

Mining the effector repertoire of the biotrophic fungal pathogen *Ustilago hordei* during host and non-host infection

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SUMMARY

The success of plant-pathogenic fungi mostly relies on their arsenal of virulence factors which are expressed and delivered into the host tissue during colonization. The biotrophic fungal pathogen *Ustilago hordei* causes covered smut disease on both barley and oat. In this study, we combined cytological, genomics and molecular biological methods to achieve a better understanding of the molecular interactions in the *U. hordei*–barley pathosystem. Microscopic analysis revealed that *U. hordei* densely colonizes barley leaves on penetration, in particular the vascular system. Transcriptome analysis of *U. hordei* at different stages of host infection revealed differential expression of the transcript levels of 273 effector gene candidates. Furthermore, *U. hordei* transcriptionally activates core effector genes which may suppress even non-host early defence responses. Based on expression profiles and novelty of sequences, knockout studies of 14 effector candidates were performed in *U. hordei*, which resulted in the identification of four virulence factors required for host colonization. Yeast two-hybrid screening identified potential barley targets for two of the effectors. Overall, this study provides a first systematic analysis of the effector repertoire of *U. hordei* and identifies four effectors (Uvi1–Uvi4) as virulence factors for the infection of barley.

Keywords: barley, biotrophy, effectors, gene expression, *Ustilago hordei*

INTRODUCTION

Ustilago hordei is a facultative biotrophic fungal pathogen that causes covered smut disease on both barley and oat plants (Gaudet et al., 2010). *Ustilago hordei* is a member of the order Ustilaginales, which contains pathogens of several economically important crops, such as maize, wheat, oat, barley and sugar cane. Similar to other phytopathogenic smut fungi, the pathogenic development of *U. hordei* is coupled with sexual

development. For successful infection, two haploid sporidia of opposite mating types fuse and form the infectious dikaryotic filament that subsequently penetrates the host seedling (Hu et al., 2002). On penetration, *U. hordei* proliferates both extracellularly and intracellularly in or just below the shoot meristem. The covered smut of barley proceeds in a mostly symptomless manner; however, at the late stage of infection, the proliferation of *U. hordei* in the spikelet of the barley inflorescence and the kernels of infected plants is manifested by masses of dark brown smut spores (Hu et al., 2002).

Secreted proteins are at the front line of host–microbe interactions. The secretome of pathogens varies considerably based on their pathogenic life style (Lowe and Howlett, 2012). Although necrotrophic plant pathogens typically secrete plant cell wall-degrading enzymes and toxins to induce cell death and subsequently feed on host dead cells (Kubicek et al., 2014), the virulence of biotrophic pathogens is largely built on the secretion of effector proteins, which manipulate host physiology and immunity to promote disease establishment (Schuster et al., 2017). Effectors show diverse modes of action that are required at different stages of disease development; thus, their transcript levels fluctuate during colonization of the host (Kleemann et al., 2012; O'Connell et al., 2012). For example, *Phytophthora sojae* expresses the SNE1 effector to suppress the Necrosis- and Ethylene-inducing Peptide 1-Like Proteins (NLP1)-mediated cell death activity during the biotrophic stage; by doing so it can fine-tune the transition from the biotrophic to the necrotrophic stage (Kelley et al., 2010). Another example is the necrosis inducing protein 1 (NIP1) of *Rhynchosporium commune*, which is specifically induced on transition from the biotrophic to necrotrophic stage (Kirsten et al., 2012). Sequential expression of genes encoding putative effectors has also been observed for the obligate biotrophic rust fungus *Melampsora larici-populina* (Duplessis et al., 2011). *Ustilago maydis*, a close relative of *U. hordei*, is considered as a model system for biotrophs. It has been reported that *U. maydis* deploys distinct sets of effector genes in a stage-, host organ- and cell type-specific manner (Lanver et al., 2018; Matei

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et al., 2018; Schilling *et al.*, 2014; Skibbe *et al.*, 2010). However, some effectors are constitutively expressed during all stages of colonization in order to modulate plant defence. Examples are the conserved core effector Pep1, which inhibits POX12 activity (Hemetsberger *et al.*, 2012), and Pit2, which inhibits host plant papain-like cysteine proteases (Mueller *et al.*, 2013).

The genomics revolution of recent decades has increased our understanding of the genetic composition of a wide range of plant-pathogenic fungi (Dean *et al.*, 2005; Kämper *et al.*, 2006; O'Connell *et al.*, 2012; Schuster *et al.*, 2017; de Wit *et al.*, 2012). Several computational pipelines have been developed to identify the secretomes of phytopathogenic fungi, according to common hallmarks of fungal effectors, including the presence of a signal peptide for extracellular secretion, absence of transmembrane domains, molecular weight and cysteine content (Sperschneider *et al.*, 2015, 2015; de Wit *et al.*, 2012). Comparative genome analysis has revealed that, although closely related phytopathogenic fungi have core sets of effectors, a great number of fungal

effectors are still lineage specific (Schuster *et al.*, 2017). This makes the identification and characterization of effectors a challenging endeavour.

For the model system *U. maydis*, a rich toolbox for functional genetics is available (Kämper, 2004; Matei and Doehlemann, 2016; Spellig *et al.*, 1996). Several of these tools have been successfully adapted for *U. hordei* in this and other studies, including targeted gene deletion. The availability of a complete genome sequence (Laurie *et al.*, 2012) and the amenability to reverse genetics define the *U. hordei*-barley system as a good model to study the molecular basis of a fully biotrophic plant-pathogen interaction. In this study, we have analysed the effector repertoire of *U. hordei* by a transcriptome analysis at different stages of *U. hordei* infection. We functionally studied 14 effector candidates with regard to their contribution to *U. hordei* virulence. In addition, yeast two-hybrid (Y2H) screens identified potential host targets of two effectors with significant virulence function.

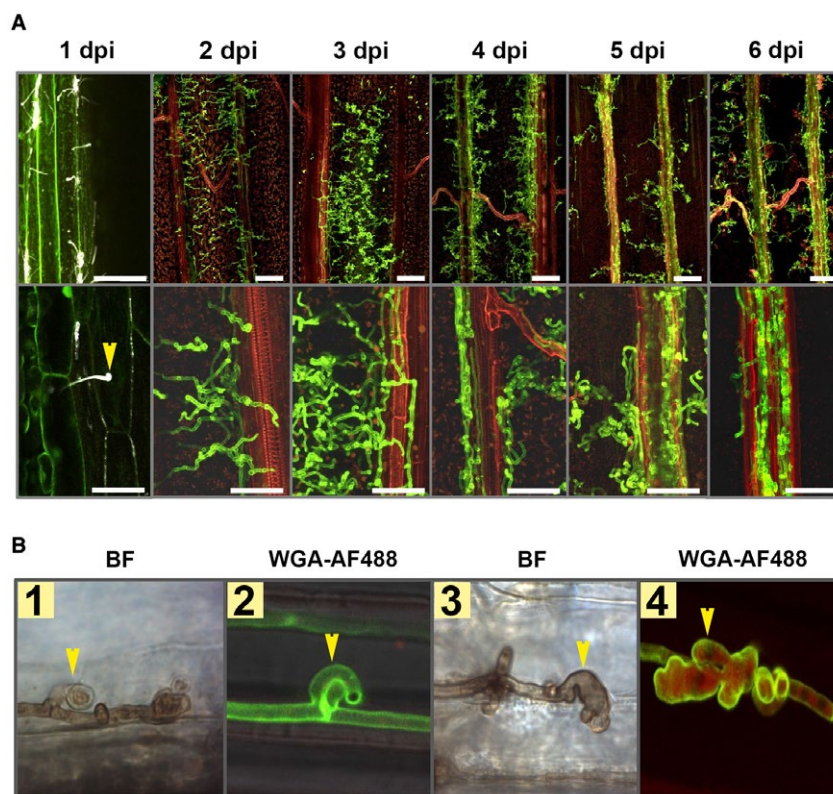


Fig. 1 *Ustilago hordei* disease progression on barley leaves. (A) Wild-type *U. hordei* (two mating types) was inoculated onto 10-day-old barley seedlings and disease progression was observed at 1, 2, 3, 4, 5 and 6 days post-inoculation (dpi). Microscopic observations were performed with calcofluor white at 1 dpi and with WGA-AF488/propidium iodide staining for the rest of the time points to visualize *U. hordei* colonization. Yellow arrow indicates *U. hordei* appressorium formation and host penetration. Scale bar at the top indicates 100 μ m and at the bottom indicates 50 μ m. Red represents plant cells and green represents fungal hyphae. (B) *Ustilago hordei* grows intracellularly in barley leaves and forms haustorium-like structures during barley colonization. WGA-AF488/propidium iodide staining and 3,3'-diaminobenzidine (DAB) staining were performed to visualize *U. hordei*. Yellow arrows indicate clamps (photographs 1 and 2) and haustorium-like structures (photographs 3 and 4). BF, bright field. The photographs were taken at 7 dpi. [Colour figure can be viewed at wileyonlinelibrary.com]

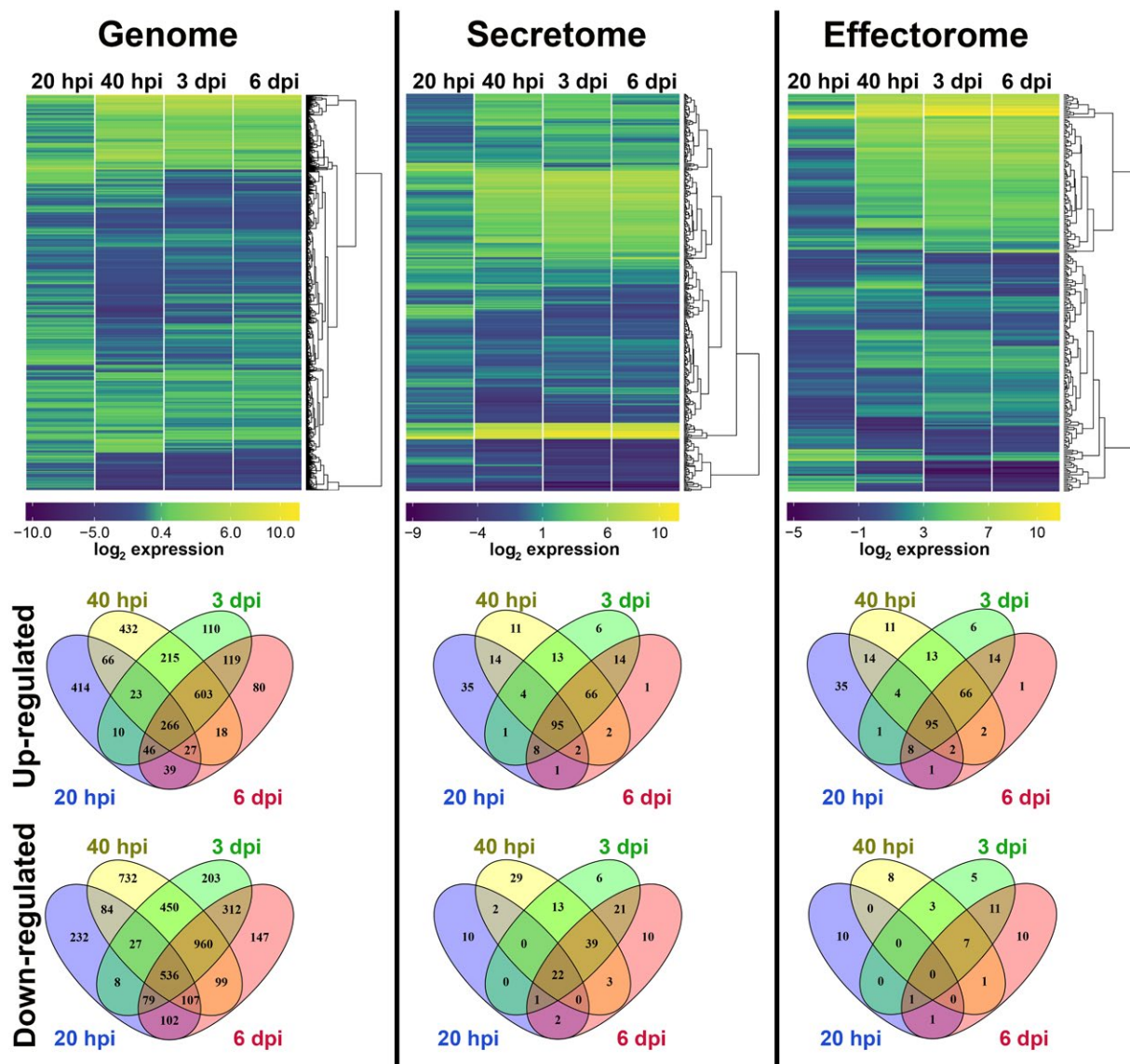


Fig. 2 The hierarchical relation heat map for microarray analysis of *Ustilago hordei* in planta. The expression profiles of the whole genome, secretome and effectorome of *U. hordei* are depicted. Differentially expressed *U. hordei* genes, both up-regulated (yellow) and down-regulated (blue), in planta are shown as log₂-fold changes relative to *in vitro* axenic culture. Differentially expressed genes are plotted as Venn diagrams. [Colour figure can be viewed at wileyonlinelibrary.com]

RESULTS

Infection of barley leaves by *U. hordei*

Ustilago hordei is a surface-borne smut fungus that initially penetrates the coleoptile of barley seeds and grows just below the apical shoot meristem without causing any visible symptoms until formation of the inflorescence (Hu et al., 2002). Therefore, on a macroscopic level, successful *U. hordei* infection can only be evaluated at the very late stage of disease (2–3 months after inoculation). In order to set-up a more rapid read-out for *U. hordei* infection, we inoculated *U. hordei* into leaf whorls of 10-day-old

barley seedlings, similar to the infection procedure that has been established for *U. maydis* on maize (Redkar et al., 2015). Fungal infection was monitored at 1, 2, 3, 4, 5 and 6 days post-inoculation (dpi) via confocal microscopy, using calcofluor white for investigation at 1 dpi and WGA-AF488/propidium iodide staining for the subsequent time points. The microscopic observations revealed that, at 20 h post-inoculation (hpi), *U. hordei* can successfully form appressoria to penetrate barley leaves (Fig. 1A). At 2 dpi, the fungal hyphae grow extracellularly and intracellularly inside the leaf and move from one cell to another. At 3 dpi onwards, *U. hordei* increasingly colonizes the plant's

vascular system (Fig. 1A). Interestingly, we observed that, when *U. hordei* hyphae grow inside barley cells, they form structures, which structurally resemble haustoria (Fig. 1B). Unlike fungal clamps (Fig. 1B, first two photographs), these haustorium-like structures (photographs 3 and 4) form multiple lobes and are found from 5 dpi onwards, but not at earlier time points.

Transcriptome analysis of *U. hordei* during infection of barley leaves

To analyse the gene regulation of *U. hordei* at different stages of host infection, a whole-genome transcriptome analysis was performed. The first time point for analysis was chosen at 20 hpi, when appressoria had formed and the fungus had started to penetrate epidermal host cells (Hof *et al.*, 2014). In addition, samples were taken for RNA preparation at 40 hpi (early accommodation), 3 dpi (growth to vasculature) and 6 dpi (massive fungal proliferation). The transcriptome analysis was performed using a custom-designed Agilent microarray, representing 7124 of the 7234 annotated *U. hordei* protein-encoding open reading frames (for details, see Experimental procedures). Changes in *U. hordei* gene expression on the host plant were calculated relative to *U. hordei* yeast cells grown in axenic culture. Overall gene expression patterns at different transition stages during host colonization relative to axenic culture were visualized in heat maps (Fig. 2). Genes showing a two-fold change in expression level were considered to be differentially expressed (\log_2 -fold change ≥ 1 ; false discovery rate (fdr) < 0.05 ; $P < 0.005$) (Fig. 2; Table S1-All genes, see Supporting Information). The genome of *U. hordei* was analysed for genes that were differentially regulated in at least one of the tested time points. This analysis revealed that 5978 genes (83.9% of the total *U. hordei* genes) were differentially expressed in at least one of the tested infection time points (Fig. 2-Genome; Table S1-All genes). Although 2468 (34.6%) of the differentially expressed *U. hordei* genes were up-regulated, 4078 (57.2%) were down-regulated in at least one time point of barley colonization (Fig. 2-Genome; Table S1-All genes). During the very early time points of pathogenic development at 20 and 40 hpi, 29% (12.5% up- and 16.5% down-regulated) and 65.2% (23.2% up- and 42% down-regulated), respectively, of the total *U. hordei* genes underwent differential expression. The high numbers of differentially regulated genes at the very early stages of infection probably reflect the fundamental switch from saprophytic to biotrophic growth. In particular, at the 20-hpi time point (penetration stage), the observed changes in gene expression may be partially linked to the developmental switch of *U. hordei* from yeast-like growth to filamentous growth on the leaf surface. At 3 and 6 dpi of *U. hordei* colonization, 55.7% (19.5% up- and 36.2% down-regulated) and 49.7% (16.8% up- and 32.9% down-regulated), respectively, of the total *U. hordei* genes were differentially expressed. The highest

differential expression ratio was observed at the 40-hpi time point (Fig. 2; Table S1-All genes). To predict the biological function of *U. hordei* differentially expressed genes, a Blast2Go (gene ontology, GO) analysis was performed (Table S1-Blast2Go). In addition, a GO enrichment analysis was performed for all differentially expressed *U. hordei* genes for their molecular function (Fig. S1, see Supporting Information) (Zheng and Wang, 2008). In particular, two GO groups, including proteins with hydrolytic activity (GO: 0016798) and proteins with carbohydrate-binding ability (GO: 0030246), were significantly enriched amongst the induced genes at all tested time points. The proteins of these GO groups were mainly sugar-cleaving enzymes, such as xylanases, glucosidases, arabinofuranosidases, glucanases, cellulases and galactosidases (Table S1), which are involved in fungal or plant cell wall modification/degradation. These results are consistent with both the dimorphic and biotrophic life style of *U. hordei*. During the transition between saprobic yeast cell and filamentous infectious form, *U. hordei* remodels its cell wall and, in order to penetrate its host cell, also degrades plant cell walls. Amongst the negatively regulated genes in Fig. S1 (green boxes), four GO groups showed a significant enrichment, including enzymes with oxidoreductase activity (GO: 0016651, mainly NADH oxidoreductases), translation factors (GO: 0008135 and GO: 0003746), components of the ribosome (GO: 0003735) and transmembrane proteins of ion transport (GO: 0015077). In addition, 75 of 149 *U. hordei* transporters, including 11 sugar transporter, three ABC and 14 MFS multidrug transporters, were up-regulated in at least one time point of colonization (Table S1-All genes).

Plant-pathogenic fungi secrete different types of protein based on their growth environments. Although, in axenic culture, fungi mostly express and secrete catalytic enzymes to utilize carbon and nitrogen sources, *in planta* phytopathogenic fungi additionally secrete proteins (called effectors) that are known to be involved in fungal virulence with roles in the manipulation of host physiology. The types of secreted effector during host colonization determine the pathogenic life style of the fungus. For example, although necrotrophic fungi secrete more carbohydrate/plant cell wall-degrading enzymes or toxins to kill their host cell, biotrophic fungi secrete proteins to avoid or suppress host defence responses to keep their host cell alive (Kämper *et al.*, 2006; Lyu *et al.*, 2015; Sprockett *et al.*, 2011; de Wit *et al.*, 2012). Thus, the cataloguing of the secreted proteins of fungi can provide hints about their life style. In this study, the catalogue of *U. hordei* secreted proteins was constructed using a computational pipeline (using SignalP, TargetP and TMHMM software to predict secreted proteins and exclude proteins with transmembrane domains) (Table S1-Secretome). In total, 413 proteins (5.8%) were classified as secreted in *U. hordei*. BLAST homology searches against publicly available sequence databases at the National Center for Biotechnology Information (NCBI) revealed that 128 of these secreted proteins were novel, uncharacterized proteins, whereas

25 (19 Ustilaginaceae-specific) were conserved, uncharacterized proteins (Table S1-Secretome). The remaining secretome members had known or predicted functional domains, or had homology to proteins with characterized biological functions.

As there is no conserved sequence feature for the identification of effectors, fungal effector prediction relies on relatively broad criteria, such as secreted, small and cysteine-rich proteins (de Jonge, 2012; Stergiopoulos and de Wit, 2009). However, several studies have shown the presence of effectors with a relatively large size and low/no cysteine content (Gout et al., 2006; Mueller et al., 2013; Ökmen et al., 2013). Because of this, more studies have started to include other criteria, such as *in planta* induction, in order to construct an effector catalogue (Guyon et al., 2014; Saunders et al., 2012; Sperschneider et al., 2014, 2014). So far, almost all described effectors are differentially up-regulated in at least one time point of host colonization. Therefore, the effectorome of *U. hordei* was constructed solely based on the presence of a secretion signal and whether the transcript levels of the genes encoding the selected secreted proteins were different in at least one time point of barley colonization in comparison with growth in axenic culture. During barley infection, 273 *U. hordei* genes encoding secreted proteins were differentially up-regulated in at least one time point of colonization (Table S1-Effectorome). Thus, these 273 predicted secreted proteins (3.8% of the total gene number) were selected as effector candidates in the *U. hordei*-barley pathosystem, regardless of their size, cysteine content or absence/presence of known putative protein domains (Table S1-Effectorome). Moreover, 130 *U. hordei* effector candidates were predicted to be apoplastic using ApoplastP (Sperschneider et al., 2018) (Table S1-Apoplastome). The successful identification of all *U. hordei* orthologues of characterized *U. maydis* effectors, including UmPep1 (UMAG_01987; Hemetsberger et al., 2012), UmPit2 (UMAG_01375; Mueller et al., 2013), UmCmu1 (UMAG_05731; Djamei et al., 2011) and UmSee1 (UMAG_02239; Redkar et al., 2015), as well as the functionally characterized *U. hordei* effector Avr1 (UHOR_10022; Ali et al., 2014), in this *U. hordei* effectorome supports the reliability of this approach (Table S1-Effectorome). Although 43.2% of the effectorome consisted of either hypothetical or uncharacterized proteins, 155 putative effector candidates showed conserved protein domains or similarity to characterized proteins (Table S1-Effectorome). In total, the *U. hordei* secretome contained 55 carbohydrate-degrading enzymes, 31 of which were up-regulated *in planta* (Tables 1 and S1-Secretome and Effectorome). Moreover, two proteins (UHOR_03469 and UHOR_07804) with a predicted carbohydrate-binding module were also identified (Table 1). In addition, four proteins that were annotated as ribonuclease and one endo/exo nuclease were found to be secreted (Table 1). Furthermore, the effectorome of *U. hordei* was mined

for proteins that had at least one predicted nuclear localization signal (NLS), resulting in 21 effector candidates with a predicted NLS signal (Table S2, see Supporting Information). The presence of a predicted NLS signal in these secreted proteins indicates that they may target the host cell nucleus.

Expression of *U. hordei* effector candidate genes is adapted to the host plant during epidermal penetration

For the obligate biotroph *Blumeria graminis* f. sp. *hordei* (powdery mildew), similar gene expression patterns were found during the early stages of infection of either the host plant barley or the non-host plant *Arabidopsis thaliana* (Hacquard et al., 2013). As our previous findings on organ- and cell type-specific gene expression in the maize smut *U. maydis* showed high plasticity of the fungal transcriptome depending on the host tissue (Matei et al., 2018; Skibbe et al., 2010), we analysed *U. hordei* gene expression on penetration (20 hpi) of the non-host plant (maize) relative to the host plant (barley). Comparison of the gene expression during host and non-host penetration events revealed differential gene expression of *U. hordei* (Fig. 3). In total, 2066 and 2237 *U. hordei* genes were differentially expressed on barley and maize plants, respectively (Fig. 3). Among these genes, 249 and 317 *U. hordei* genes were specifically up-regulated only on barley and maize, respectively, and 295 and 398 *U. hordei* genes were specifically down-regulated only on barley and maize, respectively (Fig. 3). For the genes encoding predicted secreted proteins, 197 and 197 *U. hordei* genes were differentially expressed on barley and maize plants, respectively (Fig. 3). For the genes encoding effector candidates, 37 and 27 *U. hordei* genes were specifically up-regulated on barley and maize plants, respectively; 14 and 24 *U. hordei* genes encoding effector candidates were specifically down-regulated on barley and maize, respectively (Fig. 3). Moreover, 14 secreted proteins that were not differentially expressed in any tested time point of barley infection showed significant induction on maize (Table S1-Secretome). Amongst these secreted proteins were two carboxypeptidases (UHOR_02798 and UHOR_05981), one protein that showed similarity to plant PR-1 (pathogenesis-related protein 1) (UHOR_01820), one hydrophobin 3 (UHOR_06926), one ribonuclease (UHOR_04170), one member of glycosyltransferase family 24 (UHOR_02240) and one protein that contained a glucan-binding domain (UHOR_07386). However, *U. hordei* homologues for the previously identified effectors Pep1 (UHOR_02965), Pit2 (UHOR_02064) and Cmu1 (UHOR_07433), which all are required for the suppression of early defence responses, showed a remarkably similar expression pattern on host- and non-host plants (Table S1-Effectorome) (Djamei et al., 2011; Hemetsberger et al., 2012; Mueller et al., 2013).

Table 1 Carbohydrate-degrading enzymes and nucleases present in the *Ustilago hordei* effectorome.

Carbohydrate-degrading enzymes present in the <i>U. hordei</i> effectorome			
Protein ID	UMAG_Orthologue	Description	Amino acid Length
UHOR_03500	UMAG_02111	α,β -Hydrolase	320
UHOR_00039	UMAG_10000	Glycosyl hydrolase	857
UHOR_08862	UMAG_10975	Glycoside hydrolase	354
UHOR_07381	UMAG_05704	Glycoside hydrolase	371
UHOR_06098	UMAG_04032	Glycoside hydrolase	803
UHOR_00370	UMAG_00235	Glycoside hydrolase family 5	464
UHOR_05270	UMAG_03411	Glycoside hydrolase family 10	342
UHOR_00509	UMAG_00330	Glycoside hydrolase family 16	353
UHOR_03204	UMAG_05223	Glycoside hydrolase family 30	661
UHOR_07931	UMAG_05528	Glycosyl hydrolase 53 domain-containing	424
UHOR_07793	NO	Glycoside hydrolase family 93	383
UHOR_08685	UMAG_06120	Glycoside hydrolase family 115	13128
UHOR_02067	UMAG_01377	Extracellular cellulase allergen Asp F7	583
UHOR_07788	UMAG_12007	Related to cellulase	468
UHOR_04296	UMAG_05792	Related to chitin deacetylase precursor	490
UHOR_01593	UMAG_01022	Barwin-like endoglucanase	160
UHOR_03357	UMAG_06332	Related to Endoglucanase 1 precursor	385
UHOR_07693	UMAG_04816	Related to Endoglucanase 1 precursor	323
UHOR_04056	UMAG_02523	Related to Endoglucanase 1 precursor	289
UHOR_06823	UMAG_04364	Related to EXG1-exo- β -1,3-glucanase	482
UHOR_04077	UMAG_04503	Arabinanase levansucrase invertase	466
UHOR_02718	UMAG_01829	Related to α -L-arabinofuranosidase I precursor	707
UHOR_06745	UMAG_04309	Probable α -L-arabinofuranosidase precursor	327
UHOR_02584	UMAG_01734	Glucan 1,3- β -glucosidase	331
UHOR_00702	UMAG_00446	Probable β -glucosidase	819
UHOR_08622	UMAG_06075	Related to β -glucosidase	839
UHOR_05461	UMAG_03551	Related to glucose oxidase	631
UHOR_02907	UMAG_01957	Related to mannosyl-oligosaccharide α -1,2-mannosidase	528
UHOR_03214	UMAG_05229	Related to β -mannosidase precursor	1059
UHOR_06909	UMAG_04422	Related to endo-1,4- β -xylanase b	637
UHOR_07547	UMAG_10671	Pectin lyase B precursor	888
Putative carbohydrate-binding protein found in <i>U. hordei</i> effectorome			
UHOR_07804	UMAG_05439	Chitin-binding	302
UHOR_03469	UMAG_11464	Carbohydrate-binding module family 50	374
Putative nucleases found in <i>U. hordei</i> effectorome			
UHOR_08127	NO	Related to small nuclear ribonucleosome d3	225
UHOR_04748	UMAG_03023	Related to ribonuclease Trv	298
UHOR_03174	NO	Related to ribonuclease T1	119
UHOR_02675	UMAG_10881	Probable ribonuclease T1	128
UHOR_05247	UMAG_03381	Endonuclease exonuclease phosphatase family	300

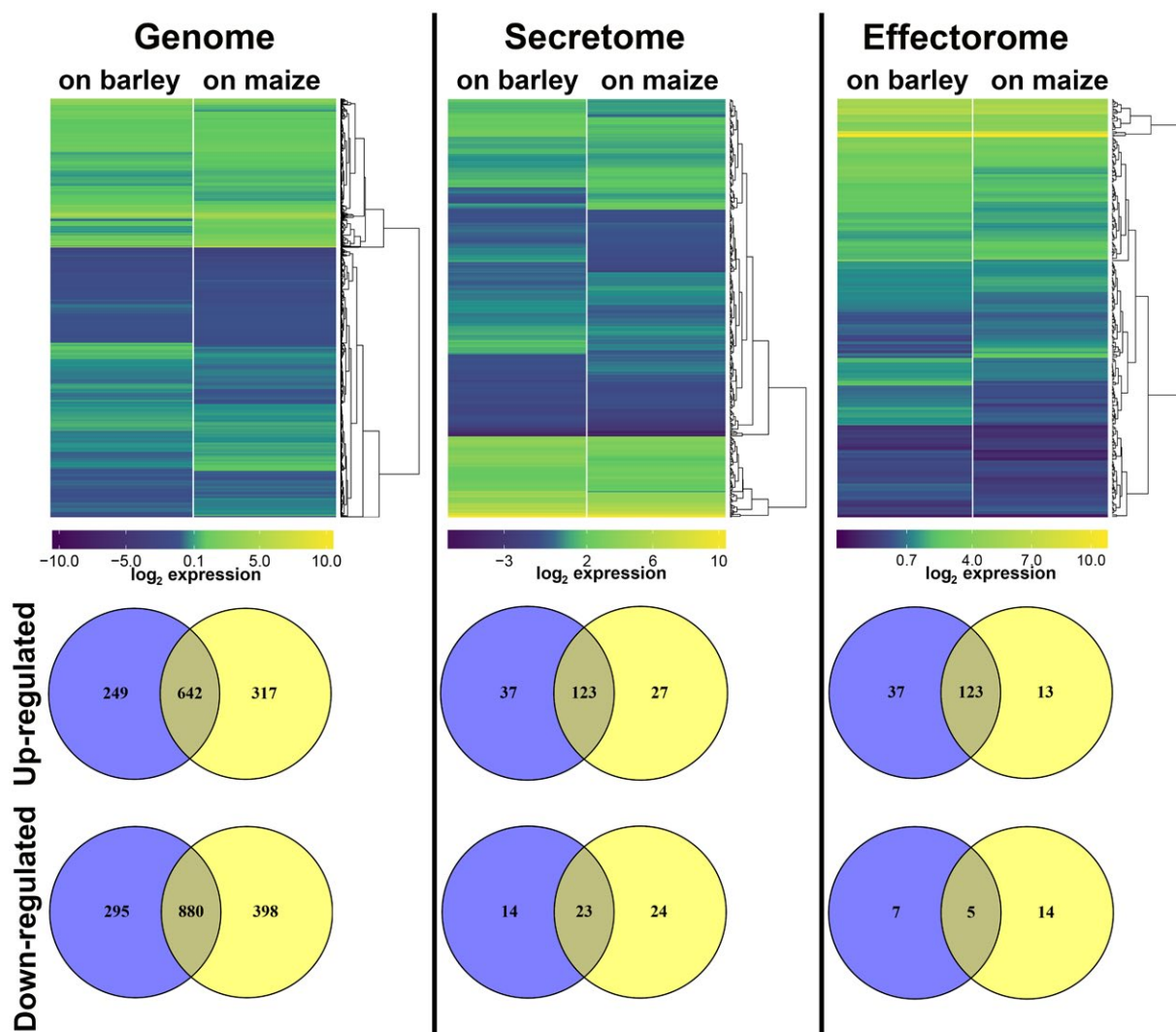


Fig. 3 The hierarchical relation heat map for microarray analysis of *Ustilago hordei* wild-type on host barley and non-host maize plants. The expression profiles of the whole genome, secretome and effectorome of *U. hordei* are depicted. Differentially expressed *U. hordei* genes which are up-regulated (yellow) and down-regulated (blue) *in planta* are shown as \log_2 -fold changes relative to *in vitro* axenic culture. Differentially expressed genes are plotted as Venn diagrams. [Colour figure can be viewed at wileyonlinelibrary.com]

Identification of *U. hordei* effectors with virulence functions

To confirm the transcriptomic data, real-time quantitative polymerase chain reaction (RT-qPCR) was performed for 16 effector gene candidates, which were selected for further characterization (Table S1-Effectors for KO). The RT-qPCR results confirmed the transcriptional induction of the selected effector candidates (Figs 4, S2, see Supporting Information). The selection of candidate effector genes was performed according to their expression patterns during barley and maize colonization. The selection criteria were as follows: (a) effector genes only expressed at the very early time point of barley penetration (20 hpi); (b) effector genes only expressed at early time points (20 and 40 hpi); (c)

effector genes more strongly expressed at very early time points (20 and 40 hpi) compared with late time points (3 and 6 dpi); (d) effector genes more strongly expressed at late time points (3 and 6 dpi) compared with early time points (20 and 40 hpi); (e) effector genes only up-regulated on barley, but not on non-host maize, or more highly expressed on barley compared with maize plants; and (f) effector genes with genome-wide highest induced transcript levels (top 25 genes) (Table S1-Effectors for KO). To test the eventual role of the selected 16 effector candidates for *U. hordei* virulence on barley, they were targeted for gene deletion via homologous recombination. The effector candidate genes were replaced by a *hygromycin* resistance cassette in the two compatible *U. hordei* mating strains (4857-4 Mat-1 and 4857-5 Mat-2). The

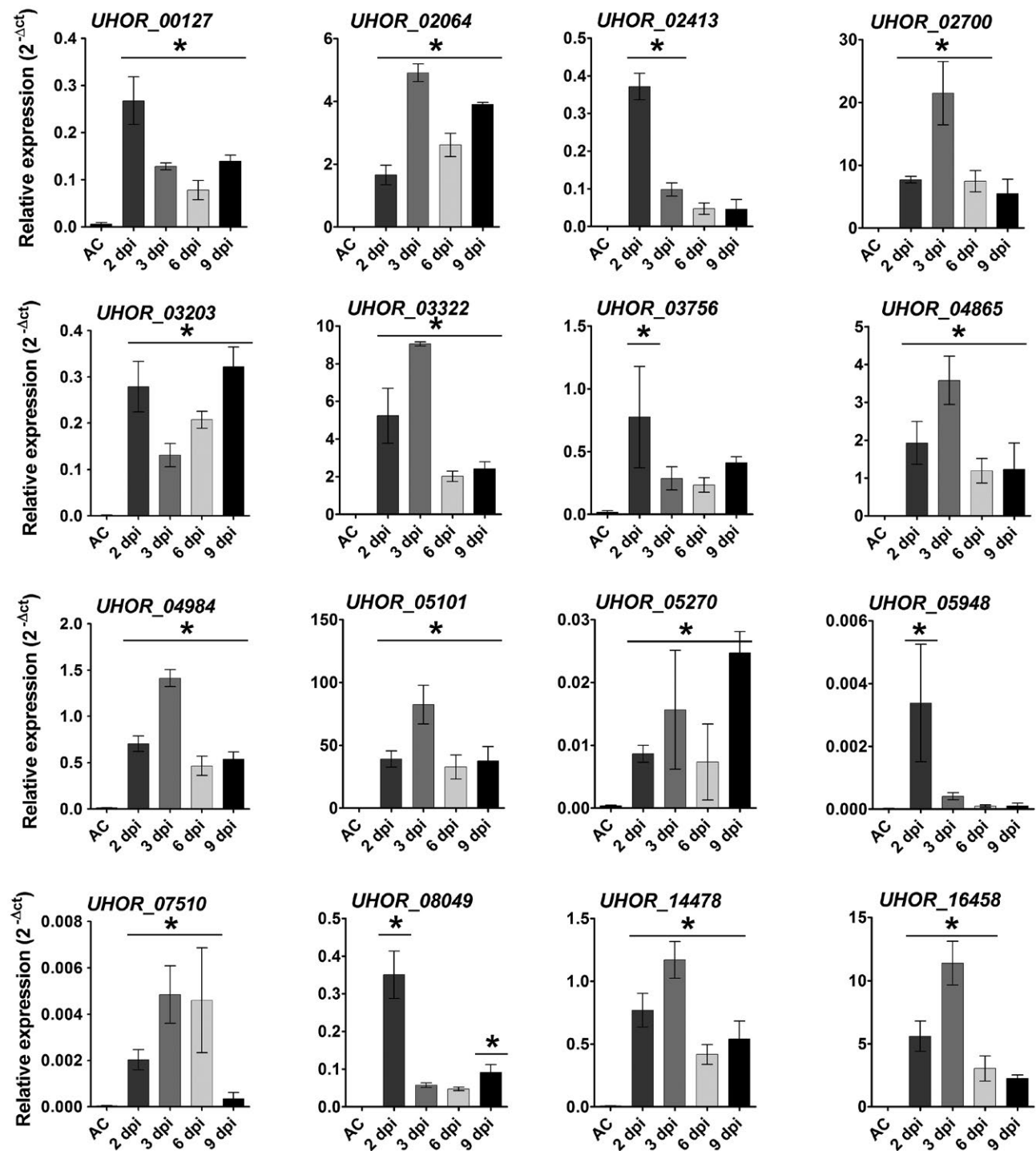


Fig. 4 Expression profile of *Ustilago hordei* effector candidates in axenic culture (AC) and *in planta*. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the expression profiles of the 16 *U. hordei* effector candidates. Expression levels were normalized using the *U. hordei* peptidylprolyl isomerase (*Ppi*) gene. A one-way analysis of variance (ANOVA) was performed. Significant differences compared with axenic culture are shown with an asterisk (**P*<0.05). Error bars represent the standard deviation of three biological replicates.

targeted gene deletion in the mutants was confirmed via PCR using genomic DNA (Fig. S3A–F, see Supporting Information). In addition, RT-qPCR analysis was performed to confirm the absence of transcripts resulting from the deleted genes during the infection of barley (Fig. S3B,F). The *U. hordei* deletion mutants could be generated for only 14 of the selected genes, whereas, for two genes (*UHOR_07510* and *UHOR_14478*), no gene deletion could be confirmed. For all 14 genes, at least two independent mutants of *U. hordei* were tested in plant infections. All mutant strains were tested for growth in liquid culture and filament formation assays on charcoal plates compared with *U. hordei* wild-type (Fig. S4A,B, see Supporting Information). To quantify host colonization of the mutant strains relative to *U. hordei* wild-type, fungal biomass was quantified via qPCR at 6 dpi using a standardized amount of genomic DNA isolated from infected barley leaves (Fig. S5, see Supporting Information and Experimental procedures). The *U. hordei* mutant for the *pep1*

effector gene (*Δuhpep1*; *UHOR_02965*), which has been described previously to be essential for barley infection (Hof et al., 2014), was used as an additional control in all experiments. This approach revealed a significant reduction in infection for four effector candidate genes, including *UHOR_02413*, *UHOR_02700*, *UHOR_03756* and *UHOR_05101* (Fig. 5), whereas deletion of the other 10 candidates did not result in significantly reduced virulence (Fig. S5). Interestingly, a traditional disease scoring of floral symptoms which appeared 3–4 months after infection (for details, see Experimental procedures) showed drastically reduced symptoms caused by mutants for *UHOR_02413*, *UHOR_02700*, *UHOR_03756* and *UHOR_05101* (Fig. S4C). This observation suggests that the *U. hordei* effectors required for the colonization of seedling leaves are also important for the systemic infection of barley that ultimately results in floral symptoms. The four effectors that were identified as virulence factors were renamed as *Ustilago hordei* virulence factors 1–4 (Uvi1–Uvi4).

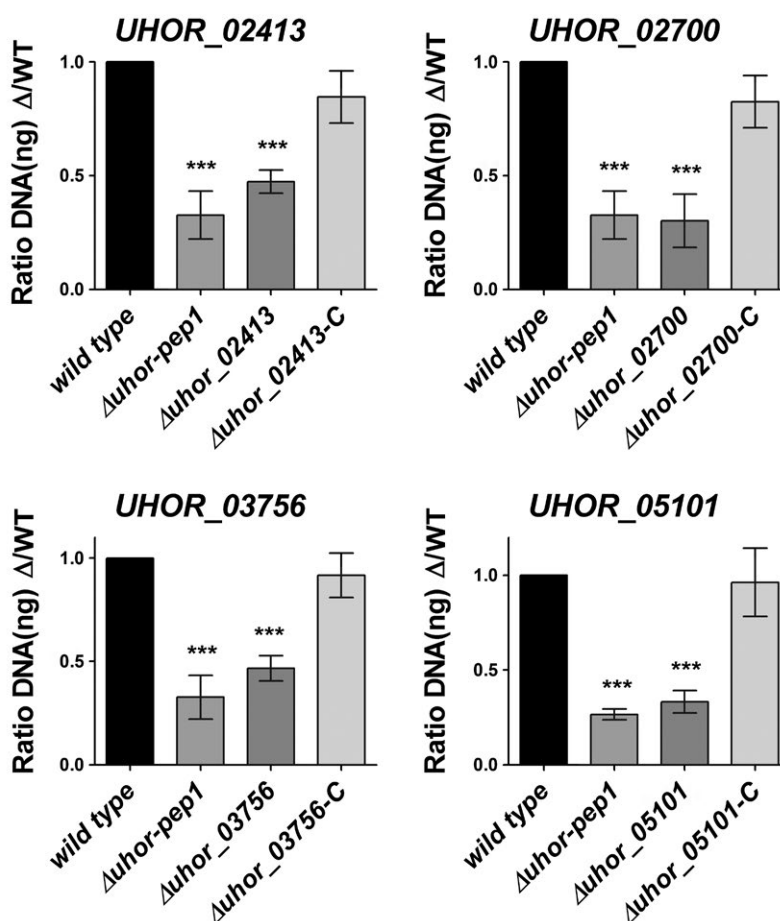


Fig. 5 Gene deletion and complementation of *Ustilago hordei* effector genes. The virulence of the *U. hordei* wild-type (WT), four effector mutants and four complementation strains was assessed by the quantification of fungal biomass at 6 days post-inoculation (dpi). The *U. hordei* *Pep1* deletion mutant was used as a positive control for virulence reduction. The infected barley leaves at 6 dpi were harvested and analysed via quantitative polymerase chain reaction (qPCR) using genomic DNA and the *Ppi1* genes from *U. hordei* as a standard. The fungal biomass was deduced from a standard curve. A one-way analysis of variance (ANOVA) was performed and followed by a Bonferroni test. Significant differences are indicated with asterisks (***) $P < 0.001$. Error bars represent the standard deviation of at least three biological repeats.

To confirm that the observed phenotype was specifically caused by deletion of the targeted effector gene candidates, genetic complementation was performed for the four effector genes whose deletion resulted in virulence reduction. In all cases, the complemented mutants showed wild-type virulence (Fig. 5). As *UHOR_02413* (*Uvi1*), *UHOR_02700* (*Uvi2*), *UHOR_03756* (*Uvi3*) and *UHOR_05101* (*Uvi4*) are significant virulence factors for the *U. hordei*–barley pathosystem, further functional characterization was focused on these four effectors.

Functional analysis of the four *U. hordei* virulence effectors

On pathogen invasion, host plants induce several layers of defence responses [pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI)] to restrict further pathogen growth. Successful pathogens must evade or suppress these defence barriers. To assess whether any of the four characterized effectors (*Uvi1*–*Uvi4*) had the ability to suppress programmed cell death (PCD), two established assays were deployed. First, a BAX1-mediated cell death suppression assay (Hückelhoven, 2004; Watanabe and Lam, 2009) was performed. Both *BAX1* and each of the selected effector candidates (*Uvi1*, *Uvi2*, *Uvi3* and *Uvi4*) were co-expressed in yeast cells. The expression of these four *U. hordei* effectors in yeast cells was confirmed via western blot analysis (Fig. S6A, see Supporting Information). Suppression of cell death was monitored on galactose-containing medium to induce the expression of *BAX1* and *U. hordei* effectors. The known inhibitor of BAX1-mediated cell death, BCL-XL, was used as a positive control. As expected, BCL-XL suppressed BAX1-mediated cell death; however, none of the selected effectors resulted in the suppression of BAX1-mediated cell death in this assay (Fig. S7A, see Supporting Information). As the mechanisms of cell death induction in yeast differ significantly from the situation in plants, these four effectors were also tested for INF1-mediated cell death suppression in *Nicotiana benthamiana* via an *Agrobacterium*-mediated transient transformation assay. Although the expression of C-terminally tagged *Uvi1*, *Uvi2* and *Uvi3* in *N. benthamiana* leaves was confirmed via western blot analysis, the *Uvi4* protein could not be detected (Fig. S6C). However, when C-terminally eGFP-tagged *Uvi4* was expressed in tobacco, a green fluorescent protein (GFP) signal was visible under the GFP channel, suggesting that the C-terminal haemagglutinin (HA) tag could be removed in tobacco (Fig. S6D). INF1 is an extracellular elicitor from *Phytophthora infestans* which acts as a PAMP to induce the hypersensitive response (HR) in *N. benthamiana* (Kamoun *et al.*, 1997, 1997). The *P. infestans* effector *Avr3aK1* and a mutated version, *Avr3aΔY147*, were used as positive and negative controls, respectively (Bos *et al.*, 2010). However, although the co-expression of *INF1* with *Avr3a* and *Avr3aΔY147* confirmed the

previously published suppression of INF1-mediated cell death by *Avr3aK1*, none of the tested effectors showed INF1-mediated cell death suppression activity in this assay (Fig. S7B). In summary, this indicates that, at least in the tested conditions, none of the four virulence factors suppresses plant cell death at either the PAMP-induced level (INF1) or via mitochondrial PCD (BAX1).

An alternative strategy to gain insights into effector function is the search for interacting host proteins. The effectors *Uvi1*, *Uvi2*, *Uvi3* and *Uvi4*, which contribute significantly to *U. hordei* virulence, were chosen for an interaction screen via the well-established Y2H assay. To this end, a custom cDNA library was generated from *U. hordei*-infected barley leaves (for details, see Experimental procedures). Prior to the library screens, correct expression of the four effectors in yeast was tested via western blotting. Although *Uvi1*, *Uvi2* and *Uvi3* were successfully expressed in yeast cells, no signal for *Uvi4* could be detected from yeast cells (Fig. S6B). Therefore, *Uvi4* was excluded from the Y2H experiment. For *Uvi1*, screening of the barley–*U. hordei* cDNA library revealed three potential interaction partners: the barley heavy metal-associated (HvHMA) protein (id: BAK00981.1), a barley domain unknown function protein (HvDuf866) (id: BAJ98443.1) and a lipid transfer protein (HvLTP) (id: CAA65680.1). For all three proteins, SignalP analysis predicted an N-terminal signal peptide for extracellular secretion. The coding regions of the three genes were cloned from barley for verification of the screening result in one-to-one Y2H assays. Although this confirmed the interaction of *Uvi1* with HvHMA and HvDuf866 (Fig. 6), HvLTP showed autoactivation and was therefore considered to be a false positive candidate. For *Uvi2*, two potential interaction partners were identified: one thaumatin-like protein 4 (HvTLP4, id: AF355455_1) and a NAC transcription factor (id: AM500855.1). However, in one-to-one interaction assays, only the interaction of HvTLP4 with *Uvi2* was confirmed using the full-length version of the respective barley genes (Fig. 6). For *Uvi3*, an SNF1-related protein kinase (SnRK1) was identified as a possible interactor partner. However, this interaction could not be confirmed in the one-to-one interaction assay using the full-length version of the respective barley gene.

In summary, we have identified two possible barley interaction partners, i.e. HvHMA and HvDuf866, for *Uvi1*, whereas HvTLP4 is the only possible interactor partner for *Uvi2*.

DISCUSSION

Ustilago hordei is a seed-borne pathogen that initially penetrates barley coleoptiles; the fungus grows just below the apical shoot meristem without demonstrating any visible symptoms until the occurrence of sori filled with dark teliospores in flowers (Hu *et al.*, 2002). Therefore, macroscopic evaluation of disease symptoms is only possible at the very late stages of the disease (2–3 months after inoculation) (Hu *et al.*, 2002). In this study, we

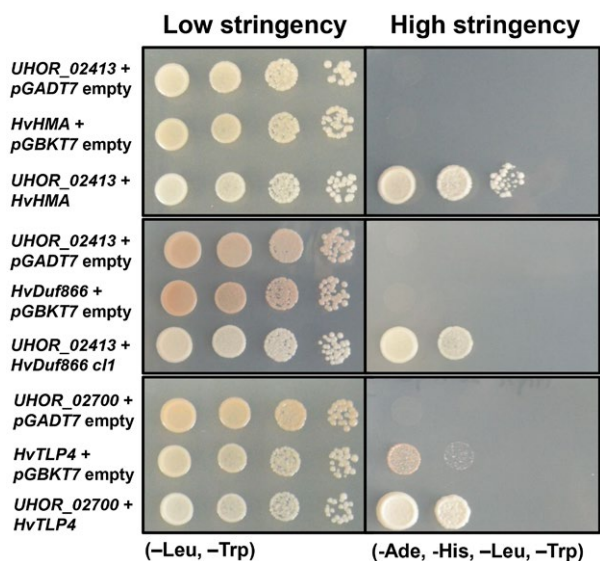


Fig. 6 *In vivo* interaction of *Ustilago hordei* effectors with *Hordeum vulgare* target proteins via a yeast two-hybrid (Y2H) system. Interaction of pGBKT7-UHOR_02413 and pGADT7-HvHMA, pGBKT7-UHOR_02413 and pGADT7-HvDUF866, and pGBKT7-UHOR_02700 and pGADT7-HvTLP4 was confirmed via Y2H assay. The empty vectors were used as negative controls for the *Saccharomyces cerevisiae* AH109 strain. Different dilutions (10^0 – 10^{-3}) of each sample were dropped on low- [-leucine (-Leu) and -tryptophan (-Trp)] and high- [-Leu, -Trp, -adenine (-Ade) and -histidine (-His)] stringency plates. The photographs were taken after 3–4 days of incubation at 28 °C. Growth on high-stringency plates indicates interaction.

have developed a more rapid readout assay to evaluate the disease symptoms of *U. hordei* during barley infection. Two compatible *U. hordei* strains were needle inoculated into leaf whorls of 12-day-old barley seedlings, similar to the inoculation procedure established for the maize smut *U. maydis* (Redkar et al., 2015). Microscopic observation of *U. hordei* on barley inoculation showed that the infectious filaments penetrate the leaf epidermis via appressorium formation and, subsequently, the fungus proliferates both extracellularly and intracellularly. At 3 dpi, the fungal filaments grow towards vascular tissue and, at 6 dpi, *U. hordei* massively proliferates around and inside the host vascular tissue. Biomass quantification of *U. hordei* on barley via qPCR was stable at 6 dpi, which represents a dramatic reduction in experimental workflow compared with the traditional disease scoring of floral symptoms (which takes around 3–4 months). Interestingly, *U. hordei* develops intracellular structures, which show morphological similarity to haustoria. These special feeding structures are typically formed by biotrophic fungi or oomycetes to utilize nutrients from their hosts (Slusarenko and Schlaich, 2003; Yi and Valent, 2013). Although it has been reported that the smut fungus *Ustacystis waldsteiniae* can form haustoria in its host plant *Waldsteinia geoides* (Bauer et al., 1995), to our knowledge, no previous studies have reported the formation of haustoria-like structures in *U. hordei*.

To facilitate host infection, biotrophic pathogens deploy large repertoires of effectors, which often show specific transcriptional induction during host colonization. Our transcriptome analysis showed the significant induction of 273 *U. hordei* effector genes, which is comparable with previous findings for the maize smut *U. maydis* (261 effector genes) (Skibbe et al., 2010). Some effectors (95 genes) showed constitutively high expression during all the tested stages of *U. hordei* infection. This group contains homologues of the known *U. maydis* effectors Pep1 and Pit2, which are both essential for fungal virulence (Hemetsberger et al., 2015, 2015; Mueller et al., 2013). However, the majority of *U. hordei* effector genes (178 genes) showed transient transcriptional induction, suggesting a stage-specific activity of these putative virulence factors. Sequential expression of putative effector genes has been observed for other plant pathogens, including the rust fungus *Melampsora larici-populina* and the hemibiotrophic anthracnose pathogen *Colletotrichum higginsianum* (Duplessis et al., 2011; Kleemann et al., 2012; O'Connell et al., 2012). Kleemann et al. (2012) proposed that *C. higginsianum* expresses effectors that are required for the suppression of host immune responses to maintain host cell viability during the early biotrophic stage; however, on switching from the biotrophic stage to the necrotrophic stage, the expression of NLP1 is increased to induce host cell death (Kleemann et al., 2012). Although *U. hordei* is strictly biotrophic, it may express different effector waves that are infection stage specific, thus eventually resembling organ- or cell type-specific interactions, as recently described for the *U. maydis*–maize interaction (Matei et al., 2018; Schilling et al., 2014; Skibbe et al., 2010).

Predictions of fungal effectors mostly rely on whether the proteins contain an extracellular signal peptide, have a relatively large size and low/no cysteine content (de Jonge, 2012; Sperschneider et al., 2016; Stergiopoulos and de Wit, 2009). However, recent studies have revealed that not all effectors fit into these criteria (Gout et al., 2006; Mueller et al., 2013; Ökmen et al., 2013). As almost all characterized effectors are differentially up-regulated in at least one time point of host colonization, more and more studies have prioritized *in planta*-expressed secreted proteins as effector candidates (Guyon et al., 2014; Saunders et al., 2012; Sperschneider et al., 2014). In this study, we defined the *U. hordei* effectorome solely based on the presence of a secretion signal and significant up-regulation during host colonization compared with axenic growth. The presence of all functionally characterized *U. maydis* effector orthologues, including UmPep1 (UMAG_01987) (Hemetsberger et al., 2012), UmPit2 (UMAG_01375) (Mueller et al., 2013), UmCmu1 (UMAG_05731) (Djamei et al., 2011) and UmSee1 (UMAG_02239) (Redkar et al., 2015), in this effector catalogue indicates the reliability of the constructed *U. hordei* effectorome.

Previous comparative genomic analyses have shown that the numbers of plant cell wall-degrading enzymes in necrotrophic or

hemibiotrophic fungi are significantly greater than in biotrophic fungi (Lyu *et al.*, 2015; Sprockett *et al.*, 2011; de Wit *et al.*, 2012). Lyu *et al.* (2015) reported that, in the necrotrophic phytopathogenic fungus *Sclerotinia sclerotiorum*, the expression of 73 of a total number of 186 genes encoding for plant cell wall-degrading-associated enzymes was significantly up-regulated during infection. The combination of microarray and Blast2Go analyses revealed the presence of 31 putative carbohydrate-degrading enzymes that were significantly up-regulated on host penetration. This result also confirms that biotrophs have a smaller repertoire of carbohydrate-degrading enzymes than more aggressive plant pathogens. The effector repertoire of *U. hordei* contains cellulases, xylanases, β -glucosidase, pectinases, α -L-arabinofuranosidases and exo- and endo-glucanases, which can hydrolyse the main components of either plant or fungal cell walls. Recently, several studies have shown that fungal-derived cellulases, endo-1,4- β -xylanases and α -L-arabinofuranosidases are required for full virulence of *Magnaporthe oryzae* during rice colonization (Nguyen *et al.*, 2011; Van Vu *et al.*, 2012; Wu *et al.*, 2016). *Ustilago hordei* may secrete these glycosyl hydrolase proteins into the host apoplast to degrade the host cell wall in order to facilitate host penetration or cell-to-cell passage by loosening the plant cell wall, or to possibly release nutrients from the host cell wall to support its own growth. We have also identified one chitin-binding protein and one LysM (CBM family 50) protein in the effectorome of *U. hordei*. These chitin-binding proteins and other cell wall-degrading enzymes could be used to suppress or avoid host immunity by sequestering fungal- or plant-derived microbe-associated molecular pattern (MAMP) or damage-associated molecular pattern (DAMP) molecules, respectively (de Jonge *et al.*, 2010; Marshall *et al.*, 2011; Mentlak *et al.*, 2012).

In addition to carbohydrate-degrading enzymes, *U. hordei* also differentially expresses several proteases, including carboxypeptidases, metalloproteases, aspartyl and serine proteases. Although there have been many studies on the function of secreted fungal protease inhibitors that inhibit host serine or cysteine proteases to suppress host defence responses (Lozano-Torres *et al.*, 2012; Mueller *et al.*, 2013; Rooney *et al.*, 2005; Tian *et al.*, 2004, 2004), the roles of secreted fungal proteases and their substrate specificities in host–microbe interactions are still elusive. A knowledge of the substrate specificities of these enzymes will provide new insights into our understanding of their contribution to virulence.

Although many studies have been performed to understand the mechanism of non-host resistance in host plants, only a few studies have investigated how plant pathogens behave on non-host plants. Recently, Hacquard *et al.* (2013) reported that the obligate biotroph *B. graminis* f. sp. *hordei* (powdery mildew) shows similar gene expression patterns on two very divergent hosts, barley and Arabidopsis (on the immunocompromised *pen2/pad4/sag101* triple mutant), at very early time points

on penetration (at 6–24 hpi). In contrast, we have shown that *U. hordei* transcriptionally activates different sets of genes during host (barley) and non-host (maize) penetration at 20 hpi. In total, *U. hordei* expresses 160 and 150 genes encoding secreted proteins on host and non-host plants, respectively. Moreover, 37 and 27 of these genes are specifically expressed only on barley and maize plants, respectively. These results indicate that *U. hordei* transcriptionally activates its secretome even during penetration attempts on a non-host plant in order to suppress the plant immune system. Furthermore, *U. hordei* keeps some effectors ‘up its sleeve’ for non-host penetration events, which are not expressed on the host plant. Consistent with these results, it has been reported that the generalist root endophyte *Serendipita indica* also transcriptionally activates different sets of effectors during the colonization of different host plants in a host-dependent manner (Lahrman *et al.*, 2013). By expressing its virulence factors, which are involved in the suppression of the host plant immune system, on non-host plants, pathogens may obtain an evolutionary potential for possible host-jump events. To check whether *U. hordei* also attempts to suppress early non-host defence responses, the expression levels of the characterized core effectors of smuts were checked on both barley and maize plants (Schuster *et al.*, 2017). Surprisingly, the *U. hordei* genes encoding homologues of Pep1, Pit2 and Cmu1 proteins, which are required for suppression of early defence responses, including reactive oxygen species (ROS) accumulation (Hemetsberger *et al.*, 2012), host cysteine protease activity (Mueller *et al.*, 2013) and salicylic acid accumulation (Djamei *et al.*, 2011), respectively, in the *U. maydis*–maize system, show remarkably similar expression patterns on both barley and maize plants. These data suggest that *U. hordei* attempts to penetrate and suppress very early non-host defence responses on maize. Consistent with these results, Hof *et al.* (2014) have reported that *U. maydis* can perform *pep1*-mediated suppression of barley ROS accumulation on non-host barley plants at 20 hpi.

In order to obtain further insights into the roles of highly expressed effector candidates during barley infection, 14 *U. hordei* effector candidates were selected for gene deletion. The selection was performed on the basis of their expression level and pattern at different time points on host or non-host plants. Biomass quantification of the 14 *U. hordei* mutants on infected barley leaves revealed four effector genes (*Uvi1*, *Uvi2*, *Uvi3* and *Uvi4*) that were required for full virulence. BLAST analysis showed that, although *Uvi2* and *Uvi4* are uncharacterized proteins, *Uvi1* shows some similarity to expansins, proteins involved in plant cell wall loosening (Cosgrove, 2000). Therefore, *Uvi1* may be involved in barley cell wall loosening during cell-to-cell passage. The *Uvi3* protein has six predicted Ish1 (induced in stationary phase) domains. Proteins containing this domain are putative stress-responsive nuclear envelope proteins that are localized in the nuclear envelope and plasma membrane (Taricani *et al.*, 2002).

It has been reported that the Ish1 protein from *Saccharomyces pombe* interacts with the nuclear Bis1 protein, which is widely conserved in a wide range of organisms, including humans, *Drosophila* and *Arabidopsis*. Bis1 could be involved in RNA processing and protein degradation (Taricani et al., 2002). Uvi3 may also interact with a homologue of the Bis1 protein in barley to manipulate host RNA processing or protein degradation.

The Y2H library screen revealed barley TLP4, a member of the PR-5 class of proteins with a predicted extracellular secretion signal (Liu et al., 2010), as a potential host interaction partner of Uvi2. It has been shown that TLPs play a role in antifungal activities in several plant-pathogenic fungi, including *Trichoderma viride*, *Botrytis cinerea*, *Fusarium oxysporum*, *Physalospora piricola* and *Mycosphaerella arachidicola* (Chu and Ng, 2003; Ho et al., 2007; Singh et al., 2013). Consistently, heterologous expression of TLP in tobacco plants induced enhanced resistance against *Rhizoctonia solani* and higher levels of PR gene expression (Chu and Ng, 2003). These results suggest that barley TLP4 may also have antifungal activity to inhibit *U. hordei* colonization. If this is the case, by interacting with TLP4, Uvi2 may inhibit its antifungal activity to support *U. hordei* growth. The Y2H screen for Uvi1 gave two potential unrelated interactor candidates with a predicted extracellular secretion signal. One of the candidates had a heavy metal-associated domain (HMA domain) and the other had a Duf866 domain. According to the integrated decoy hypothesis, during the arms race between hosts and their pathogens, some plants have integrated protein domains targeted by effector proteins into their receptor proteins to lure effectors and, subsequently, to induce the immune system (Kroj et al., 2016; Maqbool et al., 2015). Recently, it has been reported that a rice resistance protein has an integrated HMA domain, indicating that the HMA-containing host protein could be targeted by pathogen effectors (Kroj et al., 2016; Maqbool et al., 2015). Although cell death suppression assays, including BAX1-mediated and Inf1-mediated cell death suppression assays, were performed for further characterization of these four effectors, we could not identify any effector that could suppress either of these cell death mechanisms.

In this study, we systematically investigated the putative effector candidates of *U. hordei* during barley colonization. We identified 273 secreted effector candidates which were differentially up-regulated *in planta*. The results revealed that, in order to establish colonization, *U. hordei* transcriptionally activates consecutive effector waves to manipulate host metabolism at different infection stages. Moreover, we have also shown that *U. hordei* up-regulates core effectors to suppress early non-host defence responses, including ROS accumulation, PLCP activation and salicylic acid production. This indicates that *U. hordei* attempts to infect non-host plants with full intention, but is blocked by further non-host immune responses. Deletion studies for highly expressed effector candidates revealed four effectors

that contribute significantly to *U. hordei* virulence on barley, and potential interacting host proteins were identified for two of these virulence factors.

EXPERIMENTAL PROCEDURES

Fungal and plant materials

Ustilago hordei (4857-4 Mat-1 and 4857-5 Mat-2) cultures were incubated in YEPS_{light} (0.4% yeast extract, 0.4% peptone and 2% saccharose) liquid medium at 22 °C with shaking at 200 rpm. The susceptible barley cultivar (Golden Promise) was grown in a glasshouse at 70% relative humidity, 22 °C during the day and night, a light/dark regime of 15 h/9 h and 100 W/m² supplemental light when the sunlight influx intensity was less than 150 W/m².

Nicotiana benthamiana plants were grown in a growth chamber at 22 °C and 70% relative humidity with a photoperiod of 12 h.

Nucleic acid methods

Total RNA was isolated from axenic culture and *U. hordei*-infected barley plants (at 20 and 40 hpi, and 3 and 6 dpi). The third leaf of infected barley plants was collected by cutting 1 cm below the injection needle site and approximately 3 cm of the leaf was taken for RNA extraction. For the 20-hpi time point, melted latex was used to collect penetrated fungal materials. Samples were then frozen in liquid nitrogen and ground using a mortar and pestle under constant liquid nitrogen cooling conditions. Total RNA was isolated from the fine powder using the TRIzol[®] extraction method (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Total RNA was then cleaned with a Turbo DNA-Free[™] Kit (Ambion/Applied Biosystems, Darmstadt, Germany), according to the manufacturer's instructions, to remove any DNA contamination.

The synthesis of cDNA was performed with 1 µg of total RNA using the First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. RT-qPCR analysis was performed using SYBR[®] Green Supermix (BioRad, Munich, Germany) according to the manufacturer's instructions. RT-qPCR oligonucleotide pairs were designed with Primer3 Plus (Table S3, see Supporting Information) (Untergasser et al., 2007). The efficiency and specificity of the oligonucleotide pairs were determined with a dilution series of genomic DNA before use. The reaction was performed in a Bio-Rad iCycler system using the following conditions: 2 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, 30 s at 61 °C and 30 s at 72 °C. The expression levels of *U. hordei* effector genes were calculated relative to the *U. hordei* *peptidylprolyl isomerase* gene (*UhPpi*, *UHOR_05685*). The RT-qPCR data were analysed using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001). Error bars in all figures showing RT-qPCR data represent the standard

deviation of the raw data from at least three biological replicates. The primers used for RT-qPCR are listed in Table S3.

Fungal biomass quantification was performed using qPCR analysis as described previously, but employing genomic DNA isolated from *U. hordei* wild-type- and mutant-infected barley leaves. DNA isolation from infected barley leaves was performed using a MasterPure™ Complete DNA&RNA Purification Kit (Epicentre®, Illumina®, Madison, Wisconsin, USA) according to the manufacturer's instructions. The *U. hordei* *UhPpi* gene (*UHOR_05685*) was used as a reference gene. A standard curve was constructed using serial dilutions of *U. hordei* genomic DNA (100, 10, 1, 0.1, 0.01 and 0.001 ng/μL) employing *UhPpi* as a reference gene. Logarithms (base 10) of DNA concentrations were plotted against the crossing point of Ct values. The results are the average of three biological repeats.

All PCRs were performed in 25-μL reaction mixtures using Phusion® DNA polymerase (Thermo Scientific, Bonn, Germany), following the manufacturer's instructions, with 100 ng of genomic DNA or cDNA as template. All primers introduced into the reaction are listed in Table S3. The amplified DNA was then used for the cloning process. All PCRs were run in a PTC-200 (Peletier Thermal Cycler, MJ Research, Quebec, Canada) PCR machine. Nucleic acids (i.e. deriving from PCRs or restriction digests) were purified from 1% TAE agarose gels with a 'Wizard SV Gel and Purification System Kit' (Promega, Fitchburg, Wisconsin, USA) according to the manufacturer's instructions.

Plasmid DNA isolation from bacterial cells was performed using a QIAprep Mini Plasmid Prep Kit (QIAGEN, Venlo, Netherlands), according to the manufacturer's information, after the principle of alkaline lysis.

Cloning and fungal transformation

For knockout and complementation plasmid constructions, standard molecular biology methods were used according to the molecular cloning laboratory manual of Sambrook *et al.* (1989). *Ustilago hordei* gDNA was used for the amplification of PCR products for the construction of deletion and complementation constructs, and cDNA was employed for the amplification of genes for expression constructs, using appropriate primer pairs (Table S3). After restriction of the PCR fragments with appropriate restriction enzymes, they were ligated into a vector that was digested with the same restriction enzymes using T4-DNA ligase (New England Biolabs, Frankfurt-am-Main, Germany) according to the manufacturer's instructions. The sequences of all constructs were confirmed via sequencing at the GATC-Biotech Company (Cologne, Germany). Gateway cloning was performed with a pENTR™/D-TOPO™ Cloning Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) to obtain *pDONR* vectors according to the manufacturer's instructions, and Gateway™ LR Clonase™ Enzyme mix was used to

construct *pK2GW7* and *pEZY45* destination constructs according to the manufacturer's instructions. All vector constructs, primer pairs and restriction sites are indicated in Table S3. For all heterologous gene expression assays, *UHOR_02413*^{24-226aa} (Gene Bank: CCF53559.1), *UHOR_02700*^{22-224aa} (Gene Bank: CCF53255.1), *UHOR_03756*^{28-503aa} (Gene Bank: CCF51752.1) and *UHOR_05101*^{24-703aa} (Gene Bank: CCF51983.1) were cloned without the signal peptide sequences.

Escherichia coli transformation was performed via heat shock assay according to standard molecular biology methods (Sambrook *et al.*, 1989). Transformation of *Saccharomyces cerevisiae* ESM356.1 was performed as described in the yeast protocol handbook (Clontech, Mountain View, CA, USA). Gene replacement and transformation of *U. hordei* protoplasts were conducted according to Kämper (2004). For both gene deletion and complementation studies, a minimum of two colonies was selected for phenotypic observation. Only when all independent mutants showed the same virulence reduction phenotype was the observed phenotype considered to be true. Complementation of deletion mutants was performed in the native locus of the gene using a *nourseothricin* (*Nat*) resistance cassette (final concentration of 25 μg/mL).

Virulence assay

For virulence assay, both wild-type and mutant strains of *U. hordei* were grown in YEPS_{light} liquid medium at 22 °C with 200 rpm shaking to obtain an optical density at 600 nm (OD₆₀₀) of 0.6–0.8. The cells were then centrifuged at 2744 × g for 10 min at room temperature and resuspended in sterile water supplemented with 0.1% Tween-20 to obtain OD₆₀₀ of 3.0. Then, the *U. hordei* suspension was injected into the centre of the leaf whorl of 10–12-day-old barley seedlings (three-leaf stage) with a syringe with a needle. All infection assays were performed in three biological replicates with at least 16 plants. The quantification of *U. hordei* fungal biomass in infected barley plants was performed at 6 dpi using qPCR with a standardized amount of genomic DNA (200 ng/μL) as the template. Before genomic DNA isolation, infected barley leaves were cleaned (wiped) with 1% Tween-20 water to remove *U. hordei* sporidia from the leaf surface. Genomic DNA was isolated from barley leaves infected by the wild-type fungus and mutants, diluted to a final concentration of 200 ng/μL. A standard curve was constructed using serial dilutions of *U. hordei* genomic DNA (100, 10, 1, 0.1 and 0.01 ng/μL), employing *UHOR_05685* (*UHOR_Ppi*) as a reference gene. The base 10 logarithms of DNA concentrations were plotted against the crossing point of Ct values obtained from qPCR. The *Δuhpep1* mutant of *U. hordei* was used as a positive control for biomass reduction. For classical *U. hordei* infection assay, two haploid cultures of opposite mating types were mixed (1 : 1, v/v) to obtain a

final concentration of $OD_{600} = 1.0$. Barley seeds were dehulled and surface sterilized by incubation with 70% ethanol for 3 min, followed by incubation with 1% bleach for 10 min, and rinsed four times with sterile water. Subsequently, the surface-sterilized barley seeds were treated with *U. hordei* cell suspension and a vacuum of 413 kPa was applied twice for 15 min. After removal of excessive fungal inoculum, the barley seeds were incubated for 6 h at room temperature before sowing in soil. Approximately 2–3 months after inoculation, disease symptoms were scored at barley heading.

Bioinformatics analysis

Conventionally secreted *U. hordei* proteins were predicted using SignalP (Nielsen et al., 1997) to identify the N-terminal signal peptide and, subsequently, the TMHMM software program (Krogh et al., 2001) was used to exclude proteins with one or more transmembrane domains. The proteins whose transmembrane domains (sequence length of less than 30 amino acids) overlapped with their signal peptides were not excluded from the list. The protein sequences were then analysed with TargetP (Emanuelsson et al., 2000) to predict the extracellular localization.

The Blast2GO program was used to predict multiple associated GO categories for each protein with default parameter settings and given the BLAST results against the non-redundant database of NCBI as input (GO term database was last updated on 25 April 2017) (Conesa et al., 2005). In addition, each protein sequence was scanned for the presence of protein domains using InterProScan v4.4, including domain profiles from Pfam.

Microarray analysis

Samples for microarray analyses were processed using the One Color Quick Amp Labeling Kit, One Color Spike-In Kit, Gene Expression Hybridization Kit, Gene Expression Wash Buffer Kit (Agilent, Santa Clara, California, USA) and RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturers' instructions. One-hundred nanograms of total RNA were used as input for reverse transcription. Microarray analyses were performed on $8 \times 15k$ microarrays (Agilent) and scanned using the following parameters: profile, AgilentHD_GX_1Color; slide ID, AutoDetect; channels, G; scan region, Agilent HD (61 mm \times 21.6 mm); resolution, 2 μ m; Tiff, 20 bit; RPMT, 100%; GPMT, 100%; XDS, no XDR. The image was processed in Agilent Feature Extraction, and data analysis was performed using the Partek[®] Genomics Suite Software (Partek Inc., St. Louis, Missouri, USA). Changes in *U. hordei* gene expression *in planta* (at 20 and 40 hpi, and 3 and 6 dpi) were calculated relative to *U. hordei* yeast-like cells grown in axenic culture. Expression data were submitted to Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) (Accession Number: GSE117325).

Y2H assay

The *U. hordei*–barley Y2H library was constructed using a Make Your Own 'Mate&Plate™' Library System (Clontech Laboratories, Takara Bio Company, Shimogyo-ku, Kyoto, Japan) according to the manufacturer's instructions. To construct the Y2H library, total RNA was isolated from 20-hpi, 40-hpi, 3-dpi and 6-dpi *U. hordei*-infected barley plants.

To identify the host targets of UHOR_02413^{24-226aa}, UHOR_02700^{22-243aa} and UHOR_03756^{28-503aa} effectors, the corresponding genes were cloned without their signal peptide sequences into *pGBKT7* to be in frame with the Gal4 DNA-binding domain and a Myc tag. Using the 'Mate&Plate™' Library System, the *U. hordei*–barley Y2H library was screened with these three effectors according to the manufacturer's instructions.

To confirm the results of the Y2H screen, all potential interaction partners were cloned into *pGADT7* to be in frame with the Gal4 activation domain and an HA tag. *pGBKT7-UHOR_02413*, *pGBKT7-UHOR_02700* and *pGBKT7-UHOR_03756*, and their potential interaction partners in *pGADT7*, were co-transformed into the *S. cerevisiae* AH109 strain. As a negative control, co-transformation was also performed with empty *pGADT7* and *pGBKT7*. *Saccharomyces cerevisiae* transformation was performed according to Yeast Transformation System II protocol. Drop assays for Y2H interaction were performed according to Mueller et al. (2013). From each dilution, 5 μ L were dropped onto low-stringency [–leucine (-Leu) and –tryptophan (-Trp)] and high-stringency [–Leu, -Trp, –adenine (-Ade) and –histidine (-His)] SD plates, and the plates were incubated at 28 °C for 5 days. Photographs were taken after 5 days. Growth on high-stringency plates indicates candidates for protein–protein interaction.

Cell death suppression assays

To assess whether any of these selected effector candidates possess activity to suppress PCD, both BAX1-mediated cell death and INF1-mediated cell death suppression assays were performed according to Kawai et al. (1999) and Bos et al. (2010), respectively.

For the BAX1-mediated cell death suppression assay, the corresponding effector genes were cloned without their signal peptide sequences into the *pEZY45* Gateway expression vector to be in frame with the B42 transcription activator domain and an HA tag. BAX1-mediated cell death suppression assays were performed according to Kawai et al. (1999). The ESM356.1 yeast strain was co-transformed with an effector candidate and BAX1 under the control of a galactose inducible promoter. Drop assay was performed with 5 μ L of different dilutions of cultures on SD solid medium (0.67% w/v yeast nitrogen base, 0.06% v/v dropout solution without uracil and tryptophan, and 2% agar, pH 5.8) supplemented with 2% glucose or 2% galactose and 1% raffinose to induce the expression of the effector candidate

and *BAX1* genes. Yeast cells were incubated for 4 days at 28 °C to monitor BAX1-mediated cell death induction. The BAX1-mediated cell death inhibitor, BCL-XL, and an empty vector were used as positive and negative controls, respectively, for cell death suppression.

For the INF1-mediated cell death suppression assay, all selected effector candidates were cloned into the *pK2GW7* Gateway binary expression vector for *Agrobacterium*-mediated transient transformation assays (ATTAs). The INF1-mediated cell death suppression assay was performed according to Bos et al. (2010). *Phytophthora infestans* cell death inducing *INF1* (*pGR106-FLAG*) and *pK2GW7-UHOR_02413*, *pK2GW7-UHOR_02700*, *pK2GW7-UHOR_03756* and *pK2GW7-UHOR_05101* were co-infiltrated into 5–6-week-old *N. benthamiana* leaves via ATTA. *Phytophthora infestans Avr3aKI* (*pGR106-FLAG*) and *Avr3aΔY* (*pGR106-FLAG*) were used as positive and negative controls for INF1-mediated cell death suppression, respectively (Bos et al., 2010). INF1-mediated cell death suppression was observed at 4 dpi. The *pGR106-INF1*, *pGR106-Avr3aKI* and *pGR106-Avr3aΔY* constructs were kindly provided by the group of Sophien Kamoun (The Sainsbury Laboratory, Norwich Research Park, Norwich, UK).

Extraction of proteins from yeast cells for western blot

A single colony of the respective yeast strain was inoculated in 3 mL of liquid SD medium without tryptophan. The cell suspension was incubated at 28 °C with shaking at 200 rpm to obtain OD₆₀₀ = 0.8. The cell suspension was transferred to a 2-mL reaction tube and centrifuged at 9600 ×g for 3 min. The supernatant was discarded and the pellet was resuspended in 100 µL of 2 × sodium dodecylsulfate (SDS) loading dye. Glass beads (0.3 g) were added to the suspension and the sample was boiled at 98 °C for 5 min. Subsequently, the sample was shaken for 10 min at 1800 rpm using a Vibrax-VXR shaker (IKA, Staufen, Germany). The sample was again boiled at 98 °C for 5 min. Finally, 25 µL of the sample were used for western blot analysis. Western blot was performed according to Mueller et al. (2013).

Staining of leaves with WGA-AF488 and propidium iodide

WGA-AF488 (Molecular Probes, Karlsruhe, Germany) and propidium iodide (Sigma-Aldrich, St. Louis, Missouri, USA) staining were performed to monitor and track the fungal hyphae during host colonization. WGA-AF488 stains fungal cell walls and propidium iodide stains plant cell walls. After *U. hordei*-inoculated barley leaves had been bleached with pure ethanol, the samples were subsequently boiled for 2–3 h in 10% KOH at 85 °C. After boiling, the samples were neutralized with five washing steps using phosphate-buffered saline (PBS) (pH 7.4). Subsequently the staining solution (1 µg/mL propidium iodide, 10 µg/mL

WGA-AF488; 0.02% Tween-20 in PBS, pH 7.4) was vacuum infiltrated into the samples three to four times (5 min each) at 25 kPa using a desiccator. The infiltrated leaves were stored in PBS at 4 °C in the dark until microscopic analysis. WGA-AF 488 used excitation at 488 nm and detection at 500–540 nm.

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SUPPORTING INFORMATION

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Fig. S1 Gene ontology enrichment analysis of differentially expressed *Ustilago hordei* genes by using GOEAST software. Red boxes represent GOs of gene groups that were significantly induced at all four time points. Green boxes represent GOs of gene groups that were significantly repressed at all times. More intense color indicates the higher statistical significance for the particular molecular function. p1 and p2 represent *p*-values of the induced and repressed genes, respectively. A detailed list of the GO assignments is presented in Supplemental Table S1.

Fig. S2 Comparison of expression pattern of microarray vs. qRT-PCR. Quantitative real-time PCR (qRT-PCR) was performed with 16 *U. hordei* effectors to confirm the microarray data. Expression levels of each gene used in RT-qPCR were normalized by using the *U. hordei Ppi* gene. Relative gene expressions have been shown as log₂-fold changes relative to expression in axenic culture. Error bars represent standard deviation of three biological replicates.

Fig. S3 Confirmation of *Ustilago hordei* knock-out strains via PCR. A) Schematic representation of *U. hordei* wild type (wt) and knock-out (KO) gene locus before and after homologous recombination. The *U. hordei* UHOR_02413, UHOR_02700, UHOR_03756 and UHOR_05101 genes were replaced by *hygromycin* cassette. Since the length of inserted *hygromycin* cassette is bigger than replaced UHOR_02413, UHOR_02700 and UHOR_03756 genes, primer pairs that bind to flanking region of left border (LB) and right border (RB) of each gene were used to confirm knock-out (Table S3). PCRs were performed with gDNAs isolated from both mating type of (Mate1:M1; Mate2:M2) wild type (wt) and knock-out (KO) strains. Gene replacement of wt UHOR_02413 (B), UHOR_02700 (C) and UHOR_03756 (D) gene locus with *hygromycin* cassette was confirmed via PCR by using locus specific primer set (Table S3). RT-qPCR was also performed for each gene to show the loss of expression in KO background compared to wt (B-C). E) Since the length of inserted *hygromycin* cassette and UHOR_05101 genes was similar, F1-R1 primer pair was used to confirm deletion of UHOR_05101 gene and F2-R2 primer pair was used to confirm the locus integration (Table S3). F) While wt strains show PCR products with F1-R1 primer pair, KO strains do not. On the other hand, while KO strains show PCR products with F2-R2 primer pair, wt strains do not. RT-qPCR was also performed to show loss of UHOR_05101 gene expression in KO background compared to wt. Error bars in RT-qPCR results represent standard deviation of three biological replicates. Expression levels were normalized using *U. hordei Ppi* gene.

Fig. S4 A) *Ustilago hordei* filamentation test on charcoal plate. To opposite mating types of both wild type and effector deletion strains were grown in YEPS liquid medium till OD:0.8-1.0. Subsequently, two mating type were mixed (1:1, v/v) to get OD:1.0 and 5 µl of the mixture was dropped on charcoal plate. Single mating type cultures were used as negative controls. Pictures were taken after 3 days incubation at room temperature. B) Growth rate of the *U. hordei* wild type and mutant strains in liquid YEPS_{light}. The OD₆₀₀ was measured at the indicated time points. There was no significant difference between the *U. hordei* wild type and Δ*uhor_02413*, Δ*uhor_02700*, Δ*uhor_03756* and Δ*uhor_05101* mutant strains. C) Classical *Ustilago hordei*-barley infection assay. Dehulled and surface sterilized barley seeds were inoculated with the *U. hordei* wild type and Δ*uhor_02413*, Δ*uhor_02700*, Δ*uhor_03756* and Δ*uhor_05101* mutant strains. Approximately 2-3 months after inoculation, disease symptoms

were scored at barley heading. This experiment was only performed one time.

Fig. S5 Biomass quantification of 14 *Ustilago hordei* effector mutants on barley. The virulence of the *Ustilago hordei* wild type and 14 effector mutants was performed by the quantification of fungal biomass at 6 dpi on barley leaf. The *U. hordei* *Pep1* deletion mutant was used as a positive control for virulence reduction. The infected barley leaves at 6 day post-inoculation (dpi) were harvested and analyzed via quantitative PCR by using genomic DNA and using the *U. hordei* *Ppi1* genes as a standard. The fungal biomass was deduced from a standard curve. A one-way ANOVA was performed and followed by a Bonferroni test. Samples that showed significant differences compared to wild type are marked with asterisks (*, $P < 0.001$). Error bars represent the standard deviation of at least three biological repeats.

Fig. S6 Heterologous production of *Ustilago hordei* Uvi1-4 in yeast cell and *Nicotiana benthamiana*. A) Western Blot (WB) analysis was performed with total proteins extract of ESM356 yeast strains transformed with *pEZY45-UHOR_02413-HA*, *pEZY45-UHOR_02700-HA*, *pEZY45-UHOR_03756-HA* and *pEZY45-UHOR_05101-HA* to confirm the expression of these effectors. Empty *pEZY15* transformed yeast cell was used as negative control. α -HA antibody was used for WB. Expected protein sizes were indicated below figure. B) Western Blot (WB) analysis was performed with total proteins extract of AH109 yeast strains transformed with *pGBKT7-Myc-UHOR_02413-HA*, *pGBKT7-Myc-UHOR_02700-HA* and *pGBKT7-Myc-UHOR_03756-HA* to confirm the expression of these effectors. Empty *pGBKT7-BD-Myc* transformed yeast cell was used as negative control. α -HA antibody was used for WB. Expected protein sizes were indicated below figure. C) Western Blot (WB) analysis was performed to confirm expression of *pK2GW7-UHOR_02413-HA*, *pK2GW7-UHOR_02700-HA* and *pK2GW7-UHOR_03756-HA* in *Nicotiana benthamiana* leaves transformed with *Agrobacterium*

tumefaciens. α -HA antibody was used for WB. Expected protein sizes were indicated below figure. Red arrows indicate respective protein bands. D) Heterologous expression of *pK2GW7-GUS-GFP*, *pK2GW7-UHOR_02413-GFP*, *pK2GW7-UHOR_02700-GFP*, *pK2GW7-UHOR_03756-GFP* and *pK2GW7-UHOR_05101-GFP* in *Nicotiana benthamiana* leaves transformed with *A. tumefaciens*.

Fig. S7 Cell-death suppression activity of four *Ustilago hordei* effectors. A) BAX1-mediated cell death suppression assay. Cell death inducing *pEZY45-BAX1* and *pEZY45-UHOR_02413*, *pEZY45-UHOR_02700* and *pEZY45-UHOR_03756* and *pEZY45-UHOR_05101* were co-expressed under the control of a galactose inducible promoter in the ESM356.1 yeast strain. Empty *pEZY45* and *pEZY45-Bcl-XL* were used as negative and positive controls for BAX1-mediated cell death suppression, respectively. Pictures were taken after 4 days of incubation. The figure is representative of three biological replicates. B) INF1-mediated cell death suppression assay. *Phytophthora infestans* cell death inducing *INF1* (in *pGR106-FLAG*) and *pK2GW7-UHOR_05101*, *pK2GW7-UHOR_02413*, *pK2GW7-UHOR_02700* and *pK2GW7-UHOR_03756* were co-infiltrated into 5-6 week-old *Nicotiana benthamiana* leaves via *Agrobacterium*-mediated transformation. *P. infestans* *Avr3AKI* (*pGR106-FLAG*) and *Avr3a Δ Y* (*pGR106-FLAG*) was used as positive and negative control for INF1-mediated cell death suppression, respectively. Pictures were taken at 4 dpi. The figure is representative of three biological replicates.

Table S1 Microarray analysis of the whole genome of *Ustilago hordei*. Blast2Go analysis of All genes, Secretome, Effectorome and Effectors for knock-out (KO) microarray analyses are depicted in different excel sheets. Genes showing a two-fold change in expression level were considered as differentially expressed (\log_2 fold change ≥ 1).

Table S2 Proteins with predicted NLS signal found in the *Ustilago hordei* effectorome.

Table S3 Constructs and oligonucleotides used in this study.