Selection of Differential Isolates of *Magnaporthe oryzae* for Postulation of Blast Resistance Genes

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ABSTRACT

A set of differential isolates of *Magnaporthe oryzae* is needed for the postulation of blast resistance genes in numerous rice varieties and breeding materials. In this study, the pathotypes of 1,377 *M. oryzae* isolates from different regions of China were determined by inoculating detached rice leaves of 24 monogenic lines. Among them, 25 isolates were selected as differential isolates based on the following characteristics: they had distinct responses on the monogenic lines, contained the minimum number of avirulence genes, were stable in pathogenicity and conidiation

during consecutive culture, were consistent colony growth rate, and, together, could differentiate combinations of the 24 major blast resistance genes. Seedlings of rice cultivars were inoculated with this differential set of isolates to postulate whether they contain 1 or more than 1 of the 24 blast resistance genes. The results were consistent with those from polymerase chain reaction analysis of target resistance genes. Establishment of a standard set of differential isolates will facilitate breeding for blast resistance and improved management of rice blast disease.

Rice blast, caused by the ascomycete fungus *Magnaporthe oryzae*, is one of the most devastating diseases worldwide (Dai et al. 2010; Ou 1980; Valent 1990; Wilson and Talbot 2009) and can cause yield losses ranging from 20 to 50% (Huang et al. 2014; Savary et al. 2000). The interactions between blast fungus (Ling et al. 2004) and rice have been considered to follow the gene-for-gene hypothesis (Li et al. 2009). The most effective and economically viable approach to control blast disease is to plant cultivars that have major blast resistance (*R*) genes (Valent and Chumley 1991; Zhu et al. 2000). This disease management strategy is effective only when the deployed cultivars that carry major *R* genes are infected by the strains of *M. oryzae* population that contain the corresponding avirulence (*AVR*) genes. However, the resistance mediated by major genes may be overcome (Zeigler et al. 1994) by new virulent races shortly after deployment as a result of rapidly evolving *M. oryzae* populations (Bonman et al. 1992). Therefore, deployment of blast resistance cultivars requires monitoring of the dynamics of races or gaining information on the current and future frequency of *AVR* genes in *M. oryzae* populations because it is not practicable to track numerous races at the same time. During the past few decades, huge efforts have been made in this direction. Commonly, this was done by sampling isolates from *M. oryzae* populations and inoculating them onto a set of differential rice varieties (Levy et al. 1993). The differential varieties used in this work also evolved, from rice cultivars with complex genetic background to monogenic lines, and from local various differentials used by particular research groups to an internationally shared set of monogenic lines. These latter lines

have been developed by the International Rice Research Institute (IRRI)–Japan Collaborative Research Project and are composed of 31 monogenic lines carrying 24 major blast *R* genes (Kobayashi et al. 2007; Tsunematsu et al. 2000). A Japonica-type variety, Lijiangxintuanheigu (LTH), found in Yunnan province and highly susceptible to all of the evaluated blast isolates (Ling et al. 2001), has been used as the recurrent female parent, and varieties with known *R* gene(*S*) have been used as donors in development of this set of differentials. These monogenic lines with the genetic background of LTH were the first international standard differential varieties to monitor the pathotypes of blast isolates. The results from different research groups revealed that the rice blast fungal populations have a complex genetic background (Choi et al. 2013; Xue et al. 2012; Zhang et al. 2016) and huge numbers of physiological races (Le et al. 2010; Odjo et al. 2014). In the previous work, an accurate, rapid, and large-scale wounding inoculation method was established to identify the pathotypes of *M. oryzae* (Zhang et al. 2014).

Successful management of rice blast via deployment of resistant varieties requires not only information on pathogen populations but also a large number of varieties with desired *R* genes, to deploy blast-resistant rice cultivars to match their *R* genes with the high-frequency *AVR* genes in the pathogen population. Some efforts have been made in postulation of blast *R* genes carried in cultivars to be deployed and in breeding materials which provide the basis of *R* genes for future cultivars (Deng et al. 2017; Shi et al. 2015; Wang et al. 2017). According to the gene-for-gene hypothesis, an *R* gene in a host can only be revealed from its reaction to pathogen isolates carrying the corresponding *AVR* gene.

Postulation of blast resistance genotypes was usually done by inoculating the test cultivars with unknown resistance genotypes and the differential monogenic lines with selected isolates, and then comparing their reactions to the isolates with those on differential monogenic lines (Ling 1984; Ling et al. 1990). It has long been recognized that the key to *R* gene postulation is selection of appropriate isolates. However, thus far, due to lack of an established set of differential isolates, individual researchers have had to start from scratch by screening isolates and then using them in inoculation tests. Screening of proper isolates is time consuming,

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labor intensive, and often not successful due to limited availability of discriminatory isolates. The lack of the appropriate isolates has slowed the postulation of blast *R* genes in rice cultivars and breeding materials, and using different isolate sets also makes it difficult to compare results from different studies.

Molecular tools have also been developed for postulation of blast *R* genes in a cultivar during the past decades (Jeung et al. 2007). To date, 24 *R* genes have been cloned (Liu et al. 2015). With the advance of bulked segregant analysis and, more recently, next-generation sequencing techniques, whether a cultivar carries these major *R* genes can be determined with molecular techniques quickly. However, molecular methods have some limitation in identifying *R* genes. First, transposable element (TE) insertions, TE-mediated genome reorganization, and TE-associated methylation evolution (Takahashi et al. 2010) make it difficult to determine whether an *R* gene functions or not solely based on sequencing of a small DNA fragment. Compared with the nonfunctional allele *Pit^{Npb}*, the functional allele *Pit^{K59}* contains four amino acid substitutions, and has the long terminal repeat retrotransposon renovator inserted upstream (Hayashi and Yoshida 2009). Moreover, the extremely similar sequence among different functional *R* genes such as alleles at *Pi2/9* (Su et al. 2015) makes it difficult to develop gene-specific markers for determining known genes from diverse varieties. Few single nucleotide polymorphisms (SNPs) of *R* genes could lead to differences in the function; for example, the resistance specificities of *Pi2*, *Pi9*, and *Piz^t* are determined by a few amino acid changes (Zhou et al. 2006). Therefore, inoculation will be a useful method for identifying *R* genes against rice blast for a long time in the future.

In this study, our objectives were to select a set of differential isolates for postulation of blast *R* genes in rice varieties, to make better use of those varieties.

MATERIALS AND METHODS

Ideal differential isolates should have the following characteristics: (i) stable, with little variation in growth rate and conidiation; (ii) distinct reactions on susceptible and resistant monogenic lines; (iii) each isolate containing as few *AVR* gene(S) as possible, ideally a single *AVR* gene; and (iv) when used together, they can differentiate all of the possible combinations of the 24 major *R* genes in the international differential monogenic lines. Therefore, our efforts were concentrated on these four aspects.

Plant materials and isolates. The 24 monogenic lines developed from LTH, which was considered susceptible to all isolates of *M. oryzae* (Kobayashi et al. 2007; Ling et al. 2001; Tsunematsu et al. 2000), were used as differential varieties to screen isolates of *M. oryzae* (Table 1), and LTH was also included as a susceptible control. Seeds of all of these lines were planted in silty clay soil in plastic pots (40 by 20 by 5 cm) with holes at the bottom, maintained in the greenhouse at approximately 26°C and 70% relative humidity, with a regime of 14 h/day and 10 h/night, and watered regularly. At the four- to five-leaf stage (3-week-old seedlings), the middle and upper leaves of LTH and 24 monogenic lines were detached and transferred into the lab for inoculation.

Isolates of *M. oryzae* were obtained via single-spore isolation from diseased leaves of LTH plants grown in Farm 856 in Heilongjiang Province and Donggang and Dawa in Liaoning Province during 2010 to 2014. In total, 1,377 single-spore isolates, including 451, 529, and 397 isolates from Farm 856, Donggang, and Dawa, respectively, were used for screening in this study (Table 2).

Isolates of *M. oryzae* were maintained on oatmeal tomato agar (OTA) (30 to 40 g of oatmeal, 20 g of agar, 150 ml of tomato juice, and approximately 850 ml of H₂O) (Yang et al. 2010) at 26°C and further subcultured onto new OTA plates. For long-term storage, autoclaved filter paper discs (1 cm in diameter) were placed on the growing colony for 7 days to allow the pathogen hyphae to cover the discs; then, the discs were collected in a sulphuric acid paper bag, air dried, and stored at -20°C.

Inoculation of differential monogenic lines and LTH with isolates of *M. oryzae*. The 7-day-old OTA colonies of the *M. oryzae* isolates were induced to produce conidia, which were harvested and used to prepare inoculum as described previously (Peng and Shishiyama 1988). The detached leaves were cut into 5- to 7-cm leaf fragments, placed in a 90-mm Petri dish, scratched using a pin along the main vein (2 by 2 mm), and inoculated with a drop of 10 µl of conidium suspension of 1 × 10⁵ spores/ml in 0.025% Tween 20 solution (Zhang et al. 2014). The three inoculated leaf fragments were sealed in a Petri dish and incubated in darkness at 90% relative humidity for 24 to 36 h before being transferred to a chamber maintained at 26°C. The reactions were examined and photographed at 4 to 7 days postinoculation (dpi). The reaction

TABLE 1. Monogenic lines with different resistance genes

Code	Monogenic line	Resistance gene	Donor	Origin
K01	IRBLA-A	<i>Pia</i>	Aichi Asahi	Philippines
K03	IRBLI-F5	<i>Pii</i>	Fujisaki5	Philippines
K04	IRBLKs-F5	<i>Piks</i>	Fujisaki5	Philippines
K06	IRBLk-Ka	<i>Pik</i>	Kanto51	Philippines
K07	IRBLkp-K60	<i>Pik^p</i>	K60	Philippines
K08	IRBLkh-K3	<i>Pikh</i>	K3	Philippines
K09	IRBLz-Fu	<i>Piz</i>	Fukunishiki	Philippines
K10	IRBLz5-CA	<i>Piz⁵</i>	C101A51	Philippines
K11	IRBLzt-T	<i>Piz^t</i>	Toride 1	Philippines
K12	IRBLta-K1	<i>Pita</i>	K1	Philippines
K14	IRBLb-B	<i>Pib</i>	BL1	Philippines
K15	IRBLi-K59	<i>Pit</i>	K59	Philippines
K17	IRBLsh-B	<i>Pish</i>	BL1	Philippines
K18	IRBLI-CL	<i>Pil</i>	C101LAC	Philippines
K19	IRBL3-CP4	<i>Pi3</i>	C104PKT	Philippines
K20	IRBL5-M	<i>Pi5</i>	Moroberekan(RIL249)	Philippines
K21	IRBL7-M	<i>Pi7</i>	Moroberekan(RIL29)	Philippines
K22	IRBL9-W	<i>Pi9</i>	WHD-1S-75-1-127	Philippines
K23	IRBL12-M	<i>Pi12</i>	Moroberekan(RIL10)	Philippines
K24	IRBL19-M	<i>Pi19</i>	Aichi Asahi	Philippines
K25	IRBLkm-Ts	<i>Pik^m</i>	Tsuyake	Philippines
K26	IRBL20-IR24	<i>Pi20</i>	ARL24	Philippines
K27	IRBLta2-Pi	<i>Pita2</i>	Pi No.4	Philippines
K30	IRBL11-Zh	<i>Pi11</i>	Zhaiyeying 8	Philippines

TABLE 2. Origins of field isolates used in this study

Location	GPS	Number of isolates by year					Total isolates
		2010	2011	2012	2013	2014	
Farm 856	45.62°N, 132.72°E	96	99	66	90	100	451
Donggang	39.54°N, 124.08°E	126	89	107	89	118	529
Dawa	41.02°N, 122.04°E	0	100	95	90	112	397

Fig. 1. Reaction types of field isolates on monogenic lines: resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S).

types were scaled as one of four categories: resistant reaction (R), moderately resistant reaction (MR), moderately susceptible reaction (MS), and susceptible reaction (S) (Fig. 1). In the R reaction, the lesions were dark brown and restricted to the wounded area without distinctive center. In the MR reaction, the biggest lesion was less than 3 mm long whereas, in the MS reaction, the biggest lesion was more

than 3 mm long. In S reactions, the lesions were spindle shaped, with gray centers, brown margins, and yellow haloes.

Identification of AVR genes. In gene-for-gene systems, a resistance reaction of a plant to a pathogen is due to the “recognition” by the plant surveillance system of a pathogen AVR determinant encoded by an AVR gene to eventually result in triggering of plant

TABLE 3. Matrix of the preliminary candidate isolates and the data of their growth rate and conidiation^a

Isolate	Pita2	Piz ^t	Pi19	Pi20	Pita	Pi7	Pik ^m	Piz ^s	Pik ^s	Pit	Pi3	Pii	Pi9	Pish	Pi1	Pi5	Pik	Pik ^p	Pia	Pi11	Pib	Pik ^h	Pi12	Piz	Colony diameter (mm)	log ₁₀ (conidiation)
10-856-44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.7 ± 0.6	6.69 ± 0.07
10-856-33	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36.1 ± 0.5	5.96 ± 0.27
11-856-35	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.4 ± 0.5	6.53 ± 0.06
10-856-37	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.0 ± 1.1	6.47 ± 0.12
13-856-39	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35.6 ± 0.9	6.44 ± 0.05
10-856-41	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36.4 ± 0.7	5.73 ± 0.20
10-856-32	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35.3 ± 0.7	6.30 ± 0.05
13-DG-17	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.7 ± 1.0	5.62 ± 0.15
10-856-57	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.3 ± 0.4	6.24 ± 0.07
14-856-47	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.1 ± 0.6	5.88 ± 0.09
11-PJ-12	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.4 ± 0.3	5.62 ± 0.15
11-856-07	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.0 ± 0.6	6.33 ± 0.11
11-856-19	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36.2 ± 0.6	6.85 ± 0.03
11-856-10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32.7 ± 0.8	6.36 ± 0.06
12-856-02	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34.4 ± 0.4	6.14 ± 0.09
11-856-12	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35.9 ± 0.9	6.51 ± 0.07
11-856-25	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	40.9 ± 0.6	6.88 ± 0.03
13-DG-44	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	35.7 ± 0.4	5.82 ± 0.16
11-856-70	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	40.2 ± 0.3	6.60 ± 0.08
11-856-79	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	39.1 ± 0.3	6.00 ± 0.14
14-856-79	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	35.6 ± 0.4	5.68 ± 0.15
13-DG-36	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	36.6 ± 0.6	6.17 ± 0.07
12-DG-36	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	35.5 ± 0.5	5.95 ± 0.12
11-856-74	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	35.8 ± 0.2	5.67 ± 0.15
10-856-16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	36.3 ± 1.0	5.62 ± 0.15
11-856-97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	36.7 ± 0.8	6.48 ± 0.10
11-856-58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	29.2 ± 0.8	6.62 ± 0.08
11-856-59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	39.3 ± 0.4	6.97 ± 0.03
11-856-48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	39.6 ± 0.4	7.27 ± 0.02
11-856-52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	38.0 ± 0.2	7.17 ± 0.03
10-856-63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	36.2 ± 0.2	6.71 ± 0.03
13-856-56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	39.4 ± 0.5	6.94 ± 0.03
11-PJ-40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	36.0 ± 0.2	7.17 ± 0.03
12-856-64	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	38.6 ± 0.8	6.73 ± 0.04
11-856-49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	37.4 ± 0.4	6.09 ± 0.09
13-856-53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	38.0 ± 0.6	6.62 ± 0.04
11-856-21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	37.8 ± 0.5	6.36 ± 0.06

^a Reactions: 0 = susceptible and 1 = resistant. Lijiangxintuanheigu was susceptible to all of the assayed isolates.

TABLE 4. Matrix of avirulence genotypes of differential isolates^a

Isolate	Avr-Pita2	Avr-Piz ^t	Avr-Pi19	Avr-Pi20	Avr-Pita	Avr-Pi7	Avr-Pik ^m	Avr-Piz ^s	Avr-Pik ^s	Avr-Pit	Avr-Pi3	Avr-Pii
CRB-01	–	–	–	–	–	–	–	–	–	–	–	–
CRB-02	+	–	–	–	–	–	–	–	–	–	–	–
CRB-03	–	+	–	–	–	–	–	–	–	–	–	–
CRB-04	–	–	+	–	–	–	–	–	–	–	–	–
CRB-05	+	–	–	+	+	–	–	–	–	–	–	–
CRB-06	+	–	–	–	+	–	–	–	–	–	–	–
CRB-07	+	–	–	–	–	+	–	–	–	–	–	–
CRB-08	–	–	–	–	–	–	+	–	–	–	–	–
CRB-09	+	–	–	–	–	–	–	+	–	–	–	–
CRB-10	+	–	–	–	–	–	–	–	+	–	–	–
CRB-11	–	+	–	–	–	–	–	–	–	+	–	–
CRB-12	+	–	–	–	–	–	–	–	–	–	+	–
CRB-13	+	+	–	–	–	–	–	–	–	–	–	+
CRB-14	+	–	–	–	–	–	–	–	–	–	–	–
CRB-15	+	+	–	–	+	–	–	–	–	–	–	–
CRB-16	+	+	–	–	+	–	–	–	–	–	–	–
CRB-17	+	–	–	–	+	+	–	–	–	–	–	–
CRB-18	+	–	–	+	–	–	–	–	–	–	–	–
CRB-19	+	–	–	–	–	–	–	–	–	+	–	–
CRB-20	–	+	–	–	+	–	–	–	–	+	–	–
CRB-21	–	+	–	–	–	–	–	–	–	–	–	–
CRB-22	+	–	–	–	–	–	–	–	–	–	–	–
CRB-23	–	–	–	–	–	+	–	+	–	–	–	–
CRB-24	+	–	+	–	–	–	–	–	–	–	–	–
CRB-25	+	–	–	–	+	–	–	–	–	–	–	–

(Continued on next page)

^a Symbols: + and – indicate that the isolate contains or does not contain, respectively, the avirulence gene in the top line.

Preliminary selection of candidate differential isolates. First, a matrix was constructed for the reactions of all 1,377 isolates on the 24 monogenic lines and LTH, with each isolate as a row. To avoid inconsistent or ambiguous reactions, all of the rows with MR and MS were removed; then, R and S reactions were converted into “1” and “0”, respectively, and a column (“Sum”) of row sum was added to the right to store the total number of *AVR* genes carried by each isolate. The rows with a value greater than 6 were removed to minimize the number of *AVR* genes carried by selected isolates. The following row and column transformations were done on the matrix to search for a set of candidate isolates that, as a whole, can differentiate all possible combination of the 24 major *R* genes carried by the monogenic lines:

1. Sorted the rows in order from the smallest to greatest “Sum”;
2. Moved the whole column (other than “Sum”) that had a value “1” in the top row that had a value “1” in column “Sum”, and was in the area that was not yet arranged, into the left-most column that was not occupied by a “1” in the arranged area, and all rows from the top to this row were then considered arranged. If more than one row was exactly the same, then they were considered alternative choices; if there were multiple rows with sum “1” but their “1” values were not in the same column, all columns were moved;
3. Changed the value of cells in nonarranged rows to “0” if there had already been a “1” in the same columns in the arranged area;
4. Recalculated the column “Sum” and repeated steps 1 through 3 until the top matrix (excluded the column “Sum” and alternative rows) became an identity matrix.

5, 12, 35, 37, 66, and 79 isolates had 0, 1, 2, 3, 4, 5, and 6 AVR genes, respectively, based on their reactions on all 24 monogenic lines. Isolate 10-856-44 caused an S reaction on all 24 monogenic lines (Table 3), which suggested that the isolate contains none of the 24 AVR genes. Although six other isolates also had none of the 24 AVR genes, these isolates caused MS reactions (Fig. 1) on some monogenic lines; therefore, only isolate 10-856-44 was retained (Table 3). If a rice variety showed resistance to this isolate, the variety should contain at least one *R* gene different from the 24 *Pi* genes in the monogenic lines used.

After removing isolates with ambiguous reactions (MR or MS) (Fig. 1), an identity matrix with 37 preliminary candidate isolates and their alternatives was obtained after matrix transformation (Table 3).

Colony growth rate and conidia production of preliminary candidate isolates. The preliminary candidate isolates showed some variation in colony growth rate and conidiation (Table 3). By selecting isolates with a high growth rate and abundant conidial production, 25 isolates were selected as the final set of differential isolates and named as China Rice Blast (CRB)

differential isolates 01 to 25 (i.e., CRB-01 to CRB-25) (Table 4). Most of these isolates have one to four AVR genes in each isolate; however, CRB-15 had five while CRB-16 and CRB-17 both had six AVR genes (Table 4). Isolates CRB-12, CRB-13, CRB-14, CRB-19, CRB-20, and CRB-22 all had similar colony growth rates, slightly faster than the other isolates (Fig. 2B). In addition, the colony margins of CRB-04, CRB-10, CRB-13, and CRB-14 were more compact and the mycelia were whiter, while the mycelia of CRB-03, CRB-18, CRB-21, and CRB-25 were darker (Fig. 2A). The majority of differential isolates produced approximately 10^5 to 10^6 conidia/plate (Fig. 2C). These isolates could conveniently be cultured in the laboratory to produce spores for inoculation, which usually requires a concentration of 1×10^5 spores/ml (Chi et al. 2009; Zhang et al. 2014).

Stability of pathogenic reaction type and conidiation for candidate isolates. The reaction patterns of the differential isolates were stable over a period of 3 years on the 24 monogenic lines in three tests, with no noticeable differences in pathotypes between the original isolates and their subcultures. The differential isolates also showed stable conidial production on OTA. A representative set of data is presented in Table 5 for four candidate isolates.

TABLE 5. Reaction patterns and conidiation of four differential isolates in several years^a

Isolate	<i>Pi1</i>	<i>Pi11</i>	<i>Pi12</i>	<i>Pi19</i>	<i>Pi20</i>	<i>Pi3</i>	<i>Pi5</i>	<i>Pi7</i>	<i>Pi9</i>	<i>Pia</i>	<i>Pib</i>	<i>Pii</i>	<i>Pik</i>	<i>Pik^h</i>	<i>Pik^m</i>	<i>Pik^p</i>	<i>Pik^s</i>	<i>Pish</i>	<i>Pit</i>	<i>Pita</i>	<i>Pita2</i>	<i>Piz</i>	<i>Piz⁵</i>	<i>Piz^t</i>	log ₁₀ (conidiation)
CRB-03(2015)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	6.53 ± 0.06
CRB-03(2016)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	6.69 ± 0.10
CRB-03(2017)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	6.53 ± 0.06
CRB-09(2015)	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	5.81 ± 0.23
CRB-09(2016)	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	6.04 ± 0.10
CRB-09(2017)	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	6.32 ± 0.11
CRB-10(2015)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	6.76 ± 0.12
CRB-10(2016)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	S	S	6.56 ± 0.09
CRB-10(2017)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S	S	6.85 ± 0.03
CRB-12(2015)	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	6.88 ± 0.03
CRB-12(2016)	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	6.44 ± 0.07
CRB-12(2017)	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	6.88 ± 0.03

^a R = resistant and S = susceptible reaction. Lijiangxintuanheigu was susceptible to all of the tested isolates.

Fig. 2. Growth rate and conidiation of candidate differential isolates. **A**, Cultural characteristics of the differential isolates (CRB-01 to CRB-25) cultured on oatmeal tomato agar (OTA) after 5 days of inoculation. **B**, Colony diameter of 25 strains on OTA for 5 days of inoculation at 26°C. **C**, Conidia production of 25 strains cultured on OTA for 7 days at 26°C. Error bars represent standard errors of the mean.

DISCUSSION

In the present study, 25 differential isolates were selected to establish a new set of differential isolates that would be stable over multiple years and retain high growth rates and high levels of conidial production. This was the first set of differential isolates that can stably differentiate combinations of all 24 *Pi* genes and this is critical for a common set to be shared among researchers. Here, differential isolates exhibited consistency and stability in *AVR* genotypes and conidiation, unlike some previous reports, where researchers found that isolates of *M. oryzae* were highly variable (Chadha and Sharma 2014; Roumen et al. 1997) and cited mechanisms of genetic variability in *M. oryzae* that included spontaneous mutations, heterokaryosis, and parasexual recombination (Genovesi and Magill 1976; Noguchi et al. 2006; Wu and Magill 1995). The difference between the previous studies and the current one might be attributable to the use of mycelium for serial transfer and storage in the current study, whereas conidia were usually used in the previous reports, where *M. oryzae* was found to be highly variable (Sharma et al. 2002). The current findings are consistent with those of Park et al. (2010), who indicated genetic stability of two isolates on an artificial medium and rice plants and observed no difference in cultural characteristics and pathogenicity (Latterell and Rossi 1986). The stability of the isolates might be attributable to the use of mycelium for serial transfer and storage in the current study, whereas conidia were usually used in the previous reports where *M. oryzae* was found to be highly variable (Sharma et al. 2002). Further studies are needed to confirm whether subculture via mycelium transfer and storage of mycelium on filter paper can ensure the stability of *M. oryzae* isolates, and whether reproduction via conidiation is a major source of variation for *M. oryzae*. The differential isolates and their subcultures also need to be tested periodically on the monogenic lines to ensure that their pathotypes remain the same and, perhaps, reisolated from the corresponding susceptible lines to avoid decline in pathogenicity.

Conventional polymerase chain reaction (PCR) is a good method to directly detect some *Pi* genes that are carried by a rice variety. This is usually conducted by genotyping, and many of the major blast *R* genes also had been amplified in PCR. Given the complexity of repetitive sequences and transposon-like elements of genes, both upstream and downstream flanking sequences (Xue et al. 2012) should be sequenced. It is even more expensive to sequence the whole genomes of rice varieties, and laborious to have them assembled into contigs and placed into scaffolds. Therefore, within the foreseeable future, postulation of *R* gene in rice varieties will still rely on inoculation with pathogen isolates carrying corresponding *AVR* genes. Even we can detect an *R* gene via a molecular method, if the gene functions under field conditions, it may still need to be confirmed by inoculation.

With this set of differential isolates, whether a rice cultivar had one or more of the 24 *Pi* genes, and even additional blast *R* genes, can be conveniently determined via wounding inoculation with these isolates. This will allow rapid and accurate postulation of the blast *R* genes of commercial rice varieties compared with a traditional method, without inoculating the differential varieties. Ma et al. (2015) successfully postulated the major blast *R* genes of 20 rice cultivars in Harbin by using an earlier set of 22 *M. oryzae* differential isolates named rice blast (RB) isolates RB01 to RB22, in which 13 *R* genes were detected, with *Pia* and *Pi19* as the most prevalent. The results were consistent with amplification of corresponding *R* genes via PCR. Jin et al. (2011) also analyzed *R* genes of 98 rice varieties in Jilin Province using the 22 differential isolates. The number of *AVR* genes was equal to or more than 5 in each of these 22 RB differential isolates, while RB03 carried 19 *AVR* genes. This made postulation of blast *R* genes complicated and, hence, the number of *AVR* genes was purposefully and dramatically reduced in this new set of CRB differential isolates. We also expanded the ability of the differential isolates to detect different

combinations of 24 *Pi* genes. Ideally, to make postulation of *R* genes quick and simple, each differential isolates should contain a single *AVR* gene. However, only 5 of 1,377 field isolates were found with a single *AVR* gene. More field isolates, especially more isolates from different regions and host plants, may be included in future screenings to ultimately obtain a set of differential isolates that each contain single unique *AVR* gene.

If a variety were to show *R* reactions to each of the 24 differential isolates, then the genotype of the variation would remain unknown. Longdao 5 has four *R* genes (specifically, *Pik^s*, *Pi1*, *Pi19*, and *Pish*) (Ma et al. 2015), although it may contain the other, unknown *R* genes that are different from any of the 24 known *R* genes. To date, 86 major rice blast genes have been characterized (Cesari et al. 2013; Huang et al. 2014). Therefore, a future goal is to develop a greater number of near-isogenic lines (Lei et al. 2014; Telebanco-Yanoria et al. 2010) to monitor new and emerging *AVR* genotypes in *M. oryzae* populations. In contrast, more field isolates from different regions and host plants may be included in future screenings to obtain a set of differential isolates that each contains a single unique *AVR* gene and can postulate more than the current 24 *R* genes in rice varieties. Although far from perfect, this selection of 25 differential isolates will be useful in postulation of *R* genes in varieties for control of rice blast via strategic deployment of *R* genes (Zhu et al. 2000).

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