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Deleterious Effect of the Q_o Inhibitor Compound Resistance-Conferring Mutation G143A in the Intron-Containing Cytochrome *b* Gene and Mechanisms for Bypassing It▽†

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The mutation G143A in the inhibitor binding site of cytochrome *b* confers a high level of resistance to fungicides targeting the bc_1 complex. The mutation, reported in many plant-pathogenic fungi, has not evolved in fungi that harbor an intron immediately after the codon for G143 in the cytochrome *b* gene, intron bi2. Using *Saccharomyces cerevisiae* as a model organism, we show here that a codon change from GGT to GCT, which replaces glycine 143 with alanine, hinders the splicing of bi2 by altering the exon/intron structure needed for efficient intron excision. This lowers the levels of cytochrome *b* and respiratory growth. We then investigated possible bypass mechanisms that would restore the respiratory fitness of a resistant mutant. Secondary mutations in the mitochondrial genome were found, including a point mutation in bi2 restoring the correct exon/intron structure and the deletion of intron bi2. We also found that overexpression of nuclear genes *MRS2* and *MRS3*, encoding mitochondrial metal ion carriers, partially restores the respiratory growth of the G143A mutant. Interestingly, the *MRS3* gene from the plant-pathogenic fungus *Botrytis cinerea*, overexpressed in an *S. cerevisiae* G143A mutant, had a similar compensatory effect. These bypass mechanisms identified in yeast could potentially arise in pathogenic fungi.

The mitochondrial bc_1 complex is a membrane-bound multisubunit enzyme that catalyzes the transfer of electrons from ubiquinol to cytochrome *c* and couples this electron transfer to the vectorial translocation of protons across the inner mitochondrial membrane. Cytochrome *b* is the central membrane-embedded subunit that forms the ubiquinol binding pockets called Q_o and Q_i.

A number of quinol antagonists are known that inhibit bc_1 complex activity. These are either specific for the Q_i site, such as antimycin, or for the Q_o site, such as myxothiazol, stigmatellin, and the strobilurins. A range of Q_o inhibitor compounds (Q_oIs) have been developed as antimicrobial agents and are now widely used in agriculture to control fungal and oomycete plant pathogens. Unfortunately, acquired resistance has rapidly emerged in field populations of the plant pathogens. The cytochrome *b* mutation G143A plays a central role in the mechanism of resistance. The mutation has been reported in most Q_oI-resistant pathogens (see http://www.frac.info/frac/index.htm and references within). G143A causes a high level of resistance (>100×) in pathogens, which are consequently controlled poorly or not at all by Q_oIs. In the model organism *Saccharomyces cerevisiae*, G143A also dramatically increases resistance to myxothiazol (18,000×) and azoxystrobin (4,000×) (8). G143 is a highly conserved residue located in the Q_o pocket, close to the inhibitor binding site. The replacement of glycine with alanine would prevent inhibitor binding through simple steric hindrance while the Q_o site remains functional, as observed in the yeast model (8).

The cytochrome *b* gene is encoded by the mitochondrial genome in all eukaryotes. In fungi, large introns are usually found in the gene. Analysis of its genomic structure in several fungal plant pathogens revealed variations in the arrangement of exons and introns between species (13), as had been observed in earlier studies, for instance, in reference 4. Of particular interest is the intron present in some species and located immediately after the codon for G143. That intron is similar to intron bi2 found in the cytochrome *b* gene of most *S. cerevisiae* laboratory strains. In several species of *Puccinia*, in *Alternaria solani*, and in other fungi, intron bi2 is present. Interestingly, in those species, the resistance mutation G143A has not been detected so far. In contrast, species where G143A has been reported do not contain intron bi2 (13, 24), for instance, *Blumeria graminis*, *Mycosphaerella fijiensis*, *M. graminicola*, *Venturia inaequalis*, *Plasmopara viticola*, *Alternaria alternata*, etc. *Botrytis cinerea* is particularly interesting, since the same species presents two types of cytochrome *b* gene; some field isolates contain intron bi2, while others do not. The mutation G143A has been reported only in the latter populations (1, 17). These observations suggested that the presence or absence of intron bi2 might affect the occurrence of the G143A mutation. It has been hypothesized that the resistance mutation might impair the correct splicing of intron bi2, leading to a reduced level of mature cytochrome *b* mRNA and a smaller amount of the bc_1 complex and to decreased respiratory function (13). Therefore, G143A, affecting the fitness of the resistant cells, would be counterselected in the field. The mutation would not evolve in pathogens containing bi2, except if com-

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pensation mechanisms can be developed that restore respiratory efficiency while keeping the resistance mutation.

In this study, we used *S. cerevisiae* as a model organism to analyze the effect of the G143A mutation in an intron containing cytochrome *b* and to investigate possible ways to bypass the defect caused by the mutation.

MATERIALS AND METHODS

Media, chemicals, and primers. The media used to grow yeast were glucose medium (1% yeast extract, 2% peptone, and 3% glucose supplemented with adenine), glycerol medium (1% yeast extract, 2% peptone, and 3% glycerol), galactose medium (1% yeast extract, 2% peptone, and 3% galactose supplemented with adenine), uracil-lacking medium (0.7% yeast nitrogen base, 3% glucose, and 0.8 g/liter complete supplement mixture minus uracil [supplied by Anachem]), and transformation medium (0.7% yeast nitrogen base, 3% glucose, 1 M sorbitol, and 0.8 g/liter complete supplement mixture minus uracil). Agar was added at 2% for solid media. Azoxystrobin was a generous gift from Syngenta. The primers used in this study are listed in Table S1 in the supplemental material.

Generation of plasmids and a mutant strain. A 717-bp DNA fragment was amplified by PCR from the intron-containing *CYTB* gene (encoding cytochrome *b*). This fragment, containing 281 bp of the 3′ end of intron bi1, 14 bp of exon B2, and 422 bp of the 5′ end of intron bi2, was blunt-end cloned into the pCRscript vector (Stratagene). The mutation G143A (corresponding to the nucleotide change GGT>GCT) was then introduced into the *CYTB* fragment inserted in the pCRscript vector using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. The resulting plasmid, carrying the mutated *CYTB* fragment, was called pGA.

pGA was used for the construction of pGACOX, where the 2.5-kb BamHI-HindIII fragment containing the *COX2* gene from pJM2 (20) was inserted into the BamHI-HindIII site of pGA. After verification, pGACOX was used for biolistic transformation. The method used for mitochondrial transformation by microprojectile bombardment was adapted from reference 3 and is described in reference 19. A mixture of pGACOX (carrying the mutated *CYTB* fragment and the *COX2* gene) and YEp352 (which contains the *URA3* gene, allowing the selection of Ura^+ nuclear transformants) was used for transformation of the [*rho*^{0}] recipient strain. Ura^+ transformants were selected on synthetic medium minus uracil. In order to identify the mitochondrial transformants, the colonies were then crossed with a respiration-deficient *cox2* mutant tester strain and replica plated onto respiratory medium. Since the *cox2* tester deficiency mutation could be corrected by recombination with the plasmid-borne *COX2* gene, mitochondrial transformants were identified by the ability to form respiration-competent diploids when crossed with the tester strain. The mitochondrial transformants (or synthetic [*rho*$^-$]) were the subcloned and tested again. The introduction of a mutation into intron-containing *CYTB* of a [*rho*$^+$] genome was achieved by homologous recombination. The synthetic [*rho*$^-$] strain was crossed with a [*rho*$^+$] wild-type (WT) intron-containing strain, and then inhibitor-resistant recombinant cells were selected. The sequence of the cytochrome *b* region bi1-B2-bi2 was then verified. The resulting mutants were in the W303-1B nuclear background (α *ade2-1 his3*$^-$ *leu2-3,112 trp1-1 ura3-1* [27]). The mutated and WT mitochondrial genomes were then transferred into the CKU nuclear background (**a** *leu1 ura3 kar1-1* [this work]) by cytoduction. All of the strains analyzed in this study were isogenic with the CKU nuclear genome.

Isolation and genetic analysis of suppressors. The respiration-deficient mutants generated by biolistic transformation were used to select revertants. The mutants were subcloned. Several subclones were grown on glucose medium and then incubated on respiratory (glycerol) medium. Alternatively, the cells were first treated overnight with 10 mM $MnCl_2$, which is known to induce mitochondrial mutations, and then incubated on respiratory medium. Respiration-competent clones appeared after 1 or 2 weeks of incubation. Independent suppressors (each obtained from different subclones) were then analyzed as described in reference 5 to determine the mitochondrial or nuclear heredity of the suppressor mutation. Briefly, the respiration-competent clones ([*rho*$^+$] suppressors) and their [*rho*^{0}] derivatives obtained by ethidium bromide treatment ([*rho*$^+$] suppressors) were crossed with a G143A mutant (of the opposite mating type). If the suppressor mutations are mitochondrial, the diploids obtained from the [*rho*^{0}] suppressor × G143A mutant cross will be respiration deficient since the suppressor mutations are lost in the [*rho*^{0}] derivatives; the diploids obtained from the [*rho*$^+$] suppressor × [*rho*^{0}] cross will be respiration competent. Standard methods of yeast mitochondrial genetics are described in reference 10.

Isolation of multicopy suppressors. The respiration-deficient mutants generated by biolistic transformation were transformed by a high-copy WT genomic library made in the *URA3* 2μ vector pFL44L (2). Ura^+ clones were selected and replica plated onto glycerol medium. Respiration-competent clones appearing after 4 to 5 days were analyzed. Plasmids were isolated from clones that showed clear cosegregation of respiratory competence and Ura^+. The chromosomal fragments present on the plasmids were identified by sequencing.

Spectroscopic analysis of cytochromes in whole cells. Spectra were generated by scanning of cell suspensions with an in-house-built spectrophotometer operating at room temperature. The optical setup consisted of an integrating sphere built in a piece of Spectralon (Labsphere, North Sutton, NH). The light input was provided by a xenon arc lamp, and detection was done at 90° from the input with an Ocean Optics (Dunedin, FL) QE65000 charge-coupled device spectrophotometer. A linear contribution was subtracted from the spectra to compensate for the effects of optical diffusion. The cells were grown for 48 h on galactose medium or uracil-lacking medium for the cells transformed with vector pFL44L or pYeDP1/10 to maintain the plasmid. The cells were then resuspended at a concentration of around 10 mg of cells in 100 μl of water and reduced by dithionite.

RNA extraction and RNA hybridization. Cells were grown in galactose medium and harvested at exponential growth phase, and total RNAs were purified by the "hot phenol" technique (21). The RNAs were quantified spectrophotometrically at 260 nm. The RNAs were separated on 1.2% agarose formaldehyde gels and transferred onto Hybond-C extra membrane (Amersham, Buckinghamshire, United Kingdom). Prehybridization and hybridization were done at 42°C in 50% formamide in the presence of Denhardt solution. PCR-amplified fragments of *CYTB* exon B1 (400 bp) and 15S rRNA (364 bp) were generated and radiolabeled by random priming (random primer DNA labeling system from Invitrogen, San Diego, CA). These probes were then used to detect the RNAs. The *CYTB* pre-mRNA and mRNA levels were quantified with ImageJ (National Institutes of Health) and normalized to the levels of 15S rRNA.

Cloning of the nuclear *MRS3* gene from *B. cinerea*. The *MRS3*-equivalent gene (accession number XM_001553578; protein, BC1G_07715) was PCR amplified from a cDNA library of *B. cinerea* (prepared from strain B05-10 and kindly provided by A.-S. Walker, BIOGER, INRA, Thiverval-Grignon, France). The PCR fragment was then cloned into a yeast multicopy expression vector under the control of the *PGK* promoter, the constitutive promoter of the phosphoglycerate kinase gene (vector pYeDP1/10, provided by B. Guiard, Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France). This plasmid was used to transform the G143A splicing-deficient mutant.

RESULTS

Effect of G143A on the splicing of intron bi2 of cytochrome *b*. The GGT codon encoding amino acid G143, is located at the exon B2/intron bi2 boundary in the mitochondrially encoded cytochrome *b* gene (Fig. 1). In the Q_oI-resistant G143A mutants, it has been replaced with a GCT codon. It is known that pairing of the 5′ exon (B2) and the so-called internal guide sequence, a short sequence at the 5′ end of the intron (bi2), is required for splice site recognition (see reference 23 for a review of intron splicing mechanisms). Thus, it seems likely that the mutation would affect the splicing of intron bi2, leading to a reduced level of mature mRNA, a decreased level of cytochrome *b*, and hence a smaller amount of the bc_1 complex and reduced respiratory function.

In order to test this hypothesis, the G143A (GGT>GCT) mutation was introduced into a WT *S. cerevisiae* mitochondrial genome by site-directed mutagenesis and mitochondria transformation (see Materials and Methods). We then analyzed the impact of the mutation on respiratory growth, the level of the bc_1 complex, and the amount of *CYTB* mRNA (Fig. 2). For comparison, we monitored the effect of the same change in an intronless cytochrome *b* gene. G143A causes a severe decrease in respiratory growth (panel A) and a significant decrease in the level of cytochrome *b* (Fig. 2B): the cytochrome *b* signal, monitored by spectrophotometry in intact cells, was decreased

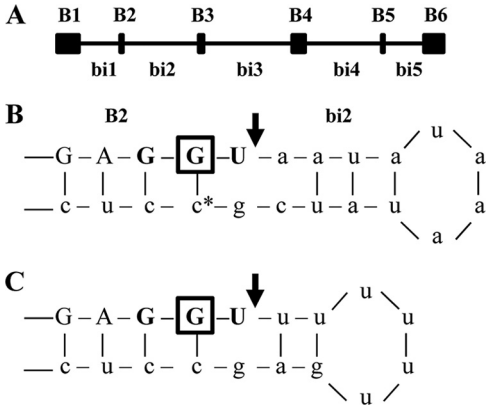

FIG. 1. Intron bi2 in the cytochrome *b* gene. (A) Structure of the exons (boxes)/introns (lines) of the cytochrome *b* gene in *S. cerevisiae*. (B) Model of the exon B2/intron bi2 pairing in *S. cerevisiae* required for efficient splicing of bi2. The position of the exon B2 (uppercase)/intron bi2 (lowercase) junction is shown by the arrow. The triplet GGU encoding amino acid G143 is in bold, and the mutated nucleotide (G>C) resulting in the amino acid substitution G143A is boxed. The asterisk indicates the position of a second mutation (C>G), present in Sup2, that restores base pairing and splicing. (C) Model of exon/intron pairing in *B. cinerea*.

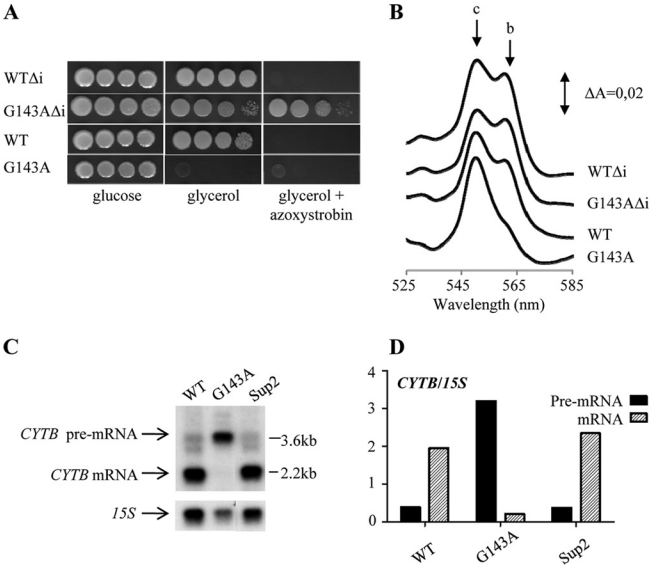

FIG. 2. Impact of G143A mutation on the respiratory growth, level of cytochrome *b*, and amount of *CYTB* mRNA in bi2-containing strains. (A) Serial dilutions of WT and G143A mutant cells (intron containing or intronless [Δi]) were spotted onto agar plates containing a fermentable medium (glucose) or a nonfermentable medium (glycerol) with or without 5 μM azoxystrobin and incubated for 5 days at 28°C. (B) Cytochrome absorption spectra of WT and G143A mutant cells (intron containing or intronless [Δi]). Optical spectra were obtained as described in Materials and Methods. Absorption maxima are indicated by arrows (c, cytochromes *c* and c_1; b, cytochrome *b*). (C) *CYTB* pre-mRNA and mRNA levels of the intron-containing WT strain, the G143A mutant strain, and strain Sup2, which combines G143A and a second compensatory mutation in bi2. RNAs were analyzed by hybridization carried out with probes specific for *CYTB* (exon B1) and the 15S rRNA as a control. The 2.2-kb RNA corresponds to the mature *CYTB* mRNA; the 3.6-kb RNA corresponds to a pre-mRNA containing bi2, as previously reported (18). (D) *CYTB* pre-mRNA and mRNA levels in WT, G143A, and Sup2 cells obtained by Northern blotting (C) were quantified with ImageJ (National Institutes of Health) and normalized for the levels of 15S rRNA.

to 40 to 50% of the WT level. In contrast, the mutation has no effect on respiratory growth and/or the cytochrome *b* level in the absence of intron bi2, as previously observed (7).

Northern blot analysis of *CYTB* pre-mRNA and mRNA (Fig. 2C and D) shows that the mutation causes a dramatic (±90%) decrease in mature mRNA. In mitochondria, mRNAs are known to be in excess, the control of gene expression being principally at a posttranscriptional level (see, for instance, reference 6). Thus, the observation that in the mutant, *CYTB* mRNA is present at 10% of the WT level while cytochrome *b* reaches 40 to 50% of the WT signal was not unexpected.

Accumulation of a pre-mRNA of around 3.6 kb, which corresponds to the size of a pre-mRNA containing intron bi2, was observed. Thus, it seems most likely that, as previously hypothesized, G143A affects the splicing of bi2 by hindering the pairing of exon B2 and the bi2 internal guide sequence. This was confirmed by the analysis of a double mutant strain called Sup2 (obtained and analyzed as described in Materials and Methods), which combines G143A and a mutation in the bi2 internal guide sequence (replacement of nucleotide C with a G) (Fig. 1B). This mutation restores the correct pairing of B2 and bi2 required for splice site recognition, which results in the correct splicing of bi2 and, in consequence, the full amount of *CYTB* mRNA (Fig. 2C and D).

In addition to a correct RNA structure, the excision of bi2 requires a maturase, encoded by bi2 itself. It has been previously reported that mutations in the bi2 maturase result in a splicing defect that could be corrected by secondary mutations at the same positions or at a distant site in the bi2 maturase (15, 18).

Bypass mechanisms of the respiratory defect. As shown in Fig. 3A, G143A causes a severe respiratory growth defect. It was therefore possible to select suppressors, i.e., respiration-competent clones obtained from the G143A mutant. The analysis of the suppressors provides interesting information on the possible mechanisms by which the respiratory defect could be bypassed. Two approaches were used to identify suppressors: direct selection of respiration-competent clones obtained spontaneously or after mutagen treatment and the selection of

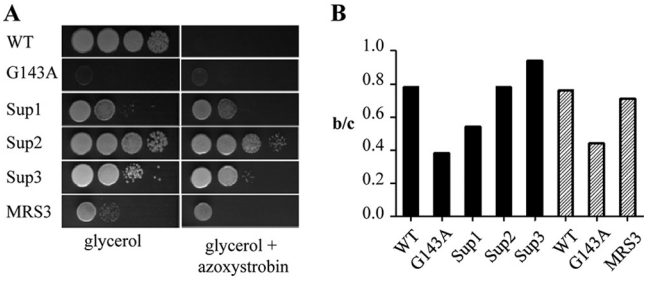

FIG. 3. Effects of suppressors on respiratory growth and on the level of cytochrome *b*. (A) Serial dilutions of each strain were spotted onto a nonfermentable (glycerol) medium with or without 5 μM azoxystrobin and incubated for 5 days at 28°C. (B) Cytochrome *b*/cytochrome *c* ratio (b/c) in intact cells obtained from optical spectra (see Materials and Methods). The cells were grown in galactose medium (black bars) or in uracil-lacking medium (hatched bars). Sup1, mitochondrial suppressor "outside bi2"; Sup2, mitochondrial suppressor "in bi2"; Sup3, mitochondrial suppressor "loss of bi1 and bi2"; MRS3, high-copy-number nuclear suppressor.

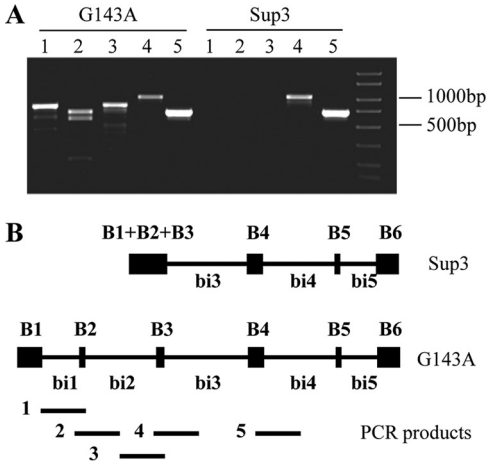

FIG. 4. PCR analysis of cytochrome *b* exon/intron structure in suppressor strain Sup3. (A) PCR amplification of regions of *CYTB* using genomic DNA extracted from the WT and Sup3 strains: lane 1, B1-bi1-B2 (836 bp); lane 2, bi1-B2-bi2 (717 bp); lane 3, bi2-B3 (850 bp); lane 4, B3-bi3 (973 bp); lane 5, B4-bi4 (715 bp). (B) Structure of the *CYTB* gene in the Sup3 and G143A strains and PCR products.

high-copy-number suppressors after transformation by using a genomic library. Using the first approach, we looked for compensatory mutations in the mitochondrial genome; using the second approach, we hoped to identify nuclear genes whose increased expression would (at least partially) restore the level of the bc_1 complex.

Compensatory mutations in the mitochondrial genome. Independent respiration-competent clones were selected on respiratory medium, and the heredity of the suppressor mutation was determined as described in Materials and Methods. For five clones, the suppressor mutation was clearly of mitochondrial origin and these were studied further.

In two suppressor clones, the complete restoration of WT respiratory growth and a WT cytochrome level was due to the reversion of G143A to the WT codon, causing a loss of Q_oI resistance. One suppressor, Sup1 (Fig. 3A and B), partially restored respiratory growth and the level of cytochrome *b*. The compensatory mutation was located by [*rho*$^-$] mapping (10) to a region comprising *COX1* and *ENS2* (encoding a putative endonuclease) but excluding *CYTB*. Sequencing of the 5′ untranslated regions of *CYTB*, *COX1*, and *ENS2* did not reveal any change. The analysis of this suppressor was not pursued.

As already mentioned above, in Sup2, a secondary mutation that replaces nucleotide C with a G in the bi2 internal guide sequence restores the correct pairing of B2 and bi2 and by consequence, the splicing of the bi2 intron and the full amount of mRNA (Fig. 2C and D). The suppressor results in an amino acid replacement of an alanine with a glycine in the bi2 maturase, which has no deleterious effect on its function: Sup2 showed WT respiratory growth and a WT cytochrome *b* level and resistance to azoxystrobin (Fig. 3A and B).

In the last type of mitochondrial suppressor, Sup3, bypass of the respiratory defect was obtained by the deletion of introns bi1 and bi2, as found by PCR analysis of *CYTB*; introns bi3 and bi4 (and presumably bi5) are retained (Fig. 4). The region including B1, B2, and B3 of the new *CYTB* gene present in Sup3 was sequenced; this showed the clean excision of bi1 and bi2 and the correct ligation of exons B1, B2, and B3. This was expected, since mitochondrial suppression leads to functional cytochrome *b*, as judged by the respiratory growth of Sup3 (Fig. 3A). The loss of the first two introns fully restores the level of cytochrome *b* (Fig. 3B).

Compensatory effect of high-copy-number nuclear genes. In order to identify nuclear genes whose increased expression would (at least partially) restore the respiratory growth of G143A, we transformed the mutant with a WT genomic library on a high-copy-number plasmid and selected respiration-competent clones as described in Materials and Methods. In 18 clones, a clear cosegregation of the plasmid with respiratory competence was observed. The plasmids were extracted from these clones, and the genomic fragments inserted into the plasmids were sequenced. In all but one clone, the inserts came from the same region of the genome that comprises *MRS3*. In one clone, the insert contained *MRS2*. *MRS2* and *MRS3* encode mitochondrial metal ion carriers. Both genes have previously been reported to be involved in the splicing of various mitochondrial introns (11, 16, 25, 31, 32). From our results, it appears that a high copy number of *MRS3* and *MRS2* compensates for the defective splicing of intron bi2, as judged by the level of cytochrome *b* (illustrated by MRS3 in Fig. 3B). Respiratory growth competence was only partially restored. This could be due to the toxic effect of a high dosage of these metal ion transporters, which could disturb the metal homeostasis of the mitochondria. It has been previously reported that overexpression of *MRS3* and *MRS4* could cause a temperature-dependent respiration deficiency phenotype (32).

Mrs2p and Mrs3p are well conserved; for instance, sequence comparison shows 45% identity between Mrs3p from *S. cerevisiae* and that from *B. cinerea* (the agent of gray mold). It is likely that Mrs3p from *B. cinerea* functions as a mitochondrial metal ion carrier, as does its yeast homolog. A higher dosage of this protein might also be able to compensate for the bi2 excision defect caused by G143A. In order to test the hypothesis, *MRS3* was amplified from *B. cinerea* cDNA and cloned into a high-copy-number plasmid under the control of a yeast constitutive promoter (*PGK*). For comparison, *MRS3* from *S. cerevisiae* was cloned into the same plasmid (controlled by the same promoter). The G143A mutant strain was transformed by the plasmids, and the respiratory growth and cytochrome b_1 level of the resulting clones were monitored. As shown in Fig. 5, *B. cinerea MRS3*, at a high copy number, has the same compensatory effect as *S. cerevisiae MRS3* and restored respiratory growth and cytochrome *b* content.

DISCUSSION

Resistance mutation and impaired intron splicing. Different factors might affect the evolution of G143A in field pathogenic fungi exposed to Q_oIs. In a previous study, we characterized the effect of small structural variations in the Q_o site using *S. cerevisiae* as a model organism (7). In this work, we investigated the impact of the exon/intron structure of the cytochrome *b* gene. The codon for G143 is located at the boundary between exon B2 and intron bi2. Intron bi2 belongs to group I of the intronic RNA structure (see reference 23 for a review). These introns catalyze their own splicing via a series of guanosine-initiated transesterification reactions. Different group I in-

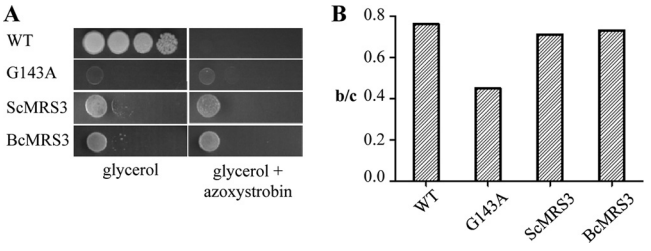

FIG. 5. Compensatory effect of the overexpressed *MRS3* gene from *B. cinerea*. (A) Dilution series of WT, G143A, and *MRS3* suppressor strains were spotted onto glycerol medium with or without 5 μM azoxystrobin and incubated for 5 days at 28°C. (B) Cytochrome *b*/cytochrome *c* ratio (b/c) in intact WT, G143A, and *MRS3* suppressor strains obtained from optical spectra (see Materials and Methods). Cells were grown in uracil-lacking medium. ScMRS3, *S. cerevisiae MRS3*; BcMRS3, *B. cinerea MRS3*.

trons have limited sequence identity but share conserved secondary and tertiary structures. The RNA secondary structures consist of a combination of short- and long-range pairings designated P1 through P10, with elements P3 to P8 forming the catalytic core of the intron. Splice site recognition relies on pairing with exon sequences. An internal guide sequence pairs with exon sequences flanking the 5′ and 3′ splice sites to form helices P1 and P10, respectively (see reference 23). Changes in the nucleotide sequence in these regions would hinder pairing and correct excision of the intron.

The change from GGT to GCT in B2, which replaces a glycine with an alanine (G143A), causing a high level of resistance to Q_oIs, is located in the pairing region between B2 and bi2 (see reference 28 and Fig. 1B). We show here that the introduction of G143A causes a severe decline in respiratory growth. This is due to the decreased level of mature *CYTB* mRNA and, in consequence, of the bc_1 complex. In field populations of pathogenic fungi, G143A mutants would be counterselected because of their reduced fitness. So far, the G143A mutation has not been reported in bi2-containing fungi. However, bypass mechanisms could be developed that would allow efficient growth combined with the resistance mutation. In order to investigate these possible mechanisms, we used the *S. cerevisiae* G143A mutant, selected respiration-competent clones, and identified the secondary event restoring respiratory function. We found that secondary mutations in the mitochondrial genomes and the overexpression of nuclear genes could bypass the defect caused by G143A in *S. cerevisiae*. Could similar mechanisms evolve in pathogenic fungi? We examine the possibility of such bypasses in *B. cinerea*.

Bypass mechanisms from the yeast model to the pathogenic fungus *B. cinerea*. It is likely that G143A would have similar effects in the model organism *S. cerevisiae* and in other bi2-containing fungi. For instance, analysis of the nucleotide sequence of the B2-bi2 region in *B. cinerea* revealed that a similar model of pairing could be drawn (Fig. 1C) and that the change from GGT to GCT (G143A) would alter the pairing and, in consequence, the excision of the intron. Similarly, it can be hypothesized that a secondary mutation in bi2 could occur that restores the pairing.

Suppression by loss of intron bi2 could also be envisaged. We showed in Results (Fig. 5) that in Sup3, the bypass of the respiratory defect was due to the deletion of bi2. This type of suppression has been previously described in *S. cerevisiae* (12). Starting from respiration-deficient mutants with bi1, bi2, or bi3 splicing defects caused by mutations in those introns, respiration-competent clones were selected. In several of them, the restoration of respiratory function was due to the clean deletion of the mutated introns. In addition to the mutated introns, neighboring introns were also deleted. In our study, we found that the deletion of bi2 (which corrects its RNA splicing defect caused by G143A) was associated with the deletion of bi1, as described for mutations in bi2 in reference 12. In that paper, the author proposed that reverse transcriptases encoded by introns ai1 and ai2 of *COX1* play a role in the deletion of *CYTB* introns. It was hypothesized that an RNA intermediate with a correctly spliced bi2 intron was involved in the process. Note that Sup3 derives from a strain containing *COX1* introns ai1 and ai2.

The populations of *B. cinerea* consist of two species (I and II) living in sympatry that can be distinguished by microsatellite markers and gene polymorphisms (17 and references within). In both species, two types of *CYTB* that differ by the presence or absence of bi2 were observed. Different lineages within each species harbor the intron that was more frequent in species I than in species II. It was postulated that species II derived from species I and that independent intron loss events in species II have occurred after the divergence (17). It could be hypothesized that a reverse transcriptase-like activity would be involved in intron loss, which facilitates the acquisition of the G143A mutation. Alternatively, in bi2-containing fungi, selective pressure—by repeated or long-term exposure to Q_oIs—might lead to the loss of bi2 and acquisition of G143A.

Finally, we found that in *S. cerevisiae*, the nuclear genes *MRS2* and *MRS3*, at a high dosage, partially compensate for the defect induced by G143A. *MRS3* and the closely related gene *MRS4* (>70% identity) encode mitochondrial carriers involved in the transport of iron (9, 11) and probably of Mg^{2+} (30). The genes are not essential, as their deletion has little effect on cell growth and mitochondrial function under standard conditions (31). It has been shown that *MRS3* and *MRS4*, at a high copy number, can suppress splicing defects in mitochondrial group II introns (25, 31). *MRS2* encodes an essential component of the mitochondrial Mg^{2+} transport system (16). The gene is required for the splicing of group II introns but is not essential for group I introns, such as bi2 (32). At a high copy number, *MRS2* also suppresses the splicing defect of group II introns. The mechanism of suppression would rely on an increased intramitochondrial concentration of Mg^{2+} caused by the overexpression of *MRS2* (14). Interestingly, the same study reported that the overexpression of *MRS3* or *MRS4* in a Δ*mrs2* mutant strain (deletion of *MRS2*) had the same effect, increased Mg^{2+} and restoration of intron splicing (14). In our study, the splicing defect of the group I intron bi2 was found to be partially compensated for by the overexpression of *MRS3* and *MRS2*. Therefore, the same mechanism of suppression could be suggested.

The *in vitro* self-splicing of group I introns has been much studied. It has been shown that the binding of Mg^{2+} in the catalytic core of the intron is required for activity (22), which was further confirmed by the resolution of the atomic structure of group I introns that revealed metal ions in the active site

(see references 26 and 29 and references therein). It can be postulated that an increased Mg^{2+} level in the mitochondria might enhance the catalytic activity of the bi2 intron and/or stabilize its structure, thus facilitating splicing despite altered exon/intron pairing.

Mrs2p and Mrs3p are well conserved among species. We showed that an increased copy number of the *B. cinerea MRS3* gene, like that of its *S. cerevisiae* homolog, at least partially restored the respiratory function impaired by G143A. It might be hypothesized that increased expression of *MRS3* in the pathogenic fungi might rescue the cell fitness of G143A mutants.

In conclusion, the presence of an intron immediately after codon G143 seems to hamper the evolution of the Q_oI resistance mutation G143A in plant-pathogenic fungi, as the mutation would be associated with a fitness penalty. This would be the current situation in field populations. However, as found in *S. cerevisiae*, bypass mechanisms might be developed to restore respiratory function. It could be hypothesized that long exposure to inhibitors might lead to the evolution of such mechanisms.

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