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Development of a high throughput transformation system for insertional mutagenesis in *Magnaporthe oryzae*

Melania F. Betts [a], Sara L. Tucker [a], Natalia Galadima [a], Yan Meng [b], Gayatri Patel [b], Lei Li [c], Nicole Donofrio [d], Anna Floyd [d], Shelly Nolin [d], Doug Brown [d], M. Alejandra Mandel [a], Thomas K. Mitchell [d], Jin-Rong Xu [c], Ralph A. Dean [d], Mark L. Farman [b], Marc J. Orbach [a,*]

[a] *Department of Plant Sciences, Division of Plant Pathology and Microbiology, University of Arizona, P.O. Box 210036, Tucson, AZ 85721-0036, USA*
[b] *Department of Plant Pathology, University of Kentucky, Lexington, KY, USA*
[c] *Department of Biological Sciences, Purdue University, West Lafayette, IN, USA*
[d] *Department of Plant Pathology, North Carolina State University, Raleigh, NC, USA*

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Abstract

Towards the goal of disrupting all genes in the genome of *Magnaporthe oryzae* and identifying their function, a collection of >55,000 random insertion lines of *M. oryzae* strain 70-15 were generated. All strains were screened to identify genes involved in growth rate, conidiation, pigmentation, auxotrophy, and pathogenicity. Here, we provide a description of the high throughput transformation and analysis pipeline used to create our library. Transformed lines were generated either by $CaCl_2$/PEG treatment of protoplasts with DNA or by *Agrobacterium tumefaciens*-mediated transformation (ATMT). We describe the optimization of both approaches and compare their efficiency. ATMT was found to be a more reproducible method, resulting in predominantly single copy insertions, and its efficiency was high with up to 0.3% of conidia being transformed. The phenotypic data is accessible via a public database called MGOS and all strains are publicly available. This represents the most comprehensive insertional mutagenesis analysis of a fungal pathogen.

Keywords: *Magnaporthe oryzae*; Insertional mutagenesis; *Agrobacterium tumefaciens*-mediated transformation; $CaCl_2$/PEG-mediated transformation; Pathogenicity

1. Introduction

Magnaporthe oryzae, an ascomyceteous fungus and causal agent of rice blast disease, has been developed as a model organism to study host–microbe interactions (Talbot, 2003; Valent and Chumley, 1991). In addition to its significant threat to worldwide rice production, blast disease impacts many other gramineous species such as finger millet, barley and wheat (Borromeo et al., 1993; Urashima et al., 1993). The disease cycle starts when a conidium lands on a leaf surface, germinates and sensing the surface, initiates formation of a penetration structure known as an appressorium (Dean, 1997). Infection begins after the accumulation of turgor pressure in the appressorium, leading to mechanical penetration by an infection peg, which is followed by the differentiation of infectious hyphae in the host cell (Howard et al., 1991; Howard and Valent, 1996; Talbot, 1995; Valent, 1990). Following intracellular growth by the pathogen, the disease cycle is completed by the production of spores from lesions on the leaf surface and their release to re-initiate infection. A full understanding of initiation and progression through the *M. oryzae* disease cycle may allow identification of targets to control the disease.

* Corresponding author. Fax: +1 520 621 9290.
E-mail address: orbachmj@cals.arizona.edu (M.J. Orbach).

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One approach to defining genes important for disease development is to identify mutants defective in pathogenicity. Insertional mutagenesis approaches have been successfully used with *M. oryzae* in order to identify several pathogenicity genes (Balhadere et al., 1999; Sweigard et al., 1998). In these cases, a limited number of insertion strains were generated using REMI (restriction enzyme-mediated insertion) and screened for defects in pathogenicity resulting in 32 mutants, with 20 of them tagged by the transforming DNA. In order to identify additional genes important for the ability of the fungus to cause disease, we have chosen to saturate the *M. oryzae* genome with random insertions of a defined DNA fragment. Through this approach, any genes of interest can be recovered because they are tagged.

We have generated a collection of >55,000 strains with DNA insertions in this fungus with a predicted gene set of ~11,000, to be screened for defects in pathogenicity, metabolism, morphology and conidiation as well as other phenotypic traits. Generation of this large collection of insertion lines allowed us to compare different transformation methods and to optimize both transformation and processing of strains for purification and phenotypic analyses. In the present study, we compare the efficiency of two different transformation methods, *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Covert et al., 2001; de Groot, 1998; Meyer et al., 2003; Mullins et al., 2001; Mullins and Kang, 2001) and standard protoplast transformation (Sweigard et al., 1995), both for ease of generating insertion lines, and analyzing tagged genes. We demonstrate that ATMT is more efficient for high throughput insertion strain generation and downstream analysis of the insertions, and present the development of a highly efficient pipeline for processing transformed lines. We show that by optimizing the ratio of *A. tumefaciens* and *M. oryzae* cells, and the time of co-cultivation between the cells, transformation can occur in up to 0.3% of the conidia. We also present initial phenotypic analyses of the strains for defects in growth and pathogenicity and molecular analysis of insertion sites and distribution. This large-scale insertional mutagenesis study is the first effort for a phytopathogenic fungus that aims to target the full genome and may serve as a model for other projects with similar goals.

2. Materials and methods

2.1. Fungal strains, growth conditions and media

Magnaporthe oryzae strain 70-15 (Chao and Ellingboe, 1991) was obtained from A. Ellingboe (University of Wisconsin) and distributed from a single stock in the Dean laboratory. It was stored on paper filters with desiccation, at −20 °C. For production of mycelium, cultures were grown on complete solid media (Crawford et al., 1986) at 28 °C with no light. For production of conidia, fungal cultures were grown at 25 °C under constant fluorescent light on either supplemented complete medium (SCM), a modified complete medium that allows *M. oryzae* cultures to sporulate (Talbot et al., 1993), or oatmeal agar (OA) (Valent et al., 1991).

2.2. Protoplast transformation and constructs

Transformations were performed as described (Sweigard et al., 1995) with the following modifications. Mycelial cultures were grown in modified Iwasaki medium (Chida and Sisler, 1987) in place of complete medium to reduce melanization. The number of protoplasts per transformation was decreased to 0.1 ml of 1×10^7 protoplasts/ml, which were mixed with 1 μg of purified DNA fragment. The regeneration top and bottom agars were modified by inclusion of 20% sucrose instead of 1 M sorbitol, as this helped to reduce the background growth. Hygromycin B (HygB) (Calbiochem, LaJolla, CA) was added to the bottom agar at a concentration of 300 μg/ml. The top agar-plating medium contained 1.5% agarose (BioWhitaker Molecular Applications) instead of 2% low melting point agarose and HygB at 100 μg/ml. Control experiments, to determine transformation efficiency, were assessed using the vector pCB1004 (Carroll et al., 1994) as the transforming DNA. For creation of insertion library strains, linear fragments of DNA were prepared from vectors pAM1120, pAM1270, pAM1273 and pAM1274, or the vectors pB-GC-Hyg or pB-AT-Hyg (see below).

Colonies were picked from transformation plates to oatmeal agar containing HygB (300 μg/ml) and grown for 5–7 days before streaking conidia onto CM agar (Crawford et al., 1986) with 300 μg/ml HygB or 4% water agar with 100 μg/ml HygB. Single germinating conidia were transferred to CM with HygB (200 μg/ml), and represent unique cultures for each transformed line.

2.3. Construction of transformation vectors

Several constructs were used for protoplast transformation. One set of vectors consisted of pCB1004 (Carroll et al., 1994) and three derivatives, pB-AT-Hyg and pB-GC-Hyg, which contain the hygromycin phosphotransferase (Hyg^R) cassette of pCB1004 with ~100 bp of AT-rich and GC-rich flanking sequences, respectively, and pAM1120. The second set of transformation vectors was pAM1270, pAM1273 and pAM1274 (Fig. 1a). Each contained the Hyg^R cassette of pCB1004 flanked by two inward facing promoterless fluorescent protein genes, EGFP and DsRed (Clontech). Like the first set of vectors, they differed in the ends of their transforming fragment, with pAM1273 and pAM1274 containing ~100 nt of AT-rich or GC-rich sequences on each end, respectively, while pAM1270 lacked these additional sequences.

The pCB1004 derivatives pB-AT-Hyg and pB-GC-Hyg were constructed by annealing pairs of oligonucleotides that were complementary at their 3′ ends, and then filling them in by PCR to create a ~200 bp fragment. For the

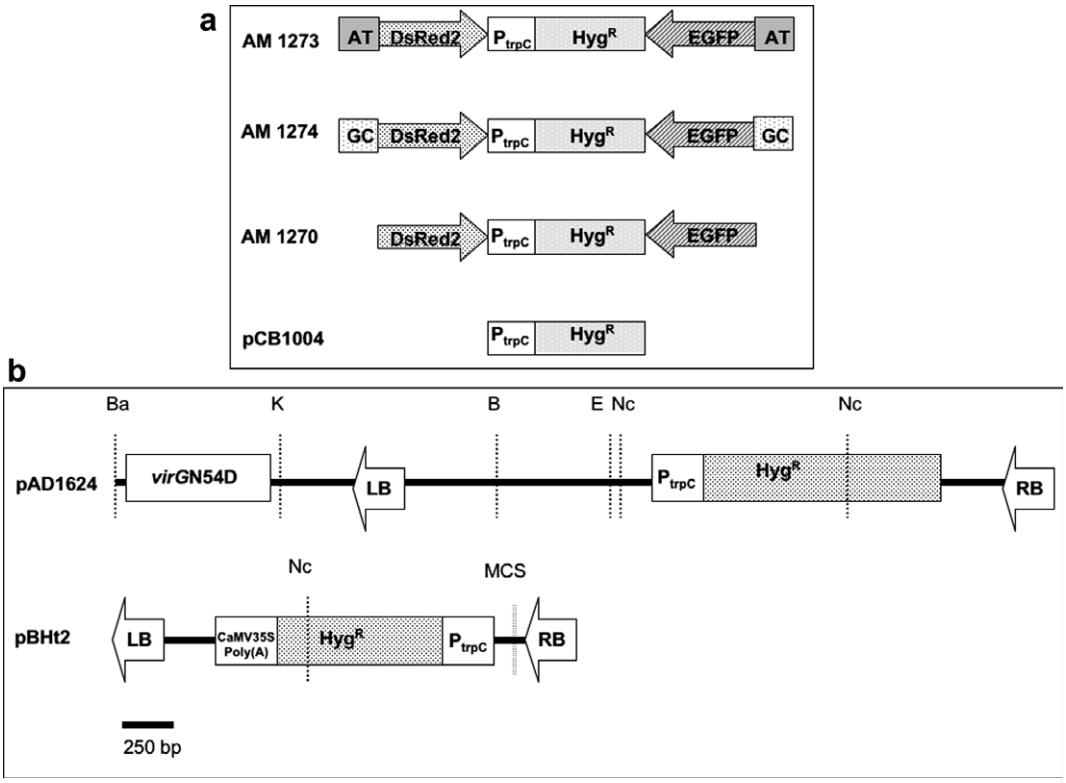

Fig. 1. (a) DNA fragments used to generate insertional mutants by protoplast transformation. The size of the AM1273 and AM1274 fragment is 3.1 kb, the AM1270 fragment is 2.9 kb and the hygromycin cassette fragment excised from pCB1004 is 1.4 kb. (b) Diagram of pAD1624 T-DNA and adjacent binary vector regions. Indicated restriction endonucleases sites are Ba: BamHI, B: BglI, E: EcoRI, K: KpnI, Nd: NdeI, Nc: NcoI. pBHt2 T-DNA (Mullins et al., 2001). Indicated restriction endonucleases sites are Nc: NcoI. MCS: EcoRI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI, and HindIII, CaMV 35S Poly(A): Cauliflower mosaic virus 35S poly(A) signal. For both diagrams, Hyg^R: hygromycin B resistance gene, P_{trpC}: *Aspergillus nidulans* trpC promoter, LB: Left border, RB: right border (arrows indicating the borders are not to scale).

pB-AT-Hyg vector, oligonucleotides, AT-rich1 and AT-rich2 were used and for pB-GC-Hyg, GC-rich1 and GC-rich2 (Supplemental Table 1). The PCR fragments were digested with SalI and KpnI to create cohesive ends and then the AT-rich fragment was cloned into pBluescript KS II+ (Strategene) to create pB-AT and the GC-rich fragment into pGEM®-T Easy (Promega) to create pB-GC. The Hyg^R cassette was then excised from pCB1004 with HpaI and cloned into the Klenow-treated NotI or SwaI sites in the middle of the AT- and GC-rich fragments of pB-AT and pB-GC, respectively, to generate pB-AT-Hyg and pB-GC-Hyg. The pCB1004 derivative, pAM1120 was constructed by cloning the HpaI Hyg^R cassette fragment into pBluescriptSK+ digested with EcoRV.

To construct pAM1270, first a BamHI-NotI fragment containing the promoterless red fluorescent protein gene from pDSRed (Clontech, San Diego, CA), was cloned into BamHI and NotI digested pCR2.1 (Invitrogen, Sorrento Valley, CA) to create pAM1266. A fragment containing the promoterless green fluorescent protein gene was isolated from pEGFP (Clontech) by digestion with NotI and XbaI, and cloned into NotI and XbaI treated pAM1266, generating plasmid pAM1267. Plasmid pAM1270 was constructed by insertion of the Hyg^R cassette from pCB1004, released as a HpaI fragment, into pAM1267 that had been digested with NotI and filled-in using Klenow DNA polymerase to create blunt ends. The AT-rich sequences and GC-rich sequences were added to both ends of pAM1270 in an approach similar to that with the pB vectors described above. Oligonucleotides OAM531 and OAM532 (Supplemental Table 1) were annealed and filled in by PCR amplification. These PCR products were cloned into pGEM®-T Easy (Promega) generating plasmid pAM1264. A BamHI fragment containing the EGFP and DsRed genes and the Hyg^R cassette was isolated from pAM1270, and cloned into pAM1264 digested with BamHI, generating pAM1271. To facilitate isolation of the insert from the vector, the ~3 kb-EcoRI fragment from pAM1271 was cloned into EcoRI digested pCR2.1 (4 kb), generating plasmid pAM1273.

To add GC-rich sequences to both ends of the transformation cassette in pAM1270, oligonucleotides GC-rich1 and GC-rich2 were annealed and extended as described above. The PCR product was cloned into pGEM®-T Easy, generating plasmid pGEM-GC. The transformation cassette was inserted between the GC-rich ends as a SmaI fragment isolated from pAM1270, and cloned into pGEM-GC digested with SwaI, generating pAM1272. To facilitate isolation of the insert from the vector, the ~3 kb-EcoRI fragment from pAM1272 was cloned into

pCR2.1 (4 kb) digested with EcoRI, generating plasmid pAM1274.

For transformation, the DNA fragments represented in Fig. 1a, were separated from the vector by electrophoresis following restriction enzyme digestion and purified using the Promega Wizard SV Gel and PCR clean-up system: pAM1270 (2896 bp) DNA was digested with BamHI, pAM1273 (3067 bp) and pAM1274 (3067 bp) were digested with EcoRI, pCB1004 (1400 bp) was digested with HpaI, and pAM1120 was digested with EcoRI and HindIII.

2.4. Agrobacterium strains and binary vectors

In this study we used two *A. tumefaciens* strains with different binary vectors. Strain AGL1 (Lazo et al., 1991) containing the pCAMBIA1300-based binary vector pBHt1 or pBHt2 (Fig. 1b) (Mullins et al., 2001). Strain EHA105 (Hood et al., 1993) containing the binary vector pAD1624 (Fig. 1b) (Abuodeh et al., 2000), constructed by A. Das and called EHA105/pAD1624, was also used. pAD1624 contains a constitutive *virG* gene (*virG*N54D) which facilitates transformation without the need to add acetosyringone for induction of the *vir* genes (Pazour et al., 1992). In both binary vectors the Hyg^R gene from pCB1004 (Carroll et al., 1994) is present between the T-DNA borders (Fig. 1b). Strains AGL1 and EHA105 lacking the binary vectors were used in the transformations as negative controls. *Agrobacterium* strains were stored at −80 °C in 15% glycerol (v/v), by addition of sterile glycerol to liquid cultures (Sambrook and Russell, 2001).

2.5. Agrobacterium tumefaciens-mediated transformation

Conidia of strain 70-15 were used as the recipient cells for transformation. Eight-day old spores were harvested with sterile distilled water (dH_2O) and filtered through sterile Miracloth (Calbiochem, LaJolla, CA). *A. tumefaciens* strains were streaked from glycerol stocks, onto Agrobacterium broth (AB) plates (1.4% agar) (Chilton et al., 1974) with appropriate antibiotics for selection, and were grown at 28 °C. A single colony was used to start each liquid culture.

When using EHA105/pAD1624 as the T-DNA donor, cells were grown in 2 ml of AB liquid medium (Chilton et al., 1974), modified by the use of 18 mM K_2HPO_4, 10 mM NaH_2PO_4, 0.2% glucose, 0.6 mM $CaCl_2$ with carbenicillin 60 μg/ml (USBiological, Swampscott, MA) for 24 h, at 28 °C, shaking at 250 rpm to reach an A_{600} of 1.0–1.5. A 300 μl aliquot of this culture was inoculated into 5 ml of Induction Medium (IM) (AB medium supplemented with 25 mM 2-(*N*-morpholino)-ethanesulfonic acid pH 5.8). Following growth at 250 rpm for 24 h at 28 °C (to reach an A_{600} of 0.5–1.0), the cells were pelleted and diluted to 1×10^8 cells/ml. For co-cultivation, 100 μl of the bacterial cells was mixed with 100 μl of conidia (10^6/ml) and the mixture was adjusted to 1 ml with IM. Under these conditions, the ratio of fungal to bacterial cells was 1:100. Other ratios were tested, as described in results. Cell aliquots (200 μl) were spread, using 3 mm sterile glass beads (VWR International), onto autoclaved black cellulose filters with 6 μm pores (catalog number, 470C10 Thomas Scientific, Swedesboro, NJ) which had been placed on IM agar Petri plates. The cells were co-cultivated for 48 h at 28 °C prior to selection (co-cultivation time was optimized as described in results). Nitrocellulose membranes (Whatman, Hillsboro, OR) were used during early transformations but were replaced by the less costly black cellulose filters in later transformations (Covert et al., 2001). The transformation efficiency using the cellulose filters was equivalent to the nitrocellulose ones and moreover the black color made it easier to distinguish the primary transformants.

For transformations using donor strain AGL1/pBHt2, bacterial cells were grown in 2 ml of minimal medium (Mullins et al., 2001) with 50 μg/ml kanamycin for 48 h, at 28 °C, shaking at 250 rpm. The cells were diluted to an A_{600} of 0.15 in 5 ml of induction medium (Mullins et al., 2001) containing 200 μM acetosyringone (Aldrich Chemical, Milwaukee, WI) and grown at 28 °C for 6 h to reach an A_{600} of 0.25. A 100 μl aliquot was mixed with 100 μl of the 10^6 conidia/ml suspension (in this mixture, the fungal cell/bacterial cell ratio was 1:250). The volume was adjusted to 1 ml and co-cultivation was carried out as described above. In the experiments where the transformation efficiency of the two *A. tumefaciens* donor strains was compared, the AGL1/pBHt2 cells were diluted to a concentration of 10^8 conidia/ml in order to use the same ratio of fungal to bacterial cells as was used for strain EHA105/pAD1624.

For selection of primary transformants the filters containing the co-cultivation mixture were transferred to CM agar (Crawford et al., 1986) supplemented with 20% sucrose, 350 μg/ml HygB (Calbiochem, LaJolla, CA) and either 100 μg/ml kanamycin (USBiological, Swampscott, MA) or 200 μM cefotaxime (USBiological, Swampscott, MA) for counter-selection against EHA105/pAD1624 or AGL1/pBHt2, respectively. Plates were incubated at 28 °C for 5–6 days at which point putative transformants were picked to oatmeal agar with 300 μg/ml HygB in 24-well plates for sporulation as described below. In some experiments, the black filters were cut into ~1 cm^2 pieces prior to plating the co-cultivation mixture. These pieces were then moved individually to selection medium and single transformants were picked from each filter following its growth into the agar, limiting the transfer of *A. tumefaciens* with the transformed culture.

2.6. Processing of transformants

Primary transformants were transferred to OA in 24-well plates to allow sporulation. The media contained 350 μg/ml HygB for selection and 100 μg/ml kanamycin or 200 μM cefotaxime to counterselect against *A. tumefaciens* growth. After 5 days of growth at 25 °C with con-

stant illumination (Fig. 2a), conidia were streaked onto CM agar containing 300 μg/ml HygB and either 100 μg/ml kanamycin or 200 μM cefotaxime to isolate monoconidial, homokaryotic cultures (Fig. 2b). After 15 h growth at 28 °C, a single germinated conidium per insertion line was picked using a sterile 1 ml tuberculin syringe with a 25-gauge needle or a No. 11 scalpel blade, and transferred to SCM in 24-well plates containing 200 μg/ml HygB. Each well of the 24-well plate contained three sterile 3 mm diameter cellulose filter discs (Whatman 3MM) generated with a paper hole punch. Monoconidial cultures were grown at 28 °C for 3 days and then shifted to 25 °C under constant illumination. Early insertion lines were grown on CM agar + $HygB_{200}$ (Crawford et al., 1986) at 28 °C without illumination, resulting in only mycelial growth on the filters used for long-term storage. However, concern about the long-term viability of stored strains led us to replace the CM agar with SCM agar. After 7 days the growth rate of each strain, relative to that of strain 70-15, was recorded as the first phenotypic characteristic of these homokaryotic isolates (Fig. 2b). Insertion lines that did not produce HygB resistant colonies following streaking for single conidial isolates, were recorded as lines with insertions in potentially essential genes. The prediction is that homokaryotic conidia with an insertion in an essential gene would be non-viable, but the mutation could be maintained in a heterokaryon containing untransformed nuclei. For these lines, long term storage cultures were generated by transfer of mycelial plugs from the original OA 24-well plate culture to CM + $HygB_{200}$ agar plates containing filter paper disks.

After 7 days of growth, filter papers were peeled from each 24-well plate and transferred to a quadrant of three separate 96-well plates resulting in three copies of each transformed line. Each 96-well microtiter plate contained strains from four 24-well plates (Fig. 2c and d). The strains were desiccated for 7–14 days and then stored in sealed containers in the presence of desiccant at −20 °C. The three 96-well microtiter plates were distributed as follows: one was sent to the Fungal Genetics Stock Center (FGSC, Kansas City, Missouri) for long term storage and distribution of strains to the research community, one was stored at the site of generation of insertion lines (either the University of Arizona or the University of Kentucky) and the third set was sent to the Dean laboratory at North Carolina State University (NCSU) for pathogenicity screening (Fig. 2c).

For production of DNA from insertion lines, a mycelial plug was transferred from the 24-well SCM plate that contained the filters for storage (Fig. 2b), into complete liquid medium (Crawford et al., 1986) in a 24-well plate, and incubated at 28 °C for 5 days prior to DNA extraction for Southern analysis (Fig. 2e and f). All phenotypic screens, were initiated with conidia from the transformed 70-15 lines, produced on oatmeal agar in a 24-well plate by transfer of a plug of fungal tissue (Fig. 2g).

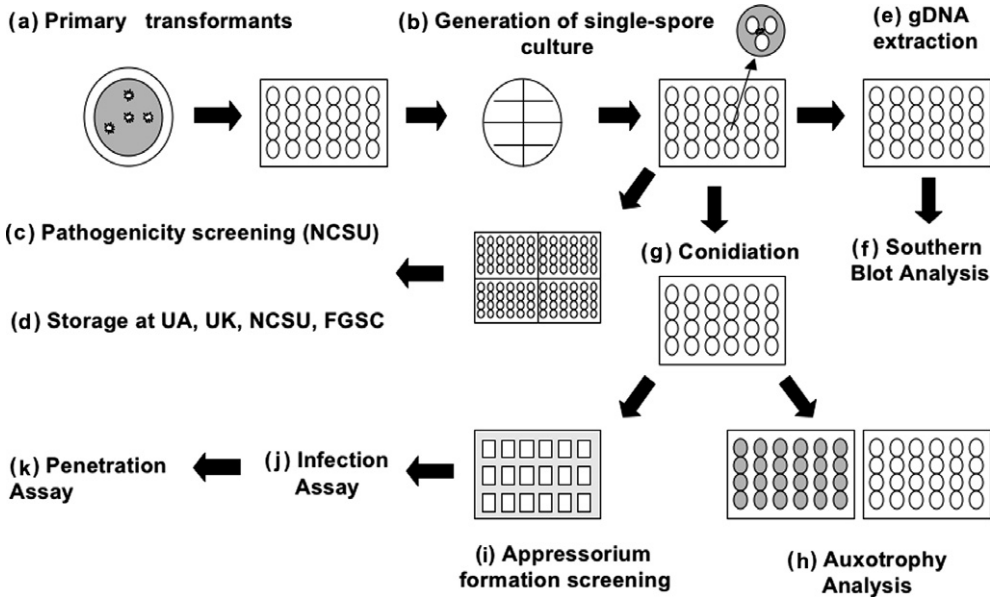

Fig. 2. Pipeline for processing and phenotypic screening of 70-15 transformed lines. (a) Primary Hyg^R colonies were picked into 24-well plates of OA with selection for transformed *M. oryzae* and counterselection against *A. tumefaciens* growth. (b) Spores from (a) were streaked onto CM plates for isolation of monoconidial, homokaryotic cultures. Monoconidial colonies were transferred to SCM agar with selection in 24-well plates containing cellulose filter discs for long-term strain storage. (c) Insertion strains in 96-well microtiter dishes were sent to NCSU for high throughput pathogenicity screening. (d) Replicas of all 96-well microtiter dishes were stored at the institution where strains were generated and were sent to the FGSC for distribution to the public. (e) Cultures were inoculated and grown in 24-well plates for gDNA extraction. (f) Southern blot analysis was carried out to characterize DNA insertion patterns. (g) Conidia of the monoconidial cultures were produced in 24-well OA plates for use in phenotypic analysis. (h) Conidia from each fungal strain were assayed for auxotrophy by parallel inoculation in CM and minimal media. (i) Conidia were also used to screen for in vitro appressorium formation on glass mirrors. (j) Insertion lines with aberrant appressorium development are tested for pathogenicity on rice plants. (k) Insertion lines that are non-pathogenic or reduced in pathogenicity, are assayed for penetration on onion epidermis.

2.7. Phenotypic screening of transformed lines

A high throughput infection assay was developed for primary screening of the 70-15 insertion lines (Fig. 2c). Fungal inoculum was produced from cultures grown from filter papers, in 24-well plates containing 8% V8 agar (per liter: 80 ml V8, 15 g agar, pH 7.0). Conidia were harvested by addition of 1 ml of Tween 20 to each well, followed by gentle scraping of the culture with a sterile cotton swab. The spore suspensions were then transferred to sterile 96-well deep-well plates. Manual sprayers (Sprayette IV, output = 0.75 ml, closures = 24–410; Saint Gobain, Calmar, Charlotte, NC) were placed into each well and used to spray the conidial suspension onto rice seedlings grown in test tubes. Seedlings of rice cultivar M202 were grown for 10 days following manual de-coating of the seeds, and surface sterilization using 2% bleach for 20 min. Three seeds were placed in 15 cm × 2.5 cm disposable culture tubes (Fisher Scientific, Pittsburgh, PA) containing MS agar; per liter, 4.3 g MS (RPI, MT. Prospect, IL), 12 g agar (EMD chemicals Inc, Gibbstown, NJ) and 30 g sucrose. Seedlings were grown at 25 °C under constant illumination, using two 40 watt Sylvania "Gro-Lux" light bulbs. After inoculation, racks containing the tubes of inoculated plants were placed in dark bags and put back in the 25 °C growth chamber for 24 h, after which the bags were removed and the plants grown under constant illumination as above. Disease symptoms were scored 7 days after infection using a scale of: 0 = no disease, 1 = reduced disease, 2 = same as wild type, 3 = more disease than wild type. Seedlings were reassessed 2 days later to verify results. For insertion lines that produced a rating of 0 or 1, spores from a second culture from the original filter paper stock were harvested to conduct a secondary pathogenicity screen. The secondary screen was performed as per the primary screen, except that two tubes of seedlings were treated with each fungal strain, and spores were suspended and inoculated in 0.25% gelatin instead of Tween 20. Tertiary screens were carried out using a quantitative infection assay where a defined spore inoculum was sprayed onto 2-week old rice seedlings grown in pots, as described previously (Valent et al., 1991). In addition to analysis of pathogenicity, several other phenotypic screens were performed on the monoconidial cultures (Fig. 2b), beginning with the assessment of colonial growth rate as described above. Assessment of pigmentation and conidiation were carried out as the cultures were growing on the OA plates (Fig. 2g) used for production of conidia for auxotroph screening (Fig. 2h) and for analysis of in vitro appressorium development (Fig. 2i see below).

Auxotrophic mutants were detected by their inability to grow on minimal medium in contrast to complete medium (Fig. 2h). Minimal medium (MM) was made either according to the recipe of Vogel (Vogel, 1964), or that of Talbot (Talbot et al., 1997). Both 1.5% agar (Sigma) and 1% agarose (BioWhitaker Molecular Applications) were used as solid base, with some indication that auxotrophs were easier to detect with agarose. Screens were initiated by transferring conidia from oatmeal agar onto MM agar and CM agar in parallel. After 7 days, growth was recorded as "normal" (equivalent to wild type), "slow" or "no growth." Lines showing non-wild type growth were re-tested twice to confirm the auxotrophy. The nutritional requirements of auxotrophs were determined using the combinatorial screen developed by Holliday (Holliday, 1956) and confirmed by testing growth on MM supplemented with the nutrient(s) thus identified.

2.8. In vitro screen for appressorium development

Conidia were collected from 24-well oatmeal agar plates using 35μl of sterile 0.25% gelatin, and were spotted on the surface of a 15.5 cm × 11.4 cm × 0.5 cm Pilkington Eclipse Blue Green Reflective Glass plate (Pilkington plc, UK) (Fig. 2g). Green glass mirrors were either a gift from Dr. Y.-H. Lee (Seoul, Korea), or were purchased from Tucson Glass (Tucson Glass and Mirror, Tucson, AZ). Plates were incubated for 6 h at room temperature in moist chambers, to allow conidial attachment and germination, prior to placing coverslips over each spore spot. Germination and appressorium development were scored for a random sample of 20 conidia, 24 h after inoculation, and compared to parental strain 70-15 in each trial (Fig. 2i). All lines that appeared to be defective either in conidial morphology, germination, germ tube development, appressorium development or appressorium morphology were re-assayed twice.

Insertion lines defined as appressorial variants after three screens were tested in rice seedling pot infection assays on cultivars M202, Maratelli and Sariceltik, or on CO39 and 51583 to screen for defects in pathogenicity (Fig. 2j) as described above for the tertiary screens (Valent et al., 1991). Quantitative analysis of conidiation was also performed on those cultures that exhibited altered appressorium development.

2.9. Appressorium penetration assay

Conidia from appressorial variant lines that were altered in pathogenicity, or that appeared to have morphological defects were assayed for ability to penetrate plant tissue using an onion epidermis screen (Balhadere et al., 1999). Conidial germination, appressorium development and growth of infectious hyphae were scored at 24 h and 48 h post inoculation (Fig. 2k). Data was recorded for a random sample of 100 conidia. Onion epidermal penetration assays were repeated three times for each strain tested.

2.10. Molecular analysis of transformants

Genomic DNA was prepared from transformants as described previously (Fig. 2e) (Sweigard et al., 1990). DNA was digested with restriction endonucleases from New England Biolabs (Beverly, MA) or Invitrogen

(Carlsbad, CA) following the manufacturers protocols and separated by gel electrophoresis. Southern hybridization analysis was carried out as described in Sambrook and Russell (Sambrook and Russell, 2001) (Fig. 2f). To detect the presence of transforming DNA in insertion lines, the 1.4 kb HpaI fragment of pCB1004 (Carroll et al., 1994) was labeled with ^{32}P-dCTP for use as a hygromycin phosphotransferase gene-specific probe. Also a 600 bp EcoRI-BglII fragment from the T-DNA in pAD1624 (Fig. 1b) was used in hybridization experiments and this enabled detection of the segments corresponding to the left and right ends of the transferred DNA, and the associated genomic DNA at the site of insertion. In addition to Southern hybridization analysis, TAIL-PCR and Inverse PCR (iPCR) were performed on selected insertion lines to rescue sequence junctions between the transforming T-DNA and the *M. oryzae* chromosome. T-DNA flanking regions were rescued by TAIL-PCR as described by Liu and Whittier (1995) with some modifications. Degenerate primers TP1, TP3, TP5, or TP6 were used with the specific primers for each insertion vector (see Supplemental Table 1) according to the conditions reported (Arie et al., 1997). The final PCR products were purified using the Qiagen PCR purification kit (Qiagen, Inc., Chatsworth, CA) and sequenced.

IPCR was performed by standard methods (Meng et al., 2007; Ochman et al., 1988), by digesting genomic DNA of transformed lines either with restriction enzymes that did not cut within the T-DNA (PstI for vector pAD1624) in order to recover both insertion junctions, or by digestion with enzymes that cut within the T-DNA, allowing targeted recovery of the left or right border junction (EcoRI or NcoI for vector pAD1624 (Fig. 1b) and MspI for pBHt2). For recovery of both junctions from a single iPCR product, primers were designed to amplify DNA sequences adjacent to the T-DNA left and right borders, OAM744 and OAM747, respectively, (Supplemental Table 1). For recovery of the left and right border junctions separately, one primer was directed towards the T-DNA border and the other towards the restriction site within the T-DNA used to generate the iPCR product. For the pAD1624 left border junction, the border primer was OAM744 and the primer associated with the internal restriction site was OAM787 (Supplemental Table 1). For the pAD1624 right border junction, the border primer was OAM747 and the primer associated with the internal restriction site was either OAM431 for EcoRI digestions, or OAM617 for NcoI digestions (Supplemental Table 1). For analysis of the pBHt2 left border junction, primers L-2 and L-1 were used, associated with the T-DNA left border and the adjacent internal MspI site, respectively (Supplemental Table 1). For analysis of the pBHt2 right border junction, primers R-2 and R-1 were used, associated with the T-DNA right border and the adjacent internal MspI site, respectively (Supplemental Table 1). The nested primers used for sequencing iPCR products are also listed in Supplemental Table 1.

2.11. Data management

A web-based data entry system was created for entering, storing and analyzing the large amount of data generated at multiple universities. 'PACLIMS' (Phenotype Assay Component Laboratory Information Management System) allows barcode scanning of plates at all stages of data collection (Donofrio et al., 2005). Barcodes were attached to plates at each step of the pipeline (Fig. 2), beginning with a "master plate" which was the 24-well plate containing the monoconidial strains (Fig. 2b) and the three filter papers used for storage and strain distribution (see above). Data entered for the master plate included information on the method of transformation, and the vector that was used to create the strain. Barcodes were scanned into PACLIMS, and menus appear for entry of detailed information pertaining to the 'type' of plate being used (i.e. media type). All plates were associated with a master plate in order to link together all information gathered for each strain. Once a plate has gone through the whole screening process, the user can track any isolate within that plate simply by entering the barcode, and following prompts. All data generated in PACLIMS was downloaded to a publicly accessible web-based database (MGOS, *Magnaporthe grisea*, *Oryza sativa*) allowing public querying of all the mutant information (www.mgosdb.org) (Soderlund et al., 2006). Data was downloaded daily into a developmental version of MGOS, where it was verified, by the laboratory that entered it, before being made available to the public.

3. Results

3.1. Protoplast transformation

A total of 17,128 insertion lines were generated by PEG-mediated transformation of 70-15 protoplasts with linear DNA fragments. Six different constructs were used for transformation, three were variants of the Hyg^R cassette in pCB1004, and three were based on pAM1270. Each set consists of the initial vector containing a basic transformation fragment and two variants that differ by the addition of ~100 bp AT-rich or GC-rich segments flanking the fragment. The set based on pCB1004 contains the Hyg^R selectable marker while the pAM1270 set additionally contains two promoterless fluorescent protein genes flanking the Hyg^R gene (Fig. 1a). The rationale for having different end sequences on the vectors (AT-rich, GC-rich and neutral) was that if the ends of linear DNA fragments aid insertion into regions of similar sequence composition by microsynteny, than having three different end types may increase the distribution of inserts through the genome. Transformation efficiency was optimized by limiting the number of protoplasts to 5×10^6/ml and varying the ratio of DNA fragment to protoplasts. All DNA fragments produced similar efficiencies of 15.5 ± 4.7 primary transformants per μg of DNA. Problems with the nature of DNA insertions (see below), and the requirement for

purification of the transforming fragment limited the utility of this approach for high-throughput generation of insertion lines.

3.2. Optimization of Agrobacterium-mediated transformation conditions

Initial ATMT experiments were plated on selection medium lacking the osmotic stabilizer sucrose because ATMT does not use protoplasts. However, high background growth was observed after transferring co-cultivation filters to the selective media containing 300 μg/ml of HygB, the level that was used for selection of protoplast transformants of strain 70-15. The appropriate amount of HygB required to select ATMT primary transformants was determined by screening on complete media in the presence or absence of 20% sucrose with HygB at concentrations of 300, 350 and 400 μg/ml. The presence of sucrose significantly decreased background growth at all HygB concentrations. For transformation of 70-15, HygB at 350 μg/ml in media containing 20% sucrose provided sufficient selection.

Two different *A. tumefaciens* donor strains were used to deliver transforming DNA to *M. oryzae* conidia, AGL1, containing either vector pBHt1 or pBHt2 (Mullins et al., 2001) and EHA105/pAD1624. ATMT was optimized for *M. oryzae* 70-15 by determining the effects of varying cell ratios between donor *A. tumefaciens* and recipient *M. oryzae* conidia, and by varying the length of cell co-cultivation. Ratios of *A. tumefaciens* cells to *M. oryzae* conidia from one conidium to 10, 20, 50 and 100 bacterial cells were tested for both *A. tumefaciens* strains, by using 10^5 conidia per incubation and varying the number of bacterial cells. Increasing efficiencies were obtained as the bacterial cell:conidium ratio increased (Fig. 3a). In early experiments a ratio of one conidium to 500 EHA105/pAD1624 cells was also tested. The transformation frequency with this ratio did not differ significantly from the efficiency when a ratio of 1:100 was used (2.3 versus 2.0 transformants per 10^3 conidia, respectively, data not shown). Based on these data, further optimization experiments for strain EHA105/pAD1624 were carried out using the ratio of one conidium to 100 *A. tumefaciens* cells.

The effect of varying the co-cultivation time from 8 to 48 h was tested by mixing induced *A. tumefaciens* EHA105/pAD1624 or AGL1/pBHt2 cells with 70-15 conidia at a ratio of one conidium/100 bacterial cells and plating on non-selective media. After this period of incubation, the cells were transferred to selective media. No transformants were observed using co-cultivation times of 32 h or less (Fig. 3b). Transformed colonies were first seen following 40 h of co-cultivation and significant improvement was seen after 48 h of non-selective growth (Fig. 3b). With EHA105/pAD1624, 3.2 transformants were obtained per 10^3 conidia after 48 h of co-cultivation (Figs. 3a and b). In contrast, AGL1/pBHt2 produced 0.48 transformants per 10^3 conidia (Fig. 3b). Background growth on all plates was similar to that observed on control transformation plates using *A. tumefaciens* strains containing T-DNA plasmids lacking the Hyg^R gene. With a ratio of one conidium to 250 bacterial cells, AGL1/pBHt2 produced 1.45 transformants per 10^3 conidia with a co-cultivation time of 48 h, indicating that its transformation efficiency continued to improve significantly as the ratio of bacterial cells/conidia increased. Still, at the 1:250 ratio of conidium to bacterial cells, AGL1/pBHt2 had a transformation efficiency ~50% that obtained with strain EHA105/pAD1624 at a ratio of 1:100.

Agrobacterium strain EHA105/pAD1624 was used to generate 12,806 transformed lines of 70-15 using the optimized co-cultivation conditions of a ratio of one conidium to 100 *A. tumefaciens* cells and non-selective growth for 48 h. AGL1/pBHt1 was used to generate 5640 insertion lines of 70-15 and AGL1/pBHt2 to produce 20,521 lines.

3.3. High throughput processing of insertion lines for purification and phenotyping

Our main goal was to create and phenotypically analyze a collection of >50,000 *M. oryzae* lines by insertional mutagenesis, at a rate of ~300 strains per week in each of two laboratories. Processing strains involved the following steps:

(1) Picking primary transformants onto medium that allowed sporulation.
(2) Streaking conidia to obtain monoconidial, Hyg^R homokaryons.
(3) Growing the monoconidial isolates to create stocks for long-term storage and distribution.

When comparing the processing efficiency between protoplast and ATMT insertion lines, we observed a dramatic difference in the percentage of primary transformants that resulted in stored strains. Only 53% of the strains initially picked for sporulation from protoplast transformations produced hygromycin resistant monoconidial strains that were preserved in long-term storage, while 86% of the ATMT-generated strains were able to be processed to storage. This difference is significant considering the labor involved in processing each strain and suggests that 47% of the strains generated from protoplast transformations were likely false positives.

3.4. Molecular analysis of transformant strains

The ability to characterize insertional mutants efficiently via rescue of the disrupted genes is dependent on having simple DNA tag integration patterns. Therefore, we performed a detailed Southern hybridization analysis of the insertion patterns in transformed lines we generated both by protoplast transformation and by ATMT.

Transformants generated from protoplasts were screened with a Hyg^R gene probe following digestion of

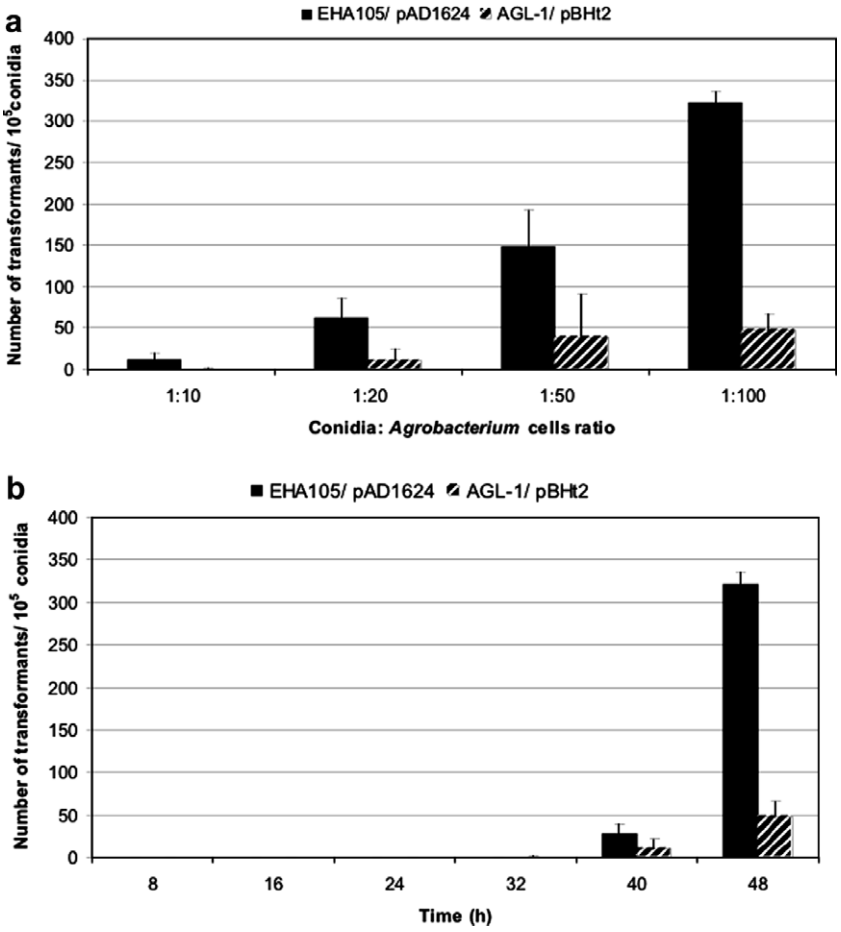

Fig. 3. (a) Effect of different conidium: *A. tumefaciens* cell ratios on the efficiency of *A. tumefaciens*-mediated transformation. Data is the average of the number of primary transformants in 12 plates for each of the cell ratios, using a co-cultivation time of 48 h. (b) Effect of different co-cultivation times on the efficiency of *A. tumefaciens*-mediated transformation. Data is the average of the number of primary transformants on 12 plates for each of the conditions. Experiments were repeated three times. Error bars represent standard error.

their genomic DNA with either XhoI or HpaI, neither of which cuts within the transforming DNA. Analysis of 122 transformants generated with the HpaI fragment corresponding to the Hyg^R gene cassette in pCB1004, revealed 86 with single, discreet hybridization signals (indicative of insertion at a single site in the genome); 32 with >1 band or one very large, intense band (suggesting insertion at more than location or a large tandem insertion, respectively); and four that lacked signals (false transformants). Interestingly, addition of AT- or GC-rich sequences to the ends of the same Hyg^R cassette resulted in a significantly higher frequency of multiple insertions, a phenomenon that was even more pronounced when promoterless DsRed and EGFP genes were added (data not shown). Analysis of 470 strains generated with either pAM1120, pAM1270, pAM1273 or pAM1274, revealed that 56% contain single intensely hybridizing high molecular weight fragments, >20 kbp in length (data not shown). These patterns suggest that the strains contain multiple copy tandem insertions at a single site. The presence of tandem insertions was confirmed by Southern analysis of selected transformants (data not shown). The genomic DNAs were digested with restriction enzymes that cut once within the transforming cassette, either PstI, for analysis of transformants generated with pAM1270, pAM1273 and pAM1274, or NcoI, for analysis of strains produced with pAM1120. Southern analysis revealed fragments that were predicted for tandem insertions of the transforming cassette in head-to-head, head-to-tail and tail-to-tail orientations (data not shown). An additional 22% of the strains had hybridization patterns indicating 2 or more insertions, with only 22% of the strains having single copy insertions.

During optimization of the ATMT procedure, genomic DNAs from 130 transformed strains were analyzed by Southern hybridization to determine whether increased co-cultivation time or varying the bacterial to conidial cell ratio had an effect on T-DNA insertion copy number. The DNAs were digested with PstI, which does not cut within the transferred DNA, and exhibited hybridization to predominantly single fragments. These fragments varied in size, but had uniform signal intensity. Neither of the optimization parameters had an effect on the T-DNA copy number, with 83% of the strains having single copy insertions, 9% of the strains having two copies of T-DNA and

8% of strains having three or more copies (Fig. 4 and data not shown).

A more detailed analysis of 283 ATMT lines indicated that a slightly lower percentage of strains have single copy insertions; 72% contained single copy T-DNA insertions, while 27% had two hybridizing fragments, indicating two insertions and the remainder displayed more complex patterns (data not shown).

4. Phenotypic analysis

Putative auxotrophs were identified by screening for strains that failed to grow, or grew poorly relative to strain 70-15 on minimal medium while exhibiting normal growth on complete medium. Of 32,192 strains 35 putative auxotrophs were identified. Among these strains, 18 are amino acid requiring, six are vitamin requiring, one requires uridine and the rest have not yet been categorized.

Initial pathogenicity screens produced a large number of strains that appeared different from strain 70-15. Of 33,943 strains tested, there were 4075 strains that showed reduced pathogenicity compared to wild type in the primary screen (N.Donofrio, personal communication). The majority of these were eliminated during secondary screens. Tertiary screens resulted in a total of 128 mutants. Of these, 38 were non-pathogenic while 18 showed a slight reduction in pathogenicity and 72, a substantial reduction in pathogenicity compared to wild type.

Of 49,248 strains screened for growth rate on complete media, 84.5% exhibited normal growth, 3.5% grew faster than 70-15, 5.6% grew slower, and 3.6% much slower than 70-15, while 2.8% failed to grow. Assessment of pigmentation on oatmeal media was performed on 48,257 strains with 99.9% appearing as wild type. There were 27 buff mutants, 9 albino mutants, and 18 strains exhibiting a reduction in pigmentation. Buff mutants are blocked at the reduction step from trihydroxynaphthalene to vermelone in the pentaketide pathway for melanin biosynthesis (Howard and Valent, 1996). Qualitative assessment of the rates of conidiation on oatmeal agar was performed on 48,264 strains and indicated that 1.6% of the strains produced more conidia than wild type and 4.9% produced fewer than wild type conidia. Initial data indicated a significant number of strains failed to sporulate; improvements to the sporulation assay eliminated these false positives. It is likely that aconidial strains would not have been recovered because strain processing required strains to sporulate to generate homokaryotic transformed strains.

To supplement the screening for pathogenicity mutants on plants, an in vitro appressorium assay was initiated by spotting conidia on reflective glass mirror and scoring after 24 h of growth. Of 12,000 strains screened, 135 were observed to be morphologically different than wild type. These differences included morphological defects in conidia, lower conidiation than wild type, failure to form appressoria, longer than normal germ tubes, germ tubes with multiple hooking attempts, branching germ tubes with multiple appressoria per conidium, delayed formation of appressoria, and abnormally shaped appressoria. The 135 abnormal strains were screened in pathogenicity assays on rice plants with 110 showing reduced pathogenicity and two being non-pathogenic. This high rate of mutant identification supplemented our total number of pathogenicity mutants. Strains exhibiting altered pathogenicity were screened for penetration on onion epidermis to characterize their post-appressorium developmental phenotypes. A range of phenotypes were observed, with many strains that exhibited reduced pathogenicity on rice showing reduced penetration on onion epidermis and extensive hyphal growth on the onion surface. However, some strains had wild type penetration on onion epidermis, indicating either host differences or post-penetration defects during the infection cycle.

4.1. Recovery of flanking sequences

The ends of 256 random insertions and their associated flanking *M. oryzae* sequences have been recovered from ATMT lines using TAIL-PCR and Inverse PCR (Meng et al., 2007). Frequently when attempting to recover sequences adjacent to the T-DNA left border, vector sequences were recovered indicating failure to cleave the T-DNA at the left border when transfer was initiated. Recovery of sequences adjacent to the T-DNA right border regularly yielded *M. oryzae* sequences, indicative of initiation of T-DNA transfer at the right border. Initial recovery of flanking sequences in a pool of 80 mutants with reduced pathogenicity identified 39 hypothetical genes, 13 ORFs without significant GenBank matches, and 11 intergenic regions (defined as insertions >1 kb from an annotated *M. oryzae* gene). The remaining T-DNA flanking regions were not informative due to the presence of tandem T-DNA insertions or transfer beyond the T-DNA left border. Table 1 presents a summary of some of the genes that were

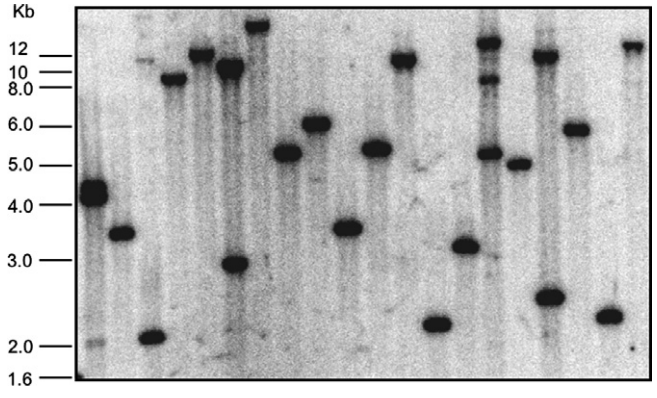

Fig. 4. Southern blot analysis of DNAs of ATMT transformed lines. Genomic DNAs of 20 random strains were digested with PstI, which does not cut within the transferred DNA fragment and hybridized with the Hyg^R gene of pCB1004. The DNA size markers are the 1 kb Plus DNA Ladder™ of Invitrogen Corp., and are listed in kilobases (kb) to the left of the figure.

Table 1
A sample of *Magnaporthe oryzae* genes identified by T-DNA flanking sequence recovery

Locus	Annotation	Conserved domains, motifs and protein families	BlastX		
			Putative function	Organism	*E*-value
MGG_03117.5	Hypothetical protein	Taurine catabolism dioxygenase TauD, TfdA family	Alpha-ketoglutarate dependent taurine dioxygenase	*Escherichia coli*	3.00E−27
MGG_05731.5	Hypothetical protein	Cyclophilin type peptidyl-prolyl cis–trans isomerase/CLD,WD domain, G-beta repeat	Cyclophilin type peptidyl-prolyl cis–trans isomerase	*Aspergillus fumigatus*	0E
MGG_01563.5	Hypothetical protein	ABC-2 type transporter	ABC transporter Adp1	*Aspergillus fumigatus*	0E
MGG_03473.5	Hypothetical protein	NmrA-like family (nitrogen metabolite repression)	NmrA-like family protein	*Neosartorya fischeri*	2.00E−25
MGG_09285.5	NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor	Respiratory-chain NADH dehydrogenase, 49 kDa subunit	NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor	*Neurospora crassa*	0E
MGG_08063.5	Pyruvate kinase	Pyruvate kinase, barrel domain, pyruvate kinase, alpha/beta domain	Pyruvate kinase	*Neurospora crassa*	0E
MGG_06361.5	Hypothetical protein	Dynamin central region, dynamin family, dynamin GTPase effector domain	Dynamin-related protein 1	*Podospora anserina*	0E
MGG_02425.5	Hypothetical protein	Kinesin motor domain	Kinesin	*Gibberella moniliformis*	0E
MGG_07065.5	Hypothetical protein	Cyclin, C and N-terminal domains	G2/mitotic-specific cyclin cdc13	*Aspergillus terreus*	3.00E−132
MGG_01588.5	DNA damage checkpoint protein rad24	14-3-3 domain	DNA damage checkpoint protein rad24	*Chaetomium globosum*	4.00E−132

identified via recovery of *M. oryzae* sequences flanking T-DNA inserts in pathogenicity mutants with additional genes presented in Supplemental Table 2. Genetic complementation and/or targeted gene disruption of a limited number of these mutants have demonstrated their role in pathogenicity. These include a putative cyclophilin (MGG_05731.5), a putative ABC transporter (MGG_01563.5) and a hypothetical protein with domains suggesting it functions in taurine catabolism (MGG_03117.5) (Table 1). Efforts to complement other mutant strains are underway.

5. Discussion

We report the construction of a comprehensive insertion library of *Magnaporthe oryzae* strain 70-15 that represents a community resource to understand gene function in this model ascomycete plant pathogen. This is the largest such resource available for any phytopathogenic fungus. The main goal is to unravel the molecular interactions that occur between *M. oryzae* and its hosts, but this library will also serve to study metabolism, morphogenesis and all other aspects of fungal biology. Since our objective was to create >50,000 fungal lines by insertional mutagenesis we needed to develop an efficient high-throughput transformation system to generate and process these strains. Two additional objectives were to develop a transformation process that produces primarily a single insertion per strain to simplify identification of disrupted genes, and that minimizes the rate of false positive transformants thus decreasing the amount of effort needed to generate each strain. Use of multiple transformation methods and multiple vectors per method was done to increase the likelihood of having insertions throughout the genome in case a single method showed sequence targeting bias. Our data show that *A. tumefaciens*-mediated transformation is clearly superior to $CaCl_2$/PEG-mediated protoplast transformation in terms of labor to generate strains, as well as the nature of the insertion patterns. ATMT had advantages in every aspect of the process. First, ATMT is less invasive to the cell since the recipient is a conidium and does not require removal of the cell wall, or any other processing prior to introduction of the transforming DNA. This also means there is less effort involved in recipient cell generation. Second, there is a much higher rate of success in processing primary transformants through to homokaryotic lines ready for storage and analysis, with nearly 90% of ATMT strains surviving the process, while only slightly more than half (53%) of protoplast lines were retained. Third and most importantly, the insertion patterns of ATMT lines resulted in primarily single insertions. The varied banding pattern of insertions and their associated flanking DNA in Southern analysis suggests that they were random (Fig. 4). The ATMT insertion patterns contrast dramatically with those of the protoplast transformants where the majority of strains generated had insertions of multiple, tandem repeats. While these apparently single site insertions still

make it possible to identify and analyze important insertion mutants, the tandem nature of the insertions makes the recovery of flanking genomic DNA by methods such as TAIL-PCR or iPCR more difficult. Due to the limitations of strains generated through protoplast transformation we produced 69.5% of our 56,095 strains by ATMT.

From the results described here, and in previous publications (Abuodeh et al., 2000; Mullins et al., 2001; Rho et al., 2001), it has been shown that the efficiency of ATMT increases with longer co-cultivation times of up to 48 h (Fig. 3b). In contrast to data from Rho and coworkers (Rho et al., 2001) also working with *Magnaporthe*, we never observed primary transformants after only 24 h of co-cultivation. We occasionally saw colonies after 32 h of co-cultivation when using EHA105/pAD1624 as the donor strain, however a six-fold improvement in numbers was observed by increasing the co-cultivation time from 40 to 48 h (Fig. 3b). When AGL1/pBHt2 was used as the donor strain as in (Rho et al., 2001) we did not see transformants unless co-cultivation was for at least 40 h, and a two-fold improvement was observed if co-cultivation continued for another 8 h (Fig. 3b). An analysis of insertion patterns indicated that there was no increase in the number of DNA insertions with extended co-cultivation time. A second factor in understanding transformation efficiency is the ratio of fungal to bacterial cells. It is reasonable to expect that an increased number of bacterial cells per conidium would increase the likelihood that a conidium would be contacted and transformed by *A. tumefaciens*. Our results suggest this is the case, with improved efficiencies of transformation for EHA105/pAD1624 up to a ratio of 1:100 fungal to bacterial cells, and improved efficiencies at least to 1:250 when using AGL1/pBHt2 as the donor strain (Fig. 3a and data not shown). The EHA105/pAD1624 transformation system appears to be more efficient than AGL1/pBHt2 for generation of primary transformants, with a six-fold difference when the cells are mixed at a ratio of one conidium per 100 bacterial cells (Fig. 3). It is unclear why EHA105/pAD1624 is a more efficient donor strain, because the two *A. tumefaciens* strains are quite similar genetically, having both been derived from EHA101, a strain that contains the hypervirulent, attenuated tumor-inducing plasmid pTiBo542, from which the T-DNA was precisely deleted and replaced with a kanamycin resistance gene (Hood et al., 1986). EHA105/pAD1624 is composed of EHA105, which is a kanamycin sensitive derivative of EHA101 (Hood et al., 1993), containing the donor plasmid pAD1624. AGL1 is a *rec*A derivative of EHA101 (Lazo et al., 1991). A significant difference in the way the strains are prepared for transformation has to do with induction of the *vir* genes. While AGL1/pBHt2 requires acetosyringone to induce *vir* genes, the binary vector pAD1624 in EHA105 contains a constitutive *virG* gene, *virG*N54D (Pazour et al., 1992), which makes the strain acetosyringone independent. Although we were concerned that optimizing transformation efficiency by increasing co-cultivation time or bacterial cell to conidium ratios might increase the number of insertions per recipient, we observed no significant difference under all the conditions tested with either *A. tumefaciens* donor strain. These results are similar to those observed for *Fusarium oxysporum* and *Beauveria bassiana* (Leclerque et al., 2004; Mullins et al., 2001) but appear to be different from what was previously reported for *Magnaporthe* (Rho et al., 2001) where it was claimed that increasing the co-cultivation time decreased the frequency of transformants containing a single copy of T-DNA. We observed that regardless of incubation time or cell/cell ratio, 72–88% of the transformed lines contained a single insertion copy, which is consistent with what has been observed in *F. oxysporum*, *B. bassiana* and *Leptosphaeria maculans* (Blaise et al., 2007; Leclerque et al., 2004; Mullins et al., 2001). The level of transformation efficiency of *M. oryzae* using AGL1/pBHt2 is comparable to that found for *B. bassiana* (Leclerque et al., 2004), and other filamentous fungi like *Aspergillus awamori, Neurospora crassa* and *Fusarium venenatum* (de Groot et al., 1998) but lower than that reported for *Magnaporthe* (Rho et al., 2001) and is slightly higher than that observed for *F. oxysporum* (Mullins et al., 2001).

One goal in creation of this insertion strain library was to identify pathogenicity mutants that have not been found previously by creating mutants first and then screening their phenotypes. Other projects on a smaller scale have used REMI to demonstrate the validity of this approach (Balhadere et al., 1999; Sweigard et al., 1998). Our results indicate that ATMT is a very amenable method for performing large-scale reverse genetics. The scale of this project and development of high throughput screens for mutant analysis should make this a model to be followed by others. To maximize the efficiency of the insertion line pipeline, minimize reagent use and limit potential recording errors, assays were done in 24-well plates, strains were placed in 96-well plates for long-term storage, and all of these plates were bar-coded and registered in our web-based PACLIMS database (Donofrio et al., 2005). A final goal of this project was to make the strains, and information analyses associated with them available to the research community. For this, the strains were deposited at the FGSC, and a web-based database, MGOS (www.mgosdb.org), was developed (Soderlund et al., 2006).

Both via the in vivo high throughput plant assay we developed, and an in vitro assay for infection structure development, we have identified more than 200 new mutant strains. Characterization of the gene defects in these strains is in progress and will be the subject of future reports. As presented in Table 1, recovery of sequences flanking T-DNA insertions from a small subset of pathogenicity mutants, revealed potential new genes important for host infection. Some of these belong to protein families that have been previously demonstrated to play a role in pathogenicity. For example, we have complemented a putative cyclophilin mutant, different from the previously characterized *cyp1* mutant (Viaud et al., 2002). *CYP1* is a cyclophilin in *M. oryzae* that is required for host penetration (Viaud

et al., 2002). The role of CYP1 in virulence supported the idea that appressorium differentiation is a process regulated by calmodulin-dependent signaling (Lee and Lee, 1998; Viaud et al., 2002). Cyclophilins have also been shown to be important for the pathogenicity of the human pathogen *Cryptoccocus neoformans* (Wang et al., 2001). Cyclophilins are present in all eukaryotes and function as peptidyl prolyl cis–trans isomerases in a variety of cellular processes such as response to environmental stresses, cell cycle control, calcium signaling and transcriptional repression (Talbot, 2003; Wang et al., 2001).

Additionally we identified an ABC (ATP binding cassette) transporter, different from the previously characterized *ABC1* (Urban et al., 1999), that is involved in pathogenicity in *M. oryzae*. Our ABC transporter mutant is significantly reduced in virulence but still occasionally is able to produce lesions. Characterization of *ABC1*, an ATP-driven efflux pump, in the rice blast fungus indicates that these proteins could constitute an important part of the mechanism by which *M. oryzae* detoxifies plant defense compounds such as rice phytoalexins (Urban et al., 1999).

Although, our initial T-DNA insertion analysis has not identified some of the already characterized pathogenicity genes in *M. oryzae*, we attribute that to the small subset of mutants analyzed. It is encouraging that several new genes have been identified since, our primary interest was to identify new pathogenicity genes. In addition to the utility of these strains for identification of pathogenicity mutants, DNA pools have been isolated from all plates of strains and could be used to screen for a mutation in a particular gene of interest. Although our primary goal was to identify strains affected in host-pathogen interactions, we also generated strains affected in growth rate, metabolism, pigmentation and morphology. Analysis of auxotrophs indicated that 0.11% of the transformed lines tested failed to grow on minimal medium but grew on complete medium. This number was lower than expected, based on the 0.35% identified by UV mutagenesis in *Magnaporthe* (Crawford et al., 1986). We believe this was due to inconsistent results in early screens using agar media. Using agarose as a support media decreased variability in later auxotroph screens.

Our initial efforts to recover T-DNA flanking regions in order to identify genes involved in pathogenicity, produced results similar to those from the analysis of insertion lines produced by plant transformation. ATMT is known to initiate transfer of DNA at the T-DNA right border and terminate transfer at the left T-DNA border, resulting in transfer of all DNA between the borders (Gelvin, 2003; Ream, 1989; Tinland, 1996). However, often transfer continues beyond the left border (De Buck et al., 2000; Forsbach et al., 2003; Ooms et al., 1982; Tinland, 1996; Tinland et al., 1995). This was in agreement with our results where recovery of *M. oryzae* sequences from the right border junction occurred at a greater frequency than adjacent to the left border.

An important question regarding insertion mutants is whether the insertion was responsible for the phenotypic defect observed. In ATMT of *Arabidopsis*, only 35–40% of mutants identified from T-DNA insertion libraries are tagged by T-DNA (AzpirozLeehan and Feldmann, 1997). We expect the rate to be higher in *M. oryzae* both based on the REMI reports (Balhadere et al., 1999; Sweigard et al., 1998) and on initial recovery of genomic sequences flanking insertions of several auxotrophic strains. Complementation experiments of nine mutant strains with wild type gene copies, demonstrated in all cases that the DNA insertion was responsible for the mutant phenotype (Meng, Betts, Farman and Orbach, unpublished). Much more recovery work needs to be done to determine the actual percentage of mutant genes that are tagged in our library. In a pilot scale ATMT experiment with the *Brassica napus* pathogen *L. maculans*, 3000 insertion lines were generated (Blaise et al., 2007). Analysis of 12 pathogenicity mutant lines by genetic crosses indicated that six mutant phenotypes co-segregated with the transforming DNA, suggesting 50% of mutants are tagged.

Analysis of insertional mutagenesis projects of *Arabidopsis* (AzpirozLeehan and Feldmann, 1997) and yeast (Bundock et al., 2002), indicate that T-DNA inserts are distributed throughout the genome. Our analysis of 256 insertions suggests that insertions are distributed throughout the genome although there may be some bias in insertion sites (Meng et al., 2007). This suggests that our collection of strains is likely to contain mutants for many *M. oryzae* genes, but the site bias may preclude some genes from mutagenesis unless a larger set of insertion lines is generated. To determine the overall randomness of insertion will require analysis of a larger set of insertion lines.

The availability of the genome sequence of *M. oryzae* (Dean et al., 2005) makes it relatively simple to identify the genes disrupted by insertions because of the ability to recover the DNA tags and their flanking genomic sequences. As more data on pathosystems like that of *M. oryzae* and rice become available more refined approaches can be integrated in disease management strategies in the agricultural field. The generation and availability of this library provides a great tool to study many of the mechanisms of disease development and the biology of *M. oryzae*. We expect that many of the techniques described here could be applied to work with other organisms. Moreover, we expect that future data obtained from the use of this library will enlighten research in other systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2007.05.001.

References

Abuodeh, R.O., Orbach, M.J., Mandel, M.A., Das, A., Galgiani, J.N., 2000. Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. Journal of Infectious Diseases 181, 2106–2110.

Arie, T., Christiansen, S.K., Yoder, O.C., Turgeon, B.G., 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved MAT HMG box. Fungal Genetics and Biology 21, 118–130.

AzpirozLeehan, R., Feldmann, K.A., 1997. T-DNA insertion mutagenesis in *Arabidopsis*: going back and forth. Trends in Genetics 13, 152–156.

Balhadere, P.V., Foster, A.J., Talbot, N.J., 1999. Identification of pathogenicity mutants of the rice blast fungus *Magnaporthe grisea* by insertional mutagenesis. Molecular Plant–Microbe Interactions 12, 129–142.

Blaise, F., Rémy, E., Meyer, M., Zhou, L., Narcy, J.-P., Roux, J., Balesdent, M.-H., Rouxel, T., 2007. A critical assessment of *Agrobacterium tumefaciens*-mediated transformation as a tool for pathogenicity gene discovery in the phytopathogenic fungus *Leptosphaeria maculans*. Fungal Genetics and Biology 44, 123–138.

Borromeo, E., Nelson, R., Bonman, M., Lueng, H., 1993. Genetic differentiation among isolates of *Pyricularia* infecting rice and weed hosts. Phytopathology 83, 393–399.

Bundock, P., van Attikum, H., den Dulk-Ras, A., Hooykaas, P.J.J., 2002. Insertional mutagenesis in yeasts using T-DNA from *Agrobacterium tumefaciens*. Yeast 19, 529–536.

Carroll, A., Sweigard, J., Valent, B., 1994. Improved vectors for selecting resistance to hygromycin. Fungal Genetics Newsletter 41, 22.

Chao, C.C.T., Ellingboe, A.H., 1991. Selection for mating competence in *Magnaporthe grisea* pathogenic to rice. Canadian Journal of Botany – Revue Canadienne De Botanique 69, 2130–2134.

Chida, T., Sisler, H.D., 1987. Effect of inhibitors of melanin biosynthesis on appressorial penetration and reductive reactions in *Pyricularia oryzae* and *Pyricularia grisea*. Pesticide Biochemistry and Physiology 29, 244–251.

Chilton, M.D., Currier, T.C., Farrand, S.K., Bendich, a.J., Gordon, M.P., Nester, E.W., 1974. *Agrobacterium tumefaciens* DNA and Ps8 bacteriophage DNA not detected in crown gall tumors. Proceedings of the National Academy of Sciences of the United States of America 71, 3672–3676.

Covert, S.F., Kapoor, P., Lee, M.H., Briley, A., Nairn, C.J., 2001. *Agrobacterium tumefaciens*-mediated transformation of *Fusarium circinatum*. Mycological Research 105, 259–264.

Crawford, M.S., Chumley, F.G., Weaver, C.G., Valent, B., 1986. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. Genetics 114, 1111–1129.

De Buck, S., De Wilde, C., Van Montagu, M., Depicker, A., 2000. T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. Molecular Breeding 6, 459–468.

de Groot, M.J.A., 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nature Biotechnology 16, 1074.

de Groot, M.J.A., Bundock, P., Hooykaas, P.J.J., Beijersbergen, A.G.M., 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nature Biotechnology 16, 839–842.

Dean, R.A., 1997. Signal pathways and appressorium morphogenesis. Annual Review of Phytopathology 35, 211–234.

Dean, R.A., Talbot, N.J., Ebbole, D.J., Farman, M.L., Mitchell, T.K., Orbach, M.J., Thon, M., Kulkarni, R., Xu, J.R., Pan, H.Q., Read, N.D., Lee, Y.H., Carbone, I., Brown, D., Oh, Y.Y., Donofrio, N., Jeong, J.S., Soanes, D.M., Djonovic, S., Kolomiets, E., Rehmeyer, C., Li, W.X., Harding, M., Kim, S., Lebrun, M.H., Bohnert, H., Coughlan, S., Butler, J., Calvo, S., Ma, L.J., Nicol, R., Purcell, S., Nusbaum, C., Galagan, J.E., Birren, B.W., 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature 434, 980–986.

Donofrio, N., Rajagopalon, R., Brown, D., Diener, S., Windham, D., Nolin, S., Floyd, A., Mitchell, T., Galadima, N., Tucker, S., Orbach, M.J., Patel, G., Farman, M., Pampanwar, V., Soderlund, C., Lee, Y.H., Dean, R.A., 2005. 'PACLIMS': a component LIM system for high-throughput functional genomic analysis. BMC Bioinformatics 6.

Forsbach, A., Schubert, D., Lechtenberg, B., Gils, M., Schmidt, R., 2003. A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. Plant Molecular Biology 52, 161–176.

Gelvin, S.B., 2003. *Agrobacterium*-mediated plant transformation: the biology behind the ''gene-Jockeying'' tool. Microbiology and Molecular Biology Reviews 67, 16–37.

Holliday, R., 1956. New method for the identification of biochemical mutants of micro-organisms. Nature 178, 987.

Hood, E.E., Gelvin, S.B., Melchers, L.S., Hoekema, a., 1993. New *Agrobacterium* helper plasmids for gene-transfer to plants. Transgenic Research 2, 208–218.

Hood, E.E., Helmer, G.L., Fraley, R.T., Chilton, M.D., 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 Is encoded in a region of pTiBo542 outside of transfer DNA. Journal of Bacteriology 168, 1291–1301.

Howard, R.J., Ferrari, M.A., Roach, D.H., Money, N.P., 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. Proceedings of the National Academy of Sciences of The United States of America 88, 11281–11284.

Howard, R.J., Valent, B., 1996. Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. Annual Review of Microbiology 50, 491–512.

Lazo, G.R., Stein, P.A., Ludwig, R.A., 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Biotechnology 9, 963–967.

Leclerque, A., Wan, H., Abschutz, A., Chen, S., Mitina, G.V., Zimmermann, G., Schairer, H.U., 2004. *Agrobacterium*-mediated insertional mutagenesis (AIM) of the entomopathogenic fungus *Beauveria bassiana*. Current Genetics 45, 111–119.

Lee, S.C., Lee, Y.H., 1998. Calcium/calmodulin-dependent signaling for appressorium formation in the plant pathogenic fungus *Magnaporthe grisea*. Molecules and Cells 8, 698–704.

Liu, Y.G., Whittier, R.F., 1995. Thermal asymmetric interlaced PCR – automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25, 674–681.

Meng, Y., Patel, G., Heist, M., Betts, M., Tucker, S., Donofrio, N., Mitchell, T., Brown, D., Li, L., Xu, J.-R., Thon, M., Orbach, M.J., Dean, R., Farman, M.L., 2007. A systematic analysis of T-DNA insertion events in *Magnaporthe oryzae*. Fungal Genetics and Biology. doi:10.1016/j.fgb.2007.04.002.

Meyer, V., Mueller, D., Strowig, T., Stahl, U., 2003. Comparison of different transformation methods for *Aspergillus giganteus*. Current Genetics 43, 371–377.

Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M., Kang, S., 2001. *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An efficient tool for insertional mutagenesis and gene transfer. Phytopathology 91, 173–180.

Mullins, E.D., Kang, S., 2001. Transformation: a tool for studying fungal pathogens of plants. Cellular and Molecular Life Sciences 58, 2043–2052.

Ochman, H., Gerber, A.S., Hartl, D.L., 1988. Genetic applications of an inverse polymerase chain-reaction. Genetics 120, 621–623.

Ooms, G., Bakker, a., Molendijk, L., Wullems, G.J., Gordon, M.P., Nester, E.W., Schilperoort, R.A., 1982. T-DNA organization in

homogeneous and heterogeneous octopine-type crown gall tissues of *Nicotiana tabacum*. Cell 30, 589–597.

Pazour, G.J., Ta, C.N., Das, A., 1992. Constitutive mutations of *Agrobacterium tumefaciens* transcriptional activator *vir*G. Journal of Bacteriology 174, 4169–4174.

Ream, W., 1989. *Agrobacterium tumefaciens* and interkingdom genetic exchange. Annual Review of Phytopathology 27, 583–618.

Rho, H.S., Kang, S., Lee, Y.H., 2001. *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. Molecules and Cells 12, 407–411.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Soderlund, C., Haller, K., Pampanwar, V., Ebbole, D., Farman, M., Orbach, M.J., Wang, G.L., Wing, R., Xu, J.R., Brown, D., Mitchell, T., Dean, R., 2006. MGOS: a resource for studying *Magnaporthe grisea* and *Oryza sativa* interactions. Molecular Plant–Microbe Interactions 19, 1055–1061.

Sweigard, J.A., Carroll, A.M., Farrall, L., Chumley, F.G., Valent, B., 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. Molecular Plant–Microbe Interactions 11, 404–412.

Sweigard, J.A., Carroll, A.M., Kang, S., Farrall, L., Chumley, F.G., Valent, B., 1995. Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. Plant Cell 7, 1221–1233.

Sweigard, J.A., Orbach, M.J., Valent, B., Chumley, F.G., 1990. A miniprep procedure for isolating genomic DNA from *Magnaporthe grisea*. Fungal Genetics Newsletter 37, 4.

Talbot, N.J., 1995. Having a blast: exploring the pathogenicity of *Magnaporthe grisea*. Trends in Microbiology 3, 9–16.

Talbot, N.J., 2003. On the trail of a cereal killer: Exploring the biology of *Magnaporthe grisea*. Annual Review of Microbiology 57, 177–202.

Talbot, N.J., Ebbole, D.J., Hamer, J.E., 1993. Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. Plant Cell 5, 1575–1590.

Talbot, N.J., McCafferty, H.R.K., Ma, M., Moore, K., Hamer, J.E., 1997. Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. Physiological and Molecular Plant Pathology 50, 179–195.

Tinland, B., 1996. The integration of T-DNA into plant genomes. Trends in Plant Science 1, 178–184.

Tinland, B., Schoumacher, F., Gloeckler, V., Bravoangel, a.M., Hohn, B., 1995. The *Agrobacterium tumefaciens* Virulence D2 Protein Is Responsible for Precise Integration of T-DNA into the Plant Genome. EMBO Journal 14, 3585–3595.

Urashima, A., Igarashi, S., Kato, H., 1993. Host range, mating type, and fertility of *Pyricularia grisea* from wheat in Brazil. Plant Disease 77, 1211–1216.

Urban, M., Bhargava, T., Hamer, J.E., 1999. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. EMBO Journal 18, 512–521.

Valent, B., 1990. Rice blast as a model system for plant pathology. Phytopathology 80, 33–36.

Valent, B., Chumley, F.G., 1991. Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. Annual Review of Phytopathology 29, 443–467.

Valent, B., Farrall, L., Chumley, F.G., 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. Genetics 127, 87–101.

Viaud, M.C., Balhadere, P.V., Talbot, N.J., 2002. A *Magnaporthe grisea* cyclophilin acts as a virulence determinant during plant infection. Plant Cell 14, 917–930.

Vogel, H.J., 1964. Distribution of lysine pathways among fungi: evolutionary implications. American Naturalist 98, 435–446.

Wang, P., Cardenas, M.E., Cox, C.M., Perfect, J.R., Heitman, J., 2001. Two cyclophilin A homologs with shared and distinct functions important for growth and virulence of *Cryptococcus neoformans*. EMBO Reports 2, 511–518.