International Journal of
Molecular Sciences

MDPI

Article

Rice *OsAAA-ATPase1* is Induced during Blast Infection in a Salicylic Acid-Dependent Manner, and Promotes Blast Fungus Resistance

Xinqiong Liu [1,*,†], Haruhiko Inoue [2,†], Xianying Tang [1], Yanping Tan [1], Xin Xu [1], Chuntai Wang [1] and Chang-Jie Jiang [2,*]

1 College of Life Science, South-Central University for Nationalities, Wuhan 430074, China; xytang@mail.scuec.edu.cn (X.T.); yanptan@mail.scuec.edu.cn (Y.T.); xinxu@mail.scuec.edu.cn (X.X.); wangchuntai@mail.scuec.edu.cn (C.W.)

2 Institute of Agrobiological Sciences (NIAS), National Agriculture and Food Research Organization (NARO), Tsukuba 305-8602, Japan; haruhiko@affrc.go.jp

* Correspondence: liuxinqiong@mail.scuec.edu.cn (X.L.); cjjiang@affrc.go.jp(C.-J.J.); Tel.: +86-189-7122-9082 (X.L.); +81-298-838-8385(C.-J.J.)

† These authors contributed equally to this work.

Received: 29 January 2020; Accepted: 18 February 2020; Published: 20 February 2020

Abstract: Fatty acids (FAs) have been implicated in signaling roles in plant defense responses. We previously reported that mutation or RNAi-knockdown (*OsSSI2*-kd) of the rice *OsSSI2* gene, encoding ta stearoyl acyl carrier protein FA desaturase (SACPD), remarkably enhanced resistance to blast fungus *Magnaporthe oryzae* and the leaf-blight bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Transcriptomic analysis identified six AAA-ATPase family genes (hereafter *OsAAA-ATPase1–6*) upregulated in the *OsSSI2*-kd plants, in addition to other well-known defense-related genes. Here, we report the functional analysis of *OsAAA-ATPase1* in rice's defense response to *M. oryzae*. Recombinant OsAAA-ATPase1 synthesized in *Escherichia coli* showed ATPase activity. *OsAAA-ATPase1* transcription was induced by exogenous treatment with a functional analogue of salicylic acid (SA), benzothiadiazole (BTH), but not by other plant hormones tested. The transcription of *OsAAA-ATPase1* was also highly induced in response to *M. oryzae* infection in an SA-dependent manner, as gene induction was significantly attenuated in a transgenic rice line expressing a bacterial gene (*nahG*) encoding salicylate hydroxylase. Overexpression of *OsAAA-ATPase1* significantly enhanced pathogenesis-related gene expression and the resistance to *M. oryzae*; conversely, RNAi-mediated suppression of this gene compromised this resistance. These results suggest that *OsAAA-APTase1* plays an important role in SA-mediated defense responses against blast fungus *M. oryzae*.

Keywords: AAA-ATPase; salicylic acid; fatty acid; rice; *Magnaporthe oryzae*; disease resistance

1. Introduction

Salicylic acid (SA) plays an important signaling role in plant defense activation against pathogens. In response to pathogen attack, SA activates a battery of defense-related genes, including pathogenesis-related (PR) genes, throughout the plant, resulting in both local and systemic resistance to the pathogen [1]. In *Arabidopsis*, NPR1 (non-pathogenesis related 1) has been demonstrated to play a master role in SA-mediated defense activation [2,3]. A loss of NPR1 function (npr1) results in loss of PR gene induction, and hypersensitivity to diseases [4]. In rice, meanwhile, it has been shown that SA signaling is mediated by two downstream factors, OsNPR1 and WRKY45, acting in parallel [5,6].

In *Arabidopsis*, genetic screening for mutations that can suppress *npr1* phenotypes (based on their ability to restore SA-induced PR expression to npr1-5 plants) resulted in isolation of several *npr1* suppressor mutants (*ssi*: suppressor of SA insensitivity), which exhibit constitutive defense activation [7]. Map-based cloning of one of the *ssi* mutants (*ssi2*) revealed that the corresponding gene (*SSI2*) encodes a stearoyl-ACP desaturase, which desaturates stearoyl (18:0)-ACP into oleoyl-ACP, and finally, into oleic acid (18:1) [7]. Disruption of this gene in *ssi2* results in a ten-fold increase in 18:0 fatty acid (FA) content, indicating involvement of FAs in plant defense reactions [7]. The *ssi2* mutant plants accumulate high levels of SA and display constitutive PR gene expression and enhanced resistance to *Peronospora parasitica*, *Pseudomonas syringae* [8], and *Cucumber mosaic virus* [7,9,10].

The orthologs of *SSI2* have also been identified in soybean (*GmSACPD-A/-B*) [11], rice (*OsSSI2*) [12], and wheat (*TaSSI2*) [13,14]. Similar to *Arabidopsis ssi2*, suppression of these ortholog genes enhanced resistance to multiple pathogens: *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae* in soybean [11]; blast fungus *Magnaporthe oryzae* and leaf-blight bacteria *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in rice [12]; and powdery mildew bacteria *Blumeria graminis* f. sp. *tritici* and Fusarium head blight fungus *Fusarium graminearum* in wheat [13,14]. These results demonstrate a common function of *SSI2* and its orthologs in defense activation in diverse plant species.

The molecular mechanisms whereby the *SSI2* family genes participate in defense reactions in plants remain to be fully elucidated. In rice, a DNA microarray analysis revealed several hundred genes differentially expressed between the wild-type and *OsSSI2*-suppressed transgenic (*OsSSI2*-kd) plants [12]. Among them was a group of six genes for AAA-ATPase (AAA: ATPases associated with diverse cellular activities) highly upregulated in *OsSSI2*-kd plants, in addition to the well-known defense-related genes, such as *WRKY45*, *PR1b*, and *PBZ1*, and a *thaumatin-like* gene [12]. These results suggest that the AAA-ATPase family genes may play important roles in defense activation in rice plants.

The AAA-ATPase family occurs in all life forms, including eukaryotes, prokaryotes, and archaebacteria, and is implicated in a variety of cellular activities, including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication, and immune responses [15–17]. Structurally, these proteins contain one or several conserved motifs, including the Walker A and Walker B motifs, which are, respectively, required for ATP binding and hydrolysis; they also contain a highly conserved amino acid sequence, referred to as the second region of homology (SRH) [16]. In plants, it has been reported that AAA-ATPase genes from *Nicotiana tabacum* (*NtAAA1*) [18,19] and *Arabidopsis* (*AtOM66*) [20] are, respectively, negatively or positively involved in the SA-signaling pathway and in the hypersensitive response (HR) upon pathogen infections. Moreover, in rice, map-based cloning of the *lesion mimic resembling* (*lmr*) mutant/*lesion resembling disease* (*lrd6-6*) mutant revealed that the corresponding gene (*LMR/LRD6-6, Os06g0130000*) encodes an AAA-ATPase, and is negatively involved in HR and disease resistance [21,22].

In this study, we conducted a functional analysis of *OsAAA-ATPase1*, one of the six AAA-ATPase genes upregulated in *OsSSI2*-kd rice plants [12]. We show that *OsAAA-ATPase1* is transcriptionally regulated by SA, and positively involved in resistance to blast fugus *M. oryzae*.

2. Results

In our previous study, a group of six AAA-ATPase family genes (hereafter *OsAAA-ATPase1–6*; (Table 1; Figure 1) was found to be significantly upregulated in *OsSSI2*-kd rice plants [12], implicating these genes in rice defense activation. From among them, we chose *OsAAA-ATPase1* for more detailed functional characterization in this study, because it showed SA-induced (Figure 2) and SA-dependent blast-induced (Figure 3) transcription responses.

Table 1. Genes and primer sequences used for qRT-PCR analysis.

Gene Name (RAP+DB ID)	Primer Sequences (5′→3′)	References
OsAAA-ATPase1 Os03g0802500	AGTGGTTGCTAGCTTCTCGT ACAACATGTGGTCAAATTATTCCA	[12]
OsAAA-ATPase2 Os01g0297200	CTAGGTTCTGCGATGGACAC CTCCTTTGCAATTGTTCCAC	[12]
OsAAA-ATPase3 Os06g0697600	GTTGTGATCGTGTCATGGTTGCG CAGAAAGCCACACACCATTGC	[12]
OsAAA-ATPase4 Os02g0697600	TTGCCTGAACGGCCAGGTGAT CCCATGTAAGGGTAAGGATTGC	[12]
OsAAA-ATPase5 Os02g0706500	GTTCCATCTCTTTGCCTGTAGC CATGCGCATCTCAGTCTTACC	[12]
OsAAA-ATPase6 Os07g0517600	TCAGTGGCCTCGTCGAGTTC CTACTTGCCTGCTTCACACAT	[12]
OsPR1b Os01g0382000	ACGGGCGTACGTACTGGCTA CTCGGTATGGACCGTGAAG	[23]
PBZ1 Os12g0555000	GCGTTTGAGTCCGTGAGAGT TCACCCATTGATGAAGCAAA	[24]
Rubq1 Os06g0681400	GGAGCTGCTGCTGTTCTAGG TTCAGACACCATCAAACCAGA	[25]
M. oryzae 28S rDNA	ACGAGAGGAACCGCTCATTCAGATAATT TCAGCAGATCGTAACGATAAAGCTACTC	[26]

2.1. *OsAAA-ATPase1 Encodes an AAA-ATPase Family Protein*

OsAAA-ATPase1 was predicted to encode a protein of 520 amino acids with a predicted mass of 58.1 kDa. OsAAA-ATPase1 shared 50%, 37.8%, and 22.3% amino acid sequence identity with previously reported defense-related AAA-ATPase proteins, namely, tobacco NtAAA1 [18], *Arabidopsis* AtOM66 [20], and rice LMR/LRD6-6 [21,22], respectively. Structural analysis revealed that OsAAA-ATPase1 contains consensus motifs that are typical of the AAA-ATPase family; these include the Walker A, Walker B, and SRH motifs (pfam00004; E-value = 4.95×10^{-17}) (Figure 1a).

To assess how *OsAAA-ATPase1–6* are related within the AAA-ATPase gene family, we performed a phylogenic comparison of the proteins predicted to be encoded by *OsAAA-ATPase1–6* and several known rice AAA-ATPase proteins, including LMR/LRD6-6 [21,22], OsCDC48 [27], RuvBL1a [28], RLS3 [29], OsSKD1 [30], OsFtsH5 [31], and RFC5 [32], together with NtAAA1 [18,19] and AtOM66 [20]. OsAAA-ATPase1 was grouped within a subclade of proteins related to plant defense, including OsAAA-ATPase2–6, NtAAA1, and AtOM66, but, unexpectedly, distally with LMR/LRD6-6 (Figure 1b).

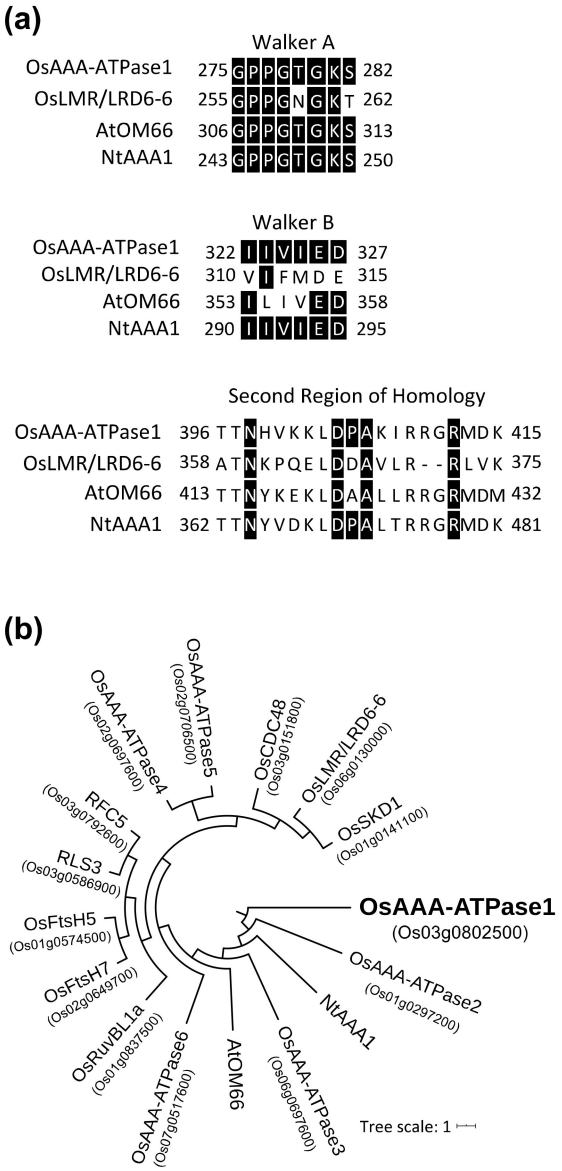

Figure 1. Amino acid sequence alignment of AAA-type ATPase proteins, and phylogenetic analysis. (**a**) Alignment of typical consensus motifs of the AAA-ATPase protein family, including the Walker A, Walker B, and SRH motifs. (**b**) Phylogeny of the AAA-ATPase proteins from rice (LMR/LRD6-6, OsCDC48, RuvBL1a, RLS3, OsSKD1, OsFtsH5, and RFC5), tobacco (NtAAA1), and *Arabidopsis* (AtOM66).

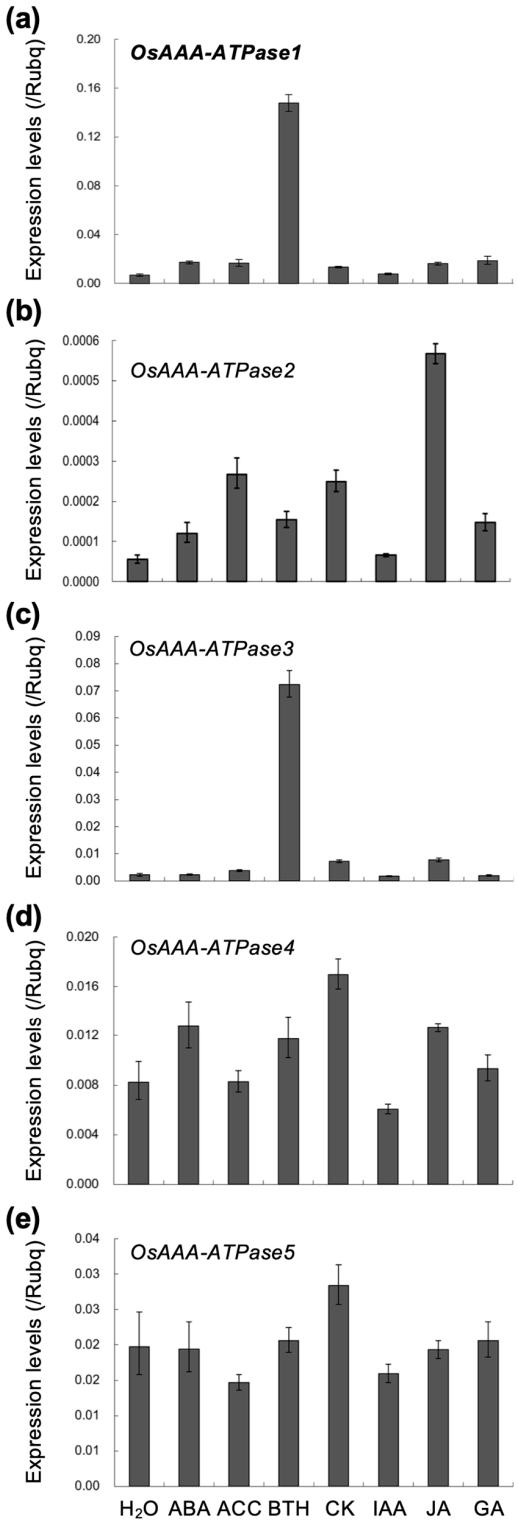

Figure 2. Expression analysis of *OsAAA-ATPase1–5* (**a**–**e**) in response to the plant hormones abscisic acid (ABA), ethylene (ACC, an ethylene precursor), benzothiadiazole (BTH, a functional analogue of SA), kinetin (CK, a synthetic cytokinin), auxin (IAA), jasmonic acid (JA), and gibberellic acid (GA), in Nipponbare rice seedlings. Data are represented as means ± SDs.

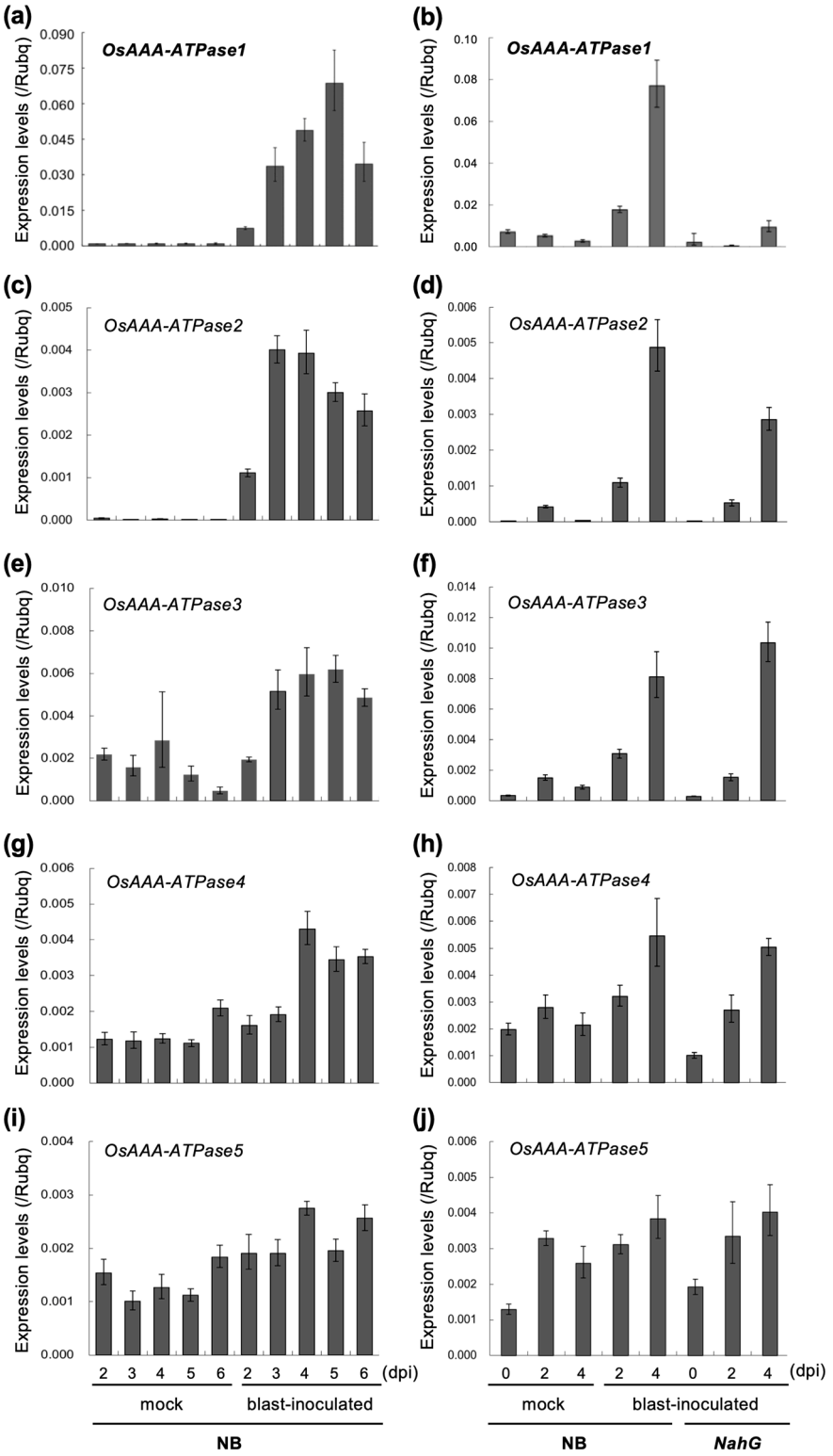

Figure 3. Expression analysis of *OsAAA-ATPase1–5* (**a**–**j**) in response to blast inoculation in Nipponbare (NB) and *narG* rice seedlings. Rice seedlings at the four-leaf stage (three true leaves) were subjected to mock treatment (mock) or blast inoculation (blast-inoculated), and the fourth leaf blades were sampled at indicated days post inoculation (dpi). Data are represented as means ± SDs.

2.2. OsAAA-ATPase1 is Induced by SA Treatment

Plant hormones have been demonstrated to play important roles in interactions between plants and pathogens. Hence, we examined the transcriptional responses of *OsAAA-ATPase1–6* to the plant hormones abscisic acid (ABA), ACC (an ethylene precursor), BTH (a functional analogue of SA), kinetin (CK, a synthetic cytokinin), auxin (IAA), jasmonic acid (JA), and gibberellic acid (GA).

OsAAA-ATPase1 (Figure 2a) and *OsAAA-ATPase3* (Figure 2c) were induced specifically by BTH treatment, and *OsAAA-ATPase2* (Figure 2b) was induced by JA treatment. Meanwhile, *OsAAA-ATPase4* (Figure 2d) and *OsAAA-ATPase5* (Figure 2e) were not specifically induced by any of the hormones, and *OsAAA-ATPase6* (*Os07g0517600*) had no detectable transcription.

2.3. OsAAA-ATPase1 is Induced in Response to Blast Infection in An SA-Dependent Manner

Rice seedlings of non-transformant Nipponbare rice (NB) and of NB expressing the *nahG* gene (*nahG*-rice), at the four-leaf stage, were subjected to blast inoculation. At 2–6 days post inoculation (dpi) of the blast, the fourth leaves were sampled to examine the expression of *OsAAA-ATPase1–5*.

All of the tested *OsAAA-ATPase* genes clearly showed transcriptional induction in response to blast inoculation (Figure 3c,e,g,i); in particular, *OsAAA-ATPase1* (Figure 3a) and *OsAAA-ATPase2* (Figure 3c) showed a high-fold transcriptional increase from the very low basal levels in the mock treatment. The induction of the genes became evident from 2 dpi and peaked at ca. 3–5 dpi.

In *nahG*-rice plants, in contrast, the induction of *OsAAA-ATPase1* was mostly attenuated relative to its induction in NB plants in response to blast inoculation (Figure 3b), demonstrating that the induction of this gene depends on the SA-signaling pathway. No appreciable attenuation of gene induction was observed for the other genes (Figure 3d,f,h,j).

2.4. OsAAA-ATPase1 is Positively Involved in Blast Resistance

To gain some insight into the role of *OsAAA-ATPase1* in disease resistance, we generated transgenic rice lines that either overexpressed the gene under maize ubiquitin promoter (*OsAAA-ATPase1*-ox; Figure 4a) or RNAi-suppressed *OsAAA-ATPase1* (*OsAAA-ATPase1*-kd; Figure 5a), and subjected these lines to blast inoculation. In order to reveal the potentially compromised resistance in *OsAAA-ATPase1*-kd plants, a half density of conidia (5×10^4/mL) was used, so as to cause blast disease moderately in NB, but more severely in OsAAA-ATPase1 plants.

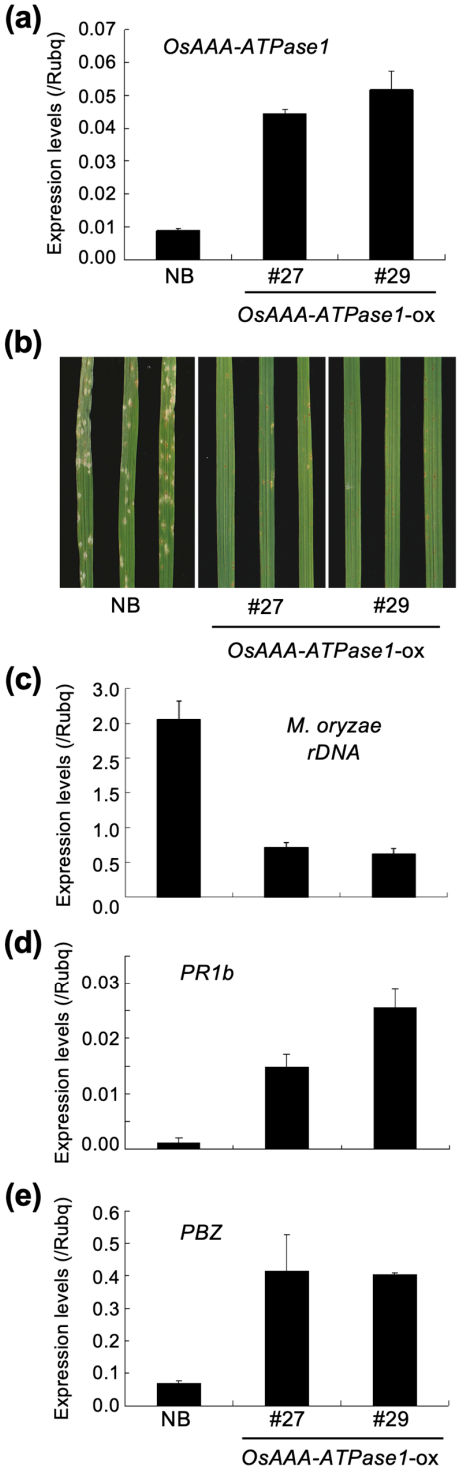

Figure 4. Blast resistance of *OsAAA-ATPase1*-ox plants. (**a**) Expression of *OsAAA-ATPase1*, (**b**) blast lesions on leaf blades, and (**c**) relative fungal growth (*Magnaporthe oryzae rDNA*). (**d**,**e**) Expression of *PR1b* (**d**) and *PBZ1* (**e**), in Nipponbare (NB) and *OsAAA-ATPase1*-ox lines (#27 and #29), respectively, at 7 days post inoculation (dpi). Data are represented as means ± SDs in (**a**,**c**–**e**).

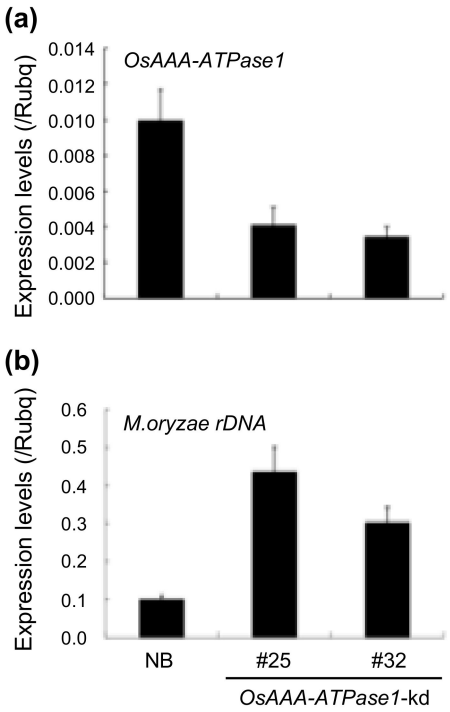

Figure 5. Compromised blast resistance in *OsAAA-ATPase1*-kd plants (#25 and #32). (**a**) Expression of *OsAAA-ATPase1*. (**b**) Relative fungal growth (*M. oryzae rDNA*) in Nipponbare (NB) and *OsAAA-ATPase1*-kd lines (#25 and #32) respectively. Data are represented as means ± SDs.

Compared with the non-transgenic control plants (NB), *OsAAA-ATPase1*-ox plants (lines #27 and #29) exhibited significantly higher resistance to blast disease, as evidenced by the fact that few susceptible blast lesions appeared on their leaf blades (Figure 4b), and that they had ca. 4-fold less fungal growth (Figure 4c). The enhanced resistance of the *OsAAA-ATPase1*-ox plants is consistent with the large increases in the expression levels of the PR genes, *OsPR1* and *PBZ1* (Figure 4d,e).

Conversely, blast resistance was significantly compromised in *OsAAA-ATPase1*-kd plants (lines #25 and #32): they had ca. 2-fold more fungal growth than the NB control plants (Figure 5b).

2.5. OsAAA-ATPase1 Has ATPase Activity and Is Localized in the Cytosol

To assess whether OsAAA-ATPase1 protein has ATPase activity, *OsAAA-ATPase1* N-terminal was fused to a His-tag and expressed in *Escherichia coli*, and purified using a high affinity Ni-resin. OsAAA-ATPase1 protein showed an ATPase activity level that was comparable to that of the positive control (potato ATPase) (Figure 6).

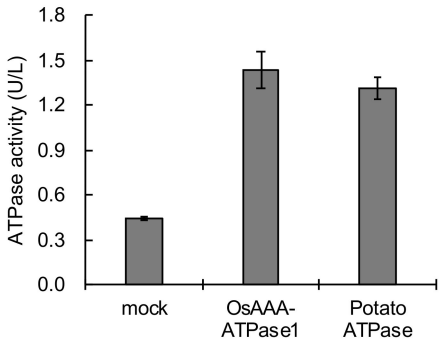

Figure 6. ATPase activity of recombinant OsAAA-ATPase1 protein. Elution buffer of Ni-resin (mock treatment), and an ATPase protein from potatoes, were used negative and positive controls, respectively.

To determine the subcellular localization of OsAAA-ATPase1 in rice cells, the EGFP-OsAAA-ATPase1 fusion protein in the rice protoplast was examined under a confocal microscope. As shown in Figure 7, EGFP-OsAAA-ATPase1 protein was co-localized with a cytosol marker, mCherry signals, indicating that OsAAA-ATPase is predominantly distributed in the cytosol.

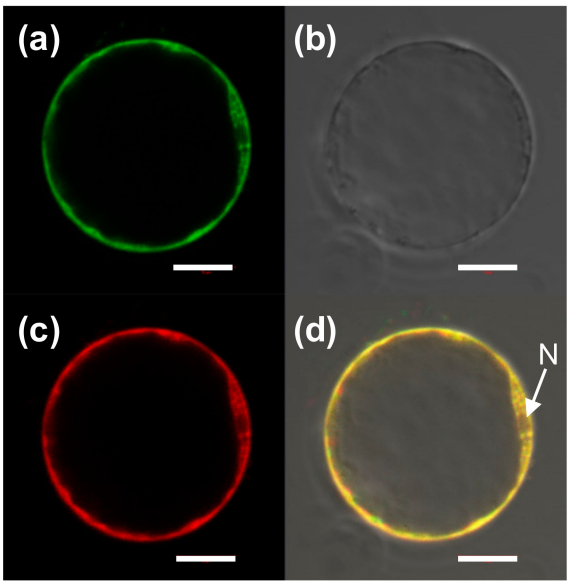

Figure 7. Subcellular localization of OsAAA-ATPase1 in rice protoplasts. (**a**) EGFP-OsAAA-ATPase1, (**b**) blight field image, (**c**) mCherry (cytoplasmic localization), and (**d**) combined image of (**a**–**c**). N, nucleus; bar, 20 μm.

3. Discussion

AAA-type ATPases constitute a large protein family in a diverse range of organisms, and thus exhibit multiple and diverse cellular functions [15,33]. In plants, AAA-ATPase genes have been implicated in proteolysis [33], male meiosis [34], vacuolar maintenance [35], peroxisome biogenesis [36], morphogenesis [37], leaf senescence [29,38], and stress [28,39] and immune responses [18–22]. In this study, we present a novel rice AAA-ATPase gene member, *OsAAA-ATPase1*. The deduced amino acid sequence of OsAAA-ATPase1 contains consensus motifs that are typical of the AAA-ATPase family; these include the Walker A, Walker B, and SRH motifs (Figure 1a) [15,17,33]. Consistent with this, biochemical analysis confirmed that there was ATPase activity in the recombinant protein of OsAAA-ATPase1 (Figure 6). Phylogenetically, OsAAA-ATPase1 was grouped within a subclade of proteins related to plant defense activation (Figure 1b), which included OsAAA-ATPase2–6 [12], tobacco NtAAA1 [18,19], and *Arabidopsis* AtOM66 [20]. These results suggest that OsAAA-ATPase1 belongs to the AAA-ATPase family. Functional analysis revealed that *OsAAA-ATPase1* is transcriptionally regulated by SA in response to blast infection (Figures 2 and 3). Overexpression or RNAi-mediated suppression of *OsAAA-ATPase1* resulted, respectively, in an increase (Figure 4) or decrease (Figure 5) in blast resistance. Taken together, our results suggest that *OsAAA-ATPase1* plays a positive role in the SA-mediated disease resistance in rice plants.

In relation to plant immune responses, several studies have shown important roles for AAA-ATPase genes. *NtAAA1* was isolated as an HR-induced gene in *Nicotiana tabacum* [18]; was found to be under the control of *N*-gene, ethylene, and jasmonate; and was localized in the cytoplasm. It was also negatively involved in the SA-signaling pathway and pathogen resistance [18,19]. In contrast, *AtOM66* (outer mitochondrial membrane protein of 66 kDa) is a stress-induced gene; overexpression of this gene increased SA content, accelerated cell death rates, and enhanced resistance to the biotrophic pathogen *Pseudomonas syringae* [20]. Recently, rice *LMR* and *LRD6-6* were map-based cloned from lesion mimic mutants *lmr* and *lrd6-6*, respectively, and were found to be the same gene (*Os06g0130000*).

LMR/LRD6-6 was shown to be localized in the multivesicular bodies (MVBs) and was negatively involved in rice immunity and cell death [21,22]. Mutation in this gene (*lmr* and *lrd6-6*) resulted in constitutive expression of *PR1* and *PBZ1*, and enhanced resistance to rice blast and bacterial blight diseases; however, no difference in SA content was determined [21,22]. By comparison, it seems that OsAAA-ATPase1 plays a role distinct from those previously reported, with respect to its association with SA-regulation and HR, its subcellular localization, and its promotion of disease resistance. Thus, our findings provide novel insights into SA-regulated defense activation in rice. Meanwhile, OsAAA-ATPase1 showed a close phylogenetic association with AtOM66 (Figure 1b); both proteins play a positive role in the SA-signaling pathway, suggesting that they may share a common cellular function.

Plants produce a variety of FAs and their derivatives, some of which have been shown to play important roles in defense activation [40,41]. In the *Arabidopsis ssi2* mutant, disruption of *SSI2*, which encodes an FA desaturase, results in an increase in the 18:0 FA content, which in turn remarkably increases SA content, PR gene expression, and resistance against multiple pathogens [42]. Similar defense-related phenotypes were observed following suppression of *SSI2*-orthologs in soybean (*GmSACPD-A/-B*) [11], rice (*OsSSI2*) [12], and wheat (*TaSSI2*) [13,14]. These results strongly suggest that *SSI2* and its orthologs serve as valuable susceptibility gene (*S* gene) resources for the development of crop cultivars with resistance to multiple pathogens, by employing targeted mutation and genome editing technologies [43–45]. In order to make such successful use of these genes in resistance breeding, it is important to understand the molecular mechanisms underlying the defense activation. In *Arabidopsis*, a mutation in the GTPase nitric oxide associated 1 (*NOA1*) gene partially restored the *ssi2* phenotype, whereas double mutations in NOA1 and either one of the two nitrate reductase isoforms (NIA1 and NIA2) completely restored the *ssi2* phenotypes; this indicates that nitric oxide (NO) is required for constitutive defense in the *ssi2* mutant [46,47]. Nevertheless, little has been reported regarding the molecular basis of defense activation in *OsSSI2*-kd rice plants. We previously identified a group of six AAA-ATPase genes (*OsAAA-ATPase1–6*) that were upregulated in *OsSSI2*-kd rice plants [12]. In this study, all of these genes tested were induced in response to blast inoculation (Figure 3), suggesting that they each play a role in resistance to blast fungus. In contrast, *OsAAA-ATPase1–5* each exhibited a distinct induction pattern in response to different plant hormone treatments (Figure 2); *OsAAA-ATPase1* and *OsAAA-ATPase3* were induced by SA, *OsAAA-ATPase2* mainly by JA, and *OsAAA-ATPase4* and *OsAAA-ATPase5* slightly by the CK treatment. These results suggest that there is functional differentiation among the *OsAAA-ATPase1–6* genes downstream of *OsSSI2* in disease resistance. Moreover, although both *OsAAA-ATPase1* and *OsAAA-ATPase3* were induced by SA treatment, only the induction of *OsAAA-ATPase1* was attenuated following blast infection in *nahG*-rice plants (Figure 3). One possible explanation for this is that *OsAAA-ATPase3* may be more sensitive to SA, allowing it to be induced even by a residual increase in the SA-signaling level in *nahG*-rice plants.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Japonica type rice cultivar Nipponbare (*Oryza sativa* L.) was grown in commercial nursery soil (Bonsol Number 2; Sumitomo Chemical Corp., Tokyo, Japan) in a greenhouse at 28 °C (day)/23 ° C (night) with ca. 50% relative humidity.

4.2. Plasmid DNA Construction and Rice Transformation

The cDNA clone for *OsAAA-ATPase1* was provided by the Rice Genome Resource Center, Japan (accession number: AK070731). To construct a plasmid for constitutive expression of *OsAAA-ATPase1* under the maize ubiquitin promoter, a DNA fragment containing a 91 bp upstream sequence followed by the full coding sequence of *OsAAA-ATPase1* (nucleotides 2–1655) was amplified by PCR and cloned into the pUCAP/Ubi-NT vector, as previously described [5]. To construct a plasmid for *OsAAA-ATPase1*

RNAi (*OsAAA-ATPase1*-kd), part of the 3′-UTR (nucleotides 1543–1845) of *OsAAA-ATPase1* cDNA was amplified by PCR and cloned into the pANDA vector, as previously described [48,49].

Nipponbare rice plants were transformed by an *Agrobacterium tumefaciens* (strain EHA105) mediated technique, as described earlier [50]. Transgenic rice lines expressing *nahG* from *Pseudomonas putida* under the control of a double 35S promoter (*nahG*-rice) were generated using the plant expression construct previously described in Yang et al. [51].

4.3. Chemical Treatments

All stock solutions were prepared at a concentration of 100 mmol/L. Indole-3-acetic acid (IAA; Sigma, St. Louis, MO, USA), gibberellin A3 (GA_3; Wako, Osaka, Japan), abscisic acid ((±)-*cis-trans*, ABA; Sigma), and methyl jasmonate (ME-JA; Wako, Saitama, Japan) were dissolved in ethanol. Kinetin (Sigma) and benzothiadiazole *S*-methyl ester (BTH; Wako) were dissolved in dimethyl sulfoxide (DMSO); and 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma) and sodium salicylate (SA; Nacalai Tesque, Tokyo, Japan) were dissolved in H_2O. The solvents did not exceed a final concentration of 0.1% in the solutions used for plant treatments, and had no effect on the expression of the rice genes examined in this study.

For plant treatments, rice seedlings at the four-leaf stage (three true leaves) were transferred to a container containing each of the plant hormone solutions at 50 μM. The rice seedlings were further grown for 1 day, and fourth leaf blades were stored in liquid nitrogen for RNA preparation.

4.4. Protein Expression, Purification, and ATPase Assay

The *OsAAA-ATPase1* sequence was amplified by PCR and cloned into the sites between BglI and HidIIIp of pET32a (Novagen) as a His-tag fusion protein; then, that was transfected into the *Escherichia coli* Origami strain BL21(Lys). The set of primers used was as follows: OsAAA1BglII 5′-GTAGATCTCTTGAGACAAATGGAGGCGACG-3′; OsAAA1HindIII 5′-GCTAAGCTTCTACTTATCCTTCCCGACCAC-3′. Expression of the protein was induced for 4 h at 25 °C with 0.5 mmol/L isopropyl β-D-1-thiogalactopyranoside. *Escherichia coli* cells were pelleted by centrifugation, resuspended in lysis buffer (20 mmol/L Tris-HCl pH 7.4, 0.1 M NaCl, 10 mmol/L imidazole), and sonicated. After the cell debris was removed by centrifugation (12,000 × g, 10 min, 4 °C), the supernatant was loaded onto a High Affinity Ni-Charged Resin (GE Healthcare, Buckinghamshire, UK), washed with washing buffer (20 mmol/L Tris-HCl pH 7.4, 0.1 M NaCl, 10 mmol/L imidazole), and eluted with elution buffer (20 mmol/L Tris-HCl pH 7.4, 0.1 M NaCl, 180 mmol/L imidazole). ATPase activity was measured by the malachite green-based colorimetric method using the QuantiChrom™ ATPase/GTPase activity assay kit (Sigma-Aldrich, St. Louis, MO, USA). The elution buffer was used as the negative control, and an ATPase from potatoes (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control. One unit is defined as the amount of enzyme that catalyzes the production of 1 μM of free phosphate per minute under the assay conditions.

4.5. Subcellular Localization

For subcellular localization of OsAAA-ATPase1, the plasmid pSAT6-AFP-C1-OsAAA1 was transformed into protoplasts prepared from etiolated seedlings as previously described [52]. As a control for cytoplasmic localization, the pSAT-mCherry construct was co-transformed. Fluorescence was examined under a confocal microscope (Leica Microsystems, Wetzlar, Germany) 16 h after transformation.

4.6. Pathogen Culture and Inoculations

Culture and inoculation of the blast fungus *M. oryzae* (compatible race 007.0) was conducted essentially as previously described [12], with slight modifications. Briefly, the fungus was grown on an oatmeal agar medium (30 g/L oatmeal, 5 g/L sucrose, and 16 g/L agar) at 26 °C for 10–12 day. After removing the aerial hyphae by washing with distilled water and a brush, conidia formation was

induced by irradiation under continuous black blue light (FL15BLB; Toshiba, Osaka, Japan) at 24 °C for 3 day. The conidia were suspended in 0.02% Silwet L-77 (a non-ionic surfactant; Nihon Unica, Tokyo, Japan) at a density of 10^5/mL, and were sprayed onto rice plants at the four-leaf stage. After incubation in a dew chamber at 24 °C for 24 h, the rice plants were moved back to the greenhouse.

Disease development was evaluated by determining the *M. oryzae* genomic 28S *rDNA* [26] by qRT-PCR [5,6], 6–7 dpi. At least 20 plants were used for each disease assay.

4.7. RNA Analyses

Total RNA was isolated from leaf blades of the 4th leaves of rice seedlings using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR (qRT-PCR) was performed on a Thermal Cycler Dice TP800 system (Takara Bio, Tokyo, Japan) using SYBR premix Ex Taq mixture (Takara Bio) as previously described [5]. The primer sequences used for qRT-PCR are listed in Table 1.

4.8. Amino Acid Sequence Alignment and Phylogenetic Analysis

The protein sequences were retrieved from the rice annotation project database (rap-db) and aligned using Clustal-X software, and the tree was constructed using iTOL software [53].

5. Conclusions

In this study, we present a novel AAA-ATPase member gene, *OsAAA-ATPase1*, one of the six AAA-ATPase genes upregulated in *OsSSI2*-kd rice plants [12]. Functional analysis revealed that *OsAAA-ATPase1* is transcriptionally regulated by SA, and plays a positive role in the SA-mediated disease resistance in rice plants. Our findings provide novel insights into SA-regulated defense activation in rice, and the molecular basis of defense activation in *OsSSI2*-kd rice plants.

Author Contributions: Conceived and designed the experiments: X.L. and C.-J.J. Performed the experiments and analyzed the data: X.L., H.I., X.T., Y.T., X.X., and C.W. Interpreted and wrote the manuscript, H.I. and C.-J.J. All authors have read and agreed to the manuscript as written.

Funding: This work was supported by the National Natural Science Foundation of China (31370306), and grants from Japan's Society for the Promotion of Science (JSPS) KAKENHI to C.J. (19K06065) and to H.I. (17K07678).

Acknowledgments: We thank the NARO Genebank for providing the cDNA clone of *OsAAA-ATPase1* (*Os03g0802500*).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Klessig, D.F.; Choi, H.W.; Dempsey, D.M.A. Systemic acquired resistance and salicylic acid: Past, present, and future. *Mol. Plant Microbe Interact.* **2018**, *31*, 871–888. [CrossRef] [PubMed]
2. Dong, X. NPR1, all things considered. *Curr. Opin. Plant Biol.* **2004**, *7*, 547–552. [CrossRef]
3. Wang, D.; Amornsiripanitch, N.; Dong, X. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *Plos Pathog.* **2006**, *2*, e123. [CrossRef]
4. Cao, H.; Glazebrook, J.; Clarke, J.D.; Volko, S.; Dong, X. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **1997**, *88*, 57–63. [CrossRef]
5. Shimono, M.; Sugano, S.; Nakayama, A.; Jiang, C.J.; Ono, K.; Toki, S.; Takatsuji, H. Rice *WRKY45* plays a crucial role in benzothiadiazole-inducible blast resistance. *Plant Cell* **2007**, *19*, 2064–2076. [CrossRef]
6. Sugano, S.; Jiang, C.J.; Miyazawa, S.; Masumoto, C.; Yazawa, K.; Hayashi, N.; Shimono, M.; Nakayama, A.; Miyao, M.; Takatsuji, H. Role of OsNPR1 in rice defense program as revealed by genome-wide expression analysis. *Plant Mol. Biol.* **2010**, *74*, 549–562. [CrossRef]
7. Kachroo, P.; Shanklin, J.; Shah, J.; Whittle, E.J.; Klessig, D.F. A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9448–9453. [CrossRef]
8. Shah, J.; Kachroo, P.; Nandi, A.; Klessig, D.F. A recessive mutation in the *Arabidopsis SSI2* gene confers SA- and *NPR1*-independent expression of *PR* genes and resistance against bacterial and oomycete pathogens. *Plant J.* **2001**, *25*, 563–574. [CrossRef] [PubMed]

9. Kachroo, P.; Kachroo, A.; Lapchyk, L.; Hildebrand, D.; Klessig, D.F. Restoration of defective cross talk in *ssi2* mutants: Role of salicylic acid, jasmonic acid, and fatty acids in *SSI2*-mediated signaling. *Mol. Plant Microbe Interact.* **2003**, *16*, 1022–1029. [CrossRef] [PubMed]
10. Sekine, K.T.; Nandi, A.; Ishihara, T.; Hase, S.; Ikegami, M.; Shah, J.; Takahashi, H. Enhanced resistance to *Cucumber mosaic virus* in the *Arabidopsis thaliana ssi2* mutant is mediated via an SA-independent mechanism. *Mol. Plant Microbe Interact.* **2004**, *17*, 623–632. [CrossRef]
11. Kachroo, A.; Fu, D.Q.; Havens, W.; Navarre, D.; Kachroo, P.; Ghabrial, S.A. An oleic acid-mediated pathway induces constitutive defense signaling and enhanced resistance to multiple pathogens in soybean. *Mol. Plant Microbe Interact.* **2008**, *21*, 564–575. [CrossRef] [PubMed]
12. Jiang, C.J.; Shimono, M.; Maeda, S.; Inoue, H.; Mori, M.; Hasegawa, M.; Sugano, S.; Takatsuji, H. Suppression of the rice fatty-acid desaturase gene *OsSSI2* enhances resistance to blast and leaf blight diseases in rice. *Mol. Plant Microbe Interact.* **2009**, *22*, 820–829. [CrossRef] [PubMed]
13. HU, L.-q.; MU, J.-j.; SU, P.-s.; WU, H.-y.; YU, G.-h.; WANG, G.-p.; Liang, W.; Xin, M.; LI, A.-f.; WANG, H.-w. Multi-functional roles of *TaSSI2* involved in Fusarium head blight and powdery mildew resistance and drought tolerance. *J. Integr. Agr.* **2018**, *17*, 368–380. [CrossRef]
14. Song, N.; Hu, Z.; Li, Y.; Li, C.; Peng, F.; Yao, Y.; Peng, H.; Ni, Z.; Xie, C.; Sun, Q. Overexpression of a wheat stearoyl-ACP desaturase (SACPD) gene *TaSSI2* in *Arabidopsis ssi2* mutant compromise its resistance to powdery mildew. *Gene* **2013**, *524*, 220–227. [CrossRef] [PubMed]
15. Vale, R.D. AAA proteins. Lords of the ring. *J. Cell Biol.* **2000**, *150*, F13–F19. [CrossRef] [PubMed]
16. Yedidi, R.S.; Wendler, P.; Enenkel, C. AAA-ATPases in protein degradation. *Front. Mol. Biosci.* **2017**, *4*, 42. [CrossRef]
17. Snider, J.; Houry, W.A. AAA+ proteins: Diversity in function, similarity in structure. *Biochem. Soc. Trans.* **2008**, *36*, 72–77. [CrossRef]
18. Sugimoto, M.; Yamaguchi, Y.; Nakamura, K.; Tatsumi, Y.; Sano, H. A hypersensitive response-induced ATPase associated with various cellular activities (AAA) protein from tobacco plants. *Plant Mol. Biol.* **2004**, *56*, 973–985. [CrossRef]
19. Lee, M.H.; Sano, H. Attenuation of the hypersensitive response by an ATPase associated with various cellular activities (AAA) protein through suppression of a small GTPase, ADP ribosylation factor, in tobacco plants. *Plant J.* **2007**, *51*, 127–139. [CrossRef]
20. Zhang, B.; Van Aken, O.; Thatcher, L.; De Clercq, I.; Duncan, O.; Law, S.R.; Murcha, M.W.; Van der Merwe, M.; Seifi, H.S.; Carrie, C. The mitochondrial outer membrane AAA ATPase AtOM66 affects cell death and pathogen resistance in *Arabidopsis thaliana*. *Plant J.* **2014**, *80*, 709–727. [CrossRef]
21. Zhu, X.; Yin, J.; Liang, S.; Liang, R.; Zhou, X.; Chen, Z.; Zhao, W.; Wang, J.; Li, W.; He, M. The multivesicular bodies (MVBs)-localized AAA ATPase LRD6-6 inhibits immunity and cell death likely through regulating MVBs-mediated vesicular trafficking in rice. *Plos Genet.* **2016**, 12. [CrossRef] [PubMed]
22. Fekih, R.; Tamiru, M.; Kanzaki, H.; Abe, A.; Yoshida, K.; Kanzaki, E.; Saitoh, H.; Takagi, H.; Natsume, S.; Undan, J.R. The rice (*Oryza sativa* L.) *LESION MIMIC RESEMBLING*, which encodes an AAA-type ATPase, is implicated in defense response. *Mol. Genet. Genom.* **2015**, *290*, 611–622. [CrossRef] [PubMed]
23. Agrawal, G.K.; Rakwal, R.; Jwa, N.S. Rice (*Oryza sativa* L.) *OsPR1b* gene is phytohormonally regulated in close interaction with light signals. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 290–298. [CrossRef] [PubMed]
24. Nakashita, H.; Yoshioka, K.; Takayama, M.; Kuga, R.; Midoh, N.; Usami, R.; Horikoshi, K.; Yoneyama, K.; Yamaguchi, I. Characterization of PBZ1, a probenazole-inducible gene, in suspension-cultured rice cells. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 205–208. [CrossRef] [PubMed]
25. Wang, J.; Jiang, J.; Oard, J.H. Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.). *Plant Sci.* **2000**, *156*, 201–211. [CrossRef]
26. Qi, M.; Yang, Y. Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and northern blot/phosphoimaging analyses. *Phytopathology* **2002**, *92*, 870–876. [CrossRef]
27. Huang, Q.N.; Shi, Y.F.; Zhang, X.B.; Song, L.X.; Feng, B.H.; Wang, H.M.; Xu, X.; Li, X.H.; Guo, D.; Wu, J.L. Single base substitution in OsCDC48 is responsible for premature senescence and death phenotype in rice. *J. Integ. Plant Biol.* **2016**, *58*, 12–28. [CrossRef]
28. Saifi, S.K.; Passricha, N.; Tuteja, R.; Tuteja, N. Stress-induced Oryza sativa RuvBL1a is DNA-independent ATPase and unwinds DNA duplex in 3′ to 5′ direction. *Protoplasma* **2018**, *255*, 669–684. [CrossRef]

29. Lin, Y.; Tan, L.; Zhao, L.; Sun, X.; Sun, C. RLS3, a protein with AAA+ domain localized in chloroplast, sustains leaf longevity in rice. *J. Integr. Plant Biol.* **2016**, *58*, 971–982. [CrossRef]
30. Xia, Z.; Wei, Y.; Sun, K.; Wu, J.; Wang, Y.; Wu, K. The maize AAA-type protein SKD1 confers enhanced salt and drought stress tolerance in transgenic tobacco by interacting with Lyst-interacting protein 5. *PLoS ONE* **2013**, 8. [CrossRef]
31. Wang, F.; Liu, J.; Chen, M.; Zhou, L.; Li, Z.; Zhao, Q.; Pan, G.; Zaidi, S.-H.-R.; Cheng, F. Involvement of abscisic acid in PSII photodamage and D1 protein turnover for light-induced premature senescence of rice flag leaves. *PLoS ONE* **2016**, 11. [CrossRef] [PubMed]
32. Furukawa, T.; Ishibashi, T.; Kimura, S.; Tanaka, H.; Hashimoto, J.; Sakaguchi, K. Characterization of all the subunits of replication factor C from a higher plant, rice (*Oryza sativa* L.), and their relation to development. *Plant Mol. Biol.* **2003**, *53*, 15–25. [CrossRef] [PubMed]
33. Santos, L. Molecular mechanisms of the AAA proteins in plants. *Adv. Agril. Food Biotechnol.* **2006**, *37*, 1–15.
34. Zhang, P.; Zhang, Y.; Sun, L.; Sinumporn, S.; Yang, Z.; Sun, B.; Xuan, D.; Li, Z.; Yu, P.; Wu, W. The rice AAA-ATPase OsFIGNL1 is essential for male meiosis. *Front. Plant Sci.* **2017**, *8*, 1639. [CrossRef]
35. Shahriari, M.; Keshavaiah, C.; Scheuring, D.; Sabovljevic, A.; Pimpl, P.; Häusler, R.E.; Hülskamp, M.; Schellmann, S. The AAA-type ATPase AtSKD1 contributes to vacuolar maintenance of *Arabidopsis thaliana*. *Plant J.* **2010**, *64*, 71–85. [CrossRef]
36. Kaplan, C.P.; Thomas, J.E.; Charlton, W.L.; Baker, A. Identification and characterisation of PEX6 orthologues from plants. *Bba-Mol. Cell Res.* **2001**, *1539*, 173–180. [CrossRef]
37. Chung, K.; Tasaka, M. RPT2a, a 26S proteasome AAA-ATPase, is directly involved in Arabidopsis CC-NBS-LRR protein uni-1D-induced signaling pathways. *Plant Cell Physiol.* **2011**, *52*, 1657–1664. [CrossRef]
38. Su, Y.; Hu, S.; Zhang, B.; Ye, W.; Niu, Y.; Guo, L.; Qian, Q. Characterization and fine mapping of a new early leaf senescence mutant es3 (t) in rice. *Plant Growth Regul.* **2017**, *81*, 419–431. [CrossRef]
39. Xu, X.; Ji, J.; Xu, Q.; Qi, X.; Weng, Y.; Chen, X. The major-effect quantitative trait locus Cs ARN 6.1 encodes an AAA ATP ase domain-containing protein that is associated with waterlogging stress tolerance by promoting adventitious root formation. *Plant J.* **2018**, *93*, 917–930. [CrossRef]
40. Siebers, M.; Brands, M.; Wewer, V.; Duan, Y.; Holzl, G.; Dormann, P. Lipids in plant-microbe interactions. *Bba-Mol. Cell Biol. L.* **2016**, *1861*, 1379–1395. [CrossRef]
41. Lim, G.H.; Singhal, R.; Kachroo, A.; Kachroo, P. Fatty Acid- and Lipid-Mediated Signaling in Plant Defense. *Annu. Rev. Phytopathol.* **2017**, *55*, 505–536. [CrossRef] [PubMed]
42. Kachroo, A.; Venugopal, S.C.; Lapchyk, L.; Falcone, D.; Hildebrand, D.; Kachroo, P. Oleic acid levels regulated by glycerolipid metabolism modulate defense gene expression in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5152–5157. [CrossRef] [PubMed]
43. Delteil, A.; Zhang, J.; Lessard, P.; Morel, J.-B. Potential candidate genes for improving rice disease resistance. *Rice* **2010**, *3*, 56. [CrossRef]
44. Haque, E.; Taniguchi, H.; Hassan, M.; Bhowmik, P.; Karim, M.R.; Śmiech, M.; Zhao, K.; Rahman, M.; Islam, T. Application of CRISPR/Cas9 genome editing technology for the improvement of crops cultivated in tropical climates: Recent progress, prospects, and challenges. *Front. Plant Sci.* **2018**, *9*, 617. [CrossRef] [PubMed]
45. Yang, W.; Dong, R.; Liu, L.; Hu, Z.; Li, J.; Wang, Y.; Ding, X.; Chu, Z. A novel mutant allele of SSI2 confers a better balance between disease resistance and plant growth inhibition on Arabidopsis thaliana. *Bmc Plant Biol.* **2016**, *16*, 208. [CrossRef] [PubMed]
46. Mandal, M.K.; Chandra-Shekara, A.; Jeong, R.-D.; Yu, K.; Zhu, S.; Chanda, B.; Navarre, D.; Kachroo, A.; Kachroo, P. Oleic acid–dependent modulation of NITRIC OXIDE ASSOCIATED1 protein levels regulates nitric oxide–mediated defense signaling in *Arabidopsis*. *Plant Cell* **2012**, *24*, 1654–1674. [CrossRef] [PubMed]
47. Moreau, M.; Lee, G.I.; Wang, Y.; Crane, B.R.; Klessig, D.F. AtNOS/AtNOA1 is a functional *Arabidopsis thaliana* cGTPase and not a nitric-oxide synthase. *J. Biol. Chem.* **2008**, *283*, 32957–32967. [CrossRef]
48. Miki, D.; Itoh, R.; Shimamoto, K. RNA silencing of single and multiple members in a gene family of rice. *Plant Physiol.* **2005**, *138*, 1903–1913. [CrossRef]
49. Miki, D.; Shimamoto, K. Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol.* **2004**, *45*, 490–495. [CrossRef]
50. Toki, S.; Hara, N.; Ono, K.; Onodera, H.; Tagiri, A.; Oka, S.; Tanaka, H. Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. *Plant J.* **2006**, *47*, 969–976. [CrossRef]

51. Yang, Y.; Qi, M.; Mei, C. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. *Plant J.* **2004**, *40*, 909–919. [CrossRef] [PubMed]
52. Inoue, H.; Hayashi, N.; Matsushita, A.; Liu, X.; Nakayama, A.; Sugano, S.; Jiang, C.-J.; Takatsuji, H. Blast resistance of CC-NB-LRR protein Pb1 is mediated by WRKY45 through protein–protein interaction. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9577–9582. [CrossRef] [PubMed]
53. Letunic, I.; Bork, P. Interactive Tree Of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Res.* **2019**, *47*, W256–W259. [CrossRef] [PubMed]