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A. tumefaciens-mediated transient expression as a tool for antigen production for *cucurbit yellow stunting disorder virus*

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The emerging importance of criniviruses, together with their limited characterisation, necessitates the development of simple tools to enable rapid detection and monitoring in case of an outbreak. While serological tools would be ideal, criniviruses are notoriously difficult to purify and traditional methods of antibody production, requiring purified virus particles, are extremely challenging. The development of a novel molecular strategy for *in planta* viral antigen preparation to bypass particle purification and allow antibody production are described. An *A. tumefaciens*-mediated transient expression system, coupled with a green fluorescent protein (GFP) purification method was employed to generate CYSDV coat protein (CP) in whole plant leaves. The CYSDV CP gene was ligated into a GFP construct, transformed into *A. tumefaciens* and agroinfiltrated into *N. benthamiana*. Expression levels of the recombinant protein were increased by co-infiltration with the viral gene-silencing suppressor P19 from TBSV. The recombinant protein, purified from plant leaves was used to immunise rats for the preparation of polyclonal antisera.

1. Introduction

Cucurbit yellow stunting disorder virus (CYSDV) causes a serious disease of cucurbits, especially cucumbers and melons (Celix et al., 1996; Wisler et al., 1998). The virus was first reported in the United Arab Emirates in 1990 (Hassan and Duffus, 1991) and has since been identified in other Middle Eastern countries and across the Mediterranean basin from Portugal and the Canary islands to Greece and Turkey (Abou-Jawdah et al., 2000; Desbiez et al., 2000; Louro et al., 2000; Ruiz et al., 2006). CYSDV is transmitted by a whitefly vector, *Bemisia tabaci*, and the disease caused by the virus remains difficult to control and continues to spread within Europe (Janssen and Cuadrado, 2001; Decoin, 2003; Marco and Aranda, 2005). Accurate diagnosis and detection of the virus is an important component of current control strategies, which include implementing quarantine regulations, preventing virus spread and establishment in new areas (Janssen et al., 2003; Wintermantel, 2004). Although nucleic acid based methods are available for the identification of CYSDV (Celix et al., 1996; Berdiales et al., 1999), specific antibodies allowing the development of diagnostic methods more suited to routine and 'in field' detection such as ELISA, tissue printing and lateral flow devices (LFDs) (Danks and Barker, 2000) remain limited (Cotillon et al., 2005; Hourani and Abou-Jawdah, 2003; Livieratos et al., 1999)

Criniviruses however, are notoriously challenging to purify (Karasev, 2000), due in part to being phloem-limited (and hence low titre) and their poor particle stability (Coffin and Coutts, 1993). Their obligate vector transmissibility to plants also results in problems in producing the large numbers of infected plants required for purification.

Conventional methods of antibody production typically require purified virus particles with which vertebrate animals are immunised. This strategy requires a sufficient quantity of relatively pure virus, substantially free of host plant contaminants (Vanslogteren and Vanslogteren, 1957; Hull, 2002). To overcome the difficulties associated with virus purification and allow molecular characterisation and antibody development, recombinant virus proteins can be produced in various expression systems including bacteria, yeasts, insect cells, mammalian cell cultures, plants, nematodes and transgenic rodents (Nørh et al., 2003). Recombinant proteins are often expressed in bacterial systems due to their ease of use, readily available vectors and the high yields that can be achieved (Baneyx, 1999). There are, however, a number of potential drawbacks to

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prokaryotic expression systems. They lack the post-translational modifications found in eukaryotic cells which can lead to different folding of the protein, creating altered secondary and tertiary structures to the native protein. Over expressed proteins often segregate into insoluble aggregates known as inclusion bodies. Proteins can be released with strong denaturing reagents but then require correct refolding (Baneyx, 1999). This process is especially difficult with large proteins (50 kDa or more) that are more prone to misfolding (Schein, 1989). This is of particular relevance to the development of antibodies, as the physical shape of discontinuous epitopes is vital for antibody recognition of native protein compared to recombinant protein.

To counter some of the potential drawbacks of prokaryotic expression systems described, an *in planta* method of antigen production was developed. The work presented in this paper, describes a novel method of antigen production in which plant virus genes, delivered by agrobacteria are expressed transiently in their natural host, in whole plant leaves (Voinnet et al., 2003). The CYSDV CP was ligated into the *A. tumefaciens* T-DNA plasmid vector pBIN 35S GFP and expressed in *N. benthamiana*. In addition, to overcome potential problems with reduced protein expression due to post-transcriptional gene silencing, a second construct consisting of P19, a silencing suppressor from *Tomato bushy stunt virus* (TBSV) was co-infiltrated (Voinnet et al., 2003). We investigated whether this approach can overcome some of the limitations of prokaryotic expression systems, and whether these proteins would produce diagnostic antibodies which would recognise native viral protein in infected plants.

2. Materials and methods

2.1. Virus maintenance

Isolates of CYSDV were maintained in a growth chamber at a controlled temperature of 18 °C with a 12-h photoperiod. Virus was transmitted to healthy cucumber plants by *B. tabaci*.

2.2. Amplification of CYSDV CP

Total plant RNA was extracted from CYSDV-infected leaf material using the KingFisher™ magnetic particle processor system (Thermo Labsystems). The CYSDV coat protein gene was initially amplified using primers CYSDV-CP-F and CYSDV-CP-R. PCR followed cDNA synthesis from total RNA, using the Expand RT-PCR system (Roche), following the manufacturer's recommended protocol. PCR was performed as follows; 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C; using the™ High Fidelity PCR System (Roche).

2.3. Construction of pBIN 35SĈP-GFP

To facilitate cloning of the amplified CYSDV CP into the green fluorescent protein (GFP) plasmid pBIN 35SĜFP (Plant Biosciences Ltd., Norwich, UK), a BamH1 recognition sequence (GGATCC) at position 168–173 of the CP, was silenced using site directed mutagenesis; resulting in the replacement of a thymine residue at position 171 with a cytosine residue. The BamH1 site in the CP gene was silenced as this was the only available site for cloning in the CP gene in front of the GFP gene. The PCR was performed on the CYSDV CP PCR product using the primers BamH1-F/CYSDV-CP-R and BamH1-R/CYSDV-CP-F as shown in Table 1 and the following cycling conditions: 94 °C for 5 min followed by 28 cycles of 1 min at 94 °C, 30 s at 60 °C, 1 min at 72 °C and one cycle at 72 °C for 5 min. The fragments of 191 and 573 nts with homologous 8 bp overlap were subjected to further PCR, using CYSDV-CP-F and CYSDV-CP-R and the following cycling condition; 2 min at 48 °C (to allow the two fragments to anneal) followed by 1 cycle of 5 min at 94 °C, 2 min at 48 °C and 2 min at 72 °C, followed by 35 cycles of 1 min at 94 °C, 30 s at 50 °C and 1 min at 72 °C with a final extension step of 5 min at 72 °C. The modified CP sequence was verified by DNA sequencing.

In order to clone the CP gene into the BamH1/Xba1 site of pBIN 35SĜFP, restriction sites BamH1 and Xba1 were added to the CP gene by PCR. Following denaturing the template DNA for 5 min at 94 °C, amplification of the modified CP gene was performed using primers Xba1-CP and BamH1-CP (Table 1) using 28 cycles of 1 min at 94 °C, 30 s at 52 °C, 1 min at 72 °C, with a final extension step of 5 min at 72 °C. The amplification product was subcloned into pGEM-T easy® vector (Promega) and digested with BamH1 and Xba1. The 790 bp CYSDV fragment, recovered following gel electrophoresis, was directionally cloned into similarly digested pBIN 35SĜFP plasmid. Sequence analysis confirmed the inserted gene was in-frame with the start codon and showed that there were no sequence re-arrangements or deletions (Fig. 1).

2.4. Expression of CYSDV CP-GFP fusion protein

The pBIN 35SĈP-GFP plasmid and an RNA silencing plasmid pBIN 35Sp̂19 (Plant Biosciences Ltd., Norwich, UK), were independently, transformed into the *A. tumefaciens* strain C58C1 (Plant Biosciences Ltd., Norwich, UK), plated onto YEP plates (10 g bacto-yeast extract, 10 g bacto-peptone, 5 g NaCl, 15 bacto-agar in 1 l) containing 5 μl/ml tetracycline and 50 μl/ml kanamycin and incubated for 3 days at 28 °C (Voinnet et al., 2003). A single colony was picked and grown up overnight in YEP broth (10 g bacto-yeast extract, 10 g bacto-peptone, 5 g NaCl in 1 l) supplemented with 5 μl/ml tetracycline and 50 μl/ml kanamycin. The bacteria were pelleted at 8000 × *g*, re-suspended in 10 mM $MgCl_2$ solution containing 100 μM acetosyringone and the OD_{600} was checked and

Table 1
Oligonucleotide primers used for PCR amplification of CYSDV CP. Primer sequences are shown in a 5′–3′ orientation and the polarity is indicated in parentheses. CYSDV-CP-F and CYSDV-CP-R were designed to amplify the entire CP, based on the nucleotide sequence published by Livieratos et al. (1999). Genebank accession number AJ24300. BamH1-CP and Xba1-CP were used to extend the CP; restriction sites for BamH1 and Xba1 are underlined. BamH1-F and BamH1-R were used to silence the CYSDV CP BamH1 sequence.

Application	Primer	Sequence 5′–3′
CYSDV CP amplification	CYSDV-CP-F	ATGGCGAGTTCGAGTGAGAATAA (+)
	CYSDV-CP-R	ATTACCACAGCCACCTGGTGCTA (−)
CYSDV CP cloning	Xba1-CP	GCTCTAGAGCGAACAATGGCGAGTTCGAGTGAGAATAAAACTTCC (+)
	BamH1-CP	CGCGGATCCCTCTACCTTCGATATTACCACAGCCACCTGGTGCTA (−)
BamH1 silencing	BamH1-F	TCACATGGACCCAACGAAATTGAAAGACAT (+)
	BamH1-R	CAATTTCGTTGGGTCCATGTGATCTGCGGTG (−)
CYSDV CP amplification for TaqMan®	CYSDV f	AGAGCAGATGTGATGAGTGATCAAG
	CYSDV r	CCAAAAACTATGGTTGCAAAATCTT
	CYSDV probe	TGAAGCAACCTTTGCTAAGTGCAT (fam)