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Visualizing double-stranded RNA distribution and dynamics in living cells by dsRNA binding-dependent fluorescence complementation

Xiaofei Cheng [a,b], Ping Deng [a], Hongguang Cui [a], Aiming Wang [a,*]

[a] Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario N5V 4T3, Canada
[b] College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, Zhejiang 310036, PR China

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ABSTRACT

Double-stranded RNA (dsRNA) is an important type of RNA that plays essential roles in diverse cellular processes in eukaryotic organisms and a hallmark in infections by positive-sense RNA viruses. Currently, no *in vivo* technology has been developed for visualizing dsRNA in living cells. Here, we report a dsRNA binding-dependent fluorescence complementation (dRBFC) assay that can be used to efficiently monitor dsRNA distribution and dynamics *in vivo*. The system consists of two dsRNA-binding proteins, which are fused to the N- and C-terminal halves of the yellow fluorescent protein (YFP). Binding of the two fusion proteins to a common dsRNA brings the split YFP halves in close proximity, leading to the reconstitution of the fluorescence-competent structure and restoration of fluorescence. Using this technique, we were able to visualize the distribution and trafficking of the replicative RNA intermediates of positive-sense RNA viruses in living cells.

Introduction

Double-stranded RNA (dsRNA) is the duplex type of RNA that plays essential roles in diverse biological processes such as viral replication, RNA silencing, viral infection recognition, RNA editing, processing and transport (Jacobs and Langland, 1996). dsRNA may be formed *in trans* by pairing of two complementary RNA molecules or small RNAs targeting to their RNA substrates, and *in cis* by pairing of complementary regions within a single RNA (Rosok and Sioud, 2004). Some RNA viruses, i.e., double-stranded RNA viruses have dsRNA as their genome. dsRNA may be biosynthesized by either viral RNA-dependent RNA polymerase (RdRp) (Dewitte-Orr and Mossman, 2011) or cellular RNA-dependent RNA polymerases (RDRs) using viral or foreign RNA as a template (Maida and Masutomi, 2011; Sijen et al., 2007). Since the vast majority of known plant and animal viruses are positive-sense single-stranded RNA (ssRNA) viruses and double-stranded viral RNA represents the replicative RNA intermediates, dsRNA has been recognized as a hallmark signature of viral infection (Dewitte-Orr and Mossman, 2011). In eukaryotic cells, dsRNA can trigger RNA silencing, a sophisticated, across-kingdom conserved, sequence-specific mechanism that has evolved against biotic invaders such as viruses (Ding, 2010). In insects and mammals, viral dsRNA or other pathogen-associated molecular patterns (PAMPs) unique to the invading microbes can also induce additional innate immune reaction (Thompson and Locarnini, 2007). In this type of the immune response, PAMPs are specifically recognized by germline-encoded pattern-recognition receptors (PRRs) to activate signaling pathways for the biosynthesis of multiple cytokines including type I interferon (IFN) and inflammatory cytokines (Akira et al., 2006). For instance, in mammals, toll-like receptor 3 (TLR3), RNA helicase RIG-I, MDA-5, and protein kinase PKR specifically recognize intracellular dsRNA, leading to the secretion of IFN or stress-induced apoptosis (Alexopoulou et al., 2001; Kumar et al., 1994; Peisley and Hur, 2013). To counteract these defense mechanisms, viruses encode one or more proteins that can block the cellular RNA silencing pathway and/or supress host immune responses (Leung et al., 2012; Li and Ding, 2001), and recruit intracellular membranes to house the virus replication complex (VRC) so as to minimize the exposure of double-stranded replicative intermediates to cellular dsRNA specific effectors (den Boon et al., 2010; Miller and Krijnse-Locker, 2008). Therefore, the precise subcellular localization of dsRNA would assist in better understanding dsRNA-involved biological processes.

Despite the importance of dsRNA in gene regulation, immune response and RNA virus replication, current methods for the

* Corresponding author. Tel.: +1 519 963 6697; fax: +1 519 457 3997.
E-mail address: aiming.wang@agr.gc.ca (A. Wang).

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visualization of dsRNA have been limited to two *in vitro* techniques, i.e., detection by dsRNA-specific monoclonal antibodies J2 and K1, and nucleic acid fluorescent *in situ* hybrid (FISH) (Bolten et al., 1998; Stollar and Stollar, 1970). Both *in vitro* methods have been employed to subcellularly localize viral replicative dsRNA of many viruses in animals, plants, and insects (Weber et al., 2006). Unfortunately, both methods are time consuming and cannot be used to study dsRNA distribution and dynamics *in vivo*. Moreover, the dsRNA signal may be dislocated during sample preparation, or seriously weakened due to the presence of the rigid and low-permeant cell wall. Therefore, an *in vivo* technology for visualizing dsRNA is highly demanded in cell biology research. Here, we report a novel, sensitive and specific dsRNA reporter system for visualizing dsRNA distribution and dynamics in living cells. As examples, we used this system to *in vivo* visualize the subcellular distribution and trafficking of dsRNA intermediates in the VRCs of *Turnip mosaic virus* (TuMV) and *Carnation Italian ringspot virus* (CIRV) in plant cells.

Results

Basic strategy

We assumed that the efficient binding of two dsRNA binding proteins (DRBs) to a common interacting partner, dsRNA, can bring in close proximity two non-fluorescent DRBs fused with the non-overlapping, N-terminal and C-terminal moieties of a fluorescent protein, respectively, to reconstitute the fluorescence-competent protein structure (Fig. 1). To achieve optimal specificity and sensitivity, the two DRBs should have the following properties: (i) highly specific recognition of dsRNA without binding to ssRNA (containing no dsRNA structure) or DNA, (ii) binding to dsRNA without sequence specific (interaction with the dsRNA backbone), and (iii) small sizes that allow free diffusion into subcellular compartments. Based on the published data, three viral proteins, the B2 protein of flock house virus (FHV), the NS1 protein of influenza A virus, and the VP35 of Marburg virus (MARV), can bind to dsRNA in a non-sequence-specific manner and cover the entire dsRNA backbone (Bale et al., 2012; Bornholdt and Prasad, 2008; Lingel et al., 2005). To minimize the side effect of these proteins, only the coding sequences for dsRNA binding domains, i.e., amino acids (aa) 1-70 of NS1, aa 1-73 of B2, and aa 199-329 of VP35 (Bale et al., 2012; Bornholdt and Prasad, 2008; Lingel et al., 2005), were cloned and used for this study.

At first, we observed the subcellular localization of these DRBs tagged by a C-terminal yellow fluorescent protein (YFP) in *Nicotiana benthamiana* leaves via *Agrobacterium*-mediated infiltration. Confocal microscopy results showed that all the three proteins localized both in the nucleus and cytoplasm (Fig. 2A–C), suggesting that these proteins, like free YFP, are distributed in both the cytoplasm and nucleus in the cell. Interestingly, we also observed some granular YFP foci in the nucleus and cytoplasm (Fig. 2A–C). These fluorescent granules likely resulted from the specific binding of the DRBs to dsRNA, whereas the evenly distributed fluorescent signal would come from the free form of DRBs. To test this assumption, the DRBs were fused with either the N- or C-terminal halve of YFP (YN or YC). As summarized in Fig. 1, the DRB-YN and -YC fusion proteins would restore the YFP signal upon co-binding to the same dsRNA, and the free form of non-dsRNA binding DRB-YN and -YC fusion proteins would not be fluorescent. Indeed, granular YFP foci were readily observed in the cells expressing either two homo or heterogeneous DRB-YN and -YC fusions, although the fluorescent foci were reduced when two heterogeneous DRBs were used (Fig. 2D–I). These results confirm that granular YFP foci result from the reconstituted YFP of DRB-YN and DRB-YC. To further test if the YFP fluorescence results from the binding of DRB-YN and -YC fusion proteins to dsRNA, we mutated DRBs by substitution of key residue(s) that are known to be indispensable for their dsRNA binding activities (Bale et al., 2012; Cheng et al., 2009; Lingel et al., 2005; Lu et al., 2005). When these mutational forms of NS1 ($NS1_{R38A}$), B2 ($B2_{R54Q}$), and VP35 ($VP35_{R271A/K299A/R301A}$) were used in the transient expression assay, fluorescent signals were not observed (Fig. 2J–L). Based on these data, we conclude that the restored YFP signal is indeed dependent on their co-binding to dsRNA. We thus name this assay as dRBFC (dsRNA binding-dependent fluorescence complementation). Since all different combinations among these protein fusions gave similar results, we used B2-YN and VP35-YC in subsequent experiments.

dRBFC signal co-localizes with cytoplasmic SGS3/RDR6 bodies and nuclear D-bodies

In order to further prove that the fluorescent foci are associated with dsRNA, we evaluated their localizations with the endogenous dsRNA processing bodies, namely, cytoplasmic bodies containing proteins Suppressor of Gene Silencing 3 (SGS3) and RDR6 (SGS3/RDR6 bodies) and Nuclear Dicer-Like 1 protein (DCL1)- and DRB4-containing dicing bodies (D-bodies). For this purpose, we cloned SGS3 (AT5G23570) and DRB4 (AT3G62800) from *Arabidopsis*. It is well established that SGS3 interacts with RDR6 to form the cytoplasmic SGS3/RDR6-bodies that are involved in RDR6-dependent *trans-acting* small-interfering RNA (ta-siRNA) pathways in *Arabidopsis* (Kumakura et al., 2009) and that DRB4 interacts with DCL1 in the nuclear D-bodies that are involved in nuclear miRNA and ta-siRNA biogenesis

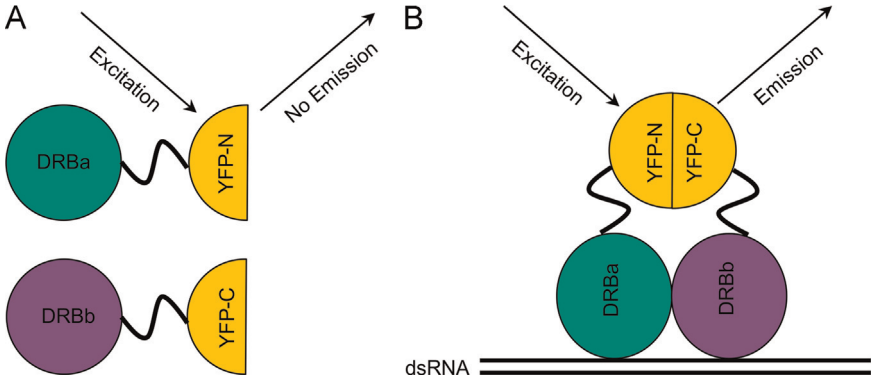

Fig. 1. Schematic illustration of the dRBFC assay. (A) Two dsRNA binding proteins (DRBa and DRBb, colored in blue and purple, respectively) are fused to one of the two non-fluorescent fragments (YFP-N and YFP-C) of split yellow fluorescent protein (YFP, in yellow). Without dsRNA, DRBa and DRBb do not interact with each other and the two non-fluorescent fragments (YFP-N and YFP-C) do not emit yellow fluorescence. (B) In the presence of dsRNA, the binding of DRBa and DRBb to dsRNA will bring in close proximity YFP-N and YFP-C to reconstitute the fluorescence-competent protein structure and restore the YFP fluorescence.