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A versatile complementation assay for cell-to-cell and long distance movements by cucumber mosaic virus based agro-infiltration

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ABSTRACT

Microinjection, bombardment or tobamovirus and potexvirus based assay has been developed to identify the putative movement protein (MP) or to characterize plasmodesma-mediated macromolecular transport. In this study, we developed a versatile complementation assay for the cell-to-cell and long distance movements of macromolecules by agro-infiltration based on the infectious clones of cucumber mosaic virus (CMV). The movement-deficient CMV reporter was constructed by replacing the MP on RNA 3 with ER targeted GFP. The ectopic expression of CMV MP was able to efficiently move the RNA3-MP::erGFP reporter from the original cell to neighboring cells, whereas CMV MP-M5 mutant was unable to initiate the movement. Importantly, the presence of CMV RNA1 and RNA2 can dramatically amplify the movement signals once the RNA3-MP::erGFP reporter moves out of the original cell. The appropriate observation time for this movement complementation assay was at 48–72 hours post infiltration (hpi), whereas the optimal incubation temperature was between 25 and 28 °C. The ectopic co-expression of MPs from other virus genera, NSm from tomato spotted wilt tospovirus (TSWV) or NSvc4 from rice stripe tenuivirus (RSV), could also facilitate the movement of the RNA3::erGFP reporter from the original cell into other cells. The chimeric mutant virus created by substituting the MP of CMV RNA3 with NSm from TSWV or NSvc4 from RSV move systemically in *Nicotiana benthamiana* plants by agro-infiltration. This agro-infiltration complementation assay is simple, efficient and reliable. Our approach provides an alternative and powerful tool with great potentials in identifying putative movement protein and characterizing macromolecular trafficking.

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1. Introduction

Plant viruses encode movement proteins (MPs) that localize to PDs and help its viral genome or particles traffic in the plant cells (Atabekov and Taliansky, 1990; Deom et al., 1992; Lucas, 2006). The MPs from a number of plant viruses are found to localize and increase the size exclusion limit (SEL) of PD to facilitate cell-to-cell or long distance movement (Ding, 1997; Fujiwara et al., 1993; Noueiry et al., 1994; Wolf et al., 1989; Xiong et al., 2008). However, the mechanisms by which MPs traffic from cell-to-cell or systemically remain largely unknown. In addition to viral MPs, several plant endogenous proteins have also been found to traffic from cell to cell or from organ to organ. These proteins include the sucrose transporter SUT1 (Kuhn et al., 1997); KNOTTED 1, which has the ability to traffic between cells, to gate PD, and to specifically traffic its mRNA (Kim et al., 2002; Lucas et al., 1995); CmPP16, a plant analog protein of the viral movement protein that can transport itself and RNA through PDs (Xoconostle-Cazares et al., 1999); and the FLOWERING LOCUS T that moves long-distance for floral induction in Arabidopsis (Corbesier et al., 2007). Cell-to-cell communication between different plant cells allows for the non-cell-autonomous regulation of plant development, including cell differentiation, morphogenesis or host defenses (Corbesier et al., 2007; Ding, 1997; Kim et al., 2002; Lucas, 1995; Lucas and Wolf, 1993; Mezitt and Lucas, 1996).

Previously, several methods have been developed for studying plasmodesma-mediated macromolecular movement, including microinjection and bombardment assays. Recombinant MPs expressed through prokaryotic cells are labeled with fluorescence probe. Then, the labeled proteins are detected in cells through the microinjection of tobacco mesophyll cells (Ding et al., 1995a; Fujiwara et al., 1993; Noueiry et al., 1994; Wolf et al., 1989). Alternatively, cell-to-cell movement can be assayed through the bombardment of GFP-fused MPs into plant cells to observe the

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MP:GFP fusion protein move from the originally delivered cells into neighboring cells (Itaya et al., 1997).

The complemented movement assay has also been developed to identify the putative movement protein (MP) from a plant virus. Tobacco mosaic virus (TMV) and potato virus X (PVX) have often been engineered and used as mutant viruses that cannot move from cell to cell. In complementation assays, the cell-to-cell or long distance movement is characterized by co-bombardment or co-agroinfiltration with a putative MP and a movement-defective virus or by the use of a chimeric virus created by substituting a putative MP into a movement-defective virus. These complementation approaches have been used for the identification and characterization of many viral movement proteins (De Jong and Ahlquist, 1992; Li et al., 2004; Sasaki et al., 2001; Tilsner et al., 2013; Xiong et al., 2008).

Cucumber mosaic virus is the type member of the genus *Cucumovirus* in the family *Bromoviridae* (Palukaitis et al., 1992). It is a single-stranded, plus-sense RNA virus and has three single-stranded genomic RNAs, designated RNAs 1, 2 and 3 (Palukaitis et al., 1992). RNA1 and RNA2 encode the 1a protein and 2a protein, respectively, which form the viral replication complex (Hayes and Buck, 1990). The subgenomic RNA of RNA2 encodes the 2b protein, which is involved in long distance movement and in the suppression of gene silencing (Brigneti et al., 1998; Ding et al., 1995b). RNA3 encodes a movement protein (MP) at the 5′ end and a coat protein (CP) by its subgenomic RNA. Both MP and CP are required for cell-to-cell movement and for systemic trafficking (Kaplan et al., 1998).

Previously, we generated the *Agrobacterium*-mediated infectious cDNA clones of cucumber mosaic virus (CMV) (Yao et al., 2011). These clones induce typical viral symptoms after agro-infiltration into *Nicotiana benthamiana*. In this study, we developed a versatile complementation assay for the cell-to-cell and long distance movements of macromolecular transport by agro-infiltration based on CMV infectious clones. We demonstrated that the movement protein from CMV or from other viruses belonging to different genera expressed ectopically by a binary vector transports the engineered movement-deficient CMV RNA3-MP::erGFP reporter from the originally delivered cell into neighboring cells. This system can also be used to generate a chimeric mutant virus for long distance movement assays. Our agro-infiltration assay is simple, efficient and reliable. It provides an alternative tool with great potentials in identifying putative movement proteins and characterizing macromolecular trafficking.

2. Materials and methods

2.1. Plant materials

All agro-infiltration experiments were performed in *N. benthamiana*. Six- to eight-week-old plants were used in all of the experiments. Agro-infiltrated plants were maintained in a growth chamber with 25 °C/23 °C day/night temperatures under a 16 h light/8 h dark photoperiod. CMV, tomato spotted wilt tospovirus (TSWV) and rice stripe tenuivirus (RSV) were propagated in *N. benthamiana* and frozen at −80 °C until use.

2.2. Plasmid constructs

2.2.1. pCB301-CMV RNA3-MP::erGFP

pCB301-CMV RNA3 (Yao et al., 2011) without MP was amplified with XT15 and XT16 (Supplemental Table S1) using PrimeSTAR High Fidelity DNA Polymerase (TaKaRa, Dalian, China). The amplified 7 kb PCR product was digested with *Nco*I and *Xba*I as a recipient vector for ER-targeted GFP (erGFP). The erGFP was amplified from genomic DNA of 16cGFP (Ruiz et al., 1998) using the forward primer XT17 and the reverse primer XT18. The PCR product was cut with *Nco*I and *Xba*I and inserted into the pCB301-CMV RNA3 vector without MP digested with the same restriction enzyme.

Supplementary table related to this article found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2014.06.013.

2.2.2. pCXSN-CMV MP and pCXSN-CMV MP:M5

The coding sequence of CMV MP was amplified using PrimeSTAR HF DNA Polymerase from the full-length infectious cDNA clone of pCB301-CMV RNA3 by the primer pairs XT758 and XT759. The PCR fragments were added with adenine by rTaq (Shengong, Shanghai, China) and introduced into binary vector pCXSN-T (digested with *Xcm*I) under the CaMV-Double-35S promoter (Chen et al., 2009). The forward insertion was selected by the primers XT307 and XT759. To generate the M5 mutant, site-directed mutagenesis was performed to alter the amino acids Tyr-144 to Ala-144 and Asn-145 to Ala-145 on CMV MP using the two-step PCR procedure described previously (Hu et al., 2012).

2.2.3. p2300S-TSWV NSm

The coding sequence of TSWV NSm was amplified from the cDNA of TSWV-YN, which was isolated from Yunnan Province, China (accession number JF960235.1) (Hu et al., 2011) by PrimeStar HF DNA Polymerase using the forward primer XT654 and the reverse primer XT655. Then, the blunt-ended PCR fragment of NSm was digested with *Xho*I and inserted into the *Sma*I and *Sal*I sites of pCambia2300 (CAMBIA, Canberra, Australia) with the CaMV-Double-35S promoter.

2.2.4. pCXSN-RSV NSvc4

The coding sequence of RSV NSvc4 was amplified from the cDNA of RSV-NJ, which was isolated from Nanjing, Jiangsu Province, China using the forward primer XT113 and the reverse primer XT114. The NSvc4 PCR fragment was added with adenine by rTaq and inserted into pCXSN-T (digested with *Xcm*I) under the CaMV-Double-35S promoter. The forward insertion was selected by XT307 and by XT114.

2.2.5. pCB301-CMV RNA3$^{TSWV\ NSm}$ and pCB301-CMV RNA3$^{RSV\ NSvc4}$

pCB301-RNA3 without MP was amplified and digested with *Nco*I and *Xba*I as described above. The TSWV NSm was amplified from the cDNA of TSWV-YN using the primers XT134 and XT135. The coding sequence of RSV NSvc4 was amplified from the cDNA of RSV-NJ using the primers XT113 and XT114. Both PCR products were cut with *Nco*I and *Xba*I and inserted into the pCB301-CMV RNA3 vector without MP digested with the same sites to generate chimeric pCB301-RNA3$^{TSWV\ NSm}$ and pCB301-RNA3$^{RSV\ NSvc4}$, respectively.

2.3. Electroporation and agro-infiltration

All recombinant constructs were transformed into *Agrobacterium tumefaciens* cells (GV3101) via electroporation. A volume of 100 μl of *Agrobacterium* competent cells was placed on ice to thaw, mixed with 2.5 μl 100 ng/μl plasmid, then transferred to a prechilled electroporation cuvette (1 mm gap) and electroporated at a field strength of 1.8 kV cm^{-1} using an Bio-Rad MicroPulse 165-2100 electroporator (Bio-Rad, Shanghai, China). One milliliter of prechilled LB medium was added to the cuvette immediately after electroporation. The mixture was incubated with shaking at 200 rpm at 28 °C for 3 h. In total, 100 μl culture was spread onto selective LB plates (kanamycin 50 mg/ml^{-1}) and incubated at 28 °C for two days. Colony PCR was performed to confirm the presence of the various DNA constructs.

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