**Comparative secretome analysis of different smut fungi and identification of plant cell death-inducing secreted proteins from *Tilletia horrida***

Aijun Wang¹,²,³, Linxiu Pan¹, Xianyu Niu²,³, Xinyue Shu²,³, Xiaojun Yi¹, Naoki Yamamoto¹,²,³, Shuangcheng Li¹,²,³, Qiming Deng¹,²,³, Jun Zhu¹,²,³, Yueyang Liang¹,²,³, Lingxia Wang¹,²,³, Ping Li¹,²,³* and Aiping Zheng¹,²,³*

**Abstract**

**Background:** *Tilletia horrida* is a basidiomycete fungus that causes rice kernel smut, one of the most important rice diseases in hybrid rice growing areas worldwide. However, little is known about its mechanisms of pathogenicity. We previously reported the genome of *T. horrida*, and 597 genes that encoded secreted proteins were annotated. Among these were some important effector genes related to pathogenicity.

**Results:** A secretome analysis suggested that five *Tilletia* fungi shared more gene families than were found in other smuts, and there was high conservation between them. Furthermore, we screened 597 secreted proteins from the *T. horrida* genome, some of which induced expression in host-pathogen interaction processes. Through transient expression, we demonstrated that two putative effectors could induce necrosis phenotypes in *Nicotiana benthamiana*. These two encoded genes were upregulated during early infection, and the encoded proteins were confirmed to be secreted using a yeast secretion system. For the putative effector gene smut_5844, a signal peptide was required to induce non-host cell death, whereas ribonuclease catalytic active sites were required for smut_2965. Moreover, both putative effectors could induce an immune response in *N. benthamiana* leaves. Interestingly, one of the identified potential host interactors of smut_5844 was laccase-10 protein (OsLAC10), which has been predicted to be involved in plant lignification and iron metabolism.

**Conclusions:** Overall, this study identified two secreted proteins in *T. horrida* that induce cell death or are involved in defense machinery in non-host plants. This research provides a useful foundation for understanding the interaction between rice and *T. horrida*.

**Keywords:** *Tilletia horrida*, Effector proteins, Cell-death, Signal peptides, RNase active site, Yeast two-hybrid

---

**Background**

The basidiomycete *Tilletia horrida* is a biotrophic fungal pathogen that causes rice kernel smut (RKS), a disease that is distributed throughout hybrid rice growing areas worldwide [1, 2]. *T. horrida* was first reported in 1896, and infects rice floral organs during the flowering stage [3]. A major feature of this pathogen is that it affects both the yield and quality of hybrid seeds by producing masses of dark powdery teliospores [4]. The incidence of *T. horrida* has been recorded to be as high as 87 and 100% in hybrid rice fields in Pakistan and China, respectively [5]. RKS is now an increasing threat to rice cultivation in Asia, Oceania, Europe, America, and Africa [6, 7].

Biotrophic fungi derive nourishment from host cells and tissues for colonization and growth, therefore, they generally do not prevent plant growth and development. On the other hand, the pathogens can secrete many effectors into host cells that suppress plant immune responses; these may be localized to different cellular compartments where they may assume diverse cellular
functions to promote infection [8, 9]. For example, host transcription, chromatin remodeling, and immune responses may be affected by secreted effectors [10].

Although plant pathogenic fungi can secrete a large number of proteins, only a small proportion of these have been characterized as effectors. *Magnaporthe oryzae* was the first plant pathogen fungus whose genome was sequenced; subsequently, several effectors in *M. oryzae* have been reported, including Slp1, MoHEG13, and MoHEG16 [11, 12]. In species of smut fungi, several effectors have been studied, including maize *Ustilago maydis* Pit2, See1, Pep1, Cmu1, and Tin2 [13–17]. Pit2 can inhibit the activity of host cellular proteases, which play an important role in plant immune responses [13]. See1 is a fungal effector that directly and specifically contributes to the formation of leaf tumors in maize [14]. Pep1 has an important role in the process by which *U. maydis* and barley covered smut fungus *Ustilago hordei* penetrates the host, and has a conserved function in establishing host-smut pathogen interaction [15]. In contrast with these models, very little is known about the mechanisms of action of *T. horrida* effectors.

Plant receptor proteins that trigger defense responses can recognize effectors, and the functions of several plant receptor proteins function have been reported. For example, the receptor-like proteins Cf-4, Cf-2, Cf-9, and Cf-4E interact with the effectors Avr4, Avr2, Avr9 and Avr4E, respectively, in the tomato pathogen *Cladosporium fulvum* [18, 19]. Previous reports have shown that non-host recognition of effectors is very important for non-host resistance during attempted inoculation by non-host pathogens [20, 21]. Eleven secreted effectors in *Ustilaginoidea virens* have been noted to induce non-host cell death when transiently expressed in *Nicotiana benthamiana* [22]. Effectors are often recognized in plant defense signaling pathways.

According to genome sequencing, *T. horrida* encodes 597 secreted proteins, of which 131 are predicted to be effectors [23]. Furthermore, many potential effector genes are arranged in clusters and transcriptome analyses during infection suggest that putative secreted effectors have an essential role in establishing successful infection in *T. horrida* [23]. No effector genes of *T. horrida* have been functionally characterized. In this study, using transient expression assays, we identified two putative effectors that could induce cell death in *N. benthamiana*. The predicted signal peptides (SPs) and RNase active sites were shown to function differently in the cell death–inducing activity of two putative effectors.

**Results**

**Comparative analysis of *T. horrida* secreted proteins with other smut pathogens**

To identify the conservation of secreted proteins between different smut pathogens, the predicted proteins of nine different smut fungi were predicted, including the three smut genera *Tilletia*, *Ustilago*, and *Sporisorium*. The results showed that *T. horrida*, *Tilletia caries*, *Tilletia controversa*, *Tilletia indica*, and *Tilletia walker* possessed more secreted proteins than *Sporisorium scitamineum*, *Sporisorium reilianum*, *U. hordei*, and *U. maydis* (Table 1; Additional file 1: Table S1). We further screened the similarities among the secreted proteins of *T. horrida* and those of eight other smut fungi through an analysis of the gene families of secreted proteins. There were 498 gene families found in 597 secreted proteins of *T. horrida* (Table 1; Fig. 1). Among them, 51 gene families were shared among *T. horrida* and eight other smut fungi; 64 gene families were shared among *T. horrida* and *S. scitamineum*, *S. reilianum*, *U. hordei*, and *U. maydis*; 177 gene families were shared among five *Tilletia* fungi; and 230 gene families appeared to be unique to *T. horrida* (Fig. 1a, b, c). The data showed that *T. horrida*, *T. caries*, *T. controversa*, *T. indica*, and *T. walker* shared more gene families than other smut genera. Further, a high level of conservation among the five *Tilletia* fungi was observed; this finding is consistent with close relationship between these species.

**Genome mining for candidate *T. horrida* small secreted protein encoding genes**

Effectors are often characterized by their small size and high cysteine content [24]. There were 367 small secreted proteins (≤400aa), and these comprised 61.47% of the *T. horrida* secretome, less than 67.19% in *T. caries*, and 66.01% in *T. controversa*; but greater than 60.06% in *T. indica* and 59% in *T. walker* (Table 1; Additional file 1: Table S1). Of these, 131 small secreted proteins that were up-regulated at 8 h after inoculation were selected as putative effectors, according to our previous study (Fig. 2) [23].

**Series-clustered analysis of secreted proteins**

In our previous report, among 36 randomly selected predicted effectors (Additional file 2: Table S2), two effector genes, smut_2965 and smut_5844, were shown to trigger cell death in *N. benthamiana* leaves. However, a negative control of the green fluorescent protein (GFP) construct did not trigger the cell death phenotype (Fig. 3a). Western blot experiments showed that these proteins were expressed in the infiltrated leaves of *N. benthamiana* (Fig. 3b). In addition, we found several homolog genes of smut_2965 in five smut species (Fig. 3c). However, the homolog genes of smut_5844 were not found; we therefore predicted that it is a novel effector in *T. horrida*. To further select high confidence candidate effectors from among these genes that encode secreted proteins, expression trend clustering analysis of 291 differentially expressed genes (P ≤ 0.05 and |log 2 fold
change) that encoded secreted proteins was performed at different infection time points, based on our previous transcriptome data [23]. This approach categorized the genes into 23 clustered profiles and enabled the selection of clusters with different sets of characteristics that may relate to different functions (Fig. 4). For example, profile 23 contained 11 genes, whose expression was up-regulated at five time points. These sustained up-regulated genes appeared to have important biological significance during infection. The clusters in profile 18, in which the expression pattern was similar to that of smut_2965; and the expression pattern of profile 19 that contained 13 putative effector genes and was similar to that of smut_5844, indicated that these genes may be involved in processes that occurred at the same stage of infection with smut_2965 and smut_5844 (Fig. 4a, b).

**Functional validation of predicted SPs of putative effectors**

We used a yeast secretion system to experimentally identify the ability of predicted SPs of the smut_2965 (guanyl-specific ribonuclease, gsr1) and smut_5844 (unannotated, uan2) effector genes in *T. horrida* according to a method used in previous studies [25]. The predicted SP nucleotide sequence of gsr1 and uan2 were each fused in frame with the truncated pSUC2 gene that encodes invertase lacking its own SP. The constructed vectors were transformed into yeast strain YTK12, which is deficient in invertase secretion. Here, the invertase with functional SPs could degrade raffinose into simple sugars. The sugars therefore supplied a carbon source to YTK12 to enable it to grow on the medium with raffinose [26, 27]. The results showed that the predicted SPs of two putative effectors and a positive control (the secretion signal of *Phytophthora sojae* Avr1b) led to the secretion of invertase. These were grown on YPRAA medium with raffinose (Fig. 5). As a negative control, the N-terminus of Mg87 in *M. oryzae* [28] did not grow on YPRAA medium (Fig. 5). These results indicate that the predicted SPs of the two putative *T. horrida* effectors were functional secreted proteins.

### Table 1 The encode gene number of predicted secreted proteins from nine smut fungal isolates

| Strains       | Number of predicted secreted proteins | Number of gene families in secreted proteins | Number of Small predicted secreted proteins (≤400aa) | Small secreted proteins/all secreted proteins (%) |
|---------------|--------------------------------------|---------------------------------------------|---------------------------------------------------|--------------------------------------------------|
| *T. horrida*  | 597                                  | 498                                        | 367                                               | 61.47                                            |
| *T. caries*   | 768                                  | 471                                        | 516                                               | 67.19                                            |
| *T. controversa* | 725                                | 660                                        | 479                                               | 66.01                                            |
| *T. indica*   | 631                                  | 569                                        | 379                                               | 60.06                                            |
| *T. walker*   | 600                                  | 542                                        | 354                                               | 59.00                                            |
| *S. scitamineum* | 416                               | 398                                        | 230                                               | 55.29                                            |
| *S. reilianum* | 505                                  | 484                                        | 313                                               | 61.98                                            |
| *U. hordei*   | 430                                  | 407                                        | 277                                               | 64.42                                            |
| *U. maydis*   | 509                                  | 471                                        | 311                                               | 61.10                                            |

**Fig. 1** Homology analysis of *Tilletia horrida* secreted proteins with other smut fungi. **a** Venn diagram showing orthologs between the nine smut fungi secreted proteins. **b** Venn diagram showing orthologs between the secreted proteins of the five different smut fungus genera. **c** Venn diagram showing orthologs between the five *Tilletia* fungi secreted proteins. tca denotes *Tilletia caries*; tco denotes *Tilletia controversa*; tin denotes *Tilletia indica*; twa denotes *Tilletia walkerii*; tho denotes *Tilletia horrida*; sre denotes *Sporisorium reilianum*; ssc denotes *Sporisorium scitamineum*; um denotes *Ustilago maydis*; uho denotes *Ustilago hordei*.
SPs are required for putative *T. horrida* effector uan2 to trigger plant cell death

According to previous reports, the SPs of many *M. oryzae* effectors are required for their ability to induce cell death in plants [29]. In this study, we identified whether two putative effectors in *T. horrida*, gsr1 and uan2, without SPs had the ability to induce cell death through transient expression assays in *N. benthamiana* leaves. The results showed that smut_5844-sp (uan2 lacking SPs) no longer caused a cell-death phenotype; however, smut_2965-sp (gsr1 lacking SPs) still had the ability to cause cell-death (Fig. 6a). Western blot experiments showed that proteins without SPs were expressed in the infiltrated leaves of *N. benthamiana* (Fig. 6b). Taken together, these results indicate that not all of the SPs of the tested secreted proteins in *T. horrida* are required to trigger cell death in *N. benthamiana*.

The predicted RNase active site of gsr1 is essential for its cell death-inducing ability

It was predicted that gsr1 has four conserved RNase domains, and uan2 was predicted to contain two conserved RNase domains. To identify whether potential RNase activity was necessary for the ability of effector genes to induce cell death, we deleted the gsr1 predicted active site (Tyr-60, Glu-79, Arg-96, and His-112) and the uan2 predicted active site (Val-68, Leu-147) of the conserved RNase domain using a sequence synthesis technique. The full-length gsr1 and uan2, as well as the uan2 mutant proteins without predicted active sites caused cell death in *N. benthamiana* (Fig. 7a). However, the expression of gsr1 mutant proteins without predicted active sites in *N. benthamiana* did not induce cell death (Fig. 7a). Western blot analysis showed that these four gsr1 mutant proteins without predicted active sites were expressed in *N. benthamiana* leaves (Fig. 7b). These results indicate that the putative RNase active site of gsr1 is essential for its ability to induce plant cell death, but that this is not the case for uan2.

Expression analysis of two putative effector genes during *T. horrida* infection of young panicles

In plant-pathogen interactions, the effector genes in filamentous pathogens are often transcriptionally induced...
To clarify the change in effector gene expression during *T. horrida* infection, the young panicles of rice male sterile lines Jiangcheng 3A (phenotype resistant to *T. horrida*), and 9311A (phenotype susceptible to *T. horrida*) were infected with the highly virulent isolate JY-521. The expression levels of these two secreted protein-encoding genes 8, 12, 24, 48, and 72 h after infection were identified by quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR). These two effector genes were up-regulated 8 h after infection with *T. horrida* JY-521, and the level of gsr1 expression peaked at 8 h, while that of uan2 peaked at 72 h (Fig. 8). These results were consistent with the transcription data, and showed that both effector genes induced up-regulated expression during the early infection stage [23]. The findings suggest that these genes have important roles in host-fungal interactions.

**Identification of the *N. benthamiana* immune response induced by gsr1 and uan2**

We evaluated the expression levels of several genes related to the activation of the immune response in *N. benthamiana* using qRT-PCR. Among the selected pathogenicity related (PR) proteins, PR1a and PR2 were induced by uan2 at 8 and 24 h, respectively, compared with the control. By contrast, neither PR3 nor PR4a showed any significant transcriptional change compared with the control during the 72 h time course of the infection.
experiment. The transcription factor WRKY12 was also strongly induced at 12 h by these two effector genes (Fig. 9a). For gsr1, PR1a and PR2 were induced at 12 h (Fig. 9a). In addition, we investigated the ability of these two effector genes to induce the hydrogen peroxide (H₂O₂) mediated activation of *N. benthamiana* leaves using 3′-diaminobenzidine (DAB) dye [32]. The results showed that both effector genes could induce H₂O₂ activation at 72 h after infection compared with the control (Fig. 9b, c, d).

![Fig. 4 Trend analysis of putative effector genes. a The expression of 12 genes in profile 18 (smut_5844 was included) and 14 genes in profile 19 (smut_2965 was included). b The 291 differentially expressed genes that encoded secreted proteins were clustered in 23 profiles (no genes were found in profile 12). In the analysis, smut_3800 was clustered in profile 18.](image)

![Fig. 5 Functional validation of signal peptides (SPs) of putative *T. horrida* effectors using a yeast invertase secretion assay. The SPs predicted two *T. horrida* putative effectors. All transformed yeast strains YTK12 were able to grow on YPRAA media with raffinose as the sole carbon source (1% yeast extract, 2% peptone, 2% raffinose, and 2 μg of antimycin A per liter). The N-terminal sequences of *Phytophthora sojae* Avr1b and *Magnaporthe oryzae* Mg87 were used as positive and negative controls, respectively. The untransformed YTK12 did not grow on either CMD-W (0.67% yeast N base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar) or YPRAA media. Yeast growth on CMD-W media was equally viable among the transformed strains. Row a: CMD-W media; row b: YPRAA media; Mg87 (−): negative controls Mg87 SPs; Avr1b (+): positive controls Avr1b SPs; sp2965: SPs of smut_2965; sp5844: SPs of smut_5844.](image)
Prediction of uan2-interacting proteins by a yeast two-hybrid analysis and transcriptome

The auto-activation test showed that positive controls (the pGBKT7–53 and pGADT7-T vector together) grew on both the SD-Trp/−Leu and SD-Trp/−Leu/−His/−Ade/Aba/X-α-gal plates; while a negative control (the pGBKT7-lam and pGADT7-T vectors together), the pGBKT7 empty vector, and the pGBKT7 vector with uan2 grew on the SD-Trp/−Leu plate, but not on the SD-Trp/−Leu/−His/−Ade/Aba/X-α-gal plate (Fig. 10a and b). These results indicate that uan2 does not exhibit auto-activation activity in yeast. To identify putative uan2-interacting proteins, the full sequence of uan2 was used as a bait to screen a rice cDNA library using yeast two-hybrid analysis and nine potential interactors were identified. The gene sequences that encoded these nine proteins were obtained and further analyzed.

Fig. 6 a The ability of truncated T. horrida secreted proteins lacking signal peptides (SPs) to induced necrotic cell-death. PMDC32–2965-sp denotes smut_2965 without SPs; PMDC32–5844-sp denote smut_5844 without SPs. Numbers, e.g., 20/20, indicate that 20 of 20 infiltrated leaves exhibited cell death or mottling phenotypes. b Expressed proteins were detected in the leaves of N. benthamiana following western blot experiments. M: marker; b1–2: smut_2965-sp (12.9 kDa) and smut_5844-sp (24 kDa).

Fig. 7 The predicted RNase active site of smut_2965 is required for its cell-death triggering ability. a The mutant proteins smut_2965–60, smut_2965–79, smut_2965–96, and smut_2965–112 lost the ability to induce cell-death, while smut_5844–68 and smut_5844–174 induced cell-death in Nicotiana benthamiana leaves. GFP refers to the PMDC32 vector containing the green fluorescent protein (GFP) gene, the same labeling is used for 2965, 5844, 2965Δ60, 2965Δ79, 2965Δ96, 2965Δ112, 5844Δ68, and 5844Δ147. Numbers, e.g., 0/35, indicate that 0 of 35 infiltrated leaves exhibiting cell-death or mottling phenotypes. b Protein expression of the smut_2965 and smut_5844 mutant proteins in the infiltrated leaves was detected by western blotting. M: marker; b1–6: smut_2965Δ60 (13.74 kDa), smut_2965Δ79 (13.74 kDa), smut_2965Δ96 (13.74 kDa), smut_2965Δ112 (13.74 kDa), smut_5844Δ68 (24.72 kDa), and smut_5844Δ147 (24.72 kDa).
proteins are listed in Additional file 3: Table S3. Nucleotide sequence analysis further revealed that these nine proteins cDNA fragment encoding laccase precursor protein (LOC_Os03g16610), ribosomal protein S2 (LOC_Os07g42450), ARF-GTPase-activating protein (OsAGAP), cholororespiratory protein (CRR6), chaperone protein dnaJ (LOC_Os05g26926), ML domain protein (LOC_Os07g06590), retrotransposon protein (LOC_Os11g46020), Os10g33350), and endo-beta-N-acetylglucosaminidase (LOC_Os07g30920), and endo-beta-N-acetylglucosaminidase (LOC_Os05g33350), respectively. However, only two genes LOC_Os03g16610 (OsLAC10) and LOC_Os07g42450 (OsRbs5) showed up-regulated expression after \( T. \) horrida inoculation according to the transcriptome data from our earlier report (Fig. 10d) [31]. In addition, laccase is associated with lignification and plays an important role in iron metabolism [33–36]. Hood et al. reported that the expression of a fungal laccase gene in transgenic maize is associated with kernel browning and limited germination [37]. These results further indicate that uan2 could interact with OsLAC10. To determine the interaction between uan2 and its potential partner, OsLAC10, a yeast two-hybrid assay, was performed via co-transformation of pGBK7-uan2 and pGADT7-OsLAC10 in the \( Y_{2}H \) Gold yeast strain. Yeast cells harboring both uan2 and OsLAC10 grew vigorously on both SD/Leu-Trp media and SD-Trp/−Leu/−His/−Ade/AbA/X-a-gal media (Fig. 10c).

**Discussion**

Plant pathogen fungal effector proteins have important roles in host-pathogen interactions [38]. In the \( T. \) horrida genome, there are 597 genes encoding secreted proteins, of which 131 are predicted to be candidate effectors [23]. Transcriptome analyses showed that many secreted protein genes in \( T. \) horrida were up-regulated during early infection [23]. To clarify the function of fungal effectors, \( N. \) benthamiana has been extensively studied as a model system using agroinfiltration [39]. Several effectors that induce non-host cell death have been identified in \( M. \) oryzae by transient expression assays in \( N. \) benthamiana using agroinfiltration [40]. Similar studies have been reported in \( P. \) sojae [41]. In this study, only 36 candidates were randomly selected for a functional assay; there two putative effectors in \( T. \) horrida were demonstrated to trigger cell death phenotypes in \( N. \) benthamiana. Further experiments showed that these two putative effectors were up-regulated during the early stages of \( T. \) horrida infection (Fig. 8), which is a common feature of these two effector proteins. In addition, transcriptome data in our study may enable the candidate effectors to be investigated, and provide useful information for the further analysis of fungus–plant interactions. Future studies should aim to identify additional effectors in \( T. \) horrida.

In our study, we found that gsr1 genes in \( T. \) horrida did not require an SP to trigger cell death in \( N. \) benthamiana. Interestingly, many oomycete RxLR effectors, such as AVR3a KI, ATR1 NdWsB, and ATR13, when directly expressed in plant, did not require an SP to trigger a hypersensitive response and were, thus, recognized inside the plant cytoplasm [42]. The requirement of an SP for secreted proteins to induce cell death in plants indicates that these proteins might function in the extracellular space. The effector protein VmE02 secreted by the necrotrophic fungus \( V. \) mali could induce cell death with or without an SP, and has been identified as a secreted apoplastic fungal protein [43]. This finding is similar to that for effector SsCP1, an extracellular protein secreted by \( S. \) sclerotiorum, which also triggers cell death without an SP [44]. The
effectors AVR3a^KI, ATR13, and ATR1^NdWaB have been found in plant cytoplasm, where SPs were not required for the triggering of cell death [45]. In addition, uan2 is a small cysteine-rich protein and SP was required for its ability of induced cell death. It is possible that the cysteine residues of uan2 form multiple disulfide bonds to stabilize their tertiary structure. This stabilization would, in turn, protect uan2 against degradation by apoplastic plant proteases, as has been shown for other secreted apoplastic fungal proteins [46]. These results indicate the importance of the extracellular space for the function of uan2. Therefore, we speculate that uan2 may be an apoplastic effector.

We also identified the effector gene gsr1 as a fungus-specific RNase-like protein that possesses the ability to trigger cell death in *N. benthamiana*. Our results indicate that RNase active sites were necessary for the ability to trigger cell death. In the transient assay, necrosis was abrogated in the mutant lacking this domain. This was also observed to be the case for the effector protein UV_1423 secreted by *U. virens*; RNase active sites were essential to its cell death–inducing activity [22]. BEC1011 and BEC1054 are secreted RNase like effectors in the powdery mildew pathogen *Blumeria graminis* f. sp. *Hordei*, which play an important role in *B. graminis* infection; however, the contribution of BEC1011 and BEC1054 to *B. graminis* infection may not involve RNase activity, with a similar situation for uan2 [47]. Furthermore, SPs were required for uan2 to induce cell death in *N. benthamiana*. A similar phenomenon has been found in *U. virens* effector proteins [22]. The eight full-length effectors in *U. virens* are able to trigger...
cell-death in leaf of *N. benthamiana*; however, proteins without SPs lack this ability [22]. The effector protein MoCDIP1 in *M. oryzae*, also requires an SP to trigger cell death [48]. This suggests that different effectors in same pathogenic fungi may function in the host-pathogen interaction via different mechanisms.

We determined the ability of these two effector genes to trigger the activation of the *N. benthamiana* immune response by confirming the expression of pathogenesis-related genes and the induction of H$_2$O$_2$ mediated activation. Gsr1 and uan2 could induce PR1a and PR2 at different inoculation times compared with controls. The induction of PR1a and PR2 was related to the activity of salicylic acid, which triggers the response to biotrophic pathogens [49]. As *T. horrida* is a biotrophic pathogen, our results indicate that the gsr1 and uan2 proteins may act as biotrophic effectors that trigger a biotrophic-specific response in plant cells. The transcription regulator gene WRKY12, which is involved in plant cell-death and known to participate in the defense against soft rot disease in both Chinese cabbage and Arabidopsis [50], was also triggered in *N. benthamiana* leaves following the transient expression of gsr1 and uan2. The induction of WRKY12 showed that the symptoms observed in *N. benthamiana* leaves are associated with plant cell death. Hydrogen peroxide has an important role in plant disease-resistance [51, 52]. In our study, the activation of H$_2$O$_2$ was also triggered by gsr1 and uan2 in *N. benthamiana* leaves. In conclusion, the activation of the *N. benthamiana* immune system was induced by these two effector genes.

---

**Fig. 10** Yeast two-hybrid analysis of the smut_5844. a-b Autoactivation activity test of smut_5844 protein in yeast. a: SD-Trp/−Leu, b: SD-Trp/−Leu/−His/−Ade/X-α-gal. pAD-WT/pBD-WT was used as positive controls, and pAD-WT/pLaminC was used as a negative control. WT, Wild-type fragment C of lambda cl repressor (aa 132–236); Mut, E233K mutated fragment of lambda cl repressor (aa 132–236); Lamin C, human lamin C (aa 67–230). c Interaction between smut_5844 and OsLAC10 in a yeast two-hybrid system. P53 and SV40 were used as a pair of positive control. Lam and SV40 were used as a negative control. SD-2, SD-Trp-Leu; SD-4, SD-Trp-Leu-His-Ade. d The expression patterns of OsLAC10 and OsRbs5 in resistant and susceptible rice male sterile lines after *T. horrida* inoculation.
Plant pathogen effectors evolved rapidly, as shown by plant resistant genes, resulting in a diverse range of effector proteins involved in host evasion [53]. For the T. horrida genome, 131 putative effectors were grouped into five clusters, demonstrating that marked sequence diversity exists among these putative effectors. On the other hand, a degree of conservation was observed in effectors among the related fungal species. For example, we found several homolog genes of smut_2965 in five smut species. We identified two candidate effectors in T. horrida that induced non-host cell-death or a defense response. However, the specific molecular mechanisms by which these proteins exert their effects rice–T. horrida interaction require further study.

**Conclusions**

We investigated the cell death–inducing ability of 131 putative effectors in T. horrida through transient expression assays. Two putative effectors were found to induce cell-death and defense machinery expression in N. benthamiana. The predicted SP is required for the cell death–inducing activity of un2, and the predicted RNase active site of gsr1 is essential for its cell death-inducing ability. Our results provide a useful foundation for understanding the mechanisms underlying rice–T. horrida interaction.

**Methods**

**Strains, plant materials, and growth conditions**

Two rice cultivars that displayed marked differences in their susceptibility to infection by T. horrida, the resistant rice male sterile line Jiangcheng 3A and the susceptible rice male sterile line 9311A [31], were used in this study. They were provided by the Department of Rice Research Institute of Sichuan Agricultural University. The T. horrida strain JY-521 was cultured in PSA medium (potato 200 g, sucrose 20 g, agar 15 g, and distilled water 1000 ml) at 28 °C. Constructed expression plasmids were transferred to Agrobacterium tumefaciens GV3101, which was cultured in LB medium (yeast extract 10 g, beef extract 5 g, NaCl 10 g, and distilled water 1000 ml). The yeast strain YTK12 was grown on YPD medium (yeast extract 10 g, peptone 20 g, glucose 20 g, agar 20 g, adenine hemisulfate 0.03 g, and distilled water 1000 ml). The yeast strain YTK12 was grown on YPD medium (yeast extract 10 g, peptone 20 g, glucose 20 g, agar 20 g, adenine hemisulfate 0.03 g, and distilled water 1000 ml). Antibiotics and the concentrations used were as follows: kanamycin 50 μg · ml⁻¹, ampicillin 100 μg · ml⁻¹, and rifampin 25 μg · ml⁻¹. N. benthamiana plants were used for transient gene expression, and were grown under a 14 h day and 10 h night cycle at 23–25 °C with 60% relative humidity.

**Homology analysis of secreted proteins**

The coding DNA sequences of predicted secreted proteins in nine smut fungal genomes were obtained and compared using BLASTP 2.6.0+ with an e-value ≤1e⁻⁷ [54]. Proteins passing this filter were clustered into families though the Markov clustering algorithm in OrthoMCL v1.4 [54, 55] with the options “–mode 3” -l as 2.0, and single-copy gene families of secreted proteins in nine smut fungal genomes were obtained. The protein sequence of each single-copy family was aligned by MUSCLE v3.8.31.

**Analysis of the expression levels of the candidate small secreted protein encoding genes**

Transcriptome data were provided in our previous report [23]. A total of 291 differentially expressed genes in the T. horrida secreted protein were classified into 23 clustered profiles based on trends observed in gene expression using the Short Time-series Expression Miner software [56]. Clustered profiles with P ≤ 0.05 were considered to be statistically significant.

**RNA isolation and plasmid construction of T. horrida putative effector genes**

Total RNA was extracted from T. horrida using a Fungal RNA kit (Omega, Biel, Switzerland), and cDNA was synthesized using a Transcriptor First strand cDNA synthesis kit (Roche, Basel, Switzerland). Secreted proteins and putative effectors in T. horrida were predicted according to a previous study [57]. The full-length secreted protein-encoding genes were amplified with Trans Start FastPfu Fly DNA Polymerase (TransGen Biotech, Beijing, China). All restriction enzymes and ClonExpress enzymes were used following the manufacturer’s instructions (Vazyme Biotech, Nanjing, China). Primers for these assays were designed using CE Design v1.03, based on our predicted gene sequences and included a BamHI site and a Stul site. The primer sequences are listed in Additional file 4: Table S4. The obtained cDNA of target genes were gel-purified with a gel purification kit (Omega) and cloned into the PMDC32 expression vector.

**Prediction and function validation of SPs**

The SPs of two candidate effector genes were predicted using http://www.cbs.dtu.dk/services/SignalP/. Functional analysis of the predicted SP of two candidate effectors in T. horrida was performed using a yeast secretion assay according to a method described in a previous report [58]. The SP sequences of two predicted effector genes were cloned into pSUC2 vectors that had a truncated invertase gene, which lacked the start codon and SP sequence, and were then transformed into the yeast strain YTK12 using Frozen-EZ yeast transformation II kit (Zymo Research, Irvine, CA, USA). A 100 μl solution of transformants was inoculated into CMD-W medium (yeast N base without amino acids 6.7 g, tryptophan dropout supplement
0.75 g, sucrose 20 g, glucose 1 g, agar 15 g, and distilled water 1000 ml, pH = 5.8). A single colony of yeast was selected, and inoculated on CMD-W and YPRAA medium (yeast extract 10 g, peptone 20 g, raffinose 20 g, antinycin A 2 μg, agar 15 g, and distilled water 1000 ml, pH = 5.8).

Site deletion by sequence synthesis
The RNase active site deletion mutant sequences of putative effector genes smut_5844 and smut_2965 were obtained using BioXp™ 3200 System DNA synthesis instruments according to the manufacturer’s instructions [59], with analysis performed at Sangon Biotech, Shanghai, China. The mutated gene sequences were relegated into the PMDC32 vector. The mutated gene sequences were listed in Additional file 5: Table S5.

Agrobacterium tumefaciens–mediated transient gene expression
We according to a method used in our previous studies to performed the N. benthamiana leaf transformation [23]. The infiltration experiment of each construct was totally for 20 leaves from different plants as repeated. The positive control using vector containing the BAX gene, and a vector containing the GFP gene inoculant was used as the negative control. Four days after infiltration, we will take note of the phenotype of cell death.

Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) and 3′-Diaminobenzidine (DAB) and trypan blue dye
The resistant rice male sterile line Jiangcheng 3A and the rice male sterile line 9311A that is highly susceptible to T. horrida were infected with the T. horrida strain JY-521 [31]. The method of T. horrida inoculation according to our former reported [23]. Mycelia of T. horrida JY-521 were collected at five time points post inoculation (8, 12, 24, 48, and 72 h). Mycelia of T. horrida JY-521, with no infected rice kernel, served as the control (infection 0 h), and were immediately frozen in liquid nitrogen, and stored at −80 °C. Total RNA of T. horrida JY-521 was isolated using the Omega Fungal RNA kit. The ubiquitin (UBQ) gene was used as an internal control for data normalization. The primers used for qRT-PCR are listed in Additional file 6: Table S6.

Protein extraction and immunoblot analysis
N. benthamiana leaves inoculated with A. tumefaciens for 72–96 h were ground using liquid nitrogen. Protein extraction was performed using a one-step plant active protein extraction kit (Sangon Biotech, Shanghai, China) according to the instructions. The extracted proteins were separated using electrophoresis with 12% sodium dodecyl sulfate-polyacrylamide. To observe sample loading, the separated proteins were electrophoretically blotted on nitrocellulose membranes and stained using 0.1% Ponceau S. Five percent skimmed milk in TBS-T buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) was used to block the membranes for 1 h at room temperature, followed by incubation for 1 h using anti-FLAG antibody solution (1:5,000 dilution) at room temperature; the membranes were then washed using TBS-T buffer. Blots were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5,000 dilution in TBS-T) at room temperature. The immunoblots were incubated using the eECL western substrate and observed with X-films.

Yeast two-hybrid analyses of protein–protein interactions
The auto-activation test and cDNA library screening were performed according to the instructions of the “HybriZAP-2.1 Two-Hybrid Libraries” system. The smut_5844 sequence was cloned to the pGBKT7 vector, and the pGBK T7 empty vector and pGBK T7 vector with smut_5844 were co-transformed into yeast strain Y2H and inoculated onto plates lacking tryptophan or leucine (SD-Trp/−Leu) for 2–3 d. Single colonies were selected and inoculated into fluid SD-Trp/−Leu medium for 2 d; Different concentrations of bacterial liquid were inoculated onto SD-Trp/−Leu and SD plates without tryptophan, leucine, histidine, and adenine, supplemented with 50 μg/ml Aureobasidin A (Ab) and 20 μg/ml X-a-gal (SD-Trp/−Leu/−His/−Ade/Ab/X-a-gal) for the auto-activation test. The pGBK T53 and pGAD T7-T vectors served as positive controls, while the pGBK T7-lam with pGAD T7-T vector served as a negative control. For cDNA library screening, the bait constructed (pGBK T7-smut_5844) and pGAD T7-cDNA plasmid libraries were sequentially transformed into Y2H strains. The transformed yeast strains were then spread on SD-Trp/−Leu/−His/−Ade/Ab/X-a-gal plates and incubated at 28 °C.
for 2–4 d until colonies appeared. The blue colonies were then selected as potentially interacting candidates. The OsLAC10 coding region was cloned into pGADT7 based on the sequencing result. Yeast transformation was conducted following the Yeast Transformation Protocol (Clontech).

Additional files

- **Additional file 1:** Table S1. The predicted secreted proteins of nine smut fungus. (XLSX 67 kb)
- **Additional file 2:** Table S2. The 36 randomly selected predicted effectors from 131 predicted effectors. (XLSX 10 kb)
- **Additional file 3:** Table S3. The sequence of smut_2965, smut_5844, and potential interacting proteins with smut_5844. (XLSX 12 kb)
- **Additional file 4:** Table S4. The primer sequence of two effector genes. (XLSX 9 kb)
- **Additional file 5:** Table S5. The sequence of mutated genes. (XLSX 9 kb)
- **Additional file 6:** Table S6. The primer sequence of qRT-PCR. (XLSX 8 kb)

Abbreviations

- GFP: Green fluorescent protein; H$_2$O$_2$: Hydrogen peroxide; PR: Pathogenicity related; qRT-PCR: Quantitative real time reverse transcription-polymerase chain reaction; RKS: Rice kernel smut; RT: Room temperature; SPs: Signal peptides; STEM: Short Time-series Expression Miner software

Acknowledgments

The authors thank all contributors for their work and would like to thank the reviewers for their valuable comments and suggestions.

Authors’ contributions

AW conceived, designed and carried out the experiments, analyzed the data and drafted the manuscript; LP performed RT-PCR analysis; XN, XS, and XY participated in the design; PL and AZ conceived the study and participated in the design. All the authors read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (31400130). The funding agent only provided the financial support and did not involve in the design of the experiment, collection, interpretation and analysis of data and in the drafting of the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1 Rice Research Institute of Sichuan Agricultural University, Chengdu, China. 2 Key laboratory of Sichuan Crop Major Disease, Sichuan Agricultural University, Chengdu, China. 3 Key Laboratory of Southwest Crop Gene Resource and Genetic Improvement of Ministry of Education, Sichuan Agricultural University, Yaan, China.

References

1. Chen Y, Yang X, Yao J, Kyaw EP, Zhang AF, Li YF, Gu CY, Zang HY, Gao TC. Simple and rapid detection of Tilletia horrida causing rice kernel smut in rice seeds. Sci Rep. 2016;6:33258.
2. Webster RK, Gunnell PS. Compendium of rice diseases. Mycologia. 1992;84:953.
3. Takahashi Y, On Ustilago virens Cooke and a new species of Tilletia parasitic on rice plant. Tokyo Bot Mag. 1896;10:16–20.
4. Tsuda M, Sasahara M, Ohara T, Kato S. Optimal application timing of simeconazole granules for control of rice kernel smut and false smut. J Gen Plant Pathol. 2006;72(5):301–4.
5. Biswas A. Kernel smut disease of rice current status and future challenges. Environ Ecol. 2003;21:336–51.
6. Carris LM, Castlebury LA, Goates BJ. Nonsystemic bunt Fungi-Tilletia indica and T. horrida: a review of history, systematics, and biology. Annu Rev Phytopathol. 2006;44:13–33.
7. Brooks SA, Anders MM, Yeater KM. Effect of cultural management practices on the severity of false smut and kernel smut of Rice. Plant Dis. 2005;93:1202–8.
8. Dou D, Zhou JM. Phytopathogen effectors subverting host immunity: different foes, similar battleground. Cell Host Microbe. 2012;12:484–95.
9. Howden AJ, Huitema E. Effector-triggered post-translational modifications and their role in suppression of plant immunity. Front Plant Sci. 2012;3:1360.
10. Quentin M, Abad P, Favery B. Plant parasitic nematode effectors target host defense and nuclear functions to establish feeding cells. Front Plant Sci. 2013;4:553.
11. Dean RA, Talbot NJ, Ebbole DJ, Farman ML, Mitchell TK, Orbach MI, Thon M, Kulkami R, Xu J, Pan H, Read ND, Lee YH, Carbone I, Brown D, Oh YY, Donofio N, Jeong JS, Soanes DM, Djonovic S, Kolmiots E, Rehmeyer C, Li W, Harding M, Kim S, Lebrun MH, Bohnert H, Coughan S, Butler J, Calvo S, Na LJ, Nicol R, Purcell S, Nutbaum C, Galagan JE, Biren BN. The genome sequence of the rice blast fungus Magnaporthe grisea. Nature. 2005;434(7036):980–6.
12. Mogga V, Delventhal R, Weidenbach D, Langer S, Bertram P, Andresen K. Magnaporthe oryzae effectors MoHEG13 and MoHEG16 interfere with host infection and MoHEG13 counteracts cell death caused by Magnaporthe-NLPs in tobacco. Plant Cell Rep. 2016;35(5):1169–85.
13. Mueller AN, Ziemann S, Tentschlie S, Albenb D, Doehlemann G. Compatibility in the Ustilago maydis maize interaction requires inhibition of host cysteine proteases by the fungal effector PIt2. PLoS Pathog. 2013;9:e1003177.
14. Redkar A, Villajuan-Bonequi M, Doehlemann G. Conservation of the Ustilago maydis effector See1 in related smuts. Plant Signal Behav. 2015;10(12):e108685.
15. Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G. The Ustilago maydis effector See1 in related smuts. Plant Signal Behav. 2015;10(12):e108685.
16. Djamei A, Schipper K, Rabe F, Ghosh A, Vincon V, Kahnt J, Osorio S, Tohge T, Kulkami R, Xu J, Pan H, Read ND, Lee YH, Carbone I, Brown D, Oh YY, Donofio N, Jeong JS, Soanes DM, Djonovic S, Kolmiots E, Rehmeyer C, Li W, Harding M, Kim S, Lebrun MH, Bohnert H, Coughan S, Butler J, Calvo S, Na LJ, Nicol R, Purcell S, Nutbaum C, Galagan JE, Biren BN. The genome sequence of the rice blast fungus Magnaporthe grisea. Nature. 2005;434(7036):980–6.
17. Mogga V, Delventhal R, Weidenbach D, Langer S, Bertram P, Andresen K. Magnaporthe oryzae effectors MoHEG13 and MoHEG16 interfere with host infection and MoHEG13 counteracts cell death caused by Magnaporthe-NLPs in tobacco. Plant Cell Rep. 2016;35(5):1169–85.
18. Mueller AN, Ziemann S, Tentschlie S, Albenb D, Doehlemann G. Compatibility in the Ustilago maydis maize interaction requires inhibition of host cysteine proteases by the fungal effector PIt2. PLoS Pathog. 2013;9:e1003177.
19. Redkar A, Villajuan-Bonequi M, Doehlemann G. Conservation of the Ustilago maydis effector See1 in related smuts. Plant Signal Behav. 2015;10(12):e108685.
20. Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G. The Ustilago maydis effector See1 in related smuts. Plant Signal Behav. 2015;10(12):e108685.
21. Tanaka S, Brefort T, Neilig N, Djamei A, Kahnt J, Venemirs W, Koenig S, Feussner K, Feussner I, Kahmann R. A secreted Ustilago maydis effector promotes virulence by targeting anthocyanin biosynthesis in maize. elife. 2014;3:e03135.
22. Luderer R, Takken FL, de Wit PJ, Joosten MH. Cladosporium fulvum overcomes Cf-2-mediated resistance by producing truncated AVR2 elicitor proteins. Mol Microbiol. 2002;45:875–84.
23. Van der Hoorn RA, Laurent F, Roth R, De Wit PJ. Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis. Mol Plant-Microbe Interact. 2000;13:439–46.
24. Schulze-Lefert P, Panstruga R. A molecular evolutionary concept connecting non-host resistance, pathogen host range, and pathogen speciation. Trends Plant Sci. 2011;16:117–25.
25. Stamm R, Mantellin S, McLellan H, Thilliez G. The role of effectors in nonhost resistance to filamentous plant pathogens. FrontPlant Sci. 2014;5:582.
26. Fang AF, Han YQ, Zhang N, Zhang M, Liu LJ, Li S, Lu F, Sun WX. Identification and characterization of plant cell death–inducing secreted proteins from Ustilaginoidea virosae. Mol Plant-Microbe Interact. 2016;29:405–16.
23. Wang AJ, Pan LX, Wang N, Ai P, Yin DS, Li SC, Deng QM, Zhu J, Liang YY, Zhu JQ, Li P, Zheng AP. The pathogenic mechanisms of Tilletia horrida as revealed by comparative and functional genomics. Sci Rep. 2018;8:15413.

24. Zhang Y, Zhang K, Fang A, Han Y, Yang J, Xue M, Bao J, Hu D, Zhou B, Sun X, Li S, Wen M, Yao N, Ma L, Liu Y, Zhang M, Huang F, Luo C, Zhou L, Li J, Chen Z, Miao J, Wang S, Lai J, Xu J, Hisagi T, Peng XL, Sun WK. Specific adaptation of Ustilaginoidea virens in occupying host florets revealed by comparative and functional genomics. Nat Commun. 2014;5:3849.

25. Jacobs KA, Collins-Racie LA, Colbert M, Duckett M, Golden-Fleet M, Kelleher K, Kim R, LaVallie ER, Merberg D, Spaulding V, Stover J, Williamson MJ, McCoy CM. A genetic selection for isolating cDNAs encoding secreted proteins: Gene. 1992;19:289–296.

26. Oh SK, Young C, Lee M, Ohlra R, Bokzurt TC, Cano LM, Win J, Bos J, Liu HY, van Damm M, Morgan W, Choi D, Van der Vossen EA, Veeshouwars VG, Kamoun S. In plant expression screens of Phytophthora infestans RLRX effector reveal diverse phenotypes, including activation of the Solanum bulbocastanum disease resistance protein Rpi-bib2. Plant Cell. 2009;21(1):2928–2947.

27. Tian M, Win J, Savoye F, Burkhardt A, Held M, Brandizzi F, Day B. 454 Genome sequencing of Pseudopanonozygus cubensis reveals effector proteins with a QXLX translocation motif. Mol Plant-Microbe Interact. 2011;24:543–553.

28. Gu B, Kale SD, Wang Q, Wang D, Pan Q, Cao H, Meng Y, Kang Z, Tyler BM, Shaw W. Rust secreted protein Ps87 is conserved in diverse fungal pathogens and contains a RLRX motif sufficient for transcription into plant cells. PLoS ONE. 2011;6:e27217.

29. Saitok H, Fujisawa S, Mitsuoka C, Hirabuchi A, Ikeda K, Irieda H, Yoshida K, Matsumura H, Tosa Y, Win J, Kamoun S, Takano Y, Teracha R. Large-scale gene expression in Magnaporthe oryzae identified MC 69, a secreted protein required for infection by monocot and dicot fungal pathogens, PLoS pathogens. 8(5):e1002711.

30. Stergiopolous I, de Wit PJ. Fungal effector proteins. Annu Rev Phytopathol. 2009;47:233–263.

31. Wang AJ, Shu YY, Niu YY, Zhao WJ, Ai P, Li P, Zheng AP. Comparison of gene co-networks analysis provide a systems view of rice (Oryza sativa L.) response to Tilletia horrida infection. PLoS ONE. 2018;13(10):e0203209.

32. Thordal Christensen H, Zhang Z, Wei YD. H2O2 accumulation in papillae and hypersensitive response during the early powdery mildew interaction. Plant J. 1997;11:1187–1194.

33. Bao W, O Malley DM, Whetten R, Sederoff RR. A laccase associated with hypocotyl cell walls. Plant Physiol. 1994;106:1095–1102.

34. Ranocha P, Chabannes M, Chamayou S, Danoun S, Jauneau A, Boudet AM, Goffner D. Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiol. 2002;129:145–155.

35. Albrecht H, Benfuss K, Magalhaes-Lundback M, Horn ME, Drees C, Delaney DE, Clough R, Howard JA. Criteria for high-level production of H2O2 in transgenic plants. Plant Physiol. 1997;115:427–435.

36. Thomma BPHJ, Eggermont K, Penninckx IAM. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis is essential for resistance to distinct microbial pathogens. Proceedings of the National Academy of Sciences of the United States of America. 1998;95:15107–15111.

37. Mehlhdy MC. Active oxygen species in plant defense against pathogens. Plant Physiol. 1994;105:467472.

38. Wu G, Barry JS, Ellen BL. Activation of host defense mechanisms by elevated production of H2O2, in transgenic plants. Plant Physiol. 1997;115:427–435.

39. Plegue C, Nowara D,Bonciani G, Gheorghe DM, Xu R, Surana P, Whigham E, Nettleton E, Bogdanove AJ, Wise RP, Schweizer P, Bindschedler LV, Spanu PD. Host-induced gene silencing in barley powdery mildew reveals a class of ribonuclease-like effectors. Mol Plant-Microbe Interact. 2013;26:633–642.

40. Stijn van D. Graph Clustering by Flow Simulation. PhD thesis, University of Utrecht. 2000.

41. Enright AJ, Van DS, Ouzounis CA. An efficient algorithm for large-scale detection of protein families. Nucleic Acids Research. 2002;30:1575–1584.

42. Ernst J, Bar-Joseph Z. STEM: a tool for the analysis of short time series gene expression data. BMC bioinformatics. 2006;7:191.

43. Zheng AP, Lin RM, Zhang DH, Qin FG, Xu LZ, Ai P, Ding L, Wang Y, Chen Y, Liu X, Sun ZG, Feng HT, Liang XX, Fu RT, Tang CQ, Li Q, Zhang J, Xie ZL, Deng QM, Li SC, Wang SQ, Zhu J, Wang LX, Liu HN, Li P. The evolution and pathogenic mechanisms of the rice sheath blight pathogen. Nature Communications. 2013;4:1424.

44. Li Y, Rose JCK. A Yeast Secretion Trap Assay for Identification of Secreted Proteins from Eukaryotic Phytopathogens and Their Plant Hosts. Plant Functional Pathogens. 2012;835:519–530.

45. Cheng Y, Chen HH, Yao KS, Yao WC, Peck K. High throughput parallel synthesis of oligonucleotides with 1536 channel synthesizer. Nucleic Acids Res. 2002;30:9e3.

46. Fernández-Bautista N, Domínguez-Núñez JA, Castellano Moreno MM, Berrocal-Lobo M. Plant Tissue Trypan Blue Staining During Phytopathogen Infection. The plant journal. 2016;24:1–7.