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The use of fluorescence microscopy to visualise homotypic interactions of tomato spotted wilt virus nucleocapsid protein in living cells

Marjolein Snippe[a], Jan Willem Borst[b], Rob Goldbach[a], Richard Kormelink[a,*]

[a] *Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands*
[b] *MicroSpectroscopy Centre, Laboratory of Biochemistry, Wageningen University, Dreyenlaan 3, 6703 HA Wageningen, The Netherlands*

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Abstract

Fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) were employed to study homotypic protein–protein interactions in living cells. To this end, the nucleocapsid (N) protein of tomato spotted wilt virus (TSWV) was expressed as a fusion protein with either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). Co-expression experiments of the two fusion proteins were carried out in baby hamster kidney (BHK21) cells. Both the wild type and the fusion proteins showed a peri-nuclear localisation pattern and were observed to form aggregates. In sensitised emission experiments, energy transfer was observed to take place from CFP to YFP when the two fluorophores were fused to TSWV N protein, indicating strongly homotypic interaction of the N proteins. This was confirmed by acceptor photobleaching studies as well as by FLIM experiments. All three methods showed interactions taking place, not only in the aggregates in the peri-nuclear region, but also throughout the cytoplasm. These experiments clearly demonstrated the potential of these fluorescence methods for studying the interactions of viral proteins in living cells.

Keywords: Fluorescence resonance energy transfer (FRET); Fluorescence lifetime imaging microscopy (FLIM); Protein–protein interaction; Tospovirus; Bunyaviridae; Virus assembly

1. Introduction

Fluorescence (or Förster) resonance energy transfer (FRET) is a relatively new technique that enables protein studies in living cells. The principle of FRET lies in the non-radiative transfer of energy between a donor fluorescent molecule and an acceptor molecule, when the molecules are in very close proximity (<10 nm) (Gadella et al., 1999; Sekar and Periasamy, 2003). This principle can be used for in vivo study of, e.g. protein folding (Rhoades et al., 2003), protein cleavage (Bastiaens and Jovin, 1996; Xu et al., 1998), phosphorylation (Sato et al., 2002; Violin et al., 2003) or protein–protein interactions (Immink et al., 2002; Larson et al., 2003). In principle, FRET-based techniques are applicable to any protein, including glycoproteins. They have the advantage, above common approaches such as co-immunoprecipitation and yeast-two-hybrid, that they not only enable a study on homo as well as heterodimeric interactions of proteins in living cells, but provide simultaneously insight into the intracellular localisation of these complexes. To evaluate the use of these methods in virological studies, the homotypic interaction of tomato spotted wilt virus (TSWV) nucleocapsid (N) proteins was studied in living mammalian cells as described in this paper using FRET and fluorescence lifetime imaging microscopy (FLIM). As the two fluorophores (cyan fluorescent protein, CFP—donor, and yellow fluorescent protein, YFP—acceptor) are fused, in this case, to two separate N molecules, FRET will in practice take place only when the two proteins tagged with the fluorophores interact. If FRET takes place, a decrease in donor fluorescence as well as an increase in acceptor fluorescence can be observed. This phenomenon is called sensitised emission. In order for energy transfer to occur, it is essential that

* Corresponding author. Tel.: +31 317 483085; fax: +31 317 484820.
E-mail address: richard.kormelink@wur.nl (R. Kormelink).

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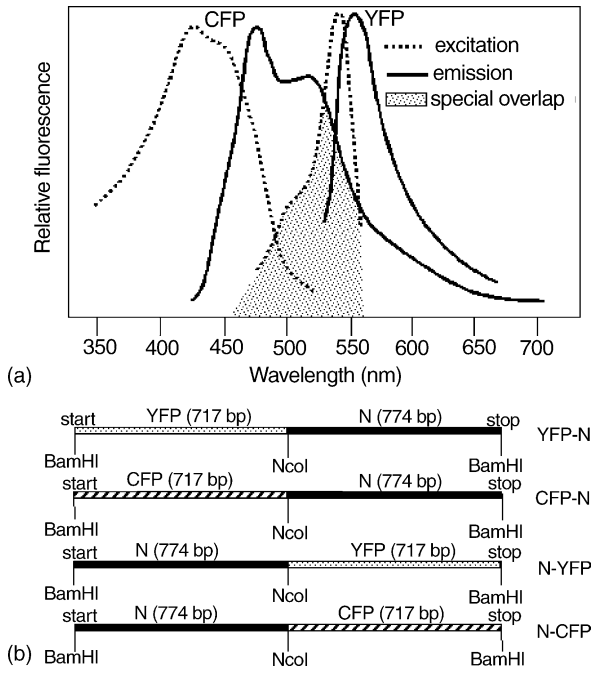

Fig. 1. (a) Absorption and emission spectra of CFP and YFP. FRET can occur because of the overlap of the donor (CFP) emission wavelength with the acceptor (YFP) excitation wavelength. Adapted from Gadella et al. (1999). (b) Schematic representation of the fusion constructs used during analyses. CFP/YFP fluorophores were fused in frame at the position of the start codon (CFP-N/YFP-N) or the stop codon (N-CFP/N-YFP) of the TSWV N protein, after translation resulting in an N protein that is, respectively, N- or C-terminally fused to the fluorophore.

the emission spectrum of the donor fluorophore has a spectral overlap with the absorption spectrum of the acceptor, as illustrated for the CFP-YFP FRET couple in Fig. 1a. In order to measure FRET, and thus observe protein–protein interaction, several methods can be used: (1) Sensitised emission: the observation of YFP emission upon excitation of CFP; (2) acceptor photobleaching, after which the increase in donor fluorescence will give an indication of the FRET efficiency; and (3) fluorescence lifetime imaging microscopy, in which the fluorescence lifetime of the donor fluorophore (CFP) is measured (Gadella et al., 1999; Hink et al., 2002; Xia and Liu, 2001).

In this study, the three approaches referred to above were used to demonstrate the occurrence of TSWV N–N interactions in vivo.

TSWV is the type species of the *Tospovirus* genus within the family Bunyaviridae (Elliott, 1990; Elliott, 1996). The tospoviruses are the only plant-infecting members of this virus family. Like all members of the Bunyaviridae, TSWV consists of enveloped, spherical particles of 80–120 nm in diameter containing a tri-partite RNA genome. The L segment is of negative polarity and codes for the viral RNA-dependent RNA polymerase (de Haan et al., 1991), whereas both the M and S segments are of ambisense polarity. The M RNA codes for the glycoprotein precursor GP in the viral complementary strand and the movement protein NSm in the viral strand (Kormelink et al., 1992). The S RNA encodes in viral complementary sense the nucleoprotein (N) and in viral sense the non-structural protein NSs, which is involved in suppression of gene silencing (de Haan et al., 1990; Takeda et al., 2002; Bucher et al., 2003). The genomic RNA molecules are tightly associated with the N protein and small amounts of the viral polymerase to form infectious ribonucleoproteins (RNPs) (van Poelwijk et al., 1993).

Infectious RNPs are assumed to concentrate at the Golgi complex where the two glycoproteins G1 and G2 are retained. Mature virus particles are then formed through enwrapment of RNPs by a Golgi stack to acquire a lipid membrane containing G1 and G2 (Kikkert et al., 1999). Prior to the assembly of mature virus particles, RNPs are formed by the association of N protein with viral RNA. TSWV N protein has been demonstrated to bind ssRNA non-specifically in vitro in a co-operative manner (Richmond et al., 1998). Moreover, N–N interactions have been shown to occur using the yeast-two-hybrid system (Uhrig et al., 1999). Studies on related Hantaviruses, both in vivo and in vitro, have yielded similar results (Alfadhli et al., 2001; Kaukinen et al., 2001).

Although it is very likely that many of the steps within the process of RNP and virus assembly require interaction between the viral structural proteins, studies on protein–protein interactions involving the viral glycoproteins have so far been hampered due to the absence of proper systems to investigate these interactions. The aim of this study was to evaluate the use of fluorescence microscopy techniques to study in vivo protein–protein interactions between the TSWV structural proteins in order to identify, on the long term, all interactions between the N, G1 and G2 proteins. As a logical first step the in vivo occurrence of homotypic interactions of the N protein are described, illustrating the potential of these methods for related research questions.

2. Materials and methods

2.1. Cell culture

Baby hamster kidney (BHK21) cells were maintained at 37 °C with 5% CO_2 in Glasgow MEM culture medium (Invitrogen) supplemented with 10% foetal calf serum, 2.6 g/l tryptose phosphate broth (Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.2. Constructs

The pCS2 plasmid (Rupp et al., 1994) was used for cloning and expression in BHK21 cells. The BamHI site in the multiple cloning site was used to insert the DNA coding for the fusion proteins described below.

The TSWV N gene was amplified from TSWV isolate BR-01 by PCR using primers carrying a BamHI restriction site and a start codon in the forward primer (p19), and an NcoI site and no stop codon in the reverse primer (Rev-Nco-