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New GATEWAY vectors for High Throughput Analyses of Protein–Protein Interactions by Bimolecular Fluorescence Complementation

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ABSTRACT Complex protein interaction networks constitute plant metabolic and signaling systems. Bimolecular fluorescence complementation (BiFC) is a suitable technique to investigate the formation of protein complexes and the localization of protein–protein interactions *in planta*. However, the generation of large plasmid collections to facilitate the exploration of complex interaction networks is often limited by the need for conventional cloning techniques. Here, we report the implementation of a GATEWAY vector system enabling large-scale combination and investigation of candidate proteins in BiFC studies. We describe a set of 12 GATEWAY-compatible BiFC vectors that efficiently permit the combination of candidate protein pairs with every possible N- or C-terminal sub-fragment of S(CFP)3A or Venus, respectively, and enable the performance of multicolor BiFC (mcBiFC). We used proteins of the plant molybdenum metabolism, in that more than 20 potentially interacting proteins are assumed to form the cellular molybdenum network, as a case study to establish the functionality of the new vectors. Using these vectors, we report the formation of the molybdopterin synthase complex by interaction of *Arabidopsis* proteins Cnx6 and Cnx7 detected by BiFC as well as the simultaneous formation of Cnx6/Cnx6 and Cnx6/Cnx7 complexes revealed by mcBiFC. Consequently, these GATEWAY-based BiFC vector systems should significantly facilitate the large-scale investigation of complex regulatory networks in plant cells.

Key words: BiFC; GATEWAY; molybdenum cofactor; nitrate reductase.

INTRODUCTION

The sequencing of an increasing number of plant genomes has provided a wealth of information about the genetic information underlying plant development, metabolism, and environmental responses. However, the regulation and execution of biological processes are achieved by specific and dynamic interactions of protein networks. The large-scale investigation of protein interactions in their native cellular environment therefore represents an important challenge for plant biology.

Bimolecular fluorescence complementation (BiFC) and fluorescence resonance energy transfer (FRET) are powerful tools to study protein–protein interactions *in situ* (Bhat et al., 2006; Kerppola, 2006, 2008). BiFC is based on the complementation of two separately expressed N- and C-terminal sub-fragments of the green fluorescent protein (GFP) or derivates, such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), which are fused to putative interacting proteins. The non-functional fluorophore fragments do not reassemble spontaneously. Only if the fused proteins interact, their tight contact mediates refolding and reconstitution of the fluorescing protein, resulting in efficient fluorescence emission (Bhat et al., 2006). One advantage of BiFC-based protein interaction analysis is the visualization of even weak or transient interactions, which are stabilized by the tight association of YFP fragments (Kerppola, 2006). However, this stable reassembling of the fluorescing protein currently prevents the investigation of protein-interaction dynamics by BiFC after the initial state of complex formation. FRET enables investigations of dynamic protein interplay processes. However, the interaction of the proteins investigated has to be stronger and monitoring of fluorescence requires sophisticated microscopic equipment and numerous technical controls. While technical constraints prevent the large-scale application of FRET for the investigation of numerous potential protein–protein interactions in

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high throughput analyses, the methodical simplicity of BiFC studies should enable the combination of numerous candidate proteins in extensive interaction analyses to explore complex regulatory or enzymatic networks. However, the generation of large clone collections to enable such systems analyses is currently hampered by the requirement of conventional cloning steps for introducing the respective cDNAs into BiFC vectors.

The molybdenum metabolism in plants represents a metabolic network in that largely unknown protein–protein interactions among the enzymes catalyzing molybdenum cofactor (Moco) biosynthesis and allocation/insertion into its user enzymes occur. In all eukaryotes studied so far, Moco is synthesized by a conserved biosynthetic pathway that can be divided into four steps, according to its biosynthetic intermediates. Six proteins have been identified to catalyze Moco biosynthesis in plants (Schwarz and Mendel, 2006) and five molybdenum enzymes (occurring with isoforms) use this cofactor among higher plants. With an additional Moco-sulfurase ABA3 (Bittner et al., 2001) and up to eight putative Moco-binding proteins, at least 20 proteins can principally interact within the cellular molybdenum network. The multitude of possible interactions within the molybdenum network requires a fast cloning system in order to generate a large number of BiFC vectors that could be easily combined in interaction assays by transient co-expression in *Nicotiana benthamiana*.

In order to facilitate such large-scale protein interaction investigations, we employed the GATEWAY-cloning technology (Invitrogen, www.invitrogen.com), which has the advantage of fast and easy cloning of different cDNAs into destination vectors without the use of restriction and ligation enzymes. We created a set of 12 destination vectors permitting protein fusions to study BiFC in all possible protein orientations. This vector set enables the investigation of dual complex formation by single-color BiFC as well as the performance of multicolor BiFC for simultaneous detection of multiple protein interactions in a single cell (Waadt et al., 2008). In this study, we used these vectors to establish the interaction of the proteins Cnx6 and Cnx7 within the molybdopterin synthase complex. Moreover, using mcBiFC, we observed the simultaneous formation of homomeric Cnx6/Cnx6 and heteromeric Cnx6/Cnx7 complexes within one and the same cell.

RESULTS AND DISCUSSION

Generation of Binary GATEWAY–BiFC Vectors

The *in vivo* identification and characterization of protein interactions within the Moco biosynthesis complex represent a current emphasis of our research. To facilitate the combination of the advantages of the BiFC technique as a tool for visualizing protein interactions in their natural cellular context with the requirement to examine a multitude of possible candidate protein interactions within the molybdenum network, we sought to create BiFC vectors that rely on GATEWAY-cloning technology. In order to warrant the full compatibility and wide applicability of such GATEWAY–BiFC vectors with previously generated BiFC vectors, we chose the advanced BiFC vector set of Waadt et al. (2008) as a source for the new constructs. These vectors are based on the yellow fluorescent protein Venus that is known to exhibit significantly brighter fluorescence (Nagai et al., 2002) and, in addition to the conventional CFP harbor, the super cyan fluorescent protein (SCFP3A (Kremers et al., 2006)). The constructs pVYNE, pVYNE(R), pSCYNE, and pSCYNE(R) express the N-terminus of Venus or SCFP3A (aa 1–173) upstream or downstream to the multicloning site (MCS), respectively. As counterparts, pVYCE, pVYCE(R), pSCYCE, pSCYCE(R), pCYCE, and pCYCE(R) were generated that allow for C- and N-terminal protein fusion to the C-terminal fragment of Venus or S(CFP)3A (aa 156–239). In these constructs, the CaMV 35S-promoter directs a strong and constitutive protein expression for BiFC between $Venus^N$/$Venus^C$, $Venus^N$/$SCFP^C$, $SCFP^N$/$Venus^C$, and $SCFP^N$/$SCFP^C$, respectively, while the MAS-promoter drives expression in the p(MAS)SCYCE and p(MAS)SCYCE(R) vectors. The defined combination of distinct C- and N-terminal fragments results in unique emission maxima for CFP and Venus or in a chimerical signal for Venus plus CFP at 515 nm, which is different from that of Venus or CFP alone (Hu and Kerppola, 2003; Waadt et al., 2008).

To identify suitable candidate proteins from the molybdenum network, we first investigated the interaction between the proteins Cnx6 and Cnx7 forming the complex of molybdopterin synthase. To this end, cDNAs for *Cnx6*- and *Cnx7*-constructs were cloned in the conventional way by restriction and ligation reactions into the binary plasmids pVYNE(R) and pCYCE(R), respectively. These constructs were transformed into *A. tumefaciens* and, after transient expression in *N. benthamiana* leaves, the interaction between Cnx6 and Cnx7 was investigated using confocal microscopy 3 d after infection (Figure 1A). This experiment indeed revealed efficient formation of Cnx6/Cnx7 heterodimers and thereby established the suitability of both proteins as positive control for further investigation in GATEWAY-based BiFC vectors.

Consequently, primers with *att*B-sites (see Supplementary Data) were used to amplify *Cnx7* to create manually cloned GATEWAY–*Cnx7*-expression vectors with a prolonged linker region between the gene and the fluorophore fragment (for details, see Methods). GATEWAY-destination vectors were created with a BP-reaction (Invitrogen) using the Donor vector pDONR/Zeo (Invitrogen) and the manually cloned GATEWAY–*Cnx7*-expression vectors, respectively. These new destination vectors, containing the N-terminal part of the fluorophores, were designated as pDEST-^{GW}VYNE, pDEST–VYNE(R)^{GW}, pDEST–^{GW}SCYNE, and pDEST–SCYNE(R)^{GW}, while the constructs expressing the C-terminal part of the fluorophore were designated as pDEST–^{GW}VYCE, pDEST–VYCE(R)^{GW}, pDEST–^{GW}SCYCE, pDEST–SCYCE(R)^{GW}, pDEST–^{GW}CYCE, pDEST–CYCE(R)^{GW}, p(MAS)DEST–^{GW}SCYCE, and p(MAS)DEST–SCYCE(R)^{GW} (Figure 2 and Table 1). These destination vectors and the entry clones harboring *Cnx6* and *Nia2* from