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CHARACTERIZATION AND RNA2 NUCLEOTIDE SEQUENCE OF BROAD BEAN WILT VIRUS 2 ISOLATE P158*

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Abstract: Broad bean wilt virus 2 (BBWV2) isolate P158 was characterized and the complete nucleotide sequence of viral genomic RNA2 was determined. P158 has 30 nm diameter isometric particles. Double immunodiffusion test suggested that P158 has high antigenicity homology with BBWV2 isolate B935. SDS-PAGE result showed that the coat protein of P158 was comprised of two types of polypeptide with molecular weight of 44.7 kD and 21.9 kD, respectively. The genome of P158 was made up of two RNA molecules with the length of 6.0 kb and 3.6 kb, respectively. cDNA of RNA2 was cloned by RT-PCR and sequenced. RNA2 was composed of 3597 nucleotide (nt) residues excluding the poly(A) tail and contained single long open reading frame extending from nt 230 to nt 3424 in the viral sense RNA, and encoded a polyprotein of *Mr* 119002 (119K). Comparison of the polyprotein and the counterpart of isolate B935 indicated that the polyprotein was cleaved at Q/G (465/466) and Q/A (867/868), to release three mature proteins: a protein of unknown function, large and small subunit. Sequence comparisons of P158 with fabaviruses showed P158 had very high sequence homology with BBWV2 isolates and patchouli mild mosaic virus, but to a less extent with BBWV1 isolates.

Key words: broad bean wilt virus, characterization, RNA2 sequence.
Document code: A **CLC number:** TU311.3, TU973

INTRODUCTION

Broad bean wilt virus (BBWV) is the type member of the genus *Fabavirus*. It has a wide host range among dicotyledons and some families of monocotyledons, and is an economically important virus in China (Zhou et al., 1994). BBWV has isometric particles, hexagonal in outline and 30 nm in diameter, and a bipartite genome of single-stranded RNA of the messenger polarity, designated B RNA (RNA1) and M RNA (RNA2). The protein shell of BBWV was built of apparently equimolar amounts of two distinct polypeptides, estimated to be 42.5 kD and 21.0 kD in size (Lisa et al., 1996; Zhou et al., 1996). BBWV isolates were divided into two serotypes (I and II) by Uyemoto and Provvidenti (Uyemoto et al., 1974), and called as BBWV1 and BBWV2 by Murphy et al (Murphy et al., 1995).

We reported before the biological characteristics and the complete nucleotide sequence of BBWV2 isolate B935 (Qi et al., 2000a; Qi et al., 2000b). To clarify the varieties among BBWV isolates, some characteristics and RNA2 sequence of BBWV2 isolate P158 is presented here.

MATERIALS AND METHODS

1. Virus sources

BBWV2 isolate P158 was obtained from Prof. Vittoria Lisa (Istitio Di Fitovirologia Applicata, Italy), and was maintained and propagated in *Chenopodium quinoa*.

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2. Virus preparation and electromicrography

Virus particles were purified as described previously (Zhou et al. 1996), and observed under electron-microscope by dip method.

3. Double immunodiffusion test

Double immunodiffusion test was conducted in 0.75% agar dissolved in phosphate buffer. The purified viruses and healthy component from non-inoculated *C. quinoa* leaves prepared by the same method were used as antigen. The antiserum against BBWV2 B935 was prepared in rabbits.

4. Determination of the components of the capsid

Purified virus particles were mixed with an equal volume of degrading buffer (100 mmol/L Tris-HCl, pH 6.8, 200 mmol/L DTT, 4%SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 2 min, then submitted to electrophoresis through a 12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (NC, 0.45 μm pore size, Bio-Rad) which was then blocked in 1% BSA in Tris-buffered saline overnight at 4℃. Transferred proteins were allowed to react with antiserum (1:1000 dilution) for 1hr, and the membrane was finally incubated for one hour after addition of alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) at 1:7500 dilution. The colorimetric reaction was then allowed to proceed after addition of alkaline phosphatase color development solution. All reactions were performed at room temperature and were finally stopped by soaking the membranes in distilled water.

5. Analysis of the components of genome

Genomic RNA was extracted from virus particles by treatment with TRIzol® reagent (Gibco BRL), and then subjected to agarose gel electrophoresis under denatured condition as described (Sambrook et al., 1989).

6. RT-PCR and cloning

The first-strand cDNA was synthesized by Expand™ reverse transcriptase (Bohringer Manhhein), with the P158 genomic RNA as template and oligo(dT) as primer. Based on the previously reported sequence of BBWV2 isolate B935, two primers were designed to amplify the whole genome of P158 RNA2. The sequences of the primers are listed in Table 1. PCR and PCR product cloning were carried out as previously described with some modification (Zhou et al., 2000).

Table 1 Primers designed for cloning the whole genome of P158 RNA2

Primer No.	Primer sequence (from 5' to 3')	Position in P158 RNA2
Primers for cloning:		
B1 - F	TGTTTTAATAAAATATTAAAA	1 - 21
B5 - R	AAAATACTATTGAAGCCTA	3597 - 3579
Primers for sequencing:		
B1 - R	TGTCTGGCAACCTGTCATC	911 - 893
B2 - F	GCGATACCAGTTGTGGAAA	854 - 872
B2 - R	TGGTCTCTGTAGCAGATTC	1694 - 1676
B3 - F	GGTTTGATGGAGGAAGAT	1625 - 1642
B3 - R	CGAATCCAAATCCCATCTA	2596 - 2578
B4 - F	GTTGCTGGAGCCTACATTG	2432 - 2450
B4 - R	AGGAAGCTTCAACCTAACA	3571 - 3553
B5 - F	AGGGAGATCACTTGATCCT	2776 - 2794

7. Nucleotide sequencing and sequence analysis

Plasmids were purified using a QIA prep spin plasmid kit (Qiagen). All the clones were sequenced using an ABI 377 DNA Sequencer (Perkin Elmer) or an ALFexpress DNA Sequencer (Pharmacia). Universal M13 (-40) primer and Reversal primer were used as well as 8 additional internal primers for sequencing, the internal primers are listed in Table 1. All the sequences were confirmed by sequencing at least two different clones in both directions. Sequenc-

es were assembled and analyzed with the aid of Wiscosin Package Version 8.1 Programs.

RESULTS AND DISCUSSION

1. Electron-micrography

In the purified preparation of isolate P158, isometric particles with diameter of 30 nm were observed under electron-microscope (Fig.1).

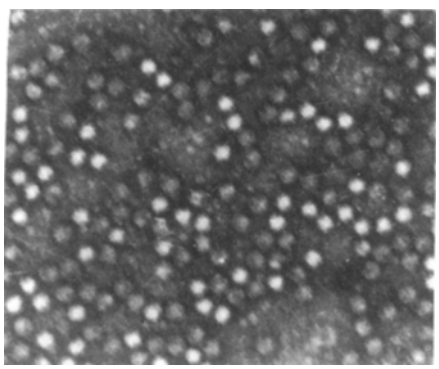

Fig.1 **Electron-photomicrograph of isolate P158 particles (×65000)**

2. Double immunodiffusion test

In double immunodiffusion test with antiserum against BBWV2 B935, both B935 and P158 formed obvious precipitation lines fused with each other (Fig.2), which suggested that P158 and B935 had high homology in antigenicity.

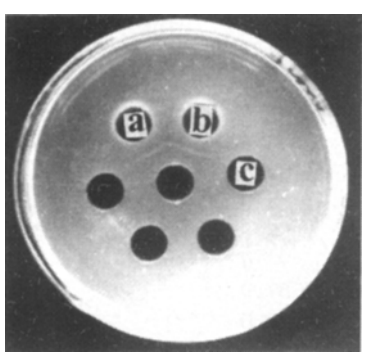

Fig.2 **Immunodiffusion reactions of two isolates of BBWV2. The central well contained antiserum against BBWV2 isolate B935, the peripheral wells contained purified preparations of P158 (a), B935 (b) and healthy leaves (c).**

3. Components of the capsid and genome

SDS-PAGE and Western blot results indicated that the capsid of P158 was made up of two types of polypeptide with molecular weight of 44.7 kD and 21.9 kD, respectively (Fig.3a). The result of agarose gel electrophoresis of the denatured genomic RNAs showed that the genome of P158 was composed of two RNA molecules with length of 6.0 kb (RNA1) and 3.6 kb (RNA2) (Fig.3b).

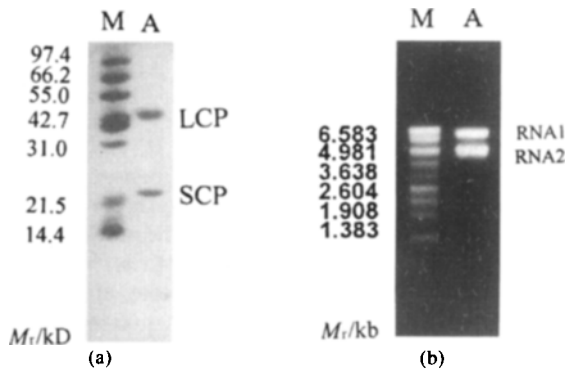

Fig.3 **a. SDS-PAGE of denatured coat proteins of isolate P158, M: mid-range protein marker; A: coat proteins of P158; b. Agarose geleletr ophoresis of denatured genomic RNAs of P158, M: standard RNA marker; A: genomic RNAs of P158**

4. Nucleotide sequence of P158 RNA2

The complete nucleotide sequence of P158 RNA2 was determined. The molecule contained 3597 nucleotides, exclusive of the polyadenylate sequence of unknown length at the 3' end (GenBank accession number: AF228423). The total base composition of RNA2 was 28.6% A, 18.5%C, 23.4%G and 29.5% U. The values were very similar (within 0.3%) to those of B935.

The RNA2 sequence contained one large open reading frame (ORF) (Fig.4). This ORF, which accounted for 89% of the RNA2 sequence, started with the AUG at position 230 and ended with a UAA stop codon beginning at position 3422. The predicted translation product had a calculated molecular mass (M_r) of 119 002 (119 kD) and contained 1064 amino acids (aa). There was a second in-phase AUG at position 614 to 616. If this second AUG codon acts as an initiation codon, the resulting

polypeptide would have an *Mr* of 103726 (104 kD) and contain 936 amino acids.

The 5' non-coding region (NCR) of P158 RNA2 consisted of 230 nucleotides, and had high U + A (69.1%) and low G + C content (30.8%). The 3' NCR of P158 RNA2 was 173 nucleotides in length, excluding the poly (A) tail, had high U + A content (68.0%) and low G + C content (32.0%). The base compositions of the P158 RNA2 5' and 3' NCRs were similar to those of BBWV2 B935.

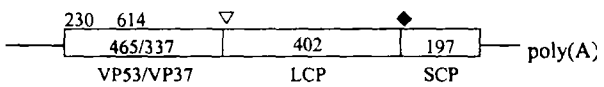

Fig.4. Genomic organization of P158 RNA2. The single line indicates non-coding regions and the open bar represents the polyprotein sequence encoded by the long ORF. The polyprotein is cleaved at Q/G (▽) and Q/A(◆) to release three mature proteins: a protein of unknown function (VP53/VP37), LCP and SCP. The number of amino acids for each protein is indicated inside the rectangle. The number above the rectangle locates the A's of the AUGs of the long ORF

5. Proteins encoded by P158 RNA2

Alignment of the 119kD polypeptide encoded by P158 with the counterpart of B935 showed the existence of two potential proteolytic processing sites, a Q/G at position 465/466 and a Q/A at 867/868. By cutting in the sites, the polyprotein could release three mature proteins. The central (44.2 kD) and C-terminal (22.3 kD) proteins had very high homology (>95%) with the large and small subunits of B935. The molecular weights deduced from amino acid sequences agreed well with the values (44.7 kD and 21.9 kD respectively) estimated by SDS-PAGE of dissociated virus particles. This suggested that the central and C-terminal proteins were coat proteins. The function of the N-terminal protein (52.5 kD/37.2 kD) remains unknown, and further work should be done to determine its function.

6. Comparisons with other fabaviruses

The complete or partial RNA2 sequences of several fabaviruses were determined recently. These viruses include BBWV2 isolates B935 (Qi et al., 2000b), K (Genbank Accession No.: AF104335), MB7 (Nakamura et al., 1998), IP, L, 1-2, E, IP (Kobayashi et al., 1999), patchouli mild mosaic virus (PaMMV) (Ikegami et al., 1998), and BBWV1 isolates PV132 and PV176 (Kobayashi et al., 1999). Comparisons of the nucleotide sequence and the amino acid sequence of BBWV2 P158 with these viruses are listed in Table 2. The results showed P158 had very high amino acid identities with BBWV2 isolates (84 - 98%) and PaMMV (89 - 94%), but to a lesser degree with BBWV1 isolates (58 - 67%).

Table 2 Percentage nucleotide or amino acid sequence identities of BBWV2 P158 isolate and other fabaviruses

Virus isolates	Nucleotide identity			Amino acid identity			Accession number
	Whole genome	5'NCR	3'NCR	LCP	SCP	VP53	
B935	91.2	92.1	93.6	99.0	95.4	96.6	AJ132844
K	83.5	85.9	86.6	96.3	95.9	91.3	AF104335
MB7	83.5	84.5	84.2	98.9	95.4	89.9	AB013616
IP	79.1	75.1	73.4	95.3	92.4	84.3	AB018698
L	—	—	—	92.0	88.8	—	AB018700
1-2	—	—	—	92.0	86.8	—	AB018701
E	—	—	—	98.3	94.4	—	AB018699
PV176	—	—	56	65.4	57.9	—	AB018703
PV132	—	—	57	64.4	67.0	—	AB018702
PaMMV	83.6	85.8	85	89.8	95.4	91.0	AB011007

—: Sequence data are not available

Fig.5 shows a comparable phylogenetic tree based on deduced amino acid sequences of SCPs of fabaviruses. BBWV2 isolates form a tight cluster that is well separated from BBWV1 isolates. SCP of P158 is much more like that of B935 than that of any other BBWV2 isolates. The comparisons of other genes also show P158 is more related with B935 than with other BB-

WV2 isolates.

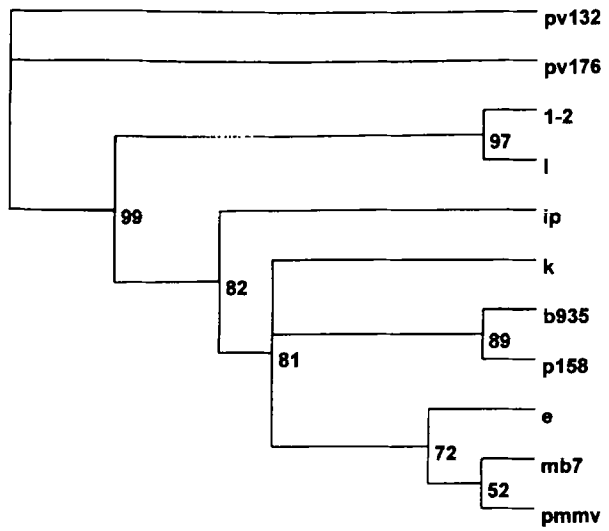

Fig.5 Phylogenetic tree obtained using the puzzle program from alignments of the deduced amino acid sequences of the SCP's of 11 fabaviruses

Based upon spur formation in agarose gel plates, Uyemoto and Provvidenti divided seven isolates of BBWV into two distinct serological type groups named serotype I and serotype II (Uyemoto et al., 1974). Now, the two serotypes are named as two different viruses (BBWV1 and BBWV2) by Murphy et al. (1995). Comparisons of nucleotide or amino acid sequence identities of BBWV isolate P158 with those of other fabaviruses confirmed that the two serotypes of BBWV should be regarded as two different viruses.

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