Benchmarks

A 2in1 cloning system enables ratiometric bimolecular fluorescence complementation (rBiFC)

Christopher Grefen and Michael R. Blatt

Laboratory of Plant Physiology and Biophysics, Institute of Molecular Cell and Systems Biology, University of Glasgow, Glasgow, UK

Gene expression and binary interaction techniques are vital tools that shape our understanding of protein complexes. An inherent flaw, however, with most current protein-protein interaction techniques is the variability in expression levels for fusion proteins when using several individual plasmids. Here, we describe a novel recombination-based cloning strategy called 2in1 that enables co-expression of fusion proteins on a cell-by-cell basis from a single plasmid. We demonstrate the utility of 2in1 through the development of a ratiometric bimolecular fluorescence complementation assay (rBiFC), in which both candidate genes are simultaneously cloned into a single vector backbone containing an internal fluorescent marker for expression control and ratiometric analysis. rBiFC significantly increases the credibility of protein-protein interaction results allowing ratiometric comparison between different protein pairs. In addition to its use in rBiFC, 2in1 can be introduced easily into other vector systems that rely on multiple gene expression and prove feasible in future synthetic biological approaches.

Keywords: bimolecular fluorescence complementation; split-YFP; protein-protein interaction; CBL-CIPK network; 2in1 cloning; Gateway technology; rBiFC

Understanding mechanistic relationships of complex signaling networks requires dissecting protein-protein interactions. Many binary interaction techniques [yeast-2-hybrid (Y2H) (1), split-ubiquitin system (SUS) (2), Förster resonance energy transfer (FRET) (3), and bimolecular fluorescence complementation (BiFC) (4)] rely on expressing two fusion proteins in the same cell at the same time. BiFC is based on splitting a fluorophore [e.g., enhanced yellow fluorescent protein (EYFP) (5)] into two nonfluorescent peptides, fusing these to proteins of interest and their subsequent fluorophore complementation (4,6). BiFC has become widely used to study protein-protein interactions in many laboratories (7,8). Advantages compared with other *in vivo* approaches are that interactions can be monitored in living cells and their subcellular localization determined. However, problems arise where co-transformations

lead to different copy numbers, expression level variations, or complete loss of interacting partners. Also, lack of internal controls makes quantitative interpretation of results difficult. These problems become increasingly important when subtle differences among different interaction partners need to be detected (9).

Here, we describe a modification to BiFC vector systems allowing to directly quantify interaction data using a ratiometric approach (rBiFC) through the introduction of multiple expression cassettes within a single vector backbone. To facilitate rBiFC experiments with different combinations of constructs, we elaborated a novel cloning system called 2in1 (Figure 1A) that utilizes recombination technology (10) to allow the simultaneous cloning of two genes into two different independent cloning cassettes on the same vector backbone. To demonstrate the utility of this 2in1 cloning system for

establishing rBiFC analysis, we integrated both cloning cassettes within the T-DNA region of a binary expression vector (Supplementary Figure S1A). Cassettes are flanked by N- or C-terminal EYFP halves [nYFP (1–155 amino acids), cYFP (156–239 amino acids)] including myc- or hemagglutinin (HA)-tags to enable biochemical detection and expression is regulated through the viral 35S promoter. To allow ratiometric analysis, the T-DNA also carries a soluble monomeric red fluorescent protein (mRFP1) (11) under control of the same promoter, thereby creating an internal marker for transformation and expression control (see Supplementary materials for details). Introducing enhanced green fluorescent protein (EGFP) and enhanced cyan fluorescent protein (ECFP) (12–15) into both cloning cassettes (Figure 1B), we measured fluorescence intensity using confocal laser scanning microscopy

Method summary:

We have devised a novel, Gateway-compatible cloning system named 2in1 that enables single-step transfer of two independent genes into two different expression cassettes on the same vector backbone. Here, we demonstrate how the 2in1 concept ensures the credibility of *in vivo* protein-protein interaction methods by its application in our ratiometric bimolecular fluorescence complementation (rBiFC) assay. The simultaneous introduction of both fusion proteins on the same plasmid ensures equal gene dosage, and the inclusion of an internal fluorescence marker allows for expression control and ratiometric quantification.

Figure 1. Ratiometric comparison of three fluorophores expressed from the same T-DNA. (A) Vector maps illustrating the 2in1 concept with its recombination reactions between two entry vectors and an exemplary 2in1 destination vector carrying the two independent cloning cassettes. (B) Illustration depicting the T-DNA of the pBiFCt-2in1-NN vector containing EGFP or ECFP in either cloning cassette. RB/LB, right/left border of T-DNA; R3R2, 2in1 cloning cassette consisting of attR3 site, lacZ expression cassette, and attR2 site; R1R4, 2in1 cloning cassette consisting of attR1 site, chloramphenicol acetyltransferase resistance gene, gyrase inhibitor gene *ccdB*, and attR4 site. (C) Exemplary CLSM image of epidermal cells from leaves of *N. tabacum* 2 days after infiltration with *Agrobacterium tumefaciens* containing pBiFCt-2in1-NN (ECFP/EGFP, above) and pBiFCt-2in1-NN (EGFP/ECFP, below). Note that the vector includes mRFP1 as a reference marker for transformation and expression. Scale bar, 30 μ m. Images were taken using an LSM510 microscope from Zeiss (Cambridge, UK) with standardized excitation intensities and photomultiplier gains and with the following excitation wavelength/emission bandwidth/dichroic filter settings: 458 nm/LP475/HFT458/543 (secondary dichroic 490) (CFP), 488 nm/BP505–530/HFT488/543 (GFP), and 543 nm/BP560–615/HFT488/543 (RFP). Settings were kept identical for both reciprocal sets of experiments. Bleed-through from CFP into the GFP channel was checked using separate controls with the same settings. (D) Quantification of the mean fluorescence showing that the fluorescence ratioed against RFP is independent of the cloning cassette. Data are means $\pm$ SEM of 20 images selected at random over the surface of four leaf samples in each case. Relative fluorescence was analyzed using Zeiss LSM510 AIM v4.2 software. (E) Immunoblot on leaf samples used for CLSM analysis and quantification in panels C and D. Top row: Ponceau S stain verifying equal loading and transfer; bottom row: immunoblot with different primary antibodies (HA, RFP, and myc; Sigma-Aldrich, Gillingham, UK) on tobacco samples that were infiltrated with pBiFCt-2in1-NN (ECFP/EGFP) (lane 1), pBiFCt-2in1-NN (EGFP/ECFP) (lane 2), or not infiltrated (lane 3). Arrows mark the expected size of tagged fluorophores.

(CLSM) after transient *Agrobacterium*-mediated transformation in *Nicotiana tabacum* leaves as described previously (16). GFP and CFP fluorescence were ratioed against RFP to estimate expression differences (Figure 1, C and D) and compared with Western blot analysis of the fusion proteins (Figure 1E), thus verifying equal expression levels within the same T-DNA and on a cell-by-cell basis in this case. However, every gene may behave differently, depending on regulatory sequences, codon-usage, protein turnover, and other factors influencing expression and/or protein stability. Hence, rBiFC experiments, like classical BiFC studies, need always to be accompanied by biochemical analysis verifying protein expression as a basis for ratiometric analysis of interaction.

Interactions of the CBL-CIPK network of calcium sensors and protein kinases in *Arabidopsis* are well documented (17,18). Calcineurin B-like proteins (CBLs) are membrane-anchored Ca^{2+} sensors that

recruit a specific set of serine-threonine kinases (CIPKs) to their site of action. CBL1 and CBL9, but not CBL6, for example, are known to interact with CIPK23 leading to phosphorylation of the potassium channel AKT1 (17,18). To analyze the functionality of rBiFC, we cloned CBL1, CBL6, and CBL9 together with CIPK23 in our expression vector pBiFCt-2in1-NC (Figure 2A) and transiently transformed *N. tabacum* leaves. For each clone, more than 20 image frames were selected at random (Figure 2B). The mean fluorescence of YFP over RFP was plotted and compared (Figure 2C). The positively interacting CBL proteins 1 and 9 were clearly identified through visualizing complemented YFP fluorescence but also through comparing the intensity with constitutively expressed mRFP1. The negative control containing CIPK23-cYFP and untagged nYFP peptide, as well as samples that coexpress CIPK23-cYFP with nYFP-CBL6, showed a weak YFP signal demonstrating the background fluores-

cence that is often associated with BiFC. However, when compared with the internal RFP signal, it becomes clear that the ratio is significantly lower than compared with the positive interaction couples and similar to the negative control. This weak YFP signal might otherwise have been recognized as evidence of interaction (Figure 2C), and the results thus emphasize the superiority of the ratiometric approach in detecting true positive interactions. For the sake of comparison, we have included immunoblot analysis confirming equal expression (Figure 2E) of the fusion proteins. To exclude artificial YFP signal detection through RFP activation, a high resolution image of the nucleus of a tobacco cell expressing CBL1-CIPK23 was taken, demonstrating that the interaction takes place outside of the nucleus and that the soluble mRFP1 protein which is abundantly present is not detected in the YFP channel (Figure 2D). It should be noted that for any protein-protein interaction couple, a different orientation of

Figure 2. rBiFC proof-of-principle. (A) Illustration depicting the T-DNA of the pBiFCt-2in1-NC vector containing CIPK23 and different CBLs. (B) Exemplary CLSM image of abaxial leaf epidermis cells of *N. tabacum* two days after infiltration with *Agrobacterium tumefaciens* containing either pBiFCt-2in1-NC (CBL1/CIPK23), pBiFCt-2in1-NC (CBL6/CIPK23), pBiFCt-2in1-NC (CBL9/CIPK23), or pBiFCt-2in1-NC (---/CIPK23). Scale bar, 30 μ m. Note the measureable fluorescence signal with CBL6 and CIPK23, although lower than evident with either of the other CBL proteins. Images were taken using an LSM510 microscope from Zeiss (Cambridge, UK) with standardized excitation intensities and photomultiplier gains and with the following excitation wavelength/emission bandwidth/dichroic filter settings: 514 nm/BP521–553/HFT458/543 (YFP) and 543 nm/BP560–615/HFT488/543 (RFP). (C) Quantification of the mean fluorescence ratioed against RFP resolves the selective interaction of CIPK23 with CBL1 and CBL9, but not with CBL6. Data are means $\pm$ SEM of 20 images selected at random over the surface of four leaf samples in each case. Relative fluorescence was analyzed using Zeiss LSM510 AIM v4.2 software. (D) High-resolution image of the nucleus of a tobacco epidermal cell transformed with pBiFCt-2in1-NC (CBL1/CIPK23). The image shows the localization of the BiFC signal to the cytosol, distinct from the RFP signal, which is evident in the nucleus, thus also verifying that the RFP signal is not detected in the YFP channel. (E) Immunoblot of leaf samples used for CLSM analysis and quantification above. Top row, Ponceau S stain verifying equal loading and transfer; bottom row, immunoblot with different primary antibodies (HA, RFP, and myc) on tobacco samples that were infiltrated with pBiFCt-2in1-NC (CBL1/CIPK23) (lane 1), pBiFCt-2in1-NC (CBL6/CIPK23) (lane 2), pBiFCt-2in1-NC (CBL9/CIPK23) (lane 3), pBiFCt-2in1-NC (---/CIPK23) (lane 4), or not infiltrated (lane 5). Arrows mark the expected size of fusion proteins and mRFP1.

the FP-tags might be necessary as protein stability or interaction might be compromised when termini are masked (19). It is important to be aware of this issue, and when performing protein-protein interaction studies, all possible tag orientations should be tested (20).

rBiFC allows ratiometric analysis of interaction partners and provides an extensive improvement toward the credibility of protein-protein interaction results obtained through fluorescence complementation. The success of rBiFC is based on the 2in1 system, which enables introduction of stoichiometric copy numbers of both interaction partners together with an internal marker. While the proof-of-concept in this manuscript

was performed in planta, rBiFC and/or the 2in1 system can be transferred to other cell and expression systems. We have created a set of helper vectors that allows conversion of any expression vector providing high flexibility for the researcher (see Supplementary materials). The use of the 2in1 cloning system is not limited to improving BiFC-based methods; this concept offers advantages for other binary interaction techniques as well, by generally circumventing issues inherent to work with individually cloned interaction partners. It will allow increasing the number of genes to be introduced in a foreign organism as it decreases the number of plasmids, and thereby auxotrophies and other selection

Monthly
Issues Now
Available
for iPad and
iPhone in
the iTunes
App Store

Scan the
QR Code
or visit
the App Store
and search for
“BioTechniques”
to download.

TAKE ACTION AGAINST CONTAMINATION WITH QMI® SAFE-SEPTUM

Sample, Inoculate Or Add Nutrients
To Your Bioreactor Aseptically !

The QMI Safe-Septum is:

- Aseptic
- Pressure & Temperature Safe
- Pre-Sterilized
- Easy To Retrofit
- Validated

651-501-2337

Email: info@qmisystems.com

www.qmisystems.com

markers, necessary for the purpose. Finally, with the 2in1 system, binary interaction assays may be expanded readily to allow screening for ternary interaction partners.

Acknowledgments

The authors are grateful to Rucha Karnik, Susan Rosser, and Gerhard Thiel for critical comments on the manuscript. M.R.B. is funded through the UK Biotechnology and Biological Sciences Research Council grant BB/HO24867/1.

Competing interests

The authors declare competing financial interests: IP rights of the 2in1 technique are assigned to PBL technology, UK.

References

1. Fields, S. and O.K. Song. 1989. A novel genetic system to detect protein protein interactions. *Nature* 340:245-246.
2. Johnsson, N. and A. Varshavsky. 1994. Split ubiquitin as a sensor of protein interactions in-vivo. *Proc. Natl. Acad. Sci. USA* 91:10340-10344.
3. Miyawaki, A., J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, and R.Y. Tsien. 1997. Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature* 388:882-887.
4. Hu, C.D., Y. Chinenov, and T.K. Kerppola. 2002. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* 9:789-798.
5. Miyawaki, A., O. Griesbeck, R. Heim, and R.Y. Tsien. 1999. Dynamic and quantitative Ca²⁺ measurements using improved cameleons. *Proc. Natl. Acad. Sci. USA* 96:2135-2140.
6. Kerppola, T.K. 2006. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nat. Protoc.* 1:1278-1286.
7. Bracha-Drori, K., K. Shichrur, A. Katz, M. Oliva, R. Angelovici, S. Yalovsky, and N. Ohad. 2004. Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. *Plant J.* 40:419-427.
8. Walter, M., C. Chaban, K. Schutze, O. Batistic, K. Weckermann, C. Nake, D. Blazevic, C. Grefen, et al. 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* 40:428-438.
9. Grefen, C., Z. Chen, A. Honsbein, N. Donald, A. Hills, and M.R. Blatt. 2010. A novel motif essential for SNARE interaction with the K(+) channel KC1 and channel gating in *Arabidopsis*. *Plant Cell* 22:3076-3092.
10. Hartley, J.L., G.F. Temple, and M.A. Brasch. 2000. DNA cloning using in vitro site-specific recombination. *Genome Res.* 10:1788-1795.
11. Campbell, R.E., O. Tour, A.E. Palmer, P.A. Steinbach, G.S. Baird, D.A. Zacharias,

and R.Y. Tsien. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* 99:7877-7882.

12. Cubitt, A.B., R. Heim, S.R. Adams, A.E. Boyd, L.A. Gross, and R.Y. Tsien. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:448-455.
13. Heim, R., A.B. Cubitt, and R.Y. Tsien. 1995. Improved green fluorescence. *Nature* 373:663-664.
14. Fricker, M., J. Runions, and I. Moore. 2006. Quantitative fluorescence microscopy: from art to science. *Annu. Rev. Plant Biol.* 57:79-107.
15. Day, R.N. and M.W. Davidson. 2009. The fluorescent protein palette: tools for cellular imaging. *Chem. Soc. Rev.* 38:2887-2921.
16. Grefen, C., N. Donald, K. Hashimoto, J. Kudla, K. Schumacher, and M.R. Blatt. 2010. A ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates temporal stability and native protein distribution in transient and stable expression studies. *Plant J.* 64:355-365.
17. Cheong, Y.H., G.K. Pandey, J.J. Grant, O. Batistic, L. Li, B.G. Kim, S.C. Lee, J. Kudla, and S. Luan. 2007. Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in *Arabidopsis*. *Plant J.* 52:223-239.
18. Xu, J., H.D. Li, L.Q. Chen, Y. Wang, L.L. Liu, L. He, and W.H. Wu. 2006. A protein kinase, interacting with two calcineurin B-like proteins, regulates K⁺ transporter AKT1 in *Arabidopsis*. *Cell* 125:1347-1360.
19. Grefen, C. and M.R. Blatt. 2012. Do calcineurin B-like proteins interact independently of the serine threonine kinase CIPK23 with the K⁺ channel AKT1? Lessons learned from a menage a trois. *Plant Physiol.* 159:915-919.
20. Stellberger, T., R. Hauser, A. Baiker, V.R. Pothineni, J. Haas, and P. Uetz. 2010. Improving the yeast two-hybrid system with permuted fusions proteins: the varicella zoster virus interactome. *Proteome Sci.* 8:8.

Received 1 July 2012; accepted 12 September 2012.

Address correspondence to Christopher Grefen, Institute of Molecular Cell and Systems Biology, University of Glasgow, Glasgow, UK. Email: Christopher.grefen@glasgow.ac.uk

Supplementary material for this article is available at www.BioTechniques.com/article/113941.

To purchase reprints of this article, contact: biotechniques@fosterprinting.com