Function of the genetic element ‘Mona’ associated with fungicide resistance in *Monilinia fructicola*

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SUMMARY

The genetic element ‘Mona’ has been shown previously to be associated with resistance to demethylation inhibitors (DMIs) in *Monilinia fructicola*. In this study, the promoter activity of the ‘Mona’ element was demonstrated genetically and the activity was narrowed down to a 20-bp active region through a series of deletions. ‘Mona’ knockout transformants (ΔMona) were generated from DMI-resistant isolate Bmpc7, and EC_{50} values and expression of the *MfCYP51* gene were found to be reduced in transformants compared with the parental isolate. When the ‘Mona’ element was inserted into the upstream region of the *MfCYP51* gene of the DMI-sensitive isolate HG3, the EC_{50} values and expression of the *MfCYP51* gene increased in the transformants compared with the parental sensitive isolate. These results indicate that the ‘Mona’ element determines the DMI fungicide resistance through the up-regulation of the expression of the downstream *MfCYP51* gene. No fitness penalty was observed in knockout and insertion transformants, i.e. transformants showed similar mycelial growth rate, sporulation and ability to cause lesions on fruit compared with their parental isolates, suggesting that the ‘Mona’ element does not affect basal life activities.

Keywords: DMI fungicide resistance, *MfCYP51* gene, ‘Mona’, *Monilinia fructicola*, peach brown rot.

INTRODUCTION

The ascomycete fungus *Monilinia fructicola* (G Wint) Honey is one of the most destructive pathogens of stone fruit worldwide, causing brown rot, blossom blight and twig canker. The control of the disease in commercial farms is largely dependent on pre-harvest applications of fungicides during bloom, ripening and fruit maturation (Ogawa *et al.*, 1995). Among the most effective fungicides are the sterol demethylation inhibitors (DMIs), which have been widely used for decades in stone fruit production. The single-site mode of action of DMIs, however, makes them vulnerable to resistance development. Resistance in *M. fructicola* was first documented in Georgia and South Carolina (Schnabel and Jones, 2001; Zehr *et al.*, 1999), and later in Ohio, New York (Luo *et al.*, 2008; Villani and Cox, 2008), New Jersey (Burnett *et al.*, 2010), Pennsylvania [illegible]

Jones, 2001), a 126- or 199-bp insertion in *Penicillium digitatum* (Ghosoph *et al*., 2007; Hamamoto *et al*., 2000), a 1.3-kb LTR retrotransposon in *Botrytis cinerea* (Kretschmer *et al*., 2009), a 2- to 5-kb truncated LINE-like retrotransposon in *Blumeriella jaapii* (Ma *et al*., 2006), a 120-bp indel in *Mycosphaerella graminicola* (Cools *et al*., 2012) and a 1.8-kb Aft1 transposon in the human pathogenic fungus *Aspergillus fumigatus* (Albarrag *et al*., 2011). We are only aware of one study providing genetic proof that the TE is associated with fungicide resistance. The 199-bp transposon sequence of *P. digitatum* located upstream of *PdCYP51B* and associated with DMI fungicide resistance has been confirmed to be a MITE-LIKE element, designated as PdMLE1. A core 20-bp sequence in PdMLE1 was deemed to be essential for promoter activity based on a series of deletion experiments (Sun *et al*., 2013b). To our knowledge, although the function of a transposon-like element with extremely small size (just 65 bp) has never been described, a regulatory mechanism may exist for the 'Mona' element in *M. fructicola*, because 'Mona' is associated with the overexpression of *MfCYP51* and DMI fungicide resistance in *M. fructicola* (Luo and Schnabel, 2008).

Here, we demonstrate the function of the 'Mona' element through a series of genetic transformations. The evidence presented in this study may shed light on the role of small transposon-like elements in fungal pathogens. The results may also be useful for the development of a simple 'Mona'-based method for the detection of DMI resistance in the future.

RESULTS

Promoter activity of the 'Mona' element

The 65-bp 'Mona' sequence was ligated with the open reading frame (ORF) of the *Npt2* gene and then cloned into vector pBHt2 to generate pMona-NPT (Fig. S1, see Supporting Information). The ORF of the *Npt2* gene was also cloned without 'Mona' into pBHt2

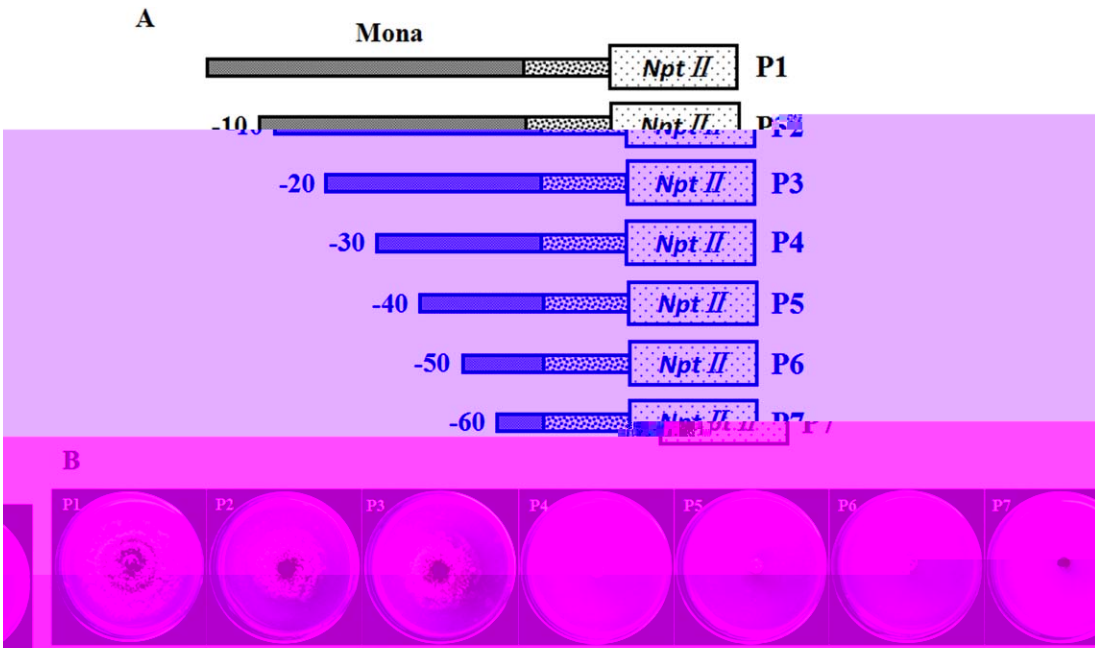

Fig. 2 Schematic diagram of truncated 'Mona' elements and the impact on *Npt2* gene expression in transformants. (A) P1 represents pMona-NPT and P2–P7 indicate the different vectors with truncated 'Mona'. (B) The activity of transformants from pMona-NPT and vectors with truncated 'Mona' was assessed on G418-amended potato dextrose agar (PDA) medium.

knockout allele (Fig. S3E). The EC_{50} values for propiconazole of the transformants varied, but, in general, were lower than the value for isolate Bmpc7 (0.216 μg/mL). The EC_{50} values of the nine transformants generated ranged from 0.005 to 0.100 μg/mL, with a mean value of 0.045 μg/mL (Table 1).

Real-time PCR was performed to determine the expression levels of the *MfCYP51* gene in the knockout transformants. The results showed that the expression of the *MfCYP51* gene in transformants was lower than that in Bmpc7 (Table 1). The expression of the *MfCYP51* gene was positively correlated with the EC_{50} values for the DMI fungicide propiconazole ($P < 0.01$, $r = 0.957$). There was no significant difference between the transformants and parental isolate in terms of mycelial growth rate, sporulation and ability to form lesions on detached fruit (Table 1).

Insertion of the 'Mona' element into the upstream region of MfCYP51

The genetic element 'Mona' was inserted into the upstream region of the *MfCYP51* gene of DMI sensitive (DMI-S) isolate HG3 (Fig. S4, see Supporting Information). Ten transformants were obtained with confirmed insertions verified by PCR (Fig. S5, see Supporting Information). Attempts to obtain transformants homogeneous in the transformed locus failed despite several rounds of single spore isolations (Fig. S5E).

The EC_{50} values to propiconazole were higher in the transformants with the inserted 'Mona' element than in the sensitive parental isolate HG3 (Table 2). *MfCYP51* gene expression was higher in all the transformants containing the inserted 'Mona' element compared with the parental isolate HG3 (Table 2). However, the expression of the *MfCYP51* gene was only moderately correlated with the EC_{50} values to the DMI fungicide propiconazole ($P < 0.05$, $r = 0.598$). No significant difference was observed between transformants with 'Mona' and the parental isolate for mycelial growth, sporulation and the ability to generate lesions on detached fruit (Table 2).

DISCUSSION

The involvement of TEs in eukaryotic resistance to fungicides has been documented in several plant and human fungal pathogens (Albarrag *et al.*, 2011; Cools *et al.*, 2012; Ghosoph *et al.*, 2007; Hamamoto *et al.*, 2000; Kretschmer *et al.*, 2009; Ma *et al.*, 2006; Schnabel and Jones, 2001). Resistance is always associated with the overexpression of fungicide target genes downstream of these transposons. However, direct genetic evidence for the involvement of promoter sequences in overexpression is often lacking.

The 65-bp genetic element 'Mona', located upstream of *MfCYP51*, is associated with DMI fungicide resistance. NNPP (Neural Network Promoter Prediction, version 2.2, http://www.fruitfly.org/seq_tools/promoter.html) predicted that the 'Mona' element was a promoter, but proof was lacking. In this study, we have shown that 'Mona' drives the expression of the *Npt2* gene and confirm that it acts, in general, as a promoter. The unaffected

Table 1 EC_{50} values, relative expression of the *MfCYP51* gene, mycelial growth, sporulation and lesion development of knockout transformants.

			Fitness parameter‡		
Isolate/transformant*	EC_{50} (μg/mL)	Relative expression†	Mycelial growth (cm/day)	Sporulation (10^6/cm^2)	Lesion size (cm)
Bmpc7	0.21	1	2.0 ± 0.6a	4.5 ± 0.6a	5.6 ± 0.6a
ΔBmpc-1	0.01	0.3 ± 0.1	1.9 ± 0.6a	3.9 ± 0.4a	5.9 ± 0.1a
ΔBmpc-2	0.07	0.4 ± 0.1	1.9 ± 0.8a	2.6 ± 0.5a	6.0 ± 0.3a
ΔBmpc-3	0.05	0.3 ± 0.1	2.0 ± 0.7a	3.6 ± 0.8a	5.7 ± 0.3a
ΔBmpc-4	0.01	0.2 ± 0.1	1.8 ± 0.7a	3.0 ± 1.4a	6.1 ± 0.2a
ΔBmpc-5	0.02	0.2 ± 0.1	1.8 ± 0.5a	3.0 ± 1.7a	5.9 ± 0.5a
ΔBmpc-6	0.09	0.5 ± 0.1	1.9 ± 0.6a	3.9 ± 1.3a	6.0 ± 0.3a
ΔBmpc-7	0.10	0.4 ± 0.1	1.9 ± 0.6a	4.0 ± 1.5a	6.1 ± 0.2a
ΔBmpc-8	0.03	0.2 ± 0.2	2.0 ± 0.6a	4.4 ± 2.0a	5.7 ± 0.1a
ΔBmpc-9	0.03	0.2 ± 0.0	2.1 ± 0.6a	4.3 ± 0.6a	6.0 ± 0.2a

*ΔBmpc-1–9 are the knockout transformants from the parental isolate Bmpc7.

†Relative expression of the *MfCYP51* gene in transformants was normalized with the *β-tubulin* gene and compared with that of the isolate Bmpc7.

‡Mean ± standard error of the mean (SEM); values within the same column followed by the same letters are not significantly different based on the analysis of the least-significant difference (LSD) test at $P = 0.05$ in SPSS.

Table 2 EC_{50} values, relative expression of the *MfCYP51* gene, mycelial growth, sporulation and lesion formation of 'Mona' insertion transformants.

			Fitness parameter‡		
Isolate/transformant*	EC_{50} (μg/mL)	Relative expression†	Mycelial growth (cm/day)	Sporulation (10^6/cm^2)	Lesion size (cm)
HG3	0.01	1	1.4 ± 0.5a	3.3 ± 0.2a	5.3 ± 0.2a
HG3:'Mona'-1	0.04	2.5 ± 1.4	1.4 ± 0.6a	3.4 ± 0.6a	5.2 ± 0.1a
HG3:'Mona'-2	0.08	12.9 ± 2.0	1.4 ± 0.6a	3.6 ± 0.4a	5.0 ± 0.1a
HG3:'Mona'-3	0.06	32.0 ± 12.4	1.3 ± 0.5a	3.1 ± 0.5a	5.3 ± 0.6a
HG3:'Mona'-4	0.10	2.0 ± 0.5	1.4 ± 0.5a	3.3 ± 0.1a	5.1 ± 0.0a
HG3:'Mona'-5	0.10	66.9 ± 18.4	1.4 ± 0.6a	3.1 ± 0.3a	5.3 ± 0.1a
HG3:'Mona'-6	0.06	11.1 ± 2.2	1.4 ± 0.5a	2.9 ± 0.6a	5.2 ± 0.3a
HG3:'Mona'-7	0.12	49.0 ± 5.8	1.4 ± 0.5a	3.1 ± 0.3a	5.1 ± 0.1a
HG3:'Mona'-8	0.14	48.2 ± 9.0	1.4 ± 0.6a	3.7 ± 0.1a	5.3 ± 0.1a
HG3:'Mona'-9	0.10	26.7 ± 4.3	1.3 ± 0.6a	3.5 ± 0.2a	5.3 ± 0.1a
HG3:'Mona'-10	0.11	7.7 ± 1.1	1.3 ± 0.6a	3.6 ± 0.1a	5.1 ± 0.1a

*HG3:'Mona'-1–10 are insertion transformants from the parental isolate HG3.

†Relative expression of the *MfCYP51* gene in transformants was normalized with the *β tubulin* gene and compared with that of the isolate HG3.

‡Mean ± standard error of the mean (SEM); values within the same column followed by the same letters are not significantly different based on the analysis of the least-significant difference (LSD) test at $P = 0.05$ in SPSS.

mycelial growth rate of 'Mona' transformants on G418-amended PDA compared with PDA suggests that the 'Mona' element is a rather powerful promoter. The only other study verifying a transposon to be a driver of a gene capable of conferring resistance to fungicides also showed a powerful performance of the inserted sequence (Sun *et al.*, 2013b). The small transposon-like element Mona acts as a powerful promoter and improves our understanding on the function of small transposon-like elements.

We have reported previously that the 'Mona' element exists in duplicate in the *M. fructicola* genome based on reverse transcription-polymerase chain reaction (RT-PCR) (Luo *et al.*, 2008). To confirm this result, next-generation sequencing was conducted for the DMI-R isolate Bmpc7. Thirty-two sequences homologous to 'Mona' were identified (E-value $< 10^{-10}$). Genome-wide localization of these elements did not reveal functional genes within 1 kb downstream of this element, suggesting that, apart from 'Mona', other homologous sequences do not have similar regulation ability (data not shown). A similar pattern was found in *P. digitatum*, where its transposon PdMLE1 was only found to cause the overexpression of *PdCYP51A* (Ghosoph *et al.*, 2007) and *PdCYP51B* (Sun *et al.*, 2013a, b). Although the PdMLE1 element was also found to be inserted into the upstream sequence of other genes, there was no evidence for these genes being involved in fungicide resistance and being overexpressed (Sun *et al.*, 2013a).

The core element of 'Mona' associated with promoter activity was located within 20 bp between P3 and P4. Sequence analysis of the core element indicated that it contained two sites for the known fungal transcriptional binding factor *ADR1* and seven sites for the vertebrate binding factors *CF2-II* and *CdxA*. A similar transcriptional factor has also been predicted by Hamamoto *et al.* (2000) in *P. digitatum* based on the analysis of a 126-bp tandem repeat fragment in the promoter region of DMI-R isolates. The 126-bp fragment contained four sites for fungal transcriptional binding factors *ADR1* and *HSF*, and 15 sites for vertebrate binding factors *HNF3-β*, *CdxA*, *MZF1*, *GATA-1*, *GATA-2*, *Pdx-1*, *Elf-1*, *c/EBPβ*, *v-Myb* and *c-Ets-1(p54)*. In both pathogens, the fungal transcriptional binding factor ADR1 was predicted to participate in regulation. It is possible that *M. fructicola* and *P. digitatum* share the same regulation model. *ADR1* has so far only been reported to encode carbon source-responsive transcriptional regulators that cooperatively control the expression of genes involved in ethanol utilization in yeast (Ciriacy, 1975; Ciriacy and Breitenbach, 1979). As *ADR1* homologues are also present in many filamentous fungi, including *M. fructicola*, further functions of *ADR1* need to be determined. To date, the transcriptional factors behind the responses to azole fungicides in filamentous fungi are still poorly understood and only a few have been identified. *AP-1*, a bZIP-type transcription factor, is known to be important for azole (DMI) resistance in both yeast and filamentous fungi (Alarco and Raymond, 1999; Alarco *et al.*, 1997; Qiao *et al.*, 2010). Another transcriptional factor, *CCG-8*, has been reported to contribute to azole adaptation by regulating the ketoconazole responses of various genes in *Neurospora crassa* and *Fusarium verticillioides* (Sun *et al.*, 2014).

As *M. fructicola* mycelium and conidia are multinucleate, a transformant may harbour mixed nuclei with various integration patterns (Lee *et al.*, 2010). In our study, at least four rounds of single spore isolation and several attempts at single hyphal tip purification failed to generate transformants homogeneous in the 'Mona' knockout locus. Protoplasts have been reported to have fewer nuclei than the undigested cells from which they are derived (da Silva and Paccola-Meirelles, 2001), which indicates that protoplast regeneration may provide an alternative method for the generation of homogeneous transformants from a heterogeneous background. Lee *et al.* (2010) successfully purified *Agrobacterium* spp.-mediated MfCUT1-GUS transformants by protoplast regeneration. However, the authors failed to obtain clean knockout mutants and indicated that *M. fructicola* was a very difficult species from which to obtain homokaryotic genes. Thus, alternative molecular genetic approaches, such as RNA-mediated gene silencing (Patel *et al.*, 2008), might be more suitable for functional analyses of genes in *Monilinia* spp.

To further verify the function of 'Mona', the 'Mona' element was inserted upstream of the *MfCYP51* gene. The increased EC_{50} value to propiconazole caused by the insertion of 'Mona' establishes a relationship between the 'Mona' element and DMI resistance. Curiously, although the expression of the *MfCYP51* gene in some transformants was higher than that of the DMI-R isolate Bmpc7, their EC_{50} values were lower than that of Bmpc7. It is possible that some of the transcribed *MfCYP51* RNA may not have been translated into mature protein.

In conclusion, the transposon-like element 'Mona' was genetically demonstrated to act as a powerful promoter. The 'Mona' element determined the DMI fungicide resistance through the up-regulation of expression of the downstream *MfCYP51* gene. A 'Mona'-based molecular tool should be established to monitor the development of the DMI fungicide in *M. fructicola* populations in the future.

EXPERIMENTAL PROCEDURES

Media and buffers

K-buffer contained 184 g K_2HPO_4 and 145 g KH_2PO_4 per litre. M-N buffer contained 30 g $MgSO_4{\cdot}7H_2O$ and 15 g NaCl per litre. Induction medium (IM) contained K-buffer (10 mL), M-N buffer (20 mL), 1 g glucose, 20% $(NH_4)_2SO_4$ (w/v) (2.5 mL), 1% $CaCl_2$ (w/v) (1 mL), 50% glycerol (w/v) (10 mL) and 7.808 g 2-(*N*-morpholino)ethanesulfonic acid (MES; 40 mM, MW195.2) per litre, pH 5.6. CO-IM buffer contained 400 μM acetosyringone in IM with 18% agarose. OM buffer contained $MgSO_4$ (1.2 M) and Na_2HPO_4 (10 mM). STC buffer contained 1.2 M sorbitol, 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, and 50 mM $CaCl_2$. Regeneration medium contained 1 M sucrose, 1 g yeast extract and 1 g casein hydrolysate per litre, 2% agar. Separation buffer A contained 0.6 M sorbitol and 100 mM Tris-HCl (pH 7.0). Separation buffer B contained 1.2 M sorbitol and 10 mM Tris-HCl (pH 7.5).

Fungal isolates, cultivation and DNA preparation

Single spore isolates of *M. fructicola* were collected from stone fruit orchards in China and the USA between 2006 and 2013. Cultures were stored on filter paper at −80°C (Table S1, see Supporting Information). Isolates were recovered by placing the filter paper on PDA at 22°C for 5 days in the dark. For DNA extraction, single agar plugs containing actively growing mycelium were transferred to 40 mL of potato dextrose broth (PDB) and incubated at 25°C on a 160-rpm orbital shaker for 3 days. Mycelium was harvested, rinsed with sterile water and genomic DNA was subsequently extracted using the Easypure Plant Genomic DNA Extraction Kit (TransGen Biotech, Beijing, China).

Plasmid construction

The *Npt2*-fused plasmid was constructed to demonstrate the function of 'Mona'. The ORF of the *Npt2* gene was amplified from pCAMBIA2300 using the primer pair nptII-F/nptII-R, digested with *Bam*HI and *Pst*I, and then cloned into the pBHt2 vector to generate the intermediary plasmid pNPT. The 'Mona' sequence was amplified using the primers MFE/MRE from genomic DNA of the DMI-R isolate Bmpc7. The PCR product was digested with *Kpn*I and *Sma*I and cloned into the upstream region of the *Npt2* gene in the plasmid pNPT to generate the plasmid pMona-NPT. The primers used to construct the vectors are listed in Table S2 (see Supporting Information).

Promoter deletion constructs

Deletion constructs were constructed for the 'Mona' element by deleting a series of 10-bp sequences from the 'Mona' element and inserting them in the upstream region of the G418 resistance gene of the vector pNPT. Six truncated 'Mona' fragments (P2–P7), together with the complete 'Mona' element (P1), were obtained (Fig. 2). Each of the fragments P2–P7 was synthesized by annealing two antiparallel oligonucleotides. Each single-strand oligonucleotide was first resuspended in TE buffer to a final concentration of 100 μM. Then, the forward strand and reverse strand oligonucleotides were mixed in a ratio of 1 : 1. Amplifications were performed in an iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) programmed for 30 s at 95°C, 2 min at 72°C, 2 min at 37°C and 2 min at 25°C. Synthesized fragments were then digested with *Kpn*I and *Sma*I, cloned into the upstream sequence of *Npt2* in the plasmid pNPT, and transformed into the isolate Bmpc7 using the ATMT method, as described below.

ATMT of *M. fructicola*

The genetic transformation of *M. fructicola* was largely performed as reported previously (Lee and Bostock, 2006). A single colony of *Agrobacterium tumefaciens*, containing the appropriate vector as described above, was incubated overnight at 28°C at 220 rpm in Luria–Bertani (LB) medium with kanamycin (50 μg/mL), streptomycin (50 μg/mL) and rifampicin (50 μg/mL). The culture was diluted to an optical density at 600 nm (OD_{600}) of 0.1 using 10 mL of liquid IM medium with acetosyringone at a concentration of 0.4 μM. Then, the bacteria were incubated under the same conditions for about 6 h until OD_{600} reached 0.5–0.6. Conidia of *M. fructicola* were mixed with the *A. tumefaciens* suspension and diluted to 10^6 conidia/mL. Then, 200 μL of the conidia and bacteria mixture were spread on a cellophane membrane placed on IM. After 2 days of co-incubation at 22°C in the dark, the cellophane membrane was transferred to a new Petri dish. PDA containing hygromycin B (50 μg/mL) and cefotaxime (300 μg/mL) was poured onto the cellophane membrane. Transformants arose after 6 days of incubation at 22°C and were individually transferred to PDA containing hygromycin B (100 μg/mL) and cefotaxime (300 μg/mL).

Construction of knockout and insertion fragments of 'Mona'

The double-joint (DJ) PCR method was applied to construct fusion PCR products for 'Mona' knockout and insertion. All primers are listed in Table S2. First, the 5′ and 3′ flanking regions of 'Mona' were amplified from the DMI-R isolate Bmpc7 using the primer pair P1/P2 to form fragment A and P3/P4 to form fragment B. Second, a hygromycin-resistant cassette (Hyp) with the *Aspergillus nidulans trpC* promoter and terminator was amplified from the pBHt2 vector using the primer pair P5/P6. Then, three amplicons (5′ and 3′ flanking sequences and Hyp) were fused by a second round of DJ PCR. A 2481-bp upstream and a 2494-bp downstream fragment were amplified from second-round PCR products with an overlapping part of 459 bp. The primers P7/P8 and P9/P10 were used to amplify the upstream and downstream fragments, respectively, which were then transformed into DMI-R isolate Bmpc7 (Fig. S2).

To construct insertion fragments of 'Mona', the hygromycin resistant cassette (Hyp) and the 'Mona' element were fused (fragment G). The Hyp fragment was amplified with the primer pair P5/P6 from pBHt2 as mentioned above, and 'Mona' was amplified with the primer pair MF/MR from the DMI-R isolate Bmpc7. The primers P5 and MR were used for the fusion of the hygromycin resistant cassette (Hyp) and 'Mona' element (fragment G). Meanwhile, the 5′ and 3′ flanking regions which correspond to the region of 'Mona' in Bmpc7 were amplified from the sensitive isolate HG3 with primer pairs P1/P11 (fragment E) and P12/P4 (fragment F), respectively. The three amplicons (fragments E, F, G) were fused by a second round of DJ PCR. Based on the fused fragment, the final PCR products were amplified by the nested primer pairs P7/P8 and P9/P10, and were transformed into the DMI-S isolate HG3 (Fig. S4).

Protoplast generation and transformation

To prepare protoplasts, *M. fructicola* conidia (10^6 conidia/mL) were incubated in 50 mL of PDB medium at 22°C for 2 days with shaking at 120 rpm. Mycelia were collected by filtration and washed with OM buffer. The suspension for digestion of the cell wall contained 10 mg/mL driselase (Sigma-Aldrich, St Louis, MO, USA), 10 mg/mL snailase (Biosharp, Shanghai, China), 10 mg/mL Lysozyme (Sigma-Aldrich) and 5 mg/mL Cellulase R-10 (Dingguo Cangsheng Biotechnologies, Co., Ltd, Beijing, China) in OM buffer. The enzyme mixture was filtered through a 0.22-μm MILLEX (Millipore, Bedford, MA) GP sterile filter membrane. A tube with 8 mL of filter-sterilized enzyme mixture and 0.8 g of mycelia was incubated at 28°C for 4 h with shaking at 80 rpm. The resulting protoplasts were mixed with 3 mL of separation buffer A and centrifuged for 15 min at 1500 ***g*** at room temperature. The resulting interface layer containing the protoplasts was then transferred to a universal tube and diluted to 10 mL with separation buffer B. After centrifugation at 1000 ***g*** for 10 min, the pellet was rinsed twice with 3 mL of separation buffer B and then resuspended gently with 0.5 mL of separation buffer B. The protoplast concentration in the suspension was determined with a haemocytometer.

DNA (1 μg) was added to 10^7 protoplasts in 1 mL of 60% polyethylene glycol (PEG), 10 mM Tris-HCl and 10 mM $CaCl_2$. The mixture was incubated at room temperature for 20 min. Then, 1 mL of STC buffer was added prior to centrifugation at 2000 ***g*** for 10 min at 4°C. The pellet was added to 100 mL of regeneration medium prior to pouring onto plates. After 48 h, the plates were overlaid with PDA amended with 150 μg/mL of hygromycin B. Hygromycin B-resistant colonies were transferred onto PDA medium amended with 70 μg/mL of hygromycin B for at least five generations. Homologous integration events were identified by the primer pair 5-MonaF/MR.

Real-time PCR

Total RNA was extracted from mycelia after 3 days of incubation in PDB using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. First-strand cDNA was synthesized from 1 μg of total RNA with a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Vilnius, Lithuania). Real-time PCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) using SYBR Green I fluorescent dye detection. Amplifications were conducted in a 20-μL volume containing 10 μL of SYBR qPCR Mix (Aidlab, Beijing, China), 1 μL of RT product and 0.2 μM of each primer CYPF and CYPR. The cycling protocol was as follows: initial denaturation for 3 min at 95°C, followed by 40 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 20 s. Specific amplification was confirmed by a single peak in the melting curve. The entire experiment starting with RNA extraction was repeated. The expression of the *MfCYP51* gene was normalized to the expression of the *β-tubulin* gene. The relative quantities of gene expression were calculated using the comparative *Ct* ($2^{-\Delta\Delta Ct}$) method (Wong and Medrano, 2005).

Sensitivity to propiconazole and fitness components

The sensitivity to propiconazole and the following fitness components were investigated in 'Mona' knockout and insertion transformants: (i) mycelial growth rate; (ii) sporulation; and (iii) the ability to form lesions on detached fruit.

Sensitivity to propiconazole was assessed on fungicide-amended PDA at 0, 0.03, 0.05, 0.1, 0.3, 0.5 and 1 μg/mL. Mycelial plugs were removed with a 5-mm cork borer from the margins of 5-day-old colonies and placed upside down on the centres of 9-cm plastic Petri dishes containing the fungicide-amended or unamended medium. Mean colony diameters were measured and expressed as the percentage growth inhibition after 5 days of incubation at 22°C in the dark. The EC_{50} values, which represent the concentration to inhibit mycelium growth by 50%, were calculated by regressing the percentage of mycelial growth inhibition against the logarithm of fungicide concentrations. Mycelial growth rates were measured

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Characteristics of the *Monilinia fructicola* isolates used in this study.

Table S2 Primers used in this study.

Fig. S1 Schematic diagram of the construction of the vector pMona-NPT.

Fig. S2 Schematic diagram of the gene replacement approach to knock out the genetic element 'Mona'. The binding sites of the primers used for polymerase chain reaction (PCR) analysis of the knockout transformants are indicated.

Fig. S3 Identification of knockout transformants by polymerase chain reaction (PCR). (A) Detection of the Hyp gene using the primer pair P5/HygR. (B) Detection of the upstream homologous fragment using the primer pair UpF/HygR. (C) Detection of the downstream homologous fragment using the primer pair HygF/DownR. (D) Detection of the 'Mona' element using the primer pair 5-MonaF/MonaR. (E) The two bands generated by the primer pair 5-MonaF/MR are evidence of heterozygous transformants.

Fig. S4 Schematic diagram of the 'Mona' element replacement approach. The binding sites of the primers used for polymerase chain reaction (PCR) analysis of the insertion transformants are indicated.

Fig. S5 Identification of the 'Mona' element insertion in transformants by polymerase chain reaction (PCR). (A) Detection of the Hyp gene using the primer pair P5/HygR. (B) Detection of the upstream homologous fragment using the primer pair UpF/HygR. (C) Detection of the downstream homologous fragment using the primer pair HygF/DownR. (D) Detection of the 'Mona' element using the primer pair 5-MonaF/MonaR. (E) The two bands generated by the primer pair 5-MonaF/MR indicate heterozygous transformants.