Identification of virulence genes in the corn pathogen *Colletotrichum graminicola* by *Agrobacterium tumefaciens*-mediated transformation

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SUMMARY

A previously developed *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol for the plant pathogenic fungus *Colletotrichum graminicola* led to high rates of tandem integration of the whole Ti-plasmid, and was therefore considered to be unsuitable for the identification of pathogenicity and virulence genes by insertional mutagenesis in this pathogen. We used a modified ATMT protocol with acetosyringone present only during the co-cultivation of *C. graminicola* and *A. tumefaciens*. Analysis of 105 single-spore isolates randomly chosen from a collection of approximately 2000 transformants, indicated that almost 70% of the transformants had single T-DNA integrations. Of 500 independent transformants tested, 10 exhibited attenuated virulence in infection assays on whole plants. Microscopic analyses primarily revealed defects at different pre-penetration stages of infection-related morphogenesis. Three transformants were characterized in detail. The identification of the T-DNA integration sites was performed by amplification of genomic DNA ends after endonuclease digestion and polynucleotide tailing. In one transformant, the T-DNA had integrated into the 5'-flank of a gene with similarity to allantoicase genes of other Ascomycota. In the second and third transformants, the T-DNA had integrated into an open reading frame (ORF) and into the 5'-flank of an ORF. In both cases, the ORFs have unknown function.

INTRODUCTION

The fungus *Colletotrichum graminicola* (Ces.) Wilson [teleomorph *Glomerella graminicola* (Politis)] is the causal agent of leaf anthracnose and stem rot of corn (Bergstrom and Nicholson, 1999). In order to invade the first epidermal cell and to establish a compatible parasitic relationship with the host plant, the pathogen differentiates an elaborate infection cell, called an appressorium (Deising *et al.*, 2000; Tucker and Talbot, 2001; Wang *et al.*, 2005). During maturation, appressoria form rigid cell walls which melanize, and the synthesis of high concentrations of compatible solutes (De Jong *et al.*, 1997) results in the generation of enormous appressorial turgor pressure (Howard *et al.*, 1991). Turgor is translated into force, which is exerted at the appressorial base, supporting penetration into the host (Bastmeyer *et al.*, 2002; Bechinger *et al.*, 1999; Küster *et al.*, 2008). In the host, *C. graminicola* forms biotrophic infection structures, i.e. the infection vesicle and primary hyphae, which develop without causing macroscopically visible damage to the host. Subsequently, the formation of secondary hyphae is initiated, which grow rapidly (Krijger *et al.*, 2008), are highly destructive and represent the necrotrophic lifestyle of the pathogen (Bergstrom and Nicholson, 1999; Horbach *et al.*, 2009; Münch *et al.*, 2008).

Depending on the lifestyle and genome size of the fungus, Idnurm and Howlett (2001) estimated that plant pathogenic fungi probably harbour between 60 and 360 virulence or pathogenicity genes. However, only some of these have been identified. Several of these genes are involved in infection structure differentiation or the synthesis of toxins or cell wall-degrading enzymes (Idnurm and Howlett, 2001; Madrid *et al.*, 2003; Möbius and Hertweck, 2009; Werner *et al.*, 2007). Other genes play a role in compromising host defence responses in order to establish a compatible pathogenic interaction. Successful suppression of the host defence depends on the secretion of fungal effector molecules and, consequently, involves the secretory machinery,

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as indicated by mutants of the rice blast fungus *Magnaporthe oryzae* deficient in a P-type ATPase, which are unable to suppress the formation of reactive oxygen species in their host, and thus show reduced virulence (Gilbert *et al.*, 2006). In *Colletotrichum gloeosporioides*, secretion of the effector molecule encoded by the gene *CgDN3* is required to avert a hypersensitive-like response of a compatible host during the establishment of a biotrophic interaction (Stephenson *et al.*, 2000). So far, a number of fungal and oomycete effector molecules have been identified (Kamoun, 2009).

Random mutagenesis has been exploited extensively as a tool to tag the fungal genes required for the establishment of a pathogenic interaction with the host plant (Hamer *et al.*, 2001; Kahmann and Basse, 1999; Mullins and Kang, 2001). In restriction enzyme-mediated integration (REMI) mutagenesis experiments performed with the plant pathogens *Ustilago maydis*, *M. oryzae* and *Cochliobolus heterostrophus*, the percentage of transformants with virulence defects ranged from 0.5 to 2% (Bölker *et al.*, 1995; Lu *et al.*, 1994; Sweigard *et al.*, 1998) and, in *C. graminicola*, only 0.3% of the transformants were affected in virulence (Epstein *et al.*, 1998; Thon *et al.*, 2000). Importantly, analyses of REMI mutants of different fungi have shown that up to 50% of the mutations may not be tagged (Kahmann and Basse, 1999; Maier and Schäfer, 1999). These findings raise doubts about whether REMI mutagenesis is a suitable tool for the efficient identification of virulence genes.

Agrobacterium tumefaciens-mediated transformation (ATMT) is thought to avoid most of the problems associated with REMI mutagenesis and related protoplast transformation techniques. For several years, ATMT has been well established for plant transformation (Escobar and Dandekar, 2003; Gelvin, 2003). After the initial application to yeast (Bundock *et al.*, 1995), de Groot *et al.* (1998) used this technique to transform filamentous fungi. ATMT has since been used to transform several plant pathogenic fungi, e.g. *Fusarium oxysporum*, *Verticillium dahliae*, *Botrytis cinerea*, *Calonectria morganii*, *M. oryzae*, *Colletotrichum falcatum* and *Colletotrichum acutatum* (Dobinson *et al.*, 2004; Jeon *et al.*, 2007; Khang *et al.*, 2006; Malonek and Meinhardt, 2001; Maruthachalam *et al.*, 2008; Rolland *et al.*, 2003; Shen *et al.*, 2008), and the oomycete *Phytophthora infestans* (Vijn and Govers, 2003). Recently, Flowers and Vaillancourt (2005) developed an efficient ATMT protocol for *C. graminicola*. However, because of the high rates of tandem integration of the T-DNA and multiple integrations of the entire Ti-plasmid, problems in rescuing flanking genomic regions and identification of the disrupted genes occurred. Therefore, these authors considered ATMT to be unsuitable for the identification of pathogenicity or virulence genes in *C. graminicola*.

Here, we report a modified ATMT protocol and the generation of a transformant collection. Approximately 70% of the transformants showed T-DNA integration at one single site. In virulence

assays on whole plants, we identified 10 transformants with virulence defects from 500 independent transformants. Seven transformants differing in virulence were studied in detail and, in all of these transformants, T-DNA integration sites were identified. In six transformants, T-DNA integration occurred into the 5'-flanks or coding regions of putative genes with unknown function. In one transformant, T-DNA integrated into the 5'-flank of a gene with similarity to allantoinase genes of other Ascomycota, such as *M. oryzae* or *Neurospora crassa*.

RESULTS

Generation of a transformant library by ATMT and the mode of T-DNA integration into the genome of *C. graminicola*

Using a modified protocol for ATMT, we generated a collection of approximately 2000 transformants of the corn pathogen *C. graminicola*. In contrast with other protocols, in which acetosyringone was added to *in vitro* cultures of *A. tumefaciens*, we added this substance during the co-cultivation of germinating falcate conidia of *C. graminicola* with *A. tumefaciens*. On average, 3.5 transformants were obtained from 10 000 conidia. No transformants were obtained in the absence of acetosyringone, indicating that this compound is indispensable for the induction of T-DNA transfer into fungal cells. The integrated T-DNA was mitotically stable, as shown by analyses of 10 randomly chosen transformants. Four successive 14-day passages of these transformants on oatmeal agar (OMA) medium without the selection marker hygromycin B did not result in the loss of integrated T-DNA, as indicated by genomic Southern blot analysis and the maintenance of the ability of all 10 transformants to grow on potato-dextrose-agar (PDA) containing hygromycin B.

In random mutagenesis experiments, single-copy T-DNA integration into one chromosomal locus of the recipient allows the efficient identification of the tagged gene and circumvents time-consuming analyses of the integration event mediating an altered phenotype in transformants harbouring two or more T-DNA insertions at different chromosomal loci. In order to characterize T-DNA integration events in our transformant collection, we investigated 105 randomly chosen transformants by genomic Southern blot analyses. Blots of *Hind*III-digested genomic DNA were probed with a digoxigenin (Dig)-labelled polymerase chain reaction (PCR) fragment corresponding to 688 bp of the *hph* gene and 321 bp of the 5'-flanking T-DNA (Fig. 1B). More than two-thirds, i.e. 69.5%, of the transformants tested showed a single-site integration of the T-DNA. In 26.7% of the transformants, two integration events occurred at different sites of the genome, and only 3.8% of the transformants exhibited more than two T-DNA integration sites. A representative selection of 40 transformants is shown in Fig. 1C. Southern blot experiments

Fig. 1 Analysis of transformants of *Colletotrichum graminicola* produced by *Agrobacterium tumefaciens*-mediated transformation (ATMT). (A) Map of pPK2 (Covert *et al.*, 2001). (B) Scheme of genomic DNA of *C. graminicola* disrupted by T-DNA integration. Probe used for hybridization and *Hind*III restriction sites are indicated. (C) Genomic Southern blot analysis of 40 transformants of *C. graminicola* (1–40) generated by ATMT. Genomic DNA was restricted with *Hind*III. One band (lanes marked with an asterisk) represents a single T-DNA integration event; two or more bands indicate two or multiple T-DNA integrations at different sites. M, size marker.

with *Eco*32I- or *Xba*I-digested genomic DNA confirmed these results. As expected, genomic DNA of the nontransformed wild-type (WT) strain did not hybridize with the probe (data not shown).

Southern blot analyses also allowed us to determine at what frequency the entire Ti-plasmid was integrated into the fungal genome, and if single or multiple copies integrated (Flowers and Vaillancourt, 2005; Li *et al.*, 2007). The integration of the entire Ti-plasmid into the fungal genome led to the detection of a fragment of at least 10.5 kb when DNA was restricted with *Hind*III. The size of the fragments exceeding 10.5 kb depends on the distance to the next *Hind*III restriction site in the flanking genomic region. In 33% of the transformants, the fragment size was sufficiently large to harbour the entire Ti-plasmid. However, this number overestimates the fraction of transformants harbouring the entire Ti-plasmid, as it also includes transformants with T-DNAs integrated into sites with long distances to the next *Hind*III restriction site. Transformants with fragment sizes smaller than 10.5 kb do not harbour the entire Ti-plasmid, which was the case in 67% of all transformants analysed.

Only in 3% of all transformants were analysed fragments of exactly 10.7 kb in *Hind*III-digested DNA and 10.0 kb in *Eco*32I-digested DNA detected, indicating that tandem integration of the entire Ti-plasmid had occurred into a single genomic site.

Identification and characterization of transformants with reduced virulence

Using detached corn leaves, we screened 500 transformants for reduced virulence. Sixty-one transformants with reduced virulence were identified and further analysed on intact living plants.

Ten transformants of these 61 exhibited either moderately or strongly reduced virulence in whole-plant assays. Three of these transformants (AT402, AT405 and AT416), exhibiting a strongly reduced virulence on intact corn plants, have been reported previously by Münch *et al.* (2008). The other seven candidates, also exhibiting a strongly reduced virulence phenotype (AT396, AT397, AT399, AT420, AT425) or moderately reduced virulence (AT039, AT171), are presented here (Fig. 2).

For further characterization, we chose transformants that were nonpathogenic (AT399) or moderately and strongly impaired in virulence (AT171 and AT039) (Fig. 2). Microscopic analyses of infection structures formed *in planta* at different time points after inoculation indicated at which stage the infection process was hampered in the transformants analysed. At 4 days post-inoculation (dpi), 85% of the conidia of the WT isolate had germinated, and 99% of the germlings had formed appressoria, the vast majority of which (97%) had melanized (Fig. 3, WT, asterisks). At this time point, 92% of the appressoria formed had penetrated the host epidermal cell and differentiated biotrophic infection vesicles and primary hyphae (Fig. 3, WT, p), as well as necrotrophic secondary hyphae (Fig. 3, WT, s).

Transformant AT399 did not cause any visible disease symptoms in detached corn leaves or on intact plants (Fig. 2). When compared with the WT isolate, germination rates on the leaf surface (66%) and rates of appressorium formation (3% of the germlings) were significantly reduced in this transformant (Fig. 3, AT399). Although melanized appressoria showed a normal morphology (Fig. 3, AT399, asterisk), host penetration rates were drastically reduced (6% vs. 92% of WT appressoria). Microscopic analyses of infected leaves (Fig. 3) showed that infection structures of AT399, formed pre- (germ tubes and

Fig. 2 Saprophytic and pathogenic development of *Colletotrichum graminicola* transformants produced by *Agrobacterium tumefaciens*-mediated transformation (ATMT). The phenotype during axenic growth on oatmeal agar is shown in the left panel. Photographs of corn leaves from whole infected plants [5 days post-inoculation (dpi)] and of detached leaves (4 dpi) indicate reduced virulence of the transformants shown, when compared with the wild-type isolate (WT). n.i.c., noninoculated control.

appressoria) and post-penetration (infection vesicles, primary and secondary hyphae), had no morphological defects (Fig. 3, AT399, p).

Transformants AT039 and AT171 germinated and differentiated appressoria at rates indistinguishable from those of the WT isolate. Appressoria exhibited normal shape and melanization (Fig. 3, AT039 and AT171, asterisks), but host penetration rates were severely reduced (39% in AT039 and 49% in AT171 vs. 92% in WT). When penetration occurred, these mutants were able to form both primary and secondary hyphae (Fig. 3, AT039 and AT171, p and s). These counts of infection structures thus suggest that the reduced severity of disease symptoms caused by these two mutants (Fig. 2) may be a result of reduced penetration competence. Reduced penetration competence, in turn, may be caused by reduced appressorial turgor pressure (Wang *et al.*, 2005).

The osmolyte concentration causing incipient cytorrhysis can be taken as a measure of appressorial turgor pressure. Appressoria formed on plastic sheets at 24 h post-inoculation (hpi) were incubated with different concentrations of glycerol, and rates of collapsed appressoria were determined (Fig. 4A). Incipient appressorial cytorrhysis in the WT isolate CgM2 and the mutants AT039, AT171 and AT399 occurred at very similar glycerol concentrations, indicating similar appressorial turgor pressures (Fig. 4B). Therefore, reduced penetration rates and symptom severity are probably not a result of insufficient turgor pressure in these three mutants.

A reduction in mutant efficiency with regard to infection structure formation could be a result of the disruption of a virulence

gene or a gene involved in general growth processes. To address this question, we investigated AT039, AT171 and AT399 in radial growth assays on differently complex carbon sources, and compared the growth with that of WT. As presented in Fig. 5, AT039 and AT399 can grow as efficiently as WT, irrespective of the type of carbon source present in the medium. AT171 showed a retardation of growth compared with WT for all different carbon sources. In addition, for all mutants and WT, we observed no difference in growth between carbon sources of low and high complexity. In summary, our data suggest that the reduction in virulence of AT039 and AT399 does not result from a retardation of growth in general. The affected genes are dispensable for normal axenic growth, but indispensable for full virulence. In contrast, the disrupted gene in AT171 is needed for both general growth processes and full virulence.

Identification of T-DNA integration sites

Genomic Southern blot experiments showed that the transformants AT039, AT171 and AT399 had single T-DNA integrations (Fig. 6A). T-DNA integration sites were identified by the amplification of genomic DNA ends after endonuclease digestion and polynucleotide tailing, using a modification of the method described by Liu and Baird (2001). This approach allowed the amplification of 390-bp and 600-bp fragments from DNA extracted from transformant AT399. Comparisons of the sequences flanking the T-DNA with fungal genome sequences showed no significant similarities. However, the gene prediction tool AUGUSTUS (<http://augustus.gobics.de/>) predicted an open

Fig. 3 Infection structure formation of *Colletotrichum graminicola* wild-type (WT) and transformants AT039, AT171 and AT399. Infection structures are given as a percentage of the preceding structure; micrographs show infection structures at 1 and 3 days post-inoculation (dpi) for the virulent WT isolate, and 2 and 4 dpi for the transformants with reduced virulence. A, appressoria; G, germ tubes; M, melanized appressoria; P, penetration. White arrowheads indicate germ tubes, black arrowheads mark *in planta* differentiated hyphae; p and s indicate primary and secondary hyphae. Appressoria are marked by asterisks. Bars represent 20 μ m.

reading frame (ORF) of 1302 bp with four introns, and suggested that the T-DNA had integrated 240 bp downstream of the start codon into the second exon (Fig. 6B). The scores for the five different exons in this hypothetical ORF range between 0.8 and 1.0.

Southern blot experiments with *Hind*III-digested DNA showed that transformants AT171 and AT039 carry single T-DNA integrations, and that the T-DNA integration sites clearly differ from those of the transformant described above (Fig. 6A). However, as transformants AT171 and AT039 were difficult to discriminate on the basis of blots with *Hind*III-digested DNA, corresponding experiments were performed with *Xba*I-digested DNA. Different fragment sizes clearly showed that integration sites differed in these transformants. Amplification and sequencing of a 184-bp fragment flanking the T-DNA integration site of transformant

AT171 was sufficient to assemble the entire gene sequence from the whole genome shotgun sequence of *C. graminicola* (Fig. S1A, B, see Supporting Information). In this mutant, T-DNA integration occurred 83 bp upstream of a gene with significant similarity to the allantoicase genes of other Ascomycota, i.e. *N. crassa* (Acc. No. XP_956494.1; *E* value: $3e^{-171}$) and *M. oryzae* (Acc. No. XP_001406661.1; *E* value: $7e^{-162}$). On the protein level, the amino acid sequences of allantoicase of *N. crassa*, and of the corresponding proteins of *M. oryzae* and *C. graminicola*, showed a homology of 70% using the CLUSTAL 2.0.12 multiple sequence alignment tool (<http://www.ebi.ac.uk>) (Fig. S1C). Using *N. crassa* or *M. oryzae* as reference organisms, one intron of 77 bp, starting 95 bp downstream of the start codon, was predicted for the putative allantoicase gene of *C. graminicola* (Fig. 6B). Furthermore, amplification and sequencing of a 263-bp DNA fragment

Fig. 4 Analysis of appressorial turgor pressure by incipient cytorrhysis. (A) Collapse of appressoria caused by hyperosmolarity of the medium is indicated by ridges of the appressorial cell wall (white arrowheads). The micrographs show appressoria in 2 M glycerol. In a control without glycerol (water control), all appressoria were turgid. Bar, 20 μ m. (B) Appressoria of mutants and wild-type (CgM2) show comparable rates of cytorrhysis at increasing osmolyte concentration.

Fig. 5 Radial growth of the wild-type (WT) strain CgM2 and of mutants AT039, AT399 and AT171 on substrata containing different carbon sources. Radial growth rates were measured on agar plates containing minimal medium without carbon sources and with 2% glucose, maltose, sucrose, starch or cellulose after 7 days. Growth rates of mutants are given relative to those of the wild-type. Growth rates on complex medium (CM) served as controls.

Fig. 6 Southern blot analysis of *Hind*III- and *Xba*I-digested genomic DNA from three virulence mutants and the wild-type isolate, and scheme showing integration sites in transformants AT039, AT171 and AT399. (A) All transformants show single-site T-DNA integration. The probe used is shown in Fig. 1B. (B) Illustration of T-DNA integration into AT039, AT171 and AT399. The integration sites were identified by polynucleotide tailing as described in the text.

adjacent to the right border and a 62-bp fragment flanking the left border of the T-DNA revealed that the integration in transformant AT039 had occurred into the promoter region 450 bp upstream of the start codon of a hypothetical ORF. The target gene encodes a hypothetical protein, is 555 bp long and has similarities with ORFs of unknown function known in *N. crassa* (*E*-value: $2e^{-28}$), *Gibberella zeae* (*E*-value: $3e^{-24}$) and *M. oryzae* (*E*-value: $3e^{-20}$).

Photosynthetic activity in infected tissue

Screening for a lack of macroscopically visible disease symptoms allows the identification of the pathogenicity mutants, but mutants affected in virulence are often overlooked. In order to introduce an independent and quantitative measure for fungal virulence, the quantum efficiency of photosystem II (Φ_{PSII}) was determined by chlorophyll fluorescence imaging of developing anthracnose lesions at 4 and 6 dpi (Fig. 7), which were rare for mutants AT039 and AT399. Φ_{PSII} was recorded at distances of 250 and 500 μ m from the lesion border to assess spatial differences in photosynthetic efficiency. Measurements at leaf areas devoid of disease symptoms and in mock-inoculated leaves served as controls. Leaf areas without disease symptoms, which were scrutinized by acid fuchsin staining, either lacked fungal infection structures or displayed single dispersed penetration events.

In comparison with mock controls, maize leaves infected with *C. graminicola* WT exhibited a more than 35% decline in Φ_{PSII} within a distance of 250 μ m to the lesion border at both 4 and

6 dpi (Fig. 7). Leaf tissue more than 500 μ m from the margin of the lesion did not exhibit significant differences in photosynthetic activity relative to mock controls. In leaves infected with the *C. graminicola* transformants AT039, AT171 and AT399, no effect on photosynthetic performance was detectable at 4 dpi at all distances to the lesion relative to mock controls (Fig. 7A). At 6 dpi, however, Φ_{PSII} adjacent to lesions caused by transformant AT171 was reduced to a similar degree as in the *C. graminicola* WT infection. In contrast, lesion borders in AT039- and AT399-infected leaves displayed only a slightly reduced Φ_{PSII} (Fig. 7B). To exclude the possibility that the changes in Φ_{PSII} between WT- and transformant-infected leaves were caused by the abundance of fungal hyphae at the margins of the lesion, we again checked the fungal colonization of these regions by acid fuchsin staining. Secondary fungal hyphae of all mutants were abundant within 500 μ m of the lesion border, comparable with that observed for WT (data not shown).

These data demonstrate that the differences in virulence between the mutant strains and WT are reflected by changes in Φ_{PSII} . Thus, chlorophyll fluorescence measurements provide an additional tool to evaluate fungal aggressiveness in the *C. graminicola*–maize pathosystem.

DISCUSSION

ATMT has been exploited extensively in random mutagenesis experiments to identify the genes involved in the pathogenic interactions of fungi with plants, insects, mammals and other fungi (Covert *et al.*, 2001; Fitzgerald *et al.*, 2003; Leclercque *et al.*,

Fig. 7 Photosynthetic performance of corn leaves infected with the wild-type and mutants of *Colletotrichum graminicola* at 4 and 6 days post-inoculation (dpi). Photosystem II efficiency in the light (Φ_{PSII}) was measured by Imaging-PAM analysis. Mean values for mock control leaves are depicted as full lines in the background, with corresponding standard deviations (SDs) shown as broken lines. Black bars, healthy leaf area; grey bars, leaf area at a distance of 500 μ m from the lesion border; white bars, leaf area at a distance of 250 μ m from the lesion border. Data are the means of 12 representative areas taken from four replicate leaves $\pm$ SD.

2004; Mullins *et al.*, 2001; Rho *et al.*, 2001; Sugui *et al.*, 2005; Talhinas *et al.*, 2008; Tsuji *et al.*, 2003; White and Chen, 2006). The frequency and mode of T-DNA integration into the genome of the recipient are the most critical factors for the subsequent identification of the genes affected. In plants, it is well known that ATMT leads to the integration of multiple copies of T-DNA into one locus, and junctions with at least one right T-DNA border occur frequently (De Neve *et al.*, 1997). Moreover, as shown recently, not only T-DNA- and Ti-plasmid sequences, but, in rare cases, also chromosomal fragments of *A. tumefaciens* are transferred into *Arabidopsis thaliana* (Ülker *et al.*, 2008). Also, in fungi, T-DNA integration may occur as single or multiple copies of either T-DNA or the entire Ti-plasmid at the same or different loci of the genome. In the rice blast fungus *M. oryzae*, for example, head-to-tail integration of two T-DNA copies and even of the entire Ti-plasmid were found, but single-copy T-DNA integration into one genomic site occurred predominantly (Li *et al.*, 2007; Meng *et al.*, 2007). In general, ATMT has been described as a method feasible for random mutagenesis in many plant pathogenic fungi, including different *Colletotrichum* species (Maruthachalam *et al.*, 2008; Mullins *et al.*, 2001; Talhinas *et al.*, 2008; Tsuji *et al.*, 2003). In contrast, in *C. graminicola*, ATMT is considered to be unsuitable for random mutagenesis because of tandem integrations of entire Ti-plasmids in the large majority of transformants, causing significant problems in the rescue of regions flanking the integration site, and thus in the identification of the gene affected.

Using the modified ATMT protocol presented here, we were able to demonstrate that this method is fully suitable for the identification of novel virulence genes in *C. graminicola*. When compared with the report by Flowers and Vaillancourt (2005), the transformation efficiency was considerably lower in our experiments, which may be a result of the reduced presence times of acetosyringone or of the *A. tumefaciens* strain and binary vector used (Flowers and Vaillancourt, 2005). In our

experiments, 69.5% of the transformants had single-site T-DNA integrations, when compared with the inexactly determined maximum rate of single-site integrations of 64% reported by Flowers and Vaillancourt (2005). These transformants, however, had multi-copy integrations of the entire Ti-plasmid in approximately 70% of all cases, with only a minor fraction of single-site T-DNA integrations. In our experiments, multi-copy integration of the entire Ti-plasmid occurred in only approximately 3% of all transformants investigated, and therefore represented rare events. The modified ATMT method, in combination with the simple amplification of genomic DNA flanking the T-DNA integration site, makes forward genetics suitable for the identification of the genes contributing to the virulence of *C. graminicola*.

A systematic analysis of T-DNA insertion events in 175 independent transformants of *M. oryzae* showed that the distribution of T-DNA inserts among the chromosomes was not random. Moreover, significant insertion bias was detected, with promoters receiving twofold more T-DNA hits than expected (Meng *et al.*, 2007). A similar pattern was detected in *Arabidopsis thaliana* (Alonso *et al.*, 2003). However, although it was not the intention of this work to characterize the randomization of integration events, the analysis of the small number of transformants of *C. graminicola* with virulence defects presented here is consistent with the findings by Meng *et al.* (2007), as the integration site was preferentially the promoter region of a gene (Fig. 6B). Interestingly, microscopic analyses of the infection process (Fig. 3) revealed that the mutants analysed had defects at the pre-penetration stages of pathogenicity. As ATMT was performed with germinating spores, one may speculate that T-DNA integration primarily occurred into the genes required during germination, and these genes may also play a role during the pre-penetration stages of plant infection. This may imply, however, that the genes specifically required during biotrophic or necrotrophic development *in planta* would be tagged by ATMT in rare cases only.

The modified ATMT protocol presented here allowed the identification of mutants clearly affected in the genes required for the infection process (Fig. 2) (Münch *et al.*, 2008). However, spatially resolved quantitative assessment of the virulence of the mutants was possible by the determination of Φ_{PSII} at different distances from the inoculation site. In WT-infected leaves, this method revealed the gradual destruction of the host tissue, as Φ_{PSII} increased with increasing distance from the centre of infection. Interestingly, transformant AT171 was affected at the appressorial penetration stage (Fig. 3), and therefore showed delayed invasion and host colonization. In contrast with the WT strain, which caused a significant reduction of 35% in Φ_{PSII} at a distance of 250 μ m from the inoculation site at 4 dpi, a significant reduction in Φ_{PSII} of 30% caused by transformant AT171 could be measured at a distance of 250 μ m from the inoculation site only at 6 dpi. In contrast, transformant AT039 only displayed a reduction in Φ_{PSII} of 17% compared with mock control leaves at 6 dpi. The transformant AT399 was nonpathogenic in the leaf segment and whole-plant infection assays, and caused only a very slight reduction (10%) in Φ_{PSII} in rare cases in which lesions were produced.

After C-tailing of completely digested DNA and the amplification of fragments with a T-DNA/genomic DNA junction, even short genomic sequences of 117 bp flanking T-DNA, as obtained from transformant AT171, were fully sufficient to locate this sequence in the genome of *C. graminicola*. The observed virulence defect in mutant AT171 is probably a result of the insertion of T-DNA into the promoter region of a gene encoding the enzyme allantoicase (Lee *et al.*, 1990). In filamentous fungi, such as *N. crassa*, purines are converted to allantoate, which is subsequently used as a substrate by the enzyme allantoicase, producing (S)-ureidoglycolate. The final products of the pathway are carbon dioxide and ammonia, which can be re-utilized as a nitrogen source (Yoo and Cooper, 1991; Yoo *et al.*, 1985).

The regulation of fungal pathogenicity by nitrogen limitation is clearly established (Talbot *et al.*, 1997). Indeed, during pre-penetration development, e.g. germ tube growth on the plant cuticle, nitrogen is hardly available and the allantoicase pathway may be involved in the provision of ammonia and compensation for nitrogen limitation. In *M. oryzae*, multiple genes, involved in both nitrogen metabolism and pathogenicity, have been identified (Meng *et al.*, 2007). For example, a gene encoding the vacuolar serine protease, *SPM1*, was shown to be indispensable for full sporulation and appressorial development (Meng *et al.*, 2007), and a mutation in one of the two regulatory genes *NPR1* or *NPR2* (for nitrogen pathogenicity regulation) resulted in a failure to use different nitrogen sources and a lack of pathogenicity (Lau and Hamer, 1996). Gene expression data also support the idea that the allantoicase pathway may play a role in pathogenicity. In *M. oryzae*, Donofrio *et al.* (2006) identified four genes in this pathway, encoding an allantoate permease, an MFS allan-

toate transporter, an allantoinase and an allantoin permease, which are upregulated under nitrogen starvation conditions, and may thus be required during plant infection.

In summary, the work described here shows that the utilization of the modified ATMT protocol is well suited for the identification of novel genes required for virulence in the corn pathogen *C. graminicola*. Thus, also in this fungus, ATMT represents an excellent alternative to REMI mutagenesis (Epstein *et al.*, 1998; Thon *et al.*, 2000).

EXPERIMENTAL PROCEDURES

Cultivation and ATMT of *C. graminicola*

The WT isolate CgM2 of *Colletotrichum graminicola* (Ces.) Wils. (teleomorph *Glomerella graminicola* (Politis)) was grown on OMA (50 g oat meal, 15 g agar per litre) under constant black light irradiation (lamps: TL-D 36 W/08; Philips, Hamburg, Germany) as described previously (Sugui and Deising, 2002). Conidia from 14-day-old cultures were used for ATMT.

In the *A. tumefaciens* strain LBA1100 (Beijersbergen *et al.*, 1992), which already contained the vir-plasmid pAL1100 carrying a spectinomycin resistance cassette, the binary vector pPK2 (Covert *et al.*, 2001) (Fig. 1A) was introduced by electroporation (2500 V, 5 ms, multiporator; Eppendorf, Hamburg, Germany). The T-plasmid pPK2 confers kanamycin resistance as a bacterial selection marker and carries the hygromycin resistance cassette between the right and left border. *Agrobacterium tumefaciens* strain LBA1100-pPK2 was grown overnight at 28 °C and 120 rpm in 7 mL of Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) containing 50 μ g/mL each of kanamycin and spectinomycin. Cells were diluted into induction medium (IM; Bundock *et al.*, 1995) to an optical density at 600 nm (OD_{600}) of 0.15, and were grown to an OD_{600} of 0.6–0.8.

Aliquots of 100 μ L of bacteria and 100 μ L of conidia suspended in sterile distilled water (10^5 – 10^6 conidia/mL) were mixed and plated onto cellulose membranes (filter no. 595; $\varnothing$ = 90 mm; Schleicher & Schuell GmbH, Dassel, Germany) placed on solidified IM containing 5 mM glucose and 200 μ M acetosyringone. After incubation in the dark for 3 days, the membranes were transferred to PDA (24 g potato dextrose, 15 g agar per litre) containing 200 μ g/mL cefotaxim and 100 μ g/mL hygromycin B, and incubated at room temperature for 14 days. Mycelia of putative transformants were transferred to selection plates [SYC, 1 M sucrose; 0.1% (w/v) yeast extract; 0.1% (w/v) casein-hydrolysate; 1.5% agar-agar] containing 100 μ g hygromycin B/mL.

To generate homokaryotic strains, mycelia were allowed to form mononucleate conidia on OMA and plated out at low densities on SYC plates containing 100 μ g/mL hygromycin B (Werner *et al.*, 2007). Single colonies were transferred to OMA

plates, and clonal conidia were used directly or stored in the presence of 40% (v/v) glycerol at -70°C .

To test for the mitotic stability of the integrated hygromycin resistance cassette, conidia of 10 transformants were cultivated on OMA without hygromycin B, with biweekly transfer to new plates. After four passages in the absence of hygromycin B, transformants were grown on OMA plates containing hygromycin B (100 $\mu\text{g}/\text{mL}$) and, for DNA isolation and Southern blot analyses, in liquid complete medium (CM; Leach *et al.*, 1982) without hygromycin B at 23°C and 100 rpm (Unitron Shaker; Infors, Bottmingen, Switzerland).

Radial growth assays were performed on solid media [0.2% (w/v) yeast extract, 6 mM $\text{Ca}(\text{NO}_3)_2$, 1.5 mM KH_2PO_4 , 2 mM MgSO_4 , 1 mM NaCl, 1.5% (w/v) agarose] containing either 2% (w/v) of different carbon sources (glucose, maltose, sucrose, starch, cellulose, pectin) or no carbon source (minimal medium; MM). In addition, a complex medium (CM) was used as described by Sugui and Deising (2002). For the quantification of growth, 5 μL of a conidial suspension (10^4 conidia/mL) were pipetted onto the centre of the plate, the plates were incubated at 23°C in the dark and radial growth was measured.

Isolation of genomic DNA and Southern hybridization

Fungal genomic DNA was isolated using the phenol–chloroform extraction procedure, combined with RNase A and proteinase K treatment, as described previously (Sugui and Deising, 2002). The digestion of DNA with *Hind*III, *Eco*32I and *Xba*I, electrophoresis on 0.7% agarose, depurination and transfer onto nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Freiburg im Breisgau, Germany) followed standard protocols (Brown, 1999; Sambrook *et al.*, 1989).

The Dig-labelled probe used in hybridization experiments, corresponding to 1 kb of the T-DNA (Fig. 1B), was amplified from plasmid pPK2 by PCR. In a total volume of 10 μL , the PCR mixture consisted of 1 $\times$ reaction buffer containing 1.5 mM MgCl_2 , 0.025 U/ μL *Taq* DNA Polymerase (Amersham Pharmacia Biotech), 0.2 mM deoxynucleoside triphosphate (dNTP), 0.7 mM alkali-labile Dig-dUTP (Roche, Mannheim, Germany), 0.5 μM each of forward (5'-GTCCCTCAGTCCCTGGTAGG-3') and reverse (5'-TGTTGGCGACCTCTATTGG-3') primers, and 10 pg of plasmid DNA. Hybridization and visualization of the hybridized Dig-labelled probe were performed as suggested by the manufacturer (Roche, Mannheim, Germany).

Identification of T-DNA integration sites

The identification of genomic regions flanking T-DNA integration sites was performed by the polynucleotide tailing method, as described previously (Liu and Baird, 2001), with some modifications. Genomic DNA of the transformants was completely

digested with *Kpn*I, *Pvu*I or *Aat*II. Depending on the restriction enzyme used, one degenerated primer (degKpn, 5'-GGAGACTGACATGGACTGAAGGAGTAAAGGGIIGGGIIGGGIIGGGTACC-3'; degPvu, 5'-GGAGACTGACATGGACTGAAGGAGTAAAGGGIIGGGIIGGGIIGGGATCG-3'; degAat, 5'-GGAGACTGACATGGACTGAAGGAGTAAAGGGIIGGGIIGGGIIGGGACGTC-3') was used in combination with a primer specific for either the left (pPK2-rev7, 5'-TCACTGCCCCGCTTTCCAGTC-3') or the right (CgSQ8, 5'-CAGATCAACGTCGTCAGAG-5') T-DNA border. One microgram of tailed genomic DNA was used as template in a total volume of 20 μL of PCR. The PCR conditions were 95°C for 2 min, followed by five cycles of 95°C for 30 s, 70°C for 30 s and 72°C for 5 min; five cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 5 min, and 20 cycles of 95°C for 30 s, 60.5°C for 30 s, 72°C for 5 min. Final elongation was at 72°C for 5 min. In the subsequent nested PCR, the anchor primer (Tn, 5'-GGA GACTGACATGGACTGAAGGAGT-3') was used in combination with either the right (CgSQ8n, 5'-ACTGAGGAATCCGCTCTTGG-3') or left (CgSQrev20, 5'-GGTTGCGTATTGGCTAGAG-3') nested T-DNA border primer. One microlitre of the first PCR served as template. The reaction volume was again 20 μL . The annealing temperature was 59°C , the extension time was 5 min, and buffer and nucleotide concentrations were as described above.

Sequencing of the PCR products was performed as described previously (Krijger *et al.*, 2008). Nucleotide sequences were compared with known protein sequences by the Basic Local Alignment Search Tool (BLASTX) (Altschul *et al.*, 1990) provided by the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>), and further analysed by the gene prediction program AUGUSTUS (<http://augustus.gobics.de>) (Stanke *et al.*, 2004).

Virulence assays

Corn (*Zea mays* cv. Nathan) leaf segments and plants were used to assess the virulence of the WT isolate and transformants of *C. graminicola* generated by ATMT.

Corn leaf segments (10 cm) of the third leaf of 14-day-old plants were inoculated with 10- μL droplets containing 5×10^3 conidia in 0.02% (v/v) Tween 20 (Mercure *et al.*, 1994). Inoculated leaf segments were incubated in the dark at 25°C in a sealed moist Petri dish. Leaves were photographed and subjected to microscopy at 1, 2, 3, 4 and 7 dpi. After bleaching in acetate–ethanol (1:3, v/v) overnight, leaf segments were stained by acid-fuchsin (de Neergaard, 1997). Microscopy was performed using a Nikon Eclipse 600 epifluorescence microscope (Nikon, Düsseldorf, Germany), as described previously (Werner *et al.*, 2007). Digital images were taken with a CCD-1300 camera (VDS Vosskühler GmbH, Osnabrück, Germany) and processed with the software package NS Elements (Nikon).

For whole-plant virulence assays, 14-day-old corn plants were evenly spray inoculated with 1.7×10^6 conidia per plant and cultivated in a growth chamber (Percival-36HIL) at 16 h/8 h day/night rhythm and a light flux of 400 μE (MASTER PL-L 55W/840/4P, Phillips, Poland and Radium-25W lamps, Radium, Frankfurt, Germany). During the first 24 hpi, the plants were kept at 25 °C and 100% relative humidity, followed by day/night conditions of 25 °C/20 °C and 50%/70% relative humidity, respectively. Symptoms were photographed daily from 3 to 7 dpi.

Cytorrhysis assays

Plastic sheets (No. 3558, Avery Dennison, Holzkirchen, Germany) of 1 cm $\times$ 1 cm were inoculated with 10 μL of water droplets containing 2000 spores and stored in a sealed moist Petri dish in the dark at 23 °C. After 24 h, each sheet was incubated for 10 min in a solution of 0, 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 M glycerol, and collapsed and noncollapsed appressoria were counted by light microscopy (Eclipse 600; Nikon).

Chlorophyll fluorescence imaging

Chlorophyll fluorescence of leaves infected with mutant and WT *C. graminicola*, and of control leaves, was recorded with a combined gas exchange/chlorophyll fluorescence imaging system (GFS-3000/MINI Imaging-PAM chlorophyll fluorometer; Walz, Effeltrich, Germany) starting at 4 dpi in daily intervals. Data analysis was performed with ImagingWin v2.30 software (Walz). Prior to the measurements, the leaves were dark adapted for 20 min and then placed into the cuvette of the GFS-3000 IRGA. CO_2 and H_2O partial pressures were set to 350 $\mu\text{g/g}$ and 10 000 $\mu\text{g/g}$, respectively, to obtain identical conditions for all measurements. An initial saturating pulse was applied to determine the maximal fluorescence (F_v/F_m) before the absorptivity (abs.) was determined using the formula $\text{abs.} = 1 - R_R/R_{\text{NIR}}$, where R_R is the signal intensity for reflected red light (660 nm) and R_{NIR} is the intensity for reflected near-infrared light (780 nm). Subsequently, the leaf was illuminated with 400 $\mu\text{E/m}^2/\text{s}$ of actinic light, and chlorophyll fluorescence images were taken in 60-s intervals on application of saturating light pulses, until Φ_{PSII} reached its maximum. Fluorescence parameters were calculated according to Horst *et al.* (2010).

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REFERENCES

- Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geraht, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C. and Ecker, J.R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.
- Bastmeyer, M., Deising, H.B. and Bechinger, C. (2002) Force exertion in fungal infection. *Annu. Rev. Biophys. Biomol. Struct.* **31**, 321–341.
- Bechinger, C., Giebel, K.-F., Schnell, M., Leiderer, P., Deising, H.B. and Bastmeyer, M. (1999) Optical measurements of invasive forces exerted by appressoria of a plant pathogenic fungus. *Science*, **285**, 1896–1899.
- Beijersbergen, A., Dulk-Ras, A., Schilperoort, R.A. and Hooykaas, P.J. (1992) Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. *Science*, **256**, 1324–1327.
- Bergstrom, G.C. and Nicholson, R.L. (1999) The biology of corn anthracnose. Knowledge to exploit for improved management. *Plant Dis.* **83**, 596–608.
- Bölker, M., Böhnert, H.U., Braun, K.H., Görl, J. and Kahmann, R. (1995) Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.* **248**, 547–552.
- Brown, T. (1999) Southern blotting. In: *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. eds), pp. 2.9.1–2.9.15. New York: John Wiley & Sons.
- Bundock, P., Dulk-Ras, A., Beijersbergen, A. and Hooykaas, P.J. (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO J.* **14**, 3206–3214.
- Covert, S.F., Kapoor, P., Lee, M., Briley, A. and Nairn, C.J. (2001) *Agrobacterium tumefaciens*-mediated transformation of *Fusarium circinatum*. *Mycol. Res.* **105**, 259–264.
- De Jong, J.C., McCormack, B.J., Smirnov, N. and Talbot, N.J. (1997) Glycerol generates turgor in rice blast. *Nature*, **389**, 244–245.
- De Neve, M., De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. *Plant J.* **11**, 15–29.
- Deising, H.B., Werner, S. and Wernitz, M. (2000) The role of fungal appressoria in plant infection. *Microbes Infect.* **2**, 1631–1641.
- Dobinson, K.F., Grant, S.J. and Kang, S. (2004) Cloning and targeted disruption, via *Agrobacterium tumefaciens*-mediated transformation, of a trypsin protease gene from the vascular wilt fungus *Verticillium dahliae*. *Curr. Genet.* **45**, 104–110.
- Donofrio, N.M., Oh, Y., Lundy, R., Pan, H., Brown, D.E., Jeong, J.S., Coughlan, S., Mitchell, T.K. and Dean, R.A. (2006) Global gene expression during nitrogen starvation in the rice blast fungus *Magnaporthe oryzae*. *Fungal Genet. Biol.* **43**, 605–617.

- Epstein, L., Lusnak, K. and Kaur, S. (1998) Transformation-mediated developmental mutants of *Glomerella graminicola*. *Fungal Genet. Biol.* **23**, 189–203.
- Escobar, M.A. and Dandekar, A.M. (2003) *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci.* **8**, 380–386.
- Fitzgerald, A.M., Mudge, A.M., Gleave, A.P. and Plummer, K.M. (2003) *Agrobacterium* and PEG-mediated transformation of the phytopathogen *Venturia inaequalis*. *Mycol. Res.* **107**, 803–810.
- Flowers, J.L. and Vaillancourt, L. (2005) Parameters affecting the efficiency of *Agrobacterium tumefaciens*-mediated transformation of *Colletotrichum graminicola*. *Curr. Genet.* **48**, 380–388.
- Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying' tool. *Microbiol. Mol. Biol. Rev.* **67**, 16–37.
- Gilbert, M.J., Thornton, C.R., Wakley, G.E. and Talbot, N.J. (2006) A P-type ATPase required for rice blast disease and induction of host resistance. *Nature*, **440**, 535–539.
- de Groot, M.J., Bundock, P., Hooykaas, P.J. and Beijersbergen, A.G. (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nat. Biotechnol.* **16**, 839–842.
- Hamer, L., Adachi, K., Montenegro-Chamorro, M.V., Tanzer, M.M., Mahanty, S.K., Lo, C., Tarpey, R.W., Skalhunes, A.R., Heiniger, R.W., Frank, S.A., Darveaux, B.A., Lampe, D.J., Slater, T.M., Ramamurthy, L., DeZwaan, T.M., Nelson, G.H., Shuster, J.R., Woessner, J. and Hamer, J.E. (2001) Gene discovery and gene function assignment in filamentous fungi. *Proc. Natl. Acad. Sci. USA*, **98**, 5110–5115.
- Horbach, R., Weihmann, F., Antelo, L., Mathea, S., Liermann, J.C., Opatz, T., Thines, E., Aguirre, J. and Deising, H.B. (2009) Sfp-type 4'-phosphopantetheinyltransferase is indispensable for fungal pathogenicity. *Plant Cell*, **21**, 3379–3396.
- Horst, R.J., Doehlemann, G., Wahl, R., Hofmann, J., Schmiedl, A., Kahmann, R., Kämper, J., Sonnewald, U. and Voll, L. (2010) *Ustilago maydis* infection strongly alters organic nitrogen allocation in maize and stimulates productivity of systemic source leaves. *Plant Physiol.* **152**, 293–308.
- Howard, R.J., Ferrari, M.A., Roach, D.H. and Money, N.P. (1991) Penetration of hard substances by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. USA*, **88**, 11281–11284.
- Idnurm, A. and Howlett, B.J. (2001) Pathogenicity genes of phytopathogenic fungi. *Mol. Plant Pathol.* **2**, 241–255.
- Jeon, J., Park, S.-Y., Chi, M.-H., Choi, J., Park, J., Rho, H.-S., Kim, S., Goh, J., Yoo, S., Choi, J., Park, J.-Y., Yi, M., Yang, S., Kwon, M.-J., Han, S.-S., Kim, B.R., Khang, C.H., Park, B., Lim, S.-E., Jung, K., Kong, S., Karunakaran, M., Oh, H.-S., Kim, H., Kim, S., Park, J., Kang, S., Choi, W.-B., Kang, S. and Lee, J.-H. (2007) Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. *Nat. Genet.* **39**, 561–565.
- Kahmann, R. and Basse, C. (1999) REMI (restriction enzyme mediated integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. *Eur. J. Plant Pathol.* **105**, 221–229.
- Kamoun, S. (2009) The secretome of plant-associated fungi and Oomycetes. In: *The Mycota V—Plant Relationships*, 2nd edn (Deising, H.B., ed.), pp. 173–180. Berlin: Heidelberg: Springer-Verlag.
- Khang, C.H., Park, S.Y., Rho, H.S., Lee, Y.H. and Kang, S. (2006) Filamentous fungi (*Magnaporthe grisea* and *Fusarium oxysporum*). *Methods Mol. Biol.* **344**, 403–420.
- Krijger, J.J., Horbach, R., Behr, M., Schweizer, P., Deising, H.B. and Wirsig, S.G. (2008) The yeast signal sequence trap identifies secreted proteins of the hemibiotrophic corn pathogen *Colletotrichum graminicola*. *Mol. Plant-Microbe Interact.* **21**, 1325–1336.
- Küster, S., Ludwig, N., Willers, G., Hoffmann, J., Deising, H.B. and Kiesow, A. (2008) Thin PTFE-like membranes allow characterizing germination and mechanical penetration competence of phytopathogenic fungi. *Acta Biomater.* **4**, 1809–1818.
- Lau, G. and Hamer, J.E. (1996) Regulatory genes controlling MPG1 expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. *Plant Cell*, **8**, 771–781.
- Leach, J., Lang, B.R. and Yoder, O.C. (1982) Methods for selection of mutants and *in vitro* culture of *Cochliobolus heterostrophus*. *J. Gen. Microbiol.* **128**, 1719–1729.
- Lecleque, A., Wan, H., Abschütz, A., Chen, S., Mitina, G.V., Zimmermann, G. and Schairer, H.U. (2004) *Agrobacterium*-mediated insertional mutagenesis (AIM) of the entomopathogenic fungus *Beauveria bassiana*. *Curr. Genet.* **45**, 111–119.
- Lee, H., Fu, Y.H. and Marzluf, G.A. (1990) Nucleotide sequence and DNA recognition elements of alc, the structural gene which encodes allantoinase, a purine catabolic enzyme of *Neurospora crassa*. *Biochemistry*, **29**, 8779–8787.
- Li, G., Zhou, Z., Liu, G., Zheng, F. and He, C. (2007) Characterization of T-DNA insertion patterns in the genome of rice blast fungus *Magnaporthe grisea*. *Curr. Genet.* **51**, 233–243.
- Liu, X. and Baird, V. (2001) Rapid amplification of genomic DNA ends by NlaIII partial digestion and polynucleotide tailing. *Plant Mol. Biol. Rep.* **19**, 261–267.
- Lu, S., Lyngholm, L., Yang, G., Bronson, C., Yoder, O.C. and Turgeon, B.G. (1994) Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proc. Natl. Acad. Sci. USA*, **91**, 12649–12653.
- Madrid, M.P., Di Pietro, A. and Roncero, M.I. (2003) Class V chitin synthase determines pathogenesis in the vascular wilt fungus *Fusarium oxysporum* and mediates resistance to plant defence compounds. *Mol. Microbiol.* **47**, 257–266.
- Maier, F.J. and Schäfer, W. (1999) Mutagenesis via insertional—or restriction enzyme-mediated-integration (REMI) as a tool to tag pathogenicity related genes in plant pathogenic fungi. *Biol. Chem.* **380**, 855–864.
- Malonek, S. and Meinhardt, F. (2001) *Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*. *Curr. Genet.* **40**, 152–155.
- Maruthachalam, K., Nair, V., Rho, H.S., Choi, J., Kim, S. and Lee, Y.H. (2008) *Agrobacterium tumefaciens*-mediated transformation in *Colletotrichum falcatum* and *C. acutatum*. *J. Microbiol. Biotechnol.* **18**, 234–241.
- Meng, Y., Patel, G., Heist, M., Betts, M.F., Tucker, S.L., Galadima, N., Donofrio, N.M., Brown, D., Mitchell, T.K., Li, L., Xu, J.-R., Orbach, M., Thon, M., Dean, R.A. and Farman, M.L. (2007) A systematic analysis of T-DNA insertion events in *Magnaporthe grisea*. *Fungal Genet. Biol.* **44**, 1050–1064.
- Mercure, E.W., Kunoh, H. and Nicholson, R.L. (1994) Adhesion of *Colletotrichum graminicola* conidia to corn leaves: a requirement for disease development. *Physiol. Mol. Plant Pathol.* **45**, 407–420.
- Möbius, N. and Hertweck, C. (2009) Fungal phytotoxins as mediators of virulence. *Curr. Opin. Plant Biol.* **12**, 390–398.

- Mullins, E.D. and Kang, S. (2001) Transformation: a tool for studying fungal pathogens of plants. *Cell. Mol. Life Sci.* **58**, 2043–2052.
- Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M. and Kang, S. (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology*, **91**, 173–180.
- Münch, S., Lingner, U., Floss, D.S., Ludwig, N., Sauer, N. and Deising, H. (2008) The hemibiotrophic lifestyle of *Colletotrichum* species. *J. Plant Physiol.* **165**, 41–51.
- de Neergaard, E. (1997) *Methods in Botanical Histopathology*. Copenhagen: Kandrups Bogtrykkeri.
- Rho, H.S., Kang, S. and Lee, Y.H. (2001) *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus *Magnaporthe grisea*. *Mol. Cells*, **12**, 407–411.
- Rolland, S., Jobic, C., Fèvre, M. and Bruel, C. (2003) *Agrobacterium*-mediated transformation of *Botrytis cinerea*, simple purification of monokaryotic transformants and rapid conidia-based identification of the transfer-DNA host genomic DNA flanking sequences. *Curr. Genet.* **44**, 164–171.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Shen, W.F., Weng, H.B., Niu, B.L., He, L.H., Liu, Y., Qi, X.P. and Meng, Y. (2008) *Agrobacterium tumefaciens*-mediated transformation of the pathogen of *Botrytis cinerea*. *Yi Chuan*, **30**, 515–520.
- Stanke, M., Steinkamp, R., Waack, S. and Morgenstern, B. (2004) AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Res.* **32**, W309–W312.
- Stephenson, S.A., Hatfield, J., Rusu, A.G., Maclean, D.J. and Manners, J.M. (2000) *CgDN3*: an essential pathogenicity gene of *Colletotrichum gloeosporioides* necessary to avert a hypersensitive-like response in the host *Stylosanthes guianensis*. *Mol. Plant–Microbe Interact.* **13**, 929–941.
- Sugui, J.A. and Deising, H.B. (2002) Isolation of infection-specific sequence tags expressed during early stages of maize anthracnose disease development. *Mol. Plant Pathol.* **3**, 197–203.
- Sugui, J.A., Chang, Y.C. and Kwon-Chung, K.J. (2005) *Agrobacterium tumefaciens*-mediated transformation of *Aspergillus fumigatus*: an efficient tool for insertional mutagenesis and targeted gene disruption. *Appl. Environ. Microbiol.* **71**, 1798–1802.
- Sweigard, J.A., Carroll, A.M., Farrall, L., Chumley, F.G. and Valent, B. (1998) *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant–Microbe Interact.* **11**, 404–412.
- Talbot, N.J., McCafferty, H.R.K., Ma, M., Moore, K. and Hamer, J.E. (1997) Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. *Physiol. Mol. Plant Pathol.* **50**, 179–195.
- Talhinhas, P., Muthumeenakshi, S., Neves-Martins, J., Oliveira, H. and Sreenivasaprasad, S. (2008) *Agrobacterium*-mediated transformation and insertional mutagenesis in *Colletotrichum acutatum* for investigating varied pathogenicity lifestyles. *Mol. Biotechnol.* **39**, 57–67.
- Thon, M.R., Nuckles, E.M. and Vaillancourt, L.J. (2000) Restriction enzyme-mediated integration used to produce pathogenicity mutants of *Colletotrichum graminicola*. *Mol. Plant–Microbe Interact.* **13**, 1356–1365.
- Tsuji, G., Fujii, S., Fujihara, N., Hirose, C., Tsuge, S., Shiraishi, T. and Kubo, Y. (2003) *Agrobacterium tumefaciens*-mediated transformation for random insertional mutagenesis in *Colletotrichum lagenarium*. *J. Gen. Plant Pathol.* **69**, 230–239.
- Tucker, S.L. and Talbot, N.J. (2001) Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annu. Rev. Phytopathol.* **39**, 385–417.
- Ülker, B., Li, Y., Rosso, M.G., Logemann, E., Somssich, I.E. and Weishaar, B. (2008) T-DNA-mediated transfer of *Agrobacterium tumefaciens* chromosomal DNA into plants. *Nat. Biotechnol.* **26**, 1015–1017.
- Vijn, I. and Govers, F. (2003) *Agrobacterium tumefaciens* mediated transformation of the oomycete plant pathogen *Phytophthora infestans*. *Mol. Plant Pathol.* **4**, 459–467.
- Wang, Z.Y., Jenkinson, J.M., Holcombe, L.J., Soanes, D.M., Veneault-Fourrey, C., Bhambra, G.K. and Talbot, N.J. (2005) The molecular biology of appressorium turgor generation by the rice blast fungus *Magnaporthe grisea*. *Biochem. Soc. Trans.* **33**, 384–388.
- Werner, S., Sugui, J.A., Steinberg, G. and Deising, H.B. (2007) A chitin synthase with a myosin-like motor domain is essential for hyphal growth, appressorium differentiation, and pathogenicity of the maize anthracnose fungus *Colletotrichum graminicola*. *Mol. Plant–Microbe Interact.* **20**, 1555–1567.
- White, D. and Chen, W. (2006) Genetic transformation of *Ascochyta rabiei* using *Agrobacterium*-mediated transformation. *Curr. Genet.* **49**, 272–280.
- Yoo, H.S. and Cooper, T.G. (1991) Sequences of two adjacent genes, one (DAL2) encoding allantoinase and another (DCG1) sensitive to nitrogen-catabolite repression, in *Saccharomyces cerevisiae*. *Gene*, **104**, 55–62.
- Yoo, H.S., Genbauffe, F.S. and Cooper, T.G. (1985) Identification of the ureidoglycolate hydrolase gene in the DAL gene cluster of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **5**, 2279–2288.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 T-DNA disruption in AT171 leads to the identification of an allantoinase gene in *Colletotrichum graminicola*. (A) Scheme of the disrupted region in AT171. (B) Partial genomic sequence of *C. graminicola* wild-type including the 5' flank (bp 1–184), the T-DNA integration site in AT171 and the allantoinase gene (start and stop codons are framed, intron with grey background). (C) Alignment of the protein sequences of allantoinase of *C. graminicola* (Cg) with the sequences of *Neurospora crassa* (Nc) and *Magnaporthe oryzae* (Mo). The protein sequence of *N. crassa* is annotated as an allantoinase and that of *M. oryzae* includes several regions of an allantoinase. The sequence homology between *C. graminicola* and *N. crassa* is 80%, between *C. graminicola* and *M. oryzae* 79%, and between all three sequences 70%.

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