Characterization of the Krish-infecting strain of Johnsongrass Mosaic Potyvirus (JGMV) and its Symptom Development in Krish Sorghums

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ABSTRACT

The aim of this research is to investigate the morphological and molecular data of Johnsongrass mosaic potyvirus (JGMV) Krish-infecting strain, whether this virus is a new variant of JGMV or another virus. In order to prove this a number of experiments were set up, including of mechanical inoculation and isolation of JGMV, observation of the morphology of the virus using Transmission Electron Microscopy and detection of viral coat protein (CP) as well as the SDS-PAGE. Phenol/chloroform method was used for the isolation of viral RNA. The results showed that characteristic symptoms produced by JGMV-Krish on Krish sorghum leaves was a red necrosis after a three to five month of post inoculation, while the size of CP JGMV-Krish were the same as the JGMV wild type (Jg). These were also confirmed by the size of full length genomic RNA which migrated at the same distance with JGMV-Jg. Based on the above, this virus was confirmed as the new strain of JGMV. Further studies on the molecular investigations were reported in next paper.

Key words: characterization, JGMV-Krish, symptom development, Krish sorghums.

INTRODUCTION

Johnsongrass mosaic virus, strain Johnsongrass (JGMV-Jg), is a single stranded positive sense RNA virus in the genus Potyvirus (Potyviridae). It is aphid transmitted and attacks a number of tropical grasses including maize and sorghum causing significant economic losses (Gough and Shukla, 1993; Shukla and Teakle, 1989). The virus can also be spread by mechanical contact that made it attractive for this project.

In 1959, a variety of sorghum, later named Krish, was introduced to Australia from India; this sorghum was found to contain a gene conferring remarkably high resistance to JGMV-Jg prevalent in Australia (Conde et al., 1976; Teakle and Pritchard, 1971; Teakle and Grylls, 1973). However, in 1985 an apparently new strain of JGMV was found to infect the sorghum line QL12 (Queensland) and cultivar WT (White Trojan) both of which contain the Krish resistance gene (Persley et al., 1986, 1987; Persley and Syme, 1990). JGMV attacks a number of graminaceous plants including maize and sorghum. The difference between JGMV-Jg and the Krish-infecting strain is that the latter is also capable of attacking Krish sorghum lines (containing a resistance gene) giving rise to typical mosaic and red necrotic symptoms. Two examples of Krish sorghum plants in which resistance has been broken by the Krish-infecting strain are line QL12 and cultivar WT. In addition, to documenting symptom development in Krish sorghums, it was thought worthwhile to confirm the similarity in morphology and some other properties between the Krish-infecting strain of JGMV and JGMV-Jg.

Since the former strain is believed to have arisen by mutation from the type strain, JGMV-Jg (Suranto et al., 1998).

MATERIALS AND METHODS

Plant materials

Seeds of Krish sorghum line QL12 and cultivar WT, containing the resistance gene, were kindly provided by Mr. D. Persley from the Department of Primary Industry, Queensland.

Virus isolates

An isolate of the JGMV Krish-infecting strain was collected by Mr. D. Persley from infected leaves of sorghum plants (cv. WT) in Darling Downs, Queensland, while the isolate of the type strain, JGMV-Jg, was kindly provided by Dr. D. Teakle of the Department of Microbiology, Queensland University. The JGMV Krish-infecting strain was propagated and maintained in both Krish sorghum line QL12 and cultivar WT, as well as in maize.

Mechanical inoculation and sampling of infected leaves

Inoculum was prepared from infected leaves by grinding in a mortar in a mixture of cold 0.01 M phosphate buffer (pH 7) and 0.1 M sodium sulphite (1:4 v/v respectively). Mechanical inoculation was conducted during the third leaf stage of development when seedlings were approximately 10-15 days old. The inoculum was rubbed onto the second leaf from the top (youngest) after
first dusting with Carborundum powder. The leaves were then briefly washed with tap water and the plants kept in insect proof cages. To confirm that Krish sorghum line QL12 and cultivar WT both of which contain the Krish resistance gene, are not susceptible to JGMV-Jg, 6 plants of each were mechanically inoculated with JGMV-Jg. Plants inoculated with the Krish-infecting strain were harvested after the symptoms appeared on the leaves, approximately 10-15 d.p.i. (day post inoculation). The infected leaf was then snap frozen in liquid nitrogen. The viral fractions were isolated from the first two or three leaves which appeared subsequent to infection and showed mosaic symptoms over one third to three quarters of each lamina.

Isolation and purification of JGMV Krish-infecting virus particles

A number of methods attempted for isolating high quality JGMV virions met with little success. The only successful procedure in our hands was that eventually provided by Dr. A. Davidson (Department of Microbiology, Monash University). The method proved to be relatively straightforward and yielded high quality particles suitable for preparing genomic RNA.

Transmission electron microscopy

Purified virus suspension (2-3 µl) was placed on carbon coated grids that were previously coated with 1% albumin to adhere the panicles. Potassium phospho-tungstate, 3 µl of a 1.5-2.0% solution (at pH 7), was added and, excess fluid removed with filter paper (Teakle and Grylls, 1973). A better method was eventually developed as described below. The minor vein of a leaf was scratched with a slightly blunt 18 gauge hypodermic needle (without forming a hole in the leaf) creating a small pool of exudate. Phosphate buffer (20-30 µl at approximately pH 7) was gently mixed with the pool of exudate. After 0.5-2 min the carbon coated side of a pioloform coated grid was brought into contact with the exudate. Phosphotungstic acid, 2-5 µl of a 2% solution, was placed on top of the grid and left to stand for 1-3 min. The excess liquid was then removed using the torn edge of a piece of filter paper and left to dry for 30-60 min before viewing.

The negatively stained preparations were observed using a Siemens 102 transmission electron microscope and photographed at x 60,000. Measuring the length and width of individual particles was basically in accordance with the method of Noordam (1973).

SDS-PAGE of viral coat protein

Recipes of sample and running buffer, as well as the protein staining solutions, were taken from Section 5 of the

Figure 1. Symptom development in Krish sorghum line QL12 and sweet corn infected with JGMV Krish infecting strain potyvirus. From left to right: (A) Sorghum QTL12: healthy, non-infected dark-green leaf; early mosaic symptoms (5 d.p.i.); mosaic symptoms (8 d.p.i.); mosaic symptoms with necrosis (14 week p.i.); and severe symptoms with necrosis (12 weeks p.i.). (B) Sweet corn: healthy, non-infected dark-green leaf; early mosaic symptoms (d.p.i.); mosaic symptoms (7 d.p.i.); mosaic symptoms (10 d.p.i.); two examples of mosaic symptomps with necrosis (12 weeks p.i.).
BIO RAD Mini-Protean II Instruction Manual, 1993 (Bio-
Rad Laboratories, Hercules, CA).

Isolation and electrophoresis of viral RNA

The isolation of the full length genomic mRNA from the
virus particles was performed using a phenol/chloro-
form method (Hammond and Lawson, 1988) modified by
Dr. A. Davidson, Department of Microbiology, Monash
University. The viral pellet was resuspended in 500 µl
of proteinase K solution (100 µg /ml) (per 40 gram of leaf
used) by fracturing the gel pellet first with a sealed Pasteur
glass pipette and then gently drawing the suspension up
and down using a broad nozzle 1 ml autopipette tip every
10 min until the gel-like pellet dispersed. The tube was
then incubated for 1.5 hr at 37°C, after which an equal
volume of phenol was added and the tube shaken
vigorously by hand, briefly vortexed, and then centrifuged
in a Heraeus benchtop centrifuge (13,000 rpm, 20 min).
The aqueous layer was transferred into a new tube and an
equal volume of phenol chloroform was added and shaken
vigorously by hand and briefly vortexed, and recentrifuged
for 20 min. The aqueous layer was transferred into a new
tube and the RNA precipitated by adding 3 M sodium
acetate (pH 5.2) to a final concentration of 0.1 M, and 3
volumes of 100% ethanol (at -20°C). Tubes were stored at
-70°C for 1 hr. The tube was then centrifuged for 20 min
(13,000 rpm). The supernatant was discarded, and the
pellet briefly dried for 2 min in a SpeedVac Concentration
unit (Savant) by starting the vacuum gently. The pellet was
resuspended in 50 µl RNase-free water (Promega) or
DEPC-treated water. The extracted mRNA (5-10 µl) was
run on an agarose/formaldehyde gel. The remaining RNA
was stored at -70°C.

Electrophoresis of viral RNA

The RNA samples (1-10 µl) were adjusted to 10 µl with
DEPC-treated water and prepared for electrophore-
sis by adding 10 µl of a mix containing 5 µl 10 x MOPS, 9 µl 12.3
M formaldehyde, 25 µl formamide and then incubating for
15 min at 65°C, followed by chilling on ice for 30 sec and
adding 2 µl of formaldehyde loading buffer (1 mM EDTA,
0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene
cyanol, 50% (v/v) glycerol). The RNA marker (GIBCO-
BRL) was prepared similarly. Prior to loading, ethidium
bromide (10 mg/ml) was added to all samples to a final
concentration of 0.5 µg/µl. Before loading the samples, the
agarose/formaldehyde gel was run for 5 min at 5 V/cm
using a 1x formaldehyde gel running buffer, and then for
approximately 2 hr at the same voltage after loading the
samples. The used running buffer from both tanks was
mixed after 1 hr and the gel run for a second hour. Gels
were visualized under UV light (Gelman Science Pty. Ltd.)
and photographed (Tominon, Japan).

RESULTS AND DISCUSSION

Symptom development on Krish sorghums

The mosaic pattern observed four days post inoculation
(d.p.i.) was typical for early systemic symptom
development on both Krish sorghums (line QLI2 and
cultivar WT) This was subsequently followed by severe
mosaic symptoms for several weeks post inoculation (d.p.i.),
and then by red necrosis after about three to five months d.p.i.
The characteristic symptoms produced by the JGMV
Krish-infecting strain on Krish sorghum leaves are shown
in Figure 1.

Symptom expression on the two Krish sorghums was
indistinguishable, except that they always appeared to be
stronger on leaves of the QLI2 line than on leaves of
cultivar WT. The mosaic symptom at the early stages of
systemic infection became noticeable at approximately 4
d.p.i., regardless of the Krish sorghum used. Red spots and
necrosis on newly developing leaves appeared after more
than 2 months p.i. Similar symptoms were observed on
maize (sweet corn, variety "Terrific") suggesting that sweet
corn could be used equally well as a host for the
JGMV Krish-infesting strain, except that it always
provided poorer material for extracting viral RNA
as compared to the Krish sorghums.

The leaves of both Krish sorghum plants
red at temperatures below 15°C even if not
inoculated, confirming that reddening can also be a
stress response, however no potyviruses could be
detected by electron microscopy in such plants.

To prove the resistance of Krish sorghums to
JGMV-Jg (type strain), sixteen seedlings, eight
plants of each of QLI2 and WT, were
mechanically inoculated with JGMV-Jg. No
systemic infections were observed on either of the
Krish sorghums even though the plants were
allowed to grow for more than two months. The
absence of virions was confirmed by electron
microscopy. As part of another early experiment,
four plants of each of the Krish sorghums were
inoculated but, again, none were infected. Their
resistance was maintained throughout the entire
project in other experiments.

Figure 2. Particles of JGMV-Krish infecting strain stained with 2%
potassium phosphotungstate at pH 7, magnified x 251.000.
Morphology of the JGMV Krish-infecting strain

The JGMV Krish-infecting strain has flexuous rods typical of potyviruses (Figure 2). Samples were diluted for the measurements of length and width of 200 virions selected at random. The average length and width were 736 ± 11 nm and 12.9 ± 0.2 nm, respectively. Approximately 82.5% of the virus particle lengths ranged between 550 and 850 nm (Figure 3A), thus there was no significant size difference between the JGMV-Krish infecting strain and the data published for JGMV-Jg (736 ± 17 nm) by Teakle and Grylls in 1973. The data for distribution of particle widths (Figure 3B) was new for JGMV, so no comparison can be made. How-ever, the observed average width in this study was in the expected range for potyviruses (Shukla et al., 1994).

Coat protein of the JGMV Krish-infecting strain

The CP monomer of the Krish-infecting strains of JGMV that migrating at approximately 34.4 kDa on a denaturing polyacrylamide gel (Figure 4) is similar to the CP of JGMV-Jg (34.0 kDa) as reported by Shukla et al. (1994). Dimmers at about 68 kDa can also often be observed on SDS-PAGE when virus particles are freshly prepared or when stored at 20°C. This result was a consistent feature in experiments using newly purified virus particles and compares well with the published data on JGMV-Jg (Gough and Shukla, 1993). However, degradation of the polypeptides can be observed by SDS-PAGE if CP samples are taken from old preparations of virus particles kept in a resuspension buffer in the cold room (instead of storage at -20°C in 50% glycerol).
The genomic RNA of the Krish-infecting strain of JGMV

The extracted genomic RNA from purified virus particles migrated corresponding to approximately 10 kb on a formaldehyde gel (Figure 5). There was no significant difference between the JGMV-Jg and the Krish-infecting strain, apart from the fact that the concentration of the Krish-infecting strain used in the gel was slightly higher than that used for the type strain.

One to two weeks p.i. was usually ideal for harvesting the virus from Krish sorghum, and generally gave reasonable yields. However, the optimum time for harvesting the propagated viruses varied from one strain to another. In this experiment, the best time for harvesting infected leaves for the Krish-infecting strain of JGMV on Krish sorghum plants was 10 d.p.i and also resulted in the highest quality of purified mRNA. Harvesting times greater than 14 d.p.i produced poor viral pellets. Optimum times may also be dependent on the host species used, such as maize for example.

Maize (variety Terrific) produced good symptom development when relatively shaded, but the amount of virus particles obtained was poor even when using the same conditions (such as seedling age, inoculation and harvesting times) as compared to the sorghums.

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