2015

Fungicide-induced transposon movement in Monilinia fructicola

Fengping Chen
Fujian Agriculture and Forestry University, Fuzhou

Sydney E. Everhart
University of Nebraska-Lincoln, everhart@unl.edu

P. Karen Bryson
Clemson University, Clemson

Chaoxi Luo
Huazhong Agricultural University

Xi Song
China Agricultural University, Beijing

See next page for additional authors

Published in *Fungal Genetics and Biology* 85 (2015), pp. 38–44. doi 10.1016/j.fgb.2015.10.006

Submitted August 20, 2015; revised October 10, 2015; accepted October 29, 2015; published online October 29, 2015.

Fungicide-induced transposon movement in *Monilinia fructicola*

Fengping Chen,[1,2] Sydney E. Everhart,[3] P. Karen Bryson,[4] Chaoxi Luo,[5] Xi Song,[2] Xili Liu,[2] and Guido Schnabel[4]

1 Key Laboratory of Biopesticide and Chemical Biology, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, China
2 Department of Plant Pathology, China Agricultural University, Beijing, China
3 Department of Plant Pathology, University of Nebraska–Lincoln, Lincoln, NE, USA
4 School of Agricultural and Environmental Sciences, Clemson University, Clemson, SC, USA
4 College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China

Corresponding authors — X. Liu, seedling@cau.edu.cn ; and G. Schnabel, schnabe@clemson.edu

Fengping Chen and Sydney E. Everhart are co-first authors.

Abstract

Repeated applications of fungicides with a single mode of action are believed to select for pre-existing resistant strains in a pathogen population, while the impact of sub-lethal doses of such fungicides on sensitive members of the population is unknown. In this study, in vitro evidence is presented that continuous exposure of *Monilinia fructicola* mycelium to some fungicides can induce genetic change in form of transposon transposition. Three fungicide-sensitive *M. fructicola* isolates were exposed in 12 weekly transfers of mycelia to a dose gradient of demethylation inhibitor fungicide (DMI) SYP-Z048 and quinone outside inhibitor fungicide (QoI) azoxystrobin in solo or mixture treatments. Evidence of mutagenesis was assessed by monitoring *Mftc1*, a multicopy transposable element of *M. fructicola*, by PCR and Southern blot analysis. Movement of *Mftc1* was observed following azoxystrobin and azoxystrobin plus SYPZ048 treatments in two of the three isolates, but not in the non-fungicide-treated controls. Interestingly, the upstream promoter region of *Mf*CYP51 was a prime target for *Mftc1* transposition in these isolates. Transposition of *Mftc1* was verified by Southern blot in two of three isolates from another, similar experiment following prolonged, sublethal azoxystrobin exposure, although in these isolates movement of *Mftc1* in the upstream *Mf*CYP51 promoter region was not observed. More research is warranted to determine whether fungicide-induced mutagenesis may also happen under field conditions.

Keywords: Transposon movement, QoI fungicide, Mutagenesis, Brown rot

1. Introduction

Sublethal exposure to fungicides is likely common under field conditions and could impact the rate and mechanism of resistance evolution. For the brown rot fungus of pome and stone fruits, *Monilinia fructicola*, the development of resistance to commonly used fungicides has been well-characterized in field isolates (Amiri et al., 2010; Lim et al., 2001; Ma et al., 2003; Schnabel et al., 2004). However, the effect of fungicide exposure on the speed of resistance development has not been examined and it may be accelerated when the fungus is repeatedly exposed to sublethal doses of fungicides. This type of resistance has been shown to occur in bacterial populations in which antibiotic stress was linked to increased mutation rates (Cirz et al., 2005; Riesenfeld et al., 1997).

Some evidence also suggests an increased rate of resistance evolution in fungal populations that have already been exposed to a fungicide of a different mode of action. In a population study of *Venturia inaequalis*, researchers found that resistance to methyl benzimidazole carbamates and demethylation inhibitors (DMIs) developed at an accelerated pace in populations already resistant (and previously exposed) to an unrelated, multisite guanidine fungicide (dodine) (Koller and Wilcox, 2001). A similar phenomenon was observed in a laboratory study, where adaptation to propiconazole was accelerated in benzimidazole-resistant isolates (Luo and Schnabel, 2008a). These observations suggest that fungicide stress may induce mutation frequencies and therefore make exposed populations and individuals able to adapt faster to other stress factors such as fungicides of another chemical class.

Although mechanisms of resistance to azoxystrobin (QoI) and propiconazole (DMI), fungicides commonly used to control brown rot, are not fully understood, the modes of action are known. Azoxystrobin inhibits mitochondrial respiration by binding to the outer quinol-oxidation site of the fungal cytochrome bc1 enzyme complex, blocking electron transfer between cytochrome

b and cytochrome c1 which disrupts the energy cycle by halting the production of ATP (Bartlett et al., 2002; Becker et al., 1981). The interruption of electron flow in the respiratory chain causes an excess of electrons leading to aberrant generation of reactive oxygen species (ROS) (Magnani et al., 2008). Such ROS have been reported in *Magnaporthe oryzae* treated with QoI fungicide SSF-126 (Mizutani et al., 1996) and in *Fusarium graminearum* treated with azoxystrobin (Kaneko and Ishii, 2009). The mechanism of lethal exposure to azoxystrobin is likely due to lack of ATP production in combination with excessive ROS production that directly causes damage to DNA, RNA, and proteins, however the effects of continuous exposure to sublethal doses of azoxystrobin is unknown. The action of the DMI fungicide SYP-Z048 (Chen et al., 2012) is by inhibition of sterol 14a-demethylase (CYP51), a cytochrome P450 required for biosynthesis of sterols of fungal cell membranes. Most DMIs bind to the active site of the cytochrome P450 (Siegel, 1981). Observed resistance to DMI fungicides in *M. fructicola* is based on overexpression of the *CYP51* gene (Luo and Schnabel, 2008b), but other mechanisms of resistance to DMIs exist (Villani and Cox, 2011).

Direct evidence of non-target, fungicide-induced mutagenic changes, caused either directly by a fungicide or indirectly as a stress response, has not been reported. Such changes could include typical mechanisms that lead to fungicide resistance, such as the movement of transposable elements into the promoter region of a target gene (Luo and Schnabel, 2008b; Ma et al., 2006) or the implementation of nucleotide sequence variations in target genes (Ishii et al., 2007; Leroux et al., 2007). The purpose of this study was to investigate whether fungicide stress can lead to genetic changes typically associated with fungicide resistance in a phytopathogenic, filamentous fungus. We utilized *M. fructicola* as a model system to assess potential mutagenic effects of two commercially applied chemical fungicides with differing modes of action (DMI and QoI). Evidence of fungicide-induced mutagenesis was examined using previously-discovered transposable element *Mftc1* (Luo et al., 2008).

2. Materials and methods

2.1. Isolates and fungicides

The three single-spore *M. fructicola* isolates SCDL28, NY9C and OH6P (Luo et al., 2008) originated from South Carolina, New York, and Ohio, respectively. These isolates were sensitive to the experimental fungicides prior to experiment initiation; EC50 values for SYP-Z048 and azoxystrobin were <0.04 and <0.03 μg/ml, respectively. Single-spore isolates Kac18, 139, and 190 were generated during a previous study where isolates were exposed continuously over 10 weeks to sublethal doses of propiconazole (DMI) and azoxystrobin fungicide (Luo and Schnabel, 2008a). In that study, mycelium of isolates was transferred weekly on V8-media amended with salicylhydroxamic acid (SHAM) or sublethal doses of azoxystrobin plus SHAM or propiconazole. These isolates were recovered from stock cultures stored with blue silica gel beads at –20 °C on dried filter paper discs (Fisher Scientific, Pittsburgh, PA).

The technical grade (84% active ingredient) of the demethylation inhibitor (DMI) fungicide SYP-Z048 (Shenyang Research Institute of Chemical Industry, Shenyang, China) and commercial formulation of the quinone outside inhibitor (QoI) fungicide azoxystrobin (Abound; Syngenta Crop Protection, Greenville, NC) were used. SYP-Z048 and azoxystrobin were chosen for *M. fructicola* isolates because they are recommended for control of brown rot of peach caused by *M. fructicola* (Horton et al., 2011). The technical grade SYP-Z048 was dissolved in methanol at 10 mg/ml to prepare stock solutions and stored at 4 °C. The commercial formulation of azoxystrobin was dissolved in sterile water, adjusted to 1 mg/ml, and stored at 4 °C.

2.2. Continuous exposure to sublethal doses of DMI and QoI fungicides

Isolates SCDL28, NY9C, and OH6P were subjected to a nonfungicide control and three fungicide treatments consisting of SYP-Z048, azoxystrobin, and a mixture of SYP-Z048 and azoxystrobin over a period of 12 weeks (Figure 1A). SYP-Z048 and azoxystrobin were applied at 50 μg/ml and 200 μg/ml stock solution, respectively, and the mixture contained 25 μg/ml SYP-Z048 and 100 μg/ml of azoxystrobin stock solution. Control plates received no fungicide. Potato dextrose agar (PDA) amended with azoxystrobin also contained 100 μg/ml of SHAM, which is added to inhibit the alternative oxidase pathway and is routinely used for QoI resistance assessment (Olaya et al., 1998). Fungicide stock solutions (54.3 μl) were spread on solidified PDA in a Petri dishes (15 cm diameter; 50 ml PDA per dish) with a spiral plater (Neu-tec Group INC, Farmingdale, NY). The exponential and slow mode settings were used to create a gradient of decreasing fungicide concentration from the center of the plate outward. Mycelial growth on the outer periphery of the dishes was uninhibited, while mycelial growth close to the center of the plate was completely inhibited. After the fungicides were spread, dishes were kept at room temperature for 2–4 h to allow penetration of the fungicide into the medium.

Inoculum for fungicide exposure plates was prepared on 53 × 4 mm Grade P8 filter paper strips (cellulose fiber; particle retention of 20–25 μm; flow rate of 160 mL/min; Fisher Scientific, Pittsburgh, PA). Aerial mycelium and conidia were scraped off a 5– 7 day old PDA plate of fungal culture and placed into 2-ml centrifuge tubes containing 500 μl sterilized water and 2 glass beads. The tube was vortexed for 1 min and 60 μl of suspension containing mycelium and spores were distributed with a pipette along and onto paper strips resting on PDA in 9 cm Petri dishes. After 3 days of incubation at 22 °C in the dark the mycelium-covered strips (two for each isolate) were transferred onto PDA amended with a fungicide gradient (see above) in 15 cm diameter dishes. Each dish contained six strips with two replicate strips per isolate on opposite sides and three isolates per dish. Aerial mycelium and conidia that developed closest to the center of each plate and stretching to the point where mycelial growth was inhibited by 50% compared to the growth on the very edge of the plate were scraped off after 4 days the experimental dishes and prepared as above for each subsequent weekly transfer. Thus the term 'sublethal exposure' is meant to reflect a dose range that inhibited 50–99.9% of mycelial growth. The mycelium of the two strips per isolate was pooled. The entire experiment was repeated.

Isolates Kac18, 139, and 190 were transferred on PDA petri dishes described in a previous study where isolates were exposed continuously for 10 weeks to sublethal doses of propiconazole (DMI) and azoxystrobin fungicide (Figure 1B) (Luo and Schnabel, 2008a).

2.3. DNA extraction

Genomic DNA was purified from *M. fructicola* isolates before transfer (parent isolates, not subjected to transfers) and after 12 transfers for SCDL28, NY9C and OH6P, or after 10 transfers for Kac18, 139, and 190. For all isolates, DNA was extracted from mycelium using a modified Cetyl Trimethylammonium Bromide method (Chen et al., 2012). Spores were scraped off the surface of a 7-day-old culture grown on tomato sauce agar (227 g canned tomato sauce with no additives and 20 g agar in 750 ml water) and placed into 2-ml centrifuge tubes containing 1000 μl sterile water.

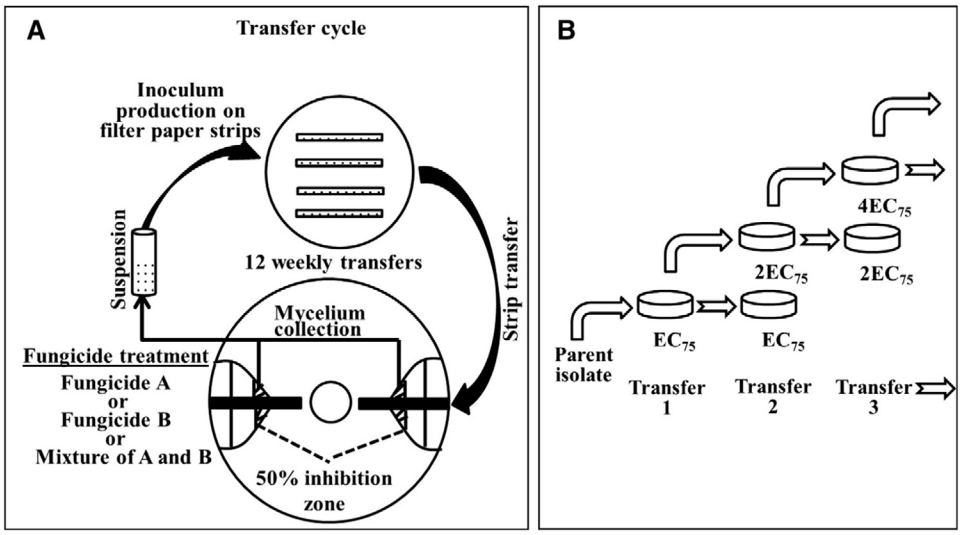

Figure 1. Schematic of weekly transfers of *Monilinia fructicola* cultures on PDA amended with a fungicide dose gradient (A) or a single inhibitory dose (B). For the dose gradient, a suspension of mycelium and spores from parental isolates was used to inoculate filter paper strips. After 3 days of incubation, the strips were transferred to unamended PDA (control) or PDA amended with a concentration gradient (highest fungicide dose in the center of the dish). For the single dose, plugs containing actively growing mycelium and spores from parental isolates were used for transfers. Isolates were maintained on two doses of the selective fungicide, one that allowed >20% growth and one that was double that concentration. Isolates were transferred individually to the next higher dose if the relative mycelial growth exceeded 20% (Luo and Schnabel, 2008a).

Spore suspension was approximately 106 spores/ml and 600 μl was transferred into 250-ml flasks containing 100 ml of potato dextrose broth. The liquid cultures were incubated at room temperature at 120 rpm. After 4 days, the mycelium was collected and flash frozen using liquid nitrogen. Frozen mycelium was ground with mortar and pestle in liquid nitrogen. Powder (0.2 g) was added to 750 μl extraction buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl) and 2 μl RNase A (100 mg/ml, Qiagen Inc., Valencia, CA), then incubated for 1.5 h at 65 °C with occasional mixing. After extraction with one volume of phenol–chloroform–isoamyl alcohol (25:24:1), DNA was precipitated with one volume of isopropyl alcohol for 10 min at room temperature (23 °C). The suspension was centrifuged at 12,000*g* for 10 min and the pellet was washed with 75% ethanol. DNA was dried in a laminar flow hood and suspended in TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) buffer, and then subjected to Southern blot analysis or used as template for PCR.

2.4. Southern hybridization analysis

About 8 μg of genomic DNA was digested with *BamH* I (NEB, Ipswich, MA), separated on a 2.0% agarose gel and transferred onto a positively charged nylon membrane (Hybond –N+, GE Healthcare, Pittsburgh, PA). Two transposable elements have been characterized in *M. fructicola,* and both were examined in this study. One mobile element designated 'Mona' located in the promoter region of *Mf*CYP51 was associated with resistance to DMI fungicide propiconazole (Luo and Schnabel, 2008b). Our efforts to assay mycelium for Mona using Southern blot analysis failed presumably because the signal generated by the full-length Mona probe fragment was too weak to be detected. Thus, remaining efforts focused on detecting movement of *Mftc1*, a 1500 bp element with 28 bp imperfect inverted terminal repeats which generates a 4 bp target site duplication. This transposon has no nucleotide similarity to Mona and can be found at GenBank accession number EU257287 and was briefly mentioned in a previous research paper (Luo et al., 2008).

A DNA fragment of 703 bp was amplified from SCDL28 parent isolate using primers mtcf1 50-GGGATATTAACCAATCACAACGT-30 and mtcr1 50-CGATTGGACAGATAAGCAATGG-30 . The product was sequenced and used for probe labeling. Probe labeling, hybridization and detection were performed using the AlkPhos Direct Labeling and Detection System with CDP-Star (GE Healthcare, Pittsburgh, PA) according to the manufacturer's instruction. Films were developed using a Mini-Med X-ray Film Processor (AFP Imaging Corporation, Elmstead, NY).

2.5. Amplification of the upstream region of MfCYP51

Primer set INS65F/INS65R was used to amplify the upstream region of *Mf*CYP51 (Luo et al., 2008) known to be a target for transposable elements (Luo et al., 2008). The PCR product was separated in a 2.0% agarose (Thermo Scientific, Rockford, IL) gel in 1× TAE buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.0). Gels were photographed under UV light with an Electrophoresis Documentation and Analysis System (KODAK EDAS 290, Eastman Kodak Company, New Haven, CT).

2.6. Data analysis

The proportions of transposon transposition in isolates were calculated for all three fungicide treatment groups (control, DMIs (SYP-Z048 and propiconazole), and azoxystrobin (AZO)) based on the results of Southern blot and shown in Table 1. Transposition was scored visually in treated isolates and their parents according to the different banding patterns after DNA hybridization. A hypothesis test was conducted to determine if there was an overall difference among the three fungicide transposition proportions, and follow-up hypothesis tests were conducted to determine specific differences among the fungicide transposition proportions. All tests were conducted using Fisher's Exact Test (to correct for the small sample sizes in each group). The results of the hypothesis tests are also shown in Table 1. The alpha level was set at 0.05 for all tests (i.e. *p*-value less than or equal to 0.05 indicated statistical significance); and all calculations were performed using SAS software 9.2 (SAS Inc, Cary, NC).

Table 1. Comparison of treatment groups according to pairwise nalysis.

Fungicide treatment group	Estimated proportion of transposition[a]
Control	0/5 = 0.0000[B]
DMIs	1/6 = 0.1667[B]
Azoxystrobin	7/9 = 0.7778[A]

a. The overall test of differences among the three proportions of transpositions resulted in a *p*-value of 0.0086 indicating significant differences among the three fungicides. Results of pairwise tests among the fungicide proportions are indicated with superscript letters; different letters indicate significant differences and similar letters indicate no significant differences.

3. Results

3.1. Movement of transposon Mftc1 at upstream region of MfCYP51

During an in vitro experiment designed to investigate the ability of *M. fructicola* strains to adapt to DMI and QoI fungicides we investigated whether the loss or gain of transposable element Mona (Luo and Schnabel, 2008b) in the promoter region of *Mf*CYP51 was associated with the adaptation process. PCR amplification of the upstream region of *Mf*CYP51 in three isolates did not reveal gains or losses of Mona following treatments; however, we found a larger insertion in azoxystrobin-exposed mycelium of isolate SCDL28 after only six transfers (Figure 2). Sequencing revealed a 1500 bp transposon, designated *Mftc1*, with 28 bp inverted terminal repeats, a degenerated transposase sequence, and a flanking 4 bp target site duplication. The TE is a member of the Tc1-*mariner* superfamily of transposable elements. The sequence of *Mftc1* was 100% identical to a transposable element that had been found in one of our previous studies at the same position in the *Mf*CYP51 the promoter in isolates from cherry fruit grown in Geneva, NY (Luo et al., 2008). Mutants containing *Mftc1* upstream *Mf*CYP51 did not increase expression of *Mf*CYP51 (data not shown). This unexpected observation led us to hypothesize that continuous exposure to sublethal doses of azoxystrobin may induce transposable element movement.

After 12 rounds of transfers, the presence or absence of *Mftc1* upstream of *Mf*CYP51 was assessed. *Mftc1* was still present in azoxystrobin-exposed SCDL28 and was also found at that location in SCDL28 mycelium treated with the mixture of azoxystrobin and SYP-Z048. Additionally, isolate NY9C which originally carried *Mftc1* within the promoter region lost the transposon following the fungicide mixture treatment (Figure 3). No insertions or deletions were found in the other treatments of isolates SCDL28 and NY9C or in any treatment of isolate OH6P.

3.2. Movement of transposon Mftc1 in the fungal genome

To get a broader view of *Mftc1* movement, *Mftc1* probe banding patterns on Southern blots of parental and transferred isolates from each treatment of two independent experiments were compared. In the first experiment, involving isolates SCDL28, NY9C and OH6P, each parental DNA produced a distinct *Mftc1* banding pattern composed of multiple bands, suggesting that many copies of *Mftc1* are present in these isolates (Figure 4A–C). The banding pattern was unaltered following control treatment or treatment with DMI fungicide; however, following azoxystrobin treatment in two isolates and the mixture of azoxystrobin and SYP-Z048 treatment in all three isolates the banding pattern was altered, suggesting transposition of *Mftc1* in these cultures. Isolate OH6P became nonviable in the non-fungicide amended control treatment and therefore it is not shown in the analysis. The entire experiment was repeated starting with the first transfer and similar southern blot results were obtained for the three isolates before and after exposure. The second experiment, involving isolates Kac18, 139, and 190, showed similar results, with multiple *Mftc1* bands suggesting multiple copies of the transposon in each isolate. An altered *Mftc1* banding pattern was present following azoxystrobin treatment in two of the three isolates, but not in any of the control treatments (Figure 4D–F). In one isolate *Mftc1* movement was observed in the propiconazole treatments. Although southern blot indicated *Mftc1* movement, this TE was not inserted in the *Mf*CYP51 upstream region in these isolates (data not shown). Pairwise analysis revealed that the proportion of altered banding patterns for azoxystrobin treatments among all isolates were significantly different from the proportion of altered patterns found in the control and DMI treatments (Table 1).

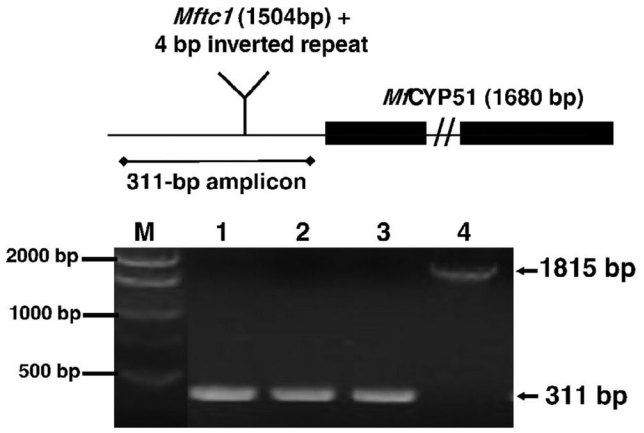

Figure 2. Gel electrophoresis analysis of DNA fragments amplified with primers INS65-F and INS65-R using DNA from isolate SCDL28 after six rounds of transfers. M = GeneRuler 1 kb plus DNA ladder (Fisher); lanes 1–4, isolate SCDL28, SCDL28 after 6 transfers, SCDL28 after 6 transfers on SYP-Z048-amended medium, and SCDL28 after 6 transfers on azoxystrobin-amended medium. The schematic above the gel indicates the location and size of transposable element *Mftc1* identified in the amplicon of the azoxystrobin treatment.

Isolates SCDL28 and NY9C were cultured on control plates lacking SHAM, whereas isolates KAC18, 139 and 190 were cultured on control plates with SHAM. Neither version of the control showed evidence for increased transposon movement (Figure 4A–F).

4. Discussion

This study shows that continuous exposure to sublethal doses of azoxystrobin can trigger movement of transposon *Mftc1* in *M. fructicola*. Transposition was observed during analysis of the *Mf*CYP51 promoter following fungicide exposure, with one isolate gaining *Mftc1* at this site and one losing it. Genome alterations, assessed as changes in the banding patterns on Southern blots caused by *Mftc1* transposition, were observed significantly more often following azoxystrobin exposure compared to the control or DMI treatments. Five out of six isolates had detectable genomic changes following at least one of the treatments which included azoxystrobin. In one case changes were observed following propiconazole treatment indicating that mutagenesis may occur in response to DMI stress, but at a reduced frequency. Whether other QoI or DMI fungicides can induce transposition was not investigated in this study.

Transposon movement is an important mechanism in natural populations of fungi to adapt to stressful environments and is possibly more important for populations lacking sexual recombination as a source of building genetic diversity in a population (Daboussi and Capy, 2003). Transposon movement has been shown to be induced by stress factors like temperature extremes or the exposure to metals such as Cu^{2+} (Ogasawara et al., 2009).

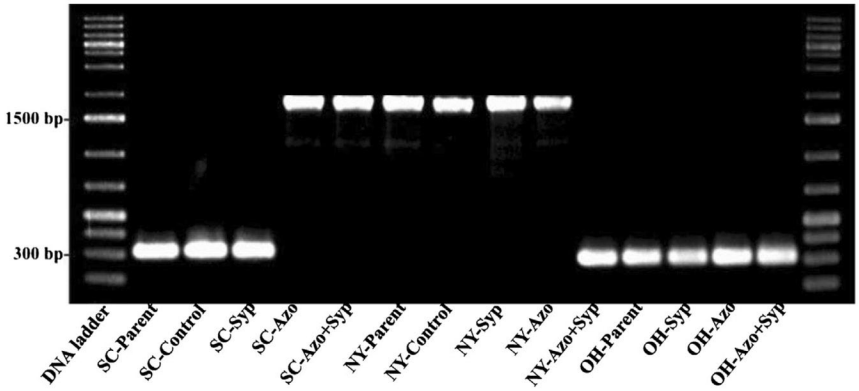

Figure 3. Gel electrophoresis analysis of DNA fragments from isolates SCDL28, NY9C, and OH6P amplified with primers INS65-F and INS65-R after 12 rounds of transfers. M1 = GeneRuler 1 kb plus DNA ladder (Fisher); Lanes 1–5 are isolate SCDL28: parental strain, control treatment, SYP-Z048 treatment, azoxystrobin treatment, azoxystrobin plus SYP-Z048 treatment; lanes 6–10 are isolate NY9C: parental strain, control treatment, SYP-Z048 treatment, azoxystrobin treatment, azoxystrobin plus SYP-Z048 treatment; lanes 11–14 are isolate OH6P: parental strain, SYP-Z048 treatment, azoxystrobin treatment, azoxystrobin plus SYP-Z048 treatment; M2 = GeneRuler.

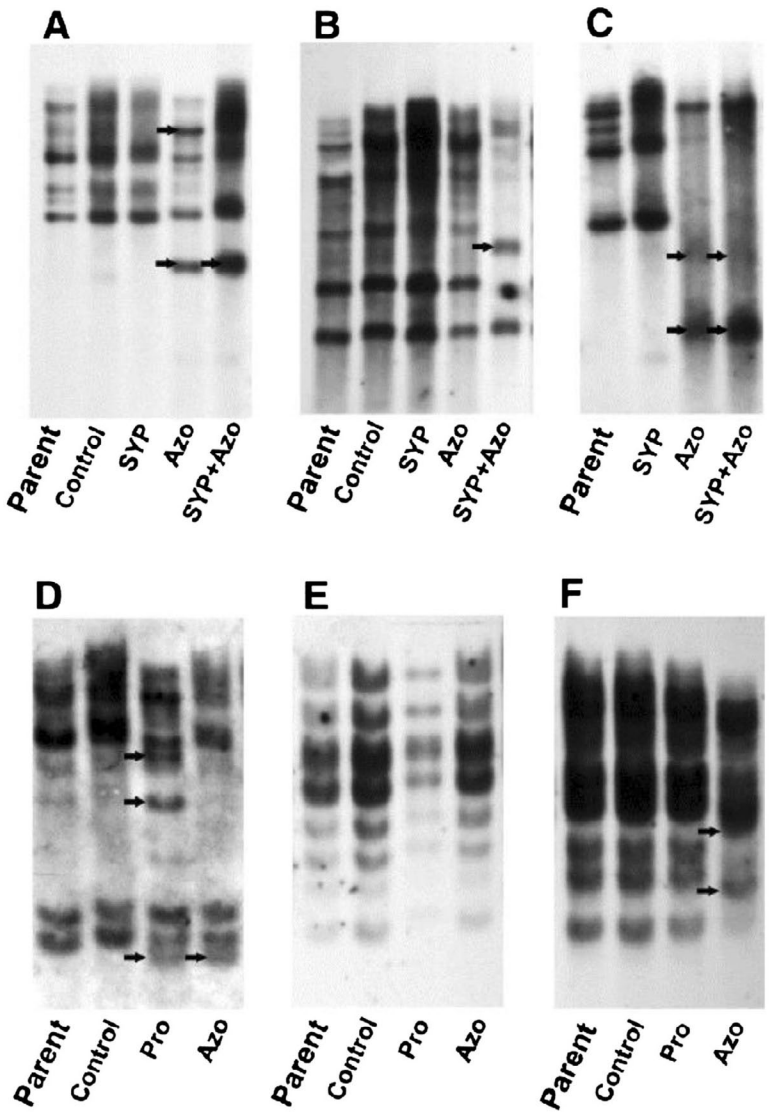

Figure 4. Southern blot analysis showing transposition of *Mftc1* in *Monilinia fructicola* isolates SCDL28 (A), NY9C (B), and OH6P (C) subjected to 12 consecutive, weekly transfers on unamended PDA (control) or PDA amended with: DMI fungicide SYP-Z048 (syp), QoI fungicide azoxystrobin (azo), and their mixture (syp + azo). Isolates KAC18 (D), 139 (E) and 190 (F) were subjected to DMI fungicide propiconazole (pro) and azoxystrobin. Parent isolates were not subjected to transfers. The OH6P control was not viable after 12 transfers and therefore is missing from the gel. Black arrows indicate new bands that were not observed in the parent or control treatments.

The movement of retrotransposon *Ty1* was induced during nutritional deficiency, which provided *Saccharomyces cerevisiae* with the ability to adapt to a new environment although the insertions had a negative effect on fitness on average (Wilke and Adams, 1992). We did not investigate the mode of action by which azoxystrobin might induce transposon movement, but inhibitors of mitochondrial respiration, are known to accelerate the generation of reactive oxygen species (ROS) (Turrens and Boveris, 1980) which could act as a trigger. This is supported by a recent study showing that ROS generation was enhanced in *F. graminearum* mycelium exposed to azoxystrobin and SHAM, compared to the azoxystrobin without SHAM treatment (Kaneko and Ishii, 2009). A direct link between the production of ROS and the activation of a transposon (*Ty1* retrotransposition) was established only recently, which represented the first evidence that ROS may have an independent and key role in the induction of *Ty1* retrotransposition in *S. cerevisiae* (Stoycheva et al., 2010).

Transposable elements have been directly associated with the development of fungicide resistance in ascomycetes. For example, DMI resistance due to overexpression of the target site gene *cyp51* was associated with a long interspersed nuclear element (LINE)- like retrotransposon in *Blumeriella jaapii* (Ma et al., 2006) and an uncharacterized 553 bp element in *V. inaequalis* (Schnabel and Jones, 2001). In this study we demonstrate for the first time a strong association between exposure to site-specific fungicides and the induction of transposon movement. The practical relevance of the observed phenomenon is not clear, since this was a study conducted under laboratory conditions where the fungus was exposed continuously over 12 weeks to sublethal doses of azoxystrobin. Such conditions are not likely to occur in the field if label rates are followed, which states to not apply more than two sequential applications of azoxystrobin-containing products before alternating with a fungicide of a different chemical class. However, according to the current label Abound 4FL (a.i. azoxystrobin) could legally be applied 10 times per season for strawberry disease management if used at the lowest label rate. Thus it would be worth investigating whether exposure of field populations to QoI fungicides at sublethal doses (low rates combined with dilution effect in plant tissue) could allow for quicker diversification and thus emergence of fungicide resistance. Clearly, more work needs to be done to investigate this novel and important hypothesis.

Plant pathogens are often exposed to sublethal doses of fungicides. Although producers routinely apply fungicides in the field at rates high enough to kill sensitive pathogens, exposure of plant pathogens to sublethal doses of fungicides occurs frequently and with every spray application. Fungicides may be diluted as they systemically enter the plant tissue, the spray coverage may be insufficient if too little water is used per hectare or only every other row is sprayed, or the fungicide may be applied at a dose lower than recommended by the label to reduce cost. It is therefore theoretically possible that a fungicide-induced increase in mutagenesis in the field creates strains with increased genotypic plasticity that can adapt quicker to new environmental stresses such as a new chemical class of fungicide. Such mutator strains were identified in bacteria (LeClerc et al., 1996; Martinez and Baquero, 2000) and yeasts (Clark et al., 1999; Glassner et al., 1998) and are believed to possess alterations in DNA repair mechanisms (LeClerc et al., 1996; Martinez and Baquero, 2000). The existence of such strains has been suggested previously to explain the observed predisposition of a pathogen population already resistant to a fungicide to accelerated resistance development to another, unrelated fungicide (Koller and Wilcox, 2001). Alternatively it is possible that fungicide-induced, accelerated mutagenesis would simply increase genetic diversity in the population thereby increasing the speed for the emergence of resistant individuals.

The addition of SHAM to medium amended with azoxystrobin for ROS generation in the fungus appears to be a prerequisite because of the alternative oxidase (AOX) pathway's protective role against oxygen stress (Magnani et al., 2008; Van Aken et al., 2009). In our study, SHAM by itself (without azoxystrobin) did not induce mutagenic responses, but it was always added to the azoxystrobin-containing treatments to suppress the AOX pathway to simulate the condition in the field during plant–pathogen interaction. The AOX pathway in pathogens is suppressed by flavonoids of the host plant according to the "flavonoid hypothesis" (Wood and Hollomon, 2003), but in vitro the AOX pathway allows the pathogen to synthesize ATP and regain metabolic activity despite fungicide exposure. It is therefore conceivable that mutagenesis of the fungus during azoxystrobin exposure may only happen during host– pathogen interactions when the AOX pathway is inhibited by flavonoids but not on spores emerging and physically separated from infected plant tissue.

In conclusion, continuous exposure of mycelium in vitro to sublethal doses of azoxystrobin induced transposon movement. More research is justified to investigate the field relevance of this phenomenon and to determine whether other QoI fungicides are also inducers of TE movement under experimental conditions.

Acknowledgments — We thank Junli Liu in Shenyang Research Institute of Chemical Industry in China for providing the fungicide SYP-Z048, Dolores Fernández-Ortuño for technical support, E. Schnabel for reviewing the manuscript and for providing helpful comments, and the China Scholarship Council. Funding was provided by the NIFA/USDA, under project number SC-1700501, the USDA Grants Contract no. 2010-34103-21000 and 2009-41534-05445, and China Postdoctoral Science Foundation (2015M571956). Clemson University Experiment Station No. 6126.

References

Amiri, A. et al., 2010. Reduced sensitivity in *Monilinia fructicola* field isolates from South Carolina and Georgia to respiration inhibitor fungicides. Plant Dis. 94, 737–743.

Bartlett, D.W. et al., 2002. The strobilurin fungicides. Pest Manage. Sci. 58, 649–662.

Becker, W.F. et al., 1981. Oudemansin, strobilurin-a, strobilurin-b and myxothiazol: new inhibitors of the *bc1* segment of the respiratory chain with an E-β-methoxyacrylate system as common structural element. FEBS Lett. 132, 329–333.

Chen, F.P. et al., 2012. Baseline sensitivity of *Monilinia fructicola* from china to the dmi fungicide syp-z048 and analysis of dmi-resistant mutants. Plant Dis. 96, 416–422.

Cirz, R.T. et al., 2005. Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol. 3, 1024–1033.

Clark, A.B. et al., 1999. Functional analysis of human MutSa and MutSb complexes in yeast. Nucleic Acids Res. 27, 736–742.

Daboussi, M.J., Capy, P., 2003. Transposable elements in filamentous fungi. Annu. Rev. Microbiol. 57, 275–299.

Glassner, B.J. et al., 1998. Generation of a strong mutator phenotype in yeast by imbalanced base excision repair. Proc. Natl. Acad. Sci. USA 95, 9997–10002.

Horton, D. et al., 2011. Southeastern peach, nectarine, and plum pest management and culture guide. University of Georgia Cooperative Extension Service, Athens, GA.

Ishii, H. et al., 2007. Molecular characterization and diagnosis of QoI resistance in cucumber and eggplant fungal pathogens. Phytopathology 97, 1458–1466.

Kaneko, I., Ishii, H., 2009. Effect of azoxystrobin on activities of antioxidant enzymes and alternative oxidase in wheat head blight pathogens *Fusarium graminearum* and *Microdochium nivale*. J. Gen. Plant Pathol. 75, 388–398.

Koller, W., Wilcox, W.F., 2001. Evidence for the predisposition of fungicide-resistant isolates of *Venturia inaequalis* to a preferential selection for resistance to other fungicides. Phytopathology 91, 776–781.

LeClerc, J.E. et al., 1996. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science 274, 1208–1211.

Leroux, P. et al., 2007. Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14 a-demethylation inhibitors in field isolates of *Mycosphaerelia graminicola*. Pest Manage. Sci. 63, 688–698.

Lim, T. et al., 2001. Fitness of dicarboximide-resistant and sensitive *Monilinia fructicola* isolated from peach in Korea. Plant Pathol. J. 17, 205–209.

Luo, C.X. et al., 2008. Occurrence and detection of the DMI resistance-associated genetic element 'Mona' in *Monilinia fructicola*. Plant Dis. 92, 1099–1103.

Luo, C.X., Schnabel, G., 2008a. Adaptation to fungicides in *Monilinia fructicola* isolates with different fungicide resistance phenotypes. Phytopathology 98, 230–238.

Luo, C.X., Schnabel, G., 2008b. The cytochrome P450 lanosterol 14a-demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. Appl. Environ. Microbiol. 74, 359–366.

Ma, Z.H. et al., 2006. Overexpression of the 14 a-demethylase target gene (*CYP51*) mediates fungicide resistance in *Blumeriella jaapii*. Appl. Environ. Microbiol. 72, 2581–2585.

Ma, Z.H. et al., 2003. Identification and characterization of benzimidazole resistance in *Monilinia fructicola* from stone fruit orchards in California. Appl. Environ. Microbiol. 69, 7145–7152.

Magnani, T. et al., 2008. Silencing of mitochondrial alternative oxidase gene of *Aspergillus fumigatus* enhances reactive oxygen species production and killing of the fungus by macrophages. J. Bioenerg. Biomembr. 40, 631–636.

Martinez, J.L., Baquero, F., 2000. Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother. 44, 1771–1777.

Mizutani, A. et al., 1996. A possible mechanism of control of rice blast disease by a novel alkoxyiminoacetamide fungicide, SSF126. Phytopathology 86, 295–300.

Ogasawara, H. et al., 2009. *Crawler*, a novel *Tc1/mariner*-type transposable element in *Aspergillus oryzae* transposes under stress conditions. Fungal Genet. Biol. 46, 441–449.

Olaya, G. et al., 1998. Differential responses of germinating *Venturia inaequalis* conidia to kresoxim-methyl. Pestic. Sci. 54, 230–236.

Riesenfeld, C. et al., 1997. Adaptive mutations produce resistance to ciprofloxacin. Antimicrob. Agents Chemother. 41, 2059–2060.

Schnabel, G. et al., 2004. Reduced sensitivity in *Monilinia fructicola* to propiconazole in Georgia and implications for disease management. Plant Dis. 88, 1000–1004.

Schnabel, G., Jones, A.L., 2001. The 14 a-demethylase (*CYP51A1*) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. Phytopathology 91, 102–110.

Siegel, M.R., 1981. Sterol-inhibiting fungicides: effects on sterol biosynthesis and sites of action. Plant Dis. 65, 986–989.

Stoycheva, T. et al., 2010. The role of reactive oxygen species in the induction of *Ty1* retrotransposition in *Saccharomyces cerevisiae*. Yeast 27, 259–267.

Turrens, J.F., Boveris, A., 1980. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem. J. 191, 421–427.

Van Aken, O. et al., 2009. Alternative oxidase: a target and regulator of stress responses. Physiol. Plant. 137, 354–361.

Villani, S.M., Cox, K.D., 2011. Characterizing fenbuconazole and propiconazole sensitivity and prevalence of 'Mona' in isolates of *Monilinia fructicola* from New York. Plant Dis. 95, 828–834.

Wilke, C.M., Adams, J., 1992. Fitness effects of *Ty* transposition in *Saccharomyces cerevisiae*. Genetics 131, 31–42.

Wood, P.M., Hollomon, D.W., 2003. A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. Pest Manage. Sci. 59, 499–511.