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Pathogen-Induced Expression of *OsDHODH1* Suggests Positive Regulation of Basal Defense Against *Xanthomonas oryzae* pv. *oryzae* in Rice

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Abstract: Bacterial leaf blight (BLB), a vascular disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), induces a significant reduction in rice yield in severe epidemics. This study investigated the transcriptional regulation of the *OsDHODH1* gene in rice cultivars exposed to the *Xoo* K3 isolate. The symptoms were monitored on a daily basis, and the lesion length of inoculated rice plants was scored 21 days post inoculation (dpi). The most resistant and the highly susceptible cultivars were used for gene expression analysis. The dihydroorotate dehydrogenase (DHODH) domain is shared by many proteins in different plant species, and in *Arabidopsis*, this protein is encoded by the *AtPYD1* gene. To investigate the functional role of the *OsDHODH1* gene under bacterial infection, we inoculated the *Arabidopsis pyd1-2* knockout (*atpyd1-2)* plants, lacking the *AtPYD1* gene (orthologous gene of the rice *OsDHODH1*), with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 *vir*, and the phenotypic response was scored 9 dpi. Results show that *OsDHODH1* was upregulated in Tunnae, the most resistant rice cultivar but downregulated in IRAT112, the highly susceptible rice cultivar. In addition, Tunnae, Sipi and NERICA-L14 exhibited a durable resistance phenotype towards *Xoo* K3 isolate 21 dpi. Moreover, the expression of *OsPR1a* and *OsPR10b* (the rice pathogenesis inducible genes) was significantly upregulated in Tunnae, while being suppressed in IRAT112. Furthermore, the *atpyd1-2* plants exhibited a high susceptibility towards *Pst* DC3000 *vir*. *AtPR1* and *AtPR2* (the *Arabidopsis* pathogenesis inducible genes) transcripts decreased in the *atpyd1-2* plants compared to Col-0 (wild type) plants. Due to the above, *OsDHODH1* and *AtPYD1* are suggested to be involved in the basal adaptive response mechanisms towards bacterial pathogen resistance in plants.

Keywords: bacterial leaf blight; *Xanthomonas oryzae* pv. *oryzae*; disease resistance; *OsDHODH1*; rice

1. Introduction

Rice is a staple food for more than half of the global population [1–3]. This important crop is cultivated for its nutritive value and economic importance [4,5]. However, rice cultivation is subjected to various abiotic [6,7] and biotic [8] stresses that reduce its productivity and quality. Among the

bacterial diseases dwelling in various parts of rice, and causing detrimental effects, bacterial leaf blight (BLB) caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most devastating and destructive bacterial diseases of rice (*Oryza sativa* L.) [9–14], in both irrigated and rainfed rice environments [15,16]. These environments provide favorable conditions for the development of BLB *Xoo* interaction with rice in a gene-for-gene relationship, making rice, a model plant for monocots, ideal for studies to depict the molecular mechanisms of disease responses in monocots [11–21]. BLB is spread worldwide [22–24] and can cause as high as 60% reduction in rice yield in severe epidemics [16,23,25]. This vascular disease starts with the infection of rice leaves or roots by the *Xoo* bacterium through hydathodes (specialized pores present at the leaf margin where vascular supply ends), natural plant openings, such as stomata, and wounds [25–27]. *Xoo* multiplies and spreads within the xylem, causing long, grey to white opaque necrotic lesions that typically spread from the tip of a rice leaf [26,28]. *Xoo* is characterized by the production of membrane-bound yellowish pigments, herein referred to as xanthomonadins, which protect the pathogen from photodamage and host-induced peroxidation damage [29,30]. Xanthomonadins are also necessary for epiphytic survival and successful infection into host plants [30].

To date, a variety of BLB resistance (*R*) genes have been identified in rice and tagged with molecular markers [31–33]. Among several identified BLB *R* genes, *Xa2* [34], *Xa4*, *xa5*, *xa13* and *Xa21* have been physically mapped and cloned [18,35–39]. The *Xa21* gene was reported to confer a broad-spectrum resistance against *Xoo* strains upon their infection into rice plants [3,11,40–42]. In Korea, the *Xoo* populations have been identified, characterized, and categorized into five pathotypes [15,43,44], of which K1, K2 and K3 races have been studied [15].

Upon pathogen infection, many of the pathways involved in the plant immune system are activated, which include the induction of a variety of pathogenesis-related genes and signaling cascades. During this event, positive or negative regulators of plants defense against pathogens are either induced or suppressed, and their interplay determines the level of resistance required for the plant triggered immunity system. [45,46]. The activation of the defense genes is mediated and controlled by an array of signal transduction pathways that include plant hormones, functioning as important signaling molecules [47,48]. These hormones give an alarm signal that results in the activation of a range of attacker-specific immune responses [49]. The classic hormones mediating activation of the plant immune system are salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), which antagonize each other while providing a balanced and appropriate response to the pathogen infection [50–52].

The dihydroorotate dehydrogenase (DHODH), in both animals and plants, is physically associated with the respiratory complex of the mitochondria, catalyzing the conversion of dihydroorotate (DHO) to orotic acid (OA), which is the fourth step and step-limiting factor in the *de novo* pyrimidine biosynthesis pathway [53–56]. Inhibition or depletion of DHODH has been shown to result in a disturbed function of the respiratory chain, thereby inducing cell growth hindrance, a decrease in the mitochondrial membrane potential, and an increase in the generation of reactive oxygen species (ROS). Additionally, the mitochondrial dysfunction due to the inhibition of the human DHODH has been reported to be responsible for a wide range of human diseases [57], accelerating aging [58,59], and inducing programmed cell death (PCD or apoptosis) [60]. In planta, however, much less is known so far about the role of the DHODH in the plant immune system, particularly the basal defense against bacterial pathogen infection.

Therefore, this study aimed at investigating the transcriptional regulation of the *OsDHODH1* gene compared to the one of the well-known pathogenesis-related (PR) genes in response to *Xanthomonas oryzae* pv. *oryzae* infection in rice leaves at the maximum tillering stage. The expression level of *OsDHODH1* was monitored by qPCR in the most resistant and highly susceptible rice cultivars, upon their exposure to K3 *Xoo* isolate. In addition, the transcriptional level of *OsPR1a* and *OsPR10b*, the well-established pathogenesis inducible genes, was measured under the same conditions. Additionally, rice (model plant for monocots) and *Arabidopsis* (model plant for dicots)

share an important genetic homology, which includes conserved domains and orthologous genes. The DHODH domain is shared by many proteins in different plant species, and in *Arabidopsis*, this protein is encoded by the *AtPYD1* gene. Moreover, the rice *OsDHODH1* gene encodes a membrane-bound protein, which is embedded in the inner mitochondrial membrane. Due to their structure, membrane-bound proteins have proven difficult to study and to clone, despite their interesting roles in diverse biological processes and metabolic pathways, including photosynthesis, respiration, signal transduction, molecular transport, and catalysis. For these reasons, we conducted a functional analysis study using the *Arabidopsis pyd1-2* knockout (*atpyd1-2*) line, lacking *AtPYD1* gene (the orthologue of the rice *OsDHODH1* gene), and we investigated its transcriptional regulation as well as its phenotypic response to *Pseudomonas syringae* pv. *tomato* (*Pst*) virulent strain (*Pst* DC3000) infection compared to the well-studied susceptible *Arabidopsis* knockout lines, *atsid2* and *atgsnor1-3*, as controls.

2. Materials and Methods

2.1. Rice Materials and Growth Conditions

Nine rice cultivars used in this study to perform the experiments included Jinbu, Odae, Tunnae (*japonica*), Lioto, IRAT112, Sipi (*indica*), and the New Rice for Africa (NERICA 4, NERICA 7, and NERICA-L14) interspecific generated from the cross between *Oryza glaberrima* and *Oryza sativa* [61], were used as genetic materials to perform the experiments. Jinbu and Odae were recently scored susceptible towards the Korean *Xoo* K1 isolate [62]. Seeds of Jinbu, Odae, and Tunnae were obtained from the Laboratory of Plant Functional Genomics (Kyungpook National University, Daegu, Korea), and those of Lioto, IRAT112 and Sipi, and NERICA4, NERICA 7 and NERICA-L14 were provided by the National Seed Service (SENASEM, Ministry of Agriculture) and the National Institute for Agronomic Study and Research (INERA, Kinshasa, Democratic Republic of Congo). Lioto and IRAT112 were both previously reported resistant to other important rice diseases, such as blast (*Pyricularia oryzae*) and leaf scald (*Monographella albescens*) [63], while Sipi was shown to be resistant to leaf scald [64]. NERICA 4 was reported for being resistant to BLB caused by *Xoo* UX00 (Ugandan) isolate [65], and NERICA 7 [66]. We further screened Tunnae, Lioto, IRAT112, Sipi and NERICA-L14 for BLB disease resistance. Plants were grown in a greenhouse at Kyungpook National University, Daegu, Republic of Korea.

Prior to germination, the seeds were surface sterilized with prochloraz (25% *v*/*v*) for 2 h, followed by rinsing three times for 1 h each to remove any traces of the prochloraz. The seeds were then germinated in petri dishes for 7 days. Germinated seeds were transferred to 50-well trays containing an enriched soil for two weeks in the greenhouse. Then, vigorous seedlings were transplanted to big pots up to 45 days prior to inoculating with *Xoo* K3 isolate. In total, 27 pots containing three plants each were used in triplicate.

2.2. Xa R Genes Tagged with DNA Markers

Bacterial leaf blight (BLB) *R* genes used in the current study included two recessive genes, *xa5* and *xa13*, and three dominant genes *Xa2*, *Xa4* and *Xa21*, the latter was reported to confer a broad-spectrum resistance to a variety of *Xoo* isolates. Primer sequences of simple sequence repeat (SSR) and sequence tagged site (STS) *Xa* marker genes and their related amplicon band sizes are given in Table 1. The STS and SSR markers were initially used to investigate the presence or absence of resistant and susceptible alleles of the selected DNA markers linked to specific *Xa R* genes in different rice cultivars.

Table 1. Simple sequence repeat (SSR) and sequence tagged site (STS) markers for Xa resistance genes in rice.

Markers	Primer Sequences (5′->3′)	Linked Gene	Distance (cM)	Chr	Expected Band Size (bp)	References
Npb197 [d], RM-317	F-CATACTTACCAGTTCACCGCC R-CTGGAGAGTGTCAGCTAGTTGA	Xa2	18.5	4	154	Singh et al., 2015
Npb181 [a], MP1	F-ATCGATCGATCTTCACGAGG R-TCGTATAAAAGGCATTCGGG	Xa4	1.7	11	150	Ma Bo-Jun et al., 1999
RM122 [b], SSR	F-GAGTCGATGTAATGTCATCAGTGC R-GAAGGAGGTATCGCTTTGTTGGAC	xa5	0.4	5	227	Blair et al., 2003
RG136 [c]- SSR	F-GGCCATGGCTCAGTGTTTAT R-GAGCTCCAGCTCTCCAAATG	xa13	3.8	8	450	Zhang et al., 1996
pTA248 [c], STS	F-AGACGCGGAAGGGTGGTTCCCGGA R-AGACGCGGTAATCGAAAGATGAAA	Xa21	0–1	11	950	Ronald et al., 1992

Chr = chromosome; source of primers sequences: [a] [14], [b] [67], [c] [68], [d] [69].

2.3. Genomic DNA Extraction and Genotyping of Rice Plants

The genomic DNA was extracted following the cetyltrimethylammonium bromide (CTAB) method [70]. Briefly, frozen leaf samples were crushed in 1.5 mL Eppendorf tubes (e-tubes) containing liquid nitrogen (N_2). Then, 300 µL of 2X CTAB buffer was added, followed by vortexing and incubation in a water bath at 65 °C for 20 min. The samples were immediately cooled down at room temperature for about 10 min. Then, 300 µL of chloroform was added, followed by gentle mixing by inversion for 5 min. All tubes were centrifuged for 3 min at 13,000 rpm to allow separation of phases. The supernatant was carefully removed and transferred to fresh micro tubes. Next, 300 µL of isopropanol was added, followed by mixing by inversion. Samples were incubated in a −20 °C freezer for 1 h to allow the DNA to precipitate, followed by centrifugation at 13,000 rpm for 7 min. The supernatant was removed, and the pellets were washed with 70% ethanol (1 mL), and the DNA pellets were completely dried at room temperature and resuspended into 100 µL nuclease-free water. Finally, the DNA concentrations of samples (ng μL^{-1}) and quality (A260/A280) were measured using NanoDrop, and samples were kept in a −20 °C freezer for further analysis.

To investigate the presence or absence of the resistant or susceptible alleles of the target *Xa R* genes in different rice cultivars, we amplified SSR and STS markers linked to *xa5*, *xa13*, *Xa2*, *X4*, and *Xa21* BLB *R* genes from the genomic DNA of samples by polymerase chain reaction (PCR). A 20 µL reaction mixture comprising 7 µL 2X F-Star *Taq* PCR Master Mix (BioFACT, Korea), 10 nM of each forward and reverse primers was used. A 3-step cycling reaction was performed including polymerase activation at 95 °C for 2 min, 95 °C strands separation for 20 sec, annealing at 56–58 °C for 40 sec for 25 cycles, extension at 72 °C for 1 min/kb, and the final extension at 72 °C for 5 min, and then visualized by the gel documentation system.

2.4. Cloning and Sequencing of Xa21

The genotyping results (Figure 1) revealed the presence or absence of resistant alleles of five Xa R genes in different rice cultivars. Further investigations were required to confirm the polymorphism and the band sizes of the *Xa21* in *indica* and *japonica* rice cultivars, which is widely known to confer a broad-spectrum resistance against *Xoo*. Therefore, we amplified *Xa21* using pTA248 STS DNA marker-specific primers, from the genomic DNA of Sipi (*indica*) and Jinbu (*japonica*) by polymerase chain reaction (PCR) using F-Star 2X *Taq* Polymerase Master Mix (Biofact, Korea); Sipi showed moderate resistance towards *Xoo* K3, while Jinbu was moderately susceptible. The PCR product was ligated into pGEM-T Easy Vector overnight under ±4 °C. The ligation reaction mixture had a total volume of 6 µL containing 2.5 µL ligation buffer, 0.5 µL vector, 2 µL PCR product, and 1 µL DNase free water. Then, ligation of the target gene was confirmed through PCR, and the construct was transformed into *Escherichia coli* (*E. coli*) DH5-α competent cells using the heat shock method [71]. After 3 h incubation

of the liquid culture at 37 °C in a sharking incubator, the culture (about 1 mL) was centrifuged at 8000 rpm for 3 min, and the cells were resuspended in 100 µL Luria–Bertani (LB) broth, and plated in duplicate on LB agar containing ampicillin as the selective agent followed by incubation overnight at 37 °C. The growing single colonies were screened using colony PCR (using the colonies as template) for confirmation of the insert. The selected positive colonies were grown in LB broth containing ampicillin, followed by extraction of plasmids using the Mini Prep Kit for plasmid purification according to the manufacturer's instructions (Qiagen, Korea). For further confirmation, we amplified the plasmid by PCR (plasmid PCR using the purified plasmid as template) using M13 forward and pTA248 STS marker reverse primers and *Taq* polymerase. Then, a final confirmation of the construct with the insert was achieved through sequencing (Figure S3).

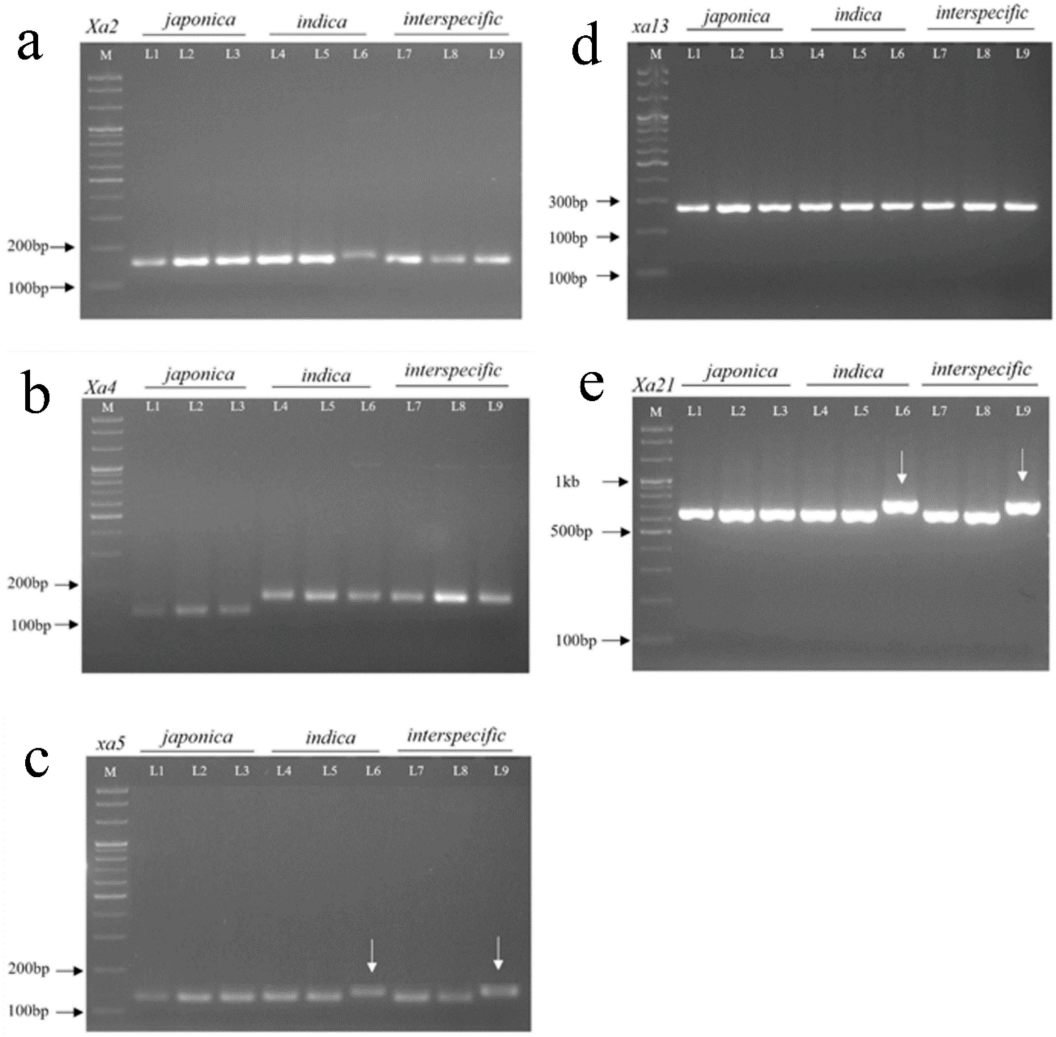

Figure 1. Amplification profile of polymorphic DNA markers tagged with Xa R genes resolved in agarose gel electrophoresis showing polymorphic bands. Five well-known molecular markers linked to dominant *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* resistance genes in rice. (**a**) *Xa2*, (**b**) *Xa4*, (**c**) *xa5*, (**d**) *xa13,* and (**e**) *Xa21* were amplified by PCR from the genomic DNA of rice cultivars and separated on gel electrophoresis and visualized under UV-light in a gel documentation system. Expected banding sizes for resistant alleles are as follows: *Xa2* (154 bp), *Xa4* (150 bp), *xa5* (227 bp), *xa13* (450 bp), *Xa21* (950 bp). M: ladder marker, lane 1: Jinbu, lane 2: Odae, lane 3: Tunnae, lane 4: Lioto, lane 5: IRAT112; lane 6: Sipi, lane 7: Nerica 4, lane 8: Nerica 7, lane 9: Nerica-L14.

2.5. Xanthomonas Oryzae pv. Oryzae Growth and Inoculation into Rice Plants

The bacterial cells (*Xoo*, K3) were cultured and maintained as described earlier [72]. Briefly, bacterial cells were grown on potato sucrose agar (PSA) petri dishes (5 g L^{-1} Bacto-peptone (Becton, Franklin Lakes, NJ, USA), 0.5 g L^{-1} sodium L-glutamate monohydrate, 5 g L^{-1} sucrose, and 8 g L^{-1} Bacto-agar) supplemented with cyclohexamide and incubated at 28 °C for 72 h. The typical *Xoo* single colonies were selected, and the cells were scraped off from the plates and resuspended in potato sucrose broth and incubated for about 48 h in a shaking incubator, until the bacterial culture reached an optical density (OD_{600}) above 1.0, which is equivalent to 8×10^8 colony forming units (CFU mL^{-1}) per mL. The actual concentration of the bacterial suspension culture had an OD_{600} equal to 1.573, equivalent to 1.3×10^9 CFU mL^{-1}, and was recorded using a spectrophotometer (AA6300C, Shimadzu, Tokyo, Japan). For inoculation to rice plants, this concentration was adjusted through serial dilutions (ratio 1:9) to the absorbance (OD_{600}) of 0.002, which corresponds to about 1.6×10^6 CFU mL^{-1}.

The leaf clip method [73] was used to inoculate plants with *Xoo* K3 at the maximum tillering stage [17]. Three topmost youngest fully expanded leaves of 60 day-old plants were clipped 5 cm from the tip (Figure S5) [74] with a sterilized scissor dipped into *Xoo* suspension culture immediately prior to each cutting; therefore, depositing the inoculum in the exposed veins across the whole cut edge near the tip. Negative controls were mock inoculated using only sterile distilled water. The inoculated leaves were closely monitored for eventual symptoms' development and progression of BLB typical lesions.

2.6. Lesion Length (LL) Measurement and Disease Scoring

Prior to conducting downstream analysis, nine rice cultivars were screened for their phenotypic response towards BLB caused by *Xoo* K3 isolate. The topmost resistant and the highly susceptible rice cultivars were selected to investigate the transcriptional response of *OsDHODH1* as well as other well-established pathogenesis inducible genes under bacterial infection. The lesion length was considered as the distance from the tip cutting edge to the leading edge of grayish to chlorotic symptoms, and was measured following the progression of the blight disease for each inoculated leaf up to 21 days post inoculation (dpi) [75,76]. The scoring for BLB resistance followed the method in Table 2.

Table 2. Standard evaluation system (SES) for bacterial leaf blight (BLB) severity.

Lesion Length (cm)	Disease Leaf Area (%)	Disease Score	Host Response
0	No disease observed	1	Highly Resistant (HR)
>0–5	Less than 1%	2	Resistant (R)
	1–3	3	Resistant (R)
	4–10	4	Resistant (R)
>5–10	11–15	5	Moderately Resistant (MR)
	16–25	6	Moderately Resistant (MR)
>10–15	26–50	7	Susceptible (MS)
>15	51–75	8	Susceptible (S)
	76–100	9	Highly Susceptible (HS)

Source: [77–79].

2.7. Arabidopsis Materials, Growth Conditions, and Genotyping

Arabidopsis Col-0 (wild type), *atpyd1-2* (AT3G17810: SALK_083897C), *atgsnor1-3*, and *atsid2* loss of function mutant lines were obtained from the Arabidopsis Biological Resource Center (ABRC) (https://abrc.osu.edu/). The *atgsnor1-3* knockout lacks the *S-nitrosoglutathione reductase 1* (*GSNOR1*),

which regulates the cellular *S*-nitrosothiols (SNO) levels, and *atsid2*, a salicylic acid (SA) deficient mutant, were used as the susceptible controls [80,81]. Plants were grown on a peat moss soil mixture at 22 °C with 16 h light and 8 h dark cycles. The *atpyd1-2* plants were genotyped to identify homozygous transfer DNA (T-DNA) insertion lines by polymerase chain reaction (PCR) for further experiments (Figure S4). The T-DNA insertion lines were identified through genotyping, using left border (LB) and gene specific reverse primers, and the DNA samples were extracted from the *atpyd1-2* plants, following the DNA extract method and PCR conditions described earlier in Section 2.3. Primers for genotyping were designed using the SALK_083897C (the Arabidopsis identification number of the target mutant line) in the iSect primer tool found in the following link: http://signal.salk.edu/tdnaprimers.2.html (SIGnAL: Salk Institute Genomic Analysis Laboratory). The list of primers is given in Table 3.

2.8. Pseudomonas Syringae pv. Tomato (Pst) Growth and Inoculum Preparation

The biotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) virulent strain (DC3000 *vir*) was grown and maintained as described [82]. Briefly, the bacterial culture was grown on Luria–Bertani (LB) agar plates containing rifampicin (50 µL/50 mL), and incubated at 28 °C for 48 h. Single colonies were picked and cultured in 5 mL LB broth in 50 mL falcon tubes for 48 h at 28 °C with continuous shaking. The overnight culture (1 mL) was centrifuged for 5 min at 8000 rpm to pellet down the cells. The bacterial cells were resuspended in 1 mL of 10 mM magnesium chloride ($MgCl_2$). Then, the absorbance (OD_{600} nm wavelength) of the bacterial culture and the blank ($MgCl_2$) [83] was read using a spectrophotometer. Plants were infiltrated with *Pst* DC3000 using a 1 mL syringe (without needle) into the abaxial side of leaf (the lower leaf surface), with a bacterial inoculum concentration of 5×10^5 CFU mL^{-1} [84] in triplicates. Mock plants were only infiltrated with 10 mM $MgCl_2$. To avoid physical damage (injury) to the leaves during infiltration, the syringe was positioned vertically to the leaf surface and low pressure was applied, knowing that the wounding effect interferes with the plant immune response, particularly through jasmonic acid-mediated signaling, which can suppress the SA-mediated defense pathways [85].

To allow the optimal pathogen proliferation and development for the most pronounced disease symptoms, particularly on susceptible genotypes, inoculation of *Pst* DC3000 *vir* was completed during light cycle hours. In addition, we tried to keep the pathogen infiltration timing consistent in order to reduce the effect of circadian rhythms and diurnal gene expression [86,87], which contribute to the reduction in variation among experimental replications.

2.9. Symptoms Development in Arabidopsis Genotypes Challenged with Pst DC3000 vir

At least three leaves were inoculated per plant in triplicate with *Pst* DC3000 virulent strain, and $MgCl_2$ was used as control [83]. For gene expression, samples were collected at 0 dpi (immediately after inoculation), 1 dpi and 2 dpi. Plants for phenotypic observations were scored 9 dpi.

Table 3. List of primers for genotyping and expression of target genes used in the study.

Gene Name/Genotype	Locus/SALK	Forward Primer (5′->3′)	Reverse Primer (5′->3′)	Gene Name
Genotyping primers of the T-DNA insertion *atpyd1-2* (Left border and right border)				
atpyd1-2	SALK_083897C	TTGGGTGGCAGAACATAGAAC	ATGAATTCAGCGGCATCATAG	Arabidopsis *pyd1-2* loss of function mutant
Primers for gene expression in rice				
OsDHODH1	LOC_Os02g50350	GAGGTCTGCGGTTGGATAAA	CTATAGGGTGCACGGCTCTC	*Dehydroorotate* dihydrogenase *encoded gene*
OsPR1a	LOC_Os07g03710	AGTTCGTCGAGCAGGTTATC	AGATTGGCCGACGAAGTTG	*Rice Pathogenesis related gene 1a*
OsPR10b	LOC_Os12g36850	ATGGCTCCGGCCTTCGTCTC	GGTTAAGCTTCATGATGTGGATGG	*Rice Pathogenesis related gene 10b*
OsUBI	LOC_Os03g03920	GCCATTAATGCTACCACTGC	GTTCTCGGATAGCTGTTGTTGC	*Rice ubiquitin encoding gene*
Primers for gene expression in *Arabidopsis*				
AtPYD1	AT3G17810	AGTGAGGATCGCTCGCTTTC	TCATCACACCGGTGCATACC	*PYRIMIDINE 1*
AtPYRD	AT5G23300	AAGACGAGTGAGGATGCTGC	GCAGTCCTGCAGTATTGGGT	*PYRIMIDINE D*
AtPR1	AT2G14610	GTGCAATGGAGTTTGTGGTC	TCACATAATTCCCACGAGGA	*Arabidopsis pathogenesis-related gene 1*
AtPR2	AT3G57260	CAGATTCCGGTACATCAACG	AGTGGTGGTGTCAGTGGCTA	*Arabidopsis pathogenesis-related gene 2*
AtACT2	AT3G18780	AGGTTCTGTTCCAGCCATC	TTAGAAGCATTTCCTGTGAAC	*Arabidopsis Actin coding gene 2*

2.10. Total RNA Isolation, cDNA Synthesis and qPCR Analysis

Total RNA was isolated from samples of leaves using the TRI-Solution™ Reagent (cat. no: TS200-001, Virginia Tech Bio-Technology, lot: 337871401001) as described by the manufacturer. Thereafter, the complementary DNA (cDNA) was synthesized as described earlier by Mun et al. [88]. Briefly, 1 µg of RNA was used to synthesize cDNA using BioFACT™ RT-Kit (BioFACT™, Republic of Korea) according to the manufacturer's standard protocol. The cDNA was then used as a template to study the transcripts accumulation of selected genes through qPCR analysis. Briefly, the reaction mixture was composed of SYBR green (BioFACT, Korea) along with 100 ng of template DNA and 10 nM of each forward and reverse primers in a final volume of 20 µL reaction. A no-template control [89] was used as a control. A 2-step reaction including polymerase activation at 95 °C for 15 min, followed by denaturation at 95 °C for 5 s and annealing and extension at 65 °C for 30 s was performed in a real-time PCR machine (Eco™ Illumina, USA). The total reaction cycles were 40 and the relative expression values of all genes were normalized with the one of the housekeeping genes (ubiquitin for rice; actin for *Arabidopsis*) (see Table 3).

3. Results

3.1. Polymorphic Bands of Amplified DNA SSR and STS Markers Linked to Xa R Genes in Different Rice Cultivars

We conducted a genotyping assay to evaluate the rice cultivars for the presence of well-known Xa R genes. Therefore, Npb197/RM-317 (SSR), Npb191 and pTA248 (STS) linked to *Xa2*, *Xa4* and *Xa21* (the dominant Xa R genes), and *xa5* and *xa13* (the recessive *xa* R genes) tagged with molecular markers RM122 (SSR) and RG136 (SSR), respectively, were used. These DNA markers amplified polymorphic bands, indicating either the presence or the absence of resistance alleles of the specific Xa R genes. pTA248 did not amplify the band size of 950 bp as reported earlier [90] or 1018 bp for the resistance allele of *Xa21* [91] in the resistant rice cultivars identified in the current study. *Xa21* is known as a major gene conferring a broad-spectrum resistance against *Xoo* strains. In the present study, unlike previous reports, the same *Xa21 R* gene amplified a band of 733 bp in Sipi (*indica*) (Figure 1e, lane 6), which exhibited a moderately resistant phenotypic response (Figures S1 and S2). Additionally, Sipi amplified polymorphic bands of *Xa2* and *xa5* R genes (Figure 1a, lane 6; Figure 1b, lane 6). Similar band sizes of *Xa21* and *xa5* were observed in NERICA-L14 (the moderately resistant interspecific rice line resulted from crosses between *Oryza glaberrima* and *Oryza sativa* ssp. *indica*) (Figure 1e, lane 9). In addition, Tunnae, a *japonica* rice cultivar scored moderately resistant 21 dpi (Figures S1 and S2, Table 4). However, Tunnae amplified a low band of around 653 bp of *Xa21 (*Figure 1e, lane three) similar to Jinbu (Figure 1e, lane 1). To further our investigations and confirm the size of the resistance allele of *Xa21*, we cloned the *Xa21* from Sipi, the resistant cultivar that amplified a high band size of *Xa21* and Jinbu, the moderately susceptible *japonica* cultivar that showed a small band size into pGEM-T Easy Vector. The sequencing results revealed that the amplified band size of *Xa21* in Sipi is 733 bp, while in Jinbu, the recorded band size is 643 bp (Figure S3).

Moreover, the highly susceptible *indica* cultivar IRAT112 showed similar *Xa21* banding pattern with the highly susceptible *japonica* cultivar Odae. All other rice cultivars, which scored either moderately susceptible, susceptible, or highly susceptible 21 dpi, amplified similar banding sizes of *Xa21* dominant and *xa5* recessive *R* genes. A study analyzing the dynamics of *Xoo* populations in Korea and their relationship to well-known BLB R genes supported that the pyramiding line carrying *Xa4*, *xa5* and *Xa21* would be a promising genotype for improving rice cultivars for BLB resistance [15]. There is now compelling experimental evidence that long-term cultivation of certain resistant rice cultivars could be attributed to the shift in the race frequency of *Xoo*, and the eventual breakdown of single *R* genes instability due to the evolution of new pathotypes [15].

Table 4. Bacterial leaf blight disease scoring and host response at growth stage (5–8).

Cultivars	Lesion length (cm) 21 dpi	SEM	% DLA [1]	Disease Score (0–9) [2]	Host Response to *Xoo* K3 Inoculation [3]
Jinbu	8.3	±4.92	29.2	7	Moderately Susceptible (MS)
Odae	14.2	±1.49	78.8	9	Highly Susceptible (HS)
Tunnae	5.4	±0.33	21.3	6	Moderately Resistant (MR)
Lioto	15.8	±3.44	71.8	8	Susceptible (S)
IRAT112	17.6	±1.04	90.1	9	Highly Susceptible (HS)
Sipi	4.6	±1.25	23.3	6	Moderately Resistant (MR)
NERICA 4	9.2	±1.92	36.2	7	Moderately Susceptible (MS)
NERICA 7	17.5	±4.05	52.7	8	Susceptible (S)
NERICA-L14	4.7	±2.11	21.5	6	Moderately Resistant (MR)

Source: [1] (our own data); [2] [78]; [3] [77]. % DLA: disease leaf area percentage. SEM: standard error of the mean. (5–8): tillering to booting stage [78].

3.2. *Differential Phenotypic Response of Nine Rice Cultivars Towards Xoo K3 Infection*

Four days after rice plants were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) K3 Korean isolate, leaf drying symptoms were observed on the cut edge of inoculated topmost fully expanded leaves in all rice cultivars. Rice plants were exposed to the inoculum (1.6×10^6 CFU mL^{-1}). The disease severity estimated by measuring the lesion length (LL) and the diseased leaf area in percentage (DLA) revealed that of all cultivars, the interspecific (generated from crosses of *Oryza. glaberrima* and *Oryza. sativa*) cultivar NERICA-L14 had the shortest lesion length (4.7cm LL; DLA: 21.5%), but IRAT112 had the lowest DLA (21.3%); therefore, being the rice cultivar with the highest level of resistance to K3 followed by Sipi of which the recorded DLA was 23.3% and the LL was 4.6cm. In contrast, Oade (*japonica*) and IRAT112 (*indica*) were identified as being highly susceptible (HS) to *Xoo* K3 race. Additionally, Lioto (*indica*) was found to be susceptible (S) to K3 upon its phenotypic response (Table 4). The recorded DLA percentage ranged between 22 (lowest = moderately resistant) and 90.1 (highest = highly susceptible) (Table 4).

The pathogenicity test of *Xoo* Korean isolates and the response of selected *indica* and *japonica* genotypes on a daily basis revealed that Jinbu, Odae and Lioto exhibited a resistance phenotype 14 dpi. Interestingly, after a prolonged period after bacterial inoculation, up to 21 days, Jinbu and Odae resulted in an increase in BLB symptoms to susceptibility (Figure S2). In general, clear symptoms were observed after 4 dpi in the majority of tested rice genotypes. The aggressiveness of *Xoo* K3 isolate was evaluated based on symptoms development and lesion length (4.6–17.6 cm), which differ between rice cultivars and time of exposure to the inoculum (Table 1). Rice cultivars Sipi and NERICA-L14 showed shorter lesions' length, 4.6cm and 4.7cm, respectively. In BLB-related studies, the evaluation period of virulence of *Xoo* strains and cultivars' resistance against BLB, and disease scoring are routinely completed early (10–15 dpi [92]), and late (21 [65,93,94] to 28 dpi [95]), which was deposited on the target leaves by cutting their tips (http://www.knowledgebank.irri.org/ricebreedingcourse/Breeding_for_disease_resistance_Blight.htm). In the current study, scoring of inoculated plants was completed 21 dpi, when the susceptibility check showed maximum symptoms of bacterial blight (Figures S1 and S2, Table 4).

The daily observation of the progress of symptoms revealed a differential aggressiveness of K3 isolate in different rice cultivars. We could distinguish here three different residual effects depending on the duration of the exposure to *Xoo* inoculum. Initially, we exposed rice cultivars to *Xoo* infection for 10 dpi, which corresponds to the initial stage of symptom development on inoculated leaves, we observed leaves drying from the tip, and the early evaluation time point showing distinctive BLB symptoms was suggested by the International Rice Research Institute (IRRI). At this time point, all tested rice cultivars scored either resistant or moderately resistant based on their phenotypic response, except IRAT112, which had the highest DLA percentage, and scored highly susceptible 7 dpi. We furthered our investigations by exposing infected plants up to 21 dpi. From 11 dpi, we observed

that the cultivars that initially scored as resistant developed symptoms and scored as susceptible over time. During this period, BLB symptoms in Odae and Lioto exponentially increased in length, resulting in an altered host response, which led to a moderately susceptible phenotype. Similarly, rice cultivars Jinbu, Lioto, NERICA 4 and NERICA 7 (earlier scored resistant cultivars at 10 dpi) scored as susceptible over time. However, Tunnae (*japonica*), Sipi (*indica*) and NERICA-L14 (interspecific line) remained resistant, and exhibited durable resistance against the *Xoo* K3 isolate, with the lowest DLA percentage and a shorter lesion length.

3.3. Xoo K3 Induced OsDHODH1 Expression in Tunnae, the Topmost Resistant, while Being Downregulated in IRAT112, the Highly Susceptible Cultivar Early after Inoculation

In the Materials and Methods section, we provided the basis for the selection of different cultivars with regard to their phenotypic response towards *Xoo* inoculation. Here, we briefly mention that based on the screening results, we selected Tunnae as resistant whereas IRAT112 was selected as a susceptible cultivar (Figure 2a). We measured the expression of *OsDHODH1* 1 h after *Xoo* K3 infection in order to investigate its transcriptional response soon after bacterial infection in the resistant (Tunnae) and highly susceptible (IRAT112) rice cultivars. The results in Figure 2b indicate that *OsDHODH1* was significantly upregulated in Tunnae, which we found to be resistant towards BLB at 21 dpi. Moreover, IRAT112, the highly susceptible cultivar, significantly downregulated *OsDHODH1* expression.

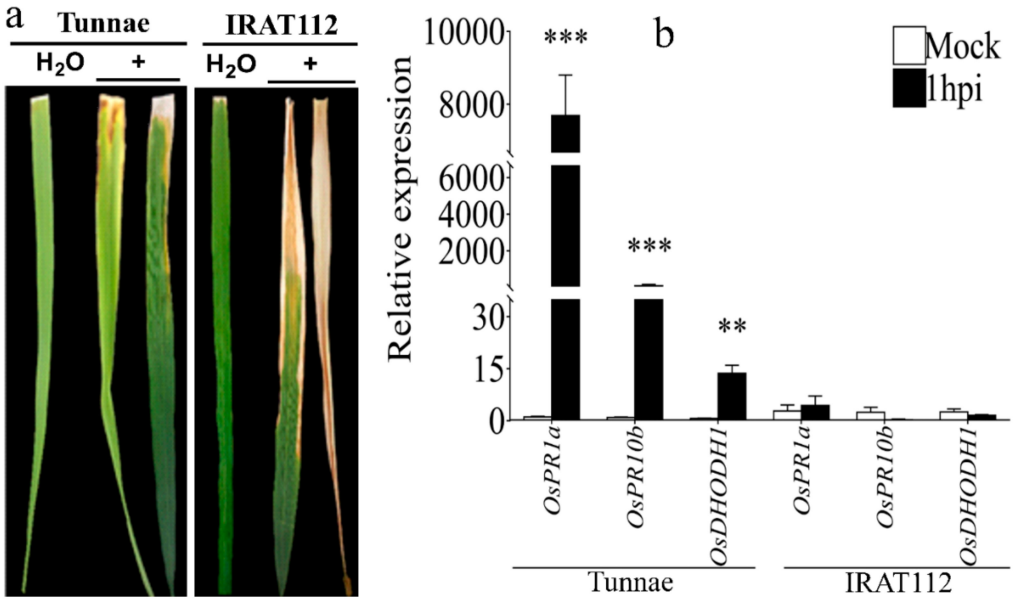

Figure 2. Phenotype at 21 days post inoculation (dpi) and transcriptional response of *OsDHODH1* 1 h after Xoo K3 inoculation in different rice cultivars. (**a**) Transcriptional level of *OsDHODH1* gene relative to the expression of the pathogen-related genes (*OsPR1a* and *OsPR10b*) under *Xoo* K3 infection in rice, and (**b**) phenotypes of the most tolerant cultivar Tunnae (*japonica*) and the highly susceptible cultivar IRAT112 (*indica*) at 21 dpi. Bars are means ±SD. *** $p < 0.001$, ** $p < 0.01$. Empty is non-significant.

3.4. The Expression of the Arabidopsis PR1 and PR2 was Differentially Regulated in atpyd1-2 Knockout Line

The expression of the *Arabidopsis PR2* significantly increased over time upon *Pst* DC3000 inoculation in Col-0, and significantly decreased in *atpyd1-2* loss of function mutant (Figure 3a,b). However, *AtPR1* showed a similar expression pattern in both Col-0 and *atpyd1-2* plants. Under the same conditions, *AtPYD1* was slightly upregulated over time in Col-0 (wild type, WT). Furthermore, *AtPYRD* expression is shown to be differentially regulated in *atpyd1-2* loss of function mutant, suggesting a negative regulation by *AtPYD1*. The phenotypes, after challenging the *atpyd1-2* with *Pst* DC3000, showed a susceptible phenotype compared to Col-0 WT (Figure 3c), suggesting that *AtPYD1* may positively regulate basal defense in *Arabidopsis*.

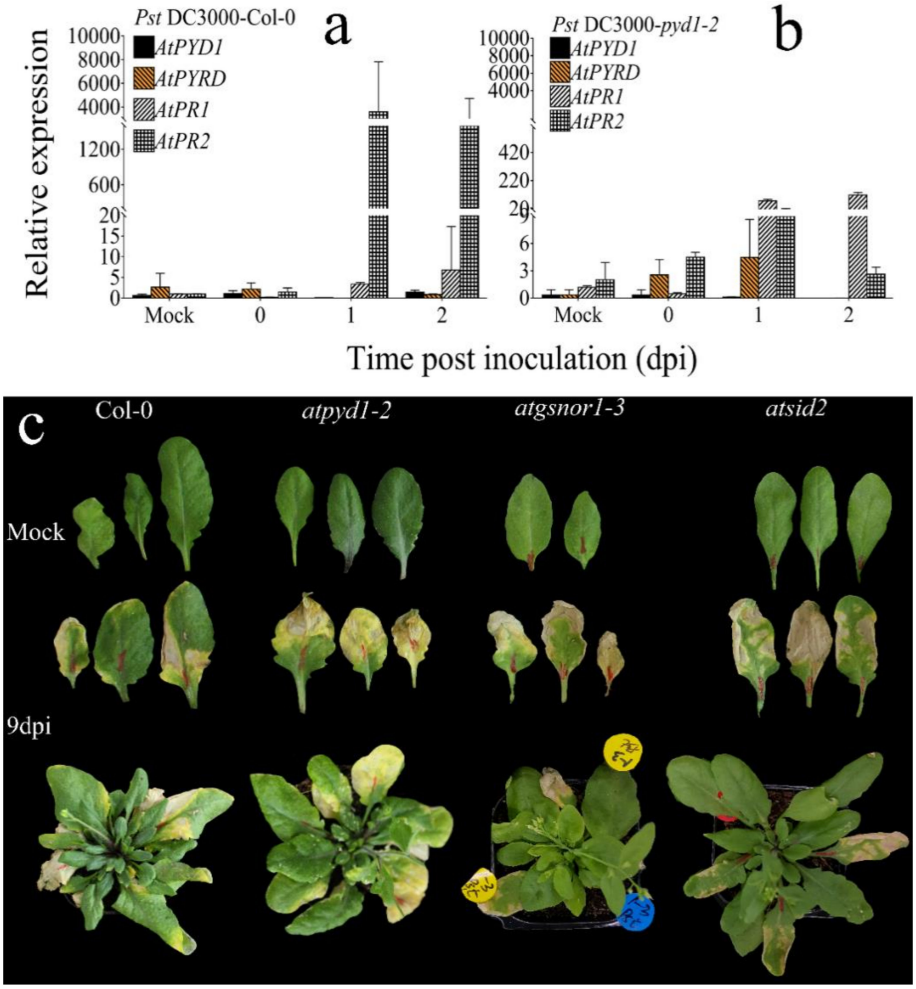

Figure 3. Transcriptional response of *AtPYD1* under bacterial *Pst* DC3000 *vir* infection. (**a**) Transcriptional response of the Arabidopsis *PYD1*, *PYRD*, *PR1* and *PR2* under *Pst* DC3000 *vir* infection over time, (**b**) expression patterns of same genes listed in the loss of function mutant *atpyd1-2* background, and (**c**) phenotype of Arabidopsis *atpyd1-2* loss of function mutant towards *Pst* DC3000 *vir* infection. The phenotype was recorded at 9 dpi.

4. Discussion

4.1. Differential Phenotypic Response of Rice Cultivars towards Xoo K3 Inoculation

Two *japonica* genotypes, Jinbu and Odae, were recently scored susceptible to the Korean *Xoo* K1 race under greenhouse and field conditions [62]. So far, no available report has mentioned screening Tunnae (*japonica*), Lioto, IRAT112, Sipi (*indica*) and Nerica-L14 (interspecific line derived from crosses of *Oryza glaberrima* and *Oryza sativa*) for their resistance to BLB. However, Lioto and IRAT112 were both previously reported as being resistant to blast (*Pyricularia oryzae*) and leaf scald (*Monographella albescens*) [63], whereas Sipi was reported as showing resistance against leaf scald [64]. A recent study has reported the upland *indica* rice cultivar New rice for Africa 4 (NERICA 4) to be resistant to BLB when the *Xoo* UX00 (African) isolate was inoculated for 21 days [65]. Furthermore, NERICA 7 was also identified as resistant genotype against a specific BLB isolate [66].

4.2. The Expression Patterns of OsDHODH1 and PR Genes in Resistant and Susceptible Rice Cultivars Suggest a Positive Regulation of Plant Basal Defense

Upon pathogen infection, plants activate the defense mechanisms, which include induction of a variety of pathogenesis-related genes and signaling cascades. During this event, positive or

negative regulators of plant defense against pathogen attack are induced or suppressed, and their interplay determines the level of resistance required for the plant triggered immunity system. Our data show that *OsPR1a* and *OsP10b* were significantly upregulated, as expected, in the most resistant rice cultivar Tunnae soon after *Xoo* K3 inoculation. A similar transcriptional pattern was observed when *OsDHODH1* was expressed in Tunnae. However, when *OsPR1a* and *OsPR10b* were expressed in IRAT112 (the highly susceptible rice cultivar), their transcriptional levels were significantly reduced. Similarly, the expression of the *OsDHODH1* gene decreased under the same conditions. Due to the recorded transcriptional response, this study suggests that *OsDHODH1* could be involved in the adaptive response mechanisms towards *Xoo* (causing BLB) resistance.

The dihydroorotate dehydrogenase (DHODH) in both animals and plants is physically and intimately associated with the respiratory complex of the mitochondria, catalyzing the conversion of dihydroorotate to orotic acid—the fourth step in the de novo pyrimidine biosynthesis pathway [53–56]. Inhibition or depletion of DHODH has been shown to result in disturbed function of the respiratory chain, thereby inducing cell growth hindrance, decreasing mitochondrial membrane potential, increasing generation of reactive oxygen species (ROS), depleting uridine and myeloid differentiation [96], and creating potential targets for anti-malarial compounds [97]. Mitochondrial dysfunction due to DHODH inhibition was reported to be responsible for a wide range of human diseases [57], accelerated aging [58,59] and induced programmed cell death (apoptosis) [60]. Recent studies have suggested that the *OsDHODH1* gene (in rice) [98,99] or *AtPYD1* gene (in *Arabidopsis*) [100,101] could play a key role in the adaptive response of plants towards drought and salinity tolerance, and nitro-oxidative stress.

4.3. AtPYD1 Positively Regulates Plant Basal Defense against Pst DC3000

In the perspective of further investigating the role of the DHODH encoding gene in the adaptive response mechanisms of plants towards bacterial pathogen resistance, we inoculated the *Arabidopsis* loss of function mutant, *atpyd1-2*, which lacks the *AtPYD1* gene (Figure 3a,b), orthologue of the rice *OsDHODH1*, with *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 *vir* strain. The phenotypic response of *atpyd1-2* after nine days revealed a highly susceptible response (Figure 3c). This would imply that *AtPYD1* could be involved in the positive regulation of plants' basal defense mechanisms towards bacterial pathogen resistance. Under *Pst* DC3000 *vir* infection, we were primarily expecting to see an enhanced resistant phenotypic response of *atpyd1-2*, rather than a susceptible response. It was unusual for us to have this situation regarding the fundamentals of the metabolism underlying plants' adaptive responses to abiotic and biotic stress conditions involving hormonal signaling such as abscisic acid (ABA) and SA, which are known to be antagonistic.

Generally, upon infection by a virulent pathogen, pathogen, or microbe-associated molecular patterns (PAMPs) activate the basal defense mechanisms [45]. Gram-negative bacterial pathogens, such as *Pseudomonas syringae,* have the capacity to deliver effector proteins to plant cells, which will interfere with PAMP-triggered resistance in order to promote the virulence of the pathogen. In many cases, some of the effectors are particularly recognized by plant resistance proteins and activate strong effector-triggered resistance [45]. Under the same conditions, both PAMP and effector-triggered resistance are shown to be associated with a wide transcriptional reprogramming of plant host genes. The molecular mechanisms underlying plants' response to bacterial pathogen infection involve a broad range of pathogenesis-related (*PR)* genes and well-organized signaling networks. Among them, *PR1* and *PR2* are salicylic acid (SA)-dependent defense signals, also considered as important markers for plants' response to pathogens [102–104]. The expression of *PR* genes is induced in response to a variety of pathogens [105].

Our data indicate that *AtPYD1* expression was upregulated (by about a 2-fold change) over time in Col-0 after *Pst* DC3000 inoculation (Figure 3a). Meanwhile, its counterpart *OsDHODH1* was upregulated by about a 17.3-fold change soon after *Xoo* K3 inoculation. Additionally, the transcriptional level of the key *PR* genes (*AtPR1* and *AtPR2*) was highly significantly induced in Col-0, with *AtPR2*

showing the highest transcriptional response. Furthermore, when expressed in *atpyd1-2* loss of function mutant, the transcript level of *AtPR2* significantly decreased compared the one recorded in Col-0, while *AtPR1* showed a similar expression pattern in Col-0 and *atpyd1-2*. Moreover, the exponential upregulation of the *AtPR1* gene (Figure 3a) indicated that the latter would prevail over the *AtPR2* gene in the adaptive response mechanisms towards *Pst* DC3000 bacterial resistance in *Arabidopsis*. In the same way, the significant downregulation of *AtPR1* and *AtPR2* in the *atpyd1-2* knockout plants exposed to the virulent *Pst* DC3000 compared to Col-0 WT suggest a possible existing transcriptional interaction with the *AtPYD1* gene.

5. Conclusions

The adaptive response mechanisms of plants towards a pathogen attack include the activation of diverse signaling cascades and pathogenesis-related genes, as part of the plant-triggered immunity system mechanism, and their interplay determines the level of resistance the plant will provide to the pathogen. In the present study, nine rice cultivars were inoculated with *Xoo* K3 race at the tillering stage. The initial bacterial leaf blight (BLB) disease symptoms appeared on the cut edge of inoculated leaves 4 dpi. The phenotypic responses of rice cultivars showed that at 10 dpi almost all rice cultivars showed a resistant response to *Xoo* K3 infection. However, a prolonged exposure to the *Xoo* inoculum revealed that some of the resistant cultivars started showing susceptibility to the BLB disease, whereas some showed a durable resistance 21 dpi, such as Tunnae, Sipi, and NERICA L14. Moreover, Tunnae (the most resistant rice cultivar) and IRAT112 (highly susceptible rice cultivar) significantly upregulated and downregulated the *OsDHODH1* gene, respectively. Therefore, due to the recorded transcriptional levels of *OsDHODH1* or *AtPYD1*, the pathogenesis-related genes in rice and *Arabidopsis*, and the enhanced susceptibility of the *Arabidopsis pyd1-2* knockout line in response to *Pst* DC3000 virulent infection, this study suggests that *OsDHODH1* or *AtPYD1* could be involved in the basal adaptive response mechanisms towards bacterial infection resistance in plants.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/10/11/573/s1, Figure S1: BLB disease phenotype on different rice cultivars 21 dpi, Figure S2: BLB daily lesion length on 9 rice cultivars, Figure S3: Alignment of *Xa21* sequences cloned from *indica* and *japonica* cultivars against the standard cultivar Shuhui498, Figure S4: Genotyping of the *Arabidopsis atpyd1-2* knockout to identify homozygous mutant plants, Figure S5: Illustration of the inoculation method by leaf cutting.

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