

Identification, characterization, and expression of *Oryza sativa* betaine aldehyde dehydrogenase genes associated with the metabolism of oxyfluorfen

Zhao Jie Chen

zhaojie-chen@gxu.edu.cn

Guangxi University

Yi Zhuo Wang

Guangxi University

Jun Jin Lu

Guangxi University

Xu Zhen Shi

Guangxi University

Ya Nan Qu

Guangxi University

Gan Ai

Nanjing Agricultural University

Li Qing Zeng

Guangxi University

Xiao Lu Wang

Guangxi University

Xi Ran Cheng

Guangxi University

Yan Hui Wang

Guangxi Academy of Agricultural Sciences

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Abstract

Betaine aldehyde dehydrogenase (BADH), a member of family 10 of the aldehyde dehydrogenase superfamily, catalyzes the second oxidation step in the biosynthesis of glycine betaine (GB), which participates in a variety of critical processes that help plants tolerate abiotic stress. Nevertheless, it is still unclear how BADH functions in rice under pesticide stress. To look at the roles that the rice BADH family plays when under pesticide stress, three BADH genes were identified in transcriptome datasets of GB + oxyfluorfen (OFF)-treated rice. Using sequence alignment and phylogenetic analysis, the two subfamilies of the BADH gene family (ALDH10 and ALDH22) among rice, Arabidopsis, soybean, wheat, maize, barley, and sorghum were found. An examination of chromosomal position revealed that segmental duplication had a role in the expansion of OsBADH genes, and that the rice BADH genes were irregularly distributed on 3 of the 12 rice chromosomes. In collinearity analyses, rice BADH genes exhibited collinearity with those of wheat, maize, barley, and sorghum. The genes also showed a variety of conserved domains, cis-elements, motif compositions, and gene architectures that made it possible for them to encode different biotic and abiotic stress response proteins. Compared to the effects of OFF alone, BADH activity in rice roots and shoots increased 1.50-fold and 1.81-fold, respectively, following treatment with GB and 0.15 mg/L OFF. Analysis of protein–protein interaction networks provided more evidence for the involvement of OsBADH proteins in OFF metabolism. Overall, these findings demonstrate that BADH genes respond effectively to OFF-induced stress by producing GB, highlighting their potential roles in regulating pesticide degradation.

Introduction

Pesticides are essential to agricultural procedures and for improving crop yields (Yu et al. 2023). They are used to increase agricultural yields while protecting plants from rot and other harms. However, pesticides pollute the environment when they spread beyond their intended plant targets. Certain pesticides have the potential to persist in soil and water, accumulate in different environmental media, and harm the ecosystem (including humans, animals, and crops) (Kaur et al. 2024).

Because they grow in a sessile manner, plants are the living organisms most often exposed to an ever-changing environment. Plants have developed various defense mechanisms to withstand diverse environmental conditions, including drought, salinity, heavy metal exposure, and pesticide contamination (Ahmad et al. 2013). Plants have evolved various mechanisms to avoid pesticide-mediated toxicity, such as the synthesis of secondary metabolites (glycine betaine [GB], phenols, and polyamines) and hormones (salicylic acid, brassinolide, and jasmonic acid), which enhance their ability to resist pesticide stress (Godoy et al. 2021; Chen et al. 2022a; Waadt et al. 2022). For example, an increase in salicylic acid content in crops can enhance the activity of antioxidant and detoxifying enzymes such as catalase (CAT), peroxidase (POD), P450, and glutathione S-transferase (GST), promoting the degradation and metabolism of isoproturon and propazine (Zhang et al. 2018; Lu et al. 2020b). An increase in jasmonic acid content in crops also promotes the activity of CAT, POD, ascorbate peroxidase (APX), and GST, reducing isoproturon-induced toxicity (Ma et al. 2018). Under environmental stressors such as dryness,

low temperature, and salt stress, the nontoxic organic molecule GB acts as an osmotic regulator (Yang et al. 2015). Numerous organisms, including bacteria, cyanobacteria, algae, higher plants, and mammals, produce GB. In GB synthesis, choline monooxygenase converts serine to choline, and choline dehydrogenase converts choline to betaine aldehyde, which is converted to GB via oxidation by choline oxidase or betaine aldehyde dehydrogenase (BADH). It was recently discovered that GB is essential for preserving cell structure because it controls metabolism in the cell microenvironment and scavenges reactive oxygen species (ROS) in response to various abiotic stressors (Ahmad et al. 2013). For example, under salt stress conditions, the application of exogenous GB significantly increases the activity of antioxidant stress enzymes involved in the ascorbate–glutathione cycle (APX, monodehydroascorbic acid reductase, dehydroascorbic acid reductase, and glutathione reductase [GR]) in tobacco plants, reducing ROS accumulation and protecting tobacco cells from salt-induced oxidative stress damage (Ahmad et al. 2013; Chen et al. 2022a; Chen et al. 2024a). Wutipraditkul et al. (2015) found that exogenous betaine alleviated salt- and drought-induced damage in rice seedlings by enhancing the activity of antioxidant stress enzymes such as superoxide dismutase (SOD), GR, and APX. However, no studies have demonstrated whether exogenous GB can alleviate pesticide-induced oxidative stress and promote pesticide degradation in rice. Additionally, regulating GB synthesis genes can effectively increase plant resistance to abiotic stress. For instance, Yang et al. (2015) successfully enhanced *Arabidopsis* tolerance to drought or osmotic stress by overexpressing the SpBADH gene, which increased proline content and enhanced the activity of antioxidant enzymes. Missihoun et al. (2015) increased the content of secondary metabolites such as GB, organic acids, and amino acids in *Arabidopsis* by overexpressing the aldehyde dehydrogenase genes ALDH10A8 and ALDH10A9, which significantly enhanced plants' ability to tolerate salt stress. Khan et al. (2016) found that overexpression of *codA* significantly increased the accumulation of GB in tomatoes while increasing their cold resistance and yield. However, there are no reports on the mechanism by which regulating GB synthesis can promote the detoxification and metabolism of pesticides in rice.

Initially categorized as substrate-specific oxidoreductases, betaine aldehyde dehydrogenases (BADHs) belong to family 10 of the aldehyde dehydrogenase superfamily (Fitzgerald et al. 2009). In species in which GB accumulates as a compatible solute under stress, BADHs oxidize betaine aldehyde to GB (Missihoun et al. 2015). The two BADH gene homologs expressed in rice are OsBADH1 and OsBADH2 (Golestan Hashemi et al. 2018). Numerous environmental conditions, including salinity, drought, cold, heat, intense light, and high CO₂ concentrations, have been revealed to trigger OsBADH1 expression in rice (Tang et al. 2014; Golestan Hashemi et al. 2018). Additionally, the OsBADH1 gene was transfected into tobacco to increase its abiotic stress tolerance and produce transgenic lines with substantially higher GB levels compared to nontransformed controls (Hasthanasombut et al. 2010). The japonica variety Nipponbare (*Oryza sativa* ssp. *japonica*) exhibited enhanced GB content and abiotic stress tolerance upon transfer of the OsBADH1 gene from indica rice (*O. sativa* ssp. *indica*) (Hasthanasombut et al. 2011). These data suggest that GB accumulation due to OsBADH1 overexpression was responsible for the increased resistance to abiotic stress. Therefore, we speculate that OsBADH can also alleviate pesticide-induced stress in plants by increasing GB synthesis.

Oxyfluorfen (OFF), a selective diphenyl ether herbicide, given its exceptional capacity to eradicate weeds, is mostly used to fields of maize, rice, and soybeans in order to manage broad-leaved weeds and sedges (Li et al. 2022). Through the accumulation of protoporphyrinogen IX, OFF induces lipid peroxidation and inhibits weeds' ability to synthesize chlorophyll (Pham et al. 2015; Phung and Jung 2015). Because of its poor biodegradability (half-life of 72–150 days in soil), OFF persists in soil and aquatic systems, resulting in pollution (Zhao et al. 2016). In rice crop areas, Sondhia (2009) states that OFF is sprayed at 240–500 g ai ha⁻¹. OFF accumulation in the ecosystem is detrimental to animal growth, including inhibition of earthworm reproduction and thyroid damage in fish and rats (Tejada et al. 2016; Abd El-Rahman et al. 2019; De Vasconcelos et al. 2019; Buckalew et al. 2020; Li et al. 2022; Chen et al. 2023a). Moreover, OFF can cause cranial fractures in infants or transverse limb abnormalities (Yang et al. 2014). Therefore, OFF has been classified as a possible human carcinogen by the US Environmental Protection Agency (Carmichael et al. 2016). Thus, the consumption of OFF-contaminated food or water could pose a risk to human health. To reduce the risk of pesticide accumulation to human health, it is necessary to clarify the mechanism by which GB synthesis genes respond to and participate in the metabolism and degradation of pesticides in plants.

At present, little is known about the responses of rice BADH genes to OFF stress and the involvement of BADH genes in pesticide metabolism in rice plants. In response to OFF exposure, we screened the genome-wide transcripts of BADH genes in order to look into the potential molecular and metabolic roles of BADHs. Comprehensive analyses were also performed on these genes' chromosomal locations, collinearity, structures, cis-elements, motif compositions, and conserved domains. Furthermore, to validate the BADH genes' reactions to OFF, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was employed. We further analyzed the overall BADH activity levels in rice plants exposed to OFF. The present study's goals were achieved through the development of an effective screening method for OFF-responsive rice BADH genes. This technique has the potential to alter toxicological reactions and OFF resistance in crops and environments. Additionally, the data supports the notion that GB synthesis genes regulate the degradation and metabolism of residual pesticides in crops.

Materials and Methods

2.1. Preparation of plant materials

After sterilization with 3% H₂O₂, wild-type rice seeds (*O. sativa* L. *japonica*, cv. Nipponbare) were germinated for 3 days at 30°C. The germinated rice seeds were grown in 50% Hoagland nutrient solution and cultured in a constant temperature incubator for 10 days with a day/night temperature of 30°C/25°C, 14-h/10-h light/dark period, light intensity of 200 μmol m⁻² s⁻¹, and relative humidity of 75% (Chen et al. 2021a). Then, the rice seedlings were transferred to fresh nutrient solution containing OFF at various concentrations and then analyzed via RNA sequencing (RNA-Seq), total BADH activity determination, and qRT-PCR. The nutrient solution was changed every 2 days. Each treatment was repeated thrice.

2.2. Construction of transcriptome libraries and RNA-Seq

Ten-day-old rice seedlings were exposed to 0.15 mg/L OFF with or without 175 mg/L GB for 2, 4, or 6 days, and the rice roots and shoots were sampled separately to analyze the genome-wide transcriptional expression of rice BADH genes during OFF exposure. Rice samples were collected at three time points, mixed together, and ground for quantitative analysis. This procedure established six rice seedling root and shoot libraries representing the control and treatment groups (0.15/mg L OFF), namely Shoot – OFF (control), Root – OFF (control), Shoot + OFF (with OFF only), Root + OFF (with OFF only), Root + OFF + GB (with OFF and GB), and Shoot + OFF + GB (with OFF and GB). Trizol was used to separate the total RNA from a sample taken from each library. DNase I was used to extract DNA (Takara, Shiga, Japan). Three biological replicates (3 × 6) of each library's RNA were sequenced using an Illumina HiSeq 2500 unit (San Diego, CA, USA). Clean bases and reads were mapped to the rice genome following the removal of poor-quality bases (<http://rice.plantbiology.msu.edu/index.shtml>) (Chen et al. 2021a, b). There were three biological replicates for each treatment.

2.3. Assays of BADH activity

Enzyme-linked immunosorbent assay (ELISA) method was carried out for the measurement of total BADH activity. Following 6 days of exposure of 0–0.23 mg/L OFF with or without 175 mg/L GB, the roots and shoots of rice seedlings (0.5 g) were separated and ground in 3 mL of ice-cold Hepes-KOH buffer solution (50 mM, pH 8.0) containing 1 mM of ethylenediaminetetraacetic acid (EDTA) and 5 mM of dithiothreitol (DTT). The mixture was centrifuged at 9000 ×g and 4°C for 10 min. The liquid supernatant was quantified using specific detection kits (MEIMIAN, Jiangsu, China). The kit's dilution buffer was used to dilute the samples five times and the 50 µl standards in equal amounts, which were then put to the 96-well ELISA plate's wells. The plate was covered with a film and incubated at 37°C for 30 minutes after being gently shaken to thoroughly mix. The plate was then cleaned five times using washing buffer, taking 30 s each time. Then, 50 µl HRP-Conjugate was added to each wall. After closing plate with Closure plate membrane, the plate was incubated for 30 min at 37°C and washed for 5 times. Then, chromaton solutions A and B were progressively added to the wells and mixed before being incubated for 10 min at 37°C in the dark. Using 50 µl of stop solution, the reaction was halted prior to the microplate reader's optical density at 450 nm measurements.

2.4. qRT-PCR analysis of BADH genes in rice

After treating 10-day-old rice seedlings with 0, 0.07, 0.11, 0.15, 0.19, or 0.23 mg/L OFF with or without 175 mg/L GB, rice root and shoot samples were collected at 2, 4, and 6 days. For the quantitative study, all root or shoot samples from various time points were merged. Following Chen et al.'s instructions (2023a), RNA was isolated from rice tissue. The reaction was started using the primers specified in Table S1, with 40 cycles of annealing (95°C for 5 s) and extension (60°C for 30 s) followed by one cycle of denaturation at 95°C for 30 s. Relative gene expression was analyzed using the $2^{-\Delta\Delta C_t}$ method.

2.5. Chromosome distribution and interspecific collinearity analysis of rice BADH genes

We downloaded the genome annotation files of rice, *Arabidopsis*, soybean, wheat, barley, maize, and sorghum from Ensembl Plants (<https://plants.ensembl.org/index.html>) (Bolser et al. 2016). Three rice BADH protein and nucleic acid sequences were downloaded from the China Rice Data Center (<https://www.ricedata.cn/gene/>). A program called TBtools- (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was utilized to plot the three rice BADH genes' chromosomal distribution (Chen et al. 2020). The collinearity of BADH genes between rice and *Arabidopsis*, soybean, wheat, barley, maize, and sorghum was analyzed and visualized using TBtools-.

2.6. Phylogenetic tree analysis of rice BADH genes

Three rice BADH genes sequences were acquired from the Rice Genome Annotation Project database (<https://rice.uga.edu/>) and employed as query sequences. The rice BADH gene sequences having a sequence similarity of $\geq 40\%$ were compared to protein sequences in *Arabidopsis*, soybean, wheat, maize, and sorghum using BLAST (<https://blast.ncbi.nlm.nih.gov/>). The phylogenetic tree, which may be used to understand how various species have evolved together, was finally constructed in Molecular Evolutionary Genetics Analysis (MEGA) 7.0 using the neighbor-joining method with Poisson correction model parameters (Kumar et al. 2016). Subsequently, EvolView (<https://evolgenius.info/evolview-v2/#login>) was used to visualize the phylogenetic tree (Chen et al. 2024b).

2.7. Cis-acting promoter elements and domain structure analysis of rice BADH genes

TBtools- was used to extract the first 2000-bp sequences of the three rice BADH genes, and PlantCARE (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to predict the cis-acting elements (Lescot et al. 2002). Multiple Expectation maximizations for Motif Elicitation (MEME, <https://meme-suite.org/meme/doc/meme.html>) were used to examine conservative motifs, with a maximum motif number of 6 (Bailey et al. 2006). The domain structure of each of the three rice BADH genes was ascertained using the NCBI CD-search Tool (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) (Lu et al. 2020a). The results were exported using TBtools.

2.8. Construction of a network of protein–protein interactions among BADH genes

The STRING database (<https://string-db.org/>) was used to create the rice BADH protein interaction network and visualized using Cytoscape (<https://cytoscape.org/>) (Chen et al. 2024b).

2.9. Protein structure and molecular docking analyses

The ExPasy program was utilized to predict the biophysical properties of proteins, including the amount of amino acids, molecular weight (MW), theoretical isoelectric point (PI), instability index, and grand average of hydropathicity (<https://web.expasy.org/protparam/>) (Duvaud et al. 2021). The secondary structure of proteins were carried out using the self-optimized prediction method with alignment (SOPMA; https://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). (Fuke et al. 2018). The SWISS-MODEL workspace (<https://swissmodel.expasy.org/interactive>) was used to predict the tertiary structure of three BADH proteins (Waterhouse et al. 2018). For molecular docking, we selected three OFF-induced rice BADH genes and constructed the corresponding proteins by eliminating H₂O molecules and heteroatoms. The 3D structure file of OFF was obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) (Kim and Bolton 2024), and the format was converted using Open Babel (O'Boyle et al. 2011). The docking model's cutoff was a binding affinity of -7 kcal mol^{-1} , and molecular docking was conducted using AutoDock Vina 1.2.5 with exhaustiveness set to 24. In the docking results, low binding energies and good conformations were selected. The interaction between residues and the ligand was visualized using Pymol 2.6.0 Open-Source, and 2D interaction pictures were generated using Discovery Studio 2019 (Chen et al. 2024b).

2.10. Statistical analysis

For every experiment, three independent replicates ($n = 3$) were used to compile all of the study's results. The statistical significance of the data was assessed using standard deviation and analysis of variance ($p < 0.05$). All analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA).

Results

3.1. Effect of exogenous GB on BADH gene expression in rice tissues under OFF stress

Using RNA-Seq, we identified GB + OFF-responsive BADH genes in rice, with the following criteria: $\log_2$ fold change > 1 and false discovery rate < 0.001 . Based on the results of pairwise comparisons of Root + OFF vs. Root + OFF + GB and Shoot + OFF vs. Shoot + OFF + GB, we identified three BADH genes (Table S2) that responded to OFF + GB treatment. In general, genes perform a specific function based on their expression in an organism, and the expression patterns of genes reflect their functions (Zhou et al. 2018). We examined the expression levels of OsBADH genes in various tissues under various treatments in order to investigate the function of OsBADH genes (different OFF concentrations [0–0.23 mg/L] with or without 175 mg/L GB; Fig. 1). The findings showed that, in contrast to the control, OsBADH genes were expressed in all tissues under various treatments, and that the expression levels of these genes differed between tissues and treatments. Compared to the control, Os4g0464200, Os07g0688800 and Os08g0424500 were highly expressed in roots (1.68-fold, 1.67-fold and 2.13- fold, respectively) under 0.15 mg/L OFF treatment for 6 days (Fig. 1). GB treatment further promoted the expression of these three OsBADH genes. In shoots, the expression of Os04g0464200 peaked after 6 days of treatment with GB + 0.15 mg/L OFF, being 1.49-fold higher than that induced by 0.15 mg/L OFF alone. In roots, the peak

expression levels of Os07g0688800 and Os08g0424500 were observed with GB + 0.15 mg/L OFF treatment (1.24- and 1.04-fold, respectively) compared to that with 0.15 mg/L OFF treatment (Fig. 1). These findings demonstrate that GB treatment in rice was associated with increases in BADH gene expression, which might be related to secondary metabolism and xenobiotic detoxification (Hasthanasombut et al. 2011; Tang et al. 2014; Golestan Hashemi et al. 2018).

3.2. Exogenous application of GB enhances the activity of BADH proteins in rice tissues under OFF stress

BADHs are essential for plants' abilities to resist xenobiotics (Hasthanasombut et al. 2011; Tang et al. 2014; Golestan Hashemi et al. 2018). To analyze the roles of BADHs under OFF stress, total BADH activity was measured in rice different tissues following various treatments (different OFF concentrations [0–0.23 mg/L] with or without 175 mg/L GB) for 6 days (Fig. 2). The results showed that OsBADH activity was enhanced in all tissues under different treatments compared to the control, and OsBADH activity varied in different tissues and under different treatments. Compared to the control, BADH activity in rice roots and shoots treated with OFF for 6 days was the highest for the 0.15 mg/L concentration (1.34- and 1.51-fold, respectively) (Fig. 2). Similar to OsBADH gene expression, GB exposure further promoted BADH activity in rice tissues. BADH activity in rice roots and shoots peaked after 6 days of GB + 0.15 mg/L OFF treatment (1.50- and 1.81-fold increases, respectively) compared to the effects of 0.15 mg/L OFF alone. The further enhancement of BADH activity under exogenous GB exposure further confirmed its involvement in responses to xenobiotic stress.

3.3. Chromosomal location and interspecies collinearity of rice BADH genes

To understand the pattern of OsBADH distribution in the rice genome, we used TBtools to construct chromosome location maps for BADH proteins. The three OsBADH genes were not evenly distributed across the 12 chromosomes. As shown in Fig. S1, Os04g0464200 was located on chromosome 4, Os07g0688800 was located on chromosome 7, and Os08g0424500 was located on chromosome 8, indicating distinct functions for the several rice BADH genes. Synteny analysis is an effective strategy for exploring the functional and evolutionary relationships between genes (Hou and Cao 2016). To further understand the evolutionary mechanisms and homologous genes of OsBADHs, a collinearity map was constructed among rice, dicotyledons (*Arabidopsis* and soybean), and monocotyledons (barley, wheat, maize, and sorghum). The numbers of orthologous gene pairs between rice and monocotyledonous plants were two (barley), nine (wheat), five (maize), and three (sorghum), whereas no orthologous events were detected between rice and the two dicots (Fig. 3), suggesting the closer phylogenetic relationships of rice BADH genes with those of monocots than those of dicots.

3.4. Phylogenetic analysis of rice BADHs

To clarify the evolutionary relationships of BADH genes between rice and six other plants, namely *Arabidopsis*, barley, soybean, wheat, maize, and sorghum, using the amino acid sequences of 3 rice

BADH proteins and 14 additional plant BADH proteins that shared > 40% similarity with OsBADH, we built an unrooted phylogenetic tree with MEGA 7.0. Interspecific phylogenetic analysis revealed that the 17 BADH proteins could be classified into two groups (ALDH10 and ALDH22). Group I contained two rice, one *Arabidopsis*, one barley, two soybean, two sorghum, three maize, and three wheat genes, and group II contained one rice, *Arabidopsis*, and soybean gene each (Fig. 4 and Table S4). Genes from the same branch were homologous because they shared motifs with other members of the same group. Furthermore, there were differences in the quantity of genes found in each branch, and phylogenetic tree analysis showed that rice, *Arabidopsis*, barley, soybean, sorghum, maize, and wheat shared interspecific similarity, indicating that the BADH families of these six species have a conserved evolutionary relationship.

3.5. Cis-acting element analysis of OsBADH promoters

Independent signal transduction pathways can trigger cis-acting promoter elements, which are significant molecular switches involved in the transcriptional control of genes under biotic and abiotic stress (Nakashima et al. 2009). The 2000-bp upstream promoter regions of OsBADHs were submitted to PlantCARE in order to identify cis-elements, with the goal of comprehending the fundamental activities of BADH genes (Fig. 5 and Table S8). The results revealed that the three BADH genes contained some light-responsive cis-acting elements (4cl-CMA2b, ACE, AE-box, Box4, CATA-motif, chs-CMA1a, G-Box, GT1-motif, I-box, Sp1, TCCC-motif, and TCT-motif); stress-responsive cis-acting elements (ARE, GC-motif, MBS, and TC-rich repeats); hormone-responsive cis-elements, including abscisic acid (ABA)-responsive cis-acting elements (ABRE), methyl jasmonate (MeJA)-responsive elements (CGTCA-motif and TGACG-motif), and gibberellin (GA)-responsive element (GARE-motif and P-box); plant development-associated cis-acting elements (CAT-box, AT-rich element, and O2-site), and other cis-acting elements. These findings indicate that OsBADH genes can be induced by exogenous factors such as hormones, light, and stress to participate in environmental stress responses.

3.6. Gene structure, conserved domain, and motif analyses of rice BADH genes

To investigate the functional relationships among BADH genes, conserved motifs, gene structures (exons/introns), and conserved domains were further studied. The differences in protein structure were compared using MEME to identify the number and distribution of motifs among rice BADH genes. In total, six distinct conserved motifs were predicted, namely motifs 1–6 (Fig. 6). Except for Os07g0688800, which lacked motif 6, the other two OsBADH genes contained all six motifs in the same order. In general, a family, clade, or group's motif composition might be exclusive, and genes with similar motif compositions share similar biological roles, although further investigation is required (Khan 2022). Plant evolution is correlated with the amount of introns; genes with fewer introns may be very efficient at responding swiftly to changes in their environment (Schmitz-Linneweber et al. 2015). In this study, the gene structure results revealed that Os04g0464200 and Os08g0424500 both contained 14 introns, whereas Os07g0688800 contained 13 introns (Fig. S2), indicating that these genes may not react

instantly to outside stimuli. Alternative splicing was possible because of the high intron density of the BADH subfamily genes. Genes can have several functions thanks to differential splicing (Ruhl et al. 2012). Thus, the BADH subfamily genes may have a variety of purposes in rice. We investigated the conserved domains of the three rice BADH genes using the NCBI Conserved Domains Database. The results showed that the BADH genes exhibited typical aldehyde dehydrogenase domains (ALDH10 and ALDH22; Fig. S2). These analyses showed that the architectures of the rice BADH genes varied even though they were located in the same branch of the gene evolutionary tree.

3.7. Physicochemical property, secondary structure, and tertiary structure analyses of rice BADH proteins

Using the ExPASy web tool, the physicochemical characteristics of the three BADH proteins were estimated. The number of amino acids in the proteins varied from 503 to 597, MW varied from 54.64802 to 66.01946 kDa, PI varied from 5.36 to 7.50, and the instability index varied from 33.42 to 35.87, thus revealing the stability of the proteins (Table S8). The grand average of hydropathicity of Os04g0464200 and Os08g0424500 varied from -0.124 to -0.02 , suggesting that they are hydrophilic, whereas the value for Os07g0688800 was 0.031 , indicating that it was possibly hydrophobic. To further understand the secondary structures of the three BADH proteins, we used the SOPMA server to predict their composition. The results revealed alpha helix, extended strand, beta turn, and random coil percentages of 43.74–45.06%, 12.23–15.64%, 5.19–7.75%, and 32.87–37.52%, respectively (Table S9). Additionally, the tertiary structures of the BADH proteins were predicted using SWISS-MODEL. The Os04g0464200 protein model was predicted using spinach BADH (4v37.1.A) as the template. The Os07g0688800 protein model was predicted using the AlphaFold DB model (Q8H5F0.1.A). The Os08g0424500 protein model was constructed using pea AMADH (3iwk.1.A) (Fig. 8).

3.8. Protein–protein interaction network analysis of OsBADH proteins

To evaluate the possible protein–protein interactions between BADH and other proteins in rice, we used the STRING database to predict the interaction network. The interaction map demonstrated the relationships of three BADH proteins with target proteins, including choline monooxygenase (A0A0P0X0M6), protein HOTHEAD (Q7XTZ0_ORYSJ, A0A0N7KGH1), tetratricopeptide repeat protein (Q2QMH0_ORYSJ), aldehyde dehydrogenase (Q69P84_ORYSJ), fructose/tagatose biphosphate aldolase (Q652S1_ORYSJ), delta-1-pyrroline-5-carboxylate synthase (P5CS1, P5CS2), proline dehydrogenase (Q336U3_ORYSJ), protein phosphatase (OJ1065_B06.20), calponin homology domain-containing protein (A0A0P0WIK5), serine–glyoxylate aminotransaminase (Q6ZFI6_ORYSJ), protein Hook homolog (A0A0P0UYD8), and tobamovirus multiplication protein (Q5JMW7_ORYSJ) (Fig. 7). In detail, both Os04g0464200 and Os08g0424500 interacted with A0A0P0X0M6, Q7XTZ0_ORYSJ, A0A0N7KGH1, Q2QMH0_ORYSJ, Q69P84_ORYSJ, Q652S1_ORYSJ, P5CS1, P5CS2, Q336U3_ORYSJ, OJ1065_B06.20, A0A0P0WIK5, Q6ZFI6_ORYSJ, A0A0P0UYD8, and Q5JMW7_ORYSJ. Meanwhile, an interaction between Os04g0464200 and Os08g0424500 was also detected (Fig. 7). Os07g0688800 interacted with

Q7XTZ0_ORYSJ, A0A0N7KGH1, Q2QMH0_ORYSJ, Q652S1_ORYSJ, P5CS1, P5CS2, Q336U3_ORYSJ, OJ1065_B06.20, A0A0P0WIK5, Q6ZFI6_ORYSJ, A0A0P0UYD8, and Q5JMW7_ORYSJ (Fig. 7). P5CS is a rate-limiting enzyme that catalyzes the biosynthesis of glutamate to proline, whose accumulation in plant cells helps maintain cell homeostasis, osmotic regulation, and redox balance, thereby alleviating oxidative damage caused by environmental stress (Signorelli and Monza 2017). Based on the interactions of BADH proteins with target proteins, this analysis of protein–protein interaction networks offered more evidence that these proteins are vital to plants' reaction to abiotic stress.

3.9. Molecular docking studies

Computational techniques represent a valuable source for predicting possible mechanisms governing biological activities, particularly molecular docking predictions (Seeliger and De Groot 2010). Molecular docking is one of the most commonly applied virtual screening methods to predict interactions between receptors and ligands, and ligand binding can alter the structural makeup of proteins, which can impact their activity (Chen et al. 2024a). In view of this, molecular docking was performed to examine the binding of OFF with the pockets of OsBADH proteins. For further examination, the model with the lowest binding energy was chosen. The results revealed strong interactions between OFF and the OsBADH protein active site, as shown in Table S11 and Fig. 8. The OFF complex interacted with Os04g0464200 through 12 interacting amino acids, with a binding energy of $-7.7 \text{ Kcal mol}^{-1}$ (Fig. 8A). Each of these amino acids exhibited a unique binding energy and bond length. The interactions between OFF and amino acid residues included conventional hydrogen bonds (SER-241, 3.13 Å; LYS-346, 3.33 Å), carbon–hydrogen bonds (GLY-220, 3.21 Å; GLY-224, 3.54 Å), pi–donor hydrogen bonds (TRP-163, 3.65 Å), halogen interactions (GLY-220, 3.21 Å; GLY-224, 3.54 Å; THR-239, 3.01 Å), pi–cation interactions (LYS-487, 4.96 Å), pi–anion interactions (GLU-190, 4.77 Å), pi–sigma interactions (PHE-397, 3.92 Å), pi–pi T-shaped interactions (TRP-163, 4.92 Å), alkyl bonds (ILE-160, 5.00 Å; PRO-162, 4.12 Å), and pi–alkyl interactions (PHE-238, 5.23 Å). Hydrogen bonds played an important role in protein docking. The OFF complex interacted with Os07g0688800 through 10 interacting amino acids, with a binding energy of $-7.5 \text{ Kcal mol}^{-1}$ (Fig. 8B). The interactions between OFF and amino acid residues included conventional hydrogen bonds (THR-63, 2.63 Å; HIS-226, 2.23 Å; ILE-377, 2.87 Å), halogen bonds (GLY-60, 3.01 Å; ALA-61, 3.50 Å; ALA-61, 3.56 Å), pi–pi T-shaped interactions (TYR-69, 5.15), alkyl bonds (PRO-71, 3.95 Å; MET-74, 4.90 Å), and pi–alkyl interactions (TYR-52, 5.10 Å; TYR-69, 5.32 Å). Hydrogen and halogen bonds were the dominant interactions between Os07g0688800 and OFF. OFF interacted with nine amino acid residues in Os08g0424500, with a binding energy of $-7.8 \text{ Kcal mol}^{-1}$ (Fig. 8C). The interactions included conventional hydrogen bonds (ARG-332, 3.20 Å), halogen bonds (GLU-280, 3.22 Å; TRP-317, 2.87 Å; TRP-317, 3.40 Å; ASN-320, 3.34 Å), pi–pi T-shaped interactions (PHE-284, 5.41 Å; TRP-288, 5.21 Å), alkyl bonds (LEU-283, 5.24 Å; ILE-321, 4.60 Å), and pi–alkyl interactions (TRP-109, 4.93 Å; PHE-284, 5.20 Å; TRP-317, 5.07 Å; ILE-321, 4.75 Å; ARG-332, 4.84 Å). The binding energy was mainly attributable to the high number of pi–alkyl interactions and halogen bonds at the active site of the enzyme. The docking results suggested that OsBADH proteins play a crucial role in pesticide metabolism and that Os08g0424500 exhibited better docking efficiency with OFF, indicating that Os08g0424500 may be more closely related to OFF metabolism.

Discussion

GB controls the growth and development of plants and strengthens their antioxidant defense mechanisms against environmental stress (Chen and Murata 2011; Chen et al. 2022a; Chen et al. 2024a). Exogenous GB has been shown in numerous studies to improve plant tolerance by upregulating the activity of detoxifying and antioxidant enzymes (Ahmad et al. 2013; Wutipraditkul et al. 2015; Chen et al. 2022a; Chen et al. 2024a). Analyzing the genes involved in GB synthesis under pesticide stress may yield significant gene discoveries and new concepts for genetic enhancement that reduce environmental concerns associated with pesticide residues. A GB synthase called BADH has been shown to improve rice's ability to withstand abiotic stress (Tang et al. 2014; Golestan Hashemi et al. 2018). In this study, we identified three BADH genes in the rice genome under GB + OFF treatment, and these genes may be crucial for mitigating OFF phytotoxicity and promoting OFF metabolism. We also performed phylogenetic and collinearity analyses in comparison to the gene sequences of Arabidopsis, soybean, wheat, maize, barley, and sorghum, and determined the structures, cis-elements, motif compositions, conserved domains, chromosome placements, and quantitative expression of these three genes. The activity and protein–protein interaction network of BADH proteins were further analyzed. Additionally,

Molecular docking was used to examine the interaction between OFF and three BADH proteins. Numerous investigations have demonstrated that gene duplication is essential to the evolution of genetic systems and genomes. Tandem and segmental duplications can therefore produce gene families, and it is through these mechanisms that plants can react quickly to environmental stress (Fraser et al. 2013). To fully understand the expansion pattern of OsBADH genes, their duplication events were examined in the current study. As shown in Fig. S1, Os04g0464200, Os07g0688800, and Os08g0424500 are located on chromosomes 4, 7, and 8, respectively, and exhibit segmental duplication events. This implied that segmental duplication had a major impact on both the evolution of rice BADH genes and their capacity for stress adaptation. Meanwhile, as evidenced by the three rice BADH genes' identical expression patterns under GB + OFF treatment, BADH genes may be implicated in the response to GB + OFF. These findings highlight the close association between the structure and function of these genes.

To explore the homologous relationships of these OsBADH genes with homologs in plant species, the interspecies collinearity between these BADH genes and those of *A. thaliana*, soybean, wheat, maize, barley, and sorghum was also examined. Significant collinearity was observed between the three rice BADH genes and those of wheat, maize, barley, and sorghum, but not between those of soybean and *A. thaliana*. The interspecies collinearity between these BADH genes and those of *A. thaliana*, soybean, wheat, maize, barley, and sorghum was also examined. Significant collinearity was observed between the three rice BADH genes and those of wheat, maize, barley, and sorghum, but not between those of soybean and *A. thaliana*. By amplification and replication, we deduce that the evolution of the BADH gene involved several species. Different BADH genes are present in plants, based on our evolutionary research of OsBADHs among plant species. In order to comprehend the evolutionary links of these genes among rice and other plants, a phylogenetic tree was built utilizing BADH genes from rice, Arabidopsis, barley,

soybean, sorghum, wheat, and maize (Fig. 4 and Table S5). Based on their evolutionary links, the three rice BADH genes were divided into two groups. Among them, Os04g0464200 and Os08g0424500 belonged to the ALDH10 family, and Os07g0688800 was categorized into the ALDH22 family. Moreover, we found close evolutionary relationships between the rice BADH gene Os07g0688800 and the wheat BADH gene TaALDH22A1-like. These close relationships were also confirmed using a map of interspecies collinearity between rice and *Arabidopsis*, barley, soybean, sorghum, wheat, and maize (Fig. 3). According to the interspecies collinearity analysis, the BADH genes of rice were not similar to those of the dicotyledonous plants *Arabidopsis* and soybean (Fig. 3).

Moreover, these findings aligned with the findings of phylogenetic analyses conducted on rice, wheat, and maize, which demonstrated that BADH genes belonging to the same ancestral branch shared the conserved motifs, functional domains, and structural characteristics of the OsBADH genes. We discovered that monocot-dicot plants, which are crucial for responding to different stresses, share relatively conserved gene family structures, evolution, and function when combined with previous research on BADH in a variety of species, including rice, *Arabidopsis*, spinach, tomato, sugar beet, and soybean (Fitzgerald et al. 2009; Golestan Hashemi et al. 2018). We can therefore plausibly surmise that these BADH genes are connected to metabolic and OFF stress responses. On the other hand, the significant variation that was found across the different groups indicates that functional differences among the rice BADH proteins evolved gradually. This understanding is consistent with the research conducted by Golestan Hashemi et al. (2018), which proved that the origins and differentiation of BADH genes may be traced back to the early stages of the evolution of monocotyledons and dicotyledons.

A motif is a brief sequence of relatively conserved proteins that recognizes particular functional protein sequences and hence contributes to a variety of biological activities (Theune et al. 2019; Jiao et al. 2022). A crucial foundation for protein functional studies can be provided by motif prediction (Schmitz et al. 2022). Further supporting their grouping in the evolutionary tree is the fact that the majority of the genes belonging to the same family in the same group share conserved domains, proteins, and motifs, according to the analysis of conserved motifs and phylogenetic trees. Moreover, conserved motifs 1–5 were present in all BADH proteins (Fig. 6), confirming domain conservation throughout the BADH family and possibly suggesting tighter evolutionary ties between OsBADH proteins and other plant species. Similarly, the distinct roles of BADH proteins in the GB signaling pathway may be influenced by the variations in group I and II motif composition patterns (Fig. 6). With motifs 1–6, Os04g0464200 and Os08g0424500 shared a closer relationship. A prior work showing that the rice conserved domain shared by the proteins BADH1 and BADH2 supports this (Golestan Hashemi et al. 2018). Moreover, rice, *Arabidopsis*, barley, maize, sorghum, soybean, and wheat BADH proteins were located in group I of the phylogenetic tree (Fig. 4), demonstrating that sequences with similar motif structures were clustered together (Cui et al. 2022). Genes that are related to each other may have comparable roles because they have conserved motifs and similar protein sequences (Tan et al. 2023; Zhou et al. 2023). Based on these results, we assume that the rice Os04g0464200, Os07g0688800, and Os08g0424500 proteins participate in the mitigation of OFF stress. Nonetheless, certain sequence and motif variations between species may have resulted from environmental effects that altered or lost genomic material in a

common ancestor, eventually leading to the expansion or limitation of genomic mutations (Anwar et al. 2023). Furthermore, a plethora of research have documented the direct impact of cis-elements on gene expression, and the transcription factors' response to cis-elements in modulating gene expression to augment plant stress tolerance (Schmitz et al. 2022). The data for the three BADH genes revealed that MeJA-responsive elements exhibited the greatest distribution in the Os08g0424500 gene, as four such elements were identified upstream of the Os08g0424500 gene. GA-responsive elements, ABREs, and AREs were widely distributed upstream of the Os07g0688800 gene (Fig. 5). The production of GA, MeJA, and ABA may be facilitated by the high frequency and wide range of MeJA-responsive elements, GA-responsive elements, ABREs, and AREs. This would make it possible for plants to withstand abiotic stresses like pesticides, low temperatures, salinity, and drought (Zhang and Yang 2021; Chen et al. 2022b). Effectively, ABA raises rice's resistance to abiotic stress (Dashevskaya et al. 2013), whereas MeJA is essential for pesticide metabolism and detoxification (Zhang and Yang 2021). For example, MeJA can further increase isoproturon resistance in wheat by accelerating isoproturon detoxification or breakdown (Ma et al. 2018). We eventually discovered that this gene stimulates fomesafen metabolism and degradation in rice plants (Chen et al. 2023b), supporting our earlier study's findings that the acetyltransferase cis-acting element responds to ABA and GA under fomesafen stress (Chen et al. 2022b). As a result, it is anticipated that OsBADH will take part in biotic and abiotic stress responses as well as potential OFF metabolism and degradation. Additionally, the OsBADH family genes also contain a large number of plant hormone-related cis-elements, including as GA-responsive elements, MeJA-responsive elements, and ABREs, which may interact or be implicated in GB signaling pathways. This could explain why all three OsBADH genes were significantly upregulated by OFF or OFF + GB treatment (Fig. 1). According to a prior study, the administration of exogenous GB activates hormone signaling pathways and stress-responsive genes to produce stress tolerance (Ahmad et al. 2013; Wutipraditkul et al. 2015; Chen et al. 2022a; Chen et al. 2024a). These findings also suggest that during plant growth and OFF tolerance, OsBADH genes may interact with plant hormones.

Prior research has indicated that the patterns of gene, cis-element, and binding site expression in the promoter may be crucial for environmental adaptation and for establishing a stable genetic framework that improves resistance to abiotic stress (Schmitz et al. 2022; Tan et al. 2023; Zhou et al. 2023). To investigate the role of OsBADH genes and proteins in OFF stress responses with or without GB, we analyzed OsBADH gene expression and OsBADH protein activity in different tissues following treatment with OFF with or without GB (Fig. 1). The results revealed that both OsBADH gene expression and protein activity were evidently enhanced under OFF stress compared to the control findings in all tissues, especially the roots. The high BADH gene expression and protein activity in rice tissues suggested that BADH plays an important regulatory role in OFF metabolism in rice seedlings. Furthermore, these findings imply that OsBADH genes interact with downstream genes by triggering cis-elements that respond to stress and are implicated in abiotic stress tolerance, including elements responsive to drought, light, and general stress (Fig. 5), and this could explain the increase in OsBADH gene expression following OFF exposure (Fig. 5).

In the promoter regions of eukaryotic genes, transcription factors have the ability to bind selectively to cis-acting regions. Stresses like low temperature, dryness, and salinity can promote the expression of genes that are involved in plant transcription, which can increase the plant's resistance to these conditions (Chen et al. 2024a). MYB transcription factors are widespread throughout plants, and they regulate development, primary and secondary metabolism, and abiotic and biotic stress responses (Wang et al. 2021). In this study, we found that an MYB binding site associated with abiotic stress, such as drought stress, was distributed upstream of BADH genes (Fig. 5). Therefore, stress-responsive cis-element activation to improve OFF metabolism may be the cause of OsBADH gene expression. Similarly, GB treatment significantly induced OsBADH gene expression and increased OsBADH protein activity (Fig. 1, Fig. 2). Meanwhile, GB biosynthesis is associated with BADH genes, while OsBADH gene promoters contain cis-elements (ARE, GC-motif, MBS, and TC-rich repeats) that may bind to transcription factors during OFF stress after GB treatment (Fig. 5). These findings align with earlier research suggesting that GB biosynthetic genes could have a role in abiotic stress reactions (Schmitz et al. 2022; Tan et al. 2023; Zhou et al. 2023).

Validating the functional role of genes requires protein-protein interactions (Theune et al. 2019; Anwar et al. 2023). In this study, OsBADH proteins were demonstrated to interact with key proteins (P5CS1, P5CS2, and Q336U3_ORYSJ) involved in proline synthesis and metabolism (Fig. 7). In previous studies, the accumulation of large amounts of proline in plants was found to play an important role in plant development and abiotic stress responses (Kaur and Asthir 2015; Kavi Kishor et al. 2015; Ghosh et al. 2022). The OsBADH promoter was shown to contain important cis-elements linked to development, metabolism, and stress responses, according to promoter analysis, which supported these findings (Fig. 5). It was therefore determined that OsBADH genes might be essential for OFF stress tolerance.

More information about the interactions between pesticides and their receptor ligands is required in order to comprehend the mechanistic role of OsBADH domains in pesticide metabolism and degradation (Qiao et al. 2022). According to Qiao et al. (2022), the stronger the reported association between a pesticide molecule and a target protein, the more likely it is that a detoxifying enzyme will be involved in the pesticide's detoxification process. In this investigation, we found that the proteins encoded by three BADH genes, particularly Os04g0464200, have significant hydrogen bond formation with OFF (Fig. 8). This discovery is essential for understanding the functions of the BADH gene family in plant metabolism of pesticides.

Conclusion

Three OsBADH genes were discovered in the rice genome after the OsBADH gene family was carefully examined in this work. Based on their structural and functional characteristics, the OsBADH genes induced by GB + OFF treatment formed two separate subfamilies (ALDH10 and ALDH22). Meanwhile, multispecific cis-elements were present in the upstream promoter regions of these genes, indicating a potential connection between the transcriptional and translational activation of BADH family genes and the stress response of plants to OFF. By interacting with different substrates, most BADH proteins possessed unique domains or patterns that allowed them to adapt to different environmental situations.

OsBADH genes in rice were distributed over three chromosomes in two groups, showing collinearity ties with those in barley, maize, wheat, and sorghum, according to classification, chromosomal distribution, and collinearity analyses. Furthermore, segmental duplication was discovered during OsBADH's genome-wide replication, indicating that one or more large-scale replication events may have played a role in the development of the OsBADH gene family. According to docking studies, different amino acid residues mediated the binding of OFF to BADH proteins. Additionally, qRT-PCR and BADH activity assessments suggested that OsBADH plays a vital role in defense against OFF. The physiological and molecular mechanisms underpinning the BADH family's action in rice can be better understood thanks to these discoveries, which also open up new avenues for research on OsBADH's role in controlling the metabolism and detoxification of OFF in rice.

Declarations

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Authorship contribution

Yi Zhuo Wang and Jun Jin Lu, conducted the RNA-seq and RT-PCR analysis of rice exposed to OFF or OFF+GB. Xu Zhen Shi and Ya Nan Qu, carried out the biochemical properties analysis of BADH genes. Gan Ai, carried out Protein interaction analysis of BADH DEGs. Li Qing Zeng and Xi Ran Cheng carried out the data analysis. Zhao Jie Chen and Yan Hui Wang, conceived and designed the study, drafted, edited and proofread the manuscript.

Data availability

Data will be made available on request.

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Declarations of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of generative AI in scientific writing

Statement: During the preparation of this work the author(s) never used the AI to write.

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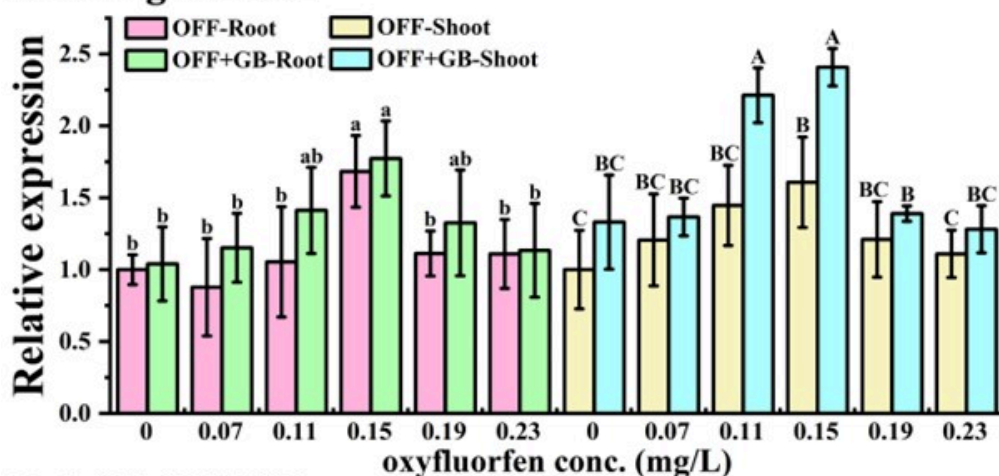
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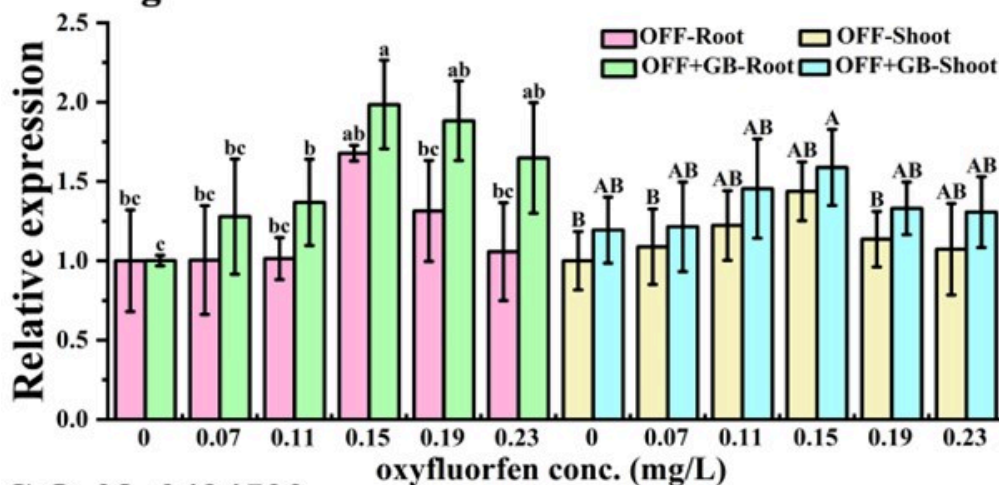
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Figures

A Os04g0464200



B Os07g0688800



C Os08g0424500

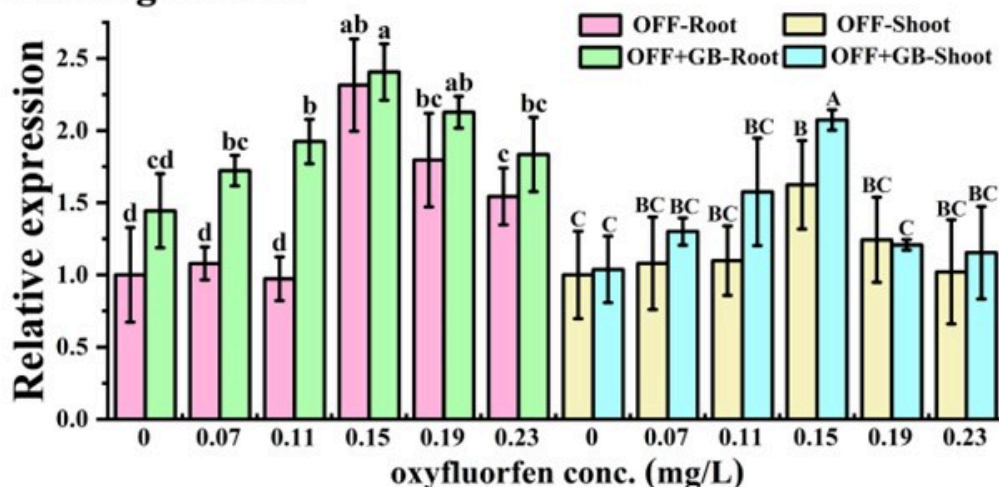


Figure 1

Effects of GB on OFF induced change in BADH expression levels of BADH genes in rice tissues. Ten-day-old rice seedlings were exposed to 0–0.23 mg/L OFF with or without 175 mg/L GB for 6 d. The results are the mean values $\pm$ SD ($n = 3$). The lowercase letters and capital letters represent significant difference of roots and shoots among different treatment ($p < 0.05$, ANOVA).

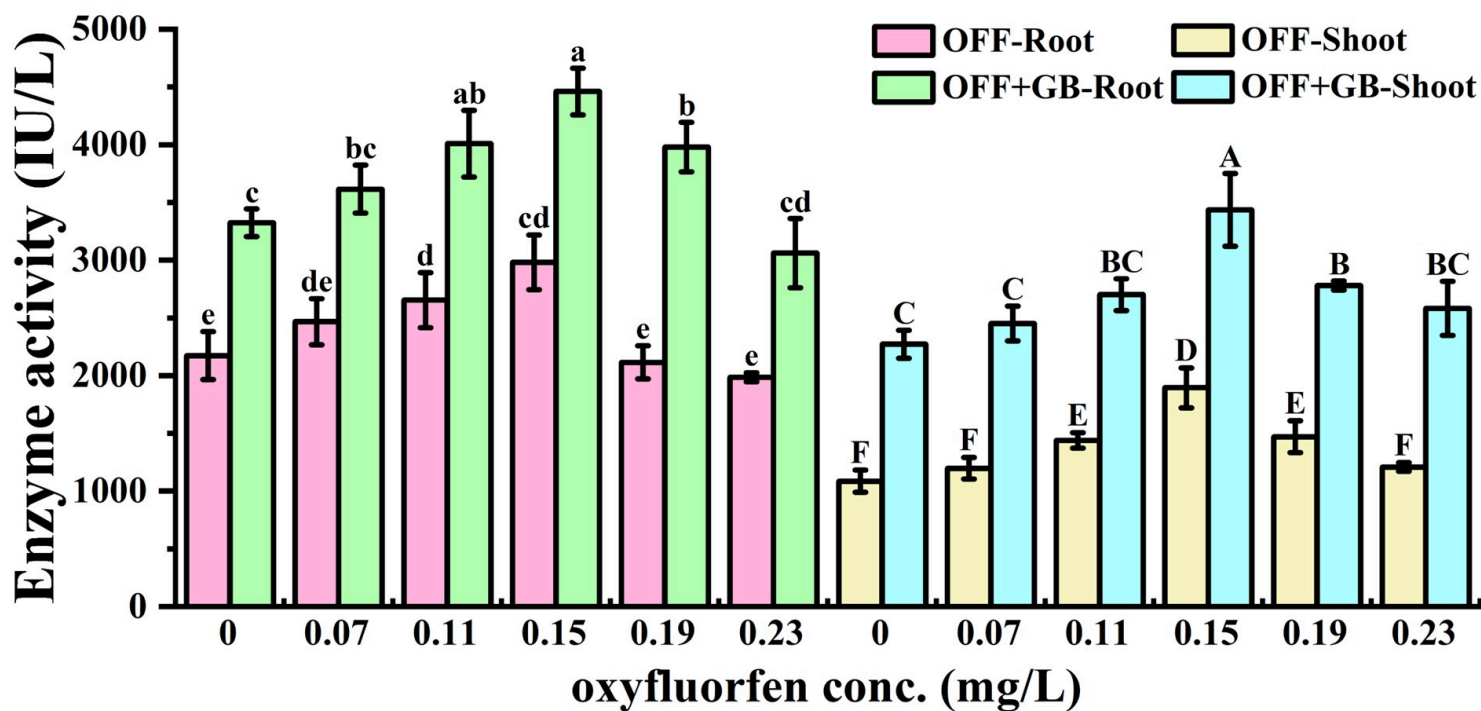


Figure 2

Effects of GB on OFF induced change in activity of BADH in rice tissues. Ten-day-old rice seedlings were exposed to 0-0.23 mg/L OFF with or without 175 mg/L GB for 6 days. The results are the mean values $\pm$ SD (n = 3). The lowercase letters and capital letters represent significant difference of roots and shoots among different OFF concentration ($p < 0.05$, ANOVA).

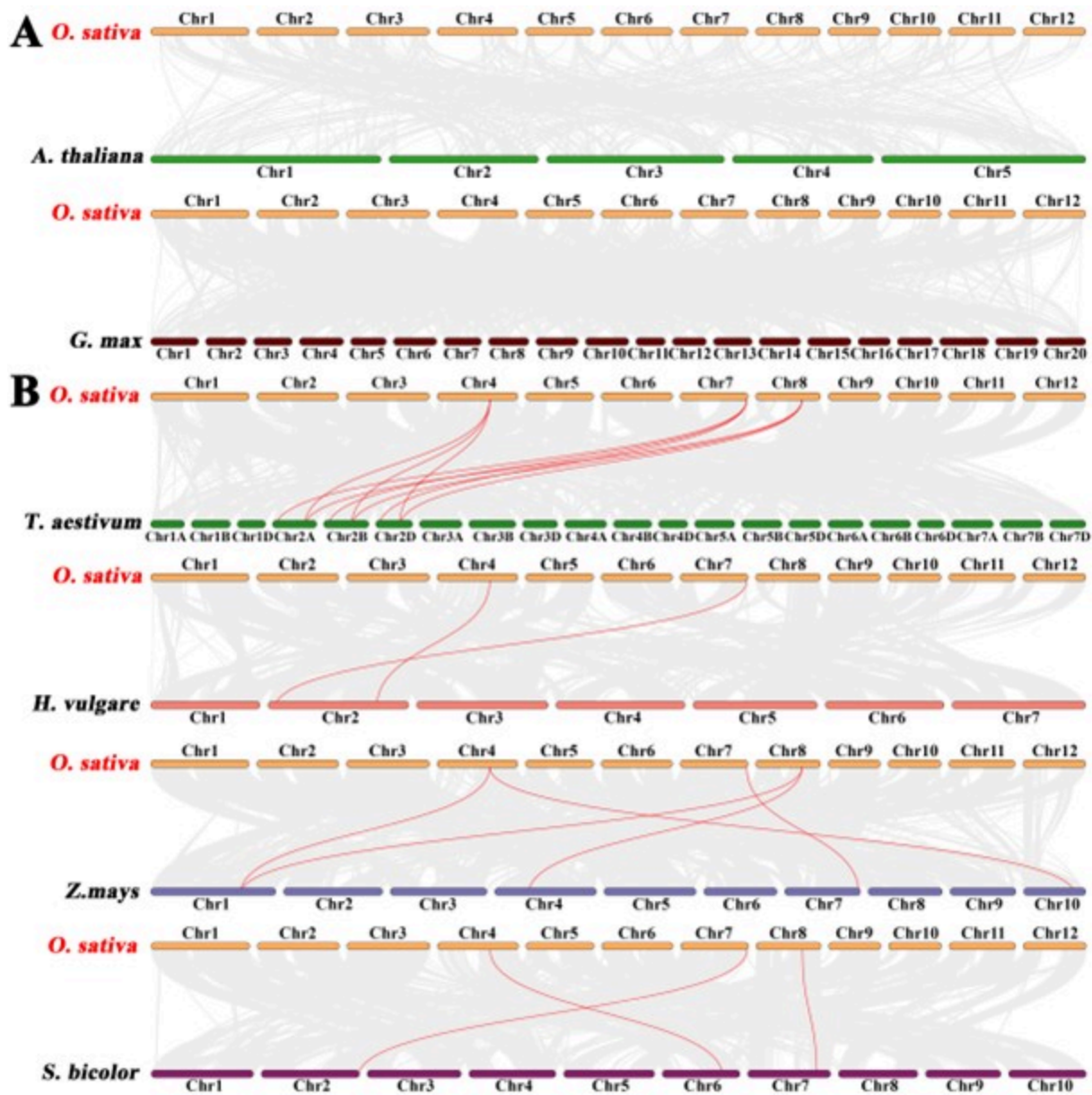


Figure 3

Syntenic analysis of BADH genes between rice and six other plant species, gray lines represent all syntenic gene pairs between two species, and red lines indicate the pairs of BADH genes. The results of the syntenic analysis between rice and dicotyledons, such as *Arabidopsis* and soybean (A), and monocotyledons, including wheat, barley, maize and sorghum (B).

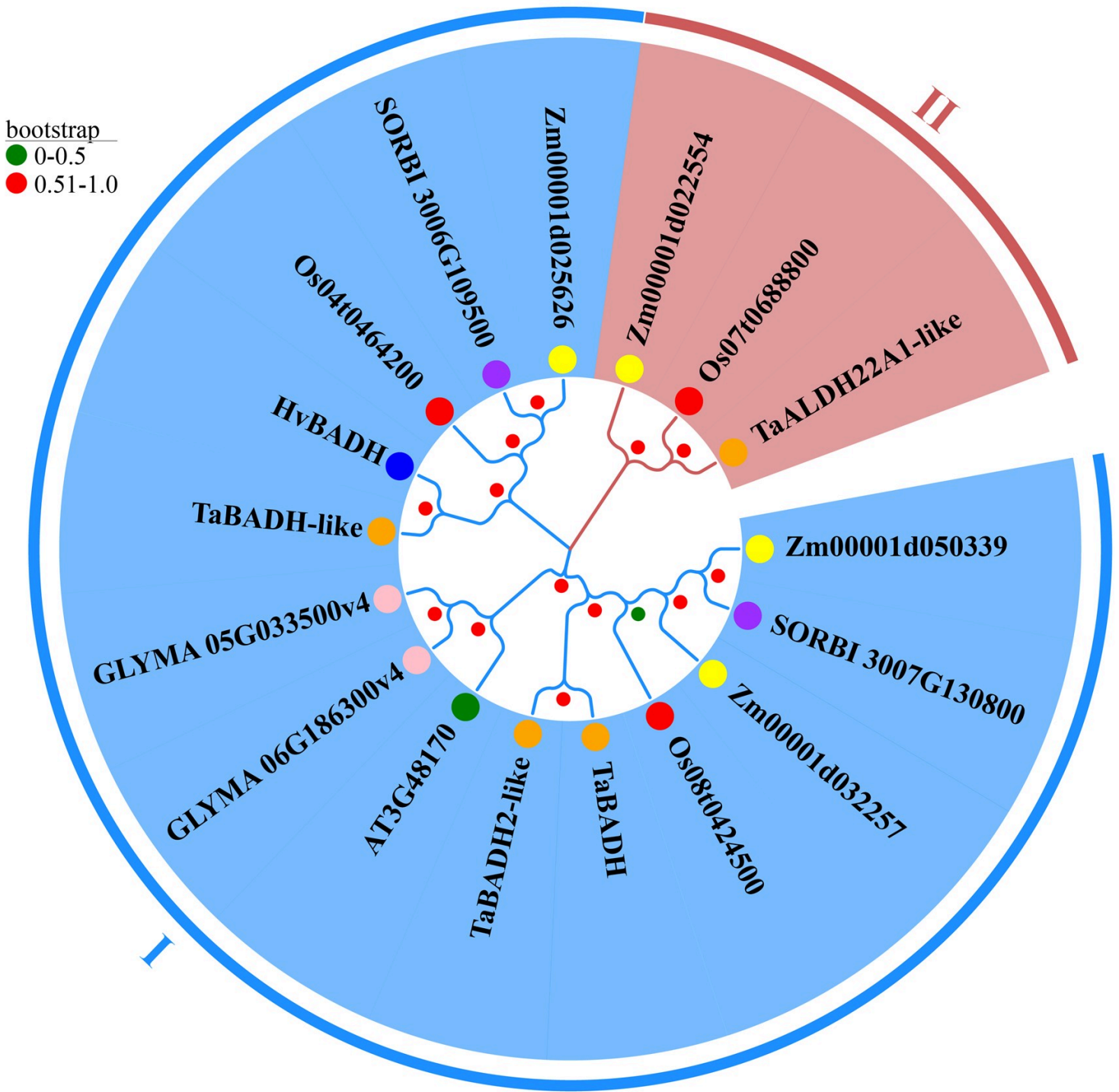


Figure 4

Phylogenetic analysis of BADH family genes in rice, Arabidopsis, barley, maize, sorghum, soybean and wheat which were marked with red circle, green circle, blue circle, yellow circle, purple circle, pink circle and orange circle, respectively. The tree split into 2 groups that named Group I and Group II. Group I were identified as ALDH10 family and Group II was identified as ALDH22 family.

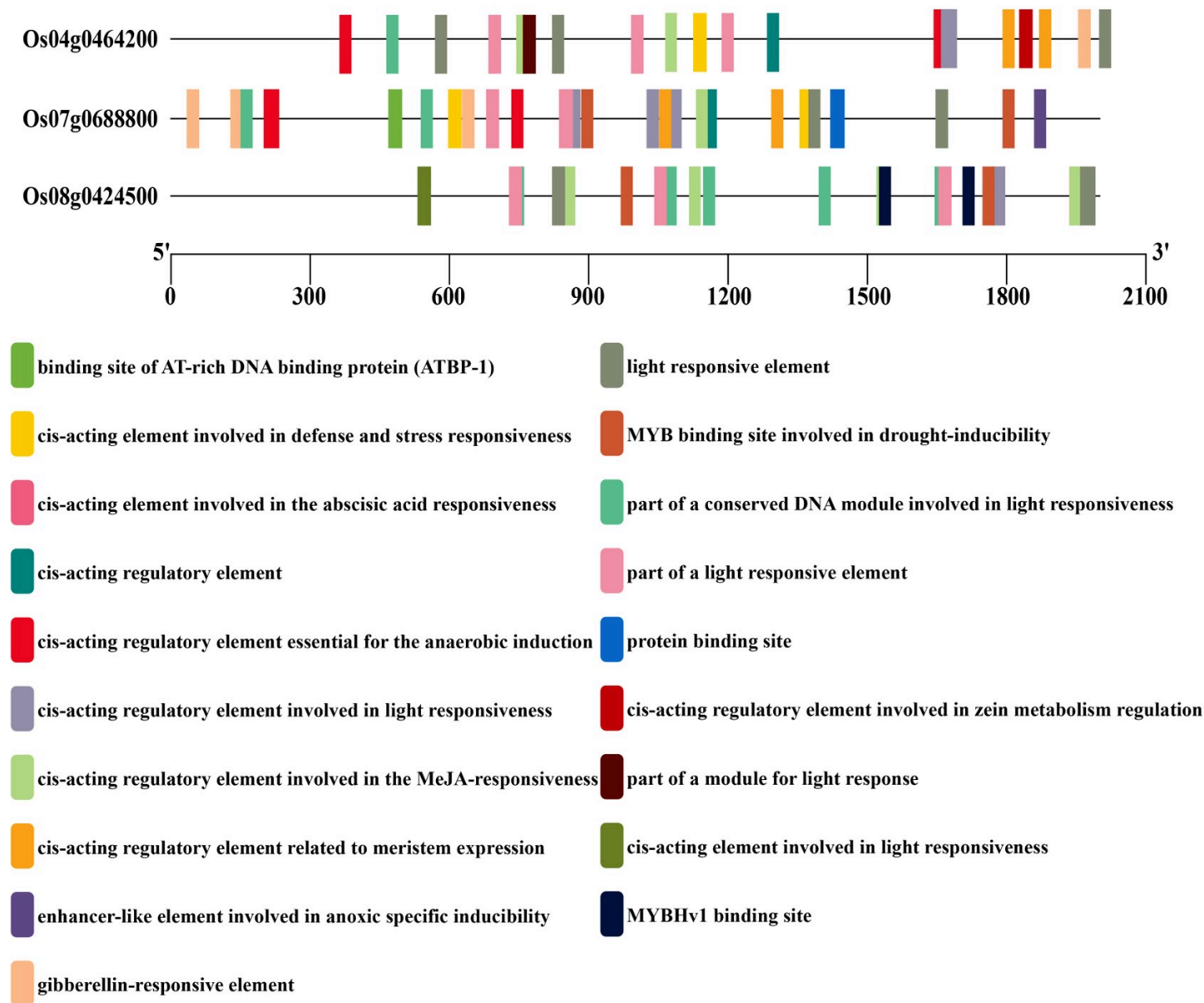


Figure 5

Cis-element analysis of rice BADH promoters. The cis-elements in the 2000-bp upstream promoter regions are mapped on each gene, with the different types of cis-elements represented by different colors.

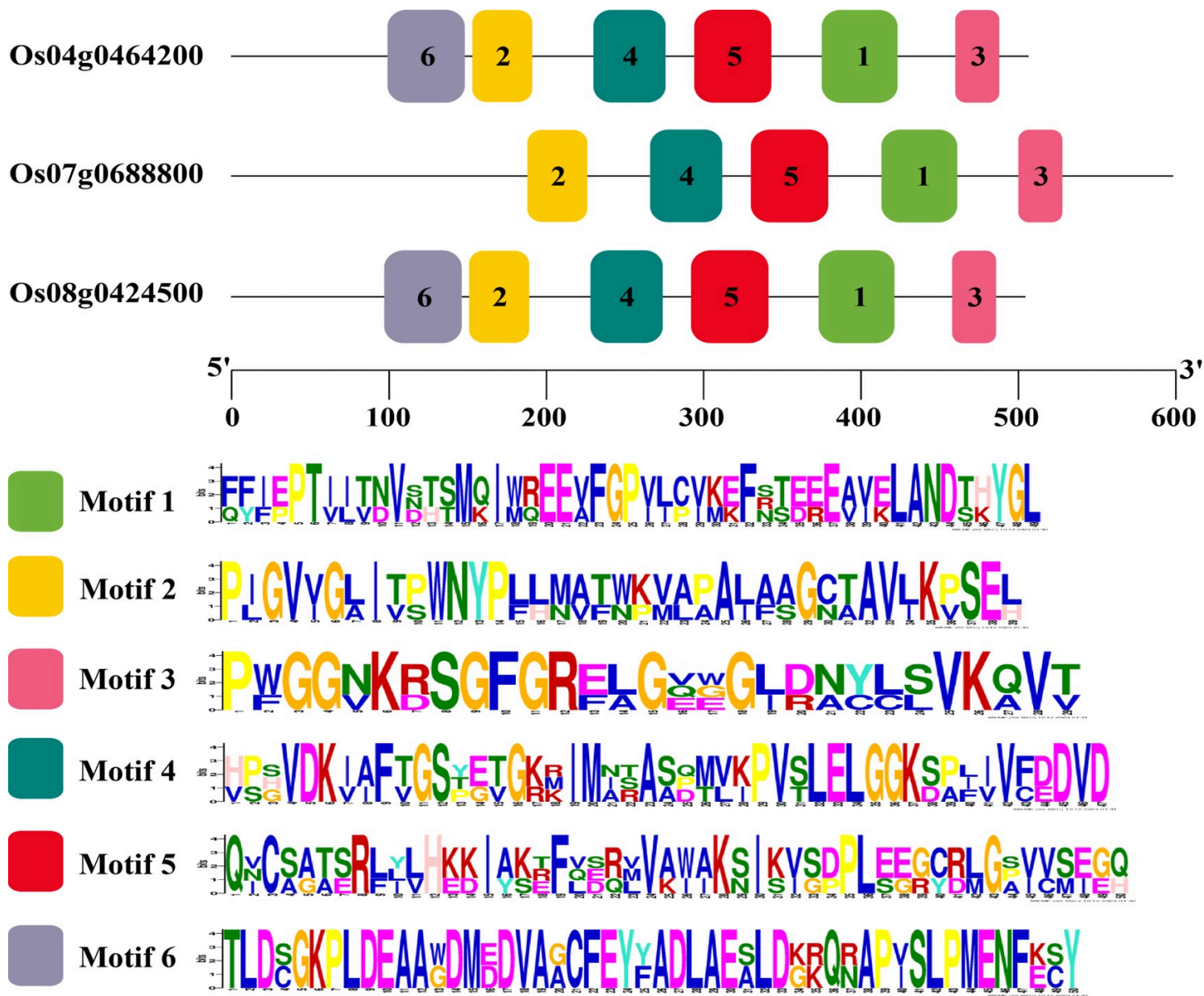


Figure 6

Analysis of conservative motif of BADH genes in rice. The 6 motifs are labeled with various colors.

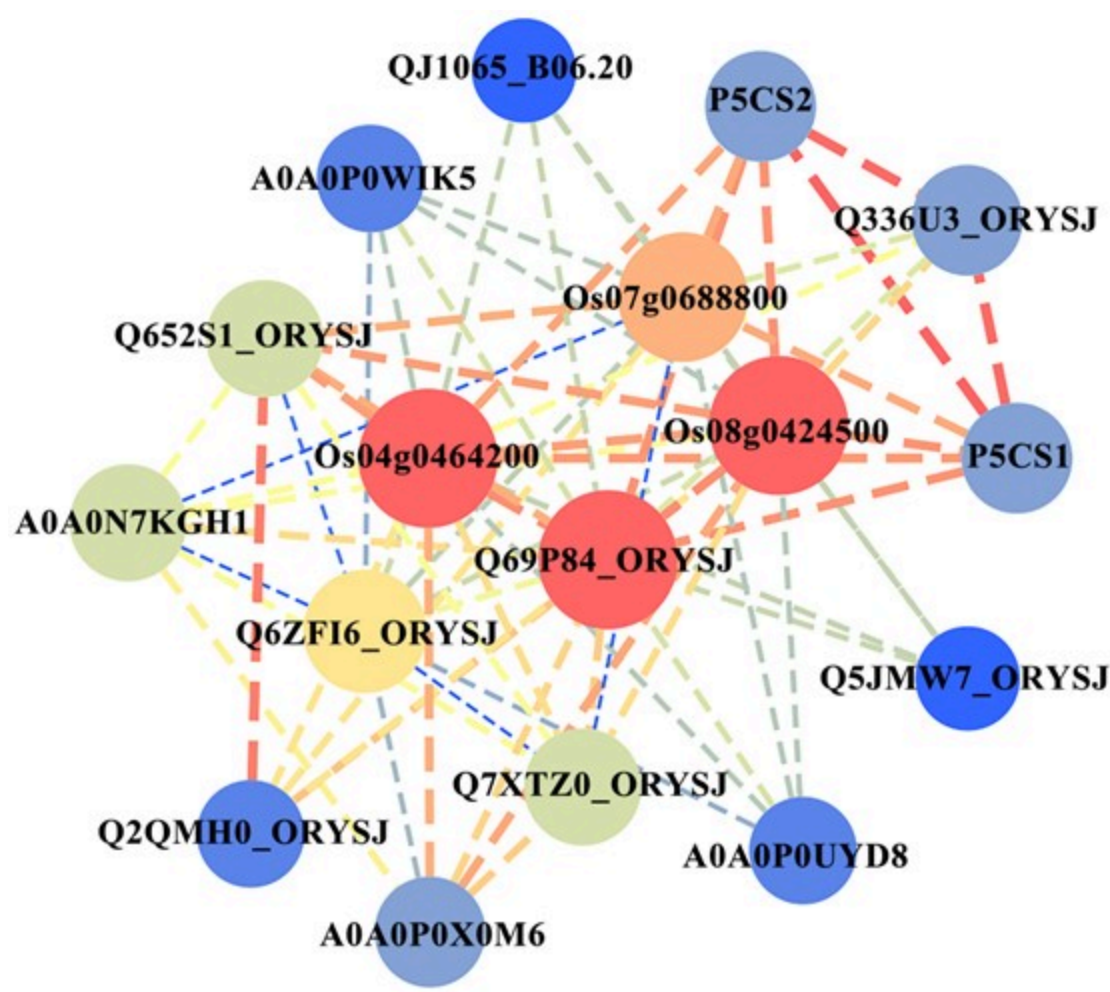


Figure 7

Protein-protein interaction network of BADH proteins in rice. The size of the nodes represents the magnitude of the degree and the width of the lines represents the size of the combined_score. The nodes were colored by the magnitude of the degree and the lines were colored by the size of the combined_score.

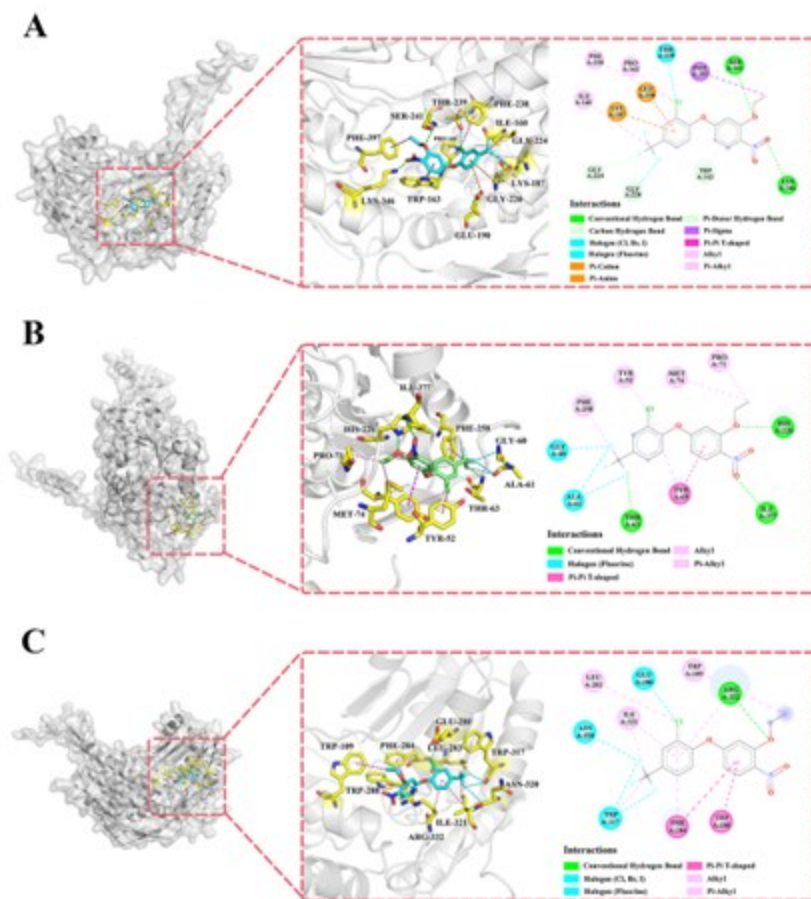


Figure 8

The receptor-ligand interaction of pesticides with the BADH proteins active site. A-C were depicted using Pymol and Discovery Studio 2019. Molecular docking of OFF with Os04g0464200 (A); Molecular docking of OFF with Os07g0688800 (B); Molecular docking of OFF with Os08g0424500 (C).

Supplementary Files

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