Virulence of the maize smut *Ustilago maydis* is shaped by organ-specific effectors

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SUMMARY

With the exception of *Ustilago maydis*, smut fungi infecting monocotyledonous hosts systemically colonize infected plants and cause symptoms exclusively in the inflorescences. *Ustilago maydis* infects primordia of all aerial organs of maize (*Zea mays* L.) and results in the formation of large plant tumours. Previously, we have found that *U. maydis* infection of seedling leaves, adult leaves and tassels causes organ-specific transcriptional changes in both the pathogen and the host. Of particular interest, *U. maydis* genes encoding secreted proteins are differentially expressed depending on the colonized maize organ. Therefore, we hypothesized that the fungus secretes virulence-related proteins (effectors) that act in an organ-specific manner. Here, we present the identification and functional characterization of 20 presumptive organ-specific *U. maydis* effector genes. *Ustilago maydis* deletion strains for these genes were generated and tested for infectivity of maize seedling leaves and tassels. This approach identified 11 effector genes required for the full virulence of *U. maydis*. In nine cases, virulence was only affected in one of the tested plant organs. These results demonstrate that individual fungal effector proteins contribute to fungal virulence in an organ-specific manner.

Keywords: effectors, maize, plant cell differentiation, tumour formation, *Ustilago maydis*.

INTRODUCTION

To cause disease, plant pathogenic microbes interfere with a broad spectrum of host cellular processes. Depending on the infection strategy, pathogens either aim to cause rapid cell death in the colonized tissue or to suppress immune responses to maintain the viability of host cells. Pathogens feeding on dead host tissues, i.e. necrotrophs, have been shown to promote host cell death by

toxins, the production of reactive oxygen species and lytic enzymes (Friesen *et al.*, 2008; Govrin and Levine, 2000; van Kan, 2006). In contrast, biotrophic pathogens, which depend on host cell survival, have evolved a plethora of strategies to modulate immune responses and manipulate metabolic processes to their own good (Glazebrook, 2005). Fundamental to this communication between pathogen and plant are effector proteins, which are secreted by the microbe to reside either in the apoplastic space or in the host cytoplasm (Stergiopoulos and de Wit, 2009; de Wit *et al.*, 2009).

The basidiomycetous smut fungi are plant pathogens that develop highly specialized biotrophic interactions. Members of the Ustilaginales parasitize monocots, including all important cereal crops, and are typically adapted to just a few host species. Most basidiomycetous smut fungi infect seedlings, often immediately following germination (Brefort *et al.*, 2009; Kämper *et al.*, 2006). Fungal hyphae growing both intra- and intercellularly colonize the host systemically and grow towards the central shoot apical meristem without inducing visible disease symptoms. On conversion of the shoot meristem to an inflorescence meristem, disease symptoms appear. Within the inflorescence floral organs, fungal spores are formed. The maize smut *Ustilago maydis*, a model organism for biotrophic fungi (Kämper *et al.*, 2006), is exceptional in the broad ability to colonize host organs. *Ustilago maydis* infects all aerial parts of the host plant and locally induces tumour formation. The tumours consist of both plant and fungal cells and, within these tumours, fungal spores develop and mature within a time span of about 2 weeks (Banuett and Herskowitz, 1996; Doeblemann *et al.*, 2008a). Transcriptome profiling, metabolite analyses and cytological studies have demonstrated that the conversion of developing plant organs into tumours involves a comprehensive reprogramming of maize gene expression, as well as both primary and secondary metabolism (Doeblemann *et al.*, 2008a, b; Horst *et al.*, 2010).

The *U. maydis* genome contains gene clusters encoding secreted effectors. The deletion mutants for these regions demonstrate participation in fungal virulence (Kämper *et al.*, 2006). So far, three effectors of *U. maydis* have been characterized functionally. Pep1, which inhibits plant peroxidases, and Pit2, which

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inhibits apoplastic cysteine proteases, are suppressors of host immunity and, as such, are essential for *U. maydis* virulence (Hemetsberger *et al.*, 2012; Mueller *et al.*, 2013). In addition, *U. maydis* chorismate mutase, encoded by the *cmu1* gene, has been found to be transferred to the cytoplasm of maize cells, where it re-channels chorismate metabolism to avoid the synthesis of salicylic acid (SA), a major signal of plant defence (Djamei *et al.*, 2011). Like the vast majority of studies on the *U. maydis*–maize interaction, the role of these effectors for *U. maydis* virulence has been analysed during the infection of maize seedling leaves. Because *U. maydis* is tumorigenic in both vegetative and floral organs, in a previous study, we tested the transcriptome of both interaction partners in seedlings, adult leaves and tassels. This approach resulted in a new concept for plant–pathogen interactions, as we demonstrated that the gene expression of both partners reflects the host organ being colonized by the fungus (Skibbe *et al.*, 2010). Moreover, deletion mutants for gene clusters showed different levels of virulence depending on the infected organ.

On the basis of these preliminary findings, we now ask whether individual effector genes of *U. maydis* are required for virulence in an organ-specific manner. Here, we present the functional analysis of 20 *U. maydis* effector genes with organ-specific expression patterns, and demonstrate for the first time that fungal effectors function in specific plant organs: approximately half of the tested effectors act in an organ-specific manner.

RESULTS

Identification of candidate genes encoding organ-specific *U. maydis* effectors

Candidates for putative organ-specific effector genes were selected from published transcriptome data (Skibbe *et al.*, 2010). The main focus was to identify genes that are specifically required for tumour formation in maize seedlings, but not in the tassel, or vice versa. To define candidates, genes were selected on the basis of either a significantly higher expression level in seedling leaves relative to tassels, or a significantly higher expression level in tassels relative to seedlings. Next, we prioritized genes encoding weakly conserved proteins in comparison with orthologues from the related species *Sporisorium reilianum* and *Ustilago hordei* (Table 1). These fungal species cause symptoms only in inflorescences, yet share 76% (*S. reilianum*; Schirawski *et al.*, 2010) and 69% (*U. hordei*; Laurie *et al.*, 2012) average similarity to *U. maydis* proteins. These criteria were based on the assumption that the exceptional ability of *U. maydis* to cause tumours in leaves depends on proteins that have gained novel functions and would have experienced accelerated divergence. In a final step, the microarray expression classification of candidates was verified by quantitative real-time polymerase chain reaction (qRT-PCR). These sequential selections resulted in a list of 17 candidate genes for putative leaf-specific effectors (Fig. 1, Table 1). Thirteen of

Table 1 Candidate genes for organ-specific *Ustilago maydis* effectors.

	Gene	Putative function*	Similarity to <i>Sporisorium reilianum</i> (%)†	Similarity to <i>Ustilago hordei</i> (%)†	Virulence leaf	Disease index leaf‡	Virulence tassel	Disease index tassel‡
Leaf induced	<i>um06222</i>	Uncharacterized protein	14	19	Wt	1.05 ± 0.09	Wt	0.99 ± 0.14
	<i>um11763</i>	Esterase (xylanase)	54	54	Wt	0.92 ± 0.09	Wt	1.03 ± 0.07
	<i>um02239</i>	Uncharacterized protein	40	34	Reduced	0.65 ± 0.05	Wt	1.03 ± 0.04
	<i>um05223</i>	β-1,6-Glucanase	67	64	Wt	1.02 ± 0.35	Wt	1.01 ± 0.08
	<i>um06127</i>	Uncharacterized protein	14	26	Wt	1.24 ± 0.32	Wt	0.94 ± 0.05
	<i>um01130</i>	Metal ion binding	62	58	Wt	0.99 ± 0.02	Wt	0.96 ± 0.04
	<i>um06223</i>	Uncharacterized protein	13	15	Reduced	0.45 ± 0.11	Reduced	0.77 ± 0.04
	<i>um05311</i>	Uncharacterized protein	22	23	Reduced	0.72 ± 0.15	Wt	1.00 ± 0.05
	<i>um01690</i>	Uncharacterized protein	45	46	Reduced	0.86 ± 0.02	Wt	1.01 ± 0.04
	<i>um01829</i>	α-L-Arabinofuranosidase	81	72	Reduced	0.69 ± 0.13	Wt	1.03 ± 0.22
	<i>um12313</i>	Uncharacterized protein	40	38	Wt	0.89 ± 0.1	Wt	0.95 ± 0.06
	<i>um10553</i>	Uncharacterized protein	34	15	Wt	0.95 ± 0.06	Wt	0.97 ± 0.02
	<i>um05318</i>	Uncharacterized protein	31	19	Reduced	0.75 ± 0.12	Wt	0.98 ± 0.02
	<i>um05306</i>	Uncharacterized protein	32	31	Reduced	0.76 ± 0.06	Wt	1.04 ± 0.11
	<i>um11060</i>	Uncharacterized protein	16	16	Reduced	0.72 ± 0.07	Wt	0.98 ± 0.03
	<i>um05495</i>	Endoglucanase	47	44	Wt	0.93 ± 0.13	Wt	0.97 ± 0.11
	<i>um12217</i>	Uncharacterized protein	19	20	Reduced	0.83 ± 0.1	Reduced	0.80 ± 0.04
Tassel induced	<i>um03046</i>	Uncharacterized protein	—	—	Wt	0.88 ± 0.09	Wt	0.95 ± 0.07
	<i>um03650</i>	Uncharacterized protein	—	26	Wt	1.08 ± 0.26	Reduced	0.64 ± 0.06
	<i>um05439</i>	Chitin-binding protein	63	63	Wt	1.03 ± 0.09	Reduced	0.84 ± 0.09

*Functions of the candidate genes were predicted using the PEDANT software tool (Helmholz-Zentrum, Munich, Germany).

†Similarity to homologues in the related species *S. reilianum* and *U. hordei*.

‡The disease indices reflect the severity of disease symptoms and are depicted in relation to SG200, which was set to unity.

Genes with significant virulence defects are printed in bold type. Wt, wild-type.

Fig. 1 Quantitative real-time polymerase chain reaction (qRT-PCR) expression profiling of organ-specific effector candidate genes selected from previous microarray data (Skibbe *et al.*, 2010). Expression levels are shown relative to the mean expression of peptidylprolyl isomerase (*ppi*) and the results were obtained using the $\Delta\Delta$ method for comparing relative expression results between treatments in real-time PCR (Livak and Schmittgen, 2001). Gene expression was analysed in axenic culture (AC), seedling at 3 days post-infection (dpi) (S 3), tassel at 3 dpi (T 3), seedling at 9 dpi (S 9) and tassel at 9 dpi (T 9). Genes highlighted in dark green are significantly leaf induced at both time points, those highlighted in light green are leaf induced at one time point and those highlighted in yellow are tassel induced. Experiments were performed in three independent biological replicates.

these 17 genes showed a sequence similarity below 50% to their closest homologues in *S. reilianum* or *U. hordei*.

Interestingly, three of the identified candidate genes (*um05306*, *um05311* and *um05318*) are situated in the *U. maydis* effector cluster 19A (Kämper *et al.*, 2006). Deletion mutants for this cluster, which encodes 24 predicted secreted effectors, have been shown previously to be blocked in tumour formation in seedling

leaves, whereas, in tassels, tumours are still formed (Kämper *et al.*, 2006; Skibbe *et al.*, 2010). Recently, in this cluster, one small gene family (Tin1-1–Tin1-4) and four individual effectors (Tin2, Tin3, Tin4, Tin5) have been found to contribute to *U. maydis* virulence in seedlings (Brefort *et al.*, 2014). The Tin4 effector is encoded by *um05318*, demonstrating the identification of this effector gene by independent approaches (Fig. 1, Table 1).

Four *U. maydis* secretome genes were found to be tassel induced in a previous microarray analysis (Skibbe *et al.*, 2010). Surprisingly, qRT-PCR indicated that one of the genes, *um06222*, was strictly leaf expressed, contradicting the previous microarray data (Fig. 1). For *um03046*, *um03650* and *um05439*, tassel-specific expression was verified by qRT-PCR: these three genes were strongly up-regulated in tassels, particularly at 9 days post-infection (dpi) when tumour formation is well underway, whereas their expression was barely detectable in infected seedlings (Fig. 1). The tassel-induced gene *um03046*, for which no homologues could be identified in *S. reilianum* or *U. hordei*, was previously identified to be regulated by the transcription factor Biz1 (Zahiri *et al.*, 2010). In contrast, neither *um03650*, which is up-regulated in tassels, nor *um05439*, which is expressed exclusively during *U. maydis* infection of tassels, has previously been analysed genetically (Fig. 1, Table 1).

In summary, our screen for organ-specific effectors identified 20 genes encoding putatively secreted proteins. Seventeen of these genes were categorized as leaf induced, because their transcription was exclusively detected in leaf infections, or they were significantly up-regulated in leaves relative to tassels for at least one of the analysed time points. In addition, three effector genes were tassel induced, with one gene (*um05439*) exclusively detected in tassel infections (Fig. 1).

Characterization of *U. maydis* deletion mutants for organ-specific effector candidates

As a first step in the functional analysis of candidate genes, deletion mutants for all genes were generated in the virulent *U. maydis* strain SG200 (Kämper *et al.*, 2006). Deletion mutants for the genes *um03046* and *um05318* were kindly provided by the laboratories of Jörg Kämper (KIT Karlsruhe, Germany) and Regine Kahman (MPI Marburg, Germany), respectively. To test whether the deletion of the organ-specific effector candidates impaired the vegetative growth of *U. maydis*, and/or its resistance to oxidative/cell wall stresses, all deletion mutants were cultured on different stress media (Fig. S1, see Supporting Information). Deletion of *um06223* resulted in a general growth defect. The deletion mutant displayed reduced growth, particularly on media containing the cell wall stress inducers Congo Red and Calcofluor. Similarly, the formation of filaments on charcoal-containing medium was impaired in this mutant (Fig. S1). In contrast, none of the other mutants analysed showed any significant difference in growth rate or colony morphology relative to the progenitor strain SG200 (Fig. S1). Therefore, with the exception of *um06223*, deletion of the selected genes did not result in pleiotropic effects, i.e. the mutants were not impaired in general fitness, stress resistance or filament formation.

All deletion mutants were tested in plant infections. Three independent replicates with 40 seedling leaves per mutant or 20

plant tassels (containing hundreds of flowers) were performed. To assess virulence in comparison with strain SG200, symptoms on seedling leaves were scored at 12 dpi, as described previously (Figs 2 and S2, see Supporting Information) (Kämper *et al.*, 2006). Maize tassel infections were scored at 14 dpi. To allow the quantitative evaluation of tassel infections comparable with that of seedling leaves, a new scoring scheme was developed. *Ustilago maydis* symptoms formed in the tassel were classified into five categories, depending on symptom severity (Fig. 3). The criteria for tassel scoring were based on both the area of the tassel converted to tumour as well as tumour size. In addition, developmental changes of infected tassels were assessed, including a growth arrest at the 1–3-cm stage, which was induced by several mutants as well as by SG200 infections (Fig. 3). Because all phenotypes observed in this study were quantitative, i.e. the mutants showed gradual differences in virulence but not complete apathogenic phenotypes, the infection data were employed to calculate a disease index which was analysed statistically using an unpaired *t*-test (Fig. 2, Table S1, see Supporting Information). Strikingly, in nine of the 17 deletion mutants for seedling-induced effectors, there was a significant reduction in virulence. Although the deletion of *um06223* and *um12217* caused a virulence defect in both organs, deletion of *um01690*, *um01829*, *um02239*, *um05306*, *um05311*, *um05318* and *um11060* specifically impaired tumour induction in seedling leaves (Fig. 2, Table 1). In addition, the deletion mutants for the tassel-induced genes *um03650* and *um05439* showed a significant reduction in virulence when infecting maize tassels, but were fully virulent on seedling leaves (Fig. 2, Table 1). Deletion of the third tassel-induced candidate gene, *um03046*, did not cause any detectable defects in *U. maydis* virulence (Table 1, Fig. S2).

The mutants impaired in seedling infection typically showed a reduction in symptom severity, i.e. at 12 dpi, tumour growth was attenuated and tumours rarely extended to 2–4 mm in diameter (Figs 2 and 4). The impact was distinctive in tassels: deletion of *um03650* and *um05439* resulted in a significantly reduced frequency of tumours in tassels, whereas the severity of the disease symptoms was comparable with SG200 (Fig. 2). To verify that the observed virulence defects were caused solely by deletion of the respective genes, the deletion mutants for *um01690*, *um01829*, *um02239*, *um03650*, *um05306*, *um05311*, *um05318*, *um05439*, *um11060* and *um12217* were complemented by introducing the respective genes with their native promoter into the ectopic *ip*-locus. Infection assays with the resulting strains showed full restoration of *U. maydis* virulence in all cases, demonstrating genetic complementation of the deletion phenotypes (Fig. 2). The result of the deletion analysis was the identification of seven seedling-specific effector genes, two tassel-specific effectors and two genes required for full virulence in both tassels and leaves (Table 1).

Fig. 2 Disease symptoms of *Ustilago maydis* mutants with a significant reduction in virulence in leaves and/or tassels. Maize seedling leaves were scored at 12 days post-infection (dpi). Disease symptoms in maize tassels were scored at 14 dpi as described in Fig. 3. SG200, virulent *U. maydis* progenitor strain; Δ, deletion mutant for organ-specific candidate gene; Δ/C, genetic complementation of deletion strain. Asterisks mark significant reduction in *U. maydis* disease index based on the data shown in Table S1: * $P \leq 0.01$; ** $P \leq 0.001$. *n*, number of plants infected. All experiments were performed in three independent biological replicates.

Fig. 3 Scoring scheme for tassel tumours based on the symptoms observed at 14 days post-infection (dpi) with *Ustilago maydis*. The symptoms were classified into five categories with increasing symptom severity, namely: I, tumours < 50% of tassel, small tumours; II, tumours < 50% of tassel, large tumours; III, tumours > 50% of tassel, small tumours; IV, tumours > 50% of tassel, large tumours; V, tassel growth stunted. The noninfected (N.I) tassel was used as a reference.

Fig. 4 Symptoms of *Ustilago maydis* organ-specific effector mutants on seedling leaves. Seven-day-old maize seedlings were infected with the *U. maydis* strain SG200, as well as with the deletion mutants for *um01690*, *um01829*, *um02239*, *um05306*, *um05311*, *um05318*, *um06223*, *um11060* and *um12217*. The pictured leaves show typical disease symptoms at 12 days post-infection (dpi). Similar to SG200, all mutant strains caused disease symptoms (chlorosis, anthocyanin accumulation) spreading along the leaf axis, but tumours are strongly diminished.

DISCUSSION

The hypothesis tested in this study was that organ-specific virulence factors exist and could be identified by transcriptome profiling. Of the 20 candidates tested, nine organ-specific effectors were identified. Therefore, we conclude that *U. maydis* deploys individual effector genes to conduct organ-specific functions, i.e. effectors can contribute specifically to fungal virulence depending on the colonized host organ. Only the deletion of *um06223* and *um12217* resulted in a significant reduction in virulence in both leaves and tassels (Fig. 2, Table 1). For *um06223*, this can be explained by the pleiotropic growth phenotype resulting from the deletion of this gene. Reduced tolerance to cell wall stress and

impaired filament formation probably lead to a general reduction in fitness, independent of the infected organ (Fig. S1). Deletion of *um12217* caused a significant reduction in tassel tumour frequency (Fig. 2, Table 1). Unlike *um06223*, however, this does not reflect a reduction in general fitness because no growth defects were observed for this mutant (Fig. S1). *um12217* is expressed not only in leaves, but also in tassels, at 3 dpi; therefore, we conclude that *um12217* encodes a significant virulence factor of *U. maydis*, but not an organ-specific effector.

Of the 11 genes whose deletion led to a reduced virulence, nine encode proteins without predicted functions or known domains (Table 1). Therefore, future functional analysis of the effectors, particularly the identification of their plant targets, will be

instrumental in understanding the molecular mechanisms of these proteins.

One of the leaf-induced genes (*um01829*) is predicted to encode an α -L-arabinofuranosidase, a xylane-degrading enzyme (Numan and Bhosle, 2006; Rahman *et al.*, 2003). Arabinoxylans are the most abundant cell wall polysaccharides in maize (Santiago *et al.*, 2013). They consist of a linear (1–4)- β -D-linked xylopyranosyl backbone, substituted with α -L-arabinofuranosyl groups at positions O-3 and/or O-2 of the (1–4)- β -D-linked xylopyranosyl residue (Aspinall, 1959). The degradation of the xylan backbone depends on endoxylanases and β -xylosidases. The arabinose residues can be removed by α -L-arabinofuranosidases (de Vries and Visser, 2001). This could provide a necessary role for *um01829* in *U. maydis* proliferation and tumour formation. However, the reason for the leaf-specific role of this effector remains unclear and will require an analysis of the cell wall composition in leaves relative to anthers.

In all cases, mutants exhibited a quantitative reduction in virulence, measured as both a reduced size and number of tumours. One reason for this partial reduction in virulence could be the functional redundancy of the deleted effectors. Nevertheless, the observed phenotypes are in clear contrast with the previously described mutants for the *U. maydis* effectors Pep1 and Pit2, which are both blocked before tumours can develop (Doehlemann *et al.*, 2009, 2011). Unlike the mutants for organ-specific effectors, deletion mutants for *pep1* and *pit2* cause host cell death responses, which appear macroscopically as necrotic lesions at the sites of infection. Pep1 and Pit2 both target central components of the plant's immune system. Suppression of the initial host defence by these effectors is essential for the establishment and maintenance of a biotrophic interaction, and effectors with immune-suppressive functions therefore form the first layer of microbial weaponry (Doehlemann *et al.*, 2008a; Takken and Tameling, 2009). Further progression of disease and, in particular, the induction of tumours requires an acclimation to growth in a particular plant organ and, possibly, cell type. The recent literature has also shown that the plant defence response acts cell specifically. For example, independent guard cell signalling elements function in stomatal defence in *Arabidopsis thaliana*, and two guard cell-specific pathways mediate biotic and abiotic stress signalling (Montillet and Hirt, 2013). Therefore, colonization of different cell types by the pathogen would require the suppression of cell-specific defence pathways.

We hypothesize that, in particular, the induction of plant tumours results from the activities of specialized effectors, which manipulate not only defence responses, but also the host cell metabolic state as well as cell cycle control. This type of effector may act in an organ to cell type-specific manner during tumour development. Hints to the presence of cell type-specific effectors were found in the human pathogen *Yersinia pseudotuberculosis*, which specifically targets phagocytic cells (Fahlgren *et al.*, 2009).

Hence, it is likely that a specialized plant biotroph, such as *U. maydis*, can deploy specialized cell-specific effectors to modulate tumour development deriving from different host cell types. In an evolutionary context, one could assume that such specific effectors have increased *U. maydis* fitness, i.e. to enhance its efficiency to colonize maize (Fig. 5). The development of such highly specialized effectors is probably a consequence of a high degree of adaptation to the host plant, and not only contributes to increased fitness but also to specialization. This model is congruent with the observation that *U. maydis* cannot colonize any host other than maize (and its wild progenitor teosinte), whereas many other smut species are able to infect two or more host species. A wider host range has been described for various smut fungi. For example, *Urocystis coralloides* (Rostr.) and *Urocystis brassicae* (Mund.) are seen to infect all the cruciferous hosts. *Ustilago striiformis* sporulates on the leaf and stem tissues of various grasses, and also occasionally sporulates on rhizomes below the ground (Holton *et al.*, 1968).

The *U. maydis* effectors identified here probably reflect different stages of host adaptation. Some of the genes described here as being leaf induced also show substantial expression in tassels. However, only the deletion mutant for *um12217* exhibited a reduction in virulence in both organs. Perfect organ specificity can be found for *um02239* which is exclusively active in leaves, as well as for *um05439*, which is only expressed in tassels, where it also affects virulence. This spectrum amongst the identified genes supports the concept of the gradual specialization of a pathogen to different environments within its host plant.

We assert that the finding of individual effectors for the promotion of organ-specific virulence adds a new level to the understanding of host manipulation by biotrophic pathogens. The identification of virulence factors that specifically act in individual host organs should allow the analysis of the mode of action of these proteins and the host processes which they target in a tissue- and, eventually, cell type-specific manner.

EXPERIMENTAL PROCEDURES

Infection of maize plants and growth of *U. maydis*

Ustilago maydis strains were grown and assayed for virulence in comparison with the *U. maydis* SG200 reference strain as described in Kämper *et al.* (2006). Infection assays were performed in three independent infection experiments. Maize variety Early Golden Bantam was infected at the three-leaf stage 7 days after sowing for leaf infections. A minimum of 40 plants per mutant and replicates were scored 12 days after infection, as described in Kämper *et al.* (2006). Tassel infections were performed 4 weeks after sowing, as described previously (Walbot and Skibbe, 2010). A minimum of 20 tassels were infected per mutant and replicate, and disease symptoms were scored 14 days after infection, following the criteria described in Fig. 3.

Fig. 5 Model for different classes of effectors acting in the *Ustilago maydis*–maize interaction. Top panel: establishment of infection during epidermal penetration requires core effectors (blue, red circles) which suppress basal host immunity. Middle panel: fungal proliferation, metabolic reprogramming and, finally, tumour induction in different host tissues are supported by the activity of organ-specific effectors (triangles, squares). Bottom panel: increasing adaptation to the host plant may coincide with the development of cell type-specific effectors, which are tailored to the specific proteome and metabolic conditions of different cell types colonized by *U. maydis*. Yellow plant cells indicate block in fungal development.

The calculation of the tumour formation index is based on the disease rating described above. For seedling infections, the number of plants sorted into categories 3 (small tumours) to 5 (large tumours) and, for tassel infections, the number of plants sorted into categories 1 (<50% of tassel with small tumours) to 5 (tassel growth stunted), were multiplied by the number of the category (1–5), summed and then divided by the number of plants: $\{[(\text{symptom category } X \times \text{number of plants in category } X)^X + (\dots)^{Y-2}]/\text{total number of plants}\}$. The resulting value for SG200 was set to unity. Indices of deletion mutants are given relative to the score for SG200. An unpaired *t*-test was used to calculate the statistical significance of the differences in disease indices between mutant strains and SG200.

Ustilago maydis strains were grown on different media. Solid CM glucose medium (Holliday *et al.*, 1974), supplemented with Calcofluor, Congo Red, H₂O₂, NaCl and sorbitol at the concentrations indicated in the respective experiment (Fig. S1), was used to generate stress conditions. To induce filament formation of *U. maydis*, charcoal (0.01 g/mL) was added to the CM glucose medium. Cells were grown in YEPSL medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v sucrose) to an optical density at 600 nm (OD₆₀₀) of 0.8 and re-suspended in H₂O to an OD₆₀₀ of 1.0. Five microlitres of serially diluted (10⁻¹) samples were spotted onto stress plates. Plates were incubated for 2–3 days at 28 °C in the dark before photographs were taken.

Sequence analyses

The accession numbers of *U. maydis* genes described in this study can be accessed at the *U. maydis* database of the Munich Information Centre for Protein Sequences (MIPS), Munich, Germany (<http://mips.helmholtz-muenchen.de/genre/proj/ustilago/>). Sequence similarities were estimated with CLUSTALW (Kyoto University Bioinformatics Centre, Kyoto, Japan) by

amino acid sequence comparison using the best-matched orthologous sequences from *U. hordei* and *S. reilianum*, if present. For this, homologous amino acid sequences were retrieved with the WUBLAST analysis tool from the MIPS *U. maydis* database. Predictions of protein functions were performed using the PEDANT software tool (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=start_method&Db=p3_t237631_Ust_maydi_v2).

Generation of deletion mutants and the respective complementation strains

All *U. maydis* strains analysed in this study [including the mutants for *um03046* (provided by Jörg Kämper) and *um05318* (provided by Regine Kahmann)] were derived from the solopathogenic strain SG200 (Kämper *et al.*, 2006). To generate constructs that introduce gene deletions via homologous recombination, a PCR-based approach using hygromycin as a resistance marker was employed (Kämper, 2004). About 1 kb of each flanking region of the gene to be deleted was amplified by PCR using SG200 DNA as template. In addition, a *Sfi*I site was introduced at the start and stop codons of the respective genes. The PCR products were digested with *Sfi*I and ligated to the *hph* cassette excised from the plasmid pBS-hhn (Kämper, 2004) via *Sfi*I. Primers used for the PCR amplification of the flanking regions are listed in Table S2 (see Supporting Information). Single homologous integration of the deletion constructs was verified by Southern blotting, and three independent isolates per gene were used for subsequent analyses. To complement *U. maydis* deletion mutants, the respective genes, including their promoter regions, were amplified by PCR from *U. maydis* wild-type DNA and ligated into the restricted vector p123. Primers used for the generation of the complementation strains are listed in Table S3 (see Supporting Information).

For integration into the *ip* locus of *U. maydis*, plasmids derived from p123 were used and linearized within the *cbx* gene before transformation into the respective deletion mutants. Integration of complementation constructs into the *ip* locus was verified by Southern blot analysis for all mutants generated in this study.

Nucleic acid isolation and qRT-PCR

DNA isolation from *U. maydis* was carried out as described by Schulz *et al.* (1990). Total RNA was extracted using the TRIZOL reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The gene expression was analysed by qRT-PCR. Liquid cultures of strain SG200 and maize leaf and tassel samples infected with strain SG200 were taken in three independently conducted experiments. The infected plant material was collected at 3 and 9 dpi, and processed for RNA extraction as described previously (Doehlemann *et al.*, 2008a; Skibbe *et al.*, 2010). For cDNA synthesis, the SuperScript III first-strand synthesis SuperMix assay (Invitrogen) was employed, using 1 µg of total RNA. qRT-PCR was performed on a Bio-Rad iCycler (Bio-Rad, Munich, Germany) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Cycling conditions were 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. Primer sequences used for qRT-PCR are listed in Table S4 (see Supporting Information).

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SUPPORTING INFORMATION

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

Fig. S1 Growth of *Ustilago maydis* on different media. Serial dilutions of exponentially growing cells of strain SG200, as well as deletion mutants for *um01690*, *um01829*, *um02239*, *um03650*, *um05306*, *um05311*, *um05318*, *um05439*, *um06223*, *um11060*, *um12217*. Cells were spotted on CM glucose plates supplemented with Calcofluor (100 µg/mL), charcoal (0.01 g/mL), Congo Red (45 µg/mL), H₂O₂ (1 mM), NaCl (1 M) or sorbitol (1 M). Plates were incubated for 2–3 days at 28 °C. Images show representative examples of three independent replicates.

Fig. S2 Disease symptoms of *Ustilago maydis* mutants without significant reduction in virulence. Maize seedling leaves were scored at 12 days post-infection (dpi). Disease symptoms in maize tassels were scored at 14 dpi as described in Fig. 3. SG200, virulent *U. maydis* progenitor strain; Δ, deletion mutant for organ-specific candidate gene.

Table S1 Calculation of the tumour formation index.

Table S2 Primers used for the polymerase chain reaction (PCR) amplification of flanking regions of deleted *Ustilago maydis* genes.

Table S3 Polymerase chain reaction (PCR) primers used for the complementation of *Ustilago maydis* deletion mutants.

Table S4 Polymerase chain reaction (PCR) primers used for quantitative real-time PCR (qRT-PCR).