J. Plant Biol. (2012) 55:43–49
DOI 10.1007/s12374-011-9188-1

ORIGINAL RESEARCH

Establishment of Glucocorticoid-Mediated Transcriptional Induction of the Rice XA21 Pattern Recognition Receptor

Chang-Jin Park · Patrick E. Canlas · Pamela C. Ronald

Received: 22 September 2011 / Accepted: 26 September 2011 / Published online: 12 October 2011

Abstract The rice pattern recognition receptor, XA21, confers robust resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). We have generated transgenic plants that express *Xa21* only in the presence of the glucocorticoid hormone, dexamethasone (DEX). DEX-mediated transcriptional induction of *Xa21* is accompanied by upregulation of *pathogenesis-related 1* gene expression and restriction of *Xoo* multiplication. The DEX-inducible system can be used to synchronize the XA21-mediated response to infection throughout the entire leaf, facilitating the study of innate immunity.

Keywords Dexamethasone · Glucocorticoid · *Oryza sativa* · Pattern recognition receptor · XA21

Abbreviations

DEX	Dexamethasone
GVG	GAL4-binding domain–VP16 activation domain–GR fusion
PRR	Pattern recognition receptor
PR	*Pathogenesis-related*
Xoo	*Xanthomonas oryzae* pv. *oryzae*

Introduction

Constitutive overexpression is frequently used to study the effect of a particular gene on metabolism, development, immunity, or other biological processes. A drawback to this approach is that constitutive overexpression can have deleterious effects on the biology of the organism, resulting in severe growth defects, sterility, or lethality (Moore et al. 2006; Padidam 2003). For these reasons, more flexible gene expression systems have been developed that allow for fine-tuning of gene expression (Aoyama and Chua 1997). For example, chemical-mediated transcriptional induction of genes has proven useful for assessing gene function and facilitating precise temporal and spatial control of the target gene (Padidam 2003; Zuo and Chua 2000). Such inducible gene expression systems can be combined with large-scale microarray or proteomics experiments to elucidate gene function (Stolarov et al. 2001; Wang et al. 2005; Widjaja et al. 2010; Zentella et al. 2007).

The glucocorticoid receptor (GR) is a member of the family of vertebrate steroid receptors (Aoyama and Chua 1997). In the presence of the steroid hormone, glucocorticoid, GR is translocated from the cytoplasm to the nucleus and activates transcription from promoters containing glucocorticoid response elements (Picard 1994). A steroid-inducible chimeric transcriptional activator has been adapted for plants using the chimeric GAL4-binding domain–VP16 activation domain–GR fusion (GVG) (Aoyama and Chua 1997). The GR system works well in plants because it is simple and because glucocorticoid itself is nontoxic, without observable adverse physiological side effects (Aoyama and Chua 1997; Joubes et al. 2004; Kinkema et al. 2000). The chimeric GVG approach has facilitated studies of transcription factors and other proteins in *Arabidopsis*, tobacco, and rice, making it by far the most widely adopted inducible system to date (Lloyd et al. 1994; Moore et al. 2006; Simon et al. 1996).

The rice PRR, XA21, confers immunity to the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of

C.-J. Park · P. E. Canlas · P. C. Ronald (✉)
Department of Plant Pathology, College of Agricultural and Environmental Sciences, University of California, Davis,
One Shields Avenue,
Davis, CA 95616, USA
e-mail: pcronald@ucdavis.edu

bacterial blight disease (Song et al. 1995). XA21 binds the AxY^S22 peptide corresponding to the N-terminal region of Ax21 (activator of XA21-mediated immunity) that is highly conserved in all *Xanthomonas* species as well as in *Xylella fastidiosa* and the human pathogen, *Stenotrophomonas maltophilia* (Han et al. 2011; Lee et al. 2009). Recognition of sulfated Ax21 by XA21 is hypothesized to activate the kinase domain leading to XA21 autophosphorylation and/or transphosphorylation of downstream target proteins (Chen et al. 2010; Park et al. 2008; Wang et al. 2006). These proteins then control WRKY transcription factors to activate *pathogenesis-related* (*PR*) genes (Peng et al. 2010, 2008).

Despite the identification of about 100 proteins that are predicted to act as positive or negative regulators of XA21-mediated immunity (Park et al. 2010b; Ronald and Beutler 2010; Seo et al. 2011), the function of only a few has been characterized in detail. One of the reasons is that the developmentally regulated immunity triggered by XA21 is generally assayed when plants are fully grown (Century et al. 1999; Park et al. 2010c). Because plants carrying *Xa21* mount a robust defense response, preventing the spread of *Xoo* from the site of inoculation, it is not possible to assay the immune response throughout the entire leaf. Comparisons of immune responses between resistant and susceptible plants upon *Xoo* inoculation are also challenging to carry out because, in contrast to in the resistant plants, *Xoo* continuously spreads slowly throughout the xylem of susceptible plants. Thus, tissue harvested several days after inoculation from susceptible plants carries a mix of cells infected at different times.

These limitations have spurred us to develop a tightly synchronized XA21 expression system that provides the flexibility needed to carry out large-scale transcriptomic and proteomic analyses. In this paper, we describe the development of a chemically regulated *Xa21* expression system that will facilitate studies of XA21-mediated immune responses.

Materials and Methods

Plant Material and Growth Conditions

Rice (*Oryza sativa* L.) plants (cultivar Kitaake) were maintained in the green house. Growth chambers were set on a 14-h light and 10-h dark photoperiod, 28°C/26°C temperature cycle, and 85%/90% humidity. Healthy and well-expanded leaves from 6-week-old (nine- to ten-leaf stage) rice plants were used for *Xoo* strain Philippine race 6 (*Xoo* strain PXO99Az carrying Ax21 activity) inoculation.

pTA7002-Myc-Xa21 Construction

To construct the inducible *Myc-Xa21* plasmid, full length *Myc-Xa21* was PCR-amplified using primers, 5′-CACCAT GATATCACTCCCATTATTGATC-3′/5′-TCAGAATT CAAGGCTCCCACCTTC-3′, and cDNA generated from Ubi Myc-XA21 transgenic plant (Park et al. 2010a). The amplified fragment was cloned into pENTR/D-TOPO/D vector (Invitrogen). Positive clones were verified by DNA sequencing. The *Myc-Xa21* was recombined, using Gateway LR Clonase (Invitrogen), into Gateway-compatible pTA7002 vector for inducible transcription (Aoyama and Chua 1997).

Rice Transformation

Rice transformation was constructed as described previously (Chern et al. 2005). *Agrobacterium* strain EHA105 was used to infect rice callus for transformation. Transformants of rice cultivars Kitaake carrying the inducible *pTA7002-Myc-Xa21* plasmid were selected using hygromycin.

Immunodetection

For immunoblot analysis, proteins were separated by an 8% SDS polyacrylamide gel (SDS–PAGE). The proteins were then blotted onto a Hybond-P membrane (Amersham Pharmacia Biotech) using a SemiPhor Semi-Dry Transfer Unit (Amersham Pharmacia Biotech). For Myc-XA21 detection, anti-Myc mouse monoclonal IgG (Santa Cruz) and anti-mouse IgG, horseradish peroxidase linked whole antibody were used as primary and secondary antibodies at a final dilution of 1:1,000 and 1:5,000 for 2 h, respectively. For actin detection, anti-actin goat polyclonal IgG (Santa Cruz) and anti-goat IgG, horseradish peroxidase linked whole antibody were used as primary and secondary antibodies at a final dilution of 1:1,000 and 1:5,000 for 2 h, respectively. Bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to standard protocol.

Expression Analysis

For reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, total RNAs were extracted from leaves using TRIzol® reagent (Invitrogen) after each treatment. The RT reaction was performed following the manual for QuantumRNA 18S Internal Standards (Ambion). PCR analyses were performed with primer pairs, 5′-GAG CAAAAGCTGATTTCTGAGGAGGAT-3′/5′-ACCACC TAG CTTGTTTTCTCTGAC-3′ (for *Myc-Xa21*) and 5′-TTATCCTGCTGCTTGCTGGT-3′/5′-GGTCGTAC

CACTGCTTCTCC-3′ (for *OsPR1a*). The amplified products were resolved by gel electrophoresis after 28 cycles.

Xoo Inoculation and Lesion Length Measurements

For *Xoo* inoculation, rice plants were grown in the greenhouse until they were 6 weeks old and then transferred to the growth chamber. The *Xoo* strain PXO99Az was used to inoculate rice by the scissors-dip method (Chern et al. 2005; Song et al. 1995). *Xoo* PXO99Az was grown on a peptone sucrose agar plate (10 gl^{-1} peptone, 10 gl^{-1} sucrose, 1 gl^{-1} glutamic acid, 16 gl^{-1} agar, and pH 7.0) containing cephalexin (100 $\mu g\ l^{-1}$) for three days and suspended with water at OD=0.5 (600 nm) for inoculation. Only the top two to three expanded leaves of each tiller were inoculated.

Smear inoculation was carried out as described previously (Nozue et al. 2011). Rice leaves were smeared three times with *Xoo*-soaked gauze. For mock treatments, water was used instead of *Xoo* PXO99Az suspension.

Dexamethasone Treatments

A glucocorticoid derivative, dexamethasone (DEX), was purchased from Sigma-Aldrich. DEX was stored as a 100-mM solution in ethanol before use. For DEX treatment, 6-week-old plants were sprayed with a 30-μM solution containing 0.01% (*w*/*v*) Tween-20 and irrigated simultaneously with a 10-μM solution without Tween-20.

Results and Discussions

Generation of Transgenic Rice Plants Carrying a DEX-Inducible *Myc-Xa21* Cassette

To generate stably transformed transgenic rice plants, we constructed *pTA7002-Myc-Xa21*, which carries a chimeric transcription factor GVG (GAL4-binding domain–VP16 activation domain–GR fusion) and *Myc-Xa21* driven by a promoter containing six copies of the GAL4 UAS (upstream activating sequence) and the −46 to +1 region of the 35S promoter (Fig. 1).

The *pTA7002-Myc-Xa21* construct was transformed into Kitaake wild-type plants, and 12 independent transgenic plants were generated. Using the scissors-dip method, we inoculated the 6-week-old TA7002-Myc-XA21 transgenic plants (T_0 generation) with *Xoo* PXO99Az in the absence of DEX. Twelve days after inoculation (DAI), lesion lengths were measured and compared with those of Kitaake wild-type and homozygous Myc-XA21 transgenic rice plants carrying *Myc-Xa21* driven by its native promoter (Nat Myc-XA21, 20–1). Five transgenic plants of pTA7002-Myc-XA21 (T_0) showed no difference in resistance compared to Kitaake, whereas seven plants displayed moderate to high levels of enhanced resistance to *Xoo* PXO99Az, suggesting leaky expression of *Myc-Xa21* (data not shown). The observed high ratio (7 out of 12 independently transformed transgenic plants) of transgenic plants displaying enhanced resistance in the absence of DEX suggests that low leaky expression of *Myc-Xa21* is enough to trigger XA21-mediated immunity. Such leaky expression has previously been demonstrated using chemical-inducible systems (Padidam 2003; Zuo and Chua 2000).

We generated T_1 progeny from each of the five T_0 transgenic plants that were susceptible in the absence of DEX and analyzed the T_1 progeny for Mendelian segregation of the transgene. Transgenic lines 6, 8, and 9 displayed a Mendelian segregation ratio (1:2:1), indicative of a single insertion of the transgene into the genome (Fig. 2a). For the chemical-inducible XA21 system, DEX was applied to Kitaake wild-type, pTA7002-Myc-XA21, and Nat Myc-XA21 (20–1) plants. The next day, plants were inoculated with *Xoo* PXO99Az using the scissors-dip method. Twelve DAI, lesion lengths of all plants were measured (Fig. 2a). Transgenic line number 6 and the Kitaake wild-type developed long water-soaked lesions (approximately

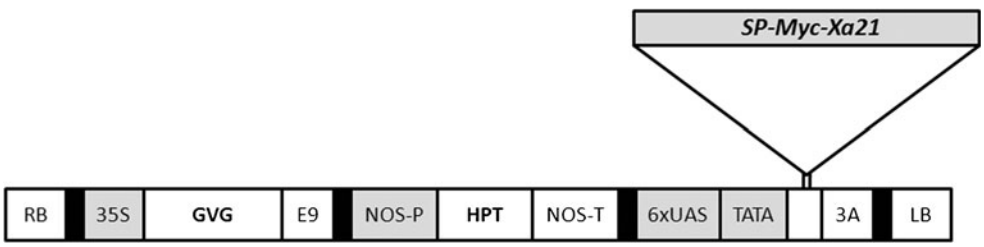

Fig. 1 The *pTA7002-Myc-Xa21* dexamethasone-inducible construct. The Myc tag was inserted in domain B of the *Xa21* gene following the putative signal peptide (domain A) as previously described (Park et al. 2010c; Wang et al. 2006). The *pTA7002-Myc-Xa21* construct is abbreviated as *Myc-Xa21* throughout the text. *RB* right border, *35S* cauliflower mosaic virus 35S promoter, *GVG* GAL4-binding domain–VP16 activation domain–glucocorticoid receptor fusion, *E9* poly (A) addition sequence of the ribulose bisphosphate carboxlase small subunit (rbcS-E9), *NOS-P* nopaline synthase promoter, *NOS-T* nopaline synthase terminator; *6× UAS* six copies of the GAL4 upstream activating sequence and the −46 to +1 region of the 35S promoter, *3A* poly (A) addition sequence of the pea rbcS-3A, *LB* left border

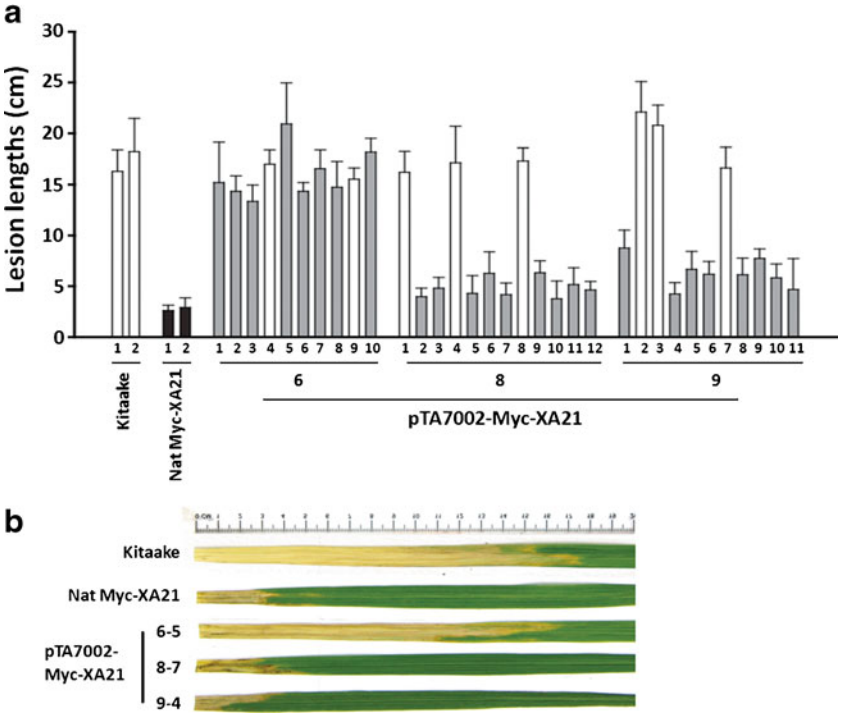

Fig. 2 Rice plants carrying *pTA7002-Myc-Xa21* confer resistance to *Xoo* strain PXO99Az in the presence of DEX. **a** DEX was applied to 6-week-old Kitaake plants, Kitaake transgenic plants carrying *Myc-Xa21* under the control of its native promoter (Nat Myc-XA21), and Kitaake transgenic plants carrying *pTA7002-Myc-Xa21* (pTA7002-Myc-XA21, lines 6, 8, and 9). The following day, plants were inoculated with *Xoo* PXO99Az. Lesion lengths were measured twelve days after inoculation. *Gray bars* in pTA702-Myc-XA21 transgenic plants represent segregants carrying the transgene. *White bars* represent segregants not carrying the transgene. *Each data point* represents the average and standard deviation of at least three independent replicates. DEX (30 μM for spraying and 10 μM for irrigating) was applied one day before inoculation. **b** High levels of resistance in DEX-treated transgenic plants 8 and 9. From *top to bottom*: Kitaake, transgenic plant (Nat Myc-XA21) carrying *Myc-Xa21* driven from its native promoter, and progeny from transgenic plants 6, 8, and 9 (pTA7002-Myc-XA21) carrying the DEX-inducible *pTA7002-Myc-Xa21*. Plants were treated with DEX one day after inoculation with *Xoo* PXO99Az. Photos were taken twelve days after inoculation

15 cm) typical of bacterial blight disease. In contrast, lines 8 and 9 were highly resistant with short lesions (approximately 3 to 5 cm). We hypothesize that *Myc-Xa21* in line 6 was not expressed because of position effects at an unfavorable chromosomal location. In Fig. 2b, we show one typical leaf from each of the inoculated rice plants after DEX treatment: Kitaake, homozygous Nat Myc-XA21 (20–1), and pTA7002-Myc-XA21 at twelve days after *Xoo* PXO99Az inoculation. Because of the high levels of resistance in lines 8 and 9 and the lack of leaky expression, these plants were chosen for further analysis.

DEX-Mediated Transcriptional Induction of *Myc-Xa21*

To examine DEX-inducible *Myc-Xa21* expression in the pTA7002-Myc-XA21 transgenic plants, we extracted total RNA from the following rice plants two days after DEX treatment: a 6-week-old Nat Myc-XA21 transgenic rice plant (20–1), a homozygous Myc-XA21 transgenic rice plant carrying *Myc-Xa21* driven by the strong maize *ubiquitin* constitutive promoter, (Ubi Myc-XA21, 7A-8-117), and T_1 progeny from pTA7002-Myc-XA21 transgenic plants, lines 8 and 9. To assess the expression level of the transgene in these plants, RT-PCR was performed with primers specifically targeting *Myc-Xa21* (Fig. 3a). 18S ribosomal RNA (*18S rRNA*) was used as an internal control in all tested transgenic plants. The protein levels of Myc-XA21 were also examined by western blot analysis using an anti-Myc antibody (Fig. 3b). Induced levels of Myc-XA21 protein in the pTA7002-Myc-XA21 transgenic plants were compared with Ubi Myc-XA21 and Nat Myc-XA21 transgenic rice plants. Non-Myc-tagged homozygous XA21 transgenic rice plants carrying *Xa21* driven by its native promoter (Nat XA21, 23A-1-14) were used as negative controls for the anti-Myc antibody. Anti-actin antibody was used as an internal loading control. We were not able to detect any induced *Myc-Xa21* in the DEX-treated Nat Myc-XA21 and Ubi Myc-XA21 plants, indicating that the DEX treatment does not affect the expression of *Myc-Xa21* under the control of its native and Ubi promoters. While the *Xa21* transcripts and the XA21 protein in the Nat Myc-XA21 transgenic plant displayed low levels of expression, tran-

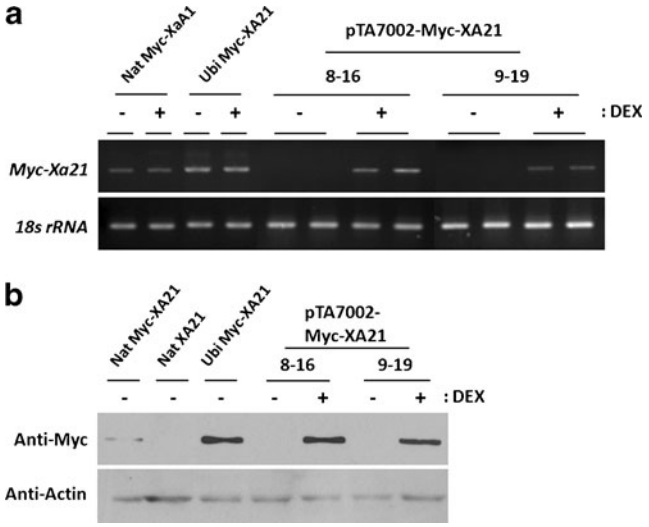

Fig. 3 Rice plants carrying *pTA7002-Myc-Xa21* induce *Myc-Xa21* after DEX treatment. **a** Total RNA was extracted from 6-week-old Nat Myc-XA21 (20–1), Ubi Myc-XA21 (7A-8-117), or pTA7002-Myc-XA21 (*lines 8–16* and *9–19*) 2 days after DEX treatment. RT-PCR was performed with primers specific for *Myc-Xa21*. Control RT-PCR reactions were carried out with *18S rRNA*. **b** Equal amounts (150 μg) of total protein from 6-week-old plants (Nat Myc-XA21, Nat XA21, Ubi Myc-XA21, pTA7002-Myc-XA21) were extracted 2 days after DEX treatment, analyzed by SDS–PAGE, and immunoblotted with anti-Myc antibody. Equal loading of total proteins was confirmed using an anti-actin antibody

scripts in the pTA7002-Myc-XA21 were not detectable in the absence of DEX, indicating that there was no detectable leaky expression of *Myc-Xa21* in lines 8 and 9. Two days after DEX treatment, the transcripts and protein were increased compared with those observed in the Nat Myc-XA21 plants. These results demonstrate that DEX-inducible GVG system allows fine-tuning expression of *Xa21* without undesirable background expression.

DEX-Mediated Transcriptional Induction of *Myc-Xa21* Confers Robust Resistance to *Xoo*

After PCR genotyping 20 progeny from each transgenic line, we identified line 9–1 as homozygous for the *pTA7002-Myc-Xa21* transgene (data not shown). To examine if DEX-induced XA21-mediated immunity can halt *Xoo* propagation, we inoculated pTA7002-Myc-XA21, Nat Myc-XA21, and pTA7002-Myc-XA21 (homozygous T_2 progeny of line 9–1) transgenic plants and the Kitaake wild-type with *Xoo* PXO99Az prior to the application of DEX. At six DAI, Kitaake displayed slightly longer lesions compared with the Nat Myc-XA21 transgenic plants (Fig. 4a; three and six DAI). The lesion lengths in the pTA7002-Myc-XA21 transgenic plants were similar to Kitaake, indicating that XA21-mediated immunity was not induced in the absence of DEX. Bacterial growth curves were carried out by monitoring *Xoo* PXO99Az populations in leaves (Fig. 4b). At six DAI, the population of *Xoo* PXO99Az in the pTA7002-Myc-XA21 plants reached approximately 1.0×10^8 colony-forming units per leaf (cfu/leaf), similar to the populations reached in the susceptible Kitaake plants. In contrast, populations of *Xoo* PXO99Az grew only to approximately 1.8×10^7 cfu/leaf in the in Nat Myc-XA21 plants. These results indicate that six DAI is an appropriate time point to investigate the effect of DEX

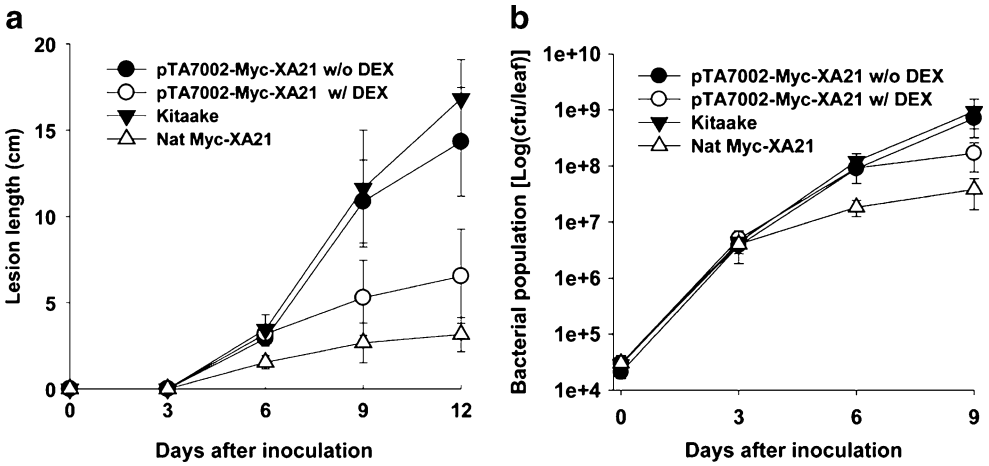

Fig. 4 DEX treatment restricts *Xoo* PXO99Az multiplication in rice transgenic plants carrying *pTA7002-Myc-Xa21*. **a** Lesion length development of Kitaake, Nat Myc-XA21 (20–1), and pTA7002-Myc-XA21 (homozygous T_2 progeny of line *9–1*) plants after *Xoo* PXO99Az inoculation. DEX was applied 6 days after inoculation (30 μM for spraying and 10 μM for irrigating). *Each data point* represents the average and standard deviation of at least three samples. **b** *Xoo* PXO99Az populations in Kitaake, Nat Myc-XA21 (20–1), or pTA7002-Myc-XA21 (homozygous T_2 progeny of line 9–1) plants 0 to 9 days after *Xoo* PXO99Az inoculation. DEX was applied 6 days after inoculation (30 μM for spraying and 10 μM for irrigating). *Capped*, *vertical bars* represent standard deviation of values (colony-forming units per leaf) from three samples. Experiments were repeated two or more times with similar results

treatment on lesion development and bacterial multiplication in the pTA7002-Myc-XA21 transgenic plant. Therefore, we treated the *Xoo* PXO99Az-inoculated rice plants with DEX at six DAI and continued to quantify the effect of *Myc-Xa21* induction over time. At 3 days after DEX treatment (nine DAI), the pTA7002-Myc-XA21 transgenic plant displayed significantly enhanced resistance to *Xoo* PXO99Az, with lesions of approximately 5 cm compared to the non-DEX-treated transgenic plants, which displayed lesion lengths of approximately 11 cm. The bacterial population measurements correlated well with lesion length development. At 3 days after DEX treatment (nine DAI), *Xoo* PXO99Az populations in the pTA7002-Myc-XA21 transgenic plant grew to only 1.7×10^8 cfu/leaf, a more than fourfold decrease compared to that observed from the non-treated transgenic plant. We found no significant difference in lesion lengths of the DEX-treated Kitaake and Nat Myc-XA21 compared to non-treated plants, demonstrating that DEX treatment itself does not have an effect on symptom developments (data not shown). These results indicate that, while *Xoo* PXO99Az multiplies normally in untreated pTA7002-Myc-XA21 transgenic plants, the application of DEX induces XA21-mediated immunity, restricting bacterial multiplication.

Establishment of a Synchronized XA21-Mediated Immune Response

We next examined if the XA21-mediated immune response can be synchronized throughout the entire leaf after DEX treatment. For these experiments, we used the smear inoculation method, which provides a useful strategy to infect the entire leaf through mild surface wounds and broken trichomes. In contrast, the traditional scissors-dip method is restricted to infection of leaf tips (Nozue et al. 2011). Therefore, for these experiments, we first inoculated pTA7002-Myc-XA21 rice leaves (9–1–2, −3, and −5) using the smear inoculation and then applied DEX four days later. Leaf tissues were harvested two days after DEX treatment (six days after smear inoculation).

Total RNA was extracted from these leaves, and accumulation of *Myc-Xa21* and *OsPR1a* transcripts was assessed. *PR1* is a defense-related marker gene which is known to be induced during the resistant response in *Arabidopsis*, rice, and other plant species (Kinkema et al. 2000; Liu et al. 2007). We have previously reported that *OsPR1* transcripts significantly accumulate following the XA21-mediated immune response (Park et al. 2010c; Ponciano et al. 2007). RT-PCR was performed with primers targeting *Myc-Xa21* and *OsPR1a* (Fig. 5). We found no significant induction of *OsPR1a* in the Nat Myc-XA21 and Kitaake plants two days after DEX treatment in the absence of *Xoo* PXO99Az (data not shown). Our result is consistent with previous reports (Kawai-Yamada et al. 2001; Kinkema et al. 2000; Spoel et al. 2003), indicating that the chemical treatment does not affect *OsPR1a* expression. The expression of *Myc-Xa21* and *OsPR1a* was not detected in the non-treated pTA7002-Myc-XA21 transgenic plants in the presence and absence of *Xoo* PXO99Az (zero and six DAI). In contrast, two days after DEX treatment (six DAI), *Myc-Xa21* and *OsPR1a* were highly induced in the *Xoo* PXO99Az-inoculated pTA7002-Myc-XA21 transgenic plants. The Nat Myc-XA21 transgenic plants displayed much lower levels of *Myc-Xa21* and *OsPR1a*. These results indicate that DEX induction triggers XA21-mediated immunity throughout the entire leaf after *Xoo* PXO99Az has already propagated and spread. Thus, this approach will be useful for transcriptomic and proteomic experiments where a precisely synchronized response is critical for identifying components of the XA21-mediated immune response.

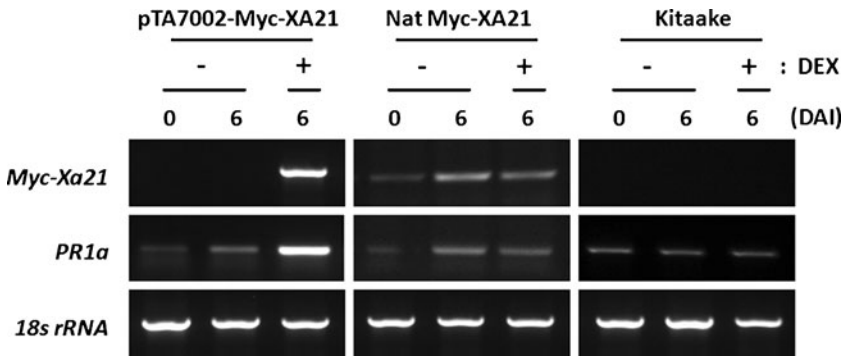

Fig. 5 Rice plants carrying *pTA7002-Myc-Xa21* upregulate *pathogenesis-related 1a* after DEX treatment. RNA accumulation of the *Myc-Xa21* and *OsPR1a* transcripts in the pTA7002-Myc-XA21 plant (9–1–2, −3, and −5) was examined before and after DEX treatment. DEX was applied 4 days after inoculation (30 μM for spraying and 10 μM for irrigating), and leaf tissues were harvested 6 days after inoculation. Total RNA was extracted, and RT-PCR was performed using *Myc-Xa21*- and *OsPR1a*-specific primers. Control RT-PCR reactions were carried out with *18S rRNA*-specific primers

Acknowledgments We thank Dr. Sang-Wook Han and Dr. Ofir Bahar for critical reading of the manuscript, Dr. Mawsheng Chen for the Gateway-compatible pTA7002 vector, and Randy Ruan for lab and greenhouse management. We also thank Seo Jung Yang and Timothy Tong for the technical assistance. This work was supported by the National Institute of Health (NIH, GM55962) and the National Science Foundation (NSF, IOS-0817738).

Conflict of Interest The authors declare no competing financial interests.

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