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Original Paper

In planta comparative analysis of improved green fluorescent proteins with reference to fluorescence intensity and bimolecular fluorescence complementation ability

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Abstract Green fluorescent protein (GFP) was discovered from the jellyfish *Aequorea victoria*, and several improvements have been carried out to change its physicochemical properties. The resulting improved GFP variants have been used as reporter proteins for bioimaging techniques in various research fields including plant science. Almost all GFP variants were developed using *Escherichia coli* to improve fluorescence properties in mammalian cells, but the impact in other organisms such as plant cells remains to be determined. In this study, we performed comparative analysis of four improved GFP variants, GFP-S65T, eGFP, frGFP and sfGFP, with reference to the fluorescence intensity in *Arabidopsis* protoplasts, and found that sfGFP is the brightest. Using non-fluorescent fragments from the GFP variants, we also conducted bimolecular fluorescence complementation (BiFC) assays to find appropriate fragment pairs of GFP-based BiFC for visualization of protein–protein interactions in living plant cells. Our observations revealed that the brightest is the sfGFP-based BiFC. Further, as an evaluation method for the sfGFP-based BiFC, a BiFC competition assay was successfully completed for the first time in planta. The present study provides useful information for selection and improvement of the GFP molecule and its application to BiFC technology in plants.

Key words: *Arabidopsis*, BiFC, BiFC competition, GFP, protein–protein interaction.

Since the first attempt to employ wild-type green fluorescent protein (wtGFP) as a reporter protein, it has been developed for bioimaging techniques in various research fields including plant science (Chalfie et al. 1994; Heim et al. 1995). wtGFP is a typical β-barrel structure harboring a chromophore spontaneously formed by the three residues (S65-T66-G67), and thereby exhibits green fluorescence without any cofactor (Tsien 1998). To date, wtGFP has been improved by targeted or random mutations to increase its fluorescence intensity, and almost all of the improvements were performed using *Escherichia coli* for suitable expression in mammalian cells (Shaner et al. 2005). The first improvement was a single mutation of serine to threonine residue at position 65 (S65T) for the chromophore (Figure 1); the mutation shifted the excitation peak to 490 nm from 395 nm and 470 nm of wtGFP (Heim et al. 1995). Compared with wtGFP, this peak-shifted GFP (psGFP) gives 6-fold brighter

Figure 1. Comparison of amino acid sequences among wtGFP, psGFP, eGFP, frGFP and sfGFP. Black box shows identical amino acids, and an arrowhead indicates a split site between positions at 154 and 155 residues for BiFC assay. The numbers above sequences denote mutation sites of psGFP, eGFP, frGFP and sfGFP.

Abbreviations: BiFC, bimolecular fluorescence complementation; eGC, the C-terminal fragment of eGFP; eGFP, enhanced GFP; eGN, the N-terminal fragment of eGFP; EYFP, enhanced yellow fluorescent protein; frGC, the C-terminal fragment of frGFP; frGFP, folding reporter GFP; frGN, the N-terminal fragment of frGFP; GFP, green fluorescent protein; MXMT, 7-methylxanthine methyltransferase; psGC, the C-terminal fragment of psGFP; psGFP, peak-shifted GFP; psGN, the N-terminal fragment of psGFP; PEG, polyethylene glycol; RC, the C-terminal fragment of DsRED monomer; sfGC, the C-terminal fragment of sfGFP; sfGFP, superfolder GFP; sfGN, the N-terminal fragment of sfGFP; wtGFP, wild-type GFP; YC, the C-terminal fragment of EYFP; YN, the N-terminal fragment of EYFP.

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fluorescence upon excitation with 490 nm in vitro (Heim et al. 1995). Subsequently, an additional mutation, F64L, was introduced to psGFP, and this protein is generally called enhanced GFP (eGFP) (Figure 1) (Cormack et al. 1996; Zhang et al. 1996). The F64L mutation successfully improved protein folding efficiency and allowed practical use in mammalian cells (Zhang et al. 1996). The next improved GFP was a folding reporter GFP (frGFP) that incorporated three more mutations, F99S/M153T/V163A, called "cycle 3", into eGFP (Figure 1) (Waldo et al. 1999). The cycle 3 mutations were discovered by using PCR-based random DNA shuffling with three cycles (Crameri et al. 1996). The cycle 3 mutations reduced protein aggregation by replacing three hydrophobic amino acids with hydrophilic amino acids, resulting in improved fluorescence intensity (Crameri et al. 1996). The superfolder GFP (sfGFP) has 6 additional mutations, S30R/Y39N/N105T/Y145F/I171V/A206V, into the frGFP to further improve folding efficiency (Figure 1) (Pédelacq et al. 2006). The sfGFP has increased resistance to denaturation in vitro, and its fluorescence is robust at 37°C in *E. coli* (Pédelacq et al. 2006). When sfGFP was expressed in *E. coli*, the fluorescence intensity was approximately 2-fold higher than frGFP (Andrews et al. 2007; Pédelacq et al. 2006). Although the impact of these GFP variants was evaluated in *E. coli*, in vitro and in mammalian cells, the impact in other organisms, such as plant cells remains to be determined.

The bimolecular fluorescence complementation (BiFC) assay is based on structural complementation of a fluorescent protein to visualize protein–protein interaction in living cells (Hu et al. 2002; Kodama and Hu 2012; Shyu et al. 2008). To date, various fluorescent proteins were employed to develop the BiFC system. Examples are EYFP-, Venus-, ECFP- and mRFP-based BiFC systems (Kodama and Hu 2012). Researchers have attempted to use several GFP variants such as psGFP, eGFP, frGFP, and sfGFP for the BiFC system (Kodama and Hu 2012). However, the most suitable GFP variant for use in plant cells has not been determined.

In this study, we performed a comparison analysis between full-length GFP variants, psGFP, eGFP, frGFP and sfGFP, with reference to fluorescence intensity using their transient expression in *Arabidopsis thaliana* protoplasts. We also evaluated the fluorescence intensity of BiFC, consisting of the non-fluorescent fragments from the GFP variants. The present study provides valuable information regarding suitable GFP variants and the BiFC technology used in plant cells.

Materials and methods

Plasmid construction

To construct expression vectors for full-length GFP variants, P35S-sGFP(S65T)-TNos vector was used as a template for the following mutagenesis strategy (Kodama et al. 2010). Note that sGFP(S65T) is termed as "psGFP" in this study. To produce genes for eGFP and frGFP, the DpnI-mediated site-direct mutagenesis method (Fisher and Pei 1997; Kodama 2011) was used to introduce appropriate mutations (see Figure 1). A gene for sfGFP was generated as a synthetic gene (Operon Technologies Inc, Alabama, USA). For BiFC experiments, PCR-amplified DNA fragments for BiFC fragments (psGN, eGN, frGN, sfGN, psGC, frGC and sfGC) were subcloned into the NcoI/BsrGI site of pBRN169-MXMT vector (Kodama and Wada 2009) by exchanging a gene for RN169 fragment.

Protoplast preparation

Arabidopsis thaliana plants were grown in vermiculite under white light at approximately 80 μmol m^{-2} s^{-1} with a light (16 h)/dark (8 h) cycle at 22°C in a growth chamber for 3 weeks. To isolate mesophyll protoplasts, the Tape-*Arabidopsis* Sandwich method (Wu et al. 2009) was used with some modifications. Detached leaves were sandwiched between vinyl tapes (Nitoms Proself J2575) and then the lower epidermis was stripped. The vinyl tape was cut along the leaf shapes, and immediately transferred into 10 ml of enzyme solution (1% cellulose R-10, 0.3% macerozyme R-10, 0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM $CaCl_2$) in a 90 mm Petri dish. Vacuum pressure was applied for 15 min to infiltrate the enzyme solution into apoplasts of the leaf. The Petri dish was incubated for 3 h at room temperature in the dark. After incubation, protoplasts were released by gently swirling the Petri dish. Fifteen ml of W5 solution (5 mM KCl, 2 mM MES pH 5.7, 125 mM $CaCl_2$, 154 mM NaCl, 0.9% Glucose) was added to the Petri dish, and the solution containing protoplasts was transferred through a nylon mesh (100 μm mesh size) into a 50 ml centrifuge tube. The protoplasts were collected by centrifugation at 50×*g* for 5 min, and gently washed with 10 ml of W5 solution. After centrifugation (50×*g* for 5 min), the protoplasts were resuspended in 5 ml of W5 solution, and kept on ice for 30 min until used in the transient expression assay.

Transient expression assay

Polyethylene glycol (PEG)-mediated protoplast transfection was performed as previously reported (Kodama et al. 2010). The protoplasts prepared in the W5 solution (see above) were collected by centrifugation at 50×*g* for 5 min, and gently washed with 8 ml of MMg solution (0.4 mM mannitol, 4 mM MES-NaOH pH 5.7, 15 mM $MgCl_2$). After centrifugation (50×*g* for 5 min), the protoplasts were resuspended in 1 ml of MMg solution to make a protoplast solution. Ten microliters of plasmid DNA (1 μg μl^{-1}) was mixed gently with 100 μl of the protoplast solution; 5 μl of each plasmid (1 μg μl^{-1}) for co-expression of GFP variants and DsRED monomer (Kodama et al. 2010), and 4.5 μl each of the appropriate two BiFC plasmids (1 μg μl^{-1}) and 1 μl of DsRED monomer plasmid (1 μg μl^{-1}) for BiFC experiments were mixed. Subsequently, 110 μl of PEG solution containing 40% (v/v) PEG4000 (Fluka, #81240), 0.4 M mannitol and 100 mM $CaCl_2$ was added, and mixed carefully.

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After incubation for 30 min at 23°C, the protoplasts were washed with 5 ml of W5 solution gently. The protoplasts were resuspended with 1 ml of W5 solution and transferred to a six-well culture plate coated with 1% BSA. The plate was incubated at 23°C in the dark for 24 h.

BiFC competition assay

Forty-five microliters of RC-MXMT plasmid ($1\,\mu g\,\mu l^{-1}$) was added to $100\,\mu l$ of protoplast solution with a plasmid mixture containing $4.5\,\mu l$ each of the two BiFC plasmids ($1\,\mu g\,\mu l^{-1}$) for sfGFP-based BiFC and $1\,\mu l$ of the DsRED monomer plasmid ($1\,\mu g\,\mu l^{-1}$) for normalization of protein expression. Transfection was carried out with $155\,\mu l$ of PEG solution as mentioned above. Instead of the RC-MXMT plasmid, $45\,\mu l$ of MMg solution or RC plasmid ($1\,\mu g\,\mu l^{-1}$) was added as a control.

Fluorescence microscopy analysis

Images of transfected protoplasts were captured using fluorescent microscopes (Axio Imager Zim, Carl Zeiss and BX60, Olympus) with specific filter sets for GFP (excitation, BP 480/40 nm; emission, BP 535/50 nm) and DsRED (excitation, BP 560/40 nm; emission, BP 630/75 nm). To measure fluorescence intensity from the images, the Java-based software ImageJ (http://rsb.info.nih.gov/ij/) (Schneider et al. 2012) was used, and the procedure was previously reported (Kodama and Hu 2013). Briefly, images were captured at the same region (cytoplasm) of a cell through the specific filters (see Figure 2A). After subtracting background signal, green (GFP) and red (DsRED) fluorescence intensities were measured from 10 randomly selected protoplasts. To normalize protein expression levels of GFP variants or the BiFC complex (e.g. psGN/psGC), the green fluorescence intensity was divided by the fluorescence intensity of DsRED, and a median was obtained. This procedure was repeated three times, and both the mean and standard deviation of three medians were obtained. Relative fluorescence intensity was calculated by dividing the mean of each benchmark experiment with psGFP in Figure 2, psGFP-based BiFC in Figures 3 and 4, or sfGFP-based BiFC in Figure 5. Each data of fluorescence intensity was compared by one-way

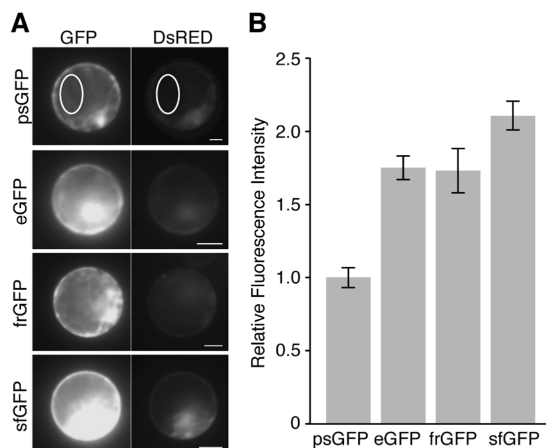
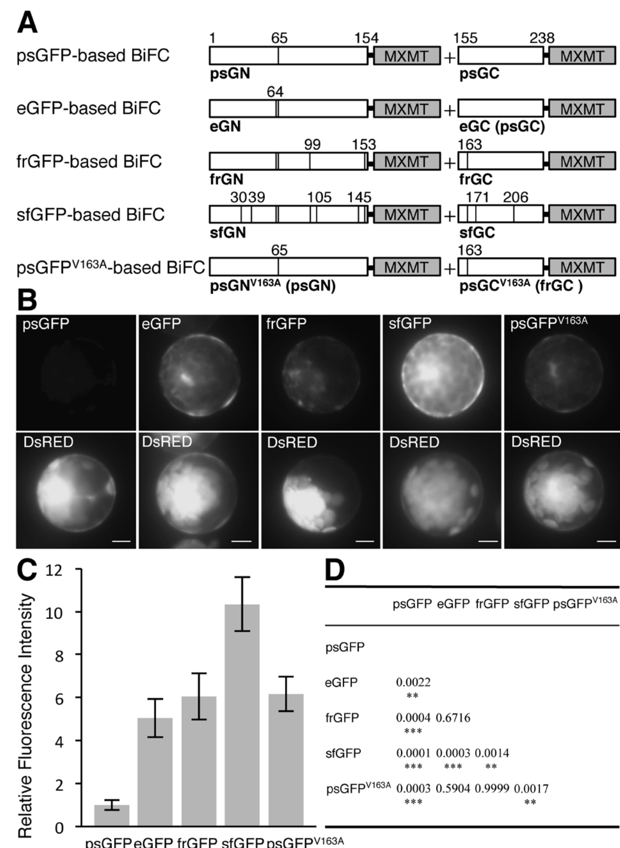


Figure 2. Comparison of fluorescence intensity among full-length GFP variants, psGFP, eGFP, frGFP and sfGFP. The full-length psGFP, eGFP, frGFP or sfGFP was co-expressed with DsRED monomer in protoplasts isolated from *Arabidopsis* mesophyll cells. The fluorescence intensity of the GFP variants was normalized to the fluorescence intensity of DsRED monomer. (A) Representative fluorescence images of a protoplast co-expressing the indicated GFP variant and DsRED. Fluorescence intensity was quantified at cytoplasmic region (an indicated oval). Bar represents $5\,\mu m$. (B) Relative fluorescence intensity of GFP variants. An averaged fluorescence intensity of psGFP was presented as 1, and relative fluorescence intensities of eGFP, frGFP and sfGFP were determined. All experiments were performed three times, and bars represent standard deviations.



	psGFP	eGFP	frGFP	sfGFP	psGFP^{V163A}
psGFP					
eGFP	0.0022 **				
frGFP	0.0004 ***	0.6716			
sfGFP	0.0001 ***	0.0003 ***	0.0014 **		
psGFP^{V163A}	0.0003 ***	0.5904	0.9999	0.0017 **	

Figure 3. Comparison of fluorescence intensity among BiFCs based on psGFP, eGFP, frGFP, sfGFP and psGFP^{V163A}. Each psGFP, eGFP, frGFP, sfGFP and psGFP^{V163A} was split into the two non-fluorescent fragments: psGN, psGC, eGN, eGC, frGN, frGC, sfGN and sfGC (see Figure 1), and subjected to the BiFC assay. Appropriate pairs of the indicated fragments were co-expressed with DsRED monomer in protoplasts isolated from *Arabidopsis* mesophyll cells. (A) A schematic diagram of fragments used for GFP-based BiFCs. The white boxes and the numbers (with the black lines) indicate non-fluorescent GFP fragments and mutation sites, respectively. (B) Representative fluorescence images of a protoplast co-expressing the indicated GFP-based BiFC complex and DsRED monomer. Bar represents $5\,\mu m$. (C) Relative fluorescence intensity of GFP-based BiFCs. The fluorescence intensity of each BiFC complex was normalized to the fluorescence intensity of DsRED monomer. An averaged fluorescence intensity of psGFP-based BiFC was presented as 1, and relative fluorescence intensities of eGFP-, frGFP- and sfGFP-based BiFCs were determined. All experiments were performed three times, and bars represent standard deviations. (D) Statistic data (p-value) on fluorescence intensities of GFP-based BiFCs. Significance was evaluated by one-way ANOVA with Tukey's HSD test. *** $p<0.001$, ** $p<0.01$.

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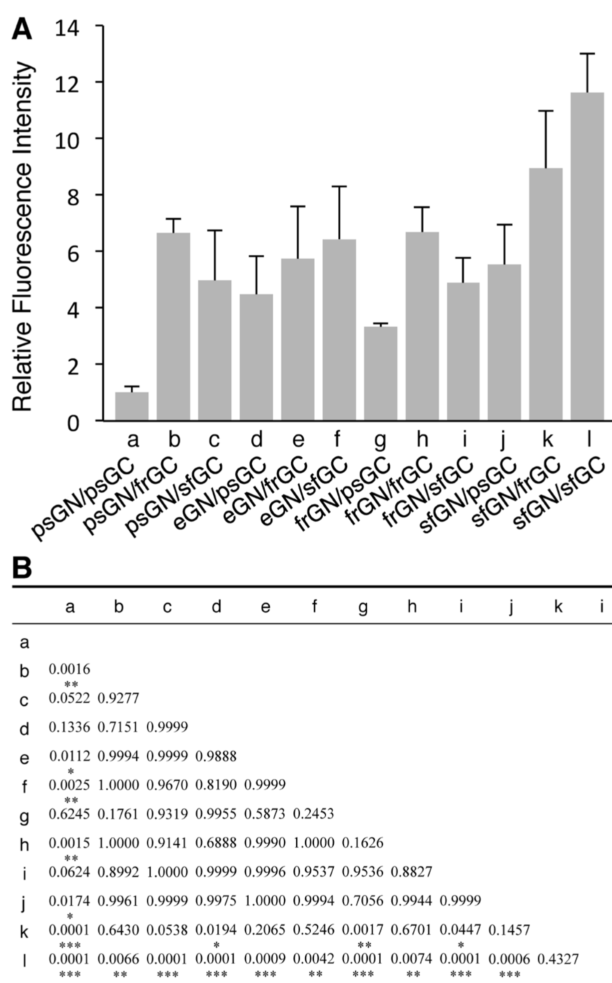

	a	b	c	d	e	f	g	h	i	j	k	i
a												
b	0.0016 **											
c	0.0522	0.9277										
d	0.1336	0.7151	0.9999									
e	0.0112 *	0.9994	0.9999	0.9888								
f	0.0025 **	1.0000	0.9670	0.8190	0.9999							
g	0.6245	0.1761	0.9319	0.9955	0.5873	0.2453						
h	0.0015 **	1.0000	0.9141	0.6888	0.9990	1.0000	0.1626					
i	0.0624	0.8992	1.0000	0.9999	0.9996	0.9537	0.9536	0.8827				
j	0.0174 *	0.9961	0.9999	0.9975	1.0000	0.9994	0.7056	0.9944	0.9999			
k	0.0001 ***	0.6430	0.0538	0.0194 *	0.2065	0.5246	0.0017 **	0.6701	0.0447 *	0.1457		
l	0.0001 ***	0.0066 **	0.0001 ***	0.0001 ***	0.0009 ***	0.0042 **	0.0001 ***	0.0074 **	0.0001 ***	0.0006 ***	0.4327	

Figure 4. Comparison of fluorescence intensity among BiFCs with all combinations of fragments from the GFP variants. Each psGFP, eGFP, frGFP, sfGFP and psGFP^{V163A} was split into the two non-fluorescent fragments (see Figure 1), and subjected to the BiFC assay using the following combinations: psGN/psGC (a), psGN/frGC (b), psGN/sfGC (c), eGN/psGC (d), eGN/frGC (e), eGN/sfGC (f), frGN/psGC (g), frGN/frGC (h), frGN/sfGC (i), sfGN/psGC (j), sfGN/frGC (k) and sfGN/sfGC (l). Appropriate pairs of the indicated fragments were co-expressed with DsRED monomer in protoplasts isolated from *Arabidopsis* mesophyll cells. (A) Relative fluorescence intensity of GFP-based BiFCs with all combination of fragments. The fluorescence intensity of each BiFC complex was normalized to the fluorescence intensity of DsRED monomer. An averaged fluorescence intensity of psGN/psGC-based BiFC (a) was presented as 1, and relative fluorescence intensities of other BiFCs (b–l) were determined. All experiments were performed three times, and bars represent standard deviations. (B) Statistic data (p-value) on fluorescence intensities of GFP-based BiFCs with all combination of fragments. Significance was evaluated by one-way ANOVA with Tukey's HSD test. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

ANOVA with Tukey's HSD test, and the results were defined to be statistically significant if the p-value was less than 0.05.

Results

Full-length GFP variants in Arabidopsis

To evaluate fluorescent intensity of full-length GFP variants in plant cells, the *GFP* genes were controlled by the 35S promoter from cauliflower mosaic virus and terminator of nopaline synthase from *Agrobacterium*. Because a synthetic gene with optimal human codons for psGFP was identified as a vital reporter (known as sGFP-S65T) in plant cells (Chiu et al. 1996), we employed a plasmid for expression of the humanized psGFP, and constructed plasmids for expression of eGFP, frGFP or sfGFP by mutagenesis of *psGFP* gene (Figure 1). The plasmid encoding each GFP variant was transfected into protoplasts of *Arabidopsis thaliana* by polyethylene glycol (PEG)-mediated transformation (Figure 2A), and the resulting 10 randomly selected transformant cells were analyzed under fluorescence microscopy. Note that an equal amount of plasmid encoding DsRed monomer (Clontech, Palo Alto, CA, USA), a monomeric red fluorescent protein, was co-transfected to normalize protein expression levels (Figure 2A). Fluorescence intensity of psGFP was much lower than those of other GFP variants, and sfGFP exhibited the highest fluorescence intensity; e.g. the fluorescence of sfGFP was approximately 2-fold brighter than that of psGFP (Figure 2B). The fluorescence intensity of frGFP was comparable to that of eGFP, although frGFP is an improved version of eGFP (Figure 2B).

BiFC with GFP variants in plant cells

To evaluate GFP-based BiFC in plant cells, a similar fluorescence microscopic analysis was carried out. Since the 7-methylxanthine methyltransferase (MXMT) protein from *Coffea Arabica* forms a homodimer that can be visualized using the BiFC assay (Kodama et al. 2008, 2009), it was fused with either the N- or C-terminal fragments of each GFP variant (abbreviated as GN and GC, respectively). Each GFP variant was split between A155 and D156: psGN, psGC, eGN, eGC, frGN, frGC, sfGN and sfGC (Figures 1, 3A). In our previous study, a V163A mutation within the C-terminal fragment of psGFP (psGC^{V163A}) was reported to improve fluorescence intensity of the BiFC complex with psGN by 7-fold in onion epidermal cells (Kodama 2011). In this study, the fluorescence intensity of BiFC between psGN and psGC^{V163A} was also compared with those of other GFP-based BiFCs in *Arabidopsis* protoplast cells (Figure 3A). Consistent with the previous report in onion cells (Kodama 2011), complemented fluorescence between psGN and psGC was barely detectable, and the V163A mutation in psGC increased the fluorescence intensity in *Arabidopsis* cells (Figure 3B–D). Similarly, we also found that an F64L mutation increased the intensity of BiFC fluorescence, in a comparison between psGFP- and eGFP-based BiFCs (Figure 3B–D). Based on a comparison between eGFP- and psGFP^{V163A}-based BiFCs, the impact of F64L is at the same level as that of V163A (Figure 3B–D). Additionally, the fluorescence

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intensity of frGFP-based BiFC that contains both V163A and F64L mutations was comparable to those of eGFP- and psGFP^{V163A}-based BiFCs (Figure 3B–D). These observations suggest that F64L and V163A improve BiFC fluorescence via the same mechanism. Among the five GFP-based BiFC systems, fluorescence of sfGFP-based BiFC was the brightest (Figure 3B–D). Fluorescence intensity of the sfGFP-based BiFC was 10-fold higher than that of psGFP-based BiFC, and 2-fold higher than those of psGFP^{V163A}-, eGFP- and frGFP-based BiFCs (Figure 3B–D). These 6 mutations (S30R/Y39N/N105T/Y145F/I171V/A206V) within sfGFP likely contributed to the significant improvement of the BiFC fluorescence intensity.

GFP-based BiFCs with all combinations of fragments

Using all combinations (12 pairs) of non-fluorescent fragments from GFP variants, GFP-based BiFCs were compared. Each fragment harboring the same mutations was termed as the same name: N-terminal fragments of psGFP and psGFP^{V163A} termed psGN, C-terminal fragments of psGFP and eGFP termed psGC, frGFP and psGFP^{V163A} termed frGC (Figures 1, 3A).

In a comparison between eGN/psGC and frGN/psGC (Figure 4A, B-d, g), it was found that F99S and M153T of the cycle 3 mutations (F99S/M153T/V163A) do not improve BiFC fluorescence. Conversely, V163A of the cycle 3 mutations (F99S/M153T/V163A) could improve the fluorescence intensity in comparisons between psGN/psGC and psGN/frGC (Figure 4A, B-a, b). Confirming the results in Figure 3, in a comparison between psGN/frGC and eGN/psGC (Figure 4A, B-b, d), the impact of V163A was comparable to that of F64L. The impact of sfGN did not improve BiFC fluorescence in comparisons between frGN/psGC and sfGN/psGC (Figure 4A, B-g, j), and between frGN/frGC and sfGN/frGC (Figure 4A, B-h, k). We also found that sfGC did not improve fluorescence intensity in comparisons between frGC and sfGC (Figure 4A, B-b, c, e, f, h, i). However, when sfGN was co-expressed with sfGC, BiFC fluorescence was significantly improved (e.g. Figure 4A, B-i, l). The results indicate that interaction(s) between the 4 mutations (S30R/Y39N/N105T/Y145F) in sfGN and the 2 mutations (I171V/A206V) in sfGC might improve BiFC fluorescence.

Competition assay for sfGFP-based BiFC

In BiFC experiments, design of appropriate control experiments is important, because undesirable BiFC reactions often occur when two non-fluorescent fragments from a fluorescent protein assemble by random collision (Kodama and Hu 2012). To address this issue, the BiFC competition assay has previously been suggested as an appropriate control experiment (Kodama and Hu 2012). In the competition assay, a competitor-induced decrease of BiFC fluorescence can be monitored, if protein–protein interaction occurred (Kodama and Hu 2012). The BiFC competition assay was originally reported using purified proteins (Hu et al. 2002), and similar experiments were performed in living cells such as mammalian cells and Drosophila cells (Grinberg et al. 2004; Hudry et al. 2011; Vidi et al. 2008). The BiFC competition assay has not been performed so far in planta. Because we found that sfGFP-based BiFC is one of the brightest systems in the above experiments, the BiFC competition assay, using sfGFP-based BiFC, was demonstrated for the first time in planta. As a competitor protein, the MXMT-fused C-terminal fragment (169–225 amino acids) of DsRED monomer (RC-MXMT) was employed (Kodama and Wada 2009). Note that the RC fragment could not be complemented with any N-terminal fragments from GFP variants (Kodama and Wada 2009). When only the RC fragment was used as a control for the competition assay, the fluorescence of sfGFP-based BiFC was kept at the same level (Figure 5). When RC-MXMT was added into the BiFC reaction, the relative fluorescence was significantly reduced to approximately 0.25 (Figure 5). Because a 10-fold excess of RC-MXMT proteins were added as a competitor, the remaining fluorescence seems to be background signal via spontaneous interactions between sfGN-MXMT and sfGC-MXMT. These results indicate a successful BiFC competition reaction between sfGN-MXMT and sfGC-

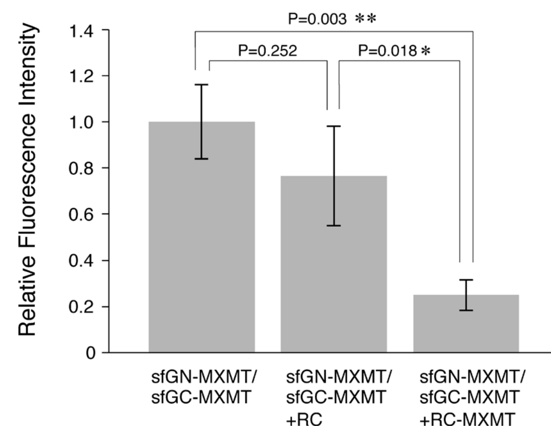

Figure 5. Competition assay for sfGFP-based BiFC. MXMT-fused RC fragment (RC-MXMT) was employed as a competitor protein, (Kodama and Wada 2009). As control experiments, cell suspension buffer (MMg solution) and only RC fragment were used. Appropriate plasmids were co-transfected with plasmid for DsRED monomer in protoplasts isolated from *Arabidopsis* mesophyll cells. The fluorescence intensity of sfGFP-based BiFC complex was normalized to the fluorescence intensity of DsRED monomer. An averaged fluorescence intensity of sfGFP-based BiFC with the MMg solution was presented as 1, and relative fluorescence intensities of other sfGFP-based BiFCs with RC and RC-MXMT were determined. All experiments were performed three times, and bars represent standard deviations. Significance was evaluated by one-way ANOVA with Tukey's HSD test. ** $p<0.01$, * $p<0.05$.

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MXMT by the RC-MXMT in planta.

Discussion

To date, many types of fluorescent proteins have been developed for various applications in biological study (Chudakov et al. 2010). In the developmental stage, practical proteins for use in *E. coli* and in mammalian cells have been screened, but not used in plant cells. Although properties of fluorescent protein would differ depending on species and/or cell types, fluorescent proteins optimized for *E. coli* and mammalian cells have been utilized for numerous studies in the plant science fields. Therefore, this paper provides original comparative information on the use of GFPs in plant cells.

We evaluated the fluorescence intensities of full-length proteins from four improved GFP variants, psGFP, eGFP, frGFP and sfGFP, by using fluorescent microscopy in plant cells, and identified sfGFP as the brightest. On the other hand, the fluorescence intensity of frGFP was comparable with that of eGFP in plant cells, although the frGFP has additional mutations (Pédelacq et al. 2006). The 3 mutations (F99S/M153T/V163A) of frGFP, called cycle 3 mutations, were reported to replace three hydrophobic amino acids on the surface of the β-barrel structure by hydrophilic amino acids for reduction of protein aggregation via the hydrophobic surfaces (Crameri et al. 1996). It was reported that the reduction of protein aggregation improves the fluorescence intensity at 37°C (Fukuda et al., 2000; Jackson et al. 2006). Because the cycle 3 mutations did not improve the fluorescence intensity in plant cells incubated at 23°C, such protein aggregation of GFP variants might not occur in low temperature conditions.

Arpino et al. (2012) reported the mechanism to improve fluorescence intensity of GFP by F64L mutation harbored by both eGFP and frGFP. The exchange of phenylalanine with leucine residues at position 64 in the central chromophore increased folding efficiency in vitro by better packing of the central hydrophobic area, thereby affecting the position of several other residues. Similarly, F64L could improve fluorescence intensity in plant cells, suggesting an increase in folding efficiency in planta. Among the 6 mutations (S30R/Y39N/N105T/Y145F/I171V/A206V) of sfGFP, S30R and Y39N could stabilize the GFP structure through the composition of an electrostatically charged network of the β-barrel (Pédelacq et al. 2006), and might work well in plant cells in the present study. These results indicate that enhancement of structural stability by specific mutations (such as F64L and the mutations in sfGFP) could contribute to improved fluorescence intensity in plant cells. Additional mutation(s) that increase structural robustness may further improve the fluorescence intensity of GFP variants for plant cells.

To date, 15 fluorescent proteins have been utilized for the BiFC assay, and several BiFC systems were used in living plant cells (Kodama and Hu 2012). Although numerous laboratories employ GFP as a marker protein, the use of GFP-based BiFC has been limited due to its low fluorescent signal (Kodama 2011). In the present study, sfGFP-based BiFC was found to be the brightest system in plant cells; e.g. the fluorescence intensity was 10-fold higher than that of psGFP-based BiFC. Based on a comparison with EYFP-based BiFC system that is widely used so far, sfGFP-based BiFC system seems to be bright enough for practical use in plants (Figure S1). We also revealed that a competition assay is an effective method to evaluate protein–protein interaction when using sfGFP-based BiFC in plant cells. Applying sfGFP to the BiFC system for plant cells may provide another benefit. Because sfGFP is able to fold correctly in oxidizing environments such as the apoplast, cell wall and endoplasmic reticulum (Aronson et al. 2011; Gjetting et al. 2013), hard-to-detect protein–protein interactions in oxidizing environments might be detectable using sfGFP-based BiFC.

Recently, we reported that the V163A mutation could improve fluorescence intensity of psGFP-based BiFC (psGFP^{V163A}-based BiFC) (Kodama 2011). The V163A improves complementation efficiency of BiFC fragments rather than full-length psGFP folding efficiency, because full-length psGFP^{V163A} protein emitted a comparable fluorescence with psGFP (Kodama 2011). During a BiFC comparison study using GFP variants, we compared psGFP^{V163A}-based BiFC with other GFP-based BiFC systems. Based on a comparison between fluorescence intensities of eGFP- and psGFP^{V163A}-based BiFCs, the impact of V163A is comparable to that of F64L. Additionally, the fluorescence intensity of frGFP-based BiFC containing both F64L and V163A mutations did not exceed that of eGFP- or psGFP^{V163A}-based BiFC, suggesting that both improvements with F64L (for eGFP-based BiFC) and with V163A (for psGFP^{V163A}-based BiFC) occurred by a similar mechanism. Because a relationship between the V163A and the F64L mutations for BiFC improvements is unknown, further study might be necessary.

In summary, the present study revealed comparative analyses of improved GFP variants with reference to fluorescence intensity and bimolecular fluorescence complementation ability in planta. We found that sfGFP and sfGFP-based BiFC are brightest for full-length protein and BiFC assays, respectively. In addition, the first attempt of the BiFC competition assay was successfully performed in living plant cells. The present study provides useful information not only to select, but also to further improve GFP molecules and their application to BiFC technology in plant science research.

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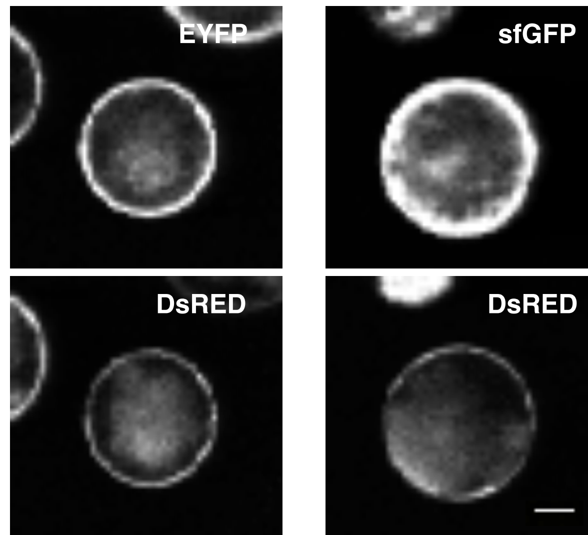

Figure S1. Demonstration of EYFP- and sfGFP-based BiFCs in *Arabidopsis* mesophyll cells. To perform EYFP-based BiFC, MXMT-fused N- and C-terminal fragments of EYFP (YN-MXMT and YC-MXMT) were employed (Kodama et al 2008), and transformed into *Arabidopsis* mesophyll cells. Fluorescence images of EYFP- and sfGFP-based BiFCs were captured using confocal laser scanning microscope (Leica TCS SP2), based on preset beam-path settings of Leica confocal software for YFP, GFP, and DsRED. Bar represents 5 µm.