effectors have been detected only in smut fungi. The finding that C. graminicola becomes more virulent on maize plants silenced for AFF1 and AFF2 but lacks an effector related to Rsp3 could suggest that this fungus has developed another strategy to provide at least partial protection against the antifungal activity of these maize proteins. In the long run, it also should be interesting to investigate whether the upregulation of DUF26-domain-containing plant antifungal proteins could provide a general strategy for increasing resistance against fungal pathogens. As secreted DUF26-containing proteins are found in monocot as well as dicot plants, successful fungi might all have evolved molecules to counteract this type of defense.

**Methods**

**Strains and growth conditions.** The *Escherichia coli* DH5α (Bethesda Research Laboratories) and Top10 (Invitrogen) were used for the cloning purposes. The haploid *U. maydis* strains FB1, FB2, as well as the haploid solopathogenic strains SG200 and SG200An1 have been described.12,23 All *U. maydis* strains used in this study are listed in Supplementary Table 3. *U. maydis* strains were grown at 28 °C in liquid complete medium (CM) with 1% glucose (Holliday, 1974), liquid YEPSL (0.4% yeast extract, 0.4% peptone, 2% sucrose), or on solid potato dextrose medium (M1.001)46 was maintained at 25 °C on oatmeal agar [Difco] plates with continuous exposure to daylight.

**Genomic DNA preparation and U. maydis transformation.** Standard molecular techniques for cloning and heterologous gene expression followed described protocols.14 Transformation of *U. maydis* and genomic DNA isolation were performed as described previously.6–9,50

**Gene expression analysis.** To analyze rrp3 expression by qRT-PCR, 6-day-old maize plants of the variety Early Golden Bantam (Urban farmer Seeds Inc., Westfield, IN, USA) were syringe inoculated with a mixture of *U. maydis* strains FB1xFB2 or *H. maydis* strains. For early time points where no disease symptoms were visible, leaf areas from the third leaf extending from the injection holes to 1 cm below were collected. For each sample, the material was ground to a fine powder and frozen in liquid nitrogen using a Retch CryoMill (Retsch GmbH, Haan, Germany) with a 50 ml grinding beaker and a 20 mm grinding ball. The machine was precooled for 30 s followed by 60 s of grinding at 20 Hz. Approximately 500 mg of powder were resuspended in 1 ml TRizol reagent (Life technologies) and total RNA extracted according to the manufacturers recommendation. The total RNA was DNAse-treated and cDNA was prepared using TURBO DNA-free kit (Invitrogen Cat#AM1907) and SuperScript® III First-Strand Synthesis SuperMix (Invitrogen Cat#1808040) respectively. The first-strand cDNA was used for real-time PCR analysis as described51 using primer pairs for indicated genes listed in Supplementary Table 2. The expression of constitutively expressed *U. maydis* peptidyl-prolyl isomerase (ppi) was used for normalization. Details of the RNAseq analysis have been described elsewhere52 and all data relevant for this analysis were extracted from this data set available at NCBI Gene Expression Omnibus under accession number GSE103876. The qRT-PCR of AFPI and AFPP2 after infection analysis was performed with the same RNA samples previously used by Lanver et al.23 and GAPDH of maize was used for gene normalization.

**Plasmid and strain constructions.** PCR reactions were performed using the Phusion® High-Fidelity DNA Polymerase (New England Biolabs). Point mutations were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Restriction enzymes were purchased from New England Biolabs. Gene replacements and integrations into the *ip locus54* were verified by Southern blot analysis. All fragments amplified by PCR were sequenced in the final plasmids. All primer sequences used are listed in Supplementary Table 2. To generate Δrrp3 mutants, the 1.0 kb fragments from upstream and downstream of *rrp3* genes were amplified by PCR using primer pairs Rps3 #1/2 and Rps3 #3/4 using FB1 genomic DNA as template. The SfiI-digested PCR fragments were ligated with a 1.9 kb SfiI-digested fragment containing a hygromycin resistance cassette isolated from pBluescript II. The linearized pBluescript II was PCR amplified using primer pair Rps3 #37 and cloned into pCR2.1-TOPOP Vector (Fermentas). Plasmid pCR2-rsp3KO. PCR-generated linear DNA of the insert was used for *U. maydis* transformation to yield strains SG200Δrrp3 and SG200An1rrp3. For the complementation of Δrrp3 mutants, linearized plasmids were integrated into the *ip locus of SG200Δrrp3 and SG200An1Δrrp3. Plasmid pMsp3 was generated by inserting a PCR fragment containing about 1.5 kb native rrp3 promoter region and the entire open reading frame (ORF) amplified by primer pair Rps3 #5/6 from FB1 DNA into Ndel and NotI sites of plasmid p12354. Plasmid pofet-rps3ΔHA was created in two steps. Afhl/NotI digested fragment from prrp3 plasmid containing ORF was ligated into Small/NotI sites of p123 and filled and ligated with Rps3ΔHA using BamH1 and NotI. The PCR product was then ligated into pCR2.1-TOPOP Vector (Fermentas). To produce an transformant, the resulting plasmid was transformed into *E. coli* DH5α to verify pofet-rps3ΔHA. The pofet-rps3-DHA was digested with Ndel/SfiI to remove the toef promoter and replaced with a Ndel/SfiI fragment containing the rps3 promoter excised from ppr3 to yield pofet-rps3ΔHA. To generate pHA-rps3, two PCR fragments were amplified using primer pairs Rps3 #7/8 and Rps3 #9/10 and inserted into HindIII/SfiI sites of ppr3 using Gibson assembly strategy. Plasmid pMsp3Δ(ACys) was generated via the same strategy using primer pairs Rps3 #11/12 and Rps3 #13/14 and inserted into HindIII/NeoMIV sites of ppr3. Plasmid pMsp3Δ(T6) was created by amplifying of the ORF of T6 rps3 from Toloca-6 genomic DNA with primer pairs Rps3 #37/16 and T6rps3ΔHA. The PCR product was digested with BamH1/NotI and replaced the rps3(FB1) ORF in p123. To generate plasmid pMsp3Δ(T6)hc, point mutations in cysteine residues were introduced using the QuikChange Lightning Site-Directed Mutagenesis Kit with primers Rps3 #17–25 and pMsp3(T6) as template. Plasmid pofet-rps3ΔHA was digested with T4 DNA polymerase and the linearized plasmid was transformed into *U. maydis* FB1ΔN10rps3Δ630, 0.5% sodium chloride, 1% glucose, 100 mM DTT, pH 6.8. Glass beads were then added to the samples prior to cell disruption using FastPrep®-24 homogenizer (MP Biochemicals). Proteins from supernatants were separated using 12% SDS-PAGE (Bio-Rad). Blots were incubated with primary antibodies, and horseradish peroxidase-conjugated secondary antibodies were used. NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-04149-0 | www.nature.com/naturecommunications

**Protein secretion assay.** Total protein fractions from cell pellets and culture supernatants of *U. maydis* were prepared as described previously. Briefly, *U. maydis* cells were grown in CM medium to an OD600 of 0.6. Cell cultures were concentrated and adjusted to an OD600 of 20 with 1x sample buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 100 mM DTT, 0.01% bromophenol blue, pH 6.8). Glass beads were then added to the samples prior to cell disruption using FastPrep®-24 homogenizer (MP Biochemicals). Proteins from supernatants were separated using 12% SDS-PAGE (Bio-Rad). Blots were incubated with primary antibodies, and horseradish peroxidase-conjugated secondary antibodies were used. NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-04149-0 | www.nature.com/naturecommunications

**The cleavage site of Rps3 by N-terminal protein sequencing.** SG200Δrrp3- 
Purified Rps3 cells were grown in CM liquid medium to an OD600 of 0.6, the supernatant fraction was collected and TCA-precipitated overnight at 4 °C. The TCA-precipitated pellet was acetate washed twice and dissolved in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, pH 8.0, 0.1% SDS, 0.1% sodium dodecyl sulfate, 0.5% β-mercaptoethanol) and an equal volume of acetate-precipitated sample was used as a control. The pellet Rps3-ΔHA on the beads was boiled in

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The in vitro induction of filamentous and appressoria of *U. maydis* was performed as described by using strains that expressed an appressorium marker (AMI1) fused to GFP. To determine appressorium formation and penetration efficiency of hyphae on the leaf surface, we established protocols as described. Briefly, infected maize leaves were harvested 18 h after inoculation with a strain expressing the appressorium marker and stained with Calcofluor white. To quantify appressoria, the percentage of filaments showing eGFP fluorescence was determined relative to filaments stained with Calcofluor white. To determine penetration efficiency of hyphae, the number of appressoria that have penetrated the epidermis was determined by visualizing eGFP fluorescence of intracellular hyphae relative to the total number of appressoria showing eGFP fluorescence.

The parafiltration buffer (50 mM Tris-HCl, 50 mM CaCl₂, and 150 mM NaCl, pH 7.5) was used to isolate the apoplastic fluid. Overall, 5 ml of apoplastic fluid was mixed with 1 ml of binding buffer (10 mM Tris-HCl, 20 mM CaCl₂, 1 M NaCl, and 0.05% NP-40, pH 7.5). The beads were then incubated with the supernatant collected from *SG200*Δm3-derived strains constitutively expressing secreted Rsp3-HA variants for 4 h at 4 °C. The beads were washed with binding buffer and eluted proteins were removed by boiling in sample buffer and subjected to western blotting analysis using anti-His-HP (1:8000 dilution, Qiagen Cat#34460) and mouse monoclonal anti-HA antibody (1:10000 dilution; Sigma Cat#H9658). Herosardial peroxidase-conjugated anti-mouse or IgG (1:10000 dilution; Sigma Cat#H9658) was then used as secondary antibody.

**Protein purification**. AFP1 protein was overexpressed in *N. benthamiana* using the TMV-based viral vector system as described and kindly provided by NOMAD. For the generation of plasmid pCH-AFP1-His in which AFP1-His is expressed including its own signal peptide, one PCR fragment was amplified using primer pairs AFP1 #1/2 with cDNA template from *SG200*-infected plants harvested 3 dpi and the other two fragments were amplified from the 3’ provector pCH31070 as template, using primer pairs AFP1 #3/4 and AFP1 #5/6, respectively. The three fragments were combined and inserted into the Pmiel/Pro3OMI sites of pCH31070 using Gibson assembly strategy. Same strategy was used to create pCH-APF2-His using primer pairs APF2#1/2, APF1#3/ APF2#3, and APF2#4/ APF1#6. To generate pCH-APF1*-His, three amino acid substitutions in the N-terminal mannose binding residues were introduced using QuikChange Site-Directed Mutagenesis Kit and primers APF1#7-9 and pCH-APF1-His as template. Plasmid pCH-APF1*-His was generated with the same strategy using primers APF1#10-12 and pCH-APF1*-His as template. The plasmids and the empty vector 5’ provector (pCH21011) were delivered by Agrobacterium tumefaciens strain GV3101 into *N. benthamiana* as described. Leaf samples harvested at 4 dpi were immersed in the binding buffer and AFP1-His was added to 30 µl mannose-agarose beads (Sigma Cat#M6400) in 1× sample buffer and separated by SDS-PAGE. Following blotting to a PVDF membrane and staining with coomassie brilliant blue R-250, the 150 kD band corresponding to Rsp3-HA was excised from the membrane and sent for N-terminal protein sequencing by Edman degradation (TopLab, Munich, Germany).

**Mannose-binding assay**. One µg of AFP1-His wild type or mutant versions or 2 µg of Cm11-His was added to 30 µl mannose-agarose beads (Sigma Cat#M6400) in 1 ml of binding buffer (10 mM Tris-HCl, 20 mM CaCl₂, 1 M NaCl, and 0.05% Tween-20, pH 7.5). After 1 h incubation at 4 °C on a rotating wheel, the beads were washed twice with binding buffer. Beads with bound proteins were boiled and subjected to western blotting analysis using antibodies conjugated to horseradish peroxidase (HRP) (1:8000 dilution, Qiagen Cat#34460).

**Growth inhibition assay**. *U. maydis* strains were grown to OD₅₅₀ = 0.8 and diluted to OD₅₅₀ = 0.001 with 10 mM Tris-HCl (pH 7.0). The AFP proteins were added to the cells to a final concentration of 1 µg/ml in a 50 µl final volume. The cells were incubated at 28 °C for 3 h before spotted on a PD agar plate. The plate was incubated on 28 °C for 1–2 days. To directly visualize cell death, AFP1-treated cells were stained with 0.1 µM SYTOX orange dye (Molecular Probes Cat#11368) for 5–10 min before visualization with epifluorescence DIC microscopy.

**Identification of Rsp3-interacting proteins**. *U. maydis* SG200Δm3-p*Δrsp3*-HA was grown in 10 ml of CM liquid medium to an OD₅₅₀ of 0.6. The supernatant fraction was collected, concentrated and buffer exchanged with 25 mM Tris-HCl (pH 7.5) using 30 kD cutoff centrifugal filters (Millipore). The supernatant was applied to NTA-matrix (Sigma Cat#1815016001) and washed twice with a binding buffer (25 mM Tris-HCl, 0.3 M NaCl, and 0.1% NP-40, pH 7.5). To collect apoplastic fluid from infected maize leaves, the leaves harvested at 3 dpi were immersed in ice-cold buffer (0.2 M CaCl₂ and 5 mM NaAcetate, pH 4.3) for 1 h before vacuum-centrifugation method was applied as described. The apoplastic fluid was loaded to the anti-HA affinity matrix-containing Rsp3-HA. After overnight incubation at 4 °C, the bound proteins to Rsp3-HA on beads were boiled and subjected to SDS-PAGE and silver stained using SilverQuest Silver Staining Kit (Invitrogen Cat#LC6707). The band of interest was excised and followed by protein extraction and digestion. The peptides were analyzed using MALDI-TOF/TOF (4800 ABI-SCIEX) for analysis.
Binding of purified Rsp3-HA to fungal surfaces. The supernatant fraction of SC200Rsp3-HA, Rsp3-HA cells grown in 100 ml CM liquid medium to an OD600 of 0.6, was collected, 100 fold concentrated and buffered exchanged with PBS (pH 7.4) using 30 kD cutoff centrifugal filters (Millipore). The supernatant was applied to anti-HA affinity matrix (Sigma Cat#B181510601). beads were washed three times with PBS buffer, and Rsp3-HA was eluted with 0.1 M glycine (pH12.5). The eluate was neutralized with 1 M Tris-CI (pH 8), concentrated and buffered exchanged with PBS buffer using 3 kD cutoff centrifugal filters. The purified Rsp3-HA was added to U. maydis SG200 (OD600 = 0.1) budding cells or SG200 hyphae induced to filament by spraying on paraformaldehyde, G. craminicola CamG2 (10'-conidial/ml), or S. cerervisiae AH109 (OD600 = 0.1) cells in a final concentration of 0.01 µg/µl. After overnight incubation at 4 °C, cells were harvested and washed with PBS buffer twice before performing the immunolocation.

Microscopy. A Zeiss Axiosplan II microscope with differential interference contrast optics was used for microscopy. The infected plant samples were treated as described. The samples were stained with WGA-AF488 (Molecular Probes, Karlsruhe, Germany) and Propidium Iodide (Sigma) to visualize fungal hyphae and plant cell wall, respectively. Confocal microscopy was performed using a TCS-SP5 confocal microscope (Leica Microsystems). Propidium iodide fluorescence was excited at 561 nm and detected at 580–630 nm. WGA-AF488 was excited at 488 nm and subsequent detected at 500–540 nm. mCherry was excitated at 561 nm and detected at 580–630 nm. Images were processed using LAS-AF software (Leica Microsystems).

Bioinformatic analyses. Signal peptide prediction was performed with the program SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP)29. Sequence alignments were generated using Clustal Omega (http://www.ebi.ac.uk/tools/msa/clustalo)30. Domain analyses were performed with Pfam (http://pfam.sanger.ac.uk/)31. Protein modeling was performed with Swiss-Model (https://swissmodel.expasy.org/interactive)32 and images were superimposed using PyMOL (https://www.pymol.org/).

Data availability. The RNAseq data were previously reported, and are available at NCBI Gene Expression Omnibus under accession number GSE103876. The data generated for this study are available in this article and the Supplementary Information Files. Other data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

L.-S.M. conceived and performed experiments. L.-S.M. and R.K. wrote the manuscript. A.M.-M., S.J., L.W., M.M., and C.T. generated mutants, identified length polymorphisms, and performed initial phenotypic analyses. A.C. did the immunostaining in planta. J.K. did the mass spectrometry analysis. B.Z. performed transmission electron microscopy. G.B. assisted in protein modeling analysis. S.R. assisted in confocal microscopy analysis. All authors discussed the results and commented on the manuscript.

Additional information

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