Brome mosaic virus Infection of Rice Results in Decreased Accumulation of RNA1

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Submitted 19 December 2014. Accepted 8 January 2015.

Brome mosaic virus (BMV) (the Russian strain) infects monocot plants and has been studied extensively in barley and wheat. Here, we report BMV can systemically infect rice (*Oryza sativa* var. japonica), including cultivars in which the genomes have been determined. The BMV capsid protein can be found throughout the inoculated plants. However, infection in rice exhibits delayed symptom expression or no symptoms when compared with wheat (*Triticum aestivum*). The sequences of BMV RNAs isolated from rice did not reveal any nucleotide changes in RNA1 or RNA2, while RNA3 had only one synonymous nucleotide change from the inoculum sequence. Preparations of purified BMV virions contained RNA1 at a significantly reduced level relative to the other two RNAs. Analysis of BMV RNA replication in rice revealed that minus-strand RNA1 was replicated at a reduced rate when compared with RNA2. Thus, rice appears to either inhibit RNA1 replication or lacks a sufficient amount of a factor needed to support efficient RNA1 replication.

Brome mosaic virus (BMV) is a model RNA virus that has taught us much about the requirements for viral translation, RNA replication, and RNA recombination in plants (Noueiry and Ahlquist 2003; Sivakumaran and Kao 2000). BMV RNAs can replicate in the budding yeast *Saccharomyces cerevisiae* (Janda and Ahlquist 1993). Studies in yeast have identified a large number of host proteins that contribute to viral RNA replication (Kushner et al. 2003). In nature, BMV infects monocots, including brome grass and fescue (Lane 1974). While the requirements for BMV infection have been extensively studied in monocot plants, this has been done primarily in plants whose genomes have not been sequenced. Thus, significant gaps exist in our understanding of the role of plant host factors that impact the BMV infection process.

BMV is a member of the alphavirus-like superfamily of RNA viruses. The BMV genome consists of three capped, messenger-sense genomic RNAs that share a common tRNA-like structure within the 3' untranslated region (UTR). Genomic RNAs 1 and 2 encode, respectively, proteins 1a and 2a, which catalyze RNA replication (Noueiry and Ahlquist 2003; Sivakumaran and Kao 2000). The 1a protein has an N-terminal portion with m⁷G methyltransferase activity and a C-terminal portion that contains helicase motifs (Ahola and Ahlquist 1999; Kong et al. 1999; Wang et al. 2005). The 1a protein recruits the RNA-dependent

RNA polymerase and viral RNAs to the membrane-associated factories in which replication takes place (Schwartz et al. 2002, 2004; Wang et al. 2005). RNA2 encodes the BMV polymerase named 2a. Genomic RNA3 is a bicistronic RNA that encodes the movement protein needed for cell-to-cell spread and the capsid protein (CP) that encapsidates the BMV RNA. The CP is translated from the subgenomic RNA4, whose synthesis is templated by minus-strand RNA3 (Miller et al. 1985).

An especially interesting property of BMV is that it encapsidates its three genomic RNAs in separate virions (Kao and Sivakumaran 2000; Rao 2006). Subgenomic RNA4 is coencapsidated with genomic RNA3 (Rao 2006; Ni et al. 2014). Vaughan et al. (2014) recently demonstrated that the virion containing RNA1 has physiochemical properties that are distinct from the other two virions. Furthermore, RNA1 virions exhibit more rapid release of the encapsidated RNA when compared with the other two BMV virions.

Rice (*Oryza sativa*) was the first cereal that had its genome sequence determined (International Rice Genome Sequencing Project 2005). The cultivars sequenced include Nipponbare and Koshihikari (Yamamoto et al. 2010). An increasing number of tools, such as a collection of full-length cDNAs, gene expression microarrays, a proteome database, and mutant lines, have been developed for *O. sativa* var. japonica (Jung et al. 2008; Komatsu et al. 2004; Rice Full-Length cDNA Consortium 2003; Suzuki et al. 2008; Till et al. 2007). Rice is now well-positioned as a system to study how the genes from a monocot can impact virus infection. For example, the rice NAC transcription factor RIM1 has been identified to affect the multiplication of *Rice dwarf virus* (Yoshii et al. 2009, 2010).

Ding et al. (2006) reported that BMV, isolated from *Festuca arundinacea* (tall fescue), could infect rice cultivars Indica and Javanica. The majority of studies of BMV infection, however, used the Russian strain of BMV, which had not been confirmed to infect rice. In this work, we establish that the Russian strain of BMV can systemically infect the majority of japonica cultivars. Interestingly, BMV-purified rice have decreased accumulation of the virions encoding RNA1 compared with BMV purified from wheat.

RESULTS

Systemic BMV infection in var. Japonica.

To examine whether the Russian strain of BMV can systemically infect *O. sativa* var. Japonica, the primary leaves of seedlings from 46 Japonica rice cultivars were challenged by mechanical inoculation with BMV purified from wheat (BMV_w) at 0.05 or 2.5 mg/ml. No symptoms were observed in any of the rice cultivars inoculated with BMV at 0.05 mg/ml, but several cultivars inoculated with BMV at 2.5 mg/ml, including Nipponbare and Tainung 67, exhibited symptoms in the uninoculated secondary leaves (Fig. 1A). Fourteen days after

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inoculation, lysates from the uninoculated secondary and the tertiary leaves from nine cultivars that exhibited symptoms were analyzed with Western blots probed with anti-CP antiserum. Eight of the nine rice cultivars contained BMV CP (Fig. 1B).

The lack of detection of CP in the majority of the rice lines could be due to the low sensitivity of detection by Western blots. Indeed, when Western blots were performed with fivefold greater amounts of proteins extracted from the secondary and tertiary leaves of 45 cultivars of rice, CP was detectable despite the majority of the cultivars not exhibiting symptoms (Supplementary Table 1). Our results suggest that BMV can systemically infect a majority of Japonica cultivars but that the level of virion production varied among the rice lines. Furthermore, systemic infection in rice cultivars does not necessarily result in obvious symptom development in the secondary and tertiary rice leaves.

The requirement for a higher concentration of inoculum suggests that BMV infection in rice is less efficient than in wheat or barley. Using the Nipponbare cultivar, we examined

the timing of BMV infection in rice compared with that in wheat. Infected wheat produced detectable CP in the secondary leaves by 2 days after inoculation. However, CP was detected in Nipponbare only starting at 8 days after inoculation (Fig. 1C). These results confirm that BMV infection in rice was delayed when compared with infection in wheat.

BMV isolated from infected Nipponbare did not possess any mutations affecting viral protein coding sequences.

BMV was shown to have changes in the sequence of the 3' UTR of RNA1 when it was purified from monocot hosts barley and wheat with inoculum from the dicot *Nicotiana benthamiana* (Ni et al. 2014). We sought to determine whether BMV virions purified from Nipponbare (to be designated BMV_R) had sequence changes in the encapsidated RNAs relative to the virions used as inoculum (BMV_W).

We first subjected BMV_W and BMV_R to mass spectrometric analysis to detect amino acid differences in the BMV CPs.

Fig. 1. *Brome mosaic virus* (BMV) can systemically infect rice seedlings. **A**, Leaves of Nipponbare infected with BMV at 2.5 mg/ml (right two leaves) and mock (left two leaves) at 14 days postinoculation. Inset: the BMV capsid protein (CP) was detected in the tertiary leaves of Nipponbare rice but not in mock-inoculated rice. BMV CP was imaged by Western blotting. Images of the large subunit of the Rubisco protein served as a loading control. The Rubisco proteins (loading controls) were detected by staining the membrane used for Western blotting with Coomassie brilliant blue. **B**, BMV CP accumulations in the second and the third leaves of nine cultivars of Japonica. The names of the rice cultivars are shown above the Western blot image. Each lane contains lysates extracted from seven independently inoculated seedlings. The plants were harvested by cutting just above the soil and were chopped to generate approximately 0.3 mm of leaf sections, and 150 mg of the leaves were used for extracting soluble material with 5% of the extracted material loaded per lane for the Western blot. Purified BMV_W CP (250 ng) was loaded in the right lane as a positive control. Total proteins were extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. BMV CP was detected by Western blots. No CP was detected in uninoculated rice or wheat seedlings (A; data not shown). **C**, Accumulation of BMV CP in wheat (upper panel) or Nipponbare (lower panel). The numbers indicate the number of days after inoculation with BMV at 0.1 mg/ml. The left lane was loaded with 250 ng of purified BMV CP for use as a positive control.

Liquid chromatography coupled to mass spectrometry could detect posttranslational modifications in the CPs from BMV virions (Vaughan et al. 2014; Ni et al. 2014). The major peak of the CP from BMV_R (to be referred to as the BMV_R CP) had a mass of 20,296 Da (Fig. 2A). This mass was expected for the CP with the N-terminal methionine removed and with covalent attachment of an acetyl group at the N-terminal amine, a common modification of eukaryotic proteins (Fig. 2B) (Polevoda and Sherman 2003). The identical modification was observed with the CP from BMV_W and BMV purified from barley and *N. benthamiana* (Fig. 2A and data not shown). A minor amount of a protein of 20,252 Da was observed, likely due to an amino acid substitution in some molecules of the CP. However, comparable amounts of this lower-weight form of the BMV CP were observed with both BMV_R and BMV_W (Fig. 2A).

CP populations with masses up to 115 Da greater than that of the WT CP were observed. These are due to various post-translational modifications in the CP (Ni et al. 2014). Although the abundances of these modified forms differed slightly between the CPs of BMV_R and BMV_W, all of the modified forms

were observed with BMV_R and BMV_W. The CP of BMV_R thus appears to have no major changes when compared with that of BMV_W.

To examine further whether infection of rice resulted in changes in the BMV genome, we extracted genomic RNAs from BMV_R, generated cDNAs, and determined their sequences. No nucleotide changes were found in RNA1 or RNA2. In RNA3, sequencing data suggests that nucleotide 1,802 located within the CP open reading frame was changed from guanine to adenine in some RNA3 molecules (Fig. 2C). However, this change does not affect the sequence of the CP, and nucleotide 1,802 is not in a region of RNA3 known to have regulatory activities (Choi et al. 2004). All of these analyses indicate that BMV isolated from infected Nipponbare did not have changes in RNA sequences encoding BMV proteins or in known regulatory elements.

Significantly less BMV RNA1 accumulates in Nipponbare than in wheat.

Since the reduced rate of BMV infection in rice was not likely due to a change in the BMV genome, we sought to characterize whether there are other changes in the BMV infection process. Given that the host plant has been shown to modulate RNA encapsidation (Ni et al. 2014), we compared the abundances of the encapsidated RNAs from BMV_R and BMV_W. The relative abundances of RNA1 and RNA2 were similar in BMV_W, and there was an approximately 2:1 stoichiometry of RNA4 relative to RNA3 (Fig. 3A) (Ni et al. 2014; Rao 2006). BMV_R had RNA3 that was modestly higher than that of RNA4. However, the most dramatic difference was that the abundance of RNA1 was substantially reduced relative to that of RNA2 (Fig. 3A). The reduced levels of RNA1 were observed in four independent preparations of BMV_R that were generated from three different rice cultivars (Fig. 3A; data not shown).

The differences in the abundances of BMV_R RNAs could be due to either selective packaging of the RNAs in rice, differential accumulation of synthesized RNA, or both. To examine this further, minus-strand BMV RNA1 and RNA2 in inoculated Nipponbare and wheat were determined using real-time reverse transcription-polymerase chain reaction (RT-PCR). Interestingly, minus-strand BMV RNA1 accumulation in infected rice was more than tenfold lower than the amount in wheat at 20 h after inoculation (Fig. 3B). This was observed in three independent samples. The accumulation of BMV RNA2 was not statistically different in BMV-inoculated rice and wheat plants. Based on this data, the slow rate of symptom production and reduced BMV virion production in rice involves reduced replication of RNA1, which then results in lower levels of RNA1 for virion formation.

BMV_R can recover RNA1 accumulation when reinoculated into wheat.

We investigated whether the BMV_R purified from rice plants was infective when reinoculated into rice or wheat. Wheat and rice seedlings were inoculated with BMV_R at 0.1 mg/ml, and the plants were harvested in a 10-day time course. Symptoms in the uninoculated leaves were observed in both rice and wheat plants by 10 days after inoculation. In wheat, the BMV CP was detected 2 days after inoculation and increased over the time course (Fig. 4A). In rice, extracts derived from fivefold more leaf tissue had to be used to detect the CP, indicating that BMV_R still infected rice inefficiently. The increased loading could have detected the inoculated virus on day 1. However, CP levels decreased by day 2 until there was an increase on day 5, followed by a slow increase during the remainder of the time course (Fig. 4A). These results suggest that the BMV_R that had

Fig. 2. Passage of *Brome mosaic virus* (BMV) through rice does not alter the capsid protein (CP) mass or the CP amino acid sequence. **A**, The CP from BMV virions contains posttranslational modifications. Liquid chromatography-mass spectrometry of CP purified from BMV_R (blue) and from BMV_W (red). The most abundant peak (20,296 Da) is from BMV_R CP, where the N-terminal methionine was removed and the N-terminal amino group was modified with an acetyl group. **B**, N-terminal amino acid sequence of BMV CP. **C**, Sequencing chromatogram of the cDNA derived from BMV from infected wheat (upper panel) or Nipponbare (lower panel) that exhibits a synonymous nucleotide change in RNA3.

Fig. 3. The encapsidation and replication of *Brome mosaic virus* (BMV) RNA1 and RNA2 in different plant hosts. **A**, The relative abundances of the four viral RNAs isolated from the cesium chloride-banded BMV_W and BMV_R purified from rice cultivars Nipponbare, Mitsuhihikari 2005, and Kijyumochi. All quantifications show the relative ratio against RNA4. The signal from ethidium bromide was normalized to the length of each BMV RNA to allow the calculation of the molar ratios of the four BMV RNAs. **B**, Quantitative reverse transcription-polymerase chain reaction results from BMV-infected rice and wheat 20 h postinoculation. Minus-strand RNA1 abundance was significantly less in rice than in wheat. The range for one standard deviation is displayed above the bar. Statistical analyses were performed with the Student's *t* test and the *P* value is displayed above the bars.

Fig. 4. BMV_R infection of wheat and rice. **A**, Western blot of capsid protein (CP) accumulation in plants inoculated with *Brome mosaic virus* (BMV) isolated from rice. The upper panel shows the CP accumulation in wheat over a 10-day time course. The bottom panel shows BMV CP accumulation in rice. The amount of protein loaded to detect BMV CP accumulation in rice was fivefold greater than that of wheat. **B**, RNA encapsidated by virions extracted from wheat or rice. The plants were inoculated with a 0.1 mg/ml concentration of BMV_R. The RNAs were detected by ethidium bromide staining of a 1.2% agarose gel loaded with denatured BMV RNA. The signal from ethidium bromide was normalized to the length of each BMV RNA to allow the calculation of the molar ratios of the four BMV RNAs.

reduced RNA1 was able to rapidly recover infectivity in wheat. However, BMV_R yield remained low with a second round of infection in rice.

To examine whether the recovery of infectivity in wheat by the inoculum BMV_R is accompanied by a change in the abundance of BMV RNA1, we purified the virions from wheat and rice inoculated with BMV_R, extracted the RNAs, and separated them on a denaturing agarose gel. Again, the levels of RNA1 were more than threefold lower than that of RNA2 in the virus recovered from rice, while the virus recovered from wheat had a more equal ratio of RNA1 to RNA2 (Fig. 4B). These results indicate that the reduced BMV RNA1 accumulation in rice could be reversed when the virus was reintroduced into wheat.

DISCUSSION

We established that BMV (the Russian strain) can systemically infect the majority of the cultivars of Japonica rice tested. Importantly, this included Nipponbare, for which the genome has been sequenced. BMV infection in rice did not change the sequence of any of the BMV-encoded proteins, but possible changes by posttranslational modifications in the CP of the virion might have occurred. Infection in rice resulted in both a lower amount of virion accumulation and delayed timing of virion accumulation in the systemically infected leaves. The reduced BMV accumulation is associated with a reduction in the accumulation of the virions that encapsidate RNA1 and the replication of RNA1.

BMV isolated from fescue plants has been shown to systemically infect rice cultivars Indica and Japonica (Ding et al. 2006). However efficient systemic infection of any Japonica cultivars by the Russian strain of BMV could not be established. Ding et al. (2006) investigated BMV infection of Japonica plants, grown in a greenhouse, using immunocapture RT-PCR at 28 days postinoculation (dpi). We used seedlings grown in a growth chamber in which the light was at either 1,500 or 3,000 lux, significantly lower than the light level typically found in greenhouses. In addition, BMV virions could be purified from systemically infected leaves in the majority of rice cultivars by 10 dpi. Differences in growth conditions and virion detection methods may account for the differences between our observations and those of Ding et al. (2006).

Ni et al. (2014) reported that host species could affect the relative abundances of the viral RNAs that accumulated and the RNAs encapsidated into virions. In this study, we found that the relative abundance of the virions containing RNA1 and RNA2 from BMV purified from rice was significantly lower than that from wheat (Fig. 3A). Quantification of minus-strand RNA, a hallmark of RNA virus replication studies, showed reduced levels of RNA1 (Fig. 3B). RNA1 plays a critical role for BMV replication to establish the BMV RNA replication factories and to recruit the BMV RNAs into the factories (Schwartz et al. 2002 2004; Vaughan et al. 2014; Wang et al. 2005). In wheat, RNA1 is preferentially replicated early in the infection process, likely to increase the amount of the 1a protein available early in infection (Vaughan et al. 2014). Our data suggest that rice may restrict RNA1 replication to decrease the efficiency of BMV propagation. Selective inhibition of replication of one component of a viral genome is a novel mechanism to inhibit viral infection. At this point, it remains to be determined whether rice lacks sufficient amounts of a factor needed for a high level of RNA1 replication or whether there are one or more rice factors that inhibit RNA1 accumulation. Analysis of BMV RNA replication suggests that these one or more factors will act at or before the level of minus-strand RNA replication (Fig. 3B). Furthermore, virions purified from rice remain infectious in wheat, indicating that the effects of any inhibitory factors that might affect BMV

molecules, such as by posttranscriptional or posttranslational modifications, were reversible (Fig. 4). It should also be pointed out that having rice as a host could impact other processes such as cell-to-cell movement or viral RNA encapsidation in addition to RNA1 accumulation. These possibilities should be the subjects of future examination.

A selective reduction in BMV RNA1 replication is particularly interesting because the BMV RNAs all contain a highly similar 3' UTR that differs only in a few nucleotides among the three genomic RNAs. However, despite the sequence similarities, previous studies of the 3' UTRs have shown that they are not functionally equivalent. First, RNAs that had exchanges of the 3' UTRs replicated poorly, suggesting that the UTRs of each BMV genomic RNA have specific roles to play for the replication (Duggal et al. 1992). Second, deletions of specific portions of the 3' UTRs had different effects on the replication and translation of the RNAs (Barends et al. 2004). Third, the deletion of the 3' terminal two nucleotides in BMV RNA2 and RNA3 were rapidly repaired, but the deletion of the last two nucleotides in RNA1 precluded repair (Hema et al. 2005). Finally, Ni et al. (2014) observed that passage of BMV in *N. benthamiana* rapidly selected for changes in the BMV RNA1 sequence but did not change the sequences of RNA2 or RNA3. Different requirements for the replication of a specific RNA in a multipartite RNA virus could contribute to the specific targeting of viral RNA replication by the host.

Successful BMV infection requires interactions between host and viral factors for viral RNA replication, cell-to-cell movement and long-distance movement through the host plant vascular system (Carrington and Whitham 1998). Yeast has been used as a powerful tool to identify and characterize host factors required for replication of BMV (Ahlquist et al. 2003; Kushner et al. 2003). The demonstration of systemic infection of rice by BMV will allow examination of host factors required for systemic infection, virion formation, and the innate immune responses in a monocot host of BMV. It will be especially interesting to identify the one or more factors that preferentially affect BMV RNA1 replication in rice. These studies will now be possible, given that the Russian strain of BMV can systemically infect *O. sativa* var. Nipponbare, in which genetic tools are increasingly available.

MATERIALS AND METHODS

Virus and plant sources.

Russian strain BMV was typically maintained in wheat. All plants were grown from seeds planted in autoclaved soil and were kept in growth chambers maintained at 24°C, with 16-h-light and 8-h-dark cycles with light set at either 1,500 or 3,000 lux. Rice seeds for Nipponbare and Tainung 67 were provided by the United States Department of Agriculture. Seeds for 46 cultivars of Japonica were gifts from Nouken Co., Ltd.

Virion purification.

Seedlings were inoculated with BMV 10 to 14 days post-germination. Primary leaves of plants were rubbed with a virus solution amended with Carborundum. The standard purification of BMV virions used the method described by Bujarski (1998). The virions were separated in 45% cesium chloride (wt/vol) and were centrifuged for 20 h at 50,000 rpm using a Beckman TLA110 rotor. Each band was collected and reconstituted into a 45% cesium chloride density gradient for a second round of centrifugation for 20 to 24 h at 50,000 rpm. The virus band was collected and dialyzed with three changes of virus buffer (50 mM NaOAc, 10 mM MgCl₂, pH 5.2). The concentration of the virions was determined by spectrophotometric analysis and by comparing the amount of protein stained

with Coomassie blue relative to known quantities of bovine serum albumin. The virions were stored at -80°C in aliquots.

Western blots.

Proteins were extracted by macerating leaf tissue with a mortar and pestle in TB buffer (50 mM Tris-acetate, pH 7.4, 10 mM MgCl_2 , 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol). The lysate was kept on ice for 20 min and was centrifuged at $15,000 \times g$ for 30 min at 4°C , to remove insoluble materials. The supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. After gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane for Western blot analysis with rabbit anti-CP antibody. Horseradish peroxidase-coupled goat antirabbit secondary antibody was used to detect the primary antibody and the signal was developed with the ECL Plus Western blot detection system (Amersham).

Mass spectrometry.

BMV CP mass measurements were made using a Waters Synapt G2 HDMS outfitted with an ion mobility cell. Each sample analyzed used $5 \mu\text{l}$ of a 1 mg/ml solution of purified virions. The spectra were deconvoluted using MaxEnt, part of the MassLynx software package that uses a maximum-entropy approach (Ferroge et al. 1992).

DNA sequencing analysis.

Total RNA (2 μg) was extracted from 28 μg of cesium chloride-purified virions obtained from 100 g of plants, using Trizol reagent (Life Technologies) and was reverse transcribed by M-MLV reverse transcription (New England BioLabs), using a primer common to the 3' terminal sequence of all BMV RNAs. The reverse-transcribed cDNA was amplified by PCR, using primer pairs that produced approximately 1 kb of overlapping fragments that spanned the entire genome. The amplified cDNA was gel-purified using a QIAquick gel extraction kit (Qiagen) and was sequenced with BMV-specific primers using a BigDye Terminator v3.1 cycle sequencing kit (Life Technologies).

Analysis of RNAs in BMV virions.

Cesium chloride-purified BMV virions were dissolved in an equal volume of $2\times$ lysis buffer (200 mM Tris, 2 mM EDTA, 2% SDS, pH 8.5) and were extracted with a 1:1 mixture of phenol/chloroform. The buffer was adjusted to 0.3 M ammonium acetate, and RNA was precipitated with an equal volume of isopropanol overnight at -20°C . The pellet was collected by a 20-min centrifugation at $16,000 \times g$. The RNAs were visualized by denaturing agarose gel electrophoresis and were stained with ethidium bromide as described by Hema et al. (2010).

Real-time RT-PCR analysis.

Total viral RNAs were extracted from 150 mg of wheat or rice leaves using Trizol (Ni et al. 2014). The lysate was extracted with a 50% volume of chloroform solution, followed by precipitating the RNAs with isopropanol. All RNA concentrations were determined by spectrometry followed by analysis using denaturing agarose gel electrophoresis.

RT and real-time PCR to quantify minus-strand BMV RNA1 and RNA2 used 1 μg of total RNA for reverse transcription with a primer corresponding to nucleotides 100 to 120 of RNA1 and 371 to 392 of RNA2 and SuperScript II reverse transcription, as described by the manufacturer (Invitrogen). PCR was performed for 32 cycles with oligonucleotide primers that will amplify nucleotides 100 to 232 of RNA1 and 371 to 499 of RNA2.

ACKNOWLEDGMENTS

We thank J. Yonemaru for providing unpublished data on the phylogenetic lineage of rice cultivars and L. Kao for editing.

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