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Research

The use of FLP-mediated recombination for the functional analysis of an effector gene family in the biotrophic smut fungus *Ustilago maydis*

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Summary

- In the *Ustilago maydis* genome, several novel secreted effector proteins are encoded by gene families. Because of the limited number of selectable markers, the ability to carry out sequential gene deletions has limited the analysis of effector gene families that may have redundant functions.
- Here, we established an inducible FLP-mediated recombination system in *U. maydis* that allows repeated rounds of gene deletion using a single selectable marker (Hyg^R). To avoid genome rearrangements via FRT sites remaining in the genome after excision, different mutated FRT sites were introduced.
- The FLP-mediated selectable marker-removal technique was successfully applied to delete a family of 11 effector genes (*eff1*) using five sequential rounds of recombination. We showed that expression of all 11 genes is up-regulated during the biotrophic phase. Strains carrying deletions of 9 or all 11 genes showed a significant reduction in virulence, and this phenotype could be partially complemented by the introduction of different members from the gene family, demonstrating redundancy.
- The establishment of the FLP/FRT system in a plant pathogenic fungus paves the way for analyzing multigene families with redundant functions.

Introduction

Plant–pathogen interactions involve secreted pathogen effectors. Such effectors can trigger virulence by suppressing pathogen-associated molecular pattern (PAMP)-triggered immunity. Alternatively, effectors can induce effector-triggered immunity (ETI) in plants that host cognate resistance proteins (Jones & Dangl, 2006). As a result of gene duplications and functional redundancy caused by an arms race between PAMP and ETI receptors in plants and effectors in pathogens, many effectors are individually dispensable (Angot *et al.*, 2006; Birch *et al.*, 2009; Kvitko *et al.*, 2009; Stergiopoulos & de Wit, 2009). Some effectors might also possess overlapping activities, which may be the case for the Avr4 and Ecp6 effectors of *Cladosporium fulvum*, both of which bind to chitin and thus could potentially protect the fungus against plant chitinases and/or suppress plant defense responses by scavenging chitin fragments released from fungal cell walls in the apoplast during infection (Bolton *et al.*, 2008).

Ustilago maydis is the causative agent of corn smut disease and has become one of the models used to study biotrophic interactions. In *U. maydis*, 426 genes code for putatively secreted proteins, and, of these, 272 encode either *U. maydis*-specific proteins or conserved proteins without recognized InterPro domains (Müller *et al.*, 2008). Many of the novel secreted proteins are encoded by gene clusters, and the respective genes are induced in infected tissue during biotrophic growth. Deletions of several effector clusters, but also of a single effector gene, have dramatic effects on virulence (Kämper *et al.*, 2006; Doehlemann *et al.*, 2009). Among the novel secreted *U. maydis* proteins, some are encoded by gene families (Kämper *et al.*, 2006). One of these *U. maydis*-specific gene families is family 9, which consists of four genes, and another, designated family 17, contains three genes (Kämper *et al.*, 2006). Neither of these

gene families has been functionally characterized because this would require the generation of multiple gene-deletion mutants. Although tools (such as a PCR-based system) for the generation of gene-replacement mutants in *U. maydis* is in place, only a limited number of dominant drug-resistance markers, such as hygromycin (Wang *et al.*, 1988), carboxin (Keon *et al.*, 1991), phleomycin and nourseothricin (Gold *et al.*, 1994) have been developed. As deletions are usually made in the engineered solopathogenic strain SG200 (Kämper *et al.*, 2006), which is already phleomycin resistant, and the link between phenotype and deletion of a particular gene needs to be established by complementation, this reduces the number of available dominant markers further. Such obstacles can be overcome by recycling the resistance marker, which can be achieved via site-specific recombination (Bucholtz, 2008; Birling *et al.*, 2009).

Site-specific recombinases, such as Cre and FLP, catalyze efficient recombination between two directly oriented recombination sites, termed LoxP and FRT, respectively. This leads to excision of the intervening DNA segment, leaving one recombination site behind. If the selectable marker is placed on the intervening DNA segment, this allows the generation of unmarked gene disruptions and the use of the same selectable marker in subsequent rounds (Wirth *et al.*, 2007). To date, such selectable marker-removal systems have been successfully established in different organisms, such as *Saccharomyces cerevisiae* (Storici *et al.*, 1999), *Candida albicans* (Morschhauser *et al.*, 1999), *Aspergillus nidulans* (Forment *et al.*, 2006), *Cryptococcus neoformans* (Patel *et al.*, 2010), maize (Kerbach *et al.*, 2005) and mouse (Wu *et al.*, 2008). The 45 kDa FLP recombinase from *S. cerevisiae* is the best characterized eukaryotic member of tyrosine recombinases. A minimal, fully functional FRT site is 34 bp long and consists of an 8 bp asymmetric spacer that defines the orientation of the FRT site, which is flanked by two, 13-bp-palindromic sequences that constitute the binding sites for FLP (Chen & Rice, 2003). As FLP-mediated recombination does not require any accessory host proteins, the FLP/FRT system has been employed in a wide range of species, ranging from prokaryotes to mammals (Luo & Kausch, 2002; Schweizer, 2003). Another advantage of the FLP/FRT system is that FLP exhibits optimal activity at 30°C (Buchholz *et al.*, 1996), a temperature at which *U. maydis* can be cultivated under laboratory conditions. As a result of the possible recombination between the FRT sites left in the genome after each round of FLP-mediated marker removal there is a potential problem of undesired chromosome rearrangements. However, this can be circumvented by introducing FRT sites with different point mutations in the core region, which restricts recombination to identical FRT sites (Storici *et al.*, 1999; Barrett *et al.*, 2008).

Here we describe establishment of the FLP/FRT system in *U. maydis*. As proof of principle we have successively disrupted 11 genes encoding related secreted effectors. We designated this group of 11 genes as the *eff1* gene family. This family combines the previously defined families 9 and 17 (Kämper *et al.*, 2006), plus four additional genes, all encoding *U. maydis*-specific secreted proteins. We show that, depending on the genes deleted, virulence can be significantly compromised when two or more genes are deleted. The virulence phenotype of the nine-gene-deletion mutant could be complemented by introducing different members of the gene family, suggesting redundancy.

Materials and Methods

Strains, plasmids, growth conditions and molecular techniques

For cloning purposes the *Escherichia coli* strains Top10 (Invitrogen) and DH5α (Gibco BRL, Eggenstein, Germany) were used and grown in yeast extract, tryptone (YT) medium. The solopathogenic *U. maydis* strain SG200 (*a1mfa2bE1bW2)* has been described previously (Kämper *et al.*, 2006). Strains were grown on potato dextrose (PD) (Difco, Becton Dickinson, Heidelberg, Germany), complete medium (CM)-glu or yeast extract peptone sucrose light (YEPSL) liquid medium (Holliday, 1974; Molina & Kahmann, 2007) or the respective solid media containing 2% agar in addition. To select transformants, hygromycin B (Duchefa, Haarlem, the Netherlands) and carboxin (Riedel de Haen, Seelze, Germany) were added to a final concentration of 200 μg ml^{-1} and 2 μg ml^{-1}, respectively. For induction of the *crg1* promoter, cells were grown in CM-glu medium to an OD_{600} of 0.7, washed twice with distilled water, resuspended in CM medium containing 1% arabinose as the sole carbon source (CM-ara) and incubated for the times indicated. To delete the hygromycin-resistance marker flanked by FRT sites, strains were transformed with the FLP-expressing plasmid pFLPexpC. A carboxin-resistant colony was grown overnight in CM-ara medium and plated on PD to obtain single colonies. Colonies were replica-plated on PD plates and PD plates supplemented with hygromycin (PD-hyg). Colonies that had lost resistance to hygromycin were subsequently tested on PD plates supplemented with carboxin to test for the loss of pFLPexpC and were analyzed by PCR to verify deletion events.

To generate deletion mutants, the single-step method developed by Kamper (2004) was adopted. The strategy for generating the codon-optimized FLP-recombinase gene was similar to that used by Stemmer *et al.* (1995) and Hale & Thompson (1998). For integrating genes into the *ip* locus, the strategy of Loubradou *et al.* (2001) was used. Plasmids pBScbx, pRU11, pNEBUH and p123 have been described previously (Keon *et al.*, 1991; Brachmann *et al.*, 2001; Weinzierl *et al.*, 2002; Aichinger *et al.*, 2003). Details for all plasmids and strain constructions can be found in the Supporting Information Methods S1.

Cloning procedures followed standard molecular techniques (Sambrook *et al.*, 1989). All primers used in this study are listed in Table S1. Transformation of *U. maydis* followed the protocol of Schulz *et al.* (1990). DNA was isolated as described previously (Hoffman & Winston, 1987). Details for additional molecular techniques are described in Methods S2.

Pathogenicity assays

Pathogenicity assays were performed as described previously (Kämper *et al.*, 2006). For maize (*Zea mays*) infections, cultures of *U. maydis* strains indicated in the respective experiments were grown to an OD_{600} of 0.7–0.8 in YEPSL, pelleted, resuspended in distilled water to an OD_{600} of 1 and injected into 7-d-old seedlings of the variety Early Golden Bantam (Olds Seeds, Madison, WI, USA). Plants were kept in the glasshouse with a light–dark cycle of 16 h (28°C) and 8 h (20°C). Disease symptoms were scored, according to severity, 14 d after inoculation (Kämper *et al.*, 2006).

FLP activity and recombination assay

To assay the efficiency of FLP-mediated recombination of wild-type and mutated FRT pairs, strains were generated where the *um11377.2* gene was deleted in SG200FLP using the *hph* resistance cassette from pHwtFRT, pHFRTm1, pHFRTm2, pHFRTm3 and pHFRTm4 plasmids, respectively. In each case, one transformant was recovered in which the *um11377.2* gene was replaced by a hygromycin resistance cassette flanked by a pair of wild-type or mutated FRT sequences. These strains were grown in CM-ara medium for 24 h and then plated onto PD plates to obtain single colonies. Colonies were replica-plated to PD and PD-hyg plates. The recombination efficiency was calculated by determining the percentage of cells that had lost hygromycin resistance.

Bioinformatics

Multiple alignments of *eff1* genes (see Table S2) were made in MACAW (Schuler *et al.*, 1991) and signal sequences were predicted with SIGNALP at http://www.cbs.dtu.dk/services/SIGNALP (Bendtsen *et al.*, 2004). All other bioinformatic analyses were performed within the MPI Bioinformatics Toolkit at http://toolkit.tuebingen.mpg.de (Biegert *et al.*, 2006). Searches for members of the Eff1 protein family were made by comparison of the profile Hidden Markov Models (HMMs) in HHPRED (Söding *et al.*, 2005). To this end, HMMs were generated for all open reading frames (ORFs) of the *U. maydis* genome and have been made publicly available for searching at http://toolkit.tuebingen.mpg.de/HHpred/. The cluster analysis was made with CLANS (Frickey & Lupas, 2004).

Results

Establishment of the FLP-mediated recombination system in *U. maydis*

To increase the probability of expression of FLP in *U. maydis*, a codon-optimized FLP recombinase gene was assembled from oligonucleotides (Methods S1). In total, 384 silent mutations were introduced (Fig. S1). To test FLP expression we generated strain SG200FLP, in which the FLP recombinase is under the control of the arabinose-inducible *crg1* promoter. By northern blot analysis, FLP recombinase gene expression was visualized 1 h after the shift to CM-ara (Fig. S2). To assess FLP activity, the self-replicating recombination reporter plasmid, pIF1, was introduced into SG200FLP and into SG200 as a control. In pIF1, *lacZ′* is disrupted by a cassette in which the FRT sites flank a constitutively expressed *egfp* gene. FLP-mediated recombination should excise the *egfp* cassette and leave a plasmid in which the *lacZ′* gene is restored (Fig. 1a). After growth in CM-glu medium, SG200pIF1 and SG200FLPpIF1 strains were shifted to CM-ara medium for up to 16 h. Starting at 4 h, a decrease of relative fluorescence units was observed in the SG200FLPpIF1 strain, indicating excision of the *egfp* gene (Fig. 1b). Neither in the control strain SG200pIF1, nor in SG200FLPpIF1 grown in CM-Glu medium was such a decrease in relative fluorescence observed (Fig. 1b). After an induction period of 16 h, DNA was prepared and introduced into DH5α by electroporation. All transformants with DNA isolated from SG200pIF1 were white on 5-bromo-4-chloroindol-3-yl β-D-galactoside (Xgal) plates, whereas 67.8 ± 15.5% (calculated from three independent experiments) of the transformants with DNA isolated from SG200FLPpIF1 were light blue (Fig. 1c), indicating partial restoration of LacZ activity. When FLP activity was not induced in SG200FLPpIF1, 1.8% of the *E. coli* transformants showed *lacZ′* expression, indicating some leakiness of the *crg1* promoter. Compared to plasmids isolated from white colonies, plasmids from light-blue colonies were reduced in size by 2.0 kb, indicative of FLP-mediated excision (Fig. S3). In five of these plasmids the expected new junction was verified by sequencing. This illustrated efficient FLP-mediated excision, when FRT sites are located on an autonomously replicating plasmid.

Next, we tested the efficiency of FLP-mediated excision in strain SG200FLP when FRT sites are present in the genome. To achieve this, *um01796* was disrupted in SG200FLP by the *hph* cassette flanked by wild-type FRT sites (Fig. 2a) to yield strain SG200FPLΔ01796$^{FRT/FRT}$. Genomic DNA was isolated at different time-points after the induction of FLP. By PCR analysis using primers flanking *um01796* the appearance of the 2 kb post-excision product was demonstrated after an induction period of only

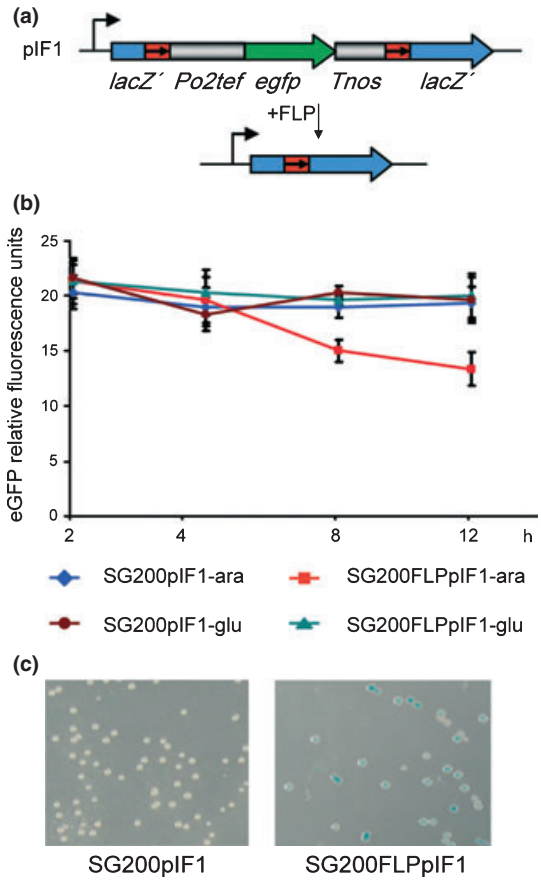

Fig. 1 Establishment of the FLP/FRT system in *Ustilago maydis*. (a) Scheme of the recombination reporter plasmid pIF1. *egfp* is inserted in the *lacZ'* gene and is flanked by directly repeated FRT sites (red bars with arrows). *egfp* expression is controlled by the constitutive *Po2tef* promoter and the *nos* terminator (upper panel). FLP-mediated recombination excises *egfp*, leaving one FRT site. This site is in-frame with the open reading frame of *lacZ'* (lower panel). (b) SG200FLPpIF1 and SG200pIF1 were either grown in CM-glu or shifted to CM-ara for the indicated time. Relative eGFP fluorescence/OD_{600} was determined in triplicate experiments. Average values are shown and SEs are indicated for SG200FLPpIF1 shifted to CM-ara (red) and CM-glu (green) and for SG200pIF1 shifted to CM-ara (blue) and CM-glu (dark red). (c) Sixteen hours after the shift of SG200pIF1 and SG200FLPpIF1 to CM-ara medium, DNA was prepared, transformed into DH5α and transformants were plated on YT/5-bromo-4-chloroindol-3-yl β-D-galactoside (Xgal) plates. All transformants with DNA recovered from SG200pIF1 yielded white colonies (left) while about two-thirds of the transformants with DNA from SG200FLPpIF1 yielded blue colonies (right).

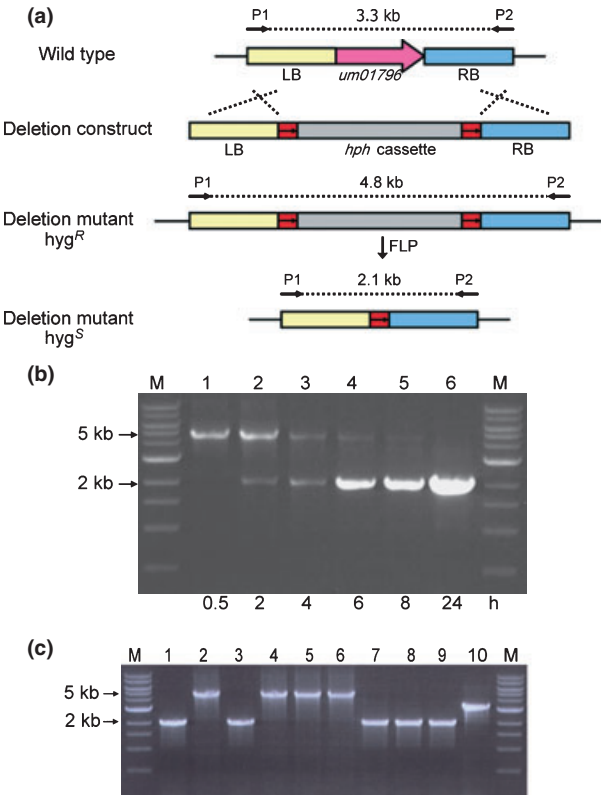

Fig. 2 Time course and efficiency of FLP-mediated excision of the *hph* cassette from a genomic location. (a) Scheme of the events leading to deletion of *um01796* and subsequent FLP-mediated excision. FRT sites are indicated as red boxes and their orientation is given by arrows. In the first step the deletion construct for gene *um01796* is integrated in the genomic locus of SG200FLP by homologous recombination (dotted crosses), yielding a hygromycin-resistant transformant. After inducing FLP expression by shifting to CM-ara medium the hygromycin resistance cassette is excised leaving one FRT site in the genome. PCR primers used for demonstrating excision of the *hph* cassette are indicated together with the length of the amplified fragments. (b) SG200FLPΔ01796$^{FRT/FRT}$ was grown in CM-glu medium and then shifted to CM-ara medium for 24 h. Samples were taken at the indicated times and DNA was prepared. Excision was monitored by PCR using primers P1 and P2. The 4.8 kb fragment corresponds to the nonexcised cassette, the 2.1 kb fragment indicates the post-excision product. (c) SG200Δ01796$^{FRT/FRT}$pFlpexpC was grown in CM-ara medium for 16 h and then plated on CM. DNA was prepared from nine single colonies (lanes 1–9) and analyzed by PCR using primers P1 and P2. Hygromycin-resistant colonies contained the nonexcised *hph* cassette (4.8 kb) while all hygromycin-sensitive colonies had excised the cassette (2.1 kb). Using the P1 and P2 primer pair a 3.3 kb PCR product is amplified from SG200 (lane 10). M, 1 kb ladder.

2 h and the intensity of this band increased with prolonged induction time (Fig. 2b).

As the low-level basal expression of FLP observed in SG200FLP could cause premature excision of the resistance cassette and complicate the identification of desired transformants, a self-replicating FLP-expressing plasmid (pFLPexpC) was generated (Fig. S4). Such plasmids containing an autonomously replicating sequence from *U. maydis* have been shown to be mitotically unstable (Tsukuda *et al.*, 1988). pFLPexpC was introduced into SG200Δ01796$^{FRT/FRT}$, and, after inducing FLP expression, colonies that had lost the hygromycin cassette, as well as the FLP donor plasmid, were identified (see the Materials and Methods section for details). In three independent experiments an excision frequency of 65.71 ± 4.34% was determined and of these cells 21.1 ± 2.6% were also carboxin sensitive (i.e. they had lost pFLPexpC). Excision of the *hph* cassette was verified by

PCR using primers flanking gene *um01796* (Fig. 2c). There was a perfect correlation between the presence of the 2 kb PCR product, indicative for deletion of the hygromycin cassette from the genome, and hygromycin sensitivity. For the subsequent experiments, selection for the simultaneous loss of hygromycin and carboxin resistance was applied.

Because the generation of successive deletion mutants was very time consuming, we modified the system in such a way that in future experiments the FLP gene could be introduced into the genome together with the *hph* cassette. To demonstrate that this is feasible we disrupted *um01796* in SG200 by introducing pYUIF-FRTm2. In the resulting strain, a cassette containing FLP and *hph* is flanked by FRTm2 sites (Fig. S5a). After inducing FLP, 34 ± 9% of the resulting single colonies were hygromycin sensitive. PCR analysis revealed that in these colonies the *hph* gene, as well as the FLP gene, had been lost (Fig. S5b). This illustrates that the experimental speed in future studies can be significantly increased.

Recombination efficiency of core-mutated FRT sequences

To minimize the likelihood of chromosome rearrangements occurring through intramolecular and intermolecular recombination between identical FRT sites left in the genome after several rounds of FLP-mediated excision, four mutated FRT sequences were designed, each with a different point mutation in the core region (Fig. S6a). For the FRTm1 sequence, functionality had previously been demonstrated (Storici *et al.*, 1999). Plasmids were generated in which the *hph* marker gene is flanked by two direct copies of these mutated FRT sequences and the left and right flanks of the *um11377.2* gene. The *hph* cassettes and flanking regions from pHwtFRT (Fig. S4), pHFRTm1, pHFRTm2, pHFRTm3 and pHFRTm4 were amplified and introduced into SG200FLP individually to disrupt gene *um11377.2*. The efficiency of FLP-mediated recombination of mutated FRT sites was assayed (see the Materials and Methods section). Relative to recombination in the strain carrying wild-type FRT sequences, the mutated FRT sequences recombined two to five times less efficiently (Fig. S6b).

To demonstrate that there is no recombination between wild-type FRT and FRT sites carrying mutations in the core region we used SG200Δ01796^{FRT}Δ11377.2$^{FRT/FRT}$ and several derivatives. In SG200Δ01796^{FRT}Δ11377.2$^{FRT/FRT}$, *um01796* on chromosome 3 has been deleted, leaving one wild-type FRT site, and *um11377.2* (also residing on chromosome 3 at a distance of 0.43 Mb from *um01796*) has been replaced with a hygromycin cassette flanked by two wild-type FRT sites. In a parallel experiment, the four strains SG200Δ01796^{FRT}Δ11377.2$^{FRTm1/FRTm1}$, SG200Δ01796^{FRT}Δ11377.2$^{FRTm2/FRTm2}$, SG200Δ01796^{FRT}Δ11377.2$^{FRTm3/FRTm3}$ and SG200Δ01796^{FRT}Δ11377.2$^{FRTm4/FRTm4}$ (Table S3), which differ from SG200Δ01796^{FRT}Δ11377.2$^{FRT/FRT}$ only by the m1, m2, m3 or m4 mutation in the FRT sites (Fig. S6a) residing in the *um11377.2* locus (before excision of *hph*), were generated. After introducing pFLPexpC and inducing FLP expression (see the Materials and Methods section) DNA was isolated and analyzed by PCR to identify deletion events that had occurred between FRT sites in the *um01796* and the *um11377.2* loci using the primer combinations shown in Fig. S7a. While recombination could be visualized between a wild-type FRT site in the *um01796* locus and wild-type FRT sites in the *um11377.2* locus in DNA isolated from SG200Δ01796^{FRT}Δ11377.2$^{FRT/FRT}$, such a 3.1 kb product was not amplified when strains harbored a wild-type FRT site and any of the mutated FRT sites (Fig. S7b). This illustrates that the FRT mutations introduced greatly reduce, or abolish, recombination with wild-type FRT sites, which should consequently help to maintain strain integrity.

An 11-gene family in *U. maydis* that codes for novel secreted proteins

U. maydis families 9 and 17 have been described to consist of four and three genes, respectively, encoding novel secreted proteins that are *U. maydis*-specific (Kämper *et al.*, 2006). Our initial interest in these groups of secreted proteins was based on the finding that three members of these two gene families contain putative nuclear localization signal (NLS) motifs (Müller *et al.*, 2008). However, these putative NLS sequences occur at nonconserved locations and are therefore not considered to be functionally relevant. Additionally, a more rigorous search for related genes using profile HMMs revealed that these seven genes in families 9 and 17 are related and four additional paralogs exist in the genome (Figs 3, S8). We designated this enlarged gene family as the *eff1* family (Table S2). The *eff1* family comprises *um01796* and *um11377* on chromosome 3, the adjacent genes *um03313* and *um03314* on chromosome 8, and the seven genes, *um02135*, *um02136, um02137*, *um02138*, *um02139*, *um02140* and *um02141* clustered on chromosome 5. All encoded proteins, except for Um11377, contain putative N-terminal secretion signals, and an analysis of the *um11377* gene region showed that sequence similarity with other Eff1 proteins extends well upstream of the predicted Um11377 start methionine and includes a putative signal sequence. This larger frame is, however, disrupted by a stop codon at position 64. We resequenced this gene from the sequenced *U. maydis* strain 521 and detected a sequencing error. The gene model was corrected and the gene is now designated *um11377.2.* Additionally, based on sequence similarity between Um02139 and Um02140 (Fig. S9) Met37 is strongly implied to be the true start codon of Um02140. The respective gene is now designated *um02140.2*.

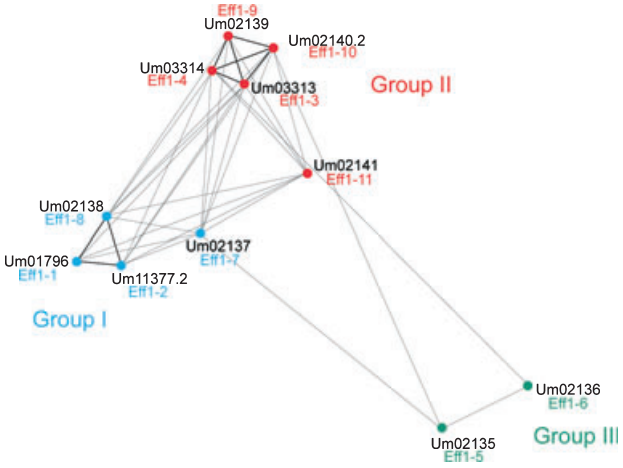

Fig. 3 Cluster map of Eff1 proteins. Sequences were compared using BLAST, seeded randomly in space and connected by forces proportional to the significance of their pairwise BLAST matches. The map was then equilibrated to convergence in a force-directed manner. BLAST matches at a *P*-value of 1.0 or better are shown as grey lines. Three groups of sequences are apparent, colored red, blue and green on the map. Group III is the most distant and loses connection with the other groups at a *P*-value cut-off of 1e-03. Groups I and II lose connection with each other at a cut-off of 1e-09. Eff1 proteins are labelled by their respective um numbers, as well as by their *eff1* gene numbers.

Sequence comparisons between Eff1 proteins showed that the family forms three subgroups (Fig. 3): group I, comprising Um01796, Um11377.2, Um02137 and Um02138; group II, comprising Um02139, Um02140.2, Um02141, Um03313 and Um03314; and group III, comprising Um02135 and Um02136. Group III sequences are highly divergent, yet should be counted as true Eff1 homologs based on the following observations: they make multiple, statistically significant connections to other Eff1 proteins in HMM comparisons (Fig. 3); they are bidirectional best-hits to the other Eff1 proteins in sequence searches by HMM; they have the same domain structure as other Eff1 proteins (Fig. S9); and they are located directly adjacent to the main cluster of Eff1 proteins on chromosome 5.

All Eff1 proteins have the same architecture, consisting of an N-terminal signal sequence, a central region predicted to be natively unstructured and a conserved C-terminal domain, which presumably represents the only folded part of these proteins (Fig. S9). We also noted that in group II sequences, the central region predicted to be natively unstructured contains a conserved segment with an area of elevated helical propensity, which is duplicated in Um02139 and Um03314 (Fig. S9b).

Expression patterns of the *eff1* effector genes

The expression patterns of all members of the *eff1* family were analyzed by quantitative real-time PCR during different

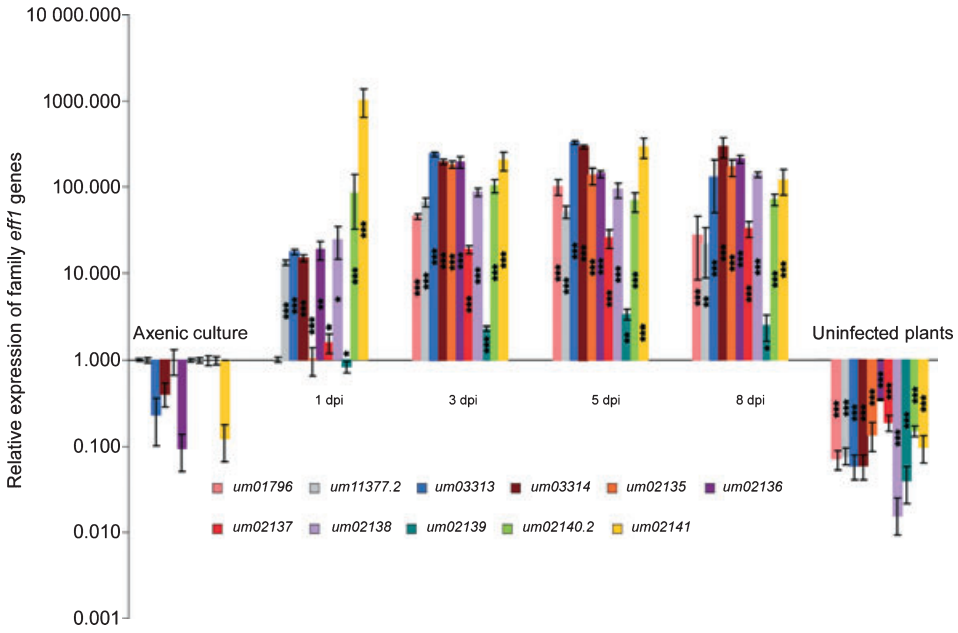

Fig. 4 Expression analysis of the *eff1* family genes by quantitative real-time PCR. RNA was prepared from SG200 grown saprophytically in YEPSL liquid medium, as well as from maize seedlings, 1, 3, 5 and 8 d postinfection (dpi). Expression levels of the *eff1* family genes was standardized relative to the constitutively transcribed *ppi* gene. Three biological replicates, with two technical replicates each, were obtained. Standard deviations are indicated. *P*-values calculated using the Student's *t*-test are shown in the Supporting Information Table S4. Asterisks indicate significant differences relative to expression values in axenic culture. *P*-values: *, < 0.05; **, < 0.01; ***, < 0.001. Expression of the *eff1* genes was also tested in uninfected plant material (depicted on the right) and in this case *P*-values were calculated relative to expression values in infected plant material 5 d postinfection (see Methods S2). The color code for individual genes is shown below the figure.

stages of fungal development using uninfected plant material as a negative control. The statistical analysis of the real-time PCR data is presented in Table S4. Gene-expression levels were quantified in reference to the constitutively expressed peptidyl-prolyl *cis-trans* isomerase gene *ppi1* (accession number EAK84904). During axenic growth of SG200 in YEPSL, expression of the 11 *eff1* genes could not be detected. Twenty-four hours after plant infection, a time-point when *U. maydis* has developed appressoria and has begun to invade the host tissue (Mendoza-Mendoza *et al.*, 2009), expression of seven *eff1* genes was evident (Fig. 4). At this time-point the *um02141* transcript was up-regulated *c.* 1000-fold compared with the *ppi1* expression level. Over the next 7 d of biotrophic growth, the expression levels of *um02141* decreased by two- to threefold (Fig. 4). The maximum transcript levels of *um01796*, *um03313* and *um02139* occurred 5 d postinfection, while *um11377.2* and *um02140.2* demonstrated the highest expression levels at 3 d postinfection. For *um02137* and *um02138* there was a continuous increase in expression over the period from 1 to 8 d postinfection. Except for *um02139*, which was, at most, up-regulated fivefold, all other genes were up-regulated at least 50-fold at one of the chosen time-points after infection (Fig. 4). This illustrates that all members of this gene family are specifically expressed during the biotrophic phase and thus qualify to be called effectors. Accordingly, they were renamed *eff1-1* to *eff1-11* (Fig. 5a, Table S2).

The generation of mutants lacking members of the *eff1* gene family

To generate mutants lacking either all or different combinations of the genes constituting the *eff1* gene family, we followed the scheme depicted in Fig. 5(a) using five successive rounds of FLP-mediated recombination (see Methods S1 for details). This allowed us to generate strains SG200eff1Δ1, SG200eff1Δ1,2, SG200eff1Δ1,2,3,4, SG200eff1Δ1,2,3,4,7,8,9,10 and SG200eff1Δ1-11 (Table S3). In addition, another five strains carrying different combinations of *eff1* gene deletions were generated. These are strains SG200 eff1Δ3,4, SG200eff1Δ1,8, SG200eff1Δ1,3,4,8, SG200eff1 Δ1,2,7,8,9,10 and SG200eff1Δ1,2,3,4,7,8,9,10,11 (Table S3, Methods S1). In SG200eff1Δ1-11, disruption of all 11 genes was verified by PCR using primers that bind to the left and right borders of the segments that were deleted (Fig. 5a,b).

Phenotypic analysis of *eff1* mutants

To test the phenotype of mutants in which different combinations of *eff1* genes were deleted, the mutant strains were grown on various stress media (sorbitol, sodium chloride, calcofluor, Congo red and H_2O_2). In no case could significant

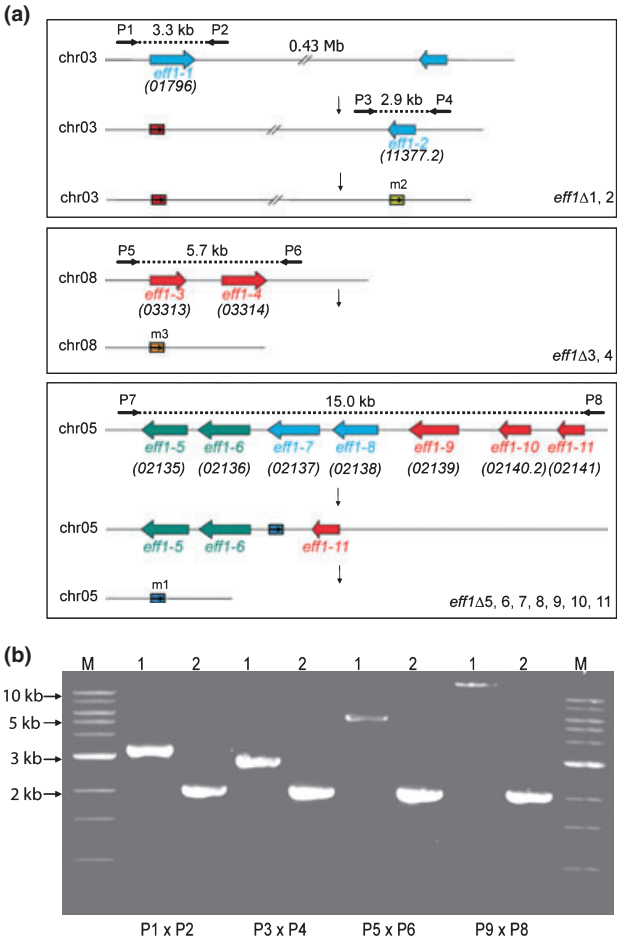

Fig. 5 Strategy for the construction of *eff1* deletion strains. (a) The arrangement of *eff1* family genes on chromosomes 3, 8 and 5 is shown in the upper part of each panel using the color code for individual groups shown in Fig. 3. The lower part of the panels shows the intermediate, as well as the final, structure of the respective chromosomes. Wild-type and mutant FRT sites remaining after excision are indicated in color. The binding sites of diagnostic primers and the sizes of the PCR fragments are indicated. (b) Verification of all deletions in SG200eff1Δ1-11. DNA was prepared from SG200 and SG200eff1Δ1-11, and diagnostic fragments were amplified from SG200 DNA (lanes 1) and SG200eff1Δ1-11 DNA (lanes 2) using the primer pairs indicated in panel (a). M, 1 kb ladder.

differences in growth between SG200 and mutants be observed (Fig. S10). In addition, all mutants developed vigorous filaments on CM-charcoal plates, comparable to those developed by SG200 (Fig. S10). Next, the 10 strains carrying different deletion combinations of family *eff1* genes (Table S3) were tested for pathogenicity. It had been shown previously that virulence can be lost completely when a single effector gene, in this case *pep1*, is deleted in SG200 (Doehlemann *et al.*, 2009). Compared with SG200 infections, significantly reduced virulence was observed when 9 or all 11 *eff1* genes were deleted (Fig. 6, see Table S5 for a statistical analysis of the data). In comparison to symptoms caused by SG200, two effects were seen with SG200eff1Δ1-

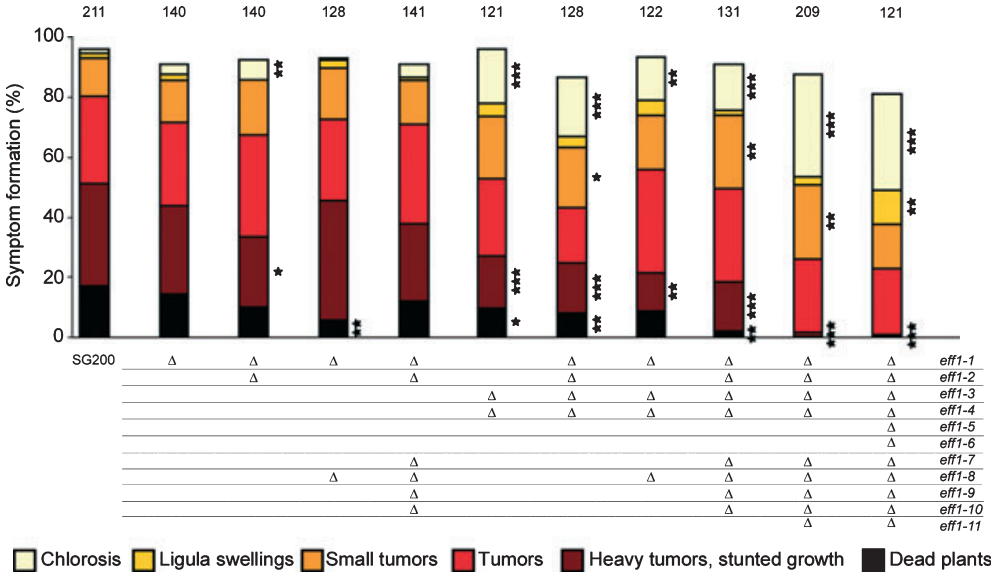

Fig. 6 Disease symptoms caused by SG200 and SG200 carrying different combinations of *eff1* gene deletions. For each strain three independent infections were performed and the total number of infected plants is given above each column. Symptoms were scored 14 d after infection and average scores from the three experiments are shown. To facilitate comparisons, the 11 *eff1* genes are listed on the right and their absence in individual strains is indicated by Δ. The color code for disease rating is given below the figure. Standard deviations and *P*-values are shown in the Supporting Information Table S5. *P*-values relative to symptoms caused by SG200: *, < 0.05; **, < 0.01; ***, < 0.001.

11: the number of plants showing symptoms decreased from > 95% to *c.* 50%; and the symptom severity was reduced. The symptoms (ligula swellings and small tumors) observed after infection with SG200eff1Δ1-11 and SG200eff1Δ1,2,3,4,7,8,9,10,11 were also significantly different, suggesting that the two distantly related genes from group III – *eff1-5* and *eff1-6* (Fig. 3) – also contribute weakly to the phenotype of the 11-gene-deletion strain. Compared with SG200eff1Δ1,2,3,4,7,8,9,10,11, SG200eff1Δ1,2,3,4,7,8,9,10 showed more large tumors and dead plants, suggesting that *eff1-11* is an important gene for tumor formation. SG200eff1Δ1,2 induced more chlorosis and fewer tumors, but no reduction in the number of plants showing symptoms (still almost 100%). Thus, *eff1-1* and *eff1-2* are important for tumor formation and they explain the elevated amount of chlorosis in all strains from which they are deleted. SG200eff1Δ1,2,3,4 and SG200eff1Δ3,4 were similar in type of symptoms, but they showed differences in the number of plants with symptoms, which was lower in SG200eff1Δ1,2,3,4. This shows again that *eff1-1* and *eff1-2* contribute positively to virulence. As SG200eff1Δ1,2,7,8,9,10 induced symptoms comparable to those of SG200eff1Δ1,2, there is no clear contribution of *eff1-7*, *eff1-8*, *eff1-9* and *eff1-10* to virulence. SG200eff1Δ1,2,3,4,7,8,9,10 showed significantly decreased tumor symptoms compared with SG200eff1Δ1,2,7,8,9,10 (Fig. 6), suggesting a contribution of *eff1-3* and *eff1-4* to virulence. This conclusion was reinforced by the attenuated virulence seen after infection with SG200eff1Δ3,4 (Fig. 6).

To assess the contribution of individual *eff1* genes to the virulence phenotype, several complementation strains were generated in which individual *eff1* effector genes were re-introduced into SG200eff1Δ1,2,3,4,7,8,9,10,11. Two strains carrying single-copy integrations of *eff1-1* or *eff1-8* were generated. Both strains showed statistically relevant elevated virulence compared with the strain carrying nine *eff1* gene deletions, with *eff1-1* showing stronger complementation than *eff1-8* (Fig. 7). Additionally, an SG200eff1Δ1,2,3,4,7,8,9,10,11 strain was generated in which the four genes *eff1-7*, *eff1-8*, *eff1-9* and *eff1-10* were introduced in single copy, and this strain also showed statistically relevant increased virulence compared with the nine-gene-deletion strain (Fig. 7). As the severely reduced virulence of the nine-gene-deletion mutant could be partially restored by complementation with all genes tested, we assume functional redundancy within the *eff1* gene family.

Discussion

In this study we successfully implemented the FLP/FRT system to generate multiple gene deletions in *U. maydis*. To achieve this, a codon-optimized FLP gene was designed and recombination was assayed using a genetic screen as well as by PCR. The subsequent scheme for the FLP-mediated marker deletion and re-use consisted of three main steps: the generation of a deletion mutant in which the selectable marker introduced is flanked by direct repeats of FRT sites; the introduction of an inducible FLP gene on an autonomously replicating plasmid; and the induction of FLP

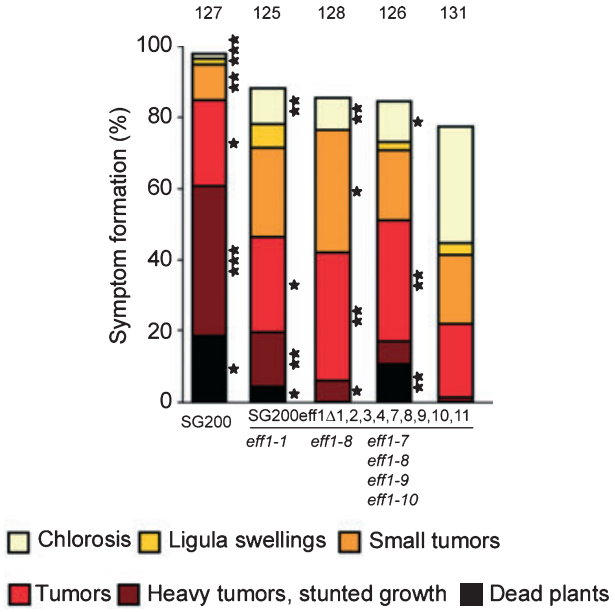

Fig. 7 Complementation of SG200eff1Δ1,2,3,4,7,8,9,10,11 with different *eff1* genes. Disease symptoms caused by SG200, SG200eff1Δ1,2,3,4,7,8,9,10,11 and three different complementation strains are shown. Genes re-introduced into SG200eff1Δ1,2,3,4,7,8,9,10,11 are listed below. The color code for disease rating is given below the figure. Standard deviations and *P*-values are shown in the Supporting Information Table S5. *P*-values relative to symptoms caused by SG200eff1Δ1,2,3,4,7,8,9,10,11: *, < 0.05; **, < 0.01; ***, < 0.001.

expression and the subsequent screening for the loss of the selectable marker as well as the FLP donor plasmid. The system was highly efficient and *c.* 21 ± 8% of the single colonies cultured after the induction of FLP had lost the marker as well as the FLP donor plasmid. For future use we have developed the system in such a way that the inducible FLP gene can become an integral part of the cassette used to generate the deletion.

The successful deletion of the 11-gene *eff1* effector family in SG200, using five consecutive rounds of gene replacements and subsequent marker excision, showed the utility of the FLP/FRT system in *U. maydis*. To eliminate possible intermolecular and intramolecular recombination events between identical FRT sites left in the genome after excision, FRT sequences with different point mutations in the core region were employed. None of these sites was found to recombine with wild-type FRT sites, but recombination could be detected in a strain where two identical FRT sites were present on the same chromosome, 0.43 Mb apart. None of the strains generated by several rounds of FLP-mediated recombination showed morphological defects and all of these strains developed vigorous filaments and were comparable to the progenitor strain with respect to growth under oxidative stress, cell wall stress and osmotic stress. This makes it likely that such strains are stable and are unlikely to have acquired additional mutations, a prerequisite for assessing small virulence phenotypes of redundant genes. We expect that the establishment of the FLP system in *U. maydis* will pave the way towards functional analysis of effector gene families as well as serving as a tool to improve genetic manipulations of plant pathogenic fungi in general.

Expression of all members of the *eff1* gene family was strongly induced during biotrophic development. This is one of the few unifying features of filamentous pathogen effectors in which the respective genes are up-regulated during host colonization, either during penetration, in haustoria, or during later stages of pathogen development inside the plant tissue (Hahn *et al.*, 1997; Catanzariti *et al.*, 2006; Kämper *et al.*, 2006; Haas *et al.*, 2009; Oh *et al.*, 2009; Skibbe *et al.*, 2010). However, information on how this regulation is connected to the growth stages inside the host is scarce. In *U. maydis* the zinc-finger protein, Mzr1, has been defined as a transcriptional activator of *mig2* effector genes during host colonization (Zheng *et al.*, 2008). However, Mrz1 was shown to regulate only two of the five *mig2* genes and thus is not a general regulator for effector genes. In addition, given the recent finding that several *U. maydis* effectors are expressed in a maize tissue-specific manner (Skibbe *et al.*, 2010), it is unlikely that such global effector regulators exist at all. In *Fusarium oxysporum*, a regulatory gene, *SGE1*, has recently been identified that was crucial for pathogenesis and affected the expression of the four tested *six* effector genes. *U. maydis* has two genes related to SGE1 (*um06496* and *um05853*) (Michielse *et al.*, 2009), which have not yet been functionally characterized. However, because SGE1 orthologs exist in all fungi (Michielse *et al.*, 2009), it is unlikely that this transcription factor will be identified as a dedicated regulator of effector genes in phytopathogenic fungi.

The fact that 9 of the 11 *eff1* genes reside in two clusters in the genome supports the assertion that they have originally arisen by a local gene-duplication mechanism followed by rapid diversification and dispersion to other chromosomes. The large *eff1* gene cluster on chromosome 5 is heterogeneous and contains genes from three different *eff1* groups (Fig. 5a) with two, two and three direct copies from each group, respectively. Intriguingly, the two dispersed copies on chromosome 3 are closely related to each other as well as to the two adjacent genes – *eff1-7* and *eff1-8* – in the large cluster on chromosome 5. In addition, the two adjacent copies on chromosome 8 are closely related to each other and to the adjacent genes – *eff1-9* and *eff1-10* – in the large cluster on chromosome 5. It will be very interesting to analyze the number, distribution and groups of *eff1* effector genes in geographically distinct isolates of *U. maydis* as this might provide insights into the evolutionary fate of the clusters as well as the dispersed copies.

Database searches with the conserved C-terminal domain identified in this protein family (Fig. S9) did not produce any statistically significant matches. However, orthologs of

the *eff1* genes were found in *Sporisorium reilianum*, the cause of head smut in maize (J. Schirawski, G. Mannhaupt & R. Kahmann, unpublished data) and in *Ustilago scitaminea*, the cause of sugarcane smut (G. Mannhaupt & R. Kahmann, unpublished data), which makes it likely that this effector family is smut specific and is not involved in determining host range. The combined deletion analysis and complementation studies conducted for members of the *eff1* family has revealed that genes *eff1-11*, *eff1-3* and *eff1-4* contribute highly significantly to virulence, whereas all other members of this gene family contribute to virulence only weakly. As we have always deleted genes *eff1-3 and eff1-4* simultaneously, we are presently unable to assess whether they both contribute to virulence. Interestingly, the *eff1* genes with the strongest effect on virulence all belong to group II (Fig. 3). By contrast, Eff1-4 is very similar to Eff1-9, but the effects of *eff1-9* on virulence were marginal. This could indicate that additional, currently unrecognized, features determine the strength of a given *eff1* gene. Alternatively, because *eff1-9* is the gene that shows the smallest increase in expression values during biotrophic growth, the differences in contribution to virulence could be attributed to these differences in expression. This could be investigated by promoter-swap experiments.

Because the observed complementation was, in all cases, partial, it remains possible that some subfunctualization in *eff1* genes (Conant & Wolfe, 2008) has occurred. In this respect it would be interesting to determine whether the separate deletion of the three different groups of *eff1* genes produces distinct phenotypic effects and whether these can then be complemented only by *eff1* genes from the same group or also by members of different groups. Such differences in virulence might become apparent when using different maize cultivars or when infecting different maize tissues such as tassel, the cob or mature leaves (Skibbe *et al.*, 2010), rather than seedlings, as used in this study.

At present, the function of Eff1 proteins after secretion remains highly speculative because the Eff1 effectors do not show any similarity to database entries. The members of this family are highly divergent but show conserved features. The central segment could represent a eukaryotic linear motif, which binds specifically to a protein of the plant host and becomes partly ordered in the process. Although the C-terminal domain also lacks similarity to known domains, as judged by HMM comparisons to the NCBI conserved domain database (CDD), the fact that it is predicted to assume a folded conformation makes it the most likely domain to confer Eff1 protein activity. This is in line with results from oomycete effectors, which revealed that functional domains reside in the C-terminus (Ellis *et al.*, 2009; Tyler, 2009). Preliminary experiments indicate that the 11-gene-deletion mutant does not elicit a hypersensitive response and is able to establish biotrophic growth (K. Schipper, Y. Khrunyk & R. Kahmann, unpublished data). This is different from the phenotype of *pep1* effector mutants that are arrested during penetration and elicit strong defense responses (Doehlemann *et al.*, 2009). The Eff1 proteins lack 'classical' RXLR motifs found in the N-terminal domains of effector proteins from oomycetes that are translocated to plant cells (Kamoun & Goodwin, 2007; Ellis *et al.*, 2009; Tyler, 2009). Further studies will reveal if the Eff1 effectors remain in the host–pathogen interface or enter plant cells by alternative, RXLR-independent, routes. Moreover, identification of interacting proteins and the results of localization studies may give valuable hints concerning the function of the family members. Given the fact that the deletion mutant lacking all 11 *eff1* genes has a strong virulence phenotype, functional studies can now be conducted. It will be of particular interest to analyze the effects of deletion of the conserved C-terminal domain as well as deletions in the central domain. This could reveal whether these domains confer functions that can be separated (i.e. on the one hand are involved in targeting, and on the other hand interact with host proteins).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Plasmid and strain constructions.

Methods S2 Molecular techniques.

Fig. S1 Nucleotide sequence of the codon-optimized FLP recombinase gene.

Fig. S2 Expression of the codon-optimized FLP gene.

Fig. S3 FLP-mediated excision assay.

Fig. S4 Schematic representation of plasmids used in recombination assays.

Fig. S5 Screen for FLP-mediated recombination leading to excision of the *hph* cassette as well as the FLP gene.

Fig. S6 FRT-dependent recombination efficiency of SG200 FlpΔ11377.2 strains, which contain different pairs of mutated FRT sequences.

Fig. S7 FLP mediated recombination in SG200 strains carrying adjacent wild-type and mutant FRT sites.

Fig. S8 Sequence relationship between Eff1 proteins.

Fig. S9 Domains of Eff1 proteins.

Fig. S10 Growth of SG200 and SG200eff1 mutant strains on different stress media.

Table S1 Primers used in this study

Table S2 The *eff1* gene family

Table S3 *U. maydis* strains used and constructed for this study

Table S4 Statistical analysis of Real-Time-PCR data

Table S5 Statistical analysis of infection data

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