Video Article

Co-expression of Multiple Chimeric Fluorescent Fusion Proteins in an Efficient Way in Plants

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Abstract

Information about the spatiotemporal subcellular localization(s) of a protein is critical to understand its physiological functions in cells. Fluorescent proteins and generation of fluorescent fusion proteins have been wildly used as an effective tool to directly visualize the protein localization and dynamics in cells. It is especially useful to compare them with well-known organelle markers after co-expression with the protein of interest. Nevertheless, classical approaches for protein co-expression in plants usually involve multiple independent expression plasmids, and therefore have drawbacks that include low co-expression efficiency, expression-level variation, and high time expenditure in genetic crossing and screening. In this study, we describe a robust and novel method for co-expression of multiple chimeric fluorescent proteins in plants. It overcomes the limitations of the conventional methods by using a single expression vector that is composed of multiple semi-independent expressing cassettes. Each protein expression cassette contains its own functional protein expression elements, and therefore it can be flexibly adjusted to meet diverse expression demand. Also, it is easy and convenient to perform the assembly and manipulation of DNA fragments in the expression plasmid by using an optimized one-step reaction without additional digestion and ligation steps. Furthermore, it is fully compatible with current fluorescent protein derived bio-imaging technologies and applications, such as FRET and BiFC. As a validation of the method, we employed this new system to co-express fluorescently fused vacuolar sorting receptor and secretory carrier membrane proteins. The results show that their perspective subcellular localizations are the same as in previous studies by both transient expression and genetic transformation in plants.

Video Link

The video component of this article can be found at https://www.jove.com/video/57354/

Introduction

Chimeric fluorescent fusion proteins have been regarded as useful tools to study intracellular dynamics and subcellular localization and further understand their physiological functions and working mechanisms[1,2,3,4]. It is especially beneficial to co-express well-known organelle reporter proteins with the protein in question to better illustrate its spatiotemporal rationale, distribution, and function(s) within the endomembrane system in cells[4,5,6,7,8].

A chimeric fluorescent fusion protein can be expressed in plants *via* transient expression and stable genetic transformation, which have their respective advantages and limitations[9,10,11]. Transient expression of a protein is a convenient approach that includes biolistic bombardment-, polyethylene glycol (PEG)-, or electroporation-mediated DNA transient expression in protoplasts and *Agrobacterium*-mediated leaf infiltration in intact plant cells, as shown in **Figure 1A,B**[12,13,14,15,16]. However, co-expression of multiple chimeric fluorescent fusion proteins in a single plant cell requires a mixture of several independent expression plasmids. Thus, the drawbacks of employing multiple plasmids for protein co-expression in plants are lower co-expression levels due to the dramatically reduced chance of several plasmids simultaneously entering the same cells when compared to a single plasmid, and the variations of protein expression levels caused by the uncontrollably random amount of each types of plasmid being transferred into the cell[17,18]. In addition, it is technically challenging to introduce several independent expression plasmids into a single *Agrobacterium* for protein co-expression[9,10,11]. Therefore, *Agrobacterium*-mediated protein transient expression by infiltration of tobacco leaves is only capable of expressing one plasmid at a time, as shown in **Figure 1B**. In contrast, generation of transgenic plants expressing fluorescent fusion proteins is usually achieved by *Agrobacterium* that carries a binary transformation vector. However, the binary vector that mediates the gene transfer and insertion into the plant genomes is only capable of expressing a single fluorescent fusion protein (**Figure 1B**)[9,10,12]. Generating a transgenic plant which expresses several chimeric fluorescent proteins simultaneously requires multiple rounds of genetic crossing and screening, which can take from months to years depending on the numbers of the genes to be co-expressed.

The employment a single expression vector for co-expression of multiple proteins in plant has been reported by several previous studies[19,20,21]. However, multiple rounds of enzymatic digestion and DNA ligation of DNA molecules and backbone vectors are usually required for generation of the final plasmid for protein co-expression or over-expression. Here, we have developed a new and robust method for co-expressing multiple

chimeric fluorescent proteins in plants. It is a highly efficient and convenient method that achieves multiple protein co-expression in plants for both transient expression and stable transformation in a time-honored fashion. It employs a single vector that contains multiple functionally independent protein expression cassettes for protein co-expression and thereby overcomes the drawbacks of the conventional methods. Moreover, it is a highly versatile system in which DNA manipulations and assembly are achieved by a simple one-step optimized reaction without extra steps of DNA digestion and ligation. The working principle is illustrated in **Figure 2**. Furthermore, it is fully compatible with current cellular, molecular, and biochemical approaches that are based on chimeric fluorescent fusion proteins.

Protocol

1. Primer Design Strategy and DNA Amplification

1. Design the primers for molecular cloning of DNA fragments. The primers comprise 20 bp gene specific binding sequences and 20 to 25 bp 5'-end overhang sequences, which are the complementary overlapping sequence of adjacent DNA molecules (see **Table 1** for example).
   NOTE: The subsequent assembly of each DNA fragments, linkage of different protein expression cassettes, and integration with the final expression vector all depend on recognition of the adjacent overlapping sequences.
2. Amplify DNA fragments, including promoter, fluorescent reporter, target gene, and terminator, that are necessary for the construction of the semi-independent protein expression cassettes by standard PCR reactions with their corresponding primers and high fidelity polymerase.
   NOTE: The templates used in this study for DNA amplification are derived from previous studies[15,22,23]. The annealing temperature and extension time of the PCR reactions are primer and gene dependent.

2. DNA Fragment Assembly and Construction of Protein Expression Cassettes

1. Examine the quality of the first-round PCR products by DNA electrophoresis and quantify by spectrophotometer. Check whether DNA degradation and contamination have occurred by 1% agarose gel electrophoresis. The OD260/OD280 of the PCR products should be between 1.6 and 1.8.
2. Mix different DNA fragments (0.05 - 0.1 pmol for each fragment) into a new PCR tube to a final volume of 5 µL.
   NOTE: Mix DNA molecules designed for the same protein expression cassette together in one PCR tube. Avoid mixing DNAs from different expression cassette together, since it will reduce the efficiency of DNA assembly due to the increasing numbers of DNA molecules that need to be linked.
   NOTE: The protocol can be paused here.
3. Prepare 5x ISO stock buffer: 500 mM Tris-HCl, pH 7.5; 50 mM $MgCl_2$; 1 mM deoxynucleotide (dNTP); 50 mM dithiothreitol; 25% polyethylene glycol (PEG) 8000; and 5 mM nicotinamide adenine dinucleotide (NAD).
4. Make 1 mL 2x master mixture from 400 µL 5x ISO stock buffer, 7.5 units of T5 exonuclease, 62.5 units of high-fidelity DNA polymerase, 5,000 units of Taq polymerase (see **Table of Materials**), and sterilized double distilled H_2O.
   NOTE: This is adapted from previous studies[24,25,26]; these volumes should be optimized.
5. Aliquot 100 µL of 2x master mixture per tube and store at -20 °C.
   Caution: Frequent freezing and thawing of 2x master mixture can cause low DNA assembly efficiency.
6. Add 15 µL of 2x master mixture to the 5 µL DNA mixture and incubate at 50 °C for 60 min.

3. Construction of the Vector for Co-expression of Multiple Chimeric Fluorescent Fusion Proteins in Plants

1. Amplify the entire semi-independent protein expression cassette by a second-round PCR using the product (0.5 - 1.0 µL) from the first-round isothermal assembly reaction as the template and the outermost primers (*e.g.,* 1-FP35S and 1-RNOS for expression cassette 1).
   1. Use 1 unit of high fidelity polymerase in a 50 µL reaction volume followed by 30 cycles (94 °C for 30 s, 55 °C for 30 s, and 68 °C for 2 min), followed by a final extension at 68 °C for 5 min.
2. Linearize the final protein expression backbone vectors pUC18 and pCAMBIA1300, which are designed for protein transient expression and genetic transformation, respectively, by adding 4 units *Sma* I into a final 10 µL reaction volume and incubating for 1 - 2 hr at 25 °C. Inactivate the restriction enzyme by incubating at 65 °C for 20 min.
3. Mix equimolar DNA molecules of protein expression cassettes and linearized final vector into a final reaction volume of 5 µL. Then, perform the second-round DNA recombination by mixing with 15 µL 2x master buffer and incubating at 50 °C for 60 min.
4. Use the final products of the second-round isothermal recombination reaction to transform competent *E. coli* cells (*e.g.,* DH5α) according to standard procedures[27]. Double-check positive colonies by colony PCR and DNA sequencing. Extract the positive plasmids from the *E. coli* by using a mini plasmid extraction kit and store at -80 °C.
   Caution: For long-term storage, plasmids dissolved in TE buffer or double distilled H_2O are more stable than keeping them in *E. coli* strains.

4. Biolistic-bombardment Mediated Transient Co-expression of Multiple Chimeric Fluorescent Fusion Proteins in Plants

1. Prepare Tobacco BY-2 suspension cells and *Arabidopsis* juvenile plants for bombardment.
   1. Culture BY-2 cells in Murashige and Skoog (MS) medium by subculturing twice per week at 25 °C in a shaker set at 130 rpm. Filter collect 30-mL 3 d cultured BY-2 cells onto a piece of 70-mm autoclaved filter paper *via* a vacuum pump by setting the vacuum pressure to 40 mbar.

2. In order to prevent the cells from drying out during the following steps, add several drops of BY-2 cell liquid cultural medium in a Petri dish before placing the filter paper (next step).
3. Transfer the filter paper with the BY-2 cells on it to a new Petri dish (85 mm x 15 mm).
4. Surface sterilize *Arabidopsis thaliana* (Col-0) seeds by vortexing a mixture with 70% (v/v) ethanol containing 0.05% Tween 20 for 10 min.
5. Spin down the seeds using a bench top centrifuge at max speed for 2 s, remove the supernatant and wash the seeds with 100% ethanol once for 30 s. Pipette out the seeds onto a new sterile filter paper in a sterile hood. Then, air-dry the seeds and spread them onto ½ MS agar plates.
6. Store the plates at 4 °C for 48 hours prior to transferring them into a plant growth chamber with the following settings: 16 h light /8 h dark with 120 - 150 μm m^{-2} s^{-1} intensity, 22 °C.
7. Transfer 7-day old sample plants into a 30 mm diameter circle in the center of a new ½ MS medium plate to increase the efficiency of bombardment.
   Caution: Avoid overlapping the plants when transferring and placing them onto the new ½ MS plate.
8. Add several drops of ½ MS liquid medium on the surface of the plants or tissues to preserve moisture and prevent drying out the plants out during the following steps.

2. Coat gold particles with plasmid DNA.
   1. Vortex gold microcarrier solution thoroughly, for 3 min. Prepare a new 1.5 mL tube.
   2. Sequentially add the following solutions into the tube and vortex: 25 μL (1.5 mg) gold particles, vortex 10 s; 10 μL of 25.46 mg/L spermidine, vortex 10 s; 5 μL of 1μg/μL plasmid DNA, vortex 3 min; 25 μL of 277.5 mg/L $CaCl_2$ solution, vortex 1 min.
      Caution: Keep vortexing during this step.
   3. Spin down the gold microcarriers using a bench top centrifuge at max speed for 5 s and carefully pipette out the supernatant without disturbing the pellet. Wash with 200 μL of absolute ethanol and re-suspend the pellet by vortex for 5 - 10 s. Spin down at max speed for 5 s and remove the ethanol.
   4. Re-suspend the gold particles in 18 μL of absolute ethanol and aliquot 6 μL particles suspension onto the middle of three macrocarriers. Let them air dry.

3. Transfer DNA into plants via particle bombardment.
   1. Set the particle delivery system as following: 1100 psi, 28-mm Hg vacuum, 1-cm gap distance, and 9-cm particle flight distance.
   2. Bombard the cells/plants on the agar medium plate for three times at three different positions and then keep the cells/plants in dark immediately after the bombardment.
      Caution: Wear safety glasses when operating the particle delivery system because of the association of high-pressure gas and high-speed particles with the system.

4. Keep bombarded cells/plants in the dark in the plant growth chamber for 6 to 72 hours prior to observation of fluorescent signals. Set the plant growth chamber to 24 h dark and 22 °C.
   Caution: The expression efficiency and fluorescent signal intensity are promoter-, gene-, and plant/tissue-dependent.

5. Generation of Stable Transgenic Arabidopsis Co-expressing Multiple Chimeric Fluorescent Proteins by *Agrobacterium*-Mediated Transformation.

1. Thaw the *Agrobacterium* competent cells (PMP90) on ice and wait for 30 min, then add 2 μL binary vector (100 - 200 ng) (prepared above) into the competent cells. Sit the mixture on ice for 10 min.
2. Transfer the mixture into a pre-chilled 0.1 cm electroporation cuvette. Insert cuvette into the electroporation system and perform electroporation with following settings: 1.6 kV, 600 ohms, 25 μF.
3. Add 1 mL of SOC liquid medium into the cuvette immediately after the electroporation, pipette the cells into a new 1.5 mL tube, and incubate at 28 °C in a horizontal orbital shaker at 200 rpm for 120 min.
4. Centrifuge the cells at 2,348 x g at room temperature for 5 min, discard the majority of the supernatant, gently re-suspend the pelleted cells with a pipette tip, spread them on a LB plate containing 50 mg/L Hygromycin B, and incubate at 28 °C for 2 - 3 days.
5. Transform *Arabidopsis thaliana* Col-0 plants with the *Agrobacterium*, which contains the binary vector pCAMBIA1300 integrated with multiple protein expression cassettes, by the floral dip[28] method as previously described to generate stable transgenic plants.
6. Sterilize the surface of the transgenic Arabidopsis seeds by mixing them with 70% (v/v) ethanol containing 0.05% Tween 20. Vortex for 10 min. Spin down the seeds using a bench top centrifuge at max speed for 2 s, remove the supernatant, and wash the seeds with 100% ethanol once for 30 s.
7. Pipette out the seeds onto a sterile filter paper in a sterile hood. Thereafter, air dry and spread them onto ½ MS agar plates containing Hygromycin B for screening positive progenies.
8. Incubate the plates at 4 °C for 2 days. Then, transfer them into the plant growth chamber and culture for 7 days.
9. Select 7-day old survival juvenile plants by checking for fluorescent signals under the fluorescent microscope, then transfer the plants into soil for further screening of homozygous plants.

6. Pharmaceutical treatments

1. For pharmaceutical treatments, dilute each drug in liquid MS medium to its appropriate working concentrations before incubation with cells or plants.
   1. Wortmannin treatment: prepare 1 mM stock solutions of wortmannin by dissolving wortmannin powder in DMSO and store the stocks at -20 °C. Transfer plant cells or juvenile plants into the liquid MS medium containing 16.5 μM wortmammin and incubate for 30 - 45 min before imaging.

2. Brefeldin A (BFA) treatment: prepare 1 mM stock solutions of BFA by dissolving BFA powder in DMSO and store the stocks at -20 °C. Transfer plant cells or juvenile plants into the liquid MS medium containing 10 µg/mL BFA for 30 - 45 min before imaging.

7. Confocal Microscope Imaging and Protein Subcellular Co-localization Analysis

1. Transfer the juvenile plants or suspension cells onto a conventional glass slide and gently put a cover slide on the top for imaging by standard confocal laser scanning microscopy. Use the following settings: 63X water objective (N.A 1.4), 0 background, 700 gain, 0.168 mm pixel size, and photomultiplier tube detector. Excite GFP-tagged proteins at 485 nm and detect fluorescence at 525 nm. For RFP-tagged proteins, excite at 514 nm and detect at 575 nm.
2. Calculate the co-localization ratio of fluorescent signals using Image J software (https://imagej.nih.gov/ij/) with the Pearson-Spearman correlation (PSC) co-localization plug-in as previously described[8]. Pearson correlation coefficients or Spearman's rank correlation coefficients are shown in the results (**Figure 4**). The produced r values will be from -1 to 1. 0 demonstrates no detectable correlation of two signals, whereas +1 and -1 show full positive and negative correlation, respectively, of two signals.

Representative Results

We have developed a robust and highly efficient method for the co-expression of multiple chimeric fluorescent fusion proteins in plants. It breaks through the barriers of the conventional approaches use multiple separated plasmids for protein co-expression, as shown in **Figure 1A,B,** *via* either transient expression or stable genetic transformation. In this new method, we generate a single expression vector that is composed of multiple protein expression cassettes to achieve protein co-expression at one time (**Figure 1C,D**). The protein expression cassette functions semi-independently with its own necessary DNA elements for protein expression. Therefore, each protein expression cassette could be customized independently according to diverse requirements for protein expression. As for the final protein co-expression vector, the protein expression cassettes in it function as basic "Lego" elements which can be modified, re-constructed. and re-placed conveniently. Furthermore, an alternative strategy for DNA molecule assembly, linkage of several protein expression cassettes, and integration of DNA fragments with the final destination vector for co-expression of chimeric fluorescent fusion proteins is simply achieved *via* an optimized isothermal *in vitro* recombination reaction without further extra steps of DNA digestion and interlinkage. The working principle of the isothermal *in vitro* recombination reaction is illustrated in **Figure 2**. The linkage of multiple DNA molecules (*e.g.,* the three representative DNA fragments 1 - 3 shown in **Figure 2**) and their integration with the final expression vector are simply and efficiently achieved by the one-step reaction (see **Figure 2**). It is adapted from previous studies by overlapping recombination of DNA molecules mediated with overlapping short sequences to achieve fusion of DNA fragments and construction of plasmids[25,26].

As a test of the method, we chose the vacuolar sorting receptor (VSR) and the secretory carrier membrane protein (SCAMP), which are two reporter proteins participating in protein secretory and endocytosis pathways, respectively[6,22,23,29,30]. VSRs are type-I integral membrane proteins that mediate biosynthetic protein traffic in the secretory pathway to the vacuole and mainly localizes in prevacuolar compartments (PVCs) in plants[6,22,23]. In contrast, secretory carrier membrane proteins (SCAMPs) are type-IV membrane proteins that participate in the plant endocytic pathway. It localizes to the plasma membrane (PM) and *trans*-Golgi networks (TGNs), which serve as early endosomes[22,29,30]. We constructed two protein expression cassettes that host the chimeric fusions of *Arabidopsis* VSR2 (AtVSR2) with RFP and *Arabidopsis* SCAMP4 (AtSCAMP4) with GFP, as shown in **Figure 3**. To ensure RFP-AtVSR2 can be translated into the ER, a signal peptide (SP) is added before RFP, as previously reported[6,31]. The two individual protein expression cassettes are further interlinked and ligate with the final protein expression vector pUC18 or pCAMBIA1300 for protein co-expression either *via* protein transient or plant stable transformation, as shown in **Figure 3**. AtVSR2 and AtSCAMP4 were successfully co-expressed in tobacco BY-2 cells *via* particle bombardment and showed correct localizations (**Figure 4A**). RFP-AtVSR2 showed a punctate pattern, which was distinct from the plasma membrane localization of AtSCAMP4-GFP with some cytosolic punctate dots. Moreover, *Arabidopsis* transgenic plants that co-express AtSCAMP4-GFP and RFP-AtVSR2 were generated *via Agrobacterium*-mediated transformation. The subcellular localizations of co-expressing RFP-AtVSR2 and AtSCAMP4-GFP in root and root hair cells were shown in **Figure 4B and 4E**. The co-expression results of RFP-AtVSR2 and AtSCAMP4-GFP obtained from transgenic *Arabidopsis* were in agreement with the ones from BY-2 cells. In addition, the transgenic *Arabidopsis* were treated with wortmannin and BFA for 30 min. Wortmmanin caused RFP-AtVSR2 labeled PVCs forming a small ring-like structure, and BFA induced AtSCAMP-GFP labeled TGN aggregation, as shown in **Figure 4C,D,F,G**. Additionally, little autofluorescent signal can be detected in tobacco BY-2 cells and Arabidopsis root and root hair cells by applying the same settings of image collection as for **Figure 4** (**Supplemental Figure 1**).

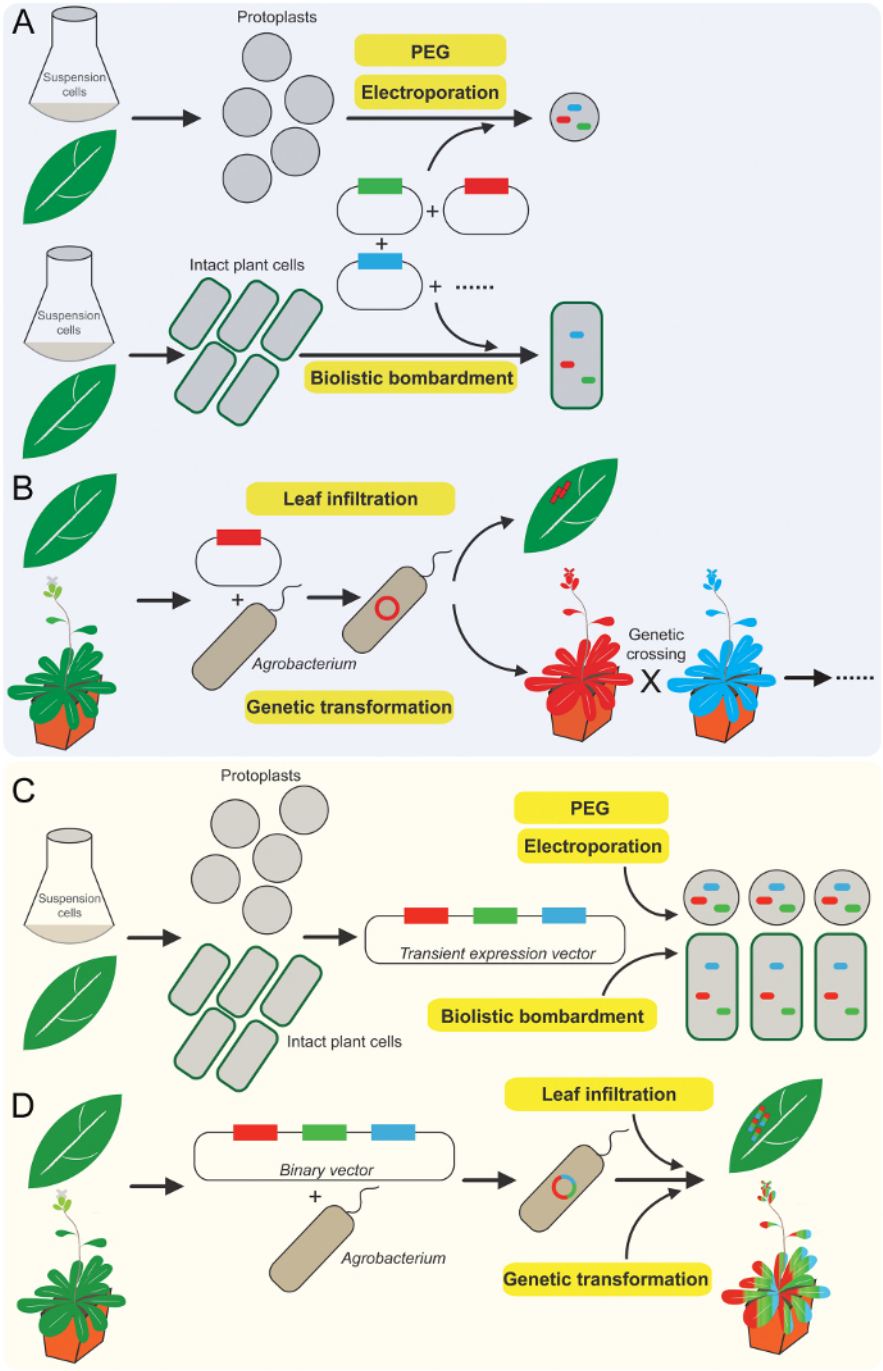

Figure 1: A robust system for co-expression of multiple chimeric fluorescent fusion proteins in plants. (A) Conventional approaches of transient co-expression of multiple fluorescent reporter proteins in plants achieved *via* electroporation, particle bombardment, and PEG-mediated transformation by mixing several independent expression vectors. **(B)** The conventional method for plant genetic transformation by *Agrobacterium* with a single fluorescent fusion protein expression vector. To co-express multiple chimeric fluorescent fusion proteins in a transgenic plant, further genetic crossing and multiple rounds of screening are required for obtaining homozygous progenies. **(C)** and **(D)** The alternative new protein co-expression method. It takes advantage of a single expression vector, which is composed of multiple protein expression cassettes and is able to co-express several chimeric fluorescent reporter proteins in plants *via* both transient expression and genetic transformation. Details of this figure were first published in Zhong *et al.* 2017[36] (reprinted with permission; copyright Frontiers in Plant Science). Please click here to view a larger version of this figure.

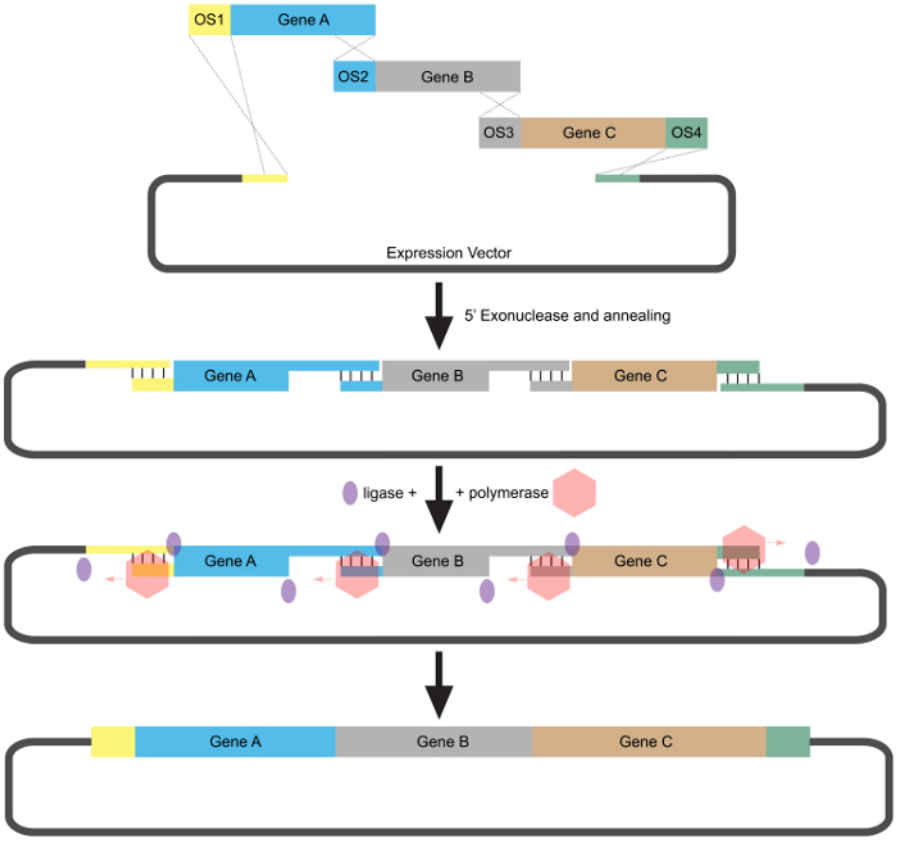

Figure 2: Demonstration of the working principle of one-step DNA assembly method by isothermal recombination reaction. The DNA fragments and linearized plasmid with overlapping sequences (OSs) are attached by base paring between the 5'-overhang overlapping regions, extending by DNA polymerase, and linking by ligase. Please click here to view a larger version of this figure.

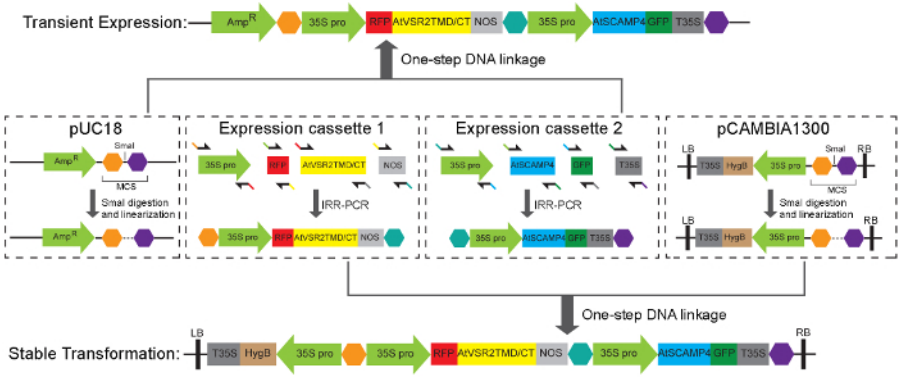

Figure 3: The strategy of constructing a single plasmid for co-expressing multiple chimeric fluorescent fusion proteins. The diagram demonstrates the strategy for constructing a single expression plasmid for the co-expression of multiple chimeric fluorescent fusion proteins either for transient expression or stable transformation in plants. The expression vector is composed of two protein expression cassettes, each of which contains its own necessary elements for protein expression, and functions in expressing its individual chimeric fluorescent fusion protein semi-independently. Assembly and Interlinkage of all the DNA molecules are conveniently achieved by an optimized isothermal *in vitro* recombination method mediated with the overlapping DNA fragments. Details of this figure were first published in Zhong *et al.* 2017[36] (reprinted with permission; copyright Frontiers in Plant Science). Please click here to view a larger version of this figure.

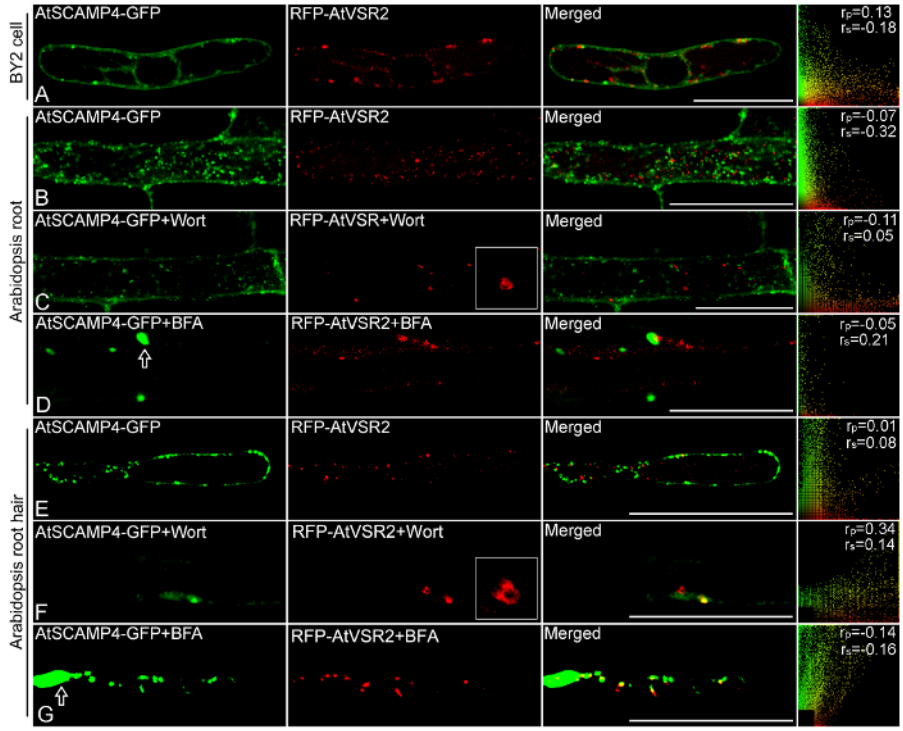

Figure 4: Representative images of co-expression of chimeric fluorescent fusion of VSR and SCAMP in plant cells.
(A) Co-expression of RFP-AtVSR2 and AtSCAMP4-GFP via particle bombardment in tobacco BY-2 suspension cells. **(B)** A representative image of a transgenic *Arabidopsis* root cell co-expressing RFP-AtVSR2 and AtSCAMP4-GFP. **(C)** and **(D)** Transgenic *Arabidopsis* roots co-expressing RFP-AtVSR2 and AtSCAMP4-GFP were treated with wortmannin and BFA for 30 min respectively. **(E)** A representative image of a transgenic *Arabidopsis* root hair co-expressing RFP-AtVSR2 and AtSCAMP4-GFP. **(F)** and **(G)** Transgenic *Arabidopsis* root hairs co-expressing RFP-AtVSR2 and AtSCAMP4-GFP were treated with wortmannin and BFA for 30 min. Arrows in **(D)** and **(G)** indicate BFA-induced protein aggregation. r_p = Pearson correlation coefficient; r_s = Spearman's rank correlation. Scale bar in **(A)**-**(D)** is 50 µm, **(E)**-**(G)** is 30 µm. Details of this figure were first published in Zhong *et al.* 2017[36] (reprinted with permission; copyright Frontiers in Plant Science). Please click here to view a larger version of this figure.

Expression cassette 1	
1-FP35S	GAATTCGAGCTCGGTACCCACATGGTGGAGCACGACACA
1-RP35S	AGGACGCGGGCGTGGGCCATTATCACATCAATCCACTTGC
1-FRFP	GCAAGTGGATTGATGTGATAATGGCCCACGCCCGCGTCCT
1-RRFP	ATTATCCATATCACTCCCCAGGCGCCGGTGGAGTGGCGGC
1-FAtVSR2	GCCGCCACTCCACCGGCGCCTGGGGAGTGATATGGATAAT
1-RAtVSR2	AAATGTTTGAACGATCGGGATTACAACTCTAGTTGAGAAG
1-FNOS	CTTCTCAACTAGAGTTGTAATCCCGATCGTTCAAACATTT
1-RNOS	GAGAATGGATGCGAGTAATGTCTAGTAACATAGATGACAC
Expression cassette 2	
2-FP35S	CATTACTCGCATCCATTCTCACATGGTGGAGCACGACACA
2-RP35S	TTAGGATCGTGTCGTGCCATTATCACATCAATCCACTTGC
2-FAtSCAMP4	GCAAGTGGATTGATGTGATAATGGCACGACACGATCCTAA
2-RAtSCAMP4	TCCTCGCCCTTGCTCACCATTAGTGCACGCATCAAGGTCG
2-FGFP	CGACCTTGATGCGTGCACTAATGGTGAGCAAGGGCGAGGA
2-RGFP	TAAAACCAAAATCCAGTGACTTACTTGTACAGCTCGTCCA
2-FT35S	TGGACGAGCTGTACAAGTAAGTCACTGGATTTTGGTTTTA
2-RT35S	GTCGACTCTAGAGGATCCCCGTCCGCAAAAATCACCAGTC

Table 1: Primer design strategy and sequences used in this study. Name of each primer is given on the left panel. The complementary overlapping sequences of the primers are underlined. Details of this table were first published in Zhong *et al.* 2017[36] (reprinted with permission; copyright Frontiers in Plant Science).

Supplemental Figure 1: Representative images of autofluorescence in BY-2 cells and Arabidopsis roots and root hairs. (A) The autofluorescence of wild type BY-2 cells was detected under the same imaging conditions with the transformed cells. **(B)** and **(C)** Arabidopsis root hair and root cells were detected under the same imaging conditions with the transformed Arabidopsis cells. Scale bar in **(A)**-**(C)** is 20 μm. DIC, differential interference contrast. Please click here to download this file.

Discussion

Here we have demonstrated a novel method to robustly co-express chimeric fluorescent fusion proteins in plants. It can be used for both transient expression and genetic transformation and is compatible with current fluorescent protein-based bio-imaging, molecular, and biochemical applications and technologies[9,10,13]. In addition, it overcomes the difficulties of the conventional methods that use several individual expression plasmids for the protein co-expression. In contrast, it employs a single expression vector that contains multiple protein expression cassettes with their own individual promoters, fluorescent tags, target proteins, and terminators. Moreover, the protein expression cassette can be managed independently to meet diverse expression requirements, such as usage of a specific promoter and the N- or C-terminal chimeric fusion of a fluorescent protein with the target protein. Therefore, the protein expression cassette functions like a basic "Lego" element that works semi-independently in the plasmid. Furthermore, it is also a highly versatile system in which gene editing, replacement, and assembly all can be easily achieved by a one-step isothermal recombination reaction without extra processes of enzyme digestion and ligation. We have optimized the efficiency of isothermal recombination reaction from previous studies, as described in step 2.4, by testing different concentrations of T5 exonuclease, Phusion DNA polymerase, and Taq polymerase. In addition, the concentration of each DNA fragments for the one-step isothermal recombination reaction is suggested to be between 0.05 and 0.1 pmol to achieve maximum ligation efficiency.

Over-expression of a transgene by replacing its endogenous promoter with a strong and continuous promoter, such as ubiquitin-10 promoter (UBQ10), and introducing additional copies of the gene is a wildly used approach to study its cellular functions and underlying working mechanism in cells[15,32]. However, unexpected down-regulation and strong inhibition of the gene expression sometimes was found as well[33,34]. The percentage of unpredictable gene silencing ranges from 2 to 100% under these situations[33,35]. Moreover, gene silencing has a higher chance to happen in expression of several genes simultaneously, transformation of high copies of DNA, and significant increases of gene transcriptional level[9,33,24,35]. In order to minimize the occurrence of gene silencing in the robust multiple fusion protein co-expression system, we chose different active promoters to drive the different protein expression cassettes when co-expressing multiple fusion proteins. Moreover, we avoided continually using the same promoter for different expression cassettes. In addition, another potential limitation of this protocol is the reduced efficiency of one-step DNA isothermal recombination caused by the increasing numbers of protein expression cassette for co-expression. Furthermore, the number of the expression cassettes that can be hosted in the final expression plasmid mainly relies on the replicon of the backbone plasmid[24,25,35].

Taken together, we have developed a powerful system for co-expressing multiple chimeric fluorescent fusion proteins conveniently in plants[36]. It overcomes the limitations of the classical methods and utilizes an optimized one-step DNA assembly reaction for DNA interlinkage and plasmid construction in a time-honored fashion. This technical advance has been validated by AtVSR2 and AtSCAMP4 co-expression in plant cells *via* both transient expression and genetic transformation. Therefore, it demonstrates a convincing and novel method for different aspects of scientific discoveries by co-expression of chimeric fluorescent fusion proteins in plants. Additionally, the concept and principle of co-expression of multiple

chimeric fluorescent fusion proteins via a single expression vector are fully compatible with CRISPR-Cas9, RNAi, and protein over-expression technologies to study the functions and interactions of multiple genes in plants[37,38,39].

Disclosures

The authors have nothing to disclose.

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