Antioxidant metabolites from riparian fungal endophytes improve the tolerance of rice seedlings to flooding

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Abstract
Endophytic fungi have the potential to enhance plant resistance to various stresses and promote the ecological adaptation of the hosts. To evaluate the effects of the riparian endophytes on rice seedlings to flooding tolerance, here we screened out two fungi from the plant Myricaria laxiflora growing in the Yangtze River zone. Through morphological characteristics and rDNA ITS (internal transcriber region) sequence, the two strains were, respectively, identified as Aspergillus fumigatus and Chaetomium globosum. Metabolites derived from both fungi were capable of increasing tolerance of rice to flooding. Systematic separation and purification coupled to bioassays revealed that two natural antioxidants, Z-N-4-hydroxystyryl formamide (NFA) and chaetoglobosin A (CheA), were effective for alleviating flooding stress. Both NFA and CheA can reverse the decline trend of oxidative parameters caused by long-term flooding, such as malondialdehyde, superoxide dismutase, ethanol dehydrogenase, and NADPH oxidase. Gene expression analyses of NADPH oxidase families indicated that OsRbohB could be involved in conferring flooding tolerance mediated by the two natural antioxidants. These findings contribute to understanding the role of the natural antioxidants in riparian endophytic fungi and providing a basis for improvement of flooding tolerance of rice and other crop plants.

Keywords Natural antioxidant · Z-N-4-hydroxystyryl formamide · Chaetoglobosin A · Endophytic fungi · Flooding tolerance · OsRbohB

Introduction
Flooding is an important factor limiting the growth and development of plants worldwide, since this disturbance reduces the supply of oxygen and light, promotes the accumulation of reactive oxygen species (ROS), and can lead to hypoxia or oxidative stress (Adkins et al. 2010; Miao et al. 2017). Global climate change is expected to aggravate extreme flood events in this century, which would adversely affect plant diversity and crop production (Voesenek and Bailey-Serres 2015). Therefore, it is of significance to better understand the adaptive mechanisms of plants in response to flooding and to develop ways to improve their tolerance of it.

Much experimental evidence demonstrates that ROS, the by-products of aerobic metabolism, have dual effects on plant’s response to flooding stress (Jorge et al. 2016; Singh et al. 2016). Mild flooding will quickly induce the production of appropriate ROS, which function as a signal transducer and trigger the adaptive response to counteract the stress (Singh et al. 2016; Foyer et al. 2017). Thus, as the main cause of ROS production, the activation of NADPH
oxidase (also known as Rboh in plants) is essential for the plant response to flooding stress. For example, AtrbohD mutants of Arabidopsis are hypersensitive to hypoxia and submergence stress because they can no longer produce ROS (Weyemi and Dupuy 2012; Liu et al. 2017).

Alternatively, a severe shortage of O2 generates the accumulation of ROS that exceeds the scavenging activity of protective enzymes (Wu and Yang 2016; Miao et al. 2017), such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). Such a scenario would give rise to lipid peroxidation and a damaged cell membrane system, together with the production of toxic malondialdehyde (MDA) that can act to crosslink macromolecules (Pucciariello and Perata 2017). It is now well understood that eliminating excessive ROS can maintain oxidative homeostasis and is conducive to plants’ flooding tolerance. A case in point is the SUB1A (submergence 1A) gene, which acts as an effective scavenger of ROS, and is closely associated with tolerance to flooding in rice (Oryza sativa L., Locke et al. 2018). Additionally, flooding up-regulates Alcohol dehydrogenase (ADH), through which ROS radicals would be partially removed by the oxidation of NAD(P)H into NAD(P)+ (Cao et al. 2017; Miao et al. 2017). Hence, ADH activity has become a screening index of flood-tolerant species in plant breeding programs (Adkins et al. 2010; An et al. 2016).

Recent studies have shown that endophytic fungi can promote the ecological adaptability of their hosts and play a key role in the response of plants to adversity (Weyens et al. 2009; Khiralla et al. 2016; Qadir et al. 2020). Work by Mei and Flinn (2010) found that endophytes inhabiting coastal mangroves are capable of enhancing the tolerance of plants there to saline–alkali conditions. More recently, root fungal endophytes were shown to enhance heavy metal stress tolerance of trees naturally distributed in several abandoned mines (Yamaji et al. 2016). To enable plants to better cope with various adverse events or conditions, endophytes may produce numerous secondary metabolites to promote the activity of specific antioxidant enzymes, or elicit particular pathways, such as those for the biosynthesis of alkaloids, terpenoids, or phenols (Mishra et al. 2016; Vinale et al. 2017; Bilal et al. 2018a, b).

In an earlier study, hundreds of endophytic fungi were obtained from plants of Myrica laxiflora (Qin et al. 2019), a species that inhabits the riparian zone of the Yangtze River and exhibits strong adaptability to flooding, in that its individuals can survive despite enduring six months of flooded conditions per year (Tian et al. 2015). To find out whether the endophytic fungi harbored in that riparian plant could improve the flooding tolerance of plants and reveal something of the underlying mechanism, we have examined the effects of two of these fungi and metabolites produced by them on flooding tolerance in rice. We found that two natural antioxidants, NFA (Z-N-4-hydroxystyrylformamide) and CheA (chaetoglobosin A), were effective at enhancing the ability of rice to tolerate flooding perhaps by affecting OsRbohB. We anticipate our findings will spur other researchers to try and better understand the ecological function of riparian endophytes and their natural antioxidants in plant response to flooding, which could yield ideas for ways to improve their flooding tolerance.

Materials and methods

Strains, medium and plant resources

In July 2014, before the flooding season, the strains of SG-17 (CCTCCM 2015286, China Typical Culture Preservation Center) and QY-1 (CCTCCM2014182) were, respectively, isolated from the root and leaves of Myricaria laxiflora, which grows at the water-level-fluctuating zone of the Three Gorges area, in Hubei Province, China. Both SG-17 and QY-1 were cultured on a potato dextrose agar (PDA) medium. Rice seeds of the ‘Nipponbare’ cultivar were used in the flooding tolerance test.

Taxonomic identification

The fungi were identified based on their morphological characteristics and molecular analysis. For the latter, genomic DNA from the fungal mycelia was extracted with an isolation kit (Karroten Life Scientific, Nanjing, China). The fungus-specific primers ITS1 and ITS4 were used to amplify the internal transcriber region (ITS). Each PCR was performed in a 20-μL volume reaction that contained 2 μL of DNA template, 1 μL of each primer, 2 μL of PCR buffer, 2 μL of dNTPs, and 0.2 μL of Ex-Taq Polymerase (Takara, Japan) and deionized water (HHitech, Shanghai, China), under these reaction conditions: 94 °C for 5 min; 33 cycles at 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. Sequences were then reassembled using the DNASTAR 12.1 software (DNASTAR Inc., WI, USA), and then deposited in GenBank. These sequences were compared with those in the NCBI database by the BLAST search algorithm, after which CLUSTAL-W in MEGA 6 software was used to construct phylogenetic trees, via the neighbor-joining and maximum likelihood methods. Bootstrap replications (n = 1000) were used to determine the statistical support for each node in the phylogenetic trees (Lubna et al. 2018).

Screening the fungi for assisting rice plants’ flood tolerance

About 6–8 rooting rice seeds of Nipponbare were sown into the plastic cups containing about 100 g of sterilized...
local soil, and then grown for 8 days in a light incubator (at 30 °C, under a 16-h/8-h light/dark photoperiod, with 75% relative humidity). The cup volume was 980 cm³ with a height of 32 cm, so the seedlings could be fully submerged. Rice seedlings of similar size were selected for the flooding stress treatment, in which the tap water level reached 3 cm above the top of rice seedlings. In the endophyte infection test, about 10⁶ spores/mL fungal solution of SG-17 or QY-1 was injected into 6-day-old rice leaves, and sterile water was used as the control. One day later, the seedlings were submerged as describe above. All the test fungi were cultured for 8–10 days, and then a little quartz was added to the fermentation broth and their cell walls disrupted for 30 min by an ultrasonic processor BX7200H (Xinnuoli Instrument Co., Ltd Beijing, China). One day before the flooding stress was imposed, 50 mL of each fermentation broth before- or after-sonication were sprayed onto the 6–8 seedlings in one cup. Each treatment in the experiments described above was replicated three times and 20 seedlings were investigated.

Plant physiological parameters measurements under flooding

Survival was investigated for 20 rice seedlings fully submerged for 8 days, whereas other physiological indicators were determined following a 4-day recovery after flooding them for 4 days to find more subtle differences (Yeung et al. 2018). Relative chlorophyll content was determined with a SPAD-502 m (Minolta, Tokyo, Japan), and the MDA and SOD contents in rice leaves were assayed using kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (Qin et al. 2019). Both the ADH and NADPH oxidase contents were determined with the one-step sandwich ELISA, using kits obtained from Jiangsu Baolai Biotechnology Co., Ltd (Jiangsu, China).

Isolation and preparation of natural substances

The fungi SG-17 and QY-1 were inoculated individually into a shaking flask after 5 days incubation on PDA solid medium at 28 °C, and then cultured for 10 days with shaking at 140 rpm. The ensuing fermented liquid (2 L) was harvested by suction and extracted thrice with an equal volume of ethyl acetate. The organic phase and aqueous phase were, respectively, combined and distilled in a vacuum at 40 °C, to harvest the crude extracts (6.24 g) for their bioassay-guided fractionation.

Identification of natural antioxidants

Thin-layer chromatography (TLC) was used to separate the crude extracts. For the SG-17 fungus, the developing agent was petroleum ether: acetone = 1:1 (V/V), but the proportion used for QY-1 was 6:4. The fractions visible at 254 nm were eluted by methanol to evaluate their antioxidant activity and whether they improved the flooding tolerance of rice. Next, the active ingredients were separated and purified by semi-preparative high-performance liquid chromatography (HPLC; 250 x 10 mm id, Cosmosil MS-II, Nacalai Tesque, Tokyo, Japan), yielding 2.33 g main active substance in SG-17 and 1.45 g in QY-1 (later proven to be NFA and CheA). The UV detection wavelength was set to 254 nm, and the mobile phase consisted of acetonitrile:water = 60:40 (V/V), with a flow rate of 3.0 mL/min for SG-17 and 1.0 mL/min for QY-1. The 1H-NMR and 13C-NMR were determined by AVANCE 400 MHz (Bruker, Switzerland) nuclear magnetic resonance (NMR) spectroscopy using DMSO-d6 as a solvent containing tetramethylsilane as the internal standard.

Antioxidant activity of the natural compounds

The total antioxidant capacity (T-AOC) kit and the 1, 1-diphenyl-2-trinitrophenylhydrazine (DPPH) radical scavenging kit (Qin et al. 2019) were used to, respectively, test the fermented broth and the compounds, following the manufacturer’s instructions. Both kits came from the Nanjing Jiancheng Bioengineering Institute, Nanjing, China (http://www.njjcbio.com/). Either NFA or CheA at 1 and 10 µg/mL were used for this investigation, for which 1 µg/mL of vitamin C (Vc) served as the positive control.

Effects of natural antioxidants on rice flooding

To evaluate whether the natural antioxidants NFA and CheA affected rice plants’ flooding tolerance, 0.01 and 0.1 mg/mL of NFA or CheA, respectively, were sprayed onto the seedlings 4 h before fully submerging them, and each cup was sprayed with 1 mL of solution. Their physiological indicators were determined during a 4-day recovery after flooding for 4 days. Each treatment was repeated three times and 20 seedlings in three cups were investigated. The growth conditions used were the same as described above.

Expression analysis of OsRbohs

After the indicated periods of imposed flooding, at least four healthy leaves in different replicates were harvested for their RNA extraction using the EASYspin kit from the Aidlab Biology Co., Ltd (Beijing, China). The samples’ cDNAs were synthesized using the PrimeScript Reagent kit with gDNA Eraser (TaKaRa, Kusatsu, Japan); then real-time quantitative RT-PCR was carried out using StarScript Two-step Kit from GenStar Co., Ltd (Beijing, China) according to the manufacturer’s instructions, with OsActin1 serving as
the internal standard. All primer sequences used are listed in Supplementary Table 1.

**Quantification of H$_2$O$_2$ in rice**

The H$_2$O$_2$ content was measured by applying a method previously described (Avramova et al. 2015). Briefly, 0.1 g of rice leaves were homogenized in 1-mL 5% (w/v) trichloroacetic acid, then centrifuged at 14 000×g for 30 min. The ensuing supernatant was incubated with xylene orange dye reagent for 45 min, and then its light absorption was measured at 595 nm.

**Data processing**

The data were analyzed, and figures drawn, in the Graphpad Prism 5 program (GraphPad Software Inc., CA, USA). One-way ANOVA and planned LSD (least significance difference) contrasts were used to evaluate treatment effects. All data are expressed as the means ± standard deviation.

**Results**

**Screening of fungus assisting rice against flooding stress**

Hundreds of endophytic fungi from riparian plants of *M. laxiflora* (Tian et al. 2015) were used in a preliminary screening for their relevance to flooding tolerance. After 8 days of fully submerged stress at room temperature, most Nipponbare rice seedlings appeared slim, withered, and eventually died. Their survival rate remained high when rice seedlings were sprayed with the sonicated fermentation broth of SG-17 or QY-1 (Table 1). To reveal more subtle effects, we adjusted the fully submerged time to 4 days, and then let the seedlings recover for another 4 days under normal growth before analysis. The corresponding SPAD values showed that they had a significantly higher relative content of chlorophyll than the control group. These results showed that SG-17 and QY-1 clearly improved the growth of rice under the experimentally imposed flooded conditions (Fig. 1a).

The colonies of fungus SG-17 were velvety, gray olive green, appearing almost round with irregular edges, and they grew rapidly on the PDA medium; the hyphae were colorless and transparent with septa; the conidiophores were smooth, their top sac was flask-shaped, with only the upper half producing spherical, light-green conidia (Fig. 1b, c). BLAST analysis revealed that the 18S ITS sequence of SG-17 (GenBank accession number: KU954091) shared a 99% identity with *A. fumigatus* (Fig. 1d). The other fungus, QY-1 grew slowly on the PDA medium, initially appearing white and fluffy, but gradually turned dark blue; the hyphae were radial, colorless, and transparent with branches and septa (Fig. 1e, f). The nearly spherical ascomycetes were scattered. The ITS sequence (MK450298) for QY-1 shared a 100% identity with *C. globosum* (Fig. 1g).

**Fungal metabolites improved flooding tolerance in rice**

Recent studies have reported on metabolites from symbiotic endophytes exerting positive effects on plants’ response to abiotic or biotic stresses (Khiralla et al. 2016; Bilal et al. 2018a, b). In the experiment, we found that the living fungi of SG-17 or QY-1 before sonication had little effect on flooding tolerance (Table 1). When these fungi were inoculated on rice seedlings, no obvious colonization was observed, and SG-17 significantly inhibited the growth, which indicated that SG-17 might be a potential pathogen of rice (Supplementary Fig. 1). Hence, in the next experiment, we prepared crude extracts of SG-17 and QY-1 to determine their active compounds. After an ethyl acetate extraction, vacuum distillation, and preparative TLC, these fractions were recovered to test their activity in rice when flooded. Both SG-17 and QY-1 had a constituent effect, in that they each significantly

### Table 1 Characteristics of the two endophytic fungi and their effects on flooding tolerance in rice

| Fungi   | SN in Genbank | The most homologous species$^a$ | Survival rate (%)$^b$ | SPAD value$^c$ |
|---------|---------------|--------------------------------|-----------------------|----------------|
|         |               |                                | Before sonication     | After sonication | Before sonication | After sonication |
| QY-1    | KU954091      | Chaetomium globosum            | 12.2 ± 3.9            | 30.8 ± 4.4**    | 18.7 ± 2.3        | 22.4 ± 3.1**    |
| SG-17   | MK450298      | Aspergillus fumigatus          | 6.3 ± 2.5             | 27.3 ± 3.7**    | 10.1 ± 3.5        | 23.8 ± 2.4**    |
| No fungi$^d$ | –             | –                              | 8.77 ± 2.4            | 14.2 ± 3.8      |                   |                 |
| No flooding | –             | 100 ± 1.1                      |                      | 36.7 ± 1.5      |                   |                 |

$^a$The most homologous species were obtained by the ITS sequence alignment in Genbank

$^b$Survival rate was calculated for 30 rice seedlings flooded for 8 days; Student’s t-test compared the means of the “No fungi” group, for which **indicates a significant difference found, at $p < 0.01$

$^c$SPAD value was determined for ten seedlings that experienced 4 days of flooding and followed by a recovery period (another 4 days)

$^d$No fungi treatment means the fermentation broth alone
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increasing the ability of rice to withstand the impact of flooding (Fig. 2a).

Through the HPLC purifications, the corresponding structures were elucidated by NMR spectroscopy (Fig. 2b, c). In SG-17, the putative active compound was suggested to be C₉H₉NO₂, on the basis of ESI–MS at m/z 186.05 [M + Na]⁺. The ¹³C-NMR spectrum indicated one carbonyl carbon at δC 160.4 (–CHO), and eight olefinic carbons at δC 126.5 (C-1’), 130.1 (C-2’), 116.0 (C-3’), 156.8 (C-4’), 116.0 (C-5’), 130.1 (C-6’), 118.2 (C-1), and 111.3 (C-2). The ¹H-NMR spectrum displayed four olefinic protons at δH 7.19 (d, J = 8.3 Hz, H-2′, and H-6′), 6.77 (d, J = 8.3 Hz, H-3′, and H-5′), 6.64 (d, J = 9.8 Hz, H-1), and 5.60 (d, J = 9.8 Hz, H-2). These spectral characteristics were similar to the NMR spectroscopy data reported for a previous compound (Anzai et al. 1962), which had been identified as (Z)-N-(4-hydroxy styryl) formamide (NFA).

Using the same approach, the molecular formula of the active substance in QY-1 was determined (Fig. 2d, e) as C₃₂H₃₆N₂O₅, by analysis of ESI–MS at m/z 551.25 [M + Na]⁺. The ¹³C NMR spectrum showed 32 carbon signals, including three carbonyl carbons, 12 olefinic carbons, three oxygenated carbons, and four methyl carbons. The ¹H-NMR spectrum displayed three methyl groups at δH 0.96 (d, J = 6.6 Hz, H-11), 1.11 (s, H-12), 0.67 (d, J = 7.2 Hz, 16-CH₃), and 1.36 (s, 18-CH₃), as well as six olefinic protons. By comparing this NMR data with that reported in the literature (Sekita et al. 1983), we identified the compound as chaetoglobosin A (CheA).
Fig. 2 Active compounds in SG-17 and QY-1. a Beneficial effects of different substances and concentrations on the flooding tolerance in rice. Each pot of seedlings was sprayed with 1 mL of solution 4 h before complete immersion, unit: mg/mL, bar = 1 cm. b, c Structure of NFA in the endophyte SG-17 and its HPLC analysis. d, e Structure of CheA in the endophyte QY-1 and its HPLC analysis.
Both NFA and CheA exhibited antioxidant activity

Riparian plants often experience flood and hypoxia stresses, so they usually engage in stronger antioxidant activity. To find out whether the endophytic fungi they harbor contributed to this antioxidant capacity, we investigated, in vitro, the crude extracts and the metabolites (using the T-AOC kit and DPPH radical scavenging kit). The compound NFA had an extremely high total antioxidant (T-AOC) value, being 16 times that of the positive control, Vitamin C (Vc, Fig. 3a). Yet at the same concentration, the DPPH free radical scavenging rate of CheA was almost equivalent to that of Vc, but exceeded it at 10 µg/mL (Fig. 3b), thus indicating its unusual anti-oxidation ability.

Physiological responses of flooded rice plants to NFA and CheA

In plants, MDA, SOD, ADH, and NADPH oxidases are the main indicators used to evaluate the flooding stress levels, and they vary significantly with the degree of oxidative stress (Qian et al. 2015). In most cases, the contents or activities of these indices would first rise then fall with prolonged inundation of plants, as found for MDA measured in our study (Supplementary Fig. 2). After the rice seedlings were flooded for 4 days, the amounts (or activity) of MDA, SOD, ADH, and NADPH oxidases diminished steadily by 50.4%, 40.9%, 35.9%, and 16.0%, respectively (Fig. 4a–d). However, this trend seemed to be reversed by the antioxidants NFA or CheA, in that the MDA contents and activities of SOD, ADH, and NADPH oxidases would be upregulated either under flooding or under normal growth conditions; hence, the decline in indices caused by flooding should be alleviated in plants by the antioxidants.

The increments of SOD induced by NFA or CheA were significantly greater after flooding than before it. Under normal conditions (i.e., no flooding) NFA and CheA at 0.1 mg/mL induced the SOD in rice to rise by 45.9% and 11.1%, but after flooding it soared by 129.2% and 75.8%, respectively (Fig. 4b), nearly attaining the level for normal growth. Similar results were also obtained for the NADPH oxidase (Fig. 4d), indicating the function of natural metabolites from these fungi are closely linked to mitigating flooding stress in rice.

The antioxidants NFA and CheA upregulated the expression of OsRbohB, rather than activity

In the rice genome, there are 11 genes encoding NADPH oxidase, among which OsRboh A–E may be involved in the pathway of ABA signaling, alleviation of hypoxia stress, or responses to drought (Li et al. 2018). The resulting expression pattern showed that OsRbohB, OsRbohD and OsRbohE were obviously induced by flooding in the early stage, but only the former diminished with longer flooding, and rebounded in the later period, while the latter two continued to strengthen with the prolongation of flooding time (Fig. 5a). In view of OsRbohB is the most homologous orthologue to AtRbohD, which plays a role in how Arabidopsis endures flooding (Kwak et al. 2003; Kadota

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**Fig. 3** Antioxidant capacity of NFA and CheA. a Total antioxidant analysis. b DPPH free radical scavenging rate. The positive control is 1 µg/mL vitamin. Data are expressed as the means ± standard deviation, and n = 3. The * indicates a significant difference at p < 0.05, and likewise *** at p < 0.001 when compared with the control group.
et al. 2015), suggesting that OsRbohB could be involved in the metabolism of rice plants exposed to flooding stress. Under normal growth, the natural metabolites NFA and CheA strongly induced the expression of OsRbohB in rice after a 4-h treatment, while OsRbohA lacked this response (Fig. 5b). After flooding for 4 days, the relative expression level of OsRbohB was significantly reduced in rice (according to real-time quantitative PCR analysis); however, this decline was evidently inhibited by NFA or CheA applications (Fig. 5c), thus maintaining OsRbohB at a relatively high level. Further, as a major product of OsRbohB, hydrogen peroxide was also elevated by NFA or CheA, both before and after flooding the seedlings (Fig. 5d). Collectively, these results demonstrated that by regulating the activity of NADPH oxidase OsRbohB, both natural antioxidants could improve the flooding tolerance of rice.

**Discussion**

The idea that endophytic fungi can improve their plant hosts’ habitat adaptability is gaining empirical support and becoming an increasingly popular subject of research. Yet surprisingly little is known about how they might enhance their hosts’ flooding tolerance, in spite of the severe damage that flood events cause to agriculture. It had been reported that fungal endophytes of A. fumigatus can produce gibberellins and regulate plant endogenous hormones (Waqas et al. 2014; Bilal et al. 2018a, b), and C. globosum from rice had antagonistic properties against fungal pathogens (Naik et al. 2007; Du et al. 2020). Here, we reported on the endophytic fungi A. fumigatus SG-17 and C. globosum QY-1 from the riparian plant M. laxiflora.
which alleviated the flooding stress of rice seedlings by producing NFA and CheA, thereby effectively reversing the tendency of MDA and SOD to accumulate under flooding conditions (Fig. 4a, b). These results indicate that the endophytic fungi under stress may have a positive effect on unnatural host plants through eco-chemistry. Moreover, as a harmful pathogen to mankind, A. fumigatus cannot be used directly (van de Veerdonk et al. 2017), and the natural compounds present in the fungus might be beneficial in agricultural production.

Both NFA and CheA were earlier considered to be selective antibiotics (Anzai et al. 1962; Sekita et al. 1983), but few reports investigated their antioxidant activity and role in improving how plants respond to stress factors. In our study, both natural compounds showed high antioxidant activity and were capable of regulating ROS homeostasis in rice. In particular, NFA and CheA were able to slightly promote the growth of rice, but its plant physiological parameters and gene expression levels evinced that NFA and CheA had more pronounced effects during submerged conditions, indicating that these natural antioxidants functioned more like anti-stress substances than growth-promoting ones. Admittedly, very few physiological indices were used to evaluate flooding impacts in this study, so it is difficult to grasp the complexity of flooding stress to plants in the field. For example, in their field production, rice plants are waterlogged rather than temporally flooded; therefore, the physiological indicators would have to be adjusted accordingly. Future research is needed to collect field data via detailed investigations, which should consider morphological and physiological plant traits affected by these natural antioxidants, such as those related to rice tillers, yields, and resistance to pests or diseases in its heading or maturity stages. Especially interesting is that the flowering period in rice is reportedly more sensitive to flooding disturbances (Adkins et al. 2010). Therefore, whether these antioxidants also play a role in the flowering process of rice awaits further verification.

Fig. 5 Effects of the two endophyte-derived antioxidants on the expression levels of OsRboh genes and H$_2$O$_2$ content in rice plants under flooding stress. a Semi-quantitative RT-PCR analysis of the OsRboh family responding to flooding in the absence of the antioxidants. b The relative contents of OsRbohA and OsRbohB with the prolongation of 0.1 mg/mL of NFA or CheA treatment under normal growth. c The relative contents of OsRbohB in the presence of antioxidants, before or after imposed flooding of rice for 4 days. Data are expressed as the means ± SD, and n = 3. The * and # denote, respectively, comparisons with the before flooding group and the after flooding group. d H$_2$O$_2$ content under the treatment of antioxidants, before or after imposed flooding of rice.
The OsRboh family plays an important role in rice against flooding, but it remains unclear which genes are responsible (Wu and Yang 2016). In our study, OsRbohD and OsRbohE might be also involved in flooding tolerance (Fig. 5a), however, OsRbohB is the key NADPH oxidase to rice regulated by natural antioxidants, which could counteract the changed expression level caused by flooding stress (Shi et al. 2020). However, we found a clear contradiction in the role of natural antioxidants in regulating the ROS content: on the one hand, NFA and CheA can reduce the content of ROS as an antioxidant, but on the other hand they can also improve the activity of NADPH oxidase in vivo to augment the ROS content in rice. The reason for this may lie in the dual functioning of ROS: inducing ROS to initiate a stress response, yet eliminating excessive ROS to improve the plant’s ability to endure flooding.

As the M. laxiflora plant is a critically endangered species, the aseptic seedlings without endophytic fungi are difficult to obtain, so the effect of natural antioxidants on the flooding tolerance trait of native hosts needs confirmation. In addition, to further reveal the molecular mechanisms by which NFA and CheA improve flooding tolerance in plants, future research could focus on these aspects: (1) the role of the NADPH oxidase family and the instantaneous quantification of ROS in various forms, for example, as fluorescently labeled ROS rather than H$_2$O$_2$; (2) using NADPH oxidase inhibitors and mutants to analyze key pathways related flooding tolerance; (3) the function of other substances isolated from the fungi in plants’ flooding tolerance, by integrating natural product chemistry, microbiology, and botany-based chemo-ecology.

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**Author contributions** XY performed most of the experiments and wrote the manuscript under the supervision of LS. LC elucidated the structure of the substances, and BX analyzed the data. CF completed the physiological experiments with the technical assistance provided by CJ. All authors read and approved the final manuscript.

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**Availability of data and material** The authors confirm that data supporting the findings of this study are available within the manuscript and Online Resource.

**Declarations**

**Conflict of interest** The authors declare no conflicts of interest.

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