All-in-one: a robust fluorescent fusion protein vector toolbox for protein localization and BiFC analyses in plants

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Summary
Fluorescent tagging protein localization (FTPL) and bimolecular fluorescence complementation (BiFC) are popular tools for in vivo analyses of the subcellular localizations of proteins and protein–protein interactions in plant cells. The efficiency of fluorescent fusion protein (FFP) expression analyses is typically impaired when the FFP genes are co-transformed on separate plasmids compared to when all are cloned and transformed in a single vector. Functional genomics applications using FFPs such as a gene family studies also often require the generation of multiple plasmids. Here, to address these needs, we developed an efficient, modular all-in-one (Aio) FFP (AioFFP) vector toolbox, including a set of fluorescently labelled organelle markers, FTPL and BiFC plasmids and associated binary vectors. This toolbox uses Gibson assembly (GA) and incorporates multiple unique nucleotide sequences (UNSS) to facilitate efficient gene cloning. In brief, this system enables convenient cloning of a target gene into various FFP vectors or the insertion of two or more target genes into the same FFP vector in a single-tube GA reaction. This system also enables integration of organelle marker genes or fluorescently fused target gene expression units into a single transient expression plasmid or binary vector. We validated the AioFFP system by testing genes encoding proteins known to be functional in FTPL and BiFC assays. In addition, we performed a high-throughput assessment of the accurate subcellular localizations of an uncharacterized rice CBSX protein subfamily. This modular UNSS-guided GA-mediated AioFFP vector toolkit is cost-effective, easy to use and will promote functional genomics research in plants.

Introduction
Defining protein subcellular localization (PSL) and protein–protein interaction (PPI) networks are crucial for studying plant gene functions and cellular processes in the post-genomics era (Cui et al., 2016; Miller et al., 2015). With the development of fluorescent proteins and high-resolution microscopes, fluorescent tagging protein localization (FTPL) and bimolecular fluorescence complementation (BiFC) technologies have become widely used for PSL and PPI analyses, providing direct visualization in living cells (Dixit et al., 2006; Tanz et al., 2013). The first step in such analyses is the preparation of constructs expressing target fusion proteins. Over the past decades, many specialized vectors have been developed for the transient or stable expression of fluorescent fusion proteins (FFPs) in plant cells (Blatt and Grefen, 2014). The first vectors developed for FTPL (such as pGDs and pSAT) and BiFC (such as pSYs and pSATNs) assays depended on traditional cloning methods using restriction enzyme digestion and ligation (Bracha-Drori et al., 2004; Citovsky et al., 2006; Goodin et al., 2002; Tzfira et al., 2005). Improved T-A cloning vectors (such as pXs/pCXs and pUC-35s-FPs/pGreen-Ubi-FPs) that enable the cloning of PCR fragments are generated for FTPL assays (Chen et al., 2009; Wang et al., 2013). A set of ligation-independent cloning-compatible vectors (pPLVs) that allow multiple fragments to be assembled in a single reaction were also developed for FTPL analysis in plants (De Rybel et al., 2011). Moreover, many Gateway cloning-based vectors (such as pGWVs, pEarleyGate and pDESTs) are widely utilized in plant research to conveniently transfer DNA fragments between vectors without the need for restriction enzyme digestion (Earley et al., 2006; Gehl et al., 2009; Karimi et al., 2007; Nakagawa et al., 2007).

For most of these applications, the basic designs of the vectors are suitable for manipulation of single gene. FTPL and/or BiFC analyses, however, often require the co-transformation of two or more plasmids (French et al., 2008; Waadt and Kudla, 2008). The efficiency of such analyses can be highly variable due to gene dosage. Single-plasmid transformation systems are, therefore, more desirable and require combination of two or more target expression units. To date, several plant transformation vector systems for multiple FFP expression units have been reported.

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(Patron, 2014; Zhu et al., 2020a, 2020b). Restriction enzyme-dependent vectors typically incorporate rare cutting sites or homing endonuclease recognition sites (such as pSAT and pPF-RC5) for multigene assembly via a protorced, low-efficiency cloning process (Goders et al., 2002; Tzifra et al., 2005). More effective site-specific recombinase-based cloning systems (such as the Cre/LoxP recombination system, the Agrobacterium-based recombination system and the Gateway-mediated cloning system) were subsequently developed. The improved TransGene Stacking II (TGSII) system utilizes modified Cre/loxp recombination based on a transformation-competent artificial chromosome and rare-cutting homing endonuclease sites to stack multiple genes (Lin et al., 2003; Zhu et al., 2017, 2018). The Agrobacterium-based GAANTRY (gene assembly in Agrobacterium by nucleic acid transfer using recombinase technology) system uses unidirectional site-specific recombinases to assemble transgene cassettes (Collier et al., 2018; Hathwaik et al., 2021). The advanced multisite Gateway cloning system allows two or three DNA fragments to be simultaneously transferred into destination vectors in a single recombination step, for use in BiFC and Forster resonance energy transfer (FRET) analyses (Grefen and Blatt, 2012; Hecker et al., 2015; Karimi et al., 2005; Martin et al., 2009). However, these multigene stacking methods require two or more rounds of cloning, and the reaction kits are expensive.

Compared to the above genetic cloning approaches, Gibson assembly (GA, also known as isothermal in vitro recombination) is a simple and versatile method for the direct, simultaneous in vitro assembly of multiple DNA fragments with overlapping complementary end sequences (Gibson, 2011; Gibson et al., 2009). With the development of unique nucleotide sequence (UNS) linkers and the improvement of in-house GA kits, GA-mediated multigene stacking now poses an efficient, single reaction and low-cost cloning method (Guye et al., 2013; Torella et al., 2014; Zhu et al., 2014). In this study, we developed a modular, cost-effective, GA-compatible all-in-one (Aio) FP (AioFP) vector toolbox for multigene co-expression in FTPL and BiFC assays. The AioFP vector system can be used to clone one or more genes of interest into customized FP vectors equipped with different fluorescence fusion genes into a plasmid via a single cloning reaction. This work was facilitated by the development of 12 subcellular localization fluorescent protein-fusion markers constructed in transient expression plasmids, which we successfully tested in *Oryza sativa* (rice) protoplasts and *Nicotiana benthamiana* leaves using known functional proteins and 12 uncharacterized members of the rice CBX5 subfamily. Our findings indicate that the AioFP vector system has potential for use in high-throughput FTPL and BiFC analyses to facilitate plant functional genomics research.

**Results**

**Development of the modular AioFP vector toolbox for FTPL and BiFC assays**

Here, we developed a versatile vector toolbox, AioFP, for the convenient assembly of multiple target genes in various FP vectors and the co-expression of two or three FFPs in transient-expression or binary vectors. We compared the excitation and emission wavelengths, brightness, photostability and oligomerization of the most commonly used fluorescent proteins (Lambert, 2019; Shaner et al., 2007), eGFP and mCherry, and selected eCFP and Venus as fluorescent markers. The N-terminal (Vn) and C-terminal (Vc) fragments of Venus are widely used in BiFC assays due to their fast and efficient maturation properties, strong fluorescence intensities, low sensitivity to environmental factors and low background fluorescence (Miller et al., 2015). The AioFP toolbox consists of four modules: mChe-labelled organelle markers, FTPL plasmids, BiFC plasmids and binary vectors (Figure 1a). These vectors utilize a modified UNS-guided GA strategy and are compatible with the Cre/loxP-based TGSII system (Zhu et al., 2017). We introduced several UNSs (Ua, Ub, Uc, Ud, Ue and Uf) (Torella et al., 2014) as cloning sites to improve the cloning efficiency of single or multiple target gene expression cassettes in a single plasmid.

Using our previously described pYL322d1 backbone of TGSII (Zhu et al., 2017), we constructed two eGFP-tagged FTPL transient expression plasmids (pYL322d1/N-eGFP and pYL322d1/eGFP-C) and two Vn-tagged BiFC vectors (pYL322d1/ N-Vn and pYL322d1/Vn-C) containing Ua-Asc I-Ub and Ub-Sbf I-Uf elements as acceptor vectors in pUC19-based plasmids (Figures S1a,d and S2). The pUC19-based FTPL plasmids (pUC19/N-mChe and pUC19/mChe-C, pUC19/eCFP and pUC19/eCFP-C) and BiFC vectors (pUC19/N-Vc and pUC19/Vc-C) with either Ua and Ub or Ue and Uf sites facilitate the transfer of expression cassettes into the same UNSs of the pYL322d1-based vectors (Figures S1b,c,e and S2). All FTPL and BiFC vectors contained a Uc-Sma I-Ud element for inserting a target gene in-frame with the N or C terminus of the fluorescent protein (Figures S1a–e and S2). We also produced a binary acceptor plasmid (pYL1300UaUf) with an Asc I-Ua-cdbb-Uf-Asc I fragment for GA-mediated multiple expression units (Figures S1f and S2).

The AioFP vector system for FTPL and BiFC analyses is illustrated in Figure 1b–e. First, the open reading frame (ORF) of the target gene is amplified using a pair of chimeric primers (containing Uc and Ud sequences) and cloned into various AioFP transient expression vectors via the Uc/Ud sites by GA (Figure 1b). In addition, multiple ORFs of different target genes could be cloned into the same AioFP transient expression vector using a GA reaction in a single tube (Figure 1c). Although the resulting individual plasmids with different FFPs can be directly mixed for PEG/Ca-mediated transient co-expression in protoplasts or co-transformation via biolistic bombardment, the stability and efficiency of co-transformation are lower than when a single vector is used. The AioFP vector system allows multiple FP expression units to be integrated into pYL322d1-based transient acceptors via Ua/Ub or Ue/UF elements to improve co-expression efficiency (Figure 1d). Moreover, multiple FP expression cassettes in pYL322d1-based plasmids can be conveniently and flexibly assembled into the pYL1300UaUf binary vectors through Ua/Uf sites using Gibson cloning or into pYL1300H by Cre/loxP-mediated recombination (Figure 1e). In summary, we generated a set of modular FFP vectors for protein colocalization and BiFC assays in plants.

**Generation of the mChe-labelled organelle marker module**

To visualize proteins of interest or interacting proteins in various subcellular compartments in vivo, the distribution of candidate fusion proteins must be compared with the distribution of known fluorescent markers or fluorescent dyes that target these subcellular structures (Tanz et al., 2013). We, therefore, developed a set of mChe-labelled subcellular compartment markers for protein colocalization (Figure S1g). This pUC19-based marker system was designed to add target proteins with differing subcellular peptide signals based on previously reported information (Table S1). The
Figure 1  Diagram illustrating the GA-based AioFFP vector toolbox for FTPL and BiFC assays. (a) The modular all-in-one (Aio) fluorescent fusion protein (FFP) vector toolbox for transient and stable gene expression in plants. (b) Open reading frames (ORFs) for multiple genes of interest are amplified with chimeric primers (Uc-PF/Ud-PR) and cloned into various FFP vectors by UNS-guided GA reaction in a single reaction to generate N- or C-terminal fluorescent fused proteins. (c) Multiple ORFs incorporating Uc and Ud cloning sites are simultaneously cloned into a FFP transient expression vector via GA reaction. (d) Multiple expression units of mChe-fused OM or ORF and eCFP- or Vc-fused ORFs are simultaneously assembled into a pYL322d1-based eGFP- or Vn-fused ORF transient expression vector via Ua-Ub and Ue-Uf sites in a GA reaction. (e) Multiple expression cassettes produced from pYL322d1-based transient expression plasmids are assembled into the pYL1300UaUf binary vector using GA or the pYL1300H binary vector using the Cre/loxP method of the TGSII system. OM: organelle marker; HPT: hygromycin selection marker gene; ccdB: encoding bacterial suicide protein. P35s: cauliflower mosaic virus 35s promoter; T35s: cauliflower mosaic virus 35s terminator; Tnos: nopaline synthase terminator. IoxP, 2R and 1L: wild-type recombination site and irreversible mutational recombination sites in the TGSII system; Pi-Scel I and I-Sce I: homing endonucleases. LB and RB: left border and right border of a T-DNA region. FTPL: fluorescent tagging protein localization; GA, Gibson assembly.
flanking Asc I-Ua and Ub-Asc I sequences in the plasmids allow for transgene integration by GA and stacking into pYL322d1-based transient acceptor plasmids (Figure 1d). As summarized in Table S1, the subcellular localization markers include those targeting proteins to the nucleus and cytoplasm (mChe), nucleus (NLS-mChe), endoplasmic reticulum (ER-mChe), plastids (Pt-mChe), tonoplast (Tm-mChe), cytoplasmic foci (CF-mChe), mitochondria (Mito-mChe), plasma membrane (PM-mChe), Golgi apparatus (Golgi-mChe), peroxisomes (Px-mChe), cytoskeleton (Cs-mChe) and plasmodesmata (Pd-mChe).

The efficacy of these localization peptide or protein fusions was confirmed by transient expression in rice protoplasts (Figure S3) and onion (Allium cepa) epidermal cells (Figure S4) followed by visualizing mChe fluorescence via confocal laser-scanning microscopy. In most cases, the subcellular localization of mChe expression in rice protoplasts was resolved at higher resolution compared to expression in onion epidermal cells. In both instances, it was better to visualize images through successive optical sections of transformed cells and analyse the maximum intensity projection image reconstructed from each optical section to clarify the subcellular localization of the mChe signal. These organelle markers are valuable for the subcellular localization of proteins in plants.

Single-step construction of multigene FFP plasmids using a modified UNS-guided GA reaction

In the GA-compatible AioFFP vector system, we introduced two UNSs (Uc and Ud) for cloning multiple target genes into a series of transient FFP vectors. As an example, the coding sequence of the rice nucleus-localized functional protein OsSAP18 (LOC_Os07g07370) was amplified using chimeric primers (Uc-SAP-F/Ud-SAP-R, Table S3) and simultaneously cloned into Sma I-digested pYL322d1/eGFP and pYL322d1/Vn or pUC19/eCFP, pUC19/eGFP and pUC19/mChe vectors by GA (Figure 2a). Transformed Escherichia coli (E. coli) cells were selected on medium containing chloromycetin (Chl, pYL322d1-based vectors) or ampicillin (Amp, pUC19-based vectors), and positive clones were identified by colony PCR using well-designed primers (Figure 2b).

The in vivo transformation of three FFP plasmids into rice protoplasts showed that OsSAP18-eGFP, OsSAP18-eCFP and OsSAP18-mChe correctly localized to the nucleus (Figure 2c), which is consistent with previous reports (Xie et al., 2018). To test the functionality of the BiFC vectors, we cloned the ORF of the OsSAP18-interacting protein OsHDA710 (LOC_Os02g12380) into pUC19/Vc using GA. Co-expression of OsSAP18-Vn with OsHDA710-Vc in rice protoplasts showed that OsSAP18 interacted with OsHDA710, which localized to the nucleus (Figure 2d); these results are consistent with previous findings (Xie et al., 2018).

The AioFFP vector system allows multiple target genes to be cloned in a single GA reaction. As summarized in Figure 3a, we amplified the ORFs of five OsCBSX genes encoding sensory relay proteins (see below) with chimeric primers (see Table S3) and cloned them together by GA in a reaction containing Sma I-digested pYL322d1/N-eGFP. We screened transformed colonies that were selected on Chl by colony PCR and obtained clones of all five OsCBSX genes (Figure 3b). Transformation of these plasmids into rice protoplasts confirmed that functional OsCBSX-eGFP fusion proteins were expressed in vivo (Figure 3c).

In summary, the AioFFP vector system enables the convenient cloning of multiple target genes into various FFP vectors as needed.

Stacking multiple FFP expression units improves their co-expression efficiencies in FTPL and BiFC assays

To improve the co-transformation efficiencies of multiple FFPs in FTPL and BiFC assays, we introduced four UNSs (Ua, Ub, Ue and Ud) into the AioFFP vector toolbox to enable the assembly of two or three FFP expression cassettes within a single plasmid. To test the functionality of this system for FTPL analysis, the Uc-OsSAP18-Ud fragment amplified with chimeric primers (see Table S3) and the Asc I fragment incorporating the nucleus-localized marker NLS-mChe expression cassette flanked with Ua and Ub sites were assembled into the Asc I and Sma I-digested pYL322d1/N-eGFP vector by GA to form the OsSAP18-eGFP-NLS-mChe plasmid (Figure 4a). We compared the number of fluorescent cells among rice protoplasts after transformation with the two plasmids (OsSAP18-eGFP and NLS-mChe) or the single plasmid OsSAP18-eGFP-NLS-mChe. The ratio of fluorescent cells expressing both eGFP and mChe fusion proteins following single-plasmid transformation was significantly higher than that following transformation using two plasmids (Figure 4b). These results indicate that the co-expression efficiency of single-plasmid transformation is higher than the efficiency of co-transformation with two individual plasmids.

To demonstrate the efficacy of the BiFC vectors, we subcloned the OsHDA710-Vc expression cassette into OsSAP18-Vn to generate OsSAP18-Vn–OsHDA710-Vc. The OsHDA710-Vc and NLS-mChe expression cassettes were simultaneously assembled into OsSAP18-Vn (Figure 4c). When we transformed rice protoplasts with these plasmids, the co-expression efficiency of the three FFPs via single-plasmid transformation (~80%) was higher than that of co-transformation using two (~58%) or three (~26%) individual plasmids (Figure 4d). These results indicate that the AioFFP vector system using the UNS-guided GA strategy is valuable for stacking multiple FFP expression units, leading to high co-expression efficiency of multiple FFPs.

The AioFFP vector toolbox enables efficient analysis of the subcellular localization of OsCBSX family members

CBSX proteins are sensor relay proteins, with only one pair of cystathionine β-synthase (CBS) domains and no other domains. These proteins play important roles in regulating the activation of thioredoxins and cellular H2O2 levels during plant growth and development in Arabidopsis thaliana (Ok et al., 2012). As the functional roles of CBSX family members in plants remain poorly understood, we performed a preliminary FTPL analysis of all 12 rice OsCBSXs. A previous study indicated that the six AtCBSXs in A. thaliana localize to different intracellular compartments, such as the chloroplast, mitochondria, cytosol, endoplasmic reticulum and vacuole (Ok et al., 2012). Of the 12 rice OsCBSXs, only OsCBSX3 and OsCBSX4 have been reported to localize to the plasma membrane (Mou et al., 2015; Singh et al., 2012).

Predictions of subcellular localizations of the OsCBSX proteins using various online software packages showed numerous disagreements (Table S2). To experimentally test these in silico predictions, we fused each OsCBSX with a C-terminal eGFP and assembled them with mChe markers with different subcellular localizations by GA (Figure 5a).Transient expression of each plasmid in rice protoplasts confirmed that some OsCBSX proteins localized to various intracellular compartments in different protoplasts (Figure 5b and Figure S5). Contrary to the previous observation that OsCBSX3 and OsCBSX4 localize...
to the plasma membrane, OsCBSX3 was found to localize to the nucleus and cytoplasm, and OsCBSX4 localized to the nucleus, cytoplasm and mitochondria (Figure 5b). The 10 other OsCBSX proteins all localized with NLS-mChe to the nucleus, and OsCBSX5, OsCBSX6, OsCBSX7, OsCBSX8, OsCBSX10, OsCBSX11 and OsCBSX12 also localized to the cytoplasm, while OsCBSX1 and OsCBSX2 localized to the mitochondria (Figure 5b). These findings are different from the predicted subcellular localizations using online software packages (Table S2).

These results indicate that the co-expression of FFPs of interest with known organelle markers is required for subcellular localization and that the accuracy of subcellular localization prediction using online programs needs to be further improved. Taken
together, these findings demonstrate that the AioFFP vector system using a UNS-guided GA strategy is a simple, modular cloning platform for multiple FFP plasmid construction that enables high-throughput subcellular localization and facilitates BiFC studies.

The binary system can be used to express multiple FFP

The AioFFP vector toolbox provides alternative binary vectors that accept multiple FFPs from transient expression plasmid to enable

Figure 3  High-throughput cloning of multiple target genes into an AioFFP transient expression vector. (a) As examples, the ORFs of five OsCBSXs were amplified with chimeric primers and cloned into Sma I-digested pYL322d1/N-eGFP by GA. Transformants were selected on Chl, and (b) positive OsCBSXs-eGFP colonies were identified by colony PCR using six primers: SAP-F/CX1-R, CX2-R, CX3-R, CX4-R and CX5-R. M: DNA ladder markers. (c) The five different OsCBSXs-eGFP plasmids were transiently expressed in rice protoplasts. BF: bright field; eGFP: eGFP fluorescent signal; Merge: overlapping of eGFP and BF images. Scale bar: 10 μm. GA, Gibson assembly; ORF, open reading frame.
both transient and stable co-expression in plants by Agrobacterium-mediated transformation (Figure 1e). To validate the capacity of the binary vector system, we assembled the OsCBSX9-eGFP–NLS-mChe expression units flanked with Ua and Uf end sites into the same UNS sites of the pYL1300UaUf binary vector by GA-mediated recombination (Figure 6a). The eGFP–mChe expression cassettes in the pYL322d1-based transient expression plasmid were recombined into the pYL1300H binary vector of TGSII using Cre/loxP-mediated recombination (Figure 6b). Both binary plasmids were transiently expressed in N. benthamiana leaves by Agrobacterium-mediated infiltration. Confocal imaging showed that OsCBSX9-eGFP and NLS-mChe both colocalized to the nucleus (Figure 6c), as was also observed in rice protoplasts (Figure 5B). By contrast, the eGFP and mChe controls colocalized to the cytoplasm and nucleus (Figure 6d), which was also observed in rice protoplasts (Figure 5D). These results demonstrate that our vector toolbox is suitable for use with the binary vector system for Agrobacterium- or biolistic bombardment-mediated transient or stable transformation.

Discussion

Plant protein interaction and subcellular localization studies are critical for understanding the functions and intracellular locations of proteins (Goodin, 2018). In post-functional genomics research, numerous genes of interest (such as gene families) must often be studied simultaneously. In this study, to facilitate the efficient and high-throughput construction of plasmids for both FTPL and BiFC assays, we generated a set of UNS-guided GA-mediated AioFFP vectors. The GA strategy is advantageous for its ease of manipulation, significantly increasing the assembly efficiency of large DNA molecules and the recombination of multiple DNA fragments (Gibson, 2011; Gibson et al., 2009). UNSs are designed for the efficient assembly of multiple DNA fragments containing repeated sequences; for instance, the same promoter is used in the study to strive for equal gene dosage expression (Ramon and Smith, 2011; Torella et al., 2014). The use of 10 transient FFP vectors, which incorporate Uc/Ud sequences in the N- and C-fusion sites of fluorescent proteins, permits the parallel

Figure 4 Stacking of multiple FFP expression units in a single plasmid improves the co-transformation efficiency of FTPL and BiFC assays. (a) Assembly of a target gene with organelle marker in a single plasmid using a one-tube GA reaction. The PCR product of OsSAP18 with Uc and Ud sites, the NLS-mChe expression unit from the Asc I-digested NLS-mChe plasmid and the Asc I- and Smal-digested pYL322d1/N-eGFP vector were mixed and assembled together by GA to generate the single plasmid OsSAP18-eGFP–NLS-mChe. (b) Transformation efficiency of two-plasmid co-transformation vs. single-plasmid transformation in an FTPL assay. The Y-axis represents the ratio of fluorescent cells to total cells. Data are shown as mean ± SE of three experiments (**P < 0.01). (c) Schematic illustration of two or three expression units stacking into a single plasmid in a BiFC assay. NLS-mChe and OsHDA710-Vc expression cassettes were inserted into the Ua-Asc I-Ub and Ue-Sfi I-Uf sites of the OsSAP18-Vn transient expression plasmid respectively. (d) Transformation efficiency of three- and two-plasmid co-transformation vs. single-plasmid transformation. The Y-axis represents the ratio of fluorescent cells to total cells. Data are shown as mean ± SE of three experiments (**P < 0.01). BiFC, bimolecular fluorescence complementation; FFP, fluorescent fusion protein; FTPL, fluorescent tagging protein localization; GA, Gibson assembly.
subcloning of different target genes under investigation in various transient expression vectors conveniently using a GA reaction in a single tube. In addition, the presence of four UNSs (Ua/Ub and Ue/Uf) in the vectors allows for the convenient assembly of multiple expression units into a single plasmid to improve co-transformation efficiency. It should be noted that the expression units for multigene stacking should be generated by PCR instead of digestion when the sequence of the target gene contains AscI, SbfI or PmeI restriction enzyme sites.

The eGFP-, eCFP- and mChe-fused transient expression vectors in the AioFFP vector system can be used to fuse target proteins to N- or C-terminal fluorescent proteins for colocalization analysis of (i) one or two target proteins with organelle markers or (ii) two or three proteins of interest. The Vn- and Vc-fused BiFC vector modules enable the N- and C-terminal fusion of two genes of interest to each tag, which could be used for (i) PPI analysis of two proteins of interest, (ii) colocalization analysis of two interacting proteins with organelle markers or (iii) colocalization analysis of two interacting proteins with another protein. The most commonly used approaches for introducing these plasmids into plant cells are PEG/Ca-mediated transient transformation of protoplasts, biolistic-mediated transient/stable transformation and Agrobacterium-mediated transient/stable transformation (Chen et al., 2006; Jeong et al., 2021; Ramkumar et al., 2020). Of these techniques, protoplast transformation is a rapid and efficient method that has become a widely used tool for plant biology.

Figure 5 Analysing the subcellular localization efficiency of OsCBSX3 and OsCBSX4 in rice protoplasts using the AioFFP vector toolbox. (a) Assembly of OsCBSX-eGFP with different organelle markers by GA in a single tube. (b) Transient transformation of OsCBSX3-eGFP and OsCBSX4-eGFP plasmids integrated with mChe, NLS-mChe, Mito-mChe and mChe-PM. The eGFP and mChe channels of mitochondria (third row) show maximum intensity projections of images of different sections. BF: bright field; eGFP: eGFP fluorescent signal; mChe: mChe fluorescent signal; Merge: overlapping of eGFP, mChe and BF images. Scale bar: 10 μm. GA, Gibson assembly.

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studies (He et al., 2016; Yoo et al., 2007). The sizes, copy numbers and promoters of vectors are critical parameters for protoplast transformation (He et al., 2016). Here, we designed several transient expression vectors with a minimal size but high copy number-based replicon and strong CaMV35S promoter to improve transformation efficiency. The AioFFP system also provides two binary vector modules for the recombination of multiple FFP expression units for Agrobacterium-mediated transient or stable expression.

Some proteins show diverse localization patterns in plants cells or are translocated into different subcellular compartments in diverse cellular environments (Duan et al., 2017; Ito et al., 2012; Meier and Somers, 2011; Zhu et al., 2020a, 2020b). In this study, most OsCBSX family members were also distributed in diverse subcellular compartments (Figure 5b and Figure S5). Except when proteins are localized to subcellular structures with typical, obvious features, such as the nucleus or plasma membrane, the localizations of most proteins that target other subcellular fractions often need to be validated by co-transformation with several organelle markers or by staining with different dyes (Nelson et al., 2007).

Several sets of organelle markers have been developed and utilized in plant cell biology and functional genomic studies (Luo and Nakata, 2012; Nelson et al., 2007; Wu et al., 2013). Most of these constructs utilize a binary vector for single-vector transformation that are not suitable for protoplast transformation, making it difficult to conveniently assemble multiple expression vectors. These limitations are avoided in the AioFFP vector system where the 12, minimally sized, mChe-labelled organelle markers are flanked with Asc I-Ua and Ub-Asc I elements in a pUC19-based vector for easy assembly into pYL322d1 derived vector to form a single, multi-gene plasmid compatible with high efficiency PEG/Ca-mediated protoplast transformation.

In summary, the UNS-guided GA-mediated AioFFP vector system described here is a versatile toolbox for investigating protein interactions and distribution in living plant cells.

Experimental procedures

Molecular cloning

Construction of transient expression vectors for FTPL and BiFC assays

Vectors were constructed by gene synthesis, Ω-PCR (Chen et al., 2013), and GA-mediated multi-type plasmid modification (Zhu et al., 2014) and verified by Sanger sequencing. The cauliflower mosaic virus 35s promoter (P35s), cauliflower mosaic virus 35s terminator (T35s) and nopaline synthase terminator (Tnos) were used as regulatory elements for gene expression. Six UNSs (Ua, Ub, Uc, Ud, Ue and Uf) were used as versatile sequences for GA or Ω-PCR. eGFP, mChe, eCFP, Vn and Vc were used as fluorescent tags. The detailed vector construction procedure is described in the Supporting Methods.

Construction of organelle markers

To construct a series of organelle markers, the DNA sequences encoding signal peptides or proteins localized to each subcellular compartment were synthesized or amplified from plant cDNA and cloned into the 5' or 3' end of mChe. For NLS-mChe and ER-mChe, two nucleus-localized or endoplasmic reticulum-localized signal peptides were fused to both the 5' and 3' ends of mChe. The peroxisome-localized signal peptide was fused to the 3' end of mChe (mChe-Px). For Pt-mChe and Tp-mChe, the truncated sequence of PsrbcS or AtCBL2 was cloned into the 5' end of mChe. The truncated sequence of AtIRFM1 was inserted into the 3' end of mChe.
end of mChe (mChe-Cs). For Mito-mChe, CF-mChe and Pd-mChe, the full-length coding sequences of OsCOX11, AtTZF1 and AtPDCB1 were cloned into the 5’ end of mChe. For mChe-PM and mChe-Golgi, the full-length OsSecA2 and AtRER1B sequences were inserted into the 3’ end of mChe.

**Plasmid construction to test the FTPl and BiFC vectors**

The coding sequence of OsSAP18 was amplified with chimeric primers (Uc-SAP-F/Ud-SAP-R, Table S3), and the resulting fragment was cloned into Sma I-digested pYL322d1/eGFP, pYL322d1/N-Vn, pUC19/N-eCFP, pUC19/N-Vc and pUC19/N-mChe via the Uc and Ud sequences. The coding vector of OsHDA710 was cloned into pUC19/N-Vc transient expression vectors. The NLS-mChe expression cassette obtained by Asc I-digestion and the Uc-OsSAP18-Ud fragment were both inserted into Asc I and Sma I-digested OsSAP18-eGFP using a GA reaction in a single tube (producing the OsSAP18-eGFP-NLS-mChe plasmid). The expression cassette of OsHDA710-Vc was generated by Asc I-digestion of OsHDA710-Vc and inserted into Sbf I-digested OsSAP18-Vn using GA (generating the OsSAP18-Vn-OsHDA710-Vc plasmid). The NLS-mChe and OsHDA710-Vc expression cassettes were simultaneously assembled into Asc I and Sbf I-digested OsSAP18-Vn (resulting in the OsSAP18-Vn-OsHDA710-Vc-NLS-mChe plasmid).

**Plasmid construction for subcellular localization analysis of OsCBSXs**

The coding sequences of OsCBSXs gene family members were amplified from rice cDNA, flanked with Uc and Ud adapters and cloned into Sma I-digested pYL322d1/eGFP via the Uc and Ud sequences. The expression cassette of different mChe-tagged organelle markers was produced by Asc I-digestion and inserted into Asc I-digested OsCBSXs-eGFP plasmids via the Ua and Ub sites. A fragment containing OsCBSX9-eGFP-NLS-mChe expression units was generated by Pme I-digestion and assembled into the Asc I-digested pYL1300UaUf binary vector by GA reaction. To construct a binary vector as a transformation control in N. benthamiana, the mChe expression cassette was inserted into pYL322d1/eGFP. A fragment containing eGFP-mChe expression units was recombined into the pYL1300H binary vector via Cre/loxP recombinase-mediated recombination.

**Isolation and transformation of rice protoplasts**

PEG/ca-mediated transformation of rice protoplasts was conducted as previously described (Chen et al., 2006; He et al., 2016). Briefly, rice seeds were sterilized and cultured on 1/2 MS medium with high osmotic pressure before bombardment. A 10-µg aliquot of each plasmid was transformed into rice protoplasts by PEG/ca-mediated transformation. Transformed protoplasts were incubated for ~16–20 h at 28°C before observation.

**Biolistic-mediated transformation**

For the particle bombardment assay, the plasmid was delivered into onion epidermal cells using the Helios Gene Gun system (BioRad, Hercules, CA, USA, Helios Gene Gun) (Hollender and Liu, 2010). Briefly, onion epidermal cells were cultivated on MS medium with high osmotic pressure before bombardment. A 10-µg aliquot of each plasmid was coated with 1.0 mg sterile gold particles and bombarded into onion epidermal cells according to the manufacturer’s instructions. After bombardment, the transformed cells were incubated on MS medium for ~16–20 h at 28°C before observation.

**Agrobacterium-mediated infiltration of N. benthamiana**

Nicotiana benthamiana seeds were germinated and grown in peaty soil at 25°C under a 14-h light/10-h dark cycle. The binary plasmids were electroporated into Agrobacterium tumefaciens strain GV3101 and transiently transformed into the leaves of 4-week-old N. benthamiana plants by syringe-mediated infiltration (Blatt and Grefen, 2014). The inoculated leaves were incubated for ~24–48 h before observation.

**Confocal laser scanning microscopy**

Cells expressing fluorescently tagged fusion protein were observed under a confocal laser-scanning microscope (Carl Zeiss LSM 710, Oberkochen, Germany). Excitation/emission wavelength were 405 nm/445–485 nm for eCFP, 488 nm/490–560 nm for eGFP, 514 nm/520–570 nm for Venus and 543 nm/580–660 nm for mChe. Images were acquired and processed using Zen software (Carl Zeiss).

**Accession numbers**

The GenBank accession numbers of the 10 transient vectors and 2 binary vectors are as follows: pYL322d1/N-eGFP (OL886686), pYL322d1/eGFP-C (OL886687), pUC19/N-eCFP (OL886688), pUC19/eCFP-C (OL886683), pUC19/N-mChe (OL962693), pUC19/mChe-C (OL962694), pYL322d1/N-Vn (OL886688), pYL322d1/Vn-C (OL886689), pUC19/N-Vc (OL886684), pUC19/Vc-C (OL8962695), pYL1300UaUf (OL962695) and pYL1300H (KY420079).

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**Conflict of interest**

The authors declare no conflict of interest.

**Authors contributions**

QZ conceived and supervised the project. QZ and JH designed the experiments. JH, KM, HL, JS, LZ, JT, SZ and YH performed the experiments. JH, KM and QZ analysed the data. JH wrote the draft. QZ, YGL and LC revised several versions of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Schematic diagrams of the plasmids designed for FTPL and BiFC assays.
Figure S2 Partial sequences of the GA-compatible vectors.
Figure S3 Confirmation of the subcellular localizations of organelle markers in rice protoplasts.
Figure S4 Transient expression of the organelle markers in onion epidermal cells.
Figure S5 Subcellular localization of OsCBSXs in rice protoplasts.
Table S1 Information about the organelle markers constructed for protein colocalization.
Table S2 Prediction and verification of the subcellular localizations of OsCBSXs.
Table S3 Primers used in this study.
Method S1 Gibson assembly (GA) protocol.
Method S2 Construction of GA-compatible vectors for FTPL and BiFC assays.