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CAPPI: A Cytoskeleton-Based Localization Assay Reports Protein-Protein Interaction in Living Cells by Fluorescence Microscopy

Dear Editor

Probing protein-protein interaction has become a routine practice in the post genomic era. Multiple *in vitro* or *in vivo* techniques have been developed to detect or report direct or indirect interactions of functionally related proteins (Lalonde et al., 2008). These techniques sometimes are technically challenging, however, because the readout would demand sophisticated detectors and/or complicated calculations. Besides, a common drawback of many of these techniques is they can render inherent false positives to various degrees so that an interaction often cannot be judged unambiguously.

One of the most popular *in planta* assays reporting protein-protein interaction is the bimolecular fluorescence complementation (BiFC) assay, which involves reconstituting green fluorescent protein (GFP)-derived YFP or Citrine from two splits upon the association of two interacting proteins fused with the two splits, respectively (Kerppola, 2008). However, the two fragments of YFP/Citrine often fold together by themselves, especially when their expression levels are elevated or when they are enriched in a particular location. To partially overcome this problem, the expression levels of both bait and prey proteins have to be reduced (Ho et al., 2012). Alternatively, a tripartite split-GFP strategy has been developed to cope with this problem (Cabantous et al., 2013).

To establish a direct and informative method that would report protein-protein interaction in living plant cells under metabolically active conditions is in urgent need. Here, we report the cytoskeleton-based assay for protein-protein interaction (CAPPI), which could report the interactions between signaling molecules as well as between nuclear transcriptional regulators encoded by genes in *Arabidopis thaliana* in the leaf cells of tobacco (*Nicotiana benthamiana*) upon transient expression. In this assay, the bait and prey proteins were tagged with distinct fluorescent proteins, and the interacting prey protein could be detected on the cytoskeletal filaments together with the bait upon transient expression in tobacco leaf cells.

To direct proteins to the cytoskeletal F-actin, we employed the 17-amino acid peptide Lifeact, which is derived from the conserved F-actin-binding proteins in fungi (Riedl et al., 2008). The bait proteins were expressed in GFP fusions with or without the cytoskeletal tag under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (Figure 1Aa). First, we chose the *A. thaliana* small GTPase ROP1 (Rho Of Plants 1) whose native form locates to the plasma membrane through the targeting motif bearing amino acids CAAX toward its C-terminus (Li et al., 1998). When GFP was fused to its C-terminus, the ROP1-GFP fusion became distributed throughout the cytoplasm and the nucleus (Figure 1Ab), indicating that the GFP tag compromised the native localization pattern of ROP1. The Lifeact-ROP1-GFP fusion protein, however, decorated a filamentous network in the cytoplasm (Figure 1Ac). To confirm that the fusion protein associated with F-actin, we used the fimbrin-derived ABD2 (actin-binding domain 2)-CFP fusion protein, which serves as a marker of F-actin (Wang et al., 2004). The two fusion proteins colocalized along actin filaments (Figure 1Ad and 1Ae).

To be prepared to deal with proteins that may associate with F-actin by themselves, we also targeted bait proteins to microtubules using a microtubule-binding domain (MBD) derived from plant microtubule-associated protein MAP65-3 (Ho et al., 2012). In contrast to Lifeact-ROP1-GFP, MBD-ROP1-GFP became targeted along cortical microtubules (Figure 1Af). The CKL6-mCherry fusion protein, previously shown to mark microtubules in plant cells (Ben-Nissan et al., 2008), was used to verify the overlap of two signals on microtubule filaments (Figure 1Ag and 1Ah). Therefore, the feasibility of the employment of both F-actin and microtubules enabled us to explore the reciprocal filaments when proteins being tested demonstrated association with one type of filament directly or indirectly.

ROP1 is specifically activated by the guanine nucleotide exchange factor ROPGEF1 through direct interaction in *A. thaliana* (Gu et al., 2006). When the prey ROPGEF1 was expressed in a fusion with the red fluorescent TagRFP, it was largely diffuse with some aggregates (Figure 1Ba and 1Bb). Because ROP1 acted on the plasma membrane, we chose the plasma-membrane-associated cation-binding protein PCaP1 (Nagata et al., 2016) as a negative control in our experiments. A PCaP1-TagRFP fusion protein exclusively decorated the plasma membrane (Figure 1Bc), confirming the earlier report. When ROP1-GFP and ROPGEF1-TagRFP were co-expressed, both fusion proteins appeared diffuse in the cytosol with noticeable aggregates (Figure 1Bd–1Bf). Based on these images, perhaps one would hesitate to claim the colocalization of the two proteins. However, when ROPGEF1-TagRFP was co-expressed with Lifeact-ROP1-GFP, they became colocalized on F-actin cables and fine filaments (Figure 1Bg–1Bi). When examined closely, the two signals overlapped (Figure 1Ca). When PCaP1-TagRFP and Lifeact-ROP1-GFP were co-expressed, however, PCaP1-TagRFP remained to be associated with the plasma

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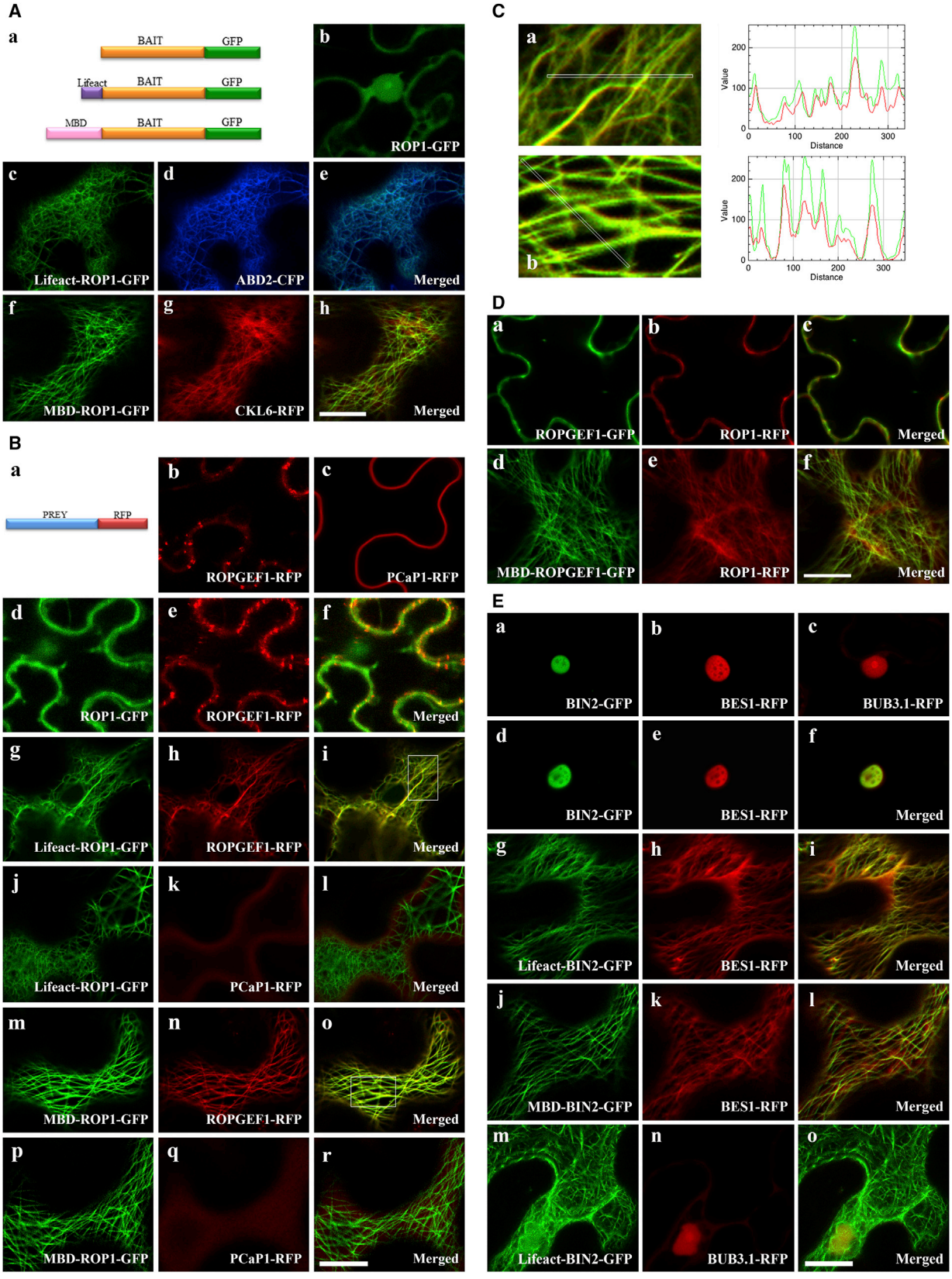

Figure 1. CAPPI Reports Protein-Protein Interaction on Cytoskeletal Filaments.

(A) Redirecting proteins to the cytoskeletal filaments. Scale bar, 20 μm. (a) Diagrams of the bait protein expressed without cytoskeletal tags and with Lifeact or the MBD domain. (b) ROP1-GFP diffuses in the cytosol and the nucleus. (c–e) Lifeact-ROP1 decorates F-actin filaments/bundles marked by

(legend continued on next page)

membrane while Lifeact-ROP1-GFP appeared on F-actin filaments (Figure 1Bj–1Bl). Conversely, in the experiment when MBD-ROP1-GFP and ROPGEF1-TagRFP were co-expressed, the latter was recruited to cortical microtubules (Figure 1Bm–1Bo). Again, such overlapped signals could be clearly viewed in an area of an enlarged image (Figure 1Cb). Similar to what was demonstrated using Lifeact-ROP1-GFP, when MBD-ROP1-GFP and PCaP1-TagRFP were co-expressed, PCaP1-TagRFP remained to be associated with the plasma membrane while MBD-ROP1-GFP decorated cortical microtubules (Figure 1Bp–1Br).

To test whether the bait selection would make a difference, we changed the bait to ROPGEF1. When ROPGEF1-GFP and ROP1-TagRFP were co-expressed, their diffuse cytosolic distributions left certain degrees of uncertainty on their colocalization (Figure 1Da–1Dc). When ROP1-TagRFP was co-expressed with MBD-ROPGEF1-GFP, however, both proteins appeared on cortical microtubules (Figure 1Dd–1Df). This result suggests that the CAPPI method is flexible in terms of bait selection.

In the plant biology field, the BiFC assay has gained great popularity in recent years. One of the concerns associated with BiFC is that the restoration of the fluorescence could be due to the self-engagement of the two split fragments but not the interaction between the bait and prey fused with the YFP/Citrine fragments. False-positive results may be more likely when the proteins are expressed at elevated levels, e.g., using the constitutive CaMV 35S promoter. When the method was used to test the interaction between ROP1 and ROPGEF1, clear positive BiFC was observed upon co-expression of ROP1-nCitrine and ROPGEF1-cCitrine (Supplemental Figure 1A). However, positive BiFC was also observed upon co-expression of ROP1-nCitrine and PCaP1-cCitrine, and the fluorescent signal appeared exclusively on the plasma membrane, perhaps in part because of their enrichment there (Supplemental Figure 1B). Therefore, BiFC would not be our method of choice when such pairs of proteins are tested for potential interaction.

It has been particularly challenging to determine specific interactions among nuclear-localized proteins because of their enrichment in the nucleus. The CAPPI method was tested in the interaction between the glucose synthase kinase 3-like protein BIN2 and its substrate of the transcription factor BES1 from *A. thaliana*. First, BIN2-GFP and BES1-TagRFP were expressed separately and their nuclear localization was reconfirmed (Figure 1Ea and 1Eb). The nuclear WD40 repeat protein BUB3.1 was used as a negative control and located in the nucleus when expressed in a TagRFP fusion (Figure 1Ec). When BIN2-GFP and BES1-TagRFP were co-expressed, they showed colocalization patterns in the nucleus (Figure 1Ed–1Ef).

We then tested whether fusions with a cytoskeletal-binding motif would redirect a nuclear protein to the cytoskeletal F-actin or microtubules. In fact, the tagging strategy worked under both circumstances as the Lifeact-BIN2-GFP and MBD-BIN2-GFP fusion proteins decorated F-actin filaments/bundles and cortical microtubules, respectively (Figure 1Eg and 1Ej). These results indicated that the fusion proteins were tethered to the cytoskeletal filaments and association out-powered their potential nuclear localization. When BES1-TagRFP was co-expressed with these two fusion proteins, they were recruited to the cytoskeletal filaments as well (Figure 1Eg–1El). When BUB3.1-TagRFP was co-expressed with Lifeact-BIN2-GFP, however, BUB3.1 remained in the nucleus while Lifeact-BIN2-GFP highlighted the F-actin filaments (Figure 1Em–1Eo). These results indicated that the CAPPI method is effective to report interactions of nuclear proteins after they are redirected to the cytoskeletal filaments and might be applicable to examining interactions of other proteins such as transcription factors that often form a complex interactive network in order to regulate the expression of developmentally important genes.

Like any other protein-protein interaction assay, the CAPPI method has a potential caveat that the fusion moieties, i.e., Lifeact, MBD, and/or fluorescent protein tags might mask the interaction domains especially when they are in close proximity.

ABD2-CFP as demonstrated by complete overlap of the signals of the two channels in the merged image. (f–h) The MBD-ROP1-GFP matches the CKL6-RFP signal, which marks cortical microtubules.

(B) Determination of specific interaction between the GTPase ROP1 and its nucleotide exchange factor ROPGEF1 in tobacco leaf cells. Scale bar, 20 μm. (a) The prey proteins are fused with the red fluorescent protein TagRFP (RFP) while the bait ROP1 is fused with GFP. (b) ROPGEF1-RFP localizes to the cytosol with aggregates at the cell cortex. (c) PCaP1 exhibits exclusive localization to the plasma membrane when solely expressed in an RFP fusion. (d–f) When ROP1-GFP and ROPGEF1-RFP are co-expressed, both fusion proteins remain in the cytosol. (g–i) When ROP1 is fused with the F-actin-binding Lifeact motif and GFP, the Lifeact-ROP1-GFP fusion protein recruits ROPGEF1-RFP to F-actin filaments, reporting their interaction. (j–l) When Lifeact-ROP1-GFP is co-expressed with PCaP1-RFP, Lifeact-ROP1-GFP decorates the F-actin filaments but PCaP1-RFP retains association with the plasma membrane because of lack of interaction. (m–o) When ROP1 is fused with the microtubule-binding domain (MBD) and GFP, the MBD-ROP1-GFP fusion protein recruits ROPGEF1-RFP to cortical microtubules, reporting their interaction. (p–r) When the MBD-ROP1-GFP fusion protein was co-expressed with PCaP1-RFP, MBD-ROP1-GFP still associates with cortical microtubules while PCaP1-RFP decorates the plasma membrane.

(C) Colocalization of ROP1 and ROPGEF1 on cytoskeletal filaments. (a, b) Enlarged view of the boxed area in (**B**i), illustrating perfect codistribution of the two signals in the fluorescence intensity scan. (c, d) Enlarged view of the boxed area in (**B**o), illustrating perfect codistribution of the two signals in the fluorescence intensity scan.

(D) ROPGEF1 recruits ROP1 to cortical microtubules. Scale bar, 20 μm. (a–c) Without the cytoskeletal tag, ROPGEF1-GFP and ROP1-RFP diffuse in the cytoplasm with some overlapped aggregates. (d–f) When MBD-ROPGEF1-GFP and ROP1-RFP are co-expressed, MBD-ROPGEF1-GFP recruits ROP1-RFP to cortical microtubules.

(E) Determination of specific interaction between the nucleus-localized BIN2 and BES1 in tobacco leaf cells. Scale bar, 20 μm. (a–c) When solely expressed in either GFP or RFP fusions, BIN2, BES1, and BUB3.1 (negative control) all localize to the nucleus. (d–f) When BIN2-GFP and BES1-RFP are expressed together, both fusion proteins remain in the nucleus. (g–i) When BIN2 is fused with the F-actin-binding Lifeact motif and GFP, the Lifeact-BIN2-GFP fusion protein recruits BES1-RFP to F-actin filaments. (j–l) When BIN2 is fused with the microtubule-binding domain (MBD) and GFP, the MBD-BIN2-GFP fusion protein recruits BES1-RFP to cortical microtubules. (m–o) While the Lifeact-BIN2-GFP fusion protein decorates F-actin, the non-interacting BUB3.1 remains in the nucleus.

Consequently, a potential interaction would not be reported by the CAPPI. To cope with this, we could easily reposition these exogenous fragments to the opposite end of either bait or prey, or both, by recombinant DNA techniques so that interaction domains could be exposed. Similarly, the fluorescent protein tag and Lifeact/MBD may be linked directly before fusing with one terminus of the bait protein in order to free up the other. Furthermore, the CAPPI may be used together with another complementary method in order to circumvent the potential caveats associated with individual methods.

In conclusion, the CAPPI method demonstrates the following advantages:

(1) Because the bait and prey proteins are tagged with distinct fluorescent proteins, they can be detected separately and their colocalization on characteristic cytoskeletal filaments reports their association.
(2) CAPPI is judged by the colocalization of bait/prey proteins on the cytoskeletal filaments. This qualitative assay does not involve optical manipulations or sophisticated calculations before drawing conclusions.
(3) When both fluorescently tagged bait and prey proteins are detected at their distinct locations, it would be clear that no interaction takes place while sufficient proteins are present.
(4) CAPPI has been demonstrated here to be applicable for cytosolic and nuclear proteins. However, it should be noted that it may be necessary to artificially remove signaling peptides for membrane- or organelle-targeted proteins if chosen for CAPPI.
(5) GFP and TagRFP can be easily swapped by others such as CFP and YFP so that they can serve as the donor and acceptor for the FRET assay when combined with CAPPI.
(6) CAPPI may be further developed into a quantitative assay to test, for example, the strength of association between a bait and a prey when the FRAP (fluorescence recovery after photobleaching) technique is used in combination to measure the turnover rate of prey proteins on the cytoskeletal filaments.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

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