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 RYMV 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 markers 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 (NaCRRRI). 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 NaCRRRI 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/indica | Aromatic | Breeding line |
| 5         | MET4   | ART34-146-1-8N-1 | ARC                      | Oryza barthii/sativa/indica | Aromatic | Breeding line |
| 6         | MET6   | ART35-49-1-4N-1 | ARC                      | Oryza barthii/sativa/indica | Aromatic | Breeding line |
| 7         | MET12  | ART34-88-1-2-B-1 | ARC                      | Oryza barthii/sativa/indica | Aromatic | Breeding line |
| 8         | MET13  | ART34-113-3-2-B-1 | ARC                      | Oryza barthii/sativa/indica | Aromatic | Breeding line |
| 9         | MET14  | ART34-256-3-1-B-2 | ARC                      | Oryza barthii/sativa/indica | Aromatic | Breeding line |
| 10        | MET15  | ART35-272-1-2-B-1 | ARC                      | Oryza barthii/sativa/indica | Aromatic | Breeding line |
| 11        | MET16  | ART35-272-1-2-B-1 | ARC                      | Oryza barthii/sativa/indica | Aromatic | Breeding line |
| No. | Accession Code | Source | Species | Description |
|-----|----------------|--------|---------|-------------|
| 12  | MET30          | ARC    | *Oryza barthii/sativa/japonica* | Aromatic Breeding line |
| 13  | MET40          | ARC    | *Oryza barthii/sativa/japonica* | Aromatic Breeding line |
| 14  | MET60          | ARC    | *Oryza barthii/sativa/japonica* | Non-aromatic Breeding line |
| 15  | MET70          | ARC    | *Oryza barthii/sativa/japonica* | Non-aromatic Breeding line |
| 16  | ARC36-2-1-2    | ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 17  | ARC36-2-P-2    | ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 18  | ARC36-4-EP-2   | ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 19  | ARC39-145-P-3  | ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 20  | ARC39-145-P-2  | ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 21  | ARC36-2-B-1-2  | ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 22  | IR64           | IRRI   | *Oryza sativa/indica* | Non-aromatic Susceptible check |
| 23  | SUPA1052       | IRRI   | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 24  | SUPA1          | IRRI   | *Oryza sativa/indica* | Aromatic Breeding line |
| 25  | SUPA3          | IRRI   | *Oryza sativa/indica* | Aromatic Breeding line |
| 26  | SUPA4          | IRRI   | *Oryza sativa/indica* | Aromatic Breeding line |
| 27  | SUPA5          | IRRI   | *Oryza sativa/indica* | Aromatic Breeding line |
| 28  | SUPA6          | IRRI   | *Oryza sativa/indica* | Aromatic Breeding line |
| 29  | SUPA126-3-B-1-2| ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 30  | Gigante        | IRRI   | *Oryza sativa/indica* | Non-aromatic Resistant check |
| 31  | Komboka        | IRRI   | *Oryza sativa/indica* | Aromatic Released variety |
| 32  | Basmati370     | IRRI   | *Oryza sativa/indica* | Aromatic Breeding line |
| 33  | WITA9          | IRRI   | *Oryza sativa/indica* | Non-aromatic Released variety |
| 34  | Supa Local     | NaCRI  | *Oryza sativa/indica* | Aromatic Breed line |
| 35  | AGRA41         | CRI    | *Oryza sativa/indica* | Aromatic Breeding line |
| 36  | AGRA60         | CRI    | *Oryza sativa/indica* | Aromatic Breeding line |
| 37  | AGRA65         | CRI    | *Oryza sativa/indica* | Aromatic Breed line |
| 38  | NamChe1        | NaCRI  | *Oryza sativa/indica* | Non-aromatic Released variety |
| 39  | NamChe2        | NaCRI  | *Oryza sativa/indica* | Non-aromatic Released variety |
| 40  | NamChe3        | NaCRI  | *Oryza sativa/indica* | Non-aromatic Released variety |
| 41  | NamChe4        | NaCRI  | *Oryza sativa/indica* | Non-aromatic Released variety |
| 42  | NamChe5        | NaCRI  | *Oryza sativa/indica* | Non-aromatic Released variety |
| 43  | NamChe6        | NaCRI  | *Oryza sativa/indica* | Non-aromatic Released variety |
| 44  | E20            | NaCRI  | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 45  | E22            | NaCRI  | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 46  | NERICA1        | NaCRI  | *Oryza sativa/japonica* | Non-aromatic Released variety |
| 47  | NERICA4        | NaCRI  | *Oryza sativa/japonica* | Non-aromatic Released variety |
| 48  | NERICA8        | NaCRI  | *Oryza sativa/japonica* | Non-aromatic Released variety |
| 49  | NERICA10       | NaCRI  | *Oryza sativa/japonica* | Non-aromatic Released variety |
| 50  | 1189 line      | ARC    | *Oryza sativa/indica* | Aromatic Breeding line |
| 51  | 1190 line      | ARC    | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 52  | 1191 line      | ARC    | *Oryza sativa/indica* | 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         | Tanzania | *Oryza sativa/indica* | Non-aromatic Breeding line |
| 56  | Jaribu         | Tanzania | *Oryza sativa/indica* | Aromatic Breeding line |

**Note.** Africa Rice Center (ARC), International Rice Research Institute (IRRI), Tanzania, National Crops Resources Research Institute (NaCRII), 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 NaCRII, 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

Phenetyping 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°31′30″ N 32°36′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 °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 × 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™ (0.8 μg/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).

| Primer   | Sequence                                                                 | Product Size             | Source                        |
|----------|--------------------------------------------------------------------------|--------------------------|-------------------------------|
| Rymv1A_EXT(F) | GTCCGATGTATGCTCAACCTTGGTCCGA                                              | 327bp (two outer primers) | This study                     |
| Rymv1A_EXT(R) | TATCAGGGGACACCACAGAGGGCTCTTGGTCCGA                                         | 204bp (A allele)          |                               |
| Rymv1A_INT(F) | TGAAGGGCGTGAGAGCCTAAGGGGATAG                                              | 177bp (G allele)          |                               |
| Rymv1B_EXT(F) | TATTIGAGAGGCTTTTTGTAGGGCCAC                                                | 450bp (1-5 allele)        |                               |
| Rymv1B_EXT(R) | CATGCTGAGAGGCCTAAGGGCCATCCAG                                             |                          |                               |
| Rymv1B_INT(F) | TGAAGGCGCTGAGAGGCTAAGGGGATAG                                              | 197/700bp (1-5 allele/wt) | Thiémélé et al. (2010)        |
| Rymv1B_INT(R) | GGTCAGGGCCAGTCAATTTTGCTATGC                                                 |                          |                               |
| *F1R1* | F: CACGTCGGCGGCGGCGATCCAAG RG: CAAACACGCTGCGGCGACCTCA                        | 725bp (*RYMV1* gene)      | Thiémélé et al. (2010)        |
| *F2R2* | F: GAGGCCCAACTTGTGCGGATG R: CAGGGCCGTCATTTTGCTATTTCC                          | 700bp (1-5 allele)        | Thiémélé et al. (2010)        |
| *F2R6* | F: GAGGCCCACTTGTGCGGATG R: CCATCTCTTGACCATGGTTGTC                           | 197/700bp (1-5 allele/wt) | Thiémélé et al. (2010)        |
| *F3R4* | F: CCTTGGTCACTAGAGAAGGGCA R: CCTGGGATACAAAACAGAGAC                            | 700/1200bp (1-5 allele/wt) | Thiémélé et al. (2010)        |
| *F4R3* | F: TGCCCTGACCAAGAGGATTGAa R: CTTCATGCTGAGGCCACCA                              | 500/700bp (1-5 allele)    | Thiémélé et al. (2010)        |
| Rymv1D  | F: CCTTGGTCAGCTAAGAGGGCA R: AGTACTGCACAAATAGAGGGCA                         | 675bp                     | Laurence Albar                |

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™ (0.8 ug−1 mL) (AMRESCO, Ohio, USA) and viewed under UV light using BioDoc-It™ Imaging System (Applied BiosystemsWaltham, Massachusetts, 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) (AGTAGCTCAACAAATTAGACGGA) 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 °C 60s (pre-read); Stage 2: 94 °C for 15 min hot start Taq activation (1 cycle); Stage 3: 94 °C for 20 s, 61 °C (61 °C decreasing by 0.6 per cycle to achieve a final annealing/extension temperature of 55 °C) for 60 s (10 cycles); Stage 4: 94 °C for 20 s, 55 °C for 60 s (29 cycles); Stage 5: 94 °C for 20 s, 57 °C for 60 s (3 cycles); Stage 6: 37 °C 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, Daegon, 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:

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

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 ($0 < \text{SD} < 1$), susceptible ($1 < \text{SD} < 2$) 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 × 10^{-16}), time of disease assessment (P-value < 2 × 10^{-16}) and the interaction between accession and time of disease assessment (P-value < 2 × 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 × 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.

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 × 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 |
| Accession   | Yield (kg/ha) | SD     | Susceptibility          |
|------------|--------------|--------|-------------------------|
| 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               |

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 Occurrence 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 rymv1-2 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.

| Primer  | Allele | Accessions                                      |
|---------|--------|-------------------------------------------------|
| 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 Occurrence 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 F2 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 marker 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 Kombo 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 F2 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.

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