Fluorescent Tagging of Full-Length Genes

I. Fluorescent tags: Citrine-YFP and CFP

We routinely tag proteins with the Citrine variant of Yellow Fluorescent Protein (YFP) (Griesbeck et al. 2001), which can be used not only to visualize a single protein but also to study protein-protein interactions *in vivo* as an energy acceptor in BRET (Xu et al. 1999) and FRET assays (Tsien et al. 1998; Pollok et al. 1999). Moreover, Citrine-YFP has enhanced photostability and is much less sensitive to pH and anions, such as chloride, compared to other YFP variants (Griesbeck et al. 2001). The reduced sensitivity to pH allows detection of proteins targeted to the extracellular matrix or to other relatively acidic subcellular compartments, thus making this reporter more suitable for tagging proteins with a wide range of targeting specificities. Some proteins are also tagged with Cyan Fluorescent Protein (CFP) (ECFP, Clontech) for comparison of localization patterns obtained with different tags and for future colocalization and interaction studies.

For tagging, Citrine-YFP/CFP coding sequences, which lack start and stop codons, are flanked by linker peptides that function as flexible tethers, minimizing potential folding interference between Citrine-YFP/CFP and the tagged protein (Doyle et al. 1996). To avoid placing identical nucleotide sequences on each side of the tag, we use two different linkers: the N-terminus of the tag is linked to a glycine-rich linker peptide $(Gly)_5Ala$, and the C-terminus is linked to an alanine-rich linker peptide $AlaGly(Ala)_5GlyAla$.

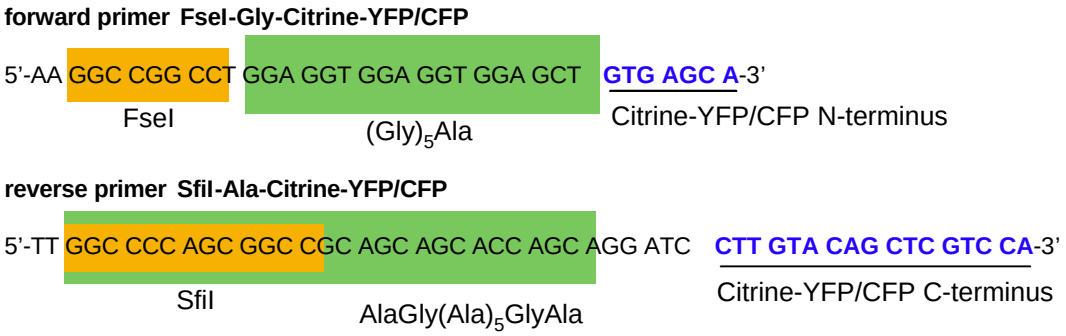

Figure 1. Forward and reverse primers for adding flanking linkers and restriction sites to citrine-YFP/CFP. Orange boxes indicate the FseI and SfiI sites in the forward and reverse primers, respectively. Green boxes indicate the ($Gly)_5Ala$ and $AlaGly(Ala)_5GlyAla$ linkers in the forward and reverse primers, respectively. N-terminal and C-terminal sequences of Citrine-YFP/CFP contained in the forward and reverse primers, respectively, are indicated in blue.

The Citrine-YFP tag is amplified from the $pRSET_B$-Citrine plasmid (Griesbeck et al. 2001) and the CFP tag from the pECFP-C1 plasmid (Clontech) using the ExTaq DNA polymerase (TaKaRa) and two primers shown in Figure 1. The products are cloned into the pTOPO TA vector (Invitrogen). The resulting cDNAs encoding the fluorescent tags contain recognition sequences for FseI and SfiI restriction endonucleases at their 5'- and 3'-ends, respectively. Plasmid with Citrine-YFP is designated as pCitrine-3 and plasmid with CFP as pCFP-3 (Figure 2).

pCitrine-3

TAA GGC CGG CCT GGA GGT GGA GGT GGA GCT **GTG AGC AAG GGC GAG**
GAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGC
CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACC
CTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCA
CCTTCGGCTACGGCCTGATGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGA
CTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAG
GACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTG
AACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCAC
AAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGA
ACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGC
TCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCG
ACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCG
ATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATG GAC
GAG CTG TAC AAG GAT CCT GCT GGT GCT GCT GCG GCC GCT GGG GCC AAA AGG

pCFP-3

TAA GGC CGG CCT GGA GGT GGA GGT GGA GCT **GTG AGC AAG GGC GAG**
GAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGC
CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACC
CTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCA
CCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACG
ACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA
GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGT
GAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCA
CAAGCTGGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAAG
AACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAG
CTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCC
GACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGC
GATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATG GAC
GAG CTG TAC AAG GAT CCT GCT GGT GCT GCT GCG GCC GCT GGG GCC AAA AGG

Figure 2. Nucleotide sequence of the Citrine-YFP and CFP tags contained in pCitrine-3 and pCFP plasmids, respectively. Citrine-YFP/CFP sequences are indicated in blue. Yellow boxes indicate forward and reverse primers for adding flanking linkers and restriction sites to Citrine-YFP/CFP (see Figure 1). Thick brown lines indicate forward and reverse primers used to amplify Citrine-YFP/CFP for TT-PCR (see Figure 3).

Next, the fluorescent tag cDNA sequences are amplified from the pCitrine-3 and pCFP-3 plasmids using the Pfu-turbo DNA polymerase (Invitrogen) and the forward and reverse Citrine-YFP/CFP primers (Figure 3) to produce the "TT-Citrine" and "TT-CFP" fragments.

PCR reaction mixture	PCR cycles	
100 ng DNA template	1 cycle:	
1x Pfu-turbo reaction buffer	94°C	3 min
0.2 mM 4 x dNTP	30 cycles:	
0.2 µM of each primer	94°C	30 sec
0.025 U/µl Pfu-turbo (Invitrogen)	70°C	2 min
total volume: 25 µl	1 cycle:	
	70°C	2 min

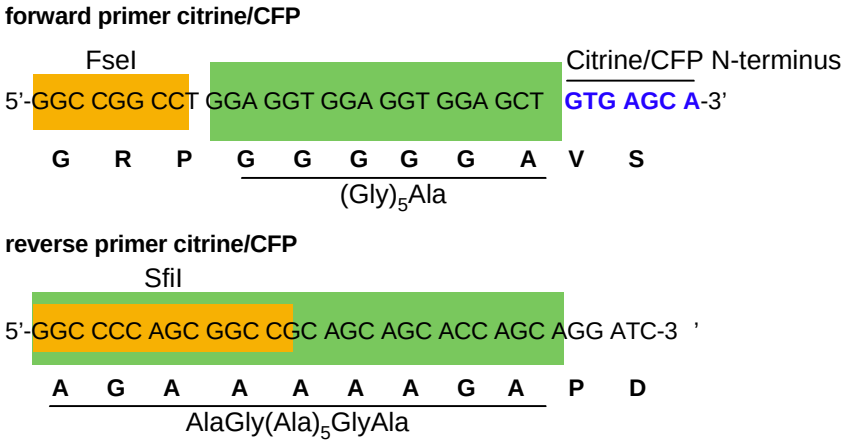

Figure 3. Forward and reverse primers for amplifying Citrine-YFP/CFP tags to use in TT-PCR. Orange boxes indicate the FseI and SfiI sites in the forward and reverse primers, respectively. Green boxes indicate the $(Gly)_5Ala$ and $AlaGly(Ala)_5GlyAla$ linkers in the forward and reverse primers, respectively. The N-terminal sequence of Citrine-YFP/CFP contained in the forward primer is indicated in blue.

The PCR products are gel-purified using the GFX PCR purification kit (Amersham) or PCR Purification Kit (Qiagen) to remove dNTPs, primers and enzyme, and used in TT-PCR (see below).

II. Gene tagging

The entire protocol is summarized in Figure 4 and described in detail below.

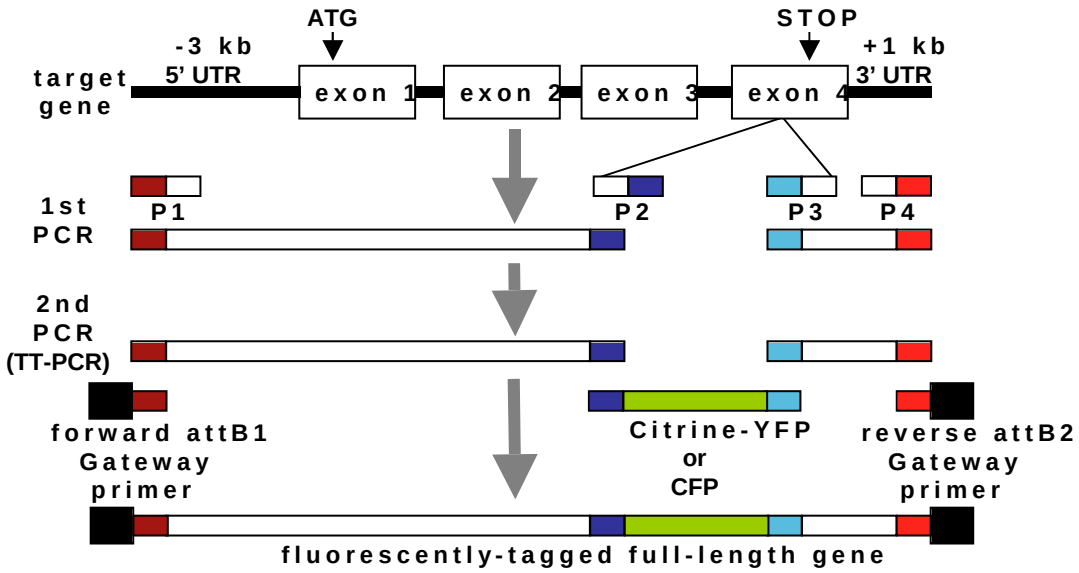

Figure 4. Flowchart for the gene tagging protocol. White boxes represent gene-specific sequences, dark and light red boxes represent P1 and P2 primer sequences overlapping the forward attB1 and reverse attB2 Gateway primers, respectively, and dark and light blue boxes represent P2 and P3 primer sequences overlapping the fluorescent tag primers (see Figure 5).

1. First PCR reaction

a. Genomic DNA template

Genomic DNA is extracted from leaf material of 6-week-old *A. thaliana* ecotype Columbia plants using the DNeasy® Plant Mini Kit (Qiagen) according the manufacturer's instructions.

b. Primers

Two sets of primers (P1/P2, P3/P4) for each gene are designed for the amplification of two genomic fragments using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Our PCR design program considers a series of criteria including the position of each primer within the genomic sequence, annealing temperature, length, and hairpin structures in an iterative fashion to determine the most suitable sets of P1/P2 and P3/P4 for each gene.

The first set of primers amplifies a fragment (P1-P2) that extends from up to 3 kb upstream of the transcription start of the gene to the tag insertion site within the coding sequence. We believe that most *Arabidopsis* promoters should be contained within 3 kb. However, some intergenic regions are <3 kb; thus, we defined a minimal size for the 5' UTR and promoter region as 1 kb, extending P1 into the upstream ORF if the intergenic region is very small.

The second set of primers amplifies a fragment (P3-P4) from the tag insertion site to 0.5-1 kb downstream of the gene to include 3' UTR and regulatory sequences. The default position for the start of the gene-specific region of P2 and P3 primers is at the 30th nucleotide (i.e. 10 amino acids) upstream of the stop codon. However, if a functional domain is predicted at this position, or it does not generate a suitable primer sequence, the positions of P2 and P3 are reiteratively shifted from the initial site until suitable priming sites are determined.

P1 and P4 contain, in addition to gene-specific sequences, sequences partially overlapping the attB1 and attB2 Gateway forward and reverse primers, respectively (used for TT-PCR, see below). P2 and P3 contain sequences partially overlapping the Citrine-YFP/CFP primers (Figure 5).

P1 primer: 5'-**GCTCGATCCACCTAGGCT+18-25 gene-specific nucleotides** -3'

P2 primer: 5'-**CACAGCTCCACCTCCACCTCCAGGCCGGCC+18-25 gene-specific nucleotides** -3'

P3 primer: 5'-**TGCTGGTGCTGCTGCGGCCGCTGGGGCC+18-25 gene-specific nucleotides** -3'

P4 primer: 5'-**CGTAGCGAGACCACAGGA+18-25 gene-specific nucleotides** -3'

Figure 5. Nucleotide sequences of P1, P2, P3, and P4 primers. Gene-non-specific sequences overlapping forward attB1 and reverse attB2 Gateway primers are indicated in dark red, and gene-non-specific sequences overlapping the Citrine-YFP/CFP primers are indicated in blue.

c. PCR conditions

Because all primers in the TT-PCR reaction (P1-P4) have gene-specific sequences, it is impossible to calculate a standard annealing temperature for all genes to be tagged. Instead, we use touch-down PCR conditions to include a range of temperatures as shown below.

PCR reaction mixture	PCR cycles
100 ng DNA template	1 cycle:
1x ExTaq reaction buffer	95°C 2 min 30 sec
0.2 mM 4 x dNTP	7 cycles touch-down:
0.2 µM of each primer	94°C 30 sec
0.025 U/µl ExTaq (TaKaRa)	64°C 30 sec; reduce t°C by 1°C per cycle
total volume: 20 µl	68°C 1 min per kb
	23 cycles:
	94°C 30 sec
	58°C 30 sec
	68°C 1 min per kb
	1 cycle:
	68°C 10 min

The PCR products are gel-purified using the GFX PCR purification kit (Amersham).

d. Polishing reaction to remove A-overhangs from ExTaq-generated fragments

PCR reaction mixture (no primers)	PCR cycle
25 µl DNA fragment	1 cycle: 72°C 30 min
1x Pfu reaction buffer	
0.2 mM 4 x dNTP	
0.01 U/µl Pfu (Invitrogen)	
total volume: 50 µl	

2. Triple template PCR (TT-PCR)

All three amplified fragments, i.e., TT-Citrine or TT-CFP, P1-P2, and P3-P4, are combined together to serve as three overlapping templates for Long Flanking Homology (LFH) PCR (Wach 1996). This second PCR reaction, designated triple-template PCR (TT-PCR), utilizes two primers containing the complete attB1 and attB2 Gateway sequences (Walhout et al. 2000) and partially overlapping the P1 and P4 primers (Figure 4). Thus, TT-PCR introduces the fluorescent tag into the selected site within the target gene without the need for conventional cloning and results in an internally-tagged full-length gene sequence flanked by attB1 and attB2 sites ready for Gateway recombination cloning.

a. Three templates

P1-P2 fragment, TT-Citrine or TT-CFP fragment, and P3-P4 fragment.

b. Primers

Universal, gene-non-specific primers carrying the Gateway attB1 and attB2 sequences that overlap with the gene-non-specific sequences of P1 and P4 primers (Figure 6).

Figure 6. Nucleotide sequences of forward attB1 and reverse attB2 Gateway primers. Blue boxes indicate attB1 and attB2 sequences. Sequences of forward and reverse primers overlapping P1 and P4 primers, respectively, are indicated in dark red.

c. TT-PCR conditions

TT-PCR reaction mixture	TT-PCR cycles
100 ng P1-P2 fragment+50ng P3-P4 fragment+50ng TT-Citrine or TT-CFP	1 cycle: 94°C 2 min 30 sec 20 cycles: 94°C 30 sec 62-65°C 30 sec* 68°C 1 min per kb 1 cycle: 68°C 10 min
1x ExTaq reaction buffer	
0.2 mM 4 x dNTP	
0.2 µM of each primer	
0.02 U/µl ExTaq (TaKaRa)	
total volume: 20 µl	

*** lowering temperature of this step to 54°C may improve the recombination of the resulting TT-PCR product into pDONR207 by 2-3 fold.**

The PCR products are gel-purified using the GFX PCR purification kit (Amersham).

3. Gateway cloning of TT-PCR products into pDONR207

The Gateway system (Invitrogen) is based on bacteriophage λ site-specific recombination (Landy 1989). Gateway cloning introduces the amplified TT-PCR product into the donor vector, pDONR207 (Invitrogen), by *in vitro* recombination between the attB1 and attB2 sequences that flank the TT-PCR product (see above) and the attP1 and attP2 sequences, respectively, of pDONR207. This attB x attP recombination is mediated by the BP reaction (Invitrogen) and produces the attL1 and attL2 sequences that flank the tagged gene within the pDONR vector.

Note that unrecombined pDONR vectors should be propagated in the DB3.1 strain of *E. coli* (Invitrogen) carrying the *gyrA462* gene which confers resistance to the *ccd*B gene [its protein product, a natural analog of quinolone antibiotics, binds to the DNA gyrase subunit A and turns it into a poison (Bahassi et al. 1999)]. Following Gateway recombination, *ccdB* is replaced by the TT-PCR product, allowing selection for the recombinant clones in bacterial strains, such as DH5α or DH10B, that do not carry *gyrA462* or F' episome (which also confers resistance to *ccd*B).

a. BP reaction and selection for recombinant clones

BP reaction mixture	BP reaction conditions
300 ng (in 1-5 µl) TT-PCR product	overnight incubation at 25ºC
150 ng (in 1 µl) pDONR207 (Invitrogen)*	***Note that unrecombined pDONR207 is toxic to most bacterial strains and should be propagated in the DB3.1 strain of *E. coli* (Invitrogen) in the presence of chloramphenicol and gentamycin**
2 µl 5x BP Clonase reaction buffer	
2 µl BP Clonase (Invitrogen)	
TE buffer (pH 8.0) to total volume of 10 µl	

Add 1 µl Proteinase K (2 µg/µl) and incubate for 10 minutes at 37ºC. Then, transform 2µl of the reaction mixture into 100 µl competent cells of the *E. coli* strain DH5α or DH10B and select for recombinants by plating on LB agar supplemented with 7 µg/ml gentamycin.

b. Identification of recombinant colonies with TT-PCR product

Pick 4 colonies per construct and analyze each by PCR for the presence of the TT-PCR product. Use the either of the following attL primers in combination with one gene specific primer.

forward attL1 primer: 5'-TCGCGTTAACGCTAGCATGGATCTC-3'
reverse attL2 primer: 5'-GTAACATCAGAGATTTTGAGACAC-3'

PCR reaction mixture	PCR cycles	
1 bacterial colony	1 cycle:	
1x Taq reaction buffer	94°C	3 min
0.2 mM 4 x dNTP	25 cycles:	
0.2 µM of each primer	93°C	30 sec
total volume: 20 µl	50°C	30 sec
incubate 10 min at 95°C to release DNA	68°C	1 min per kb
0.02 U/µl Taq (any brand)	1 cycle:	
	72°C	1 min per kb

Select positive clones, i.e., those that have the correct size insert, purify their plasmid DNA and sequence the tagged genes. *In our experiments, the efficiency of the recombination of the TT-PCR products into pDONR207 is 80-90%.*

4. Gateway transfer of the tagged genes into binary destination vectors

a. Gateway binary destination vectors

The binary destination vector was constructed by subcloning the Gateway conversion cassette C.1 (Invitrogen) into the filled-in EcoRI-HindIII sites of the promoterless T-DNA region of pBIN19. The resulting Gateway destination vector, designated pBIN-GW, has the following structure in its T-DNA region: T-DNA right border-NOS terminator<-NPTII<-35S promoter-attR1->CAT->*ccd*B->attR2-T-DNA left border. This vector has no regulatory sequences for expression of cloned genes and, thus, is useful for producing native levels and patterns of gene expression.

Using a similar strategy, the pMN20 activation tagging plasmid (Weigel et al. 2000) was converted to a Gateway vector by subcloning the Gateway conversion cassette C.1 into the filled-in HindIII site of the T-DNA region of pMN20. The resulting plasmid, pMN-GW, has the following structure of its T-DNA region: T-DNA right border-$(35S\ enhancer)_4$-attR1->CAT->*ccd*B->attR2-35S promoter-NPTII-NOS terminator-T-DNA left border. This vector has tetramerized CaMV 35S enhancers in its T-DNA region (Weigel et al. 2000) and, thus, is useful for producing elevated levels of gene expression while retaining native expression patterns.

Note that pMN20-based vectors should be prepared from fresh bacterial stocks and used immediately after transferring them to *Agrobacterium* because they tend to lose some copies of their 35S enhancers due to recombination in *E. coli* or *Agrobacterium* when stored at 4°C (Weigel et al. 2000). Also note that unrecombined destination vectors should be propagated in the *ccd*B-resistant DB3.1 strain of *E. coli* (Invitrogen) whereas, following Gateway recombination, the recombinant clones should be propagated in the *ccd*B-sensitive bacterial strains such as DH5α or DH10B (see description of pDONR207 above for more details).

b. LR reaction and selection and identification of recombinant clones

The tagged gene is transferred to the binary destination vector by *in vitro* recombination between the attL1 and attL2 sequences that flank the TT-PCR product in the pDONR vector (see above) and the attR1 and attR2 sequences, respectively, of the destination vector (Landy 1989, see also www.invitrogen.com). This attL x attR recombination is mediated by the LR reaction (Invitrogen) and produces the attB1 and attB2 sequences that flank the tagged gene within the binary vector.

LR reaction mixture	LR reaction conditions
200 ng pDONR construct	overnight incubation at 25ºC
200 ng 1:1 w/w mixture of pBIN-GW and pMN-GW*	***Note that unrecombined destination vectors are toxic to most bacterial strains and should be propagated in the DB3.1 strain of *E. coli* (Invitrogen) in the presence of chloramphenicol and kanamycin (pBIN-GW) or spectinomycin (pMN-GW)**
0.5 µl topoisomerase I (10 U/µl)	
2 µl 5x LR Clonase reaction buffer	
2 µl LR Clonase (Invitrogen)	
TE buffer (pH 8.0) to total volume of 10 µl	

Add 1 µl Proteinase K (2 µg/µl) and incubate for 10 minutes at 37ºC. Then, transform 2µl of the reaction mixture into 100 µl competent cells of the *E. coli* strain DH5α or DH10B and plate one half of the transformation mixture on LB agar supplemented with 50 µg/ml kanamycin to select for pBIN-GW recombinants and the other half — on LB agar supplemented with 100 µg/ml spectinomycin to select for pMN-GW recombinants.

Pick 2 colonies per construct and analyze each by PCR, using P1 and P4 primers, for the presence of the TT-PCR product. *In our experiments, the efficiency of the recombination of the TT-PCR products from pDONR into the binary destination vector is 90-100%.*

III. Production of transgenic *Arabidopsis* expressing the tagged genes

1. Introduction of binary constructs into *Agrobacterium*

(i) Grow *Agrobacterium tumefaciens* strain GV3101 containing the pMP90 helper plasmid (carrying gentamycin resistance) in 5 ml of LB medium overnight at 28°C.

(ii) Add 2 ml of the overnight culture to 50 ml LB medium in a 250-ml flask and shake vigorously (250 rpm) at 28°C until the culture grows to an OD_{600} of 0.5 to 1.0 (about 4-6 hrs).

(iii) Chill the culture on ice. Centrifuge the cell suspension at 3000xg for 5 min at 4°C.

(iv) Discard the supernatant solution. Resuspend the cells in 1 ml of 20 mM $CaCl_2$ solution (ice-cold). Dispense 0.1-ml aliquots into prechilled Eppendorf test tubes.

(v) Add about 3-5 µg of plasmid DNA to the cells.

(vi) Freeze the cells in liquid nitrogen.

(vii) Thaw the cells by incubating the test tube in a 37°C water bath for 5 min.

(viii) Add 1-ml of LB medium to the tube and incubate at 28°C for 2-4 hrs with gentle shaking. This period allows the bacteria to express the antibiotic resistance genes.

(ix) Centrifuge the tubes for 30 sec in an Eppendorf microfuge. Discard the supernatant solution. Resuspend the cells in 0.1 ml LB medium per tube.

(x) Spread the cells on an LB agar plate containing 50 µg/ml kanamycin and 50 µg/ml gentamycin (for pBIN-GW constructs) or 100 µg/ml spectinomycin and 50 µg/ml gentamycin (for pMN-GW constructs). Incubate the plate at 28°C. Transformed colonies should appear in 2-3 days.

Note: After step (vi), the cells frozen in liquid nitrogen can be stored at -80°C. The frozen cells can be used for future transformation experiments. Add about 3-5 µg of DNA to the frozen cells and follow the steps (vii) to (x).

2. *Agrobacterium*-mediated transformation of *Arabidopsis*

A. thaliana ecotype Columbia is genetically transformed with *Agrobacterium* using the standard flower dip method (Clough et al. 1998) or its modified version (Kim et al. 2003).

(i) Plant 3-6 *Arabidopsis* seeds/pot in 6x6x6 cm pots. Let them grow for 5-6 weeks.

(ii) For pre-culture, inoculate *Agrobacterium* colonies/glycerol stock into 2 ml LB medium with appropriate antibiotics. Grow overnight at 28°C.

(iii) Next day, around 5 pm, inoculate 0.4-1.0 ml of the overnight pre-culture into 200 ml YEP containing appropriate antibiotics. Dipping can be done on the next day between 10 am and 4 pm, depending on the growth of the *Agrobacterium* culture. For example, if you inoculated 1 ml of the pre-culture, you can do the dipping in the morning.

(iv) To the 200 ml culture add 40 ml of water containing 12 g sucrose (final concentration 5%) and 100 µl Silwett (final concentration 0.04%). Transfer the culture to a beaker and mix gently. The "wonder mix" is ready for dipping. One 200 ml culture is enough for 2-3 pots containing 3-6 plants each.

(v) Carefully take each pot containing the plants, dip them in the "wonder mix" solution for a few seconds, transfer the pots to trays and keep them covered for overnight.

(vi) Remove the cover and let the plants grow and set seed.

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