

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 endophytes 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 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 inhibited the production of factors such as malondialdehyde, superoxide dismutase, ethanol dehydrogenase, and NADPH oxidase that are associated with oxidative stress induced by flooding. 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 through the antioxidants of the fungal endophytes.

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 (Voeselek & 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, 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 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 ecological adaptability of their hosts and play a key role in the response of plants to adversity (Weyens et al. 2009; 2010; Khiralla et al. 2016). 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. 2018).

In an earlier study, hundreds of endophytic fungi were obtained from plants of *Myricaria 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.

Methods And Materials

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 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- μ L volume reaction that contained 2 μ L of DNA template, 1 μ L of each primer, 2 μ L of PCR buffer, 2 μ L of dNTPs, and 0.2 μ L 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 endophytic 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 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, a fungal solution of SG-17 or QY-1 was injected into 6-day-old rice leaves; 1 day later, the seedlings were submerged as describe the 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- and after-sonication were sprayed onto the rice seedlings. 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. Chlorophyll relative content was determined with a SPAD-502 meter (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 endophytic 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 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 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 × 10 mm id, Cosmosil MS-II, Nacalai Tesque, Tokyo, Japan). 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 TMS 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. Their physiological indicators were determined during a 4-day recovery after flooding for 4 days. Each treatment was repeated three times and 20 seedlings were investigated. The growth conditions used were the same as described above.

Expression analysis of *OsRboh*s

After the indicated periods of imposed flooding, at least four leaves 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 H₂O₂ in rice

The H₂O₂ content was measured by applying a method previously described (Avramova *et al.*, 2015). Briefly, 0.1 g of rice leaves was homogenized in 1-mL 5% (w/v) trichloroacetic acid, then centrifuged at 14 000 *g* for 30 min. The ensuing supernatant was incubated with Xylenol 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), and the corresponding SPAD values showed that they had a significantly higher relative content of chlorophyll than the control group. These results indicated that both riparian fungi might promote the ability of rice seedlings to tolerate flooding stress. 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. These results showed that SG-17 and QY-1 clearly improved the growth of rice under the experimentally imposed flooded conditions (Figure 1A).

The fungus SG-17 was isolated from the roots of *M. laxiflora*. Its colonies 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 (Figure 1B, C). BLAST analysis revealed that the 18S ITS sequence of SG-17 shared a 99% identity with *A. fumigatus* (Figure 1D).

The other fungus, QY-1, came from the leaves of *M. laxiflora*. Its colonies 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 (Figure 1E, F). The nearly spherical ascomycetes were scattered. The ITS sequence for QY-1 shared a 100% identity with *C. globosum* (Figure 1G).

Therefore, based on their morphology and 18S ITS sequences (GenBank accession numbers: KU954091 and MK450298), the strains SG-17 and QY-1 were respectively identified as *A. fumigatus* and *C. globosum*, and deposited in China Center for Type Culture Collection (respectively, as CCTCCM 2015286 and CCTCCM2014182).

Metabolites of endophytes 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. 2018). 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), a result corroborated by the inoculation of the fungus (Supplementary Figure 1). These results revealed that SG-17 might be a potential pathogen of rice, and they also implied that the endophytes were unlikely to function through living elicitors. So, 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 increasing the ability of rice to withstand the impact of flooding (Figure 2A).

Through the HPLC purifications, the corresponding structures were elucidated by NMR spectroscopy (Figure 2B, C). In SG-17, the putative active compound was suggested to be $C_9H_9NO_2$, on the basis of ESI-MS at m/z 186.05 $[M+Na]^+$. The ^{13}C -NMR spectrum indicated one carbonyl carbon at δ_C 160.4 (-CHO), and eight olefinic carbons at δ_C 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 δ_H 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-hydroxystyryl) formamide (NFA).

Using the same approach, the molecular formula of the active substance in QY-1 was determined (Figure 2D, E) as $C_{32}H_{36}N_2O_5$, by analysis of ESI-MS at m/z 551.25 $[M+Na]^+$. The ^{13}C 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 δ_H 0.96 (d, J = 6.6 Hz, H-11), 1.11 (s, H-12), 0.67 (d, J = 7.2 Hz, 16- CH_3), and 1.36 (s, 18- CH_3), 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).

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, Figure 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 μ g/mL (Figure 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 varies significantly with the degree of oxidative stress (Qian et al. 2015). In most cases, the contents or activities of these indexes would first rise then fall with prolonged inundation of plants, as found for MDA measured in our study (Supplementary Figure 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 (Figure 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 indexes 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 (Figure 4B), nearly attaining the level for normal growth. Similar results were also obtained for the NADPH oxidase (Figure 4D), indicating the function of natural metabolites from endophytes are closely linked to mitigating flooding stress in plants.

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 for *OsRbohB* showed that it was obviously induced by flooding in its early stage, but diminished with longer flooding (Figure 5A); this suggested *OsRbohB* could be involved in the metabolism of rice plants exposed to flooding stress (Figure 5A). This presumption was also supported by the fact that *OsRbohB* was the most homologous orthologue to *AtRbohD*, the latter known to play a role in how *Arabidopsis* endures flooding (Kwak et al. 2003; Kadota et al. 2015). Under normal growth, the natural metabolites NFA and CheA strongly induced the expression of *OsRbohB* in rice after a 4-hour treatment, while *OsRbohA* lacked this response (Figure 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 (Figure 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 (Figure 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. Here, we reported on the novel 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 (Figure 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, and the natural compounds present in the fungus might be hopeful in agricultural production.

Both NFA and CheA were earlier considered to be selective antibiotics (Anzai et al. 1962; Sekita et al. 1983), so 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 indexes 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.

OsRbohB may be the key NADPH oxidase involved in conferring flooding tolerance to rice mediated by natural antioxidants, which could counteract the changed expression level caused by flooding stress. 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 stress response, yet eliminating excessive ROS to improve the plant's ability to endure flooding.

Given the difficulty in obtaining aseptic seedlings without endophytic fungi, we could not confirm whether the natural antioxidants found here improve the flooding tolerance of *M. laxiflora*, the endophytic fungi SG-17's natural host. More importantly, although our study uncovered two substances that promoted the rice tolerance of adverse flooding effects via the activity-tracking method, we cannot be sure whether there were other functionally active substances in the endophytes. 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 effects of natural antioxidants on the flooding tolerance trait of native hosts; (2) The role of the NADPH oxidase family and the instantaneous quantification of ROS in various forms, for example, as fluorescently labeled ROS rather than H₂O₂; (3) Using NADPH oxidase inhibitors and mutants

to analyze key pathways related flooding tolerance; (4) The function of other substances isolated from the fungi in plants' flooding tolerance, by integrating natural product chemistry, microbiology, and botany-based chemo-ecology.

Declarations

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Author contributions Xue Yanhong performed most of the experiments and wrote the manuscript under the supervision of Liu Shiping. Liu chengxiong elucidated the structure of the substances, and Bai Xiaoxuan analyzed the data. Cheng fan completed the physiological experiments with the technical assistance provided by Chen Jianfeng. 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.

Compliance with ethical standards

Conflict of Interest The authors declare no conflicts of interest.

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Table

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	<i>Chaetomium globosum</i>	12.2±3.9	30.8±4.4**	18.7±2.3	22.4±3.1**
SG-17	MK450298	<i>Aspergillus fumigatus</i>	6.3±2.5	27.3±3.7**	10.1±3.5	23.8±2.4**
No fungi	–	–	8.77±2.4		14.2±3.8	
No flooding	–	–	100±1.1		36.7±1.5	

Notes:^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 10 seedlings that experienced 4 days of flooding and followed by a recovery period (another 4 days).

Figures

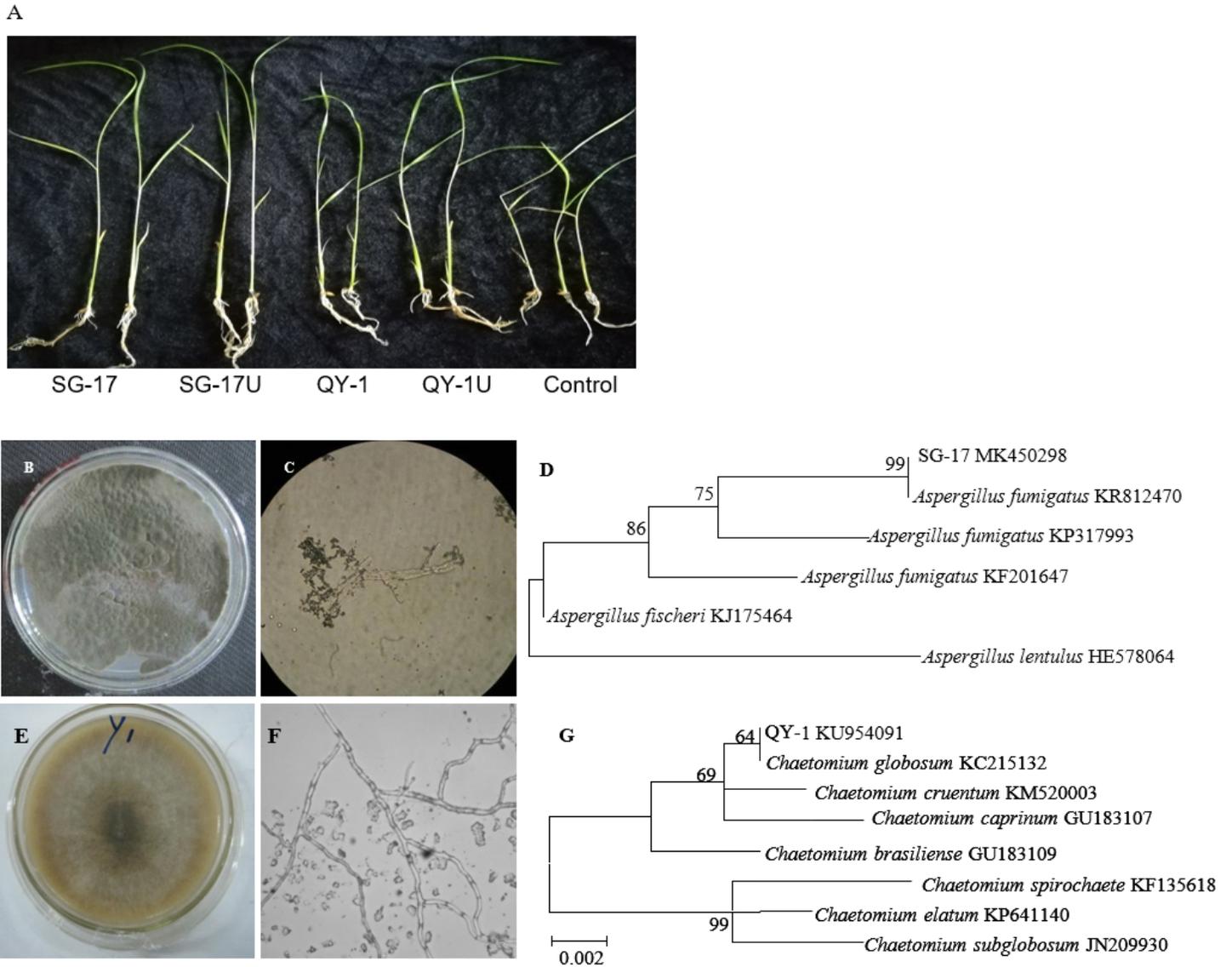


Figure 1

Preliminary screening for fungi and their taxonomic identification. (A) Rice seedlings were treated by two living fungi (SG-17 and QY-1) and their fermentation broth after ultra-sonication (SG-17U and QY-1U). All seedlings were fully flooded for 4 days, and then allowed to resume normal growth for another 4 days. (B–D) The SG-17's colony, micromorphology, and phylogenetic tree based on the ITS sequence and applying the neighbor-joining (NJ) method. Numbers above the branches are the bootstrap values. (E–G) Taxonomic identification of QY-1.

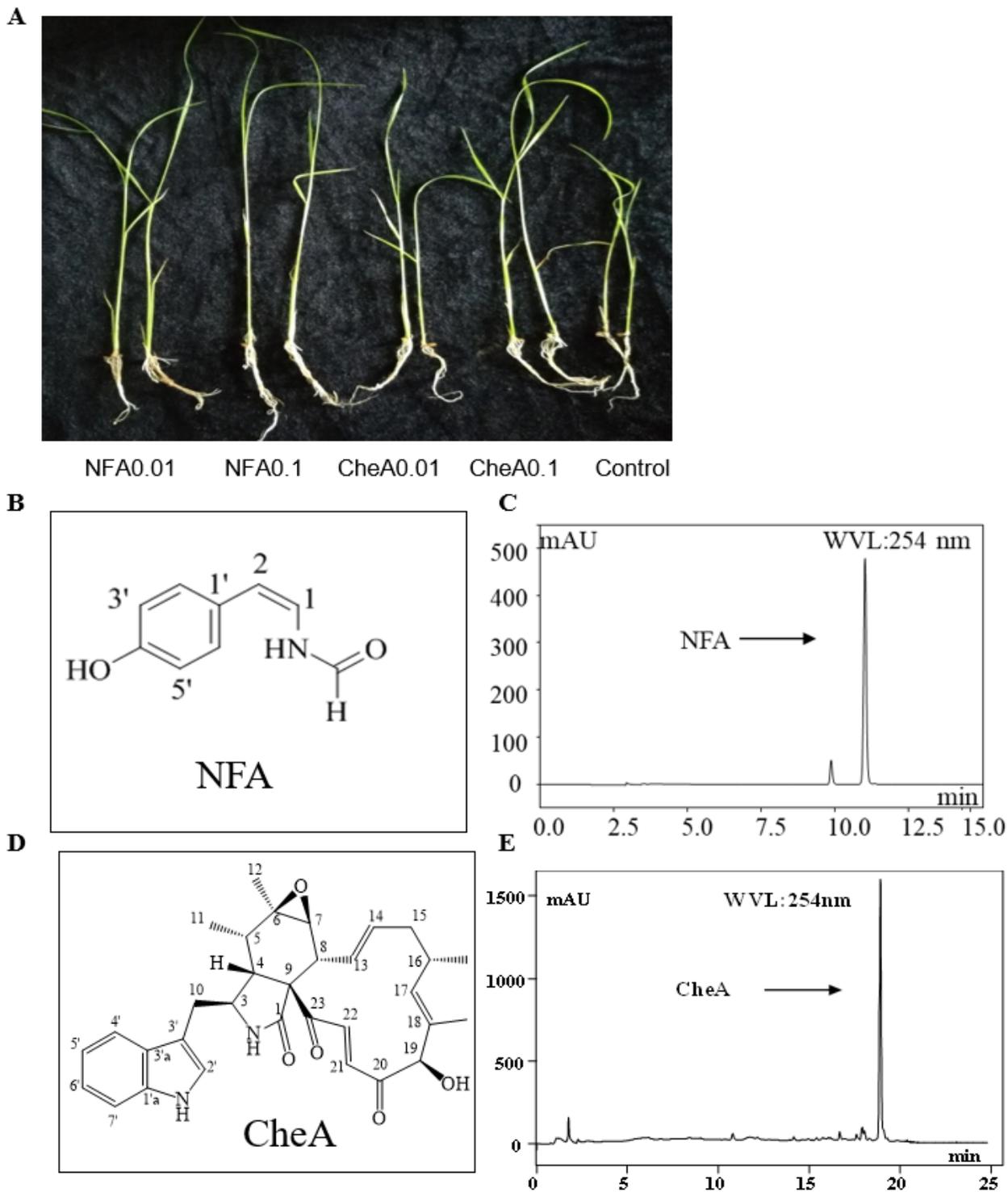


Figure 2

Active compounds in SG-17 and QY-1. (A) Beneficial effects of different substances and concentrations on the flooding tolerance in rice. (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.

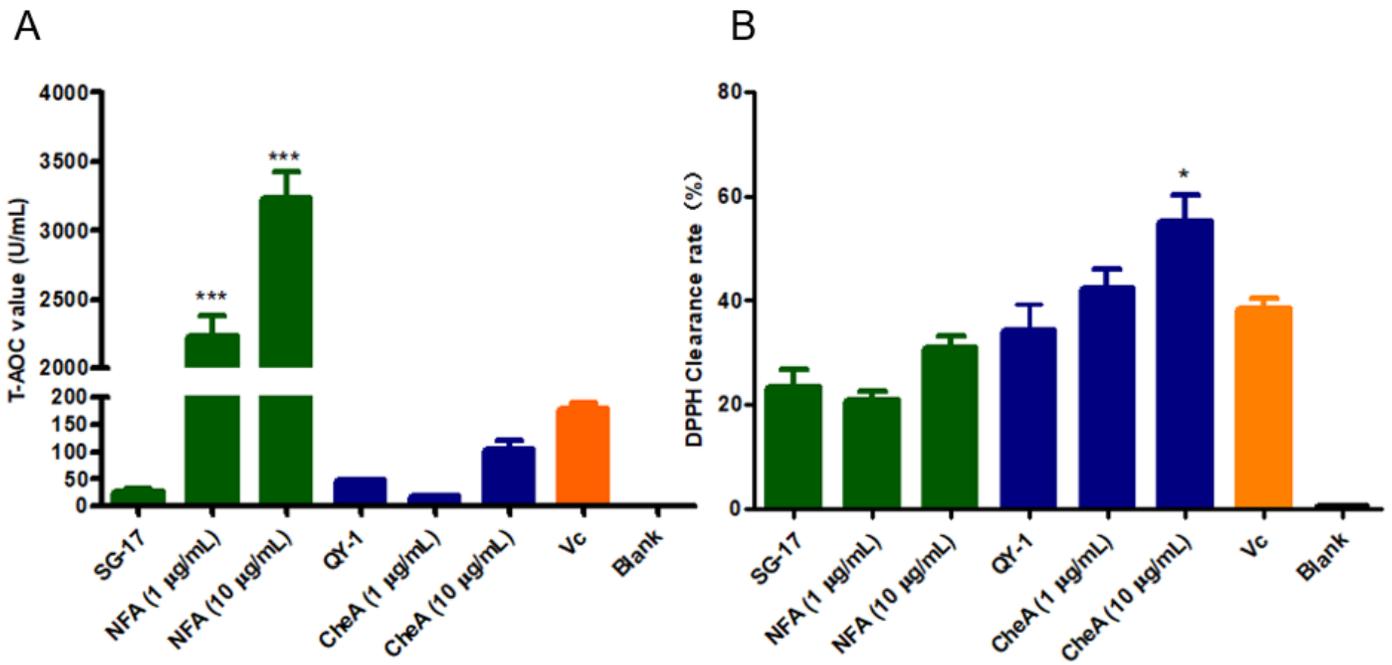


Figure 3

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.

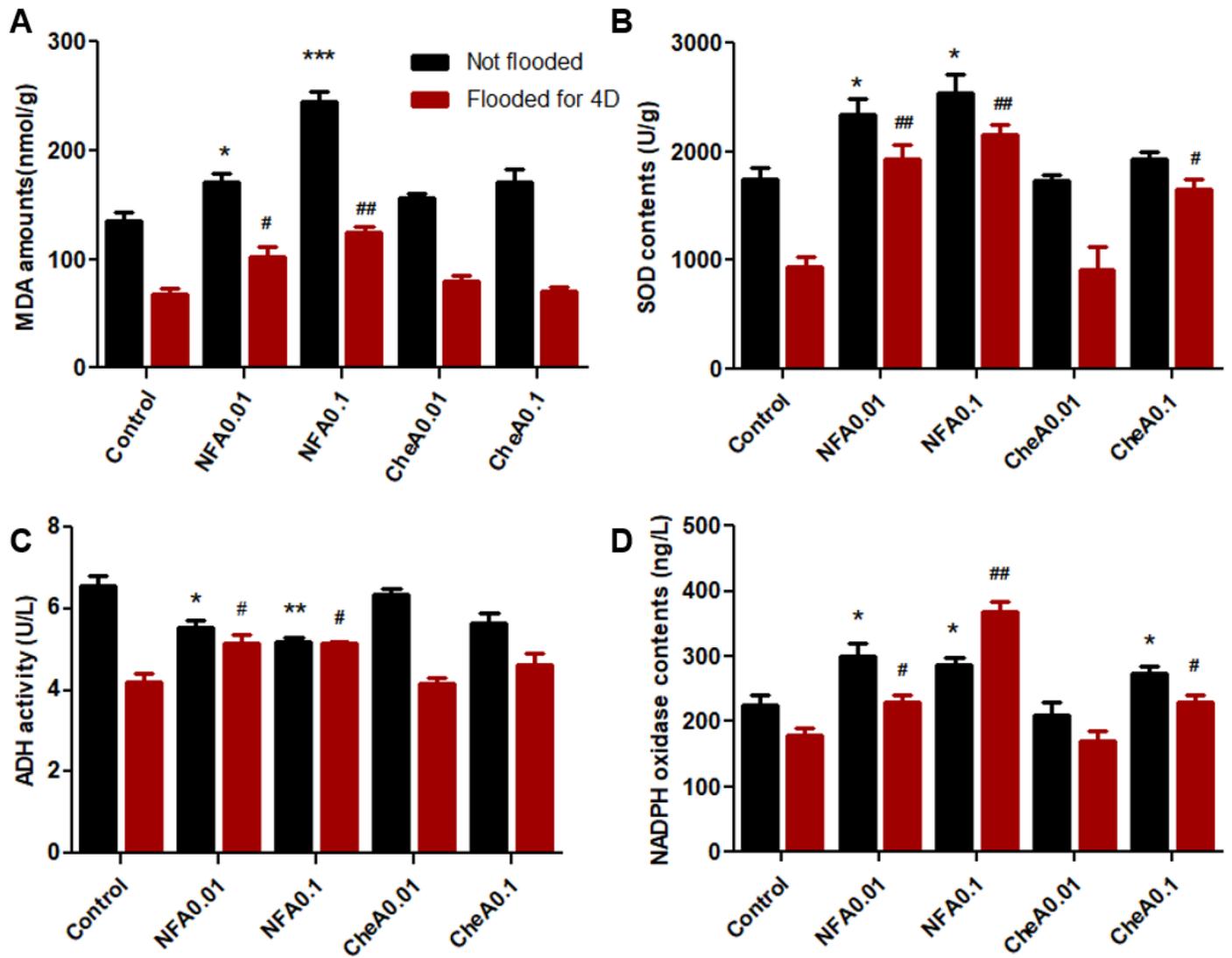


Figure 4

Physiological responses of flooded rice seedlings to NFA and CheA. (A–D) corresponds to the content or activity of MDA, SOD, ADH, and NADPH oxidase. Data are expressed as the means \pm SD, and $n = 20$. The *, ** and *** respectively represent significant differences at $P < 0.05$, < 0.01 , and < 0.001 when compared with the non-flooded control group. The # and ## indicate a significant difference at $P < 0.05$ and < 0.01 , respectively, when compared with the flooded control group.

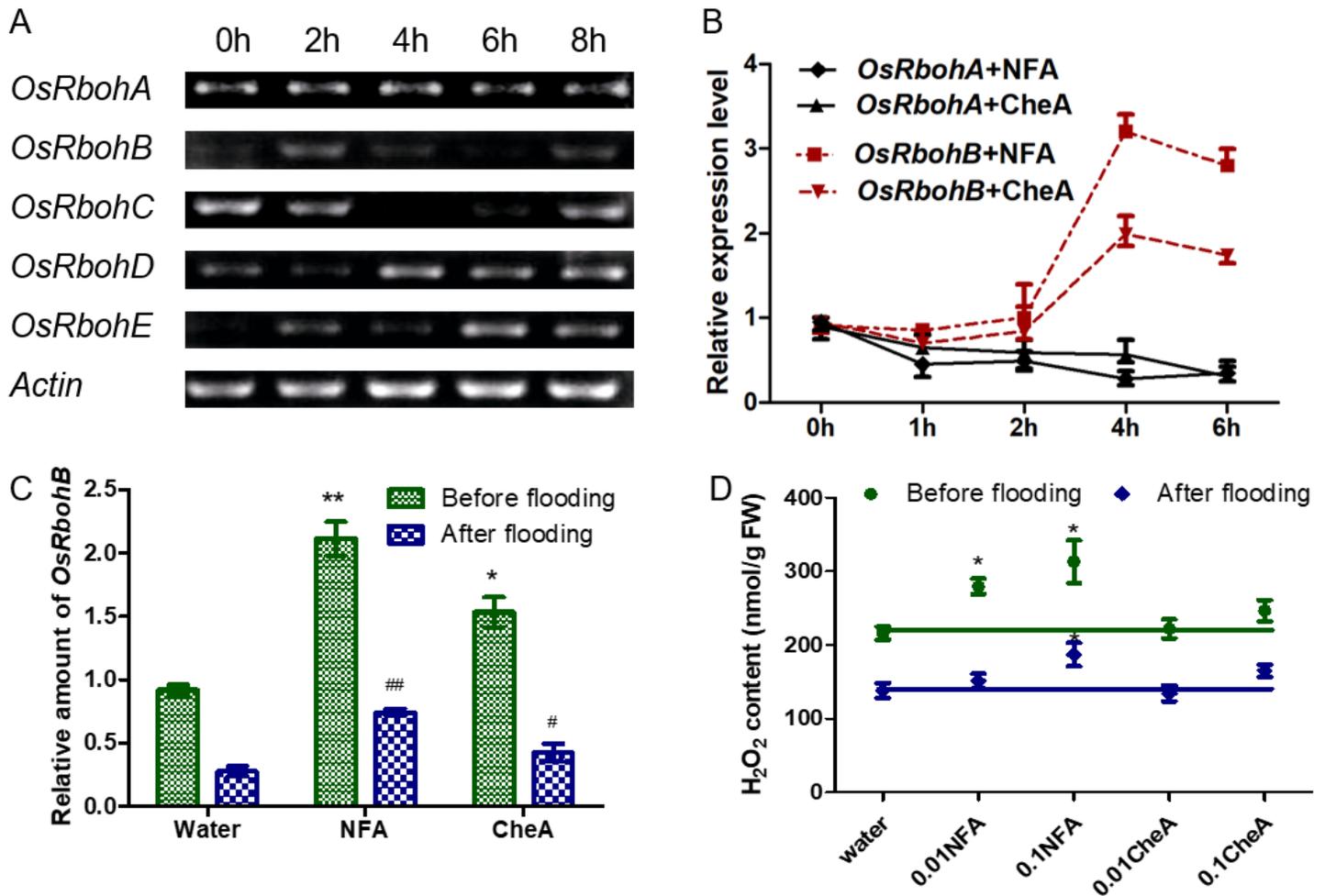


Figure 5

Effects of the two endophyte-derived antioxidants on the expression levels of *OsRboh* genes and H_2O_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 4d. Data are expressed as the means \pm SD, and $n = 3$. The * and # denote respectively comparisons with the before flooding group and the after flooding group. (D) H_2O_2 content under the treatment of antioxidants, before or after imposed flooding of rice.