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Yeast Functional Analysis Report

Bimolecular fluorescence complementation analysis system for *in vivo* detection of protein–protein interaction in *Saccharomyces cerevisiae*

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Abstract

The bimolecular fluorescence complementation (BiFC) assay has been widely accepted for studying *in vivo* detection of protein–protein interactions in several organisms. To facilitate the application of the BiFC assay to yeast research, we have created a series of plasmids that allow single-step, PCR-based C- or N-terminal tagging of yeast proteins with yellow fluorescent protein fragments for BiFC assay. By examination of several interacting proteins (Sis1–Sis1, Net1–Sir2, Cet1–Cet1 and Pho2–Pho4), we demonstrate that the BiFC assay can be used to reliably analyse the occurrence and subcellular localization of protein–protein interactions in living yeast cells. The sequences for the described plasmids were submitted to the GenBank under Accession Nos: EF210802, pFA6a-VN-His3MX6; EF210803, pFA6a-VC-His3MX6; EF210804, pFA6a-VN-TRP1; EF210807, pFA6a-VC-TRP1; EF210808, pFA6a-VN-kanMX6; EF210809, pFA6a-VC-kanMX6; EF210810, pFA6a-His3MX6-P_{GAL1}-VN; EF210805, pFA6a-His3MX6-P_{GAL1}-VC; EF210806, pFA6a-TRP1-P_{GAL1}-VN; EF210811, pFA6a-TRP1-P_{GAL1}-VC; EF210812, pFA6a-kanMX6-P_{GAL1}-VN; EF210813, pFA6a-kanMX6-P_{GAL1}-VC; EF521883, pFA6a-His3MX6-P_{CET1}-VN; EF521884, pFA6a-His3MX6-P_{CET1}-VC; EF521885, pFA6a-TRP1-P_{CET1}-VN; EF521886, pFA6a-TRP1-P_{CET1}-VC; EF521887, pFA6a-kanMX6-P_{CET1}-VN; EF521888, pFA6a-kanMX6-P_{CET1}-VC.

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Introduction

As almost every cellular process is mediated by networks of protein–protein interactions, identification of these interactions is crucial for understanding the cellular functions of proteins. Several methods have been developed to study protein–protein interactions in living cells, e.g. green fluorescent protein reassembly (Ghosh *et al*., 2000), split ubiquitin system (Johnsson and Varshavsky, 1994) and protein fragment complementation (Michnick *et al*., 2000; Wehrman *et al*., 2002; Luker and Piwnica-Worms, 2004). One of the most popular approaches for visualization of protein–protein interactions in living cells is fluorescence resonance energy transfer (FRET) between spectral variants of the green fluorescence protein fused to the associating proteins (Periasamy and Day, 1999; Pollok and Heim, 1999). However, because FRET assay relies on specific detection of spectrally similar fluorophores or even quantification of fluorophore lifetimes, it requires sophisticated and expensive instrumentation and comprehensive post-imaging data analysis. Recently,

a novel approach, termed the bimolecular fluorescence complementation (BiFC) assay, has been widely accepted, due to stronger signal and direct readout measurable with simple equipment. The BiFC assay is based on the formation of a fluorescent complex by fragments of yellow fluorescent protein, brought together by association of two interacting partners fused to the fragments. Since the BiFC assay was successfully used to detect interaction and subcellular localization of bZIP and Rel family proteins (Hu *et al*., 2002), it has been implemented for studying protein–protein interaction in several organisms, including mammalian cells (Hu and Kerppola, 2003; Hynes *et al*., 2004), plants (Bracha-Drori *et al*., 2004; Walter *et al*., 2004), yeast (Blondel *et al*., 2005; Cole *et al*., 2007) and filamentous fungus (Hoff and Kuck, 2005). However, there is so far no known report describing reliable application of the BiFC assay to studying the interaction of proteins expressed from their native promoters in any organism.

In *Saccharomyces cerevisiae*, the one-step PCR-mediated technique for modification of chromosomal genes is well established and has been used to create valuable tools for protein analyses (Longtine *et al*., 1998). This technique allows fast and efficient tagging of yeast proteins with various epitopes at the C- or N-terminus. Especially, chromosomal C-terminal tagging ensures that the tagged protein is expressed from its native promoter, so that its expression pattern is similar to that of the authentic protein. Here we describe a series of plasmids that allow single-step, PCR-based C- or N-terminal tagging of yeast proteins with yellow fluorescent protein fragments for the BiFC assay. The plasmids contain one of three selectable markers, the *kanMX6* module, the *His3MX6* module (Wach *et al*., 1997) and the *S. cerevisiae TRP1* gene (Longtine *et al*., 1998), for selection with G418, growth media lacking histidine or tryptophan. Using these tagging plasmids, we show that the BiFC assay can be used to reliably analyse *in vivo* protein–protein interactions in yeast cells.

Materials and methods

Yeast strains and growth media

All *S. cerevisiae* strains were derived from BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). Yeast cells were grown at 30 °C in YPD or appropriate synthetic complete (SC) drop-out media. Low-phosphate medium was prepared by dissolving 5 g ammonium sulphate, 0.1 g sodium chloride, 20 g glucose and 1.6 g all needed amino acids and amino bases in 1 l water (Sherman, 2002).

Microscopic analysis

Yeast cells grown to mid-logarithmic phase in SC drop-out medium were microscopically analysed in 96-well glass-bottomed microplates (Whatman), pre-treated with concanavalin A (Sigma) to ensure cell adhesion. Microscopy was performed on a Zeiss Axiovert 200M inverted microscope with a Plan-NeoFluar 100×/1.3 NA oil immersion objective. Images were recorded on a Zeiss Axiocam MRm with 2×2 binning. Fluorescence images for BiFC were taken using a standard fluorescein isothiocyanate filter set (excitation band pass filter, 450–490 nm; beam splitter, 510 nm; emission band pass filter, 515–565 nm).

Plasmid construction

To facilitate the application of the BiFC assay to yeast research, a set of yeast tagging plasmids were designed to contain N- (VN) or C-terminal fragment (VC) of Venus, a variant of yellow fluorescent protein (Nagai *et al*., 2002). For construction of N- or C-terminal VN tagging vectors, the ~560 bp PCR product obtained using pBiFC-VN173 (Shyu *et al*., 2006) as template, forward primer 5′-CCCGGGTTAATTAACAGATCCATCGCCACC-ATGGTG-3′ and reverse primer 5′-AGAAG-TGGCGCGCCCTAGGCCATGATATAGACG-TTGTG-3′, was digested with *Pac*I and *Asc*I and ligated into *Pac*I–*Asc*I-digested pFA6a-based N- or C-terminal protein tagging vectors (Longtine *et al*., 1998), thus generating a series of VN tagging vectors (Figure 1A, B). For construction of N- or C-terminal VC tagging vectors, the ~300 bp PCR product, obtained using pBiFC-VC155 (Shyu *et al*., 2006) as template, forward primer 5′-CCCGGGTTAATTAACCGTCCGGCGTGCA-AAATCCCG-3′ and reverse primer 5′-AGAA-GTGGCGCGCCCTACTTGTACAGCTCGTCCA-TGCC-3′, was digested with *Pac*I and *Asc*I, and ligated into *Pac*I–*Asc*I-digested pFA6a-based N- or C-terminal protein tagging vectors (Longtine

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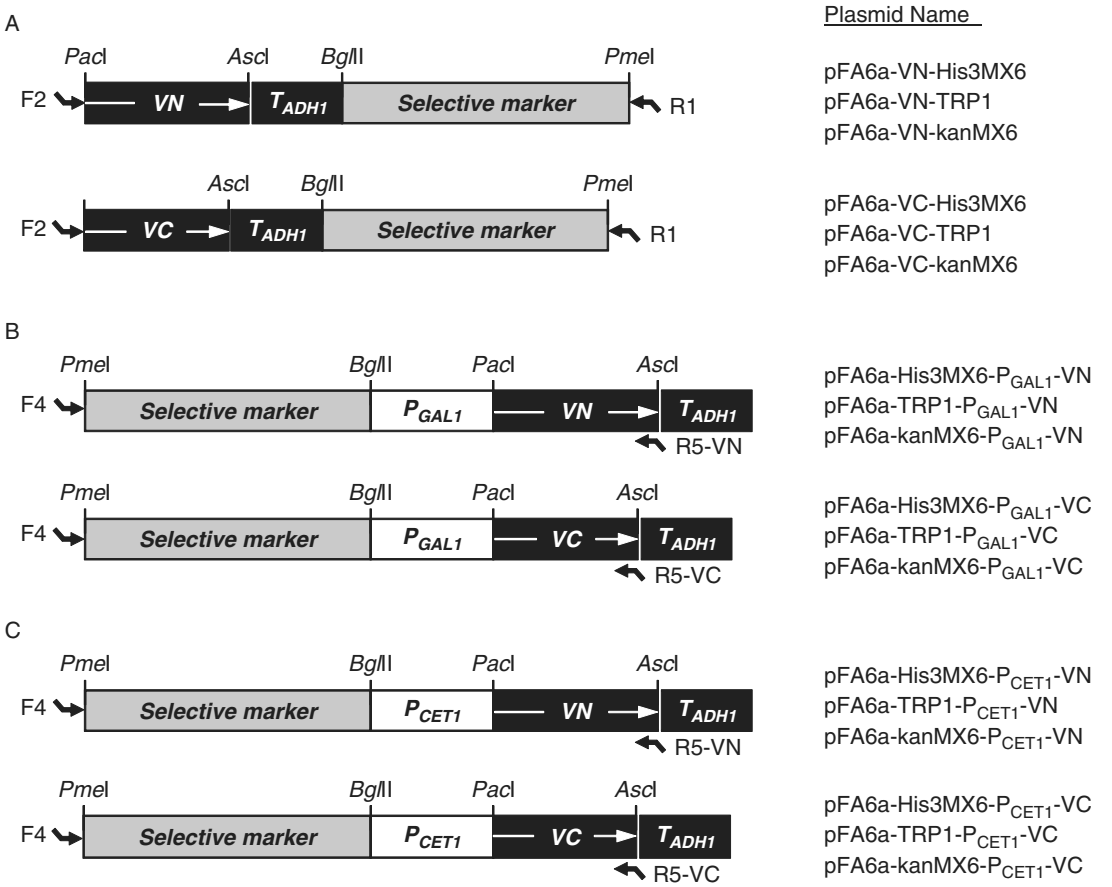

Figure 1. Schematic diagram of the yeast tagging vectors for BiFC analysis. (A) C-terminal protein tagging vectors to be used for tagging VN or VC at the C-terminus of a gene of interest. (B) N-terminal protein tagging vectors to be used for placing a gene under control of the inducible *GAL1* promoter (white boxes) with concomitant VN or VC tagging. (C) N-terminal protein tagging vectors to be used for placing a gene under control of the constitutive *CET1* promoter (white boxes) with concomitant VN or VC tagging. Black boxes indicate protein-tagging modules consisting of the sequences encoding VN or VC, together with the *S. cerevisiae ADH1* terminator. Grey boxes represent selectable markers, including the *His3MX6* module, the *S. cerevisiae TRP1* gene and the *kanMX6* module (Longtine *et al.*, 1998). Relevant restriction sites used for cloning are indicated. Arrows within the boxes indicate directions of transcription. Arrows outside the boxes indicate forward and reverse PCR primers (see Table 1) and the bent portions represent the regions of the primers homologous to the yeast target sequences

et al., 1998), thus generating a series of VC tagging vectors (Figure 1A, B). To construct N-terminal tagging vectors that allow constitutive expression of the fused proteins under the control of *CET1* promoter, the ~600 bp PCR product obtained using *S. cerevisiae* genomic DNA as template, forward primer 5′-CTGTGGATCCATGTCAAGCCGTTT-CATTTC-3′ and reverse primer 5′-GTCATTAATT-AAACTCATAGTGGGAGGATAGA-3′, was digested with *BamH*I and *Pac*I, and ligated into *Bgl*II–*Pac*I-digested N-terminal tagging vectors as described above, thus replacing the inducible *GAL1* promoter with the constitutive *CET1* promoter (Figure 1C).

Results and discussion

To validate the new tagging vector system for BiFC assay, we manipulated several genes, using the vectors shown in Figure 1 as templates and primers designed as in Table 1, according to one-step PCR-mediated gene targeting procedure as previously described (Longtine *et al.*, 1998). The

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Table 1. Oligonucleotide primers used in this study to amplify the transformation modules

Primer name	Sequence[a]
SIS1-F2	5′-*ACTAAACGACGCTCAAAAACGTGCTATAGATGAAAATTTT*GGTCGACGGATCCCCGGGTT-3′
SIS1-R1	5′-*ATTTATTTGAGTTTATAATTATATTTGCTTAGGATTACTA*TCGATGAATTCGAGCTCGTT-3′
NET1-F2	5′-*TGGTGGATTTGCATCATTAATAAAAGATTTCAAGAAAAAA*GGTCGACGGATCCCCGGGTT-3′
NET1-R1	5′-*TAGCTTTCTGTGACGTGTATTCTACTGAGACTTTCTGGTA*TCGATGAATTCGAGCTCGTT-3′
SIR2-F2	5′-*CGTGTATGTCGTTACATCAGATGAACATCCCAAAACCCTC*GGTCGACGGATCCCCGGGTT-3′
SIR2-R1	5′-*TATTAATTTGGCACTTTTAAATTATTAAATTGCCTTCTAC*TCGATGAATTCGAGCTCGTT-3′
SIK1-F2	5′-*TAAAAAGGAAAAGAAGGATAAAAAGAAGAAAAGTAAGGAT*GGTCGACGGATCCCCGGGTT-3′
SIK1-R1	5′-*AAAAAGATGGGATATACTTTATTTCGATTCATTGTTCCTTT*CGATGAATTCGAGCTCGTT-3′
CET1-F2	5′-*TTTATCATATGAAATTTTTGAAGGTTCAAAGAAAGTCATG*GGTCGACGGATCCCCGGGTT-3′
CET1-R1	5′-*CAAGGGCATTTGCTTATTTTTTTTTGAAATGATTCAAATA*TCGATGAATTCGAGCTCGTT-3′
CET1-F4	5′-*TAAAAGCGTATTCGACACTGAAAGATCTGCTGGGAATACT*GAATTCGAGCTCGTTTAAAC-3′
CET1-R5-VN	5′-*CTCTTTTTGTTTGAGGAGGGTTGTCAGTGTAACTCAT*agtaccaccagaaccCTCGATGTTGTGGCGGATC-3′[b]
CET1-R5-VC	5′-*CTCTTTTTGTTTGAGGAGGGTTGTCAGTGTAACTCAT*agtaccaccagaaccCTTGTACAGCTCGTCCATG-3′[b]
PHO2-F2	5′-*GAAGAACACTAACGAACTAACTGACGAGCATAGATGGATA*GGTCGACGGATCCCCGGGTT-3′
PHO2-R1	5′-*AAAATGCAATCGCAAAAAAAAAAAAAACAGAATTATTTTCA*TCGATGAATTCGAGCTCGTT-3′
PHO4-F2	5′-*CTGCCGGTACATCCGTCACCTACAGCAGAACGTGAGCACG*GGTCGACGGATCCCCGGGTT-3′
PHO4-R1	5′-*AGTCCGATATGCCCGGAACGTGCTTCCCATTGGTGCACGG*TCGATGAATTCGAGCTCGTT-3′

[a] Sequences in italics represent the gene-specific sequences.
[b] Sequence of the linker peptide (GSGGT) between VN (or VC) and the N-terminus of the target protein is shown in lower case letters.

resulting strains used in this study are listed in Table 2. Sis1 is a Type II HSP40 co-chaperone that interacts with the HSP70 protein Ssa1 (Luke *et al*., 1991) and has been shown to form a homodimer (Sha *et al*., 2000). We tagged the C-terminal end of *SIS1* with VN and VC in the *MAT***a** and *MAT*α cells, respectively, generating HY0186 and HY0187. The HY0186 cells were then mated to the HY0187 cells, and the resulting HY0188 cells were analysed by fluorescence microscopy (Figure 2A, top panels). The BiFC signal was clearly detected in the nucleus and the cytoplasm, indicating that the VN-tagged Sis1 interacted with the VC-tagged Sis1 in the nucleus and the cytoplasm, where Sis1 is reported to be localized (Huh *et al*., 2003). The BiFC signal was not detected in the diploid cells expressing either the VN-tagged Sis1 (Figure 2A, bottom left panels) or the VC-tagged Sis1 alone (Figure 2A, bottom right panels), demonstrating that neither VN nor VC fluoresces on its own and that the BiFC signal in Figure 2A (top panels) originates from dimerization between the VN-tagged Sis1 and the VC-tagged Sis1.

Next, we examined whether BiFC can be used to detect interaction between different proteins. Net1 and Sir2 are part of a protein complex that regulates rDNA-specific silencing and cell cycle progression, called RENT (regulator of nucleolar silencing and telophase exit). Net1 is localized to the nucleolus and recruits Sir2 to rDNA (Straight *et al*., 1999). We tagged the C-terminal end of *NET1* with VN in the *MAT***a** cells and the C-terminal end of *SIR2* with VC in the *MAT*α cells, generating HY0201 and HY0204, respectively. The HY0201 cells expressing the C-terminally VN-tagged Net1 were then mated to the HY0204 cells expressing the C-terminally VC-tagged Sir2, generating HY0211. The HY0211 cells were transformed with pRS416-SIK1-RFP plasmid to locate nucleolus, and the resulting HY0217 cells were analysed by fluorescence microscopy. Figure 2B shows that co-expression of the VN-tagged Net1 and the VC-tagged Sir2 resulted in BiFC signal localized to the nucleolus. No signal was observed from the HY0222 cells co-expressing the VN-tagged Net1 and the VC-tagged Sik1, a nucleolar marker protein (data not shown), demonstrating that BiFC assay strongly favours detection of direct protein–protein interactions.

We further examined the feasibility of BiFC assay with Cet1, another protein known to form a homodimer (Lima *et al*., 1999). Cet1 is an essential RNA 5′-triphosphatase, a subunit of the mRNA capping enzyme, and is localized to the nucleus (Huh *et al*., 2003). We first tagged the C-terminal end of *CET1* with VN and VC in *MAT***a** and *MAT*α cells, respectively, and mated them to generate the diploid cells expressing the C-terminally VN-tagged Cet1 and the C-terminally VC-tagged Cet1 together. When analysed by fluorescence

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Table 2. Yeast strains used in this study

Strain	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Research Genetics
HY0186	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIS1-VN::His3MX6*	This study
HY0187	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SIS1-VC::His3MX6*	This study
HY0188	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 SIS1-VN::His3MX6/SIS1-VC::His3MX6*	This study
HY0192	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CET1-VN::His3MX6*	This study
HY0196	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 CET1-VC::His3MX6*	This study
HY0197	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 CET1-VN::His3MX6/CET1-VC::His3MX6*	This study
HY0198	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PHO2-VN::His3MX6*	This study
HY0199	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 PHO4-VC::His3MX6*	This study
HY0200	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 PHO2-VN::His3MX6/PHO2 PHO4-VC::His3MX6/PHO4*	This study
HY0201	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-VN::His3MX6*	This study
HY0204	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SIR2-VC::His3MX6*	This study
HY0211	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 NET1-VN::His3MX6/NET1 SIR2-VC::His3MX6/SIR2*	This study
HY0217	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 NET1-VN::His3MX6/NET1 SIR2-VC::His3MX6/SIR2*, pRS416-SIK1-RFP	This study
HY0221	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SIK1-VC::His3MX6*	This study
HY0222	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 NET1-VN::His3MX6/NET1 SIK1-VC::His3MX6/SIK1*	This study
HY0255	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 His3MX6::*P_{CET1}*-VN-CET1*	This study
HY0256	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 His3MX6::*P_{CET1}*-VC-CET1*	This study
HY0257	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 His3MX6::*P_{CET1}*-VN-CET1/His3MX6::*P_{CET1}*-VC-CET1*	This study
HY0258	*MAT***a**/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 CET1-VN::His3MX6/His3MX6::*P_{CET1}*-VC-CET1*	This study

microscopy, the resulting HY0197 cells did not show any BiFC signal (Figure 3A). Considering the fact that cells expressing the C-terminally GFP-tagged Cet1 show a normal growth pattern (Huh *et al*., 2003), it is unlikely that C-terminal protein tagging disturbed the cellular function of Cet1 and caused the failure of BiFC complex formation. We then tagged the N-terminal end of *CET1* with VN and VC in *MAT***a** and *MATα* cells, respectively, and mated them together. The resulting HY0257 cells co-expressing the N-terminally VN-tagged Cet1 and the N-terminally VC-tagged Cet1 under the control of *CET1* promoter showed strong BiFC signal in the nucleus (Figure 3B). The BiFC signal was also detected in HY0258 cells expressing the C-terminally VN-tagged Cet1 and the N-terminally VC-tagged Cet1 together, although the signal intensity was much weaker than in the HY0257 cells (Figure 3C). Considering that both VN-tagged Cet1 and VC-tagged Cet1 are expressed under the control of *CET1* promoter in all the HY0197, HY0257 and HY0258 cells, the absence of BiFC signal in the HY0197 cells and the weak BiFC signal in the HY0258 cells are thought to be due to the topological constraints in BiFC complex formation, which seem to be maximized when both VN and VC are tagged at the C-terminal ends of the Cet1 homodimer and minimized when tagged at the N-terminal ends of the Cet1 homodimer. It is estimated that BiFC can occur when the fluorescent protein fragments are fused to positions that are separated by up to a distance of approximately 10 nm, provided

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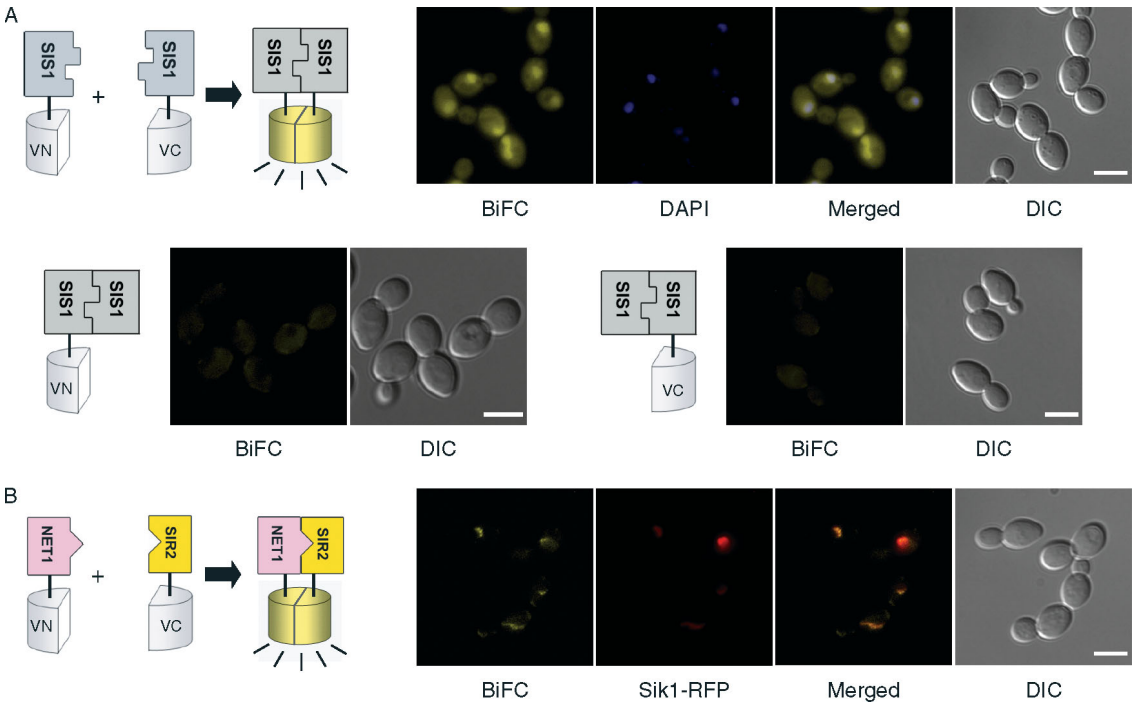

Figure 2. Visualization of protein–protein interaction using C-terminal BiFC tagging vector. (A) Fluorescence images of the diploid cells expressing the C-terminally VN-tagged Sis1 and the C-terminally VC-tagged Sis1 together (top panels). Those of the diploid cells expressing either the VN-tagged Sis1 (bottom left panels) or the VC-tagged Sis1 alone (bottom right panels) are also shown. (B) Fluorescence images of the diploid cells expressing the C-terminally VN-tagged Net1 and the C-terminally VC-tagged Sir2 together. RFP-tagged Sik1 was used as a nucleolar marker (Huh *et al*., 2003). Fluorescence images for BiFC were taken using a standard fluorescein isothiocyanate filter set (excitation band pass filter, 450–490 nm; beam splitter, 510 nm; emission band pass filter, 515–565 nm) and merged with images for DAPI or Sik1-RFP. Scale bars, 5 μm

that there is sufficient flexibility to allow association of the fragments (Hu *et al*., 2002). Based on this estimation, it is presumed that the C-terminal ends of Cet1 in the homodimeric form may stay away from each other. This presumption correlates well with the results of structural study on Cet1 homodimer (Lima *et al*., 1999), which shows that the α4 helices in each C-terminal region of the Cet1 homodimer face one another.

To examine the feasibility of the BiFC assay to detect protein–protein interactions induced under specific conditions, we analysed the interaction between Pho2 and Pho4, both of which are transcription factors involved in phosphate metabolism. When cells are grown in medium containing a high concentration of phosphate, Pho4 is fully phosphorylated and localized to the cytoplasm, and transcription of phosphate-responsive genes such as *PHO5* and *PHO84* is turned off. In response to phosphate limitation, Pho4 is dephosphorylated and accumulates in the nucleus, where it binds cooperatively with Pho2 and activates the transcription of phosphate-responsive genes (Lenburg and O'Shea, 1996). We tagged the C-terminal end of *PHO2* with VN in *MAT***a** cells and the C-terminal end of *PHO4* with VC in *MAT*α cells, generating HY0198 and HY0199, respectively. The HY0198 cells expressing the C-terminally VN-tagged Pho2 were then mated to the HY0199 cells expressing the C-terminally VC-tagged Pho4. The resulting HY0200 cells did not show any BiFC signal in medium containing a high concentration of phosphate (Figure 4A). When the HY0200 cells were starved for phosphate, we could detect BiFC signal accumulating in the nucleus (Figure 4B), indicating the occurrence of Pho2–Pho4 interaction in the nucleus. Intriguingly, background cytoplasmic fluorescence also increased under the phosphate starvation condition. This phenomenon was also observed in diploid cells expressing either the

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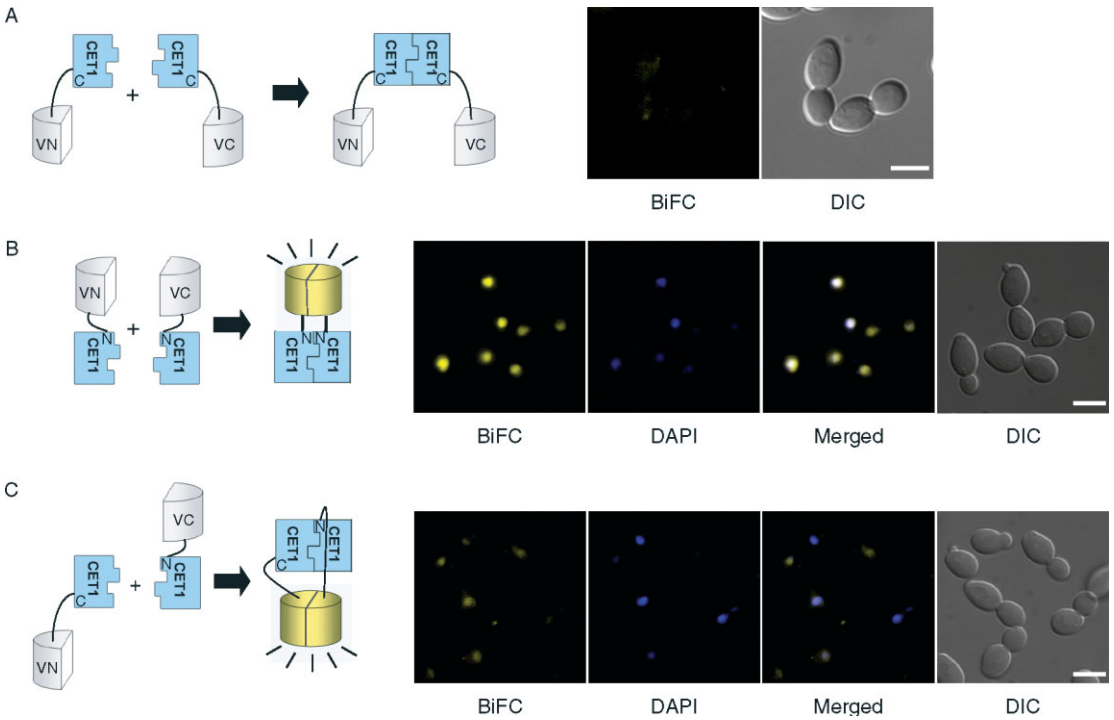

Figure 3. Visualization of protein–protein interaction using N-terminal BiFC tagging vector. (A) Fluorescence images of the diploid cells co-expressing the C-terminally VN-tagged Cet1 and the C-terminally VC-tagged Cet1. (B) Fluorescence images of the diploid cells co-expressing the N-terminally VN-tagged Cet1 and the N-terminally VC-tagged Cet1 under the control of *CET1* promoter. (C) Fluorescence images of the diploid cells co-expressing the C-terminally VN-tagged Cet1 and the N-terminally VC-tagged Cet1 under the control of *CET1* promoter. N and C in the diagrams indicate the N- and C-terminal regions of Cet1, respectively. Fluorescence images for BiFC were taken using a standard fluorescein isothiocyanate filter set and merged with images for DAPI. Scale bars, 5 μm

VN-tagged Pho2 or the VC-tagged Pho4 alone, regardless of the presence of cycloheximide (data not shown), the reason for which is not clear at present. It has been reported that the addition of phosphate to a phosphate-starved culture causes rapid phosphorylation and nuclear export of Pho4 (Komeili and O'Shea, 1999). In accordance with this report, when the HY0200 cells were transferred from medium lacking phosphate to medium containing a high concentration of phosphate, the BiFC signal disappeared from the nucleus (Figure 4C). When these cells were again subjected to phosphate starvation, the BiFC signal reappeared in the nucleus (data not shown). These results strongly indicate that BiFC complex formation can occur in a reversible manner, contrary to what is widely believed (Hu *et al*., 2002; Magliery *et al*., 2005). The discrepancy between the previous reports and our results about the reversibility of BiFC complex formation may be related to overexpression of the fluorescent protein fragments fused to the interacting proteins; contrary to the BiFC analyses in previous studies which employed highly expressed fluorescent protein fragments under the control of strong constitutive promoters, we rendered the fluorescent protein fragments expressed from the native promoters of fused proteins. The reversibility of BiFC complex formation has also been observed in interactions between phospholipase $C\beta_2$ and $C\delta_1$ (Guo *et al*., 2005) and between Cdc42 and Rdi1 in yeast (Cole *et al*., 2007). More investigation will be needed to define the reversibility of BiFC complex formation and thus the applicability of BiFC analysis to assess the dynamic process of protein–protein interaction.

In the present study, we have developed a new series of tagging vectors designed to carry out BiFC assay in yeast cells and demonstrated the functionality of these vectors with several interacting proteins. Despite the topological constraints, the

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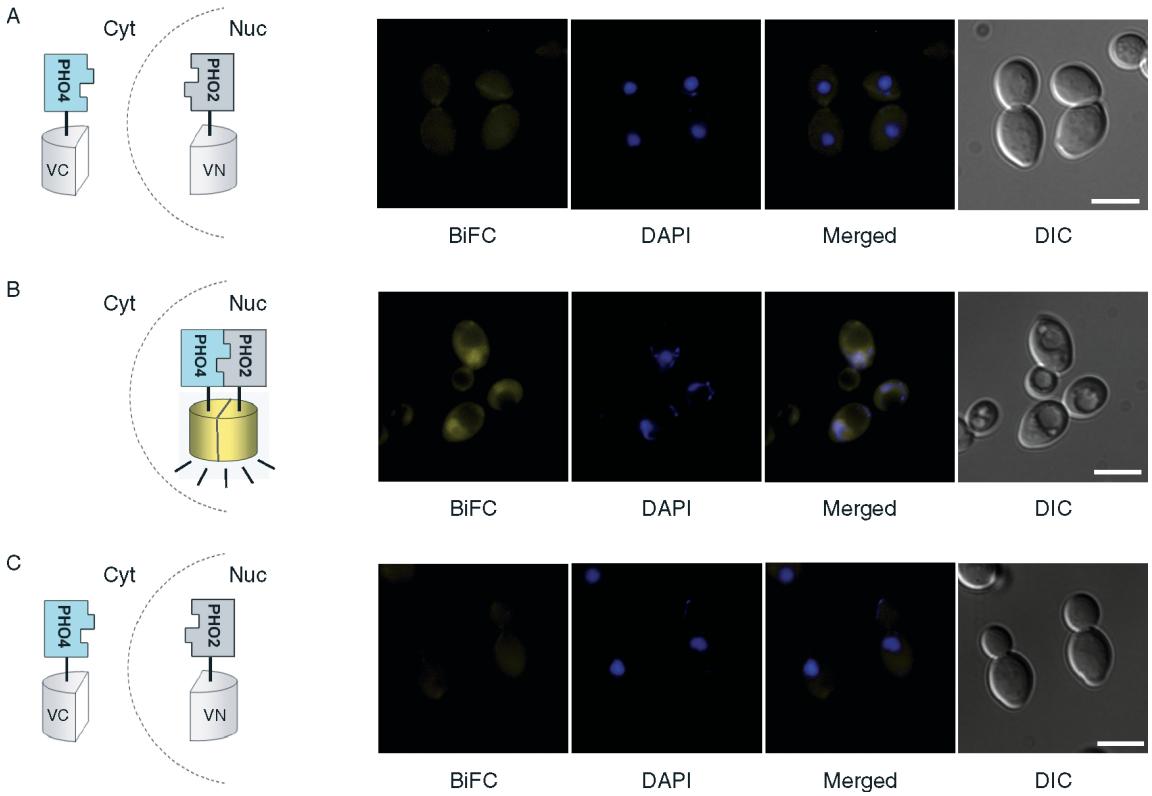

Figure 4. Visualization of induced protein–protein interaction using BiFC analysis. Diploid cells expressing the C-terminally VN-tagged Pho2 and the C-terminally VC-tagged Pho4 together were grown in medium containing plentiful or no phosphate, and analysed for BiFC. (A) Fluorescence images of cells grown in medium containing a high concentration of phosphate. (B) Fluorescence images of cells transferred from medium containing a high concentration of phosphate to medium lacking phosphate and incubated for 2 h. (C) Fluorescence images of cells transferred from medium lacking phosphate to medium containing a high concentration of phosphate and incubated for 30 min. Fluorescence images for BiFC were taken using a standard fluorescein isothiocyanate filter set and merged with images for DAPI. Cyt, cytoplasm; Nuc, nucleus. Scale bars, 5 μm

BiFC assay is now regarded as one of the most advanced and powerful tools for studying and visualizing protein–protein interactions in living cells. Our BiFC vector system maximizes the potential and merits of the BiFC assay. With the developed BiFC vectors, we can easily tag VN or VC fragment at the N- or C-terminal end of any yeast protein by a one-step PCR-mediated procedure. Because the tagging event occurs at the chromosomal level, VN or VC tag is stably maintained and cell-to-cell variation in expression of the tagged protein is minimized. Furthermore, the C-terminal tagging ensures that the protein tagged with VN or VC is expressed from its own endogenous promoter, so that the expression pattern of VN- or VC-tagged protein is comparable to that of the authentic protein. This feature of our BiFC vector system makes it possible to realize '*in vivo* detection of protein–protein interaction' in a true sense. Perhaps the most exciting application of the BiFC assay system will be genome-wide high-throughput screening of protein–protein interactions. Sharing the same principle with the vector system used for construction of a genome-wide GFP-tagged library (Huh *et al.*, 2003), our BiFC vector system will be very useful for the development of a high-throughput platform to study protein–protein interactions in living yeast cells.

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