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Short communication

PCR-based amplification and analysis of specific viral sequences from individual plant cells

Michel Yvon[a], Baptiste Monsion[a], Jean-François Martin[b], Serafín Gutiérrez[a], Stéphane Blanc[a,*]

[a] UMR BGPI, INRA-CIRAD-SupAgroM, TA A-54/K, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France
[b] UMR CBGP, INRA-IRD-CIRAD, Campus International de Baillarguet, CS 30016, 34988 Montferrier-sur-Lez Cedex, France

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A B S T R A C T

Plant virus diversity and the spatial distribution of viral strains or isolates are studied at many different scales: global, regional, local, and even within a single host in different organs or tissues. However, one level that has been totally lacking at the extremity of this scale is that of the single cell. The technical difficulties involved in isolating individual cells from infected plants, and the lack of an efficient diagnostic procedure allowing the specific detection of viral sequences with no major contamination from other cells, have precluded such single cell analysis to date. This paper describes the preparation of protoplasts from plants infected with *Cauliflower mosaic virus* (CaMV), and their decontamination and separation using a technique requiring no specialised equipment. Efficient single-cell nested-PCR procedures (both standard and high-resolution-melting) were developed to allow efficient amplification and analysis of viral sequences from isolated single cells. Moreover, the specific identification of two CaMV variants in different cells demonstrated a very low level of cross-contamination. This technique paves the way for the future development of numerous applications of broad interest in the study of viral diversity and population genetics of plant viruses at the cellular level.

Viruses have been used extensively in recent decades as models for experimental evolution and population genetics (Domingo et al., 2006; Elena et al., 2008; Sacristan and Garcia-Arenal, 2008). In parallel, an immense volume of work has accumulated on the genetic diversity of virus populations, a diversity that can be viewed at different hierarchical scales. Numerous studies investigate viral sequence variations in different hosts from different geographical locations, furthering understanding of the epidemiology and/or the history of genome evolution of a given viral species (for example see: Fargette et al., 2008). Other studies describe sequence variations within samples extracted from single hosts, providing information on mutation (for review see: Duffy et al., 2008) and recombination (Froissart et al., 2005) rates, and characterizing the genetic structure of viral populations (quasispecies). Less frequent are studies that have scaled down the analysis of viral genome variations within an individual host to the level of organs. Although rare, such studies have provided information on the demographic fluctuations of virus populations when invading new territories within a host (Kouyos et al., 2006; Sacristan et al., 2003; Monsion et al., 2008b), the diversity of viral subpopulations in different organs (Jridi et al., 2006; Frost et al., 2001; Sanjuan et al., 2004), and the extent of intra-host competition or genetic drift (Garcia-Arenal et al., 2003). One hierarchical level that is clearly missing at the extremity of this scale is that of the individual cell. Although such analysis would complement the comprehensive understanding of the biology of viruses and the intricate relationship with their multi-cellular hosts, there are very few reports on the amplification, analysis and/or quantification of viral sequences from individual cells in animal models (for a recent example see: Huang et al., 2009), and none at all in plants.

In plant cell biology, inspired by developments in animal single-cell PCR analysis (for a review, see: Todd and Margolin, 2002), increasing research efforts have allowed the amplification and analysis of both DNA and RNA sequences from isolated cells, ultimately allowing gene expression profiling at the level of organs, cell types, and individual cells (Brandt, 2005; Kryvych et al., 2008; Zhang et al., 2008; Dinneny et al., 2008). Major progress has been made based on the conjunction of techniques allowing the dissociation or dissection of tissues and the isolation of individual cells, together with techniques for detection and amplification of minute amounts of specific DNA or RNA sequences. When considering the transfer of single-cell PCR to the field of plant virology, there are two major technical bottlenecks. The first is the difficulty of isolating individual cells, either by preparing and isolating protoplasts, or by using specific equipment such as a micro-manipulator for micro-capillaries (Kryvych et al., 2008) or laser-capture micro-dissection (Nakazono et al., 2003; Day et al., 2005). The second difficulty arises when attempting to amplify very small amounts of viral targets

* Corresponding author. Tel.: +33 04 99 62 48 04; fax: +33 04 99 62 48 22.
E-mail address: blanc@supagro.inra.fr (S. Blanc).

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without contamination from neighbouring cells or from disrupted cells present in the protoplast preparations. This problem is particularly acute in virology as the virus load per cell can be extremely high and thus contaminating targets cannot be neglected.

Both these technical bottlenecks were faced upon developing a procedure for evaluating the multiplicity of infection of host plant cells by genomes of *Cauliflower mosaic virus* (CaMV) in our laboratory (to be published elsewhere). To achieve this goal required evaluation of the rate of cellular co-infection by two CaMV genetic variants, and hence a means of specifically detecting and distinguishing these variants within individual host cells. The present report describes a novel procedure allowing PCR-based amplification and analysis of CaMV sequences from individually isolated protoplasts, with minimal contamination. Moreover, the method requires no specialized technology, and can be performed with equipment and reagents readily available in any plant virology laboratory.

CaMV is the type member of the family *Caulimoviridae*, and its genome is a circular dsDNA of about 8000 bp (Franck et al., 1980). Due to size limitations of the icosahedral capsid, genes encoding fluorescent reporter proteins such as GFP or RFP cannot be incorporated into viable full-length genome clones. Short non-coding sequences of ~40 bp have been successfully inserted between genes I and II, and used as specific genetic markers in mixed populations isolated from whole plants or from leaves (Monsion et al., 2008a). In the present study, two of these genetically labelled CaMV clones, CaMV-VIT1 and CaMV-VIT3 (hereafter VIT1 and VIT3) demonstrated previously to be equally competitive in mixed infected plants (Monsion et al., 2008b) were used. These two clones were derived from the plasmid pCa37, a full-length infectious clone of the CaMV isolate Cabb-S (Franck et al., 1980). The precise insertion site as well as the sequences of the genetic markers are indicated in Fig. 1.

Young turnip plantlets (*Brassica rapa*, cv. "Just Right") were inoculated mechanically with cloned VIT1 or VIT3 DNA, as described previously (Monsion et al., 2008a); 3 weeks later, the resulting systemically infected plants were used to prepare purified virions according to the method of Hull and Shepherd (1976). These purified virions were then used as the virus source for mechanical inoculation of the plants analysed in this study. In all cases, 20 μl of a solution containing 10 μg ml^{-1} of purified virions was rubbed gently together with abrasive Carborundum powder on the surface of plantlet leaves. The inoculated plants were grown in a greenhouse at 25/19 °C and 16/8 h (days/night) for at least 21 days.

A single-cell PCR assay was developed, incorporating a *de novo* technique for isolating protoplasts that would allow them to be processed individually using only equipment and materials routinely present in any laboratory. Leaf discs (1 g; 8.5 cm Ø) from a fully expanded leaf were harvested from plants infected by VIT1, VIT3, or both, and sterilized and macerated with cellulase and macerozyme as described previously (Martiniere et al., 2009). Since numerous protoplasts are disrupted during maceration, rinsing, and associated centrifugations, it was possible that the inevitable release of virus particles and/or viral genomic DNA would contaminate the culture medium, and perhaps also become adsorbed onto the plasma membrane of intact cells. To avoid further disruption of cells and additional contamination of the preparation, protoplasts were incubated in a fixation solution (0.5 M mannitol, 1% glutaraldehyde) for 15 min. The fixed cells were rinsed three times and finally suspended in 10 ml of PBS buffer.

The subsequent steps were implemented for three specific purposes: (i) to optimize protoplast visualization (staining); (ii) to eliminate, as far as possible, virions or viral DNA in the buffer or associated externally with fixed protoplasts (decontamination); and (iii) to dilute the protoplasts throughout the staining and decontaminating process to facilitate their individual isolation (concentration adjustment). Protoplasts in a 250 μl aliquot of the above suspension were stained for 10 min by adding 5 μl of Coomassie blue R solution (100 mg/ml in water), diluted to a final volume of 2 ml prior to centrifugation at 100 × *g* for 2 min to eliminate excess colorant in the supernatant, and finally suspended in 1 ml of pure water. For decontamination, a 250 μl aliquot of this

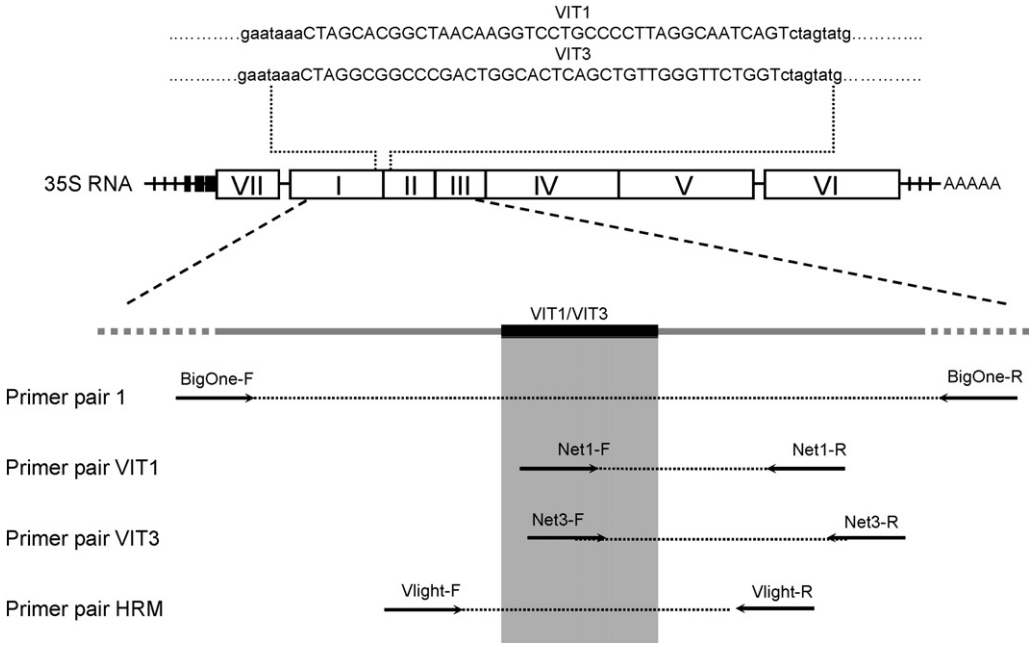

Fig. 1. Genome organization of CaMV genetic variants VIT1 and VIT3. The organization of the genome of CaMV is illustrated by the 35S RNA, with a 5′ untranslated region bearing several small ORFs (vertical bars) and a 3′ polyA tail. Viral ORFs I–VII are shown as open boxes. The genetic markers VIT1 and VIT3 are inserted between ORFs I and II (Monsion et al., 2008a). The exact sequence of the markers and the insertion site is shown at the top. A scheme of the region flanking the markers is used to indicate the relative positions of the different primer pairs used in the PCR procedures described in the text. The exact position of primers and their sequence is provided as electronic supplementary material (Table S1).