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Positively selected effector genes and their contribution to virulence in the smut fungus *Sporisorium reilianum*

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Abstract

Plants and fungi display a broad range of interactions in natural and agricultural ecosystems ranging from symbiosis to parasitism. These ecological interactions result in coevolution between genes belonging to different partners. A well-understood example are secreted fungal effector proteins and their host targets, which play an important role in pathogenic interactions. Biotrophic smut fungi (Basidiomycota) are well-suited to investigate the evolution of plant pathogens, because several reference genomes and genetic tools are available for these species. Here, we used the genomes of *Sporisorium reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum*, two closely related formae speciales infecting maize and sorghum, respectively, together with the genomes of *Ustilago hordei*, *Ustilago maydis* and *Sporisorium scitamineum* to identify and characterize genes displaying signatures of positive selection. We identified 154 gene families having undergone positive selection during species divergence in at least one lineage, among which 77% were identified in the two investigated formae speciales of *S. reilianum*. Remarkably, only 29% of positively selected genes encode predicted secreted proteins. We assessed the contribution to virulence of nine of these candidate effector genes in *S. reilianum* f. sp. *zeae* by deleting individual genes, including a homologue of the effector gene *pit2* previously characterized in *U. maydis*. Only the *pit2* deletion mutant was found to be strongly reduced in virulence. Additional experiments are required to understand the molecular mechanisms underlying the selection forces acting on the other candidate effector genes, as well as the large fraction of positively selected genes encoding predicted cytoplasmic proteins.

Keywords

Positive selection; effector evolution; smut fungi; comparative genomics; virulence

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Introduction

Plants and fungi have a long history of coevolution since the emergence of pioneering land plants approximately 400 million years ago. The development of early plants was likely supported by associations with symbiotic fungi, as suggested by analyses of ribosomal RNAs and fossil records (Remy et al. 1994; Gehrig et al. 1996; Martin et al. 2017). Different forms of plant-fungus interactions have evolved, including mutualistic symbiosis where both plant and fungus benefit (Parniske 2008), and pathogenic interactions where fungal colonization greatly reduces plant fitness (Dean et al. 2012). Pathogenic interactions play critical roles in natural and agricultural ecosystems, and understanding the evolutionary mechanisms shaping them is of great importance to plant production, food security and protection of biodiversity in natural ecosystems (Fisher et al. 2012; Bagchi et al. 2014).

Secreted fungal effector proteins are key players in pathogenic interactions as they are involved in protecting and shielding growing hyphae, suppressing plant defense responses and changing plant physiology to support growth of the pathogen (Stergiopoulos and de Wit 2009; de Jonge et al. 2011; Giraldo and Valent 2013). Many effector proteins lack known functional domains, and expression of a subset of effectors is linked to plant colonization (Lo Presti et al. 2015; Toruño et al. 2016; Franceschetti et al. 2017; Lanver et al. 2017). Effector proteins with a strong effect on virulence phenotype are thought to coevolve with their plant targets either in an arms race or a trench-warfare scenario (Brown and Tellier 2011). In the former, fungal effectors manipulating the host are under positive directional selection, and plant targets evolve in response to changes in effector proteins (Rovenich et al. 2014). In the latter scenario, sets of alleles are maintained by balancing selection in both host and pathogen populations (Brown and Tellier 2011; Tellier et al. 2014). Several methods are available for identifying genomic regions under selection (Nielsen 2005; Aguileta et al. 2009; Aguileta et

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al. 2010). It has been proposed that genes with signatures of positive selection have important functions during host pathogen interaction or have contributed to host specialization (Tiffin and Moeller, 2006). It is therefore expected that the deletion of such genes reduces virulence when tested on a susceptible host.

Depending on the aim of the investigation, studies identifying genes with signatures of positive selection are carried out within or between species. Whilst studies on the population level focus on recent and ongoing selective processes and are instrumental in the understanding of adaptation, comparative genomic studies employing different species encompass a broader time span and provide insight into the underlying genetic basis of host specialization (Plissonneau et al. 2017). The signature of positive selection in such case typically takes the form of an excess of divergence between species due to increased fixation of mutations by selective sweeps compared to a neutral expectation (Yang and Nielsen, 1998). This is commonly measured by the ratio of non-synonymous (d_N) over synonymous (d_S) divergence and a d_N / d_S ratio > 1 is taken as evidence for positive selection under the assumption that synonymous substitutions are neutral while non-synonymous are not. Positive selection studies in a number of plant pathogen systems revealed that genes encoding secreted effector proteins are enriched in signatures of positive selection (Möller and Stukenbrock 2017). Such studies include investigations in diverse plant pathogens like *Microbotryum* species causing anther-smut disease of Caryophyllaceae species (Aguileta et al. 2010), the wheat pathogen *Zymoseptoria tritici* (Stukenbrock et al. 2011), the rust fungus *Melampsora larici-populina* (Hacquard et al. 2012), the rice blast fungus *Magnaporthe oryzae* (Huang et al. 2014), the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* (Sperschneider et al. 2014) the Irish potato famine pathogen *Phytophthora infestans* (Dong et al. 2014), a group of *Fusarium* species (Sperschneider et al. 2015) and a group of smut fungi parasitizing different grasses and a dicot host (Sharma et al. 2014; Sharma et al. 2015). Yet, as only a few genes

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under positive selection have been functionally studied, the link between the selected genotypes and their corresponding phenotypes are only beginning to be understood and only a few studies used evolutionary predictions to unravel the molecular mechanisms of host adaptation. For example, the population genomics study in the wheat pathogen *Z. tritici* which identified candidate effector genes under positive selection (Stukenbrock et al. 2011) was followed up experimentally, and in this case it was shown that the deletion of three of four candidate genes reduced virulence (Poppe et al. 2015). In the grey mold fungus *Botrytis cinerea* four positively selected genes were deleted without affecting virulence, and this finding was attributed to functional redundancy, the limited number of tested host plants, or experimental conditions different from natural infections (Aguileta et al. 2012). A study of the oomycete effector protein EpiC1 showed that a single amino acid substitution at a site under positive selection affected the binding affinity of different host proteases determining host specificity (Dong et al. 2014).

Smut fungi, belonging to the division of Basidiomycota, are a group of about 550 species parasitizing mostly grasses, including important crops like maize, sorghum, oat, barley and sugarcane (Begerow et al. 2014). In smut fungi, sexual reproduction is linked to pathogenic development and smut fungi therefore depend on successful plant colonization to complete their life cycle. As biotrophic pathogens, they require living plant tissue for establishing a successful interaction (Martinez-Espinoza et al. 2002). With few exceptions like *Ustilago maydis*, smut fungi usually develop symptoms only in the female or male inflorescence of their respective host plants. During the last ten years, quality draft genome sequences of prominent species were obtained, including *U. maydis*, the causative agent of smut disease on maize and teosinte (Kämper et al. 2006), *Sporisorium reilianum* causing head smut of maize and sorghum (Schirawski et al. 2010), *Ustilago hordei* infecting barley (Laurie et al. 2012), and *Sporisorium scitamineum* parasitizing sugarcane (Que et al. 2014; Taniguti et al. 2015;

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147 Dutheil et al. 2016). The head smut fungus *S. reilianum* occurs in two formae speciales that
148 infect maize (*S. reilianum* f. sp. *zeae*) or sorghum *(S. reilianum* f. sp. *reilianum*) (Zuther et al.
149 2012). The concept of formae speciales is used in phytopathology to distinguish members of
150 the same species based on their ability to colonize a certain host plant (in this example maize
151 or sorghum) (Anikster, 1984). The divergence of *U. hordei*, *U. maydis*, *S. scitamineum* and *S.*
152 *reilianum* was inferred to have occurred in the interval of seven and 50 million years ago
153 (Munkacsi et al. 2007). The availability of genome sequences of several species with different
154 host ranges, together with established tools for genetic manipulations (Brachmann et al. 2004;
155 Kämper 2004; Khrunyk et al. 2010; Schuster et al. 2016) make this group of smut fungi
156 particularly interesting to study the evolution of effector genes as well as their contributions to
157 virulence, speciation and host specificity.
158 Here, we employed the genome of the recently sequenced strain *S. reilianum* f. sp. *reilianum*
159 SRS1_H2-8 (http://www.ebi.ac.uk/ena/data/view/LT795054-LT795076) (Zuther et al. 2012)
160 together with the genomes of *U. maydis*, *U. hordei*, *S. scitamineum* and *S. reilianum* f. sp.
161 *zeae* to identify potential effector genes with signatures of positive selection. Candidate genes
162 were individually deleted in *S. reilianum* f. sp. *zeae* and the phenotype of the deletion strains
163 was assessed after infection of maize in order to understand their function with respect to
164 virulence. We report that the deletion of one candidate gene, *pit2*, led to a strong reduction in
165 virulence and we further discuss hypotheses on the origin of positive selection for the other
166 candidate genes.
167
168
169 **Materials and Methods**
170 *Construction of homologous protein families*

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Fungal species used in this study, their number of gene models, number of predicted secreted proteins and sources of genome data are listed in supplementary table S1. The predicted proteome of the five smut fungi *U. hordei*, *U maydis*, *S. scitamineum*, *S. reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum* were used to perform an all-against-all blastp search (Altschul et al. 1990). The SiLiX algorithm was subsequently used to infer homology relationships based on the blast hits (Miele et al. 2011). Two parameters are considered to decide whether a Blast hit can be taken as evidence for homology: the percent identity between two sequences and the relative length of the hit compared to the total length of the two sequences, hereby referred to as "coverage". In order to maximize the number of families comprising 1:1 orthologues (that is families that have an equal number of members in each species), SiLiX (Miele et al. 2011) was run with a range for coverage and identity thresholds between 5 % and 95 % in 5 % steps. An identity of 40 % and coverage between 5 % and 45 % lead to the maximum number of families with 1:1 orthologues (5,394; supplementary fig. S1) while settings with 40 % identity and 80 % coverage lead to 5,326 families with 1:1 orthologues (supplementary fig. S1). Since using a higher coverage had only a cost of 68 core families, the stricter criteria were applied for family clustering. Families with at least two members were aligned on the codon level using MACSE 1.01b (Ranwez et al. 2011) and on the protein level using PRANK v.100802 (Löytynoja and Goldman 2008). The resulting alignments were subsequently compared and column scores (CS) computed for each position in the alignment (Thompson et al. 1999). Only positions with CS of 100 % (that is, alignment columns identically found by both methods) and a maximum of 30 % gaps were retained for further analysis.

Estimation of genome-wide divergence values and divergence times

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The five genomes of *U. hordei*, *U. maydis*, *S. scitamineum*, *S. reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum* were aligned using the Multiz genome aligner from the TBA package (Blanchette et al. 2004) and projected on the *U. maydis* genome as reference. The resulting multiple genome alignment had a total size of 21 Mb and was further restricted to regions with homologous sequences in the five species (total length after this step: 14.3 Mb) and processed to remove coding regions. The final non-coding sequence alignment had a total length of 2.2 Mb, for which pairwise nucleotide similarities were computed in non-overlapping windows of 10 kb.

Gene families with exactly one member in each species were concatenated and pairwise protein sequence similarities computed using the *seqinr* package for R (Charif and Lobry 2007). Protein alignments were also used to infer dates of divergence, using a relaxed clock model. The PhyloBayes version 4.1 software (Lartillot et al. 2009) was used with the auto-correlated model of Thorne et al. (Thorne et al. 1998) under a GTR + CAT model. A unique calibration point was used, based on the divergence time of the most divergent lineage *U. hordei*, previously estimated to have occurred between 27 and 21 Myr (Bakkeren and Kronstad, 2007). A uniform prior was used on this interval for the Monte-Carlo Markov Chain. As convergence issues arise when large alignments (more than 20,000 positions) are used, we followed the PhyloBayes authors' recommendation to conduct a jackknife procedure. We generated three datasets of ca 20,000 amino acids by randomly sampling families and concatenating the corresponding alignments. Two chains were run in each case and convergence was assessed. Sampling was performed after a burning of 10,000 iterations, and every 10 subsequent iterations. Chains were run to ensure that the minimum effective sample size was greater than 50 and maximum relative difference lower than 0.3 in at least one sample. Results are summarized in supplementary table S2 and supplementary fig. S2 shows the six chains for the three samples. In addition to the convergence of the two chains

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for each sample, our results reveal extremely consistent results between samples. Figure 1A shows estimates from one chain of the third data set, which shows a minimum effective sample size greater than 300.

Detection of positive selection

For gene families with at least three members, translated sequences were employed to create maximum likelihood phylogenetic trees using PhyML 3.0 (Guindon et al. 2010) with a minimum parsimony starting tree and the LG amino acid substitution model with a four-classes gamma distribution of site-specific substitution rate (Le and Gascuel 2008). The best tree topology obtained from nearest neighbor interchange (NNI) and subtree pruning recrafting (SPR) searches was kept (Guindon et al. 2010). BppML (Dutheil and Boussau 2008) was then used to re-estimate branch lengths from the codon alignment using the YN98 substitution model (Nielsen and Yang 1998). We next aimed at inferring the occurrence of positive selection for each gene family. This is typically achieved by measuring the ratio of non-synonymous vs. synonymous substitutions (d_N/d_S ratio) using models of codon sequence evolution (Yang, 2006). In particular, non-homogeneous models of sequence evolution estimate the d_N/d_S ratio independently in different lineages, yet at the cost of potential over-parametrization issues. In the manual of the PAML package, the authors state that such models should only be used for hypothesis testing and advise against using them for scans of positive selection. Dutheil et al. (2012) proposed a model selection approach (implemented in the TestNH package) allowing to select for the best non-homogeneous model supported by the data. They start by fitting the simplest (homogeneous) model and sequentially add parameters to model variation of selective regime among lineages. Because the number of

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245 possible models is large even for small data sets, two heuristic approaches have been
246 introduced: the 'free' heuristic permits unconnected branches from the tree to evolve under
247 the same regime, while the 'joint' heuristic restricts model sharing to connected branches (see
248 Dutheil et al. 2012 for details). The choice of models to test is guided by statistics on the
249 patterns of substitutions on the phylogenetic tree, an approach named *substitution mapping*
250 (Romiguier et al. 2012). Apart from the model selection approach, the underlying models of
251 codon sequence evolution are identical to the one originally described by Yang (Yang, 1998;
252 Yang and Nielsen, 1998). Model selection was performed with the TestNH software, which
253 contains two programs: (1) MapNH (Romiguier et al. 2012) was used for mapping
254 substitutions on the previously inferred phylogenetic tree and (2) PartNH (Dutheil et al. 2012)
255 was subsequently employed to fit time non-homogeneous models of codon substitutions.
256 PartNH uses the previously inferred substitution maps in order to perform model comparisons
257 and select a non-homogeneous model with minimal number of parameters. Both methods
258 'free' and 'join' were applied and compared to scan for positive selection. Finally, putative
259 secreted effector proteins were identified by predicting secretion using SignalP 4.0 (Petersen
260 et al. 2011) and proteins were considered as secreted if the program indicated the presence of
261 a signal peptide but no transmembrane domain.
262 To detect residues under positive selection in homologues of *pit2*, the branch-site model with
263 Bayes Empirical Bayes (BEB) analysis as implemented in PAML4 (Yang 2007) was applied.
264 We employed information about family composition, alignment and phylogeny as outlined
265 above and defined *sr10529* and *srs_10529* as foreground branches. A posterior probability
266 threshold of > 95 % was used for the BEB analysis.
267
268 *Association of positively selected genes with repeats in U. hordei*

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We tested whether genes under positive selection are located significantly closer to repetitive elements than average genes in the genome of *U. hordei*, which shows the highest content of repetitive elements in the group of smut fungi investigated here. For this analysis, only a group of "uncharacterized interspersed repeats" was investigated, because it was shown previously that this is the only category showing a strong association with candidate effector genes (Dutheil et al. 2016). Binary logistic regressions were conducted in R using the *rms* package (Harrell 2015). The 'robcov' function of the rms package was used in order to get robust estimates of each effect. The variable "distance to the closest interspersed repeat" was transformed by log(x+1) because of its extreme distribution.

Comparing d_N/d_S ratios of genes residing in virulence clusters

Previous work has identified several virulence gene clusters in *U. maydis* and some of them play important roles during pathogenic development (Kämper et al. 2006; Schirawski et al. 2010). In total, these clusters contain 163 genes, where 100 reside in clusters without virulence phenotype and 63 reside in clusters with virulence phenotype upon deletion. Both types of clusters contain each 32 genes for which a d_N/d_S ratio could be determined (the missing genes are part of families that do not have at least three members and were therefore not analyzed). The d_N/d_S ratios of all genes in clusters were compared between clusters with and without virulence phenotype (Wilcoxon Rank-Sum Test).

Gene Ontology terms enrichment analysis

All proteins in *S. reilianum* f. sp. *zeae*, in *S. reilianum* f. sp. *reilianum* and in *U. hordei* were considered for Gene Ontology (GO) term enrichment analyses. GO terms were assigned using iprscan 1.1.0 (http://fgblab.org/runiprscan; developed by Michael R. Thon) which links GO information provided by Interpro to each protein. In this way, 1,759 unique GO terms could

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be assigned to 4,130 proteins in *S. reilianum* f. sp. *zeae*, 1,744 unique GO terms could be assigned to 4,124 proteins in *S. reilianum* f. sp. *reilianum* and 1,757 unique GO terms could be assigned to 3,922 proteins in *U. hordei* (supplementary table S3). The Bioconductor package topGO (Alexa et al. 2006) was then used to link each GO term to the three major categories "Cellular Component", "Biological Process" or "Molecular Function". Enrichment analysis was performed by computing *P* values for each GO term using Fisher's classic test with parent-child correction (Grossmann et al. 2007). Cytoplasmic proteins with and without signatures of positive selection were compared for the three species separately, and differences were considered to be significant at the 5 % level.

Strains and growth conditions

The *Escherichia coli* derivative Top10 (Invitrogen, Karlsruhe, Germany) and the *Saccharomyces cerevisiae* strain BY4741 (*MAT*a *his*3Δ1 *leu*2Δ *met*15Δ *ura*3Δ; Euroscarf, Frankfurt, Germany; kindly provided by M. Bölker, Marburg) were used for cloning purposes. *Sporisorium reilianum* strains used in this study are listed in supplementary table 4. They are derivatives of the haploid solopathogenic strain JS161 which is capable of plant colonization without the need of a mating partner, because it expresses a compatible pheromone/receptor pair (Schirawski et al. 2010). *Escherichia coli* was grown in dYT liquid medium (1.6 % (w/v) Trypton, 1.0 % (w/v) Yeast Extract (Difco), 0.5 % (w/v) NaCl) or YT solid medium (0.8 % (w/v) Trypton, 0.5 % (w/v) Yeast-Extract, 0.5 % (w/v) NaCl, 1.3 % (w/v) agar) supplemented with 100 mg/mL Ampicillin when needed. The yeast *S. cerevisiae* was maintained in YPD solid medium (1 % (w/v) yeast extract, 2 % (w/v) Bacto-Pepton, 2 % (w/v) Bacto-Agar, 2 % (w/v) glucose) and grown on SC URA^- medium (1.7 % (w/v) Yeast Nitrogen Base without ammonium sulfate, 0.147 % (w/v) dropout-mix without Uracil, 2 % (w/v) glucose) for selecting transformants containing the plasmid pRS426 (Sikorski and

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Hieter 1989) (kindly provided by M. Bölker, Marburg) or derivatives of pRS426. Strains of *S. reilianum* were grown in liquid $YEPS_{light}$ medium (1.0 % (w/v) yeast extract, 0.4% (w/v) peptone, 0.4% (w/v) sucrose) at 28°C on a rotary shaker at 200 rpm.

Construction of S. reilianum strains

Polymerase chain reactions were performed using the Phusion High-Fidelity DNA Polymerase (New England Biolabs). Templates were either JS161 genomic or indicated plasmid DNA. Restriction enzymes were obtained from New England Biolabs. Protoplast-mediated transformation was used to transform *S. reilianum* following a method established for *U. maydis (Schulz et al. 1990).* Transformants were selected on RegAgar plates (1.0 % (w/v) yeast extract, 0.4 % (w/v) Bacto-Pepton, 0.4 % (w/v) Sucrose, 1 M Sorbitol, 1.5 % (w/v) Bactoagar) supplemented with 200 µg/mL Geneticin and true resistance was tested by growing single colonies on PD plates (3.9 % (w/v) Potato-Dextrose Agar, 1 % (v/v) Tris-HCl (1M, pH 8.0)) supplemented with 50 µg/mL Geneticin. Gene replacements with resistance markers were generated with a PCR-based method employing the previously described *Sfi*I insertion cassette system (Brachmann et al. 2004; Kämper 2004) and were confirmed by Southern blot analysis. Genomic regions residing about 1 kb upstream (left border) or downstream (right border) adjacent to open reading frames of candidate genes were PCR-amplified using the listed primer pairs (supplementary table S5) and genomic DNA of JS161 as template. The resulting fragments were used for cloning plasmids containing the respective deletion constructs.

To obtain deletion constructs for the genes *sr10529* and *sr14347*, PCR fragments containing the left and right borders of each gene were ligated to the hygromycin resistance cassette of pBS-hhn (Kämper 2004) via *Sfi*I restriction sites and cloned into pCRII-TOPO (Life Technologies) to generate pTOPO Δsr10529 #1 and pTOPO Δsr14347 #1, respectively. Since

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the use of Geneticin as selection marker resulted in much less false positive transformants compared to the use of Hygromycin B, the hygromycin resistance cassettes in these plasmids were replaced by the Geneticin resistance cassette of pUMA 1057 (Brachmann et al. 2004) by ligation via *Sfi*I restriction sites, yielding plasmids pTOPO Δsr10529 G418 and pTOPO Δsr14347 Gen #1, respectively. Deletion constructs were PCR-amplified from plasmids pTOPO Δsr10529 G418 and pTOPO Δsr14347 Gen #1 using the listed primers (supplementary table S5) and used to transform the *S. reilianum* strain JS161 to generate the gene deletion strains JS161Δsr10529 and JS161Δsr14347, respectively.

The drag and drop cloning method in yeast (Jansen et al. 2005) was used to generate plasmids pRS426 Δsr12968 Hyg #1, pRS426 Δsr14944 Hyg #2, pRS426 Δsr10059 Hyg #1, pRS426 Δsr10182 Hyg #1, pRS426 Δsr14558 Hyg #1 and pRS426 Δsr12897 Hyg #1 which contain deletion constructs for deleting the candidate genes *sr12968*, *sr14944*, *sr10059*, *sr10182*, *sr14558* or *sr12897*. These plasmids are a derivate of plasmid pRS426, which can be maintained in *E. coli* and *S. cerevisiae* (Sikorski and Hieter 1989). PCR-amplified left and right borders of each candidate gene and the hygromycin resistance cassette were integrated in pRS426 by homologous recombination in *S. cerevisiae*. Subsequently, the hygromycin resistance cassette was replaced with the Geneticin resistance cassette by ligation via *Sfi*I restriction sites, yielding plasmids pRS426 Δsr12968 Gen #1, pRS426 Δsr14944 Gen #3, pRS426 Δsr10059 Gen #1, pRS426 Δsr10182 Gen #1, pRS426 Δsr14558 Gen #1 and pRS426 Δsr12897 Gen #5, respectively. Gene deletion constructs were PCR-amplified from the respective plasmid using listed primers (supplementary table S5). The obtained deletion constructs were used to transform the *S. reilianum* strain JS161 to generate the gene deletion strains JS161Δsr12968, JS161Δsr14944, JS161Δsr10059, JS161Δsr10182, JS161Δsr14558 and JS161Δsr12897, respectively.

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The drag and drop cloning method was also used to generate plasmid pRS426 Δsr12084 Gen #1. PCR-amplified left and right borders of *sr12084* and the Geniticin resistance cassette were integrated in pRS426 by homologous recombination in *S. cerevisiae*. The gene deletion construct for deleting the candidate gene *sr12084* was PCR-amplified from plasmid pRS426 Δsr12084 Gen #1 using primers sr12084_lb_fw/sr12084_rb_rv and transformed into the *S. reilianum* strain JS161 to generate the gene deletion strain JS161Δsr12084.

Virulence assays

The solopathogenic strain JS161 and derivatives thereof were grown in $YEPS_{light}$ liquid medium to an optical density at 600 nm (OD_{600}) of 0.8 - 1.0 and cell cultures were adjusted to an OD_{600} of 1.0 with sterile water prior to injection into one-week old maize (*Zea mays*) seedlings of the dwarf cultivar 'Gaspe Flint' (kindly provided by B. Burr, Brookhaven National Laboratories and maintained by self-pollination). Plants were sowed in T-type soil of 'Fruhstorfer Pikiererde' (HAWITA, Vechta, Germany) and grown in a temperature-controlled greenhouse (14h-/10h- light/dark cycle, with 28/20°C and 25,000 – 90,000 lux during the light period). Virulence symptoms were scored nine to ten weeks post infection according to previously described symptoms (Ghareeb et al. 2011) and the following categories were distinguished: the plant did not develop ears, the plant developed healthy ears shorter or equal to 1 cm or the plant developed healthy ears longer than 1 cm, the plant developed spiky ears, phyllody in ears or phyllody in tassels. Spore formation was only observed occasionally, and rarely the plant died due to the infection. Three independent infections were carried out per strain, mock treated plants were infected with water as control and at least three independent deletion strains were tested for virulence.

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Results

Candidate effector genes are less conserved between species compared to other genes

We reconstructed families of homologous genes for the five smut fungi *U. hordei*, *U. maydis*, *S. scitamineum*, *S. reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum* using the SiLiX clustering algorithm (Miele et al. 2011). We optimized the clustering parameters to maximize the occurrence of orthologues and minimize the number of paralogues within each family. In this way, we were able to reconstruct 8,761 families, among which 5,266 had at least one gene in each species (supplementary table S6). As a consequence, we found at least one homologous sequence in four species for 78 % of all genes. 5,254 gene families are found to have exactly one member in each species and were therefore taken as true orthologues (referred to as “core orthologous set” in the following). Considering that secreted proteins are putative effectors, we used SignalP (Petersen et al. 2011) to predict secretion of the encoded protein for each gene (supplementary table S1). We report that 920 (11 %) families contained only genes encoding a predicted secreted protein, while 7,657 (87 %) contained only genes encoding a protein not predicted to be secreted. The remaining 184 (2 %) families contained both predicted secreted and cytoplasmic proteins (supplementary table S6). The occurrence of families with both secreted and cytoplasmic proteins can be explained by (1) false negative predictions for secretion, as truncated C-terminal sequences were not removed from the data set, (2) wrong gene annotations or (3) gain or loss of a secretion signal peptide during effector evolution (Poppe et al. 2015). Among all predicted secreted proteins, 52 % have at least one orthologue in all other species, which is significantly less than the global 78 % proportion for all proteins (Chi-squared test, P value < 2.2×10^{-16}). Genes encoding putative effector proteins are therefore less conserved across species than other genes, either because their sequence is

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418 evolving faster, preventing the recovery of homologous relationships, or because effector
419 genes are created or lost at a higher rate. In *U. hordei*, we observe several species-specific
420 family expansions. There were 17 families which encompassed five to 25 members, but no
421 orthologue in other species (supplementary table S6). Moreover, we identified three families
422 with up to 62 members in *U. hordei*, but only one member in up to three of the other species
423 (supplementary table S6). Gene duplications in *U. hordei* have been hypothesized to be driven
424 by mobile elements (Laurie et al. 2012).
425
426 *The genomes of S. reilianum f. sp. zeae and S. reilianum f. sp. reilianum diverged around one*
427 *million years ago*
428 To establish a frame for our comparative analysis we first calculated sequence similarity of
429 the five smut fungi for non-coding intergenic and protein sequences. In addition, we estimated
430 divergence times by performing a molecular dating analysis based on the core orthologues set
431 of the five pathogens, using advanced models of protein sequence evolution and Bayesian
432 inference as implemented in the PhyloBayes package (Lartillot et al. 2009). As calibration
433 point, we used the divergence time of *U. hordei* and *U. maydis*, previously estimated to be
434 between 27 and 21 Myr (Bakkeren and Kronstad, 2007). In alignable intergenic regions *U.*
435 *hordei* shares 57 % identity with *S. reilianum* f. sp. *reilianum* and 77 % identity in protein
436 sequences (fig. 1B). Monte-Carlo Markov chains were run for three independent gene
437 samples totaling more than 20,000 amino acid positions each, and two chains were run in
438 each case to assess convergence. The resulting posterior distribution of divergence times were
439 used to infer 95 % posterior intervals. The split between *U. maydis* and the *Sporisorium*
440 species was estimated to have occurred around 20 Myr ago (95 % posterior interval 25 to 12
441 Myr; fig 1A and supplementary table S2). *Sporisorium reilianum* f. sp. *reilianum* shares 61 %
442 nucleotide identity in alignable intergenic regions with *U. maydis*, and 79 % sequence identity

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443 at the protein level (fig. 1B). The divergence times of *S. scitamineum* and the two formae
444 speciales of *S. reilianum* were calculated to be 13 Myr ago (95 % posterior interval 19 to 7
445 Myr; fig. 1A and supplementary table S2), which is consistent with the mean divergence
446 estimated between the hosts sorghum and sugarcane (10 Myr with a posterior interval of 8 to
447 13 Myr, average over eight studies, source: timetree.org (Kumar et al. 2017)). *Sporisorium*
448 *reilianum* f. sp. *reilianum* and *S. scitamineum* share 74 % non-coding nucleotide identity and
449 88 % identity at the protein level (fig. 1B). Finally, the two *S. reilianum* strains diverged 1.1
450 Myr ago (95 % posterior interval 2.4 to 0.4 Myr; fig. 1A) and share 98 % noncoding
451 nucleotide identity and 99 % protein identity (fig. 1B). We note that the estimation of this
452 divergence date varied with the gene set used, and was in some cases found to be older (1.7
453 Myr, with a 95 % posterior interval of 4.7 to 0.6 Myr, see supplementary table S2). The
454 comparison of the five smut genomes therefore encompasses a broad evolutionary time, and
455 the divergence times obtained are compatible with previous estimates from smaller data sets
456 (Munkacsi et al., 2007). The speciation times of the investigated smut species largely predate
457 the 10,000 years of crop plant domestication, which implies that adaptation to the agricultural
458 host, if any, will be negligible when interpreting the inter-specific patterns of sequence
459 divergence, as it represents a marginal proportion of the time since the divergence from the
460 ancestral species.
461
462 *Sporisorium reilianum contains the largest number of positively selected genes*
463 To detect positive selection, 6,205 families with at least three members (orthologues and/or
464 paralogues, see supplementary table S6) were, regardless of their species composition, aligned
465 on the codon and amino acid level and a phylogenetic tree was inferred. Obtaining accurate
466 alignments is critical for detecting positive selection since alignment errors frequently inflate
467 the false discovery rate (Schneider et al. 2009; Jordan and Goldman 2012). We therefore

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developed a stringent bioinformatics pipeline for the filtering of sequence alignments by masking ambiguous alignment positions for further analysis (see Methods). To scan for positive selection, we employed a non-homogeneous model of sequence evolution allowing d_N/d_S ratios to vary along the phylogeny, in combination with two heuristic model selection procedures to avoid over-parametrization issues (Nielsen and Yang 1998; Dutheil et al. 2012). Model parameters could not be fitted by either one of the two methods in 1.7% of branches. The two model selection procedures led to highly consistent estimates of branch-specific d_N / d_S ratios (Spearman's rank correlation coefficient equal to 0.85, p-value < $2.2x10^{-16}$). The distribution of d_N / d_S was highly skewed with a median value of 0.06, demonstrating the strong predominance of purifying selection throughout lineages and genes. The mean value of d_N / d_S ratios for lineages undergoing positive selection (d_N / d_S >1) was 4.1 (median 1.9). While a d_N / d_S ratio above one is indicative of positive selection, the absolute value of the ratio is a poor indicator of the strength of undergoing selection. In particular, high ratio values can be obtained because of low d_S values, and the d_N rate might include neutral substitutions, such as non-synonymous substitution that are conservative regarding certain biochemical properties of the amino-acids involved (Sainudiin et al. 2005). The largest number of genes with signs of positive selection was found in *S. reilianum* f. sp. *zeae* (84 genes, of which 25 encode predicted secreted proteins) and *S. reilianum* f. sp. *reilianum* (111 genes of which 27 encode predicted secreted proteins) (fig. 1C). In addition, a substantial number of positively selected candidate genes was also found in *U. hordei* (49, and of these, 22 genes are predicted to code for secreted proteins), but only very few in *U. maydis* (2 genes) and *S. scitamineum* (7 genes) (fig. 1C). A list of all proteins with their associated d_N/d_S ratios in each species is provided in supplementary table S3. Predicted secreted proteins were significantly enriched in the group of proteins under positive selection in *U. hordei* and in the two investigated formae speciales of *S. reilianum* (*P* values < 10^{-5}; Fisher's exact test). This corroborates results of

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earlier studies in other pathosystems that showed that predicted secreted proteins are often under positive selection, which can be attributed to their direct interaction with host proteins (Joly et al., 2010; Wicker et al. 2013; Poppe et al., 2015). Notably, all genes found under positive selection in the two strains of *S. reilianum*, in *S. scitamineum* and in *U. maydis* share orthologous genes in the other species (supplementary tables S3 and S6). In contrast, genes with signs of positive selection in *U. hordei* belong largely (36 out of 49 genes) to families showing species-specific expansions (supplementary tables S3 and S6).

Genes under positive selection in U. hordei are associated with uncharacterized interspersed repeats

Among the species compared here, the genome of *U. hordei* shows the highest fraction of repetitive elements (Laurie et al. 2012; Dutheil et al. 2016). Such elements are known to contribute to gene family expansions (Kazazian 2004), and have been suggested to contribute to adaptation by providing advantageous mutations, for instance by repeat-induced point mutations (RIP) leakage which was revealed in a species complex of *Leptosphaeria* (Rouxel et al. 2011; Grandaubert et al., 2014). As sequence signatures of RIP were found in LTR elements of *U. hordei* (Laurie et al., 2012), we tested whether genes under positive selection in *U. hordei* are physically associated with repetitive elements. We performed a binary logistic regression with the prediction of positive selection as a response variable (that is, whether the underlying branch has a d_N/d_S ratio higher than one) and we considered three putative explanatory variables for each analyzed gene: (1) whether the gene is predicted to encode a secreted protein, (2) whether the gene is duplicated and (3) the distance of the gene to the closest interspersed repeat. The complete linear model explains 50 % of the observed variance, and the three explanatory variables are all significant at the 0.1 % level (supplementary table S7). These results suggest that positively selected genes in *U. hordei* are

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associated with duplication events, and positive selection is more likely to occur at genes encoding putative effectors. In addition, the proximity of interspersed repeats increases the odds of positive selection, independently of the two other effects, and is confirmed by a stratification approach: the effect still holds when only duplicated genes are considered, or only genes encoding a secreted protein, or the combination of the two (supplementary table S7). This finding corroborates previous results obtained in other microbial plant pathogens where it was described that effector genes tend to localize in repeat rich regions and where it was suggested that such regions contribute to the rapid evolution of effector genes (Raffaele and Kamoun, 2012).

Positively selected genes encoding cytoplasmic proteins in S. reilianum and U. hordei

While we expect effector genes to be under positive selection, we find that the majority of positively selected genes in *S. reilianum* encodes cytoplasmic proteins (fig. 1C). To assess the putative functional role of these genes, we performed a Gene Ontology term enrichment analysis, comparing cytoplasmic proteins under positive selection to cytoplasmic proteins not under positive selection (table 1). This analysis revealed that genes with a potential role in metabolic processes, like sulfur compound metabolism, molybdopterin cofactor metabolic process, RNA metabolic process, organic cyclic compound metabolic process and oxidoreductase activity, as well as responses to starvation and extracellular stimuli are significantly overrepresented at the 5% level (Fisher's classic test with Parent-Child correction; see table 1). This could indicate that cytoplasmic proteins under positive selection contribute to metabolic changes which might be needed to survive with the limited nutrients available on the surface or in the biotrophic interface of different host plants. A similar analysis for cytoplasmic proteins under positive selection in *U. hordei* was conducted, but only led to top-level categories (DNA integration, DNA metabolic process and isomerase activity; see table 1).

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543

544 *Virulence contribution of effector genes showing signs of positive selection in S. reilianum*

545 Candidate effector genes inferred to be under positive selection in a particular species could
546 play a critical role in pathogenicity. Therefore, we sought to assess the contribution to
547 virulence of such candidate genes by creating individual deletion mutants. In total, we tested
548 nine candidate genes with high d_N/d_S ratios and predicted to encode secreted proteins: three
549 with signatures of positive selection only in *S. reilianum* f. sp. *zeae*, three with signatures of
550 positive selection in *S. reilianum* f. sp. *zeae* as well as in *S. reilianum* f. sp. *reilianum* and
551 three with signatures of positive selection only in *S. reilianum* f. sp. *reilianum*. All nine
552 chosen candidate genes together with their characteristics are summarized in table 2. Deletion
553 mutants were generated in the haploid solopathogenic strain JS161 of *S. reilianum* f. sp. *zeae*.
554 This strain is capable of colonizing maize plants and cause disease without a compatible
555 mating partner (Schirawski et al. 2010) but virulence is much reduced relative to infection
556 with mating-compatible wild-type strains and spores are only rarely produced. Deletion
557 mutants were also generated in strain JS161 in cases where positive selection was only
558 detected in *S. reilianum* f. sp. *reilianum* (table 2), because no solopathogenic strain is
559 presently available for *S. reilianum* f. sp. *reilianum*. For each gene at least three independent
560 deletion mutants were generated and tested for virulence. To determine virulence, Gaspe
561 Flint, a dwarf variety of corn, was infected and symptoms were scored in male and female
562 flowers (fig. 2). Only the deletion of *sr10529*, a gene showing positive selection in both
563 formae speciales of *S. reilianum*, showed a strong reduction in virulence (table 2 and fig. 2).
564 The gene *sr10529* in *S. reilianum* f. sp. *zeae* is orthologous to the previously identified and
565 characterized gene *pit2* (*UMAG_01375*) in *U. maydis*.
566 Pit2 plays an essential role in virulence as inhibitor of a group of maize papain-like cysteine
567 proteases that are secreted to the apoplast (Doehlemann et al. 2011; Mueller et al. 2013).

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Previous work identified a conserved domain of 14 amino acids (PID14) in Pit2 as required and sufficient for the inhibition of maize cysteine proteases (Mueller et al. 2013). When the branch-site model of PAML 4 (Yang 2007) was used to identify amino acid residues under positive selection in the Pit2 orthologues of the two *S. reilianum* species, only two residues residing in the PID14 domain were found under positive selection. However, 24 positively selected residues were detected outside this domain in the 57 amino acid long C-terminal part (fig. 3).

Discussion

We used evolutionary comparative genomics of five related smut fungi infecting four different host plants to identify genes with a signature of positive selection during species divergence, with a focus on genes encoding predicted secreted proteins, as such genes were suggested to contribute to virulence in various plant pathogenic microbes (Aguileta et al. 2010; Stukenbrock et al. 2011; Hacquard et al. 2012; Dong et al. 2014; Huang et al. 2014; Sharma et al. 2014). Our analysis revealed that positive selection is found between paralogous genes in *U. hordei,* where they belong to families with species-specific expansions. In contrast, genes under positive selection in the other four species belong to families of orthologous sequences. While we find evidence for a large set of genes under positive selection in the *S. reilianum* species, signatures for positive selection are hardly detectable in the more distant relatives *U. hordei, U. maydis* and *S. scitamineum* that diverged earlier. Finding evidence for positive selection over time spans of several millions of years is notoriously difficult (Gillespie 1994) because of two main reasons: (1) periods where genes

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593 are evolving under positive selection may occur episodically and may be followed by long
594 episodes of purifying selection, leading to an average d_N/d_S below 1 on long periods of time
595 and (2) fast evolving genes may diverge to an extent where their homology is difficult to infer
596 and where they can no longer be aligned reliably. To overcome this problem, more genome
597 information of species with intermediate branching points is needed (Gillespie 1994).
598 Predicted secreted proteins were about three times overrepresented in the set of positively
599 selected genes, which illustrates the importance of secreted proteins in adaptation processes of
600 smut fungi. This also corroborates results in other plant pathogenic microbes like *Melampsora*
601 *sp.*, *Z. tritici* and the wheat powdery mildew *Blumeria graminis* (Joly et al. 2010; Stukenbrock
602 et al. 2011; Wicker et al. 2013). However, the majority of positively selected genes encodes
603 cytoplasmic proteins (fig. 1C), suggesting that both secreted and non-secreted proteins are
604 important targets of adaptation. A Gene Ontology analysis in *S. reilianum* showed that mainly
605 processes related to metabolism and its regulation as well as responses to starvation and
606 external stimuli are enriched in cytoplasmic proteins under positive selection. This points at a
607 role of these proteins in adaptation to differences in nutrient availability in the respective host
608 plants maize and sorghum as well as responses to cues originating from the respective host
609 (Haueisen and Stukenbrock 2016). A study conducted in *U. maydis* has shown that the fungus
610 induces major metabolic changes in the host plant upon infection during establishment of
611 biotrophy and undergoes a series of developmental transitions during host colonization that
612 are likely influenced by the host environment (Doehlemann et al. 2008). It is thus conceivable
613 that the two *S. reilianum* accessions have adapted to their different hosts that differ
614 significantly for example in their amino acid and vitamin composition (Etuk et al. 2012).
615 Furthermore, recent studies in *U. maydis* suggested that intracellular changes of metabolism
616 influence virulence, and therefore the underlying proteins could be targets of positive
617 selection (Kretschmer et al. 2012; Goulet et al. 2017).

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618 Out of nine deletions of positively selected genes, only one mutant, lacking *sr10529,* was
619 affected in virulence. While six of the deleted genes are single genes in *S. reilianum* f. sp.
620 *zeae* for which we failed to identify paralogs, *sr12084* has two paralogs, *sr14347* has five
621 paralogs and *sr10182* has ten paralogs. We restricted our analyses to generating deletion
622 mutants in some of the genes under positive selection. This leaves open the possibility that the
623 paralogous genes have redundant functions in virulence. Adapting the CRISPR-Cas9
624 technology allowing multiplexing (Schuster et al. 2017) to *S. reilianum* will be instrumental
625 in testing this hypothesis in future studies. Alternatively, the candidate effectors we
626 investigated may be needed under conditions which differ from those tested here. For
627 example, *S. reilianum* f. sp. *zeae* can also systemically colonize maize plants via root
628 infection (Mazaheri-Naeini et al. 2015), a colonization route we have not assessed in our
629 experimental setup. Moreover, we employed only one maize cultivar for infection assays.
630 Results from other pathosystems suggest that virulence effects can strongly depend on the
631 host and pathogen genotypes, in particular in the presence of *R* and *avr* genes (Petit-Houdenot
632 and Fudal, 2017). No *avr*-*R* gene interaction was described so far in the *S. reilianum* f. sp.
633 *zeae*-maize pathosystem. Instead, quantitative virulence differences are observed when
634 different host cultivars are infected (Lübberstedt et al. 1999). Knowing the expression profile
635 of effector genes may assist the identification of differences in development of the mutants
636 compared to wild type strains. Since we lack this information, we scored disease symptoms
637 only in the inflorescences about nine weeks after infection. Additionally, it may be possible
638 that small differences in virulence between JS161 and deletion mutants of candidate genes
639 remain undetected due to the weak infection behavior of JS161. For example, spore formation
640 is only rarely observed after infecting maize plants with the solopathogenic strain. In contrast,
641 infections resulting from infections with two compatible haploid strains show spores in about
642 40 % of the infected plants (Zuther et al. 2012). This means that defects related to spore

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643 formation will not be evident in mutants of JS161. In three cases positive selection was
644 detected in orthologous genes in *S. reilianum* f. sp. *reilianum* while candidate effector genes
645 were for experimental reasons deleted in *S. reilianum* f. sp. *zeae*. Therefore, it cannot be
646 excluded that these effectors might have a virulence function in *S. reilianum* f. sp. *reilianum.*
647 In this case, the positively selected effector genes might have evolved during adaptation to the
648 sorghum host and present host specificity genes. In summary, our virulence assays leave open
649 the possibility that the eight candidate genes which did not show a contribution to virulence
650 could play a role in pathogenicity under conditions not tested here. Alternatively, candidate
651 effector proteins might also be positively selected for traits that are not directly linked to
652 pathogenicity. Such traits could for instance involve competition with large numbers of other
653 plant colonizing microbes (Zhan and McDonald 2013; Rovenich et al. 2014). Secreted
654 proteins of *S. reilianum* could act for example as toxin or could efficiently utilize resources
655 from the environment and thereby limit the growth of other microbes. In these cases, a
656 contribution to virulence is not expected to be observed in the employed infection assay with
657 the effector gene mutants. Moreover, our molecular dating analysis showed that the common
658 ancestors of the investigated smut species originated before the beginning of crop
659 domestication. Therefore, positive selection, whose signs we detect by our approach, has most
660 likely occurred on ancestral host plants and not on the domesticated host maize.
661 Consequently, some of the candidate effector genes under positive selection might not be
662 important for the colonization of crop plants, but for infection of related wild species.
663 In *U. maydis*, we note that effector genes residing in clusters whose deletion affected
664 virulence (Kämper et al. 2006; Schirawski et al. 2010) have similar d_N/d_S ratios as effector
665 genes in clusters where the deletion had no effect on virulence (median d_N/d_S ratio 0.0619
666 vs.0.1094; Wilcoxon rank test with *P* value = 0.1848). Furthermore, orthologues of the
667 effectors Pep1, Stp1 and Cmu1, which were shown to have important roles in pathogenicity of

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668 *U. maydis* (Djamei et al. 2011; Doehlemann et al. 2009; Schipper 2009) showed no signature
669 of positive selection. These observations could suggest that certain fungal effector proteins
670 are under evolutionary constraint and are therefore not free to accumulate non-synonymous
671 mutations. Such effectors are conserved over long time spans (Schirawski et al. 2010;
672 Hemetsberger et al. 2015; Sharma et al. 2015) and this illustrates that they are instrumental
673 for successful infections in a large group of smut fungi. They probably target molecules
674 shared by several host plants, for example housekeeping functions that cannot easily evolve in
675 response to the binding of an effector.
676 One candidate gene (*sr10529*) under positive selection in both formae speciales of *S.*
677 *reilianum* showed a strong contribution to virulence upon deletion. It is orthologous to the
678 previously described protease inhibitor Pit2 in *U. maydis*, where the deletion also abolished
679 virulence (Doehlemann et al. 2011; Mueller et al. 2013). Positively selected residues in the
680 PID14 domain of Pit2 might reflect that different proteases need to be inhibited in maize and
681 sorghum. Pit2 might thus contribute to determining the host range of the respective species. A
682 role of cysteine protease inhibitors in host specificity was demonstrated in *Phytophthora*
683 *infestans*, a pathogen of potato and its sister species *Phytophthora mirabilis*, which infects the
684 ornamental plant *Mirabilis jalapa*. Positively selected orthologous protease inhibitors were
685 shown to inhibit proteases specific to the respective host plants and this specificity could be
686 traced back to a single amino acid substitution (Dong et al. 2014). Surprisingly, 24 positively
687 selected sites in Pit2 were detected outside the PID14 domain in the 57 amino acid long C-
688 terminal part in both *S. reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum*. This finding
689 raises the intriguing possibility that the C-terminus of Pit2 might possess a second function
690 that is independent of protease inhibition. Earlier work has shown that the *pit1* gene encoding
691 a transmembrane protein is located next to the *pit2* effector gene and both genes contribute
692 similarly to virulence (Doehlemann et al. 2011). Furthermore, *pit1* and *pit2* are divergently

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693 transcribed, which makes it likely that the expression of *pit1* and *pit2* is co-regulated. In
694 addition, this gene arrangement of *pit1* and *pit2* is conserved in *U. hordei*, *U. maydis*, *S.*
695 *scitamineum* and *S. reilianum* (Sharma et al. 2015). This finding has led to the speculation
696 that Pit1 and Pit2 somehow act together to govern virulence of *U. maydis* and related smut
697 fungi. It was hypothesized that that Pit2 shuttles apoplastic maize proteins towards Pit1,
698 thereby scavenging damage-associated molecules (Doehlemann et al. 2011). In this scenario,
699 the positively selected amino acids in the C-terminus of Pit2 could have been selected for
700 scavenging such molecules as adaptation to the two hosts. In future studies it will be highly
701 interesting to complement the *pit2* mutant of *S. reilianum* f. sp. *zeae* with the *pit2* orthologue
702 of *S. reilianum* f. sp. *reilianum* to see if this promotes virulence on sorghum.

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705 **Conclusions**

706 Screens for genes with signs of positive selection are commonly used to identify candidate
707 effector genes in various plant pathogenic microbes. However, it is currently largely open
708 whether positively selected effector genes play indeed a role in virulence. Here, we used
709 comparative genomics of five smut fungi and showed that only one out of nine genes under
710 positive selection contributes to virulence of *S. reilianum*. Moreover, the majority of
711 positively selected genes did not encode predicted secreted proteins. Our results leave open
712 the possibility that many genes with signatures of positive selection contribute to virulence
713 under conditions not tested in this study or are selected in traits that are not directly related to
714 pathogenicity.

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962 **Tables**

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964 **Table 1**: Gene Ontology terms significantly overrepresented in positively selected genes
965 encoding cytoplasmic proteins in *S. reilianum* and *U. hordei*

Gene Ontology Id	Gene Ontology Description	Category[a)]	*P* values[b)]	Species[c)]
GO:0030532	small nuclear ribonucleoprotein complex	CC	0.023	Srr
GO:0045263	proton-transporting ATP synthase complex, coupling factor F(o)	CC	0.038 and 0.036	Srr and Srz
GO:0044425	membrane part	CC	0.047	Srr
GO:0031668	cellular response to extracellular stimulus	BP	0.015 and 0.013	Srr and Srz
GO:0051186	cofactor metabolic process	BP	0.015	Srr
GO:0042594	response to starvation	BP	0.017 and 0.012	Srr and Srz
GO:0009267	cellular response to starvation	BP	0.020 and 0.014	Srr and Srz
GO:0006790	sulfur compound metabolic process	BP	0.022	Srr
GO:0051301	cell division	BP	0.023	Srr
GO:0006777	Mo-molybdopterin cofactor biosynthetic process	BP	0.027	Srr
GO:0009605	response to external stimulus	BP	0.027 and 0.012	Srr and Srz
GO:0043545	molybdopterin cofactor metabolic process	BP	0.034	Srr
GO:0043413	macromolecule glycosylation	BP	0.036	Srr
GO:0022402	cell cycle process	BP	0.037	Srr
GO:0051189	prosthetic group metabolic process	BP	0.039	Srr
GO:0006139	nucleobase-containing compound metabolic process	BP	0.012	Srz
GO:0016070	RNA metabolic process	BP	0.023	Srz
GO:1901360	organic cyclic compound metabolic process	BP	0.016	Srz
GO:0006725	cellular aromatic compound metabolic process	BP	0.024	Srz
GO:0046483	heterocycle metabolic process	BP	0.023	Srz
GO:0035383	thioester metabolic process	BP	0.041	Srr
GO:1902589	single-organism organelle organization	BP	0.048	Srr
GO:0015074	DNA integration	BP	0.039	Uh
GO:0006259	DNA metabolic process	BP	0.023	Uh
GO:0010181	FMN binding	MF	0.047 and 0.022	Srr and Srz
GO:0030515	snoRNA binding	MF	0.021	Srz
GO:0016491	oxidoreductase activity	MF	0.028	Srz
GO:0016853	isomerase activity	MF	0.029	Uh

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968 [a)] CC, Cellular Component; BP, Biological Process; MF, Molecular Function.

969 [b)] calculated by Fisher's classic test with Parent-Child correction. Only entries with *P* value ≤
970 0.05 are shown.

971 [c)] Species in which GO terms were found enriched. Srr, *S. reilianum* f. sp. *reilianum*; Srz, *S.*

972 *reilinaum* f. sp. *zeae*; Uh, *U. hordei*.

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Table 2: Positively selected genes that were deleted in *S. reilianum* f. sp. z*eae* and their selection criteria

Family	Gene Id	Gene description	d_N/d_S	Species with detected positive selection[a)]	Number of Paralogs in Srz[b)]	Virulence phenotype of candidate gene deletion	Closest ortholog in *U. maydis*[c)]
FAM001428	*sr10529*	conserved hypothetical protein	31.1469	Srz and Srr	0	virulence abolished	*UMAG_01375* (*pit2*)[6)]
FAM005472	*sr10059*	conserved hypothetical *Ustilaginaceae*-specific protein	6.53881	Srz and Srr	0	virulence unaffected	*UMAG_05306* (cluster 19A)[7)]
FAM000532	*sr10182*	conserved hypothetical protein	1.57473	Srr	10[3)]	virulence unaffected	*UMAG_00492*
FAM002067	*sr12968*	conserved hypothetical protein	37.9007	Srr	0	virulence unaffected	*UMAG_02006*
FAM003728	*sr14558*	conserved hypothetical protein	24.355	Srz	0	virulence unaffected	*UMAG_03564*
FAM004113	*sr14944*	conserved hypothetical *Ustilaginaceae*-specific protein	4.30527	Srz and Srr	0	virulence unaffected	*UMAG_04034* (cluster 11-16)[8)]
FAM003465	*sr14347*	conserved hypothetical protein	544.37	Srz	5[4)]	virulence unaffected	*UMAG_03349*
FAM001868	*sr12897*	conserved hypothetical protein	infinite[1)]	Srr	0	virulence unaffected	*UMAG_01820*
FAM000842	*sr12084*	conserved hypothetical protein	infinite[2)]	Srz	2[5)]	virulence unaffected	UMAG_00792 (cluster 1-32)[8)]

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985 [a)] species are *S. reilianum* f. sp. *zeae* (Srz) and *S. reilianum* f. sp. *reilianum* (Srr)

986 [b)] based on blastp search with an e-value cutoff of 0.001

987 [c)] based on blastp search

988 [1)] infinity due to low value of d_S

989 [2)] infinity due to long branch for *sr12085*, a species-specific duplicate in *S. reilianum* f. sp.
990 *zeae*

991 [3)] the ten paralogs include: *sr13431*, *sr11876*, *sr16607*, *sr11405*, *sr10621*, *sr16723*, *sr16877*,
992 *13293*, *sr11163.2* and *sr15970*

993 [4)] the five paralogs include: *sr12257*, *sr11661*, *sr13976*, *sr14607* and *sr11273*

994 [5)] the two paralogs include: *sr12085* and *sr12086*

995 [6)] as described in Doehlemann et al., 2011 and Mueller et al., 2013

996 [7)] as described in Kämper et al., 2006

997 [8)] as described in Schirawski et al., 2010

998

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999 **Figure legends**

1000 **FIG. 1.** Phylogeny, divergence estimates and number of genes under positive selection in five
1001 related smut fungal species parasitizing different host plants. A) Chronogram of the five
1002 fungal pathogens as estimated under a relaxed molecular clock. Boxes represent 95%
1003 posterior intervals, with corresponding values indicated below. B) Pairwise sequence
1004 differences, for both the non-coding genome and the proteome (non-synonymous differences).
1005 C) Number of positively selected genes on each terminal branch (total number of genes and
1006 genes predicted to encode a secreted protein).

1007

1008 **FIG. 2.** Virulence phenotypes of single deletion mutants of positively selected genes in *S.*
1009 *reilianum* f. sp. *zeae*. Plants of the maize variety 'Gaspe Flint' were infected with the
1010 solopathogenic strain JS161 or independent deletion mutants of candidate genes as indicated
1011 below each bar. Deletion of *sr10529* led to a strong reduction in virulence (A). In contrast,
1012 deletion of the candidate genes *sr12968* (B), *sr14944* (C), *sr10059* (D), *sr10182* (E), *sr14558*
1013 (F), *sr14347* (G), *sr12897* (H), and *sr12084* (I) did not alter virulence. Symptoms were scored
1014 about 9 weeks post infection and categorized according to severeness as illustrated in the
1015 legend below the bar plots. Results are shown as mean of three independent experiments in
1016 relation to the total number of infected plants, which is indicated above each bar (n). Note that
1017 strains JS161ΔSr10529 #G4 and #G5 (A) were only infected in one replicate.

1018

1019

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FIG. 3. Distribution of positively selected amino acids in the cysteine protease inhibitor Pit2. The alignment shows the protein sequences of orthologues in *U. hordei* (UHOR_02064), *U. maydis* (UMAG_01375), *S. scitamineum* (SPSC_03677), *S. reilianum* f. sp. *zeae* (sr10529) and *S. reilianum* f. sp. *reilianum* (srs_10529). Sites under positive selection detected by a branch-site model are indicated by colored bold letters. Residues colored in red indicate positive selection detected in the respective species and purple residues indicate sites found under positive selection in both species. The yellow shaded area is orthologous to the previously identified conserved PID14 domain, which is required and sufficient for inhibition of a group of papain-like cysteine proteases. Green sequences indicate secretion signal peptides and bold numbers above the alignment indicate positions in UHOR_02064.

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List of supplementary tables

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Supplementary table S4: List of strains of *S. reilianum* f. sp. *zeae* used in the present study

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Supplementary figure 1. Number of families consisting of 1:1 orthologues in relation to varying settings for coverage and identity in the clustering program SiLiX. The maximum number of families containing 1:1 orthologues can be obtained with a coverage between 5 % and 45 % and an identity of 40 %.

Supplementary figure 2. Trace of the Monte-Carlo Markov chains for 3 gene samples (see Methods). Vertical lines show the burning phase (10,000 iterations)

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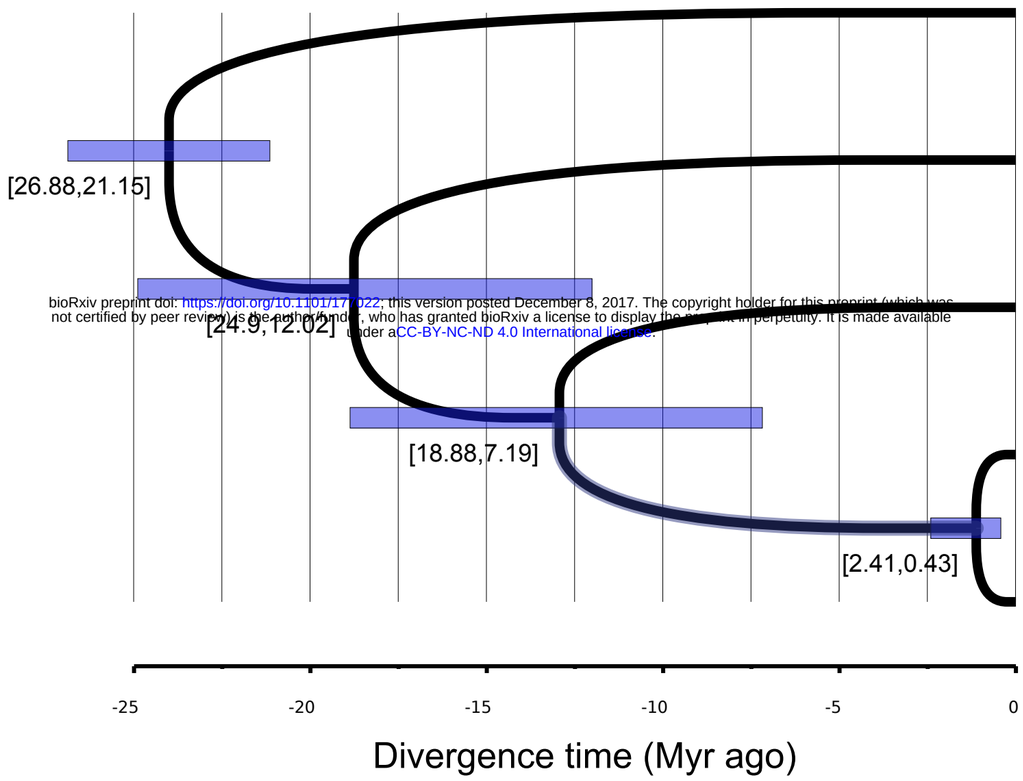
A



U. hordei

U. maydis

S. scitamineum

S. reilianum f. sp. *zeae*

S. reilianum f. sp. *reilianum*

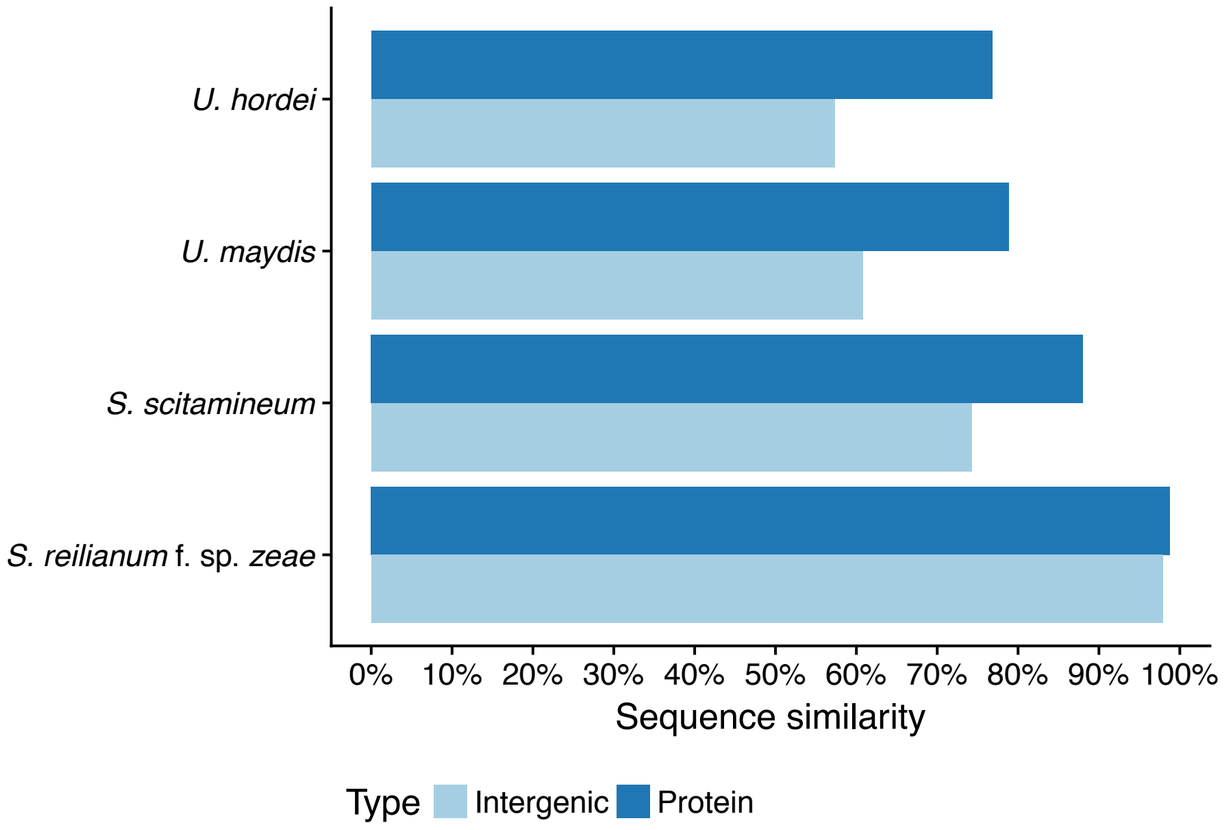
Barley

Maize

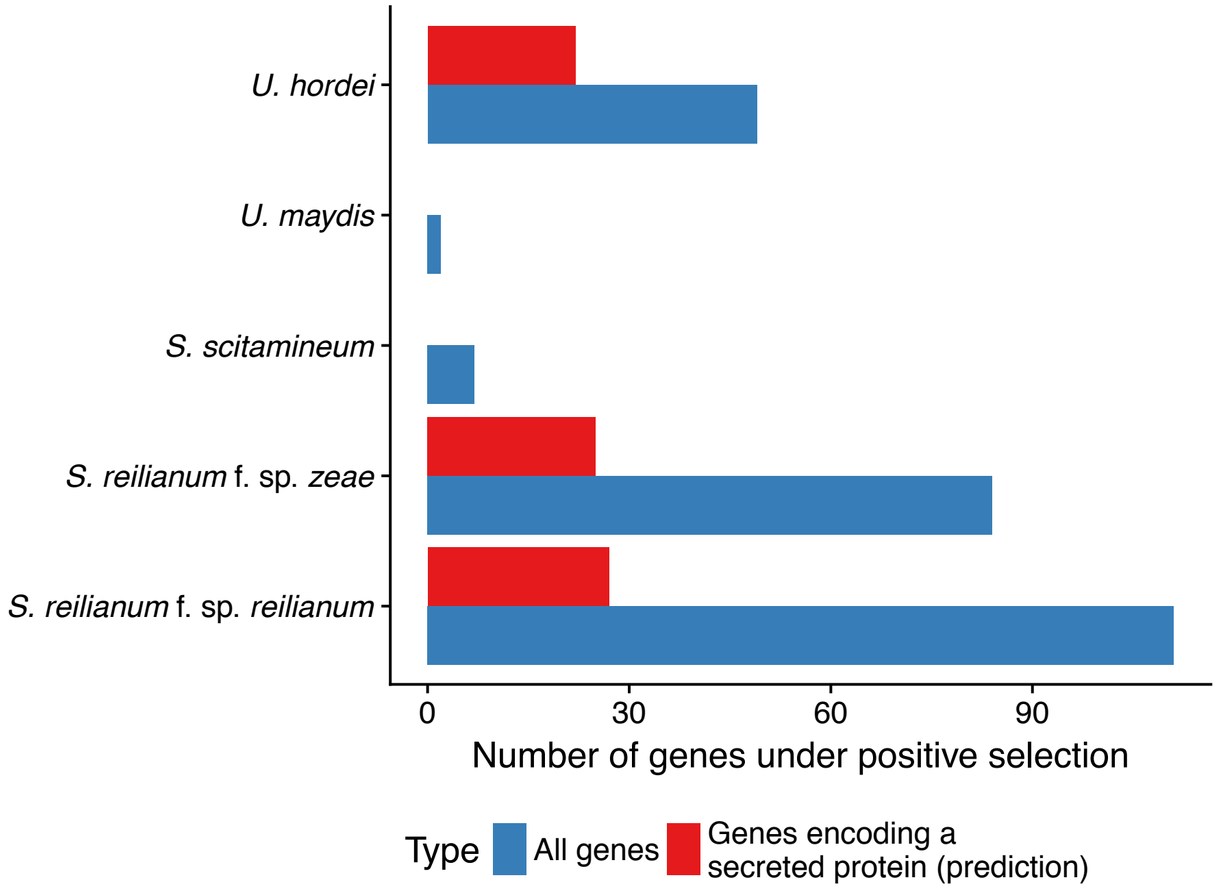
Sugar cane

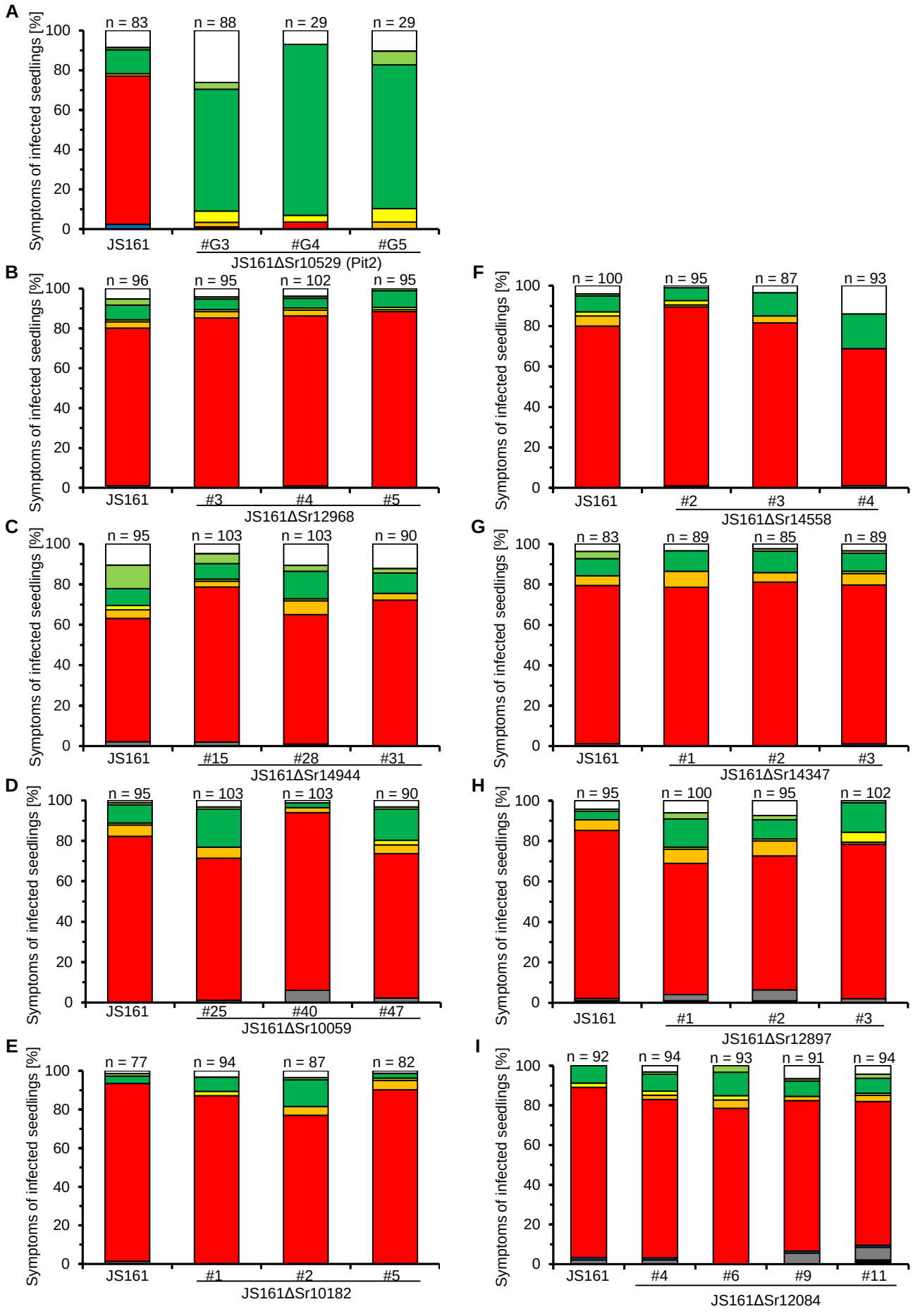
Sorghum

B



C




A
Symptoms of infected seedlings [%]
n = 83
n = 88
n = 29
n = 29
JS161
#G3
#G4
#G5
JS161ΔSr10529 (Pit2)
B
n = 96
n = 95
n = 102
n = 95
JS161
#3
#4
#5
JS161ΔSr12968
C
n = 95
n = 103
n = 103
n = 90
JS161
#15
#28
#31
JS161ΔSr14944
D
n = 95
n = 103
n = 103
n = 90
JS161
#25
#40
#47
JS161ΔSr10059
E
n = 77
n = 94
n = 87
n = 82
JS161
#1
#2
#5
JS161ΔSr10182
F
n = 100
n = 95
n = 87
n = 93
JS161
#2
#3
#4
JS161ΔSr14558
G
n = 83
n = 89
n = 85
n = 89
JS161
#1
#2
#3
JS161ΔSr14347
H
n = 95
n = 100
n = 95
n = 102
JS161
#1
#2
#3
JS161ΔSr12897
I
n = 92
n = 94
n = 93
n = 91
n = 94
JS161
#4
#6
#9
#11
JS161ΔSr12084
plant without ears
healthy ears ≤ 1 cm
healthy ears > 1 cm
spiky ears
phyllody in ears ≤ 1 cm
phyllody in ears > 1 cm
phyllody in tassels
spore formation
dead plants

```
                                  20                                  40
UHOR_02064   MLHYLGRLFLVAALAVACLR PATQ---NRPLRRAIV---- -GDNNDNYITKLHRRWYFLW
UMAG_01375   MLFRSAFVLLIVAFASACLV QHVQAI---PVRRSLSTDAS M----SSAAGKLNRRWWFGF
SPSC_03677   MLVHSAP-AFIATLVALCLA QHVQAIQLPAIRRSLTHNDD A--------ANLERRWFWNF
srs_10529    MLVHSAR-AFVAALL-LGLV LHVHAIQMPAMRRSLSSHAD AGAAGGSTLGKLARRWFFNF
sr10529      MLVHSAR-AFVAALL-LGLV LHVHA--MPAMRRSLSSHAD AGAAGGSTLGKLARRWFFDF

                 60                  80                 100
UHOR_02064   PGSLAPKPPREGEEHKIIYA DWIVHHDPAYNSNVQKEIEL ARLQNPTFIQVSVGESSSSS
UMAG_01375   TGSLGKEPDNGQVQIKIIPD ALIIKNPPANKDDLNKLIEN LKRKHPRFKTVVMPTDPNGD
SPSC_03677   GSSLGRSPDNN---ALIVPE DMIKKHTAALVTEWQTYLNE MHRQHPNWKRIDWRDDGPAG
srs_10529    GGSLAPLDAVP---IFEIPK SLIKTHKPAEVTKWEVFLQR VHRKHPDWTHVHWTTDGPVG
sr10529      GGALSRWDVAP---ILRIPE DVAKAHSRAEVARWEVYLER VHREHPDWQYVHWTDNGPIG

                 120
UHOR_02064   SSSSSKKS----- 120
UMAG_01375   VVIWE-------- 118
SPSC_03677   FARWESEKQGRSH 121
srs_10529    YKGH--------- 119
sr10529      YKSH--------- 117
```