First report of an isolate of Japanese iris necrotic ring virus from Australia

Stephen J. Wylie · Hua Li · Michael G. K. Jones

Received: 24 March 2012 / Accepted: 14 May 2012 / Published online: 30 May 2012
© Australasian Plant Pathology Society Inc. 2012

Abstract An isolate of Japanese iris necrotic ring virus (JINRV) was detected from a plant of *Iris ensata* Thunb. growing in Western Australia, and the sequence of its complete genome (4019 nucleotides) was determined. The deduced replicase and coat proteins shared 75 % and 72 % amino acid identities, respectively, with those of the only other sequenced isolate of JINRV, from Japan. This is the first report of JINRV in Australia, and the first beyond Japan.

Keywords Flower bulb · Virus incursion · High throughput sequencing · Horticulture

Japanese iris necrotic ring virus (JINRV) (family *Tombusviridae*, genus *Carmovirus*) was first described in Japan in 1982 from Japanese iris (*Iris kaempferi* Sieb.) (Yasukawa et al. 1982). It possessed spherical particles of about 35 nm in diameter with linear, monopartite, single-stranded plus-sense RNA. The virus was transmitted mechanically but not by aphids or through soil. It caused necrotic spindle-shaped streaks or rings in the leaves of Japanese irises and latent local infection in Siberian iris (*I. sanguinea* Hornem. ex Donn) and rabbit-ear iris (*I. laevigata* Fisch.) (Yasukawa et al. 1991). The complete genome sequence of the type isolate from Japan (GenBank accession NC_002187) is 4014 nucleotides (nt) long and predicted to encode five or six overlapping open reading frames (ORF). Until now, JINRV was described only from Japan, and only one

sequence (Takemoto et al. 2000), that of the type isolate, was available.

Iris plants were analysed as part of a project to determine incidence and sequence diversity of potyviruses and flexiviruses in the Perth region, Western Australia. Total RNA was extracted from leaf samples of five Spanish iris (*I. xiphium* Desf.) and five Japanese water iris (*I. ensata* Thunb.) plants using the Qiagen Plant RNeasy kit. RNA samples from each species were pooled. Purification of polyadenylated RNA from total RNA using Oligo-dT labeled beads, nebulization of RNA prior to adaptor ligation, library construction, amplification, and paired-end sequencing using Illumina HiSeq 2000 technology was done by Macrogen Inc (Seoul). *De novo* assembly of contigs was carried out using CLC Genomics Workbench v4.8 (www.clcbio.com) and Geneious Pro v5.5.6 (Drummond et al. 2011). Parameters for the assembly of contigs were minimum overlap of 50 % of read length (50 nt), 10 % maximum gaps per read, 85 %, 90 % and 95 % minimum overlap identity. Resulting contigs were sorted according to length. Batches of remaining contigs were subjected to Blastn, Blastx and Blastp analysis against appropriate GenBank databases. The JINRV sequences identified were edited manually to remove gaps and aberrant reads. The final virus consensus sequence was constructed using contigs from both assemblers. ORFs and identities of mature peptides, and domains encoded by them were predicted by homology after alignment with available carmovirus genomes. Pairwise identities were calculated after alignment of nt and amino acid (aa) sequences in ClustalW (Thompson et al. 1994). The phylogenetic tree of amino acid sequences was constructed using Neighbor-joining (NJ), Maximum Likelihood (ML), and Maximum Parsimony (MP) methods in MEGA5 (Tamura et al. 2011) after pairwise alignment of sequences with a Gonnet protein weight matrix, a gap open

S. J. Wylie (✉) · H. Li · M. G. K. Jones
Plant Virology Group, Western Australian State Agricultural
Biotechnology Centre, School of Biological Sciences and
Biotechnology, Murdoch University,
Perth, WA 6150, Australia
e-mail: s.wylie@murdoch.edu.au

Table 1 Comparison of replicase and coat protein genes of carmovirus isolates

Virus ^a	JINRV- Marijuiniup10	JINRV- Japan	AnFBV	CbMV	CCFV	CarMV	CpMoV	GMV	HCRSV	HnRSV	MNSV	NLVCV	PSNV	PFBV	SCV	SYMMV	TPGV1
a. Distance matrix of amino acid sequence identities of the large read-through proteins (putative replicase) of 17 carmovirus isolates for which complete genome sequences are available.																	
JINRV-Japan	75																
AnFBV	39	39															
CbMV	39	39	48														
CCFV	45	45	40	38													
CarMV	40	39	51	48	41												
CpMoV	37	37	39	37	40	38											
GMV	32	31	33	34	33	36	34										
HCRSV	47	47	41	41	49	40	42	34									
HnRSV	41	41	52	53	40	56	39	34	43								
MNSV	39	37	40	39	42	38	41	35	41	39							
NLVCV	38	39	47	51	38	49	36	34	40	51	39						
PSNV	42	39	40	40	41	38	40	36	43	39	57	38					
PFBV	38	38	51	54	39	57	39	33	42	65	40	48	40				
SCV	39	39	49	51	38	52	38	32	42	54	39	47	39	54			
SYMMV	40	39	37	38	39	38	66	35	42	38	41	37	41	40	37		
TPGV1	33	34	39	37	35	38	34	30	37	38	35	36	35	38	38	34	
TuCV	46	47	41	41	67	41	43	34	52	41	41	40	42	41	40	42	37
b. Distance matrix of amino acid sequence identities of the coat proteins of 17 carmovirus isolates for which complete genome sequences are available																	
JINRV-Japan	72																
AnFBV	28	28															
CbMV	26	26	27														
CCFV	37	37	24	30													
CarMV	28	29	34	34	27												
CpMoV	26	24	17	19	22	16											
GMV	25	19	23	21	22	22											
HCRSV	30	33	25	28	33	31	24	26									
HnRSV	28	29	33	37	28	39	21	22	27								
MNSV	24	22	18	19	22	18	19	28	24	20							
NLVCV	25	27	29	31	27	35	17	19	26	34	20						
PSNV	22	22	19	18	20	19	20	25	20	19	30	18					
PFBV	30	30	35	38	28	38	20	22	31	55	22	37	19				
SCV	29	30	30	43	32	41	20	24	31	42	23	33	19	43			
SYMMV	26	24	18	20	22	19	61	24	24	22	20	19	19	23	20		
TPGV1	27	28	33	28	25	30	17	19	28	31	18	31	18	35	28	18	
TuCV	37	36	25	28	52	25	23	24	30	24	23	25	21	26	28	24	23

^a *AnFBV* angelonia flower break virus (NC_007733); *CbMV* calibrachoa mottle virus (GQ244431); *CCFV*, cardamine chlorotic fleck virus (NC_001600); *CarMV*, carnation mottle virus (NC_001265); *CpMoV* cowpea mottle virus (NC_003535); *GMV* galinsoga mosaic virus (NC_001818); *HCRSV* hibiscus chlorotic ringspot virus (NC_003608); *HnRSV* honeysuckle ringspot virus (NC_014967); *JINRV* Japanese iris necrotic ring virus (isolate Japan NC_002187, isolate Marijuiniup10 JQ807998); *MNSV* melon necrotic spot virus (AB232925); *NLVCV* nootka lupine vein-clearing virus (NC_009017); *PSNV* pea stem necrosis virus (NC_004995); *PFBV* pelargonium flower break virus (NC_005286); *SCV* saguaro cactus virus (NC_001780); *SYMMV* soybean yellow mottle mosaic virus (NC_011643); *TGPV1* TGP Carnovirus 1 (NC_015227); *TuCV*, turnip crinkle virus (NC_003821)

penalty of 10, a gap separation distance of 4, and a gap extension penalty of 0.2. The test of phylogeny was done using 1,000 bootstrap replications.

The genome sequence of the isolate of JINRV was 4019 nt in length, excluding the polyA tail. It was predicted to encode five ORFs. ORF1 and ORF2 encode subunits of the replicase. ORF1 encodes a protein of 27 kDa that terminates with an amber stop codon. Read-through of this amber codon results in the synthesis of ORF2, in this case a protein of 85 kDa. The third and fourth overlapping ORFs encode proteins of 8 kDa and 12 kDa, respectively. Both proteins are thought to be involved in cell-to-cell movement (MP) of the virus. The overlapping 3'-proximal ORF5 encodes a 37 kDa capsid protein (CP). The isolate was named Marijiniup10 after the suburb in which it was found, and its genome sequence was granted GenBank accession JQ807998.

When the genome sequence of JINRV-Marijiniup10 was aligned with those of other carmoviruses for which a complete genome was available, it shared greatest nt identity (69 %) with the type isolate of JINRV from Japan. It shared lower nt identities (53–57 %) with the genome sequences of other carmoviruses. The deduced aa sequence of the large read-through protein subunit of the replicase shared 75 % pairwise identity with that of JINRV-Japan and only 32–47 % identity with other carmoviruses (Table 1a). Similarly,

the CP sequence had greatest aa identity (72 %) with JINRV-Japan, and shared much lower identities (22–37 %) with other carmoviruses (Table 1b). Although aa identity between the two JINRV isolates is low, the sequence identity criteria determined by the International Committee on the Taxonomy of Viruses for species demarcation for the replicase and coat proteins are <57 % and <52 % respectively (King et al. 2012). Therefore, the sequence identities between replicase and CP sequences of isolate Marijiniup10 and JINRV-Japan, and shared features of their genomic architecture, confirm them as members of the same taxon. When the replicase sequences (aa) of carmoviruses were aligned to deduce their phylogeny using NJ, ML, and MP methods, essentially the same evolutionary relationships between them were revealed with each algorithm. The NJ tree is shown (Fig. 1).

All the iris plants tested were screened individually using primers JINRVF1 (5'-CAGGGG CGCTCAGGCGACTA-3') and JINRVr1 (5'-CAACCCCCGACGCTGCCAAT-3') in an RT-PCR assay as described previously (Wylie et al. 2012). Primer JINRVF1 annealed to the 3' end of the MP and primer JINRVr1 annealed to the 5' end of the CP to yield a product of 376 bp. The product was sequenced directly by the Sanger method to confirm that it was derived from JINRV. The virus was detected in a single *I. ensata* plant. As others have noted

Fig. 1 Neighbor-joining tree of amino acid sequences of the large read-through protein (putative replicase) of an isolate of each species of carmovirus for which this sequence was available. Isolate names (where available) and GenBank accessions are shown. JINRV isolate

Marijiniup10 is boxed. 1,000 bootstrap replicates were done and values >60 % are shown. The tree was drawn to scale, with evolutionary distance used to infer the branch length in nucleotide substitutions per site

for other iris host species (Yasukawa et al. 1991), the virus did not induce visible symptoms in *I. ensata* typical of virus infection.

Of interest is that the reads that mapped perfectly to the 3' terminal 30–80 nucleotides of the JINRV genome had contiguous 3' tracts of adenines of up to 43 nucleotides. Carmoviruses, including the type isolate of JINRV, are not reported to be polyadenylated (Sit and Lommel 2010; Takemoto et al. 2000). The existence of the poly (A) region cannot be explained as a cDNA synthesis or library preparation artifact because neither process involved addition of adenines. In fact, the presence of a poly (A) is required for RNA to be sequenced using this method because poly (T) beads are utilized to capture polyadenylated RNAs while ribosomal RNAs are removed prior to cDNA library construction and sequencing. A possible explanation is that a small number of copies of the genome are, in fact, polyadenylated, but until now these have not been detected using conventional cloning and sequencing techniques. As more plant viruses are sequenced using high throughput technologies, this issue may be clarified.

To our knowledge this is the first report of Japanese iris necrotic ring virus occurring outside of Japan, and within Australia. The virus has a narrow host range and was probably inadvertently introduced to Australia on imported iris propagules, and if they were grown and underwent quarantine inspection, it was passed because no obvious symptoms of infection were evident on the leaves. Plants that are vegetatively propagated, such as irises, accumulate viruses, and when they are traded internationally, viruses infecting them are spread to new locations (Wylie et al. 2010; Wylie et al. 2011; Wylie and Jones 2012; Wylie et al. 2012). The sequencing method used was designed to detect only polyadenylated RNA viruses, so additional viruses may be present in the plant tested. This study highlights the need for more stringent examination of imported plant propagation materials at pre- and post-entry quarantine to prevent new viruses entering Australia, and the requirement for Australian producers to source virus-free stock and to also have their stocks tested for viruses prior to distribution.

Acknowledgment This study was funded by Australian Research Council Linkage Grant LP110200180

References

- Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2011) Geneious Pro v5.5.6. <http://www.geneious.com>
- King AMQ, Lefkowitz E, Adams MJ, Carstens EB (2012) Virus taxonomy: IXth report of the International Committee on Taxonomy of Viruses. Academic, London
- Sit TL, Lommel SA (2010) Tombusviridae. In: Encyclopedia of Life Sciences. John Wiley & Sons Ltd, Chichester. DOI: [10.1002/9780470015902.a0000756.pub2](https://doi.org/10.1002/9780470015902.a0000756.pub2)
- Takemoto Y, Kanehira T, Shinohara M, Yamashita S, Hibi T (2000) The nucleotide sequence and genome organization of *Japanese iris necrotic ring virus*, a new species in the genus *Carmovirus*. *Arch Virol* 145:651–657
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. doi:[10.1093/molbev/msr121](https://doi.org/10.1093/molbev/msr121)
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nuc Acid Res* 22:4673–4680
- Wylie SJ, Jones MGK (2012) Complete genome sequences of seven carlavirus and potyvirus isolates on *Narcissus* and *Hippeastrum* plants in Australia, and proposals to clarify their naming. *Arch Virol* (in press 21 Mar 2012 AVIROL-D-12-00014)
- Wylie SJ, Nouri S, Coutts BA, Jones MGK (2010) *Narcissus* late season yellows virus and *Vallota speciosa* virus found infecting domestic and wild populations of *Narcissus* species in Australia. *Arch Virol* 155:1171–1174
- Wylie SJ, Li H, Jones MGK (2011) Phylogenetic analysis of allexiviruses identified on garlic from Australia. *APDN*. doi:[10.1007/s13314-011-0038-2](https://doi.org/10.1007/s13314-011-0038-2)
- Wylie SJ, Luo H, Li H, Jones MGK (2012) Multiple polyadenylated RNA viruses detected in pooled cultivated and wild plant samples. *Arch Virol* 157:271–284
- Yasukawa K, Ohki ST, Osaki T, Inouye T (1982) Japanese iris necrotic ring disease, a newly recognized disease caused by Japanese iris necrotic ring virus. *Ann Phytopathol Soc Jpn* 48:113–114
- Yasukawa K, Osaki T, Inouye T (1991) Necrotic Ring Disease of Japanese Iris (*Iris kaempferi* Sieb.), A new disease caused by Japanese iris necrotic ring virus. *Bull U Osaka Pref Ser B Agric Biol* 43:21–28