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Host specificity in *Sporisorium reilianum* is determined by distinct mechanisms in maize and sorghum

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SUMMARY

Smut fungi are biotrophic plant pathogens that exhibit a very narrow host range. The smut fungus *Sporisorium reilianum* exists in two host-adapted formae speciales: *S. reilianum* f. sp. *reilianum* (SRS), which causes head smut of sorghum, and *S. reilianum* f. sp. *zeae* (SRZ), which induces disease on maize. It is unknown why the two formae speciales cannot form spores on their respective non-favoured hosts. By fungal DNA quantification and fluorescence microscopy of stained plant samples, we followed the colonization behaviour of both SRS and SRZ on sorghum and maize. Both formae speciales were able to penetrate and multiply in the leaves of both hosts. In sorghum, the hyphae of SRS reached the apical meristems, whereas the hyphae of SRZ did not. SRZ strongly induced several defence responses in sorghum, such as the generation of H_2O_2, callose and phytoalexins, whereas the hyphae of SRS did not. In maize, both SRS and SRZ were able to spread through the plant to the apical meristem. Transcriptome analysis of colonized maize leaves revealed more genes induced by SRZ than by SRS, with many of them being involved in defence responses. Amongst the maize genes specifically induced by SRS were 11 pentatricopeptide repeat proteins. Together with the microscopic analysis, these data indicate that SRZ succumbs to plant defence after sorghum penetration, whereas SRS proliferates in a relatively undisturbed manner, but non-efficiently, on maize. This shows that host specificity is determined by distinct mechanisms in sorghum and maize.

Keywords: defence responses, fluorescence microscopy, host specificity, phytopathogenic fungi, RNA sequencing, *Sporisorium reilianum*, transcriptome.

INTRODUCTION

The smut fungus *Sporisorium reilianum* causes head smut disease of maize and sorghum, two of the most important commercial cereals in the world. Although the fungus is a biotroph, the disease is devastating for the plant because it leads to complete harvest loss of the affected individual. In the field, the main sources of inoculum are soil-borne diploid teliospores which, under favourable conditions, undergo meiosis, germinate and give rise to haploid sporidia of different mating types (Halisky, 1963; Martinez *et al*., 2002). Compatible sporidia recognize each other through a pheromone–pheromone receptor system and form conjugation hyphae that grow towards each other and fuse at their tips (Schirawski *et al*., 2005). From then on, the fungus grows as a dikaryotic filament that penetrates and colonizes the plant without causing obvious symptoms (Martinez *et al*., 1999; Prom *et al*., 2011; Schirawski *et al*., 2010). When the fungus invades the undifferentiated floral tissue, the emerging inflorescence or flowers are replaced by sori containing black masses of teliospores (Wilson and Frederiksen, 1970). In this way, sexual proliferation of the fungus depends on successful host plant colonization.

Sporisorium reilianum exists in two formae speciales with different host preferences (Halisky, 1963; Zuther *et al*., 2012). *Sporisorium reilianum* f. sp. *reilianum* (SRS) is highly virulent on sorghum, but does not produce spores on maize, whereas *S. reilianum* f. sp. *zeae* (SRZ) is virulent on maize, but does not lead to head smut of sorghum. The inoculation of sorghum with SRZ induces the generation of the sorghum-specific phytoalexins luteolinidin and apigeninidin, of which luteolinidin has been shown to inhibit the vegetative growth of both formae speciales of *S. reilianum in vitro* (Zuther *et al*., 2012). This suggests that the formation of phytoalexins inhibits the spread of SRZ in sorghum. In contrast, no information is available regarding the inability of SRS to cause smut disease on maize.

New fungal diseases emerge as a result of host switching events and host range extensions of phytopathogenic fungi (Friesen *et al*., 2006; Giraud *et al*., 2010). It is thought that the original host of *S. reilianum* was sorghum and that the disease only later spread to maize (Halisky, 1963). The mechanism and conditions that allowed this host jump are unknown. For a few fungi, the mechanisms of host adaptation have been elucidated. In the necrotroph *Alternaria alternata*, virulence is dependent on the production of particular toxins, and the transfer of biosynthesis genes from one pathovar to another alters the host range (Akagi *et al*., 2009; Masunaka *et al*., 2005; Miyashita *et al*., 2001; Morisseau *et al*., 1999; Walton, 2000), whereas, in the hemibiotroph *Fusarium oxysporum*, lineage-specific

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regions containing four chromosomes occur (Ma *et al*., 2010). In *F. oxysporum* f. sp. *lycopersici*, host specificity has been associated with the presence of SIX (secreted-in-xylem) proteins that are secreted by the fungus into the plant xylem (Lievens *et al*., 2009; Takken and Rep, 2010). In *Magnaporthe oryzae* and *Phytophthora infestans*, host specificity has been related to PWL (pathogenicity on weeping lovegrass) and RXLR (arginine, any amino acid, leucine, arginine) effector proteins, respectively (Kang *et al*., 1995; Lee *et al*., 2014; Sweigard *et al*., 1995). Strains that are unable to cause disease on a particular plant typically induce a variety of plant defence responses, which include the generation of reactive oxygen species, the reinforcement of the cell wall and the generation or mobilization of antimicrobial substances (Ahuja *et al*., 2012; Heller and Tudzynski, 2011; Underwood, 2012). The accumulation of hydrogen peroxide (H_2O_2) can lead to cell wall strengthening, signal transduction or programmed cell death (Gadjev *et al*., 2008; Gilchrist, 1998; Hückelhoven, 2007; Kuźniak and Urbanek, 2000). In addition, the deposition of callose and lignin at sites of fungal penetration and cell-to-cell crossings acts as a physical barrier to block fungal entry and spread (Bhuiyan *et al*., 2009; Luna *et al*., 2011). The biosynthesis of antimicrobial compounds, such as phytoalexins, is also a very efficient strategy, protecting plant tissues against pathogen spread (Hammerschmidt, 1999).

The factors involved in the host specificity of *S. reilianum* are yet undiscovered. For the SRS–sorghum interaction, at least six different races are known (Prom *et al*., 2011), indicating the possible presence of a gene-for-gene resistance mechanism. A gene-for-gene resistance mechanism does not seem to be active in the SRZ–maize interaction, as no fully resistant maize lines have been described (Lübberstedt *et al*., 1999). The SRZ genome shows a marked paucity of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes potentially involved in the production of host-specific toxins (Schirawski *et al*., 2010). Instead, genome sequencing reveals the presence of hundreds of small secreted proteins suggested to contribute to host selection (Laurie *et al*., 2012; Schirawski *et al*., 2010; Wollenberg and Schirawski, 2014).

In this study, we investigated the differences between the two formae speciales of *S. reilianum* during plant infection. Using fluorescence microscopy and the quantification of fungal DNA, we showed that both SRS and SRZ are able to spread in both hosts, but to different extents. On sorghum, SRZ is strongly challenged by multiple plant defence compounds, whereas these compounds are only marginally induced by SRS. On maize, neither SRS nor SRZ induced strong plant defences, and both formae speciales proliferated well. Whole transcriptome analysis of colonized maize leaves revealed that SRZ induced more plant defence genes than did SRS. This excludes a mechanism of plant defence in the leaf for the inability of SRS to sporulate on maize and indicates that the host specificity of *S. reilianum* is achieved by different mechanisms in maize and sorghum.

RESULTS

SRS reaches the apical meristems in maize, whereas SRZ does not enter sorghum nodes

To identify the exact point at which SRS and SRZ cease to proliferate in the non-favoured host plant, we macro- and microscopically investigated maize and sorghum plants after inoculation with SRS or SRZ. We observed that SRS induced chlorosis on sorghum leaves, sori replacing apical meristems and spore-filled sori replacing inflorescences (Fig. S1A–C, see Supporting Information) in all investigated plants ($n = 19$). In contrast, SRZ induced phytoalexin deposition in sorghum leaves, whereas the apical meristems and inflorescences of all samples analysed ($n = 20$) were healthy (Fig. S1D–F). On maize, SRS induced chlorosis on leaves, and young cobs showed phyllody (Ghareeb *et al*., 2011) on about 70% of evaluated plants ($n = 20$), but there were no spores and the tassels were healthy (Fig. S1G–I). SRZ induced chlorosis and necrosis on maize leaves and resulted in about 60% of the young cobs carrying fungal spores and some tassels showing phyllody and spores (Fig. S1J–L).

Fluorescence microscopy of samples stained with wheat germ agglutinin (WGA)-Alexafluor revealed that both formae speciales efficiently colonized sorghum leaves at 4 days after inoculation (dai) ($n = 12$, Fig. 1A, B). However, whereas SRS clearly seemed to prefer bundle sheath cells (Fig. 1A), the hyphae of SRZ did not show such a preference and were abundant in mesophyll areas (Fig. 1B). At 9 dai, hyphae of SRS colonized bundle sheath cells and vascular bundles in inoculated leaves (Fig. 1C), whereas hyphae of SRZ were found in bundle sheath cells, but rarely in vascular cells (Fig. 1D). When the stem tissue was investigated at 15 dai, hyphae of SRS were readily visible in vascular bundles near the growing point of the plant (Fig. 1E), whereas the stem tissues of plants inoculated with SRZ were completely healthy (Fig. 1F).

Inoculated maize leaves showed colonization by SRS and SRZ at 4 dai ($n = 12$, Fig. 1G, H). However, in samples infected with SRS, apparently smaller amounts of hyphae were observed (Fig. 1G) when compared with SRZ (Fig. 1H). In maize stems, fungal proliferation was observed for both SRZ (Fig. 1J) and SRS (Fig. 1I), and SRS-infected stems showed several hyphae stained by propidium iodide (Fig. 1I, white arrows), indicating that they were dead or damaged (Hickey *et al*., 2004). These results indicate that both strains are able to proliferate from the inoculated leaves up to the nodes and meristems in maize, whereas only SRS can do so in sorghum.

SRS and SRZ show better proliferation in their compatible hosts

To confirm and quantify the differences observed by microscopy, we analysed the content of fungal genomic DNA relative to total

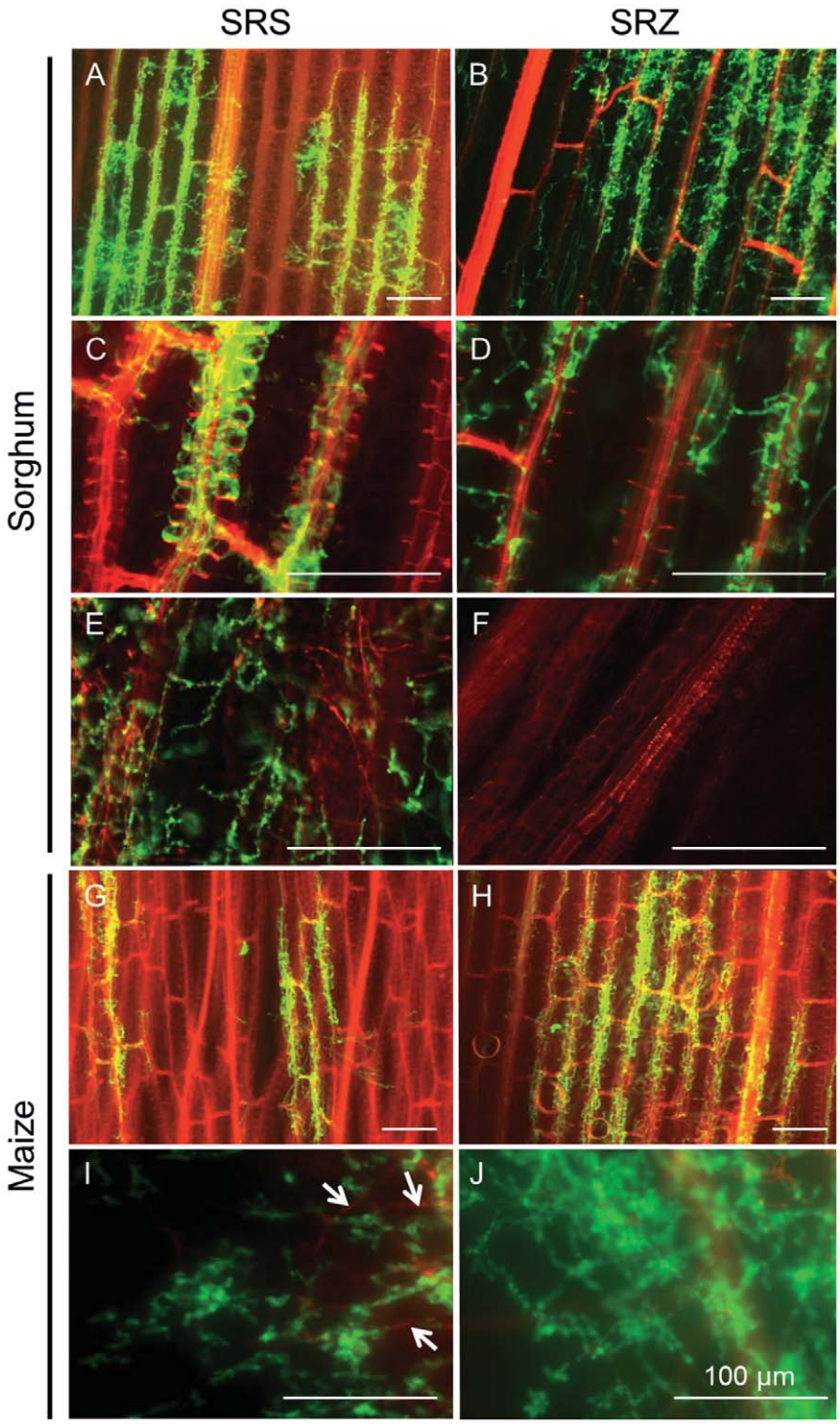

Fig. 1 Microscopic characterization of sorghum and maize infection by *Sporisorium reilianum*. Sorghum (A–F) and maize (G–J) seedlings were syringe inoculated with *S. reilianum* f. sp. *reilianum* (SRS) (left) or *S. reilianum* f. sp. *zeae* (SRZ) (right). Samples were collected at 4 days after inoculation (dai) (A, B, G, H), 9 dai (C, D) or 15 dai (E, F, I, J). Plant material and dead hyphae were stained with propidium iodide and appear red; fungal hyphae were stained with wheat germ agglutinin (WGA)-Alexafluor-488 and appear green. Sorghum samples inoculated with SRS show hyphae colonizing leaf tissues (A) and presenting a preference for vascular bundles (C), later reaching the nodes and apical meristems (E). SRZ infects leaves (B) and leaf sheaths (D), without showing such a preference for vascular bundles, and without reaching the nodes and apical meristems (F). Maize plants inoculated with SRS show hyphae colonizing leaf tissues (G) and reaching the nodes and apical meristem (I), where some dead hyphae are also observed (white arrows). Leaves inoculated with SRZ show extensive fungal growth (H) and the pathogen reaches the nodes and apical meristem (J). Bars, 100 µm; $n = 12$ plant samples.

plant DNA at 9 dai by quantitative polymerase chain reaction (qPCR). In sorghum, a similar quantity of fungal DNA was observed at the inoculation site, whereas a lower quantity of SRZ relative to SRS was measured in the ligules, leaf sheaths and stems (Fig. 2A). The relative amount of genomic DNA of SRZ decreased with increasing distance from the inoculation site, thus being undetectable in the stems (Fig. 2A). Therefore, the proliferation of SRZ seems to be effectively blocked during its spread in the inoculated leaf, and the fungus does not reach the nodes or apical meristems of sorghum.

In maize, the reverse result was obtained: although the level of genomic DNA was not statistically significantly different at the

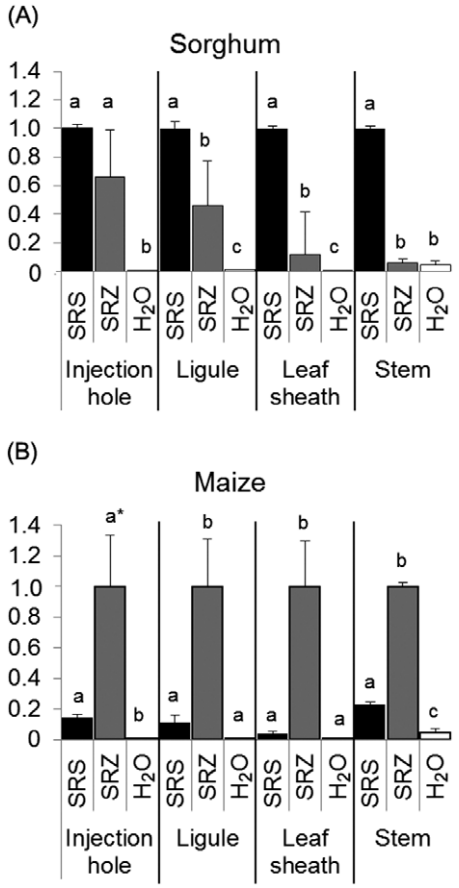

Fig. 2 Quantification of fungal biomass *in planta* by quantitative polymerase chain reaction (qPCR). Fungal DNA was quantified relative to plant DNA and relative to *Sporisorium reilianum* f. sp. *reilianum* (SRS) DNA (A) or to *S. reilianum* f. sp. *zeae* (SRZ) DNA (B). Samples from the inoculation site, ligule, leaf sheath and stems were collected at 9 days after inoculation (dai) from sorghum and maize. (A) In sorghum, a greater amount of DNA from SRS than from SRZ was detected in ligules, leaf sheaths and stems. (B) In maize, SRZ was dominant in ligules, leaf sheaths and stems. The presence of SRS was observed in maize stems, whereas SRZ could not be detected in sorghum stems. The experiment was performed in three biological replicates of 10 plants each that were inoculated with SRS, SRZ or water. Error bars represent the standard error of the mean (SEM). Different letters above the bars indicate a significant difference (*t*-test with $P \leq 0.05$) within one tissue. **P* value comparing the data of SRS and SRZ at the injection hole is slightly above 0.05.

inoculation site, the relative amount of SRZ DNA was higher at all other sites analysed. However, SRS DNA could still be detected in maize stems (Fig. 2B), indicating a relatively small, but clearly observable, proliferation of SRS in maize, confirming the microscopic analysis of the nodes (Fig. 1I) and the visual observations of the meristems (Fig. S1H). Taken together, fungal DNA quantification in maize and sorghum indicates that both SRS and SRZ can grow in both plants, with a clear proliferation preference for the compatible host. In contrast with SRS on maize, SRZ is unable to spread systemically in sorghum.

SRS and SRZ show differences in the plant–fungus interaction zones

To determine whether the differences in the colonization of SRS and SRZ in maize and sorghum were reflected by variations in the regions of fungal–plant interaction, we observed the ultrastructure of fungal cell walls (FCWs) and interfacial matrix (IM) in sorghum and maize leaves by transmission electron microscopy (TEM). Most fungal occurrences were characterized by a dark layer of FCW that was surrounded by a grey sheath of IM (Yi and Valent, 2013), which separated the fungal hyphae from the plant plasma membrane (Fig. 3). In sorghum, IM was roughly twice as thick as FCW for both SRS and SRZ, and no obvious differences between FCWs could be observed (Fig. 3A, C, E). IM appeared to be very similar for the hyphae of SRS that grew in maize (Fig. 3B). In contrast, the hyphae of SRZ had a thicker FCW when compared with SRS, and had an extremely thin or seemingly lacking IM which, on average, only reached about half the thickness of FCW (Fig. 3D, F). This indicates that the contact zones between fungi and hosts differ between maize and sorghum for SRZ and differ between SRS and SRZ in maize.

H_2O_2 occurs more often at penetration sites of SRZ in sorghum

Sorghum responds to SRZ infection by the generation of phytoalexins at 3 dai (Poloni and Schirawski, 2014). To investigate the occurrence of earlier plant defence responses, we evaluated the production of H_2O_2 at penetration sites. On sorghum, 20% of the appressoria of SRS showed the accumulation of H_2O_2 at 1 dai (Fig. 4B). Unexpectedly, for SRZ on sorghum, we observed a strong accumulation of H_2O_2 at the majority (61%) of penetration sites at 1 dai (Fig. 4B). Interestingly, in spite of a strong H_2O_2 response, the hyphae of SRZ continued to spread in sorghum leaves after penetration (Fig. 1B).

On maize, the penetrating hyphae of SRZ exhibited the accumulation of similar small amounts of H_2O_2 to SRS on sorghum (Fig. 4B, C). In contrast, the penetrating hyphae of SRS showed a much lower production of H_2O_2 at penetration sites in maize than did SRS in sorghum (Fig. 4B, C). This indicates that the H_2O_2 deposition response of sorghum to SRZ is distinct from and much stronger than that of maize to SRS.

Callose deposition occurs more frequently and more strongly at cell-to-cell crossings of SRZ in sorghum

We determined the occurrence of callose at attempted hyphal cell-to-cell crossings in maize and sorghum at 2 dai. In sorghum leaves, for about 6% of the cell-to-cell crossings, the hyphae of SRS induced callose deposition that was localized at the tip of the

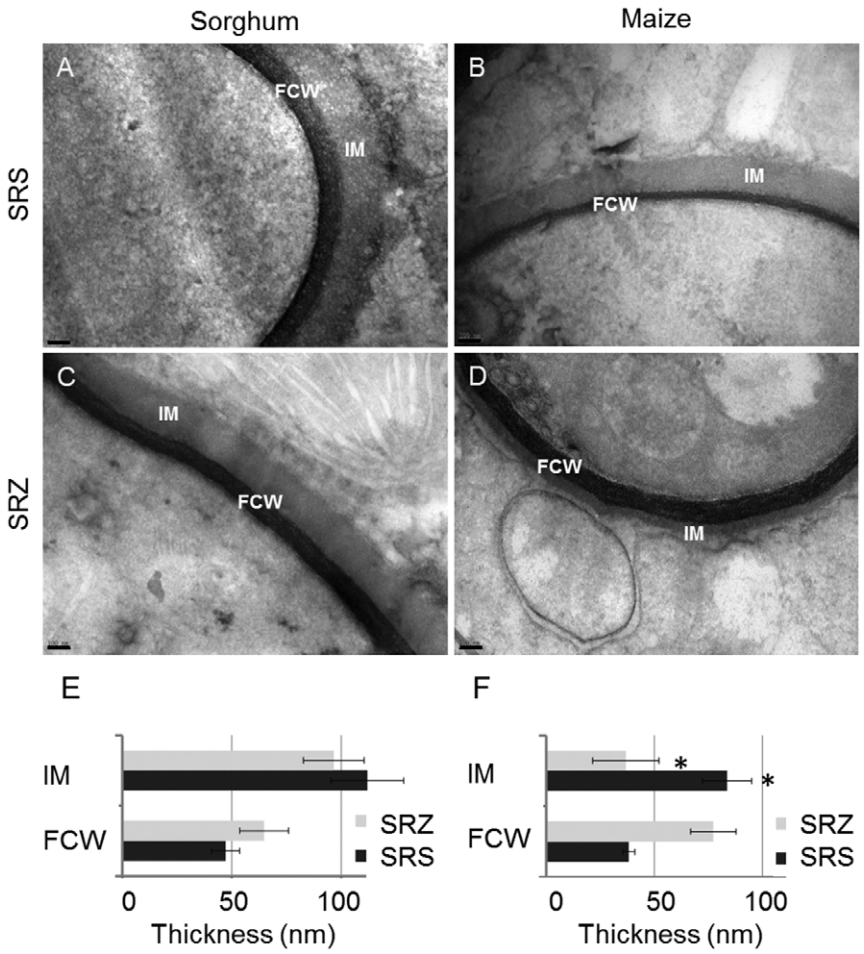

Fig. 3 Electron micrographs of sections obtained from sorghum (A, C) and maize (B, D) infected with *Sporisorium reilianum* f. sp. *reilianum* (SRS) (A, B) or *S. reilianum* f. sp. *zeae* (SRZ) (C, D). Photographs show transverse cuts of fungal hyphae with black fungal cell wall (FCW) and grey interfacial matrix (IM). Measurements were performed for FCW and IM in sorghum (E, $n = 10$) and maize (F, $n = 8$). Error bars give the standard error of the mean (SEM); size bars, 100 nm.

crossing hyphae (Fig. 5A, E). In contrast, about 25% of the hyphal cell-to-cell crossings of SRZ induced callose deposition that was stronger—often affecting the complete cell wall of the responding cell (Fig. 5B, E).

In maize, cell-to-cell crossings of SRZ hyphae induced callose deposition with the same low percentage and localized response as the hyphae of SRS in sorghum (Fig. 5C, E, F). In contrast, the hyphae of SRS showed much weaker signs of callose deposition in maize than did SRZ in sorghum (Fig. 5D, E, F). This indicates that the callose deposition response of sorghum to SRZ is distinct from and much stronger than that of maize to SRS.

Expression of marker genes reveals differential plant responses in maize and sorghum

To investigate the occurrence of additional defence responses, we determined the expression of several plant defence marker genes in inoculated leaves at 3 dai using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). In sorghum, we selected the gene Sb03g030800 as a marker for callose deposition, as it is the best homologue of the *Arabidopsis thaliana* glucan synthase-like 5 (GSL5, Jacobs *et al*., 2009). In addition, we selected the genes Sb03g030100, encoding a chitinase, Sb01g037970, encoding the pathogenesis-related protein 10 (PR10), *SbDFR3* (Sb02g000220), encoding the enzyme dihydroflavonol 4-reductase (DFR3) involved in phytoalexin biosynthesis in sorghum (Liu *et al*., 2010), Sb05g018800, encoding a leucine-rich repeat (LRR)-containing extracellular glycoprotein precursor, and Sb08g022440, potentially encoding a thaumatin-like protein. Of these, genes encoding the putative chitinase, DFR3, LRR-containing protein and thaumatin-like protein all showed specific up-regulation in SRZ-colonized sorghum leaf samples, when compared with SRS-infected samples (Fig. 6A).

In maize, we investigated the expression of the gene GRMZM2G430680, encoding a 1,3-β-glucan synthase, a putative chitinase (GRMZM2G005633), a putative *PR10* (GRMZM2G112538), the gene *AN2* (GRMZM2G044481) involved in kauralexin biosynthesis, the putative *PR5* gene (GRMZM2G402631) and a gene putatively encoding a thaumatin-like protein (GRMZM2G149809). Of these six genes, the glucan synthase and *AN2* gene did not show differences in

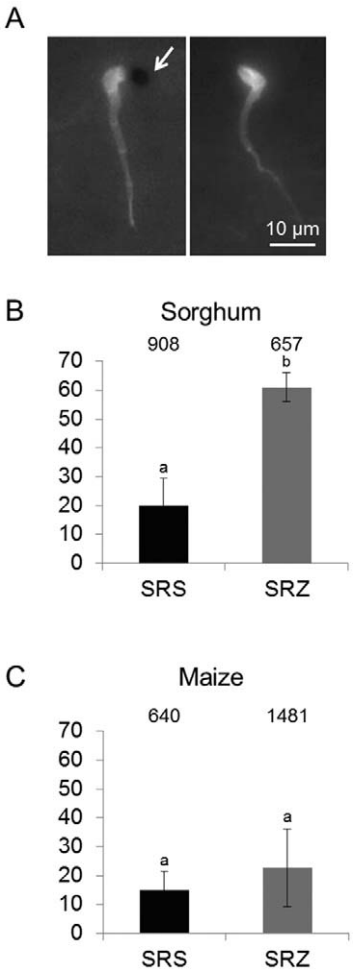

Fig. 4 H_2O_2 accumulation at *Sporisorium reilianum* infection sites in maize and sorghum. Samples were collected at 1 day after inoculation (dai) and were stained with 3,3'-diaminobenzidine (DAB) and calcofluor. (A) Examples of appressoria showing H_2O_2 deposition (left, white arrow) and without H_2O_2 (right). (B, C) Percentage of appressoria with H_2O_2 deposition for sorghum (B) and maize (C). Three biological replicates containing three to four plants each were analysed. Data given are the mean values and standard deviations. The total number of counted appressoria is indicated above the bars. Different letters above the columns indicate significant differences (*t*-test with $P \leq 0.05$).

expression among H_2O, SRZ and SRS, whereas the chitinase and *PR5* gene were similarly up-regulated by both SRZ and SRS (Fig. 6B). In contrast, *PR10* was significantly up-regulated in maize leaves colonized by SRZ in comparison with SRS, whereas the gene encoding the thaumatin-like protein was up-regulated only in SRS-colonized maize leaves (Fig. 6B). In summary, a large variety of plant defence marker genes are up-regulated in sorghum colonized with SRZ, whereas, in maize, both strains induce particular plant defence genes, indicating a mechanistic difference in host response to the two formae speciales of *S. reilianum* in maize and sorghum.

Transcriptome analysis reveals distinct gene sets induced by SRS and SRZ in maize

As no clear differences were observed in maize with regard to the evaluated plant defence responses, we analysed the gene expression profile of infected maize leaves by Illumina RNA sequencing. *Sporisorium reilianum*-inoculated samples were compared with water-inoculated samples (Zm-SRS vs. Zm-H_2O, and Zm-SRZ vs. Zm-H_2O) and with each other (Zm-SRS vs. Zm-SRZ). In SRS-inoculated samples compared with control (Zm-SRS vs. Zm-H_2O), 1450 maize genes were differentially regulated, 1279 (88%) of which showed up-regulation and only 171 (12%) of which were down-regulated (Fig. 7A). In SRZ-infected samples compared with control (Zm-SRZ vs. Zm-H_2O), 1563 genes showed differential regulation, 1443 (92%) of which were up-regulated and 120 (8%) of which were down-regulated (Fig. 7A). Among the differentially regulated genes, 698 were present in both SRS- and SRZ-infected samples compared with control. Of these, 670 were up-regulated and 28 were down-regulated in plants infected with SRZ compared with control plants. This was very similar for SRS, and only three genes (GRMZM2G113378, GRMZM2G502350 and GRMZM2G333022) that were up-regulated in SRZ-infected samples showed down-regulation for plants infected with SRS. Therefore, SRZ infection led to the regulation of an exclusive set of 865 genes, 773 (89%) of which were up-regulated and only 92 (11%) of which were down-regulated. Infection with SRS led to the regulation of a different set of 752 genes, 612 (81%) of which were up-regulated and 140 (19%) of which were down-regulated (Fig. 7A). This shows that, although the presence of both SRS and SRZ resulted in a large set of genes commonly regulated, the presence of each forma specialis induced or decreased an even larger set of distinct genes.

To determine which functional processes were activated by each strain, the 1279 and 1443 genes up-regulated by SRS and SRZ, respectively, were subjected to a gene ontology (GO) term enrichment analysis using the Singular Enrichment Analysis (SEA) tool of the agriGO platform (Zhou *et al*., 2010). This analysis revealed 62 and 100 GO terms as significantly enriched for SRS and SRZ, respectively, which were reduced to 55 and 78 GO terms using REVIGO (Supek *et al*., 2011; Tables S1, S2, see Supporting Information). GO terms enriched for both SRS and SRZ included apoplast, chitinase activity, heme binding, response to oxidative stress, nutrient reservoir activity and amino acid transport (Fig. 7B). The GO terms protein kinase activity and protein amino acid phosphorylation were up-regulated by both SRS and SRZ, but almost twice as many genes were present for SRZ compared with control (Fig. 7B, Tables S1, S2). GO terms specifically enriched in SRZ-induced genes included DNA replication, post-translational protein modification, glutathione transferase, ATP binding, L-phenylalanine metabolic process, lipoxygenase activity

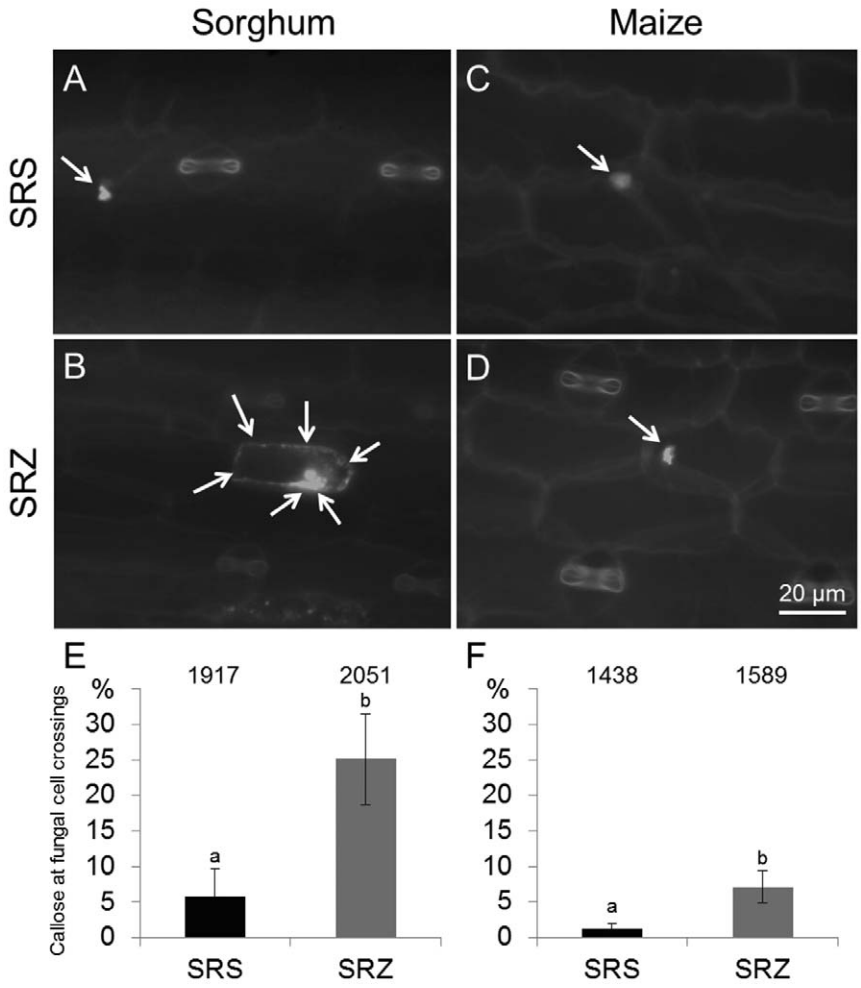

Fig. 5 Callose deposition in infected sorghum and maize. Sorghum (A, B, E) and maize (C, D, F) leaves were collected at 2 days after inoculation (dai). Sorghum infected with *Sporisorium reilianum* f. sp. *reilianum* (SRS) showed weak and localized callose deposition (A, E), whereas stronger and more frequent levels of callose were observed for *S. reilianum* f. sp. *zeae* (SRZ) (B, E). In maize, callose was observed in small and localized amounts for SRS (C) and SRZ (D), and was slightly more frequent for SRZ (F). (E) and (F) show the percentage of occurrence of callose at hyphal cell-to-cell crossings in sorghum and maize, respectively. Hyphal cell-to-cell crossings were counted for three biological replicates consisting of two leaves of two plants each. Data given are the mean values and standard deviations. The total number of counted cell-to-cell crossings is given above the bars. Different letters above the columns indicate significant differences (*t*-test with $P \leq 0.05$).

and pollination. GO terms specifically enriched for SRS-induced genes included lipid transport, fatty acid metabolic process, acetyltransferase activity and cell wall organization or biogenesis (Fig. 7B).

The comparison of SRS-infected samples with SRZ-infected samples (Zm-SRS vs. Zm-SRZ) revealed 500 differentially regulated maize genes. Of these, 270 and 230 genes were up-regulated in SRZ- and SRS-infected samples, respectively (Fig. 8A). The logarithmic fold changes of these genes were used for Parametric Analysis of Gene Set Enrichment (PAGE) from agriGO (Zhou *et al*., 2010). Of the 500 genes, only 242 (48%) were annotated and appeared in the query list. Of these, 171 (63% of 270 genes) were up-regulated in SRZ-inoculated samples and 71 (31% of 230 genes) in SRS-inoculated samples. The analysis resulted in 55 GO terms that were all significantly up-regulated in maize colonized with SRZ relative to SRS, 18 of which belonged to the main ontology 'molecular function', 37 to 'biological process' and none to 'cellular components' (Fig. 8B; Table S3, see Supporting Information). The genes strongly up-regulated by SRZ relative to SRS belonged to functionally connected GO terms that culminated in the four GO terms 'regulation of transcription', 'protein amino acid phosphorylation', 'ATP binding' and 'protein serine/threonine kinase activity'.

As, in agriGO, annotation was available for only one-half of the differentially regulated genes, we manually annotated each of the 500 genes by comparison with the non-redundant databases using BLAST (Altschul *et al*., 1990). Among the 270 genes induced by SRZ, at least 70 were predicted to be involved in defence responses, and included four peroxidases, one chitinase, one PR10, three β-glucosidases, seven genes related to calmodulin, one protease inhibitor, four glutathione-*S*-transferases, three genes involved in the biosynthesis of toxins and seven genes associated with flavonoid biosynthesis (Fig. 8C). Moreover, 30 genes coding for plant receptors or protein kinases were up-regulated, as well as 11 heat shock proteins mainly belonging to the group of Hsp70. DNA binding or transcription factors comprised 23 genes, five of which belonged to the WRKY transcription factor group. Other genes included 21 membrane transporters, seven hormone-related genes, 12 genes

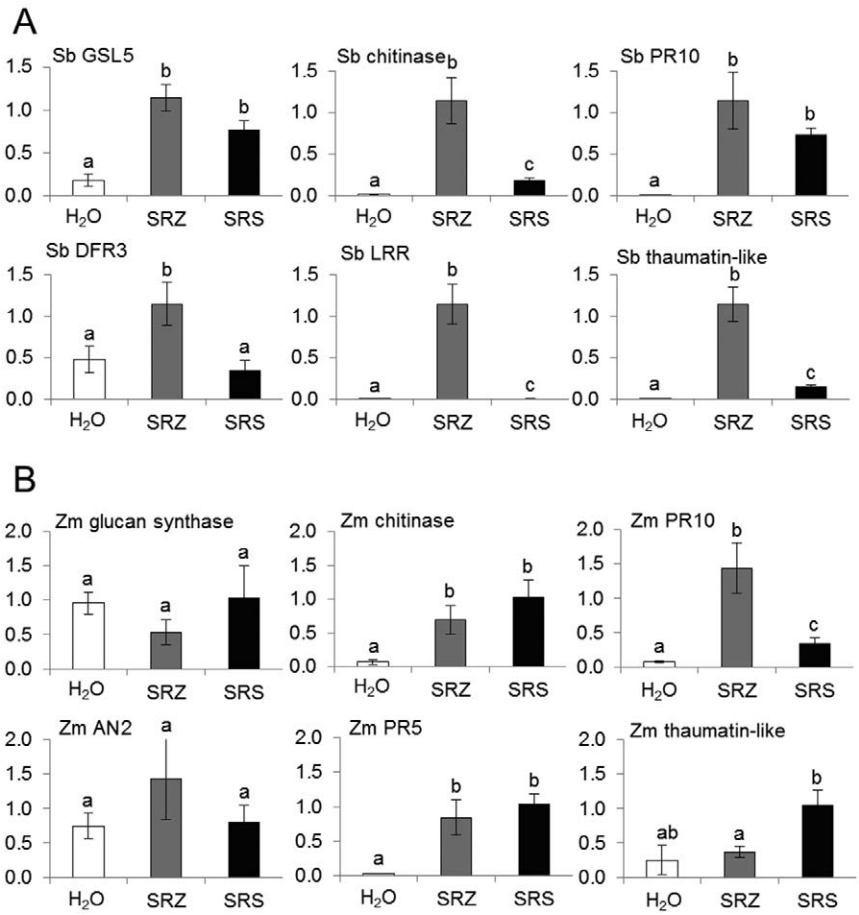

Fig. 6 Quantification of defence gene expression in sorghum and maize inoculated with *Sporisorium reilianum* f. sp. *reilianum* (SRS) (black), *S. reilianum* f. sp. *zeae* (SRZ) (grey) or water (H_2O, white). Changes in transcript levels of marker genes of sorghum (Sb) are shown in (A), whereas maize (Zm) genes are shown in (B). Mean values and standard deviations of three biological replicates containing eight plants each are shown. Different letters above the columns show significant differences (*t*-test with $P \leq 0.05$). AN2, involved in kauralexin biosynthesis; DFR3, dihydroflavonol 4-reductase; GSL5, glucan synthase-like 5; LRR, leucine-rich repeat; PR5, pathogenesis-related protein 5; PR10, pathogenesis-related protein 10.

associated with metal binding, four genes related to photosynthesis, three genes involved in the regulation of actin and three genes involved in the regulation of ubiquitin (Fig. 8C; Table S4, see Supporting Information).

Of the 230 maize genes induced by SRS, at least 30 were potentially implicated in plant defences (Fig. 8C, Table S5, see Supporting Information). These genes included four peroxidases, one thaumatin-like gene, one calmodulin-related gene, one protease inhibitor, two β-glucosidases, one toxin and two genes encoding glutathione-*S*-transferases. Receptors and protein kinases comprised five genes. Furthermore, eight membrane transporters, three hormone-related genes, nine genes associated with metal binding, two genes involved in cell wall remodelling and 10 genes representing DNA-binding proteins and transcription factors were up-regulated. Interestingly, 11 pentatricopeptide repeat (PPR) proteins and three genes involved in thiamine biosynthesis were induced (Fig. 8C). Transcriptome analysis demonstrated that the presence of either SRS or SRZ in maize leaves led to a clear transcriptional response in maize which involved a greater number of genes in response to SRZ than to SRS. Defence genes were induced in both cases, but, here also, a larger number of genes were induced by SRZ.

DISCUSSION

The smut fungus *S. reilianum* exists in two host-adapted formae speciales that infect sorghum (SRS) or maize (SRZ) (Fig. S1). We found that both formae speciales could colonize both plants, with SRS being more proliferative on sorghum and SRZ on maize. SRS was able to colonize maize and reach meristematic tissues, whereas the proliferation of SRZ was restricted to the inoculated leaves in sorghum (Figs 1, 2). The proliferation behaviour correlated with the observed levels of plant defence responses that were strong only for SRZ in sorghum (Figs 5, 6), and included H_2O_2 formation at 1 dai, callose deposition at 2 dai and phytoalexin induction at 3 dai. Obviously, discrete mechanisms exist that determine the host specificity of SRS and SRZ. In sorghum, SRZ is recognized at least as soon as it penetrates, and multiple successive layers of defence responses were shown to be induced during the first 3 dai. Together, these defences are effective in restricting the growth of SRZ to the inoculated leaf, and the prevention of node and meristem colonization is efficient in inhibiting smut disease.

The specific recognition of SRZ in sorghum may be induced by microbe-associated molecular patterns (MAMPs), such as chitin

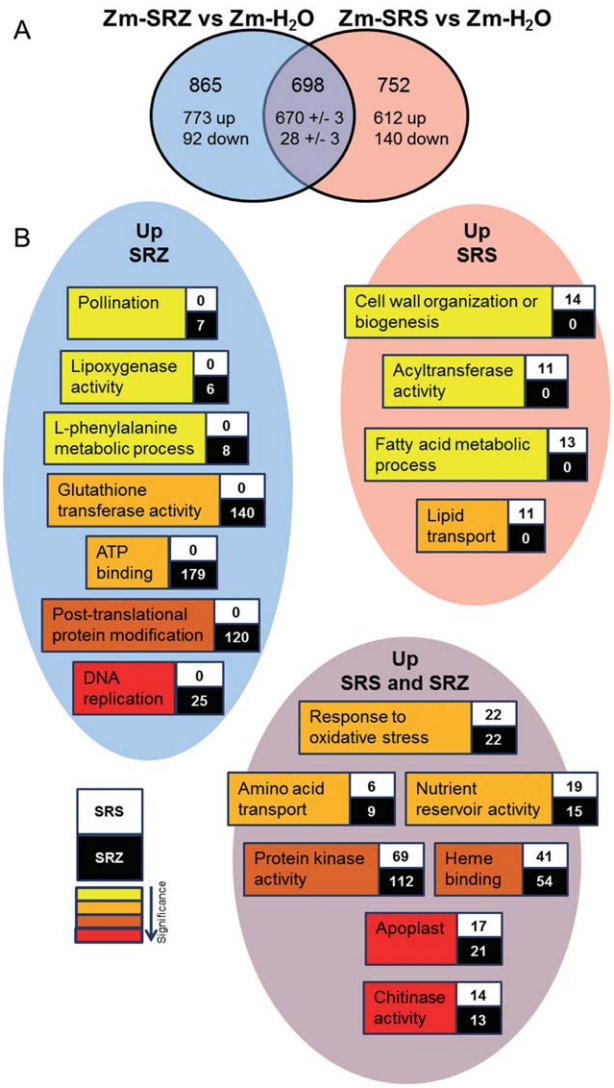

Fig. 7 Comparison of transcriptome analysis of maize leaves infected with *Sporisorium reilianum* f. sp. *reilianum* (SRS) and *S. reilianum* f. sp. *zeae* (SRZ) vs. water control samples. (A) Venn diagram showing the distribution of genes differentially regulated ($P \leq 0.05$) in the comparison of SRS-infected samples vs. water-inoculated control samples (Zm-SRS vs. Zm-H_2O) and SRZ-infected samples vs. water-inoculated control samples (Zm-SRZ vs. Zm-H_2O). (B) Examples of gene ontology (GO) terms up-regulated by SRS, SRZ or both fungi when compared with water-inoculated control samples. Boxes contain the number of genes induced by SRS (white box) or by SRZ (black box) belonging to each associated GO term. Background colours of the GO terms indicate significance according to the key.

and β-1,3-glucan, which are known to induce H_2O_2, callose, phytoalexins and PR proteins. Although SRS is also expected to contain these MAMPs, recent studies have shown that plant pathogens can hide or escape from MAMP recognition through various mechanisms (De Jonge *et al*., 2010; El Gueddari *et al*., 2002; Fujikawa *et al*., 2009, 2012; Oliveira-Garcia and Deising, 2013; Van den Burg *et al*., 2006), some or all of which could also be used by *S. reilianum*. Alternatively, the distinct responses encountered by SRS and SRZ in sorghum may indicate the existence of specific avirulence effectors in SRZ that are recognized by resistance receptors in sorghum and trigger defence reactions, as described for other plant pathogens, such as *Melampsora lini*, *M. oryzae* and *Cladosporium fulvum* (Ellis *et al*., 2007; Sweigard *et al*., 1995; Tosa *et al*., 2005; Van der Does and Rep, 2007).

In the compatible interaction of *Ustilago maydis* and maize, microarray analysis of plant genes showed a differential regulation of 575 genes at 2 dai, including genes associated with stress, secondary metabolism, cell wall metabolism, protein, transcription and RNA processing (Doehlemann *et al*., 2008). In addition, *U. maydis* induced Bax-inhibitor 1 and several cystatin genes expected to be involved in cell death suppression (Doehlemann *et al*., 2008). These genes were not among the genes induced by SRZ in maize (Table S4). A different maize response to the two compatible maize pathogens might reflect the different lifestyle of the fungi. In the leaf, SRZ proliferates without causing symptoms, whereas *U. maydis* induces tumours to assist spore formation.

In maize, SRS does not proliferate to the same level as SRZ (Fig. 2), which could be caused by compatibility issues, such as the lack of virulence factors or the misexpression of factors needed for growth, or to the presence of enhanced plant resistance. However, no or only very weak phenotypic defence responses were elicited by either fungus, and the weak defence responses observed were similar or even stronger for SRZ than for SRS on maize (Figs 4, 5). Analysis of maize gene expression largely corroborated a weaker defence gene expression response for SRS (Fig. 8). Transcriptome analysis revealed that the presence of both fungi is clearly detected by the plant, and commonly induced a large set of genes (Fig. 7). However, each forma specialis also induced a specific set of genes that could be involved in the observed differences in fungal proliferation in maize leaves. Notably, a group of 11 PPR proteins was up-regulated for SRS-infected plants. In Arabidopsis and soybean, specific PPR proteins have been shown to be involved in resistance against various pathogens (Laluk *et al*., 2011; Park *et al*., 2014; Wong *et al*., 2014) and have been reported to function in RNA binding (Fujii and Small, 2011; Lurin *et al*., 2004; Saha *et al*., 2007; Yagi *et al*., 2014). As the function of PPR proteins is still unknown in maize, further work is required to determine whether they are involved in slowing the growth of SRS in maize. Interestingly, hyphae of SRZ that colonize maize leaves show a thinner IM (Fig. 3). Possibly, a thinner IM is necessary for efficient colonization of maize. If this is so, the lack of efficient proliferation of SRS in maize might be a result of compatibility issues caused by an inconveniently thick IM.

It is interesting that the transcriptional plant responses of maize to SRZ involve many more genes than its response to SRS (Fig. 7), including a much larger number of defence-related genes (Fig. 8C). This corroborates the suggestion that SRS is not successful on maize because of incompatibility rather than increased

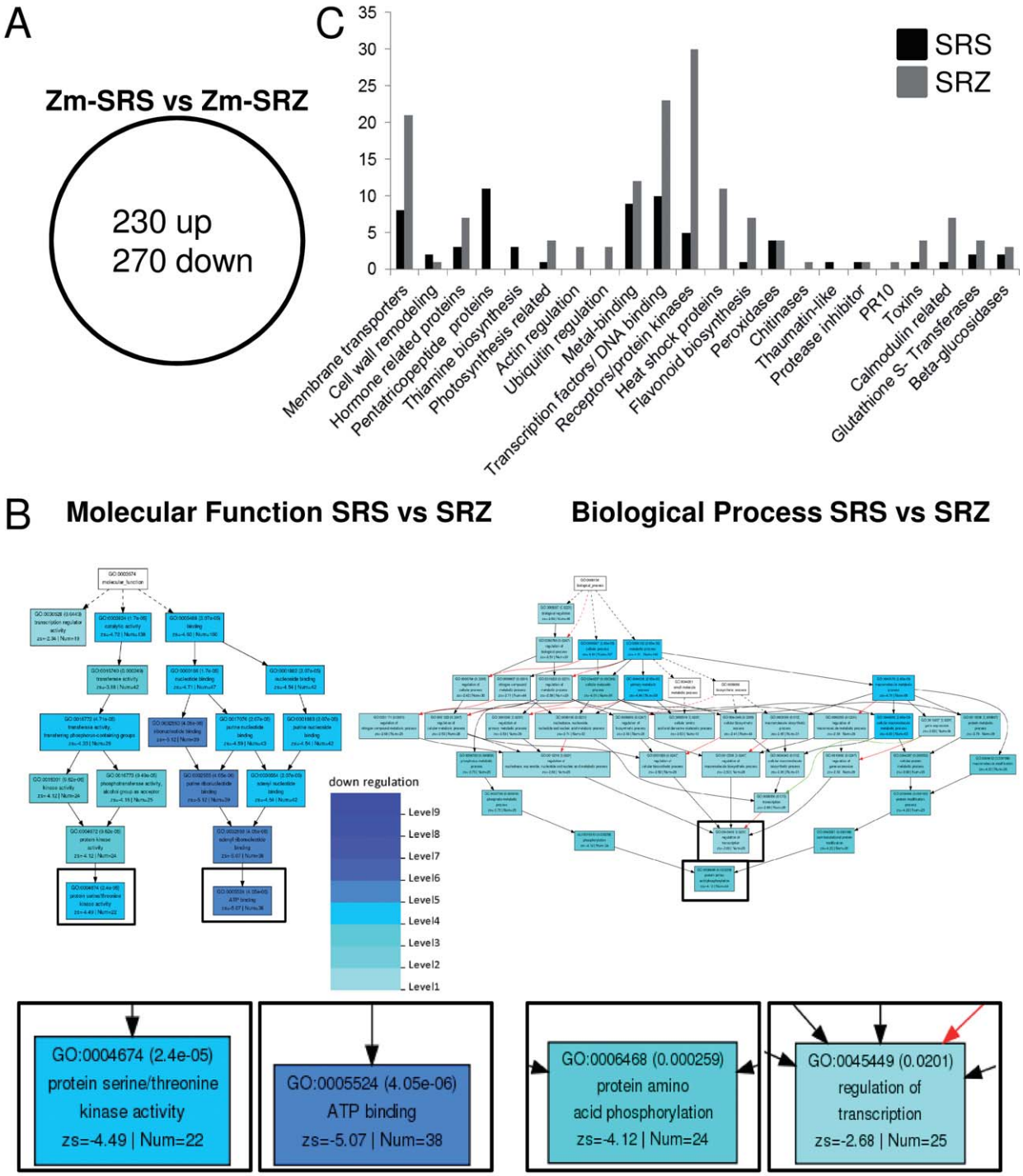

Fig. 8 Comparison of transcriptome analysis of maize leaves infected with *Sporisorium reilianum* f. sp. *reilianum* (SRS) relative to *S. reilianum* f. sp. *zeae* (SRZ)-infected samples. (A) Venn diagram showing the distribution of genes differentially regulated ($P \leq 0.05$) in the comparison of SRS- with SRZ-infected samples (Zm-SRS vs. Zm-SRZ). (B) Pathway mapping of the significantly enriched gene ontology (GO) terms in the comparison of Zm-SRS vs. Zm-SRZ showing the categories 'Molecular function' and 'Biological process'. Four culminating GO terms are enlarged below for better readability. Boxes with GO terms have the GO ID, the false discovery rate (FDR), the GO term, the *z* score and the number of submitted maize genes belonging to the respective GO term. Background colours of GO terms indicate significance according to the key. (C) Summary of gene identity of differentially up-regulated annotated genes of (A) in samples infected with SRS (black) and SRZ (grey). The graph shows the annotation and number of genes belonging to each function (212 genes are represented; complete gene lists are available in Tables S2 and S3, see Supporting Information).

plant defence. Such a scenario would also be in support of a proposed host jump from sorghum to maize. If ancient SRS strains colonized maize, they would not have met with a strong defence response, allowing persistent colonization and spread into the meristematic tissues, which, under certain environmental conditions, might have allowed spore formation on maize and the subsequent evolution of strains specific for maize. A host jump of SRZ strains from maize to sorghum would not have been possible because of excessive plant defence responses met by SRZ strains in sorghum.

In this study, we performed a detailed analysis of the fungal growth behaviour and plant responses induced by the two formae speciales of *S. reilianum* on maize and sorghum. We showed that host specificity is determined after plant penetration, and different plant responses and fungal growth patterns are observed in each pathosystem. In sorghum, the spread of SRZ is inhibited by the induction of plant defence responses that limit fungal growth to the inoculated leaf. In maize, SRS does not meet with stronger defence responses than SRZ and seems to show an altered thickness of the plant–fungus interaction zone, which indicates the existence of more subtle compatibility issues. It would be interesting to compare the gene expression profiles of SRS and SRZ in maize in order to identify compatibility-related fungal genes.

EXPERIMENTAL PROCEDURES

Plant material, fungal strains and seedling inoculation

Seeds of *Zea mays* Gaspe Flint and *Sorghum bicolor* Tall Polish were grown for 7 and 14 days, respectively, under glasshouse conditions of 15 h daylight, at 28 °C and 50% relative humidity, and 9 h night, at 22 °C and 60% relative humidity.

The compatible wild-type *Sporisorium reilianum* strains SRZ1_5-2 (*a1b1*) and SRZ2_5-1 (*a2b2*), originally isolated from maize (Schirawski *et al*., 2010), and SRS1_H2-8 (*a1b1*) and SRS2_H2-7 (*a2b6*), isolated from sorghum (Zuther *et al*., 2012), were used in this study. Fungal strains were inoculated in 2 mL of YEPS light medium (1% tryptone, 1% yeast extract and 1% sucrose) and incubated at 28 °C with shaking at 200 rpm for 8 h. The cultures were used to inoculate 50 mL of potato dextrose broth (BD, Heidelberg, Germany), and were incubated at 28 °C overnight, until they reached an optical density at 600 nm (OD_{600}) of 0.6–0.8. The cultures were centrifuged at 5700 g for 5 min in a multifuge X3R centrifuge (Thermo Fischer Scientific, Dreieich, Germany), and the cell pellets were suspended in water to reach an OD_{600} of 2.0. Cell suspensions were mixed at a 1 : 1 ratio and mixtures of SRZ1_5-2 and SRZ2_5-1 (SRZ), or SRS1_H2-8 and SRS2_H2-7 (SRS), were used for syringe inoculation of leaf whorls of maize or sorghum seedlings.

Macroscopic and microscopic characterization of plant infection

Leaf blades, ligules, leaf sheaths of inoculated leaves and stems containing the nodes and floral meristems were used for microscopic analysis. The tissues were collected at several time points between 4 and 75 dai. Samples were used directly for light microscopy, or were stained with propidium iodide and WGA-Alexafluor-488 prior to analysis by fluorescence microscopy, as described by Ghareeb *et al*. (2011).

Sample preparation and TEM

Infected leaves were cut into small pieces (0.5–1 cm), fixed in a solution of glutaraldehyde 2.5% and then kept in a solution of osmium tetroxide (1%) for 90 min. Samples were washed in water and dehydrated in six successive rinsing concentrations of ethanol for 30 min each. Samples were incubated in LR White (SPI Supplies, West Chester, PA, USA)–ethanol (2 : 1) for 2 h and embedded in pure LR White resin overnight. After polymerization at 50 °C for 24 h, ultra-thin cuts of 90 nm were prepared and stained with 4% uranyl acetate.

Quantification of fungal genomic DNA

Sorghum and maize plants inoculated with SRS, SRZ or water were harvested at 9 dai. Samples were obtained from the leaf blade, ligule and leaf sheath of the inoculated leaf, and from the inner stem containing nodes and meristems of the inoculated plant. Ten plants were collected and pooled, and the experiment was repeated three times. The plant material was frozen and ground in liquid nitrogen until a fine powder was obtained, and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Oligonucleotides oSP101 (GTGCATCAACTGCCAGAAGG) and oSP102 (TCGTAGCCGTAGTACCAAGC) were used to amplify a 396-bp fragment of *S. reilianum* genomic DNA derived from the gene *sr16559*. As reference genes, the sorghum actin gene *SbActin* was amplified using oligonucleotides HEL794 and HEL795 (Du *et al*., 2010), whereas, in maize, the actin gene *ZmActin* was amplified with primers oBH73 (ACCTCACCGACCACCTAATG) and oBH74 (ACCTGACCATCAGGCATCTC). A 25-μL reaction mixture was composed of 1 × NH_4 reaction buffer, 2 mM $MgCl_2$, 0.25 U BIOTaq DNA polymerase, 100 μM deoxynucleoside triphosphates (dNTPs) (all from Bioline, Luckenwalde, Germany), 0.2 × SYBR Green solution (Invitrogen, Karlsruhe, Germany), 0.25 μM of each primer (Sigma-Aldrich, Taufkirchen, Germany) and 1 μL of template DNA. PCR amplification was performed in a CFX Connect cycler (Bio-Rad Laboratories, München, Germany) with an initial denaturation of 95 °C for 6 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min, followed by a plate reading step. Finally, a product melting curve was performed at 55–95 °C. Expression ratios in samples of inoculated plants compared with water-inoculated plants were calculated using CFX Manager 3.0 (Bio-Rad Laboratories).

Staining of plant material for H_2O_2 and callose

For the detection of H_2O_2, leaf samples were collected at 1 and 2 dai, vacuum infiltrated for 3 min in 3,3'-diaminobenzidine solution (DAB, 1 mg/mL, Sigma Aldrich, Taufkirchen, Germany; D3939) and stained for 6 h in the dark. Sections were destained in ethanol and soaked for 30 s in a solution of calcofluor white (10 mg/mL, Fluorescence Brightener 28, Sigma). The generation of H_2O_2 was visualized as a reddish-brown coloration, whereas fungal surface structures were fluorescent under UV illumination using a

fluorescence microscope (DM6000B, Leica Bensheim, Germany) and a DAPI (4',6-diamidino-2-phenylindole) filter (EX BP 365, BS FT 395, EM BP 445/50). For the detection of callose, infected leaves were collected at 1 and 2 dai, soaked overnight in ethanol and incubated for 1 h in a staining solution containing aniline blue (0.005%) in 50 mM sodium phosphate buffer (pH 8.2). The material was analysed by fluorescence microscopy (DM6000B, Leica) using a DAPI filter set.

RNA extraction and cDNA synthesis

Sorghum and maize leaf pieces of about 3 cm inoculated with SRS, SRZ or water control were collected at 3 dai in three biological replicates containing eight plants each. Samples were macerated in liquid nitrogen, and 100 mg of the resulting powder was used for RNA extraction using Trizol (Sigma). A clean-up step was performed using a Qiagen RNeasy Plus Mini Kit. The final RNA concentration was determined using a NanoDrop Spectrophotometer (Peqlab, Erlangen, Gemany), and RNA integrity was confirmed through denaturating agarose gel electrophoresis. One microgram of total RNA was subjected to cDNA synthesis using oligo$(dT)_{18}$ oligonucleotides (First Strand cDNA Synthesis Kit, Fermentas, St. Leon-Rot, Germany) following the manufacturer's protocol.

Expression of plant defence marker genes by qRT-PCR

Real-time PCR was performed in a CFX 96 Thermal Cycler (Bio-Rad Laboratories) in a 25-μL reaction mixture composed of $1 \times NH_4$ reaction buffer (Bioline), 3 mM $MgCl_2$, 100 μM dNTPs, 0.4 μM of each gene-specific primer, 0.25 units BIOTaq DNA polymerase (Bioline), 1 μL of cDNA and 100 000 times diluted SYBR Green I solution (Cambrex, Wiesbaden, Germany). Primers used for the amplification of marker genes in maize and sorghum are listed in Table S6 (see Supporting Information). As reference genes in sorghum, actin, ubiquitin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were selected, whereas, for maize, ubiquitin, GAPDH and tubulin were used. The PCR conditions consisted of an initial denaturation at 95 °C for 6 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min, followed by a plate reading step and a final product melting curve at 55–95 °C. Expression ratios in samples of inoculated plants compared with water-inoculated plants were calculated using CFX Manager 3.0 (Bio-Rad), and statistical calculations were performed using Graph Pad software.

Transcriptomic analysis

Total RNA was extracted as described above from maize leaves inoculated with SRS, SRZ or water (three biological replicates containing samples of 10 plants each were pooled prior to sequencing). Illumina sequencing was performed by Beckman Coulter Genomics (Danvers, MA, USA) using the paired-end sequencing protocol. Read files were imported into CLC genomics workbench version 6 and trimmed by ambiguous bases, quality and Illumina adapter sequences. The resulting reads were mapped to the reference genomes of *Zea mays* (maize B73; Schnable *et al*., 2009). Logarithmic fold changes, *P* values and false discovery rates (FDRs) were calculated with the extension package edgeR 1.6.5. Comparisons were made between three groups of samples: SRS- and SRZ-infected plants and water-inoculated control samples. Genes with a corrected $P \leq 0.05$ were considered to be significantly regulated. Gene annotation was obtained from maize GDB (http://www.maizegdb.org/), Plant GDB (http://www.plantgdb.org/ZmGDB/) or Phytosome (http://www.phytozome.net), when available. Genes that did not present annotation were subjected to BLAST search at the National Center for Biotechnology Information (NCBI) (Altschul *et al*., 1990; http://blast.ncbi.nlm.nih.gov/Blast.cgi). GO enrichment was performed for genes that showed significant expression change using the agriGO tool kit (http://bioinfo.cau.edu.cn/agriGO/), and redundant GO terms were reduced using the REVIGO tool (http://revigo.irb.hr/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Macroscopic symptoms on sorghum and maize after inoculation with *Sporisorium reilianum*. Seedlings of sorghum (A–F) or maize (G–L) were syringe inoculated with *Sporisorium reilianum* f. sp. *reilianum* (SRS) (A–C, G–I) or *S. reilianum* f. sp. *zeae* (SRZ) (D–F, J–L). Samples of plants were analysed at 4 days after inoculation (dai) (A, D, G, J), 21 dai (I, L), 35 dai (B, E), 50 dai (H, K) or 75 dai (C, F). Sorghum plants inoculated with SRS showed only chlorosis on leaves (A), but resulted in meristems wrapped in a white peridium (B) and spore formation in the apical inflorescences (C). Penetrating hyphae were colourless (A, inset). However, SRZ-inoculated sorghum plants displayed red phytoalexin accumulation in leaves (D), and phytoalexins also stained fungal hyphae red (D, inset). SRZ-inoculated sorghum plants developed healthy meristems (E), and inflorescences containing normal seeds (F) were produced. In maize, SRS-inoculated plants showed weak chlorosis on leaves (G) and leaf-like structures in cobs (H), but tassels were healthy (I). SRZ-inoculated maize presented strong chlorosis on leaves (J), and produced spores and leaf-like structures in cobs (K) and tassels (L). Bars: 1 cm in A–L, 20 μm in the insets. Insets in (A) and (D) are bright field micrographs of untreated leaf samples. Arrows in the insets of (A) and (D) point to fungal hyphae. Insets in (H) and (I) represent inflorescences of mock-inoculated plants.

Table S1 List of significant gene ontology (GO) terms in *Sporisorium reilianum* f. sp. *zeae* (SRZ)-infected samples vs. water control samples.

Table S2 List of significant gene ontology (GO) terms in *Sporisorium reilianum* f. sp. *reilianum* (SRS)-infected samples vs. water control samples.

Table S3 List of significant gene ontology (GO) terms in *Sporisorium reilianum* f. sp. *reilianum* (SRS)-infected samples vs. *S. reilianum* f. sp. *zeae* (SRZ)-infected samples.

Table S4 List of maize genes significantly up-regulated in the comparison Zm-SRZ vs. Zm-SRS. SRS, *Sporisorium reilianum* f. sp. *reilianum*; SRZ, *S. reilianum* f. sp. *zeae*; Zm, *Zea mays*.

Table S5 List of maize genes significantly up-regulated in the comparison Zm-SRS vs. Zm-SRZ. SRS, *Sporisorium reilianum* f. sp. *reilianum*; SRZ, *S. reilianum* f. sp. *zeae*; Zm, *Zea mays*.

Table S6 List of oligonucleotides used for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).