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Evaluation of parameters affecting *Agrobacterium*-mediated transient expression in citrus

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Abstract

Agrobacterium-mediated transient expression assays are a convenient alternative to stable expression because they are simple, easy to perform, and achieve gene expression rapidly. This study investigated the factors affecting transient gene expression efficiency in citrus by observing the cryo-sectioning of leaf samples under a laser confocal microscope. These factors included the composition of the infiltration buffer, the *Agrobacterium* cell density, the leaf development stage, the incubation temperature, and plant genotype. The highest transient expression level of yellow fluorescent protein (YFP) was detected in Mexican lime (*Citrus aurantifolia*) on the third day after the intermediate-aged leaves were infiltrated with the improved infiltration buffer 1 (15 mmol L^{-1} 2-(N-morpholino) ethanesulfonic acid, 10 mmol L^{-1} $MgCl_2$, and 200 μmol L^{-1} acetosyringone), which had an optical density of 0.8 and was incubated at 22°C. Additionally, this transient expression assay was applied to other citrus genotypes. Of note, trifoliate orange (*Poncirus trifoliata*) and kumquat (*Fortunella obovate*) had higher expression efficiency than other six genotypes of the *Citrus* genus. Our study provides research basis for the selection of optimization strategies in transient gene expression and improves the method for available genome investigation in citrus.

Keywords: citrus, *Agrobacterium*, transient expression efficiency, yellow fluorescent protein (YFP)

1. Introduction

Citrus is an important global crop. The genes cloned from citrus genome require functional identification. Gene functions are generally identified *via* stable or transient gene expression. The transient gene expression assays are better than stable gene expression in terms of time and labor efficiency (Kapila *et al.* 1997; Jones *et al.* 2009). Thus, it can expedite functional gene identification in citrus.

The optimization of transient analysis factors would increase gene expression efficiency and help gene function analysis. Transient gene expression assays on citrus plants have been performed *via Agrobacterium* infiltration (Figueiredo *et al.* 2011; Sendín *et al.* 2012). And the technical factors, including infiltration medium, *Agrobacterium* concentration, leaf development stage, and environmental conditions,

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have potential to be improved because they have been optimized in other plants (Wroblewski *et al.* 2005; Santos-Rosa *et al.* 2008; Kim *et al.* 2009). The transient expression efficiency of citrus has been explored in young and mature leaves (Figueiredo *et al.* 2011), and the pretreatment of *Xanthomonas citri* subsp. citri (Jia and Wang 2014a, b). Optimal transient expression assay conditions were different for different plants (Batra and Kumar 2003; Wroblewski *et al.* 2005; Yasmin and Debener 2010; Brückner and Tissier 2013; Xu *et al.* 2014). For example, the highest level of transient gene expression was reached when the acetosyringone (AS) concentration was 450 μmol L^{-1} in tobacco leaves (Wydro *et al.* 2006). And Debnath *et al.* (2014) found that 100 mmol L^{-1} AS was considered to be an optimized concentration for the transient expression in sesame.

Plant genotype is a key factor that influences the efficiency of agro-infiltration (Sheludko *et al.* 2007; Zottini *et al.* 2008; Bhaskar *et al.* 2009). Bhaskar *et al.* (2009) found that the effect of the plant genotype on transient gene expression is consistent with that of the stably transformed potato plants. The genetic transformation of citrus is also cultivar dependent (Dutt *et al.* 2009), which encouraged us to test transient gene expression efficiency of different citrus genotypes.

This paper describes various factors which influence transient gene expression assay of citrus. And an optimal procedure for Mexican lime was established, which would provide technical help for transient gene expression of citrus.

2. Materials and methods

2.1. Plant materials

The seedlings of trifoliate orange (*Poncirus trifoliata*) were two-year-old. Other cultivars that were grafted on trifoliate orange were also two-year-old. These cultivars included Mexican lime (*Citrus aurantifolia*), Dahong sweet orange (*C. sinensis*), seedless ponkan (*C. reticulata*), Duncan grapefruit (*C. paradisi*), Ovale di Sorrento lemon (*C. limon*), Chandle pummelo (*C. grandis*), and Changshou kumquat (*Fortunella obovate*). All plants were grown at 22°C in a greenhouse.

2.2. Binary plasmids and bacterial strain

Three expression vectors (P1C35, P2A1, and PAp*Ptcor8::YFP*) were utilized in the transient expression experiments (Fig. 1). The vectors, P1C35 and P2A1, were provided by the University of Maryland, USA. P1C35 was constructed by inserting a C*auliflower mosaic virus* 35S (CaMV 35S) promoter into pZP 211. P2A1 was created by inserting a yellow fluorescent protein (YFP) gene into P1C35 in which *yfp* gene expression was under the control of a CaMV 35S promoter. PAp*Ptcor8::YFP* was created by replacing the CaMV 35S promoter of P2A1 with a p*Ptcor8* promoter isolated from the identified trifoliate orange cold stress-responsive gene, *Ptcorp* (Long *et al.* 2012). All vectors were induced into the *Agrobacterium* strain, EHA105, using the freeze-thaw method.

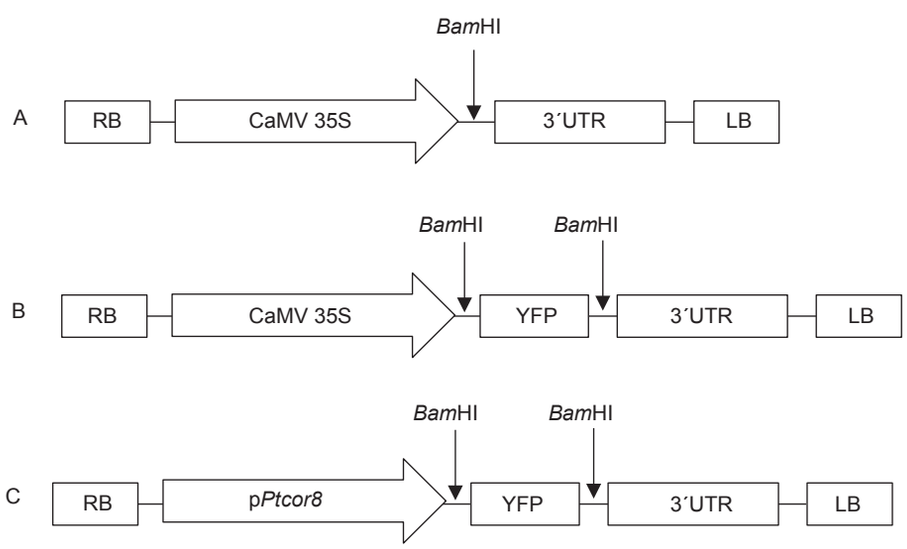

Fig.1 Schematic representations of the T-DNA region of the vector used in this study. A, organization of vector P1C35 as control. B, organization of vector P2A1 with the *yellow fluorescent protein* (*yfp*) gene was under the control of a *Cauliflower mosaic virus* 35S (CaMV 35S) promoter. C, organization of vector PAp*Ptcor8::YFP* with the *yfp* gene was under the control of a p*Ptcor8* promoter. RB, the right border; LB, the left border; YFP, yellow fluorescent protein.

2.3. Infiltration buffers

This study evaluated five infiltration buffers of the following chemical formulas: buffer 1: 10 mmol L^{-1} 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.6, 10 mmol L^{-1} $MgCl_2$, and 150 μmol L^{-1} AS (Cote *et al.* 1996; Ghosh *et al.* 2012; Sendín *et al.* 2012; Jia and Wang 2014a, b); buffer 2: 10 mmol L^{-1} MES, pH 5.6, 10 mmol L^{-1} $MgSO_4$, and 100 μmol L^{-1} AS (Yang *et al.* 2000); buffer 3: 10 mmol L^{-1} $MgCl_2$ and 150 μmol L^{-1} AS (Llave *et al.* 2000); buffer 4: 50 mmol L^{-1} MES, pH 5.6, 2 mmol L^{-1} Na_3PO_4, 0.5% (v/v) glucose, and 100 μmol L^{-1} AS (Zottini *et al.* 2008); buffer 5: Murashige and Skoog medium (Murashige and Skoog 1962), 30 g L^{-1} sucrose, 50 mg L^{-1} myoinositol, 10 mL L^{-1} of vitamins (40 mg L^{-1} glycine, 100 mg L^{-1} nicotinic acid, 200 mg L^{-1} pyridoxine HCl, and 200 mg L^{-1} thiamine), and 100 μmol L^{-1} of AS (Figueiredo *et al.* 2011).

2.4. *Agrobacterium* infiltration

Agrobacterium was grown in yeast extract broth (YEB; 1% yeast) at 28°C for 18–24 h. The cells were harvested *via* centrifugation and suspended to a final concentration in infiltration buffer. Then the bacterial suspension remained static at room temperature and the leaves were superficially wounded with a needle to improve infiltration. The bacterial suspension (300 μL) was delivered to the entire leaf using a 1-mL syringe.

2.5. YFP images

To evaluate each parameter, three independent experiments were performed to measure the expression of YFP. The infiltrated leaf was randomly cut into a 1-cm^2 area and then cryo-sectioned (15 μm in thickness) using a freezing microtome (Model CM1950, Leica, Germany). The YFP fluorescence of the cryo-section was monitored using a laser scanning confocal microscope (Model LSM 700, Zeiss, Germany) with a 10× objective len and quantified using the Image J program (ver. 1.48, National Institutes of Health, USA). Statistical analyses were performed using SPSS software (ver. 9, IBM, USA).

3. Results

3.1. YFP transient expression variations with time

The *Agrobacterium* bacterium that contained vectors P2A1 and P1C35 were suspended with the infiltration buffer 1 to an optical density at 600 nm (OD_{600}) of 0.8 and used to monitor the YFP fluorescence trend over 10 days in the Mexican lime leaves. For P2A1, the YFP fluorescence was observed on the 1st day of post agro-infiltration (DPA), thereafter increased, and its intensity reached the highest value on the 3rd day. Then it decreased sharply on the 4th day and decreased slowly until the 10th day, when the experiment was ended (Fig. 2). For P1C35 without the *yfp* gene, the YFP fluorescence could not be observed at all during the entire experiment. The YFP fluorescence of the agro-infiltrated leaves was observed at 3 DPA in the following studies.

3.2. Evaluation of infiltration buffers in a transient expression assay

In addition to the infiltration buffers 1 and 5 that were described in the reported transient expression assays of citrus, other three types of infiltration buffers were adopted also. The differences between these buffers motivated us to test which infiltration buffer was the best for citrus. After the agro-infiltration with the P2A1 binary plasmids (OD_{600}=0.8) in the Mexican lime leaves, no abnormal symptom like browning or abscission of leaves, reported by Figueiredo *et al.* (2011), was observed. The YFP fluorescence could also be visualized on all of the leaves infiltrated with five infiltration buffers at 3 DPA. The highest YFP fluorescence level was achieved when the P2A1 binary plasmids were suspended in the buffer 1 (Fig. 3), and the lowest level appeared in the buffer 3. The other three infiltration buffers 2, 4, and 5 exhibited no significant difference and remained in the middle levels between buffers 1 and 5. Then the infiltration buffer 1 was chosen for the further experiments.

3.3. Effects of the AS and MES concentrations on the transient transformation assay in Mexican lime leaves

In this study, different concentrations of AS (0, 100, 150, 200, and 250 μmol L^{-1}) were added in the infiltration buffer 1. YFP fluorescence observation results showed that AS could enhance the transient transformation of YFP fluorescence when AS concentration in the buffer 1 increased from 0 to 200 μmol L^{-1} (Fig. 4-A). That is, the *yfp* gene expression could be enhanced when AS concentration in the buffer 1 increased from 0 to 200 μmol L^{-1}. YFP fluorescence signals were weak in the leaves when the infiltration buffer 1 was prepared without AS. The highest YFP fluorescence level was observed when AS concentration in the buffer 1 was 150 μmol L^{-1}, and maintained up to 200 μmol L^{-1}, which was 3.8 times higher than that when the buffer 1 was without AS. However, the YFP fluorescence level decreased when AS concentration in the buffer 1 was up to 250 μmol L^{-1}, which was comparable to its level when AS concentration in the buffer 1 was 100 μmol L^{-1}.

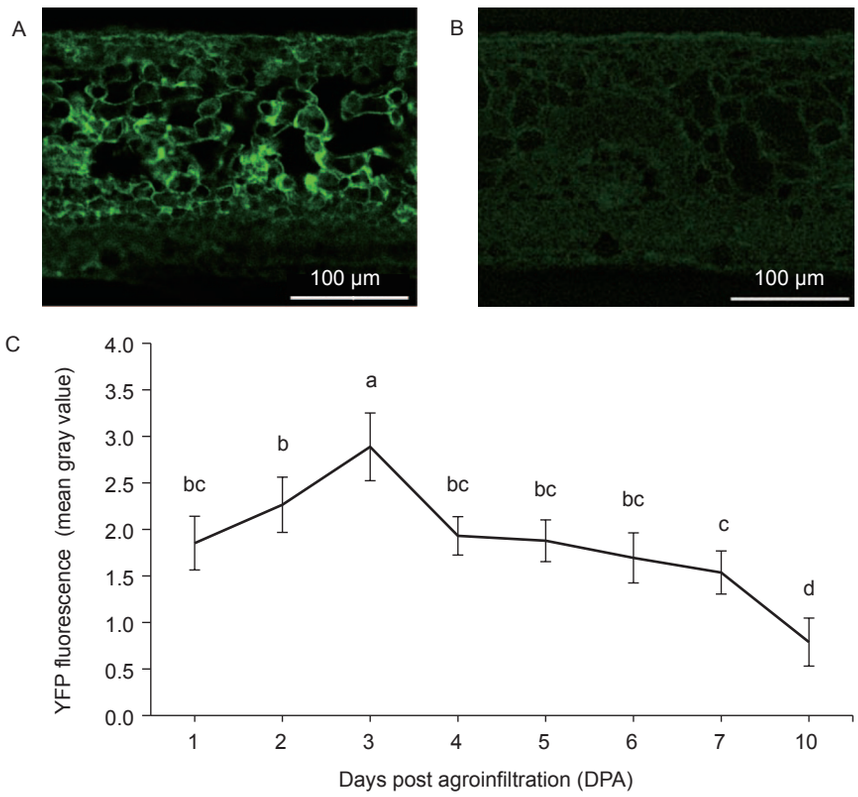

Fig. 2 Gene transient expression in leaves of Mexican lime. A, YFP expression after agro-infiltration with P2A1. B, no YFP expression after agro-infiltration with P1C35. C, trend of the YFP fluorescence transient expression with time. *Agrobacterium*-containing vectors were suspended using the infiltration buffer 1 at a concentration of OD_{600}=0.8. The mean values with the same letters were not significantly different based on Duncan's multiple range test (P=0.05). The error bar means standard error (SE). The same as below.

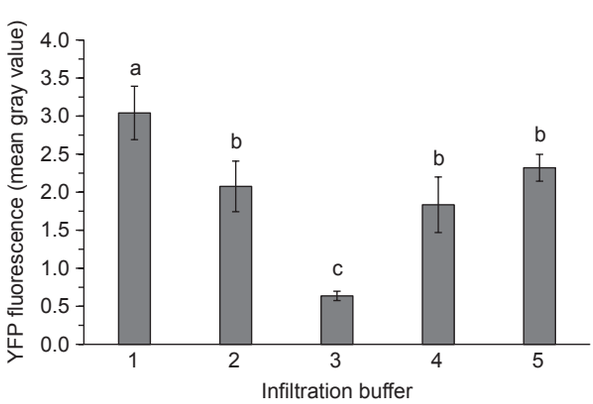

Fig. 3 Effect of infiltration buffers on transient *yfp* gene expression.

Ogaki *et al.* (2008) found that MES could influence the *Agrobacterium*-mediated genetic transformation of *Lilium*×*formolongi*. Therefore, different MES concentrations (5, 10, 15, and 20 mmol L^{-1}) were added in buffer 1 to test its effects on citrus transient analysis. 10 to 15 mmol L^{-1} of MES together with 200 μmol L^{-1} of AS in the buffer 1 resulted the highest YFP fluorescence level (Fig. 4-B). However, 15 mmol L^{-1} of MES significantly reduced the transient gene expression efficiency when the AS concentration reduced to 150 μmol L^{-1}. Compared with the original infiltration buffer, the improved infiltration buffer 1 (15 mmol L^{-1} MES, 10 mmol L^{-1} $MgCl_2$, and 200 μmol L^{-1} AS) had higher efficiency.

3.4. Effect of the *Agrobacterium* concentration on the transient gene expression

This study analyzed the effect of five different densities (OD_{600}=0.1, 0.4, 0.8, 1.0, and 1.2) of bacterial suspension on the YFP fluorescence level in the Mexican lime leaves. The results indicated varied transient *yfp* gene expression levels between leaves with different *Agrobacterium* cell densities (Fig. 5). An increasing trend of YFP expression was observed at an increasing *Agrobacterium* concentration ranging from 0.1 to 0.8. The higher density (OD_{600}=1.0–1.2) failed to promote gene expression. Then, OD_{600}=0.8 was adopted as the bacterial suspension density that was chosen for infiltration throughout the experiments reported in this paper.

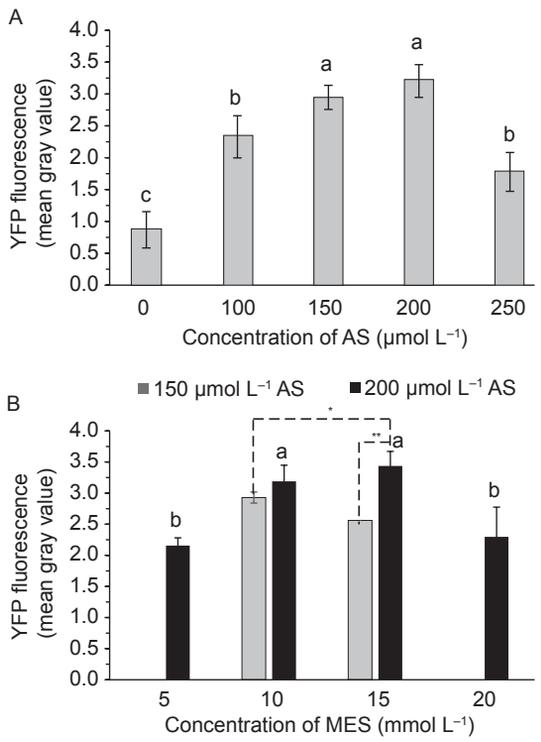

Fig. 4 Effect of different acetosyringone (AS) and 2-(N-morpholino) ethanesulfonic acid (MES) concentrations on transient *yfp* gene expression, based on infiltration buffer 1. A, five different AS concentrations. B, four different MES concentrations. *t*-test was generated by ANOVA and denoted as follows: *, $P<0.05$; **, $P<0.01$. The same as below.

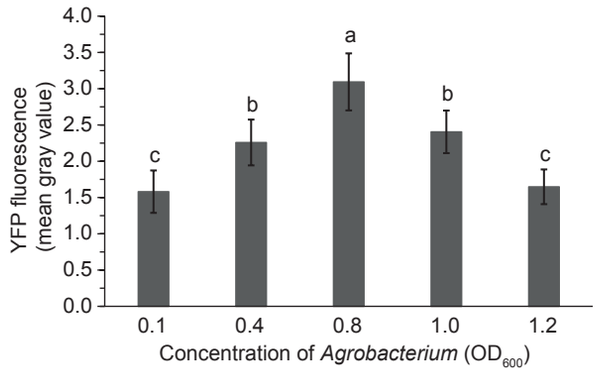

Fig. 5 Effect of different *Agrobacterium* concentrations on transient *yfp* gene expression.

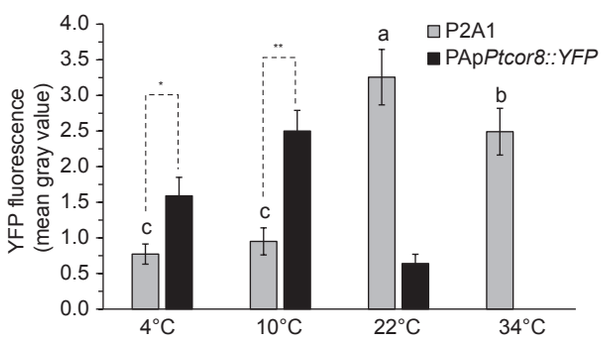

Fig. 6 Effect of different temperatures on transient *yfp* gene expression. The *Agrobacterium*-containing vectors P2A1 and PAp*Ptcor8*::*YFP* were suspended using the improved infiltration buffer 1 (15 mmol L^{-1} MES, 10 mmol L^{-1} $MgCl_2$, and 200 μmol L^{-1} AS) at the concentration of OD_{600}=0.8, respectively.

3.5. Effect of temperature on the transient transformation assay

We examined the effect of different temperatures (4, 10, 22, and 34°C) on the YFP levels in the Mexican lime leaves that were infiltrated with the P2A1 vector containing the CaMV 35S promoter. The highest expression among the examined temperatures was recorded at room temperature (22°C, Fig. 6). The YFP levels were significantly reduced by decreasing or increasing the room temperature. The decreased degree of transient expression, which was caused by low temperature (10°C), was 3.01 times higher than that caused by high temperature (34°C). No significant difference between 4 and 10°C was observed because of the low fluorescence activity at low temperature. These observations indicated that 22°C was the most ideal temperature for transient expression in citrus.

Meanwhile, we also analyzed the transient gene expression from a p*Ptcor8* promoter at low temperature (Fig. 6). The *yfp* gene was expressed at high levels under cold stress conditions, and the level of expression under severe stress (4°C) was lower than that under slight stress (10°C). Compared with the control leaves (P2A1 containing a CaMV 35S promoter), the YFP fluorescence level of the leaves that were infiltrated with PAp*Ptcor8*::*YFP* containing a p*Ptcor8* promoter increased by 2.06 times ($P<0.05$) when the incubation temperature was 4°C and by 2.63 times ($P<0.01$) at 10°C. Therefore, this finding indicates that p*Ptcor8* improves the transient expression efficiency at low temperature.

3.6. Effect of different leaf development stages

To determine the optimal leaf development stage for transient expression, we tested Mexican lime leaves of four ages: tender leaves (two-week-old first leaves developed after pruning back the branches), young leaves (three-week-old first leaves developed after pruning back the branches), intermediate-aged leaves (four-week-old first leaves developed after pruning back the branches), and mature leaves (more than five-week-old leaves following pruning). The collected YFP fluorescence data suggest that the age of the infiltrated leaves can influence the transient expression

(Fig. 7). Furthermore, the accumulation level increased with the gradual maturity of the leaf but significantly decreased once the leaf matured. Thus, it was more optimal to choose intermediate-aged leaves for the transient gene expression.

3.7. Adaptation of the transient expression assay for citrus genotypes

To analyze the influence of genetic factors on the transient expression efficiency, the YFP expression levels were investigated in the following genotypes: trifoliate orange of the *Poncirus* genus in Swingle's system, Changshou kumquat of the *Fortunella* genus, and six types from the *Citrus* genus, including Mexican lime, Duncan grapefruit, seedless ponkan, Dahong sweet orange, Ovale di Sorrento lemon, and Chandle pummelo. The results showed YFP fluorescence intensities were higher in plants from the *Poncirus* and *Fortunella* genus than that from the *Citrus* genus (Fig. 8). Various types in the *Citrus* genus were found to have the same influence on the transient expression except for Ovale di Sorrento lemon, which was detected at a lower fluorescence level. The assay established here could suitably cause transient expression in all of the genotypes used in this study.

4. Discussion

Various infiltration buffers were used in previous studies. This study found that the different infiltration buffers caused different transient expression efficiencies, and infiltration buffer 1 was optimal for citrus. The highest efficiency for this infiltration buffer could be obtained in *Nicotiana benthamiana* by increasing the AS concentration at least three times (Wydro *et al.* 2006). Although changes in the AS and MES concentrations could help improve the transient expression efficiency in citrus, these changes were little.

Kim *et al.* (2009) showed that the effect of *Agrobacterium* concentration on the transient expression efficiency was determined by the genetic background of the *Agrobacterium* strain. In this work, we observed that plants were also a determinant. In the transient expression of *Arabidopsis* (Kim *et al.* 2009) and citrus, the optimal concentrations for the EHA 105 strain were 0.3 and 0.8, respectively, whereas *Agrobacterium* concentration (OD_{600}=0.1–1.5) indicated no difference in *N. benthamiana* (Wydro *et al.* 2006). This observation indicated that optimization of the *Agrobacterium* concentration for the maximum transient efficiency was required for each plant species.

The *Agrobacterium*-mediated transient expression efficiency was also influenced by the plant's physiological factors (Wroblewski *et al.* 2005). The physiological condition of the leaves varied with different developmental stages. Published studies on the agro-infiltration of leaves reported that young leaves have a higher expression than older leaves (Wroblewski *et al.* 2005; Santos-Rosa *et al.* 2008; Figueiredo *et al.* 2011). This study further extended the previous observations by comparing more leaf developmental stages. And it also showed that leaf approaching full maturity was the optimal stage for *Agrobacterium*-mediated transient expression. Furthermore, temperature was an important environmental factor because temperature stress certainly reduces the physiological activities of the leaf cells. Dillen *et al.* (1997) reported that post-infiltration incubation at 22°C had a higher transient efficiency compared with that at 29°C. This paper found that transient efficiency was reduced by both hot and cold stress. However, environmental conditions have never been mentioned in transient expression assays of citrus described in previous studies (Figueiredo *et al.* 2011; Sendı´n *et al.* 2012; Jia and Wang

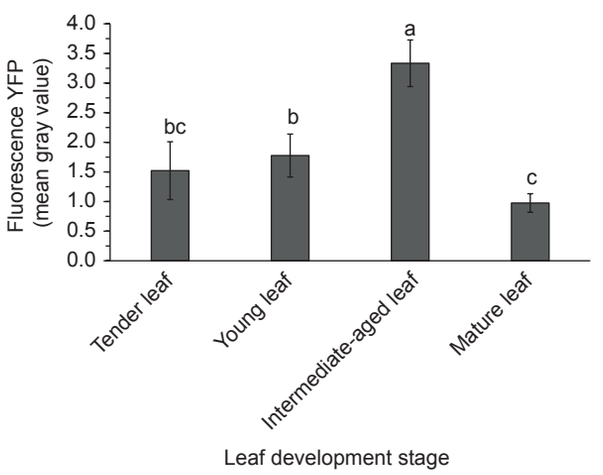

Fig. 7 YFP accumulation in leaves of Mexican lime at different development stages.

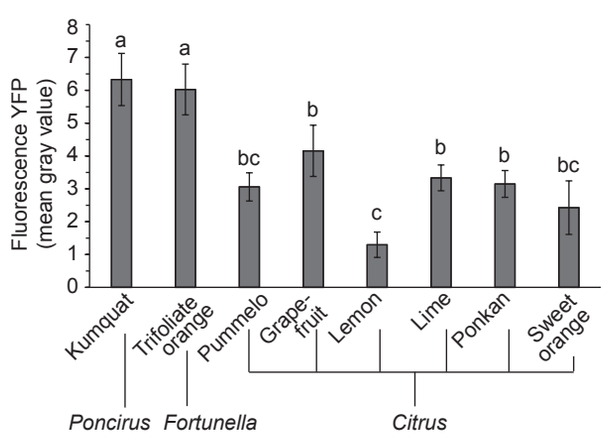

Fig. 8 Effect of different citrus genotypes on transient *yfp* gene expression.

2014a, b). These results indicated that it is necessary to consider the optimization of the physiological condition of plants to improve the *Agrobacterium*-mediated transient expression assay.

Our results suggested that the enhancement of *Agrobacterium*-mediated transient expression also involved optimization of the citrus genotypes. This strategy was similarly effective in the transient expression of other plants, including potato (Bhaskar *et al.* 2009), grapevine (Zottin *et al.* 2008), and *Arabidopsis* (Wroblewski *et al.* 2005). However, the transient expression efficiency of citrus cultivars is not in accordance with the genetic transformation efficiency described in the research of Dutt *et al.* (2009). For example, Duncan grapefruit exhibited a higher efficiency of genetic transformation than trifoliate orange and a lower efficiency of transient gene expression. Jung *et al.* (2015) suggested that the overall level of transient gene expression is determined by protein synthesis in addition to T-DNA transfer, which determines the genetic transformation efficiency. Thus, the consistency observed in the research of Bhaskar *et al.* (2009) may not be absolute between the transient expression efficiency and genetic transformation efficiency causing by plant genotype.

Low temperature is the main factor limiting the development of the citrus industry in subtropical areas. A number of low-temperature stress responsive genes have been isolated in citrus (Peng *et al.* 2012; Sahin-Cevik 2013). Previous research identified the expression level of these genes under cold stress. However, it is lack of in *vivo* proof regarding the genes improving the tolerance of citrus to cold stress. Generally, the promoters of stress-inducible genes could strongly increase gene expression under stress conditions (Kasuga *et al.* 1999; Lin *et al.* 2015). The *Ptcorp* gene is up-regulated by cold stress (Long et al. 2012), and *pPtcor8* has the same ability which is determined using the transient expression assay in this study. This ability possibly results from special elements in the promoter region, such as the low-temperature responsive element, ABA-responsive element (Yamaguchi-Shinozaki and Shinozaki 2006), TC-rich repeat (Wang *et al.* 2011), and MYB binding site (Shi *et al.* 2011). These elements are considered to be responsible for the regulation of cold stress-related gene expression. Compared with the constitutive CaMV 35S promoter, p*Ptcor8* overcame the negative influence of low temperature on transient gene expression and made it possible to use the transient expression assay to identify low-temperature stress responsive genes.

5. Conclusion

Agrobacterium-mediated transient expression is a rapid process for gene expression analysis. We observed transient gene expression in citrus on the first day after *Agrobacterium* infiltration. Gene expression was maintained until our final observation. We established an optimal protocol for Mexican lime by investigating the effects of various factors (e.g., infiltration buffer, *Agrobacterium* concentration, plant incubation temperature, and leaf development stage) on the transient expression efficiency. And this protocol successfully applied to other citrus genotypes. The highest efficiencies were achieved in trioliate orange and in Changshou kumquat. This study provides an empirical basis for the selection of optimization strategies in *Agrobacterium*- mediated transient expression in citrus. Furthermore, our work have relevance for the development of applications to characterize cold-tolerance genes *in vivo*.

Acknowledgements

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