Original article

A protoplast-based transient expression system for melon (*Cucumis melo* L.)

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Summary

Melon (*Cucumis melo* L.), an important horticultural and economic crop, is widely cultivated all over the world. Functional analysis of genes related to important agronomic traits will greatly promote the development of basic research in melon. The transient transformation system is a convenient method to analyze gene function *in vivo* and has been developed and applied in many species. Our objective was to develop an effective transformation system in melon. By comparing cotyledons and first true leaves, and balancing protoplast yield and protoplast vitality, optimal sampling tissue and enzymolysis time were obtained. Through analyzing effects of main parameters including PEG concentration, plasmid DNA amount and transfection time, on transformation efficiency, the optimal procedure for PEG-mediated transformation was obtained. Moreover, the subcellular localization of a GLK2-like transcription factor CmAPRR2 was analyzed by using this system. Results showed that first true leaves could yield more isolated protoplast than did cotyledons. The optimal time of enzymatic digestion is 6 h. The PEG concentration and plasmid DNA amount have a greater effect than incubation time on transformation efficiency. The peak transformation efficiency is approximately 47% when PEG4000 concentration is 40% and 20 µg plasmid DNA is injected. In addition, subcellular localization showed that CmAPRR2 is a nuclear-localized protein. This research shows that the developed transient expression system based on protoplast isolation from first true leaves and PEG-mediated transformation is efficient and can be applied for functional gene analysis research in melon.

Keywords

melon (*Cucumis melo* L.), protoplast isolation, PEG-mediated transformation, subcellular localization, CmAPRR2

Introduction

Melon (*Cucumis melo* L.) is a diploid plant species ($2n = 2x = 24$) with small genome (about 450M) and rich phenotypic diversity, which make it a valuable material for biological research (Arumuganathan and Earle, 1991; Yano et al., 2020). It belongs to the Cucurbitaceae family, which also includes cucumber (*Cucumis sativus* L.) and watermelon (*Citrullus lanatus*). In recent years, many genes associated with important agronomic traits in melon were identified along with the progress in genome sequencing, resequencing,

Significance of this study

What is already known on this subject?

- The protoplast-based and PEG-mediated transformation is a convenient and effective transient expression system for functional analysis, such as gene expression, subcellular localization, protein intracellular transport, protein interaction and promoter activities.

What are the new findings?

- By optimizing protoplasts isolation and several key parameters in PEG-mediated transformation, an efficient protoplast-based transient expression system was developed. The application of this system on subcellular localization was investigated by transfecting a rind color gene CmAPRR2.

What is the expected impact on horticulture?

- This study provided an effective transient transformation method that assists functional genomics research on melon.

and transcriptomics, such as CmACS7 and CmWIP1 for sex determination (Boualem et al., 2008; Martin et al., 2009a), CmAPRR2 and CmKFB for rind color (Oren et al., 2019; Feder et al., 2015), CmOr for flesh color (Tzuri et al., 2015), and CmPH for fruit acidity (Cohen et al., 2014). However, due to lacking an efficient genetic transformation system in melon, the biological functions of these genes remain unclear. Although some progress has been made in recent years in stable genetic transformation in melon, there remain many challenges such as limited varieties and genotype, difficulty in differentiation and regeneration, time-consuming and laborious, and low transformation efficiency (Galperin et al., 2003; Akasaka-Kennedy et al., 2004; Bezirganoglu et al., 2014; García et al., 2017; Bezirganoglu, 2018; Hooghvorst et al., 2019).

Transient expression system is a rapid and efficient expression method compared to stable genetic transformation (Yoo et al., 2007; Zhang et al., 2011). Owing to convenient operation and high-throughput transformation, transient expression system has become one of the most efficient techniques for functional gene analysis, such as gene expression (Abel and Theologis, 1994), subcellular localization (Marion et al., 2008), protein intracellular transport (Vogel et al., 2007), protein interaction and promoter activities (Martin et al., 2009b; Agius et al., 2005).

Protoplasts are cells with removed cell walls but intact plasma membrane and intercellular organelles, which offer a perfect cell-based experimental space for exploring pro-

tein function (Davey et al., 2005). With various ways, macromolecules such as DNA, RNA and proteins can be sent into protoplasts. *Agrobacterium tumefaciens*-mediated infiltration (Zottini et al., 2008), biolistic bombardment (Wang and Jiang, 2011) and PEG-mediated protoplast transfection (Miao and Jiang, 2007) are commonly used for transient transformation. Compared with biolistics and agro-infection, PEG-mediated transformation is more convenient and effective. This system has been performed in many plants, such as *Arabidopsis* (Abel and Theologis, 1994), rice (*Oryza sativa* L.) (Hiei et al., 1997), maize (*Zea mays* L.) (Cao et al., 2014), strawberry (*Fragaria × ananassa* Duch) (Gou et al., 2020), cucumber (*Cucumis sativus* L.) (Huang et al., 2013), and so on. However, research about the isolation of protoplasts and the establishment of transient transformation system is rare in melon.

Here, we develop a relatively effective protoplast-based and PEG-mediated transient transformation system with melon true leaves. Several critical factors including tissue material, enzymatic digestion time, PEG concentration, plasmid amount and transfection time are investigated. In addition, we utilize this system to investigate the subcellular localization of CmAPRR2 which is a GLK2-like transcription factor (Oren et al., 2019). Finally, this transient expression system is considered convenient and efficient for future molecular research.

Materials and methods

Plant materials

Cantaloupe M7 used in this research was kindly provided by Prof. Feishi Luan of Northeast Agricultural University. Seeds were soaked at 30°C for 24 h and then germinated on nutrient medium in a climate chamber under 16-h light/8-h dark with the humidity of 60%.

Protoplast isolation

7-days old (fully expanded) cotyledons and 14-days old leaf (the first true leaf) were utilized for protoplast isolation. The acquisition of protoplast was performed as described previously with some modification (Huang et al., 2013). The enzyme solution contained 1.5% (w/v) cellulase R-10, 0.4% (w/v) macerozyme R-10, 0.4 M mannitol, 20 mM MES and 10 mM CaCl₂. Firstly, the enzyme solution was bathed in 55°C water for 10 min and then cooled down. 0.1% (w/v) bovine serum albumin (BSA) was added into the solution and the pH was adjusted to 5.8. Flesh cotyledons and true leaves were divided into two parts along the main vein and then were cut into fine strips. These strips were rapidly transferred to enzymolysis solution (~0.5 g 10 mL⁻¹), and then vacuum infiltrated for 30 min under dark. The solution was shaken in darkness at 40 r min⁻¹. To investigate the optimized enzymolysis time, the tissues were incubated for 2, 4, 6, and 8 h.

After different digestion time, an equal volume of W5 solution (2 mM MES, 154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, pH 5.8) was added into the solution, mixed gently, and then filtered with 200 mesh (75 μm) sieves. The mixture was centrifuged at 106×g for 2 min to obtain the pellet protoplasts. The protoplasts were washed with W5 once and then 200 μL W5 was added to resuspend the protoplasts. With a hemacytometer, the W5 resuspended protoplasts were counted under the microscope to calculate protoplast yield. Protoplast yield was calculated as follows: protoplast yield (protoplasts g⁻¹ FW) = total number of the protoplasts in enzymolysis (protoplasts)/fresh weight of the samples used (g FW).

FDA staining

5 mg mL⁻¹ FDA stock solution was added into the W5 resuspended protoplasts to the final concentration of 50 μg mL⁻¹. After 5 min incubation in the dark, W5 solution was added to wash away unbound FDA molecules on the cell surface twice and 200 μL W5 solution was added to resuspend the protoplasts and protoplast viability was calculated. Protoplast viability (%) = (fluorescent protoplast number / total protoplast number) × 100%.

Transient transfection

The resuspended protoplasts were first placed on ice for 30 minutes and then removed the supernatant. The purified protoplasts were resuspended with 1 mL MMG solution (4 mM MES, 0.4 M mannitol, 15 mM MgCl₂, pH 5.8) for the plasmid transfection (about 4~5 × 10⁵ protoplasts). Several amounts of plasmids (total content: 5 μg, 10 μg, 15 μg, and 20 μg) were added into 100 μL protoplasts and mixed gently. 110~130 μL PEG4000-Ca²⁺ solutions [(20%, 30%, 40% and 50%, w/v) PEG4000, 0.2 M mannitol and 100 mM CaCl₂] were added and mixed carefully to induce plasmid transfer. To get the best transfection time, the mixture was stand at 25°C for 5, 10, 20 and 30 min in the dark. After that, 650 μL W5 solution was added to terminate the transfection and then centrifuged at 106×g for 5 min. Finally, the protoplasts were resuspended in 750 μL WI solution and were incubated at 25°C for 20 h in the darkness. Microscopic observation was operated after centrifuging protoplasts at 106×g for 5 min, then resuspended with 50 μL WI solution. Fluorescence was observed using a confocal laser microscope and the transformation efficiency was calculated with randomly 6~8 fields of view: transformation efficiency (%) = (fluorescent protoplast number in view/total protoplast number in view) × 100%.

Plasmid construction

To monitor the transfection system effect, we used a control plasmid pAN580 which carries a GFP signal for transformation. In addition, we constructed a CmAPRR2 (MELO3C003375) protein fused the 5' end of GFP to explore

TABLE 1. The effect of material source on protoplast yield. Date is given as means ± SD (n≥3 biological replicates). The significant differences were evaluated by Student's *t* test.

Enzymolysis time (h)	Protoplasts yield (10 ⁵ g ⁻¹)		P
	Cotyledon	First true leaf	
2	5.3± 2.1	30.6± 2.4	0.089
4	14.0± 4.0	157.5±10.5	0.0047
6	31.0± 6.6	1,237.5±60.4	0.008
8	86.7±15.3	2,125.0±57.0	2.27E-07

FIGURE 1. Protoplast isolation of cotyledon and first true leaf. A. The complete (left) and shredded (right) phenotype of cotyledon (upper) and the first true leaf (lower) of M7. B. Enzyme solution after vacuum infiltration. C. Enzyme solution after 6 h of digestion. D. Isolated protoplasts of cotyledon (upper) and first true leaf (lower) after 6 h enzymolysis.

FIGURE 2. Protoplast yield and viability of cotyledon and first true leaf. A–B. The effect of enzymolysis time on protoplast yield and viability from cotyledons (A) and first true leaves (B). The histograms indicate the number of protoplast; the lines indicate the ratio of viable protoplast. C. Protoplasts from true leaves after 6 h of enzymolysis without (left) and with (right) FDA staining. D. The isolated protoplasts from first true leaves after 6 h (left) and 8 h (right) enzymolysis. The red arrow indicates the ruptured protoplast. Data is given as means $\pm$ SD ($n \geq 3$ biological replicates). Bars represent standard errors. These letters (lowercase letters for protoplast number and capital letters indicate the ratio of viable protoplast) represent statistical differences ($P \leq 0.05$) among samples according to Duncan's multiple range tests.

its subcellular location. The *CmAPRR2* coding region was amplified and recombined into the pAN580 with ClonExpress®II One Step Cloning Kit (Vazyme). The primer sequences were as below: 580-APRR2-F: 5'-AAGTCCGGAGCTAGCTCTAGAATGGTTTGCCTGCGGACG-3' and 580-APRR2-R: 5'-GCCCTTGCTCACCATGGATCCGGGTGATCTGGAGCCGTCG-3'. The vector pAN580 and *CmAPRR2*-pAN580 were transformed into DH5 α and the plasmid DNA was extracted according to the EndoFree Maxi Plasmid Kit (Tiangen). Before the transformation, the plasmid was concentrated to 1 $\mu\text{g } \mu\text{L}^{-1}$ with a rotary evaporator.

Results

Isolation of protoplasts from cotyledons and true leaves

One-week-old cotyledons and two-weeks-old first true leaves of Cantaloupe M7 were used to detect the effect of enzyme solution (Figure 1A). With the shredding method, the tissues were immediately digested in the enzyme solution. After 6 hours of enzymolysis, the solution color changed from grayish-yellow to yellow-green, indicating that the protoplasts had been dissociated (Figure 1B–C). Microscopic observation confirmed this result (Figure 1D). In addition, the protoplast production of true leaves of Cantaloupe M7 was found to be higher than that of cotyledons after the same enzymolysis time (Figure 1D; Table 1). This result suggested the first true leaves were more suitable material for protoplast isolation than young cotyledons.

Optimized enzymolysis time for protoplast isolation

To optimize the enzymolysis time, the protoplast yield and viability of cotyledons and true leaves were analyzed at 2, 4, 6 and 8 h. The result showed that the protoplast production of cotyledons and true leaves both increased along with increased enzymolysis time (Figure 2A–B). However, the protoplast number derived from cotyledons was 14×10^5 , while that from true leaves was 15×10^6 , which was nearly 10 times higher than that of cotyledons (Table 1). This further confirmed that true leaves were appropriate tissue for protoplast isolation. In addition, the protoplast state and viability were evaluated by FDA staining. For true leaves, the highest protoplast viability occurred at 6 hours enzymolysis although the protoplast production was less than 8 hours (Figure 2B–C). The ratio of abnormal protoplasts after 8 hours was higher than that after 6 hours (Figure 2D), which might cause protoplasts rupture and result in low viability. This phenomenon had also been found in cotyledons enzymolysis (Figure 2A). In general, the optimized protoplasts could be obtained by enzymatic hydrolysis of true leaves for 6 hours.

Effect of PEG4000 concentration, plasmid DNA amount and transfection time on transformation efficiency

To optimize the PEG concentration, plasmid DNA amount and transfection time, transient expression vector pAN580-GFP was used to evaluate transfection efficiency. The result showed that transformation efficiency increased along with increased PEG4000 concentration (10%, 20%, 30% and

FIGURE 3. Influences of PEG concentration, plasmid DNA amount, and transformation time on transformation efficiency. A. The transformation efficiency under different PEG concentrations. The transformation efficiency was calculated after 20 min incubation of 20 μg plasmid and 100 μL protoplasts isolated from the first true leaves. B. The transformation efficiency with different plasmid amounts. The transformation efficiency was calculated with 40% PEG after 20 min incubation. The efficiency was assessed after 20 h cultivation. C. The transformation efficiency at different incubation times. The transformation efficiency was calculated with 40% PEG-mediated transfection of 20 μg plasmid and 100 μL protoplasts isolated from first true leaves. D. The fluorescence of pAN580 plasmid. The efficiency was assessed, and the fluorescence was observed after 20 h cultivation. Data is given as means $\pm$ SD ($n \geq 5$ biological replicates). Bars represent standard errors. The letters represent statistical differences ($P \leq 0.05$) among samples according to Duncan's multiple range tests.

FIGURE 4. Subcellular localization of the CmAPRR2 protein. The white arrow indicates ectopic expression.

40%, initial concentration) under uniform conditions (20 μg plasmid DNA, incubated for 20 min). Additionally, the transformation efficiency with 20%–40% PEG4000 was close to 40%, which was significantly higher than that with 10% PEG4000. The maximum transformation efficiency appeared at 40% PEG4000, approximately 47% (Figure 3A). However, when PEG4000 concentration reached 50%, the transformation efficiency was reduced to 39%. Together, 40% PEG4000 was the most appropriate transformation concentration.

To explore the effect of plasmid DNA on transformation frequency, the total amount of plasmid (5 μg , 10 μg , 20 μg and 30 μg) were transfected into protoplasts with a consistent 1 μg μL^{-1} plasmid concentration, 40% PEG4000 and 20-min incubation. The results showed that when the amount of plasmid was 20 μg , the transformation efficiency reached the peak, approximately 53% (Figure 3B). The transformation efficiency of 5 μg plasmid was higher than that of 10 μg plasmid and when the amount of plasmid reached 30 μg , the transformation efficiency decreased to 35%. In summary, 40% PEG4000 and 20 μg plasmid DNA was the optimized parameter for transient transformation.

To observe the effect of incubation time on transformation efficiency, 40% PEG4000 and 20 μg plasmid DNA were kept consistent. The incubation time was set at 5, 10, 20 and 30 min. The result showed that transformation efficiency of diverse incubation time was generally around 50%, indicating the incubation time had little effect on the transformation efficiency (Figure 3C). Due to the small size (5 kb) and GFP tag contained, pAN580 is commonly used as a control vector for transient expression (Zhang et al., 2015). In this work, pAN580 was used to evaluate transformation efficiency with several key parameters. Consistent with the previous study, fluorescence observation displayed that the pAN580 was located both in the nucleus and cytoplasm, indicating that the protocol for a system of transient expression was effective (Figure 3D).

Application of protoplast-based transient expression system on subcellular localization

According to the annotation, CmAPRR2 (MELO3C003375) is a transcription factor which belongs to the GLK2-like transcription factor family. Previously, we cloned the full length

CDS of CmAPRR2 in the “MR-1” muskmelon. To further confirm the effectiveness of the system, the CDS was fused to the 5' end of the GFP tag in the pAN580 vector. The result showed that CmAPRR2 was located in the nucleus, verifying it a transcription factor (Figure 4). Moreover, fluorescence signals were detected on some punctate structures, which might be due to ectopic expression caused by strong promoter 35S. In short, the transient expression system was an efficient system to study protein localization.

Discussion

Plant protoplasts are widely applied in cell engineering and molecular biology research because they contain complete genetic material and organelles. For example, Sidorov et al. (1987) obtained genetically novel plants with pigment-related by protoplast-mediated somatic hybridization in potato. Several plants like *Arabidopsis* (O'Neill and Mathias, 1993), rice (Yamada et al., 1986), strawberry (Barceló et al., 2019), and wheat (Vasil et al., 1990) have achieved the regeneration by protoplasts isolation and culture, which will be helpful for germplasm preservation and mutant creation. In molecular biology research, protoplast-based transient expression system is frequently used to study protein localization, protein-protein interaction, gene silencing, and gene editing. In model plants such as *Arabidopsis*, tobacco and rice, efficient transient expression systems have been established early (Yoo et al., 2007; Pröls et al., 1988; Chen et al., 2006). In recent years, plants such as cucumber (Huang et al., 2013), tomato (Ruf et al., 2001), and strawberry (Agius et al., 2005) have developed transient systems. Although the transformation efficiency is lower than that of model plants, it avoids mislocalization caused by heterologous expression. However, an effective transformation system has not yet been developed on melon. In this work, we have initially developed an effective protoplast-based transient expression system by selecting optimal tissues and adjusting transformation parameters.

A high yield and viability of protoplasts is a prerequisite for developing a successful transient transformation system. The choice of tissue samples is particularly important. Young and healthy leaves are ideal materials in several plants, such as *Arabidopsis* and maize (Yoo et al., 2007; Cao et al., 2014).

However, Chen isolated many protoplasts from rice stems and leaf sheaths (Chen et al., 2006). We selected cotyledons and first true leaves to separate the protoplasts and found that the yield of protoplasts from true leaves far exceeded that from cotyledons. This suggests that first true leaves are suitable materials. In addition to yield, the protoplast viability and digestion time are also crucial (Jiang et al., 2015). The effective transfection depends on vigorous protoplasts to undergo the shaking and cultivation during transformation. Interestingly, the protoplast viability of cotyledons was higher than that of true leaves at all designated time points in our study (Figure 2A–B). Prolonging enzymolysis time promotes the release of protoplast. However, varied plant species have distinct optimal enzymolysis times. For example, optimal digestion time is 3 h for *Arabidopsis* (Yoo et al., 2007) and rice (He et al., 2016), 4 h for wheat (Jia et al., 2016), 5 h for sugarcane (Wang et al., 2020), and 8 h for cucumber (Huang et al., 2013). In our study, the optimal digestion time was 6 h for melon, and the yields of viable protoplasts were 12.3×10^7 protoplasts g^{-1} fresh weight (Figure 2B). Together, the balance between yield, viability and digestion time must be considered to obtain high-quality protoplasts for transient expression.

For transient expression, transfection procedure is crucial to transformation efficiency. Several key parameters like PEG concentration, transformation time, concentration and amount of plasmid all have a great influence on transfection. As the commonly used transfection reagent, PEG mediates the transfer of plasmid DNA into protoplasts. Accordingly, 10%–50% PEG is recognized as effective transfection concentrations and the optimal concentration varies in the plant species. For example, it is found that 20% is the appropriate concentration for *Arabidopsis* (Yoo et al., 2007), 25% for grapes (Wang et al., 2015), and 40% for cucumber (Huang et al., 2013). Unlike the PEG concentration which has a great influence on transformation efficiency in cucumber, the efficiency of 20%–40% PEG in melon is close, approximately 40%. Similarly, the transfection time has little effect on the transformation efficiency. The 5-minutes incubation pushes the transformation efficiency to nearly 30% and prolonging time has not promoted the transfection more, which implies that the transformation is a rapid process (Figure 3C). Previous studies generally focused on the effect of PEG concentration on transformation efficiency (Huang et al., 2013; Wang et al., 2020). However, among the parameters that we investigated, the total amount of plasmids was the most important factor that determined the transformation efficiency. The transformation efficiency of 20 μg plasmid was significantly higher than that of 5 μg and 10 μg plasmid (Figure 3B). We successfully established a transient expression system in melon, but the transformation efficiency with optimized parameters was only 50%, which was much lower than the model plants such as *Arabidopsis* or rice (Yoo et al., 2007; He et al., 2016).

Conclusion

By optimizing protoplasts isolation and several key parameters in PEG-mediated transformation, an efficient PEG-mediated transient expression system was developed with high-yield protoplast derived from first true leaves and highest transformation efficiency occurred when PEG4000 concentration was 40% and 20 μg plasmid DNA was transfected. This system supplied a convenient method for melon molecular biology but could be improved for transformation efficiency.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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