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

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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-ATPase1 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 archaeabacteria, 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 (*lr6*-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 fungus *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 | AGTTGGTGTGCAGCTCTCGT ACAAACATGGTCAAATTACCA | [12] |
| OsAAA-ATPase2 Os01g0297200 | CTTAGTTCGGCATGACAC CTTCTTGCATTTGCCAC | [12] |
| OsAAA-ATPase3 Os06g0697600 | GGGTGATCGTGTCATGGTCG CAGAAAGCACCACACCACATGC | [12] |
| OsAAA-ATPase4 Os02g0697600 | TTGCCTGAACGGCCAGGTGAT CCCATGCAAGGATTGCCAC | [12] |
| OsAAA-ATPase5 Os02g0706500 | TTGCATCTCTTTGCTGTCAG CATGGCATCTCTTTTACC | [12] |
| OsAAA-ATPase6 Os07g0517600 | TACAGGGCGGCTCGACGTCAT CTCAGTGCTGCACACATC | [12] |
| OsPR1b Os01g0382000 | ACCGCGTACGTACTGGCTA CTTCCCATCTCTTTGCTGTCAG | [23] |
| PBZ1 Os12g0555000 | GCGTTTGAGTCCGTGAGATT CTCGGTATGGTAGCAGG | [24] |
| Rubq1 Os06g0681400 | GCCAGCTGCTGCTGAGTTC GTCCATGCTGAAGCAGA | [25] |
| M. oryzae 28S rDNA | ACGAGAGGAGCGCTATTCCAGTTATTTCTTCTC | [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 phylogenetic 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 ($5 \times 10^4$/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, OsPRI 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.](image)

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. NIAA1 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 lmrm 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 (GmSACP-D-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 (GA3; Wako, Osaka, Japan), abscisic acid (cis-trans, ABA; Sigma), and methyljasmonate (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 H2O. 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 BglII 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.

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