



# The versatile functions of *OsALDH2B1* provide a genic basis for growth–defense trade-offs in rice

Yinggen Ke (柯应根)<sup>a,b</sup>, Meng Yuan<sup>a,b</sup>, Hongbo Liu<sup>a,b</sup>, Shugang Hui<sup>a,b</sup>, Xiaofeng Qin<sup>a,b</sup>, Jie Chen<sup>a,b</sup>, Qinglu Zhang<sup>a,b</sup>, Xianghua Li<sup>a,b</sup>, Jinghua Xiao<sup>a,b</sup>, Qifa Zhang (张启发)<sup>a,b,1</sup>, and Shiping Wang (王石平)<sup>a,b,1</sup>

<sup>a</sup>National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China; and <sup>b</sup>National Center of Plant Gene Research, Huazhong Agricultural University, Wuhan 430070, China

Contributed by Qifa Zhang, December 19, 2019 (sent for review October 30, 2019; reviewed by Xuewei Chen and Jong-Seong Jeon)

**In plants, enhanced defense often compromises growth and development, which is regarded as trade-offs between growth and defense. Here we identified a gene, *OsALDH2B1*, that functions as a master regulator of the growth–defense trade-off in rice. *OsALDH2B1* has its primary function as an aldehyde dehydrogenase and a moonlight function as a transcriptional regulator. Loss of function of *OsALDH2B1* greatly enhanced resistance to broad-spectrum pathogens, including fungal blast, bacterial leaf blight, and leaf streak, but caused severe phenotypic changes such as male sterility and reduced plant size, grain size, and number. We showed that its primary function as a mitochondrial aldehyde dehydrogenase conditions male fertility. Its moonlight function of transcriptional regulation, featuring both repressing and activating activities, regulates a diverse range of biological processes involving brassinolide, G protein, jasmonic acid, and salicylic acid signaling pathways. Such regulations cause large impacts on the morphology and immunity of rice plants. The versatile functions of *OsALDH2B1* provide an example of the genic basis of growth–defense trade-offs in plants.**

*Oryza sativa* | disease resistance | growth | development | male sterility

**G**rowth, development, reproduction, and defense are basic processes for all organisms. Although defense is essential for an organism to survive, frequently, however, enhanced defense is achieved at the cost of other processes. In plants, for example, enhanced defense is often accompanied by a “syndrome” of compromised morphology such as growth retardation, male sterility, reduced plant size, etc. In other words, such compromised morphology often results in immunity activation as observed in transgenic rice overexpressing *OsWRKY45* and *Ideal Plant Architecture1* or knockdown of miR156 and knockout of *OsDOF11*, which is often referred to as trade-offs between growth and defense (1–5). Such a trade-off has posed great difficulty for crop genetic improvement in the effort to keep the balance between defense and productivity. In the last few decades tremendous progress has been made in the understanding of the genes, pathways, and regulatory networks underlying defense, growth, and reproduction, respectively (1, 3–14). However, much less is known on how these processes are coordinated or whether there is a master regulator that may modulate these processes.

The protein moonlight function, a second function in addition to the presumed regular one, has been proposed as an adaptive mechanism to yield a novel regulator meeting the needs of organismal complexity (15, 16). Such moonlighting proteins have been found in a wide range of organisms (17), and about two-thirds of them are enzymes, which play diverse biological roles (18, 19). A second function of enzymes as transcription regulators or factors has been found in multiple organisms (20–26). Moonlighting proteins playing roles in disease development and as new therapeutic targets for disease treatments have been extensively studied in mammals (27, 28). However, little is known about whether moonlighting proteins play any roles in plant disease response or whether such a function may impact plant growth and development.

Here we identified a gene, *OsALDH2B1*, in rice with a primary function as an aldehyde dehydrogenase and a moonlight function as a regulator at multiple tiers of regulatory activities. The activities of this gene integrate multiple processes and pathways governing defense, growth, and reproduction. The results unraveled the mechanistic connections of the various processes underlying the growth–defense trade-off in this special case of rice.

## Results

**Mutation in *OsALDH2B1* Impeded Rice Growth but Increased Defense.** We identified a T-DNA (Ti plasmid deoxyribonucleic acid) insertion mutant, RMD\_05Z11EQ94, in which the T-DNA was inserted in the first intron of the *OsALDH2B1* gene (*SI Appendix, Fig. S1A*). The transcript of *OsALDH2B1* was severely reduced in the mutant relative to the wild type (WT) (*SI Appendix, Fig. S1B*). We designated the mutant *osaladh2b1*.

The *osaladh2b1* mutant exhibited a defect phenotype in growth and development, including plant height, tiller number, lamina joint angle, panicle morphology, grain number, and seed setting (Fig. 1 *A* and *B* and *SI Appendix, Fig. S1 E–J*). Compared with WT, *osaladh2b1* showed increased tiller number (188.9%) and lamina joint angle (55.8%) and decreased plant height (–20.4%), panicle length (–18.6%), and grain number per panicle (–29.1%). The mutant was completely male sterile, leading to zero seed setting. Furthermore, seeds produced by the *osaladh2b1* plant pollinated with WT pollen were substantially smaller than seeds of WT (Fig. 1*C* and *SI Appendix, Fig. S1K*).

To investigate whether such a compromised phenotype is associated with increased immunity response as reported in previous studies, we inoculated *osaladh2b1* with the bacterial pathogens *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Xanthomonas oryzae* pv.

## Significance

**Crops’ defense activation often causes growth inhibition and yield reduction, which is referred to as trade-offs between growth and defense. In this study, we identified a gene, *OsALDH2B1*, that functions as a master regulator of the growth–defense trade-off in rice. The findings provide an example for the genic basis of growth–defense trade-offs in plants and may also have important implication for crop genetic improvement by exploring and modulating these components to achieve a balance between high yield and disease resistance.**

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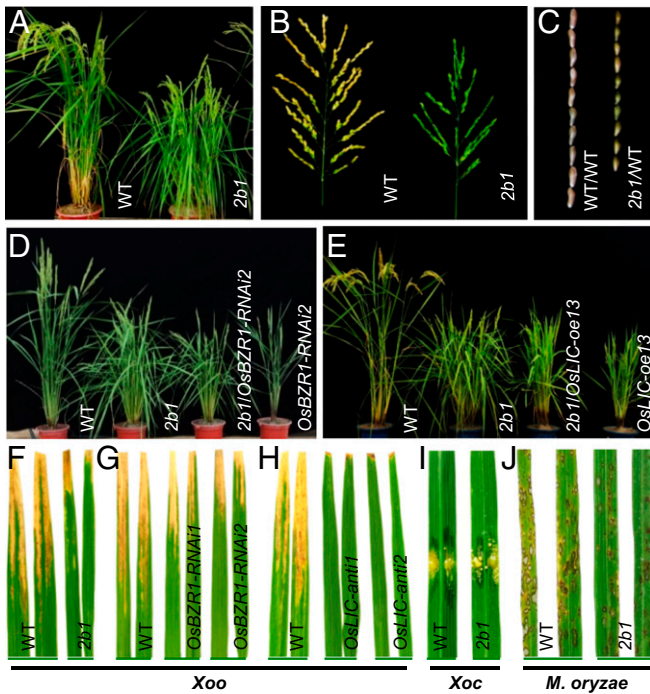
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<sup>1</sup>To whom correspondence may be addressed. Email: qifazh@mail.hzau.edu.cn or swang@mail.hzau.edu.cn.

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**Fig. 1.** Phenotypic effects of *OsALDH2B1*, *OsBZR1*, and *OsLIC*. Plants (A), panicles (B), and seeds (C) of WT and the *osaldh2b1* (*2b1*) mutant. (D) Plants of WT, *osaldh2b1*, *OsBZR1-RNAi2*, and *osaldh2b1/OsBZR1-RNAi2* double mutants segregated from F<sub>2</sub>. (E) Plants of WT, *osaldh2b1*, *OsLIC-oe13*, and *osaldh2b1/OsLIC-oe13* double mutants segregated from F<sub>2</sub>. (F–H) Increased resistance of the *osaldh2b1* mutant (F), *OsBZR1-RNAi* lines (G), and *OsLIC-anti* lines (H) to *Xoo* shown with leaves at 14 d after inoculation with *Xoo* strain PXO341. (I) Increased resistance of *osaldh2b1* mutant to *Xoc* shown with leaves at 7 d after inoculation with *Xoc* strain RH3. (J) Increased resistance of *osaldh2b1* mutant to *M. oryzae* shown with leaves at 7 d after inoculation with *M. oryzae* isolate N2-2.

*oryzicola* (*Xoc*) and the fungal pathogen *Magnaporthe oryzae* (*M. oryzae*). *Xoo* and *Xoc* cause rice bacterial leaf blight and bacterial leaf streak, respectively, and *M. oryzae* causes blast. All three diseases are highly devastating and cause heavy yield losses worldwide. The results showed that *osaldh2b1* enhanced resistance to all of the pathogen strains we tested. The lesion lengths of *osaldh2b1* plants resulting from inoculation of *Xoo* and *Xoc* were 66.5% and 43.1% shorter than those of WT (Fig. 1 F and I and *SI Appendix, Fig. S1 L and M*), and the disease index of *M. oryzae* was reduced by 37.1% relative to WT (Fig. 1J and *SI Appendix, Fig. S1N*). The amounts of bacterial growth of *Xoo* and *Xoc* and fungal biomass of *M. oryzae* were smaller, corroborating the observation of enhanced disease resistance of *osaldh2b1* (*SI Appendix, Fig. S1 L–N*).

The *osaldh2b1* complementary plants (*OsALDH2B1-C*) complemented the mutant phenotype (*SI Appendix, Fig. S1Q*), suggesting that *OsALDH2B1* was the gene responsible for the *osaldh2b1* mutant phenotype. Putting these findings together, these results indicated that mutation in *OsALDH2B1* hindered rice growth and development but enhanced disease resistance.

***osaldh2b1* Mutant Constitutively Activated Brassinolide- and Jasmonic Acid-Related Signaling Pathways.** The enlarged leaf angle in *osaldh2b1* is a typical phenotype caused by overdose of brassinolide (BR) or activated BR signaling in rice (29). As BR biosynthesis is negatively feedback regulated by BR signaling (30), we measured the expression levels of BR biosynthetic genes, *D4*, *D11*, and *BRD1*, in WT and the *osaldh2b1* mutant and found that these genes were down-regulated in the *osaldh2b1* mutant (*SI Appendix, Fig. S1B*). In rice, the lamina joint angle is an indicator highly sensitive to BR

and has been used as a BR-sensitive marker (31, 32). Lamina joint angle was increased by eBL (24-epibrassinolide, an active form of BR) treatment both in WT and the *osaldh2b1* mutant and was induced to a much larger degree in the *osaldh2b1* mutant (*SI Appendix, Fig. S1P*). Thus, in terms of lamina joint angle, the *osaldh2b1* mutant was hypersensitive to BR. Collectively, these results indicated that the BR signaling was highly activated in the *osaldh2b1* mutant.

*OsBZR1* positively regulates rice BR signaling, while *OsLIC* is a negative regulator of rice BR signaling; *OsBZR1-RNAi* plants and *OsLIC-oe* lines displayed a similar BR-deficient phenotype with reduced lamina joint angle and plant height (33). To genetically determine the position of *OsALDH2B1* in the BR signaling pathway, we crossed the *osaldh2b1* mutant with the *OsBZR1-RNAi2* plants and *OsLIC-oe* plants. In the F<sub>2</sub> population, the *osaldh2b1/OsBZR1-RNAi2* plants showed a phenotype similar to that of the *osaldh2b1* mutant, although they accumulated an amount of *OsBZR1* transcripts similar to that of *OsBZR1-RNAi2* plants (Fig. 1D and *SI Appendix, Fig. S2D*), suggesting that *OsALDH2B1* functioned genetically downstream of *OsBZR1*; *osaldh2b1/OsLIC-oe13* plants showed a phenotype similar to that of *OsLIC-oe13* plants, except that *osaldh2b1/OsLIC-oe13* plants were highly sterile and produced no seeds, similar to *osaldh2b1* (Fig. 1E and *SI Appendix, Fig. S3G*). Thus, *OsALDH2B1* functioned genetically upstream of *OsLIC* in the BR-related signaling pathway. Taken together, these results suggested that *OsALDH2B1* was a negative regulator of BR signaling and functioned genetically downstream of *OsBZR1* and upstream of *OsLIC* in the BR-related signaling pathway. However, the male fertility mediated by *OsALDH2B1* might not be regulated by the BR-related signaling pathway.

Immune response in plants usually occurs with the activation of defense-related genes, such as *AOS2*, involved in jasmonic acid (JA) biosynthesis; *JAZ8*, involved in the JA-dependent signaling pathway; and *PR1a*, which is related to the salicylic acid (SA)- and JA-dependent signaling pathway (34). The expression levels of these three genes were significantly up-regulated in *osaldh2b1* compared with WT (*SI Appendix, Fig. S1C*). Since SA and JA are two major defense signaling molecules, we quantified SA and JA contents, and the results showed that the *osaldh2b1* mutant accumulated a much higher level of JA and a slightly but significantly higher level of SA (*SI Appendix, Fig. S1O*). These results demonstrated that the broad-spectrum resistance of the *osaldh2b1* mutant to the pathogens was associated with activation of both JA- and SA-related signaling pathways.

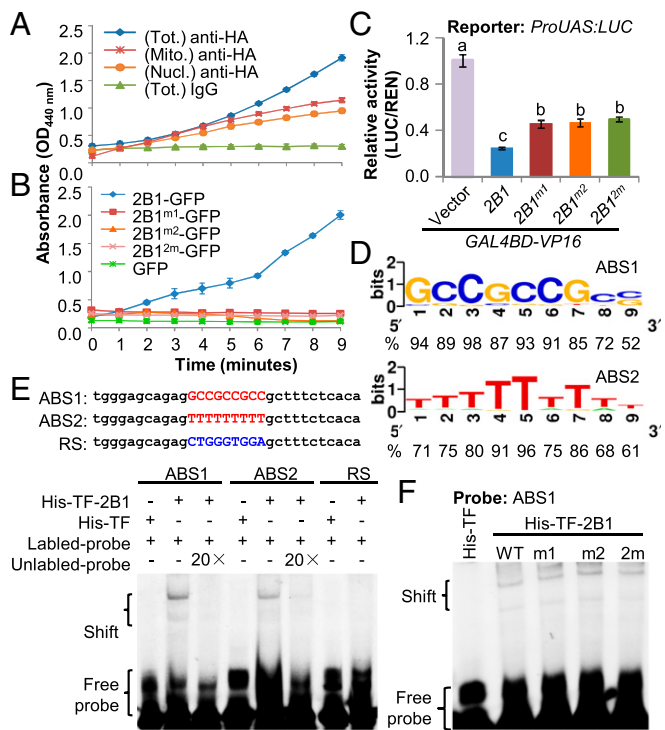
***OsALDH2B1* Functions as an Aldehyde Dehydrogenase in Both Nuclei and Mitochondria.** The DNA sequence of *OsALDH2B1* was predicted to encode an aldehyde dehydrogenase (ALDH) with two putative active sites (Glu<sup>316</sup> and Cys<sup>350</sup>) and putatively localize in mitochondrion (*SI Appendix, Fig. S4A*) (35). In rice protoplasts transiently expressing assays, the *OsALDH2B1*-GFP protein was detected in both the nucleus and mitochondria (*SI Appendix, Figs. S4 B and C and S6A*).

It was previously shown that *OsALDH2B1* expressed in bacteria had ALDH activity (36). To detect the ALDH activity of mitochondrial and nuclear *OsALDH2B1* in vivo, the *OsALDH2B1*-HA protein was isolated with anti-HA (anti-hemagglutinin) antibody from total, nuclear, and mitochondrial fractions from *OsALDH2B1-C* plants expressing the *OsALDH2B1*-HA protein and subjected to ALDH activity assay by monitoring the reduction of NAD<sup>+</sup> to NADH with a fluorescence spectrophotometer at 440 nm. The absorbance value increased with the reaction time (Fig. 2A), suggesting that both nuclear and mitochondrial *OsALDH2B1* had ALDH activity. *OsALDH2B1* is highly similar to the maize gene *ZmALDH2B1* (also known as *rf2a*), which is a functional mitochondrial ALDH regulating male sterility in maize (37). Thus,

it is highly likely that the male sterility of *osalhd2b1* is due to the deficiency of the enzyme activity of OsALDH2B1 in mitochondria.

Previous results showed that Pro<sup>323</sup> is required for the ALDH activity of ZmALDH2B1 (38). To investigate whether Glu<sup>316</sup> and Pro<sup>322</sup> (corresponding to the Pro<sup>323</sup> in ZmALDH2B1) in OsALDH2B1 are required for its ALDH activity, we mutated Glu<sup>316</sup> to Arg (referred to as OsALDH2B1<sup>m1</sup>), Pro<sup>322</sup> to Ser (referred to as OsALDH2B1<sup>m2</sup>), and both (Glu<sup>316</sup> to Arg and Pro<sup>322</sup> to Ser, referred to as OsALDH2B1<sup>2m</sup>). To analyze the ALDH activity of these OsALDH2B1 variants in vivo, we transiently expressed the proteins that were fused with GFP (*SI Appendix, Fig. S6A*). We did not detect ALDH activity in any of these variants (Fig. 2B). Thus, both Glu<sup>316</sup> and Pro<sup>322</sup> are critical for its ALDH activity.

**OsALDH2B1 Had the Characteristics of a Transcription Factor.** The moonlight function of several enzymes has been identified as transcription regulators or factors (16, 26, 39, 40). Until now, no nuclear localization of ALDH has been found in plants. The nuclear localization of OsALDH2B1 suggests that it may function as a transcription factor or regulator. To investigate this



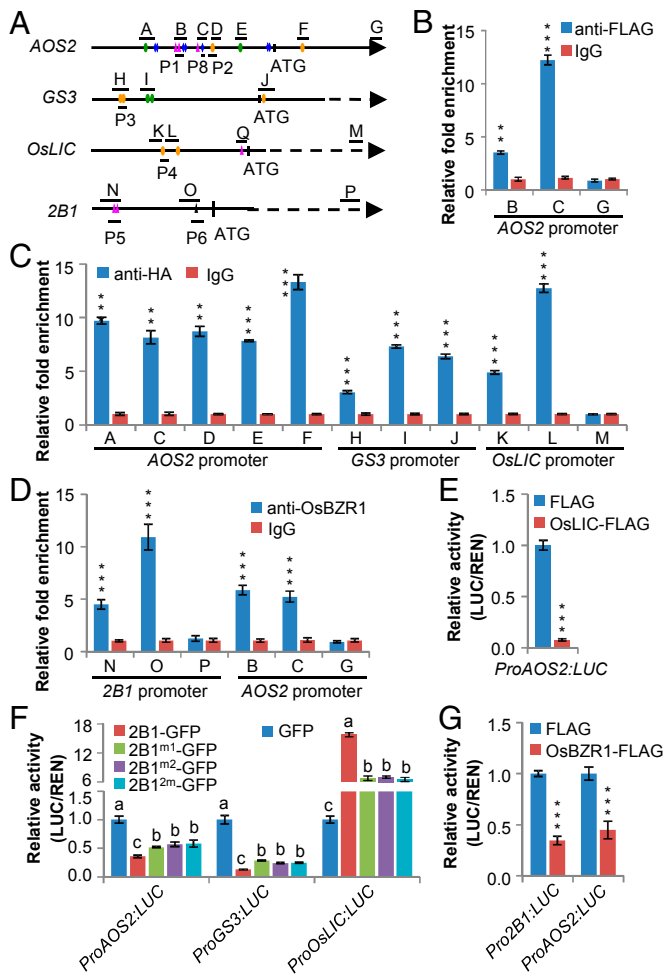
**Fig. 2.** Activity assays of the OsALDH2B1 protein. Data represent mean  $\pm$  SEM ( $n = 3$ ). 2B1, OsALDH2B1. (A) Aldehyde dehydrogenase activity of OsALDH2B1 in total homogenate (Tot.), mitochondria (Mito.), and nuclear (Nucl.) fractions. Protein isolated from total (Tot.) homogenate with IgG (immunoglobulin G) was used as a negative control. (B) Aldehyde dehydrogenase activity assay of OsALDH2B1 mutant proteins. Protein isolated from the total homogenate from rice protoplast transiently expressing GFP with anti-GFP antibody was used as a negative control. (C) The role of Glu<sup>316th</sup> and Pro<sup>322nd</sup> in the transcriptional repression activity of OsALDH2B1. *OsALDH2B1* variants were fused to GAL4DB-VP16 as effector constructs. Transient gene expression assays were performed in rice protoplast cells. Different letters above the bars indicate differences by a multiple range test at  $P < 0.05$ . (D) Putative OsALDH2B1 binding sequences identified using WebLogo. (E) DNA binding activity assay of OsALDH2B1 by EMSA. The red capital letters indicate the putative OsALDH2B1 binding sequences. The blue capital letters represent a random sequence (RS). (F) DNA binding activity of the Glu<sup>316th</sup> and Pro<sup>322nd</sup> mutant proteins of OsALDH2B1.

possibility, we tested the transcriptional regulation activity of OsALDH2B1 using dual-LUC (luciferase) activity assays. We observed that the reporter LUC activity was repressed by OsALDH2B1 (*SI Appendix, Fig. S6 A and B*), indicating a transcriptional repression function of OsALDH2B1. We also analyzed the roles of Glu<sup>316</sup> and Pro<sup>322</sup> in OsALDH2B1 transcriptional repression activity. We found that mutation of Glu<sup>316</sup> or Pro<sup>322</sup> impaired the transcriptional repression activity of OsALDH2B1, and double mutations of Glu<sup>316</sup> and Pro<sup>322</sup> did not further reduce its transcriptional repression activity (Fig. 2C and *SI Appendix, Fig. S6A*). This suggests that ALDH activity is associated with the transcriptional repression of OsALDH2B1.

We then checked the DNA binding activity of OsALDH2B1 and the potential DNA sequence specificity using systematic evolution of ligands by exponential enrichment assays (41). The analysis showed that 46 and 44% of the selected and amplified sequences contained core sequences GCCGCCGCC/G and TTTTTTTTTT, which might be OsALDH2B1 binding sequences (ABSs) (Fig. 2D). To test this inference, we conducted an electrophoretic mobility shift assay (EMSA). The results showed that the OsALDH2B1 protein bound to ABS1 (GCCGCCGCC/G) and ABS2 (TTTTTTTTTT), while OsALDH2B1 did not bind to a random sequence (Fig. 2E). We also found that neither mutation of Glu<sup>316</sup> nor mutation of Pro<sup>322</sup> could reduce the DNA binding activity of OsALDH2B1 in an EMSA (Fig. 2E), suggesting that mutations of Glu<sup>316</sup> and Pro<sup>322</sup> singly or in combination did not affect the DNA binding activity of OsALDH2B1. Thus, mutations of Glu<sup>316</sup> and Pro<sup>322</sup> reduced the transcriptional repression activity of OsALDH2B1 without affecting the DNA binding activity. Taken together, these data indicated that OsALDH2B1 may function as a transcription factor.

**Differential Regulations of OsALDH2B1 on Expression of Target Genes.** We searched for the targets of OsALDH2B1 in the rice genome by quarrying the two ABS sequences; three genes were found to contain two to four putative ABSs in the promoters (Fig. 3A). The first was *AOS2*, a defense gene that was up-regulated in the *osalhd2b1* mutant. The second was *OsLIC*, a negative regulator of rice BR signaling that we found to be genetically downstream of OsALDH2B1. Interestingly, *GS3*, a major QTL (quantitative trait locus) for grain size that encodes a  $G\gamma$  protein (13, 42, 43), was also found to contain ABSs. We performed chromatin immunoprecipitation (ChIP)-qPCR assays on the binding of OsALDH2B1 to *AOS2*, *OsLIC*, and *GS3* promoters in vivo. OsALDH2B1 binding was enriched on positions A, C, D, E, and F in the *AOS2* promoter; H, I, and J in the *GS3* promoter; and K and L in the *OsLIC* promoter, which contained or was near the ABS (Fig. 3C), suggesting that OsALDH2B1 may bind to these promoters in vivo. EMSA showed that OsALDH2B1 could bind in vitro to the *AOS2* promoter at P2 containing a sequence similar to ABS1, the *GS3* promoter at P3 containing two ABS1s, and the *OsLIC* promoter at P4 containing one ABS1 (*SI Appendix, Fig. S5A*). These results indicated that OsALDH2B1 bound to the promoters of the *AOS2*, *GS3*, and *OsLIC* genes both in vitro and in vivo.

We further analyzed OsALDH2B1 action on *AOS2*, *GS3*, and *OsLIC* expression in a transient expression assay with the LUC reporter driven by  $\sim 2$  kb putative promoter regions of *AOS2* (*ProAOS2:LUC*), *GS3* (*ProGS3:LUC*), and *OsLIC* (*ProOsLIC:LUC*) by cotransformation of the relevant constructs (*SI Appendix, Fig. S6A*). The LUC activity of *ProAOS2:LUC* and *ProGS3:LUC* was repressed by OsALDH2B1-GFP, while the LUC activity of *ProOsLIC:LUC* was enhanced by OsALDH2B1-GFP (Fig. 3F). Moreover, Glu<sup>316</sup> and Pro<sup>322</sup> were required for the full transcriptional regulation activity of OsALDH2B1 (Fig. 3F). Consistent



**Fig. 3.** In vivo DNA binding and transcriptional regulation activity assays. (A) Identifying binding sites in promoters by different transcription factors. The orange ellipses indicate OsALDH2B1 binding site AB51, and the green ellipses indicate AB52. The pink triangles indicate OsBZR1 binding site BRRE, and the black triangle indicates E-box. Blue diamonds indicate the OsLIC binding motif. P1, P5, and P6 indicate the probes used in EMSA bound by OsBZR1; P2, P3, and P4 indicate the probes used in EMSA bound by OsALDH2B1, and P8 indicates the probe used in EMSA bound by OsLIC. A, C–F, and H–L indicate sequences tested in the ChIP–qPCR assay bound by OsALDH2B1; B and C indicate those bound by OsBZR1 and OsLIC, and N, O, and Q indicate those bound by OsBZR1. G, M, and P are negative controls. (B) Binding assay of OsLIC to the promoter of *AOS2* by ChIP–qPCR using *OsLIC*-*oe* plants. (C) Binding assays of OsALDH2B1 to the promoters of *AOS2*, *GS3*, and *OsLIC* by ChIP–qPCR with the *OsALDH2B1*-C plants using the anti-HA antibody. (D) Binding assays of OsBZR1 to the promoters of *AOS2* and *OsALDH2B1* by ChIP–qPCR with WT using anti-OsBZR1 antibodies. (E) Activity assay of OsLIC in regulating *AOS2* expression. (F) Activity assays of OsALDH2B1 in regulating target gene expression and the role of Glu<sup>316th</sup> and Pro<sup>322nd</sup> in the transcriptional regulation. (G) Activity assays of OsBZR1 in regulating *OsALDH2B1* and *AOS2* expression. OsBZR1-FLAG was used as an effector. In (B)–(G) data represent mean  $\pm$  SEM ( $n = 3$ ). \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , obtained with a one-tail Student's  $t$  test. Different letters above bars indicate differences by a multiple range test at  $P < 0.05$ . Transient gene expression assays were performed in rice protoplast cells.

with these transient assays, *osaldh2b1* plants accumulated more *AOS2* and *GS3* transcripts but fewer *OsLIC* transcripts (SI Appendix, Fig. S1 B–D). These results indicated that OsALDH2B1 differentially regulated expression of the target genes.

*OsALDH2B1* had relatively high expression during the whole life cycle, while *GS3* and *AOS2* had relatively low expression

(SI Appendix, Fig. S7A). *GS3* expression was significantly negatively correlated with that of *OsALDH2B1* (SI Appendix, Fig. S7B). Particularly, *GS3* had a relatively high expression in the young panicle, and the expression decreased gradually with panicle development (SI Appendix, Fig. S7A) (43), while *OsALDH2B1* had relatively low expression in the young panicle and the transcripts gradually increased with panicle development (SI Appendix, Fig. S7A). *GS3* reduces grain length by competing with two other Gy proteins, DEP1 and GGC2, both of which increase grain size, in forming the complex with the G $\beta$  protein RGB1 in a G protein pathway (13). These results suggested that the grain size regulation of *OsALDH2B1* might be via the G protein pathway.

**OsALDH2B1 Antagonized OsBZR1 Function.** Since *OsLIC* expression is directly repressed by OsBZR1 (33) and activated by OsALDH2B1 (Fig. 3F and SI Appendix, Fig. S1D), we investigated the relationship between OsALDH2B1 and OsBZR1 in the regulation of *OsLIC* expression. We performed a transient expression assay in which we cotransformed a fixed amount (2  $\mu$ g) of *OsBZR1*-FLAG (SI Appendix, Fig. S64) with varying amounts (0.5 to 2  $\mu$ g) of *OsALDH2B1*-GFP into rice protoplast cells using *ProOsLIC*:LUC as the reporter. The LUC activity increased gradually, in accordance with the amounts of *OsALDH2B1*-GFP (SI Appendix, Fig. S6C), indicating that OsALDH2B1 antagonized the repression of OsBZR1 on *OsLIC* expression. Conversely, we also transformed a fixed amount of *OsALDH2B1*-GFP with varying amounts of *OsBZR1*-FLAG. The LUC activity gradually decreased with the increased amounts of *OsBZR1*-FLAG (SI Appendix, Fig. S6C), indicating that OsBZR1 reduced the activation function of OsALDH2B1 on *OsLIC* expression. These results suggested that OsALDH2B1 and OsBZR1 antagonized each other to mutually reduce the activity of their counterparts in regulating *OsLIC* expression.

We investigated the possibility of physical interaction between OsALDH2B1 and OsBZR1 using a bimolecular fluorescence complementation assay. The yellow fluorescent protein (YFP) signals indicated that OsALDH2B1 interacted with OsBZR1 in the nucleus (SI Appendix, Fig. S84). The interaction was further confirmed by LUC complementation activity and Co-IP (coimmunoprecipitation) assays (SI Appendix, Fig. S8 B and C), suggesting that OsALDH2B1 directly interacted with OsBZR1 in rice. We then analyzed the DNA binding activity of OsBZR1 and OsALDH2B1 in the presence of OsALDH2B1 and OsBZR1, respectively. The DNA binding activity of OsBZR1 was dramatically reduced in the presence of OsALDH2B1 (SI Appendix, Fig. S5C); in vivo assay showed that OsBZR1 displayed higher binding activity to the *OsLIC* promoter on position Q in *osaldh2b1* plants (SI Appendix, Fig. S6D), suggesting that OsALDH2B1 greatly inhibited the DNA binding activity of OsBZR1. Similarly, the DNA binding activity of OsALDH2B1 was gradually reduced with increased OsBZR1 (SI Appendix, Fig. S5D). Taken together, these results suggested that OsALDH2B1 and OsBZR1 may form a complex in inhibiting their DNA binding activity reciprocally.

**OsALDH2B1 and AOS2 Were Direct Targets of OsBZR1.** To determine whether *OsBZR1* plays a role in rice disease resistance, we inoculated *OsBZR1*-RNAi plants with the bacterial pathogen *Xoo*. This assay showed that *OsBZR1*-RNAi plants developed shorter lesion length and grew fewer bacteria relative to WT (Fig. 1G and SI Appendix, Fig. S24). Consistently, *OsBZR1*-RNAi plants accumulated a higher level of JA (SI Appendix, Fig. S2B). These results indicated that the increased disease resistance by suppressing *OsBZR1* expression was associated with activation of the JA-related signaling pathway.

OsBZR1 can directly bind to the promoter of a number of genes to regulate their expression via the BR response element (BRRE) and the E-box (enhancer-box) element (33, 44, 45).

*OsALDH2B1* functioned genetically downstream of *OsBZR1* (Fig. 1D), and *OsBZR1* expression was negatively correlated with that of *OsALDH2B1* (SI Appendix, Fig. S7B); there are two BRREs and one E-box element in the *OsALDH2B1* promoter and three BRREs in the *AOS2* promoter (Fig. 3A). These prompted us to test whether *OsBZR1* could bind to the promoters of *OsALDH2B1* and *AOS2*. We thus conducted EMSA and found that the His-TF-*OsBZR1* protein could bind to *OsALDH2B1* and *AOS2* promoters in vitro (SI Appendix, Fig. S5B). We then confirmed the binding of *OsBZR1* to *OsALDH2B1* and *AOS2* promoters using ChIP-qPCR, and the results revealed that *OsBZR1* binding to the *OsALDH2B1* promoter was enriched on positions N and O containing BRRE and E-box in the *OsALDH2B1* promoter and on positions B and C containing BRRE in the *AOS2* promoter (Fig. 3D). We then conducted transient transcriptional regulation activity assay to check the effect of *OsBZR1* on *OsALDH2B1* and *AOS2* expression with *ProOsALDH2B1:LUC* and *ProAOS2:LUC* as reporters (SI Appendix, Fig. S6A). We found that *OsBZR1* transiently repressed the LUC activity (Fig. 3G), indicating that *OsBZR1* could repress *OsALDH2B1* and *AOS2* expression. Consistent with the transient expression analyses, *OsBZR1-RNAi* plants accumulated more *OsALDH2B1* and *AOS2* transcripts relative to WT (SI Appendix, Fig. S2C). These data indicated that both *OsALDH2B1* and *AOS2* were targets of *OsBZR1*.

**OsLIC Repressed AOS2 Expression.** *OsLIC* can directly bind to the promoter of a number of genes to regulate their expression via the “TCGC” element (33). The expression of *OsLIC* was negatively correlated with, but substantially higher than, those of *GS3* and *AOS2* (SI Appendix, Fig. S6), suggesting that *OsLIC* might repress *GS3* and *AOS2* expression. There are seven *OsLIC* binding elements in the *AOS2* promoter (Fig. 3A), indicating that *OsLIC* might bind to the promoter of *AOS2*. ChIP-qPCR assays showed that *OsLIC* binding was enriched on positions B and C containing the “TCGC” element in the *AOS2* promoter (Fig. 3B). The binding of *OsLIC* to the *AOS2* promoter was further confirmed in vitro by EMSA (SI Appendix, Fig. S5E). A transient transcriptional regulation assay using *ProAOS2:LUC* as a reporter also showed that *OsLIC* transiently repressed the LUC activity (Fig. 3E). Consistent with the transient expression analysis, *OsLIC-oe* plants accumulated fewer *AOS2* transcripts than WT, while the antisense transgenic plants of *OsLIC* (*OsLIC-anti*) accumulated more *AOS2* transcripts (SI Appendix, Fig. S3D and E). These results indicated that *OsLIC* repressed *AOS2* expression.

To assess the function of *OsLIC* in rice resistance, we inoculated *OsLIC* transgenic plants with the bacterial pathogen *Xoo*. Overexpressing *OsLIC* could partially suppress the resistance of the *osaldh2b1* mutant (SI Appendix, Fig. S3F and G), while *OsLIC-anti* plants significantly reduced lesion length and bacterial growth (Fig. 1H and SI Appendix, Fig. S3B). Consistently, *OsLIC-anti* plants accumulated a higher level of JA (SI Appendix, Fig. S3D), indicating that the increased disease resistance of *OsLIC-anti* plants was associated with activation of JA biosynthesis.

To investigate whether BR has any direct effect on rice defense, we treated WT seedlings with eBL and BRz (brassinazole, a BR biosynthesis inhibitor) and inoculated the treated seedlings with *Xoo*. Seedlings treated with eBL developed increased lesion length and bacterial growth compared to mock treatment, while the reverse was the case for plants treated with BRz (SI Appendix, Fig. S9A). *AOS2* expression was suppressed by eBL treatment (SI Appendix, Fig. S9B), which is consistent with a previous report (46), while BRz induced *AOS2* expression (SI Appendix, Fig. S9B). *JAZ8*, which is induced by pathogens and JA in the JA-dependent signaling pathway, showed an expression pattern similar to *AOS2* (SI Appendix, Fig. S9B). Accordingly, the JA level was reduced with eBL treatment but increased with BRz compared to mock

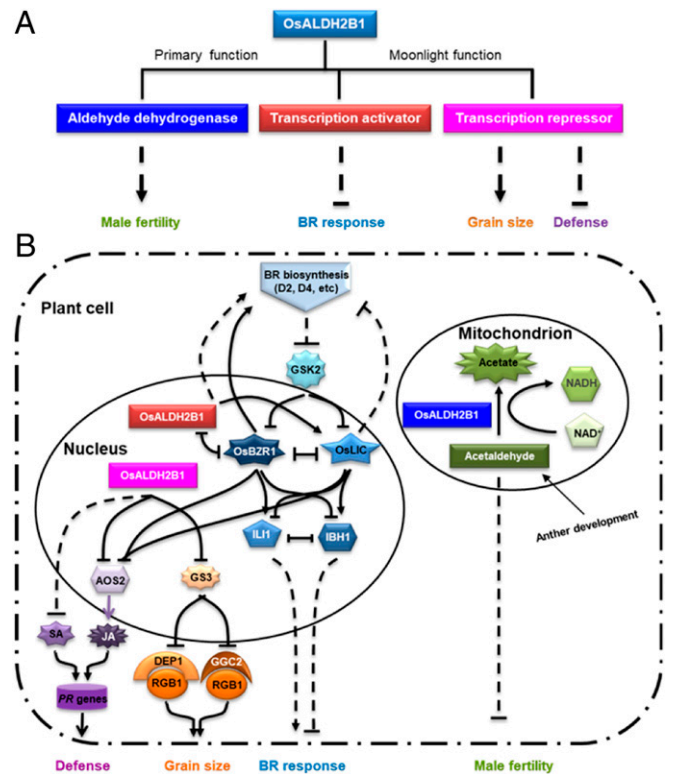
treatment (SI Appendix, Fig. S9C), suggesting that BR negatively regulates rice defense via the JA pathway.

## Discussion

We summarize the results of this work in Fig. 4 to illustrate how *OsALDH2B1* works, allowing the possibility that *OsALDH2B1* regulates different processes to be expanded in future studies.

*OsALDH2B1* possesses both primary and moonlight functions (Fig. 4A). Its primary function as a mitochondrial enzyme regulates male fertility as observed in this study. Its moonlight function is transcriptional regulation in nuclear, featuring both repressing and activating activities at both transcription and posttranscription levels, which regulates a diverse range of biological processes and pathways (Fig. 4B).

Functioning as a transcription factor, *OsALDH2B1* regulates the BR signaling pathway through both activation and repression and is also regulated by the BR pathway. *OsALDH2B1* activates *OsLIC* expression. *OsALDH2B1* is repressed by *OsBZR1* at the transcriptional level; *OsALDH2B1* and *OsBZR1* reciprocally inhibit each other by forming a complex at the protein level. Mutation in *OsALDH2B1* reduces *OsLIC* expression and increases *OsBZR1* activity, resulting in activation of the BR signaling pathway, involving genes both upstream and downstream. The net outcome indicates a negative role of *OsALDH2B1* in BR signaling. *OsALDH2B1*, *OsBZR1*, and *OsLIC* have different roles in BR signaling, but they show similar negative roles in



**Fig. 4.** A summary of the functions of *OsALDH2B1* found in this study. The light blue *OsALDH2B1* shows ALDH activity. The brown *OsALDH2B1* shows transcription activation activity. The pink *OsALDH2B1* shows transcription repression activity. Arrows and T lines indicate promoting and inhibiting effects, respectively. Solid and dotted lines indicate direct action and indirect actions, respectively. (A) The main functions of *OsALDH2B1*. (B) Pathways and processes regulated by *OsALDH2B1*. The G protein pathway, including DEP1, GGC2, and RGB1, was summarized by Sun et al. (13). The BR pathway, including D2, GSK2, IL1, and IBH1, was summarized by Zhang et al. (29).

JA signaling. Simultaneously suppressing *OsBZR1* and *OsLIC* expression and knockout of *OsALDH2B1* activate both BR and JA signaling. The perception of BRs by membrane-localized coreceptors promotes *OsBZR1* and *OsLIC* dephosphorylation and nucleus translocation (33, 47), which eventually activates the BR signaling and represses the JA signaling.

*OsALDH2B1* regulates JA signaling pathway in two ways. It directly represses the expression of *AOS2* via the transcriptional repression activity and indirectly represses the expression of *AOS2* via the BR signaling pathway through *OsBZR1* and *OsLIC*, both of which repress *AOS2* expression. Reducing the expression of *OsBZR1* and *OsLIC* increases *AOS2* transcripts, leading to JA accumulation. In addition, *OsALDH2B1* also has an effect on SA via an as yet uncharacterized pathway. Together, these impact immune response and disease resistance.

The regulation of *OsALDH2B1* on *GS3* may have a fundamental effect on growth and development. A previous study suggested that *GS3* was installed as a check to prevent grain size from becoming too large by expressing it only in the young panicle (13). The reduced expression of *OsALDH2B1* only in the young panicle triggers *GS3* to work as the check for grain size not to affect plant morphology; constitutive overexpression of *GS3* leads to a range of alterations in plant morphology such as increasing tiller number and reducing plant size, including grain size (43), which may largely account for the observed phenotype of the *osalhdh2b1* mutant.

The multiple functions of *OsALDH2B1* are thus coordinated in two fundamental ways: the biochemical versatility and dynamic expression. In particular, the relatively low expression of *OsALDH2B1* in the young panicle only results in the relatively

high expression of *GS3* to regulate grain size. Sufficient transcripts of *OsALDH2B1* in the stamen provide ALDH activity to detoxify acetaldehyde produced by ethanolic fermentation to produce fertile pollen. The dynamic expression of *OsALDH2B1* in nonreproductive organs keeps the balance of the biological processes, while alteration of its normal function causes an imbalance among defense, growth, development, and reproduction. These results may have implications for crop genetic improvement by modulating the *OsALDH2B1* activities to balance defense and growth.

## Materials and Methods

The rice variety ZH11 was used for transformation in most of this study. Rice plants were grown in the field for most of the studies and were grown in growth chambers for *Xoc* and *M. oryzae* inoculation. Details of experimental methods, including bioinformatic analysis, pathogen inoculation, subcellular localization, ALDH enzyme activity assay, systematic evolution of ligands by exponential enrichment, EMSA, transient expression assay in protoplasts, ChIP-qPCR, hormone treatment, and gene expression analysis, are given in [SI Appendix, SI Materials and Methods](#).

**Data Availability Statement.** The vectors and genetic materials will be available upon request.

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