Further Characterization and Detection of Indian Citrus Ringspot Virus

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ABSTRACT. Indian citrus ringspot virus (ICRSV) from a Kinnow mandarin orchard at the IARI, New Delhi, India (isolate K1) has flexuous particles 650 nm long, with clearly visible cross-banding. It can be mechanically transmitted to and multiplied in *Phaseolus vulgaris* cv. Saxa. The genome consists of one ssRNA of about 7.5 kb and the coat protein (CP) is 34 kd in size. The virus has been purified and an antiserum prepared suitable for DAS ELISA. Partial sequencing of the genome, revealing the CP ORF near the 3' end and further sequences upstream, indicates that ICRSV, while morphologically resembling a capillovirus, has no homology with this genus. Absence of serological relationship confirms this. Short amino acid motifs in the CP sequence reveal limited similarities with the genera *Potexvirus, Carlavirus, Foveavirus* and *Allexivirus*, but no strong similarity to any one of these, so that the virus does not fall into any known genus. RNA and cDNA probes that detect K1 are available, and also primers suitable for RT-PCR. EM decoration tests with an antiserum to K1 revealed the presence, in a Kagzi Kalan lime-lemon hybrid of a virus (KK) with similar morphology but differing consistently from K1 by one serological step. Moreover, in the original Kinnow orchard, we detected a further virus (K2), again with similar morphology, serologically related to K1 but differing by five steps. K2 now in turn appears, in preliminary results, to be a mixture containing yet a further morphologically similar virus, K3. Thus, Indian citrus ringspot disease may be caused by a series of viruses, and while diagnostic methods are now available for K1, these may not immediately solve the problem of field diagnosis.

Citrus ringspot disease is widely distributed in India and affects the fruit quality of Kinnow mandarins (1, 5, 6) but the causal agent or agents have not been well identified. A virus was found (2) with capillovirus-like morphology in extracts of Kinnow mandarin leaves showing typical ringspot symptoms in an experimental orchard at IARI, New Delhi. Two other particle types, filamentous or tubular, were also seen in these preparations but were later identified as host components (Milne, unpublished data). The virus with capillovirus-like morphology, named Indian citrus ringspot virus (ICRSV), has flexuous particles with a modal length of 650 nm, and clearly visible cross banding (2). We now report further studies on the virus. The original isolate is named K1, with further isolates identified as KK, K2 and possibly K3 (see below).

K1 is sap-transmissible to *Chenopodium quinoa*, *C. amaranticolor*, soybean, cowpea and French bean cv Saxa. The best experimental host is Saxa bean, and the virus could be purified from it by the following procedure. Systemically infected leaves were homogenized in 10 volumes (w/v) 0.05 M phosphate buffer, pH 7.8, 0.005 M DIECA, 0.01 M EDTA, 0.02 M sodium sulfite. The extract was expressed through nylon mesh, and clarified by addition of 10% chloroform, with stirring for 10 min at room temperature. After phase separation by centrifugation at 12,000 rpm for 10 min, the supernatant was centrifuged at 45,000 rpm for 2 h and the pellets resuspended in the extraction buffer. The virus was further purified by rate zonal centrifugation into a 10 to 40% cesium sulfate density gradient, prepared in extraction buffer.

A rabbit antiserum was made and its titer determined by EM decoration and gel double diffusion tests using virus from K1-infected Saxa bean as antigen. A DAS-ELISA kit was prepared. Protein from purified ICRSV was analyzed on 12% SDS polyacrylamide (SDS-PAGE) and transferred to PVDF-membranes for Western blotting.

Nucleic acid was extracted with phenol/chloroform from purified K1 and its size estimated by electrophoresis using a glyoxal denaturing system.

For cloning the 3' terminal part of the virus genome, cDNAs were synthesized and cloned from viral RNA using an oligo d(T) primer and the Universal Riboclone cDNA Synthesis System (Promega). The resulting cDNA was ligated into the plasmid pBluescript and the recombinant plasmids were purified, then sequenced. The sequence was compiled using PC/Gene (IntelliGenetics) software, and was analyzed using the National Center for Biotechnology Information Blast Network server. The sequences of some possibly related viruses were used for pairwise sequence comparison with the K1 sequence.

The best K1 antiserum had a gel-diffusion titer of 1/64 against the virus and an EM decoration titer of 1/4,096. The relatively low titer in gel diffusion may have been due to the poor diffusion of this filamentous virus in agar. K1 could also be detected by DAS-ELISA at a tissue dilution of over 1/1,000. Experimentally infected greenhouse citrus reacted similarly with the antiserum, proving that the virus purified from Saxa bean was the same as that in the citrus. However, it was important that the virus used to raise the antiserum was not multiplied in and purified from citrus, because virus-like and highly antigenic fibers (protein aggregates) present in all citrus varieties we tested are easily co-purified with the filamentous virus particles (Milne, unpublished data), and antisera raised by injecting such material into rabbits can give and have given misleading results.

When the protein of purified ICRSV was analyzed by SDS-PAGE, a single band of 34,000 Da was detected; in Western blots this band clearly reacted with the antiserum when diluted 1/40,000, and was therefore likely to be that of the coat protein (CP).

Denaturing gel electrophoresis revealed one RNA of about 7.5 kb. The cloning procedure yielded eight clones that covered a stretch of 1,585 nucleotides (excluding the poly-A tail) at the 3' end of the RNA. An open reading frame was found with potential to encode a polypeptide of 325 amino acids (Mr 34,000). Cloning this gene in a Gene Fusion System (Pharmacia), we obtained a fusion protein detectable by the K1 antiserum in Western blots. Thus, the fusion protein contained the viral CP and identification of the gene encoding it was confirmed. The Blast search revealed that some short amino acid motifs of the coat protein are similar to those of certain viruses in the genera *Potexvirus, Carlavirus, Allexivirus* (these last are mite-borne filamentous viruses of *Allium* species), and *Foveavirus* (e.g., apple stem pitting virus), but not *Capillovirus* (for example citrus tatterleaf virus). For descriptions of these genera, see (3, 7).

PCR primers based on conserved sequences of capilloviruses (4) were constructed and tested to see if they would amplify sequences in ICRSV-K1. There was no amplification, although two isolates of apple stem grooving capillovirus, used as controls, gave positive results. These data confirm that ICRSV is not a capillovirus.

Western blotting showed that isolate K1 reacted faintly with antisera to some potexviruses. Tests for possible serological reactions with allexiviruses, run by Dr. D. E. Lesemann, BBA, Braunschweig, Germany, were negative.

Digoxigenin-labeled cDNA and RNA probes, both complementary to the sequence, were prepared and were able to recognize K1 in graft-infected glasshouse citrus. Two sets of primers could also detect the virus by RT-PCR.

The K1 isolate of ICRSV thus appears to be a new virus not

belonging to any recognized genus, and serological and molecular methods are now available that should make possible its detection in field and nursery material.

However, use of the antiserum to K1 showed, in EM decoration tests, that other morphologically indistinguishable but serologically distinct viruses are present in Indian citrus. For example, a lime-lemon hybrid cv Kagzi Kalan in a New Delhi private garden contained an isolate, named KK, that consistently differed from isolate K1 by one two-fold dilution step, when tested with the K1 antiserum. More significantly, trees in the original Kinnow mandarin orchard from which K1 was isolated were found to contain a further virus, named isolate K2, that was still morphologically the same but differed from K1 in decoration tests (using the same antiserum) by five steps. This virus was not detected by the DAS-ELISA prepared with antiserum to K1, or by K1 primers in RT-PCR.

An antiserum to K2 is now available, with an EM decoration titer of 1/512 to the virus in citrus, and an ELISA kit is in preparation, but this procedure is again complicated by the apparent presence, mixed with K2, of a different virus (K3), again morphologically similar, but distinct from K1 and K2 in decoration tests. These findings, still preliminary, suggest that Indian citrus ringspot disease may be more complex than at first anticipated, and that preparing effective diagnostic kits will be correspondingly more difficult.

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