Host Plant Reaction to Rice Yellow Mottle Virus and Allelic Diversity of *RYMV1* Gene in Rice Cultivars in Uganda

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Abstract

Rice Yellow Mottle Virus (RYMV) disease is endemic to Africa where it affects rice production. Host plant resistance would form a cost effective and sustainable option for managing the disease. However, there is still lack of knowledge on the reaction of rice germplasm and the genetic basis of their resistance/susceptibility to RYMV coupled with lack of molecular markers to facilitate the development of RYMV resistant varieties. We screened 56 rice accessions drawn from breeding lines and varieties commercially cultivated in Uganda for their resistance/susceptibility to RYMV. We also sought to develop and validate allele specific markers for *RYMV1* alleles. The rice accessions showed variation in their reaction to RYMV; 14, 12, 17 and 13 accessions were categorized as resistant, moderately resistant, moderately susceptible and susceptible respectively. Among the resistant accessions, five possessed a single SNP (G/A) corresponding to the *rymv1-2* allele. The new accessions can be deployed as resistant cultivars and/or used to introgress *rymv1-2* allele into susceptible adapted cultivars throughout Africa. We developed two functional allele specific markers, which co-segregated with the *rymv1-2* resistance allele in an F2 population and clearly differentiated between the susceptible and resistant individuals in the F2 population. The functional allele specific marker developed in this study can be used in MAS programs for introgression of *rymv1-2* resistance allele.

Keywords: rice, Rice Yellow Mottle Virus, RYMV1 gene, functional allele specific markers

1. Introduction

In Uganda, rice is important both as a food security crop and a source of income for many smallholder farmers. The total rice produced in Uganda has steadily increased from 177,857 tonnes in 2008 to 200,000 tonnes in 2020 (FAOSTAT, 2020). Rice production is still below the projected quantity of 680,000 Mt required to make Uganda self-sufficient in rice (Arouna et al., 2021). This target has not been achieved to date due to many abiotic and biotic stresses affecting rice production in Uganda. Rice Yellow Mottle Virus (RYMV) disease caused by a sobemovirus is a major disease limiting rice production in lowland and irrigated ecologies in Africa (Kouassi et al., 2005). The virus is not transmissible via seed (Konate et al., 2001) but can be transmitted by insects (Koudamiloro et al., 2015) and mechanically by rubbing inoculum onto the leaves by hand (Pinel-Galzi et al., 2018) or by wind-mediated leaf contact (Sarra et al., 2004). The disease causes mottling and yellowing of leaves to varying intensities depending on genotype, strain of the virus and time of infection (Onwughalu et al., 2010). Yield loss ranges from 10% to 100%, depending on the timing of the infection, virulence of the virus strain and the genotype (Kouassi et al., 2005). In Uganda, RYMV disease incidences as high as 75% have been reported in major rice growing districts (Ochola & Tusiime, 2011).

The management of RYMV disease has mainly centered on cultural practices that prevent build-up of vectors and virus. However, development and deployment of resistant varieties is deemed the most economically viable option. Three RYMV resistance genes (*RYMV1*, *RYMV2* and *RYMV3*) have so far been identified from various rice accessions (Orjuela et al., 2013). *RYMV1*, located on chromosome four encodes a translation initiation

factor, eIF(iso)4G1 (Albar et al., 2006) and four independent *RYMV1* resistance alleles (*rymv1-2*, *rymv1-3*, *rymv1-4* and *rymv1-5*) have been reported in diverse *Oryza sativa* and *Oryza glaberrima* accessions.

Preliminary breeding for RYMV resistance has focused on introgressing the *rymv1-2* allele from *O. sativa* background (Ndjiondjop et al., 2013) as it produces fertile F1 hybrids. This has made the *RYMV1* locus a major focus of analysis in the past decade (Albar et al., 2006; Ndjiondjop et al., 2013; Pidon et al., 2020; Rakotomalala et al., 2008; Thiémélé et al., 2010).

RYMV1 resistance gene and its alleles have been tagged by genetically linked DNA markers or other PCR based markers (Albar et al., 2003, 2006; Thiémélé et al., 2010). As such, indel markers corresponding to 10-100 base pair insertion/deletions and cleaved amplified polymorphic sequences (CAPS) markers corresponding to single nucleotide polymorphisms (SNPs) within the *RYMV1* gene were developed (Albar et al., 2006; Thiémélé et al., 2010). However, indel markers and CAPS require digestion of PCR amplicons for genotyping, making them costly and time consuming; they, thus, cannot be used for routine germplasm screening for RYMV resistance. In addition, owing to genetic recombination, the genetically linked markers may give rise to false positives (Frisch et al., 1999). Allele specific markers that target polymorphism within a gene of interest provide more efficient selection of desired genotypes compared to DNA markers at a nearby but functionally irrelevant site (Andersen & Lübberstedt, 2003). There is, therefore, need to develop alternative, rapid, accurate and affordable SNP-based tools for screening *RYMV1* alleles.

Attempts have been made to screen some of the rice accessions used in this study for resistance to RYMV using visual assessment of symptoms progress (Mogga et al., 2012; Ndikuryayo et al., 2020). However, there is lack of knowledge on the genetic basis of RYMV resistance of most of the accessions with reference to the *RYMV1* gene allele diversity. In addition, there is a limited number of molecular makers to facilitate/accelerate routine screening for RYMV resistant accessions and breeding improved varieties that are resistant to RYMV. The objectives of this study were to: 1) to screen rice accessions for their reaction to RYMV; 2) determine the allele diversity of the *RYMV1* resistance gene in selected rice accessions that have shown resistance or tolerance to RYMV in Uganda, and 3) develop and validate SNP markers that are useful for high throughput genotypic selection for RYMV resistance.

2. Method

2.1 Source and Description of Materials

Fifty-six rice accessions were selected from a collection of rice germplasm that is currently held at the National Crops Resources Research Institute (NaCRRI). The germplasm collection was assembled from rice breeding centers around the world to support rice breeding efforts in Uganda (Lamo et al., 2021) and were selected based on aroma and resistance to RYMV (Table 1). The checks included IR64 (susceptible to RYMV) and Gigante (resistant to RYMV) for phenotypic evaluation, and Gigante, Tog5681, Tog5672, and Tog5674 for genotypic evaluation (Table 1). Gigante, Tog5681, Tog5672, and Tog5674 were chosen because they carry the *rymv1-2*, *rymv1-3*, *rymv1-4* and *rymv1-5* recessive alleles, respectively. An F2 population was developed from the cross between accession ARS126-3-B-1-2 and SUPA5 for the purpose of validating new markers. ARS126-3-B-1-2 is resistant to RYMV and carries the *rymv1-2* allele while SUPA5 is susceptible to RYMV and lacks the *rymv1-2* allele but is also highly aromatic, a trait that is highly sought after by consumers.

Table 1. List and origin of rice accessions evaluated for reaction to RYMV at NaCRRI in 2018 and 2019

Entry No.	Name	Designation	Origin/Center of breeding	Species/subspecies	Aromatic status	Remarks
1	Tog5674	Tog5674	ARC	*Oryza glaberrima*	Non-aromatic	Resistant check
2	Tog5672	Tog5672	ARC	*Oryza glaberrima*	Non-aromatic	Resistant check
3	Tog5681	Tog5681	ARC	*Oryza glaberrima*	Non-aromatic	Resistant check
4	MET3	ART35-114-1-6N-2	ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
5	MET 4	ART34-146-1-8N-1	ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
6	MET 6	ART35-49-1-4N-1	ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
7	MET12	ART34-88-1-2-B-1	ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
8	MET 13	ART34-113-3-2-B-1	ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
9	MET14	ART34-256-3-1-B-2	ARC	*Oryza barthii/sativa/indica*	Aromatic	Breeding line
10	MET15		ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
11	MET16	ART35-272-1-2-B-1	ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line

12	MET30		ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
13	MET40	ART27-190-1-4-2-1-1-3	ARC	*Oryza barthii/sativa/japonica*	Aromatic	Breeding line
14	MET60	PCT-4\0\0\1>295-2-3-1-3-3-M	ARC	*Oryza barthii/sativa/japonica*	Non-aromatic	Breeding line
15	MET70	PCT-4\SA\5\1>1754-5-1-5-3-1-M	ARC	*Oryza barthii/sativa/japonica*	Non-aromatic	Breeding line
16	ARC36-2-1-2	ARC36-2-1-2	ARC	*Oryza sativa/indica*	Non-aromatic	Breeding line
17	ARC36-2-P-2	ARC36-2-P-2	ARC	*Oryza sativa/indica*	Non-aromatic	Breeding line
18	ARC36-4-EP-2	ARC36-4-EP-2	ARC	*Oryza sativa/indica*	Non-aromatic	Breeding line
19	ARC39-145-P-3	ARC39-145-P-3	ARC	*Oryza sativa/indica*	Non-aromatic	Breeding line
20	ARC39-145-P-2	ARC39-145-P-2	ARC	*Oryza sativa/indica*	Non-aromatic	Breeding line
21	ARS126-3-B-1-2	ARS126-3-B-1-2	ARC	*Oryza sativa/indica*	Non-aromatic	Breeding line
22	IR64	IR64	IRRI	*Oryza sativa/indica*	Non-aromatic	Susceptible check
23	SUPA1052	SUPA V88*2/IR09F154	IRRI	*Oryza sativa/indica*	Aromatic	Breeding line
24	SUPA1	IR 97011-6-2-4-1	IRRI	*Oryza sativa/indica*	Aromatic	Breeding line
25	SUPA2	IR 97011-7-5-2-B	IRRI	*Oryza sativa/indica*	Aromatic	Breeding line
26	SUPA3	IR 97011-7-7-3-1-B	IRRI	*Oryza sativa/indica*	Aromatic	Breeding line
27	SUPA4	IR 97011-7-7-3-1-B	IRRI	*Oryza sativa/indica*	Aromatic	Breeding line
28	SUPA5	IR 97011-16-2-4-B	IRRI	*Oryza sativa/japonica*	Aromatic	Breeding line
29	SUPA6	IR 9712-4-1-2-1-1	IRRI	*Oryza sativa/indica*	Aromatic	Breeding line
30	Gigante	Gigante	IRRI	*Oryza sativa/indica*	Non-aromatic	Resistant check
31	Komboka	IR 05N 221	IRRI	*Oryza sativa/indica*	Aromatic	Released variety
32	Basmati370	Basmati370	IRRI	*Oryza sativa/japonica*	Aromatic	Breeding line
33	WITA9	WITA9	IRRI	*Oryza sativa/indica*	Non-aromatic	Released variety
34	Supa Local	Supa Local	NaCRRI	*Oryza sativa/indica*	Aromatic	Landrace
35	AGRA41	AGRA-CRI-UPL-3-4	CRI, Ghana	*Oryza sativa/japonica*	Aromatic	Breeding line
36	AGRA60	AGRA-CRI-UPL-4-13	CRI, Ghana	*Oryza sativa/japonica*	Aromatic	Breeding line
37	AGRA65		CRI, Ghana	*Oryza sativa/japonica*	Aromatic	Breeding line
38	NamChe1	WAB95-B-B-40-HB	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Released variety
39	NamChe 2	NM7-8-2-B-P-11-6	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Released variety
40	NamChe 3	NM7-29-4-B-P-80-8	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Released variety
41	NamChe 4	ART3-11L1P1-B-B-2	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Released variety
42	NamChe 5	NM7-27-1-B-P-77-6	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Released variety
43	NamChe 6	NM7-5-2-B-P-79-7	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Released variety
44	E20	IRAT 325/WAB 365-B-1H1-HB	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Breeding line
45	E22	NM7-22-11-B-P-1-1 (WAB 450-1-BL1-136-HB/ WAB 450-B-136-HB)	NaCRRI	*Oryza sativa/japonica*	Non-aromatic	Breeding line
46	NERICA1	WAB 450-1-B-P-38-HB	ARC	*Oryza sativa/japonica*	Non-aromatic	Released variety
47	NERICA4	WAB450-1-B-P-91-HB	ARC	*Oryza sativa/japonica*	Non-aromatic	Released variety
48	NERICA8		ARC	*Oryza sativa/japonica*	Non-aromatic	Released variety
49	NERICA10	WAB 450-11-1-1P41-HB	ARC	*Oryza sativa/japonica*	Non-aromatic	Released variety
50	1189 line	IR77454-22-B-20-2-2-B-TGR1	ARC	*Oryza sativa/japonica*	Non-aromatic	Breeding line
51	1190 line	IR77454-22-B-20-2-2-B-TGR2	ARC	*Oryza sativa/japonica*	Non-aromatic	Breeding line
52	1191 line	IR77454-13-B-18-2-B-B-ARC3	ARC	*Oryza sativa/japonica*	Non-aromatic	Breeding line
53	Kafaci326104		Korea	*Oryza sativa/indica*	Non-aromatic	Breeding line
54	Sande		Tanzania	*Oryza sativa/indica*	Aromatic	Breeding line
55	TXD306	TOX 3058-28-1-1-1	Tanzania	*Oryza sativa/indica*	Non-aromatic	Breeding line
56	Jaribu	Jaribu	Tanzania	*Oryza sativa/indica*	Aromatic	Breeding line

Note. Africa Rice Center (ARC), International Rice Research Institute (IRRI), Tanzania, National Crops Resources Research Institute (NaCRRI), Crop Research Institute (CRI).

2.2 Source of RYMV Isolates

Isolates of RYMV were obtained from rice fields in RYMV hotspots: Iganga in the eastern region of Uganda at coordinates 00°37′960″N; 33°32′996″E (Iganga isolate) and from NaCRRI, Namulonge in Central Uganda at coordinates 00°31′834″N; 32°37′443″E (Namulonge isolate). The isolates were multiplied and maintained separately on the standard susceptible cv. IR64.

2.3 Description of Study Site

2.3.1 Phenotypic Evaluation

Phenotyping for Rice Yellow Mottle Virus was done at NaCRRI, Namulonge, Wakiso district. NaCRRI is about 30 km northeast of Kampala in the central region of Uganda at the coordinates $0^o31'30''$ N $32^o36'54''$ E (Latitude: 0.5250 and Longitude: 32.6150). The area has a tropical climate with a bimodal rainfall regime; the first rainy season begins in March and ends in May and the second from August to December. The rest of the months are relatively dry and hot. The average annual rainfall and temperature are 1242 mm and 21.7 ^{o}C, respectively. The elevation is 1160 m above sea level, with undulating topography. The soils are mainly oxisols (ferralsols) in the plains and hills, and vertisols in the swamps and valleys (Ndikuryayo et al., 2020).

2.3.2 Genotypic Evaluation

Genotyping for *RYMV1* alleles was done at the Biotechnology Laboratory at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK) in Kampala, Uganda.

2.4 Multiplication and Maintenance of Isolates

The isolates were multiplied using rice cv IR64 which was planted in six 15-litre rectangular plastic buckets filled with black forest soil. Two weeks post germination, rice plants in three buckets were inoculated with RYMV isolate from Iganga and the plants in the other three buckets were inoculated with isolates from Namulonge and kept in separate screenhouses. Symptomatic plants were used as sources of inoculum.

2.5 Experimental Set up

Individual rice accessions were planted in 15-litre rectangular plastic buckets filled with black forest soil. Nine seeds were planted per bucket in two separate screen houses following a randomized complete design with two replications. The plants were watered every other day to keep the soil moist. Weeding was done by hand picking. Fertilizer application followed two regimes; Urea (10 g per bucket) applied at 21 days after planting (DAP) and NPK (10 g per bucket) applied 42 DAP to coincide with booting.

2.6 Inoculation of Test Materials

Two weeks post germination; all nine plants per bucket were inoculated with RYMV virus isolates following the procedure of Pinel-Galzi et al. (2018). Infected leaves of cv IR64 on which the isolates were maintained were picked and cut in 0.5 to 1 cm pieces and placed in a mortar and finely ground. Distilled water was then added at a ratio of 10 ml of water to 1 g of leaves. Using a piece of paper towel dipped into the inoculum, each leaf was rubbed twice from the base to the tip.

2.7 Phenotyping for Resistance to Rice Yellow Mottle Virus Disease

Symptom intensity on leaves was monitored weekly from 14 days post inoculation (dpi) until anthesis of the earliest maturing accession (35 dpi). The standard evaluation system (SES) for rice was used to phenotype for RYMV disease using a scale of 1-9 where; 1 = No symptom observed; 3 = Leaves green but with sparse dots or streaks; 5 = Leaves green or pale green with mottling; 7 = Leaves pale yellow or yellow and 9 = Leaves turn yellow or orange, no flowering or some plants dead (IRRI, 2013).

2.8 Genotyping for RYMV1 Alleles

2.8.1 DNA Extraction and Analysis

Two weeks post germination just before inoculation, one leaf was picked from each of the nine plants per accession, wrapped in aluminum foil and stored at-80 °C. Leaf tissues of equal sizes were pooled from all nine plants to make a composite sample per accession. Genomic DNA was extracted from these leaves using the cetyl trimethylammonium bromide lysis buffer. Briefly, ground leaf powder was suspended in 1 ml of buffer solution kept at 65 °C for 1 hr. Upon cooling for 5 minutes a 24:1 chloroform:isoamyl alcohol mixture was added and mixed thoroughly, then span for 20 minutes at 10,000 rpm. The aqueous layer was transferred to a fresh tube and an equal volume of ice-cold isopropanol was added and inverted 10 times and then span for 30 minutes to precipitate the DNA. The supernatant was discarded, and the pellet was washed with 70% ethanol and air dried. The dry pellet was then dissolved in 250 μL of $1 \times$ TE buffer solution. The quality of DNA was assessed by running an aliquot of 5 μL of each extracted DNA sample in a 1% agarose gel electrophoresis stained with EZ^{TM} (0.8 μg^{-1} mL) (AMRESCO, Ohio, USA). The concentration and purity of DNA was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA USA) at wavelengths 230, 260 and 280 nm.

2.8.2 Primers and Primer Design

Primers that amplify the regions of *rymv1-2*, *rymv1-3*, *rymv1-4*, and *rymv1-5* alleles of the *RYMV1* resistance gene were designed from nucleotide sequences retrieved from the NCBI website corresponding to the *RYMV1* locus in cvs. Gigante (carrying the *1-2 allele*), Tog5681 (carrying the *1-3 allele*), Tog5672 (carrying the *1-4 allele*) and Tog5674 (carrying the *1-5 allele*). Primers Rymv1A and Rymv1B were designed using Primer1, a web primer design program (Collins & Ke, 2012). Additional primers obtained from Thiémélé et al. (2010) and kindly provided by Professor Laurence Albar (Institute of Research for Development (IRD), Marseille, France) were also used to study the *RYMV1* allele diversity (Table 2).

Table 2. Primers used to assess allele variation of *RYMV1* gene, their sequences, product size and source

Primer	Sequence	Product Size	Source
Rymv1A_EXT(F)	GTCCGATGTATGCTCAACTTTGTTCCGA	327bp (two outer primers)	
Rymv1A_EXT(R)	TATCAGGTCCAGACCCCAAGAGTTCCTG		
Rymv1A_INT(F)	TGAAGGCGCTGAGAGCCTAAGGGATA	204bp (A allele)	This study
Rymv1A_INT(R)	GGTCAGGGCCAGTCAATTTTGCTATGTC	177bp (G allele)	
Rymv1B_EXT(F)	TATTTGAGAAGGCTGTTTTTGAGCCCAC		
Rymv1B_EXT(R)	AATAGCCTCAACGTTTTCCTCTTCAGGG		
Rymv1B_INT(F)	ATTGACTGGCCCTGACCAAGAGACGGA	450bp (1-5 allele)	This study
Rymv1B_INT(R)	TTTGACAATCCTTTCTTTGTCCCTTCTATC		
*F1R1	F: CACGTCGGCGGCGCATCCAAG	725bp (*RYMV1* gene)	Thiémélé et al. (2010)
	R: CGAACACGCTCGCGCACCTCA		
F2R2	F: GAGCCCACCTTCTGTCCGATG	700bp (1-5 allele)	Thiémélé et al. (2010)
	R: CAGGGCCAGTCAATTTTGCTATTTC		
F2R6	F: GAGCCACCTTCTGTCCGATG	197/700bp (1-5 allele/wt)	Thiémélé et al. (2010)
	R: CCATCTCTTGGTCAGGGTTTGC		
F3R4	F: CCTTGGTCAGCTAGAAGAGGCA	700/1200bp (1-5 allele/wt)	Thiémélé et al. (2010)
	R: CCTCGGTACAACCAAGAGAC		
F4R3	F: TGGCCCTGACCAAGAGATGAa	500/700bp (1-5 allele/wt)	Thiémélé et al. (2010)
	R: CTCTTCACGTCGAGGCACCCA		
Rymv1D	F: CCTTGGTCAGCTAGAAGAGGCA	675bp	Laurence Albar
	R: AGTAGCTCACCAATTAGACGGA		

Note. *F1R1 is an internal control primer for the entire *RYMV1* gene.

2.8.3 Polymerase Chain Reaction Using Allele Specific Markers

Genomic DNA from fifty-six accessions was subjected to Polymerase Chain Reaction (PCR) amplification each performed in a final volume of 10 μL containing 1 μL of 50 ng template DNA, 1 μL of each primer, 5μL of AccuPower PCR Premix (Bioneer Corporation, Daejeon, South Korea) and topped up using sterile distilled water. Primer Rymv1D was excluded in this stage because it was not producing clear bands separation after digestion and electrophoresis. The PCR cycle was programmed for an initial 5-min at 95 °C; the annealing temperatures were progressively reduced from 64 to 56 °C for 8 cycles and then maintained at 56 °C for the remaining 27 cycles and a final extension of 5 min at 72 °C. The amplification products were separated using a 1.5% agarose gel stained with EZ^{TM} (0.8 ug^{-1} mL) (AMRESCO, Ohio, USA) and viewed under UV light using BioDoc-It^{TM} Imaging System (Applied BiosystemsWaltham, Massachusettes, USA). A 100 bp ladder (Bioneer Corporation, Daejeon, Korea) was used to estimate the PCR fragment sizes.

2.8.4 Polymerase Chain Reaction Using primer Rymv1D

Based on the RYMV resistance phenotyping and genotyping, 38 accessions that were resistant or tolerant to either RYMV isolates (disease score: 1 to 5) were selected for further molecular screening to confirm the allelic status of their *RYMV1* gene and to assess the frequency of *RYMV1* alleles. Four susceptible accessions (IR64, SUPA5, Supa Local and Basmati370) were added as controls. Genomic DNA from the 42 accessions were further subjected to PCR using primer Rymv1D following the same PCR conditions as the allele specific markers.

2.8.5 DNA Sequencing and Sequence Analysis

PCR products of primer Rymv1D were shipped to Macrogen Europe B.V (Amsterdam, the Netherlands) for sequencing. The PCR products were sequenced in the reverse direction using primer Rymv1D (R) (AGTAGCTCACCAATTAGACGGA) to obtain partial sequences of the *RYMV1* gene. Each fragment was sequenced at least four times and high-quality consensus sequences were used for data analysis.

2.8.6 Analysis of Sequences for Presence of RYMV1 Alleles

Sequences were manually edited by trimming off the trace data and reverse complements obtained using MEGA version X (Kumar et al., 2018) before aligning using Clustal W (Thompson et al., 1994). Multiple sequence alignments were done to identify the presence of insertions/deletions and SNPs in the accessions. The allele sequence obtained from each accession was compared with that of the gene sequences from Gigante, Tog5672, Tog5674, Tog5681 and IR64 (possessing the susceptibility allele). Allelic and nucleotide diversity were then analyzed for percentage sequence identity between alleles by comparing the percentage sequence identity between allele and reference sequence by pairwise alignment using NCBI BLAST (Johnson et al., 2008).

2.9 Development and Application of Functional Markers in Segregating F₂ Population

Sequence divergences distinguishing the *rymv1-2* allele from other alleles were used to develop markers by designing flanking primers, which were then used to genotype the F2 population. The genome sequence portion of ~50 bp flanking the target SNP on either side was sent to LGC genomics (Hoddesdon, UK) to design the Kompetitive Allele-Specific PCR (KASP) primer Rymv1-2kbd. The KASP assay was used to interrogate the SNP (G/A) corresponding to the *rymv1-2* allele of the *RYMV1* gene in 40 individuals of an F2 segregating population generated from a cross between ARS126-3-B-1-2 and SUPA5. Primer Rymv1A was also converted to a marker as it gave rise to unambiguous polymorphic PCR products and used to genotype 71 individuals of the same F2 population.

KASP assay genotyping was performed using the LGC SNPline system following standard KASP protocols (LGC Genomics) at the Makerere University Agricultural Research Institute Kabanyolo (MUARIK) Biotechnology Laboratory using the Real Time PCR machine 7500 (Applied Biosystems, Waltham, Massachusetts, USA). The KASP genotyping mix was prepared in a 96 well plate containing 5uL of DNA, 5uL of 2x-KASP master mix, 0.14 KASP assay mix and topped up to 10 μL using sterile water. The following cycling conditions were used: Stage 1: 30 ℃ 60s (pre-read); Stage 2: 94 ℃ for 15 min hot start *Taq* activation (1 cycle); Stage 3: 94 ℃ for 20 s, 61 ℃ (61 ℃ decreasing by 0.6 per cycle to achieve a final annealing/extension temperature of 55 ℃) for 60 s (10 cycles); Stage 4: 94 ℃ for 20 s, 55 ℃ for 60 s (29 cycles); Stage 5: 94 ℃ for 20 s, 57 ℃ for 60 s (3 cycles): Stage 6: 37 ℃ for 60 s (1 cycle cooling) followed by an end point florescent read. The quality of genotyping cluster plot was visually assessed and only samples in distinct clusters were considered for manual SNP calling using 7500 v 2.3 software (Life Technologies Corporation, 2019) incorporated in the 7500 real time PCR machine.

An endpoint multiplexed PCR amplification was performed using the functional marker Rymv1A in a final volume of 10 μL containing 1 μL of DNA template (50 ng), 1 μL of each primer and 5 μL of AccuPower PCR Premix (Bioneer Corporation, Daejeon, South Korea). The final reaction volume was completed to 10 μL using sterile distilled water. The PCR cycle was programmed for an initial 5 min at 95 °C; the annealing temperatures were progressively reduced from 68 °C to 60 °C for 8 cycles and then maintained at 60 °C for the remaining 27 cycles and a final extension of 5 min at 72 °C.

2.10 Data Analyses

Analysis of variance (ANOVA) was performed to determine the effect of accession, time of symptom assessment and isolate on the severity of RYMV using R version 3.6.3 software (R Core Team, 2017). Severity data were also used to compute the area under symptom progress curve (AUSPC) using the method of Thiémélé et al. (2010) as:

$$AUSPC = \sum \frac{(S_i + S_{i+1} - 2)(t_{i+1} - t_i)}{2} \qquad (1)$$

where, S_i corresponds to the severity score at the date t_i, in days, S_{i+1} corresponds to severity score at date two and $t_{(i+1)}$ corresponds to date two. The AUSPC values were then arranged in descending order and the mean and standard deviation calculated. The accessions were then categorized into various groups based on the standard deviation values. Accessions with SD values falling to the right of the mean (positive) on the mean distribution curve were considered either as moderately susceptible ($0 < SD < 1$), susceptible ($1 < SD < 2$) or highly susceptible ($SD > 2$) while accessions falling to the left (negative) of the mean were considered either as

moderately resistant (0 < |SD| < 1), resistant (1 < |SD| < 2) or highly resistant (|SD| > 2) (Ariyo et al., 2002; Ohunakin et al., 2019).

3. Results

3.1 Reaction of Rice Accessions to Rice Yellow Mottle Virus in Uganda

There were significant differences between rice accessions (P-value $< 2 \times 10^{-16}$), time of disease assessment (P-value $< 2 \times 10^{-16}$) and the interaction between accession and time of disease assessment (P-value $< 2 \times 10^{-16}$) on the severity of RYMV. The resistant accessions were ARC36-4-EP-2, ARC39-145-P-3, ARC39-145-P-2, ARS126-3-B-1-2, MET14 and NamChe1, while the susceptible accessions were, WITA9, TXD306, Komboka, Jaribu and the supa series (SUPA1, SUPA3, SUPA4, SUPA5, SUPA6) and SupaLocal among others.

Accessions displayed significantly different (P-value $< 2 \times 10^{-16}$) reaction patterns over the time of disease assessment (Figure 1). The resistant accessions did not develop RYMV symptoms even after 35 dpi while the most susceptible accessions; IR64, SUPA1, Jaribu and Komboka developed initial symptoms of RYMV before 14 dpi (as early as 10 dpi). By 14 dpi some accessions from IRRI Supa series (2, 3, 4, 5, and 6) including IR64 and Komboka had already registered a score of 7 on the severity scale of 1-9 (Figure 1). At 35 dpi, most of the susceptible accessions had a score of 5 and above with varying symptoms ranging from delayed flowering or incomplete emergence of the panicles to no flowering, plant height reduction and in extreme cases, death of plants.
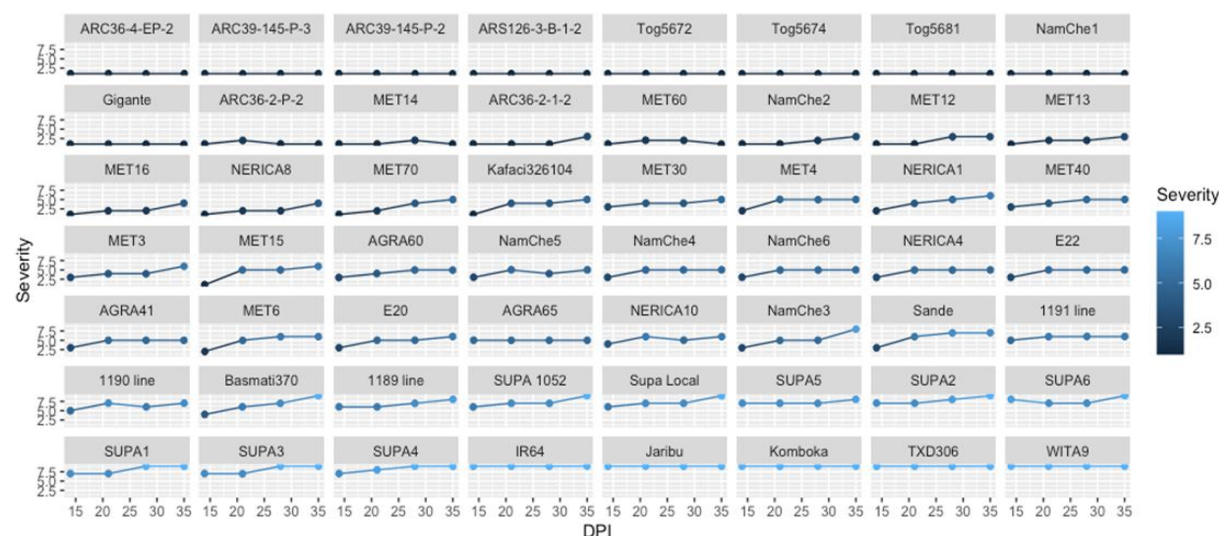

Figure 1. Graphs showing the reaction to and progress of RYMV symptom severity on individual accessions over time in days post inoculation (DPI)

The isolates and their interaction with accessions did not influence the severity of RYMV disease and AUSPC (P > 0.05). The AUSPC was significantly affected by accessions (P-value $< 2 \times 10^{-16}$) but was not affected by isolate (P-value = 0.916) and the interaction between accession and isolate (P-value = 1.000). The AUSPC values of the different accessions ranged from 0 to 168 with a mean of 73.437 and standard deviation of 55.342 (Table 3). There were 14, 12, 17 and 13 accessions categorized as resistant, moderately resistant, moderately susceptible and susceptible respectively (Table 3).

Table 3. The reaction of rice accessions for resistance to Rice Yellow Mottle Virus Disease

Genotypes	AUSPC	SD	Reaction
IR64	168	1.73	Susceptible
Jaribu	168	1.73	Susceptible
Komboka	168	1.73	Susceptible
TXD306	168	1.73	Susceptible
WITA9	168	1.73	Susceptible
SUPA4	154	1.47	Susceptible

SUPA1	147	1.35	Susceptible
SUPA3	147	1.35	Susceptible
SUPA2	140	1.22	Susceptible
SUPA6	136.5	1.15	Susceptible
SUPA 1052	129.5	1.03	Susceptible
Supa Local	129.5	1.03	Susceptible
SUPA5	129.5	1.03	Susceptible
1189 line	119	0.84	Moderately Susceptible
Basmati370	115.5	0.77	Moderately Susceptible
1190 line	112	0.71	Moderately Susceptible
Sande	105	0.58	Moderately Susceptible
1191 line	101.5	0.52	Moderately Susceptible
NERICA10	91	0.33	Moderately Susceptible
NamChe3	87.5	0.26	Moderately Susceptible
AGRA65	84	0.20	Moderately Susceptible
MET6	84	0.20	Moderately Susceptible
E20	80.5	0.14	Moderately Susceptible
AGRA41	77	0.07	Moderately Susceptible
E22	77	0.07	Moderately Susceptible
NamChe4	77	0.07	Moderately Susceptible
NamChe6	77	0.07	Moderately Susceptible
NERICA4	77	0.07	Moderately Susceptible
MET15	73.5	0.01	Moderately Susceptible
MET4	73.5	0.01	Moderately Susceptible
AGRA60	70	-0.05	Moderately Resistant
MET40	70	-0.05	Moderately Resistant
NamChe5	70	-0.05	Moderately Resistant
NERICA1	70	-0.05	Moderately Resistant
MET3	66.5	-0.12	Moderately Resistant
MET30	63	-0.18	Moderately Resistant
Kafaci326104	56	-0.31	Moderately Resistant
MET70	42	-0.56	Moderately Resistant
MET16	24.5	-0.88	Moderately Resistant
NERICA8	24.5	-0.88	Moderately Resistant
MET12	21	-0.95	Moderately Resistant
MET13	21	-0.95	Moderately Resistant
MET60	14	-1.07	Resistant
NamChe2	14	-1.07	Resistant
ARC36-2-1-2	7	-1.20	Resistant
ARC36-2-P-2	7	-1.20	Resistant
MET14	7	-1.20	Resistant
ARC36-4-EP-2	0	-1.33	Resistant
ARC39-145-P-3	0	-1.33	Resistant
ARC39-145-P-2	0	-1.33	Resistant
ARS126-3-B-1-2	0	-1.33	Resistant
Gigante	0	-1.33	Resistant
NamChe1	0	-1.33	Resistant
Tog5672	0	-1.33	Resistant
Tog5674	0	-1.33	Resistant
Tog5681	0	-1.33	Resistant
Grand mean	**73.4375**		
Standard deviation	**55.3418**		

Accessions with positive SD between zero and one ($0 < SD < 1$) were considered as moderately susceptible, those with positive SD between 1 and 2 ($1 < SD < 2$) were considered susceptible while accessions with negative

SD falling between zero and negative one (0 < |SD| < 1), were considered as moderately resistant and those with negative SD falling between 1 and 2 (1 < |SD| < 2) were considered as resistant.

3.2 Occurence of RYMV1 Alleles Using Allele Specific Primers

Using Primer Rymv1A, we identified five accessions (ARC36-2-1-2, ARC36-4-EP-2, ARC39-145-P-3, ARC39-145-P-2 and ARS126-3-B-1-2) that possessed the *rymv1-2* allele previously identified in Gigante. Five other primers also confirmed the presence of the *rymv1-5* allele in Tog5674 as previously identified by Thiémélé et al. (2010). Surprisingly, two primers; F3R4, and F4R3 detected the presence of *rymv1-5* allele in a susceptible accession Supa Local and a resistant accession MET12, respectively (Table 4). The *rymv1-3* and *rymv1-4* alleles were not detected by any of the primers used in this study.

Table 4. Rice Yellow Mottle Virus resistance alleles detected in different accessions using allele specific primers

Primer	Allele				Accessions
	rymv1-2	*rymv1-3*	*rymv1-4*	*rymv1-5*	
Rymv1A	+	-	-	-	Gigante, ARC36-2-1-2, ARC36-4-EP-2, ARC39-145-P-3, ARC39-145-P-2, ARS126-3-B-1-2
Rymv1B	-	-	-	+	Tog5674
F2R2	-	-	-	+	Tog5674
F2R6	-	-	-	+	Tog5674
F3R4	-	-	-	+	Tog5674, Supa Local
F4R3	-	-	-	+	Tog5674, MET12

Note. "-" and "+" indicates absence or presence respectively of a given resistance allele.

3.3 Occurence of RYMV1 Resistance Alleles Using Sequence Analysis of PCR Products

Eight out of the 42 accessions had poor quality sequences and were excluded from further analysis. For the remaining 34 accessions, sequence lengths ranged between 612 and 622 nucleotides after trimming. Percentage identity of the nucleotide sequences of the alleles ranged between 98.32 to 100% in comparison to alleles sequences from Tog5672, Tog5674, Tog5681 and Gigante. Alignment of sequences of PCR products obtained from primer Rymv1D found five accessions (ARC36-2-1-2, ARC36-4-EP-2, ARC39-145-P-3, ARC39-145-P-2 and ARS126-3-B-1-2) possessing a single SNP (G/A) corresponding to the *rymv1-2* allele profile of Gigante. The results also revealed the presence of a 9 bp deletion in the genotype Tog5681 and Tog5674 corresponding to the *rymv1-3* allele and *rymv1-5* allele, respectively (Figure 2). The SNP associated with genetic basis of RYMV resistance in Tog5672 previously identified by Thiémélé et al. (2010) could not be determined. None of the other accessions had the profiles of Tog5681, Tog5672 and Tog5674. Supa Local and MET12 did not contain the *rymv1-5* allele as earlier indicated by primer F3R4 and F4R3.

Although all mutations characteristic of the resistant accessions occurred in a very small interval of 45 nucleotides, a section of the sequence alignment between 490 and 610 nucleotides is depicted to include polymorphisms that are not associated with RYMV resistance but rather have been reported (Pidon et al., 2020) to distinguish between the different rice species and subspecies (Figure 3). A single SNP (C/A) characterized the *O. glaberrima* and two SNPs (C/T) characterized the *O. sativajaponica* accessions while a single SNP (A/G) characterized the *O. sativa indica* accessions (Figure 3).

Figure 2. Sequence alignment showing the allelic diversity in *RYMV1* gene. The alleles were named as cited in Thiémélé et al. (2010) and Pidon et al. (2020). (a) corresponds to *rymv1-2* allele in Gigante, ARC36-2-1-2, ARC39-145-P-2, ARC39-145-P-3, ARC36-4-EP-2 and ARS126-3-B-1-2; (b) *rymv1-5* allele in Tog5674; (c) *rymv1-3* allele in Tog5681

Figure 3. Variants of *RYMV1* gene at nucleotide level. Number and position of nucleotides is indicated at the top of the figure. Mutation characteristics of the resistant accessions are indicated in red while mutations that are not associated with RYMV resistance are indicated in blue. Alleles and species are indicated on the left while numbers of accessions are indicated on the right of the figure

3.4 Application of Functional Markers in the Segregating F_2 Population

End point multiplexed PCR analysis detected a 204-bp fragment in either a homozygous or heterozygous pattern in all resistant accessions. In contrast, PCR amplification yielded a 177-bp fragment in all susceptible plants (Figure 4). Of the 71 F2 individuals genotyped, 20 were confirmed to be genotypically homozygous at the *RYMV1* gene locus (*rymv1-2* allele), and the remaining 33 individuals were heterozygous at the *RYMV1* gene locus (containing alleles from both parents). The segregation ratio, 1:2:1, perfectly matched that of co-dominant monogenic inheritance.

Using the KASP primer Rymv1-2kbd, 12 individuals were homozygous for allele A, six individuals were homozygous for allele G and 18 individuals were heterozygous for both alleles (Figure 5). The SNP identity of four individuals was undetermined.
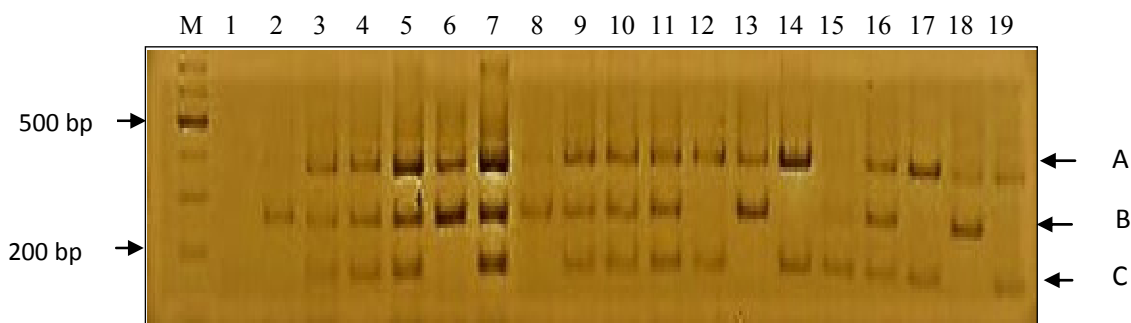
Figure 4. PCR amplification patterns of allele specific maker Rymv1A that differentiated *rymv1-2* alleles in a random selection of F2 segregating population (1 to 17) and parents (18: Gigante, 19: Supa 5). A: 327 bp (two outer primers) external control band, B: 204 bp (*rymv1-2* allele/A allele); C: 177 bp (G allele); M: DNA ladder
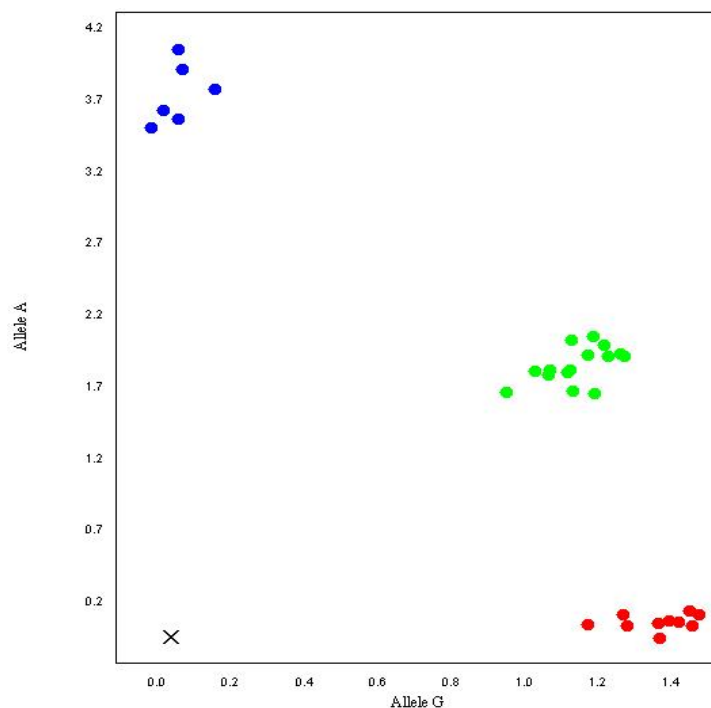
Figure 5. Allele discrimination plot of KASP primer Rymv1-2kbd distinguishing *rymv1-2* allele in a F2 population. Blue represents homozygous A allele, red represents homozygous G allele, green represents heterozygous A/G allele and X represents no template negative control

4. Discussion

We combined phenotype-based methods with molecular-based methods to identify new sources of resistance to RYMV. RYMV disease severity and AUSPC were significantly affected by accessions and the interaction between accession and time of disease scoring. Isolates and time of disease scoring had no significant effect on both RYMV severity and AUSPC. *rymv1-2* allele was detected in five accessions and two new alleles specific markers clearly identified individuals carrying the *rymv1-2* allele in a segregation F2 population. We developed two functional allele specific markers, which co-segregated with the *rymv1-2* resistance allele in an F2 population and clearly differentiated between the susceptible and resistant individuals in the F2 population.

The significant effects of accession and interaction between time of disease score and accession (*i.e.*, differences in the disease progression in the accessions over time) can be explained by the genetic differences between the accessions. Some accessions (ARC36-2-1-2, ARC36-4-EP-2, ARC39-145-P-3, ARC39-145-P-2, ARS126-3-B-1-2) did not develop symptoms even after 35 dpi due to the presence of *rymv1-2* allele of the *RYMV1* gene. The *RYMV1* gene acts by restricting cell to cell movement of the virus from the point of inoculation thus preventing symptom development (Ndjiondjop et al., 2001). Four accessions, NamChe1, NamChe2, MET14 and MET60 were resistant and did not either develop any symptoms or had very mild symptoms of RYMV and yet they had no *RYMV1* gene. Their resistance could possibly be due to the presence of other resistance genes or loci. Thiémélé et al. (2010) and Pidon et al. (2017) reported the existence of two other resistance genes; *RYMV2* and *RYMV3*, respectively associated with RYMV resistance in rice. In our study, however, we limited ourselves to *RYMV1* gene. Subsequent studies will be required to identify the alleles of other RYMV resistance genes existing in these accessions.

The significant difference between the AUSPC of the different accessions indicated that accessions displayed different symptom intensities. Accessions IR64, Supa1, Jaribu and Komboka were the most susceptible accessions recording the highest value of AUSPC irrespective of the isolate used while ARC36-2-1-2, ARC36-4-EP-2, ARC39-145-P-3, ARC39-145-P-2, ARS126-3-B-1-2 and NamChe1 were resistant to RYMV with lowest AUSPC values (zero) recorded. With exception of NamChe1 which has already been released as a commercial variety in Uganda, these accessions can be evaluated further in multilocation trials and released as new varieties or be used as parents for breeding for resistance to RYMV.

While lack of symptoms on leaves after virus inoculation has been associated with high resistance, delayed symptom onset and lower symptom severity has been associated with partial resistance (moderately resistant). Partial resistance has previously been associated with upland rice *O. sativa, japonica* subspecies (Ioannidou et al., 2000). Indeed, with the exception of Kafaci326104, 11 out of the 12 moderately resistant accessions that showed partial resistance (moderately resistant) were upland *O. sativa, japonica* subspecies. Partial resistance to plant viruses has been attributed to incomplete penetrance of resistance (Gallois et al., 2018) or possible resistance breakdown (Hébrard et al., 2018). *Oryza glaberrima* shows more frequent high resistance and less frequent partial resistance (Pidon et al., 2020) as all three *glaberrima* accessions in this study were highly resistant to the RYMV isolates. Resistance in these accessions was confirmed to be due to the presence of the *RYMV1* gene also reported in other literature (Thiémélé et al., 2010).

The non-significant differences in both RYMV severity and AUSPC between the two isolates and the consistent reactions of the accessions when subjected to different isolates suggest that the two isolates may be similar in terms of virulence. Recently, Ugandan RYMV isolates were serotyped and grouped in Ser 4 based on polymorphism in amino acid sequences of the coat protein (CP) gene (Uke et al., 2016). The polymorphism of the R domain of the CP and near the conserved position 151-154 of the S domain is reported to determine the differences and aggressiveness among isolates (Pinel et al., 2000). In this study we used RYMV isolates that were obtained from symptomatic rice plants from RYMV hotspots in Uganda and thus were not characterized into serotypes.

The utilization of wild relatives of a crop is indispensable in crop improvement as they host a lot of diversity that is not available in cultivated species. For instance, *Oryza barthii* hosts a lot of diversity that is not available in *O. glaberrima*. *O. glaberrima* was domesticated from its wild progenitor *O. barthii* (Wang et al., 2014), therefore the majority of resistance genes in this species originated from *O. barthii*. In this study, accessions from the MET series were derived from introgression with *O. barthii*, therefore, the resistance or partial resistance observed in MET accessions could be due to the *O barthii* gene pool leading to different gene and allele interactions each contributing to variations in phenotype resistance.

To date, *rymv1-2* allele has only been found in *O. sativa* species. This allele was previously identified in rice cultivars Gigante and Bekarosaka (Albar et al., 2006; Rakotomalala et al., 2008). In this study, five more sources

of resistance carrying the *rymv1-2* allele were identified. *RYMV1* has only been very recently deployed in the field; specifically, resistance allele *rymv1-2* was introgressed into four elite cultivars from AfricaRice NARS partners through Marker Assisted Selection (MAS) (Bouet et al., 2013; Ndjiondjop et al., 2013). Four of the accessions in the current study that possessed the *rymv1-2* allele originated from the AfricaRice center and thus could be the elite cultivars. The accession ARS 126-3-B-1-2 that also carried the *rymv1-2* allele was developed through shuttle breeding. This makes *rymv1-2* very important for use in crop improvement because: 1) *O. sativa* is the most widely cultivated species worldwide while *O. glaberrima* is restricted to West Africa. 2) The existence of reproductive barriers between these two rice species limits utilization of other *RYMV1* alleles (*rymv1-3*, *rymv1-4* and *rymv1-5*) found in *glaberrima* species. The reproductive barriers can be overcome by combining backcross and MAS.

Being co-dominant, the newly developed markers FMRymv1A and Rymv1-2kbd can distinguish between homozygotes and heterozygotes, thus, eliminating the extra laborious step and reduces the potential of error. FMRymv1A marker and Rymv1-2kbd KASP primer were designed to target the *rymv1-2* resistance allele indicated by G/A SNP within the *RYMV1* gene. This makes these allele-specific markers more efficient in selection of desired genotypes compared to DNA makers. FMRymv1A and Rymv1-2kbd are, thus, advantageous for MAS breeding programs targeting the *rymv1-2* resistance allele especially in large scale population screening. The two primers were validated in an F_2 segregating population, which showed that the *rymv1-2* allele genotype co-segregates with the RYMV resistance phenotype.

5. Conclusion

This study revealed resistance sources with resistance alleles at the *RYMV1* locus in a collection of cultivated rice in Uganda. We report five RYMV resistant accessions possessing the *rymv1-2* allele that can be deployed as resistant cultivars and/or used to introgress *rymv1-2* allele into susceptible adapted cultivars throughout Africa. The two allele specific markers do not require digestion of PCR product and thus will be useful especially in large scale screening of materials and in MAS programs for introgression of *rymv1-2* resistance allele.

Acknowledgements

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