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Technical Note

A bright green-colored bimolecular fluorescence complementation assay in living plant cells

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Abstract Protein-protein interactions are important for various biological and cellular processes. To analyze protein-protein interactions in living cells, the bimolecular fluorescence complementation (BiFC) assay, based on structural complementation of two non-fluorescent N- and C-terminal fragments from a fluorescent protein, has been developed and widely used in various research fields. Here I report a bright green-colored BiFC assay in living plant cells by using the N-terminal fragment (GN) of green fluorescent protein-S65T (GFP-S65T) and the C-terminal fragment (CC) of cyan fluorescent protein (CFP), but not GN and the C-terminal fragment (GC) of GFP. Fluorescence intensity of the GN/CC-based BiFC was 7-fold higher than that of the GN/GC-based BiFC. The emission spectrum of the GN/CC-based BiFC *in planta* was identical to that of full-length GFP-S65T. Ala163 residue within the CC fragment was found to be responsible for the improvement of the BiFC efficiency. These findings provide a BiFC method for *in vivo* protein-protein interaction studies to many GFP users in various research fields.

Key words: *Allium cepa*, BiFC, bimolecular fluorescence complementation, GFP, protein-protein interaction.

For the past several years, the bimolecular fluorescence complementation (BiFC) assay has been widely used for protein-protein interaction studies in various research fields including plant science (Kerppola 2008). The BiFC assay is based on the structural complementation of two non-fluorescent N- and C-terminal fragments, from a fluorescent protein, that are fused to a pair of interacting proteins (Shyu and Hu 2008). When the two fusion proteins interact, the complemented fluorescent protein exhibits fluorescence. The BiFC assay was originally reported by using an enhanced yellow fluorescent protein (EYFP) to visualize protein-protein interactions in living mammalian cells (Hu et al. 2002). To date, over 10 fluorescent proteins have been used for the BiFC assay (Shyu and Hu 2008). For example, cyan (Hu and Kerppola 2003; Shyu et al. 2006), green (Ghosh et al. 2000), red (Chu et al. 2009; Fan et al. 2008; Jach et al. 2006; Kodama and Wada 2009) and photoswitchable (Lee et al. 2010) fluorescent protein-based BiFC assays are available. Using these fluorescence complementation technologies, advanced BiFC-based techniques such as multicolor BiFC and BiFC-based fluorescence (Förster) resonance energy transfer (FRET) assays have also been developed. The multicolor BiFC assay is a method for visualization of two alternative (Hu and Kerppola 2003; Waadt et al. 2008) or distinct (Chu et al. 2009; Kodama and Wada 2009) protein complexes in a single cell using two different colored BiFCs (e.g. green and red). The BiFC-based FRET assay was developed, by combining BiFC and FRET assays, to analyze a ternary protein complex in living cells (Shyu et al. 2008). Therefore, advances in BiFC technology not only facilitate identification of protein-protein interactions, but also aid the development of such new methods for protein-protein interaction studies in living cells and organisms.

Green fluorescent protein (GFP) has been widely used as a fusion protein tag in numerous studies because the GFP-tagged protein is easily detected using readily available devices with fluorescence detection capabilities. Due to the widespread use of GFP, most laboratories are equipped with devices (e.g. fluorescent microscope) to analyze fluorescent proteins optimized for GFP rather than other fluorescent proteins such as cyan fluorescent protein (CFP) and YFP. Although some studies with a GFP-based BiFC were reported (Ghosh et al. 2000; Magliery et al. 2005), a GFP-based BiFC assay has not been accepted. The reason why GFP is not used for the BiFC assay might be because of its low fluorescence complementation efficiency. For example, although Hu and Kerppola tested a GFP-based BiFC assay by using

Abbreviations: BiFC, bimolecular fluorescence complementation; CC, C-terminal fragment of CFP; CFP, cyan fluorescent protein; FRET, fluorescence (Förster) resonance energy transfer; GC, C-terminal fragment of GFP; GFP, green fluorescent protein; GN, N-terminal fragment of GFP; MXMT, 7-methylxanthine methyltransferase; S65T, serine to threonine at amino acid residue 65; YC, C-terminal fragment of YFP; YFP, yellow fluorescent protein.

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N- and C-terminal fragments of EGFP (GN and GC, respectively) in mammalian cells, they could not detect any fluorescence complementation (Hu and Kerppola 2003). Although I observed fluorescence from a GN/GC-based BiFC in plant cells, using fragments from GFP-S65T (serine to threonine at amino acid residue 65) (Kodama and Wada 2009), its complemented fluorescence was barely detectable (Kodama, unpublished observation). These observations suggest the fluorescence complementation efficiency of a GFP-based BiFC assay is extremely low and produces a fluorescent signal that is not readily detected. Thus, development of a bright green-colored BiFC assay, with high complementation efficiency, is important for providing a BiFC method to many GFP users.

Previous work on three GN-based BiFCs showed that the C-terminal fragments of CFP, GFP and YFP (CC, GC and YC, respectively) had similar fluorescent properties (Kodama and Wada 2009). Hu and Kerppola also reported on two GN-based BiFCs with CC and YC in mammalian cells, although fluorescence from the GN/GC-based BiFC could not be observed (Hu and Kerppola 2003). The GN/CC-based BiFC had an emission spectral peak at 512 nm (Hu and Kerppola 2003) similar to the GFP emission spectral peak at 510 nm (Tsien 1998), and the emission spectral peak of the GN/YC-based BiFC was at 521 nm (Hu and Kerppola 2003).

Based on these previous observations, I predicted that the GN and CC fragments would be applicable to a bright green-colored BiFC assay, instead of the fragments from a GN and GC pair. To test this notion, the BiFC efficiency of GN and CC was compared with that of GN and GC. The details of the BiFC fragments used in this study are listed (Table 1). Because the 7-methylxanthine methyltransferase (MXMT) protein from *Coffea arabica* (Uefuji et al. 2003) forms a homodimer that can be visualized by the BiFC assay (Kodama et al. 2008; Kodama and Wada 2009), it was fused with either GN, GC or CC (Table 2). Combinations of appropriate BiFC plasmids (1 mg each) were co-bombarded with gold particles into onion (*Allium cepa*) epidermal cell layers. A *DsRED monomer* gene (Clontech) driven by the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator was co-bombarded as an expression control. After incubation at 25°C for 24 h in the dark, images of the epidermal cells were captured using fluorescent microscopes (Axio Imager Z1m, Carl Zeiss and BX60, Olympus) with specific filters for GFP (excitation, 470–490 nm; emission, 515–550 nm) and DsRED (excitation, 530–550 nm; emission, >570 nm). To normalize protein expression levels and estimate BiFC efficiency, the raw fluorescence intensity of the BiFC complex (GN/GC or GN/CC) was divided by the raw fluorescence intensity of DsRED. Fluorescence of the GN/CC-based BiFC was much brighter than the GN/GC-based BiFC (Figure 1A), with a fluorescence intensity (BiFC efficiency) 7-fold higher than that of the GN/GC-based BiFC (Figure 1B).

To verify the emission spectrum of the GN/CC-based BiFC, it was measured *in planta* with full-length GFP-S65T used as a control. After the proteins were transiently expressed in onion epidermal cells, emission spectra of the GN/CC-based BiFC and GFP-S65T were measured using a confocal microscope with a 488 nm laser. The emission spectral peak of the GN/CC-based BiFC was identical to that of GFP-S65T (Figure 2A), at 510 nm. In contrast to my result, an emission spectral peak of GN/CC-based BiFC in mammalian cells was reported to be at 512 nm (Hu and Kerppola 2003).

Table 1. Details of BiFC fragments used in this study

Designation	Fragment of fluorescent protein[a]	Mutation vs. wild-type GFP
GN	GFP-S65T (1-154)	S65T
GC	GFP (155-238)	None
CC	CFP (155-238)	V163A, H231L
GC-V163A	GFP-V163A (155-238)	V163A

[a] Parenthesis indicate amino acid positions within the fluorescent proteins

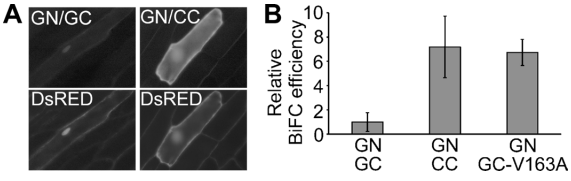

Figure 1. Improvement of GFP-based BiFC assay. (A) Representative images of GN/GC- and GN/CC-based BiFC. Appropriate BiFC vectors were co-introduced with a control vector encoding a DsRed monomer (DsRED) into onion epidermal cells via particle bombardment. The resulting cells were viewed under fluorescent microscopes. (B) Relative BiFC efficiency of GN/GC-, GN/CC- and GN/GC-V163A-based BiFCs. To estimate relative BiFC efficiency, fluorescence intensities of GN/CC- and GN/GC-V163A-based BiFC were divided by fluorescence intensity of GN/GC-based BiFC. Bars indicate standard deviations.

Table 2. A list of BiFC vectors used in this study

Designation of vector	Expressed protein	Reference
pBGN155-MXMT	GN155-MXMT	Kodama and Wada 2009
pBGC155-MXMT	GC155-MXMT	Kodama and Wada 2009
pBCC155-MXMT	CC155-MXMT	Kodama and Wada 2009
pBGC155(V163A)-MXMT	GC155(V163A)-MXMT	This study

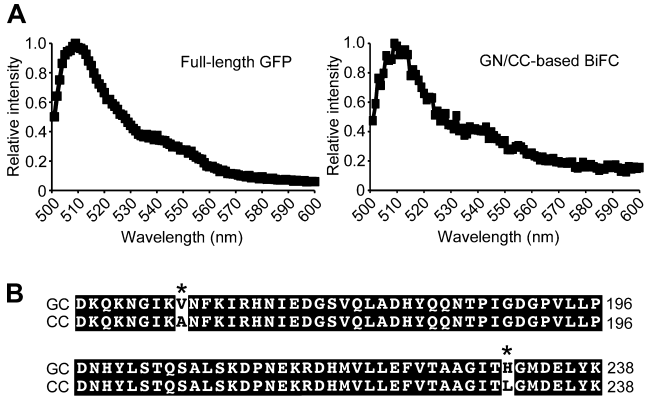

Figure 2. Emission spectrum of GN/CC-based BiFC *in planta*, and amino acid comparison between GC and CC fragments. (A) Emission spectra of full-length GFP-S65T and GN/CC-based BiFC *in planta.* After GFP-S65T and the GN/CC complex were transiently expressed in onion epidermal cells, their emission spectra were measured by Leica SP2 confocal microscope with a 488 nm laser, according to the instruction manual of Leica Microsystems. (B) Alignment of amino acid sequences of GC and CC fragments. Black boxes and asterisks indicate identical and different residues, respectively.

Differences in experimental conditions such as proteins and/or organisms might explain this inconsistency.

There are only two differences between the sequences of the GC and CC fragments in the BiFC plasmids: Val163 in GC; Ala163 in CC and His231 in GC; Leu231 in CC (Figure 2B). These differences do not affect their spectral properties (Figure 2A). The V163A mutation is known as one of three mutations (F99S, T153M and V163A) that improve the protein folding of GFP at high temperature (37°C), whereas H231L is considered a neutral mutation (Tsien 1998). Residue 231 is unlikely to affect any properties of the fluorescent protein, because residues 230–238 are extremely disordered (Tsien 1998). In the previously reported GN/CC-based BiFC assay, only Ala163, but not Leu231, existed within the CC fragment (Hu and Kerppola 2003). Note that the GN/CC-based BiFC assay in mammalian cells was performed at low temperature (30°C) (Hu and Kerppola 2003). These previous observations suggested that the Ala163 residue within the CC fragment increases the BiFC efficiency between GN and CC fragments under low temperature (25–30°C) conditions. In fact, fluorescence intensity of GN/GC-V163A-based BiFC was increased, similar to the GN/CC-based BiFC (Figure 1B). To compare the effect of the Ala163 residue on GFP folding under low temperature, a gene for GFP-S65T/V163A was constructed from the *GFP-S65T* template using site-directed mutagenesis and its fluorescence intensity was estimated *in planta* at 25°C. The fluorescence intensity of GFP-S65T/V163A was comparable with that of GFP-S65T (data not shown), suggesting that V163A is a valuable substitution to improve complementation efficiency of the GFP-based BiFC rather than GFP folding under low temperature. Based on the structure of GFP-F99S/T153M/V163A (Battistutta et al. 2000) and my GN/CC-based BiFC system (Kodama and Wada 2009), Ala163 is located on the 8th β-strand of the CC fragment. This allows its side chain to interact with Tyr151 in the GN fragment and with Glu183, Lys162 and Asn164 in the CC fragment. Because these interactions are thought to increase complementation efficiency, alterations of these amino acid interactions may lead to further improvements of the GFP-based BiFC assay.

Since several different colored fluorescent proteins have become available for the BiFC assay, users can select a suitable BiFC system, depending on their research purpose. It remains to be determined whether my improved GFP-based BiFC system has comparable brightness to that of other fluorescent protein-based BiFC systems, because intensities of their fluorescence with various spectra could not be compared *in vivo.* Despite this, bright fluorescence from the improved GFP-based BiFC was readily observed using several commercial microscopes: Leica SP2, Carl Zeiss Axio Imager Z1m, Olympus BX60 and Leica MZ16. Thus, the improved GFP-based BiFC assay in this study seems to be a practical BiFC system.

In summary, this report describes the development of a bright GFP-based BiFC assay, in living plant cells, by using GN and CC (or GC-V163A) fragments. The BiFC efficiency of GN and CC was much higher than that of GN and GC, and the fluorescence emission spectrum was identical to that of GFP-S65T. These findings provide a BiFC method for protein-protein interaction studies that can be utilized by many GFP users in various research fields.

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