Microarray Analysis of *bacterial blight resistance 1* mutant rice infected with *Xanthomonas oryzae* pv. *oryzae*

So Young Yi^{1†}, Ha Yeon Lee^{1†}, Hyun A Kim¹, Chan Ju Lim¹, Woong Bom Kim¹, Hyun A Jang¹, Jong-Seong Jeon², Suk-Yoon Kwon^{1*}

¹Plant Systems Engineering Research Center, KRIBB, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea

²Crop Biotech Institute and Graduate School of Biotechnology, Kyung Hee University, Yongin 446-701, Korea

ABSTRACT We analyzed the transcriptional profile of the *Xoo* infected *bbr1* mutant using a commercial rice gene chip containing 51,279 transcripts. Microarray revealed 92 genes with increased levels of expression and 22 genes with decreased levels of expression in *bbr1*. Some of the differentially expressed genes were validated by qRT-PCR. Higher expression of defense-related genes and AP2 domain containing transcription factors along with lower expression of reactive oxygen scavenging enzymes may be responsible for defense signaling in the *bbr1* upon *Xoo* infection. The putative target genes of AP2 domain containing transcription factors also showed differential gene expression during *Xoo* infection, some of which encoded bacterial pathogen resistance-related protein. Induction of AP2 domain containing transcription factors along with up-regulation of their putative target genes during *Xoo* infection may inhibit pathogen spread in the *bbr1*. This observation supports the hypothesis that AP2 domain containing transcription factors is involved in the regulation of differentially expressed genes in *bbr1*.

Keywords Bacterial blight resistant rice, Gamma-ray mutant, *Xanthomonas oryzae* pv. *oryzae*, ERF and DREB/CBF

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important crop in the world as it feeds half of the world's population. The stable production of rice is affected by biotic and abiotic stress. The rice diseases caused by plant pathogenic fungi, bacteria and viruses are capable of causing heavy loss on rice crop, the global yields of which is annually decreasing by 10-15% (Dai *et al.* 2007). The use of resistant cultivars is one of the most important factors used to control diseases.

Because worldwide rice production has been severely affected by bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), early research efforts have been focused on the utilizing disease resistance (*R*) genes in rice. Since the first cloning of the *R* gene *Xa21* which confers resistance to *Xoo* almost twenty years ago (Song *et al.* 1995), nearly 30 major *R* genes for resistance to *Xoo* have

been identified (Kurata and Yamazaki 2006) and five of them: *Xa1*, *Xa3/Xa26*, *xa5*, *xa13* and *Xa27*, have been cloned: (Yoshimura *et al.* 1998; Iyer and McCouch 2004; Sun *et al.* 2004; Gu *et al.* 2005; Chu *et al.* 2006; Jiang *et al.* 2006). Genetic studies of BB resistance have resulted in the development of donor lines carrying major *R* genes. A number of these donor lines have been used in rice breeding programs around the world. Using transgenic approach, agronomically important cultivars such as IR64 and IR72 have been transformed with the *Xa21* gene and field trials of selected lines were successfully undertaken in China (Zhang *et al.* 1998; Tu *et al.* 2000). Further, the *Xa21* gene has been introduced into a widely used restorer of hybrid rice in China, Minghui 63, in order to produce BB-resistant hybrid rice with elite agronomic characters (Zhai *et al.* 2002).

Mutation breeding is also an important tool for crop improvement. During the past 75 years, more than 3,100

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*Corresponding author Suk-Yun Kwon, sykwon@kribb.re.kr, Tel: +82-42-860-4340, Fax: +82-42-860-4349

[†]They are equally contributing authors.

mutant varieties have been produced worldwide (The FAO/IAEA Mutant Varieties Database, <http://mvgs.iaea.org/AboutMutantVarieties.aspx>). More than 501 new varieties in rice have been obtained by applying different mutagenic agents. In Asia Pacific, there are approximately 343 mutants of rice released (Ahloowalia *et al.* 2004).

DNA microarray can measure the individual transcript level of tens of thousands of genes simultaneously, thus providing a high-throughput means for analyzing gene expression levels at the whole-genome scale (Schena *et al.* 1995; Chu *et al.* 1998) that may help elucidate the network of defense response. For example, microarray has been used to characterize the rice-pathogen interaction, such as the interaction of rice-*Xoo* (Li *et al.* 2006; Kottapalli *et al.* 2007), -flagellin (Fujiwara *et al.* 2004), -lipopolysaccharides (Desaki *et al.* 2006), -fungal elicitor (Kim *et al.* 2005), -rice dwarf virus (Shimizu *et al.* 2007) and -plant hopper (Cho *et al.* 2005).

In previous study, six rice mutant lines (M5 generation); TILL300-534, TILL300-537, TILL300-1212, TILL300-793, TILL300-693 and TILL300-651, which present resistant phenotype against *Xoo* KXO85 were selected from 3000 lines of gamma-ray mutated M3 plants (Lim *et al.* 2010). Among selected six mutant lines TILL300-651 (*Bacterial Blight Resistance 1*; *bbr1*) showed relatively strong induction of rice *R* gene *Xa21* which confers resistance to *Xoo* and other rice *R* genes such as *Pi36* and *Pi-ta* against *Magnaporthe oryzae* (Lim *et al.* 2010). To further understand defense response in the *bbr1*-*Xoo* interaction, we profiled the expression of rice genes involved in the phenotype of rice mutant, *bbr1*, using a commercial rice gene chip containing 51,279 transcripts representing two rice cultivars, indica and japonica.

MATERIALS AND METHODS

Plant materials and pathogen inoculation

'Dongan' seeds (*Oryza sativa* L. cv. *japonica*) were allowed to imbibe water overnight at 22°C and kept on moistened filter paper until germination. Germinated seeds were grown in soil in a greenhouse for 40 days prior to *Xoo* inoculation.

Bacterial cells of *Xoo* were suspended in sterile distilled water and the inoculation concentration was adjusted to OD₆₀₀ = 1.0 (Song *et al.* 1995). Rice leaves were inoculated with the bacterial suspension using the leaf clipping method (Kauffman *et al.* 1973). For gene expression analysis, leaf samples were collected from untreated control and infected leaves at 10 days after inoculation. The samples were flash frozen in liquid nitrogen and stored at -80°C.

RNA Isolation and qRT-PCR analysis

Total RNA was isolated from the collected samples using RNeasy mini kit (Qiagen) according to the manufacturer's instruction. Approximately 1 µg DNA-free RNA was used for first-strand cDNA synthesis using the Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase for quantitative real-time polymerase chain reaction (qRT-PCR; Fermentas). The qRT-PCR reactions were performed using a Thermal Cycler Dice Real Time System TP850 (TaKaRa, <http://www.takara-bio.com>) and SYBR Premix Ex Taq (TaKaRa). Primer sets prepared at 0.1 µM final concentration were used for a final volume of 25 µL. The thermal profile of the qRT-PCR reactions was 10 min at 95°C, 40 cycles of 5 s at 95°C/20 s at 60°C. Subsequently, a dissociation curve was generated. All reactions were carried out in triplicate. Primers used for qRT-PCR are listed in the Table 1.

Microarray analysis

The microarray experiment was conducted by the DNA Link Corporation (Seoul, Korea) according to the Affymetrix technical manual (<http://www.affymetrix.com/support/index.affx>). Total RNAs were extracted from the samples stored at -80 °C and gene expression was compared between the wild type 'Dongan' and *bbr1* mutant with two replicates. Hybridization, washing, staining and scanning procedures were performed as described in the Affymetrix technical manual. The analysis software was the Affymetrix Command Console, R affy-package (2.9.2), Expression Console1.1, DAVID.

Table 1. Summary table displaying Arabidopsis orthologous of differentially expressed genes with known roles in disease resistance (R-), transcript regulation (TF-) and oxidative stress (POD-) and sequences of forward and reverse primers used in quantitative RT-PCR to validate the 17 selected gene expression changes determined by microarray analysis. TF, Transcription factor; R, Resistance; POD, Peroxidase.

Primer name	Gene title	Arabidopsis orthologous	Forward-primer	Reverset-primer
TF-1	Os02g0527300	AT2G26150	gtggcactagtcagcaagca	tactctcccaagctgcgttt
TF-2	Os06g0127100	AT4G25480	ctacgcgtactacggcaaca	gaggagcaaagctggttgag
TF-3	Os08g0474000	AT4G34410	gagacaggggaccagctct	ttcaattagtacaccagagccaat
TF-4	Os04g0583900	AT5G37260	ctccacaaaacaggagtg	tgtttcttagctcgctgt
TF-5	Os03g0327800	AT3G04070	ctaggtcgatcatgc	ccggctttatgatcttgaca
TF-6	Os07g0558100	AT4G21440	gcaacaaccacaacgtcaac	agtgttcgattcgctctgt
TF-7	Os01g0975300	AT5G59780	cagccagaggatgagtcgt	gcgaataatccgagcagaag
TF-8	Os01g0141000	AT1G13260	atcagcgtagctcctgccta	tgcaatctctgacctgacaaa
R-1	Os01g0944900	AT4G16260	gcttactaccggagcgtcaa	atgacggatgggttggtg
R-2	Os02g0194700	AT3G45140	gctgcatttgggacaagatt	atccgtccgcatgacatact
R-3	Os03g0129100	AT2G39200	aaagggtgaggtcggaagat	ggccatcaccgtgtacact
R-4	Os06g0698300	AT4G31750	ctgcaaagaagctcctccag	tctgcttgccacaagacaac
R-5	Os10g0490800	AT2G15130	gtggactacgcgaagcaggt	gtcacctccgtcctcacg
POD-1	Os03g0235000	AT5G06720	ggcaactccatggtcaagat	gcgctccacaacacattaaa
POD-2	Os07g0677100	AT5G05340	atcaggcttagctgtccaa	tcggtacataacatgggttc
POD-3	Os07g0677200	AT5G05340	agctgtccaaggtgaactc	atggctgtctgtctccatac
POD-4	Os08g0113000	AT4G33420	ctgaattgccgccttag	cctccatgccacaatacaaa
ACTIN	Os03g50890	AT3G18780	ggaactggataggtcaaggc	agtctcatggatacccgag

RESULTS AND DISCUSSION

Isolation of the *bbr1* mutant with enhanced *Xoo* resistance

To examine whether the *Xoo* resistant phenotype can be maintained in the next generation, progeny from the TILL300-651 (M5) were tested for the *Xoo* resistance phenotype. Figure 1a shows a picture of typical leaves from each of the following inoculated wild type ‘Dongan’ and *bbr1* mutant. While all *bbr1* mutant progenies tested were resistant, showing relatively short lesions, inoculated leaves of wild type ‘Dongan’ developed water soaked long lesions (Fig. 1a, b). This result demonstrates that the *bbr1* mutant enhanced resistance to *Xoo*.

Differential gene expression in the leaves of *bbr1* mutant plants

Leaf samples were collected from the uninoculated (no

treatment; NT) and *Xoo*-inoculated (10 DAI) wild type ‘Dongan’ (WT) and *bbr1* mutant (M6), to detect differential gene expression under both conditions. To reduce experimental variation we separately pooled the leaf samples from eight inoculated/uninoculated WT and *bbr1* mutant of individual biological replicate. Total RNA was then isolated from pooled samples and used for labeling. To identify the significantly differential expression of *Xoo* responsive genes in *bbr1* compared to WT, we applied the following criteria; (a) the gene expression change occurred at the same direction (increase or decrease) in replication; and (b) the average ratio (fold change of *bbr1*/WT signal intensity) of expression levels in all microarray analyses was greater than 4 or less than -4. Applying these criteria, we detected 92 genes with increased (up-regulated) levels of expression and 22 genes with decreased (down-regulated) levels of expression in *bbr1* (Fig. 2). These genes were considered as characteristics of the *bbr1* mutant during *Xoo* infection.

Fig. 1. Phenotypic analysis of the rice *bbr1* mutant. (a) Water-soaked disease lesions on three leaves from wild type ‘Dongan’ (WT) and *bbr1* mutants (M6). 40-day-old plants were inoculated with *Xoo*. Image was taken two weeks after *Xoo* inoculation. (b) Leaf lesion lengths of eight *bbr1* M6 progeny. Lesion lengths were measured at two weeks post *Xoo* inoculation. Experiments were repeated two times with similar results. Each data point represents the average and standard deviation of at least three leaves.

Fig. 2. Gene ontology classification of differentially expressed genes in rice leaves at 10 day post inoculation. Functional categories are derived from the primary annotation of biological process retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg). Differentially-regulated genes were expressed at 4-fold higher or lower levels in the *bbr1* mutant compared to wild type from two independent microarray analyses. White bars indicated the number of four-fold down regulated genes and black bars indicated the number of four-fold up-regulated genes compared to the WT.

These 114 differentially expressed genes (DEGs) were further analyzed and annotated using public access databases (<http://www.genome.jp/kegg>). According to predicted functions of their homologous genes in *Arabidopsis*, these genes could be assigned to eleven biological process

categories (Fig. 2), including defense and/or stress response, carbohydrate metabolic process, hormone responses, lipid metabolic process, nucleic acid metabolism, oxidation reduction, phosphate metabolism, protein metabolism, structure, transport and unknown function. Major categories

Table 2. Highlight of differentially expressed genes with known or putative roles in stress response and transcript regulation. Differentially-regulated genes were expressed at 4-fold higher or lower levels in the *bbr1* mutant compared to wild type from two independent microarray analyses.

Description	Gene Title	Average fold change ^a
Transcription factor activity		
TF-1; Similar to Heat shock transcription factor 31	Os02g0527300	11.52
TF-2; AP2, Similar to CBF-like protein	Os06g0127100	43.92
TF-3; AP2, Similar to AP2 domain containing protein RAP2.6	Os08g0474000	51.32
TF-4; MYB, Similar to LHY protein	Os04g0583900	6.12
TF-5; NAM, No apical meristem (NAM) domain containing protein	Os03g0327800	5.37
TF-6; MYB, Similar to Myb-related transcription factor LBM1	Os07g0558100	6.04
TF-7; MYB, Similar to Typical P-type R2R3 Myb protein	Os01g0975300	-7.52
TF-8; AP2, RAV-like protein	Os01g0141000	-4.17
Response to biotic stress		
R-1; Similar to Beta-1,3-glucanase-like protein	Os01g0944900	19.29
R-2; Similar to Lipxygenase 2.3, chloroplast precursor	Os02g0194700	10.9
R-3; Seven transmembrane protein MLO2	Os03g0129100	8.27
R-4; Protein phosphatase 2C family protein	Os06g0698300	7
R-5; Similar to NtPRp27	Os10g0490800	14.22
Similar to Iron-phytosiderophore transporter protein yellow stripe 1	Os02g0649900	-6.28
Similar to Pathogen-related protein	Os01g0731100	-8.17
Response to oxidative stress		
POD-1; Peroxidase	Os03g0235000	-42.2
POD-2; Peroxidase	Os07g0677100	-7.73
POD-3; Peroxidase	Os07g0677200	-4.79
POD-4; Similar to Peroxidase 47 precursor	Os08g0113000	-14.6
Response to abiotic stress		
Late embryogenesis abundant (LEA) group 1 family	Os04g0589800	25.25
Similar to Low-temperature induced protein lt101.2	Os05g0122700	16.92
Similar to Allyl alcohol dehydrogenase	Os04g0497000	11.48
Similar to 1-Cys peroxiredoxin;	Os07g0638300	9.16
Similar to Small heat stress protein class CIII	Os02g0782500	8.33
GRAM domain containing protein;	Os12g0478100	7.53
Similar to Acyl-CoA-binding protein 2 (ACBP 2)	Os06g0115300	6.89
Hly-III related proteins family protein	Os06g0652200	6.54
Similar to Dehydrin DHN1 (B8)	Os01g0702500	6.42
Heat shock protein DnaJ, N-terminal domain containing protein	Os01g0606900	6.34
EFA27 for EF hand, abscisic acid, 27kD	Os04g0511200	4.86
Glycoside hydrolase, family 17 protein	Os01g0860800	4.44
Similar to germin-like protein 8	Os08g0189850	-6.45
Similar to germin-like protein 12	Os08g0189900	-6.92

^a Average values of *Xoo* inoculated *bbr1* samples, compared to WT samples, from two independent microarray analysis. Numbers show the factor of change between wild type and mutant after *Xoo* inoculation; positive values represent up-regulation (e. g. 3 = 3-fold increase) negative values down-regulation (e. g. -3 = 3-fold decrease).

of DEGs were biotic and abiotic stress related and transcription factors (Table 2). Thirty-three genes were classified into these categories and only considered for functions on specific pathways to further illustrate the differential response of WT and *bbr1* mutant to *Xoo* infection (Table 2).

Defense-related genes induced in *bbr1* mutant

Higher expression of β -1,3-glucanase-like protein (Os01g0944900; >19 fold), lipoxigenase 2.3, chloroplast

precursor (Os02g0194700; >10 fold), seven transmembrane protein MLO2 (Os03g0129100; >8 fold), protein phosphatase 2C family protein (Os06g0698300; 7 fold) and basic secretory protein (Os10g0490800; >14 fold) genes were detected at 10 DAI in *bbr1* mutant plant. On the other hand, lower expression of iron-phytosiderophore transporter protein yellow stripe 1 (Os02g0649900; >6 fold) and pathogen-related protein (Os01g0731100; >8 fold) were detected in *Xoo*-infected *bbr1* leaf tissue (Table 1). qRT-PCR analysis further confirmed the expression of

Fig. 3. Altered expressions of genes related to *Xoo* inoculation in *bbr1*. The rice *ACTIN* gene was used as an internal positive control and transcript levels of the tested genes were normalized with that of *ACTIN*. Relative expressions of the tested genes were compared with that in WT plants. Bar represents standard deviation (three replicates) (a) qRT-PCR analysis was performed to study the transcript levels of peroxidase genes in the leaves of WT and *bbr1* plants at the 40-day-old stage. The tested genes include *POD-1*, Os03g0235000; *POD-2*, Os07g0677100; *POD-3*, Os07g0677200; *POD-4*, Os08g0113000. (b) qRT-PCR analysis of the transcripts level of defense-related genes in the leaves of WT and *bbr1* plants at the 40-day-old stage. The tested genes include *R-1*, Os01g0944900; *R-2*, Os02g0194700; *R-3*, Os03g0129100. (c) qRT-PCR analysis of the transcripts level of transcription factor genes in the leaves of WT and *bbr1* plants at the 40-day-old stage. The tested genes include *TF-1*, Os02g0527300; *TF-2*, Os06g0127100; *TF-3*, Os08g0474000; *TF-7*, Os01g0975300; *TF-8*, Os01g0141000.

representative five genes (R-1 ~ R-5; Table 1) that were highly up-regulated in *bbr1* mutant 10 DAI. Among the five genes, three [Os01g0944900 (β -1,3-glucanase-like protein; R-1), Os02g0194700 (lipoxygenase 2.3 chloroplast precursor (R-2); and Os03g0129100 (seven transmembrane protein MLO2; R-3) were expressed at higher levels in *bbr1* than in WT after *Xoo* inoculation (Fig. 3a).

As a member of the PR-2 group of pathogenesis-related (PR) proteins, β -1,3-glucanase is induced by pathogen infection and plays an active antifungal role in hydrolyzing β -1,3-glucan, a major structural component of fungal cell walls (Sela-Buurlage *et al.* 1993; Jach *et al.* 1995). Lipid peroxidation, triggered by lipoxygenases and reactive oxygen species (ROS), is a hallmark of plant pathogen responses, both in signal transduction processes and during the execution of programmed cell death. Lipoxygenases oxidize free fatty acids in the cytosol or chloroplasts, thereby initiating several oxylipin pathways including the jasmonic acid and hydroperoxide lyase pathway (Mosblech *et al.* 2009). Twelve predicted *Mlo* homologs were identified in the rice genome (Goff *et al.* 2002). The seven transmembrane MLO protein (Devoto *et al.* 1999) is thought to mediate defense suppression against *Blumeria graminis* f. sp. *hordei* attack via direct Ca^{2+} -dependent interaction with calmodulin (Kim *et al.* 2002). MLO-mediated defense suppression also likely involves one or several small GTP-binding proteins of the ROP (Rho-related GTPases from plants) family (Schultheiss *et al.* 2002). Consistent with its involvement in plant-microbe interactions, *MLO* expression is induced upon biotic and abiotic stress stimuli (Piffanelli *et al.* 2002). Induction of defense related genes during *Xoo* infection may inhibit pathogen spread in the *bbr1*.

Peroxidase genes reduced in *bbr1* mutant

Four class III peroxidases genes [Os03g0235000 (POD-1), Os07g0677100 (POD-2), Os07g0677200 (POD-3) and Os08g0113000 (POD-4) were expressed at lower levels in the *bbr1* mutant than in WT 10 DAI (Table 2). qRT-PCR also confirmed lower level of four peroxidases expression (Fig. 3b). Peroxidases belong to well-known class of PR-9 proteins (van Loon *et al.* 2006). They are expressed to limit cellular spreading of the infection through the establishment

of structural barriers or generation of highly toxic environments by massively producing ROS and reactive nitrogen species (RNS) (Passardi *et al.* 2005). Since POD scavenges the ROS, a reduced activity of this enzyme will increase the levels of ROS and result in hastened cell death. Down-regulation of *PODs* in *bbr1* may therefore result in enhanced resistance against the invading pathogen. A lower expression of four *PODs* in *bbr1* mutant implies the ROS involvement in defense against *Xoo* invasion.

Induced and suppressed genes encoding transcription factors

WRKY, myeloblastosis (MYB), APETALA2 (AP2) domain containing transcription factors (ethylene-responsive element-binding proteins; EREBPs, C-repeat-binding proteins; CBFs, related to ABI3/VP1; RAV), basic region/leucine zipper motif (bZIP), no apical meristem (NAM), zinc-finger proteins, and heat shock factors (HSFs) are encoded by large gene families and have been intensively studied for their roles in stress responses (Kagaya *et al.* 1999; Singh *et al.* 2002; Saibo *et al.* 2009; Hirayama and Shinozaki 2010; Santos *et al.* 2011; Scharf *et al.* 2012).

Higher expression of heat shock transcription factor 31 (Os02g0527300; >11 fold), RAP2.6 (Os08g0474000; >50 fold) and CBF-like protein (Os06g0127100; >40 fold), MYB domain containing protein LHY (Os04g0583900; >6 fold) and LBM1 (Os07g0558100; >6 fold) and NAM domain containing protein (Os03g0327800; >5 fold) genes were detected in *bbr1* mutant plant 10 DAI. On the other hand, lower expression of typical P-type R2R3 MYB protein (Os01g0975300; >7 fold) and AP2 domain containing protein RAV (Os01g0141000; >4 fold) were detected in *Xoo*-infected *bbr1* leaf tissue (Table 2). qRT-PCR analysis further confirmed the expression of eight representative transcription factor genes (TF1-1 ~ TF-8; Table 1) that were highly up- and down-regulated in *bbr1* mutant 10 DAI. The result showed that expression levels of TF-1 (heat shock transcription factor 31), TF-2 (CBF-like protein) and TF-3 (RAP2.6) were increased in *bbr1* whereas TF-7 (typical P-type R2R3 Myb protein) and TF-8 (RAV-like protein) were suppressed in *bbr1* which are in agreement with the microarray results (Fig. 3c). Due to the central role of ERF and CBFs/DREBs in biotic and abiotic

stress responses and their ability to regulate a large number of stress-responsive target genes, induction of these transcription factors are involved in the regulation of differentially expressed genes in *bbr1*.

AP2 domain containing transcription factors is involved in the regulation of differentially expressed genes in *bbr1*

On the basis of the number of AP2/ERF domains encoded and the gene function, the AP2/EREBP gene family has been divided into four subfamilies: AP2, RAV, DREB and ERF (Sakuma *et al.* 2002). Both ERF and DREB/CBF subfamilies are of particular interest owing to their involvement in plant responses to stresses. *ERF* subfamily genes encode a large number of ERFs (Fujimoto *et al.* 2000), which have been shown to participate in the plant response to biotic stress such as pathogens by recognizing the *cis*-acting element GCCGCC, known as the GCC-box (Hao *et al.* 1998). The DREB subfamily genes play an important role in the resistance of plants to abiotic stresses by recognizing the dehydration responsive element (DRE), which has a core motif CCGAC (Liu *et al.* 1998). The transcription factors, RAV1 and RAV2, contain an AP2 domain in the N-terminal regions and a B3 domain in the C-terminal regions (Kagaya *et al.* 1999). Using binding site selection assays, the AP2 and B3 domains of RAV1 were found to bind to the CAACA and CACCTG motifs (Kagaya *et al.* 1999). Ectopic expression of the

CaRAV1 gene in transgenic *Arabidopsis* plants resulted in the induction of some pathogenesis related (PR) genes, enhancing resistance against infection by bacterial pathogens, and tolerance to osmotic stresses caused by high salinity and dehydration conditions (Sohn *et al.* 2006).

Among the differentially expressed transcription factor genes observed in this study, the average expression fold change of RAP2.6 (51.32 fold higher than wild type) and CBF like protein (43.92 fold higher than wild type) are the highest in the *bbr1*. Another AP2 domain containing transcription factor RAV-like protein (4.17 fold lower than wild type) is also suppressed in the *bbr1* after *Xoo* infection (Table 2). The extent of induction or repression of an individual gene by *Xoo* infection in *bbr1* depends on a complex interaction between its transcription apparatus and associated regulation related sequences. To analyze the promoter region of target genes we selected 117 candidates which are more than two fold up- or down-regulated genes in *bbr1* and used sequences located 2 kb upstream of the 5' termini of each candidate genes. Among 117 candidate genes, GCC-box (GCCGCC; Table S1), DRE-related core motifs (CCGAC; Table S2) and AP2+B3 binding motif (CAACA and CACCTG; Table S3) are found in the promoter regions of 90 (73.77%) putative target genes (Fig. 4). Among the genes we detected, several were reported to be related to disease resistance, such as F-BOX STRESS INDUCED 2 (Maldonado-Calderon *et al.* 2012), PEN3 (Xin *et al.* 2013), RPM1-INDUCED PROTEIN KINASE

Fig. 4. Venn diagram of more than two folds up- and down-regulated genes with different AP2 domain binding motif in *bbr1* mutant. A total 117 genes were differentially expressed before and after *Xoo* infection, among which 90 putative target genes contain DRE-related core motifs (80 genes), GCC-box (36 genes) and AP2+B3 binding motif (51 genes) in their putative promoter regions.

(Feng *et al.* 2012), WRKY108 (Higashi *et al.* 2008) and BETA-1,3-GLUCANASE 2 (Silipo *et al.* 2005). This observation supports the hypothesis that AP2 domain containing transcription factors are involved in the regulation of differentially expressed genes in *bbr1*.

CONCLUSION

Japonica rice mutant *bbr1* which is selected by mutant screening in this study was characterized. The up- and down-regulated genes identified in *Xoo* resistant *bbr1* may play a role in resistance. To investigate the *Xoo* resistance of the gamma-ray irradiated mutant line, *bbr1*, we performed the commercial rice gene chip analysis. Through DNA microarray analysis, we found significantly elevated expression of AP2 domain containing transcription factor genes, *RAP2.6* (Os08g0474000) and *CBF* (Os06g0127100). Because the primary role of ERF and CBFs/DREBs is the regulation of a large number of target stress-responsive genes in biotic and abiotic stress, the enriched expression of the transcription factors suggests that they might be involved in the regulation of differentially expressed genes in *bbr1*. Also, the suppression of ROS scavenging enzyme, class III POD genes (Os03g023500, Os07g0677100, Os07g0677200, and Os08g0113000) was detected from DNA microarray analysis of *bbr1*. Down-regulation of *PODs* in *bbr1* may contribute to the resistance against the invading pathogen. A lower expression of four *PODs* in *bbr1* mutant than wild-type implies that increased ROS may have influence on the defense against *Xoo* invasion. These observations support the hypothesis that the genes mentioned above, contribute to bacterial blight resistance in *bbr1* mutant.

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Table S1. Thirty-six GCC-box containing differentially expressed genes in *bbr1* mutant compared to wild type.

Gene Description	Gene Title	AGI Number	Number of GCC-box in putative promoter region ^a		Total No	Average fold change ^b	
			GCCGCC	GGCGGC		NT	Xoo
Transcription factor activity							
MYB family transcription factor	Os01g0863300	AT2G38090	3	3	6	7.5	8.72
MYB family transcription factor	Os02g0104500	AT1G76890	5	10	15	-5.62	-2.1
Response to stress							
HSF-type DNA-binding domain containing protein	Os06g0553100	AT3G24520	0	4	4	2.69	24.93
VQ domain containing protein	Os01g0278000	AT2G41010	1	1	2	4.84	7.29
oxidoreductase/ transition metal ion binding protein	Os09g0445600	AT5G19875	1	5	6	3.57	6.87
Similar to F-BOX STRESS INDUCED 2	Os07g0561300	AT4G21510	5	3	8	3.64	6.57
Calmodulin-related calcium sensor protein	Os01g0955100	AT1G76640	1	1	2	7.24	6.32
phosphate carrier protein	Os09g0454600	AT3G48850	4	2	6	3.86	6.19
diacylglycerol kinase 1	Os12g0224000	AT5G63770	1	1	2	2.31	2.75
PR (pathogenesis-related) peptide	Os12g0437800	AT2G38870	12	5	17	-3.81	1.06
Protein modification process							
protein phosphatase 2C	Os03g0268600	AT2G29380	1	2	3	7.29	9.99
STE_MEKK_ste11_MAP3K.6 - STE kinases	Os01g0699500	AT5G55090	2	0	2	9.48	9
receptor protein kinase CRINKLY4 precursor	Os08g0374600	AT3G55950	2	0	2	4.94	8.88
protein kinase	Os02g0165100	AT1G16670	2	0	2	6.63	8.63
STE_MEKK_ste11_MAP3K.4 - STE kinases	Os01g0699100	AT5G55090	2	2	4	3.1	7.44
protein phosphatase 2C	Os01g0583100	AT1G17550	1	1	2	2.93	3.77
Nucleotide binding							
WD domain containing protein	Os01g0383700	AT4G03020	2	0	2	4.42	5.19
Development							
late embryogenesis abundant protein D-34	Os06g0341300	AT3G22490	4	3	7	5.39	15.56
senescence-associated gene 29	Os02g0513100	AT3G48740	2	0	2	4.47	10.93
Auxin regulated protein?	Os01g0851100	AT2G37980	1	1	2	3.86	6.41
Metabolic process							
fringe-related protein	Os03g0269900	AT2G37730	3	2	5	3.86	6.94
gibberellin receptor GID1L2	Os03g0790500	AT5G06570	1	1	2	4.76	6.82
Anthranilate synthase alpha 2 subunit	Os03g0264400	AT2G29690	2	0	2	3.4	3.23
starch synthase	Os06g0133000	AT1G32900	1	1	2	13.45	1.23
Unknown function							
transposon protein	Os01g0186900		0	3	3	17.03	15.89
expressed protein	Os01g0305200	AT1G69510	1	9	10	4.3	9.88
RPGR, putative	Os03g0296200		1	1	2	6.77	8.97
expressed protein	Os02g0527200	AT2G27830	2	4	6	5.1	8.57
expressed protein	Os02g0601000		4	0	4	4.99	6.54
expressed protein	Os06g0133300		4	2	6	4.3	4.13
expressed protein	Os01g0138500	AT2G01260	0	1	1	2.88	4.13
DUF966 domain containing protein	Os01g0975000	AT5G59790	2	3	5	3.48	4.04
cyclase/dehydrase family protein	Os01g0772400	AT4G17650	1	3	4	3.36	3.38
expressed protein	Os12g0209700	AT4G10930	2	3	5	-2.31	-2.19
expressed protein	Os11g0307600		2	0	2	-7.41	-2.37
Similar to pnn protein	Os12g0516700		1	23	24	-3.88	-3.67

^a Occurrence of GCC-box (GCCGCC or GGCGGC) in 2 kb upstream region of differentially expressed genes in *bbr1* mutant compared to wild type.

^b Average values of *Xoo* inoculated *bbr1* samples, compared to WT samples, from two independent microarray analysis. Numbers show the factor of change between wild type and mutant after *Xoo* inoculation or no treatment (NT); positive values represent up-regulation (e. g. 3 = 3-fold increase), negative values down-regulation (e. g. -3 = 3-fold decrease).

Table S2. Seventy-nine DRE binding domain containing differentially expressed genes in *bbr1* mutant compared to wild type.

Description	Gene Title	AGI Number	Number of DRE-box in putative promoter region ^a			Average fold change ^b	
			CCGAC	GTCGG	Total No.	NT	Xoo
Transcription factor activity							
Tify domain containing protein	Os10g0391400	No	5	3	8	2.98	85.24
transcription factor bHLH92-like	Os03g0741100	No	1	1	2	2.4	35.02
Heat shock transcription factor 31	Os02g0527300	AT2G26150	0	1	1	3.05	26.01
WRKY DNA -binding domain	Os01g0821300	No	2	6	8	2.14	25.72
Similar to MCB2 protein (Myb-type)	Os01g0863300	AT5G04760	4	0	4	3.39	19.37
Chitin-inducible gibberellin-responsive protein	Os07g0545800	AT5G48150	1	3	4	2.08	12.32
RING/FYVE/PHD-type domain containing protein	Os02g0682300	No	1	0	1	2.65	8.86
Tify domain containing protein	Os09g0439200	No	2	1	3	3.07	8.84
MYC/bHLH transcription factor-like	Os06g0164400	AT5G67110	2	1	3	2.51	6.05
NAM protein domain containing protein	Os12g0123800	AT5G18270	0	1	1	2.85	4.36
Response to stress							
Avr9Cf-9 rapidly elicited protein 74	Os06g0248500	No	4	1	5	3.24	95.27
hypothetical protein	Os01g0186900	AT5G12010	1	1	2	2.97	92.16
Avr9Cf-9 rapidly elicited protein 74	Os03g0240600	No	1	0	1	2.65	57.38
EF-hand Ca2+-binding protein CCD1	Os06g0683400	AT4G27280	8	1	9	2.33	23.83
GLYCINE-RICH PROTEIN 8	Os01g0278000	AT4G39260	2	0	2	2.56	13.9
RPM1-INDUCED PROTEIN KINASE	Os09g0442100	AT2G05940	2	0	2	2.58	13.33
phosphate transport protein	Os09g0454600	AT5G14040	1	2	3	2.46	9.71
expressed protein	Os01g0582600	AT5G12010	2	1	3	2.18	9.36
putative beta-1,3 glucanase	Os09g0542900	AT1G76070	1	1	2	2.23	9.32
Similar to Protein phosphatase 2C	Os01g0583100	AT5G57050	1	1	2	2.93	5.22
heat shock protein Oshsp18.0	Os03g0267000	AT3G46230	0	5	5	2.54	4.68
Dehydrin Rab25	Os01g0702500	No	2	1	3	4.59	3.68
subtilisin-chymotrypsin inhibitor-2A-like	Os12g0437800	No	3	6	9	-3.81	1.06
Protein modification precess							
protein kinase domain containing protein	Os01g0699600	No	0	1	1	3.65	70.53
protein kinase domain containing protein.	Os01g0699500	No	2	0	2	3.25	26.42
HIGHLY ABA-INDUCED PP2C GENE 3	Os03g0268600	AT2G29380	3	1	4	3.24	22.68
Protein kinase	Os02g0165100	No	2	1	3	2.76	20.92
Similar to Receptor kinase-like	Os08g0374600	No	1	1	2	3.02	14.39
MAPKKK18	Os01g0699400	AT1G05100	2	0	2	2.93	11.11
Oryza sativa MAP kinase BIMK1	Os03g0285800	No	3	2	5	2.61	10.7
protein kinase domain containing protein	Os01g0699100	No	4	3	7	3.61	6.43
diacylglycerol kinase	Os12g0224000	No	2	1	3	2.04	3.19
Similar to Chaperone protein dnaJ 1	Os03g0822800	AT5G59610	1	1	2	2.6	-1.12
Biosynthetic process							
Similar to Viviparous-14	Os07g0154100	No	0	1	1	2.48	16.14
Mog1/PsbP	Os01g0934400	AT3G05410	1	2	3	4.89	8.9
GRANULE BOUND STARCH SYNTHASE 1	Os06g0133000	AT1G32900	2	2	4	13.45	1.23
Similar to Cytochrome P450	Os07g0635500	AT2G46960	6	1	7	2.82	6.25
Development							
zinc finger protein ZFP15 mRNA	Os03g0820400	No	4	2	6	3.91	34.75
Seed maturation protein domain containing protein	Os06g0341300	AT3G22490	1	2	3	4.37	19.29
CALMODULIN LIKE 39	Os01g0955100	AT1G76640		1	1	2.53	18.25
Late embryogenesis abundant protein	Os01g0705200	No	1	0	1	3.21	6.62

Table S2. Continued.

Description	Gene Title	AGI Number	Number of DRE-box in putative promoter region ^a			Average fold change ^b	
			CCGAC	GTCGG	Total No.	NT	Xoo
Gene regulation							
arginine/serine-rich 12	Os12g0516700		1	17	18	-3.84	-3.63
Metabolic process							
hypothetical protein	Os01g0952900	No	1	3	4	3.73	248.65
Nuclease, Phosphatase	Os01g0716800	AT1G71710	1	2	3	2.87	15.91
α/β hydrolase fold-3 domain containing protein	Os03g0790500	AT5G06570	2	0	2	2.83	11.52
putative beta-1,3 glucanase	Os03g0792800	AT1G64760	1	2	3	2.91	6.35
putative 4-coumarate-CoA ligase	Os01g0901600	AT5G63380	1	1	2	2.25	5.04
Similar to H-ATPase	Os03g0689300	AT5G62670	0	1	1	3.53	4.88
Transport							
peptidylprolyl isomerase ROC7	Os06g0708300	AT4G39220	1	1	2	2.65	10.09
Similar to MtN3 protein precursor	Os02g0513100	AT5G50800	1	1	2	5.73	8.59
putative axi 1 protein	Os01g0851100	AT2G37980	2	0	2	3.62	6.9
Anthranilate synthase component I family protein	Os03g0264400	AT5G05730	2	2	4	2.24	4.94
dehydrase family protein	Os01g0772400	AT4G17650	2	1	3	4.09	2.8
Similar to GTP-binding nuclear protein Ran1B	Os06g0600301	AT5G55190	1	0	1	2.15	2.12
Nucleotide binding							
WD-40 repeat family protein	Os01g0383700	AT4G03020	4	2	6	3.56	6.49
Unknown function							
ZIM domain containing protein	Os03g0181100	No	3	5	8	2.73	56.55
hypothetical protein	Os06g0133500	No	1	0	1	3.01	45.58
hypothetical protein	Os02g0733900	No	1	1	2	3.47	44.9
DWN domain domain containing protein	Os03g0659400	No	1	2	3	2.91	19.86
hypothetical protein	Os02g0527200	No	4	4	8	2.55	17.3
hypothetical protein	Os02g0601000	No	3	5	8	2.47	13.29
hypothetical protein	Os01g0305200	No	4	5	9	3.23	13.21
hypothetical protein	Os01g0121600	No	0	2	2	2.51	10.85
hypothetical protein	Os03g0296200	No	4	0	4	6.64	9.28
hypothetical protein	Os07g0115500	No	1	1	2	2.58	9.08
hypothetical protein	Os06g0133300	No	0	1	1	2.34	7.59
DUF604 family protein	Os03g0269900	AT2G37730	1	0	1	3.6	7.49
DUF966 family protein	Os01g0975000	No	2	2	4	2.24	6.31
Cyclin-like F-box domain containing protein	Os07g0561300	No	5	2	7	3.9	6.17
hypothetical protein	Os09g0445600	AT2G31940	0	2	2	4.22	5.75
DUF789 family protein	Os01g0138500	AT2G01260	4	4	8	2.28	5.22
TonB box domain containing protein	Os09g0532000	No	1	0	1	2.35	4.15
Conserved hypothetical protein	Os01g0121500	No	1	0	1	2.88	3.07
hypothetical protein	Os07g0516400	No	1	2	3	4.83	3
metallothionein-like type 2 (OsMT-2) mRNA	Os01g0149200	No	1	3	4	2.77	2.78
hypothetical protein	Os12g0209700	No	4	2	6	-2.36	-2.14
Hydroxyproline-rich glycoprotein DZ-HRGP	Os11g0307600	No	3	0	3	-4.59	-2.89
similar to GT-2 factor	Os02g0104500	No	2	0	2	-5.62	-2.1
hypothetical protein	Os01g0303800	No	3	3	6	-2.08	-14.42

^a Occurrence of DRE binding domain (CCGAC or GTCGG) in 2 kb region up stream of differentially expressed genes in *bbr1* mutant compared to wild type.

^b Average values of *Xoo* inoculated *bbr1* samples, compared to WT samples, from two independent microarray analysis. Numbers show the factor of change between wild type and mutant after *Xoo* inoculation or no treatment (NT); positive values represent up-regulation (e. g. 3 = 3-fold increase), negative values down-regulation (e. g. -3 = 3-fold decrease)

Table S3. Fifty-one RAV1 binding domain containing differentially expressed genes in *bbr1* mutant compared to wild type.

Gene Description	Gene Title	AGI Number	Number of RAV-binding site in putative promoter region ^a			Average fold change ^b	
			CAACA+ CACCTG	TGTTG+ CAGGTG	Total NO	NT	Xoo
Transcription factor activity							
WRKY108, expressed	Os01g0821300	AT4G11070	1	1	2	5.86	9.35
HSF-type DNA-binding domain containing protein	Os06g0553100	AT3G24520	1	0	1	2.69	4.03
Response to stress							
U-box protein CMPG1	Os06g0248500	AT5G37490	0	1	1	14.72	20.82
cytochrome P450	Os12g0150200	AT2G27690	1	1	2	14.57	18.57
Similar to ATL31 and ATL6	Os02g0759400	AT5G27420	3	0	3	9.25	12.55
HSF-type DNA-binding domain containing protein	Os02g0527300	AT5G03720	1	1	2	6.84	11.51
Similar to RAP 2.4	Os03g0191900	AT1G78080	1	2	3	6.15	9.38
late embryogenesis abundant protein, group 3	Os01g0705200	AT3G15670	1	0	1	2.87	7.34
oxidoreductase/ transition metal ion binding protein	Os09g0445600	AT5G19875	1	0	1	3.57	6.87
phosphate carrier protein	Os09g0454600	AT3G48850	1	0	1	3.86	6.19
Similar to RPM1-induced kinase	Os09g0442100	AT2G05940	1	0	1	5.76	5.96
Similar to PEN3	Os01g0609300	AT1G59870	1	0	1	5.68	5.62
expressed protein	Os01g0582600	AT5G12010	1	0	1	4.24	4.81
Similar to 4CL	Os08g0448000	AT3G21240	0	1	1	4.55	4
glutamate decarboxylase	Os03g0236200	AT5G17330	2	1	3	4.27	3.46
Similar to PR-6 proteinase inhibitor family	Os12g0437800	AT2G38870	1	0	1	-3.81	1.06
heat shock protein DnaJ	Os03g0822800	AT5G59610	1	0	1	2.6	-1.12
Protein modification process							
STE_MEKK_ste11_MAP3K.7 - STE kinases	Os01g0699600	AT2G32510	1	2	3	18.13	14.07
STE_MEKK_ste11_MAP3K.6 - STE kinases	Os01g0699500	AT5G55090	0	3	3	9.48	9
receptor protein kinase CRINKLY4 precursor	Os08g0374600	AT3G55950	0	1	1	4.94	8.75
protein kinase	Os02g0165100	AT1G16670	1	0	1	6.66	8.63
protein phosphatase 2C	Os09g0325700	AT2G29380	1	0	1	4.76	7.59
STE_MEKK_ste11_MAP3K.4 - STE kinases	Os01g0699100	AT5G55090	1	0	1	3.1	7.44
STE_MEKK_ste11_MAP3K.5 - STE kinases	Os01g0699400	AT5G55090	1	0	1	4.63	6.99
protein phosphatase 2C	Os01g0583100	AT1G17550	2	1	3	4.03	3.77
Nucleotide binding							
CCHC-type zinc finger	Os03g0659400	AT5G47430	0	1	1	6.23	9.25
WD domain	Os01g0383700	AT4G03020	1	0	1	4.42	5.19
Development							
EF hand family protein	Os06g0683400	AT2G46600	1	0	1	7.94	6.94
growth regulator related protein	Os01g0851100	AT2G37980	1	0	1	3.86	6.41
glucan endo-1,3-beta-glucosidase precursor	Os03g0792800	AT2G19440	1	0	1	3.13	5.9
senescence-inducible chloroplast stay-green protein 1	Os09g0532000	AT4G11910	0	1	1	2.72	3.57
Metabolic process							
luminal PsbP	Os01g0934400	AT3G05410	0	2	2	5.08	12.04
expressed protein	Os06g0203600	AT2G26310	1	0	1	9.19	11.35
AMP-binding domain containing protein	Os01g0901600	AT5G63380	1	0	1	2.69	4.2
Transport							
white-brown complex homolog protein 7	Os01g0121600	AT2G01320	1	2	3	4.87	5.54
Rer1 protein	Os06g0708300	AT4G39220	1	0	1	4.58	5.8
ras-related protein	Os06g0600301	AT5G55190	0	1	1	2.17	2.06

Table S3. Continued.

Gene Description	Gene Title	AGI Number	Number of RAV-binding site in putative promoter region ^a			Average fold change ^b	
			CAACA+ CACCTG	TGTTG+ CAGGTG	Total NO	NT	Xoo
Unknown function							
expressed protein	Os01g0952900	AT5G12340	1	0	1	27.86	33.01
transposon protein	Os01g0186900		2	0	2	17.09	15.89
expressed protein	Os06g0133500		1	0	1	10.78	12.64
expressed protein	Os01g0305200	AT1G69510	1	0	1	4.3	9.88
RPGR, putative,	Os03g0296200		1	0	1	6.82	9
expressed protein	Os02g0527200	AT2G27830	1	0	1	5.1	8.57
expressed protein	Os02g0601000		1	1	2	4.99	6.5
expressed protein	Os07g0516400		1	0	1	2.27	6.34
expressed protein	Os01g0138500	AT2G01260	1	0	1	2.88	4.07
expressed protein	Os09g0542900	AT1G76070	1	0	1	5.26	3.93
transposon protein	Os01g0872900		1	0	1	5.68	3.64
cyclase/dehydrase family protein	Os01g0772400	AT4G17650	0	1	1	3.36	3.38
expressed protein	Os12g0209700	AT4G10930	0	1	1	-2.31	-2.13

^a Occurrence of RAV1 binding domain (CAACA--CACCTG or TGTTG--CAGGTG) in 2 kb region upstream of differentially expressed genes in *bbr1* mutant compared to wild type.

^b Average values of *Xoo* inoculated *bbr1* samples, compared to WT samples, from two independent microarray analysis. Numbers show the factor of change between wild type and mutant after *Xoo* inoculation or no treatment (NT); positive values represent up-regulation (e. g. 3 = 3-fold increase), negative values down-regulation (e. g. -3 = 3-fold decrease).