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Full Length Research Paper

Construction of a bimolecular fluorescence complementation (BiFC) platform for protein interaction assays in plants

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Protein–protein interactions are essential for signal transduction in cells. Bimolecular fluorescence complementation (BiFC) is a novel technology that utilises green fluorescent proteins to visualize protein–protein interactions and subcellular protein localisation. BiFC based on pSATN vectors are a good system for high-level expression of fused protein. A series of pCAMBIA vectors were most widely used in plant transgene and transient expression. To provide multiple options in the study of protein interactions that utilise BiFC, we reconstructed a new pair of BiFC vectors, pCAMBIA1301-nEYFP and pCAMBIA1301-cEYFP. These vectors were generated by eliminating restriction enzyme cutting sites (*Ban*II, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Sal*I, *Pst*I and *Sbf*I) at the multiple cloning sites (MCSs) of pCAMBIA1301 (p1301), and introducing cEYFP/nEYFP cassettes containing MCSs generated from pSATN medium. Fluorescence can be imaged when AtCBL1 and AtCIPK23 are co-injected, but imaging cannot be done when co-injecting AtCBL1 and AtCIPK23-NAF-deleted (AtCIPK23m), suggesting that the proposed modified vector system is effective for the study of protein interactions.

Key words: Protein–protein, bimolecular fluorescence complementation (BiFC), vector reconstruction.

INTRODUCTION

Protein–protein interactions play a key role in most physiological processes and occur frequently in all sub-cellular compartments and organelles. Elucidating the when, where and how of protein interactions with corresponding partners provides valuable clues about how they function in signal transduction and adapt to and modulate physiological processes (Brady and Provart, 2009). So far, various methods have been developed and modified for the study of protein interactions, such as co-immunoprecipitation, surface plasmon resonance, yeast two-hybrid technology, fluorescence resonance energy transfer, bioluminescence resonance energy transfer and bimolecular fluorescence complementation (BiFC) (Bartel and Fields, 1995; Xu et al., 2006, 2007; Citovsky et al., 2006; Walter et al., 2004; Hu et al., 2002).

BiFC is a novel technology that is used for identifying and visualising dynamic protein interactions in living cells (Hu et al., 2002). This technology is based on the principle that single N- and C-terminal sub-fragments of yellow fluorescent protein (YFP) do not spontaneously reconstitute a functional fluorophore. However, if the two non-functional halves of the fluorophore are brought into tight contact through external force which results from the interaction of the targeted protein with its putative partner, they refold and generate fluorescence (Bhat et al., 2006). Because this technique is easily implemented and results can be visualised without dyestuff, BiFC has become one of the most powerful and widely-adopted tools in the study of protein–protein interactions (Lee et al., 2007; Lumbreras et al., 2010; Xu et al., 2006). Recently, vectors with N- or C- terminal subfragments of YFP have

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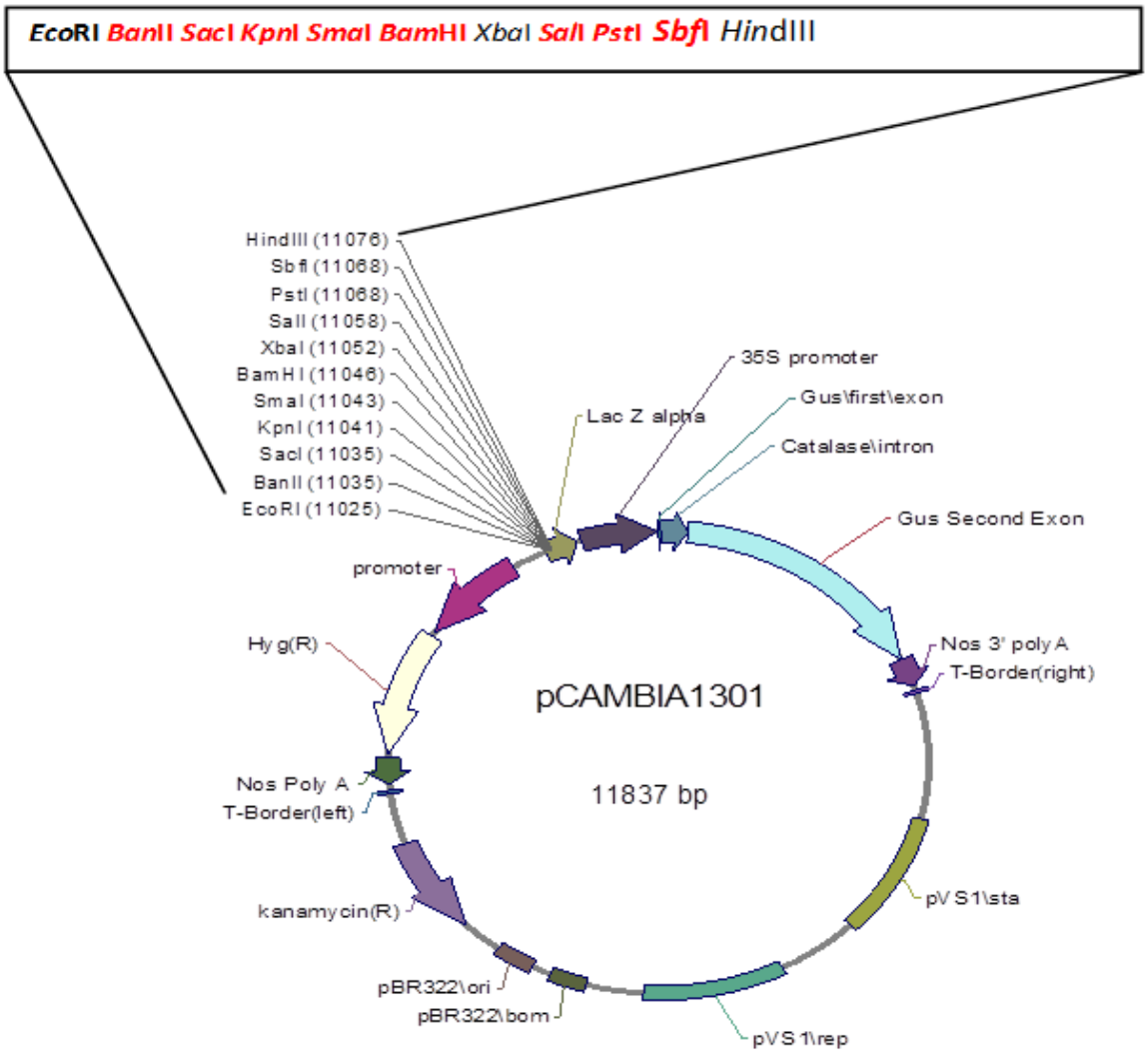


Figure 1. Map of p1301. p1301 was used as a backbone for BiFC construction. BiFC vectors were generated by eliminating restriction enzyme cutting sites (*Ban*II, *Sac*I , *Kpn*I, *Sma*I, *Bam*HI, *Sal*I, *Pst*I and *Sbf*I) (marked in red) from the MCSs of p1301, and introducing 35S terminator and nEYFP/cEYFP cassettes instead.

been developed for fusion targeted or putative proteins (Citovsky et al., 2006; Walter et al., 2004). BiFC based on a pSATN series of vectors are a good system because they carried constitutive expression cassettes with expanded multiple cloning sites (MCSs) to facilitate the fusion of targeted protein to the YFP fragments and double 35S promoters to facilitate the high-level expression of YFP fragments and targeted protein (Tzfira et al., 2005; Citovsky et al., 2006).

In addition, p1301 vectors were invented by CAMBIA Co., Ltd. and were most widely applied in plant transgene and transient expression. The advantages to utilising this vector include minimal heterologous sequences for plant transformation, small sequences and numerous copies in the host cell for easy construction. Because of *hptII* encoding resistance to hygromycin, most targeted genes are generated by constructing into p1301 and transformed into plants. If the interacting partner of the targeted gene needs to be identified using BiFC, these genes should be constructed as mentioned previously to avoid differing vectors and results. Therefore, it is necessary to construct BiFC vectors using p1301 as a backbone and thus, provide various options for vector construction.

Here, we reconstructed a BiFC platform based on pSATN, p1301-nEYFP-c and p1301-cEYFP-c, which were generated by eliminating restriction enzyme cutting sites (*Ban*II, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Sal*I, *Pst*I and *Sbf*I) at the MCSs of p1301, and introducing 35S terminator and cEYFP/ nEYFP cassettes into the MCSs (Figure 1). The viability of the reconstructed BiFC vectors was tested by analysing the interactions between *AtCBL*1

Table 1. Primers.

Name	Targeted sequence	Size (bp)	Primer
wcc216 wcc217	*CaMV 35S terminator*	210	TCTAGAGTCCGCAAAAATCACCAGTCT AAGCTTCGTCACTGGATTTTGGTTTTA
wcc218 wcc219	nEYFP/cEYFP cassette	1482	TCTAGAGTGGAGCACGACACACTTGTCT TCTAGATCAGGTGGATCCCGGG
wcc274 wcc275	*AtCIPK23*	1446	CTGCAGGCTTCTCGAACAACGCCTTC GGTACCTTATGTCGACTGTTTTGCAATTG
wcc276 wcc277	*AtCIPK23m*	1398	AGGCGAGAAGAAGGACTCAATCTCAATCTCGGTTCACTTTTC GAAAAGTGAACCGAGATTGAGATTGAGTCCTTCTTCTCGCCT
wcc278 wcc279	*AtCBL1*	642	GTCGACATGTATGCTGTTTCTGGTTG CTGCAGTCACAACAAATGGATTTTCT

Underlined sequence is the restriction site.

and *AtCIPK*23.

MATERIALS AND METHODS

Strains and media

pSAT4-nEYFP-c1 and pSAT1-cEYFP-c1 (B) (State University of New York, Stony Brook, NY) contained the N-part of EYFP (nEYFP) and the C-part of EYFP (cEYFP), respectively. p1301 was used as the backbone (Figure 1). *Escherichia coli* (*E. coli*) DH5α and *Agrobacterium* strain GV3101 were used for general cloning. Luria-Bertani medium was used for *E. coli* cultures and Yeast Extract Peptone medium was used for GV3101.

Primer synthesis, DNA cloning and DNA sequencing

Primers were obtained from Shanghai SBS Gene Technology Co., Ltd. (Shanghai, China). Taq DNA polymerase, dNTPs, pMD-18T vector, DL 2000 Marker and T4 ligase were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China), while the restriction endonucleases were purchased from Promega Biotechnology Co., Ltd. (Beijing, China). DNA was sequenced by the Sangon Biotechnology Co., Ltd. (Shanghai, China).

Experimental procedures

Production of the p1301-terminator

The p1301-terminator was generated by eliminating the restriction enzyme cutting sites (*Sal*I, *Pst*I and *Sbf*I), which are between the *Xba*I and *Hin*dIII markers of the p1301 vector (Figure 1), and by introducing a 35S terminator. The 35S terminator was amplified via polymerase chain reaction (PCR) from pSAT4-nEYFP-c1 using primers wcc216 and wcc217. The PCR cycling parameters were as follows: an initial denaturation step at 94°C for 5 min followed by 32 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min.

Production of the p1301-nEYFP-terminator (p1301-nEYFP-c) and p1301-cEYFP-terminator (p1301-cEYFP-c)

Other restriction enzyme cutting sites (*Ban*II, *Sac*I, *Kpn*I, *Sma*I and *Bam*HI) on the MCSs were eliminated from the p1301-terminator through cleavage by *Xba*I and *EcoR*I at 37°C. The corresponding cohesive termini were blunted by the Klenow fragment. At the same time, the nEYFP/cEYFP cassettes which had been digested by *Xba*I and subsequently blunted was inserted into p1301-terminator which was eliminated restriction enzyme cutting sites. Direct insertion was confirmed by PCR amplification with primers wcc217 and wcc218. If the 1.5 kb fragment was amplified, direct insertion was confirmed. The recombinant vectors were named p1301-nEYFP-c and p1301-cEYFP-c (henceforth referred to as p1301-nEYFP-c/cEYFP-c). nEYFP and cEYFP were amplified from pSAT4-nEYFP-c1 and pSAT1-cEYFP-c1(Table 1) using the same primers since they had the same sequence on both sides. The forward and reverse primers were wcc218 and wcc219, respectively. The PCR products were purified and ligated to pMD-18T vector at 16°C and then transformed into the *E. coli* strain DH5α. The p1301-nEYFP/cEYFP vectors were verified through restriction enzyme analysis and sequencing. The PCR cycling parameters were the same as previously mentioned, except that the annealing temperature was 62°C for 30 s and the extending time at 72°C was 75 s.

Cloning and construction of *AtCIPK23*, *AtCIPK23*m and *AtCBL*

AtCIPK23 was amplified from root cDNA using primers wcc274 and wcc275 and subsequently sequenced. The PCR cycling parameters were the same as those for the 35S terminator amplification, except that the annealing temperature was 54°C for 30 s and the extending time at 72°C was 90 s. *AtCIPK23*m was amplified using a 200x dilution of pMD-18T–*AtCIPK23* as template. First, one fragment of *AtCIPK23* was amplified using primers wcc274 and wcc277. The annealing temperature was 58°C for 30 s and the extending time at 72°C was 60 s. Secondly, another fragment was amplified using the forward and reverse primers wcc275 and wcc276, respectively. The annealing temperature was 56°C for 30 s and the extending time at 72°C was 30 s. Thirdly, *AtCIPK23*m was amplified using two fragments that were mixed at a 1:1 (molecule/molecule) ratio as a

template and the forward and reverse primers wcc274 and wcc275, respectively, and subsequently sequenced. The PCR cycling parameters were the same as those used for *AtCIPK23* amplification. In the final step, *AtCIPK23* and *AtCIPK23m* were digested by *Pst*I and *Kpn*I, respectively, and inserted into p1301-cEYFP-c. *AtCBL* was amplified, using root cDNA as a template and forward and reverse primers wcc278 and wcc279, respectively, and subsequently sequenced. The annealing temperature was 55°C for 30 s and the extending time at 72°C was 45 s. *AtCBL*-digested by *BamH*I and *Sal*I was inserted into pCAMBIA 1301-nEYFP-c.

BiFC assay

AtCBL1–AtCIPK23 protein interactions were examined to test the applicability of the reconstructed BiFC platform, p1301-nEYFP/cEYFP. We constructed *AtCBL1* into p1301-nEYFP-c and *AtCIPK23* as well as *AtCIPK23m* into p1301-cEYFP-n. These plasmids were then transformed into the *Agrobacterium* strain GV3101. AtCBL1-nEYFP-GV3101 and AtCIPK23-cEYFP-GV3101 were mixed at a 1:1 (w/w) ratio and injected into the leaf of *Nicotiana benthamiana*. The injected *N. benthamiana* were kept at 25°C for 3 days and then assessed.

RESULTS

BiFC platform p1301-nEYFP-c1 and p1301-cEYFP-c1

The BiFC platform (Figure 2) was generated by eliminating restriction enzyme cutting sites (*Ban*II, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Sal*I, *Pst*I and *Sbf*I) from the MCSs of p1301 and subsequently cloning in both the 35S terminator and nEYFP/cEYFP cassettes. Direct insertion must be confirmed because nEYFP/cEYFP cassettes have a blunt-end ligation. The 1.5 kb fragments that were amplified using primers wcc217 and wcc218 suggest this vector is the targeted vector. Finally, the p1301-nEYFP/cEYFP-c construct was sequenced and the result was as expected; no mutant nucleotides were present. The available and unavailable restriction enzyme cutting sites are shown in Figure 2. Furthermore, both nEYFP and cEYFP contained 2 CaMV promoters which enhance targeted gene expression.

AtCBL1–AtCIPK23 protein interaction

Plants can increase their ability to resist environmental stress through the calcineurin B-like proteins and their interacting protein kinase (CBLs–CIPKs) pathway in which the CBLs sense a change in Ca^{2+} concentration and activate CIPK and Whether the CBL–CIPK interaction is a pre-conditioned function of CIPK (Luan 2009). Interaction between AtCBL1 and AtCIPK23 increases tolerance to low K^+ concentrations (Xu et al., 2006; Cheong et al., 2007). To test the applicability of the reconstructed BiFC vectors, we employed p1301-nEYFP/cEYFP-c and studied AtCBL1–AtCIPK23 interactions. *AtCBL1* and *AtCIPK23* were cloned from the cDNA of *Arabidopsis thaliana* root to construct a BiFC vector. Three days after 1301-nEYFP-AtCBL1 and 1301-cEYFP-AtCIPK23-GV3101 were mixed and injected into the leaf of *N. benthamiana*, visible fluorescence were observed using confocal microscopy (Figure 3). This suggests that the interaction between AtCBL1 and AtCIPK23 did occur as expected. Fluorescence only appeared at the injection site.

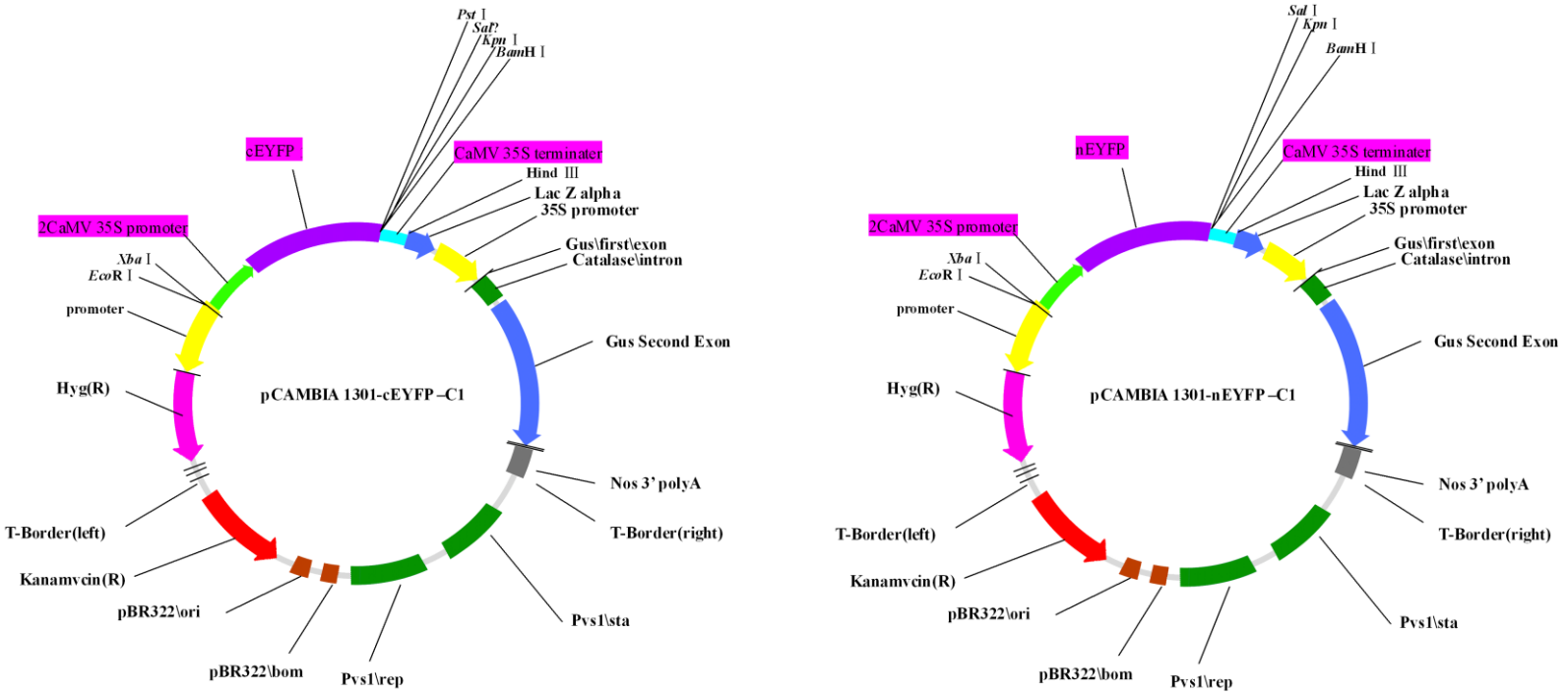
Furthermore, the fluorescence was strong and easily observed. Since the NAF domain of CIPK is responsible for the interaction between CBL and CIPK (Albrecht et al., 2001; Shi et al., 1999), we constructed chimeric AtCIPK23 (considered as the negative control) by deleting the NAF domain. The resulting fluorescence at the injection site was barely discernible. Even though occasional fluorescence was observed, it was very weak and there was a very low occurrence probability. This means that intensity and probability of fluorescence is distinct between the interaction and non-interaction between CBL and CIPK. Thus, our BiFC platform is suitable for the study of protein interactions.

DISCUSSION

Protein–protein interactions occur in all subcellular compartments and organelles and play an important role in various cellular physiological processes. Identification and characterisation of protein–protein interactions are essential for understanding and studying the various aspects of cell biology (Brady and Provart, 2009). Various technologies have been developed to identify and analyse protein–protein interactions *in vivo* and *in vitro* (Bartel and Fields, 1995; Citovsky et al., 2006; Xu et al., 2007; Walter et al., 2004; Hu et al., 2002). The reconstructed BiFC platform based on pSATN, p1301-nEYFP-c/cEYFP-c, provides various options for studying protein interactions.

p1301-nEYFP-c/cEYFP-c can efficiently detect protein interactions. Strong fluorescence appeared after co-injecting *Agrobacterium* with *AtCBL*1 and *AtCIPK*23, suggesting that there is an interaction between AtCBL1and AtCIPK23. When the NAF domain of AtCIPK23 was deleted, fluorescence did not usually appear. If on rare occasion it did, fluorescence was very weak. On the contrary, fluorescence was very strong and easily observed in the positive control. The appearance of fluorescence was not the sole indicator of interaction; strength of fluorescence was also significant. In fact, if fluorescence was strong and the occurrence probability was high, two proteins may have actually interacted. But if fluorescence was weak and the occurrence probability was low, two proteins may not have interacted.

When constructing targeted genes into p1301-nEYFP-c/cEYFP-c, it is noteworthy that the commonly used restriction endonucleases *Sal*I, *Pst*I, *Kpn*I and *BamH*I were very inexpensive and highly efficient. Once the targeted protein nEYFP/cEYFP is successfully constructed



A B

(A) Map of p1301-cEYFP-c. The MCS sequence is as follows:

TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA
AGG CCT GAG TCT AGA GCT CGA GTT CGA AGC TTA AGT CGT

SalI KpnI BamHI

GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC TGA TCT AGA
CAG CTG CCA TGG CGC CCG GGC CCT AGG TGG ACT AGA TCT

(B) Map of p1301-nEYFP-c. The MCS sequence is as follows:

PstI

TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA
AGG CCT GAG TCT AGA GCT CGA GTT CGA AGC TTA AGT CGT

SalI KpnI BamHI

GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC TGA TCT AGA
CAG CTG CCA TGG CGC CCG GGC CCT AGG TGG ACT AGA TCT

Figure 2. Maps of the reconstructed BiFC platforms.

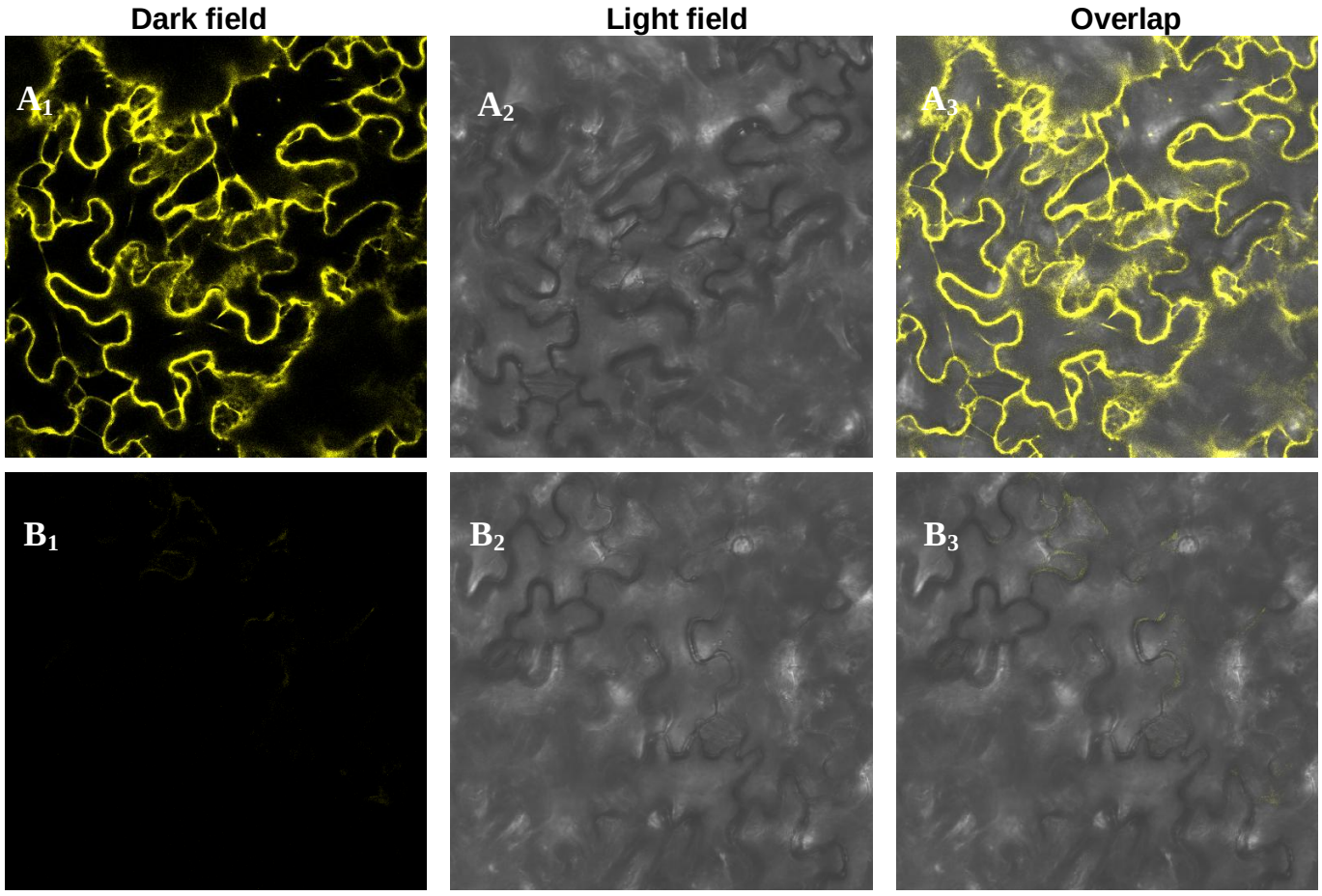


Figure 3. BiFC detection of AtCBL1–AtCIPK23 interactions. A1 and B1 are images of fluorescence microscopy in dark field, A2 and B2 are images of fluorescence microscopy in light field. A3 and B3 are overlapped. The A group shows a positive result for LeCBL1–LeCIPK23 and the B group shows a negative result for AtCBL1–AtCIPK23m (AtCIPK23m is AtCIPK23 with a deleted NAF domain).

into p1301-nEYFP-c/cEYFP-c, it can recombine with potential interacting proteins by co-injecting with two fusing vectors. This is an economical means that avoids the need for repeated construction of fusion vectors. On the other hand, though BiFC using a pSATN series of vectors is widely used (Jaedicke et al., 2011; Tsuchiya and Eulgem, 2011; Krichevsky et al., 2011), we found that it is difficult to freely clone and recombine them in the actual operation process. pSATN cannot be used as a mediating vector in transient expression, so EYFP expressing cassettes for fusing targeted proteins must be inserted into binary vectors. It is easy for targeted proteins to be cloned into pSATN but laborious to construct cassettes into binary vectors (Tzfira et al., 2005; Citovsky et al., 2006).

There are two ways to construct BiFC vectors from pSATN carrying targeted protein. The first employs Gateway-mediated cloning (Citovsky et al., 2006). Construction by *in vitro* recombination is efficient if economy is not a requirement. The second is constructing the gene encoding the N/C YFP fusion targeted protein into pZIP, which is a set of modular plant transformation vectors that allow flexible insertion of up to six expression units (Goderis et al., 2002). This method ensures an exact 1:1 ratio of N/C YFP expression requires simple injection of *Agrobacterium* and is efficient for transforming two expression cassettes into plants. However, using rare restriction endonucleases, such as octanucleotide endonucleases results in two limitations. Octanucleotide endonucleases are expensive and the N/C YFP fusion targeted protein cannot be freely combined. Therefore, the 1301-nEYFP/cEYFP BiFC vectors are not only suitable for the study of protein interactions in a wide range of plants, but also provide various options for studying these interactions.

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