In vitro expression studies of three proteins of Iranian wheat stripe virus

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Abstract
The genome of Iranian wheat stripe virus (IWSV), a tentative member of the genus *Tenuivirus*, is comprised of three ambisense and one negative sense RNA segments. The coat and non-structural proteins encoded by the vcRNA3 and vRNA4 genes, respectively, were efficiently translated *in vitro*. Translated proteins of vcRNA3 and vRNA4 transcripts were approximately 35000 and 22000 in M_r, respectively, which could be attributed to the corresponding open reading frames (ORFs). The pc4 protein encoded by the IWSV-vcRNA4 was also translated by the same method. Translation product of vcDNA3 transcript gave a band at the approximate position of 37 kDa. The direct translation product of the DNA clone from IWSV-RNA4 gave a band for pc4 similar to that obtained when transcript RNA was used as a messenger. Translation of NS4 and pc4 in viral and viral complementary strands of IWSV RNA4 confirm its ambisense coding strategy.
Keywords: Iranian wheat stripe virus; *Tenuivirus*; protein expression; ambisense genome segments.

INTRODUCTION

The genus *Tenuivirus* comprises of five species of *Rice stripe virus* (RSV) (type), *Maize stripe virus* (MStV), *Rice hoja blanca virus* (RHBV), *Rice grassy stunt virus* (RGSV), and *Echinochloa hoja blanca virus* (EHBV). Iranian wheat stripe virus (IWSV) which was first reported in southern Iran in 1989 (Heydarnejad and Izadpanah, 1989), is a tentative member of this genus (Haenni *et al*., 2005).

The genome of tenuiviruses comprises 4-6 segments which contain 7-12 ORFs encoded by the viral sense (v) or complementary viral sense (vc) RNA strands. Segments 2, 3 and 4 of all tenuiviruses use an ambisense coding strategy, each having one ORF at the 5′ proximal region on both the vRNA and vcRNA strands (Haenni *et al*., 2005; Heydarnejad *et al*., 2006). However, EHBV RNA2 has not been sequenced so far. In the case of RGSV, all six segments have this type of coding strategy and RNA5 and RNA6 correspond to RNA3 and RNA4 of other tenuiviruses (Toriyama *et al*., 1997). The RNAs belonging to the first segment of RSV and the fifth segment of MStV and EHBV are negative (Haenni *et al*., 2005).

Nucleotide and deduced amino acid sequence analyses have allowed identification of specific tenuivirus-encoded proteins and their mapping to specific ORFs. The ORF in vcRNA3 of tenuiviruses (vcRNA5 of RGSV) encodes a 35 kDa putative protein (pc3) that has been identified as the coat protein also known as the N protein. This protein together with the genomic RNA forms the ribonucleoprotein complex (reviewed in Falk & Tsai, 1998). The ORF in the vRNA4 of tenuiviruses (vRNA6 of RGSV) encodes the major non-structural protein that accumulates in infected plants. This protein has been referred to as a disease protein, major non-capsid protein, and non-structural RNA4 protein (NS4) in the RSV, MStV and RHBV, respectively (Kakutani *et al*., 1990; Huiet *et al*., 1992; Hamamatsu *et al*., 1993; Ramirez *et al*., 1993; Miranda and Koganezawa, 1995; Toriyama *et al*., 1997). The ORF located in the vcRNA4 encodes

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the NSvc4 (pc4) protein. (Ramirez and Haenni, 1994). The role of the p5 and p2 proteins has been studied in RGSV. The p5 protein may have an essential role in virus infection in both plant and insect hosts, whereas p2 may be responsible for cell-to-cell movement in plants (Chomchan *et al*., 2003). Other proteins including the pc4 have been mapped to specific ORFs but their functions remain unknown. In some cases, potential properties can be predicted from computer analyses of amino acid sequences. For example, pv2 is predicted to be a small hydrophobic protein that may be membrane-associated and pc2 is likely to be a polyprotein that may be processed to yield two glycoproteins (de Miranda *et al*., 1996; Estabrook *et al*., 1996).

For tenuiviruses both virion RNA and cDNA clones have been used in order to translate their ORFs in the rabbit reticulocyte lysate and wheat germ extract (Falk *et al*., 1987; Hamamatsu *et al*., 1993; Ramirez *et al*., 1993). Protein production in these systems can be monitored in a variety of ways. Usually a radioactive amino acid is added to the translation reaction and, after its incorporation, the gene product is identified by autoradiography following SDS-polyacrylamide gel electrophoresis (Promega, 1996). Translation products produced by several ORFs have been studied in the RSV, MStV and RHBV. For example, *in vitro* translation of N, pv3 (NS3) and NS4 proteins have been analysed for these viruses (Falk *et al*., 1987; Huiet *et al*., 1991; Hamamatsu *et al*., 1993; Ramirez *et al*., 1993). N, pv3 (NS3) and NS4 proteins of MStV have been translated from either viral RNA or specific cloned cDNAs, in either rabbit reticulocyte lysate or wheat germ extract (Falk *et al*., 1987; Huiet *et al*., 1991). Hamamatsu *et al*. (1993) were successful in obtaining translation products of the RSV cDNA clones of RNA segments 2, 3 and 4 in both directions using the two systems. Neverthless, they were not able to detect the N protein when using the purified RSV RNA3.

Although IWSV shares some properties with members of the genus *Tenuivirus*, the N and NS4 proteins of this virus have been partially characterized. According to Sharzei and Izadpanah (1998), like other tenuiviruses, an NS protein accumulates in IWSV infected plants. Sequence analyses of IWSV RNA 2, 3 and 4 have shown that these segments use an ambisense coding strategy and the three putative proteins of 40.9 kDa (N), 37.3 kDa (pc4) and 23.1 kDa (NS4) are encoded by vcRNA3, vcRNA4 and vRNA4, respectively (Heydarnejad *et al*., 2006). The aim of this work was *in vitro* transcription and translation of three IWSV ORFs in vcRNA3, vcRNA4 and vRNA4 and to ascertain the ambisense nature of segment 4 by experimentation.

MATERIALS AND METHODS

Source and propagation of the virus: Both virus-free planthoppers (*Unkanodes tanasijevici*) and naturally IWSV-infected wheat were collected from cereal fields in Badjgah (15 km north of Shiraz, Iran). Infection of collected plants was confirmed by testing them against the IWSV antiserum in agar gel diffusion test (Heydarnejad and Izadpanah, 1992). IWSV was propagated under greenhouse condition by inoculation of wheat seedlings using *U. tanasijevici*. One viruliferous planthopper was used per seedlings at one-leaf stage. Leaf tissues were harvested when symptoms of infection appeared. The tissues were used freshly or stored at -20ºC until further use. IWSV was purified according to the Heydarnejad and Izadpanah method and viral RNA was extracted as previously described (Heydarnejad *et al*., 2006).

Cloning of the IWSV segments 3 and 4: Two recombinant pCR 2.1-TOPO plasmids (Invitrogen), each containing the full length IWSV segments 3 or 4 cDNAs were used for *in vitro* transcription and translation studies. Recombinant plasmids were prepared as described previously (Heydarnejad *et al*., 2006). Due to the absence of promoters at the 5′ and 3′ ends of the multicloning site of the pCR 2.1-TOPO plasmid, transcription and *in vitro* translation studies were carried out using the vector pBluescript SK^+ (Stratagene, USA), a phagemid with both promoter sequences for T_3 and T_7 RNA polymerases flanking the polylinker. This plasmid was first digested using *Not* I and treated with calf intestinal alkaline phosphatase (CIAP, New England BioLabs, UK) (Fig. 1). The digested sample was extracted using phenol-chloroform. Each recombinant pCR 2.1-TOPO plasmid containing the complete IWSV segment 3 or 4 cDNAs (D6 and D2, respectively) was separately digested using *Not* I and the resulting digested samples were extracted using phenol-chloroform. The Two digested samples were then electrophoresed in 1% (w/v) agarose gel and a band corresponding to each IWSV segment was electroeluted using dialysis tubing. For electroelution, the block of agarose containing the DNA of interest (IWSV segments 3 or 4) was placed in a dialysis tube together with 200-300 µl Tris-acetate-EDTA (TAE) buffer. The DNA was eluted from the gel slice electrophoretically

in TAE buffer for 10-20 min at 5 V/cm. After elution, the sample was transferred to a fresh microfuge tube and the DNA was extracted using phenol-chloroform.

Recovered IWSV segments 3 and 4 cDNAs were ligated into pBluescript SK^+. Two recombinant plasmids containing full length IWSV segments were separately digested by different restriction enzymes, some of which cut the IWSV sequence internally. This allows determining orientation of the insert and finding an appropriate enzyme for linearizing recombinant plasmids for *in vitro* transcription. Hence, following this procedure, *Pvu* II was selected to linearize recombinant plasmids.

***In vitro* transcription of IWSV cDNA clones:** Three IWSV ORFs in vcRNA3, vcRNA4 and vRNA4 which encoded the N, pc4 and NS4 proteins, respectively, were used for *in vitro* transcription and translation studies. Five hundred ng of each linearized DNA was added to a tube containing 2 µl of 5X T_7/T_3 buffer, 1 µl of 100 mM DTT , 1 µl of 5 mM rNTPs (Pharmacia, Uppsala, Sweden), 40 U/µl of RNasin (Pharmacia, Uppsala, Sweden), 1 µl of T_7 or T_3 RNA polymerase enzyme (Promega Corp., Madison, USA) and di-ethyl-propyl carbonate (DEPC)-treated water to a final volume of 10 µl. The mixtures were incubated for 60 min at 37ºC. After phenol-chloroform extraction, each sample was treated with 5 U of DNase (Promega Corp., Madison, USA) and reextracted using phenol-chloroform. Four µl of each transcription product, as well as the original DNA template, were run on 1.5% agarose gel and visualized by ethidium bromide.

***In vitro* translation of IWSV mRNAs:** The following reactions were set up in a microfuge tube in order to study *in vitro* translation of IWSV mRNAs. Translations were carried out using rabbit reticulocyte lysate and [35S] methionine (Promega Corp., Madison, USA) as a labeled substrate to detect synthesized proteins. The mixture contained 25 µl of rabbit reticulocyte lysate, 5 µl of 10X reaction buffer, 1 µl of amino acid mixture minus methionine (1 mM), 4 µl [35S] of methionine (1,000 Ci/mmol, at a concentration of 10 mCi/ml), 40 U/µl RNasin, 1 µg of transcription products, and DEPC water to a final volume of 50 µl. A negative control was included containing the same components but without the template. In one case a cDNA clone of IWSV RNA4 was used in place of the preformed RNA during the translation study. In this case 1 µl of T_7 RNA polymerase was added to the above mixture. Rainbow [^{14}C] methylated protein low molecular weight markers (Amersham Pharmacia Biotech, UK) were used for comparison. After addition of all components, the lysates were gently mixed and the reactions were incubated at 30ºC for 120 min. The translation products were analysed using polyacrylamide gel electrophoresis, in order to detect expressed proteins according to the method by Sambrook *et al*. (1989).

RESULTS

Cloning of recovered IWSV segments into pBluescript SK^+: After digestion of recombinant pCR 2.1-TOPO plasmids containing the full length IWSV segments 3 or 4 (D6 and D2, respectively), two bands were recovered for each sample. These corresponded to the linear forms of the pCR 2.1-TOPO plasmids and IWSV segments 3 or 4 cDNAs (Fig. 1).

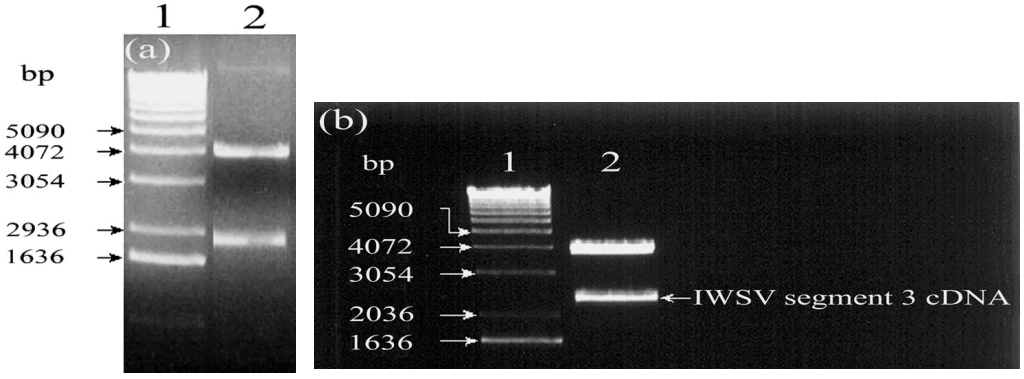

Figure 1. Band patterns of recovered IWSV cDNA segments and pCR 2.1-TOPO plasmid after digestion of recombinant plasmids by *Not* I in 1% agarose gel. (a): Lane 1, DNA ladder; lane 2, cut recombinant plasmid containing IWSV segment 4 cDNA. (b): Lane 1, DNA ladder; lane 2, cut recombinant plasmid containing IWSV segment 3 cDNA.

Table 1. Molecular weights of predicted and *in vitro* translated products of three IWSV open reading frames (ORFs).

RNA	Encoded protein	Number of amino acids	Predicted MW (kDa)	Approximate MW (kDa)
RNA3c	pc3 (N)	317	40.9*	37
RNA4c	pc4	284	37.3	35
RNA4v	pv4 (NS4)	174	23.1	22

*Information for coding capacity of IWSV segments 3 and 4 is derived from Heydarnejad *et al*. (2006).

The two recovered IWSV cDNAs were successfully ligated into CIAP-and *Not* I-treated pBluescript SK^+. Linearized recombinant plasmid containing a full length of IWSV segment 3 or 4 cDNAs was separately used for *in vitro* transcription, after digestion of recombinant plasmid by *Pvu* II. The IWSV cDNA clones were transcribed *in vitro* and the products electrophoresed on an agarose gel. For each sample two bands were obtained, one derived from plasmid DNA and the other being the segment transcript.

***In vitro* translation of IWSV cDNA clones:** A translation reaction was carried out for each IWSV cDNA using RNA generated with the T_3 or T_7 RNA polymerases. Results of polyacrylamide gel electrophoresis showed that three IWSV ORFs were efficiently translated *in vitro* and no band was detected in the negative control. Products of vcDNA4 and vDNA4 transcripts (pc4 and NS4, respectively) gave approximate M_rs of 35000 and 22000, respectively (Table 1 and Fig. 2). In addition, the direct translation product of the cDNA clone from IWSV RNA4, using T_7 RNA polymerase gave a band for pc4 similar to that obtained when transcript RNA was used as a template. The translation product of the vcDNA3 transcript gave a band at the approximate position of 37 kDa (Table 1 and Fig. 2).

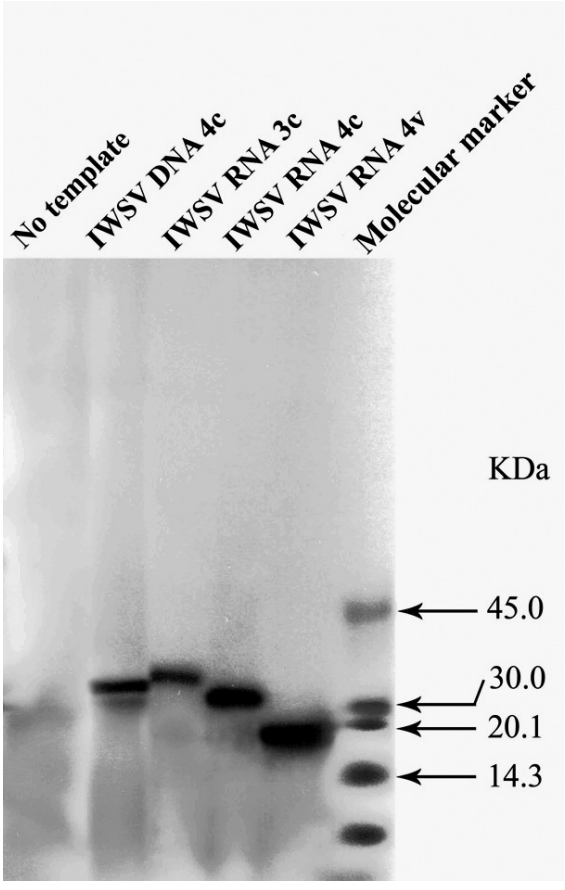

Figure 2. Polyacrylamide gel electrophoresis pattern of the *in vitro* translation products of IWSV RNAs 3 and 4 using rabbit reticulocyte lysate and [35S] methionine as a labelled substrate.

DISCUSSION

In vitro translation of vcRNA3, vRNA4 and vcRNA4 from IWSV in the rabbit reticulocyte lysate system led to the detection of 3 products (N, pc4 and NS4, respectively) that could be attributed to the ORFs identified. Since translation products were detected for vRNA4 and vcRNA4, the ambisense coding strategy was verified for IWSV RNA 4. Among the tenuiviruses, several ORFs and related proteins have been studied. According to Hamamatsu *et al*., (1993), the ambisense nature of RSV segments 2, 3 and 4 was shown by translation *in vitro* of the ORFs present in the 5′ proximal regions of both senses of these segments. The cDNA of RSV RNA segments 2, 3 and 4 have been transcribed *in vitro* in both directions using T_7 RNA polymerase and have been efficiently translated *in vitro* using either rabbit reticulocyte lysate or the wheat germ system. In the case of MStV, the ambisense coding strategy was shown for vRNA segments 3 and 4 by analysis of subgenomic RNA from infected plants (Huiet *et al*., 1991, 1992) or *in vitro* translation of cDNA transcripts (Huiet *et al*., 1991). In addition, *in vitro* translation of RHBV RNA resulted in the pv3 (NS3) and NS4 proteins but not the N protein (Ramirez *et al*., 1992).

The M_r of the three proteins detected (*i.e*., 37000,

35000 and 22000) from transcripts of IWSV vcRNA3, vcRNA4 and vRNA4, respectively, are close to putative products of other tenuiviruses (Falk and Tsai, 1998). The veracity of the estimation for the M_r of N, pc4 and NS4 proteins in this study can be confirmed by the deduced amino acid sequence extracted from the IWSV genome, *i.e*., 40900, 37300 and 23100, respectively (Heydarnejad *et al.*, 2006). However, according to Sharzei and Izadpanah (1998), IWSV produces an NS protein of 15.8 kDa but the location of the gene coding for this protein is unknown. One possible reason for this difference might be inaccuracy of the size markers employed. A coupled transcription/translation system is an alternative method to the separate transcription/translation system for *in vitro* translation.

In this study a DNA clone from IWSV RNA4 was successfully transcribed and translated to produce a similar band for pc4 when compared to that obtained when RNA transcript was used as the template. This data indicates that the usage of a coupled transcription/translation system greatly simplifies the process and reduces the time required to obtain *in vitro* translation results.

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