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ORIGINAL RESEARCH PAPER

Enhanced resistance of *Trichoderma harzianum* LZDX-32-08 to hygromycin B induced by sea salt

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Abstract
Objectives To determine the effect of sea salt on the resistance of *Trichoderma harzianum* LZDX-32-08 to hygromycin B and speculate the possible mechanisms involved via transcriptome analysis.
Results Sea salt addition in media to simulate marine environment significantly increased the tolerance of marine-derived fungus *Trichoderma harzianum* LZDX-32-08 to hygromycin B from 40 to 500 µg/ml. Meanwhile, sea salt addition also elicited the hygromycin B resistance of 5 other marine or terrestrial fungi. Transcriptomic analyses of *T. harzianum* cultivated on PDA, PDA supplemented with sea salt and PDA with both sea salt and hygromycin B revealed that genes coding for P-type ATPases, multidrug resistance related transporters and acetyltransferases were up-regulated, while genes coding for Ca^{2+}/H^{+} antiporter and 1,3-glucosidase were down-regulated, indicating probable increased efflux and inactivation of hygromycin B as well as enhanced biofilm formation, which could jointly contribute to the drug resistance.
Conclusions Marine environment or high ion concentration in the environment could be an importance inducer for antifungal resistance. Possible mechanisms and related key genes were proposed for understanding the molecular basis and overcoming this resistance.

Keywords Antifungal resistance · Hygromycin B · Sea salt · Transcriptome · *Trichoderma harzianum*

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Introduction

About 1.5–2 million people die of fungal infection each year (Denning and Bromley 2015). These numbers increased steadily in recent decades (Sekyere and Asante 2018). Worse still, antifungal resistance became a big challenge due to consistent use of limited antimicrobials (Denning and Bromley 2015). Thus, it is extremely urgent to obtain a better understanding of resistance mechanisms in order to reverse the resistance or provide novel targets for drug development. Previous studies revealed three main mechanisms

including changes in drug target sites, activation of drug efflux and formation of biofilms (Sekyere and Asante 2018). Hygromycin B (HYG) is an aminoglycoside antibiotic that inhibits protein synthesis by stabilizing the tRNA-ribosomal acceptor site (Kaster et al. 1983). HYB resistance was mainly based on modification of ribosome binding sites (Honore et al. 1995), decreasing intracellular HYG concentrations (Karlowsky et al. 1997), and inactivation of HYG via structure modification by aminoglycoside phosphotransferase, acetyltransferase, or nucleoside adenosine transferase (Rao et al. 1983). Besides, addition of KCl and mutation of *pma1* by UV-treatment was found to improve the HYG tolerance of *Saccharomyces cerevisiae*, which indicated that the HYG transport was associated with the electrochemical proton gradient and cells were unable to uptake HYG, when the H^+-ATPase Pma1 was mutated (Perlin et al. 1988). Later, Barreto et al. hypothesized that any mutation resulting in changes of the electrochemical gradient could give rise to anomalous sensitivity to cationic drug independently of its toxicity mechanism. They made a genome-wide screen for mutants that demonstrated altered tolerance to HYG, spermine, and tetramethylammonium in *S. cerevisiae* and lots of genes related to potassium homeostasis were identified (Barreto et al. 2011). Furthermore, Alao et al. found that KCl can suppress the sensitivity of *Schizosaccharomyces pombe* to a wide range of antibiotics, which might depend in part on changes to electrochemical gradient of membrane and membrane transport proteins. However, the specific mechanism of ion induced drug resistance remains largely elusive (Alao et al. 2015). In this study, the effects of sea salt on HYG tolerance of *Trichoderma harzianum* and five other fungi were investigated and the possible mechanisms were proposed thereof.

Materials and methods

Strains, media and growth conditions

Fungal strains used were listed in Supplementary Table 1 and cultivated at 28 °C on PDA (Potato Dextrose Agar, Difco) or PDA with sea salt (3.33%, w/v) or PDA with both sea salt and 100 µg/ml HYG. Sea salt was from Instant Ocean Reef Crystals®. HYG solution (50 mg/ml) was purchased from VWR. Each plate was inoculated with 3 µl spore suspension (10^5 conidia/ml).

Transcriptome analysis

Trichoderma harzianum were cultivated on PDA supplemented with sea salt (A), PDA supplemented with sea salt and 100 µg/ml HYG (B), and PDA (C) for 6 days at 28 °C. The mycelia were collected respectively and sent to Sangon Biotech (Shanghai) for total RNA extraction, library construction, sequencing and analysis. Total RNA was extracted using Total RNA Extractor (Trizol). Sequencing libraries were generated using VAHTSTM mRNA-seq V2 Library Prep Kit for Illumina® and index codes were added to attribute sequences to each sample. Raw reads were filtered by Trimmomatic (version 0.36) and the remaining clean data were used for further analysis. The direct expression of a gene expression level was quantified by StringTie (version 1.3.3b), which was used to calculate Transcripts Per Million (TPM) of protein-coding genes in each sample. DESeq (version 1.12.4) was used for differential expression analysis and significant DEGs were obtained (p-value < 0.05 and $|FoldChange| > 2$). TopGO (version 2.24.0) was used for GO (Gene Ontology) enrichment, and the function was thought to be a significant enrichment when the correct p-value (q-value) < 0.05 (Database: https://www.geeontology.org). ClusterProfiler (version 3.0.5) was used for euKaryotic Ortholog Groups (KOG) enrichment analysis (Database: https://www.ncbi.nlm.nih.gov/COG/). The raw sequence data have been submitted to the National Center for Biotechnology information (NCBI) Sequence Read Archive (SRA) with accession number PRJNA623072.

Results

Hygromycin B resistance induced by sea salt

Hygromycin B is commonly used in lab as selection marker for positive transformants. During the genetic manipulation of marine-derived fungus *T. harzianum* LZDX-32–08, we observed that sea salt addition (3.33%) in PDA could significantly increase its resistance to HYG (Fig. 1a). *T. harzianum* could only survive 40 µg/ml HYG on PDA, in contrast to 500 µg/

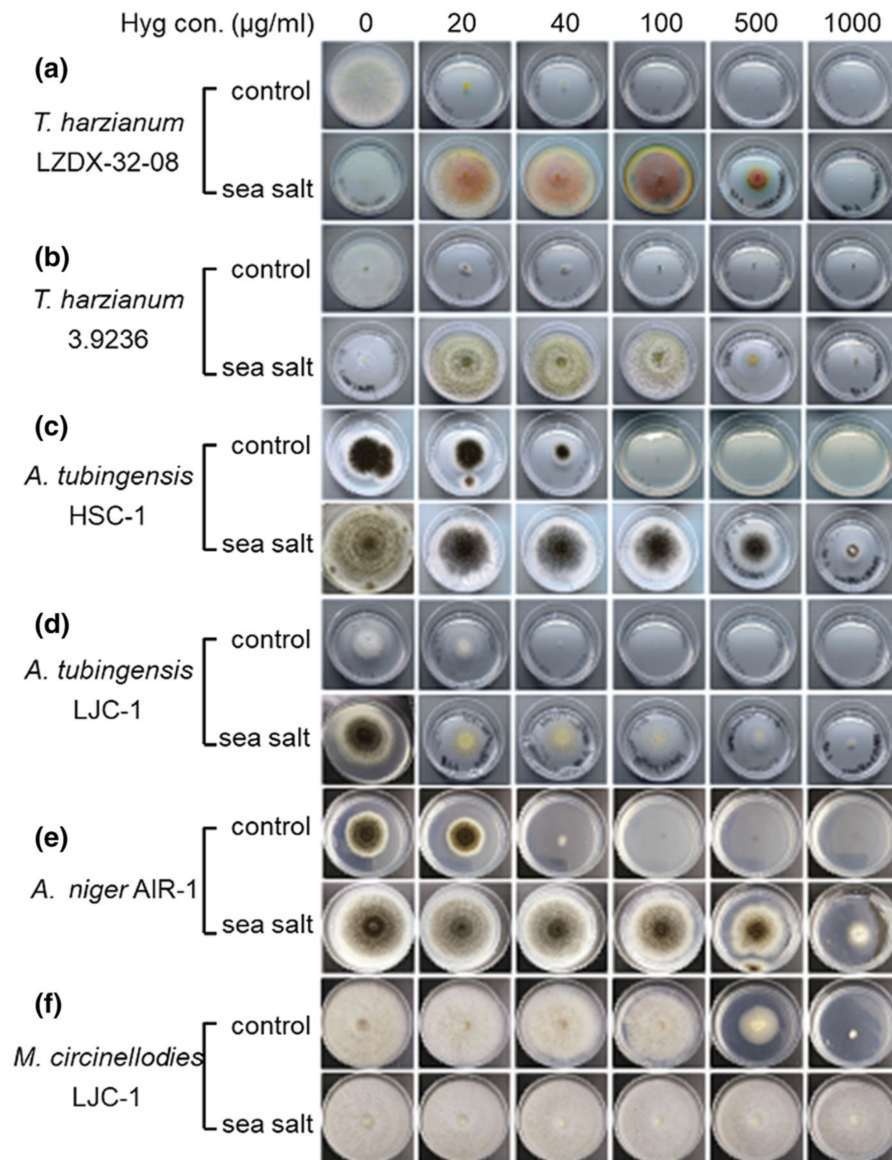

Fig. 1 Fungi cultivated on PDA or PDA supplemented with sea salt cultivated at 28 °C for 5 days, with HYG concentration (hyg con.) of 0, 20, 40, 100, 500, 1000 μg/ml, respectively. The figures represent the average of 4 parallel experiments

ml HYG in the presence of sea salt. The addition of sea salt seemed to be beneficial for the marine-derived fungus to adapt to harsh environment of HYG exposure. To examine whether this effect is limited to *T. harzianum* LZDX-32-08, other commonly used fungal strains were tested under the same condition, including terrestrial fungus *T. harzianum* 3.9236, marine-derived *Aspergillus tubingensis* HSC-1, terrestrial *A. tubingensis* 3.6402, *A. niger* AIR-1 and *Mucor circinelloides* LJC-1. Similar to *T. harzianum* LZDX-32-08, *T. harzianum* 3.9236, *A. tubingensis* HSC-1, *A. tubingensis* 3.6402 and *A. niger* AIR-1 only survived 40 μg/ml HYG on PDA, while on PDA supplemented with sea salt, they could even survive 1000 μg/ml HYG (Fig. 1b–e). *M. circinelloides* LJC-1 was not as sensitive to HYG as other selected fungi and could withstand 1000 μg/ml HYG on both PDA and PDA with sea salt. Even though, the growth diameter of *M. circinelloides* on PDA decreased with the increased concentration of HYG, while the growth

of *M. circinelloides* LJC-1 with sea salt addition was almost uninhibited (Fig. 1f). Thus, all six fungi demonstrated increased resistance to HYG with sea salt addition in media, no matter it is from marine or terrestrial.

Effect of different ions on hygromycin B resistance

Since the cations in sea salt could be responsible for the increased resistance of fungi to HYG, four cations that have high content in sea salt, K^+, Na^+, Mg^{2+}, and Ca^{2+} were individually investigated to figure out which ion might play a major role. 0.57 M $CaCl_2$, KCl, $MgCl_2$, and NaCl could increase the HYG tolerance of *T. harzianum* up to 1000, 500, 500 and 200 μg/ml, respectively, among which $CaCl_2$ demonstrated the strongest effect (Fig. 2). Both divalent and monovalent cations could increase the tolerance of *T. harzianum* to HYG, suggesting that this effect might not base on the chelation inactivation of HYG by ions. This suggestion was further proved by the ^{1}H NMR spectrum of a mixture of HYG and sea salt, which was almost identical to that of HYG alone (Supplementary Fig. 1). In addition, different osmotic pressures of PDA media created with sorbitol cannot induce the HYG resistance of *T. harzianum* (Supplementary Fig. 2). Therefore, the mechanism for the sea salt induced HYG resistance was neither inactivation of HYG by chelation nor osmotic changes, and the remaining possibility would lie on the sea salt-induced transcriptomic changes in *T. harzianum*.

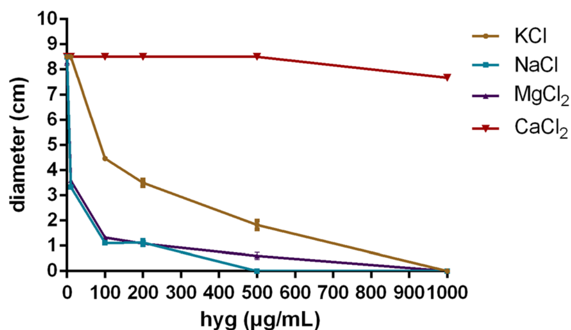

Fig. 2 The growth diameter of *T. harzianum* cultivated on PDA supplement with different salts and different concentrations of HYG for 6 days. Error bars represent standard deviation from three independent measurements

Transcriptomic changes of *T. harzianum* in response to sea salt and hygromycin B

To speculate the molecular basis of HYG resistance induced by sea salt, Illumina RNA-sequencing technology (RNA-seq) was used to characterize *T. harzianum* transcripts under three conditions, i.e. PDA supplemented with sea salt (A), PDA supplemented with sea salt and 100 μg/ml HYG (B) and PDA (C). The numbers of differentially expressed genes (DEGs) were shown in Fig. 3. Most of DEGs were detected when compared B to A, indicating that the expression of many genes under sea salt stress (A) was converse with the extra addition of HYG (Fig. 3).

GO enrichment for DEGs

The clustering analysis based on Gene Ontology (GO) categories was performed to analyze the DEGs of *T. harzianum* response to sea salt and HYG (Fig. 4).

In the presence of sea salt, the most abundant enriched GO terms in "Biological Process" were related to cell wall disassembly (GO:0044277, GO:0071853 and GO:1904541), cell wall organization (GO:0070871), cell septum edging catabolic process (GO:0030995) and extracellular polysaccharide catabolic process (GO:0071999) (Fig. 4a). All of DEGs related to these GO terms were significantly down-regulated for about 13–209.9 times. In the ontological category "Molecular Function", the DEGs were related to glucosidase activity (GO:0015926, GO:0051118), among which one gene was up-regulated for about eight times and six genes were down-regulated for 4.6–207.9 times (Fig. 4a). These results

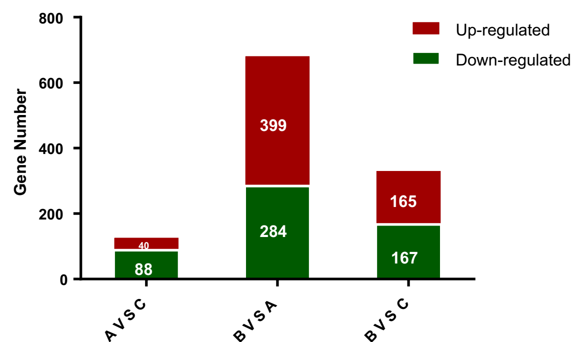

Fig. 3 Number of DEGs in the pair-wise comparison (p-value < 0.05 and $\log_2$[FoldChange] > 2)

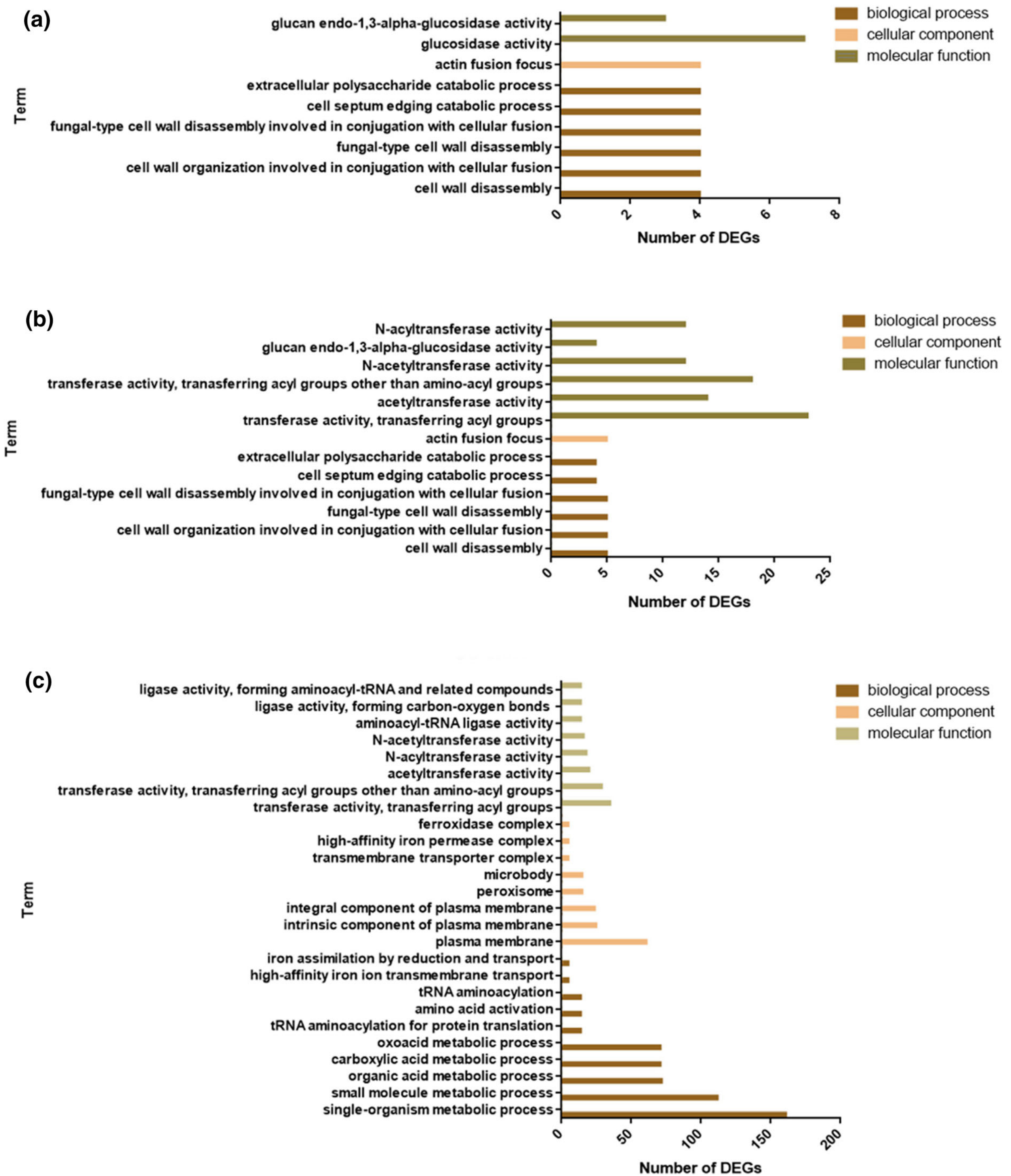

Fig. 4 GO enrichment analysis of DEGs of *T. harzianum* when cultivated at 28 °C for 6 days on PDA supplemented with sea salt (**a**), PDA supplemented with sea salt and HYG (**b**) and PDA (**c**)

imply that the cell wall of *T. harzianum* might experience a great change under sea salt stress due to the extracellular polysaccharide catabolic variation.

Comparing B to C, the most abundant enriched GO terms in the "Biological Process" were also related to cell wall formation, same as the group of A vs C

(Fig. 4a and b), which might affect the cell wall phenotypes of *T. harzianum*. Therefore, the cell walls of *T. harzianum* cultivated under these 3 conditions (A, B and C) were examined with transmission electron microscope (TEM). Much thicker cell walls were observed in B and A comparing to C, which was in agreement with the transcriptomic changes (Fig. 5). While in the ontological category "Molecular Function", the result seemed to be a combination of the outcome of A vs C and B vs A, with significantly up-regulated terms as acyltransferase (GO:0016746 GO:0016747 GO:0016410), acetyltransferase (GO:0016407 GO:0008080), and significantly down-regulated term as glucan endo-1,3-alpha-glucosidase (GO:0051118) (Fig. 4b).

Compared between B and A (Fig. 4c), the most abundant enriched GO terms in the "Biological Process" were related to single-organism metabolic process (GO:0044710), followed by small molecule metabolic process (GO:0044281), organic acid metabolic process (GO:0006082, GO:0019752 and GO:0043436), and tRNA aminoacylation (GO:0006418 and GO:0043039), which were among the significantly up-regulated terms. "Cellular Component" related to transmembrane transporter complex (GO:1902495), high-affinity iron permease complex (GO:0033573) and ferroxidase complex (GO:1905862) were significantly up-regulated. In the ontological category "Molecular Function", DEGs were mostly associated with transferase activity, including N-acyltransferase (GO:0016746, GO:0016747 and GO:0016410), acetyltransferase (GO:0016407 and GO:0008080) and tRNA related ligase (GO:0004812, GO:0016875 and GO:0016876), which were among the significantly up-regulated terms as well (Fig. 4c).

DEGs found in all three groups included ion related transporters as well as genes coding for multidrug resistance protein (MDR), multidrug resistance associated protein (MPR), pleiotropic drug resistance protein (PDR) and other transporters of ATP binding cassette (ABC) superfamily or major facilitator superfamily (MFS) (Supplementary Table 2). The results revealing that *T. harzianum* might employ diverse transport systems for sea salt and HYG, which were likely involved in the enhanced resistance. Further venn analysis of ion related transporters indicated that 2 DEGs coding for putative Na^{+}/K^{+}-ATPase were considerably up-regulated with sea salt and HYG addition (Fig. 6a A vs C and B vs C), which might be mainly induced by sea salt and independent from HYG (Fig. 6a B vs A). Meanwhile, there were 2 down-regulated overlapping DEGs coding for putative Ca^{2+}/H^{+} antiporter (M431DRAFT_5034) and Na^{+}/phosphate symporter (M431DRAFT_93229) with HYG addition (Fig. 6a, B vs A and B vs C), indicating that HYG might inhibit the expression of these two genes. 6 DEGs demonstrated that HYG addition with sea salt had adverse effects as that of sole sea salt addition (Fig. 6a, B vs A). Apart from M431DRAFT_495547, the expressions of the other 5 DEGs were inhibited by HYG, including putative solute carrier (SLC), potassium uptake protein, Ca^{2+}/H^{+} antiporter, and cation transporting ATPases (Fig. 6a). The venn analysis diagram of MDR/MRP-related DEGs showed that the expression of one putative MDR (M431DRAFT_487806) gene was inhibited by sea salt addition (Fig. 6b, A vs C), which can be induced

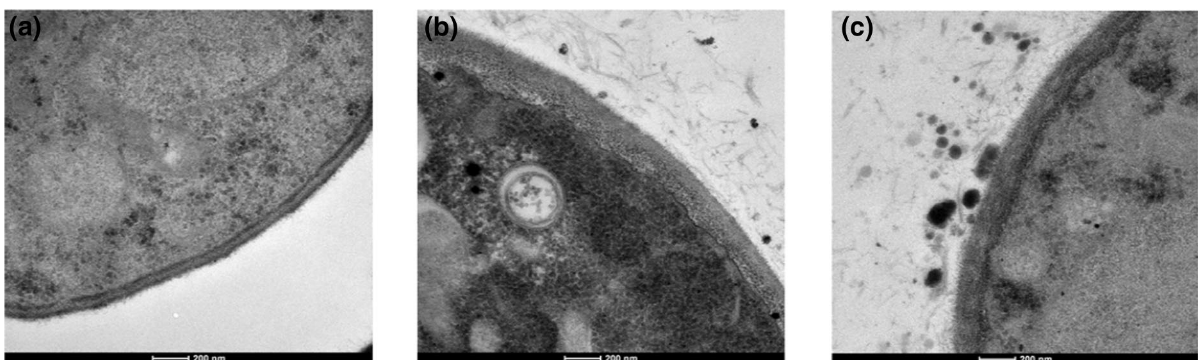

Fig. 5 The transmission electron microscope (TEM) images of the *T. harzianum* cultivated at 28 °C for 6 days on PDA, PDA with sea salt and PDA with sea salt and HYG

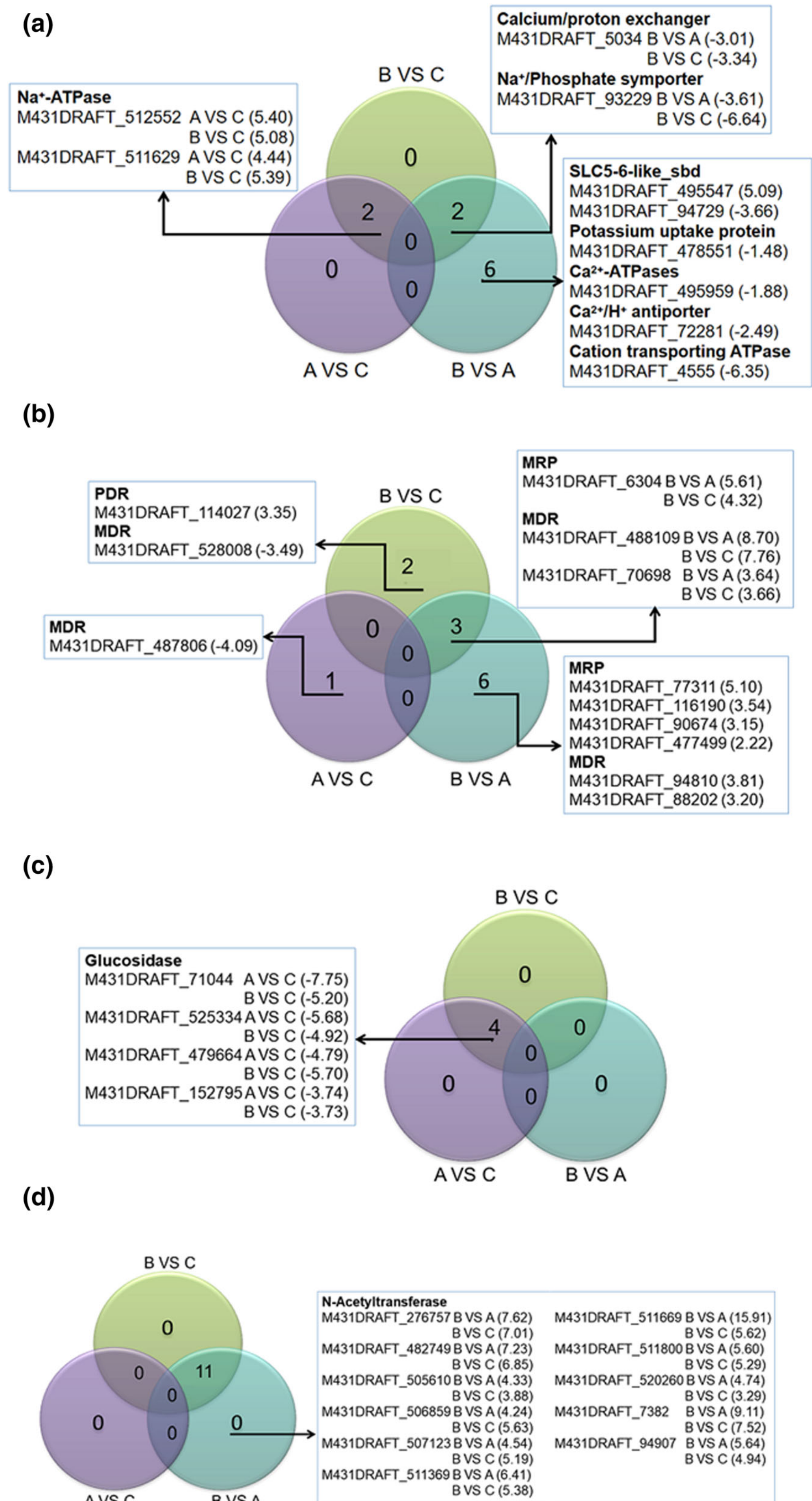

Fig. 6 Overlap of DEGs among comparison between control (C), *T. harzianum* under sea salt stress (A) or sea salt together with HYG (B): **a** ion transport-related DEGs; **b** multidrug resistance (MDR/MRP)-related DEGs; **c** glucosidase-related DEGs and **d** N-acetyltransferase related DEGs

by HYG addition (Fig. 6b, B vs C). Furthermore, there were 6 MRP/PDR genes (M431DRAFT_6304, 77311, 116190, 90674, 477499 and 114027) and 4 MDR genes (M431DRAFT_488109, 70698, 94810 and 88202) elicited by HYG (Fig. 6b). Moreover, the venn analysis diagram of DEGs concerning glucosidase suggested that sea salt addition in the media could remarkedly inhibit the expression of 4 glucosidases (M431DRAFT_479664, 152795, 525334, and 71044), and the extra addition of HYG demonstrated no obvious difference (Fig. 6c). In addition, the expression of 11 acetyltransferases was not affected by sea salt (Fig. 6d, A vs C), but significantly induced by HYG, including M431DRAFT_7382, 276757, 482749, 506859, 511669, 511369, 520260, 511800, 507123, 94097 and 505610 (Fig. 6d, B vs A and B vs C).

Discussion

Ions play a vital role in various living organisms, which have evolved efficient regulatory systems for ions uptake and export to keep ion homeostasis (Han et al. 2017). Halophilic bacteria were found to show natural resistance to antibiotics, whose mechanism have remained loosely defined (Tokunaga et al. 2004). Previous study found that a Na^+/ATPase plasma membrane transporter Ena1 in *S. cerevisiae* mediated toxic cations efflux by hydrolyzing ATP and inhibition of Ena1 dramatically enhanced susceptibility of *Cryptococcus neoformans* to antifungal drugs, such as amphotericin B (Jung et al. 2012). RNA-seq analysis of *T. harzianum* LZDX-32-08 revealed two putative Na^+/K^+ ATPases (coded by M431DRAFT_511629 and 512552), which show 42.64% and 50.05% identity to Ena1, respectively. The significantly increased expression of these two genes with sea salt (Fig. 6a, A vs C) or sea salt and HYG addition (Fig. 6a, B vs C) indicated that they could play a critical role in both ion homeostasis and the induced HYG resistance of *T. harzianum*. Besides, calcium is a second messenger involved in growth, development, apoptosis, and stress response. Enhanced cytosolic Ca^{2+} transient was found to increase the azole resistance of *A. fumigatus* by up-regulating a series of calcineurin-dependent–response-element genes (Li et al. 2020). In our study, calcium addition in PDA displayed the strongest effect to confer the antifungal resistance (Fig. 2). Therefore, Ca^{2+} transporters were of great interests. Two putative Ca^{2+}/H^+ antiporters (coded by M431DRAF_5034 and 72281), shared 48.91% and 33.96% identity to Vcx1 from *S. cerevisiae,* respectively, were clearly down-regulated with HYG and/or sea salt addition (Fig. 6a), which could contribute to enhanced cytosolic Ca^{2+} and consequently elicit the HYG resistance (Stathopoulos-Gerontides et al. 1999). In addition, MDR is vital for antifungal resistance. 4 putative MDR transporters of MFS family and 6 MRP/PDR genes of ABC family were considerably up-regulated (log_2FoldChange up to 8.70) mainly induced by HYG in the media (Fig. 6b). Among them, M431DRAFT_88202 shows 34.27% identity to Flu1 in *Candia albicans*. The expression of Flu1 in *S. cerevisiae* made it resistant to fluconazole and cycloheximide (Calabrese et al. 2000). Besides, putative PDR protein (coded by M431DRAFT_114027) shows 34.65% identity to the Pdr5 of ABC family in *S. cerevisiae* (Hadiar Rahman et al. 2018). Since these transporters utilize electrochemical potential as energy to transport substrate and require the involvement of cations (Na^+ or H^+) in the transport of drugs (Fluman et al. 2014), the addition of sea salt might facilitate these transporters and accelerate the efflux of HYG to confer fungal resistance.

In addition to transporters, the formation of biofilms has been shown to be the main reason of drug resistance in *Candidia* spp. (Silva et al. 2017; Huang et al. 2020). β-1,3-glucan is an important component of biofilm. The expressions of 4 α/β-1,3-glucosidases were significantly down-regulated with sea salt addition, which might reduce the hydrolyzation of β-1,3-glucan and contribute to thicker cell wall and formation of biofilm. Furthermore, microbes could produce enzymes to inactivate antibiotics. For instance, Eis from *Mycobacterium smegmatis* could acetylate aminoglycoside antibiotics to confer resistance (Pan et al. 2018). In our case, 11 putative N-Acetyltransferase genes were up-regulated mainly induced by HYG, which could inactivate HYG by acetylation (Rao et al. 1983). Among them, a putative N-Acetyltransferase coded by M431DRAFT_482749 shows 37.93% identity to Eis (Pan et al. 2018). Further characterization of these genes might provide novel types of inactivation enzymes.

The aforementioned DEGs are promising candidate genes responsible for the antifungal resistances. Taken together, 3 major mechanisms were proposed for the

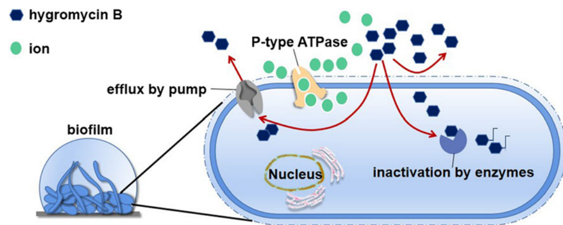

Fig. 7 Proposed mechanisms of sea salt induced HYG resistance in *T. harzianum*

enhanced HYG resistance of *T. harzianum* LZDX-32-08 under sea salt stress (Fig. 7). Firstly, sea salt addition induced the expression of P-type ATPases to facilitate ionic transport in order to equilibrium the intracellular osmotic pressure. Along with this process, the electrochemical potential or proton gradient was changed, which might affect the MDR related transporters induced by HYG and promoted the efflux of HYG. Secondly, sea salt addition significantly affected the cell wall phenotype and reduced the expression of 1,3-glucosidase genes, leading to thicker cell wall and forming of biofilms, which could decrease the uptake of HYG. At last, HYG induced the expression of acetyltransferases and resulted in its inactivation. The synergistic effects of the above mechanisms could jointly contribute to the increased resistance of *T. harzianum* to HYG under sea salt environment.

Further investigation on the relationship between ion transporters and MDR related transporters could provide more detailed information for the resistance mechanism. Meanwhile, functional characterization of the candidate genes in the future could shed light on new targets for overcoming this resistance.

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Supplementary Information Supplementary Table 1- Strains used in this study.

Supplementary Table 2- Other transporter-related genes in DEGs of *T. harzianum* when cultivated at 28 °C for 6 days on PDA supplemented with sea salt (A), PDA supplemented with sea salt and HYG (B) and PDA (C).

Supplementary Fig. 1- NMR spectra of HYG (a) and mixture of HYG and sea salt (b).

Supplementary Fig. 2- *Trichoderma harzianum* LZDX-32-08 cultivated at 28 °C for 4 days on PDA supplemented with 0, 0.57, 1 or 2 M sorbitol and 0, 10, 50, 100 or 200 µg/mL HYG, respectively.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human and animal participants This article does not contain any studies with human participates or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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