

# Cell Penetrating Peptides Mediated DNA-Free Transfection in Arabidopsis and Chinese Cabbage

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## Research

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# Abstract

**Background:** The third-generation genome editing system CRISPR/Cas had shown strong application prospects in crop genetic improvement. However, this technology largely depends on genetic transformation. Public concerns on GMO (genetically modified organisms) safety, as well as related regulations, have restricted the application. Therefore, establishing DNA-free transfection system is important to promote CRISPR/Cas genome editing in agriculture.

**Results** In this paper, cell penetrating peptides fusion protein (CPP-mCherry) was found to be effective on DNA-free transfection. DNA sequences of nine tandem arginine (R9), one cysteine (cys), reporter mCherry and histidine label were sequentially constructed into pET 45B+ expression vector and transformed into *Escherichia coli* BL21(DE3) strain. CPP-mCherry fusion protein can be induced by 1mM IPTG for at least 1 hour in 28 °C. CPP-mCherry fusion protein can be obtained by 200W ultrasonication, then purified by Ni column and MWCO dialysis. The *Arabidopsis thaliana* root tips and leaves, as well as Chinese cabbage microspores and 3-week-old microspore embryo can be used as transfected recipient. Concentration can be selected between 10-100µg/ml and incubated overnight at room temperature. R9-cys-mCherry protein can be translocated into the nucleus of microspore. The transfection efficiency of root tips reached 100% and of microspore and MDE was 8.13% and 94.79%, respectively.

**Conclusions:** Here, a CPP mediated DNA-free transfection system was built in dicots. These results lay a technical foundation of DNA-free genome editing.

## Background

In recent years, the third generation of site-specific genome editing system (CRISPR) is becoming one of the core technologies in molecular breeding. However, this technology largely depends on the genetic transformation system. Public concerns on the safety of GMOs and relevant laws restrict the application. Therefore, DNA-free transfection is of great significance for promoting the application.

Cell penetrating peptides (CPPs) belong to a special class of short peptides, which generally contains several amino acids. It was first discovered in 1988 in the study of Tat protein of HIV-I virus (Frankel et al., 1988). Since then, researchers have found that some peptides also have the ability of intracellular delivery. Although these peptides are short, they contain various hydrophilic or hydrophobic motifs, which is helpful for transmembrane (Derakhshankhah et al., 2018). Cell penetrating peptide is an ideal transfection tool, whereby biomacromolecules can be transferred into recipient cells (Chugh et al., 2009, 2010; Ziemienowicz et al., 2012; Bilichak et al., 2020, Geng et al., 2020). On one hand, synthesized CPPs can combine with exogenous DNA non-covalently in vitro, forming a cargo complex, and enter the recipient cells by endocytosis. The exogenous DNA is released in cytoplasm and eventually integrated into the genome (Chugh et al., 2009, 2010). The CPP synthesis service is tradable. On the other hand, in order to form a covalent protein, the DNA sequence of CPPs can also be expressed together with target gene in prokaryotic system, forming a complex by disulfide bridge in Vitro, and eventually transfected into

the recipient cells. Bilichak carried out a beneficial attempt to transfect wheat microspore with CPP-mCherry fusion protein and eventually edit the genome by delivering ZNF protein (Bilichak et al., 2015, 2020). Compared with other chemical transfection methods, CPPs method has low cytotoxicity, which is suitable for plant experiments (Chugh et al., 2010; Bilichak et al., 2015, 2020; Huang et al., 2015; Derakhshankhah et al., 2018, Alizadeh et al., 2020, Geng et al., 2020).

Cell penetrating peptide fusion protein is an alternative method for CRISPR RNPs DNA-free intracellular delivery. Ramakrishna fused Cas9 with 9 consecutive arginine (9R) and then incubated with sgRNA to form a transfection complex. With the help of 9R, human HEK293T was successfully edited (Ramakrishna et al., 2014). In recent years, the research on DNA-free gene editing is in ascendant (Svitashev et al., 2016; Liang et al., 2017; Ran et al., 2017; Bilichak et al., 2020).

In order to develop a DNA-free transfection system on dicotyledon, Arabidopsis somatic cells and Chinese cabbage microspores and MDE were used as experimental materials in this study. We offered a DNA-free transfection method mediated by cell penetrating peptide, that can be used in DNA-free genome editing in the future.

## Materials And Methods

### 1 CPP-mCherry constructs

The structures of CPP vectors used in this study are shown in Fig. 1. The R9-cys-mCherry, cys-mCherry DNA sequences are guided by T7 promoter and built in Pet45b + backbone sequence. The constructs were transformed into BL21 (DE3) competent cell.

### 2 Induction of CPP-mCherry fusion protein

5  $\mu$ l glycerol stock were activated in 10 ml LB medium containing 0.5% glucose and 100 mg / L carboxybenzyl penicillin, pre cultured overnight at 28 °C. On the next day, 2 ml of pre culture was added to 20 ml LB medium, to make the OD value reaches 0.5, kept culture at 28 °C. In the IPTG gradient experiment, 0,0.5,1.0,1.5,2.0 mm IPTG were presented. The samples were collected after 5 h induction. Centrifuge immediately, resuspend the bacterium in the equilibrium lysis buffer B (hispur Ni NTA spin columns kit). Then, sonification with 200 W for 10 s on ice, repeat 6 times. After 15 000 g centrifugation for 15 min, the supernatant was the crude protein sample for SDS-PAGE detection.

### 3 Purification of CPP-mCherry fusion protein

CPP-mCherry-6xHistag fusion protein was purified by Ni column, which was taken out from 4 °C and balanced at room temperature for 20 min before use. Ni column was centrifuged at 700 g for 2 min to remove the storage liquid. Make sure the equilibrium solution fully contacts with the column, centrifuge 700 g for 2 min to remove the equilibrium solution. The crude protein from bacterial was added into the Ni column, fully mixed, and incubated at room temperature for 30 min. 700 g centrifuge for 2 min. Rinse the Ni column with washing buffer, centrifuged at 700 g for 2 min. Elusion buffer was added to replace

the target protein from the Ni column. 700 g centrifuged for 2 min. The filtrate was the target protein solution. 280 nm OD value was detected to determine the concentration by Bicinchoninic acid (BCA) method, then measured by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Non target proteins were removed by MWCO dialysis column.

#### 4 Plant material

The mesophyll, root tip of *Arabidopsis* 'Col-0' and microspores of *Brassica rapa*. 'FT' were used as transfection targets. 'FT' is a double haploid (DH line) from microspore culture. Plantlets were grown in kilma, an artificial climate zone in Wageningen University Research Center (WUR). 16 h photoperiod ( $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ ), 20 °C/18 °C.

#### 5 DNA-free transfection recipients of *Arabidopsis*

*Arabidopsis* seeds were dried at 30% RH, and then stored at - 80 °C (Wu et al., 2019). Dry seeds were surface-sterilized with 75% ethanol and 2% bleach for 20 minutes. After rinsing, the seeds were suspended in 0.1% agarose solution, carefully sowed on 1/2 MS solid medium, placed on vertical scaffolds in artificial climate chamber 16 h photoperiod ( $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ ), 20 °C/18 °C. 7 days later, root tip and leaf samples were removed and placed in 1/2 MS liquid medium for transfection.

#### 6 DNA-free transfection recipients of Chinese cabbage

The microspore isolation protocol is modified from Sato et al., (1989). Buds of 'FT' were selected from healthy plantlet for surface-sterilize. After rinsing, crush the buds to press out the microspores in 5 ml NLN liquid medium. removed the debris by nylon mesh, the yellow precipitates (microspores) were used as transfection target.

#### 7 DNA-free transfections

R9-cys-mCherry fusion protein was used for DNA-free transfection, and cys-mCherry without cell penetrating peptide was used as negative control. Fusion protein were diluted as concentration gradients: 0, 10, 50, 100  $\mu\text{g} / \text{ml}$ . The transfection target tissue was transferred to 1.5 ml centrifuge tube, discarded the medium, incubate with CPP-mCherry gradient solution overnight at room temperature. Centrifuge at 5000 rpm for 3 min, remove the protein solution, add 100  $\mu\text{l}$  Renaissance solution to stain the cell wall for 1 h, then fix the material with MTSB solution overnight at 4 °C. Transfected material was Z stacked by Leica dm5500 Q laser confocal microscope.

## Results

#### 1 IPTG induction of CPP-mCherry

The induction of CPP-mCherry expression was conducted by IPTG gradient, the results are shown in Fig. 2. The obvious pink mCherry protein was clearly visible in the bacterial precipitation (Fig. 2A). The

bacterium was then broken by sonication to release the protein. The molecular weight of R9-cys-mCherry protein was about 30 kD as expected (Fig. 2B). With the increase of IPTG concentration, there was no significant difference in the expression of target protein (Fig. 2B).

In addition to R9-cys-mCherry and cys-mCherry vectors, we tried others for induction, such as penetratin-cys-mCherry, transportan-cys-mCherry and Map-cys-mCherry. R9-cys-mCherry is productive.

## 2 SDS-PAGE

The target protein with 6x histag was purified by Ni column. After SDS-PAGE electrophoresis, the results are shown in Fig. 3. Lane 1–3 were from BL21 (DE3) competent cell as negative control. Lane 4–6 are from CPP-mCherry transformants. The negative control had no target protein as expected. In contrast, the transformants contain large amount of CPP-mCherry fusion proteins. SDS-PAGE results suggest that the cys-mCherry and R9-cys-mCherry protein could be induced from 1.0 mM IPTG induction for 5 h. After Ni column purification, the non-target protein was removed by MWCO dialysis.

Protein concentration was detected by Bicinchoninic acid (BCA) method. The concentrations of R9-cys-mCherry and Cys-mCherry protein were about 0.13 mg/ml (Table 1), which is sufficient for the following experiments.

Table 1  
concentration of isolated CPPs-mCherry fusion protein

CPP fusion protein	Concentration(mg/ml)
R9-mCherry	0.13
cys-mCherry	0.13

### 3 R9-cys-mCherry transfected into *Arabidopsis* somatic cells

In the beginning, 7-days *Arabidopsis* mesophyll cells were found to be transfected by R9-cys-mCherry accidentally (Fig. 4A). Leaves were incubated with 30 µg/ml R9-cys-mCherry overnight at room temperature. In the second day, strong mCherry red fluorescence signal was observed in mesophyll cells, Fig. 4A. These results suggest that CPP-mCherry fusion protein was successfully transfected into the cytoplasm. *Arabidopsis thaliana* mesophyll can be used as the CPP mediated transfection recipient.

Later, *Arabidopsis* root tip were used as DNA-free transfection recipient. Root tips were incubated with 50 µg/ml R9-cys-mCherry overnight at room temperature Fig. 4B. Strong red fluorescence signal was found in root tip cells. These results suggest that the somatic cells of *Arabidopsis* can be used as the transfection recipient. Mesophyll and root cells were usually used for protoplast isolation (Ikeuchi et al., 2016), our result also provides a technical basis for protoplast DNA-free transfection and genome editing.

### 4 Dose dependent effect

Cell penetrating peptide mediated transfection normally shows dose dependent (Cardozo et al., 2007; Ru et al., 2013; Bilichak et al., 2015; Guidotti et al., 2017) In order to optimize the best transfection condition, the concentration gradient was set as 1, 10 and 100 µg / ml, and incubated overnight at room temperature Fig. 5.

There is no mCherry signal in low concentration treatment. When R9-cys-mCherry concentration increased to 100 µg/ml, strong mCherry signal was found in root tip cells (Fig. 5B). No fluorescence signal was found in negative control, as expected (Fig. 5A). The results suggest that the transfection shows dose-dependency, the concentration of fusion protein can be selected between 10–100 µg / ml.

In order to further determine R9-cys-mCherry distribution in recipient cell, we used high resolution confocal microscopy, Fig. 6A. In the cys-mCherry negative control, no fluorescence signal was found in root tip cells Fig. 6B. In contrast, the strong red fluorescence signal was found in the R9-cys-mCherry treatment. These results indicate that R9-cys-mCherry can be transfected into root tip cells in the range of 10–100 µg/ml, with 100% transfection efficiency, Table 2.

Table 2  
CPPs-mCherry transfection efficiency in *Arabidopsis* root tip

CPP-mCherry Fusion protein	mCherry positive root tip	total number of root tip	Transfection efficiency
<b>50 µg/ml</b>			
R9-mCherry	98	98	100%**
Negative control cys-mCherry	0	93	0%
Note: Student's <i>t</i> test (*indicate difference $p < 0.05$ ; ** indicate significant difference $p < 0.01$ )			

5 R9-cys-mCherry transfected into microspores of *Brassica rapa*.

DNA-free transfection was also carried out in Chinese cabbage microspores. The microspores are nearly spherical cells with thick exine, different orientation would interfere laser scanning. Therefore, the “face-up” microspores were selected for observation, whereby germination furrows are vertical to the glass slide. The thick exine was stained by Renaissance dye. The concentration gradient of R9-cys-mCherry and cys-mCherry was set as 1, 10, 50, 100 µg / ml, respectively, Fig. 6. No cys-mCherry signal was detected at any concentration as expected (Fig. 7A). In contrast, the positive results were found in R9-cys-mCherry transfection. With the increase of concentration, strong red fluorescence signals were found at 50 and 100 µg/ml (Fig. 7B). These results suggest that 'FT' microspore can be used as DNA-free transfection recipient cell. The concentration can be selected between 10–100 µg/ml. The transfection efficiency was about 8.13% (Table 3).

Table 3  
 CPPs-mCherry transfection efficiency in *B. rapa* 'FT' microspores

CPP-mCherry Fusion protein 50 µg/ml	mCherry positive microspores	Observed microspores	Transfection efficiency
R9-mCherry total	27	332	8.13%**
Negative control cys-mCherry total	0	312	0.00%

Note: Student's *t* test (\*indicate difference  $p < 0.05$ ; \*\* indicate significant difference  $p < 0.01$ )

Table 4  
 CPPs-mCherry transfection efficiency in *B. rapa* 'FT' MDE

CPP-mCherry Fusion protein 100 µg/ml	mCherry positive MDE	total number of MDE	Transfection efficiency
R9-mCherry	91	96	94.79%**
Negative control cys- mCherry	0	102	0.00%

Note: Student's *t* test (\*indicate difference  $p < 0.05$ ; \*\* indicate significant difference  $p < 0.01$ )

CRISPR RNP protein complex needs to enter the nucleus, then performing gene editing. In order to clarify the distribution of R9-cys-mCherry in microspores, we observed transfected microspores by high resolution confocal, Fig. 8. Strong fluorescence was clearly visible in the nucleus of microspore, indicating that CPP-mCherry was successfully transfected into microspore nucleus.

6 R9-cys-mCherry transfected into MDE of *Brassica rapa*.

DNA-free transfection of microspore derived embryos (MDE) was carried out in this study. The concentration of CPP-mCherry was 100 µg / ml. After incubation overnight at room temperature, confocal microscopy was performed, Fig. 9.

With 100 µg / ml of R9-cys-mCherry incubation, strong red fluorescence signal was found in MDE epidermal cell, as the medium section is too thick for the laser went through. MDE treated with cys-mCherry had no fluorescence signal as expected. The transfection frequency was over 94.79%. The results showed that, 21-day microspore embryos of 'FT' could be used as the DNA-free transfection recipient.

Many successful attempts have been made to transfect Chinese Cabbage (Boulter et al., 1990; Zhang et al., 1998; 2000; Tang et al., 2003; Yang et al., 2004; Baskar et al., 2016; Li et al., 2018). However, these techniques showed difficult to regenerate after transfection. Microspore embryo is an important haploid transfection recipient, whereby pure homozygous Lines DH line would be obtained. In this study, we

found that, transfected MDEs are able to regenerate into plantlets due to low cytotoxicity (Chugh et al., 2010; Bilichak et al., 2015; Huang et al., 2015; Derakhshankhah et al., 2018)

In general, CPP-mCherry can be used as a DNA-free transfection tool in crops. Microspore and 3-week-old MDEs, as well as Arabidopsis somatic cells, could be used as transfection recipient.

## Discussion

Firstly, select the suitable Cell penetrating peptides (CPPs) for transfection is vital. CPPs are a class of short peptides with transmembrane activity, which are often used as effective nano carriers (Chugh et al., 2010; Ziemienowicz et al., 2012; Bilichak et al., 2015; Borrelli et al., 2018). Cell penetrating peptides can bind to biomacromolecules covalently or non-covalently (Chugh et al., 2010), then penetrate the cell membrane through pinocytosis or direct pathway (Derakhshankhah et al., 2018). In terms of physical and chemical properties, cell penetrating peptides can be divided into cationic peptide (cationic CPP), hydrophobic peptide (hydrophobic CPP), amphiphilic peptide (amphiphilic CPP), etc. (Derakhshankhah et al., 2018). The penetration efficiency of CPP varies with different recipient cell. In addition to R9-cys-mCherry and cys-mCherry vectors, other CPPs were also tried for transfection, such as penetratin, transportan and Map, R9-cys-mCherry is the most effective.

Technically, the preparation of CPP fusion protein is also important. CPP can be linked as fusion protein through disulfide bond (Liu et al., 2014; Bilichak et al., 2015); or its sequence can also be constructed in a vector and translated in prokaryotic expressing system (Ru et al., 2013; Bilichak et al., 2015). In this study, the latter method was utilized. The fusion protein vector mainly includes T7 promoter, cell penetrating peptide, target protein (mCherry used in this study) and purification tag. Pet series vectors contain T7 promoter from  $\lambda$  phage, which can be used in BL21 (DE3) strains for prokaryotic expression (Yoon et al., 2009). In this study, several vectors were used for the expression of CPP-mCherry, and pet 45 + R9-cys-mCherry was the most productive one (data not shown). The location and numbers of cell penetrating peptide in the fusion protein are crucial for the transfection effect. In the vector, cell penetrating peptide can be placed at the C-terminal or N-terminal (Ru et al., 2013). The more CPP appears on the surface of the fusion protein, the more favorable it is to cross the cell membrane (Numata et al., 2012; Ng et al., 2016). CPP is located on C terminal in this study, and our transfection result is in line with previous reports.

CPPs mediated transfection is also an important method for DNA-free genome editing (Ran et al., 2017). We found that root tips, mesophyll cells, microspores, 3-week-old microspore embryos could be used as transfection target, which offered more types of transfection targets (Ramakrishna et al., 2014; Bilichak et al., 2015). The nuclei localization of transfection complex is important. Only when the complex was transfected into the nucleus could it perform gene editing. Arginine rich peptides (such as tat, R9) can interact with nuclear pore complexes (NPCs), and finally take the complex into the nucleus (Wender et al., 2000; Chugh et al., 2007, 2010). Different from previous reports (Bilichak et al., 2015), we found that R9-cys-mCherry fusion protein could be transfected into 'FT' microspore nucleus, not only on the surface

(Fig. 8). Maybe because the structure and chemical composition of microspore exine are different between Cruciferae and Poaceae. There are three germinal furrows on 'FT' microspore, where the exine is thin. During microspore culture, some have double or even three furrows rupture, and most are prone to single rupture (Tang et al., 2013). R9 is a typical cationic peptide, but sporopollenin on the exine is negative charge (Salter et al., 2002; Quilichini et al., 2010; Bilichak et al., 2015). The R9-cys-mCherry would first accumulate on the exine (Fig. 7), then enter the microspore through the furrows rupture, and finally located to the nucleus (see Fig. 8).

Cell penetrating peptide mediated DNA-free transfection has obvious advantages. Firstly, the transfection is dose dependent (Cardozo et al., 2007; Ru et al., 2013; Bilichak et al., 2015; Guidotti et al., 2017). The transfection of R9-cys-mCherry is also dose-dependent. We found that the working concentration ranged from 10 to 100  $\mu$ g / ml. With the increase of dosage, the fluorescence signal increased gradually. which is in line with previous report (Ru et al., 2013). Furthermore, compared with other transfection methods, CPPs method has low cytotoxicity (Chugh et al., 2010; Bilichak et al., 2015; Huang et al., 2015; Derakhshankhah et al., 2018), and has less inhibition on the regeneration process. Therefore, cell penetrating peptides have been used in genetic transformation (Chugh et al., 2009) and DNA-free genome editing (Ramakrishna et al., 2014; Svitashv et al., 2016; Liang et al., 2017; Ran et al., 2017; Bilichak et al., 2020). CPP showed no toxic effect in mammalian cells (Chugh et al., 2010). Taking TAT and R9 as examples, even at a high dose of 30  $\mu$ g / ml, there was no obvious toxic effect (Jones et al., 2005). Trypan blue and FDA staining results also showed that cell penetrating peptide had little effect on cell viability of plant cells (Chang et al., 2005; Chugh et al., 2007). In haploid transfection, the CPP had little effect on the microspore viability (Chugh et al., 2009). Generally speaking, R9 or TAT at lower doses does not strongly inhibit regeneration. Consistent with previous studies, we found that the microspore embryos transfected with CPP-mCherry could regenerate plantlets.

Our results indicate that CPPs is an ideal DNA-free transfection tool to transfer the target protein into the nucleus, that provides theoretical and technical basis for DNA-free CRISPR RNP intracellular delivery and further genome editing.

## Conclusion

CPP-mCherry fusion protein is an efficient DNA-free transfection complex. After incubation at room temperature, the R9-cys-mCherry can be successfully transfected into somatic cells of *Arabidopsis thaliana* and fresh microspore, as well as MDE, of Chinese cabbage.

## Declarations

### Ethics approval and consent to participate:

Not applicable

### Consent for publication:

Not applicable

### **Availability of data and materials:**

Not applicable

### **Competing interests:**

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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### **Authors' contributions:**

H.W. designed and performed experiments. H.W., Y.Z., H.F. wrote this manuscript and all other authors provided feedback on the manuscript.

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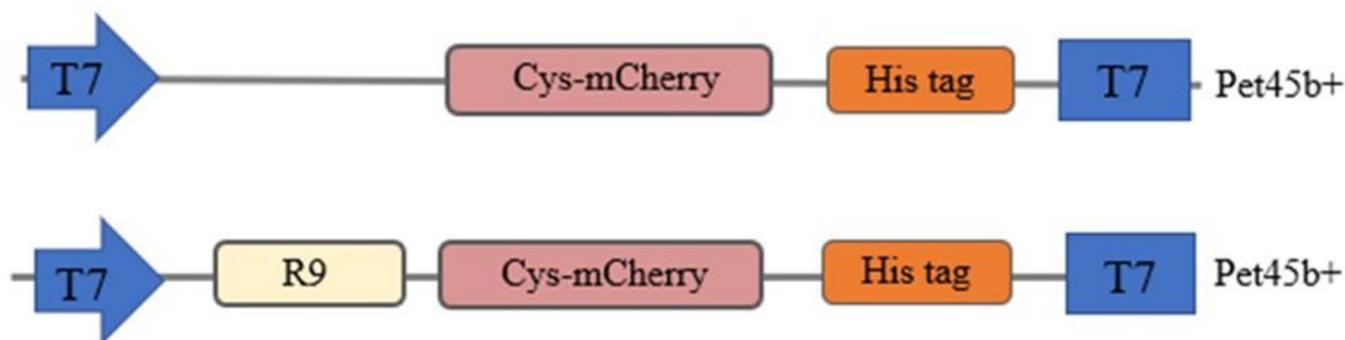
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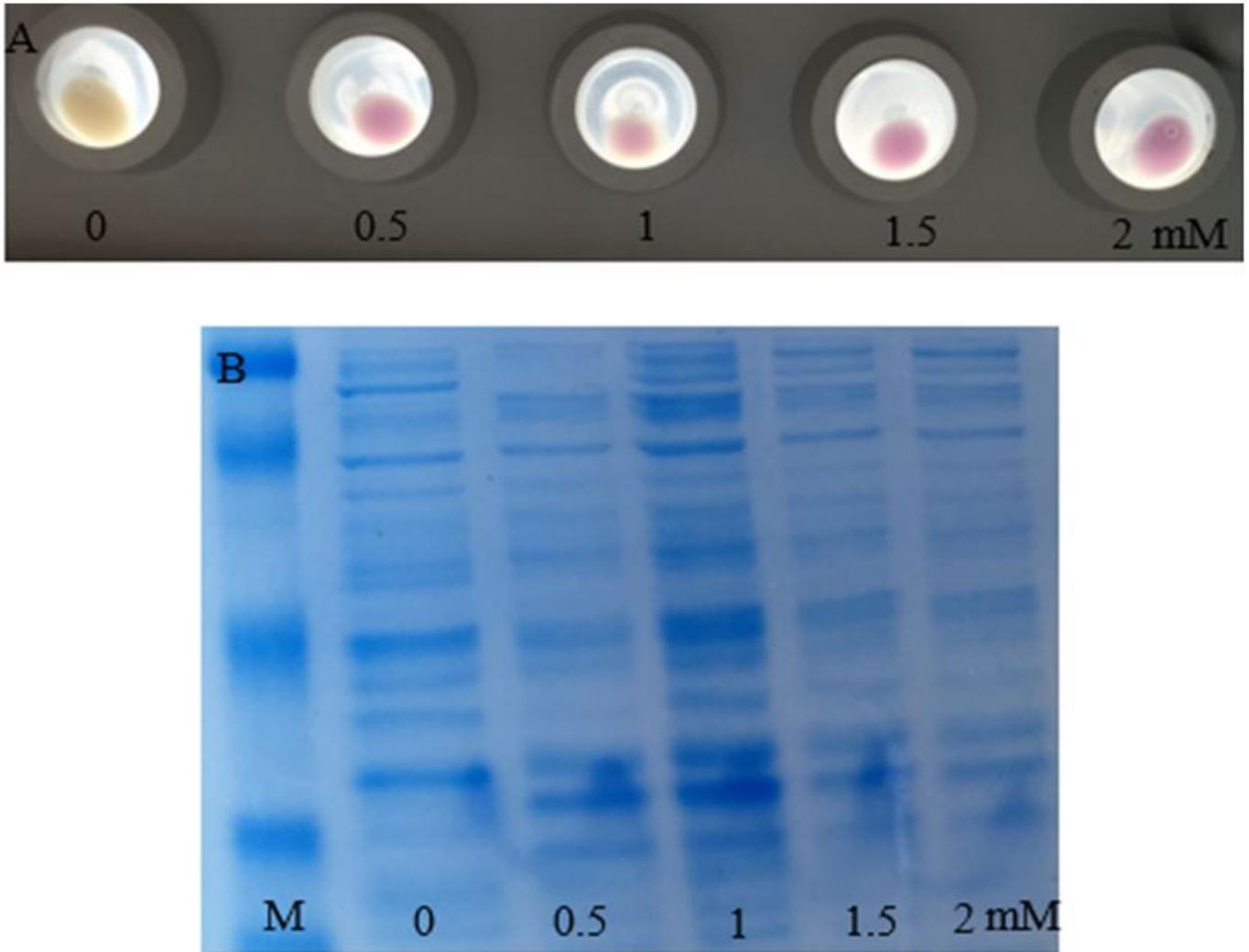
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## Figures



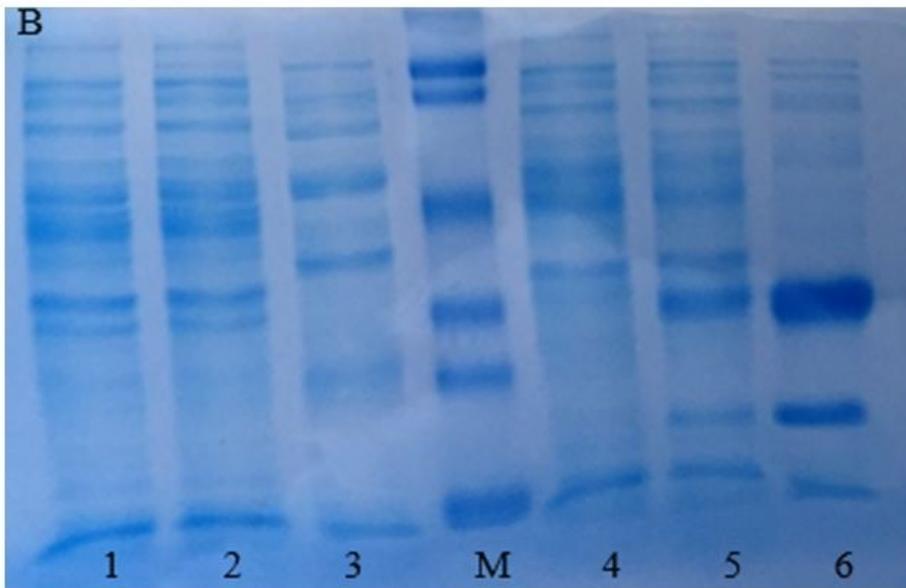
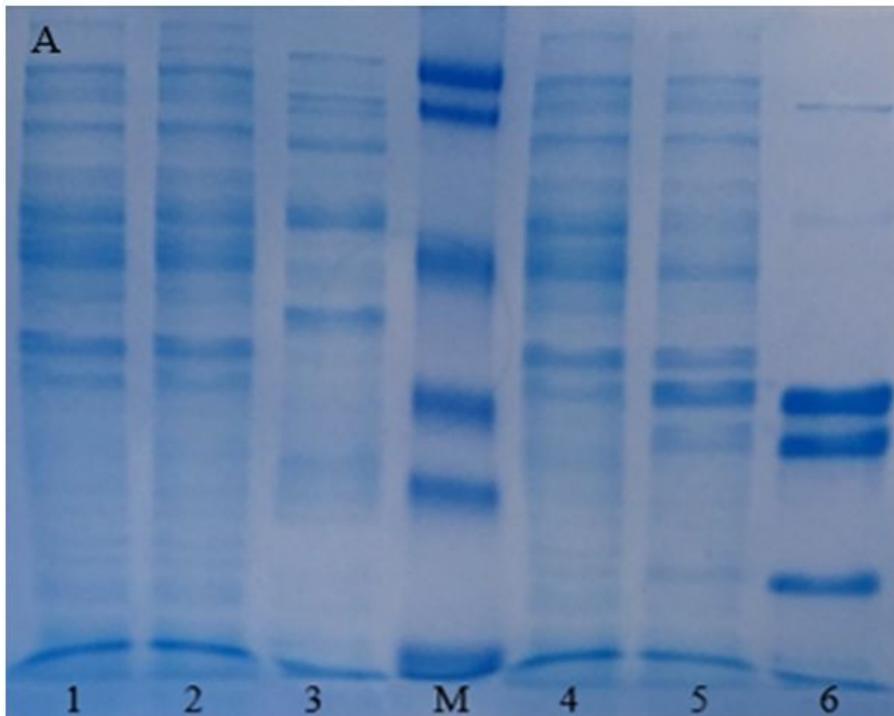
**Figure 1**

CPPs-mCherry constructs used in this experiment.



**Figure 2**

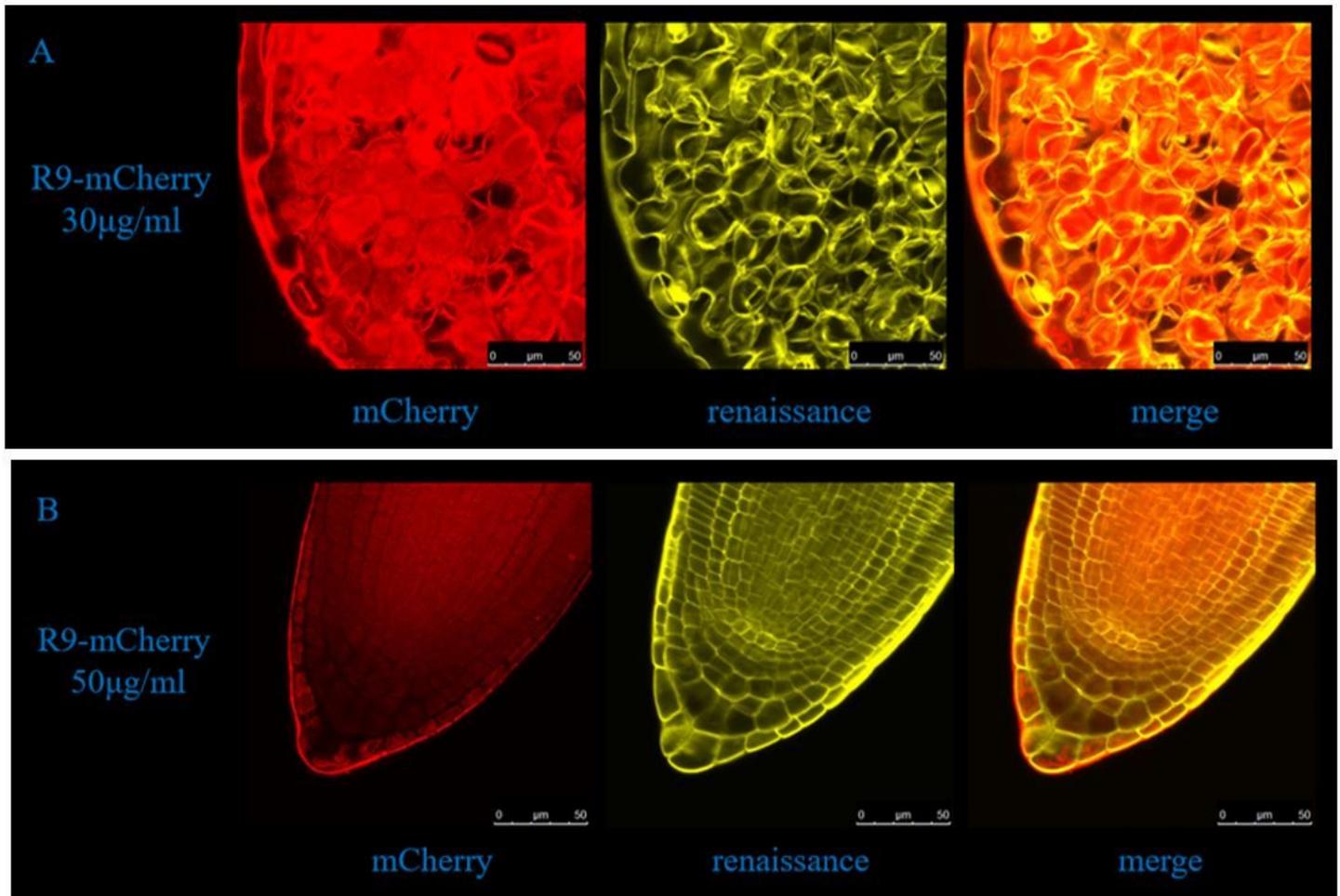
CPP-mCherry induced by IPTG gradient A.centrifuged IPTG gradient induced colony, the pink color is from mCherry. B. SDS-PAGE of IPTG gradient induced CPP-mCherry.



**Figure 3**

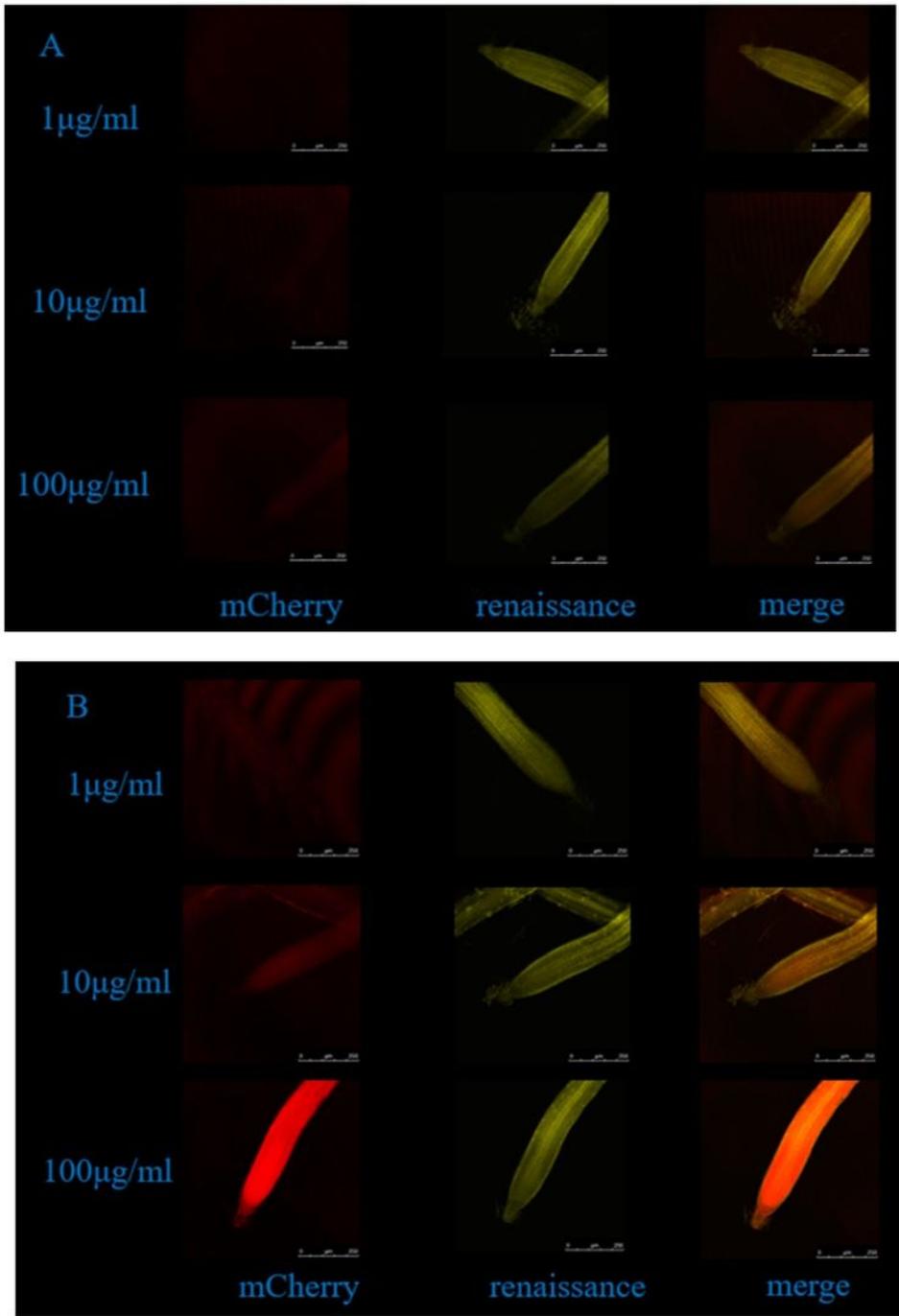
SDS-PAGE of CPP-mCherry fusion protein before and after Ni column purification. A SDS-PAGE of R9-cys-mCherry fusion protein before and after Ni column purification. lane1, 2, 3 are from BL21-DE3 competent cell as negative control. 1, before IPTG induction 2, after IPTG induction 3, after Ni column purification, respectively. Lane4, 5, 6 are from R9-cys-mCherry transformants. 4, before IPTG induction 5, after IPTG induction 6, after Ni column purification. B SDS-PAGE of cys-mCherry fusion protein before and after Ni

column purification. lane 1, 2, 3 are from BL21-DE3 competent cell as negative control. 1, before IPTG induction 2, after IPTG induction 3, after Ni column purification, respectively. Lane 4, 5, 6 are from cys-mCherry transformants. 4, before IPTG induction 5, after IPTG induction 6, after Ni column purification, respectively.



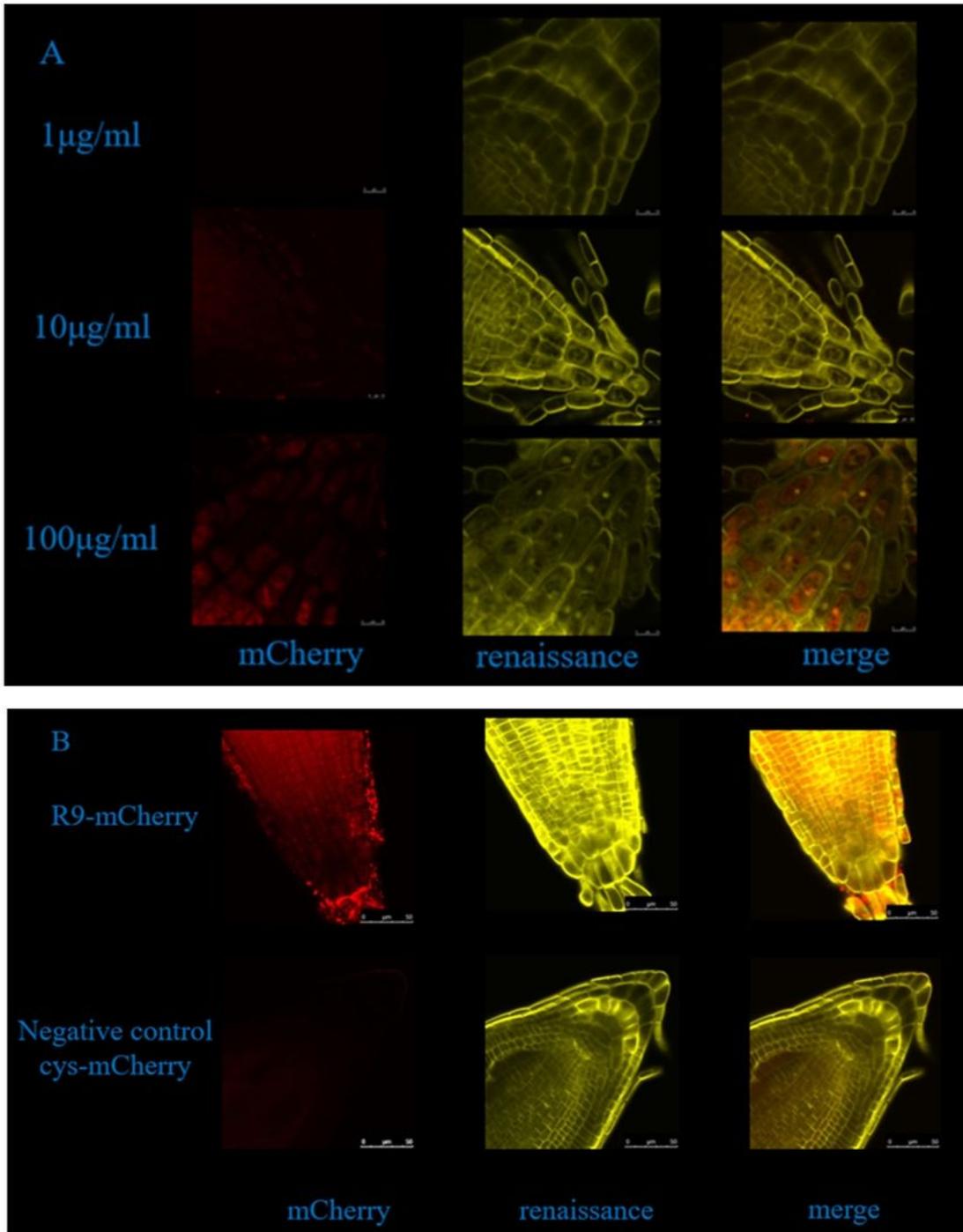
**Figure 4**

R9-cys-mCherry translocated into Arabidopsis somatic cells A. translocated into Mesophyll cells. B. translocated into root tip cell



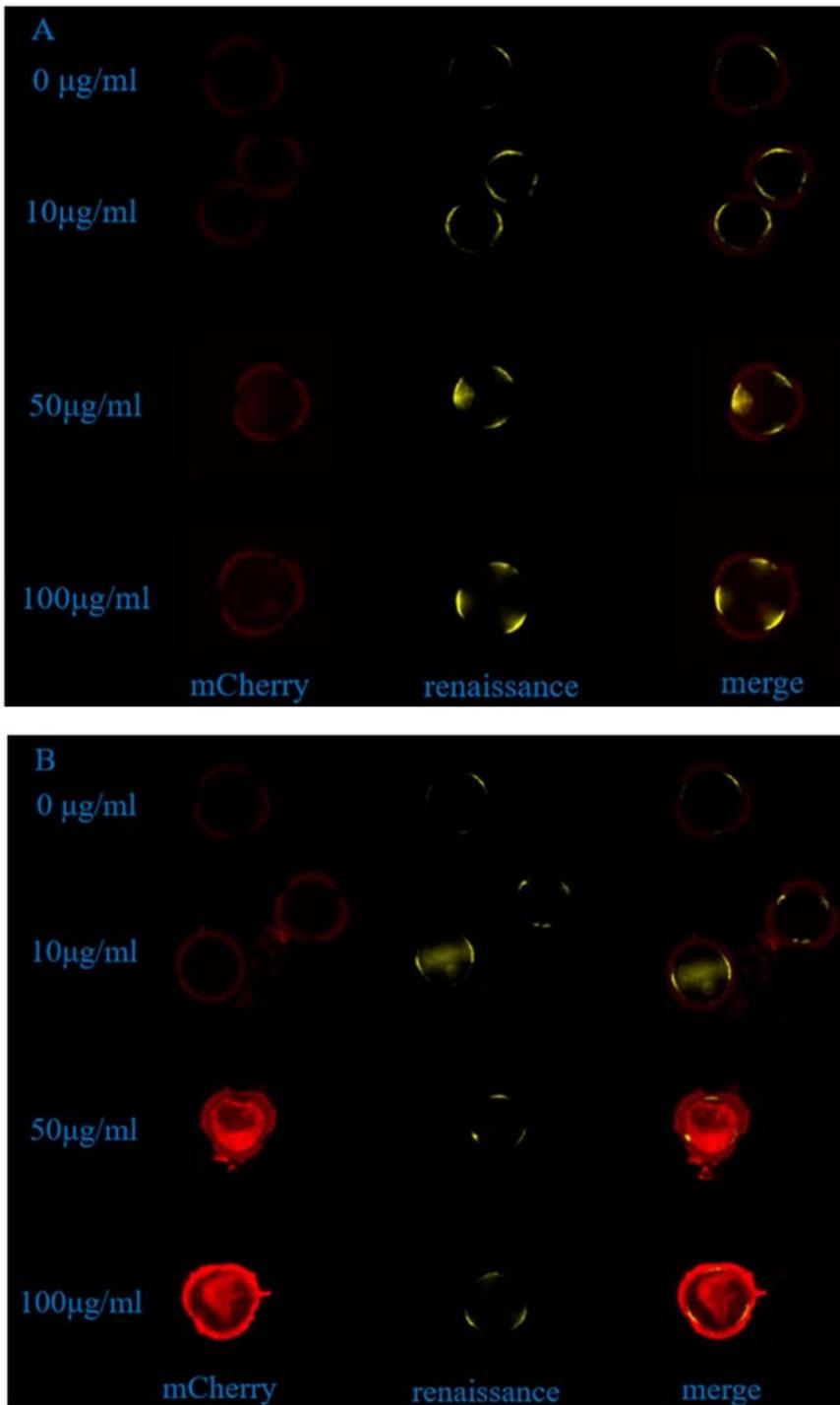
**Figure 5**

Dose dependent effect of CPP-mCherry transfection A. Negative control cys-mCherry gradient (1,10,100µg/ml) cannot translocated into Arabidopsis root tip B. R9-cys-mCherry gradient (1,10,100µg/ml) translocated into Arabidopsis root tip



**Figure 6**

High resolution detection of CPP-mCherry transfection in Arabidopsis root tip. A. R9-cys-mCherry dose dependent effect. B. Comparison of R9-cys-mCherry and cys-mCherry transfection.



**Figure 7**

Dose dependent effect of R9-cys-mCherry transfection in *B. rapa* 'FT' microspore A. negative control cys-mCherry transfection. B. R9-cys-mCherry transfection.

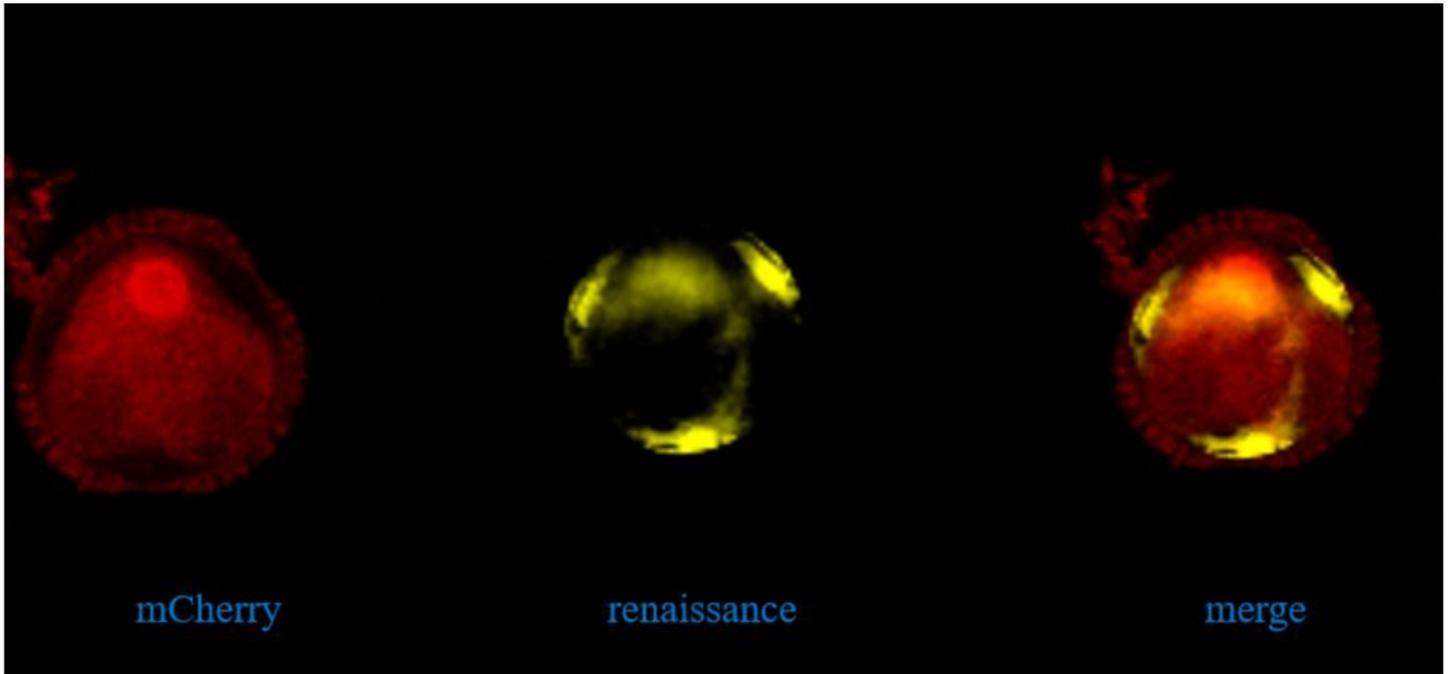


Figure 8

R9-cys-mCherry fusion protein translocated into B.rapa 'FT' microspore nuclei

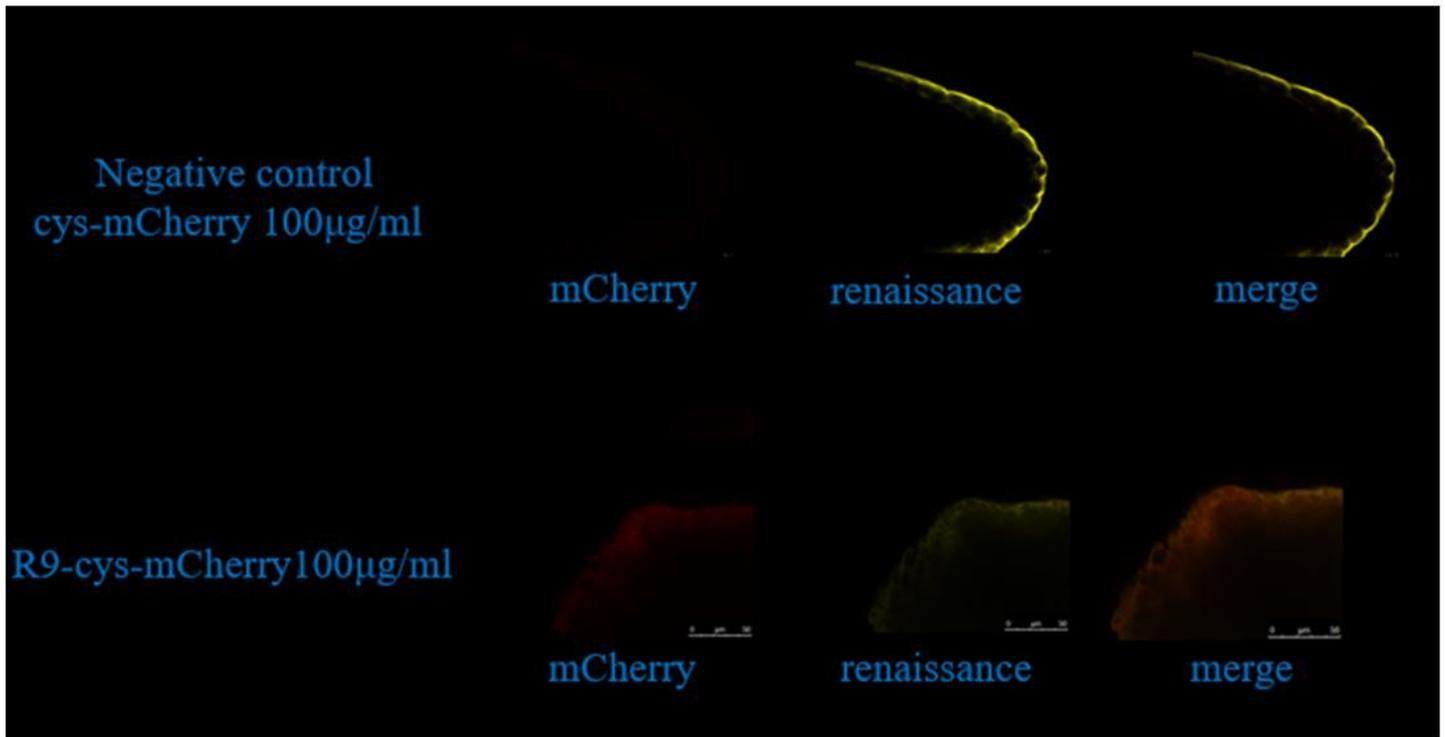


Figure 9

comparison of 100µg/ml CPP-mCherry translocated into B.rapa MDE.