A novel strategy for the expression of foreign genes from plant virus vectors

Rachel L. Toth, Sean Chapman, Fiona Carr, Simon Santa Cruz*

Mylnefield Research Services Ltd and Division of Biochemistry and Cell Biology, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Received 25 October 2000; revised 3 January 2001; accepted 4 January 2001

First published online 15 January 2001

Edited by Marc Van Montagu

Abstract Potato virus X (PVX)-based vector constructs were generated to investigate the use of an internal ribosome entry site (IRES) sequence to direct translation of a viral gene. The 148-nucleotide IREScp sequence from a crucifer-infecting strain of tobacco mosaic virus was used to direct expression of the PVX coat protein (CP). The IRES was inserted downstream of the gene encoding green fluorescent protein (GFP) and upstream of the PVX CP, in either sense or antisense orientation, such that CP expression depended on ribosome recruitment to the IRES. Stem–loop structures were inserted at either the 3′- or 5′-end of the IRES sequence to investigate its mode of action. In vitro RNA transcripts were inoculated to *Nicotiana benthamiana* plants and protoplasts: levels of GFP and CP expression were analysed by enzyme-linked immunosorbent assay and the rate of virus cell-to-cell movement was determined by confocal laser scanning microscope imaging of GFP expression. PVX CP was expressed, allowing cell-to-cell movement of virus, from constructs containing the IRES sequence in either orientation, and from the construct containing a stem–loop structure at the 5′-end of the IRES sequence. No CP was expressed from a construct containing a stem–loop at the 3′-end of the IRES sequence. Our results suggest that the IRES sequence is acting in vivo to direct expression of the 3′-proximal open reading frame in a bicistronic mRNA thereby demonstrating the potential of employing IRES sequences for the expression of foreign proteins from plant virus-based vectors.

Key words: Potato virus X; Virus vector; Internal ribosome entry site; Internal initiation

1. Introduction

Plant virus-based vectors have a number of advantages as gene expression tools including the ability to direct rapid and high-level expression of foreign genes in mature, differentiated, plant tissue and have been used for a number of different applications. Reporter proteins expressed by viral genomes allow localisation of virus-infected cells [1–3] and can be used to study mutant phenotypes [4,5]. Plant virus-based vectors are also used for the production of valuable foreign peptides and proteins in plants [6]. Plant virus-based vectors offer advantages over other more costly and less flexible protein production systems such as fermentation, and much research has focused on the development of DNA and RNA viruses as vectors for gene expression in plants. Most approaches for the expression of foreign genes from viral vectors rely on either expression of the foreign protein as a fusion to a viral protein [2,7,8], or from a duplicated subgenomic mRNA promoter [9,10]. However, a disadvantage of the latter approach is that the duplicated sequence is prone to homologous recombination with the consequent loss of the inserted sequence [11].

Translation of most eukaryotic mRNAs occurs by the scanning mechanism in which the 40S ribosome subunit binds to a 5′-cap structure and then 'scans' the mRNA until it reaches an AUG translation initiation codon in a favourable sequence context where translation begins [12]. An alternative to cap-dependent initiation of translation, involving direct recruitment of ribosomes to internal tracts within mRNAs, has been observed for some cellular and viral mRNAs. Specific sequences, termed internal ribosome entry sites (IRES), located upstream of AUG codons have been found to be involved in this process, however, the mechanisms of IRES action are not fully understood [13]. IRESs have been found in capped as well as uncapped viral RNA, they show no strong sequence homology and direct the translation of mRNAs with different functions, under different physiological conditions. Although some reports of IRES sequences in plant viruses have proven controversial [14], Ivanov et al. [15] demonstrated that the 148-nucleotide sequence (IREScp) upstream of the coat protein (CP) gene of a crucifer-infecting tobamovirus (crTMV) is capable of promoting internal initiation of translation of the CP in vitro, acting as an IRES. Skulachev et al. [16] subsequently showed that this sequence, and sequences originating from the region upstream of the movement protein gene in both crTMV and tobacco mosaic virus strain U1, mediated expression of a 3′-proximal reporter gene in vivo, on transfection of tobacco protoplasts and particle bombardment of *Nicotiana benthamiana* leaves with bicistronic RNA transcripts.

Potato virus X (PVX) is a single-stranded RNA virus [17] that has been used successfully as a vector for gene expression in plants using both protein fusion and duplicated promoter expression strategies [1,18]. Here we investigate the potential of a novel strategy for the expression of proteins in plants using viral vectors containing an IRES sequence. The strategy employed exploited the fact that, for PVX, cell-to-cell movement is completely dependent on the presence of viral CP [1,5,19]. In order to test the ability of the crTMV IREScp

*Corresponding author. Present address: Department of Entomology and Plant Pathology, Horticulture Research International, East Malling, Kent ME19 6BJ, UK. Fax: (44)-1732-849067.
E-mail: simon.santacruz@hri.ac.uk

0014-5793/01/$20.00
PII: S0014-5793(01)02091-9

to direct the expression of an internal open reading frame (ORF) from a heterologous viral vector we have assessed a series of viral constructs that produce a bicistronic mRNA carrying the green fluorescent protein (GFP) ORF positioned upstream of the IREScp sequence and followed by the PVX CP coding sequence. Using this strategy the infectivity of the viral constructs could be determined by the expression of GFP, and expression of CP could be determined by cell-to-cell movement, resulting in multicellular infection foci expressing GFP.

2. Materials and methods

2.1. Plasmid constructions

The plasmid pTXS, carrying a cDNA of the wild-type PVX genome, pTXS.GFP, in which GFP expression is under the transcriptional control of a duplicated subgenomic mRNA promoter and pTXS.GFPΔCP, in which the CP gene is replaced with that of GFP, have been described previously [5]. The plasmid pIREScpGUS (the gift of J. Atabekov and Y. Dohrokov) carries the IREScp sequence positioned upstream of the gene encoding β-glucuronidase [15]. Polymerase chain reaction (PCR) amplification was performed using pTXS as template with an upstream mutagenic primer, designed to introduce an *Nco*I restriction site across the initiating AUG codon of the viral CP gene, and the universal reverse primer as the downstream oligonucleotide. Following digestion of the amplification product with *Nco*I and *Sac*I, the resulting fragment, which spans the CP and 3′-untranslated region of PVX, was inserted into pIREScpGUS from which the GUS coding sequence had been removed by digestion with *Nco*I and *Sac*I. The resulting plasmid, pIRES-XCP, was the basis for all subsequent plasmid constructions. PCR amplification of pIRES-XCP was performed with a non-mutagenic upstream primer and a mutagenic downstream primer that introduced a *Sac*I site between the 3′-end of the IREScp sequence and the *Nco*I site. The amplification product was digested with *Eco*RI, which cuts naturally at the 5′-end of the IREScp sequence, and *Nco*I prior to cloning of the fragment between the same sites, flanking the IREScp sequence, of pIRES-XCP to produce pIRESs-XCP. PCR amplification of pIRES-XCP was performed with mutagenic primers that introduced *Nco*I and *Eco*RI sites at the 5′- and 3′-ends of the IREScp sequence respectively. The amplification product was digested with *Nco*I and *Eco*RI prior to cloning of the amplified fragment in reverse orientation between the same sites of pIRES-XCP to produce pSERI-XCP. A sequence encoding a stem–loop was introduced 5′ of the IREScp sequence by digestion of pIRES-XCP with *Eco*RI and ligation to a self-annealed oligonucleotide (AAT-TCG-GAT-CCC-GGG-GGG-CCC-TAC-CGC-CGC-GGC-GGT-TAA-CCG-CCG-CGG-CGG-T-AG-GGC-CCC-CCG-GGA-TCC-G) producing pHIRES-XCP. Full-length clones were produced by digestion of the subclones pIRES-XCP, pIRESs-XCP, pSERI-XCP and pHIRES-XCP with *Sal*I and *Spe*I, and cloning of the released fragments, encompassing the IREScp sequence, CP and 3′-untranslated region of PVX, between the same sites of pTXS.GFP to produce pTXS.GFP-IRES-CP, pTXS.GFP-IRESs-CP, pTXS.GFP-SERI-CP and pTXS.GFP-HIRES-CP, respectively. The plasmid pHIRES-XCP was digested with *Eco*RI to release the fragment encoding the stem–loop and, after T4 DNA polymerase treatment to fill in the overhangs, this fragment was cloned into pTXS.GFP-IRESs-CP that had been digested with *Sac*I and T4 DNA polymerase-treated. The resulting plasmid, pTXS.GFP-IRESH-CP, thus contained the sequence encoding the stem–loop 3′ of the IREScp sequence. A derivative of pTXS.GFP-IRESs-CP, containing the 148 nucleotides from upstream of the TMV strain U1 CP gene in sense orientation in place of the IREScp sequence, was produced by PCR amplification of a U1 wild-type cDNA clone. Mutagenic primers designed to introduce flanking *Sal*I and *Sac*I were used and the digested amplification product cloned between the same sites of pTXS.GFP-IRESs-CP to produce pTXS.GFP-IRE-Su1-CP. A further construct, lacking IREScp, was prepared by restriction enzyme digestion of pTXS.GFP-IRESs-CP with *Sal*I and *Sac*I. After polishing the DNA termini with T4 DNA polymerase the plasmid was recircularised to give pTXS.GFP-ΔIRES-CP. Virus derived from each of the above plasmids is referred to by deleting the prefix p from the plasmid name.

2.2. In vitro transcription and plant inoculation

All plasmids were linearised with *Spe*I prior to in vitro transcription using a T7 mMESSAGE mMACHINE™ kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Transcription reaction products were inoculated directly to aluminium oxide dusted leaves of *N. benthamiana* as described previously [5]. Two leaves were inoculated per plant, and each leaf was inoculated with the transcript products derived from 0.2 μg plasmid template.

2.3. Detection of fluorescence and measurement of infection foci

Leaves were viewed under UV illumination (365 nm) generated from a Blak Ray B100-AP lamp (Ultraviolet Products, San Gabriel, CA, USA). For confocal imaging 0.5 cm^2 squares of leaf tissue were cut, mounted in water and imaged using an MRC 1000 confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA) as described previously [5]. The size of fluorescent infection foci was measured using COMOS software (Bio-Rad). An analysis of variance was carried out on area measurements of 20 separate infection foci for each construct, 5 days post inoculation (dpi). The least significant difference at a 5% level was calculated and used to identify groups of data showing statistically significant differences.

2.4. Protoplast preparation and transfection

N. benthamiana plants (4–5 weeks old), grown for at least 10 days in a controlled environment room (16 h light, 22°C), were used for preparation of mesophyll protoplasts according to the method of Power and Chapman [20]. Approximately 6×10^5 protoplasts were electroporated with 10 μl of an in vitro transcription reaction as previously described [21]. Electroporated protoplast samples were incubated at approximately 20°C with 16 h light (200 lx) and harvested after 48 h for protein quantification by enzyme-linked immunosorbent assay (ELISA).

2.5. ELISA

Accumulation of GFP and CP in transfected protoplasts was determined by indirect triple antibody sandwich ELISA essentially as described by Clarke and Bar-Joseph [22]. ELISA plate wells (MaxiSorp, Nalgene Nunc International, Rochester, NY, USA) were coated with a monoclonal antibody raised against either GFP (antibody 3E6, Molecular Probes, Eugene, OR, USA) or PVX CP (antibody MAC58, the gift of Lesley Torrance). Protoplasts from triplicate wells of a tissue culture plate were pooled, pelleted and the pellets ground in 400 μl phosphate-buffered saline. Supernatants collected after brief centrifugation were used for ELISA. The total soluble protein in each sample was measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Bound CP and GFP were probed for with polyclonal antiserum raised against either PVX (Adgen, Auchincruive, UK) or GFP [18] and subsequently alkaline phosphatase-conjugated antibody raised against rabbit IgG (Sigma, St. Louis, MO, USA). All antibodies were diluted 1:2000 for use. The ELISA reaction product (*p*-nitrophenyl) was quantified colourimetrically. Levels of CP and GFP were calculated using Biolinx™ software (Dynatech Laboratories, Chantilly, VA, USA) with standards of PVX virion or His-tagged GFP purified from yeast.

3. Results

Seven constructs, five of which carried the IREScp, were produced (Fig. 1). In pTXS.GFP-IRES-CP, the IRES was introduced in the sense orientation between the GFP and CP coding sequences in order to allow the synthesis of a bicistronic subgenomic mRNA with CP expression dependent on recruitment of ribosomes to the IREScp sequence. A derivative of this clone, pTXS.GFP-IRESs-CP, with a unique *Sac*I restriction enzyme recognition site between the IRES sequence and the PVX CP gene, was prepared to aid subsequent plasmid constructions.

In the clone pTXS.GFP-HIRES-CP, a sequence encoding a stem–loop structure, comprising a 36-bp stem and 4-nucleotide loop (ΔG-90 kcal/mol), was inserted between the 3′-end of the *gfp* gene and the 5′-end of the IRES sequence, in order

to block leaky scanning of ribosomes through the *gfp* gene. In pTXS.GFP-IRESH-CP the stem–loop structure described above was positioned between the 3′-end of the IRES sequence and the CP coding sequence, in order to block scanning ribosomes and prevent translation of the CP. Thus, if the crTMV-derived sequence was acting as an IRES, the introduction of the stem–loop at the 3′-end but not the 5′-end of the IRES would be expected to block CP expression. In the plasmid pTXS.GFP-SERI-CP, the IRES was placed in the opposite orientation in the expectation that IRES activity would be blocked and CP expression would not occur. The plasmids pTXS.GFP-IRESu1-CP and pTXS.GFP-ΔIRES-CP were produced as negative controls. In pTXS.GFP-IRESu1-CP the 148-nucleotide IREScp sequence from the crucifer strain of TMV was replaced with the equivalent region from the U1 strain of TMV, which has been shown to lack IRES activity [15], and in pTXS.GFP-ΔIRES-CP the IREScp sequence was deleted leaving a 9-bp DNA fragment between the GFP and CP coding sequences.

In vitro run-off transcripts, synthesised from the above plasmids and the control plasmids pTXS.GFP and pTXS.GFPΔCP, were infectious as determined by the expression of GFP, giving rise either to individual GFP-expressing cells (TXS.GFP-IRESH-CP, pTXS.GFP-ΔIRES-CP, pTXS.GFP-IRESu1-CP, pTXS.GFPΔCP) or multicellular infection

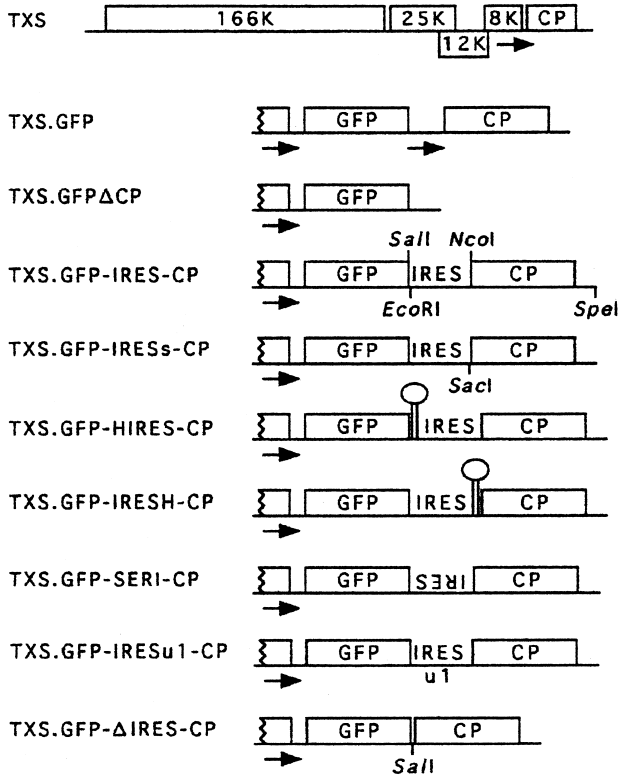

Fig. 1. Schematic representation of the genome organisation of the PVX wild-type clone, TXS, and its derivatives. ORFs are indicated by boxes with the size of their predicted products or the names of the proteins they encode (GFP, green fluorescent protein; CP, coat protein). The promoter that directs expression of the PVX CP is indicated by an arrow and stem–loops are indicated by hairpins. The IRES originating from upstream of the CP in the crucifer-infecting TMV strain and the equivalent region from the U1 strain are indicated by IRES and IRESu1, respectively. Positions of restriction enzyme sites used to produce the constructs are indicated.

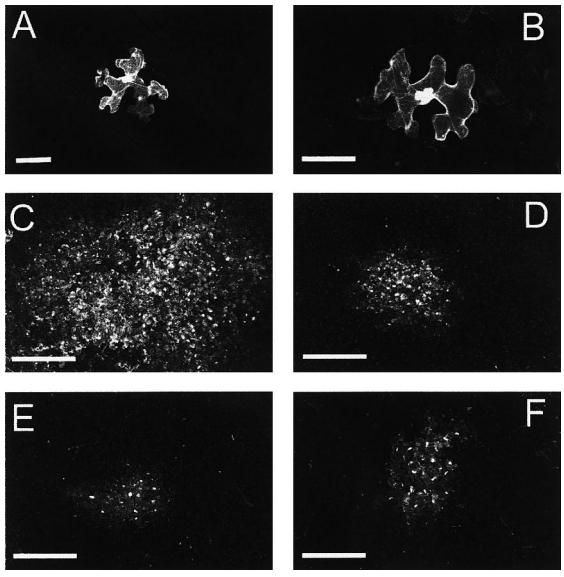

Fig. 2. Confocal images of inoculated *N. benthamiana* leaf tissue, 5 dpi, showing either single epidermal cell infections with TXS.GFPΔCP (A) and TXS.GFP-IRESH-CP (B) or multicellular infection foci with TXS.GFP (C), TXS.GFP-IRES-CP (D), TXS.GFP-HIRES-CP (E) and TXS.GFP-SERI-CP (F). In (A) and (B) bar = 150 μm. In (C)–(F) bar = 750 μm.

sites (pTXS.GFP-IRES-CP, pTXS.GFP-IRESs-CP, pTXS.GFP-HIRES-CP, pTXS.GFP-SERI-CP and pTXS.GFP, Fig. 2 and data not shown).

Area measurements of multicellular, fluorescent, infection foci obtained with the different CP-expressing constructs, representative examples of which are shown in Fig. 2, indicated that the size of infection foci varied between the different constructs. The mean area of 20 infection foci, measured for each construct showing cell-to-cell movement at 5 dpi are shown in Table 1. Statistical analysis of the data showed that all the IRES-containing constructs displayed significantly slower cell-to-cell movement than the control TXS.GFP, in which the CP is translated directly from the 5′-end of a subgenomic mRNA. Furthermore, the infection foci produced by TXS.GFP-HIRES-CP were significantly smaller than those produced by TXS.GFP-IRES-CP, TXS.GFP-IRESs-CP and TXS.GFP-SERI-CP (Table 1).

The constructs containing IREScp were further analysed in transcript-inoculated *N. benthamiana* protoplasts with TXS,

Table 1
Measurement of fluorescent infection foci

Inoculum	Mean area of fluorescent infection foci (mm^2)
TXS.GFP	4.90
TXS.GFP-IRES-CP	0.516
TXS.GFP-IRESs-CP	0.514
TXS.GFP-SERI-CP	0.461
TXS.GFP-HIRES-CP	0.257
TXS.GFP-IRESH-CP	0.0 (single cells)
TXS.GFP-IRESu1-CP	0.0 (single cells)
TXS.GFP-ΔIRES-CP	0.0 (single cells)

The areas of fluorescent lesions on inoculated leaves of *N. benthamiana* were measured 5 dpi. Mean areas in mm^2 are presented. Analysis of variance gave a value of 0.122 for the least significant difference at the 5% level.

Table 2
GFP and CP accumulation in *N. benthamiana* protoplasts

Inoculum	ng GFP/μg TSP	ng CP/μg TSP
TXS	0.0	0.492
TXS.GFP	0.0369	0.442
TXS.GFPΔCP	0.0603	0.0
TXS.GFP-IRES-CP	0.0360	0.1518
TXS.GFP-IRESs-CP	0.0342	0.1080
TXS.GFP-RESI-CP	0.0415	0.1081
TXS.GFP-HIRES-CP	0.0347	0.0504
TXS.GFP-IRESH-CP	0.0	0.0

Protoplasts were inoculated with transcripts and levels of GFP and CP accumulation were assayed by ELISA, 2 dpi. TSP = total soluble protein.

TXS.GFP and TXS.GFPΔCP as controls. The levels of GFP and CP accumulation in infected protoplasts were measured by ELISA at 2 dpi (Table 2). The results from this quantitative study of CP and GFP accumulation correlated well with the in planta observations. All of the constructs carrying the *gfp* gene, with the exception of TXS.GFPΔCP, gave rise to similar levels of GFP accumulation, while levels of CP expression were more variable. There was no detectable CP expression in infections with TXS.GFP-IRESH-CP, as expected from the in planta experiment where no cell-to-cell movement was observed. The other IREScp-containing constructs accumulated lower levels of CP than the TXS and TXS.GFP controls. The construct TXS.GFP-SERI-CP accumulated similar levels of CP to constructs TXS.GFP-IRES-CP and TXS.GFP-IRESs-CP. In addition, TXS.GFP-HIRES-CP, which showed slightly reduced cell to cell movement on plants relative to the other IRES-containing constructs, showed the lowest level of CP accumulation in protoplasts out of the constructs that produced any CP.

4. Discussion

Most approaches to foreign gene expression using virus-based vectors have relied either on the synthesis of a polyprotein, which is proteolytically processed to release the foreign protein, or depend on a viral promoter to direct expression of a foreign gene at the 5′-end of a subgenomic mRNA. Here we have investigated the use of internal initiation of translation as an alternative approach for the expression of genes from virus vectors. The use of an IRES sequence to direct gene expression avoids the need to duplicate promoter sequences in gene insertion strategies, circumventing the possibility of homologous recombination, but not precluding the possibility of non-homologous recombination. We used PVX-based vector constructs that produce a bicistronic mRNA containing the IREScp sequence previously described by Ivanov et al. [15] in which expression of the 3′-proximal CP gene was dependent on internal ribosome entry (Fig. 2, Tables 1 and 2). All constructs carrying the *gfp* gene accumulated similar levels of GFP with the exception of TXS.GFPΔCP, which accumulated two- to three-fold more GFP than the other vectors.

A stem–loop structure inserted 3′ of the IREScp sequence prevented expression of the downstream CP ORF in the bicistronic mRNA indicating that the stem–loop was able to effectively block scanning ribosomes. In contrast, insertion of the stable stem–loop structure at the 5′-end of the IREScp sequence did not abolish expression of the CP indicating that leaky scanning of ribosomes through the *gfp* gene or reinitiation of translation, a phenomenon previously reported to occur with low efficiency in plants [23], were unlikely. In addition TXS.GFP-ΔIRES-CP, in which the IREScp sequence was completely deleted, gave rise to single cell infection sites (Table 1), a result that also argues against leaky ribosome scanning. These results indicate that the IREScp sequence is functioning as a site for direct recruitment of ribosomes for initiation of translation. Our results support observations by Skulachev et al. [16] that 3′-proximal gene expression was obtained from bicistronic transcripts, separated by the IREScp sequence, even when translation of the first gene was abolished by a stem–loop structure inserted upstream of the 5′-proximal ORF. However, in our experiments the presence of the stem–loop immediately upstream of the IRES sequence led to a reduction in CP expression levels (Fig. 2, Tables 1 and 2). This observation could be explained if the upstream loop sequence interfered with direct ribosome landing at the IRES, a conclusion consistent with the known importance of tertiary structure in some animal viral and cellular IRES sequences [13], resulting in reduced levels of CP expression. Another explanation for the observed expression of CP is that there is a cryptic promoter element within the IREScp sequence that is able to initiate transcription of an additional subgenomic RNA, however, we obtained no evidence of extra subgenomic RNAs in Northern blotting experiments (data not shown) and there is no reason to expect a tobamovirus-derived sequence to function as a promoter when present in a heterologous virus. This view is strengthened by the fact that inclusion of the equivalent region from the U1 strain of TMV did not result in CP expression and multicellular infections.

Surprisingly, the IREScp sequence appears to initiate translation effectively in either orientation indicating that either the IRES activity is not orientation specific or that there may be a functional structure that is conserved in both strands. This ability of the IREScp sequence to function in either orientation raises the possibility that this sequence does not function in the same fashion as previously described IRES sequences from animal viruses [13] and further experiments are required to define precisely the mechanism(s) by which the IREScp acts. Whatever the mechanism by which the IREScp sequence is functioning, the data presented demonstrates the possibility of using a plant virus-derived IRES to direct translation of a protein from a heterologous viral vector. However, the levels of CP expression and rates of cell to cell movement achieved with the IREScp-containing constructs were lower than achievable with the progenitor construct in which CP expression is driven from a duplicated subgenomic promoter. This results in impaired infection phenotypes and increased selection pressure for in planta recombinants arising through non-homologous recombination events. Therefore, if IRES-containing vectors are to be of greater utility than currently available vectors it will be necessary to enhance activity of the IRES to promote more efficient translation of the downstream ORF. In the future, greater understanding of the mechanisms by which IRES sequences work will hopefully allow increased levels of gene expression by internal initiation of translation from virus-based vectors, enabling further improvements in vector design.

Acknowledgements: We thank Yuri Dorokhov and Josef Atabekov for providing the plasmid pIREScpGUS, Lesley Torrance for supplying the antibody MAC58, Jim Haselhoff for supplying purified GFP

and Graham Belsham for helpful discussion. R.L.T. and S.C. acknowledge the financial support of Biosource Genetics Corporation. The Scottish Crop Research Institute is grant-aided by the Scottish Executive Rural Affairs Department.

References

[1] Chapman, S.N., Kavanagh, T. and Baulcombe, D.C. (1992) Plant J. 2, 549–557.
[2] Dolja, V.V., McBride, H.J. and Carrington, J.C. (1992) Proc. Natl. Acad. Sci. USA 89, 10208–10212.
[3] Scholthof, H.B., Morris, T.J. and Jackson, A.O. (1993) Mol. Plant–Microbe Interact. 6, 309–322.
[4] Dolja, V.V., Haldeman, R., Robertson, N.L., Dougherty, W.G. and Carrington, J.C. (1994) EMBO J. 13, 1482–1491.
[5] Baulcombe, D.C., Chapman, S.N. and Santa Cruz, S. (1995) Plant J. 7, 1045–1053.
[6] Turpen, T.H. (1999) Philos. Trans. R. Soc. Ser. B 354, 665–673.
[7] Gopinath, K., Wellink, J., Porta, C., Taylor, K.M., Lomonossoff, G.P. and van Kammen, A. (2000) Virology 267, 159–173.
[8] Hamamoto, H., Sugiyama, Y., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y. and Okada, Y. (1993) BioTechnology 11, 930–932.
[9] Donson, J., Kearney, C.M., Hilf, M.E. and Dawson, W.O. (1991) Proc. Natl. Acad. Sci. USA 88, 7204–7208.
[10] Kumagai, M.H., Turpen, T.H., Weinzettl, N., della-Cioppa, G., Turpen, A.M., Donson, J., Hilf, M.E., Grantham, G.L., Dawson, W.O., Chow, T.P., Piatak, M. and Brill, L.K. (1993) Proc. Natl. Acad. Sci. USA 90, 427–430.
[11] Dawson, W.O., Lewandowski, D.J., Hilf, M.E., Bubrick, P., Raffo, A.J., Shaw, J.J., Grantham, G.L. and Desjardins, P.R. (1989) Virology 172, 285–292.
[12] Kozak, M. (1991) J. Biol. Chem. 266, 19867–19870.
[13] Houdebine, L.M. and Attal, J. (1999) Transgenic Res. 8, 157–177.
[14] Belsham, G.J. and Lomonosoff, G.P. (1991) J. Gen. Virol. 72, 3109–3113.
[15] Ivanov, P.A., Karpova, O.V., Skulachev, M.V., Tomashevskaya, O.L., Rodionova, N.P. and Dorokhov, Yu.L. (1997) Virology 232, 32–43.
[16] Skulachev, M.V., Ivanov, P.A., Karpova, O.V., Korpela, T., Rodionova, N.P., Dorokhov, Yu.L. and Atabekov, J.G. (1999) Virology 263, 139–154.
[17] Koenig, R. and Leseman, D.-E. (1989) in: AAB Descriptions of Plant Viruses (Murrant, A.F. and Harrison, B.D., Eds.), No. 354, pp. 1–5, Association of Applied Biologists, Warwick.
[18] Santa Cruz, S., Chapman, S., Roberts, A.G., Roberts, I.R., Prior, D.A.M., Chapman, S. and Oparka, K.J. (1995) Proc. Natl. Acad. Sci. USA 93, 6286–6290.
[19] Santa Cruz, S., Roberts, A.G., Prior, D.A.M., Chapman, S. and Oparka, K.J. (1998) Plant Cell 10, 495–510.
[20] Power, J.B. and Chapman, J.V. (1985) in: Plant Cell Culture (Dixon, R.A., Ed.), pp. 37–66, IRC Press, Oxford.
[21] Gal-On, A., Kaplan, I.B., Roossink, M.J. and Paulukaitis, P.F. (1994) Virology 205, 280–289.
[22] Clarke, M.F. and Bar-Joseph, M. (1984) in: Methods in Virology (Maramorsoch, K. and Koprowski, H., Eds.), Vol. 7, pp. 51–85, Academic Press, San Diego, CA.
[23] Futterer, J. and Hohn, T. (1996) Plant Mol. Biol. 32, 159–189.