Nuclear status and leaf tumor formation in the *Ustilago maydis*–maize pathosystem

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**Summary**

- *Ustilago maydis* is a biotrophic fungus causing smut disease in corn. The infectious forms are dikaryotic hyphae. Here we analyze mutants lacking the *nlt1* transcription factor and investigate why these mutants are unable to induce leaf tumors.
- The study involved reverse genetics, complementation, epistasis analysis, microscopy, gene expression analysis by quantitative reverse transcriptase PCR and virulence assays.
- We show that *nlt1* mutants colonize maize leaves efficiently but fail to undergo karyogamy and are attenuated in late proliferation. Nlt1 activates transcription of *rost1*, a transcription factor controlling karyogamy, and represses *see1*, an effector previously shown to contribute to leaf tumor induction. In mononucleate solopathogenic strains, *nlt1* mutants cause attenuated leaf tumor formation. In actively dividing maize organs, *nlt1* mutants undergo karyogamy and induce tumor formation. *Sporisorium reilianum*, a smut fungus unable to induce leaf tumors, possesses an ortholog of *nlt1* that controls the fusion of dikaryotic nuclei late in infection during cob colonization.
- Our results have established a regulatory connection between *nlt1, rost1* and *see1* and suggest the existence of two stages contributing to leaf tumor formation, one before nuclear fusion and involving *nlt1* and one after karyogamy that is *nlt1* independent.

**Introduction**

The biotrophic fungus *Ustilago maydis* is responsible for smut disease in corn. One of the striking features of this disease is the induction of plant tumors, which at the end of the infection cycle are filled with huge amounts of diploid spores. The disease is initiated by fusion of haploid budding cells that differ in their *a* and *b* mating types, an event which occurs on the leaf surface. The *a* locus controls the cell fusion step via its encoded pheromones and receptors while the *b* locus encodes a pair of homeodomain proteins (*bE* and *bW*) which function as active transcription factors when *bE* and *bW* proteins from different alleles dimerize, an event that occurs in the dikaryon (Gillissen et al., 1992). The dikaryon is able to differentiate an infection structure from which infective hyphae penetrate the leaf surface and enters epidermal cells as a filament completely encased by the host plasma membrane. The dikaryotic biotrophic hyphae rapidly traverse the epidermal layer and mesophyll tissue with only small increases in fungal biomass and accumulate and proliferate close to and within the veins. Around 4–5 d after infection leaf bundle sheath cells begin to resume cell divisions and surrounding mesophyll cells enlarge (Matei et al., 2018), eventually forming the tumor. In tumor tissue around day 5 and 6 after infection, the nuclei of the dikaryon undergo karyogamy followed by a step of massive proliferation and the formation of aggregates where cells become embedded in a mucilaginous matrix. These hyphae then swell, fragment and develop into the diploid spores which are decorated by a dark stained spore coat and are released when the tumors dry up and rupture (Banuett & Herskowitz, 1994; Banuett & Herskowitz, 1996; Tottor et al., 2016; Snetselaar & McCann, 2017).

*Ustilago maydis* can induce tumor formation in all above-ground parts of the plant. Disease can also be induced by diploid *U. maydis* strains provided that at least the *b* loci are heterozygous (Puhalla, 1968; Cummins & Day, 1977; Banuett & Herskowitz, 1989; Banuett & Herskowitz, 1994). In addition, haploid strains have been engineered which are pathogenic because they show autocrine pheromone stimulation and express an active *bE*–*bW* heterodimer (Kamper et al., 2006). Such strains, which do not need a mating partner to infect, are termed solopathogenic. The life cycle of most other smut fungi is very similar to that of *U. maydis* and in all cases they are linked to sexual development and host colonization. However, with a few exceptions these related smut fungi do not induce tumors, they colonize their hosts systemically and they differentiate diploid spores exclusively in male and female flower organs. One such example is *Sporisorium reilianum*, the corn head smut fungus.

How tumors are induced by *U. maydis* remains a mystery. To allow colonization, plant immune responses which are triggered by pathogen-associated molecular patterns such as fungal chitin need to be downregulated and the metabolism of the host needs to be redirected to support fungal growth. This is accomplished by a large set of secreted effector proteins (Kamper et al., 2006;
Lanver et al., 2017). Among these is See1, an organ-specific effector contributing to tumor formation in leaves. In parts of the leaf containing postmitotically differentiated cells, See1 is required for the reactivation of plant DNA synthesis during leaf tumor progression, but See1 is not required for tumor formation in immature tassel (Redkar et al., 2015), an actively dividing tissue (Gao et al., 2013). Ustilago maydis produces 456 predicted secreted proteins that are expressed in specific waves during coloniztion (Lanver et al., 2018). Three of the respective modules are particularly enriched in effector genes and, one of these, the cyan module, is associated with tumor formation and fungal aggregate formation (Lanver et al., 2018). For the cyan module an APSES-domain containing transcription factor Nlt1 (no leaf tumors 1) was identified on the basis of strong intramodular connectivity. nlt1 mutants are able to colonize maize but are unable to induce leaf tumors while they can induce rare tumors at the stem of infected plants. In addition, it was demonstrated that Nlt1 is a positive regulator of several effector genes (Lanver et al., 2018), which raised the possibility that among these might be genes contributing to leaf tumor formation. Another transcription factor residing in the cyan module is Ros1, which is required for karyogamy and spore formation but only attenuates tumor formation (Tollot et al., 2016).

In this study we present a detailed analysis of the nlt1 mutant. We show that dikaryotic nuclei of the nlt1 mutant do not fuse in leaf tissue but undergo fusion in infected stems, tassels and cobs and in these locations tumors develop. nlt1 mutants of solopathogenic strains that are monokaryotic can induce leaf tumor formation. With this we have uncovered a previously unrecognized connection between nuclear status and tumor formation.

Materials and Methods

Strains and growth conditions

The Escherichia coli strain DH5α (Life Technology, Darmstadt, Germany) was used for cloning purposes. All U. maydis and S. reilianum strains used in this study are listed in Supporting Information Table S1. Ustilago maydis and S. reilianum 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 on PD plates consisting of 39 g l−1 Difco™-Potato Dextrose-Agar (BD Biosciences, Heidelberg, Germany) and 0.01 M Tris-HCl, pH 8.0.

For growth and filamentation assays, U. maydis strains were grown to an absorbance at 600 nm (OD600) of 1 in YEPSL medium. Subsequently, the cells were harvested by centrifugation and diluted in H2O to a final OD600 of 1.0. Then, 10 μl of the cell suspension was spotted on PD plates to assess growth, or on PD-charcoal plates (PD-agar containing 1% activated charcoal) to assess formation of aerial hyphae (Day et al., 1971).

Plasmid and strain constructions

The plasmids used and generated for this study are listed and described in Table S2. Oligonucleotides are listed in Table S3. PCRs were performed using the Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) for gene construction, and the BioMix™ Red Taq DNA polymerase (Meridian Life Science Inc., Memphis, TN, USA) for preliminary screening. Restriction enzymes were all supplied by New England Biolabs. An established protoplast-mediated method was used for U. maydis and S. reilianum transformation (Brachmann et al., 2004). For the generation of U. maydis and S. reilianum deletion strains, a PCR-based method (Brachmann et al., 2004; Kamper, 2004) was used. Detailed procedures for the construction of plasmids and mutant strains are described in Methods S1.

Virulence assays

Detailed procedures for conducting virulence assays are described in Methods S2.

Fungal biomass determination

Quantification of relative fungal biomass in infected maize leaves was performed as described previously, with some modifications (Tollot et al., 2016; Lanver et al., 2018). Details of the experimental procedures are described in Methods S3.

qRT-PCR analysis

Gene expression analysis from infected plant material was performed as described previously, with some modifications (Brefort et al., 2014; Tanaka et al., 2014). Infected plant tissues were ground to a fine powder in liquid nitrogen using a Retsch CryoMill (Retsch GmbH, Haan, Germany) in a 50 ml grinding beaker and a 20 mm grinding ball, and total RNA was extracted with TRIzol (Thermo Fisher Scientific, Dreieich, Germany). DNA contaminations were removed with the Turbo DNA-free Kit (Thermo Fisher Scientific). The Super III First Strand Synthesis Super Mix Kit (Thermo Fisher Scientific) was used to reverse-transcribe 1–2 μg of total RNA with oligo (dT) primers. Quantitative PCR (qPCR) analysis was performed using the Platinum SYBR Green Supermix (Thermo Fisher Scientific) and an iCycler (Bio-Rad, Munich, Germany). Experiments were done in three biological and three technical replicates and gene expression levels were calculated relative to the expression levels of the constitutively expressed fungal ppi (UMAG_03726) gene. Student’s t-test was used to assess statistically relevant differences among strains, and the P value was denoted as *P<0.05. Primers used for quantitative reverse transcription PCR (qRT-PCR) are listed in Table S3.

Spore germination assay

Ustilago maydis and S. reilianum spores were collected and germinated according to published procedures (Schirawski et al., 2005; Nadal et al., 2016). Details of the experiments are described in Methods S4.
DAPI staining and epifluorescence microscopy

To observe the nuclear status of hyphae in *U. maydis*- or *S. reilianum*-infected plant tissue, staining with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was performed as described previously (Snetselaar & Mims, 1994) with some modifications. To remove hyphae from the leaf surface, leaves were treated with liquid latex (XUR®, Berlin, Germany) and air dried for 45 min. The dry latex layer was peeled off before DAPI staining. For DAPI stained, infected tissues were cut into small pieces and directly put on a microscope slide, adding DAPI solution (20 μg ml⁻¹ DAPI in 50% 0.2 M phosphate buffer (pH 7.4), 50% glycerol and 1 mg ml⁻¹ p-phenylene-diamine (Sigma-Aldrich)), then covered with a coverslip and placed in a 60°C oven for at least 40 min to aid penetration of the DAPI solution into fungal hyphae. Epifluorescence microscopy was performed with a Zeiss Axioplan 2 imaging microscope (Zeiss) equipped with a CoolSNAP-HQ charge-coupled device camera (Photometrics, Tucson, AZ, USA) and controlled by the imaging software MetaMorph (Universal Imaging, Downingtown, PA, USA). A standard DAPI filter was used to detect the DAPI signal for nuclei observation by epifluorescence microscopy.

Confocal microscopy

The proliferation of *U. maydis* in infected maize leaf tissue was visualized by confocal microscopy as previously described (Tanaka *et al.*, 2018), and detailed in Methods S5.

cDNA analysis of nlt1

To validate the gene models for *nlt1* in *U. maydis* and *S. reilianum*, infected plant material was collected after infection with *U. maydis* FB1 × FB2 and *S. reilianum* JS161 at 8 dpi. The material was ground to a fine powder using a mortar and pestle under liquid nitrogen. The ground material was used for total RNA extraction with TRIzol reagent (Thermo Fisher Scientific). DNA contaminations were removed with the Turbo DNA-free Kit (Thermo Fisher Scientific). The Super III First Strand Synthesis Super Mix Kit (Thermo Fisher Scientific) was used to reverse transcribe 3 μg of total RNA with oligo (dT) primers. The complete *nlt1* coding region of *U. maydis* was amplified using primers oAN76/oS43. To obtain the complete *S. reilianum* nlt1 cDNA sequence, the 5’ end of the gene was amplified with primers oPH261/oPH257. To obtain a 3’ cDNA fragment the 3’ end was subjected to a 3’RACE experiment (3’RACE Kit: Thermo Fisher Scientific) and then subjected to a PCR using primers oPH265/AUAP. The products were sequenced and assembled against the full-length cDNA. The amino acid sequences translated from cDNA sequences of the *U. maydis* and *S. reilianum nlt1* genes are aligned in Fig. S2.

Accession numbers

The *nlt1* genes and encoding protein sequences from *U. maydis* and *S. reilianum* are available at NCBI under the following accession numbers: *U. maydis* ppi (UMAG_03726), XM_011391885.1; *U. maydis actin* gene (UMAG_11232), XM_011394329.1; *U. maydis nlt1* (UMAG_04778), MT905395; *S. reilianum Snlt1* (SrI15650), MT905396; maize glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, gene number: GRMZM2G046804), NP_001105413.1.

Results

Detailed characterization of the phenotype of *nlt1* mutants

The *nlt1* gene encodes an APSES-type transcription factor, and frame-shift mutations in FB1 × FB2 strains caused a strong reduction of virulence and abolished leaf tumor formation (Lanver *et al.*, 2018). We have now generated gene deletion mutants of *nlt1* in FB1 and FB2 for further characterization. The virulence phenotype and inability to cause leaf tumors of FB1Anlt1 and FB2Anlt1 strains were comparable to strains carrying frame-shift mutations in *nlt1*, and could be fully complemented (Fig. S1). In addition, by sequencing cDNA, we have revised the gene model for *nlt1* (Fig. S2). To answer the question of why *nlt1* mutants cannot induce tumors in leaves while mutants are able to do so in the stem of infected maize plants, we characterized at which stage of development the *nlt1* mutants are blocked. To this end, we performed wheatgerm agglutinin-Alexa Fluor 488 (WGA-AF488) staining of leaf tissue infected with a mixture of FB1 × FB2 wild-type strains as well as with FB1Anlt1 × FB2Anlt1 mutants. A time course experiment revealed similar colonization levels between wild-type and mutants up to day 4. However, the mutant failed to produce the large fungal aggregates which are typically found in wild-type infections between days 6 and 8. Furthermore, the *nlt1* mutants were unable to produce spores in leaves while mature spores were detected in large quantities 12 days post infection (dpi) with FB1 × FB2 (Tollot *et al.*, 2016) (Fig. 1a). An analysis of fungal biomass supported these observations and showed that initial stages of colonization up to 4 dpi were comparable between wild-type and *nlt1* mutant while no significant increase in fungal biomass was detected in *nlt1* mutant infections at later time points (Fig. 1b).

A solopathogenic haploid strain of *U. maydis* was shown to induce new cell divisions in bundle sheath cells as well as an enlargement of surrounding mesophyll cells (Matei *et al.*, 2018). To study whether the *nlt1* mutant is able to induce such alterations, we tried to detect the formation of new cell walls in infected maize leaf tissue was visualized by confocal microscopy as previously described (Tanaka *et al.*, 2018), and detailed in Methods S5. For DAPI staining, infected tissues were cut into small pieces and directly put on a microscope slide, adding DAPI solution (20 μg ml⁻¹ DAPI in 50% 0.2 M phosphate buffer (pH 7.4), 50% glycerol and 1 mg ml⁻¹ p-phenylene-diamine (Sigma-Aldrich)), then covered with a coverslip and placed in a 60°C oven for at least 40 min to aid penetration of the DAPI solution into fungal hyphae. Epifluorescence microscopy was performed with a Zeiss Axioplan 2 imaging microscope (Zeiss) equipped with a CoolSNAP-HQ charge-coupled device camera (Photometrics, Tucson, AZ, USA) and controlled by the imaging software MetaMorph (Universal Imaging, Downingtown, PA, USA). A standard DAPI filter was used to detect the DAPI signal for nuclei observation by epifluorescence microscopy.

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Fig. 1 *nlt1* mutants colonize plants efficiently, but are attenuated in massive late proliferation. (a) Investigation of plant colonization by *Ustilagomaydis* wild-type strains (FB1 × FB2) and *nlt1* mutants (FB1Δnlt1 × FB2Δnlt1) by confocal microscopy. Leaf samples of maize seedlings of the variety Early Golden Bantam infected by the indicated strains were observed at 2, 4, 8 and 12 days postinfection (dpi) by confocal microscopy. Fungal hyphae were stained with WGA-AF488 (green). Plant cell walls were stained with propidium iodide (red), and bright field is superimposed in gray. For the experiment, two or three independently infected plants were analyzed with similar results. Bars, 50 µm. (b) Fungal biomass determination. Maize seedlings of the variety Early Golden Bantam were infected with the indicated strains and samples were collected at 2, 4, 6, 8, 10 and 12 dpi. Relative fungal biomass (blue columns: FB1 × FB2; gray columns: FB1Δnlt1 × FB2Δnlt1) was determined by qPCR using plant-specific (GAPDH) and *U. maydis*-specific (*ppi*) primers. Columns give mean ratios of fungal DNA to plant DNA ($2^{-\Delta\Delta C_t}$). The experiment was performed in three biological and three technical replicates. Error bars indicate the standard deviation of three biological replicates. Asterisks denote statistically significantly differences (t-test; *, $P < 0.05$; ns, not significant).
Collectively, these results show that the nlt1 mutant is blocked after colonization, is unable to induce late fungal development and is also unable to induce the events associated with tumor formation in leaves (i.e. plant cell division and enlargement).

We had previously shown that despite their inability to induce tumors in leaves, nlt1 mutants can induce tumors containing spores in the stem of infected maize plants (Lanver et al., 2018) (Fig. 3a). As *U. maydis* is able to induce tumors on all above-ground organs of maize, we next tested whether the nlt1 mutant is able to induce tumors in tassels and cobs. Indeed, nlt1 mutants produced tumors containing dark-colored spores (Figs 3b,c, S3a,b). However, compared with FB1 × FB2 infections, fewer spikelets and fewer kernels developed tumors and these were smaller compared to those induced by FB1 × FB2 (Fig. 3b,c). The spores produced by the nlt1 mutant appeared mis-shaped and had a significantly lower ability to germinate than wild-type spores (Fig. S3b,c), suggesting that Nlt1 plays important roles during spore formation as well as spore viability. The finding that nlt1 mutants can complete the life cycle and induce tumors not only in the stem but also in other plant organs suggests that their defect in development is a leaf-specific phenomenon.

*nlt1* mutants remain dikaryotic in leaves but become diploid in stem, cob and tassel tumors

With respect to the defects in late proliferation, aggregate formation and spore formation in leaves, the nlt1 mutant is similar to mutants lacking the *ros1* transcription factor (Tollot et al., 2016). However, in contrast to *nlt1* mutants, *ros1* mutants still induce leaf tumors (Tollot et al., 2016). As *ros1* mutants fail to undergo karyogamy (Tollot et al., 2016), we next investigated whether this developmental step is affected in *nlt1* mutants. Nuclei were visualized by DAPI staining after FB1 × FB2 infection using leaf sections only showing chlorosis as well as in areas developing leaf tumors (Fig. 4a). Chlorotic leaf sections contained dikaryotic fungal hyphae at all time points (Fig. 4b). Leaf tumors as well as stem tumors induced by FB1 × FB2 became visible at 6 and 8 dpi respectively and in these structures we detected only hyphae with a single nucleus per cell compartment, indicating that nuclei had fused (Fig. 4b). Comparable to wild-type infections, *nlt1* mutants showed only dikaryotic hyphae in leaf regions below the injection holes at all time points (Fig. 4a,c). However, in contrast to wild-type hyphae, *nlt1* mutant hyphae remained dikaryotic at 6–10 dpi, also in leaf regions where nuclei in wild-type hyphae fuse and develop leaf tumors (Fig. 4a,c). To support these data we also generated FB1, FB2 as well as FB1Δnlt1 and FB2Δnlt1 strains expressing histone H1-mCherry to visualize nuclei. With respect to virulence, these strains were comparable to the respective progenitor strains (i.e. the *nlt1* mutant did not develop leaf tumors; Fig. S4). When analyzed for their nuclear status, wild-type strains showed two nuclei per cell compartment in hyphae colonizing chlorotic leaf areas while a single nucleus was detected in hyphal compartments from leaf as well as stem tumors (Fig. S5, upper panel). *nlt1* mutant strains expressing the histone H1-mCherry marker showed only dikaryotic hyphae even if samples were analyzed from leaf parts where the wild-type developed tumors (Fig. S5, lower panel). Interestingly, in stem tumors as well as in tassel and cob tumors induced by *nlt1* mutants we observed only hyphae with a single nucleus per compartment (Figs 4c, S5, S6) and we were able to detect spores (Figs 3, S3a,b).

The *nlt1* ortholog of *S. reilianum* complements the defect of a *U. maydis nlt1* mutant

*Sporisorium reilianum* causes kernel smut disease in maize. In this system plants are colonized systemically but development of disease symptoms and spore formation are restricted to infected flowers (Ghareeb et al., 2011). *Sporisorium reilianum* encodes an ortholog of *nlt1* (*Sr15650*) designated *Srnt1*. Nlt1 of *S. reilianum* shows 67.9% identity to *U. maydis* Nlt1 at the amino acid level and identity is most prominent in the N-terminal half containing the putative APSES helix–turn–helix DNA-binding domain while the C-terminal halves of the proteins are more divergent but also contain several blocks of sequence identity (Fig. S2). We next tested whether *Srnt1* is able to complement the function of *nlt1* in *U. maydis*. To this end the coding region of *Srnt1* was fused to the *U. maydis* *nlt1* promoter sequence and the *nos* terminator and integrated in single copy into the *ip* locus (Loubraudou et al., 2001) of FB1Δnlt1 and FB2Δnlt1. Maize infections with these strains revealed full complementation of the leaf tumor and virulence phenotype of the *U. maydis nlt1* mutants (Fig. 5a). This result was unexpected, because *S. reilianum* is unable to induce tumors. Therefore, we next generated *nlt1* mutants as well as complementation strains in *S. reilianum* and studied their virulence phenotype. *Sporisorium reilianum* *nlt1* mutants were significantly attenuated in virulence and displayed an unexpected phenotype which we call the ‘crazy cob’ phenotype (Figs 5b,c, S7, S8) following a similar phenotype in corn that is induced by the soil-borne oomycete *Sclerophthora macrospora* and is designated ‘crazy top on corn’ (Ullstrup, 1952). The crazy cob phenotype was fully complemented by reintroducing the *Srnt1* gene in *S. reilianum nlt1* mutant strains (Fig. 5b,c).

The ‘crazy cob’ phenotype is characterized by the development of hard wire-like structures (Fig. S8) that develop in places where wild-type *S. reilianum*-infected cobs develop phyllody (Ghareeb et al., 2011). *Sporisorium reilianum* infection has also been shown to promote the outgrowth of subapical ears (Ghareeb et al., 2011) and we observed this also in the *nlt1* mutant (Fig. S7; Table S4). The ‘crazy cob’ phenotype developed most strongly in apical ears and we observed this also in the *S. reilianum nlt1* mutant (Fig. S7; Table S4). Cobs harvested from plants infected with the *S. reilianum nlt1* mutant rarely produced spores (Figs S7–S9) but these were viable and produced haploid progeny upon germination (Fig. S9), indicating that there was no block in meiosis. These results illustrate that *nlt1* mutants of *S. reilianum* have a unique virulence phenotype that appears morphologically unrelated to the phenotype of *U. maydis nlt1* mutants after cob infection.
Fig. 2  *nlt1* mutants are blocked before mesophyll cell enlargement in leaves. Seven-day-old *Zea mays* seedlings of the variety Early Golden Bantam were inoculated with H2O (mock), FB1 × FB2 and FB1Δnlt1 × FB2Δnlt1 strains and leaf samples were collected at 3, 4, 5, 6 and 8 days postinfection (dpi) and representative pictures of cross-sections are shown. In the upper rows overview pictures are depicted, while in the corresponding lower rows enlargements of the areas marked with a white broken line in the upper rows are shown. Leaf samples were stained and observed with a confocal laser-scanning microscope. Plant cell walls were visualized by autofluorescence (cyan). Fungal hyphae were stained by WGA-AF488 (pink). Examples of epidermal cells (Ec), mesophyll cells (Mc) and bundle sheath cells (Bc) are labeled. Examples of enlarged mesophyll cells are indicated by white asterisks. Examples of new cell walls in dividing bundle sheath cells are indicated by yellow arrowheads. The necrotic autofluorescing areas seen after FB1Δnlt1 × FB2Δnlt1 infection are indicated by white arrowheads. The experiment was performed in two biological replicates with similar results. Bars, 50 µm.
Fig. 3  nilt1 mutants are able to induce tumors and spores in stems, tassels and cobs. (a) Seven-day-old Zeamays seedlings of the variety Early Golden Bantam were infected with Ustilagomaydis wild-type strains (FB1 × FB2) and nilt1 mutants (FB1Δnlt1 × FB2Δnlt1) or maintained without infection as indicated. Representative pictures of uninfected lower parts of the stems as well as of stem tumors induced by FB1 × FB2 were taken 14 days postinfection (dpi). Stem tumors induced by FB1Δnlt1 × FB2Δnlt1 were photographed 17 dpi. In all cases did stem tumors contain dark-colored spores. (b) Developing cobs of 5-wk-old Gaspé Flint plants were infected with U. maydis wild-type strains (FB1 × FB2) and nilt1 mutants (FB1Δnlt1 × FB2Δnlt1) or maintained without infection. At least 20 cobs were infected for each combination of strains. Representative pictures of cobs were taken 14 dpi. Tumors with black spores were induced by FB1 × FB2 as well as FB1Δnlt1 × FB2Δnlt1. The experiment was performed in three biological replicates with comparable results. (c) Developing tassels of 3-wk-old Gaspé Flint plants were infected with U. maydis wild-type strains (FB1 × FB2) and nilt1 mutants (FB1Δnlt1 × FB2Δnlt1) or maintained without infection. At least 20 tassels were infected for each combination of strains. Representative pictures of tassels were taken 14 dpi. Upper panels show overview pictures of selected tassels, while the row below shows enlargements of three selected tassels as well as further enlargements of the areas marked with a white broken line below. White arrowheads point to areas containing spores. The experiment was performed in three biological replicates with comparable results.
Fig. 4 nlt1 mutants are unable to perform karyogamy in leaves but undergo karyogamy in stem tumors. Seven-day-old Zeamays seedlings of the variety Early Golden Bantam were infected with Ustilagomaydis wild-type strains (FB1 × FB2) and nlt1 mutants (FB1Δnlt1 × FB2Δnlt1). (a) Representative leaves infected with the indicated strains at 8 days postinfection (dpi) are shown to illustrate how leaf areas were selected for microscopy. Chlorotic leaf areas with anthocyanin are highlighted by a red broken line, and the area of tumor development of FB1 × FB2 infection as well as the comparable leaf chlorotic area of FB1Δnlt1 × FB2Δnlt1 infection are highlighted by a black broken line. (b,c) Visualization of fungal nuclei. Plants were infected with a combination of strains indicated on the left and samples obtained from areas with leaf chlorosis, leaf tumor and stem tumor were collected at the indicated time points. The nuclear status of biotrophic hyphae from different plant tissues was assessed by imaging fungal nuclei using epifluorescence microscopy after DAPI staining. Nuclei in at least 25 hyphae were observed per sample. Samples from at least two or three infected plants were analyzed with comparable results. White arrows indicate the position of nuclei. Bars, 10 μm.
Sporisorium reilianum colonizes plants as the dikaryon and undergoes karyogamy only in infected flowers.

As S. reilianum is unable to induce leaf tumors, we next determined the nuclear status of hyphae throughout the infection cycle by DAPI staining. To be able to directly compare potential differences in nuclear status between U. maydis and S. reilianum during biotrophic development, infections with U. maydis wild-type strains FB1 × FB2 and S. reilianum wild-type strains SRZ1 × SRZ2 were done in Gaspé Flint, a maize variety.
flowering after about 3 wk and reaching a height of only about 70 cm at the flowering stage. Similar to development in the maize variety Early Golden Bantam, we observed dikaryotic fungal hyphae at all time points in chlorotic leaf sections after FB1 × FB2 infections while leaf tumors as well as stem tumors only contained fungal hyphae with a single nucleus per cell compartment (Fig. 6a). By contrast, between 2 and 32 dpi with S. reilianum SRZ1 × SRZ2, we could detect only hyphae with two nuclei per cell compartment in leaves or leaf sheaths. When infected husks, stamens and kernels were analyzed between 25 and 38 dpi we observed dikaryotic hyphae in husks, while stamens and kernels, the organs in which S. reilianum develops spores, contained hyphae with a single nucleus per compartment (Fig. 6b). In crazy cob tissue induced by the S. reilianum nlt1 mutant, hyphae remained dikaryotic even at 8 wk after infection (Fig. S10). These data show that nuclear fusion in S. reilianum is a very late event occurring only shortly before spore formation and requires nlt1.

Nlt1 is not essential for leaf tumor formation in solopathogenic strains

Dikaryotic nlt1 mutant hyphae of U. maydis display a nuclear fusion defect in infected leaves while this developmental step is not blocked in hyphae that develop in other plant organs. This could indicate that karyogamy is required for tumor formation. We next studied the phenotype of nlt1 mutants in solopathogenic strains which have a single nucleus per cell compartment and can cause disease without a mating partner. To this end, nlt1 was deleted in the solopathogenic haploid strain SG200 (Kamper et al., 2006) as well as in the two diploid strains FBD11 and FBD12 (Bauuett & Herskowitz, 1989). Disease symptoms were classified within nine disease categories (Fig. S11). We subsequently simplified this representation by placing all plants that developed leaf tumors together in the category ‘tumors on leaf and other places’ (Fig. 7). The results showed that compared to the respective solopathogenic parental strains, all nlt1 mutants were less virulent. However, in contrast to infections by FB1Δnlt1 × FB2Δnlt1 where no plants were scored in the category ‘tumors on leaf and other places’, between 39% and 61% fell into this category after infection with solopathogenic nlt1 mutants. This illustrates that the requirement of nlt1 for leaf tumor formation can be bypassed in solopathogenic haploid as well as diploid strains.

Generation and phenotypic characterization of kar7 mutants

To elucidate whether the link between nuclear status and tumor induction in leaves can be substantiated independently, we generated kar7 (UMAG_02833) mutants in FB1, FB2 and SG200. In Saccharomyces cerevisiae, nuclear fusion involves three discrete steps, outer membrane fusion, inner membrane fusion and spindle pole body fusion (Melloy et al., 2007), and Kar7p/Sec66 was shown to be involved in nuclear membrane fusion (Kurihara et al., 1994), the step initiated after pairing the two nuclei. The choice to focus on U. maydis kar7 was based on the observation that the kar7 ortholog in Cryptococcus neoformans also functions in nuclear fusion while orthologs of several other genes involved in karyogamy in S. cerevisiae are either missing or do not show a karyogamy phenotype when deleted in C. neoformans (Lee & Heitman, 2012). Similar to the situation for kar7 mutants in C. neoformans, U. maydis kar7 mutants were attenuated in growth (Fig. S12a). In addition, formation of dikaryotic hyphae after mating compatible kar7 mutants was strongly attenuated and this defect could be fully complemented (Fig. S12b). The few hyphae produced after mating were dikaryotic (Fig. S12c). SG200Δkar7 also showed attenuated growth and aerial hyphae development (Fig. S12a,b).

In virulence assays, FB1Δkar7 × FB2Δkar7 and SG200Δkar7 induced only leaf chlorosis while the complemented strains showed virulence comparable to wild-type strain combinations (Fig. S13a). WGA staining of biotrophic FB1Δkar7 × FB2Δkar7 revealed strongly attenuated leaf colonization (Fig. S13b). DAPI staining of hyphae at 6 and 8 dpi detected only dikaryotic hyphal segments (Fig. S13c). To rule out that these dikaryotic hyphae were still on the leaf surface, surface-attached cells were removed with the help of liquid latex before DAPI staining (Fig. S13c). As kar7 mutants displayed strong defects in plant colonization and failed to induce disease symptoms, we were unable to draw conclusions about their ability to induce leaf tumors.

A possible connection between Nlt1, See1 and Ros1

While the data presented so far suggest a connection between the ability to induce tumors in leaves and being a monokaryon, ros1 mutants (which are unable to undergo karyogamy but induce leaf tumors (Tollot et al., 2016)) do not fit this scheme. We therefore tried to investigate whether ros1 and nlt1 are connected. With respect to the expression of nlt1 and ros1 during plant colonization, both genes display a very similar expression profile with an expression peak at 8 dpi (Lanver et al., 2018) (Fig. 8a). Next, we generated nlt1,ros1 double mutants in FB1 and FB2 and compared them phenotypically with the respective single mutant strains. nlt1 mutants ros1 mutants and the nlt1,ros1 double mutants were not affected in growth and mating (Fig. S14a,b). With respect to virulence, the double mutant displayed the nlt1 mutant phenotype, was unable to induce leaf tumors and cells remained dikaryotic in leaf tissue (Fig. S15a,b), suggesting that nlt1 is upstream of ros1. However, in rare stem tumors the nuclei of the nlt1,ros1 double mutant did not fuse and this strain behaved like the ros1 single mutant (Fig. S15b), suggesting that ros1 is upstream of nlt1. To resolve this, we considered a regulatory connection between ros1 and nlt1 and studied this by qRT-PCR. This revealed that Nlt1 acts as a direct or indirect activator of ros1 (i.e. in the nlt1 mutant ros1 is poorly expressed; Fig. 8b). This could explain why karyogamy is affected in the nlt1 mutant. In ros1 mutants, nlt1 expression was about 3-fold reduced compared to expression levels in wild-type cells at 8 dpi (Fig. 8e) (Tollot et al., 2016).

To explain why the ros1,nlt1 double mutant behaves like the nlt1 mutant in leaves and fails to induce tumors but behaves like...
Fig. 6 *Sporisorium reilianum* undergoes karyogamy only late in infected flowers. Seven-day-old maize seedlings of the variety Gaspé Flint were infected with a combination of *Ustilagomaydis* wild-type (FB1 x FB2) and *S. reilianum* wild-type (SRZ1 x SRZ2) strains. (a) Visualization of nuclei after infection by FB1 x FB2. Samples obtained from infected areas showing leaf chlorosis, leaf tumors and stem tumors were collected at the indicated time points (days postinfection (dpi)). The nuclear status of biotrophic hyphae from different plant tissues was assessed by imaging fungal nuclei inside hyphae using epifluorescence microscopy after DAPI staining. (b) Visualization of nuclei after infection by *S. reilianum* wild-type SRZ1 x SRZ2. Samples obtained from chlorotic leaves, leaf sheath, stamen, husk and kernel after *S. reilianum* SRZ1 x SRZ2 infection were collected at the indicated time points. Nuclei were visualized as in (a) analyzing at least 25 hyphae per sample from two or three infected plants at each time point, which yielded comparable results. White arrows indicate fungal nuclei. Bars, 10 µm.
the ros1 mutant in stem tumor induction and is unable to undergo karyogamy there, we considered the possibility of an involvement of see1. The See1 effector is required for the resumption of plant DNA synthesis in leaves but is not needed for tumor formation in proliferating tissue such as the tassel and is not even expressed there (Redkar et al., 2015). Expression of the see1 gene is required for leaf tumor formation in dikaryotic strains. Virulence of nlt1 mutants in different strain backgrounds. nlt1 was deleted in solopathogenic diploid strains (FBD11 and FBD12), the solopathogenic haploid strain SG200 as well as in haploid strains. The indicated strains or strain combinations were injected into 7-d-old maize seedlings of the variety Early Golden Bantam and symptoms were scored at 12 days postinfection (dpi). The color code for each disease category is given below. Disease symptoms were quantified based on three biological replicates. The mean percentage of plants placed in a certain disease category is indicated. The number of infected plants as well as the ratio of leaf tumor formation are indicated at the top of the bar respectively. Statistically significant differences between strains are indicated (***, P < 0.001; ns, not significant). The ratio of leaf tumor formation in the respective progenitor strains was set to 100% compared to each nlt1 mutant. Detailed information of disease symptoms from the same experiment is presented in Supporting Information Fig. S11.

![Karyogamy is required for leaf tumor formation in dikaryotic strains. Virulence of nlt1 mutants in different strain backgrounds. nlt1 was deleted in solopathogenic diploid strains (FBD11 and FBD12), the solopathogenic haploid strain SG200 as well as in haploid strains. The indicated strains or strain combinations were injected into 7-d-old maize seedlings of the variety Early Golden Bantam and symptoms were scored at 12 days postinfection (dpi). The color code for each disease category is given below. Disease symptoms were quantified based on three biological replicates. The mean percentage of plants placed in a certain disease category is indicated. The number of infected plants as well as the ratio of leaf tumor formation are indicated at the top of the bar respectively. Statistically significant differences between strains are indicated (***, P < 0.001; ns, not significant). The ratio of leaf tumor formation in the respective progenitor strains was set to 100% compared to each nlt1 mutant. Detailed information of disease symptoms from the same experiment is presented in Supporting Information Fig. S11.](image-url)
see1 occurs early during leaf colonization, peaks at 2 dpi and then declines to a barely detectable level at 8 dpi (Lanver et al., 2018) (Fig. 8a). To analyze nuclear fusion in see1 mutants, we deleted the see1 gene in FB1 and FB2 and performed plant infections. DAPI staining revealed that see1 mutants are not affected in karyogamy in leaves (Fig. S16b) and we did not observe a reduction in virulence (Fig. S16a), contrary to what had been observed when see1 is deleted in SG200 (Redkar et al., 2015) (Fig. S16a). However, in nlt1 as well as ros1 mutants, see1 expression was significantly upregulated (Fig. 8c,d). These data demonstrated...
regulatory connections between nlt1, ros1 and see1 during plant colonization.

Discussion

In our study of Nlt1 we have uncovered a connection between this transcription factor and Ros1, a transcription factor controlling karyogamy and spore formation and show that the need of Nlt1 for tumor formation in leaves can be bypassed in haploid or diploid solopathogenic strains. Nlt1 is an APSES domain-containing transcription factor, and in fungi transcription factors of this class act as global regulators of a large number of traits (Zhao et al., 2015). The best studied example is StuA in ascomycete fungi. StuA orthologs have also been functionally analyzed in several plant pathogenic fungi such as Fusarium species, Magnaporthe oryzae, Glomerella cingulata and Stagonospora nodorum, where they were shown to regulate appressorium as well as subsequent pathogenic development, spore formation, condiation, secondary metabolite production, carbon metabolism, mycotoxin production and effector gene expression (Ohara & Tsuge, 2004; Tong et al., 2007; Nishimura et al., 2009; IpCho et al., 2010; Lysoe et al., 2011; Qi et al., 2012; Pasquali et al., 2013; Zhao et al., 2015). In U. maydis Ust1 is an StuA ortholog and plays a role in regulating dimorphism, sporulation and pathogenic development (Garcia-Pedrajas et al., 2010).

In the following we first discuss the role of Nlt1 during leaf tumor development. During leaf infections, nlt1 mutants fail to undergo karyogamy and fail to accumulate biomass after infection, two phenotypes shared with ros1 mutants (Tollot et al., 2016). This similarity is probably explained by the observation that Nlt1 functions as an activator of ros1 transcription, which is also consistent with nlt1 being epistatic to ros1 during leaf colonization. However, ros1 mutants produce leaf tumors – albeit at reduced severity compared to the wild-type (Tollot et al., 2016) – while nlt1 mutants do not. We consider it likely that this is due to the fact that nlt1 is expressed in the ros1 mutant (Fig. 8e). For leaf tumor development it has been established that in U. maydis the See1 effector is deployed in an organ-specific manner for the reactivation of cell division in leaves (Redkar et al., 2015). See1 induces cell division in leaf bundle sheath cells, while it is not required for induction of mesophyll hypertrophy and endoreduplication (Matei et al., 2018). The latter might explain why the see1 mutants still induce small leaf tumors. Because the function of see1 had only been analyzed in the solopathogenic haploid strain SG200 (Redkar et al., 2015) we have studied the contribution of see1 to virulence also in the dikaryon and failed to see leaf specific virulence defects there and observed fusion of the nuclei. Conversely, in nlt1 mutants we have not observed mesophyll hypertrophy or bundle sheath cell divisions, despite see1 being expressed at a level comparable/higher to wild-type infections. This makes it likely that Nlt1 controls a function distinct from see1 which is necessary for leaf tumor formation and which might involve a combination of several additional effectors whose expression is controlled by nlt1 (Lanver et al., 2018).

Why is the need for nlt1 partially bypassed in solopathogenic strains which are monokaryons and is this linked to the step of karyogamy itself or to processes ensuing after the nuclei have fused? In contrast to S. cerevisiae, where nuclear fusion occurs immediately after fusion of compatible cells, karyogamy in U. maydis occurs at a late stage of infection (Tollot et al., 2016; Snetselaar & McCann, 2017) and is followed by an unusual diploid mitotic stage that accompanies rapid tumor enlargement (Snetselaar & McCann, 2017). During the earlier biotrophic plant colonization phase, dikaryotic hyphae develop clamp connections to ensure that during cell division all cells receive a single copy of both nuclei (Scherer et al., 2006). During this phase, there is very little increase in fungal biomass (Tollot et al., 2016) (Fig. 1b). Therefore, we consider it likely that mitotic divisions via clamp connections slow down growth while allowing sufficient biomass accumulation for the induction of leaf tumors. The ability of the karyogamy-deficient ros1 mutants to induce tumors at a lower incidence than the wild-type (Tollot et al., 2016) fits this proposition. Mitotic divisions of the diploid might be facilitated if the complex clamp cell program is turned off. Evidence for such a scenario has been gained in C. neoformans, where it has been observed that self-fertile diploid strains produce hyphae with unfused clamp connections while the dikaryon produces hyphae with fused clamps (Sia et al., 2000).

As see1 transcripts decline at the stage of karyogamy, the massive mitotic growth of the diploid might accompany tumor enlargement in a see1-independent manner. This could suggest that full tumor development is regulated in two phases, an early phase controlled by nlt1 and involving the see1 effector before karyogamy. This program would be responsible for tumor formation by the ros1 mutant. A second phase inducing tumor formation/enlargement would be initiated after karyogamy. This program would bypass the need for see1 and nlt1. In this scenario see1 mutants in FB1 × FB2 would be unaffected in virulence because the postnuclear fusion tumor program is active and nlt1 mutants in solopathogenic strains would induce leaf tumors because there is no need for nuclear fusion and the postfusion program is active. To explain why see1 mutants in the solopathogenic strain are attenuated in virulence (Redkar et al., 2015) (Fig. S16a) we consider this to result from the severely compromised late proliferation of SG200 in infected tissue (F. Fukada, personal communication).

The signal triggering karyogamy is unknown and probably challenging to identify because so far it has not been possible to propagate the dikaryon in axenic culture. We speculate that karyogamy is induced in plant cells which are actively dividing. Previously, it has been demonstrated that U. maydis is only able to induce tumor development in zones with actively dividing cells found at the base of the stem, at the ligular region located at the base of the leaf blade, in a specific zone in immature tassels and in developing kernels (Christensen, 1963; Wallbot & Skibbe, 2010). Our observation that dikaryotic hyphae are detected in tissue adjacent to tissue where tumors containing diploid hyphae develop suggests that tumor formation and karyogamy are restricted to specific cell types. This is in line with previous experiments which have shown that maize anthers show cell type-specific responses to U. maydis infection (Gao et al., 2013).
Karyogamy has most extensively been studied in yeast and higher eukaryotes. In the budding yeast \textit{S. cerevisiae} mating pheromones induce cell polarization and cell fusion, which is immediately followed by karyogamy. Karyogamy occurs in two major steps: the migration of the two nuclei toward each other (termed nuclear congression) and the subsequent fusion of the nuclear envelope membranes (Rose, 1996). A number of \textit{kar} genes involved in this process have been identified and analyzed with respect to how they contribute to karyogamy molecularly (Gibieux & Knop, 2013). The repertoire of \textit{kar} genes in \textit{U. maydis} is comparable to that found in \textit{C. neoformans} and we therefore have deleted the ortholog of \textit{kar7}, the only nonessential \textit{kar} gene in \textit{C. neoformans} for which mutants display a karyogamy defect (Lee & Heitman, 2012). Unfortunately, \textit{U. maydis} \textit{kar7} mutants were already attenuated in mating and produced only few dikaryotic hyphae. Plant colonization was poor and only few intracellular dikaryotic hyphae could be detected which remained dikaryotic also at late times of the infection. Virulence symptoms were completely abolished and it is currently unknown whether this is due to the defect in karyogamy or the defect in colonization. Because the solopathogenic strain lacking \textit{kar7} also did not induce any disease symptoms, \textit{KAR7} which in \textit{S. cerevisiae} is allelic to \textit{SEC66}, a gene involved in protein translocation into the endoplasmic reticulum, probably affects transport processes unrelated to karyogamy (Kurihara \textit{et al}., 1994). \textit{kar7} mutants in \textit{C. neoformans} also exhibited some detrimental phenotypes during budding growth (Lee & Heitman, 2012), which also supports this possibility.

The \textit{nlt1} ortholog from \textit{S. reilianum} was able to complement the \textit{nlt1} function in \textit{U. maydis}, and \textit{nlt1} mutants of \textit{S. reilianum} also failed to undergo karyogamy. This indicates that the karyogamy-related function of this regulator, presumably conferred via the downregulation of \textit{ros1}, is conserved in the two smut species. However, in \textit{S. reilianum}, a smut fungus unable to induce tumors, \textit{nlt1} mutants induced the crazy cob phenotype. Instead, symptoms developed exclusively in floral tissue where phyllody phenotypes such as leafy ears and cary ears are induced by wild-type strains (Ghareeb \textit{et al}., 2011). We speculate that the crazy cob phenotype of \textit{S. reilianum} \textit{nlt1} mutants may result from high levels of \textit{see1} expression, analogous to what was observed in \textit{U. maydis} \textit{nlt1} mutants. In \textit{U. maydis} it has been shown that constitutive overexpression of \textit{see1} has no effect during leaf colonization but leads to abnormal, greenish looking tumors on vegetative parts of the tassel (Redkar \textit{et al}., 2015).

Why do \textit{nlt1} mutants induce tumors in actively dividing tissue (i.e. the stem, the developing cob and the developing tassel)? We consider it likely that in such tissue karyogamy is induced and this then allows mitotic proliferation of the diploid in a process which could be further enhanced by high levels of \textit{see1} expression.

In the present study we have analyzed in detail the phenotype of an \textit{nlt1} mutant during biotrophic development and provide evidence for two stages of tumor formation, one initiated before karyogamy and a second one initiated once the nuclei are fused. We implicate \textit{nlt1} only in the program before karyogamy.

Additionally, if Nlt1 primarily controls the expression of a set of effectors essential for tumor formation in leaves, pathogenic development of the \textit{nlt1} mutant might be halted before \textit{ros1} expression and karyogamy could be activated by Nlt1. This could also explain why \textit{nlt1} mutants cause tumors in actively dividing tissue like the base of the stem, cobs and tassels, where leaf-specific effectors may not be needed (i.e. the effects on karyogamy/regulation of nuclear status could be secondary effects). It will be interesting to identify the Nlt1 targets required for leaf tumor induction. To do this one could envision a comparative RNA sequencing approach to identify those fungal genes differentially regulated in \textit{nlt1}-infected tissue which are not affected by the downregulation of \textit{ros1} and elucidate their contribution to leaf tumor formation. Particularly interesting in this regard could be the largest \textit{U. maydis} gene cluster encoding 24 effectors because deletions of the entire cluster abolish tumor formation (Kamper \textit{et al}., 2006; Befort \textit{et al}., 2014).

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Author contributions

RK directed the project. J-SL and RK designed the concept of the study. J-SL performed data analysis. J-SL and PH generated strains, designed and performed all experiments. J-SL 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** Virulence assay of *Ustilago maydis nlt1* deletion mutants.
**Fig. S2** Amino acid sequence alignment of Nlt1 orthologs.

**Fig. S3** nlt1 mutants are able to induce spore-filled tumors in cobs and tassels.

**Fig. S4** Virulence assay of *Ustilago maydis* strains expressing histone H1-mCherry.

**Fig. S5** *Ustilago maydis* nlt1 mutants are unable to perform karyogamy in leaves but undergo karyogamy in stem tumors.

**Fig. S6** *Ustilago maydis* nlt1 mutants undergo karyogamy in cob and tassel tumors.

**Fig. S7** Overview of ears developing after *Sporisorium reilianum* infection.

**Fig. S8** Ear infection symptoms after *Sporisorium reilianum* infections.

**Fig. S9** Compatibility assay of haploid progeny isolated from *Sporisorium reilianum* spores.

**Fig. S10** *Sporisorium reilianum* nlt1 mutants are unable to perform karyogamy in crazy cob tissue.

**Fig. S11** Karyogamy is required for leaf tumor formation after infection with compatible haploid strains.

**Fig. S12** Growth and aerial filament production of *Ustilago maydis* kar7 mutants.

**Fig. S13** Virulence, plant colonization and nuclear status of *Ustilago maydis* kar7 mutants.

**Fig. S14** Growth and aerial filament production of various *Ustilago maydis* mutants.

**Fig. S15** Virulence and nuclear status of various *Ustilago maydis* mutants.

**Fig. S16** Virulence and nuclear status of see1 mutants after plant infection.

**Methods S1** Plasmid and strain constructions.

**Methods S2** Virulence assays.

**Methods S3** Fungal biomass determination.

**Methods S4** Spore germination assay.

**Methods S5** Confocal microscopy.

**Table S1** *Ustilago maydis* and *Sporisorium reilianum* strains used in this study.

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

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

**Table S4** *Sporisorium reilianum* nlt1 mutant can promote the outgrowth of subapical ears.

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