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Article

Improvement and application of vacuum-infiltration system in tomato

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Abstract

The *Agrobacterium*-mediated transient expression system has been developed and applied to various plants as an alternative to stable transformation. However, its application in tomatoes is still limited due to low expression efficiency. In this study, we describe an improved vacuum-infiltration system that can be used in both tomato fruits and leaves. Notably, this study is the first report of vacuum infiltration in attached tomato fruits. The feasibility of the improved vacuum-infiltration system in Micro-Tom tomato was confirmed by various assays, including multiple fluorescent protein expression analysis, β-glucuronidase activity analysis, and *RUBY* reporter visualization. Subsequently, the improved vacuum-infiltration system was successfully applied to tomato biotechnology research. Herein, a trichome-specific promoter in tomato was identified that can drive the directional synthesis of specific plant natural products (PNPs). Additionally, based on the assessment results of the improved vacuum-infiltration system, we obtained a flavonoid-rich tomato variety through the stable transformation of *AmRosea* and *AmDelila*. In a significant practical application, we successfully synthesized the high-value scutellarin in tomato, which provides an alternative route for the production of PNPs from plants. In addition, the improved vacuum-infiltration system has been demonstrated to be suitable for commercial tomato varieties ('Emerald' and 'Provence') as well. The improved vacuum-infiltration system not only speeds up fundamental and applied research in tomato but also offers an additional powerful tool for advancing tomato synthetic biology research.

Introduction

Tomato (*Solanum lycopersicum* L.) is an important economic crop and the second most cultivated vegetable in the world. It is rich in various high-value secondary metabolites, which make a substantial nutritional contribution to the human diet [1]. Over the past decades, the characterization of tomato gene function has been based mainly on establishing stable transgenic plants. With the development of plant biotechnology research, stable genetic transformation methods have been implemented in several tomato varieties, such as M82, Ailsa Craig, Money Maker, Micro-Tom, etc [2–4]. Nevertheless, a stable genetic transformation method is not suitable for all tomato varieties, especially for some commercially important ones (such as those serving as excellent chassis for plant biotechnology research) as they are recalcitrant to tissue culture and *Agrobacterium*-mediated transformation. In addition, the entire manipulation process remains laborious, time-consuming, and costly. These factors have restricted the development of tomato biotechnology research and tomato synthetic biology research. Therefore, developing fast, efficient, and high-throughput gene characterization strategies can help overcome these challenges.

The *Agrobacterium*-mediated transient expression system was initially applied to *Nicotiana benthamiana* leaves and has now been described in a variety of plant species such as *Populus davidiana* × *P. bolleana* [5], *Caragana intermedia* [6], *Theobroma cacao* [7], *Citrus macrophylla* [8], *Fragaria ananassa* [9], *Artemisia annua* [10], and *Persea americana* [11]. When compared to stable genetic transformation, the vacuum-infiltration system does not involve tedious tissue culture process and enables the results to be presented within 3–5 days, which particularly is suitable for plants with difficult regeneration establishment systems and those with long growth cycles. The vacuum-infiltration system has been widely used for promoter analysis [12], plant-pathogen interaction [13], transcription factor (TF) activity analysis [14], protein–protein interaction [15], protein subcellular localization [16], study of metabolic pathways [17], secondary metabolites production [18], and pharmaceutical proteins production [19]. Also, with the development of plant synthetic biology, vacuum-infiltration system is widely used for screening elements and testing biosynthetic pathways in plants [20]. To date, syringe infiltration is one of the most commonly used methods for the vacuum-infiltration system. However, the transformation efficiency of syringe infiltration depends on plant characteristics, so that thick stratum corneum and wax coat, low stomatal density or small aperture of stomatal pores, complex vein network arrangement, overall fragility of plant tissues, and small cellular gaps within the plant tissue often lead to low efficiency of syringe infiltration [2, 6, 21]. To address these issues, vacuum infiltration is gradually being developed for these species. For instance, an *Agrobacterium*-mediated vacuum

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Beijing, China) at an OD_{600} of 0.8. The *A. tumefaciens* suspension was kept at room temperature for 3 h before infiltration.

Efficient agroinfiltration of tomato leaves and fruits

Well-expanded tomato leaves (9-week-old) were selected for agroinfiltration. Prior to agroinfiltration, *A. tumefaciens* suspension was supplemented with Silwet L-77 (1/5000 v/v, Coolaber, China) and transferred into a 50-mL sterile centrifuge tube. As shown in Fig. 1a, the leaves were immersed in the centrifugal tube, which is secured in the clamp of the iron support stand. Subsequently, the vacuum transformation device was used for agroinfiltration, which was designed and manufactured by our team in collaboration with Shanghai Wonbio Biotechnology Co., Ltd (Model: PTS-50, Shanghai, China, Fig. 1b). In this study, plant tissues were kept in the vacuum chamber at 100 mbar for 20 s to ensure that the air inside the tissues was completely expelled. Finally, the release valve was opened (turn counter-clockwise) to release pressure, allowing *Agrobacterium* to penetrate the interior of the plant tissue, and the above operation was repeated twice. Before the corresponding experimental analyses, infiltrated plants were incubated in a dark room for 24 h and then in a greenhouse for 3–5 days (Fig. 1c and d). The abovementioned process is also applicable to fruits, with the exception that the fruits need to be pierced with a needle prior to agroinfiltration to facilitate the infiltration of *A. tumefaciens*.

Histochemical GUS activity assay

The GUS staining assay was performed by incubating tissues in GUS staining solution (1/1000 v/v Triton X-100, 0.1 mol L^{-1} sodium phosphate buffer, pH 7.0, 2 mM X-Gluc, TargetMol, USA) for 12 h at 37°C in dark conditions. Tissues were then destained with an ethanol solution at 60°C to remove the chlorophyll. Stained tissues were photographed using a Leica microscope (Olympus, Tokyo, Japan) coupled with an insight digital camera.

Confocal microscopy

The infiltrated leaves were used for fluorescence observation. An upright confocal microscope, the Leica TCS SP5-II (Leica Microsystems Inc., Bufalo Grove, IL, USA), was used to visualize the protein expression intensity. The excitation and emission detection wavelengths for YFP, CFP, EGFP, and mCherry were set as follows: 514 nm–527 nm, 456 nm–480 nm, 484 nm–510 nm, and 587 nm–610 nm, respectively.

Total flavonoids extraction and content determination

Total flavonoids were extracted according to the method described by Yao *et al.* [17], with some modifications. In brief, 0.02 g of lyophilized powder was sonicated in 1 mL of 100% methanol for 30 min and then centrifuged at 12 000 *g* for 10 min. The supernatant was transferred to a new tube, and the precipitate was resuspended with 1 ml of 100% methanol and centrifuged again (12 000 g, 10 min). The combined supernatants (2 mL) were used for flavonoid chromogenic reactions and content determination assays. For TFC, a plant flavonoids assay kit (A142–1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used.

UPLC-MS analysis of scutellarin

A total of 0.05 g of lyophilized tomato leaves were ground and then extracted twice with 100% methanol. The combined supernatants (2 mL) were filtered by 0.22 μm syringe filter (Sartorius, Göttingen, Germany) and analysed by UPLC-MS (Acquity UPLC I-class/VION IMS QTOF, Waters Corp., Milford, MA, USA). The separation of the target substances was achieved on a BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters), which was kept at 45°C in the column oven. For the gradient elution procedure, 0.1% formic acid in ddH_2O was used as solvent A, 0.1% formic acid in MeCN as solvent B, and the flow rate was set at 0.4 mL/min. The elution process is as follows: 0–3 min, 5–20% B; 3–10 min, 20–100% B; 10–12 min, 100% B; 12–15 min, 100–95% B; 15–19 min, 95% B. For the detection of scutellarin, 2 μL of sample was injected, and the wavelength was set to 335 nm. The setting of the mass spectrometry parameters and the data analysis were performed according to Xia *et al.* [65].

Accession numbers

AmRosea (KP311682.1), *AmDelila* (M84913.1), *AtMYB12* (At2g47460), *AtMYB75* (AY519563.1), *SmMYB1* (KF727476.1), *ZmLC* (M26227.1), *ZmC1* (M37153.1), *EbFNS II* (KC521362), *EbF6H* (KU237240), *EbF7GAT* (KU237242), *SlMYB114* (Solyc10g086260), *SlMYB12* (Solyc01g079620.2.1), *SlMYB75* (Solyc10g086250.2.1), *SlAN11* (Solyc03g097340), *SlAN2-Like* (Solyc10g086290).

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Author contributions

X.Y. and K.T. conceived and designed the research; H.L., X.Y., Y.Z., and X.H. performed the experiments; A.T., L.L., W.H., K.W., J.S., and Q.M. analysed the data; X.Y. and A.T. wrote the manuscript; all the authors revised the manuscript.

Data availability

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary data.

Conflict of interest statement

The authors declare no competing interests.

Supplementary data

Supplementary data is available at *Horticulture Research* online.

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