Study on the Identification of a Disease Resistance Gene *BSR1* and Its Broad-Spectrum Resistance

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Abbreviation

AK gene	*Aspartate kinase* gene
BSR1	*BROAD-SPECTRUM RESISTANCE 1*
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
EF-Tu	Elongation factor-Tu
ELISA	Enzyme-linked immunosorbent assay
ET	Ethylene
FOX hunting	Full-length cDNA OvereXpressor gene hunting
HR	Hypersensitive response
JA	Jasmonic acid
KB medium	King's B medium
KOME	Knowledge-based Oryza Molecular biological Encyclopedia
MAP kinase	Mitogen-activated protein kinase
MS medium	Murashige and Skoog medium
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NG	Non-germinated
NPR1 gene	*N*on-expressor of *PR* gene
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PG	Pre-germinated
PR genes	*pathogenesis-related* genes
PSA	Peptone sucrose agar
Pst DC3000	*Pseudomonas syringae* pv. *Tomato* DC3000
PTI	Pattern-triggered immunity
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real-time reverse transcription-polymerase chain reaction
QTLs	Quantitative trait loci
RAP-DB	Rice Annotation Project Database
R gene	Resistance gene
rice-FOX *Arabidopsis*	Rice-full-length cDNA OvereXpressor *Arabidopsis*
RLCK	Receptor-like cytoplasmic kinase

RLK	Receptor-like kinase
ROS	Reactive oxygen species
RPD genes	Resistance to *Pst* DC3000 genes
RSV	Rice stripe virus
RT	Represents retransformed
SA	Salicylic acid
SD	Standard deviation
SSA	Sulphonated SA
TAIL-PCR	Thermal asymmetric interlaced-polymerase chain reaction
T-DNA	transfer DNA
UV	Ultraviolet
Xoo	*Xanthomonas oryzae* pv. *oryzae*

Chapter 1

General Introduction

Preventing crop diseases is an important issue in overcoming hunger and ensuring the stability of food supply as the world's population increases. Pathogenic bacteria and filamentous fungi are major causes of various crop diseases, and are responsible for about 80% of plant diseases in Japan (Sato 2013). In rice, fungal blast and bacterial leaf blight are the most important diseases and seriously damage yield. For this reason, they are controlled with pesticides, despite concerns about cost and effects on human health and the environment. An alternative solution is breeding using disease resistance genes, which must first be isolated. In conventional methods of gene isolation and identification, genes introduced by crossing with resistant cultivars are identified through the use of gene mapping (Fig. 1A). However, the multiple crosses needed are very time consuming. In addition, although resistance (*R*) genes are frequently used in this method, the resistance that they confer is restricted to specific races of pathogens and often breaks down with the emergence of new races after a few years of field cropping (Bonman *et al.* 1992). Further, it is possible only when disease resistance genes are available. Instead, it can be highly effective to use genes that confer broad-spectrum disease resistance in rice breeding. However, few such genes have been isolated by conventional methods.

Rice is a major cereal and is the most representative model plant for the study of

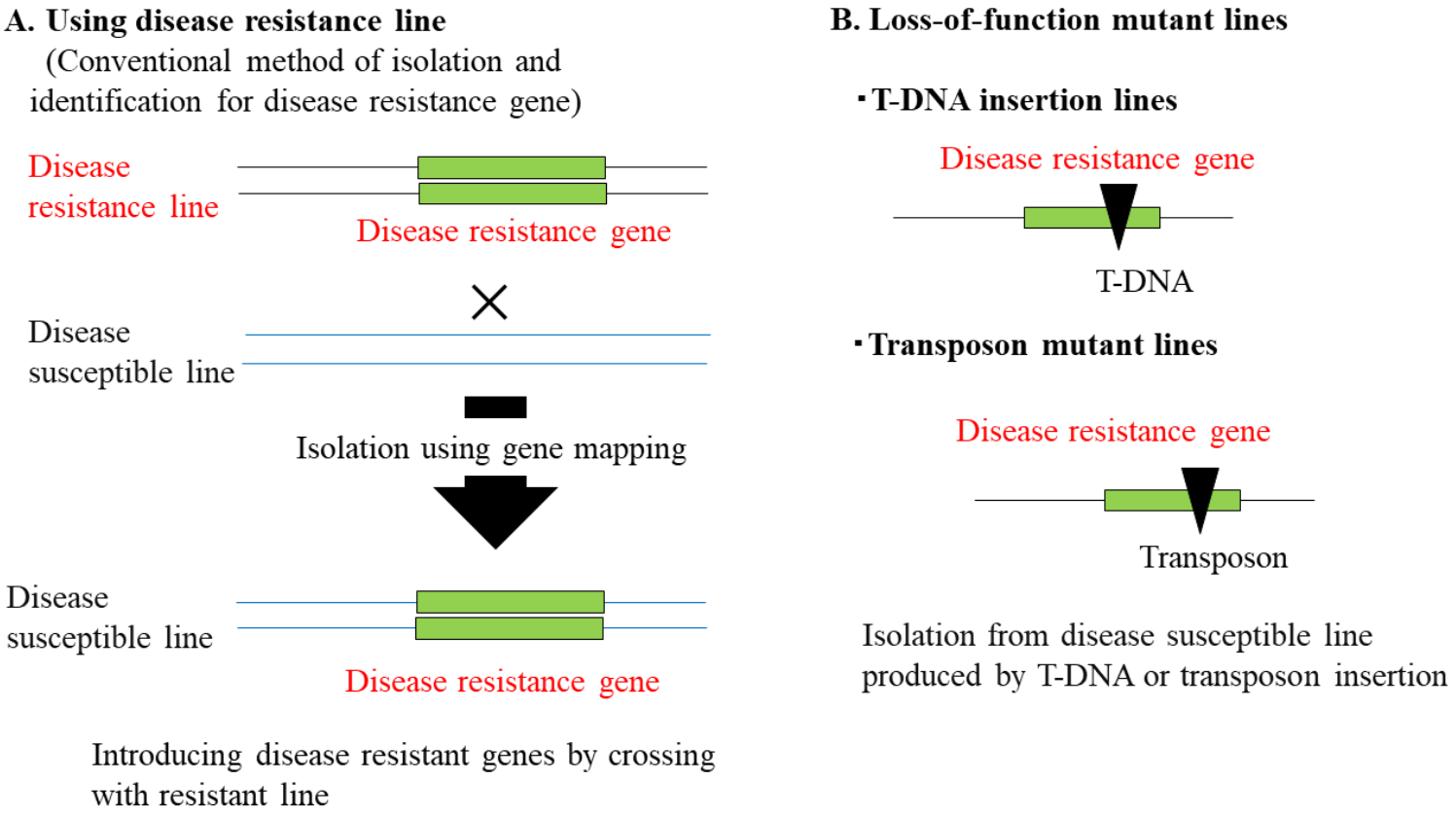
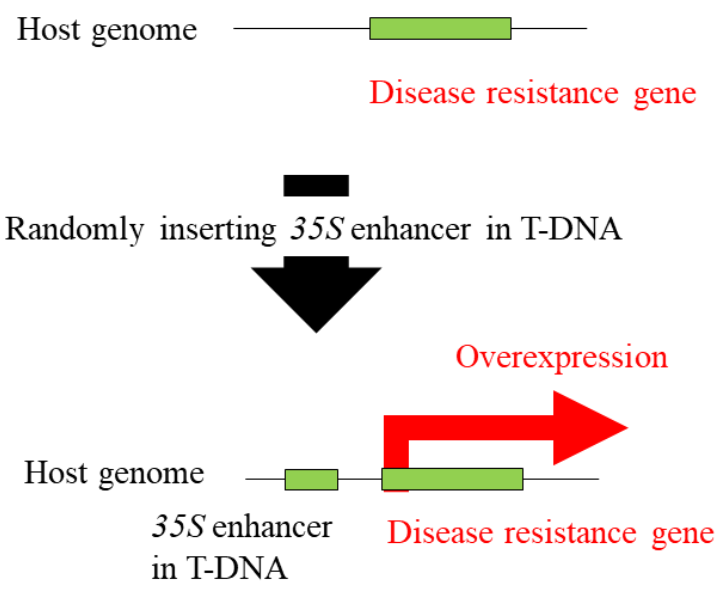


Figure 1. Method of isolation and identification for disease resistance gene

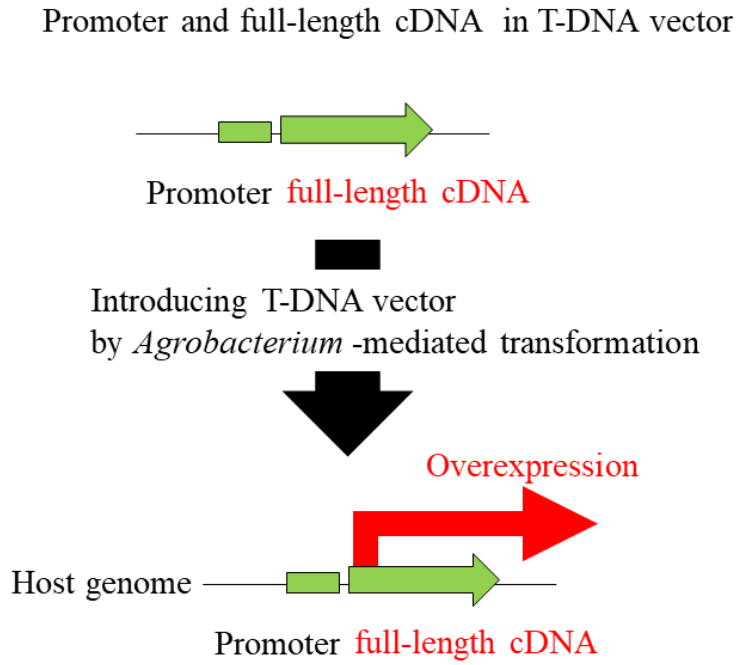
C. Gain-of-function lines

▪ Activation tagging lines



Producing disease resistance phenotype by enhancing expression of disease resistance gene near T-DNA insertion point.

▪ FOX hunting (Full-length cDNA OvereXpressor gene hunting) lines



Possible to visually observe various function of the gene because of overexpression of full-length cDNA

Figure 1. Continued

monocots. Since the release of the whole rice genome sequence (cultivar Nipponbare; IRGSP 2005), approximately 32,000 rice genes have been predicted (Itoh *et al.* 2007, Tanaka *et al.* 2008), and approximately 28,000 full-length cDNA clones have been collected (Rice Full-Length cDNA Consortium 2003). As the functions of most of these genes have not been elucidated yet, tools to knock out or overexpress those genes have been developed to reveal the function of each gene (Tsuchida-Mayama *et al.* 2010). In the process, many mutant lines specific to many individual genes have been developed, advancing research.

Among tools frequently used to analyse loss-of-function mutants, transfer DNA (T-DNA) insertion lines (An *et al.* 2005) and *Tos17* (retrotransposon)-insertion lines (Hirochika *et al.* 2004) allow the large-scale analysis of rice gene functions (Fig. 1B). Disrupted genes can be identified from phenotypes of loss-of-function mutants (Fig. 1B). For example, Takahashi *et al.* (2010) isolated *Pish*, an *R* gene conferring resistance to rice blast disease, from *Tos17* mutant lines. However, estimated 29% of genes in the rice genome are redundant (IRGSP 2005), and are thus difficult to isolate.

To isolate redundant genes, gain-of-function methods were developed such as activation tagging and FOX hunting system (Fig. 1C). Activation tagging is a method of randomly inserting *35S* enhancers into a genome to overexpress genes near the insertion point (Hsing *et al.* 2007, Mori *et al.* 2007, Nakazawa *et al.* 2003a). This method is possible to produce disease resistance phenotype by enhancing expression of disease resistance gene near T-DNA insertion point. However, it is difficult to saturate a genome with T-DNA tags because the insertion sites are limited in the genome. Moreover, because gene activation can occur as long as 12 kb upstream or downstream of the insertion site, this can lead to complex phenotypes due to upregulation of neighbouring genes (Ichikawa *et*

al. 2003). Furthermore, insertion of an activator cassette into a gene, especially into a coding region, can lead to gene disruption or other unexpected outcomes.

To remove these disadvantages, Ichikawa *et al.* (2006) developed a new tool for functional genomics, the full-length cDNA overexpressor gene hunting (FOX hunting) system, in which they inserted 10,000 full-length *Arabidopsis* cDNAs downstream of a CaMV promoter (Table 1) and, using the *Agrobacterium*-mediated floral dip method, generated about 15,000 transgenic *Arabidopsis* lines that expressed the full-length cDNAs. They found about 1,500 morphological mutants and identified some causal genes (Ichikawa *et al.* 2006). Subsequently, Fujita *et al.* (2007) made a small-scale pool of FOX lines to characterize the possible roles of transcription factors in plant salt tolerance.

In rice, Nakamura *et al.* (2007) reported the generation of a population of transgenic lines overexpressing 13,980 independent full-length rice cDNAs under the control of the maize ubiquitin-1 promoter (Table 1). Similarly, Kondou *et al.* (2009) developed a rice-FOX population of 23,000 *Arabidopsis* (ecotype Columbia) lines by introducing 13,000 full-length rice cDNAs under the control of the CaMV *35S* promoter (Table 1, Fig. 2). An advantage of using rice-FOX *Arabidopsis* lines is the rapid and easy identification of rice genes (e.g., disease resistance genes) for various functions, because *Arabidopsis* has a short lifespan and can be grown in a small space (Fig. 2).

Many plants can recognize pathogens and mount a defensive reaction, a phenomenon called basal resistance. Because it does not depend on the type of pathogen, it may be possible to confer resistance to various diseases by enhancing basal resistance. To identify novel, broad-spectrum disease resistance genes effective in various plants, I screened rice-FOX *Arabidopsis* lines. This dissertation describes my studies; chapter 2 describes screening for resistance to *Pseudomonas syringae* and characterization of the

Table 1. Three types of FOX-hunting lines

FOX lines	Source of FL-cDNA	Host plant	Number of used FL-cDNAs	Number of lines	Reference
Arabidopsis FOX	*Arabidopsis*	*Arabidopsis*	10,000	15,547	Ichikawa *et al.* 2006
Rice-FOX rice	Rice	Rice	13,980	12,000	Nakamura *et al.* 2007
Rice-FOX Arabidopsis	Rice	*Arabidopsis*	13,000	23,000	Kondou *et al.* 2009

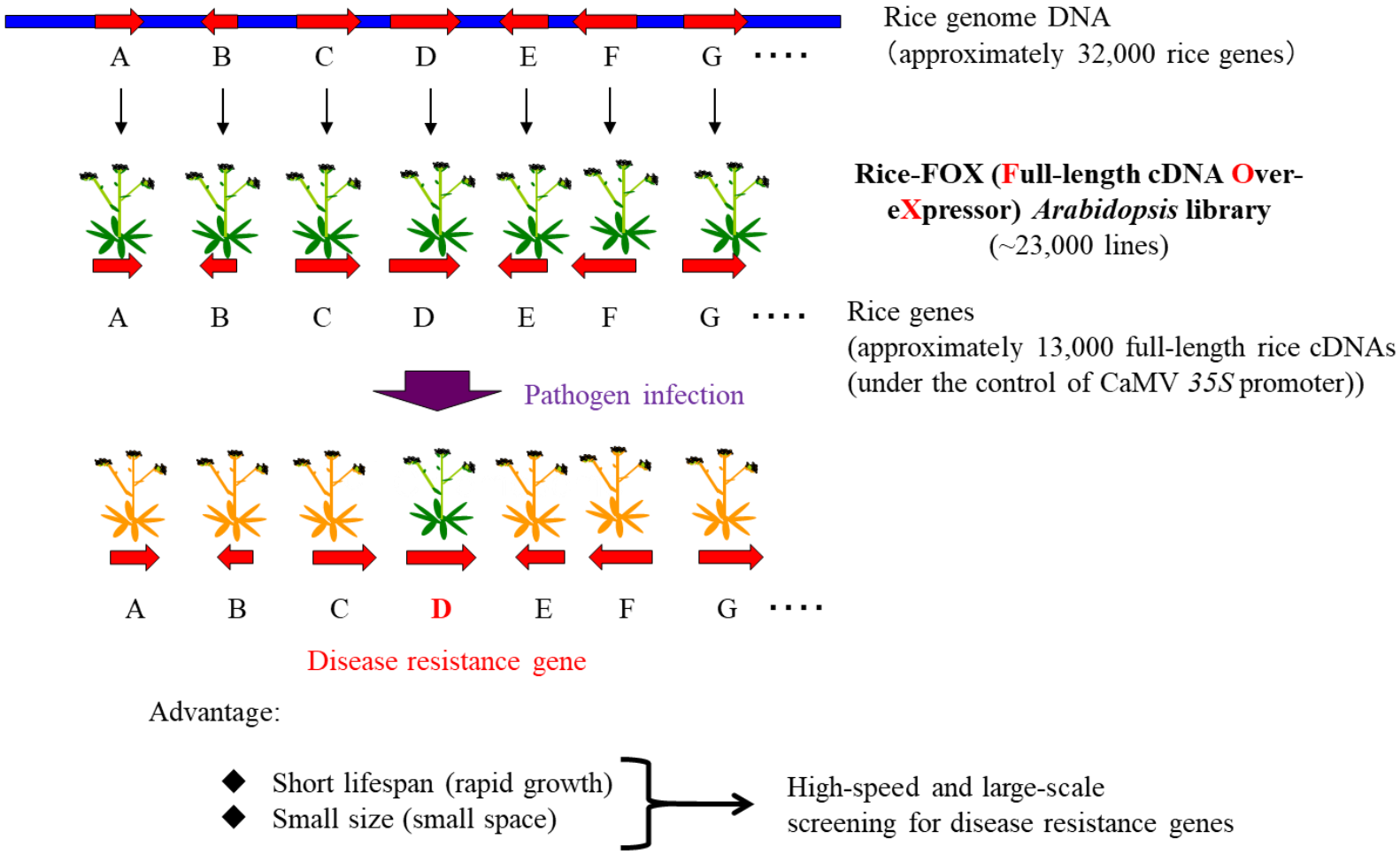


Figure 2. Advantage for using the rice-FOX *Arabidopsis* lines

resistant lines; chapter 3 describes a putative receptor-like cytoplasmic kinase gene, *BSR1*, which confers resistance to major bacterial and fungal pathogens in *Arabidopsis* and rice; and chapter 4 describes how overexpression of *BSR1* confers broad-spectrum resistance to two bacterial diseases and two major fungal diseases in rice.

Chapter 2

Screening for resistance against *Pseudomonas syringae* in rice-FOX *Arabidopsis* lines and characterization of the resistant lines

Introduction

In recent years, several gain-of-function transgenic mutant populations have been developed in rice (Hsing *et al.* 2007, Jeong *et al.* 2002, Mori *et al.* 2007, Nakamura *et al.* 2007). Meanwhile, Kondou *et al.* (2009) produced more than 20,000 independent *Arabidopsis* transgenic lines overexpressing rice full-length cDNAs (rice-FOX *Arabidopsis* lines) to enable high-throughput screening for rice genes. By using these rice-FOX *Arabidopsis* lines, several rice genes were identified that are involved in heat stress tolerance (*OsHsfA2e*; Yokotani *et al.* 2008), salt tolerance (*OsSMCP1*; Yokotani *et al.* 2009a, *OsNAC063*; Yokotani *et al.* 2009b, *OsCEST*; Yokotani *et al.* 2011 and *JAmyb*; Yokotani *et al.* 2013), secondary metabolism (*OsLBD37*; Albinsky *et al.* 2010) and photosynthesis (*OsLFNR1* and *OsLFNR2*; Higuchi-Takeuchi *et al.* 2011). Since there were such examples of identifying various rice genes using rice-FOX *Arabidopsis* lines, I also tried to identify disease resistance genes using the FOX lines.

Because rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causing bacterial leaf blight and rice fungal pathogen *Pyricularia oryzae* causing rice blast cannot infect *Arabidopsis* plants, I had to select a bacterial pathogen used for screening in *Arabidopsis*. I used *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 which is often used as a model system of defence mechanism in interaction between plants and pathogens in *Arabidopsis* (Mansfield 2009). *P. syringae* has the type III secretion machinery similarly to *Xoo* has (Furutani *et al.* 2009).

In this chapter, I screened the rice-FOX *Arabidopsis* lines for resistance to *Pst* DC3000 with the aim of rapidly identifying a number of defence related genes of rice in a heterologous plant screening system.

Materials and Methods

Pst DC3000 culture

Pst DC3000 was obtained from Dr. B. J. Staskawicz (UC Berkeley, USA). All *Pst* DC3000 cultures were started from stocks containing 50% glycerol and 50% KB medium (King *et al.* 1954) stored at - 80°C. One hundred millilitres of KB medium with 50 μg／mL of rifampicin (WAKO Pure Chemicals, Osaka, Japan) was inoculated with 0.5 mL of glycerol stock and then cultured for 16–18 h (until OD_{600} = 1) in a rotary shaker set at 180 rpm and kept at 28°C in the dark. The bacterial cells were harvested by centrifugation and resuspended at a concentration of 0.5 to 2 × 10^8 cfu／mL in an inoculation medium consisting of 10 mM $MgCl_2$ and 0.05% Silwet L-77(Lehle Seeds, Round Rock, TX, USA).

Arabidopsis culture and *Pst* DC3000 screening protocol

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type (negative control), whereas the positive control was *cpr5-2* (a gift from Dr. B.N. Kunkel, Washington University, USA), a mutant line showing very high resistance to *Pst* DC3000. The rice-FOX Arabidopsis lines (Kondou *et al.* 2009) were sown in two replications at five seeds per well in 60-well plates containing pre-sterilized moist black peat moss (Super Mix A; Sakata, Yokohama, Japan). After a 2-day cold (4°C) exposure, the seeds were germinated and grown in aseptic condition for 3 weeks under a 9／15 h light／dark

regime at 22°C.

The plants were dipped for 30 s in a suspension containing *Pst* DC3000 at 0.5 to 2×10^8 cfu/mL supplemented with 0.05% Silwet L-77 and incubated for 3 days in the dark and 3 days under light prior to evaluation of survival. Photographs of the plants were taken 6 days after inoculation under white fluorescent illumination and evaluated for recovery of green colour because of de novo chlorophyll synthesis using images in a computer screen. Screening of the candidate resistant lines was repeated at least twice for verification. During the initial stages of screening, I also took photographs under UV (365 nm) illumination as the red fluorescence of chlorophyll offered greater contrast. However, in the third screening, I opted to use higher inoculum and selected only the lines that had survived for 6 days after the dip inoculation for further screening and evaluation.

Quantification of resistance by bacterial counts

To determine the degree of resistance, T_2 seeds of the FOX lines were selected by growing them on half MS media with 1% sugar, B5 vitamins (0.04% myo-inositol, 0.0004% nicotinic acid, 0.0004% pyridoxine hydrochloride, 0.004% vitamin B1 hydrochloride), 0.05% MES, 10 mM hygromycin and 0.8% agar (adjusted to pH 5.7).

The negative control plants (wild type Columbia) were sown in half MS media without the antibiotics. The 3-week-old seedlings were transferred to sterile 60-well plates containing moistened black peat moss and allowed to recover for another 2 weeks prior to dip inoculation with *Pst* DC3000 at 10^6 cfu/mL as described previously. Three days after inoculation, aerial parts of 4–5 plants were harvested and weighed. Bacterial counting was performed using a procedure described by Katagiri *et al.* (2002). Each biological replicate plant sample was surface sterilized with 70% ethanol, washed with

sterile water and dried. The samples with 100 μL of sterile water was homogenized in 2 mL tube, added 900 μL of sterile water and thoroughly vortexed. The sample was continuously diluted 1:10. Then 10 μL of the samples and the serial dilutions were spread on KB plates. After the plates were left at 28°C for 2 days, colonies on plates were counted.

Insert identification

DNA was extracted from selected FOX lines using Qiagen DNeasy Plant mini kit (Valencia, CA, USA) and then amplified by PCR using the following primers: GS17, 5'-GTACGTATTTTTACAACAATTACCAAC-3', and GS18, 5'- GGATTCAATCTTAA GAAACTTTATTGC-3'. The number of inserts and their sizes were estimated by electrophoresis of the PCR products in agarose gels. The identity of each fragment was determined by sequencing the first 400–600 bp from the 5' and 3' ends and comparing the resultant data with those kept at the Knowledge-based Oryza Molecular biological Encyclopedia (KOME) website (Rice Full-Length cDNA Consortium 2003).

Generation of transgenic *Arabidopsis* for verification of resistance

For retransformation, the candidate rice full-length cDNAs identified by *Pst* DC3000 screening were obtained from the Rice Genome Resource Center (NARO, Japan) as *E. coli* plasmids, digested with *Sfi*I (Takara, Tokyo, Japan) and inserted downstream of 2 × CaMV *35S* promoter at the compatible *Sfi*I sites of the binary vector, pBIG2113SF (Ichikawa *et al.* 2006). The engineered plasmids were subsequently introduced into *Agrobacterium* GV3101 by electroporation. Transgenic *Arabidopsis* lines were obtained by floral dip transformation (Clough and Bent 1998). Transformed T_1 seedlings were selected on a medium containing 1 mM KNO_3, 10 μg／mL hygromycin and 0.8% agar

(Nakazawa and Matsui 2003b). After 4 weeks, seedlings that survived and showed sufficient root development were individually transferred to pots containing black peat moss and grown at 22°C in a 9⁄15 h light⁄dark regime prior to bolting and then transferred to 16⁄8 h light⁄dark regime at the start of flowering. Seeds from these T_1 plants were used to verify the resistance phenotypes of the original T_2 FOX lines.

Results

Screening for resistance to *Pst* DC3000

Conventional protocols for screening and evaluation of disease resistance traits (see Fig. 3A) are difficult to apply to a large population of plants. In conventional methods that use plants grown in non-aseptic conditions, the plant's responses to pathogen inoculation can be modulated by abiotic factors like humidity and ventilation, and biotic factors like insects and microbes. The elimination of those extraneous factors is an essential requirement to attain repeatability and reliability in the screening of large populations. Therefore, I developed a new system to avoid these problems (Fig. 3B). *Arabidopsis* plants were grown in an aseptic condition free from drought and pest infestation. They were dip inoculated with bacteria for 30 s and this was their only exposure to non-sterile conditions. The inoculated plants usually turned yellow after the 3-day incubation in the dark, but control resistant plants *cpr5-2* (Boch *et al.* 1998), in which salicylic acid (SA) signalling pathway is constitutively activated, recovered its green colour presumably as a result of de novo chlorophyll synthesis. Hence, the ability to recover from the chlorosis induced by *Pseudomonas* and generate healthy green tissue after stringent inoculation and incubation was deemed as an indicator of plant resistance. This protocol was used to screen 20,000 FOX lines for resistance to *Pst* DC3000 to

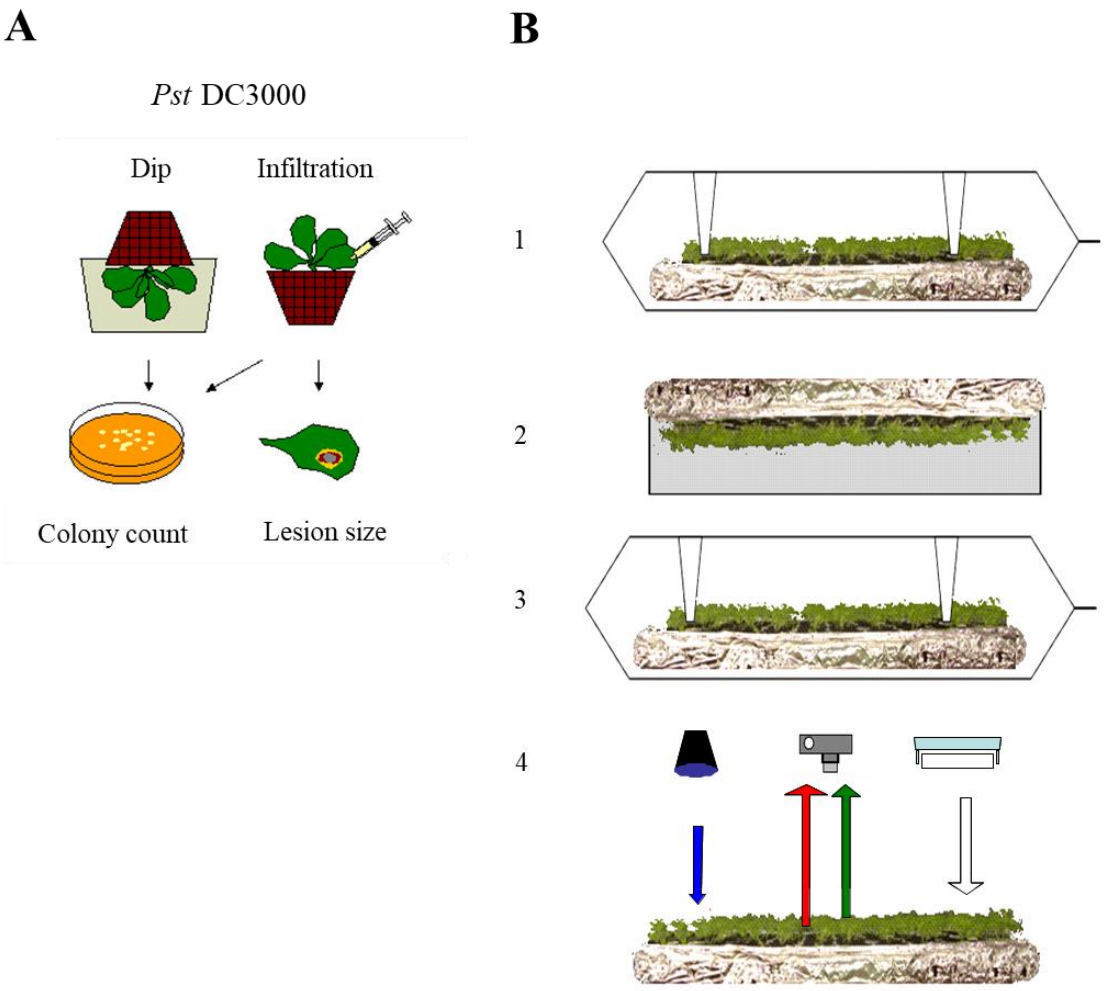


Figure 3. Screening for resistance to *Pst* DC3000.
(A)Standard methods. *Arabidopsis* is usually inoculated with *Pst* DC3000 by dip or infiltration method. Resistance level is usually evaluated by bacterial counts or lesion size in the infected plants.
(B) High throughput screening of the FOX lines employed in this study. Steps 1. Grow the FOX lines in 60-well plates for three weeks in aseptic condition, 2. Dip inoculate with *Pst* DC3000 or *C. higginsianum*, 3. Incubate, 4. Record image by digital photography of reflected light (green) under white light or fluorescent light (red) under UV.

discover novel genes involved in rice defence mechanism to pathogens. Typical examples of the FOX lines that were resistant to *Pst* DC3000 are shown in Fig. 4. Three week-old T_2 plants (upper photographs) were inoculated with 10^8 cfu/mL of *Pst* DC3000, and disease symptoms were evaluated 6 days after inoculation (lower photographs). Wild type (Col-0) and vector control (VC#1) plants were apparently killed by the screening method (Fig. 4). In contrast, the plants from some FOX lines (AK070024-OX, AK102525-OX, AK102125-OX, AK072201-OX and AK070417-OX) showed many green healthy leaves 6 days after inoculation, similar to the resistant control plants, *cpr5-2* (Fig. 4A). Two independent retransformed lines for AK070024, RT:AK070024-OX#1 and RT:AK070024-OX#2, also showed phenotypes similar to *cpr5-2* (Fig. 4B).

This high-throughput screening enabled the evaluation of 20,000 transgenic lines for resistance to *Pst* DC3000. Screening at inoculum levels ($0.5–2 \times 10^8$ cfu/mL) led to the isolation of 1,620 lines in the first screening and 204 lines in the second screening (replicated twice each time). The third screening, at inoculum levels of $1–2 \times 10^8$ cfu/mL, resulted in the selection of 72 lines (Table 2).

Validation of the high-throughput screening procedure by bacterial count

My binomial (survive or die) screening protocol identified FOX lines that survived three independent dip inoculations with *Pst* DC3000 at relatively high inoculum levels under conditions that favoured successful infection. As my selection was based on the reinitiation of de novo chlorophyll production in erstwhile chlorotic leaves, it can be argued that the observed 'resistance' was because of factors other than suppression of bacterial growth. Hence, I also counted bacteria numbers in inoculated plant tissues of a few selected lines to examine whether the plant survival was because of the repression of

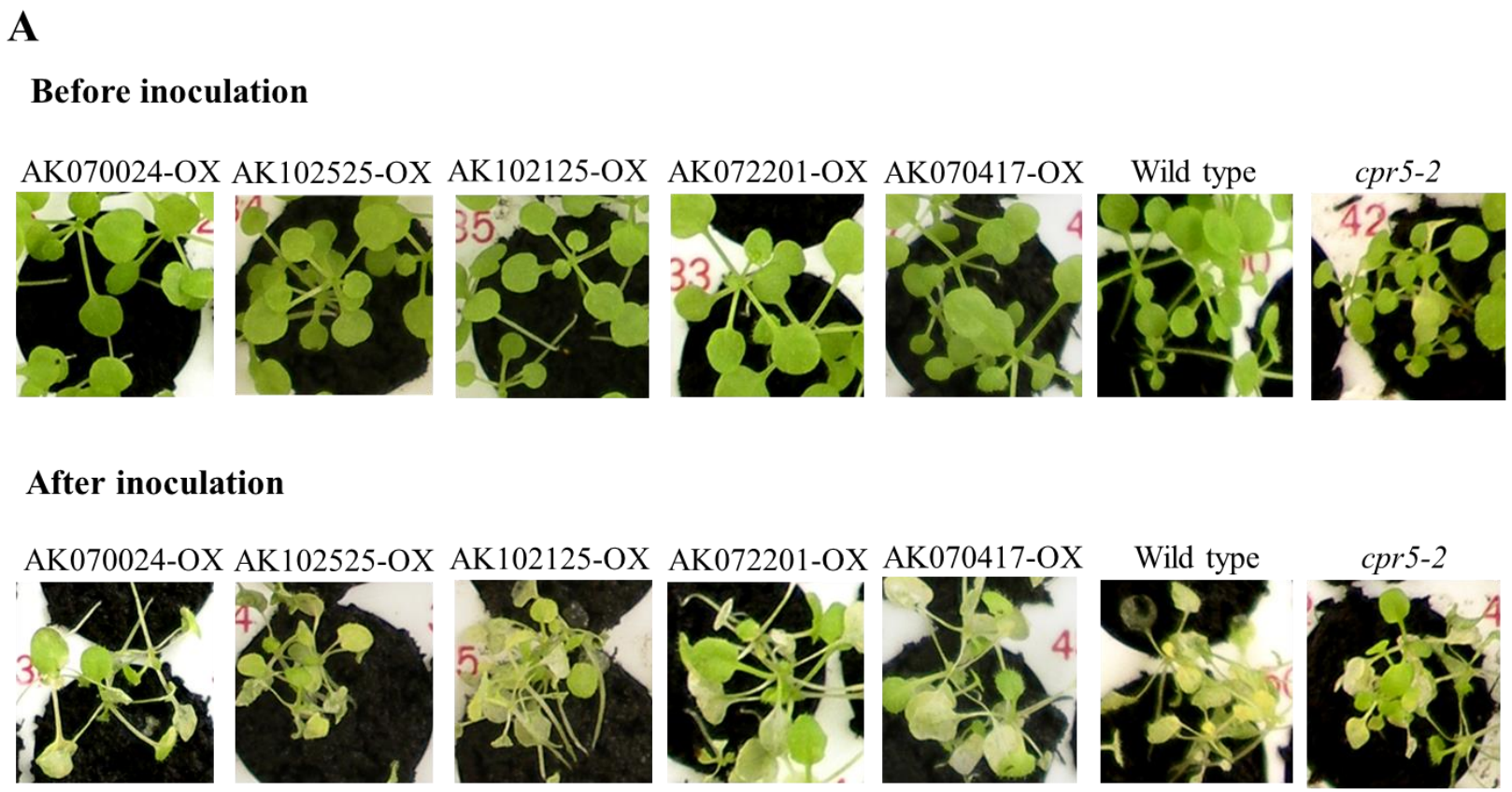


Figure 4. Phenotypic responses to *Pst* DC3000 dip inoculation.

Typical example of the *Pst* DC3000 resistance FOX lines (A) and *Pst* DC3000 resistance of two independent retransformed lines (B). Upper panel show 3-week-old T_2 plants used for *Pst* DC3000 inoculation. Lower panel show typical disease symptoms 6 days after inoculation with 10^8 cfu/mL of *Pst* DC3000. The wild type (Col-0) and vector control (VC#1) plants died, but the AK070024-OX, AK102525-OX, AK102125-OX, AK072201-OX, AK070417-OX and *cpr5-2* (resistance mutant to *Pst* DC3000) plants survived. RT:AK070024-OX#1 and RT:AK070024-OX#2 also survived the *Pst* DC3000 screen. RT:AK070024-OX#1 and RT:AK070024-OX#2 are independent retransformed lines for AK070024.

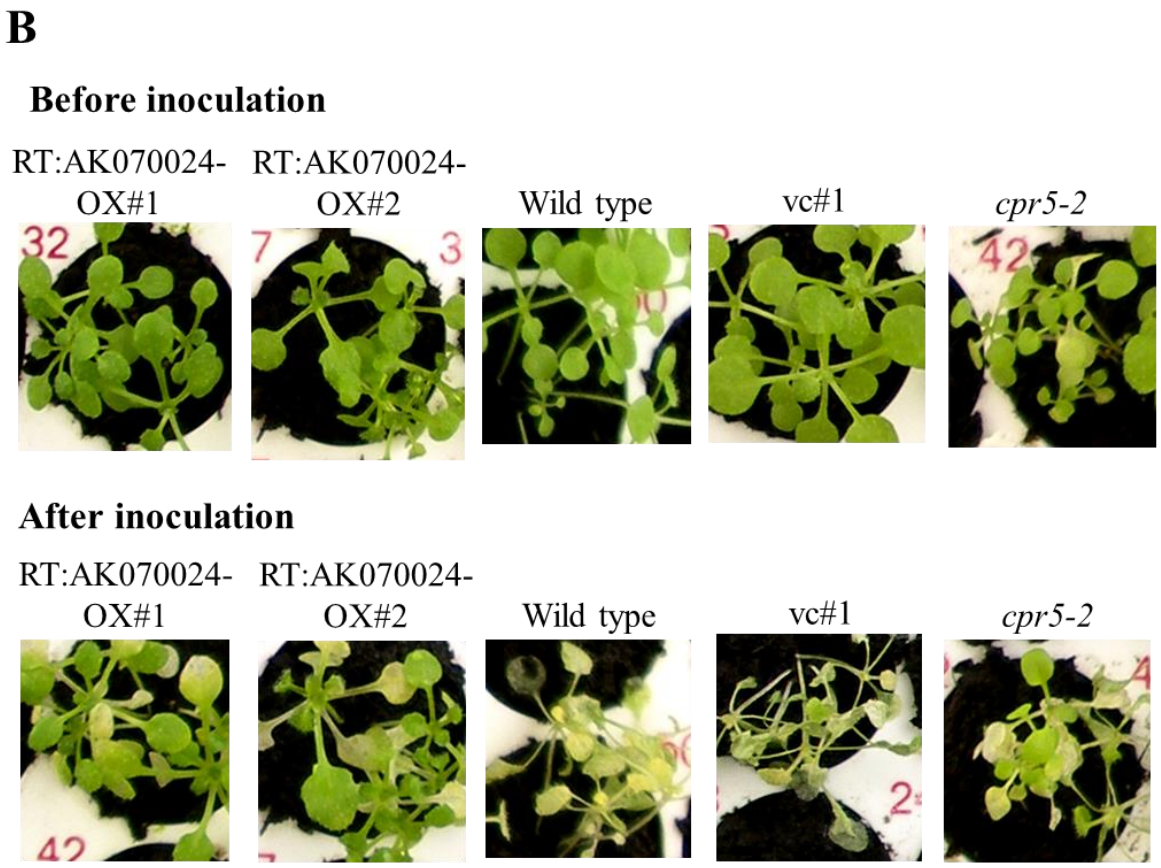


Figure 4. Continued.

Typical example of the *Pst* DC3000 resistance FOX lines (A) and *Pst* DC3000 resistance of two independent retransformed lines (B). Upper panel show 3-week-old T_2 plants used for *Pst* DC3000 inoculation. Lower panel show typical disease symptoms 6 days after inoculation with 10^8 cfu/mL of *Pst* DC3000. The wild type (Col-0) and vector control (VC#1) plants died, but the AK070024-OX, AK102525-OX, AK102125-OX, AK072201-OX, AK070417-OX and *cpr5-2* (resistance mutant to *Pst* DC3000) plants survived. RT:AK070024-OX#1 and RT:AK070024-OX#2 also survived the *Pst* DC3000 screen. RT:AK070024-OX#1 and RT:AK070024-OX#2 are independent retransformed lines for AK070024.

Table 2. Number of rice-FOX *Arabidopsis* lines that survived dip inoculation with *Pst* DC3000

	First Screening	Second Screening	Third Screening
Inoculum level (cfu/ml)	$0.5\text{-}2\times10^8$	$0.5\text{-}2\times10^8$	$1\text{-}2\times10^8$
Screened	20,000	1,620	204
Resistant[a]	1,620	204	72 [b]
% Resistant lines	8.10	1.02	0.36

[a]Lines that survived 6 days after dipping in inoculum containing *Pst* DC3000.

[b]Fifty nine lines with unique gene inserts plus 13 independently transformed lines with gene inserts identical to one of the 59 lines.

the bacterial population. I counted the bacteria according to the protocol of Katagiri *et al.* (2002). As expected, the bacterial counts (in colony-forming units/mg plant tissue) in two vector control lines were similar to that in the wild type, Col-0 (Fig. 5A). The bacterial count in the resistant FOX line AK070024-OX was significantly lower than those in the wild type and vector control (Fig. 5A). In another experiment, the bacterial counts in four randomly selected resistant lines, which were shown in Fig. 4A, were significantly lower as well (Fig. 5B). In particular, the bacterial counts in AK102525-OX plants were about 1/100 of that in wild type. This protocol was also applied to one of retransformed plant (RT:AK070024-OX#1), which also showed significantly lower bacterial count compared to the wild type (Fig. 5B).

FOX lines with single rice cDNA insert showing resistance to *Pst* DC3000

The *Pst* DC3000-resistant phenotype observed in T_2 population could be because of gene disruption or other unexpected mutations that may have occurred during the *Agrobacterium*-mediated transformation process. Hence, it is important to verify the phenotypes in independent transformant lines overexpressing the same cDNA, either by finding these in the existing T_2 population of the FOX lines or by retransformation. With this criterion, 19 single insert rice cDNAs have so far been confirmed to enable the survival of transgenic *Arabidopsis* after exposure to my screening protocol (Table 3). These cDNAs conferred *Pst* DC3000 resistance to the corresponding transgenic lines in at least five independent screens, i.e., in three screens with the original FOX population and at least two screens with retransformed lines. Another group of 16 lines that consistently showed high resistance to *Pst* DC3000 still need verification through repeat screening of independently transformed lines (Table 3). I currently refer to these 35 genes

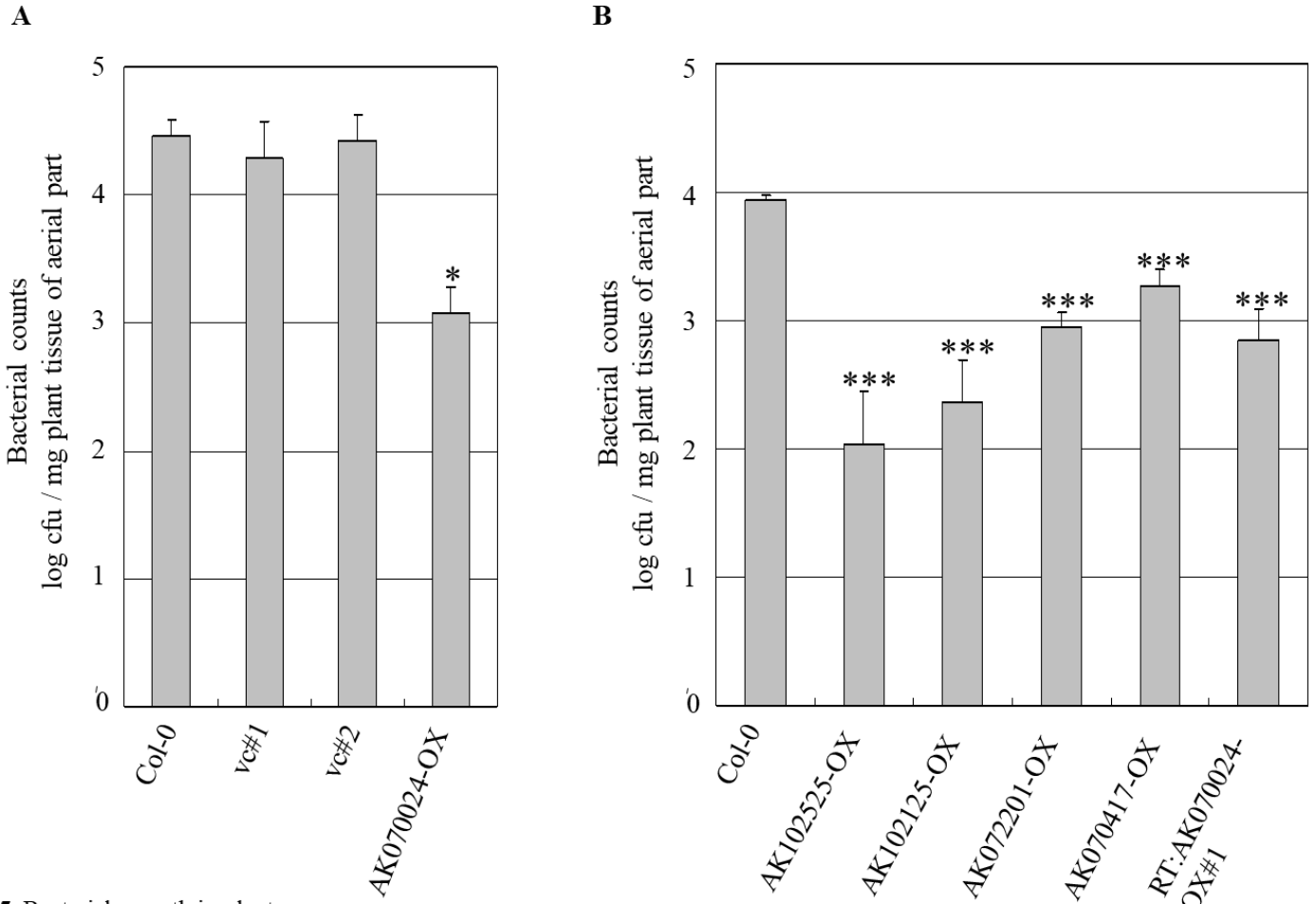


Figure 5. Bacterial growth in plants.

Five-week-old *Arabidopsis plants* were inoculated with *Pst* DC3000 by dipping in a bacterial suspension (10^6 cfu/mL), and the numbers of bacteria in the aerial part of the plants were counted after 3 days. Bars indicate the SD (n=4). (A) The differences in bacterial count between Col-0 (or vector controls) and AK070024-OX plants are significant at 5% (*) by t-test. Col-0, wild type; vc, vector control. (B) The differences in bacterial counts in plants between Col-0 and the selected *Pst* DC3000 resistant transgenic *Arabidopsis* lines are significant at 5% (*) or 0.1% (***) by t-test, respectively. RT:AK070024-OX#1 is the same retransformed line as that used in Fig. 4.

Table 3. *Pst* DC3000 resistant lines with one cDNA insert

Line No.	Original screened line	Independently transformed line(s)[a]	Accession No.[b]	RAP ID[c]	RAP description[c]	Sequence in comparison to genomic DNA[d]		*Pst* count[e]
						cDNA	Protein	
Resistance confirmed by independent line(s) (19 lines)								
1	K00714	RT	AK068846	Os01t0127300-01	SufBD family protein.	Ok	Ok	
2	K21617	RT	AK103699	Os10t0530900-01	Similar to Glutathione S-transferase GST 30	1 ins	Ok	
3	K00841	RT	AK072201	Os01t0503400-04	Similar to metal transporter Nramp6	1 del	62% shorter	Yes
4	K15424	R06015, RT	AK070024	Os09t0533600-01	Similar to Avr9/Cf-9 induced kinase 1	Ok	Ok	Yes
5	K04135	K20450, RT	AK100547	Os02t0145600-01	Conserved hypothetical protein	Ok	Ok	
6	K25904	K18218(2inserts)	AK072899	Os09t0363900-01	Similar to HOTHEAD protein precursor	Ok	Ok	
7	K02342	K23019	AK102525	Os12t0619000-01	IQ calmodulin-binding region domain containing protein	Ok	Ok	Yes
8	K21204	K17730	AK102125	Os08t0250700-01	Thioredoxin domain 2 containing protein	Ok	Ok	Yes
9	K29409	RT	AK099032	Os03t0240500-01	Similar to Toc34-2 protein	Ok	Ok	
10	R04214	RT	AK069592	Os01t0232100-01	Similar to Tonoplast membrane integral protein ZmTIP4-3	1 ins	Ok	
11	K03301	RT	AK070417	Os03t0197100-01	Similar to Sugar transporter protein	Ok	Ok	Yes
12	K03216	K18912	AK101795	Os04t0382300-01	Similar to SNF1-related protein kinase regulatory gamma subunit 1	Ok	Ok	
13	K37838	K25231	AK070720	Os03t0563300-03	Similar to Mg-chelatase subunit (Fragment)	1 bs	Ok	
14	R05946	K02535(2inserts)	AK099542	Os06t0639550-01	Non-protein coding transcript	Ok	no protein	
15	R06202	K04148	AK071866	Os03t0738300-01	Hypothetical protein	1 bs	Ok	
16	R06736	K13623	AK101242	Os04t0346800-02	EAR repeat containing protein	1 ins	2x longer	
17	R06848	K13001	AK073641	Os06t0623600-01	Similar to Cinnamoyl-CoA reductase	Ok	Ok	
18	R03132	K19414, K30128	AK069465	Os07t0622100-01	Similar to Ribosomal protein s6 RPS6-2	1 bs	Ok	
19	K09018	RT	AK071510	Os06t0556200-01	Similar to Amino acid permease I	Ok	Ok	

Table 3. Continued

Line No.	Original screened line	Independently transformed line(s)[a]	Accession No.[b]	RAP ID[c]	RAP description[c]	Sequence in comparison to genomic DNA[d]		*Pst* count[g]
						cDNA	Protein	
No independent lines available for confirmatory screening (16 lines)								
20	K17110		AK101316	Os07t0435100-01	Similar to 26S proteasome subunit RPN12	1 del 2 bs	Ok	
21	K19720		AK072674	Os03t0333300-02	Similar to eukaryotic translation initiation factor 2 beta subunit	2 ins	40% longer	
22	K08435		AK068205	Os06t0661600-01	Zinc finger, DPH-type domain containing protein	1 bs	Ok	
23	K17109		AK111889	Os10t0160000-01	Similar to Ubiquitin carboxyl-terminal hydrolase 12	Ok	Ok	
24	K04020		AK066139	Os09t0461700-01	Alpha/beta hydrolase fold-3 domain containing protein	1 bs	1 ac	
25	K37931		AK071286	Os01t0803300-01	Protein of unknown function DUF6	1 bs	1 ac	
26	K39531		AK099196	Os02t0590400-02	Lecithin:cholesterol acyltransferase family protein	Ok	Ok	
27	K40223		AK065007	Os01t0978100-01	Similar to Cysteine synthase, mitochondrial precursor	Ok	Ok	
28	K40946		AK103235	Os02t0829100-01	Replication protein A 30kDa	Ok	Ok	
29	R04016		AK102402	Os02t0489400-01	Similar to 40S ribosomal protein S8	2 bs	1 ac	
30	R06007		AK103707	Os01t0160800-01	Similar to Protein synthesis inhibitor II (Ribosome-inactivating protein II)	1 bs	Ok	
31	R05018		AK111775	Os01t0313300-01	Similar to EREBP-3 protein (Fragment)	Ok	Ok	
32	K03221		AK070873	Os04t0103100-01	Glycosyl transferase, family 43 protein	2 ins 1 del 2 bs	8.4% shorter	
33	K17538		AK070457	Os10t0190900-01	Multi antimicrobial extrusion protein MatE family protein	1 del 1 bs	1 ac	
34	K31418		AK101216	Os10t0573900-01	NMD3 family protein.	1 del 2 bs	37% shorter	
35	K30521		AK073206	Os10t0573900-03	Similar to Nonsense-mediated mRNA decay protein 3	2 del 1 bs	Ok	

[a]RT represents retransformed.

[b]Accession No. provided by KOME

[c]ID and predicted protein annotation provided by RAP-DB (http://rapdb.dna.affrc.go.jp/)

[d]KOME cDNA sequences compared to genomic DNA sequences in RAP-DB . Ok, sequence identical to either genomic DNA sequence or the predicted protein sequence based on the genomic DNA data; bs, base substition; ins, insertion; del, deletion; ac, amino acid change.

[e]Yes indicates that bacterial population count was perfomed after inoculation of plants; data are shown in Fig. 5.

as *RPD* (Resistance to *Pst* DC3000) genes.

The rice cDNAs inserted in the FOX hunting lines were derived from those listed in KOME. However, the cDNA sequences curated at the KOME site had a few errors, possibly because of errors in reverse transcription, when compared to their genomic sequences described in the Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp/). About 51% (18 of 35) of the cDNAs listed in Table 3 had some mutations, although most of them did not have any effect on the putative protein product.

I used blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the closest protein homologues of those encoded by rice inserts (Table 3) in the hope of finding some of the possible underlying reasons for the observed resistance phenotype. Interestingly, most of the genes in Table 3 (or their close homologues in other species) have not been reported previously as defence-related genes.

FOX hunting lines with zero or multiple cDNA inserts showing resistance to *Pst* DC3000

My screening for resistance to *Pst* DC3000 identified 35 FOX lines that overexpressed single rice cDNA (Table 3). The rest of the *Pst* DC3000 resistant lines (Table 4) had no cDNA inserts (eight lines), a chimeric insert (one line), an unreadable insert (two lines), two independent inserts (four lines), two independent inserts with more than two cDNAs in one insert (one line) and more than two cDNAs at one insertion locus (eight lines).

The resistance in plants having T-DNA with no cDNA insert may be because of a disruption of a functional host gene during the transformation process with an empty

Table 4. *Pst* DC3000 resistant lines with none or more than one cDNA insert

Line No.	Original Line		Sequenced region	Accession No.	RAP ID	RAP description[a]
No cDNA insert (8 lines)						
36	K20031					
37	K31235					
38	K37936					
39	R05434					
40	R06639					
41	R06746					
42	K30208					
43	K30718					
cDNA fragment chimera (based on full sequence) (1 line)						
44	K02809		5'	AK071280	Os10t0110800-02	Similar to Nitrate transporter (Fragment)
			3'	AK071222	Os10t0539400-01	Similar to MCE-family protein MCE2C
Single PCR fragment but unreadable (2 lines)						
45	K29426					
46	R06632					
Two independent cDNA inserts (4 lines + 2 independent lines [b])						
47	R05622	A	5', 3'	AK111700	Os01t0113800-01	Protein kinase, core domain containing protein
		B	5', 3'	AK103090	Os01t0958100-02	Similar to Chloroplast SRP receptor cpFtsY precursor
48	K26225	A	5', 3'	AK068060	Os12t0605400-01	Similar to CROC-1-like protein (Fragment)
		B	5', 3'	AK065583	Os10t0438600-01	Similar to Family II lipase EXL3
49	R07445	A	5', 3'	AK102786	None (Similar to Os05t0440250-01)	Similar to Histone deacetylase superfamily protein
		B	5', 3'	AK071245	Os03t0608800-01	PDZ/DHR/GLGF domain containing protein
50	K17801	A	5', 3'	AK066933	Os06t0178900-01	Vacuolar H+-pyrophosphatase
		B	5', 3'	AK101159	Os04t0528400-01	Similar to ABC transporter
Two independent inserts, more than two cDNAs in one insert (1 line)						
51	K35251	A	5'	AK073249	Os05t0456900-01	Conserved hypothetical protein
			3'	AK067396	Os01t0368700-01	Protein of unknown function DUF679 family protein
		B	5', 3'	AK105874	Os01t0268800-01	Ubiquitin-associated/translation elongation factor EF1B

Table 4. Continued

Line No.	Original Line	Sequenced region	Accession No.	RAP ID	RAP description[a]
More than two cDNAs at one insertion locus (total length of cDNAs at 5' and 3' shorter than the observed PCR fragment) (8 lines)					
52	K02851	5'	AK071506	Os03t0383600-01	Thiolase-like, subgroup domain containing protein
		3'	AK065536	Os09t0244200-01	Conserved hypothetical protein
53	K37919	5'	AK073569	Os07t0175600-01	Plant lipid transfer protein and hydrophobic protein
		3'	AK067825	Os03t0201600-02	Similar to ischemia/reperfusion inducible protein
54	K35008	5'	AK102635	Os06t0273800-01	Similar to Signal peptidase 18 subunit (Fragment)
		3'	AK067216	Os01t0868200-01	Zinc finger, DHHC-type domain containing protein
55	K36312	5'	AK072635	Os02t0532900-02	Similar to H0717B12.10 protein
		3'	AK071613	Os12t0555500-01	Probenazole-inducible protein PBZ1
56	K41633	5'	AK072747	Os04t0657100-01	Similar to Farnesyl diphosphate synthase (Fragment)
		3'	AK102417	Os12t0540900-01	Similar to Tryptophanyl-tRNA synthetase
57	R05917	5'	AK100760	Os12t0123600-01	Similar to Nucleoside-triphosphatase
		3'	AK069004	Os03t0220700-02	Peptidase, trypsin-like serine and cysteine domain containing protein
58	R05945	5'	AK065044	Os03t0749300-01	Similar to Exoglucanase precursor
		3'	anti-sense of AU069314 (vector fragment)		
59	R06201	5'	AK064875	Os03t0369800-01	Similar to Novel plant SNARE 13 (AtNPSN13)
		3'	AK071002	Os12t0518000-01	Hypothetical conserved gene

[a]Predicted protein annotation provided by RAP-DB (http://rapdb.dna.affrc.go.jp/)

[b] Two independent lines with two independent cDNA inserts, K18218 and K02535, are listed in Table 3.

vector. Therefore, it would be interesting to sequence the DNA adjacent to the T-DNA insertion site via TAIL-PCR. Multiple insertion events are expected when a pool of cDNAs is transformed en masse, via floral dip protocol, into a population of *Arabidopsis* plants. The presence of more than one cDNA at one insertion locus has been explained by Nakamura *et al.* (2007). In this report, since *Sfi*I-digestion of the FOX vector and the rice cDNA clones generated different cohesive ends, it is probable that multi different *Sfi*I-digested cDNA fragments were inserted into the *Sfi*I-digested FOX vector simultaneously. The causatory gene for the resistance found in these lines must be determined by independent transformation to verify the function of each candidate gene.

Discussion

Advantages of the rice-FOX *Arabidopsis* system

In this study, I screened 20,000 of rice-FOX *Arabidopsis* lines for resistance to *Pst* DC3000 infection and obtained 72 resistant lines. The rice-FOX *Arabidopsis* system has several characteristic features as a resource for screening gene function in rice. Rice is widely used as a model plant of monocots; however, the lifespan of rice is much longer than that of *Arabidopsis*, which is one of the disadvantages for using rice. In addition, large-scale functional genomics using transgenic rice is constrained by space limitations especially in countries where experimental transgenic rice need to be grown under isolated or glasshouse conditions. The rice-FOX *Arabidopsis* system is a system that is able to overcome these disadvantages. The small size and short lifespan of *Arabidopsis* enable high-speed and large-scale screening, and this is especially useful for the screening of disease resistance genes that involve complicated pathogen infection mechanisms.

Plant phenotypes resulting from the overexpression of genes do not necessarily

reflect the inherent functions of the genes. It is generally accepted that such neomorphic phenotypes frequently occur when regulatory genes, such as those for protein kinases and transcription factors, are overexpressed. Because of this problem, the phenotypes in the rice-FOX *Arabidopsis* lines should be interpreted carefully. However, this issue seems to be less serious when the FOX lines are used for the screening of genes that potentially improve crops, because useful phenotype, but not the elucidation of gene functions, is the final goal in this case.

Another factor that should be considered is that the overexpression of a gene of foreign origin can yield phenotypes different from those resulting from the overexpression of the corresponding endogenous gene. This is because proteins, even if they have the same catalytic activity, may undergo different regulation in their respective genomic backgrounds. For example, the tobacco *aspartate kinase* (*AK*) gene is regulated by feedback inhibition; however, its counterpart gene in *E. coli* does not undergo regulation when overexpressed in tobacco. This enabled enhanced accumulation of free methionine in transgenic tobacco seeds expressing *E. coli AK* gene (Karchi *et al.* 1993).

In addition, the overexpression of genes in their native host sometimes induces gene silencing, which hampers the screening of gene function. In this regard, overexpression in a foreign genomic background is less likely to induce gene silencing because of lower sequence homology with corresponding endogenous genes.

For these reasons, the FOX lines offer a unique opportunity to find previously unknown functions of rice genes. In fact, I identified a number of genes that enhance plant resistance to disease, most of which (including their orthologs in other species) have not previously been associated with resistance to any disease.

Relationships between *Pseudomonas, Arabidopsis* and foreign rice genes

Many reports on the resistance to *P. syringae* in *Arabidopsis* concentrated on genes involved in recognition and regulation (Katagiri *et al.* 2002). Establishment of recognition and interaction between gene products of two interacting organisms presumably require a significant period of co-evolution, probably measured by millions of years, during which both host and pathogen evolve new gene products by random mutations. In the case of the compatible (pathogenic) relationship between *Pst* DC3000 and *A. thaliana* 'Columbia', evolution has enabled the pathogen to overcome host defences when conditions are optimized for colonization. My screening strategy is based on the principle of optimized pathogenesis: grow the plants in humid conditions and infect at high inoculum density to enable pathogenesis to run its full course (i.e., kill the wild type plant). Under these conditions, only the most resistant transgenic lines can survive. Therefore, some of the genes that had been introduced into the surviving lines may be potential major disruptors of the basic host–pathogen relationship. The *RPD* genes may be explained in the context of an evolutionary arms race, where the overexpressed rice cDNA represent major mutations, some of which are potent enough to overcome the screening conditions that had been tilted in favour of the compatible pathogen. These rice genes could be sufficiently novel (in the *Arabidopsis* genome) to disturb the attack and colonization machinery of *Pst* DC3000.

Chapter 3

An identified putative receptor-like cytoplasmic kinase gene, *BSR1*, confers resistance to major bacterial and fungal pathogens in *Arabidopsis* and rice

Introduction

Utilizing genes involved in plant defence mechanisms is an approach to develop disease-resistant crops. Resistance (*R*)-gene-mediated resistance has been widely used in breeding; however, the resistance is limited to specific races of pathogens and often breaks down because of the outgrowth of mutated pathogens after a few years of commercial cultivation in the field (Bonman *et al*. 1992). Thus, disease resistance that is durable and effective against broad spectrum of pathogens (or pathogen races) is of invaluable agronomical importance (Kou and Wang 2010). Therefore, identifying new genes that can confer such disease resistance traits to crops is among the subjects of top priority in plant science.

Breeding crops with broad-spectrum disease resistance using genetic resources is one of the principal goals of crop improvement. However, only a few genes have been identified as genetic resources for broad-spectrum disease resistance in rice. It is desirable to have broad-spectrum disease resistance of bacterial leaf blight caused by bacterial pathogen *Xoo*, and rice blast caused by fungal pathogen *P. oryzae*, which are the major diseases in rice.

In chapter 1, I performed screening successfully and identified many *RPD* genes. In this chapter, to select broad-spectrum disease resistance genes, I further re-screened the lines screened by *Pst* DC3000 for resistance to the fungal pathogen *Colletotrichum higginsianum* in *Arabidopsis*. The infection mechanism of *C. higginsianum* in

Arabidopsis is similar to that of the fungus *P. oryzae*: this pathogen forms appressoria and penetration pegs during infection. Then, I tested whether the selected causative genes can confer resistance to bacterial pathogen *Xoo* and fungal pathogen *P. oryzae*, when overexpressed in rice.

Materials and Methods

Test for resistance to *C. higginsianum*

Fungal *C. higginsianum* was cultured on PDA agar plates (0.39% potato extract, 2.1% glucose and 1.41% agar, adjusted to pH 5.6, Nissui, Tokyo, Japan) for 2 weeks at 28°C under dark conditions and used for inoculation.

I used a screening procedure almost identical to that applied for *Pst* DC3000, except that I used 0.25 to 2×10^6 conidia/mL of *C. higginsianum* (MAFF305635 supplied by Genetic Resources Center, NARO) and incubated the plants for 6 days under short-day (9 h) conditions. The response to infection (R, resistant, S, susceptible) was based on qualitative evaluation of residual green portions of infected leaves in comparison with a positive control, Eil-0, an *Arabidopsis* ecotype highly resistant to *C. higginsianum* (Narusaka *et al.* 2004).

Generation of transgenic rice lines overexpressing screened full-length rice cDNAs

To generate overexpression rice lines, full-length cDNAs, provided by the Rice Genome Resource Center, NARO (Japan), were cloned into the *Sfi*I site between the maize *Ubiquitin* promoter and the nopaline synthase terminator in a binary vector, pRiceFOX (Nakamura *et al.* 2007). The plasmids were introduced into rice (*O. sativa* ssp. *japonica* cv. Nipponbare) by an *Agrobacterium*-mediated procedure (Toki *et al.* 2006).

Xoo culture

Xoo (MAFF311018, T7174, race I) was obtained by Dr. Hirokazu Ochiai (Institute of Agrobiological Sciences, NARO (NIAS), Japan). *Xoo* was grown on PSA agar plate (1% proteose peptone, 1% sucrose and 1.5% bacto agar) at 28°C in the dark for 2 days. The bacterial cells were harvested with sterilized water and suspended at a concentration of OD_{600} = 0.3 in an inoculation medium.

Screening for resistance to *Xoo* and *P. oryzae* in transgenic rice lines

Transgenic rice seedlings were selected by hygromycin resistance (50 μg／mL). The selected rice plants were grown in a growth chamber until 6-7 leaf stage at 25°C under a 16／8 h light／dark regime. The plants were inoculated with *Xoo* (T7174), and symptoms were evaluated 2 weeks after inoculation as described previously (Mori *et al.* 2007). In this experiment, the 6th leaf blades of the tested plants were cut with scissors pre-wetted with inoculum (OD_{600} = 0.3) at about 5 cm from the tip, and the cut ends (about 1 cm from the ends) were dipped in a suspension of *Xoo* for 10 s.

For screening resistance to the blast fungus, I used isolate Kyu89-246 (MAFF101506, race 003.0) of *P. oryzae* obtained by Dr. Nagao Hayashi (NIAS, Japan). Kyu89-246 is compatible to Nipponbare. It was grown on oatmeal agar plates (3% oatmeal, 0.5% sucrose and 1.6% bacto agar) at 25°C in the dark for 12 days and under continuous illumination for 2 days to induce sporulation. To make the conidial inoculum (5×10^4 spores／mL), gel surface was flooded with sterile water containing 0.01% Tween 20 and the mycelia of *P. oryzae* were scraped. The rice plants were grown in a greenhouse at 28°C in the day and 24°C at night until 4-leaf stage and spray-inoculated as described previously (Mori *et al.* 2007). Evaluation of resistance was based on the total number of

the compatible lesions that appeared on the 3rd and 4th leaf blades of each plant 6 days after inoculation.

Sequence alignment and phylogenic analysis

Amino acid sequence alignments were generated by the CLUSTALX computer program (Thompson *et al.* 1997). The phylogenic tree was constructed by the neighbour-joining method from the deduced amino acid sequences. Bootstrap mode (1,000 replications) was used for estimating the confidence that could be assigned to particular nodes on the tree. The result was illustrated by NJ plot (Perrière and Gouy 1996).

Results

Resistance to *C. higginsianum*

To determine whether the overexpression of the *RPD* genes is also effective against other types of pathogens, I tested the *RPD* lines for resistance against the fungal pathogen, *C. higginsianum*. *Colletotrichum* species are hemibiotrophic fungi that initially feed on living tissues and continue feeding on the nutrients released from dead tissues (Perfect *et al.* 1999). More importantly, *Colletotrichum* species produce appressoria, whose walls contain melanin, and its infection mechanism is similar to *P. oryzae*, the most important rice pathogen that causes rice blast. *C. higginsianum*-resistant lines are shown in Fig. 6. Six days after inoculation, plants of the AK070024-OX line, which was originally selected for resistance to *Pst* DC3000, survived stringent inoculation with *C. higginsianum*, whereas the wild type plants were obviously dead (Fig. 6, photographs under white light). This indicated that AK070024-OX plants are also resistant to *C. higginsianum*. Under black light illumination (UV 365 nm), healthy tissues with intact

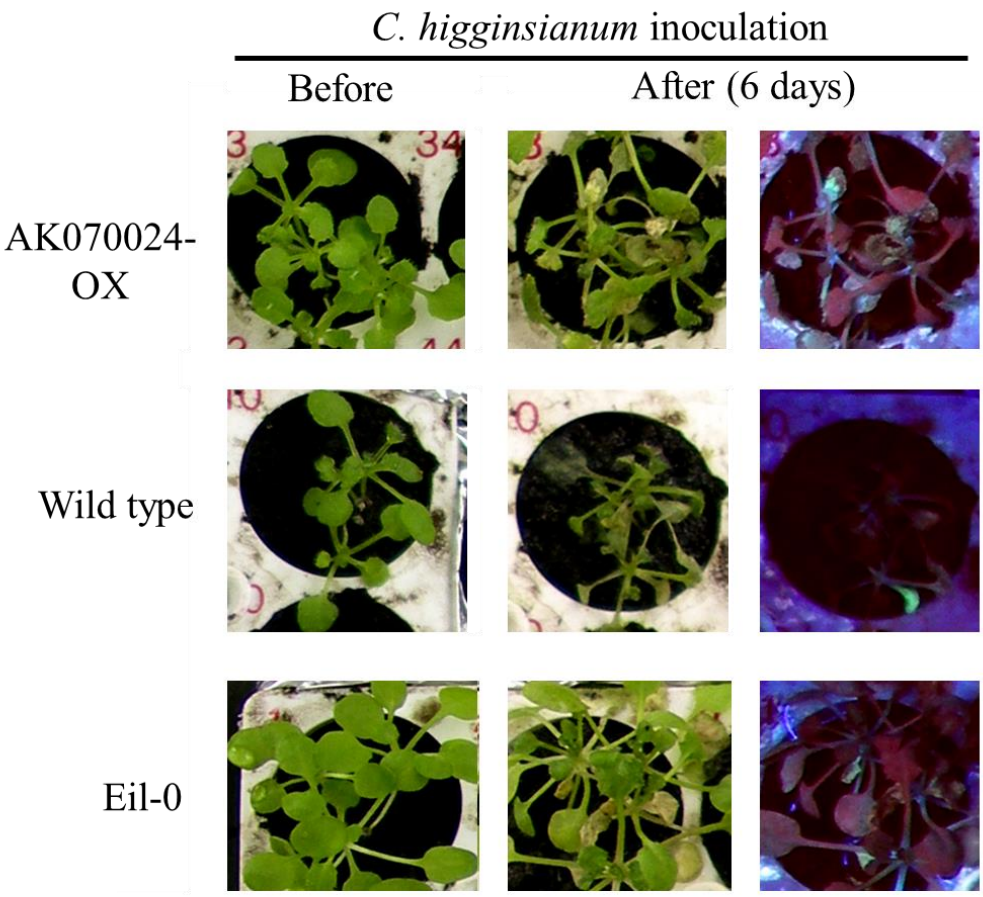


Figure 6. Phenotypic responses to *C. higginsianum* ($2.5x10^5$ conidia/mL) dip inoculation.
Three-week-old plants were used for inoculation. Compared with wild type (Col-0), AK070024-OX and Eil-0 (resistant ecotype to *C. higginsianum*) plants were still surviving 6 days after inoculation. The amounts of residual chlorophyll, which appear green under white light and red under UV, were used to assess resistance. Resistant plants appear green under white light and red under UV. AK070024-OX is the same line as that used in Fig. 4.

chlorophyll exhibited red fluorescence (Eil-0 and AK070024-OX), whereas dead ones (wild type) had no red fluorescence (Fig. 6, photographs under UV light). Surviving leaves of AK070024-OX and wild type with severe fungal growth emitted a silvery fluorescence under UV light. In *C. higginsianum*-inoculated wild type plants, only a part of the leaf emitted a silvery fluorescence. On the basis of these results, I used red fluorescence as a direct indicator of the health (resistance) of the inoculated tissues when it was difficult to determine whether the tissues were 'dead or alive' under white light.

Many of the FOX hunting lines selected for resistance to *Pst* DC3000 also showed resistance to *C. higginsianum* (Table 5). Of the 35 lines in Table 3 that were tested for *C. higginsianum* resistance, 11 (31.4%) were considered as resistant. Of the 16 lines with cDNA inserts in Table 4 that were tested for *C. higginsianum* resistance, only two were considered as resistant. All of the lines with no cDNA insert were susceptible to *C. higginsianum*.

Resistance to bacterial leaf blight in the transgenic rice

I examined whether *RPD* genes also extended resistance to *Xoo*, the bacterial pathogen for rice leaf blight. The cDNAs of some *RPD* genes were inserted downstream of the constitutive maize *ubiquitin* promoter, and the constructs were used to generate transgenic rice lines. Screening was performed by inoculating *Xoo* by cut-dip method at the T_1 generation (plants from seeds of plants regenerated from transgenic calli). Overexpression of inserted cDNAs was confirmed in T_0 plants (plants regenerated from transgenic calli) by qPCR, and T_1 seeds derived from them were used for screening. Table 6 shows that, of eight transgenic lines tested so far, only one has shown strong resistance under my screening conditions. The detailed resistance phenotype using

Table 5. *C. higginsianume* resistant lines selected for resistance to *Pst* DC3000

Line No.	Original screened line		Sequenced region	Accession No.[a]	RAP ID[b]	RAP description[b]	Response to *C. higginsianum*[c]
one cDNA insert (11 lines)							
2	K21617			AK103699	Os10t0530900-01	Similar to Glutathione S-transferase GST 30	R
3	K00841			AK072201	Os01t0503400-04	Similar to metal transporter Nramp6	R
4	K15424			AK070024	Os09t0533600-01	Similar to Avr9/Cf-9 induced kinase 1	R
6	K25904			AK072899	Os09t0363900-01	Similar to HOTHEAD protein precursor	R
7	K02342			AK102525	Os12t0619000-01	IQ calmodulin-binding region domain containing protein	R
12	K03216			AK101795	Os04t0382300-01	Similar to SNF1-related protein kinase regulatory gamma subunit 1	R
20	K17110			AK101316	Os07t0435100-01	Similar to 26S proteasome subunit RPN12	R
21	K19720			AK072674	Os03t0333300-02	Similar to eukaryotic translation initiation factor 2 beta subunit	R
24	K04020			AK066139	Os09t0461700-01	Alpha/beta hydrolase fold-3 domain containing protein	R
26	K39531			AK099196	Os02t0590400-02	Lecithin:cholesterol acyltransferase family protein	R
31	R05018			AK111775	Os01t0313300-01	Similar to EREBP-3 protein (Fragment)	R
cDNA fragment chimera (based on full sequence) (1 line)							
44	K02809		5'	AK071280	Os10t0110800-02	Similar to Nitrate transporter (Fragment)	R
			3'	AK071222	Os10t0539400-01	Similar to MCE-family protein MCE2C	
Two independent inserts, more than two cDNAs in one insert (1 line)							
51	K35251	A	5'	AK073249	Os05t0456900-01	Conserved hypothetical protein	R
			3'	AK067396	Os01t0368700-01	Protein of unknown function DUF679 family protein	
		B	5' , 3'	AK105874	Os01t0268800-01	Ubiquitin-associated/translation elongation factor EF1B	

[a]Accession No. provided by KOME

[b]ID and predicted protein annotation provided by RAP-DB (http://rapdb.dna.affrc.go.jp/)

[c]*Colletotrichum higginsianum* on the FOX hunting lines; R, resistant; S, susceptible

Table 6. *Xoo* resistant of selected line with one cDNA insert for resistance to *Pst* DC3000

Line No.	Original screened line	Accession No.[a]	RAP ID[b]	RAP description[b]	Response to	
					C. higginsianum[c]	*Xoo*[d]
1	K00714	AK068846	Os01t0127300-01	SufBD family protein.	S	S
2	K21617	AK103699	Os10t0530900-01	Similar to Glutathione S-transferase GST 30	R	S
3	K00841	AK072201	Os01t0503400-04	Similar to metal transporter Nramp 6	R	S
4	K15424	AK070024	Os09t0533600-01	Similar to Avr9/Cf-9 induced kinase 1	R	R
5	K04135	AK100547	Os02t0145600-01	Conserved hypothetical protein	S	S
6	K25904	AK072899	Os09t0363900-01	Similar to HOTHEAD protein precursor	R	S
7	K02342	AK102525	Os12t0619000-01	IQ calmodulin-binding region domain containing protein	R	S
21	K19720	AK072674	Os03t0333300-02	Similar to eukaryotic translation initiation factor 2 beta subunit	R	S

[a]Accession No. provided by KOME

[b]ID and predicted protein annotation provided by RAP-DB (http://rapdb.dna.affrc.go.jp/)

[c]*Colletotrichum higginsianum* on the FOX hunting lines; R, resistant; S, susceptible

[d]*Xoo*, *Xanthomonas oryzae* pv *oryzae* on transgenic Nipponbare overexpressing the pertinent full-length rice cDNA; R, resistant, S, susceptible

T_2 plants is shown in Fig. 7. While Nipponbare (wild type) and the vector control plants developed extended lesions from the cut (inoculated) end of the leaf, AK070024-OX plants showed restricted lesion development similar to the resistant control, cv. Asominori (Fig. 7A). Lesion lengths in inoculated AK070024-OX and Asominori were about 1 cm long, whereas those in Nipponbare and vector control were about 13 cm long (Fig. 7B). These results indicate that AK070024 cDNA selected for *Pst* DC3000 resistance in *Arabidopsis* also conferred strong *Xoo* resistance in transgenic rice. Interestingly, lesions in AK070024-OX showed a dark brown colour that is likely associated with cell death (Fig. 7A).

Resistance to rice blast in AK070024-OX rice

In *Arabidopsis*, overexpression of AK070024 also conferred resistance to the fungal pathogen *C. higginsianum* (Fig. 6 and Table 5). Therefore, I investigated the resistance of AK070024-OX rice lines to the rice fungal pathogen *P. oryzae* in comparison with Nipponbare (wild type) and the highly *P. oryzae* -resistant cultivar, Sensho (Fig. 8). Compatible isolate of *P. oryzae* was inoculated by spraying. Lesion numbers in AK070024-OX plants were markedly lower than those in Nipponbare plants, and even less than those in Sensho, which has a strong non-race-specific resistance to *P. oryzae* associated with *pi21* (Fukuoka *et al.* 2009). Thus, overexpression of AK070024 cDNA conferred resistance to the major bacterial and fungal pathogens in both *Arabidopsis* and rice. I designated AK070024 (Os09g0533600) gene as *BROAD-SPECTRUM RESISTANCE 1* (*BSR1*) accordingly. So far, I have not observed any notable growth defect or morphological changes both in *Arabidopsis* and in rice plants overexpressing *BSR1*, except that rice plants overexpressing *BSR1* displayed a decreased

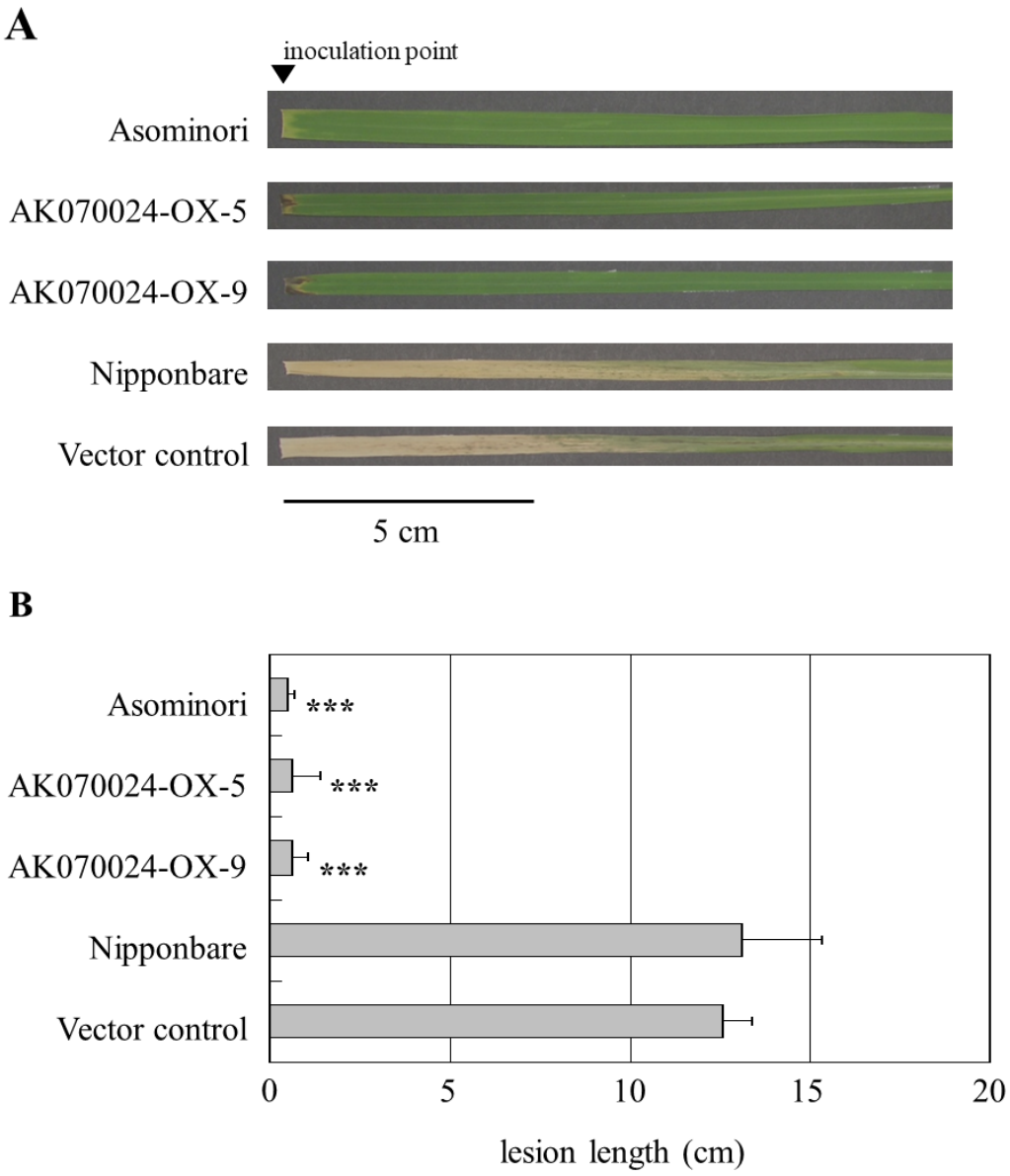


Figure 7. *X. oryzae* pv. *oryzae* (*Xoo*) resistance of AK070024-OX rice.
Photos (A) and lesion lengths (B) of the 6th leaf blades of AK070024-OX (T_2), Nipponbare (wild type), vector control and Asominori (*Xoo* resistant cultivar) 2 weeks after inoculation with *Xoo*. An arrowhead indicates the point of inoculation. Lesion lengths in AK070024-OX and Asominori plants were significantly lower than those in Nipponbare and vector control plants (*** $P<0.001$ by t-test). Error bars indicate standard deviations (n=4-8).

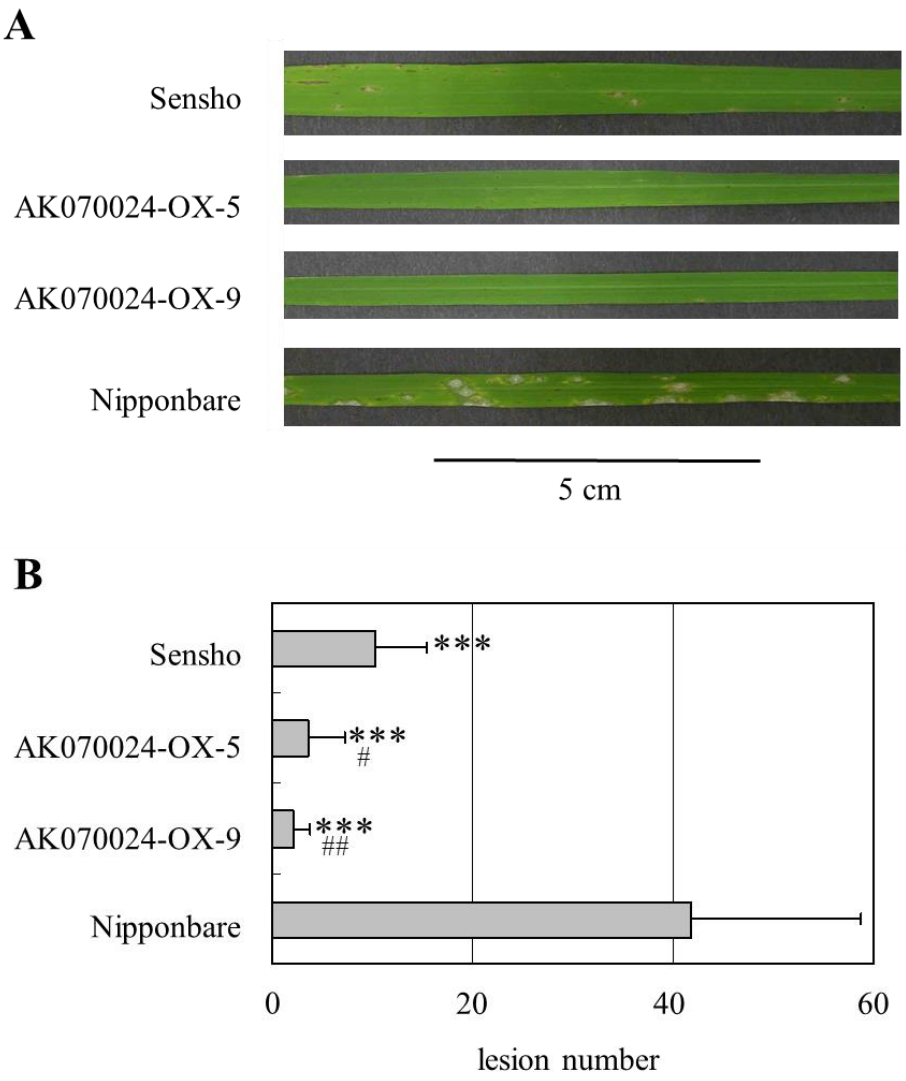


Figure 8. *P. oryzae* resistance of AK070024-OX rice.
(A) Photos of 4th leaf blade. (B) Number of compatible lesions. Nipponbare (wild type), Sensho and AK070024-OX (T_2) plants were grown until 4-leaf stage and spray-inoculated with compatible *P. oryzae*. Lesion numbers in AK070024-OX and Sensho plants were significantly lower than those in Nipponbare plants (*** P<0.001 by t-test). In addition, lesion number in AK070024-OX-5 and AK070024-OX-9 plants were significantly lower than those in the resistant cv. Sensho ($^{\#\#}$ P<0.01 and $^{\#}$ P<0.05). Error bars indicate standard deviations (n=6-8).

germination rate and brownish seeds.

BSR1 (AK070024) encodes a putative receptor-like cytoplasmic kinase

BSR1 (AK070024) codes for a functionally uncharacterized protein of 406 amino acid residues similar to Avr9／Cf-9-induced kinase 1 according to the Rice Annotation Project Database (RAP-DB) description (Table 3). It belongs to a family of receptor-like cytoplasmic kinases (RLCKs) and was previously named as OsRLCK278 according to the phylogenetic analyses of 187 OsRLCKs (Vij *et al.* 2008). RLCKs are a subgroup of receptor-like kinases (RLKs) that do not contain an extracellular domain or transmembrane domain but share a common monophyletic origin with RLKs (Shiu and Bleecker 2001). Sequence alignments and phylogenic tree for BSR1 and representative RLCKs are shown in Fig. 9. BSR1 (OsRLCK278) is classified into the RLCK-VIIb subfamily (Fig. 9B). *Arabidopsis* RLCKs closest to BSR1 are At5g47070 and At4g17660 according to the phylogenic analyses by Shiu *et al.* (2004). To my knowledge, no gene in the RLCK-VIIb subfamily has been characterized; however, some genes in RLCK-VIIa, the closest subfamily of RLCK-VIIb, have been characterized. NAK (At5g02290) is a novel *Arabidopsis* protein kinase (Moran and Walker, 1993), for which no putative function nor patterns of expression have been described so far. REFSEQ (NCBI) reports that NAK has two conserved domains; viz., STKc, the catalytic domain of serine／threonine protein kinases, and PTKc, the catalytic domain of the protein tyrosine kinase (PTK) family. *Arabidopsis* BIK1 (At2g39660) and tomato TPK1b are involved in plant defence against necrotrophic fungal pathogens (Abuqamar *et al.* 2008, Veronese *et al.* 2006). PTO and PBS1 are well-characterized RLCKs involved in race-specific resistance to bacterial pathogens in tomato and *Arabidopsis*, respectively (Martin *et al.* 1993, Swiderski and Innes 2001).

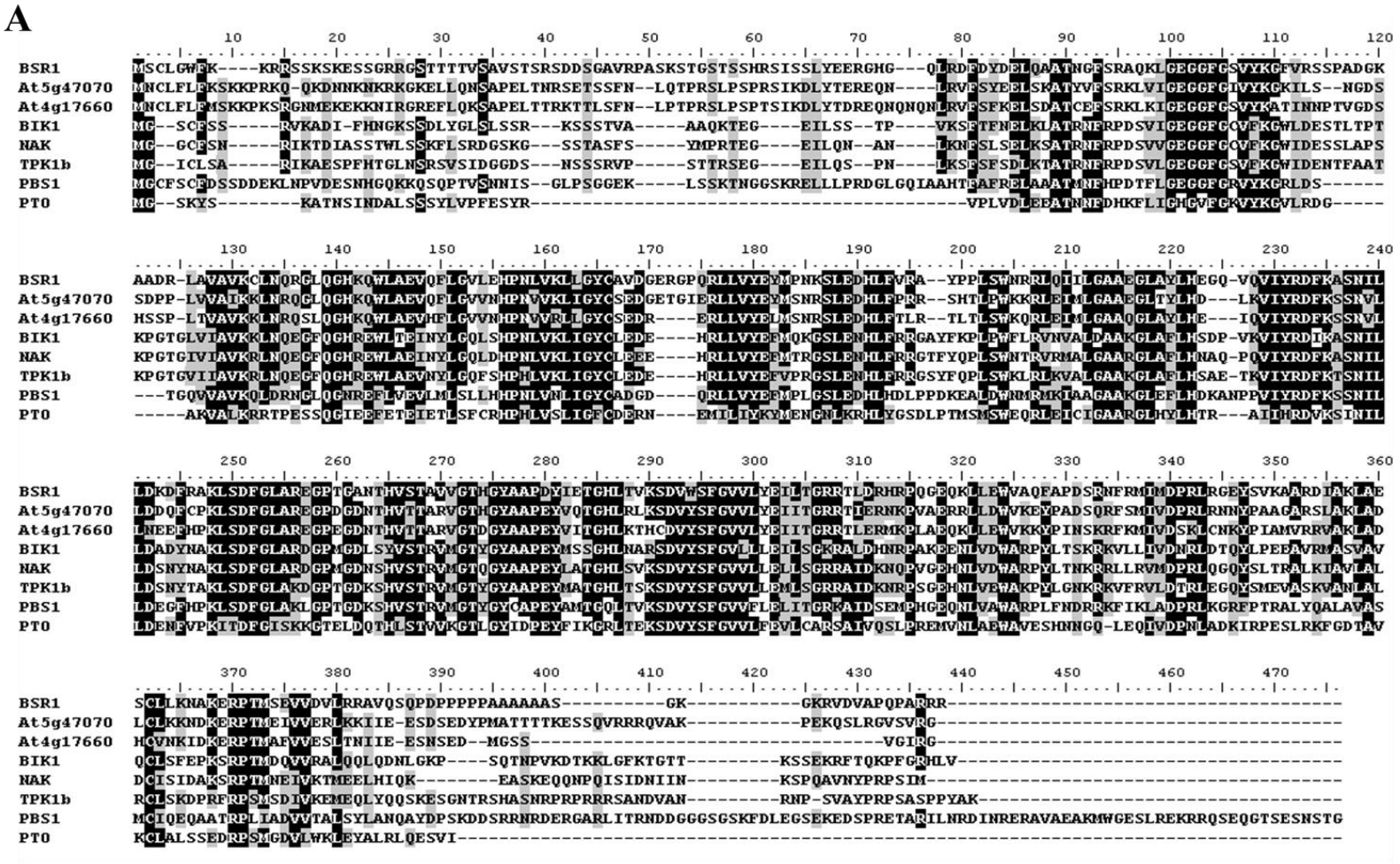


Figure 9. Protein sequence analyses between BSR1 and related RLCKs.
(A) Alignment for BSR1 and related RLCKs. Protein sequence data are from rice (BSR1/AK070024), *Arabidopsis* (NAK/At5g02290, BIK1/At2g39660, and PBS1/At5g13160) and tomato (TPK1b/EU555286, and PTO/DQ019170). Black and gray backgrounds indicate identical and similar amino acids, respectively.

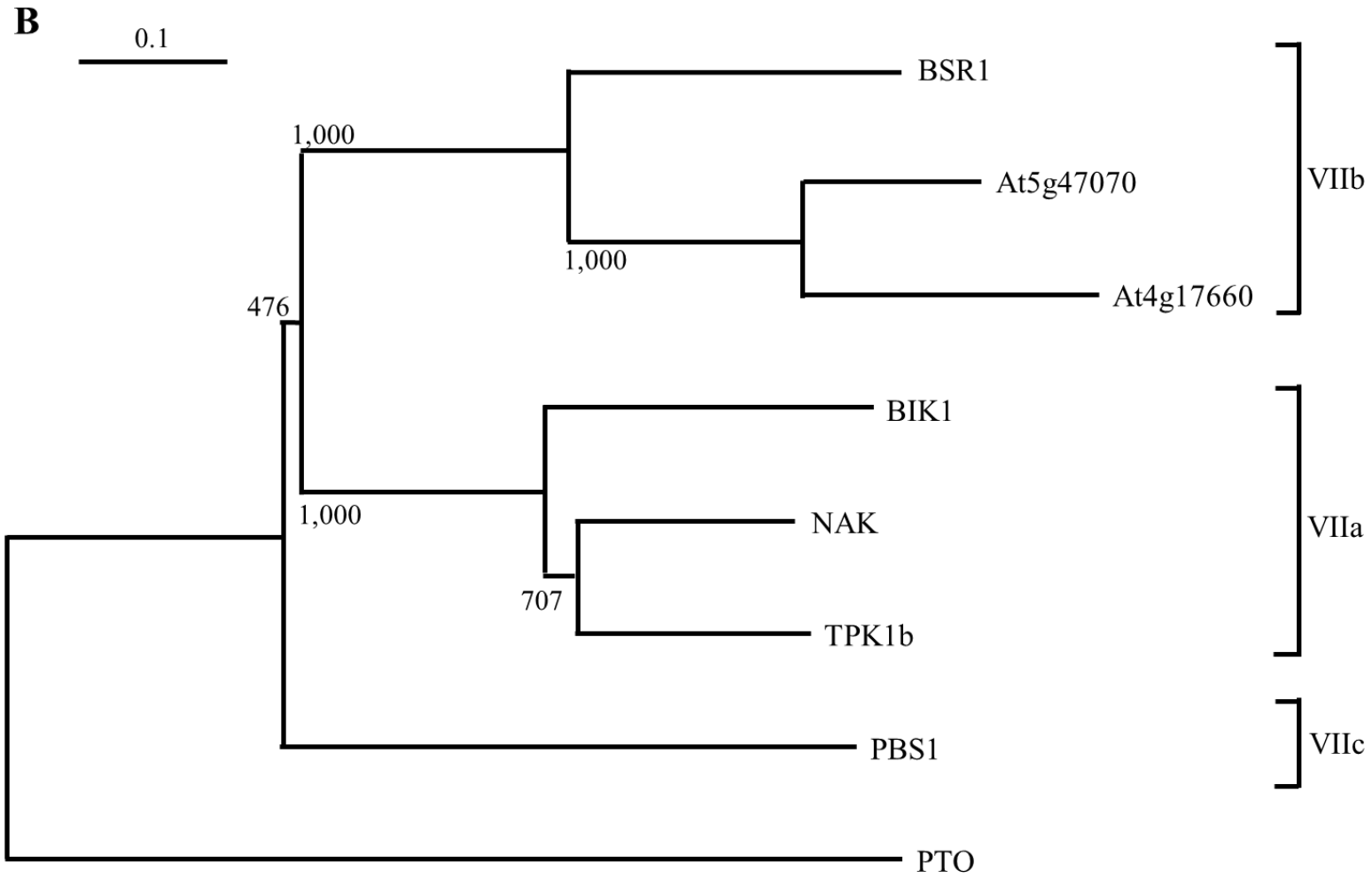


Figure 9. Continued.
(B) Phylogenic tree for BSR1 and related RLCKs. Numbers at nodes indicate bootstrap values. The bar corresponds to 0.1 amino acid substitutions per site. VIIa VIIb and VIIc represent the subfamilies of RLCKs.

Discussion

In this chapter, more than ten lines showed resistance to the fungal pathogen *C. higginsianum* in addition to the bacterial *Pst* DC3000. One of the selected genes, *BSR1*, encoding a RLCK family protein, conferred remarkable resistance to both bacterial and fungal pathogens when overexpressed in *Arabidopsis* and rice. Thus, this screening system allowed us to identify at least one potentially very useful gene that can confer multiple or broad-spectrum disease resistance to both dicot and monocot plants. Further characterization of the remaining candidate genes may identify more genes of scientific and practical importance.

Broad-spectrum disease resistance in plant

I found that 11 of 35 single cDNA inserts identified by the *Pst* DC3000 screen also provided resistance to *C. higginsianum* (Table 5). Broad-spectrum resistance against 2 or more different pathogens is an agronomically desirable trait. Overexpression of *Arabidopsis NPR1* (non-expressor of *PR* genes), a transcriptional cofactor involved in the SA pathway, conferred broad-spectrum disease resistance to *Arabidopsis*, tomato, rice, carrot and cotton (Cao *et al.* 1998, Lin *et al.* 2004, Parkhi *et al.* 2010, Quilis *et al.* 2008, Wally *et al.* 2009). However, constitutive expression of *NPR1* also rendered plants susceptible to viral infection and hypersensitive to abiotic stresses (salt and drought) in rice (Quilis *et al.* 2008). Zheng *et al.* (2006) reported that ectopic overexpression of WRKY33 in *Arabidopsis* made the plants resistant to necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* but more susceptible to *P. syringae*. Recently, it was reported that the expression of a pathogen-associated molecular pattern (PAMP) receptor of *Arabidopsis*, EFR, conferred broad-spectrum bacterial resistance in *Nicotiana*

benthamiana and tomato (Lacombe *et al.* 2010). Thus, most of the genes heretofore reported as providing broad-spectrum disease resistance are associated with signal transduction pathways. Compare with genes that conferred resistance to only *Pst* DC3000 (Tables 3 and 4), many of the genes that conferred resistance to both *Pst* DC3000 and *C. higginsianum* in Table 5 appear to encode signalling components from their annotations. They are likely to be involved in one or more signal transduction pathways, considering their transcriptional responses to signaling molecules (Dubouzet *et al.* 2011). *Arabidopsis* genes corresponding to these rice genes for putative signaling components may be those involved in defence signaling pathways that are generally effective against particular types of pathogens. This speculation needs to be verified by silencing of the endogenous genes or similar approaches.

Of eight transgenic rice lines tested, only one showed high resistance to *Xoo* (Table 6 and Fig. 7) and *P. oryzae* (Fig. 8), although more resistant lines are expected to be identified when I finish screening the rest of the rice lines. The *BSR1* gene is notable because it conferred broad-spectrum disease resistance in *Arabidopsis* (*Pst* DC3000 and *C. higginsianum*) and in rice (*Xoo* and *P. oryzae*). To my knowledge, no other monocot gene has been reported to confer disease resistance in both monocot and dicot to both bacterial and fungal pathogens, respectively.

The rarity of resistance to *Xoo* compared to *C. higginsianum*

The rarity (1／8) of resistance to *Xoo* among the transgenic rice lines contrasts with the higher portion (11／35) of cDNAs showing resistance to *C. higginsianum* in the *RPD* genes (Table 5). I surmise that the reason may lie in the different backgrounds of the hosts and the corresponding pathogens. In the case of *Arabidopsis*, overexpression of

the foreign rice gene represents a major mutation in both quantitative (high transcript level) and qualitative (foreign gene) terms. Such mutations may have critical effects on the *Arabidopsis–Pst* DC3000 or *Arabidopsis–C. higginsianum* interaction if they corresponded to a 'weak parts' in the defensive mechanisms of the host plant or produced novel products that confound the attack and colonization machinery of those pathogens. Mere overexpression of seven of the eight genes (see Table 6) in Nipponbare did not lead to resistance to *Xoo*; this can only indicate that these genes may not be part of the defence mechanisms that the rice plant deal with an invasion by *Xoo*. In other words, most of these rice genes did not play effects to the rice–*Xoo* pathosystem. One probable reason is because *Arabidopsis* and rice, and *Pst* DC3000 and *Xoo* are not closely related. Meanwhile, inoculation of wild type Nipponbare with *Xoo* (or *P. oryzae*) led to transcriptional repression of some of these genes (Dubouzet *et al.* 2011). Furthermore, there is no report that pathogens which can infect to *Arabidopsis* can also infect to rice plants. I guess that these rice genes would function in a defence mechanism of closely related crops to *Arabidopsis* that can be infected by *Pst* DC3000.

Chapter 4

Overexpression of *BSR1* confers broad-spectrum resistance against two bacterial diseases and two major fungal diseases in rice

Introduction

In the natural environment, plants encounter many species of pathogenic microorganisms, such as fungi, bacteria and viruses. The damage caused by microbial diseases is one of the most important limiting factors for crop production. To solve this problem, improvement of host resistance against these pathogens is the most economical and environmentally friendly approach. Rice is one of the most important food crops and is a staple food for approximately 50% of the world's population (Liu *et al.* 2014). Moreover, it is a model plant of monocotyledonous species. Bacterial leaf blight caused by bacterial pathogen *Xoo* and blast by fungus *P. oryzae* are the major rice diseases worldwide and result in serious losses of rice production.

To breed blast-resistant rice, efforts have aimed to introduce the resistance (*R*) genes into susceptible cultivars. The *R* gene is a key component of disease resistance to a particular pathogen and is often associated with a hypersensitive response (HR) (Flor 1971). In most cases, the resistant cultivars with *R* genes remain effective for only a few years in agricultural production (Dean *et al.* 2005) because new biotypes of the pathogen that can overcome the *R* gene often appear after release of the resistance cultivar. By contrast, although large numbers of quantitative trait loci (QTLs) (or quantitative genes) for bacterial leaf blight or blast resistance have been identified, these sources have not been used effectively in rice improvement because the genetic control of quantitative resistance is complex. Therefore, breeding for cultivars that exhibit broad-spectrum and

durable disease resistance is a top priority in rice improvement programs around the world.

In addition to bacterial leaf blight and blast, there are several other important diseases in rice, such as brown spot, bacterial seedling rot and rice stripe virus disease. Brown spot disease is caused by the fungus *Cochliobolus miyabeanus*, a representative necrotrophic pathogen, and is one of the most prevalent diseases in all rice-growing areas. *C. miyabeanus* infects plant tissues such as leaves and spikelets in all development stages. Fungicides, such as iprodione and propiconazole, are effective means to manage this disease (Moletti *et al.* 1997). However, the use of resistant varieties would be preferable because fungicides are expensive and not environmentally friendly. Meanwhile, rice cultivar 'Tadukan' offers quantitative resistance to brown spot disease. However, no major genes conferring immunity to this disease have been identified, although three QTLs for disease resistance have been identified (Sato *et al.* 2008). So far, genetic studies of resistance to brown spot disease have remained the many issues.

Bacterial seedling rot and bacterial grain rot (bacterial panicle blight) in rice are caused by bacterial pathogen *Burkholderia glumae*, which is also a necrotrophic pathogen (Iwai *et al.* 2002, Mizobuchi *et al.* 2013b). Recently, these diseases have become an increasingly serious problem in global rice production because of global warming and climate change; *B. glumae* prefers high temperature and humidity (Ham *et al.* 2011). Some studies reported partially resistant varieties for these diseases and several QTLs for resistance to bacterial seedling rot and bacterial grain rot have been identified (Mizobuchi *et al.* 2013a, 2013b, 2015, Pinson *et al.* 2010). However, these resistances are not strong. Meanwhile, Iwai *et al.* (2002) reported that transgenic rice lines overproducing *Asthi1*, an oat leaf thionin gene, showed enhanced resistance to *B. glumae*. However, it has not been applied to actual breeding.

Rice stripe disease, caused by rice stripe virus (RSV), is one of the major viral diseases in East Asia. The majority of *japonica* cultivars, e.g. Nipponbare and Koshihikari, in East Asia are highly susceptible to RSV although a few rice cultivars/lines that show resistance to RSV have been described (Noda *et al.* 1991, Zhang *et al.* 2011). Recently, Wang *et al.* (2014) reported that *STV11*, which confers durable resistance to RSV, encodes a sulfotransferase (OsSOT1) catalysing the conversion of SA into sulphonated SA (SSA).

In this chapter, I report that overexpression of *BSR1* not only conferred non-race-specific resistance to *Xoo* and *P. oryzae*, but also extended resistance to *B. glumae* and *C. miyabeanus*. Moreover, overexpression of *BSR1* is likely to confer partial resistance to RSV.

Materials and Methods

Plant materials

Rice wild type cultivar 'Nipponbare', *Xoo*-resistant cultivar 'Asominori', RSV-resistant cultivar 'Sainokagayaki' and two transgenic plant lines (*BSR1*-OX-5 and -9) were grown under greenhouse conditions at 27°C to 30°C. *BSR1*-OX-5 and *BSR1*-OX-9 correspond to the previously described *AK070024*-OX-5 and *AK070024*-OX-9, respectively.

For disease resistance tests, except for *B. glumae*, dehusked seeds were surface sterilized, sown on one-half strength MS medium (Wako Pure Chemicals, Osaka, Japan), containing 3% (w/v) sucrose and 0.4% (w/v) Gelrite (Wako Pure Chemicals), in Agripots and grown in the growth chamber at 28°C in the dark for 3 days, then at 25°C under long-day conditions (16 h light [60–70 μmol m^{-2} s^{-1}]/8 h dark) for 4–7 days. For transgenic

seeds, Hygromycin B (30–50 μg/mL; Wako Pure Chemicals) was added to the medium. Wild type seedlings and hygromycin-resistant transgenic seedlings were transplanted into soil (Bonsol No. 2, Sumitomo Kagaku Kougyo, Osaka, Japan) and used for disease resistance tests.

Pathogens and pathogen cultures

The bacterial isolates used in this study were T7147 (MAFF311019, race II) and T7133 (MAFF311020, race III) of *Xoo* and AZ8204 (MAFF301682) of *B. glumae*, and the fungal isolates were Hoku1 (MAFF101512, race 007.0) of *P. oryzae* and H11-42-1 of *C. miyabeanus*.

Culture procedures for the various pathogens for inoculum were as follows. *Xoo* were cultured on PSA agar plates for 2 days at 28°C under dark conditions. *B. glumae* were cultured on King B agar plate (2% proteose peptone, 0.15% K_2HPO_4, 0.15% $MgSO_4$, 1% glycerin and 1.5% agar, Eiken Chemical, Tochigi, Japan) at 28°C for 2 days under dark conditions. *P. oryzae* was grown on oatmeal agar plates at 25°C in the dark for 10 days, then under continuous illumination for 4 days to induce sporulation. *C. miyabeanus* was grown on V8 agar plates (20% V8 juice (Campbell soup company, Camden, NJ, USA), 0.3% $CaCo_3$ and 1.5% bacto agar) at 25°C in the dark for 5 to 6 days, and then under a 12/12 h light/dark regime for 3 to 4 days to induce sporulation. The cultured pathogens were scraped and used to produce inocula.

Expression analysis of *BSR1* by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from rice leaves using Isogen (Wako Pure Chemicals)

followed by further purification with the RNeasy mini kit (Qiagen, Valencia, CA, USA). First-strand cDNAs were synthesized from equal amounts of total RNA using a ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo, Osaka, Japan) in a total volume of 10 μL, as described by the manufacturer. qRT-PCR was performed with the Thermal Cycler Dice TP800 system (Takara, Tokyo, Japan) using a Kapa SYBR FAST qPCR kit (Kapa Biosystems, Cape Town, South Africa) as described by the manufacturer. The primers used for qPCR were as follows: *BSR1* 5′-AGGTGAGGTTGCACTCTGCT-3′ and 5′-CCAAGAATCCACCAACTCGT-3′; those for *Rubq1* were 5′-GGAGCTGCTGCTGTTCTAGG-3′ and 5′-TTCAGACACCATCAAACCAGA-3′ as an internal control, as described Jiang *et al.* (2010). Transcript levels of *BSR1* were normalized to the endogenous rice reference gene (*Rubq1*). The relative expression level of each gene was calculated using an expression ratio adjusted for gene-specific PCR amplification efficiencies and derived from 2^{-DDCt} (Yuan *et al.* 2006).

Test for resistance to *Xoo*

Rice seedlings transplanted in soil were grown in a growth chamber until the 6–8-leaf stage at 25°C under long-day conditions (16 h light/8 h dark) and then used for evaluation. The suspensions of *Xoo* (isolate T7147 or T7133) for inocula were adjusted to OD_{600} = 0.3 with sterile water. The top leaf blades of the tested plants were cut with scissors pre-wetted with inoculum at about 5 cm from the tip, and the cut ends (about 5 mm from the end) were dipped in inoculum for 10 s. The disease symptoms (lesion length) of inoculated plants were assessed 2 weeks after inoculation, as described in chapter 3.

Test for resistance to *P. oryzae*

Rice seedlings transplanted in soil were grown in a greenhouse until the four-leaf stage at 28°C under a natural photoperiod. The plants were inoculated by spraying a spore suspension of *P. oryzae* (isolate Hoku1 (MAFF101512, race 007.0)). The detailed procedure for producing spore suspension is as follows chapter 3: The mycelia of *P. oryzae* were scraped and the gel surface was flooded with sterile water containing 0.01% Tween 20. The suspension was filtered through a Kimwipe, and the resulted spore suspension was collected. The spore suspension was adjusted to a concentration of 6.7×10^5 spores/mL and used for inoculation. After inoculation, the plants were placed in a dark chamber at 26°C and 100% humidity for 20 h, and then further cultured in the greenhouse. Evaluation of resistance was based on the total number of compatible lesions that appeared on the 3rd and 4th leaf blades of each plant 5 days after inoculation.

Evaluation of bacterial pathogen *B. glumae* resistance

Wild type 'Nipponbare' rice seeds and $T_{3–5}$ seeds of *BSR1*-OX-5 and -9 were sterilized by soaking in 70% ethanol for 30 s and Antiformin (available chlorine 5%) for 20 min. The seeds were then rinsed with sterilized water. The sterilized seeds were soaked in sterilized water at 28°C for 2 to 3 days in the dark and pre-germinated to 1–2 mm of sprout. The pre-germinated seeds were soaked in suspensions of *B. glumae* adjusted to $OD_{520} = 0.004$ and held under a vacuum for 1 min. The inoculated seeds were dried and sown in sterilized soil (Bonsol No. 2, Sumitomo Kagaku Kougyo). Then, the inoculated seeds were incubated in a growth chamber at 28°C with 100% humidity under a 14-h photoperiod. Plant phenotypes were classified as 'healthy' or 'diseased' at 7–10 days after inoculation and the percentage of healthy plants among the total seeds used for

inoculation was calculated as the survival ratio.

Evaluation of fungal pathogen *C. miyabeanus* resistance

Rice seedlings transplanted in soil were grown in a greenhouse until the six-leaf stage at 28°C under a natural photoperiod. The inoculation method of *C. miyabeanus* was the same as that of *P. oryzae*, except as follows. The concentration of the spore suspension was adjusted to 10^4 spores/mL. Evaluation of resistance was based on the total number of compatible lesions that appeared on the 5th and 6th leaf blades of each plant 5 days after inoculation.

Assessment of resistance to RSV

BSR1-OX seedlings selected by Hygromycin resistance (30–50 μg/mL), wild type and cv. 'Sainokagayaki' seedlings were transplanted into soil at about 10 seedlings per pot, and grown in a greenhouse until the two-leaf stage at 28°C under a natural photoperiod. The rice seedlings were exposed to approximately 10 viruliferous small brown planthoppers (*Laodelphax striatellus* Fallén) per plant in an inoculation cage for 1 day to inoculate RSV, as described previously (Satoh *et al.* 2010). After the inoculation period, the insects were killed with insecticide and the seedlings were transferred to the same greenhouse. The virus infection was evaluated by enzyme-linked immunosorbent assay (ELISA) using antiserum against RSV nucleocapsid protein, as described previously (Shimizu *et al.* 2011). The pieces (about 1 cm) of leaf sheath plus stem tissue from inoculated seedlings were harvested for ELISA at 16 days after inoculation. Resistance to RSV was calculated by the ratio of diseased seedlings detected by ELISA among all inoculated seedlings.

Results

Transcript level of *BSR1* in *BSR1*-OX rice lines

The cDNA of *BSR1* was inserted downstream of the constitutive maize *ubiquitin* promoter (Fig. 10A), and the construct was used to generate transgenic rice lines overexpressing *BSR1*. The resulting two transgenic lines, *BSR1*-OX-5 (former name, *AK070024*-OX-5) and -9, were used for various disease resistance tests. To gain sufficient seeds for the disease resistance tests, the two transgenic lines were subjected to acceleration of advanced generations. To confirm overexpression of *BSR1*, I examined the transcript level of *BSR1* by qRT-PCR in $T_{3–4}$ generations of the *BSR1*-OX lines (Fig. 10B). Transcript levels of *BSR1*-OX-5 and -9 lines were 159- and 130-fold higher than that of 'Nipponbare' (wild type), respectively. Thereafter, I used the plants of $T_{3–5}$ lines for various disease resistance tests.

Overexpression of *BSR1* confers resistance to multiple races of *Xoo* and *P. oryzae* in rice

I have reported that *BSR1*-OX rice shows strong resistance to isolate T7174 (race I) of *Xoo*, a bacterial pathogen for rice bacterial leaf blight, and to isolate Kyu89-246 (race 003.0) of *P. oryzae*, a fungal pathogen for rice blast as described in chapter 3. Hence, it would be plausible that *BSR1* also confers resistance to other races of *Xoo* and *P. oryzae*. First, I examined whether *BSR1-OX* rice extended resistance to isolates T7147 (race II) and T7133 (race III) of *Xoo*. The wild type and the resistant control, cv. 'Asominori', inoculated with isolate T7147 (race II) developed extended lesions from the cut (inoculated) end of the leaves, whereas *BSR1*-OX-5 and -9 plants showed restricted lesions (Fig. 11A). Lesion lengths in the inoculated *BSR1*-OX-5 and -9 were

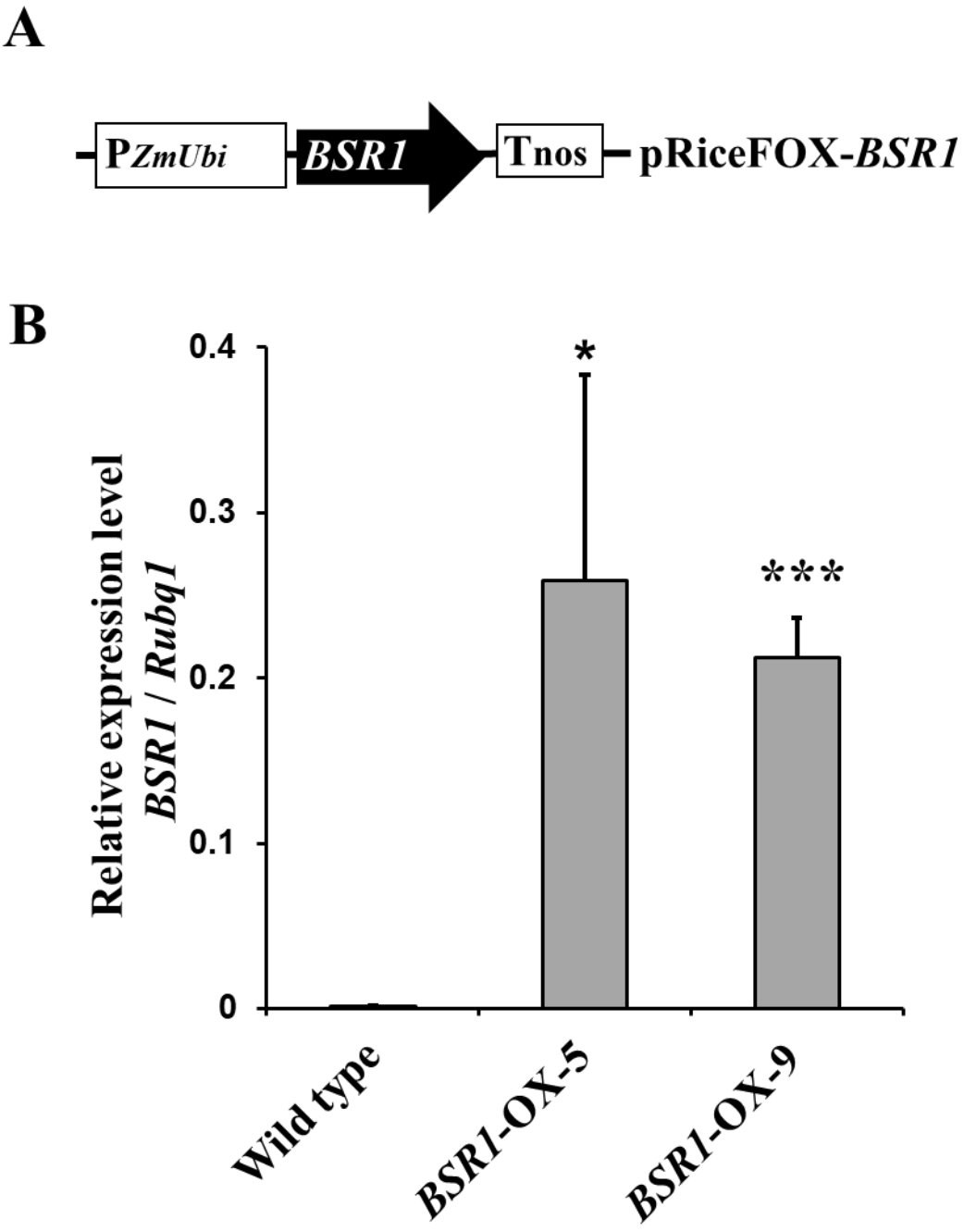


Figure 10. Schematic representation of pRiceFOX-*BSR1* and the transcript level of *BSR1* in *BSR1*-OX rice.

(A) The pRiceFOX-*BSR1* construct for overexpression of *BSR1*. (B) Transcript levels of *BSR1* in $T_{3\text{-}4}$ generations of *BSR1*-OX lines. Second youngest leaf blades of *BSR1*-OX and wild type plants at the eight-leaf stage were used for measurement. Transcript levels of *BSR1* were normalized to that of an endogenous *Rubq1* housekeeping gene (Jiang *et al.* 2010). Values are means ± SD (n=4). Transcript level of *BSR1*-OX-5 and *BSR1*-OX-9 plants were significantly higher than those of wild type (* $P<.05$ and *** $P<0.001$ by t-test).

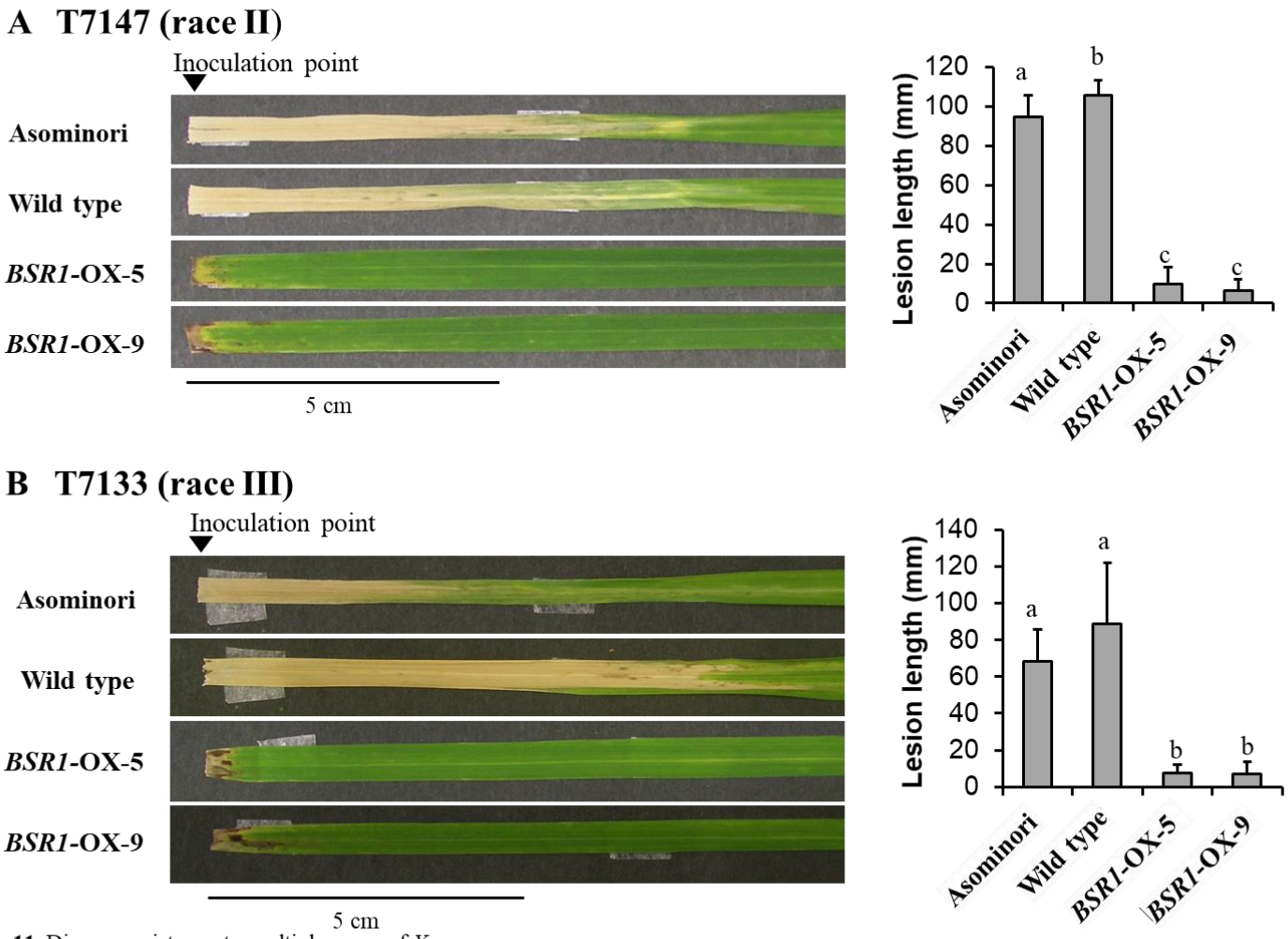


Figure 11. Disease resistance to multiple races of *X. oryzae* pv. *oryzae*.
Isolates T7147 (race II; A) and T7133 (race III; B) of *Xoo* were used for infection. Arrowheads indicate the point of inoculation. Lesion lengths in *BSR1*-OX plants were significantly shorter than those in wild type and 'Asominori', the resistance control cultivar. Values are means ± SD, n=6–18. Different letters indicate significant differences ($P<0.05$ by Tukey-Kramer's test).

about 10 mm and 7 mm long, whereas those in wild type and ‘Asominori’ were about 106 mm and 95 mm long (Fig. 11A). Two *BSR1*-OX lines showed 10- and 15-fold reductions in lesion length compared with the wild type. Similarly, the results of inoculation of isolate T7133 (race III) are shown in Fig. 11B. The two *BSR1*-OX lines showed 10- and 12-fold reductions in lesion length compared with the wild type. These results indicated that overexpression of *BSR1* confers strong resistance to both T7147 (race II) and T7133 (race III), as well as to previously shown T7174 (race I, Fig. 7). The results suggested that overexpression of *BSR1* conferred non-race-specific resistance to *Xoo*. Although ‘Asominori’ has very strong resistance to the isolate T7174 (race I), the resistances to T7147 (race II) and T7133 (race III) were moderate (Kaku and Kimura 1989). Resistance levels of ‘Asominori’ shown here were similar to the report and ‘Asominori’ showed more race-specific resistance.

Next, to examine whether overexpression of *BSR1* also confers resistance to another race of *P. oryzae* in rice, isolate Hoku1 (MAFF101512, race 007.0) was used for inoculation. Lesion numbers in the *BSR1*-OX-5 and -9 lines were significantly smaller than those in the wild type and the resistant control, cv. Sensho plants (Fig. 12). Thus, because *BSR1*-OX lines conferred strong resistance to isolate Hoku1 (race 007.0) in addition to the previously shown isolate Kyu89-246 (race 003.0, Fig. 8), I hypothesized that overexpression of *BSR1* conferred non-race-specific resistance to *P. oryzae*.

Extended resistance to another bacterial pathogen, *Burkholderia glumae*

Bacterial seedling rot and bacterial grain rot (bacterial panicle blight) are caused by the bacterial pathogen *B. glumae*. The latter is an increasingly important disease problem in global rice production (Ham *et al.* 2011). Many genetic studies for resistance

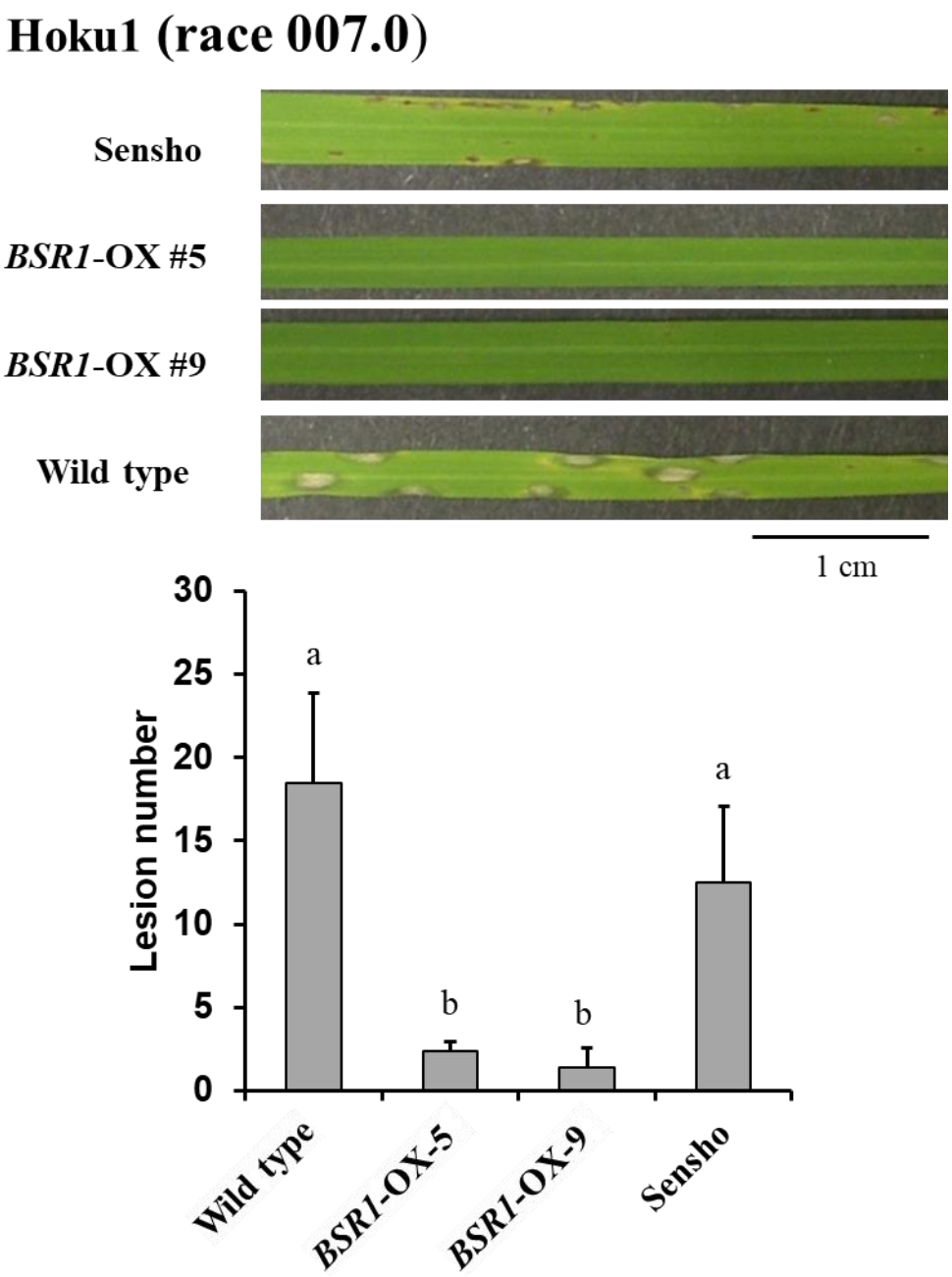


Figure 12. Disease resistance to another race of *P. oryzae*.
Isolate Hoku1 (race 007.0) of *P. oryzae* was used. Lesion numbers in *BSR1*-OX plants were significantly lower than those in wild type and Sensho plants. Different letters indicate significant differences ($P<0.05$ by Tukey-Kramer's test). Values are means ± SD, n=3–7.

to bacterial grain rot have been reported (Mizobuchi *et al.* 2013a, 2015, Pinson *et al.* 2010, Sayler *et al.* 2006, Wasano and Okuda 1994). However, there are few reports on resistance to bacterial seedling rot, because such resistance is a complex characteristic influenced by environmental factors (Iwai *et al.* 2002, Mizobuchi *et al.* 2013b). I was interested in whether overexpression of *BSR1* conferred resistance to bacterial seedling rot in rice. In the test for resistance to bacterial seedling rot, nongerminated seeds are usually used for inoculation by soaking. However, it was difficult to evaluate the resistance by this method because *BSR1*-OX rice displayed a decreased germination rate. Therefore, I gathered only pre-germinated seeds for use in the disease resistance test. To determine the condition for inoculation of pre-germinated seeds, I performed a preliminary experiment using various concentrations of *B. glumae* suspension and wild type seeds. The disease symptoms were classified as shown in Fig. 13A. Browning of the leaf sheath was usually detected together with a dwarf phenotype in diseased plants. Disease resistance was evaluated by the survival ratio, indicating the ratio of healthy plants to total seeds used for infection and shown as a percentage. When non-germinated (NG) seeds were inoculated by soaking in suspensions of three different concentrations of *B. glumae*, no healthy plant survived in all concentrations (Fig. 13B). In contrast, when pre-germinated seeds were used, 98%, 43% and 2% of healthy plants survived after soaking in suspensions of low (OD_{520} = 0.0004), medium (OD_{520} = 0.004) and high (OD_{520} = 0.04) concentrations of the bacteria, respectively. Thus, the optimal concentration range to evaluate resistance in this experiment was OD_{520} = 0.004–0.04. In subsequent experiments, I evaluated disease resistance by this method using pre-germinated seeds.

Resistance to *B. glumae* was evaluated for *BSR1*-OX pre-germinated seeds.

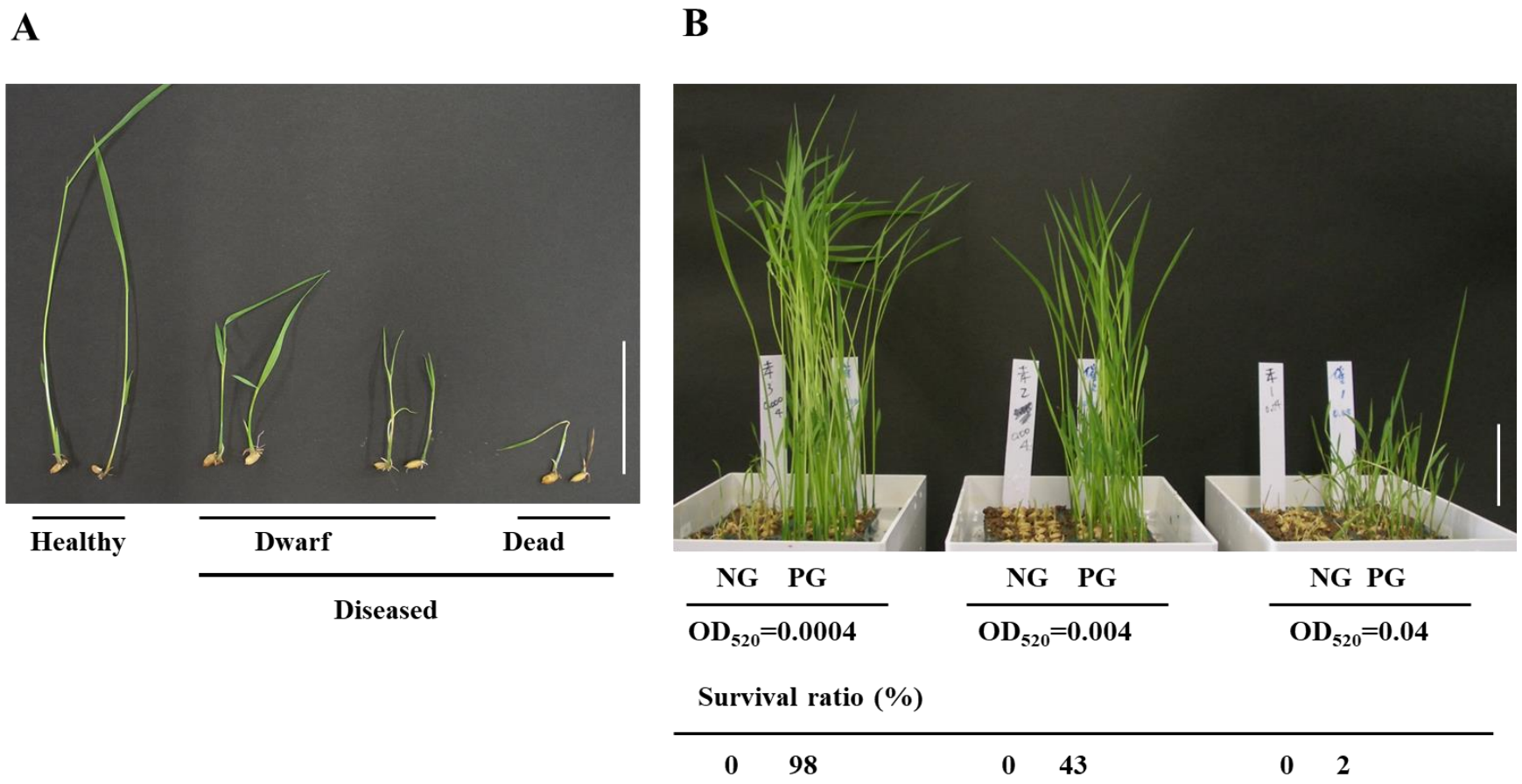


Figure 13. Preliminary experiments to determine the optimal concentration of bacterial pathogen, *Burkholderia glumae*, for inoculation.
(A) The classification of disease symptoms at 7 days after inoculation. Healthy, normal phenotype; Diseased, dwarf or dead phenotype. Bar=50mm. (B) Results of the preliminary experiment. Photograph shows plants at 7 days after inoculation (n=50–60). Non-germinated seeds (NG) and pre-germinated seeds (PG) were used for inoculation. Bar=50mm. Survival ratio (%) = (number of healthy plants / number of total seeds) × 100

Survival ratios in *BSR1*-OX-5 and -9 lines were two times higher than that in the wild type (Fig. 14). These results indicated that *BSR1*-OX lines displayed resistance to *B. glumae* in rice.

Extended resistance to another major fungal pathogen, *Cochliobolus miyabeanus*

Brown spot disease is caused by the fungal pathogen, *C. miyabeanus*. I hypothesized that *BSR1* conferred resistance to brown spot disease as well as to rice blast in rice. First, I investigated whether the expression of *BSR1* changed by inoculation with *C. miyabeanus* in wild type plants (Fig. 15A). *C. miyabeanus* was spray-inoculated onto wild type plants, and the transcript level of *BSR1* was measured by qRT-PCR. As a result, inoculated plants showed inducible expression of *BSR1* compared with mock control after inoculation (Fig. 15A), although the transcript levels of *BSR1* in the inoculated plants were much lower than those in *BRS1*-OX lines. This result suggested that *BSR1* is involved in innate immunity against brown spot in rice. Hence, I examined whether overexpression of *BSR1* conferred resistance to *C. miyabeanus* (Fig. 15B). Lesion numbers in *BSR1*-OX-5 and -9 plants were significantly lower than those in the wild type plants (***$P < 0.001$ by t-test, Fig. 15B). Thus, overexpression of *BSR1* conferred significant resistance to *C. miyabeanus*.

***BSR1*-OX rice were slightly resistant to rice stripe virus (RSV)**

I examined whether overexpression of *BSR1* could confer resistance against a viral pathogen, RSV, in rice, because the majority of *japonica* cultivars, including cv. 'Nipponbare', are susceptible to RSV. The results are shown in Fig. 16. After inoculation of RSV, the percentages of diseased seedlings detected by ELISA in *BSR1*-OX-5 and -9

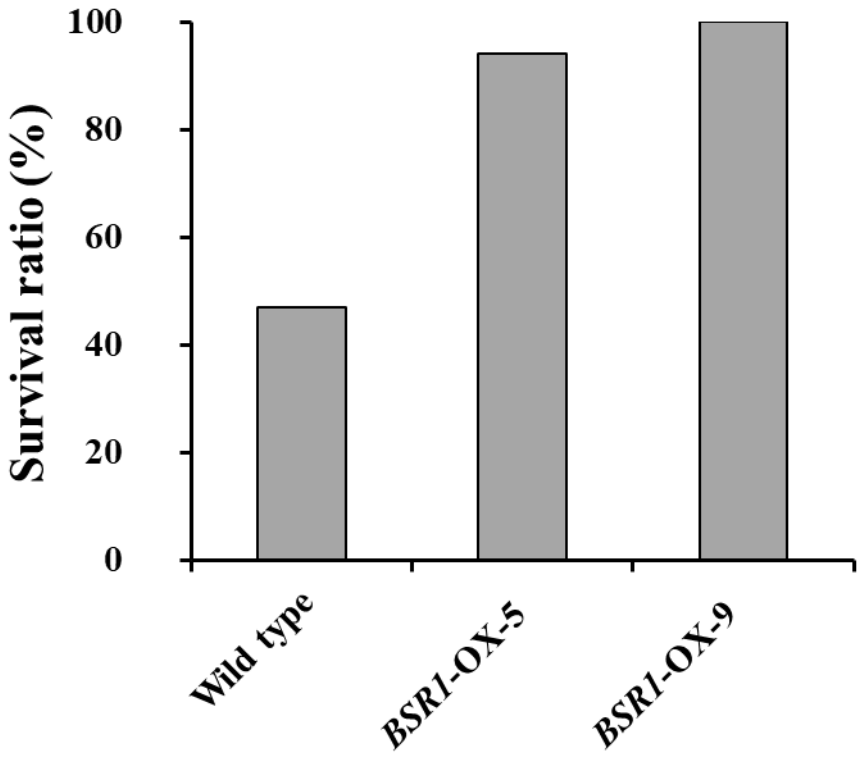


Figure 14. Disease resistance to another bacterial pathogenic species, *Burkholderia glumae*.

Pre-germinated seeds of *BSR1*-OX and wild type were inoculated with *B. glumae*. Concentration of inoculation was OD_{520}=0.004. Survival ratio was calculated 8 days after inoculation (n=17). Tests were performed three times with similar results.

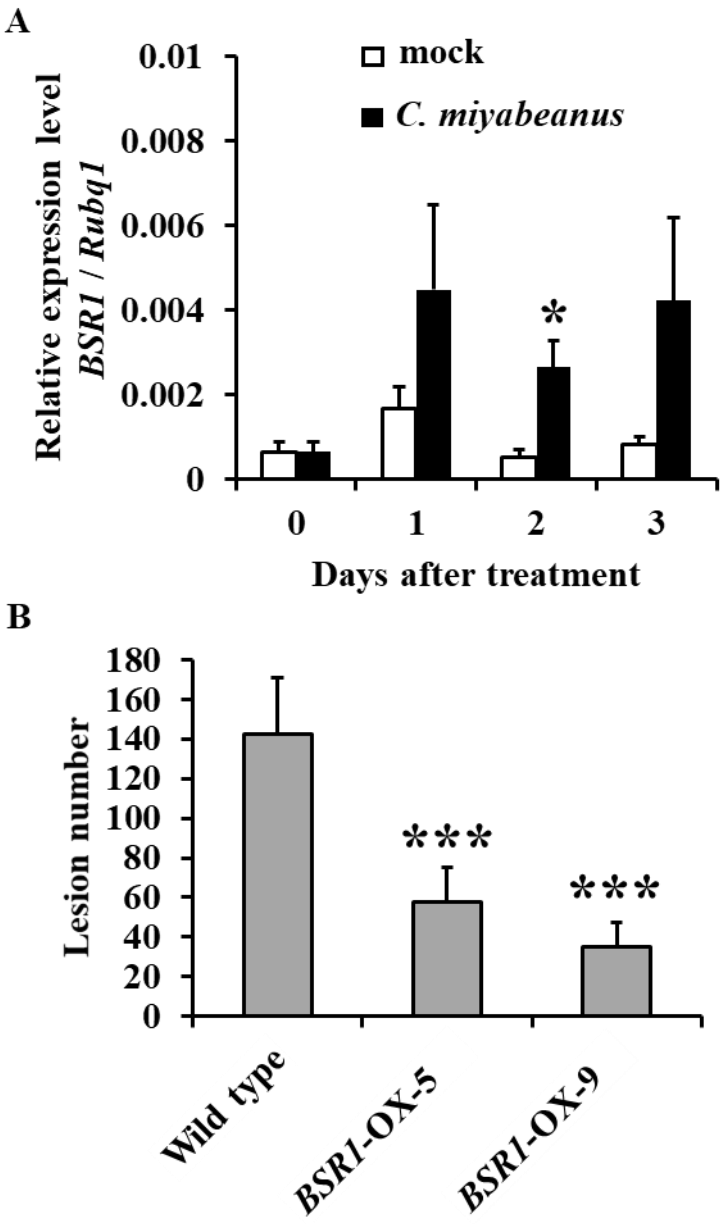


Figure 15. Disease resistance to another fungal pathogenic species, *Cochliobolus miyabeanus*.

(A) Relative expression levels of *BSR1* in wild type plants on inoculation with mock or *C. miyabeamus.* Seventh-leaf blades were used for inoculation. Total RNAs at 0 to 3 days after inoculation were extracted. Transcript levels of *BSR1* were normalized to that of *Rubiq1*. Expression levels of *BSR1* in inoculated leaves were up-regulated compared with mock at 1 to 3 days. Values are means ±SD, n=3. An asterisk indicates a statistically significant difference from the mock at 2 days ($P<0.05$ by t-test).

(B) Resistance to *C. miyabeamus* in *BSR1*-OX rice. Lesion numbers in *BSR1*-OX plants were significantly lower than those in wild type plants (***$P<0.001$ by t-test). Values are means ±SD, n=4–12.

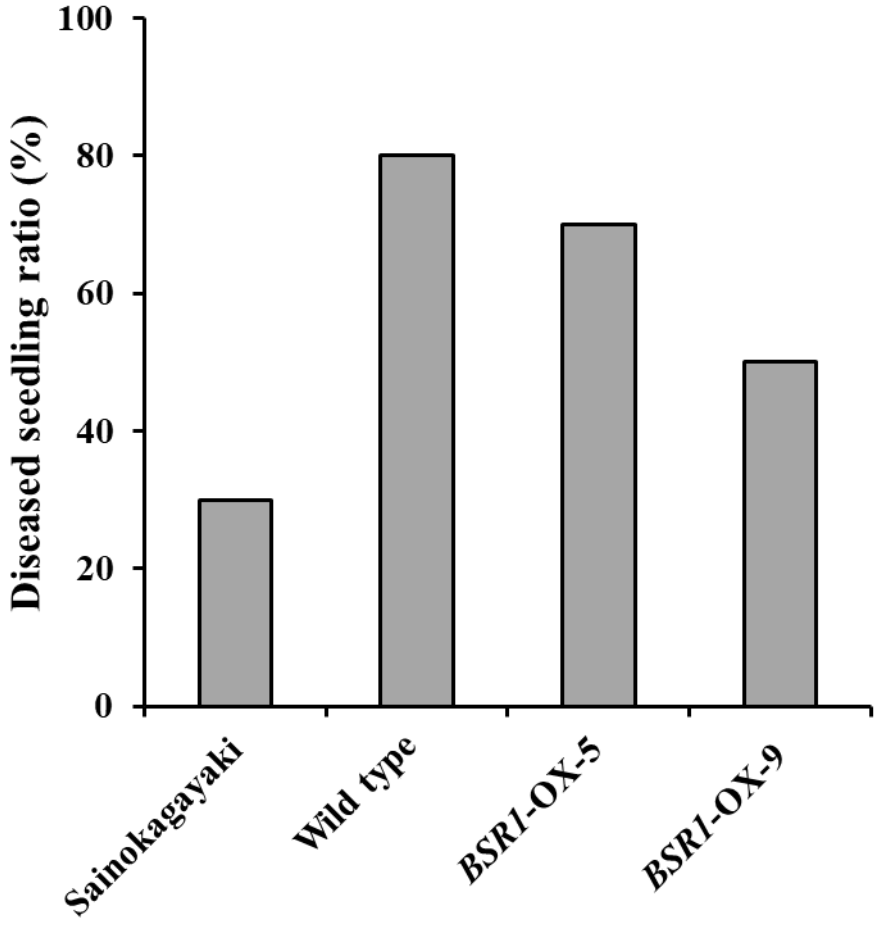


Figure 16. Disease resistance to a viral pathogen, rice stripe virus.
Diseased seedling ratio, percentage of diseased seedlings detected by enzyme-linked immunosorbent assay (ELISA) in all inoculated seedlings at 16 days after inoculation (n=8 to 10). Tests were performed three times with similar results.

seedlings were slightly lower than those in wild type seedlings, but were higher than that in cv. ‘Sainokagayaki’, the RSVresistant control cultivar (Fig. 16). Thus, overexpression of *BSR1* could confer slight resistance to RSV, although its resistance level was weaker than that of ‘Sainokagayaki’, which possesses the highly resistant *Stvb-i* gene to RSV (Shimizu *et al.* 2011).

Discussion

Broad-spectrum disease resistance in rice

From the point of view of breeding, the quality of broadspectrum resistance against two or more different pathogen species is an agronomically important trait. In this study, I first showed that *BSR1* confers resistance to multiple races of *Xoo* and *P. oryzae* in overexpressing rice. In addition, *BSR1*-OX rice showed extended resistance to another bacterial pathogen, *B. glumae*, and another fungal pathogen, *C. miyabeanus*. Thus, overexpression of *BSR1* conferred broad-spectrum disease resistance to at least two bacterial and two fungal pathogenic species in rice. Therefore, it would be natural to consider that the resistance to bacterial and fungal pathogens by *BSR1* is non-race specific. Furthermore, *BSR1*-OX rice showed slight resistance to RSV. To the best of my knowledge, there are no other genes that confer such multi-disease resistance.

Currently, several genes have been reported to confer broad-spectrum disease resistance in rice. For example, overexpression of *OsWRKY13* or *OsWRKY45* enhanced resistance to *Xoo* and *P. oryzae* in rice by mediating SA signaling (Qiu *et al.* 2007, Shimono *et al.* 2007, 2012, Tao *et al.* 2009). Unlike *BSR1*-OX rice, however, there are few reports that overexpressed genes enhanced resistance to necrotrophic pathogens, *B. glumae* and *C. miyabeanus* in rice. Meanwhile, overexpression of *OsWRKY30* or *OsACS2*

encoding a key enzyme of ethylene (ET) biosynthesis enhanced resistance to fungal pathogens *P. oryzae* and necrotrophic *Rhizoctonia solani* in rice. The resistance conferred by *OsWRKY30* was associated with the activation of jasmonic acid (JA) synthesis-related genes and the increased accumulation of endogenous JA (Peng *et al.* 2012), and that by *OsACS2* was associated with the increased level of endogenous ET (Helliwell *et al.* 2013). Hence, I speculate that the broad-spectrum disease resistance against two hemibiotrophs (*Xoo* and *P. oryzae*) and two necrotrophs (*B. glumae* and *C. miyabeanus*) in *BSR1*-OX rice is based on an SA and JA/ET combined pathway or another pathway.

Non-race-specific resistance to *Xoo* and *P. oryzae i*n *BSR1*-OX rice

Here, *BSR1*-OX rice displayed strong resistance to three tested races (races I to III) of *Xoo*. In contrast, 'Asominori' showed more race-specific resistance. Although the resistance to isolate T7174 (race I) on 'Asominori' was very strong (Fig. 7), the resistances to T7147 (race II) and T7133 (race III) were moderate (Fig. 11), almost similar to the report of Kaku and Kimura (1989). 'Asominori' has been reported to have an *Xa1-as*(t) gene at the *Xa1* locus (Ise *et al.* 1998). *Xa1-as*(t) is implicated in the strong resistance to T7174 (race I). 'Asominori' has also been suggested to have minor-affected loci that are involved in the quantitative resistance to T7133 (race III) (Yoshimura *et al.* 1996). *BSR1-OX* rice showed strong resistance to both T7174 (race I) and T7133 (race III); therefore, the defence mechanism of *BSR1*-OX rice would be different from that of 'Asominori'. From the viewpoint of breeding, overexpression of *BSR1* could confer more useful non-race-specific resistance to *Xoo* in rice compared with using the resistance genes of 'Asominori'.

In the previous chapter, *BSR1*-OX rice displayed strong resistance to race 003.0

(isolate Kyu89-246) and the resistance level was stronger than that in cv. ‘Sensho’ (Fig. 8), which has a strong non-race-specific resistance to *P. oryzae* associated with *pi21* (Fukuoka *et al.* 2009). In this chapter, *BSR1*-OX rice displayed extended strong resistance to race 007.0 (isolate Hoku1) and the resistance level was also stronger than that in cv. ‘Sensho’ (Fig. 12). Hence, non-race-specific resistance or field resistance against *P. oryzae* is also promising in *BSR1*-OX rice. Taken together, overexpression of *BSR1* could confer more promising leaf blight and blast resistances compared with the resistant cultivars ‘Asominori’ and ‘Sensho’, respectively, in many useful *O. sativa* varieties. Furthermore, it is plausible that *BSR1*-OX rice also shows non-race-specific resistance to *B. glumae* and *C. miyabeanus*, and exhibits resistance to other pathogen species, because *BSR1*-OX rice showed resistance to all pathogens tested.

Conclusion

In conclusion, *BSR1*, when overexpressed in rice, conferred broad-spectrum disease resistance against at least four diseases: bacterial leaf blight, blast, bacterial seedling rot and brown spot, and slight resistance against rice stripe disease by RSV. *BSR1* represents a highly valuable and convenient genetic resource because it confers resistance to various diseases by a single gene. In the future, the defence mechanism conferred by *BSR1* will be clarified to use the *BSR1* gene effectively.

Chapter 5

General Discussion

Advantage of rice-FOX *Arabidopsis* lines

I screened for resistance to *Pst* DC3000 in a rice-FOX *Arabidopsis* population of more than 20,000 lines, and selected 72 resistant lines. As previously described chapter 3, one gene, *BSR1*, conferred resistance to bacterial and fungal pathogens in both *Arabidopsis* and rice, with the same disease resistance phenotype. Higuchi-Takeuchi *et al.* (2011) reported that *OsLFNR1* and *OsLFNR2*, two photosynthesis genes, showed the same phenotype in *Arabidopsis* and rice. I expect that other genes isolated in my study will also show the same disease resistance phenotypes in *Arabidopsis* and rice, as the evaluation of *RPD* genes in rice is still in progress. The rice-*Arabidopsis* FOX hunting system, which allows high-throughput screening for rice genes, revealed several genes with the same phenotype in *Arabidopsis* and rice. This result supports the use of the system as a valuable tool for large-scale screening of agronomically useful rice genes.

Eleven cDNAs used to create the rice-FOX *Arabidopsis* lines (one per line) selected by screening for resistance to *Pst* DC3000 and *C. higginsianum* contain many genes associated with signal transduction (Chapter 3, Table 5). These could be unidentified disease resistance genes. One of these genes is *BSR1*. Disease resistance includes basal resistance, true resistance and field resistance. Because genes for true resistance and field resistance confer resistance to specific pathogens of the host plant, the unknown genes found in this study are likely to be involved in common basal resistance. Therefore, this screening has revealed many unidentified disease resistance genes that conventional tools such as loss-of-function and gain-of-function tools haven't

isolated. The broad-spectrum disease resistance in both *Arabidopsis* and rice conferred by *BSR1* shows that it is not necessary to replace rice *BSR1* with a homologous *Arabidopsis* gene to analyse its function in *Arabidopsis* and that it is possible to screen specific rice genes by using rice-FOX *Arabidopsis* lines. The FOX hunting system may thus be applicable for identifying agronomically useful genes of various crops.

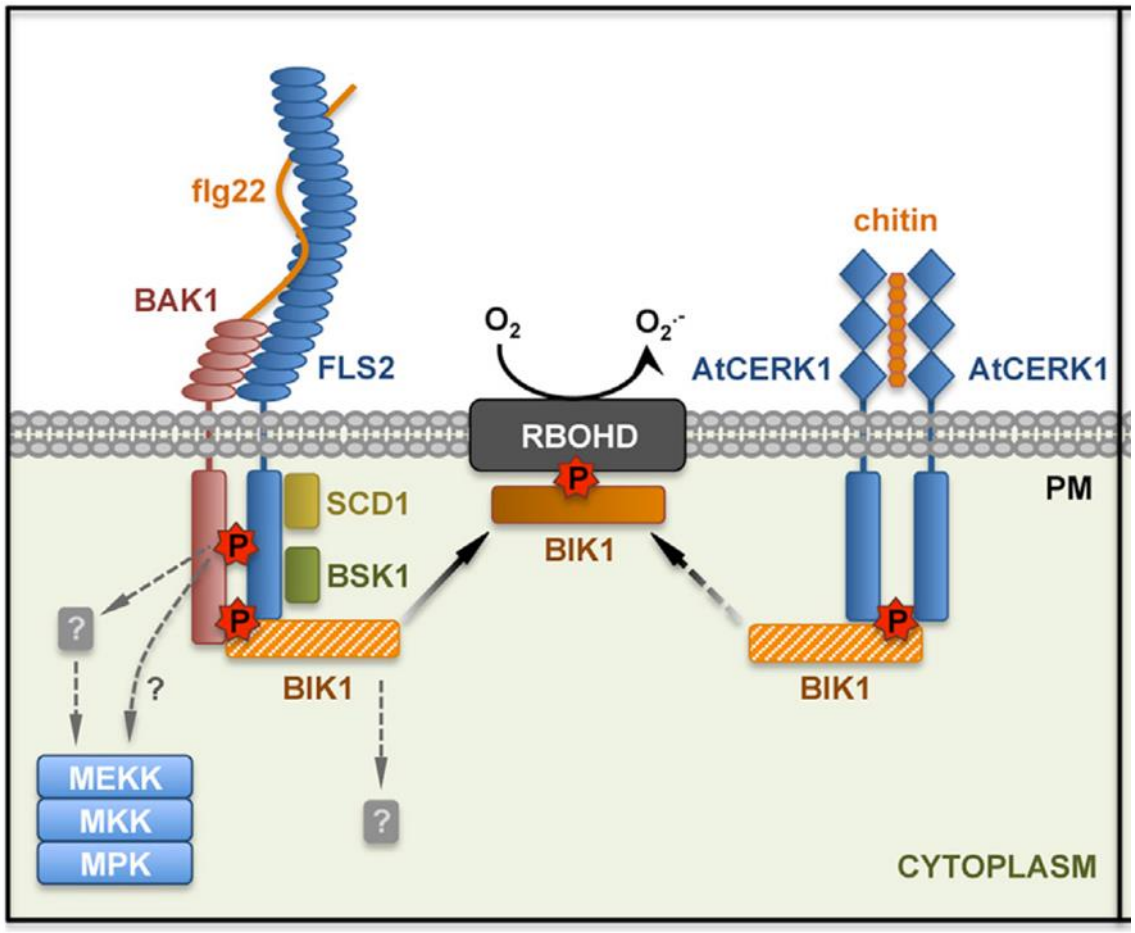
Possible function of *BSR1* in plant defence

As a typical example of interactions between host plants and pathogens, plant defence against pathogens is provided through cell-surface-localized pattern recognition receptors, which detect PAMPs and promote pattern-triggered immunity (PTI) (Boller and Felix 2009). To counteract this innate immunity, pathogens deliver effector proteins into the host cell that suppress PTI (Jones and Dangl 2006). In some cases, plants use intracellular resistance (R) proteins to detect race-specific effectors, which results in effector-triggered immunity, often associated with hypersensitive response and programmed cell death.

BSR1 encodes a putative RLCK, OsRLCK278, and is one of 379 RLCK genes in rice (Chapter 3). BSR1 is classified into the RLCK-VII protein family, along with PBS1, PTO and BIK1, well characterized RLCKs involved in plant defence (Chapter 3, Fig. 9). Tomato PTO and *Arabidopsis* PBS1 are involved in race-specific resistance to *P. syringae* (Ade *et al.* 2007, Ntoukakis *et al.* 2009). PTO recognizes a *Pseudomonas* effector protein, AvrPtoB, and inactivates its E3 ligase activity via phosphorylation to induce *P. syringae* resistance (Ntoukakis *et al.* 2009). PBS1 is complexed with a resistance protein, RPS5, and becomes cleaved by AvrPphB, a *P. syringae* effector with protease activity, leading to the activation of RPS5 and induction of programmed cell

death in host cells (Ade *et al.* 2007). In both cases, the RLCKs play direct roles in the recognition of bacterial effectors. *Arabidopsis BIK1* was originally isolated as a gene involved in defence against necrotrophic fungal pathogens (Veronese *et al.* 2006). BIK1 also associates with a flagellin receptor complex, FLS2/BAK1, which is rapidly phosphorylated upon perception of flagellin (Fig. 17). BIK1 is also phosphorylated upon perception of another PAMP, translation elongation factor (EF-Tu). Hence, in response to multiple PAMP-receptor complexes, BIK1 mediates PTI signal transduction, such as production of a reactive oxygen species (ROS) burst, activation of mitogen-activated protein (MAP) kinases and calcium-dependent protein kinases, transcriptional reprogramming and, ultimately immunity (Chinchilla *et al.* 2007, Heese *et al.* 2007, Lu *et al.* 2010, Roux *et al.* 2011, Schulze *et al.* 2010, Schwessinger *et al.* 2011, Sun *et al.* 2013; Fig. 17). Furthermore, BIK1 regulates calcium influx, phosphorylates and activates the NADPH oxidase RBOHD (Fig. 17), and not only induces the defence response (including the ROS burst that directly attacks pathogens), but also prevents invasion of pathogenic bacteria by closing the stomata (Kadota *et al.* 2014, Li *et al.* 2014). Thus, BIK1 plays pivotal roles in the recognition of PAMPs and subsequent signal transduction. Interestingly, it has been shown that PBS1 and PBS1-like kinases also contribute to PTI defences and play some roles in signal integration from multiple surface-localized receptors in plants lacking *RPS5* (Zhang *et al.* 2010). Thus, plant RLCKs play important roles in direct and indirect recognition of pathogen-derived molecules and subsequent signal transduction.

In rice, OsRLCK185, also in the RLCK-VII family, interacts with a pattern recognition receptor, OsCERK1, which recognizes chitin and peptidoglycan at the plasma membrane, and regulates a MAP kinase cascade that leads to a PTI defence response



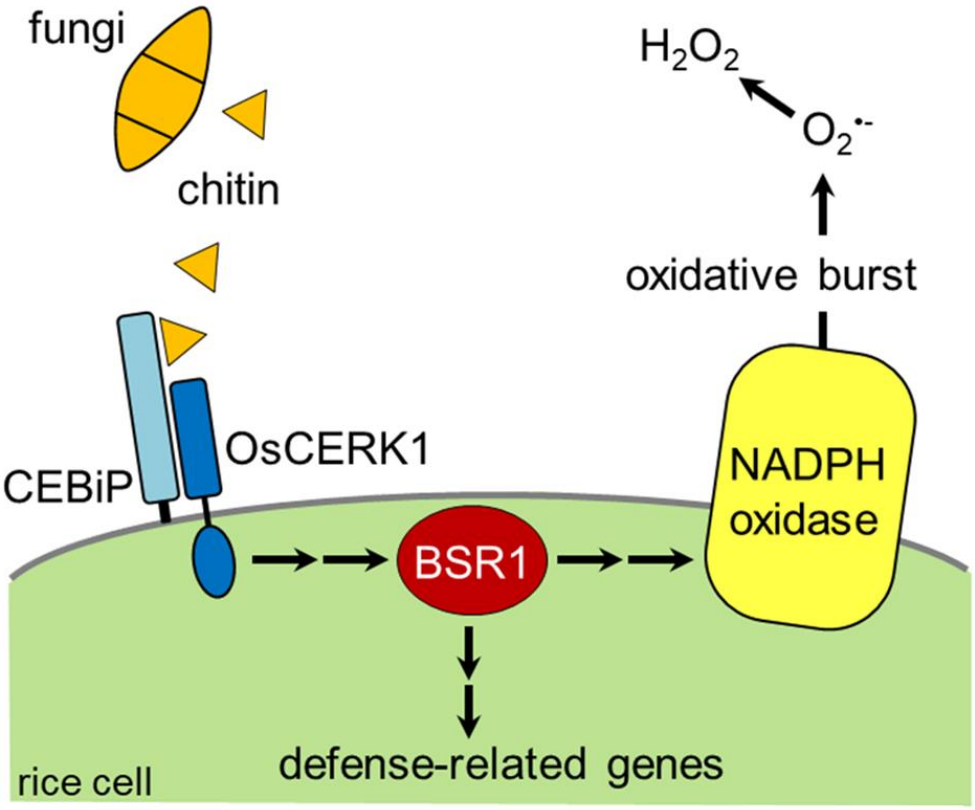
Macho and Zipfel 2014

Figure 17. Phosphorylation of BIK1 by MAMPs initiates plant innate immunity in *Arabidopsis*.

(Yamaguchi *et al.* 2013). Our research group recently reported that BSR1 is important in rice innate immunity triggered by chitin, which functions as a PAMP of the rice blast fungus and is involved in the pathogen-responsive ROS burst and the expression of defence-related genes (Kanda *et al.* 2017, Fig. 18). However, it has not been clear whether BSR1 interacts directly with OsCERK1 or NADPH oxidase. Taken together from chapter 4, it is likely that BSR1 interacts with various transmembrane receptors that recognize PAMPs of *Xoo*, *P. oryzae*, *B. glumae* or *C. miyabeanus*, and links multiple PAMP receptor complexes to downstream intracellular signalling, enhancing PAMP-mediated defence (Fig. 19). Further, it may be possible that BSR1 directly phosphorylates and activates NADPH oxidase, leading to the ROS burst (Fig. 19). Elucidation of the defensive role of BSR1 is a task for future study. Since much of how pathogens interact with rice remains unclear, BSR1 will prove useful for clarifying the mechanisms.

Relationship between typical defence hormones and feeding strategies of pathogens used in this study

The pathogens used in this study are classified as either hemibiotrophs or necrotrophs. The blast fungus, *P. oryzae*, and the bacterial leaf blight pathogen, *Xoo*, are considered hemibiotrophs (Van Bockhaven *et al.* 2013). Hemibiotrophs are characterized by an initial infectious period (the biotrophic stage), in which the pathogen grows within host cells before switching to the necrotrophic growth stage, when lesions become apparent (Wilson and Talbot 2009). *Xoo* has been considered mostly a biotroph but is probably best classified as a hemibiotroph (De Vleesschauwer *et al.* 2013). In contrast, the pathogens of bacterial seedling rot, *B. glumae*, and of brown spot, *C. miyabeanus*, are classified as necrotrophs (Iwai *et al.* 2002, Su'udi *et al.* 2012). Necrotrophs kill the host



Kanda *et al.* 2017

Figure 18. The role of BSR1 in response to the perception of chitin elicitor.

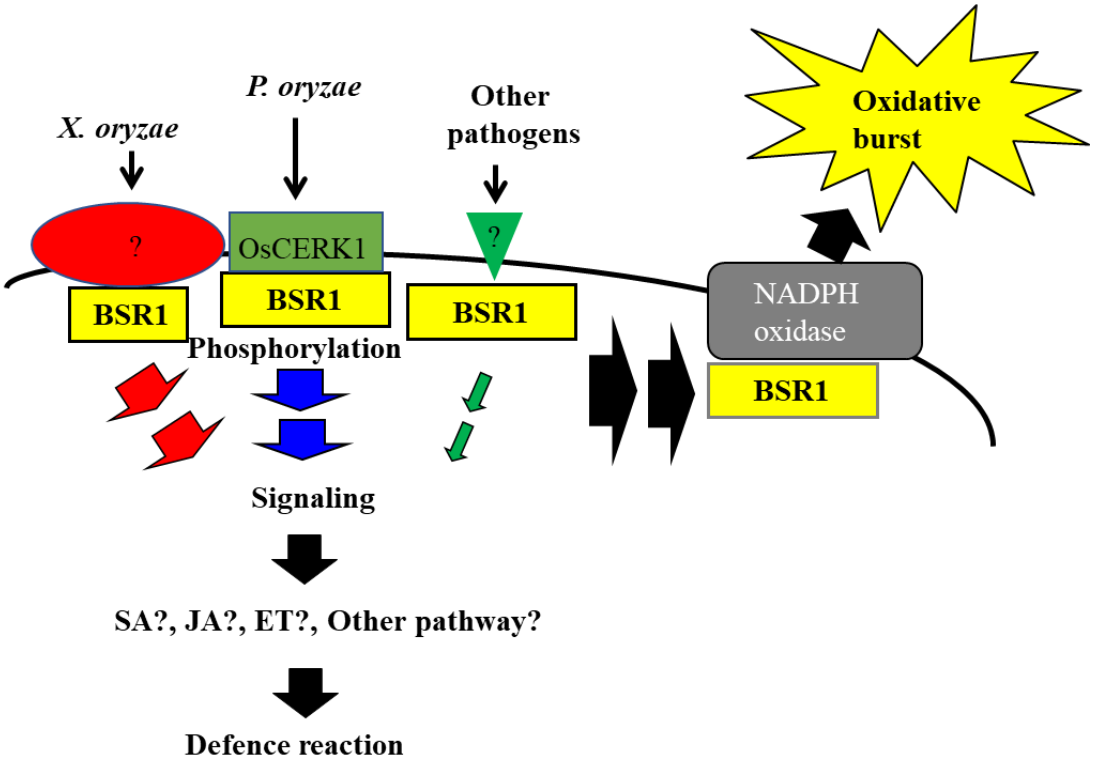


Figure 19. Possible function of BSR1 in rice.

cell and feed on the contents. My results show that *BSR1*-OX rice was resistant not only to hemibiotrophs, but also to necrotrophs. Since necrotrophs kill host cells, the host cells must present a resistance reaction while still alive. Because BSR1 is involved in PAMP-mediated defence (Fig. 19), I assume that the resistance reaction occurs in the early stage of infection by these pathogens.

Signal transduction plays an important role in plant–microbe interactions, whereby molecular signals from pathogens are perceived by specific receptors in the host, leading to a series of signalling cascades and defence responses, consequently resulting in either host colonization or suppression of pathogenesis. SA, JA and ET are typical defence hormones, and the regulation of their signalling networks in response to pathogen infection is well established, especially in *Arabidopsis* (Van Bockhaven *et al.* 2013). In *Arabidopsis*, SA is a key signalling molecule involved in plant defence against biotrophic pathogens, such as the oomycete *Hyaloperonospora arabidopsidis*, or hemibiotrophic pathogens, such as *P. syringae* (Tsuda and Katagiri 2010). By synthesizing SA, the accumulation of a set of *pathogenesis-related* (*PR*) genes are induced in plants. JA and ET mediate the defence signalling that is generally effective against necrotrophic pathogens such as *Alternaria brassicicola*. The SA and JA signalling pathways generally act antagonistically (Thomma *et al.*, 1998, Glazebrook 2005). Recently, however, it has been reported that each of the SA, JA and ET signalling sectors can contribute positively to immunity against both biotrophic and necrotrophic pathogens (Tsuda *et al.* 2009). In addition, ROS, such as H_2O_2, play a major role in cellular signalling pathways and in the regulation of gene expression networks in plants (Li *et al.* 2009). The production of ROS is one of the earliest cellular responses following either pathogen infection or elicitor treatment, and leads to strengthening of the host cell walls via cross-linking of

glycoproteins and activation of *PR* genes (Liu *et al.* 2010). The defence system in rice is considered to be controlled by a more complicated signalling network than that in *Arabidopsis* (De Vleesschauwer *et al.* 2013). SA-, JA- and ET-dependent defences all enhanced resistance to hemibiotrophic *P. oryzae* (Ahn *et al.* 2005, Helliwell *et al.* 2013, Iwai *et al.* 2006, Mei *et al.* 2006, Peng *et al.* 2012, Qiu *et al.* 2007, Schweizer *et al.* 1998, Shimono *et al.* 2007, 2012). SA- and JA-dependent defences were involved in disease resistance against hemibiotrophic *Xoo* (Babu *et al.* 2003, Qiu *et al.* 2007, Shimono *et al.* 2012, Tanaka *et al.* 2014, Taniguchi *et al.* 2014), whereas ET-dependent defence played a negative role (Shen *et al.* 2011). Hence, ET has opposite roles between *Xoo* and *P. oryzae*. ET-dependent defence is also involved negatively in resistance to *C. miyabeanus*, a necrotrophic pathogen (De Vleesschauwer *et al.* 2010), but no reports have implicated SA and JA in defence against *C. miyabeanus* (Van Bockhaven *et al.* 2013). More interestingly, abscisic acid, which antagonizes the SA pathway, is involved in resistance to *C. miyabeanus* (De Vleesschauwer *et al.* 2010), although it promotes susceptibility to *Xoo* and *P. oryzae* (Jiang *et al.* 2010, Xu *et al.* 2013). The network of plant hormone defence against *B. glumae* is unknown in rice. RSV resistance is implicated in enhancing the SA signal (Wang *et al.* 2014). These facts indicate the complexity of the mechanism of broad-spectrum resistance conferred by *BSR1* in relation to plant hormones (Fig. 19). It will be important to reveal the components of intracellular signalling of PAMP-mediated defence in the disease resistance mechanism of BSR1 (Fig. 19).

Potential uses of *BSR1*

My high-throughput analysis of rice genes heterologously expressed in *Arabidopsis* showed that *BSR1* confers resistance to bacterial and fungal pathogens in

both *Arabidopsis* and rice. This method can find genes that conventional methods can't. To my knowledge, no other monocot gene has been reported to confer disease resistance in both a monocot and a dicot to both bacterial and fungal pathogens. Although the activation of plant defence generally comes at the cost of plant growth (Hout *et al.* 2014), I have not observed any notable growth retardation or differences in plant type or fertility in plants of either species overexpressing *BSR1* (data not shown). These results indicate that heterologous rice genes can be expressed and function in different plant species. I propose that *BSR1* should be introduced into other monocot crops, such as wheat and sugarcane, and dicot crops, such as tomato, soybean and ornamentals, where it may confer disease resistance.

BSR1 overexpression (*BSR1*-OX) decreased the germination rate of rice seeds to <80%, and partly coloured husked seeds brownish (data not shown). With these exceptions, *BSR1*-OX rice has similar agriculturally important traits as the wild type. If such undesirable phenotypes can be removed, *BSR1*-OX rice will be applicable for practical use. The *WRKY45* transcription factor overexpressed under the control of the maize *ubiquitin* promoter confers strong resistance to *P. oryzae* and *Xoo* in rice (Shimono *et al.* 2007, 2012), but reduces growth and yield. In contrast, *WRKY45* driven by the moderately expressed *OsUbi7* promoter (Goto *et al.* 2015) or the pathogen-inducible *PR1b* promoter (Goto *et al.* 2016) improved growth and yield to the same level as in the wild type while maintaining resistance. Therefore, I propose changing the driven promoter of *BSR1* to minimize undesirable phenotypes. Since the maize *ubiquitin* promoter is very strong in rice, it may enhance undesirable effects. Moderately expressed or pathogen-inducible promoters may minimize such effects, making *BSR1*-OX applicable for use in rice.

General conclusion

Overexpression of *BSR1*, encoding a receptor-like cytoplasmic kinase, confers remarkable resistance to both bacterial and fungal pathogens in *Arabidopsis*, and to the bacterial pathogen *Xoo* and the fungal pathogen *P. oryzae* in rice (Chapter 3). Further, *BSR1*-OX rice showed resistance to two other races of *Xoo* and to at least one other race of *P. oryzae*, in addition to *B. glumae*, which causes bacterial seedling rot and bacterial grain rot, and to *C. miyabeanus*, a fungus that causes brown spot (Chapter 4). *BSR1* is a potentially very useful gene that can confer broad-spectrum disease resistance in both dicots and monocots.

This study demonstrates the utility of rice-FOX *Arabidopsis* lines in finding novel genes that conventional loss-of-function and gain-of-function tools cannot find. The results suggest the applicability of genes isolated by a FOX hunting system using dicot and monocot plants to other dicot and monocot crops. The FOX hunting system could also be used to identify agronomically useful genes in crops which do not have genome information or pose difficulties in crossing (e.g., in sugarcane, trees). Finally, I expect *BSR1* to be used as a genetic resource that can confer broad-spectrum disease resistance.

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Summary

Broad-spectrum disease resistance against several types of pathogen species is desirable for crop improvement. However, to my knowledge, such disease resistance genes have not been isolated until now in rice.

In recent years, a Full-length cDNA OvereXpressor gene hunting system (FOX hunting system) has been developed. By using the system, genes having desired functions can be efficiently searched. In addition, a rice-FOX *Arabidopsis* system has been developed by inserting approximately 13,000 rice full-length cDNAs downstream of the CaMV *35S* promoter and introducing into *Arabidopsis* (ecotype Columbia) via *Agrobacterium*. By using this system, high-speed and large-scale screening can be possible because of the small size and short lifespan of *Arabidopsis*, which is especially useful for the screening of disease resistance genes that involve complicated pathogen infection mechanisms. In this study, I performed screening for broad-spectrum disease resistance rice genes by using this system, isolated a prominent gene and evaluated the resistance in rice.

First, approximately 20,000 of the rice-FOX *Arabidopsis* transgenic lines were screened for bacterial disease resistance by dip inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). The identities of the overexpressed genes were determined in 72 lines that showed consistent resistance after three independent screens. *Pst* DC3000 resistance was verified for 19 genes by characterizing other independent *Arabidopsis* lines for the same genes in the original rice-FOX hunting population or obtained by reintroducing the genes into ecotype Columbia by floral dip transformation.

Next, these 72 selection lines were screened for fungal pathogen *Colletotrichum higginsianum* to isolate broad-spectrum disease resistance genes. Thirteen lines of these

72 selections were also resistant to *C. higginsianum*. Eight genes that conferred resistance to *Pst* DC3000 in *Arabidopsis* have been introduced into rice for overexpression, and transformants were evaluated for resistance to the rice bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). One of the transgenic rice lines was highly resistant to *Xoo*. Interestingly, this line also showed remarkably high resistance to *Pyricularia oryzae*, the fungal pathogen causing rice blast, which is the most devastating rice disease in many countries. The causal rice gene, encoding a putative receptor-like cytoplasmic kinase, was therefore designated as *BROAD-SPECTRUM RESISTANCE 1* (*BSR1*). BSR1 protein has similarity to *Arabidpsis* BIK1 protein. My results demonstrate the utility of the rice-FOX *Arabidopsis* lines as a tool for the identification of genes involved in plant defence and suggest the presence of a defence mechanism common between monocots and dicots.

Then, I further examined disease resistance against other races of *Xoo* and *P. oryzae*, and three other pathogens in *BSR1*-OX rice. As the results, *BSR1*-OX rice showed extended resistance against two other different races of *Xoo*, and to at least one other race of *P. oryzae*. In addition, the rice showed resistance to another bacterial species, *Burkholderia glumae*, which causes bacterial seedling rot and bacterial grain rot, and to *Cochliobolus miyabeanus*, another fungal species causing brown spot. Furthermore, *BSR1*-OX rice showed slight resistance to rice stripe disease, a major viral disease caused by rice stripe virus. Thus, I demonstrated that *BSR1*-OX rice shows remarkable broad-spectrum resistance to at least two major bacterial species and two major fungal species, and slight resistance to one viral pathogen.

日本語要約

イネにおける耐病性品種を育成する上で、広範な病害抵抗性を有する遺伝子を利用することは極めて有用である。しかし、現在までにそのような複合病害抵抗性の原因となる遺伝子はほとんど単離されていない。

近年、Full-length cDNA OvereXpressor gene hunting system (FOX ハンティングシステム)が開発された。FOX ハンティングシステムを用いることにより、期待する機能をもつ遺伝子の探索が効率的に行える。約13,000種類のイネの完全長cDNAsをCaMV *35S* プロモーター下流に連結し、アグロバクテリウムを介してシロイヌナズナ(エコタイプ Columbia)に導入することによりイネ FOX シロイヌナズナ系統が作出された。シロイヌナズナは植物体のサイズが小さく、生活環が短いので、このシステムを用いることによって、高速かつ大規模に様々な機能の遺伝子をスクリーニングすることができる。特に、複雑な病原菌感染メカニズムに関連する病害抵抗性遺伝子のスクリーニングには有利である。本研究において、私はイネFOXシロイヌナズナ系統を用いてイネの広範な病害抵抗性遺伝子のスクリーニングを行い、重要な遺伝子を単離し、単離した原因遺伝子の高発現イネで病害抵抗性の評価を行った。

最初に、イネ FOX シロイヌナズ系統約 2 万系統を用いてシロイヌナズナの病原細菌 *Pseudomonas syringae* pv *tomato* DC3000(*Pst* DC3000)に対する感染抵抗性系統の選抜を行った。*Pst* DC3000 のスクリーニングは 3 回行い、最終的に 72 系統を選抜した。19 の候補遺伝子は、その原因遺伝子を有する別の独立したシロイヌナズナ系統、すなわちイネ FOX シロイヌナズナ 2 万系統中の別系統や花序浸し形質転換法によりエコタイプ Columbia に原因 cDNA を再導入した系統で *Pst* DC3000 抵抗性を確認した。

次に、複合抵抗性遺伝子を単離するために、72の*Pst* DC3000抵抗性系統につい

て病原糸状菌*Colletotrichum higginsianum*に対する感染抵抗性を調べた。72系統のうち13系統は*C. higginsianum*感染抵抗性を示した。*Pst* DC3000抵抗性を付与した8個の原因cDNAをそれぞれイネに再導入して高発現イネを作出し、白葉枯病を引き起こす病原細菌*Xanthomonas oryzae* pv. *oryzae*に対する抵抗性の評価をした。これらのうちの1系統は高度白葉枯病抵抗性を示した。さらにこの系統は、多くの国でイネの最重要病害となっているいもち病を引き起こす病原糸状菌*Pyricularia oryzae*に対しても顕著な高度抵抗性を示した。以上のことから、受容体様細胞内リン酸化酵素をコードするこの原因遺伝子を*BROAD-SPECTRUM RESISTANCE 1*（*BSR1*）と命名した。BSR1タンパク質はシロイヌナズナのBIK1タンパク質に類似していた。これらの結果は植物の防御遺伝子の同定のための1つのツールとしてイネFOXシロイヌナズナ系統を用いることは有効であることを示し、単子葉および双子葉植物に共通した防御機構の存在を示唆することができた。

さらに、私は*BSR1*の高発現（*BSR1*-OX）イネが白葉枯病菌およびいもち病菌の別のレースや3つの他の病原体に対しても抵抗性を示すかどうかについて調べた。その結果、*BSR1*-OXイネは白葉枯病菌の他の2つのレースやいもち病菌の他のレースに対して少なくとも抵抗性を示しした。また、*BSR1*-OXイネは籾枯細菌病を引き起こす病原細菌*Burkholderia glumae*およびごま葉枯病を引き起こす病原糸状菌*Cochliobolus miyabeanus*に対しても抵抗性を示した。加えて、*BSR1*-OXイネは縞葉枯病ウィルスによって引き起こされる縞葉枯病に対してもやや抵抗性を示した。以上のように、*BSR1*-OXイネは少なくとも2つの重要な細菌病および2つの重要な糸状菌病に対して顕著で広範な病害抵抗性を示し、また1つのウィルス病に対してやや抵抗性を示すことを明らかにした。

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