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Over-expression in the nucleotide-binding site-leucine rich repeat gene *DEPG1* increases susceptibility to bacterial leaf streak disease in transgenic rice plants

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Abstract Bacterial leaf streak of rice (BLS) caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) is a widely-spread disease in the main rice-producing areas of the world. Investigating the genes that play roles in rice–*Xoc* interactions helps us to understand the defense signaling pathway in rice. Here we report a differentially expressed protein gene (*DEPG1*), which regulates susceptibility to BLS. *DEPG1* is a nucleotide-binding site (NBS)-leucine rich repeat (LRR) gene, and the deduced protein sequence of *DEPG1* has approximately 64% identity with that of the disease resistance gene *Pi37*. Phylogenetic analysis of *DEPG1* and the 18 characterized NBS-LRR genes revealed that *DEPG1* is more closely related to *Pi37*. DEPG1 protein is located to the cytoplasm, which was confirmed by transient expression of *DEPG1-GFP* (green fluorescent protein) fusion construct in onion epidermal cells. Semi-quantitative PCR assays showed that *DEPG1* is widely expressed in rice, and is preferentially expressed in internodes, leaf blades, leaf sheaths and flag leaves. Observation of cross sections of leaves from the transgenic plants with a *DEPG1*-promoter::glucuronidase (GUS) fusion gene revealed that *DEPG1* is also highly expressed in mesophyll tissues where *Xoc* mainly colonizes. Additionally, *Xoc* negatively regulates expression of *DEPG1* at the early stage of the pathogen infection, and so do the three defense-signal compounds including salicylic acid (SA), methyl jasmonate (MeJA) and 1-aminocyclopropane-1-carboxylic-acid (ACC). Transgenic rice plants over-expressing *DEPG1* exhibit enhanced susceptibility to *Xoc* compared to the wild-type controls. Moreover, enhanced susceptibility to *Xoc* may be mediated by inhibition of the expression of some SA biosynthesis-related genes and pathogenesis-related genes that may contribute to the disease resistance. Taken together, *DEPG1* plays roles in the interactions between rice and BLS pathogen *Xoc*.

Keywords Stress · Susceptibility · Defense response · Pathogenesis-related gene · Bacterial leaf streak · *Oryza sativa*

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
CTAB	Cetyltrimethyl ammonium bromide
ET	Ethylene
GFP	Green fluorescent protein
JA	Jasmonic acid
MeJA	Methyl jasmonate
MALDI-TOF-MS	Matrix assisted laser desorption ionization-time of flight-mass spectrometry
NBS-LRR	Nucleotide binding site-leucine rich repeat
ORF	Open reading frame
QTL	Quantitative trait locus
RT-PCR	Reverse transcription PCR
SA	Salicylic acid
Xoo	*Xanthomonas oryzae* pv. *oryzae*
Xoc	*Xanthomonas oryzae* pv. *oryzicola*

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Introduction

Rice is the staple food for over half the people in the world. Some devastative rice diseases, such as blast disease caused by fungal pathogen *Magnaporthe grisea*, bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and bacterial leaf streak (BLS) caused by *X. oryzae* pv. *oryzicola* (*Xoc*), lead to the heavy losses of rice yield. Breeding disease-resistance varieties is an important strategy to control the diseases and to improve rice yield. To date, a number of rice blast resistance (R) genes and rice bacterial leaf blight R genes have been cloned and identified [1].Conversely, only a few quantitative trail locus (QTLs) have been reported to contribute to resistance to *Xoc*, but these QTLs remain to be characterized [1, 2]. In recent years, some defense-related genes have been reported to confer resistance to *Xoc*. For example, *GH3-2* encoding an indole-3-acetic acid-amido synthetase mediates a broad-spectrum resistance to bacterial pathogens *Xoo* and *Xoc* [3]. Similarly, a pair of allelic WRKY genes that were designated as *OsWRKY45-1* and *OsWRKY45-2*, have opposite functions in rice–bacteria interactions. *OsWRKY45-1* negatively regulates resistance against *Xoo* and *Xoc*, while *OsWRKY45-2* positively regulates the resistance [4]. Additionally, knock-out of *OsMPK6* helps strengthen resistance to *Xoo* and *Xoc* [5]. Besides these genes, a non-host R gene *Rxo1* that confers resistance to the maize bacterial stripe pathogen *Burkholderia andropogonis*, also regulates resistance against *Xoc* in rice [6]. These findings will help us to breed rice varieties with better resistance to *Xoc*; however, it seems that the explored genetic resources for controlling BLS of rice remain too limited to breed the rice varieties with excellent resistance to *Xoc*. Hence, more and continuous efforts should still be made to exploit the resistance-related genes for control of BLS.

Dissecting the molecular mechanism of the interactions between rice and *Xoc*, would be of great benefit in exploring the genes involved in defense response upon *Xoc* challenge, including the genes play pivotal roles in the defense network. Therefore, we carried out the corresponding work previously based on the above opinion. Some differentially expressed protein genes upon infection of the BLS pathogen *Xoc*, were obtained by analysis of rice proteomes using the technologies of two-dimensional gel electrophoresis and matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Among of these differentially expressed protein genes, there are several nucleotide binding site-leucine rich repeat (NBS-LRR) genes. It is reported that the NBS-LRR genes play roles in disease resistance and plant development. For example, lots of the rice blast R genes such as *Pi-ta* [7], *Pib* [8], *Pi2/Piz-t* [9], *Pi9* [10], *Pi37* [11], *Pi36* [12], *Pikm* [13], *Pit* [14], *Pid3* [15] and *Pb1* [16], and the rice bacterial blight R gene *Xa1* [17] are NBS-LRR genes. In addition, at least two studies reported that the NBS-LRR genes are involved in plant development. The NBS-LRR gene *CSA1* along with other NBS-LRR genes including *RPS4*, contribute to the photomorphogenic development in *Arabidopsis* [18]. Similarly, antisense expression of a NBS-LRR sequence causes developmental abnormalities in transgenic sunflowers, and results in severe alteration in seed development of the transgenic tobacco plants and enhanced susceptibility to *Phytophthora parasitic* [19]. Some R genes cloned in the past few years contain two NBS-LRR gene members, no one of the two members could confer resistance independently, but do both members together. For instance, the rice blast R gene *Pikm* contains *Pikm1-TS* and *Pikm2-TS*, both members are required to confer *Pikm*-specific rice blast resistance, and no one could do that independently [13]. The other rice blast R gene *Pik* [20] functions in the same manner. These studies indicate that the NBS-LRR genes have diverse biological function and modes of action in plants. Thus, it would be interesting to investigate the function of the NBS-LRR genes, especially their roles in the interactions between host and pathogens.

As mentioned above, among our identified differentially expressed protein genes, there is a NBS-LRR gene designated as *DEPG1*. Proteomic analysis showed that the expression levels of the NBS-LRR protein DEPG1 decreased at 12 and 48 h post inoculation with *Xoc* as compared to 0 h post inoculation, implying that it may participate in the interaction between rice and *Xoc*. To investigate the role of *DEPG1* in the rice–*Xoc* interactions, we examined its tissue-expression pattern and expression in response to *Xoc* and the defense-signal compounds, and assessed the resistance of the *DEPG1*-overexpressing rice plants to *Xoc* in this paper.

Materials and methods

Plant materials, growth conditions and treatments

The rice seeds of two rice varieties of Zhonghua11 and Nipponbare were germinated at 28°C, then the seedlings were cultured by aquaculture in the green house for 16 h light/8 h dark cycle at 28°C. Some seedlings at the three-leaf stage were transplanted to the soil in pots, moving to outdoors and maintaining their normal growth.

The seedlings of Zhonghua11 at the three-leaf stage were subjected to different treatments. The seedling leaves were sprayed with 2 mM salicylic acid (SA), 100 μM methyl jasmonate (MeJA), 2 mM 1-aminocyclopropane-1-carboxylic acid (ACC), respectively, while the seedlings treated with 0.1% ethanol solution were chosen as control. The leaves under these treatments were collected at different times after treatments (0, 0.5, 3, 6, 9, 12 and 24 h). The roots and leaves from the seedlings of Zhonghua11 at three-leaf stage, internodes, leaf sheaths, leaf blades, flag leaves, and inflorescences from the rice plants of Zhonghua11 at booting stage were harvested for tissue-specific expression analysis. All the collected samples were frozen in liquid nitrogen and kept at −80°C.

Pathogen inoculation

A virulent *Xoc* strain, RS105 [21] was used in this study. The isolate was incubated on nutrient agar adding 100 mg/l rifampicin at 28°C for 3 days. The inoculum was prepared by suspending the bacterial mass in sterile water containing 0.05% Tween 20 to a concentration of approximately 1×10^8 cells/ml. The fully expanded leaves at the booting stage were inoculated with the pathogen suspension or the sterile water containing 0.05% Tween 20 by a pinpricked method [22]. The tip, middle and end part of each leaf was pricked, respectively.

To analyze the expression of *DEPG1* gene in *Xoc*-challenged rice, the fully expanded leaves at booting stage, which inoculated with the pathogen suspension or sterile water containing 0.05% Tween 20 (mock treatment), were collected at different time points (0–4, 6, 8, 10 days post inoculation), respectively. These collected samples were immediately frozen in liquid nitrogen, and stored at −80°C.

The disease level was scored by measuring lesion lengths at 2 weeks post inoculation. Statistic analysis of the lesion lengths was performed by using "one way ANOVA" program with Origin 8.0, and Tukey test was used for means comparison.

RNA extraction, cDNA synthesis and DNA preparation

Total RNA was extracted from the collected samples with the Trizol reagent (Invitrogen, USA) according to the manufacture's protocol, and then was treated with RNase-free DNase I (TaKaRa, Japan) to remove the potential genomic DNA contamination. The first-strand cDNA was synthesized with PrimeScript® RT reagent Kit (TaKaRa, Japan) according to the manufacture's protocol. Genomic DNA was isolated from rice seedlings using the cetyltrimethyl ammonium bromide (CTAB) method [23].

RNA expression analysis

Semi-quantitative reverse transcription PCR (RT-PCR) was performed using gene-specific primers (Table 1) to analyze tissue-specific expression of *DEPG1*. The expression level of *Actin* gene was used to standardize the RNA sample. For each gene, the semi-quantitative RT-PCR assays were repeated at least twice.

Quantitative real-time RT-PCR (qPCR) was conducted using SYBR®*Premix Ex Taq*™ II (TaKaRa, Japan) and StepOne™ Real-Time PCR System (Applied Biosystems, USA) with the gene-specific primers (Table 2), some primers were described previously [4]. The expression level of *Actin* gene was used to standardize the RNA sample for each qPCR. For each gene, the qPCR assays

Table 1 The primers for the construction of the plant expression vectors and semi-quantitative RT-PCR assays

Gene (GenBank accession number)	Primer name	Primer (5′–3′)	Use
DEPG1 (AK120289)	DEPG1F	CAGATACTCAAAGAGAGAGAACAGGGAGACAG[a]	Amplifying the coding resion of *DEPG1*
	DEPG1R	GAGAATTCACAAACAACGACACAGAGCTTC[b]	
Promoter of *DEPG1*	DEPROF	CACCCTATTTGGTCGACTGTCAG	Amplifying the promoter of *DEPG1*
	DEPROR	ACAAGGACCGATGGGAACAAG	
DEPG1	DEGFPF	CACCATGGCGGAAGTGGCATTGGC	Subcellular localization analysis
	DEGFPR	GTGGATATGCTCACTATGATCATG	
DEPG1	DE-sRTF	CTGGACAAATGGATACGGGA	Semi-quantitative RT-PCR asaay
	DE-sRTR	AATGGAGACAACGAAACGGC	
Actin (X15865)	Act-sRTF	GACCTTCAACACCCCTGCTA	Semi-quantitative RT-PCR asaay
	Act-sRTR	GAATGTGCTGAGAGATGCCA	

[a] The *underlined* nucleotides are the digestion site of *Eco*R V

[b] The *underlined* nucleotides are the digestion site of *Eco*R I

Table 2 The specific primers for quantitative real-time RT-PCR assays

Gene name	GenBank accession number	Forward primer (5′–3′)	Reverse primer (5′–3′)
Actin	X15865	TGTATGCCAGTGGTCGTACCA	CCAGCAAGGTCGAGACGAA
DEPG1	AK120289	GATTGGAGCTGTCACGGATT	TCGTTCATGTTCCTGAGCTG
ICS1	AK120689	TATGGTGCTATCCGCTTCGAT	CGAGAACCGAGCTCTCTTCAA
PR5	U77656	ACGACCAGACGAGCACCTTCA	TCGATCAGATGCCAGCTAACAAG
PR1a	AJ278436	CTACGTGTTTATGCATGTATGGACA	ACACTAAGCAAATACGGCTGACA
PR1#12	AK121108	ATCGTCCCTCGCTCGTTACAT	CGATCGTGGTTTTGTCTTTATTTC
PR1b	AJ278436	CACTCCAGCAACCGGAACA	AGGCGTAGTCGTAGTCGCTCTT
PAD4	CX118864	GCCAGCTCCCCTACGACTTC	CGTGTGCGGTGTAGGTTGTT
PAL1	X16099	GGGCAACCCAGTGACCAA	CGATTGCCTCGTCGGTCTT
PR10/PBZ1	D38170	CCCTGCCGAATACGCCTAA	CTCAAACGCCACGAGAATTTG
RSPR10	AK061606	GCCAAGATAAACGTGGAGTATGA	GTAGGGTGAGCGACGAGGTAG

were repeated at least twice, with each repetition having three replicates. Standard deviation was calculated for technical replicates.

Construction of plant expression vectors and transformation of rice

A 3,368 bp DNA including the open reading frame (ORF) of *DEPG1* was obtained by PCR amplification with the specific primers DEPG1F and DEPG1R (Table 1), using the *DEPG1* cDNA clone (http://cdna01.dna.affrc.go.jp/cDNA/, clone ID: 215008) as template. The PCR product was fused with 2× CaMV (cauliflower mosaic virus) 35S promoter, a translation enhancer and a napoline synthase polyadenylation signal (NOS) terminator to form an overexpression cassette, the cassette was inserted into the multiple cloning sites of pCAMBIA1301 vector. Then, the constructed overexpression vector of *DEPG1* was introduced into *Agrobacterium tumfaciens* strain EHA105 by the freeze–thaw method [24].

For a green fluorescent protein (GFP) fusion expression construct, the ORF of *DEPG1* without the termination codon was obtained by PCR amplification using specific primers DEGFPF and DEGFPR (Table 1). The PCR products were cloned into pENTR™/D-TOPO® vector (Invitrogen, USA), and then fused into the vector pMDC85 [25] by performing a recombination reaction using Gateway LR Clonase II Enzyme Mix (Invitrogen, USA). The target DNA of *DEPG1* was fused to 5′-terminus of the *GFP* gene and under the control of 35S promoter in this construct. The plasmids of the fusion construct *35S*::*DEPG1-GFP* and the empty vector *35S*::*GFP* were introduced into *A. tumfaciens* strain EHA105 by the method described above, respectively.

To construct a promoter-glucuronidase (GUS) expression vector, a 1,277 bp DNA with the promoter region of *DEPG1* gene was amplified by PCR with the specific primers DEPROF and DEPROR (Table 1), using the genomic DNA of Nipponbare plants as template. The PCR products were cloned into pENTR™/D-TOPO® vector, and then fused into the vector pMDC164 [25] by the method used for a GFP fusion expression construct described above. The combination construct P_{DEPG1}::*GUS* containing the promoter of *DEPG1* gene and *gusA* gene was introduced into *A. tumfaciens* strain EHA105 as described above.

Agrobacterium-mediated transformation was performed using calli derived from mature embryos of japonica variety Zhonghua11 or Nipponbare according to a published protocol [26].

Subcellular localization of *DEPG1*

The transient transformation of onion epidermal cells was carried out by *Agrobacterium*-mediation method as described by Yuan et al. [27] with the positive clones of *A. tumfaciens* carrying the fusion construct *35S*::*DEPG1-GFP* and the empty construct *35S*::*GFP*, respectively. The transformed onion epidermal cells were plasmolyzed by treating with 1 M sucrose solution for about a half hour before observation. Expression of the genes transformed into the onion epidermal cells was observed using confocal laser scanning microscope (LSM 510 META, ZEISS, Germany).

Sequences analysis

The structure of *DEPG1* was analyzed by comparing the genomic DNA (GenBank accession number AC135927) and complementary DNA (GenBank accession number AK120289) sequences, and sequence alignment was done using the "MegAlign" program with DNASTAR version

7.0. Additionally, a BLAST search was performed using the deduced protein sequence of *DEPG1* by the program "protein blast" online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The online tool PSORT program (http://wolfpsort.org) was used to predicate the localization of DEPG1 protein. The phylogenetic tree of DEPG1 protein and the other 18 characterized NBS-LRR R proteins was constructed using the program with MEGA version 4 [28].

Results

Molecular characterization of *DEPG1 gene*

Comparative analysis of the genomic and complement DNA sequences of *DEPG1* gene showed that it contains a 2,631 bp coding region, interrupted by two introns and flanked by a 499 bp 5′ and a 1,780 bp 3′ untranslated region (UTR). The first intron is located within the 5′ UTR and is 140 bp in length, while the second intron is positioned within the 3′ UTR, and is 121 bp in length. The transcript length of *DEPG1* is 4,910 bp (Fig. 1). The ORF of *DEPG1* gene encodes an 876-residue polypeptide with an estimated molecular mass of 100.3 kDa, and a calculated isoelectric point of 7.21. The N-terminal section of *DEPG1* protein contains three typical NBS family motifs [11]. The GGMGKS sequence (beginning at residue 222) corresponds to the kinase 1a (P-loop) consensus, while the LLVLDDI (beginning at residue 297) and GATKVLVTSRS (beginning at residue 327) conform to the kinase 2, and the kinase 3a consensus motifs [11, 29], respectively. The C-terminal region of the protein is composed of about 10 irregular LRRs between residues 592 and 876 (Fig. 2). Additionally, a BLAST search showed that the deduced DEPG1 protein sequence shares about 64% identity with that of the rice blast R protein Pi37.

The rice blast resistance (R) genes such as *Pi37*, *Pib*, *Pita*, *Pi9*, *Pi2*, *Piz-t*, *Pi36*, *Pb1*, *Pikm1*, *Pikm2-TS* and rice bacterial blight R gene *Xa1* are all NBS-LRR genes. Besides these genes, maize rust R gene *rp1-dp3* [30], maize stripe disease R gene *Rxo1*, barley rust R gene *Mla1* and *Mla7* [31, 32], and the R genes *N*, *RPP5* and *L6* from the dicotyledonous species [33–35] also belong to this type. Therefore, the deduced amino acid sequences of these R genes were included in the phylogenetic analysis. Altogether, these 18 R proteins and DEPG1 can be clustered into 14 clades: Mla1 and Mla6 (I), Pi36 (II), Pikm-1 (III), Pita (IV), Pikm-2TS (V), Pib (VI), Piz-t, Pi2 and Pi9 (VII), Pb1 (VIII), Rxo1 (IX), Xa1 (X), rp1-kp3 (XI), DEPG1 and Pi37 (XII), L6 (XIII), and N and RPP5 (XIV) (Fig. 3). This analysis suggests that *DEPG1* is more closely related to *Pi37* than to any other characterized R genes.

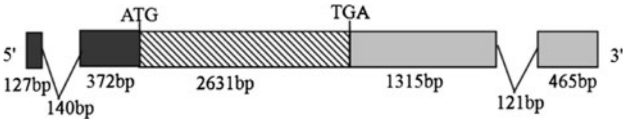


Fig. 1 The DNA structure of DEPG1 gene. *Hatched box*, coding region; *black box*, 5′-UTR; *gray box*, 3′-UTR; *fold lines*, 5′ and 3′ introns. The translation start codon (ATG) and stop codon (TGA) are also indicated

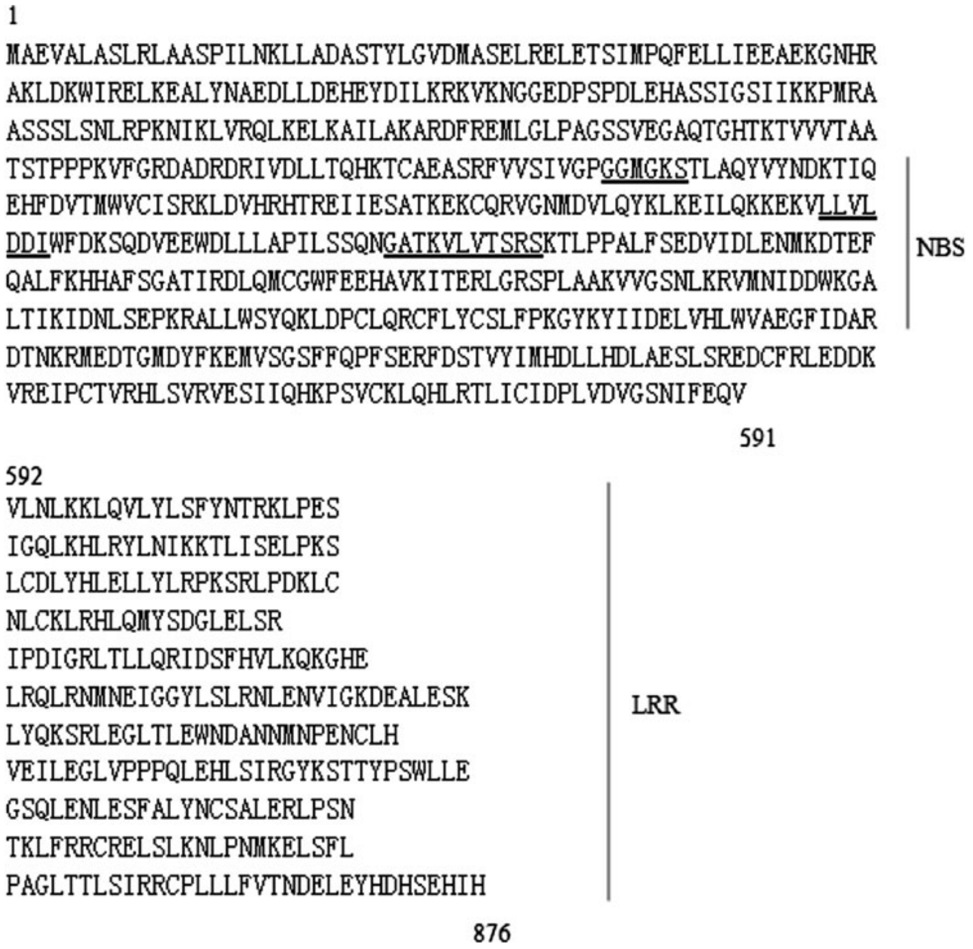
Tissue-specific expression of *DEPG1*

The expression profile of *DEPG1* in different rice organs and tissues detected by semi-quantitative RT-PCR showed that *DEPG1* gene is predominantly expressed in internodes, leaf blades, flag leaves and leaf sheaths from the plants at booting stage. A basal level of expression was also detected in roots and leaves of the seedlings at the three-leaf stage, roots and inflorescences of the plants at booting stage (Fig. 4). The basal expression level in inflorescences was lower than that in roots or leaves of the seedlings. These data indicated that expression of *DEPG1* is developmentally regulated, and is in a tissue and organ preferential manner.

The expression of the *DEPG1*-promoter (P_{DEPG1}) and *gusA* (GUS) fusion gene(P_{DEPG1}::*GUS*) in T$_1$ transgenic plants revealed that *DEPG1* is expressed in all tissues and organs of rice plants, including internodes, leaf blades, leaf sheaths, flag leaves and inflorescences of plants at booting stage and the seedlings (Fig. 5a–f). Observation of cross sections of leaves from the transgenic plant with P_{DEPG1}::*GUS* fusion gene, we found that *DEPG1* is highly expressed in the mesophyll cells, but is expressed at a relatively low level in the parenchyma cells and the vascular vessels (Fig. 5g). These results indicated that the expression of *DEPG1* is of tissue preference.

The expression of the six pathogenesis-related genes and *DEPG1* in response to *Xoc*

Some pathogenesis-related genes (PR genes) are considered the marker genes of the defense signals such as SA and jasmonic acid (JA) signaling pathway, and inducible expression of a set of the PR genes is associated with activation of defense responses [36–38]. To determine whether the defense response is activated during *Xoc* attack, we examined the expression patterns of the six PR genes including *PR1a*, *PR1*#12, *PR1b* [36], *PR10*/*PBZ1* [37, 39], *PR5* [40], and *RSPR10* [41] in rice plants of Zhonghua11 upon *Xoc* infection, respectively. The expression levels of the six PR genes in the leaves upon *Xoc* infection were higher than that of the leaves with mock treatment at the early stage of *Xoc* infection (1–2 days post



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1
MAEVALASLRLAASPILNKLLADASTYLGVDMASELRELETSIMPQFELLIEEAEKGNHR
AKLDKWIRELKEALYNAEDLLDEHEYDILKRKVKNGGEDPSPDLEHASSIGSIIKKPMRA
ASSSLSNLRPKNIKLVRQLKELKAILAKARDFREMLGLPAGSSVEGAQTGHTKTVVVTAA
TSTPPPKVFGRDADRDRIVDLLTQHKTCAEASRFVVSIVGPGGMGKSTLAQYVYNDKTIQ
EHFDVTMWVCISRKLDVHRHTREIIESATKEKCQRVGNMDVLQYKLKEILQKKEKVLLVL
DDIWFDKSQDVEEWDLLLAPILSSQNGATKVLVTSRSKTLPPALFSEDVIDLENMKDTEF   NBS
QALFKHHAFSGATIRDLQMCGWFEEHAVKITERLGRSPLAAKVVGSNLKRVMNIDDWKGA
LTIKIDNLSEPKRALLWSYQKLDPCLQRCFLYCSLFPKGYKYIIDELVHLWVAEGFIDAR
DTNKRMEDTGMDYFKEMVSGSFFQPFSERFDSTVYIMHDLLHDLAESLSREDCFRLEDDK
VREIPCTVRHLSVRVESIIQHKPSVCKLQHLRTLICIDPLVDVGSNIFEQV
                                                     591

592
VLNLKKLQVLYLSFYNTRKLPES
IGQLKHLRYLNIKKTLISELPKS
LCDLYHLELLYLRPKSRLPDKLC
NLCKLRHLQMYSDGLELSR
IPDIGRLTLLQRIDSFHVLKQKGHE
LRQLRNMNEIGGYLSLRNLENVIGKDEALESK              LRR
LYQKSRLEGLTLEWNDANNMNPENCLH
VEILEGLVPPPQLEHLSIRGYKSTTYPSWLLE
GSQLENLESFALYNCSALERLPSN
TKLFRRCRELSLKNLPNMKELSFL
PAGLTTLSIRRCPLLLFVTNDELEYHDHSEHIH
                               876
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Fig. 2 The structure of *DEPG1* product. The three conserved motifs forming the NBS region are underlined. The C-terminal LRR is shown detached from the rest of the sequence

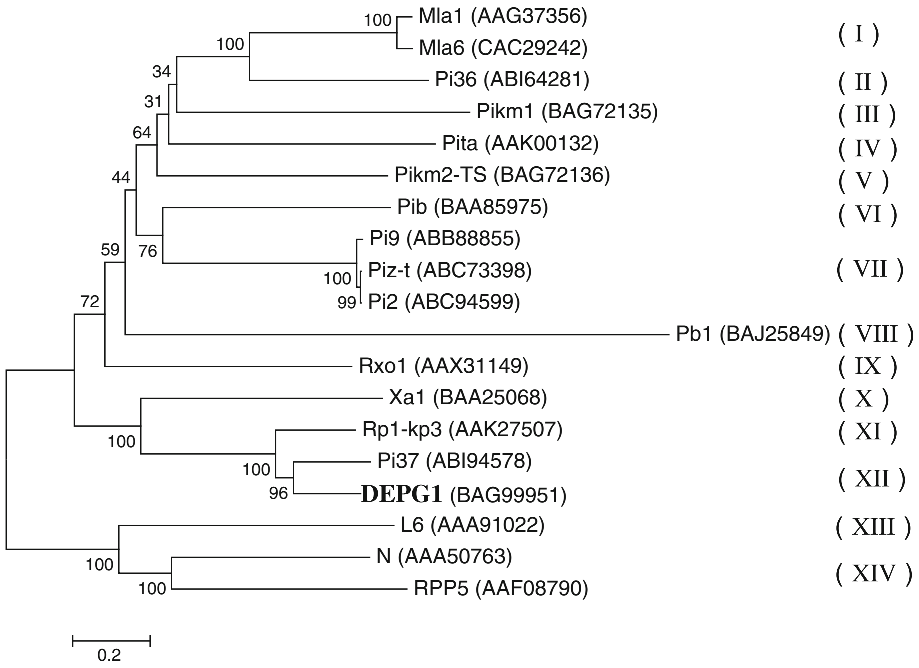
100
Mla1 (AAG37356)
Mla6 (CAC29242)
(I)
100
34
Pi36 (ABI64281)
(II)
31
Pikm1 (BAG72135)
(III)
64
Pita (AAK00132)
(IV)
44
Pikm2-TS (BAG72136)
(V)
Pib (BAA85975)
(VI)
59
76
Pi9 (ABB88855)
100
Piz-t (ABC73398)
(VII)
99
Pi2 (ABC94599)
72
Pb1 (BAJ25849)
(VIII)
Rxo1 (AAX31149)
(IX)
Xa1 (BAA25068)
(X)
100
Rp1-kp3 (AAK27507)
(XI)
100
Pi37 (ABI94578)
96
DEPG1 (BAG99951)
(XII)
L6 (AAA91022)
(XIII)
100
N (AAA50763)
100
RPP5 (AAF08790)
(XIV)
0.2

Fig. 3 Phylogenetic analysis of *DEPG1* with the 18 NBS-LRR R genes based on their amino acid sequences. *Numbers on the branches* indicate bootstrap percentages. GenBank accession numbers of these proteins sequences were listed in *brackets* behind their names. The unit branch length is equivalent to 0.2 amino acid residue substitutions per site, as indicated by the *bar* in the *lower left corner*

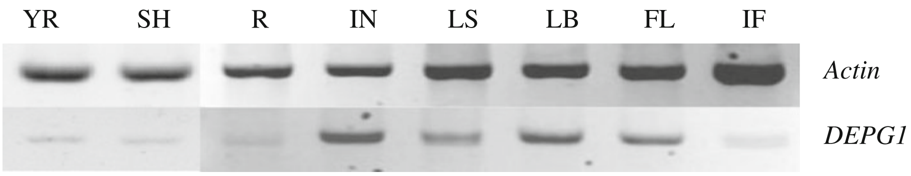

Fig. 4 Tissue-specific expression profiles of *DEPG1* in rice. Total RNA was extracted from roots (YR) and leaf blades (SH) of the seedlings, roots (R), internodes (IN), leaf sheaths (LS), leaf blades (LB), flag leaves (FL) and inflorescences (IF) of the plants at booting stage, respectively. The *Actin* gene was used as the standard control to show the normalization of the amount of the templates in semi-RT-PCR reactions

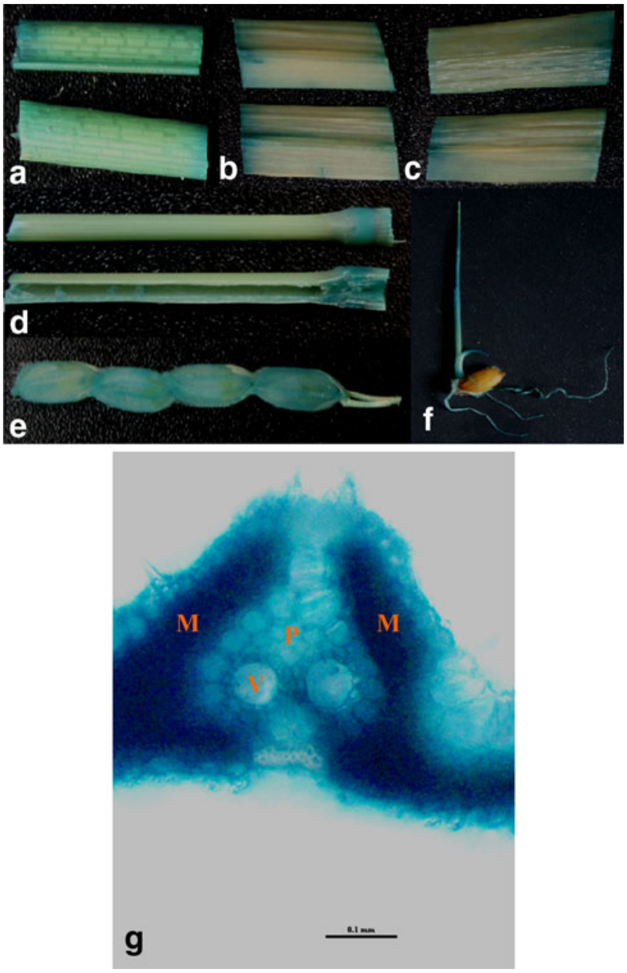

Fig. 5 Histological analysis of *DEPG1* expression in transgenic plants. The tissues and organs including leaf sheaths (*a*), leaf blade (*b*), flag leaves (*c*), internodes (*d*), inflorescences (*e*), seedling (*f*) and cross section of leaves (*g*) from T$_1$ P_{DEPG1}::*GUS* transgenic plant were subjected to histological GUS analysis; *M* mesophyll cells; *V* vascular bundles; *P* parenchyma cells. Because similar expression profiles were obtained in three independent transgenic lines, the data from a representative line is shown

inoculation, DPI) (Fig. 6c–h). The transcript levels of the PR genes in the leaves upon *Xoc* infection or with mock treatment decreased gradually over time (from 3 to 10 DPI). These results indicated that *Xoc* infection activates the defense response and induces the expression of the PR genes at the early stage; the defense response weakens and the expression of the PR genes recovers to the normal levels gradually over time.

To understand whether *DEPG1* is involved in the defense response triggered by *Xoc*, the expression patterns of *DEPG1* in rice plants of Zhonghua11 and Nipponbare upon *Xoc* challenge were examined by qPCR. As shown in Fig. 6, the expression level of *DEPG1* was lower in rice leaves of Zhonghua11 and Nipponbare inoculated with *Xoc* than that in leaves inoculated with water at 1 and 2 DPI. The expression of *DEPG1* was induced at 6 and 10 DPI in rice plants of Zhonghua11, and at 4 and 8 DPI in rice plants of Nipponbare. In addition, the expression level of *DEPG1* in leaves upon *Xoc* infection was lower than or comparative with that in leaves under mock treatment at the other marked time points. These data indicated that the expression of *DEPG1* is suppressed at the early stage of *Xoc* infection (1 and 2 DPI) when the defense response is activated, and then varies with the weakened defense response over time.

The expression profiles of *DEPG1* in response to the defense-signal compounds

Several phytohormones such as SA, JA and ethylene (ET), are implicated in complex signaling pathway and play pivotal roles in plant defense responses [42]. The expression pattern of *DEPG1* at the transcript level in rice seedlings under the treatments of the three defense-signal compounds SA, MeJA (methyl ester of JA) and ACC (precursor of ET synthesis) was examined by qPCR to assess their effects on the expression of *DEPG1*. The transcript level of *DEPG1* decreased quickly within 0.5 h after SA treatment, reduced to the lowest level at 12 h after treatment (HAT) and maintained a relatively low level at 24 HAT (Fig. 7a). The expression of *DEPG1* was also repressed by MeJA at 0.5 HAT and maintained a relatively low level at 24 HAT (Fig. 7b). Additionally, the expression of *DEPG1* in leaves with the ACC treatment was similar to that with MeJA and SA treatments (Fig. 7c). In summary, the three defense-signal compounds down regulated expression of *DEPG1*.

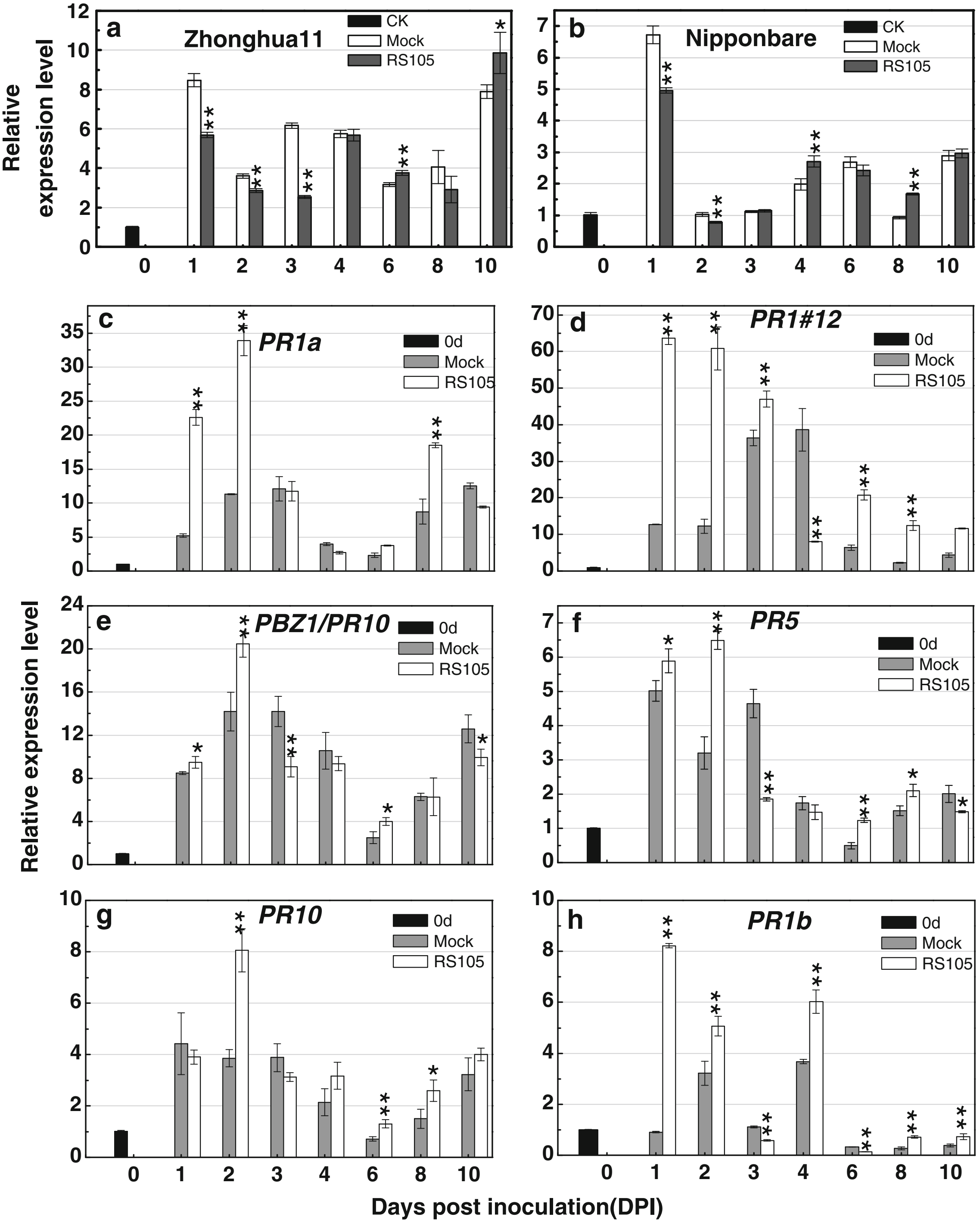

Fig. 6 The expression profiles of *DEPG1* and the six pathogenesis-related genes upon *Xoc* challenge. The leaves inoculated with the pathogen *Xoc* RS105 strain (RS105) or inoculated with sterile water (Mock) were collected at different days post inoculation, respectively. RNA extracted from these samples was used for the quantitative real-time RT-PCR (qPCR) analysis. **a** and **b** The expression of *DEPG1* in rice plants of Zhonghua11 and Nipponbare, respectively. **c**–**h** The expression of the six pathogenesis-related genes including *PR1a*, *PR1#12*, *PBZ1/PR10*, *PR5*, *RSPR10* and *PR1b* in Zhonghua11 plants upon *Xoc* challenge. "0" represents before inoculation; "1–4, 6, 8 and 10" represents the corresponding days post inoculation. *Bars* represent mean (3 replicates) ± standard deviation. "*" and "**" Indicate a significant (*t* test, $P < 0.05$) and an extremely significant (*t* test, $P < 0.01$) differences detected in the average relative expression levels between Mock and *Xoc*-inoculation treatments, respectively

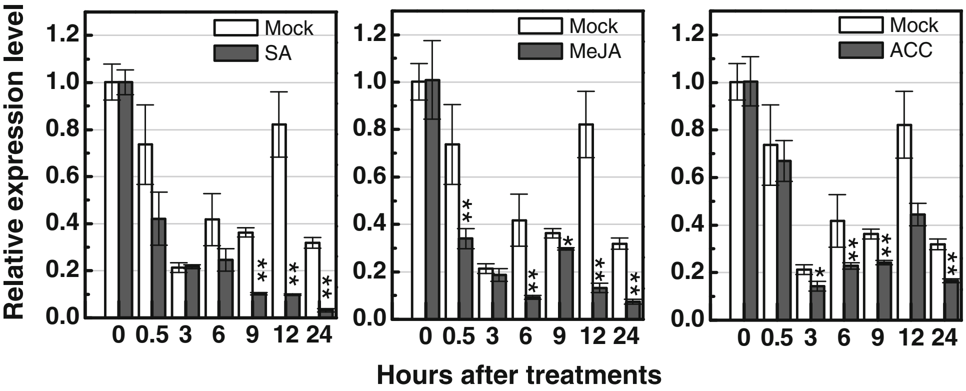

Fig. 7 The expression profiles of *DEPG1* in rice seedlings treated by the defense-signal compounds SA, JA and ACC. The seedlings of Zhonghua11 at three-leaf stage were sprayed with the defense-signal compounds 2 mM SA, 100 μM MeJA and 2 mM ACC, respectively. Total RNA extracted from the leaves collected at the successive time points after treatments was used for the qPCR analysis. *Bars* represent mean (3 replicates) ± standard deviation. "*" and "**" Indicate a significant (*t* test, $P < 0.05$) and an extremely significant (*t* test, $P < 0.01$) differences detected in the average relative expression levels between Mock and the different defense-signal compounds treatments, respectively

Subcellular localization of DEPG1 protein in the onion epidermal cells

DEPG1 protein was predicted to be localized in the cytoplasm by PSORT program due to the absence of the putative nuclear localization signal (PKRR) and signal peptide. To confirm the subcellular localization of DEPG1 protein, the *DEPG1-GFP* fusion gene and the gene encoding GFP were transiently expressed in onion epidermal cells, respectively. The fluorescence due to GFP in onion epidermal cells was monitored. As shown in Fig. 8, fluorescence signals due to DEPG1-GFP can be detected in the cytoplasm (Fig. 8a–c), while signals due to GFP can be detected in the cytoplasm and the nucleus (Fig. 8d–f). These results suggested that *DEPG1* is a cytoplasm protein.

Overexpression of *DEPG1* increases susceptibility to *Xoc* in transgenic plants

We over expressed *DEPG1* in rice then examined resistance of the transgenic plants to *Xoc* to determine the roles of *DEPG1* in rice–*Xoc* interactions. *DEPG1* driven by a double CaMV 35S promoter was transformed into the susceptible rice variety Zhonghua11. Six independent

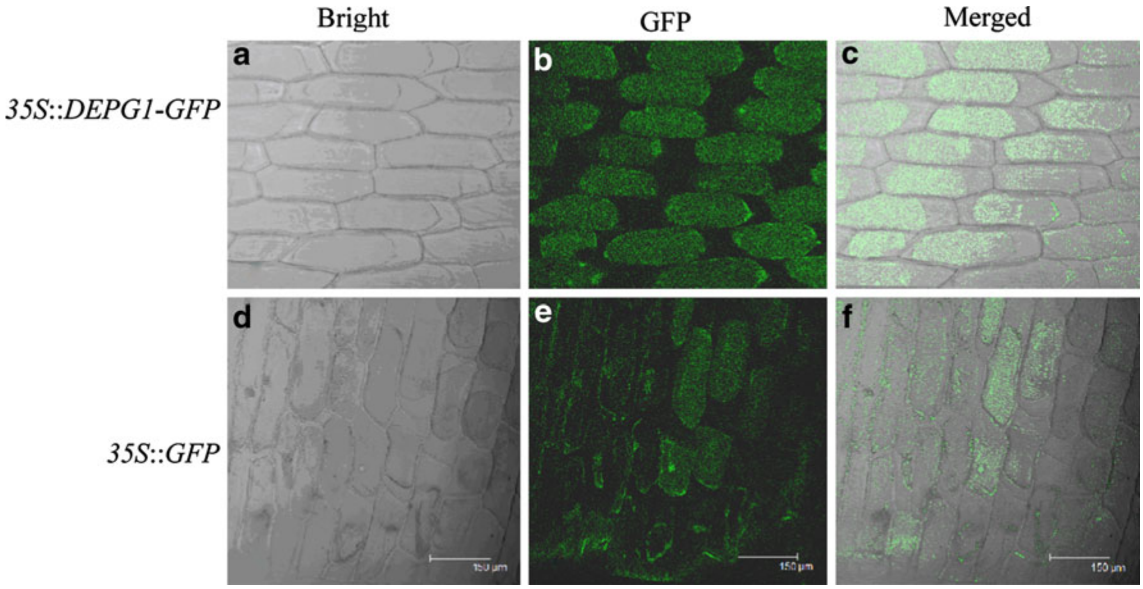

Fig. 8 Subcellular location of DEPG1 protein in onion epidermal cells. Onion epidermal cells were transformed with vector carrying *35S*::*DEPG1-GFP* or *35S*::*GFP* by *Agrobacterium*-mediated transformation and plasmolyzed by treating with 1 M sucrose. **a** and **d** Bright-field transmission images; **b** expression of DEPG1-GFP in the cytoplasm; **c** transmission images overlaying **a** and **b**; **e** expression of GFP (control) in the cytoplasm and the nucleus; **f** transmission images overlaying **d** and **e**. *Scale bars* 150 μm

transformants were obtained, and the expression of *DEPG1* was significantly increased in two of them (data not shown). Six T_1 transgenic plants from these transformants were inoculated with *Xoc* at booting stage. All of them showed increased expression of *DEPG1*, and the relative expression level in four of them was over 5 times that in wild-type plants (Fig. 9a). Moreover, the average lesion lengths of these transgenic plants were ranging from 6.4 to 9.6 mm, versus 5.1 mm for wild-type plants (Fig. 9b); the average lesion length of all six *DEPG1*-overexpressing transgenic plants (8.2 mm) was significantly (*t* test, $P < 0.01$) longer than that of wild-type plants (5.1 mm) (Fig. 9c). Therefore, these results suggested susceptibility to *Xoc* is enhanced in *DEPG1*-overexpressing transgenic plants.

The *DEPG1-GFP* fusion gene was transformed into the susceptible rice variety Nipponbare, and 13 independent transformants were obtained. Ten of the 13 transformants showed significantly increased expression of *DEPG1*, and their relative expression level was above 10 times that of wild-type plants (Fig. 10a). Additionally, the average lesion lengths of the 13 transformants were ranging from 5.5 to 7.8 mm versus 5.1 mm for wild-type plants (Fig. 10b); the average lesion length of all 13 *DEPG1*-overexpressing plants (6.7 mm) was significantly ($P < 0.01$) longer than that of wild-type plants (5.1 mm) (Fig. 10c). These results indicated that susceptibility of *DEPG1-GFP* transgenic plants to *Xoc* is increased. The similar results were also observed in T_1 *DEPG1-GFP* transgenic plants. The relative expression levels of all nine T_1 *DEPG1-GFP* transgenic plants were over 10 times that of wild-type plants (Fig. 10d). More, the average lesion lengths of the nine T_1 *DEPG1-GFP* transgenic plants were ranging from 8.5 to 12.1 mm versus 7.5 mm for wild-type plants (Fig. 10e); the average lesion length of all nine T_1 plants (10.2 mm) was significantly (*t* test, $P < 0.01$) longer than that of wild-type plants (7.5 mm) (Fig. 10f). Taken together, all results showed that enhanced susceptibility to *Xoc* is associated with overexpression of *DEPG1* in transgenic plants.

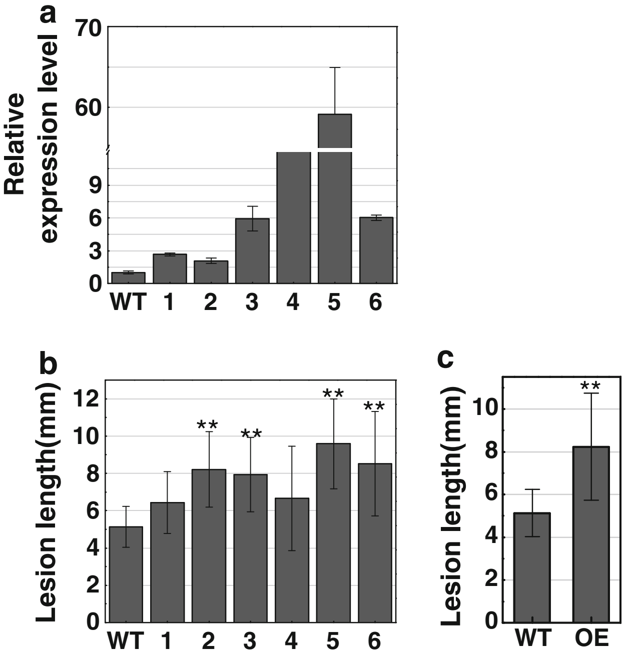

Fig. 9 The expression level of *DEPG1* and performance to *Xoc* RS105 strain of *DEPG1*-overexpresssing T_1 plants. **a** and **b** represent the relative expression levels of *DEPG1* and average lesion lengths of the six different *DEPG1*-overexpresssing T_1 plants, respectively; **c** the average lesion lengths of all the six *DEPG1*-overexpressing T_1 plants(OE) and wild-type plants (WT). *Bars* represent mean ± standard deviation. "**" Indicates that an extremely significant difference (*t* test, $P < 0.01$) detected in the lesion lengths between wild type and transgenic plants

Expression analysis of defense-related genes in the *DEPG1*-overexpressing transgenic plants

Lots of evidences support that SA plays key roles in disease resistance of monocotyledonous plants [43], and some PR genes are reported to contribute to the disease resistance [44]. To analyze the molecular mechanisms by which the *DEPG1*-overexpressing transgenic plants enhance susceptibility to *Xoc*, the expression of SA biosynthesis-related genes [38] including *PAD4*, *PAL1* and *ICS*, and some PR genes including *PR1a*, *PR1#12*, *PR5*, *PBZ1* and *PR10*, was examined by qRT-PCR. As shown in Fig. 11, the expression of *ICS* and *PR1#12* was significantly (*t* test, $P < 0.01$) down regulated, and the transcription of *PAD4* and *PBZ1* was repressed in both *DEPG1*-overexpressing transgenic plants, compared to wild-type plants. Besides, the expression of *PAD4* and *PR10* was comparable with wild-type plants, while *PAL1* and the other PR genes *PR1a* and *PR5* were differently expressed in the two examined *DEPG1*-overexpressing transgenic plants, compared to wild-type plants. In summary, the expression of SA biosynthesis-related genes *ICS* and *PAD4*, and the PR genes *PR1#12* and *PBZ1* was down regulated, while the expression of the other defense-related genes was in distinct ways in the two *DEPG*-overexpressing transgenic plants.

Discussion

The NBS-LRR genes play pivotal roles in disease resistance [45, 46] and plant development [18, 19]. There are about 480 NBS-LRR genes in the rice genome of Nipponbare [47], and only a small number of NBS-LRR genes have been characterized and identified to mediate disease resistance [1]. Moreover, most of these known NBS-LRR

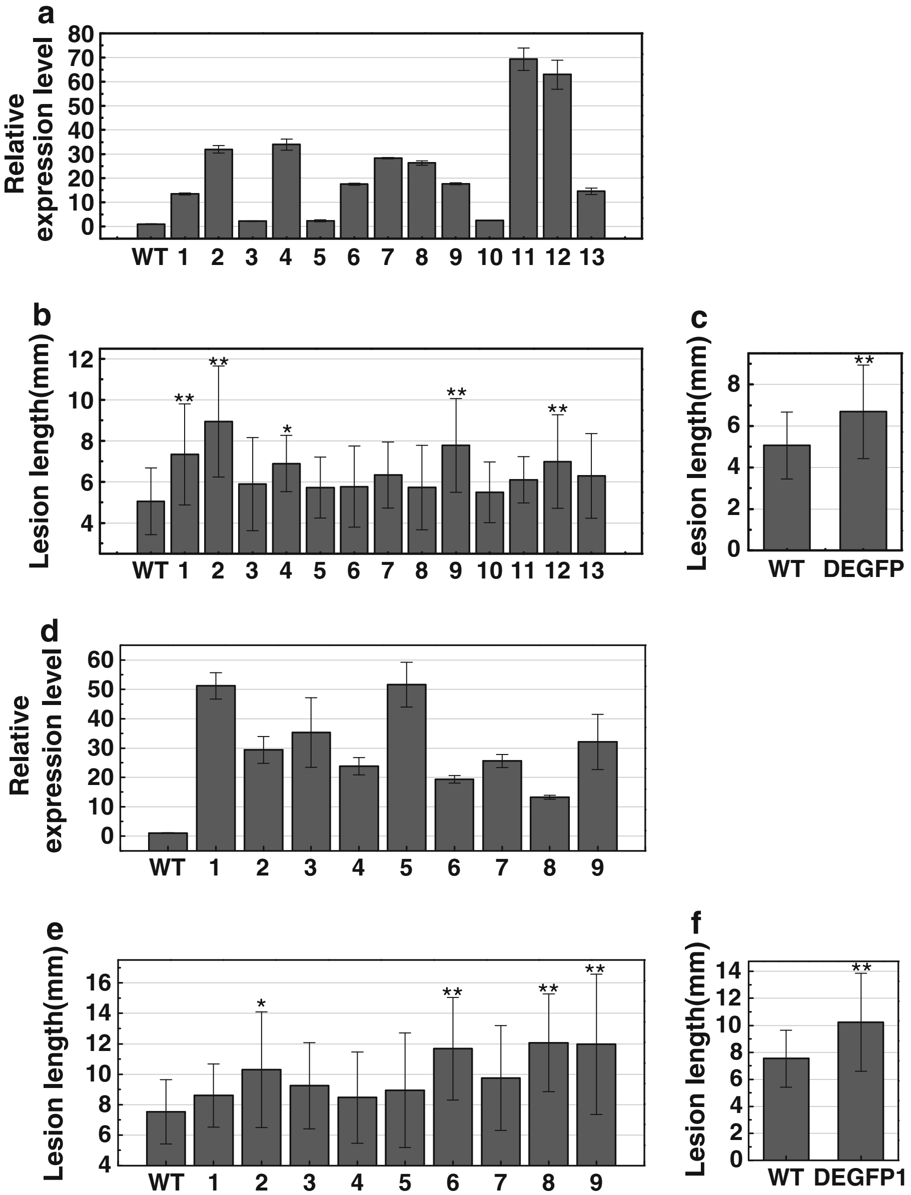

Fig. 10 The expression level in *DEPG1* and performance to *Xoc* RS105 strain of the T_0 and T_1 transgenic plants containing the *DEPG1-GFP* fusion gene. **a** and **b** Denote the relative expression levels of *DEPG1* and the average lesion lengths of the 13 different T_0 transgenic plants containing the *DEPG1-GFP* fusion gene, respectively; **c** the average lesion lengths of all the 13 T_0 transgenic plants (DEGFP) and the wild type plants(WT). **d** and **e** Refer to the relative expression levels of *DEPG1* and the average lesion lengths of the nine different T_1 transgenic plants containing the *DEPG1-GFP* fusion gene, respectively; **f** the average lesion lengths of all the nine T_1 transgenic plants (DEGFP1) and wild-type plants(WT). *Bars* represent mean ± standard deviation. "*" and "**" Indicate a significant (*t* test, $P < 0.05$) and an extremely significant (*t* test, $P < 0.01$) differences detected in the lesion lengths between wild-type and transgenic plants, respectively

genes confer resistance to the rice blast pathogen *M. grisea* or bacterial leaf blight pathogen *Xoo*. To our knowledge, the NBS-LRR genes that participate in the interaction between rice and *Xoc* have not been reported until now.

DEPG1 is a differentially expressed protein gene obtained by analysis of the proteomes from the rice leaves upon *Xoc* challenge in our previous studies (unpublished). The deduced DEPG1 protein contains a NBS and several LRR in carboxyl terminal (Fig. 2), and has approximately 64% sequence identity with the rice blast resistance gene *Pi37* product. Additionally, phylogenic analysis revealed that *DEPG1* is more closely related to *Pi37* than to the other 17 characterized NBS-LRR genes (Fig. 3). DEPG1 protein is located in the cytoplasm, which is consistent with that of most NBS-LRR R protein including Pi37 (Fig. 8). Taken together, all these results suggest that *DEPG1* is a NBS-LRR gene, and is highly similar to the rice blast R gene *Pi37*, and may play roles in rice–*Xoc* interactions.

To determine whether *DEPG1* gene takes part in the interactions between rice and *Xoc*, we evaluated resistance of *DEPG1*-overexpressing plants to *Xoc*, and observed that both *DEPG1* and *DEPG1-GFP* overexpressing plants show enhanced susceptibility to *Xoc* (Figs. 9, 10). These results suggest that *DEPG1* positively regulates susceptibility to *Xoc*. Based on these results, we speculated that *DEPG1* participates in interaction between rice and *Xoc*.

The activation of defense response always accompanies with induced expression of the pathogenesis-related genes such as *PR1a*, *PR1b, and PR10/PBZ1* [36, 38]. The six PR genes (including *PR1a*, *PR1b*, *PR10/PBZ1*, *PR5*, *RSPR10*

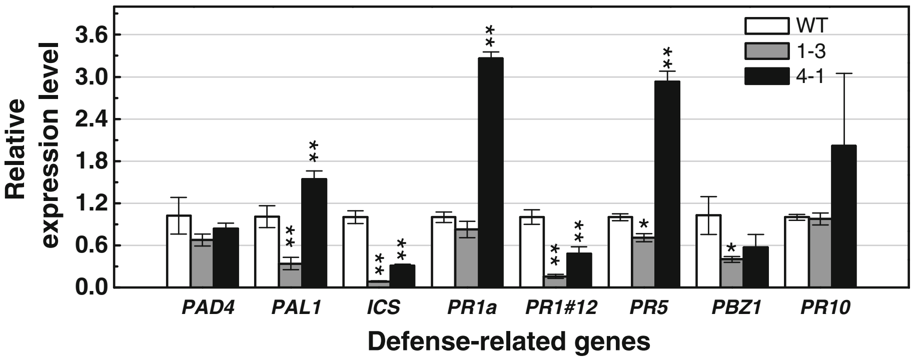

Fig. 11 Expression analysis of defense-related genes in *DEPG1*-overexpressing transgenic plants. RNA from the *Xoc*-inoculated leaves of transgenic plants and wild-type plants was used for expression analysis of defense-related genes; *WT* wild-type plants; *1-3* and *4-1* the two different *DEPG1*-overexpressing transgenic plants; "*" and "**" Indicate a significant (*t* test, $P < 0.05$) and extremely significant (*t* test, $P < 0.01$) difference detected in the average relative expression levels between wild-type and transgenic plants, respectively

and *PR1#12*) were induced in *Xoc*-inoculated rice at the first 2 days post inoculation (Fig. 6), implying that the defense response is activated at this stage. By contrast, the expression of *DEPG1* in both rice plants of Zhonghua11 and Nipponbare was repressed at both 1 DPI and 2 DPI. These results suggested that *DEPG1* is repressed in the defense response triggered by *Xoc*. The reduced expression of *DEPG1* in defense response was further confirmed by analysis of the expression of *DEPG1* in rice seedlings treated with the three defense-signal compounds including SA, MeJA and ACC (Fig. 7). Thus, we concluded that *DEPG1* participates in the defense response triggered by *Xoc* through down regulating its expression.

The expression profile of *DEPG1* in rice organs and tissues revealed that the expression of *DEPG1* is of tissue preference, the high expression level appeared in internodes, leaf sheaths, leaf blades and flag leaves (Fig. 4). Moreover, *DEPG1* was also highly expressed in the mesophyll tissues (Fig. 5g). In addition, the BLS pathogen *Xoc* principally infects the leaves of rice, and mainly colonizes the mesophyll tissues of rice leaves [48]. Thus, it seems that the high expression of *DEPG1* in the mesophyll tissues may help rice cells to detect the effectors of the pathogen *Xoc*. Since the disease resistance genes belonged to the NBS-LRR gene family are thought to take part in detecting the pathogens by interacting with the effectors of the pathogens directly or through monitoring the host proteins modified by the effectors [46]. If *DEPG1* functioned as the other known R gene that belonged to NBS-LRR genes in rice–*Xoc* interactions, the resistance phenotype would be observed. However, susceptibility to *Xoc* was observed in rice leaves of Nipponbare in which *DEPG1* is expressed at a high level. Therefore, we inferred that *DEPG1* functions in a different way. The bacterial pathogen always injects various effectors into plant cells during pathogenesis to overcome the host plant [48]. Thus, it is possible that *DEPG1* product may be the targets of the effectors of the BLS pathogen *Xoc*, and high level of *DEPG1* products in mesophyll cells may provide the pathogen effectors with enough targets, and advance colonization of *Xoc*. In current study, enhanced susceptibility of *DEPG1*-overexpressing plants to *Xoc* (Figs. 9, 10) supports this assumption well. In summary, the bacterial pathogen *Xoc* preferentially colonizes in the mesophyll tissues where *DEPG1* is expressed at a high level, supporting that *DEPG1* is involved in interactions between rice and *Xoc*.

Zhou et al. [22] reported that the non-host resistance gene *Rxo1* that mediates resistance to *Xoc*, could specifically activate several defense pathways such as SA and ET-dependent pathways and lots of genes involved in signaling pathways leading to hypersensitivity reaction during rice and *Xoc* interactions. Similarly, some other researches reported enhanced resistance to bacterial blight accompanies with SA accumulation and activation of several defense related genes including pathogenesis-related genes [3–5, 38]. In this study, overexpressing *DEPG1* increases susceptibility to *Xoc*, is the change in the expression of the components involved in SA defense signaling pathway responsible for enhanced susceptibility to *Xoc*? To answer this question, we examined the expression of several SA biosynthesis-related genes and some PR genes, and found that the expression of the SA biosynthesis-related genes *ICS* and *PAD4*, and the PR genes *PR1#12* and *PBZ1* was down regulated. Thus, inhibition of the SA biosynthesis pathway together with some PR genes might partially explain enhanced susceptibility to *Xoc*. However, whether the expression of the components involved in other defense signaling pathways (such as JA/ET signaling pathways) is altered in the *DEPG1*-overexpressing transgenic plants, and whether overexpression of *DEPG1* induces silence of its highly similar NBS-LRR genes that may contribute to the disease resistance, remain to be determined.

Due to the facts that *DEPG1* responds to defense signaling compounds and BLS pathogen *Xoc*, and enhanced susceptibility of the *DEPG*-overexpressing plants to *Xoc*, *DEPG1* might play pivotal roles in the rice–*Xoc* interactions. In addition, *Xoc* preferentially colonizes in mesophyll tissues where *DEPG1* is highly expressed, which may support that *DEPG1* plays roles in rice–*Xoc* interactions. For further study, the detailed molecular mechanism of susceptibility of the *DEPG1*-overexpressing plants to *Xoc* and assessment of resistance of the *DEPG1*-suppressing plants or knockout plants to *Xoc* will be achieved to further dissect the role of *DEPG1* in rice–*Xoc* interactions.

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