Relative efficiency of split-marker versus double-crossover replacement protocols for production of deletion mutants in strain PH-1 of *Fusarium graminearum*

Sladana Bec[1], Gabdiel E. Yulfo-Soto[1], and Lisa Vaillancourt[1*]

[1] Department of Plant Pathology, University of Kentucky, Lexington KY 40546, USA.
*Corresponding author

Abstract

The split-marker (SM) transformation protocol is popular for production of gene deletion mutants in fungi. It has been suggested that this method is more efficient than the more traditional double-crossover method (intact-marker: IM), producing a higher percentage of targeted deletions versus ectopic integrations. We compared the performance of both protocols in *F. graminearum* strain PH-1. The SM method resulted in a higher percentage of deletions for sequences between 1 and 6 kb, but was similar to IM for a smaller 865 bp sequence. Both methods produced additional ectopic integrations in 12-13% of the deletion strains. SM produced more independent ectopic integrations in these strains than IM. This may result in more variability among individual transformants due to off-site mutations, as well as complicating their removal by backcrossing. Southern hybridizations are helpful for the identification of strains with fewer ectopic integrations.

Introduction

Inactivation of a gene by deletion or disruption is an important way to study its function (Shafran et al., 2008; Turgeon et al., 2010). Gene deletion via double-crossover replacement is usually preferred because it avoids the possibility of residual or restored gene function. Gene deletion constructs typically consist of varying amounts of sequence from the flanks upstream and downstream of the gene of interest fused to a selectable marker gene (hereafter referred to as "intact-marker" (IM) constructs). Successful gene deletion depends on homologous recombination (HR) at double-strand breaks (DSB) within each flanking sequence (Hynes, 1996; Krappman, 2007; Weld, 2006). Recombination at DSB occurs by one of two DNA repair pathways: homology-directed repair (HDR) requires the presence of DNA sequence homology, while non-homologous end joining (NHEJ) does not (Cahill et al., 2006; Kanaar et al., 1998; Krappman 2007). *Saccharomyces cerevisiae* uses primarily HDR for DNA repair, and the generation of gene deletion mutants by HR is consequently very efficient in this organism (Hua et al., 1997). In contrast, many filamentous fungi tend to use the NHEJ mechanism more often. This results in a reduced number of transformants in which the gene of interest has been replaced by HR, and more strains in which the marker has integrated randomly at an ectopic location in the genome (Chaveroche et al., 2000).

The split-marker (SM) protocol, first applied to yeast (Fairhead et al., 1996), is an alternative approach for gene deletion in filamentous fungi (Catlett et al. 2003; de Hoogt et al. 2000). For this procedure, fungal protoplasts are transformed with a mixture of two DNA fragments, each comprised of DNA flanking one end of the gene of interest fused to overlapping segments of a selectable marker gene. To reconstitute a functional selectable marker, the SM fragments must undergo HR at three points, and this has been reported to increase the efficiency of gene deletion (Fairhead et al., 1996; Catlett et al., 2003). However, the performance of IM versus SM protocols has been directly compared

in very few cases (e.g. Fu et al. 2006), and a detailed examination of integration sites by use of Southern hybridization has been rare. The objective of the current study was to use Southern hybridization to compare SM and IM protocols and evaluate their relative efficiency for generating gene deletions versus ectopic integrations in strain PH-1 of *F. graminearum*. For this work we targeted the mating-type locus of *F. graminearum* which has a previously characterized deletion phenotype (Lee et al. 2003).

Materials and Methods

Fungal strains and growth conditions: *Fusarium graminearum* strain PH-1 (NRRL 31084) was cultured on mung bean agar (MBA) (Bai and Shaner 1996) for 7–10 days at 23°C under continuous fluorescent light to produce macroconida. Spore suspensions were prepared by adding 2 ml of sterile water to the culture and gently rubbing the surface with a sterile plastic micro-pestle. Harvested spores were filtered through glass wool, washed twice with sterile water, and adjusted to the desired concentration.

DNA extraction: Five ml of YEPD medium (20 g dextrose, 20 g bacto-peptone, 10 g yeast extract) was inoculated with an 8-mm agar plug taken from the edge of an actively growing colony. Cultures were incubated at 25°C for 5–7 days at 250 rpm. Recovered mycelia were flash-frozen in liquid nitrogen, lyophilized, and pulverized in individual 2 ml Eppendorf tubes by using a mini-pestle, or in deep 96-well plates with a 2000 GENO/Grinder® (Spex Certiprep) (500 strokes/sec for 30 sec). One ml of warm lysis buffer (0.5 M NaCl; 1% SDS; 10 mM Tris HCl, pH 7.5; 10 mM EDTA) was added per 100–200 mg fungal tissue, and samples were incubated at 65°C for 30 min, vortexing once during the incubation. After incubation the samples were transferred into individual tubes containing 660 µl PCI (25 parts phenol; 24 parts chloroform; 1 part isoamyl alcohol), mixed by inverting 4–6 times, then incubated at 65°C for an additional 30 min. The contents were mixed once during incubation. The samples were centrifuged in a tabletop centrifuge for 20 min at maximum speed to separate the phases. DNA was precipitated from the aqueous phase by using 1 volume of isopropanol, and the pellet was washed twice with 70% ethanol. The pellet was resuspended in 100 µl of TE, pH 7.9, with 2 µl of a 5 mg/ml concentration of RNase A, at 65°C for 1 h.

Southern hybridizations: PCR amplicons used for probes were gel-purified and labeled with radioactive isotope P^{32} using the Prime-a-gene labeling system (Promega, cat. #U1100). Between 1 and 5 µg of the genomic DNA isolated from the fungal strains was digested with 20 U of the appropriate restriction enzyme (RE) in a 50 µl reaction. DNA was precipitated and resuspended before loading the entire sample onto a 0.8% agarose gel made with 0.5X TBE buffer (20X TBE, 1L: 216g Tris base; 110g boric acid; 80 ml 0.5M EDTA, pH 8.0). The gel was electrophoresed at 35V in 0.5X TBE for 18-20 hours, followed by staining for 30 min in 0.5X TBE plus 5µg/ml EtBr for imaging, and then destaining for 30 min with fresh 0.5X TBE. DNA fragments were transferred from the gel to a charged nylon membrane (PALL Life Sciences) by electroblotting with a GENIE electroblotter (Idea Scientific) for 2 h at 12V. The DNA on the membrane was denatured in 0.4N NaOH for 10 min, then neutralized for 10 min in 2X SSC (20X SSC, 1 L; 175.3g NaCl; 88.2g $Na_3C_6H_5O_7$; adjusted to pH 7.0, autoclaved). DNA was fixed to the membrane by UV-crosslinking in a Spectrolinker (Spectronics Corporation). The membrane was prehybridized at 65°C for 30 min in hybridization buffer (100 mls; 25 ml

0.5 M Na_2HPO_4 pH 7.2; 7 g sodium dodecyl sulfate; 0.2 ml 0.5M EDTA, pH 8.0; stir to dissolve, heating if necessary) and then fresh hybridization solution was added together with the radioactively labeled probe, and the membrane was hybridized overnight at 65°C. The membrane was then washed three times with 2X SSC at 65°C for 20 min each, blotted dry, wrapped in plastic wrap, and exposed on a Storage Phosphor Screen (Molecular Dynamics) at room temperature for up to 3 d. The screen was scanned by using a Typhoon Phosphorimager (GE Healthcare). Membranes were stripped by washing for 30 min in 0.4N NaOH, followed by 15 min in 0.1X SSC, 0.1% SDS, at 45°C. Stripped membranes were rehybridized with probes for the mating-type genes and for the *hyg* gene. The resulting images were false-colored and overlaid to create the figures by using Adobe imaging software.

Plasmid construction: The strategy for construction of plasmid templates is shown in Figure 1. For each of the genes of interest, 0.6–1.2 kb of each flank was amplified. The PCR primers are listed in Table 1, and the PCR parameters used are shown in Table 2. The primers were designed manually from the published *F. graminearum* genome (Cuomo et al., 2007). Primer P2 incorporated a 3' *Eco*RI recognition site, and primer P3 included a 5' *Bam*HI recognition sequence. These same RE were used to isolate the hygromycin phosphotransferase (*hph*) selectable marker gene from the donor plasmid pBSHyg2. To produce pBSHyg2, the *hph* gene with the TrpC promoter was first recovered from the plasmid pCB1636 (Sweigard et al., 1997) by digesting with *Sal*I. Then, the *Sal*1 fragment was blunt-ended and ligated into the pBluescript plasmid digested with *Sma*I. PCR amplicons and the restricted *hph* gene fragment from pBSHyg2 were gel-purified with a gel extraction kit (QIAquick Gel Extraction Kit, Qiagen). Amplicons were digested with the appropriate RE and then gel-purified. The restricted fragments were ligated in a 1:2:1 molar ratio with T4 ligase (Invitrogen) at 16°C overnight. The ligase was inactivated at 70°C for 20 min, and the reactions were diluted 1000-fold and used as PCR templates with primers P1 and P4. The resulting amplicons were gel-purified and cloned into the pGEM T-easy plasmid (Promega #PR-A1360). Before cloning, "A-tails" were added by incubating 15 µl of each gel-purified amplicon with 0.4 µl Taq polymerase (Invitrogen 5U/µl #18-038-042), 2 µl 10x PCR buffer, 0.6 µl 50 mM $MgCl_2$ and 2 µl of 10 mM dATP for 30 min at 72°C. The plasmids were introduced into electro-competent DH5α *E. coli* cells, and clones were confirmed by restriction digestion and sequencing, then stored as 15% glycerol stocks at -80°C.

PCR amplification of split-marker fragments, and intact-marker replacement cassettes: The plasmids were used as templates for generation of SM fragments and IM replacement cassettes (Figure 1). PCR reactions consisted of a 25 µl total reaction volume including 2.5 µl of 10X Phusion PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTP mix, 1µl of each primer (20 nM) and 1 µl of template DNA (20-50 ng/µl). The thermocycling parameters were as follows: initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 20 sec, and extension at 72°C for 1 min/kb, with a final extension for 7 min at 72°C.

Preparation of fungal protoplasts: Protoplast isolation and transformation protocols were modified from methods used for transformation of *Aspergillus parasiticus* (Skory et al. 1990; F. Trail, personal communication). Macroconidia were harvested from 7-day-old

cultures, washed, and resuspended at a concentration of $1x10^6$ spores/ml. A 100 ml aliquot of YEPD was inoculated with 2 ml of the spore solution. The culture was incubated for 10–14 hours at 25°C with constant agitation (175 rpm). Mycelia were harvested by filtration, washed with sterile water, and treated with 20 ml of protoplasting buffer [500 mg Driselase D9515 (MilliporeSigma); 100 mg lysing enzyme from *Trichoderma harzianum* L1412 (MilliporeSigma); in 20 ml of 1.2 M KCl, filter sterilized]. Mycelium was incubated in the protoplasting buffer for 45–90 minutes at 37°C with gentle agitation (50–80 rpm). When most hyphae had released protoplasts, the solution was filtered through a 30 µm Nitex nylon membrane into sterile 50 ml centrifuge tubes. Protoplasts were pelleted by centrifugation (1729 x *g*) at room temperature for 5 min, then gently resuspended in STC buffer (1.2 M sorbitol; 10 mM Tris-HCl, pH 8.0; 50 mM $CaCl_2$). Protoplasts were pelleted once more, and then resuspended in STC at a concentration of 1×10^8 protoplasts/ml.

Fungal transformation: For each transformation, 100 µl of the freshly prepared protoplast suspension was mixed with 100 µl STC buffer, 50 µl freshly prepared and filter-sterilized 30% PEG solution (8000 polyethylene glycol, MilliporeSigma, P2139) dissolved in a buffer (10 mM Tris-HCl, pH 8.0; 50 mM $CaCl_2$) and 10 µl of DNA (1-3 µg). Protoplasts were transformed by IM or SM protocols, or with linearized MAT1 and MAT1-2-1 plasmids. For SM fragments, 6 µl of both fragments were combined first, and then 10 µl of the mixture was added to the transformation. The reactions were incubated at room temperature for 20 min. Two ml of 30% PEG Solution was added, and incubation continued for an additional 5 min. Four ml of STC buffer was added, and gently mixed. The transformation reaction was then added to 250 ml of cooled regeneration medium (RM) [1.0 g yeast extract; 1.0 g N-Z-Amine AS (N4517 MilliporeSigma); 7.4 g agar; and 271 g sucrose per liter]. The medium was mixed gently and aliquoted into three 100 mm Petri plates. Protoplasts were regenerated for 12–15 hours, and then plates were overlaid with 15 ml of RM amended with 150 µg/ml hygromycin B. Transformants usually appeared within 4–7 days, when they were transferred to potato dextrose agar (Difco®) containing 450 µg/ml hygromycin B.

Analysis of transformants: Transformants were single-spored, and genomic DNA extracted from each strain was evaluated by using Southern hybridization (Southern, 2006) to characterize integration events. The fertility phenotypes of gene deletion mutants were assessed on carrot agar (Leslie and Summerell, 2006). The colonies were incubated for 5 days at 23°C with constant fluorescent light. When the colonies covered the plate, 0.5–1.0 ml of 2.5% Tween 60 was applied to the surface of the plate, and the mycelia were flattened by rubbing gently with a sterile glass rod to induce perithecial production. Presence and appearance of perithecia, as well as presence of asci containing ascospores, were assessed 14 days post-induction.

Results and Discussion

The mating-type locus of *F. graminearum*, known as MAT1, is a complex locus that contains four individual open reading frames (Yun et al., 2000). In this study, three DNA intervals from the MAT1 locus were targeted for deletion. These included the entire MAT1 locus, the MAT1-1-1 gene, and the MAT1-2-1 gene. SM and IM constructs were produced for each interval and used in transformation experiments. Each experiment

generated between 50 and 70 hygromycin-resistant transformants within 4–7 days, from which 20-25 individual transformants were arbitrarily chosen for further analysis. There were no noticeable differences among the constructs in the number or appearance of the transformants they produced on regeneration medium.

Southern hybridization analysis revealed the presence of three different types of integrations among the recovered transformants; these included double-point HR resulting from HDR and producing a gene deletion (D-HR), single-point HR producing an integration of the transformation construct adjacent to the original gene (S-HR), and ectopic integration of the transformation construct by NHEJ (Figure 2).

Both types of construct (SM and IM) generated gene deletion (D-HR) strains at rates ranging from 24–75%. The SM protocol produced more deletion strains than IM for the two longest sequences, MAT1 (6070 bp) and MAT1-1-1 (1091 bp). However, deletion efficiencies for the smallest MAT1-2-1 sequence (865 bp) were similar for SM versus IM (Table 3). In yeast, increase in GC content of the targeted DNA, comprising the ORF to be deleted and the flanking DNA included in the disruption construct, resulted in increased representation of HR transformants (Gray and Honigberg, 2001). We noticed a similar trend in our study. Overall, MAT1 (48.6% GC) resulted in 32% D-HR, MAT1-1-1 (51.2% GC) produced 56% D-HR, while MAT1-2-1 (52.7% GC) yielded 67% D-HR.

Among the strains with D-HR events resulting in gene deletions, approximately one in ten had additional ectopic (NHEJ) integrations of the DNA (Table 3, Figure 2). Such strains may be less desirable since additional integrations cause off-site mutations that could independently affect the phenotypes of the transformants (Weld et al., 2006). The SM and IM protocols both generated a similar proportion of strains with additional ectopic integrations (13% for SM, 12% for IM).

SM produced more strains with S-HR events than IM (Table 3). Separate hybridizations with each flank of the SM construct pairs (not shown) suggested that SM also produced more independent NHEJ integrations overall than IM. Of the NHEJ integrations involving SM, 18% comprised just one of the SM flanks. Of those single flank integrations, 74% were of the upstream flank. One possible explanation for the larger relative frequency of NHEJ events with SM is the increased number of recombinogenic ends in the transforming DNA. Transformation experiments in which the template plasmids were linearized within the plasmid sequence, thus avoiding creation of homologous recombinogenic ends, produced fewer integrations, supporting this hypothesis (Table 3). Single ectopic insertions can be more easily removed by backcrossing than multiple integrations, making them less problematic for research purposes. However, we do not know whether transformation with either method also produces unmarked off-site mutations. This question will require further study.

Deletion of any of the three MAT sequences by either SM or IM produced transformants that were completely infertile and could reproduce only asexually, as expected. There were no apparent differences related to the deletion approach used. MAT1 and MAT1-1-1 deletion strains produced numerous small perithecial initials containing no ascospores after induction (Figure 3). Most MAT1-2-1 deletion strains produced few or no perithecial initials and had a thick layer of aerial mycelium that was easily removed from the agar (Figure 3). These phenotypes were consistent with other descriptions of similar

knockouts in *F. graminearum* (Lee et al., 2003). Strains without HR events, whether with single or multiple ectopic integrations, had wild type homothallic fertility phenotypes. The perithecia were of normal size and contained ascospores (not shown).

The SM and IM methods both efficiently produced gene deletion mutants in *F. graminearum* strain PH-1. SM produced deletions with a higher efficiency for the two largest sequences but was similar to IM for the smallest. *Fusarium graminearum* has a relatively high rate of HR compared to other fungi (Maier et al., 2005; Trail, 2009). In cases where HR is less efficient, SM could provide more of an advantage. The SM protocol facilitates high throughput gene deletion studies, and that convenience provides an additional compelling reason to use SM versus IM. However, we observed that SM produces higher numbers of independent ectopic integrations, including integrations that involved only one of the two flanks, and this may result in more variability among individual transformants due to off-site mutations, as well as complicating their removal by backcrossing. Although PCR is more convenient for screening transformants for strains in which the gene of interest has been successfully deleted, Southern hybridizations are helpful for the identification of strains with fewer ectopic integrations for experimental purposes.

Acknowledgements

This research was supported by a cooperative project with the U.S. Wheat and Barley Scab Initiative. Any opinions, findings or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture.

References

Bai, G. H., and Shaner, G., 1996, Variation in *Fusarium graminearum* and cultivar resistance to wheat scab: Plant Disease v. 80, p. 875-979, doi: 10.1094/PD-80-0975.

Cahill, D., Connor, B., and Carney, J. P., 2006, Mechanisms of eukaryotic DNA double strand break repair: Frontiers in Bioscience: a Journal and Virtual Library v. 11, p. 1958-1976, doi: 10.2741/1938.

Catlett, N. L., Lee, B. N., Yoder, O. C., and Turgeon, B. G., 2003, Split-marker recombination for efficient targeted deletion of fungal genes: Fungal Genetics Reports, v. 50, no.1, p. 9-11, doi: 10.4148/1941-4765.1150.

Chaveroche, M. K., Ghigo, J. M., and d'Enfert, C., 2000, A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*: Nucleic Acids Research, v. 28, no. 22, p. e97-e97, doi: 10.1093/nar/28.22.e97

Cuomo, C. A., Güldener, U., Xu, J. R., Trail, F., Turgeon, B. G., Di Pietro, A., ... and Kistler, H. C., 2007, The *Fusarium graminearum* genome reveals a link between

localized polymorphism and pathogen specialization: Science, v. 317, no. 5843, p. 1400-1402, doi: 10.1126/science.1143708

Fairhead, C., Llorente, B., Denis, F., Soler, M., and Dujon, B., 1996, New vectors for combinatorial deletions in yeast chromosomes and for gap-repair cloning using 'split-marker' recombination: Yeast, v. 12, no. 14, p. 1439-1457, doi: 10.1002/(SICI)1097-0061(199611)12:14%3C1439::AID-YEA37%3E3.0.CO;2-O

Fu, J., Hettler, E., and Wickes, B. L., 2006, Split marker transformation increases homologous integration frequency in *Cryptococcus neoformans*: Fungal Genetics and Biology, v. 43, no. 3, p. 200-212, doi: 10.1016/j.fgb.2005.09.007

Gray, M., and Honigberg, S. M., 2001, Effect of chromosomal locus, GC content and length of homology on PCR-mediated targeted gene replacement in *Saccharomyces*: Nucleic Acids Research, v. 29 no. 24, p. 5156-5162, doi: 10.1093/nar/29.24.5156

de Hoogt, R., Luyten, W. H., Contreras, R., and De Backer, M. D., 2000, PCR-and ligation-mediated synthesis of split-marker cassettes with long flanking homology regions for gene disruption in *Candida albicans*: Biotechniques, v. 28, no. 6, p. 1112-1116, doi: 10.2144/00286st01

Hua, S. B., Qiu, M., Chan, E., Zhu, L., & Luo, Y., 1997, Minimum length of sequence homology required for in vivo cloning by homologous recombination in yeast: Plasmid, v. 38, no. 2, p. 91-96, doi: 10.1006/plas.1997.1305

Hynes, M. J., 1996, Genetic transformation of filamentous fungi: Journal of Genetics, v. 75, no. 3, p. 297-311, doi: 10.1007/BF02966310

Kanaar, R., Hoeijmakers, J. H., and van Gent, D. C., 1998, Molecular mechanisms of DNA double-strand break repair: Trends in Cell Biology, v. 8, no. 12, p. 483-489, doi: 10.1016/S0962-8924(98)01383-X

Krappmann, S., 2007, Gene targeting in filamentous fungi: the benefits of impaired repair: Fungal Biology Reviews, v. 21, no. 1, p. 25-29, doi: 10.1016/j.fbr.2007.02.004

Lee, J., Lee, T., Lee, Y. W., Yun, S. H., and Turgeon, B. G., 2003, Shifting fungal reproductive mode by manipulation of mating type genes: obligatory heterothallism of *Gibberella zeae*: Molecular Microbiology, v. 50, no. 1, p. 145-152, doi: 10.1046/j.1365-2958.2003.03694.x

Leslie, J. F., and Summerell, B. A., 2008, The Fusarium laboratory manual: Hoboken New Jersey, John Wiley & Sons, 355 p.

Maier, F. J., Malz, S., Lösch, A. P., Lacour, T., and Schäfer, W., 2005, Development of a highly efficient gene targeting system for *Fusarium graminearum* using the

disruption of a polyketide synthase gene as a visible marker: FEMS Yeast Research, v. 5, no. 6-7, p. 653-662, doi: 10.1016/j.femsyr.2004.12.008

Shafran, H., Miyara, I., Eshed, R., Prusky, D., and Sherman, A., 2008, Development of new tools for studying gene function in fungi based on the Gateway system: Fungal Genetics and Biology, v. 45, no. 8, p. 1147-1154, doi: 10.1016/j.fgb.2008.04.011

Skory, C. D., Horng, J. S., Pestka, J. J., and Linz, J. E., 1990, Transformation of *Aspergillus parasiticus* with a homologous gene (*pyrG*) involved in pyrimidine biosynthesis: Applied and Environmental Microbiology, v. 56, no. 11, p. 3315-3320, doi: 10.1128/AEM.56.11.3315-3320.1990

Southern, E., 2006, Southern blotting: Nature Protocols, v. 1, p. 518-525, doi: 10.1038/nprot.2006.73

Sweigard, J.A., Chumley, F., Carroll, A., Farrall, L, and Valent, B. 1997. A series of vectors for fungal transformation: Fungal Genetics Newsletter v. 44, p. 52-53, doi: 10.4148/1941-4765.1287

Trail, F., 2009, For blighted waves of grain: *Fusarium graminearum* in the postgenomics era: Plant Physiology, v. 149, no. 1, p. 103-110, doi: 10.1104/pp.108.129684

Turgeon, B. G., Condon, B., Liu, J., and Zhang, N., 2010, Protoplast transformation of filamentous fungi, *in* Sharon A., eds., Molecular and Cell Biology Methods for Fungi. Methods in Molecular Biology, Humana Press. v. 638, p. 3-19, doi: 10.1007/978-1-60761-611-5_1

Weld, R. J., Plummer, K. M., Carpenter, M. A., and Ridgway, H. J., 2006, Approaches to functional genomics in filamentous fungi: Cell Research, v. 16, no. 1, p. 31-44, doi: 10.1038/sj.cr.7310006

Yun, S. H., Arie, T., Kaneko, I., Yoder, O. C., and Turgeon, B. G., 2000, Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species: Fungal Genetics and Biology, v. 31, no. 1, p. 7-20, doi: 10.1006/fgbi.2000.1226

Table 1. List of primers used in this study. *Eco*RI sites are highlighted in red and *Bam*HI sites in blue in the primer sequences.

Gene of Interest	Primer	Amplicon	Primer sequence 5'- 3'
MAT1	GzMAT1P1	MAT1 5' flank* forward	GCGCTTATATCGGGCATAGA
	GzMAT1P2EcoRI	MAT1 5' flank reverse + *EcoRI* site	AAAGGAATTCGGCGTTCTGAGAGTTGGA
	GzMAT1P3BamHI	MAT1 3' flank forward + *BamHI* site	AAAAGGATCCTGCATTGATTTGAGCCAG
	GzMATP4	MAT1 3' flank* reverse	TCTCACAACGGCAACTGTTC
MAT1-1-1	GzMAT111F	MAT111 internal probe forward	AGTCCGAATGAAGCCCCAATACC
	GzMAT111R	MAT111 internal probe reverse	CAGAACTTGCAGGTGCTGGGAGT
	GzMAT111P1	MAT1 5' flank forward	GGCCGATAATCTCCTCGACT
	GzMAT111P2EcoRI	MAT1 5' flank reverse + *EcoRI* site	AAGCGAATTCGCACGGAATCGTTCCAGA
	GzMAT111P3BamHI	MAT1 3' flank forward + *BamHI* site	AAGGGGATCCTTAAATTGCAGAGGTGTGTAAGG
	GzMAT111P4	MAT1 3' flank reverse	TCTATGTTAGTAGGCAGCAGTGG
MAT1-2-1	GzMAT121F	MAT121 internal probe forward	TCTTCCACCCCCTGTGTCTACCA
	GzMAT121R	MAT121 internal probe reverse	TGCGAATGTCAGGATGCTCCA
	GzMAT121P1	MAT121 5' flank forward	GGCATAGAGTCGTCCCAGAA
	GzMAT121P2BamHI	MAT121 5' flank reverse + *BamHI* site	GTAAGGATCCTCAGATGAAGTTGGCAGGTG
	GzMAT121P3EcoRI	MAT121 3' flank forward + *EcoRI* site	TCGAGAATTCCTCAGGCCCTACGTTTTGTT
	GzMAT121P4	MAT121 3' flank reverse	CCTGCAAGTTCTGATGTGGA
Hyg. marker cassette	HY	Internal hygromycin probe forward	GGATGCCTCCGCTCGAAGTA
	YG	Internal hygromycin probe reverse	CGTTGCAAGACCTGCCTGAA

*5' flank = upstream flank, 3' flank = downstream flank

Table 2. Summary of PCR protocols used in this study.

Locus	Flank	Primer Pair	Initial denaturation	PCR amplification Cycles			Final extension	
				Denaturation	Annealing	Elongation		
MAT 1	upstream flank	GzMAT1P1 / GzMAT1P2EcoRI	94	94	60	72	72	temp. (ºC)
			3:00	0:30	0:20	1:00	7:00	time (min)
			1	40			1	# of cycles
	downstream flank	GzMAT1P3BamHI / GzMAT1P4	94	94	58	72	72	temp. (ºC)
			3:00	0:30	0:20	1:00	7:00	time (min)
			1	40			1	# of cycles
MAT 1-1-1	upstream flank	GzMAT111P1 / GzMAT111P2EcoRI	94	94	58	72	72	temp. (ºC)
			3:00	0:30	0:20	0:35	7:00	time (min)
			1	40			1	# of cycles
	downstream flank	GzMAT111P3BamHI / GzMAT111P4	94	94	60	72	72	temp. (ºC)
			3:00	0:30	0:20	0:35	7:00	time (min)
			1	40			1	# of cycles
MAT 1-2-1	upstream flank	GzMAT121P1 / GzMAT121P2BamHI	94	94	60	72	72	temp. (ºC)
			3:00	0:30	0:20	0:50	7:00	time (min)
			1	40			1	# of cycles
	downstream flank	GzMAT121P3EcoRI / GzMAT121P4	94	94	62	72	72	temp. (ºC)
			3:00	0:30	0:20	1:00	7:00	time (min)
			1	40			1	# of cycles

Table 3. Characteristics of transformants generated by different gene replacement approaches.

Gene	Gene Replacement Strategy	Number of Transformant Strains/Type of Integration						Integration Events		Total Number of Transformants Examined by Southern Blot
		D-HR			NHEJ		S-HR			
		single	D-HR +NHEJ	Percent HR	single	multiple		Total	Avg	
MAT1	SMC*	10	2	48%	0	8	5	101	4	25
MAT1	IMC**	5	1	24%	12	5	2	53	2	25
MAT1	IMC(lp)***	6	0	24%	13	4	2	29	1	25
MAT1-1-1	SMC	11	6	68%	0	5	3	95	4	25
MAT1-1-1	IMC	7	4	44%	3	9	2	76	3	25
MAT1-2-1	SMC	12	1	65%	1	0	6	54	3	20
MAT1-2-1	IMC	15	3	75%	2	4	0	42	2	24
MAT1-2-1	IMC(lp)	8	4	60%	3	1	4	38	2	20

*SMC = Split Marker Cassette Method

**IMC = Intact Marker Cassette Method

***IMC(lp) = Template plasmid linearized within plasmid sequence.

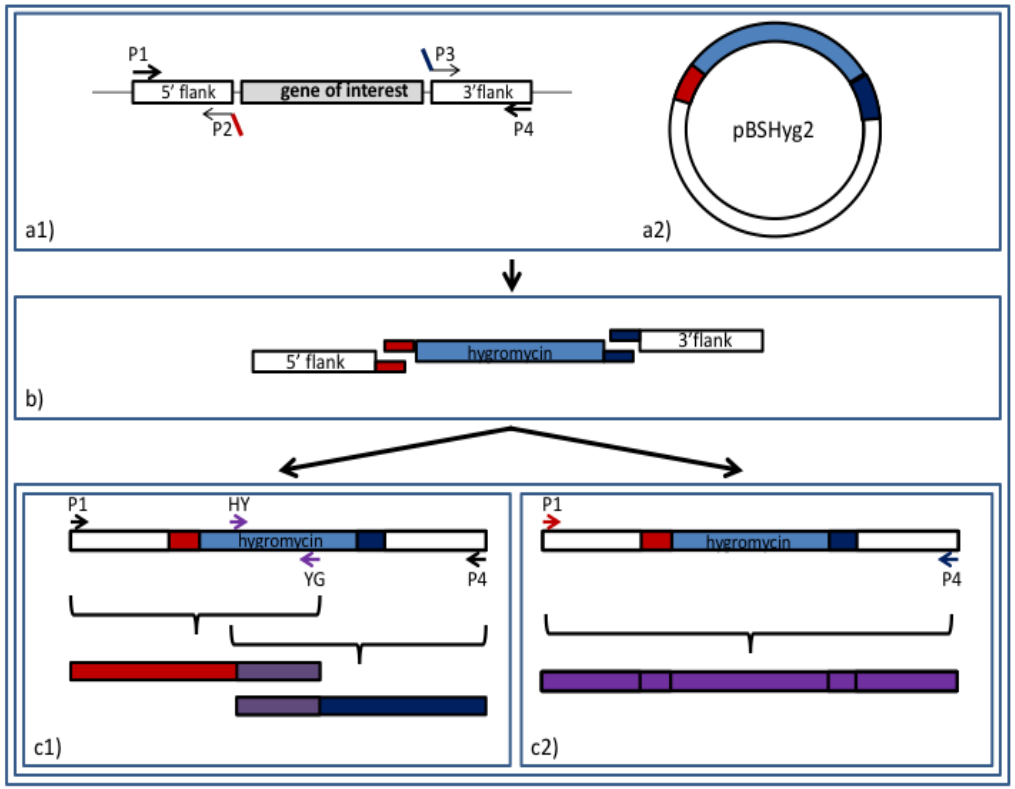
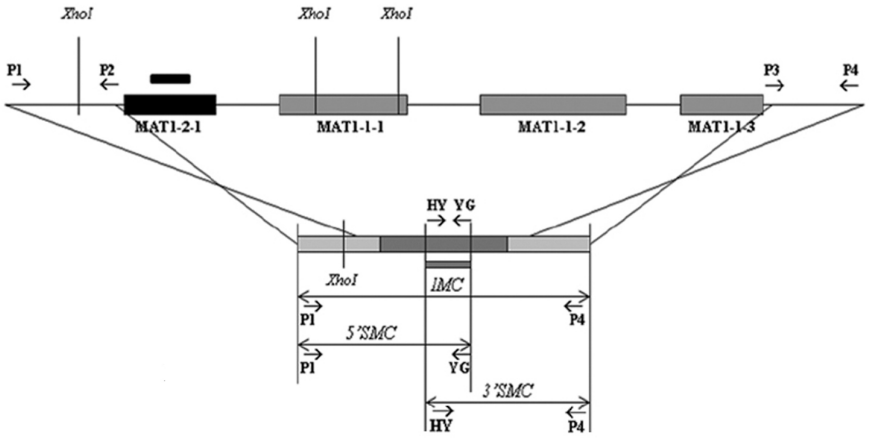


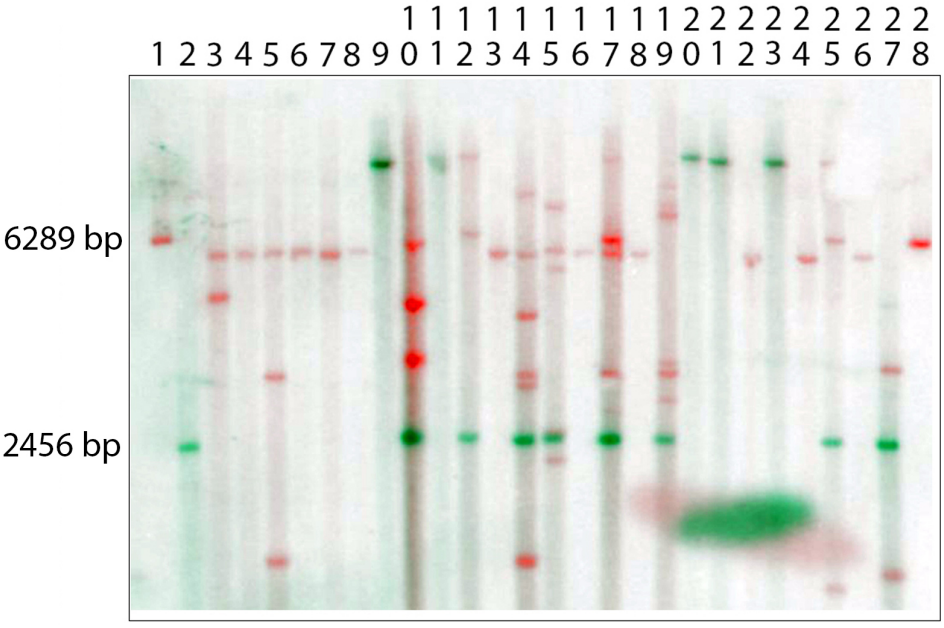
Figure 1. Split-marker and intact-marker gene-replacement strategies. a1) PCR amplification of flanking regions with overhangs containing RE recognition sequences; a2) release of hygromycin resistance gene from pBSHyg2 by digestion with *Eco*RI and *Bam*HI; b) Ligation of PCR-amplified flanking regions with the hygromycin gene released from pBSHyg2; c1) PCR amplification of split-marker fragments with combination of flanking region (5'flankP1 or 3'flankP4) primer and hygromycin-gene specific primer (HY or YG); c2) PCR amplification of an intact marker cassette with gene specific 5'flankP1 and 3'flankP4 primers.

1

MAT1


XhoI
XhoI
XhoI
P1
P2
P3
P4
MAT1-2-1
MAT1-1-1
MAT1-1-2
MAT1-1-3
HV YG
XhoI
1MC
P1
P4
5'SMC
P1
YG
3'SMC
HV
P4

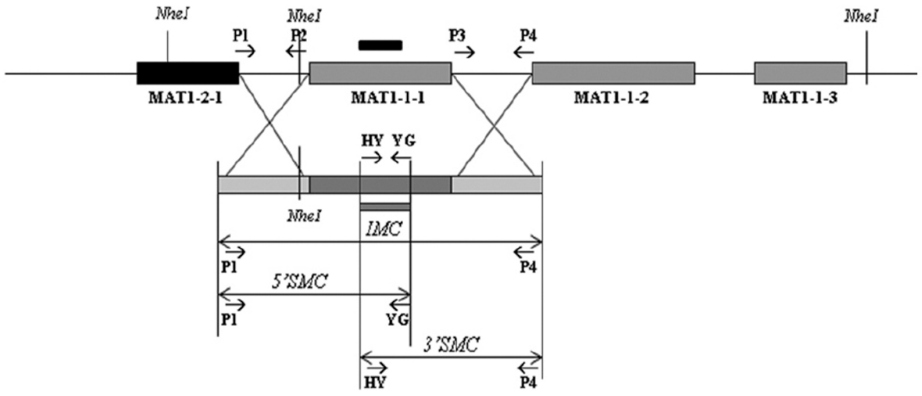
a


1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8
6289 bp
2456 bp

b

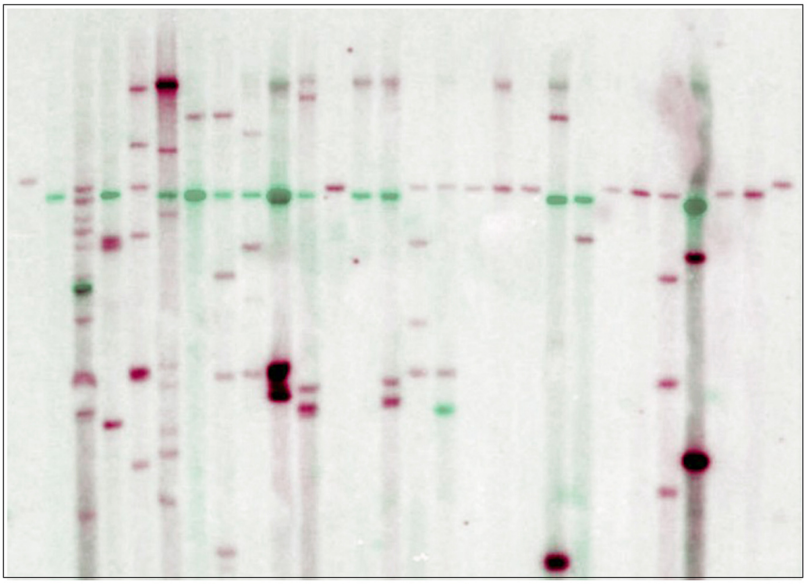
2

MAT1-1-1



a

1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8

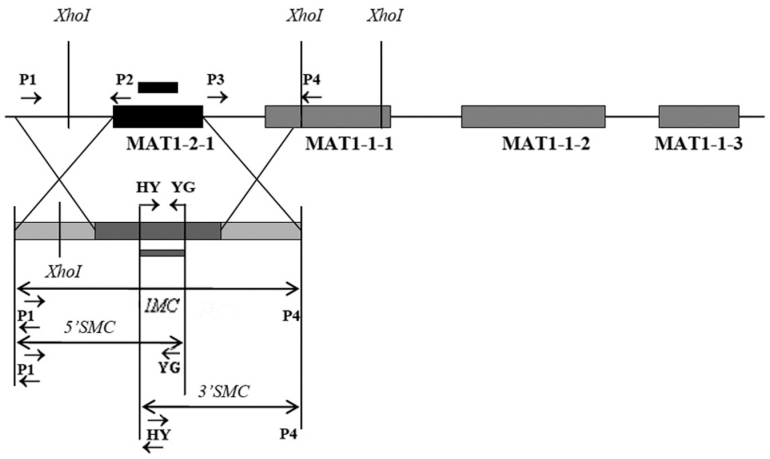


5011bp

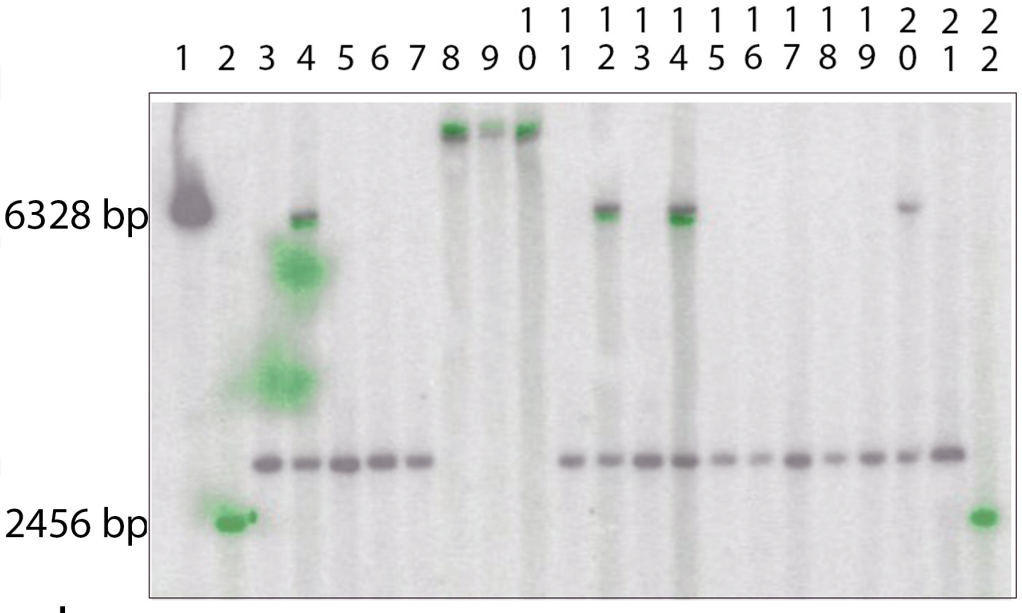
b

3

MAT1-2-1


XhoI
XhoI
XhoI
P1
P2
P3
P4
MAT1-2-1
MAT1-1-1
MAT1-1-2
MAT1-1-3
HY
YG
XhoI
IMC
P1
P4
5'SMC
P1
YG
3'SMC
HY
P4

a


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
6328 bp
2456 bp

b

Figure 2. Diagrams illustrating the Southern blot strategies used to characterize *F. graminearum* MAT gene deletion transformants. Only SM results are shown here as examples. Genomic DNA from MAT1 and MAT1-2-1 transformant strains (**1, 3**) was digested with *Xho*I, while MAT1-1-1 transformant DNA (**2**) was digested with *Nhe*I. Blots were hybridized with MAT-1-2-1 or MAT1-1-1 gene-specific probes (black bars in panels **1-3a**) and with an *hph* gene-specific probe (gray bar in panels **1-3a**). In the blot overlays (panels **1-3b**), fragments hybridizing to the *hph* probe are shown in red or gray, and to MAT1-1-1 or MAT1-2-1 in green. The first lane in each blot contained linearized *hph* plasmid DNA while lane 2 in each case contained genomic DNA from untransformed PH-1. **1a.** MAT1 whole locus deletion map. **1b**. Homologous recombination gene deletion (D-HR) without additional ectopic integrations (lanes 4, 6, 7, 8, 13, 16, 18, 22, 24, 26); D-HR with additional ectopic integrations (lanes 3 and 5); ectopic integration (NHEJ) without HR (lanes 10, 12, 14, 15, 17, 19, 25, 27); and single point HR recombination (S-HR) (lanes 9, 11, 20, 21, and 23)**.** In lanes 20, 21, and 23, the MAT probe and the *hph* probe both hybridized to the same fragment: the green band is obscuring the underlying red band in these cases. **2a.** MAT1-1-1 gene deletion map. **2b**. D-HR without additional ectopic integrations (lanes 12, 17, 19, 22, 23, 26, 27); D-HR with additional ectopic integrations (lanes 5, 15, 18, and 24); NHEJ without HR (lanes 4, 6, 7, 8, 9, 10, 11, 13, 14, 20, 21, 25); and S-HR (lanes 3 and 16). **3a.** MAT 1-2-1 gene deletion map. **3b**. D-HR without additional ectopic integrations (lanes 3, 5, 6, 7, 11, 13, 15, 16, 17, 18, 19, and 21); D-HR with additional ectopic integrations (lane 20); and S-HR (lanes 4, 8, 9, 10, 12, and 14). There are no examples of transformants with NHEJ without HR shown here.

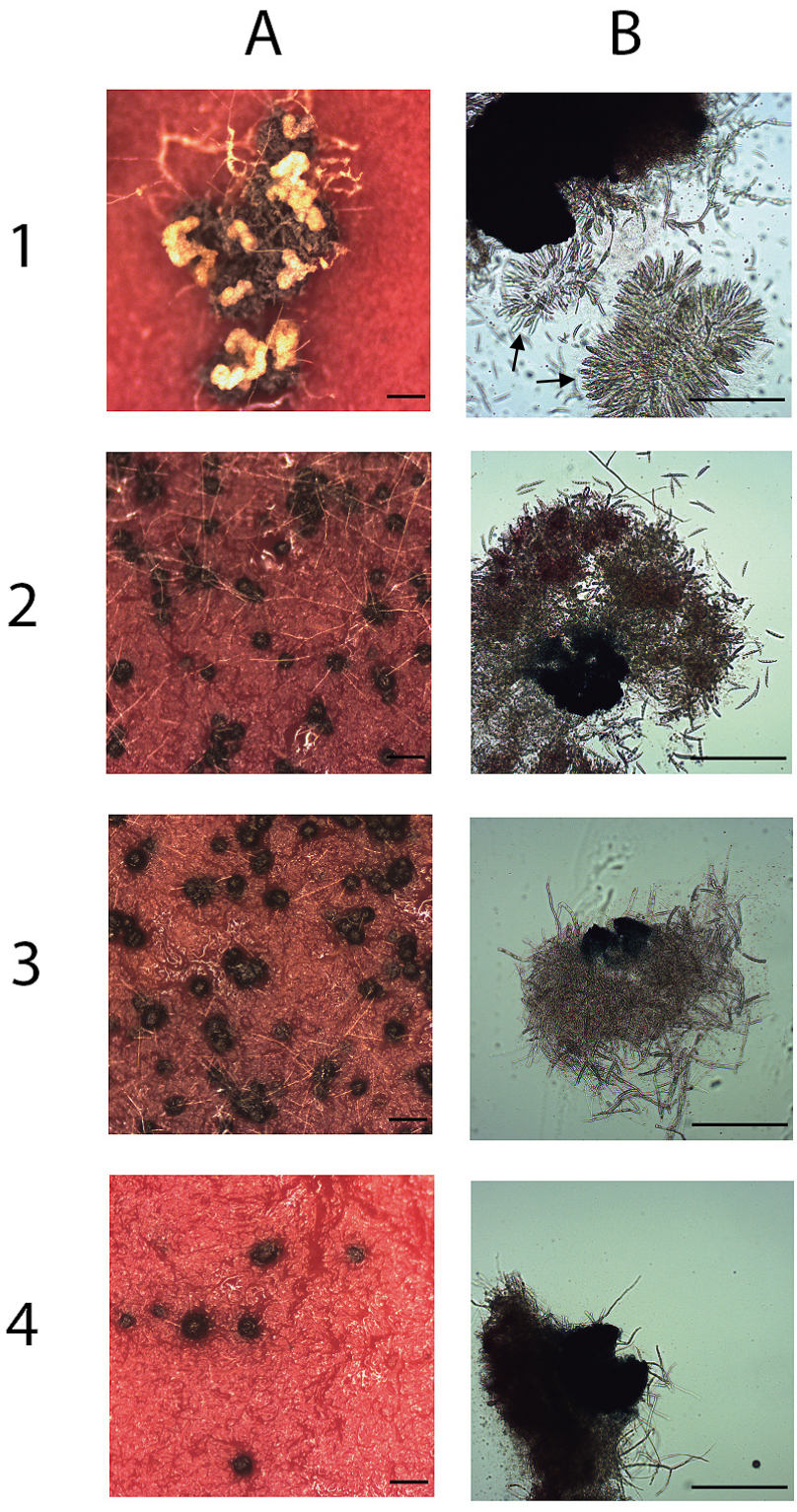

A
B
1
2
3
4

Figure 3. Fertility phenotypes of MAT deletion mutants on carrot agar. Scale bars = 200 microns. **1A:** Wild type strain PH-1 perithecia with cirrhi, 100X magnification. **1B:** A single perithecium of PH-1, squashed to reveal mature asci and ascospores (black arrows), 400X magnification. **2A:** Perithecia of MAT1 deletion mutants were less than half the size of WT perithecia and never produced cirrhi. 100X magnification. **2B:** Perithecia of the MAT1 deletion mutants did not release asci or ascospores when squashed. The spores visible in this photo are asexual conidia. **3A:** Perithecia of the MAT1-1-1 deletion mutants were similar in size, number, and appearance to those of the MAT1 deletion mutants. 100X magnification. **3B:** Perithecia of MAT1-1-1 deletion mutants did not contain asci or ascospores. **4A:** MAT1-2-1 deletion mutants in general produced fewer perithecia than MAT1 or MAT1-1-1 deletions strains, although they were similar in appearance at less than half the size of the WT perithecia. 100X magnification. **4B:** MAT1-2-1 perithecia also did not contain asci or ascospores. 400X magnification.