Transposition of the Zorro2 Retrotransposon Is Activated by Miconazole in *Candida albicans*

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Zorro2 is a member of a non-long terminal repeat (LTR) retrotransposon family in *Candida albicans*, but as yet no clear evidence has been provided to establish either transcription or transposition activity for Zorro2. In this study, the relative expression changes of two open reading frames in Zorro2, ORF19.7274 and ORF19.7275, were examined in response to miconazole (MCZ), and were found to be increased by this treatment. As well, the copy number and the transcripts of Zorro2 in MCZ-induced resistant daughter strains were increased compared to the parental strain, indicating that transposition of Zorro2 occurred during long-term MCZ treatment. Intriguingly, the transcription activity of Zorro2 retrotransposons was significantly inhibited when the cells were treated with MCZ together with antioxidant *N*-acetyl-L-cysteine (NAC). As both the level of intracellular reactive oxygen species (ROS) and the expression of genes involving DNA repair activated by MCZ were reduced when combined with the treatment of NAC, we propose that the damage caused by accumulation of ROS under MCZ stress is a major reason for the transcription and transposition activation of the Zorro2 retrotransposon.

Key words *Candida albicans*; retrotransposon; miconazole; Zorro2

Candida albicans is a pleiomorphic fungus that can exist as either a commensal or an opportunistic pathogen and is capable of causing superficial to life-threatening infection in human beings. Nowadays, with the widespread application of immunosuppressive therapy, clinical infections by *C. albicans* have become more common.^{1,2)} *C. albicans* cells can be cleared through phagocytosis and the respiratory burst involving reactive oxygen species (ROS) in phagocytes.³⁾ However, an impressive ability to adapt to environmental insults, including oxidative stress, helps *C. albicans* escape the immune system and facilitates a high incidence of resistance to azole antifungal agents in *C. albicans*.⁴⁾

In recent years, transposons, particularly retrotransposons, have been found to play an important role in genetic evolution. Retrotransposons are mobile genetic elements capable of transposition *via* RNA intermediates.⁵⁾ The autonomous retrotransposons, which are capable of independent movement, consist of two sub-types, the long terminal repeat (LTR) retrotransposons and the non-LTR retrotransposons. Both of these classes contain one or two open reading frames (ORFs), which encode all the protein functions needed for retrotransposition, such as reverse transcriptase, endonuclease, integrase, *etc.* The most important difference between the two classes is that the latter lacks the LTR sequence. The non-LTR retrotransposons don't carry directly repeated sequences at their ends. Instead, they are often characterized by 3'-poly-A tails or 3' tandem repeats.

There are a large number of retrotransposons in eukaryotic genomes. However, the incidence of transposition is very low.

It is estimated that the frequency of spontaneous transposition of Ty1 elements in *Saccharomyces cerevisiae* varies among strains at rates of 10^{-5} – 10^{-7} /element per generation.⁶⁾ The stability is due to the evolution of defense system. Researchers have shown that DNA methylation, covalent modification of histones, and small molecule interfering RNAs can restrict the activity of transposons.^{7–9)} Currently it has been reported that DNA damage is an important reason for activating transcription and transposition of yeast Ty retrotransposons.¹⁰⁾ Stress conditions such as UV light, X-rays, nitrogen starvation, carcinogens and low temperature may increase the transposition activity of Ty1.^{11–14)}

In *C. albicans*, the activation of retrotransposons has so far been found only under conditions of temperature shifts. Tca2 and Tca5 are transcriptionally activated by a temperature shift from 27°C to 37°C and the transposed copies of Tca2 are integrated at several new positions in the genome.^{15,16)} Recently, non-LTR retrotransposons, the Zorros, have been identified in *C. albicans*. Only Zorro3 was full length; the other two, Zorro1 and Zorro2, were truncated in 5' or degenerate and without long poly-A tracts or 3' tandem repeat in the 3' UTR. Interestingly, unlike most non-LTR retrotransposons, the Zorros are present in few copies per genomic. Zorro1 and Zorro3 are also temperature-dependent retrotransposons in *C. albicans*. However, though Zorro1 and Zorro2 are found closer to each other,¹⁷⁾ no clear evidence has been provided to prove the transcription or transposition activity for Zorro2 retrotransposon.

In this study, the activation of the Zorro2 retrotransposon was explored. The expression of the protein-encoded regions of Zorro2, ORF19.7274 and ORF19.7275, were activated by the treatment of miconazole (MCZ). The copy number and the transcripts of Zorro2 in laboratory-induced MCZ-resistant strains were also increased. The antioxidant *N*-acetyl-L-cys-

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teine (NAC) inhibited the transcription activity of Zorro2. Both the level of intracellular ROS, and the expression of genes involving DNA damage repair activated by MCZ, were reduced when combined with NAC treatment. Our findings suggested that the treatment of MCZ resulted in the transcription and transposition activation of the Zorro2 retrotransposon.

MATERIALS AND METHODS

Strains and Culture Conditions *C. albicans* SC5314 was kindly provided by Dr. William A Fonzi from Department of Microbiology and Immunology Georgetown University (Washington, U.S.A.). Three strains (sc-m1, sc-m2, and sc-m3) were derived from SC5314 under the pressure of MCZ for a month in liquid complete medium consisting of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose (YPD). The strategy for induction of drug resistance was performed as described previously¹⁸⁾ with some modifications. A single colony of *C. albicans* SC5314 was inoculated into 10 mL of YPD broth and incubated overnight at 30°C with shaking (200 rpm). An aliquot of this culture containing 10^6 cells was then transferred to 10 mL of YPD broth containing MCZ at twice the most recently measured 80% minimum inhibitory concentration (MIC_{80}), and incubated at 30°C with shaking (200 rpm). When the cultures reached a density of about 10^8 cells/mL, aliquots containing 10^6 cells were transferred into fresh YPD broth also containing MCZ at twice the most recently measured MIC_{80} and incubated as described above.

Sample Preparation Hydrogen peroxide (H_2O_2); Azoles; Hypertonic treatment: *C. albicans* SC5314 cells in early-logarithmic phase at an OD of 0.2 in YPD medium were subjected to 5 mM H_2O_2 , 24 μ g/mL of MCZ, 250 μ g/mL of fluconazole (FLC), 250 μ g/mL of itraconazole (ICZ), 156 μ g/mL of ketoconazole (KCZ), 0.6 M NaCl or 1 M sorbitol, respectively. Cells without any treatment were used as a control. Cells were grown at 30°C and collected after 3 h and 6 h treatment.

The combination of NAC and MCZ: *C. albicans* SC5314 cells in early-logarithmic phase at an optical density (OD) of 0.1 were transferred to fresh YPD medium containing 40 mM NAC. Cells without NAC treatment were used as a control. Both the control and NAC-treated cells were grown to an OD_{600} of 0.2, followed by resuspension in an equal volume of fresh YPD medium with or without 24 μ g/mL of MCZ. Cells were collected after 6 h culture.

RNA Isolation and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis Total RNA was extracted from *C. albicans* SC5314 with or without the treatments using Fungal RNAout kit (TIANDS, China). Reverse transcription was performed in a total volume of 20 μ L with Avian Myeloblastosis Virus reverse transcriptase (TaKaRa, Dalian, China), random primer (6-mer) (TaKaRa) and 1 μ g of total RNA, followed by the condition of 30°C for 10 min, 45°C for 15 min, and 99°C for 2 min, as recommended by the manufacturer. Real-time PCR reactions were performed with SYBR Green I (TaKaRa), using the LightCycler Real-Time PCR system (Roche Molecular Biochemical). The oligos used in the real-time RT-PCR assay are listed in Table 1. The thermal cycling conditions comprised an initial step at 95°C for 10 s, followed by 40 cycles at 95°C for 10 s, 62°C for 20 s, and 72°C for 15 s. The C_T value of 18S rRNA was subtracted

Table 1. Primers Used in This Study

Primer name	Sequence (5'→3')	Amplicon size (bp)
18S rRNA-F	TCTTCTTGATTTGTGGGTGG	150
18S rRNA-R	TCGATAGTCCCTCTAAGAAGTG	
ORF19.7274-F	AGCCAAGTCCCAAAAAGAC	174
ORF19.7274-R	AATGCTGGACCACACACACC	
ORF19.7275-F	ACCTTGCCACTGATAGAC	143
ORF19.7275-R	GTTAGTAGCCAGATTTT	
MSH2-F	CAAGCAATCCGAAGTTAT	150
MSH2-R	AATTTGGGTTGGGTGTAT	
RAD50-F	CGAAGATTGAGTGGGTTTG	138
RAD50-R	CGTTGTGCCTTGTATTGCT	
MRE11-F	AAGATTGAGTGGGTTTGG	135
MRE11-R	GTTGTGCCCTTGTATTGCT	
RAD6-F	CCTAATAAACCAACCACA	147
RAD6-R	TAATGATTGAACCGATG	
RAD52-F	GGATTCTATGGTTGTTTCG	127
RAD52-R	GTGAGTGCTTCCTTTTAC	
DDR48-F	CGACGACAAAGACAGAAGAC	100
DDR48-R	AGAGCCAAATGAAGAGGAT	
YKU80-F	AGATGACGATGAAGAGGG	152
YKU80-R	GGGATGTTGGTAAAGTGG	
SPO11-F	TATCAAAGAAACCGAGAC	199
SPO11-R	TAATGACCACCTAACCC	

from that of the gene of interest to obtain a ΔC_T value. The ΔC_T value of an arbitrary calibrator was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta C_T}$.

Southern Blotting *C. albicans* genomic DNA samples were digested with *Xba*I, electrophoresed through 0.8% agaroseD-5 (Sigma) for 2.5 h, and then vacuum-transferred to hybond-N+ nylon membranes (Roche). Hybridization was performed with a 688 bp Zorro2-containing DNA fragment at 42°C for 16 h. The DNA fragment was obtained by PCR amplification using the primer, 5'-TGTGTTGGCGTATGCGAAGT-3' and the primer 5'-GTTGCCGAGAGTGCCCTTGTC-3'. The purified PCR product was labeled with DIG-High Prime (Roche). The detection was carried out using DIG-High Prime DNA Labeling and Detection Starter kit II according to the producer's manual (Roche).

Antifungal Susceptibility Test The broth microdilution method for *in vitro* antifungal sensitivity testing of *C. albicans* was performed as standard Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (M27-A2).¹⁹⁾ Briefly, cells were inoculated into wells of a 96-well plate containing serial two-fold dilutions of antifungal drugs in RPMI-1640 medium buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS). The initial concentration of the fungal suspension in RPMI-1640 medium was 1×10^3 cells/mL, and the final concentration of MCZ, FLC, ICZ, and KCZ in wells was ranged from 0.0195 μ g/mL to 10 μ g/mL, 0.125 μ g/mL to 64 μ g/mL, 0.039 μ g/mL to 20 μ g/mL, or 0.039 μ g/mL to 20 μ g/mL, respectively. Optical density was measured at 630 nm after incubation for 48 h at 30°C. The endpoints for MIC_{80} were determined as the lowest concentration that resulted in an 80% reduction in turbidity as compared to the drug-free control wells.

Fig. 1. The Transcriptional Activation of Two Predicted Protein-Encoding ORFs, *ORF19.7274* and *ORF19.7275*, for Zorro2 Is Dependent on MCZ Treatment

C. albicans SC5314 cells were collected upon the treatment of azoles (24 μ g/mL of MCZ, 250 μ g/mL of FLC, 250 μ g/mL of ICZ, 156 μ g/mL of KCZ), 5 mM H₂O₂, or hypertonic stresses (0.6 M NaCl and 1 M sorbitol) after 3 h and 6 h incubation. Relative fold changes were calculated with ΔC_T values of the targets with or without the treatments compared to those without the treatment after 3 h incubation. Data are shown as mean $\pm$ S.D. ($n=3$). * $p < 0.05$ vs. not-treated 3 h.

Spot assays were used to test the antifungal sensitivity as well. Briefly, *C. albicans* strains in mid-logarithmic phase were collected by centrifugation at 3000 $\times g$ for 5 min, counted, and resuspended in 0.9% sterile normal saline at a concentration of 10⁷ cells/mL. A total of 4 μ L of these cultures was spotted in serial 10-fold dilutions on drug-containing agar plate: MCZ 2 μ g/mL, FLC 4 μ g/mL, ICZ 4 μ g/mL, KCZ 2 μ g/mL. Growth differences were recorded after incubation of plates for 48 h at 30°C.

Measurement of Intracellular ROS Production Intracellular ROS level was determined by 2',7'-dichlorodihydrofluorescein diacetate, Sigma (DCFH-DA) as previously described.¹⁸⁾ Briefly, *C. albicans* cells were collected by centrifugation and resuspended in phosphate buffered saline (PBS) at an OD₆₀₀ of 1.0. The cell suspension was incubated with 20 μ g/mL DCFH-DA at 30°C for 30 min. The cells were exposed to MCZ (24 μ g/mL), NAC (40 mM), or MCZ (24 μ g/mL) together with NAC (40 mM), and then incubated at 30°C with shaking (200 rpm). At specified intervals, 100 μ L of cell suspensions were transferred to the wells of a flat-bottomed microplate (BMG Microplate, Black) to detect fluorescence intensity on the POLARstar Galaxy (BMG, Labtech, Offenbourg, Germany) with excitation at 485 nm and emission at 520 nm. Triplicate experiments were performed to generate a mean value. Alternatively, after treatments of the above agents for 3.5 h, cell samples were examined with a 63 $\times$ oil immersion lens under a TCS SP5 confocal laser-scanning microscope

(Leica Microsystems Trading Ltd.) with excitation at 485 nm and emission at 520 nm. Micrograph pictures were acquired using LAS AF Lite program (Version 2.1.1 build 4443). Cells treated with 5 mM H₂O₂ were performed as a control.

RESULTS

The Active Transcription of the Zorro2 Retrotransposon Depends on MCZ

Transcriptional activity is required for transposition for all retro-elements. To test whether elements in Zorro2 are transcribed in response to treatments including H₂O₂, azoles, hypertonic NaCl and sorbitol, semi-quantitative RT-PCR was performed to analyze the relative mRNA expression changes of Zorro2 in strain SC5314. Two predicted protein-encoding regions required for retrotransposition in the Zorro2 retrotransposon were selected: *ORF19.7274*, with similarity to reverse transcriptase, and *ORF19.7275*, with similarity to endonuclease. The relative expressions of *ORF19.7274* and *ORF19.7275* increased by more than 4 to 5 folds in response to 24 μ g/mL of MCZ for 6 h (Fig. 1A), and didn't increase after 6 h incubation in the absence of MCZ (Fig. 1A) or in the presence of the other stresses including 5 mM H₂O₂, 0.6 M NaCl or 1 M sorbitol (Fig. 1B). However, the relative expressions of the two ORFs increased twice with the treatment of FLC or ICZ for 3 h, but decreased after 6 h, while those didn't changed with KCZ at 3 h, but decreased at 6 h (Fig. 1C). These results suggested that the transcription of Zorro2 depended on stress

Fig. 2. Azole Sensitivity Testing for the Parental Strain SC5314 and the Three Derivative Strains of Long-Term MCZ Treatment (Sc-m1, Sc-m2, and Sc-m3)

(A) The MIC₈₀ for azoles was tested by using broth microdilution in 96-well microtiter plates according to the Clinical and Laboratory Standards Institute. (B) The spot assay was performed to profile the growth difference with the treatment of MCZ, FLC, KCZ, or ICZ. Four microliters of ten-fold serial dilutions of each strain were spotted onto the plates in the presence of MCZ (2 μg/mL), FLC (4 μg/mL), ICZ (4 μg/mL), and KCZ (2 μg/mL). Growth differences were recorded after incubation for 48 h at 30°C.

Fig. 3. The Zorro2 Retrotransposon Is Capable of Transposition

(A) Southern blotting analysis. *Xba*I-digested genomic DNA from the parental strain SC5314 and the three derivative strains of long-term MCZ treatment (sc-m1, sc-m2, and sc-m3) were hybridized to the internal region of Zorro2. Line 1: SC5314; line 2: sc-m1, line 3: sc-m2, line 4: sc-m3. (B) The transcription of the Zorro2 was activated in the derivative strains. Relative fold changes were calculated with ΔC_T values of the derivative strains compared to the parental strain SC5314. * $p < 0.05$ vs. SC5314.

Fig. 4. Oxidative Damage Contributes to the Induction of Zorro2

(A) NAC rescued the growth of SC5314 in the presence of MCZ. A total of 40 mM NAC was added to the cell suspension at an initial OD₆₀₀ value of 0.1. The cells were grown to an OD₆₀₀ of 0.2, followed by the addition of 24 μ M of MCZ. OD₆₀₀ values were then recorded at the indicated time points. * p <0.05 vs. control group, # p <0.05 vs. MCZ-treated group. (B) The increased endogenous ROS caused by MCZ in SC5314 was inhibited in the presence of NAC. Inter-cellular ROS level was detected under continuous treatment for 6h and 12h on the POLARstar Galaxy. * p <0.05 vs. control group, # p <0.05 vs. MCZ-treated group. (C) The intracellular ROS of each group were observed with confocal microscopy with excitation at 485nm and emission at 520nm after 3.5h of treatment. Bar=5 μ m. The increased expression levels of genes involved in the DNA damage repair (D) and the transcription activity for *ORF19.7274* and *ORF19.7275* (E) caused by MCZ were reduced in the presence of 40 mM NAC. Samples were collected under the continuous treatment for 6h. Relative fold changes were calculated with ΔC_T values of genes in the strain with treatments compared to those in the strain without treatments. Data are shown as mean $\pm$ S.D. (n =3). * p <0.05 vs. MCZ-treated group.

caused by the antifungal MCZ and was independent of ergosterol biosynthesis pathway.

The Zorro2 Retrotransposon Is Capable of Transposition To evaluate the function of a retrotransposon, it is necessary to analyze whether the retrotransposon element is capable of performing all the steps of the replication cycle, including the generation of full-length double-stranded DNA molecules. In this study, considering the low incidence of transposition for retrotransposons during only one passage, three strains were selected after ca. 25 d of continuous MCZ-induction. The induced daughter strains (sc-m1, sc-m2, and sc-m3) possessed high resistance to MCZ with MIC₈₀ value

increases of 800 to 1000 fold (Fig. 2A). Low concentrations of derivative strains still survived in the presence of 2 μ M of MCZ in contrast to the parental strain (Fig. 2B). Moreover, the MIC values of FLC, ICZ, and KCZ against the derivative strains were increased as well (Fig. 2A). The derivative strains survived in the presence of 4 μ M of FLC, 4 μ M of ICZ, and 2 μ M of KCZ, while the parental strain did not (Fig. 2B). These results indicated that the MCZ-induced daughter strains were cross resistant to other azole agents.

Southern blotting was then performed in these three strains to investigate whether or not retrotransposon of Zorro2 occurred. As shown in Fig. 3A, with the internal probe of

the Zorro2 retrotransposon, additional bands ranging from 20000 bp to 5000 bp were detected in the three induced MCZ-resistant strains, in comparison with the parental SC5314, which suggested that Zorro2 was capable of transposition in response to MCZ. Furthermore, the relative expressions of *ORF19.7274* and *ORF19.7275* in the derivative resistant strains were increased (Fig. 3B), suggesting a high transcriptional activity of Zorro2.

Oxidative Damage Contributes to the Induction of the Zorro2 Retrotransposon As the transcription and transposition activity of Zorro2 have been shown, we investigated whether the oxidative damage caused by MCZ was the reason for the transposition of Zorro2. The growth of SC5314 was first monitored to investigate the effect of NAC, an antioxidant, which is capable of eliminating cellular ROS, particular superoxide and hydrogen peroxide. There was no difference in growth between with or without treatment of NAC only. However, the sustained inhibition of growth caused by MCZ treatment was significantly rescued in the presence of both NAC and MCZ after 4h incubation (Fig. 4A). We then examined whether or not the addition of NAC influenced intracellular ROS levels. The endogenous ROS level increased 15 to 24 fold after MCZ treatment for 6 to 12h (Fig. 4B). As expected, with the addition of NAC together with MCZ, the generation of endogenous ROS was significantly inhibited (Fig. 4B). These results were also confirmed by confocal microscopy. The fluorescence signals indicating intracellular ROS were observed inside of almost each MCZ-treated cell. However, the number of cells carrying fluorescence signal was significantly decreased in cells treated with MCZ and NAC. And their fluorescence intensity was relatively weak as well (Fig. 4C). These results provided evidence that the ROS were just generated inside the cells.

Furthermore, semi-quantitative RT-PCR was performed to analyze the expression change of genes involved in damage repair. As shown in Fig. 4D, the expression of repair-related genes including *MSH2*, *RAD50*, *MRE11*, *RAD52*, *YKU80*, *DDR48* and *SPO11* were induced with the treatment of MCZ only, while the induction of those genes were significantly decreased when treated with MCZ in the presence of NAC. These results suggested that the addition of NAC reduced the degree of damage caused by MCZ. The effect of antioxidant NAC on the transcription activity of Zorro2 induced by MCZ stress was also observed. As expected, the induction of *ORF19.7274* and *ORF19.7275* by MCZ were reduced with the addition of NAC (Fig. 4E). Taken together, these results suggested that oxidative damage contributed to the transcriptional activation of Zorro2 retrotransposon.

DISCUSSION

Previous studies have reported that retrotransposons, including *Tca2*, *Tca5*, *Zorro1* and *Zorro3*, can be activated by temperature changes in *C. albicans*,^{15–17,20} but no evidence has yet been provided to prove the transcription or transposition activity for Zorro2 in *C. albicans*. In this study, we showed that the Zorro2 retrotransposon in *C. albicans* was transcriptionally activated and transposed in response to MCZ, probably caused by the induction of endogenous ROS. This is the first time to report that an antifungal azole could participate in the regulation of retrotransposon activity in *C. albicans*.

To be functional, retrotransposons are transcriptionally activated first, and then are inserted at new positions in the host genome. The Zorro2 retrotransposon is different from the commonly studied LTR retrotransposons in structure, as the LTRs usually contain signals for promotion and termination of transcription that are required in the reverse transcription process. However, the lack of an LTR doesn't affect the process to transposition of Zorro2. We previously revealed that the copy numbers of *ORF19.7274* and *ORF19.7275* of the Zorro2 retrotransposon were increased in several clinical isolates compared with SC5314 by array-based comparative genomic hybridization analysis.²¹ In this study, the transcription of Zorro2 was found up-regulated in strains in response to short-time MCZ stress. Additional copies of Zorro2 were observed in the genome of derivative strains under long-term treatment of MCZ, suggesting that the Zorro2 has been transposed in the derivative strains with resistance to azole drugs. Our unreported data has shown that the transcription of *ORF19.6469* in *TCA11* retrotransposon and *ORF19.2219* in *TCA3* were also increased by more than 4 to 8 folds with the treatment of MCZ for 6h. But there were no additional copies of them observed in the genome of the derivative MCZ-resistant strains. These results further confirmed the correlation between MCZ and the transcriptionally activated and transposed Zorro2. It is reported that retrotransposons impart evolutionary plasticity to the genome, thereby contributing to the ability of cells to adapt to new environments.²² Our results indicated that the transposition of Zorro2 could contribute the strains ability to survive under the pressure of the antifungal MCZ for genetic evolution.

The potential mechanism for Zorro2 transcription and transposition activity in response to MCZ stress was further investigated. As has been reported, the induction of endogenous ROS in *C. albicans* cells is one of the functions of MCZ, besides inhibition of the 14 α -demethylation of lanosterol.^{23,24} The increase of intracellular ROS production may block DNA replication and promote chromosomal loss.²⁵ Retrotransposons can patch together broken chromosome ends, and provide dispersed regions of homology that facilitate chromosomal rearrangement.^{26,27} It has been shown that Tyl is capable of transposition through a branch of the DNA-damage checkpoint pathway.²⁸ In this study, the addition of NAC reduced the transpositional activity of Zorro2 induced by the MCZ stress. Meanwhile, the widely up-regulated genes involved in DNA-damage repair in response to MCZ stress were all decreased when treated together with NAC. A plausible reason is that NAC could strongly inhibit endogenous ROS generation and protect the cells from oxidative damage. Therefore, it is possible that the generation and accumulation of intracellular ROS upon MCZ stress has a key role in activating the transcription level and transposition of Zorro2.

The increased transposition of Zorro2 could be a result of the MCZ treatment. But whether or not the increased copies of Zorro2 actually contribute to the increased azole resistance, further mechanistic experiments are required. Directly deleting the copy number of Zorro2 genetically or characterizing the specific locations of the transposed Zorro2, in the evolved MCZ-resistant strain, would be investigated in the future.

In conclusion, our study revealed that activation of Zorro2 retrotransposon in transcription and transposition upon MCZ treatment in *C. albicans* was caused by an oxidative damage

repair mechanism. We propose that the Zorro2 in *C. albicans* genome is a potential internal element allowing genetic evolution.

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