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A Nonhomologous End-Joining Mutant for Neurospora sitophila Research

Abstract

Disruption of the nonhomologous end-joining (NHEJ) pathway has been shown to increase the efficiency of transgene integration into targeted genomic locations of *Neurospora crassa* and other fungi. Here, we report that a similar phenomenon occurs in a second Neurospora species: *N. sitophila*. Specifically, we show that deletion of *N. sitophila mus-51* increases the efficiency of targeted-transgene integration, presumably by disrupting NHEJ. Researchers interested in obtaining the *N. sitophila mus-51*$^{\Delta}$ strains described in this study can obtain them from the Fungal Genetics Stock Center (FGSC, Kansas State University, Manhattan, KS).

Keywords

Neurospora, NHEJ, nourseothricin, sitophila, transformation

A nonhomologous end-joining mutant for *Neurospora sitophila* research

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Abstract
Disruption of the nonhomologous end-joining (NHEJ) pathway has been shown to increase the efficiency of transgene integration into targeted genomic locations of *Neurospora crassa* and other fungi. Here, we report that a similar phenomenon occurs in a second Neurospora species: *N. sitophila*. Specifically, we show that deletion of *N. sitophila mus-51* increases the efficiency of targeted-transgene integration, presumably by disrupting NHEJ. Researchers interested in obtaining the *N. sitophila mus-51*$^{\Delta}$ strains described in this study can obtain them from the Fungal Genetics Stock Center (FGSC, Kansas State University, Manhattan, KS).

Introduction
The eukaryotic DNA repair machinery can be a significant obstacle to fungal genome editing efforts. For example, while working with the model organism *N. crassa*, Ninomiya *et al.* (2004) found that targeted-transgene integration (TTI) efficiency was quite low (~20%) in standard laboratory strains, but TTI efficiency was nearly perfect in strains lacking a functional copy of the *mus-51* gene. MUS-51 is a critical NHEJ protein and the increase in TTI efficiency was likely due to the inability of MUS-51-deficient strains to preform NHEJ (for review of NHEJ, please see Chang *et al.* 2017).

Several NHEJ-mutants have been constructed for use in *N. crassa* (Ninomiya *et al.* 2004; Colot *et al.* 2006; Smith *et al.* 2016) and are readily available from the FGSC (McCluskey *et al.* 2010). While these mutants are useful, the genus *Neurospora* contains many species (Nygren *et al.* 2011), some of which possess unique genetic elements and/or biological processes. The study of these "non-*crassa*" species could be made more efficient by the availability of species-specific NHEJ mutants. For example, we are interested in *Spore killer-1* (*Sk-1*), a meiotic drive element found in natural populations of *N. sitophila* (Turner and Perkins 1979). To facilitate our work on *Sk-1*, and to help others who wish to perform molecular genetic research with *N. sitophila*, we have constructed two *N. sitophila mus-51*$^{\Delta}$ strains. In the report below, we describe how the strains were constructed and we show that deletion of *mus-51* increases TTI efficiency in *N. sitophila*.

Materials and Methods

Strains and growth media
The strains used in this study are listed in Table 1. Vegetative cultures were maintained on Vogel's Medium N (VM) (Vogel 1956) with 2% sucrose and 1.5% agar unless otherwise indicated. Other media used in this study are BDS (VM with 2.0% L-sorbose, 0.05% D-fructose, 0.05% D-glucose, and 1.5% agar; Brockman and De Serres 1963), SC-IAA (synthetic crossing medium with iodoacetate; Ebbole and Sachs, 1990), TA (VM with 2.0% L-sorbose, 0.05% D-fructose, 0.05% D-glucose, 0.02% myo-inositol, 1 M sorbitol, and 1.0% agar); BA (VM with 2.0% L-sorbose, 0.05% D-fructose, 0.05% D-glucose, 0.02% myo-inositol, and 1.5% agar); and RM (VM with 2% sucrose and 1 M sorbitol).

Table 1 Strains used in this study

Strain Name	Alternate names	Species / genotype
FGSC 4746		*N. sitophila A* (Tahiti)
W1426		*N. sitophila A* (Italy)
FGSC 26716	ISU-4636 / HNR101.14.11	*N. sitophila mus-51$^{\Delta}$::nat A*
FGSC 26717	ISU-4637 / HNR126.14.1	*N. sitophila mus-51$^{\Delta}$::nat A*
P8-65		*N. crassa csr-1$^{\Delta}$::hph a*

Vector construction

Vector 166 (v166) was designed to replace *mus-51* in FGSC 4746 and W1426 with a nourseothricin-resistance cassette (*nat*). The vector was constructed by Double-Joint (DJ)-PCR (Yu *et al.* 2004) using primers listed in Table 2. For v166, the left and right flanks were 782 bp and 1119 bp, respectively. DJ-PCR was also used to construct vector 238 (v238), which was designed to replace *csr-1* in FGSC 4746 and ISU-4636 with a hygromycin-resistance cassette (*hph*). For v238, the left and right flanks were 1262 bp and 980 bp, respectively.

Table 2. Primers used in this study

Primer Name	Sequence (5' > 3')	Purpose
P1400	GTTGGCATAGCGGAAAGCGACATAG	v166 LF
P1401	ATGCTCCTTCAATATCAGTTATCTGCAACACGAGAGGAGGGGTGAGAAGTTG	v166 LF
P1396	TGCAGATAACTGATATTGAAGGAGCAT	v166 CF
P1397	TGTGCATTCTGGGTAAACGACTCA	v166 CF
P1402	TGAGTCGTTTACCCAGAATGCACAGAGTCCTGCCATCTTGGGTTTGAGT	v166 RF
P1403	GTTGGTTTCCGTTGACATTGCTGTT	v166 RF
P1404	CATGTTGAGGAAGACCATTGCTGTG	v166 NP
P1405	ACGCCTCCTTCTCCTGATACAGACG	v166 NP
P579	GCCTTGATGTCGTAGAGTGTCAGGT	v238 LF
P580	CTATAGTGAGTCGTATTAAGGGCGTGGACGGATGTGGGCTTCGGATTGGTTCGT	v238 LF
P585	CCGTCCACGCCCTTAATACGACT	v238 CF
P586	CTTGATTGACAGCGAACGAAACC	v238 CF
P581	GTTTCAGGGGTTTCGTTCGCTGTCAATCAAGCACCCACCCTTCTTCACTGCATC	v238 RF
P582	GCATCTGGAACCGGACCATCAA	v238 RF
P583	ACGTGTCTATCGCCGGAGTTGCT	v238 NP
P1794	CGGCCTTTATCAACGAGCTACAGG	v238 NP
P198	CAAGACCTGCCTGAAACCGAACTG	*hph* chk
P199	CTGCTGCTCCATACAAGCCAACC	*hph* chk

The center fragment (*nat*) for v166 was amplified from pNR28.12 (GenBank MH553564.1) with primers P1396 and P1397. The center fragment (*hph*) for v238 was amplified from pTH1256.1 (GenBank MH550659.1) with primers P585 and P586. Left and right flanks were amplified from genomic DNA of the transformation host. Underlined bases mark 5′ "overhang" sequences for fusion of PCR products. The v238 primers were initially designed for use with the *N. crassa csr-1* locus. Despite a few nucleotide differences between these primers and their binding sites in *N. sitophila* (double-underlined bases in P580 and P581), these primers were used successfully to amplify the desired products. LF, left flank; CF, center fragment; RF, right flank; NP, nested primers; *hph* chk, primers for detecting the *hph* cassette.

Transformation and homokaryon isolation

Transformation of conidia was adapted from the method of Margolin *et al*. (1997). Approximately 1-week old conidia were collected into 30 ml of ice-cold 1 M sorbitol and filtered through a 100 µm cell strainer (Corning 352360). The filtered conidia were pelleted by centrifugation in a swinging bucket rotor at 2000 × g, 4°C. The supernatant was removed, and the conidial pellet was resuspended to 1 billion conidia/ml in ice-cold 1 M sorbitol. A 100 µl aliquot of resuspended conidia was mixed with approximately 300 ng of transformation vector (which was dissolved in 10 mM Tris-HCL, pH 8.5). The conidia/DNA mixture (<110 µl) was transferred to a 0.1 cm gap-width electroporation cuvette (BTX model 613) and electroporated at 1500 volts with an Eppendorf Eporator. A 750 µl aliquot of ice-cold 1 M sorbitol was added to the electroporated conidia immediately after the pulse. The electroporated conidia were transferred to a 50 ml conical tube containing 4 ml of RM. This "recovery culture" was incubated at 32°C for 3.5 hours with gentle rotation (75 rpm), after which 500 µl and 1000 µl aliquots were transferred to 10 ml of molten TA (~47.5–50°C) for plating on 20 ml BA containing nourseothricin sulfate (55 µg/ml) or hygromycin B (300 µg/ml) in 100 mm culture dishes. The transformation cultures were incubated at room temperature for 12–16 hours before transfer to a 32°C incubator for an additional incubation period of 3–4 days. Colonies were transferred from the transformation dishes to culture tubes (16 × 100 mm) containing 2 ml of slanted VM plus nourseothricin sulfate (45 µg/ml) or hygromycin B (200 µg/ml). Homokaryons were derived from heterokaryotic *mus-51*$^{\Delta}$ transformants by culturing on SC-IAA to generate microconidia for purification by the filtration method of Ebbole and Sachs (1990).

Results and Discussion

The *mus-51* gene in *N. sitophila* is flanked by genes *ncu08291* and *ncu08289* (Figure 1A), which is similar to situation in *N. crassa* (Galagan *et al.* 2003). We designed a transformation vector (v166) to replace *mus-51* with *nat*. Transformation of FGSC 4746 with v166 produced 47 nourseothricin-resistant transformants. A preliminary screen of the transformants by conidial PCR (Henderson *et al.* 2005) with primers designed to detect replacement of *mus-51* with *nat* identified seven *mus-51*$^{\Delta}$ candidates (data not shown). A homokaryotic strain, ISU-4636, was obtained from one of these candidates and PCR was used to confirm that ISU-4636 possesses the *mus-51*$^{\Delta}$ genotype (Figure 1B).

To determine if *mus-51* deletion increases TTI efficiency in *N. sitophila*, we used a cyclosporin A (CSA)-resistance assay (Smith *et al*. 2016). The CSA-resistance assay involves transforming *N. sitophila* with a vector designed to replace the *csr-1* gene with *hph*. Previous research in *N. crassa* has shown that strains with a functional *csr-1* allele are sensitive to CSA (5 µg/ml), while those with a null allele are resistant (Bardiya and Shiu, 2007). Predicting that this relationship between *csr-1*-deletion and CSA-resistance would hold true for *N. sitophila*, we transformed *mus-51*$^{+}$/FGSC 4746 and *mus-51*$^{\Delta}$/ISU-4636 with a vector designed to replace *csr-1* with *hph* (v238) and selected transformants for resistance to hygromycin only (*i.e.*, CSA was not included in the selection medium). We isolated fifty hygromycin-resistant transformants from each transformation host. The transformants were then screened for resistance to CSA on CSA-containing BDS medium. While only 9 of 50 transformants from *mus-51*$^{+}$/FGSC 4746 demonstrated significant growth on CSA-containing medium (Figure 2A), nearly all (49 of 50) of the *mus-51*$^{\Delta}$/ISU-4636-derived transformants were resistant to CSA (Figure 2B).

To confirm that the CSA-resistant phenotypes detected in the CSA-resistance assays were caused by replacement of *csr-1* with *hph* and not "spontaneous resistance" to CSA, the *csr-1* locus was analyzed by PCR in eight transformants from each transformation host. For the *mus-51*$^{+}$/FGSC 4746-derived transformants, we examined four that were sensitive to CSA and four that were CSA resistant and found a perfect correlation between resistance to CSA and the presence of *csr-1*$^{\Delta}$ (Figure 3, A and B). For the *mus-51*$^{\Delta}$/ISU-4636-transformants, we only isolated one that appeared to be CSA sensitive (#31), therefore we examined this transformant along with seven that were resistant to CSA. Interestingly, all eight of these transformants possess the *csr-1*$^{\Delta}$ genotype, even the one that was scored as susceptible to CSA in the media-based screen. Although we have not investigated transformant #31 further, it may possess a growth defect that contributed to a false negative on the media-based CSA-resistance screen (*e.g.*, note transformant #31's atypical/slow growth pattern on BDS with and without CSA in Figure 2B).

The results described above show that deletion of *mus-51* increases TTI efficiency in *N. sitophila*. FGSC 4746, which is the parent strain of ISU-4636, is an *N. sitophila* isolate from Tahiti. To facilitate research on *N. sitophila* strains from different populations, we also constructed a *mus-51*$^{\Delta}$ strain called ISU-4637 with methods nearly identical to those used to construct strain ISU-4636. The one difference is that we used W1426 (Jacobsen *et al.* 2006) as the transformation host (instead of FGSC 4746). *N. sitophila* W1426 was isolated in Italy and it will be described in more detail in a future work (H. Johannesson, personal communication). While we have used ISU-4637 in transformation experiments, we have not measured TTI efficiency; however, we have no reason to suspect that ISU-4637 is inferior to ISU-4636 for use in experiments requiring the integration of a transgene into a specific location of the *N. sitophila* genome.

Conclusion

In summary, our results demonstrate that deletion of *mus-51* increases TTI efficiency in *N. sitophila*. Strains ISU-4636 and ISU-4637 can be obtained from the Fungal Genetics Stock Center as FGSC 26716 and FGSC 26717, respectively.

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Figure 1

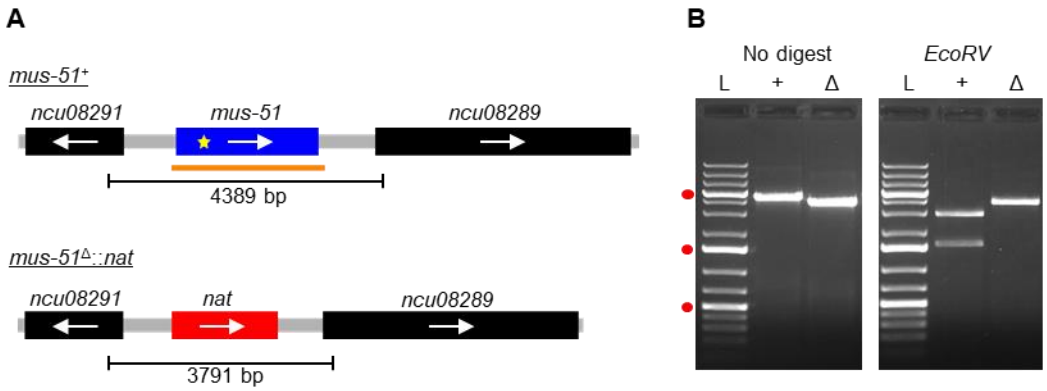

Figure 1. Deletion of *mus-51* from *N. sitophila* FGSC 4746. A) A diagram of the *N. sitophila mus-51* locus is shown above a diagram of the same locus after replacement of *mus-51* with *nat*. Primers P1400 and P1403 amplify a 4389 bp PCR product from the *mus-51*$^{+}$ allele (*Eco*RV digest of the product produces 1557 bp and 2832 bp DNA fragments) and a 3791 bp PCR product from the *mus-51*$^{\Delta}$ allele (*Eco*RV does not cut the product from the *mus-51*$^{\Delta}$ allele). The orange bar marks the region replaced with *nat*. The location of a diagnostic *Eco*RV-restriction site within *mus-51*$^{+}$ is marked with a yellow star. B) Primers P1400 and P1403 were used to analyze the *mus-51* locus in FGSC 4746 and ISU-4636 by PCR. PCR products were analyzed before *Eco*RV-digestion (left image) and after *Eco*RV-digestion (right image). The results are consistent with *mus-51*$^{+}$ and *mus-51*$^{\Delta}$ genotypes for FGSC 4746 and ISU-4636, respectively. Lane symbols: L, GeneRuler 1kb Plus DNA Ladder (Thermo Scientific); +, FGSC 4746; Δ, ISU-4636. Red circles denote locations of 5000 bp, 1500 bp, and 500 bp size markers (top to bottom).

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Figure 2

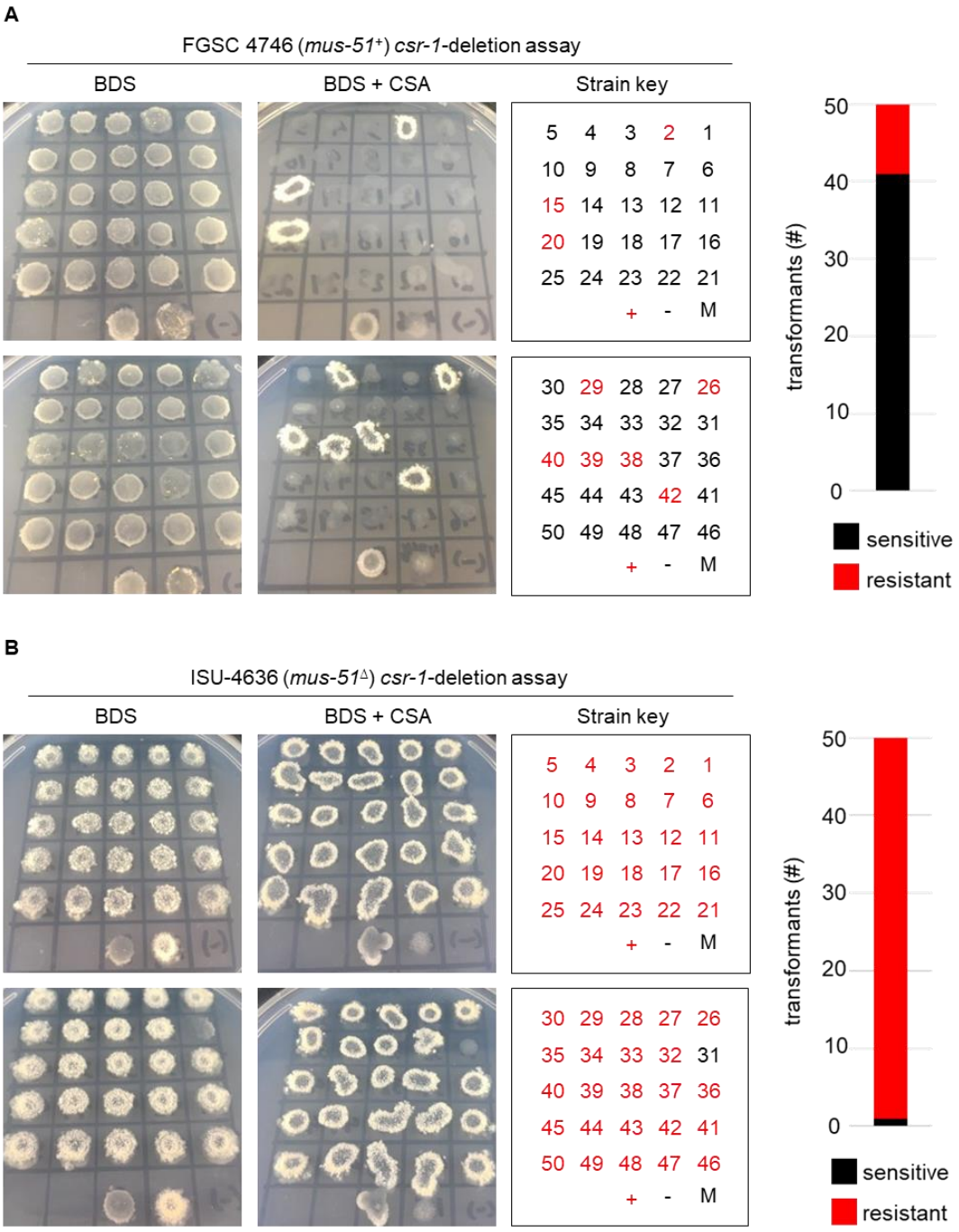

Figure 2. *N. sitophila* TTI efficiency increases fivefold after deletion of *mus-51*$^{+}$. A) FGSC 4746, an *N. sitophila mus-51*$^{+}$ strain, was transformed with vector 238, a vector designed to replace *csr-1*$^{+}$ with *hph*. Fifty hygromycin-resistant transformants were isolated and examined in a cyclosporin A (CSA)-resistance assay. Conidial suspensions (3 µl) of each transformant were transferred to culture dishes containing BDS with or without CSA. Images of the culture dishes were taken two days after incubation

at 32°C. Strain designations: +, P8-65; -, FGSC 4746; 1-50, transformants. Red font is used for strains that were scored as resistant to CSA. The stacked column chart at the right side of the panel summarizes the data. B) Similar to panel A except that ISU-4636 was used as the transformation host. Strain designations are like those in panel A except that "-" refers to ISU-4636.

Rhoades, Webber, and Hammond (2019)
Figure 3

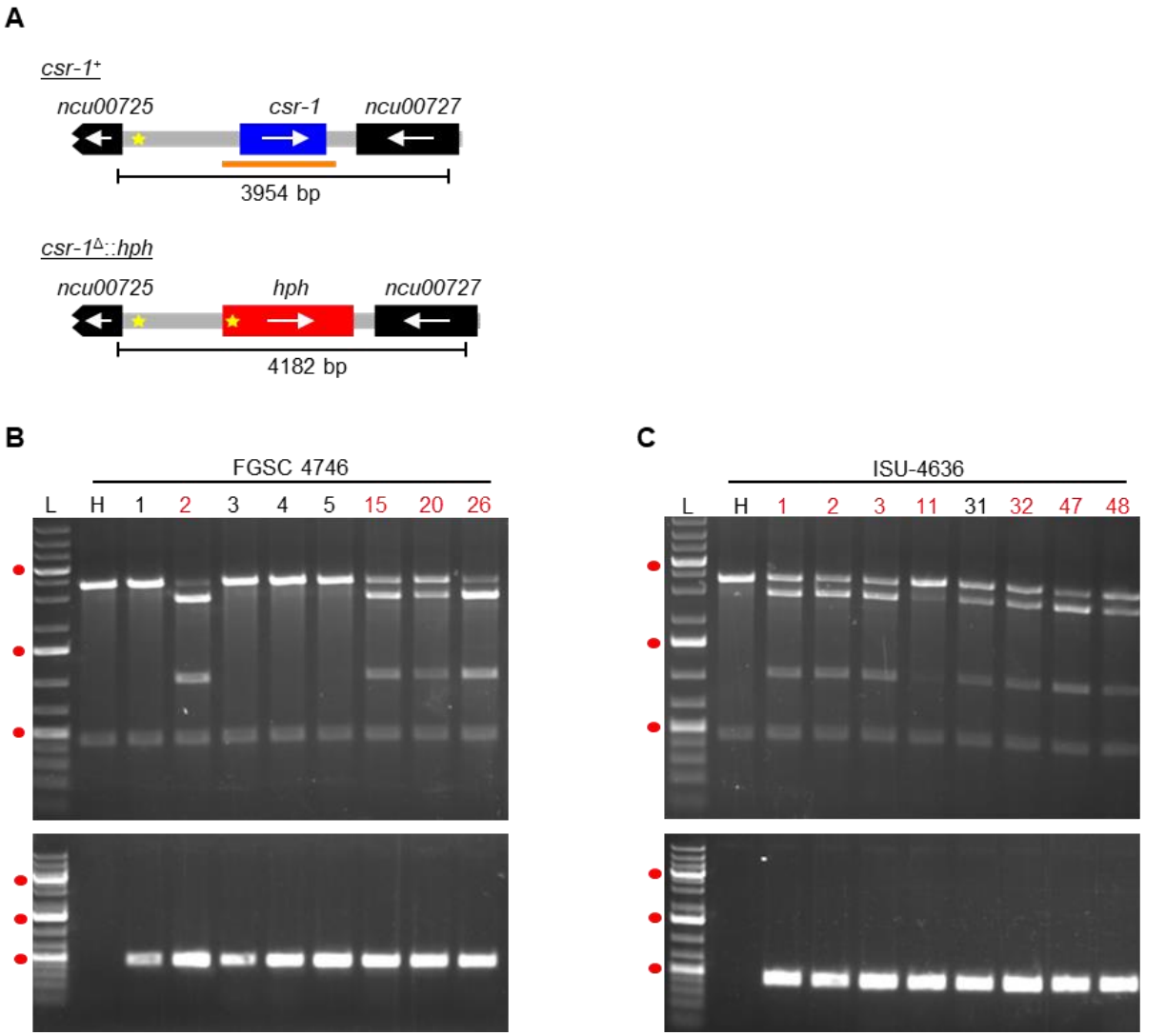

Figure 3 Deletion of *mus-51* correlates with increased TTI efficiency in *N. sitophila*. A) A diagram of the *csr-1* locus with the *csr-1*$^{+}$ allele is shown above a diagram of the same locus after replacement of *csr-1*$^{+}$ with *hph.* The orange bar marks the region replaced with *hph*. The locations of diagnostic *Eco*RI-restriction endonuclease recognition sites are marked with yellow stars. Primers P579 and P582 amplify a 3954 bp PCR product from the *csr-1*$^{+}$ allele (*Eco*RI digest produces 435 bp and 3519 bp fragments) and a 4182 bp PCR product from the *csr-1*$^{\Delta}$ allele (*Eco*RI digest produces 435 bp, 990 bp, and 2757 bp fragments). B) [Top] Primers P579 and P582 were used to analyze the *csr-1* locus in FGSC 4746 (H) and eight hygromycin-resistant transformants (1, 2, 3, 4, 5, 15, 20, and 26) obtained by transformation of FGSC 4746 with v238. PCR products were digested with *Eco*RI before analysis by gel electrophoresis. An image of the ethidium bromide-stained gel is shown. [Bottom] Primers P198 and P199 were used to confirm the presence of *hph* in all transformants. These primers amplify a 435 bp PCR product from the *hph* cassette. An *hph*-specific product was amplified from all transformants but not the transformation host, as expected. L, GeneRuler 1kb Plus DNA Ladder (Thermo Scientific). Red circles mark positions of

5000 bp, 1500 bp, and 500 bp size markers. Red font is used for lane numbers of transformants that were found to be resistant to cyclosporin A (see Fig. 2). C) [Top] Primers P579 and P582 were used to analyze the *csr-1* locus in ISU-4636 (H) and eight hygromycin-resistant transformants (1, 2, 3, 11, 31, 32, 47, and 48). PCR products were digested with *Eco*RI before analysis by gel electrophoresis. Labeling is as in Panel B. [Bottom] Primers P198 and P199 were used to check for the presence of the *hph* cassette. As expected, a 435 bp *hph*-specific product was amplified from all transformants but not the transformation host.