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Araştırma Makalesi

Domateste Agrobacterium Aracılı Geçici Gen İfadesi için Vakum Destekli Düzenek Tasarımı

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ÖZ

Hedef genleri bitkilere aktarmak için çeşitli yöntemler kullanılmaktadır. Bunlardan, *Agrobacterium* aracılı transformasyonda, bitkiye genler kalıcı olarak ikili vektör sistemi ile aktarılabilmektedir. Ancak bu yöntem ile transgenik bir bitki elde etmek için birkaç aylık bir zamana ihtiyaç duyulduğu düşünüldüğünde, süreç oldukça yoğun iş gücü, emek ve zaman gerektirmektedir. Geçici gen ekspresyonu (örn., vakum-infiltrasyon) sistemleri, özellikle zamanla ilgili dezavantajların üstesinden gelmek için, kalıcı transformasyona alternatif olarak kullanılabilmektedir. Ancak bu yöntemde, vakum odaları gibi pahalı ekipmanlara ihtiyaç duyulmaktadır. Bu çalışmada, domates fidelerinde bir raportör gen olan modifiye yeşil floresan proteinin (mGFP) geçici ifadesini gösteren bir vakum-infiltrasyon protokolü rapor edilmektedir. Çalışma sonucunda, uygun fiyatlı ekipmana sahip temel bir deney düzeneği (özel yapım bir büyüme odası dahil) ile 6 dakika boyunca ve sadece 200 milibarlık bir vakum uygulanarak tüm yaprağa ilgili geni taşıyan *Agrobacterium* solüsyonunun infiltre edilebileceği, böylece domateste geçici gen ifadesinin sağlanabileceği gösterilmiştir. Sonuç olarak, T-DNA, PCR ile tespit edilmiş, mGFP'nin varlığı hem SDS-page analizi ile analitik olarak hem de floresan mikroskobu ile görsel olarak belirlenmiştir.

Anahtar Kelimeler: Bitkide geçici gen ifadesi, vakum infiltrasyonu, Agrobacterium aracılığıyla gen aktarımı, *Solanum lycopersicum*

The Basic Experimental Setup for the Vacuum-assisted, Agrobacterium-mediated Transient Expression in Tomato

ABSTRACT

Several methods are available for use to deliver the gene of interest (GOI) into plants. Among these, *Agrobacterium*-mediated transformation utilizes binary vector systems to achieve the stable transformation of plants. However, this process is labor-intensive and time-consuming as several months are needed to obtain from a true transgenic plant. Transient gene expression (e.g., vacuum-infiltration) systems were offered as an alternative over stable transformation, specifically to overcome time-related drawbacks. However, this method requires expensive equipment such as vacuum chambers. In this study, we report a vacuum-infiltration protocol for the transient expression of a reporter gene, modified green fluorescent protein (mGFP), in tomato seedling. With a basic experimental setup (including a custom-built growth chamber) with affordable equipment, we showed that the entire leaf can be infiltrated by applying a mere 200 millibar vacuum for 6 minutes, and thus the transient expression can be achieved in tomato plant, evidenced by PCR-based detection of the T-DNA, detection of mGFP both analytically with SDS-page analysis, and visually by the images acquired by fluorescence microscopy.

Key words: Plant transient expression, vacuum infiltration, Agrobacterium-mediated gene delivery, *Solanum lycopersicum*

INTRODUCTION

A common practice in modern biotechnology is the employment of virulent *Agrobacterium* strains to deliver the gene of interest (GOI) into plants (Hellens et al., 2005). Although several other methods (i.e., electroporation, biolistic methods, etc.) are available for use, these methods often fall short as electroporation can cause cell damage (Young and Dean, 2015), potentially harm the ability of regeneration, and biolistic methods simply require expensive equipment. In *Agrobacterium*-mediated transformation, using binary vector systems, researchers are able to hijack virulent *Agrobacterium* species machinery and perform the molecular transformation of plants (Mardanova et al., 2017). However, this process is time-consuming, and several months (Van Eck et al., 2019) are needed to obtain a transgenic plant . While true-transgenic plants are essential for some studies, the required time is a drawback for other studies (i.e., recombinant protein production). Transient gene expression is offered as an alternative over stable integration, specifically to overcome this problem.

One method for transient expression of plants is the infiltration of intercellular space in plant leaf by a suspension containing a virulent *Agrobacterium* strain armed with a GOI, hence the term *Agroinfiltration* is used for this technique. Transient gene expression by agroinfiltration allows plants to accumulate desired recombinant protein after the first couple of days (Sparkes et al., 2006; Sheludko, 2008) and thus, the time required to express GOI is no longer an issue. Two methods are employed to perform the Agroinfiltration: *i)* the syringe infiltration and *ii)* the vacuum infiltration. For the former, a simple needless-syringe is placed at the abaxial side of the leaf, by using the index finger as a counter-pressure, *Agrobacterium* suspension is injected into the intercellular space (Liu et al., 2018). This method requires an expert to execute the infiltration, as it is common to deal extensive damage to the tissues. In addition, certain types of leaves such as tomato are recalcitrant to syringe-infiltration regardless of the expertise. While single infiltration point is enough for tobacco leaves, multiple infiltration points are required for tomato leaves. Also, specific equipment (i.e., face protector) are needed to protect both the scientist and the environment from bacterial contamination. For the latter, typically, a vacuum chamber (e.g., Abbess Instruments) is used to apply vacuum to the plant tissue submerged into the *Agrobacterium tumefaciens* suspension. With this method, following the re-pressurization after the depressurization, leaves are infiltrated by the *Agrobacterium* suspension (Mariashibu et al., 2013). The drawback of this method is the necessity of expensive equipment.

In addition to the delivery method, certain factors such as ambient temperature, light source, bacterial strain, and density can influence the transient expression (Norkunas et al., 2018). Often growth rooms are used to control these variables, which are expensive to build and maintain, not immediately accessible to most researchers. Furthermore, virulent *Agrobacterium* suspension can easily contaminate the growth room environment and thus endangering experiments running simultaneously.

Both Agroinfiltration methods, the syringe-infiltration being the dominant one, are extensively applied to tobacco (*Nicotiana benthamiana*) and brassica species (Zhao et al., 2017; Hu et al., 2019; Norkunas et al., 2018). Reports on the use of these methods in crop species, such as tomato (*S. lycopersicum*), are available in the literature (Yamamoto et al., 2018; Beihaghi et al., 2018). But to the best of our knowledge, these reports are almost exclusively for syringe-infiltration. The cost of setting up a vacuum experiment is most likely the main reason behind this choice. Tomato (*S. lycopersicum*), is one of the most important fruit crops, perhaps the most important after potato (Quinet et al., 2019), attributed to its use in the culinary world (Piscitelli et al., 2020). Besides its importance as a food product, tomato is also extensively studied as a model organism due to its unique features (i.e., fleshy fruit, a sympodial shoot, and compound leaves) (Kimura and Sinha, 2008). This crop can certainly benefit from transient expression studies (e.g., plant-pathogen interactions, functional genetics, and recombinant protein production). Furthermore, transient expression studies can also help researchers to obtain transgene-free plants of agronomic importance (Veillet et al., 2019). While all focus diverted to syringe based agroinfiltration; cheap, repeatable, scalable, and labor-less vacuum infiltration experiment can certainly offer a valuable alternative.

In this study, we report a vacuum-infiltration protocol for the transient expression of a reporter gene, modified green fluorescent protein (mGFP), in young tomato plantlets. With a basic experimental setup (including a growth chamber) built with affordable equipment, we show that *Agrobacterium* can infect and transform tomato cells transiently, which is evidenced by PCR-based detection of the transferred DNA, detection of reporter gene both analytically with SDS-page analysis, and visually by the images acquired with fluorescence microscopy. Quantification of the fluorescence images helped us to identify the best *A. tumefaciens* strain to use with this setup.

MATERIALS AND METHODS

Plant material, *Agrobacterium tumefaciens* strains and binary vector

Four-week-old indeterminate type, F1 tomato plantlets were kindly provided by Proto Seeds, Antalya, Turkey. Plantlets were potted into peat moss and perlite mix (1:1) and were grown at 24-28ºC under 20 hours light – 4 hours dark photoperiod; previously reported to increase the protein content (Khoeyi et al., 2012), and in a custom-built growth chamber (Figure 1) for an additional week. The day before the infiltration, watering was withheld to facilitate better intake (i.e., the bacterial suspension). For vacuum infiltration process, plantlets were covered with three layers of protective coating, first a filter paper, then an aluminum foil, and finally a wrapping film (see Protocol for details) to protect the infiltration suspension from potting material. Also, the oldest leaves (and the youngest leaves, if needed) were trimmed to minimize cluttering, which was found to influence efficiency of intake.

A custom growth chamber was used for this experiment built by using easily affordable materials (Figure 1). This growth chamber deserves a dedicated paragraph for itself since it presents an opportunity for researchers to build their own simple growth chamber. First, a supporting frame (70x75x50cm) was built by connecting aluminum sigma profiles. Then, forex boards (2.7 mm) cut in an appropriate dimension were mounted to each surface of the frame, except the front-surface; this surface provided access to the chamber. A silicon-sealant was used to isolate the inner-chamber from its surroundings. Using reflective-adhesive tape (aluminum) the inner-chamber walls were covered to reflect light. Simple 12 V stripe blue and red LED light sources (3 stripes each) were attached to each side of the inner chamber walls, as reports indicate blue-red LED lights positively contribute to the vegetative growth. LED light stripes were relayed into a 12V power supply using a simple 5V relay card and a Raspberry Pi minicomputer was used to control the photoperiod with in-house built Python scripts. This growth chamber under normal room temperature conditions (i.e., 24-28 ºC) was stable at 28-29 ºC.

Figure 1. Exterior (on the left) and interior (on the right) of the custom-build growth chamber. The growth chamber was built in the specified dimensions (70x75x50cm) and was connected to a Raspberry Pi minicomputer. The minicomputer controls the photoperiod (20:4) through a simple 5V relay card with in-house built Python scripts. 12V blue and red LED as the light sources and were attached to either side of the growth chamber.

Four *A. tumefaciens* strains; namely EHA105, AGL1, C58C1, and LBA4404 armed with binary vector pBIN-mGFP5-ER (Haseloff et al., 1997) were used. pBIN-mGFP5-ER carries a modified GFP gene (product: 29.85 kDa) under the control of CaMV 35S promoter. These modifications include an incorporation of ER-retention signal and mutations to allow better visualization at UV (395 nm) and blue light (473 nm). Single colony were selected for each *Agrobacterium* strain carrying pBIN-mGFP5-ER, and liquid cultures were grown at 28C°, 225 rpm with appropriate antibiotics (25 mg/L rifampicin and an extra 50 mg l-l streptomycin for LBA4404). An additional *A. tumefaciens* strain (AGL1) carrying a second, helper plasmid p19 gene of Tomato Bushy Stunt Virus (TBSV) (kindly provided by Dr. Mamedov, Akdeniz University) was grown under the same conditions (except for the antibiotic choice, 50mg/L kanamycin). Previous reports show that using the p19 gene increases the production of recombinant protein (Heidari-Japelaghi et al., 2020). Bacteria were collected by centrifugation at 4000 rpm for 15 minutes at room temperature once they reach OD600 of 2.0. Following the centrifugation, bacteria were resuspended to a final OD_{600} of 0.8 with the infiltration medium (10 mM MES buffer, 10 mM MgCl2, 200 µM acetosyringone, and few drops of Tween20). Several other OD_{600} values were sampled on our previous studies (data not shown), greater values of OD600 (>1.0) can cause extensive necrosis in tomato leaves after 4-5 days. Thus, we opted to use OD600 value 0.8, which was found to be a good balance for survival, expression, and

suspension intake. Infiltration mediums (each carrying a different binary vector) were mixed in a 9:1 ratio (pBIN-mGFP5-ER:p19), so for each strain (i.e., EHA105, AGL1, C58C1, and LBA4404), a final infiltration medium was prepared. Each infiltration medium was incubated at 28℃ and 225 rpm for the acetosyringone activation of *Agrobacterium* strains.

The vacuum assisted, *Agrobacterium*-mediated gene delivery protocol

In our experiments, an affordable setup for vacuum-assisted, *Agrobacterium*-mediated gene delivery was used. Mainly, two components of this experimental setup determine its cost: *i)* the vacuum pump, *ii)* the vacuum chamber. For the former, previous studies suggest a vacuum pump that can produce a 100 millibar (or less) vacuum (Yamamoto et al., 2018), as expected, the capacity of the pump increases with its cost. In this study, a simple vacuum pump (ISOLAB Cat#622.12.001) was proved to be sufficient. For the latter, various applications require different solutions, but any chamber that maintains its structural integrity under a 200 millibar vacuum (maximum capacity of ISOLAB vacuum pump) is suitable to use. A custom-built vacuum chamber built from simple materials (i.e., glass) can greatly increase the scale of the experiment in conduct. For this study, a glass desiccator (ISOLAB Cat#039.02.300) was used as a vacuum chamber.

Tomato plantlets prepared for agroinfiltration (see. Materials and Methods) were immersed into at least 250 ml of the infiltration solution. Chamber was sealed airtight with the help of petroleum jelly. Approximately 200 millibar vacuum applied to the chamber. After several seconds, bubbles started to form as a clear indication of air-evacuation from the intercellular space. Plantlets were kept under vacuum for different durations (1, 2, and 6 minutes). Upon the relief, the infiltration medium rushed into the leaves as expected. Three plantlets for each strain were infiltrated using the same medium. After the infiltration, excessive medium was removed using filter papers and protective coating was removed. Plantlets were then grown for 12 days inside the custom-built growth chamber.

Visualization and quantification of fluorescence

For each *A. tumefaciens* strain used in our experiments, leaf slides were prepared using three thin slices cut from 3 to 12 days post infiltration leaves. Measurements were taken from sections of 3 different plants for each day. The slides were then evaluated by exciting leaf samples with a laser beam at 488 nm and applying the FITC filter on Leica DMi8 inverted microscope. For image acquisition, the in-built camera system was used. Cell fluorescence was measured using the ImageJ program and Equation 1. The mean values for the 3 plants measured for each separate day are given in Table 1.

(1) $$CF = ID - (A\ x\ f)$$

Using tools supplied by ImageJ, *ID* (integrated density), *a* (area), and *f* (mean fluorescence) values were calculated for each day. Calculated *CF* value was then used to quantify fluorescence. One-way analysis of variance (ANOVA) was applied to define the differences in *Agrobacterium* strains. The data were normalized prior to analysis by lg10 (x) where x represents the *CF* values. The general linear model procedure of SPSS (Statistics 20) software (IBM Corp., Armonk, NY, USA) was used for data analyses. All main effects were considered as fixed effects. Multiple comparisons of the strains were performed by using Tukey's multiple range post hoc test at an alpha 0.05 level.

Protein extraction and SDS-page analysis

Total soluble proteins were extracted by previously reported procedure with modification (Mamedov et al., 2017). Briefly, tomato leaves (100 mg) were grounded in a microcentrifuge tube with a pestle after PBS (phosphate buffered saline) buffer (4 µl μg^{-1}) was added. Grounded leaves were then centrifuged for 20 minutes at 4°C, and the resulting supernatant was mixed with the Laemli buffer in a 1:4 ratio. The samples were boiled at 100°C for 5 minutes and were then loaded to 12% SDS-PAGE gel.

Each protein sample (extracted from a different *Agrobacterium* strain treated leaf, on 4 and 8 dpi) were loaded into the same gel, and RGA (relative GFP amount, GFP/RuBisCo) were calculated by measuring band intensities using software ImageJ and Photoshop, and Equation 2 after gel electrophoresis.

(2) $$RGA = \frac{Sample\ Intensity_{GFP} / Sample\ Intensity_{RuBisCo}}{Control\ Intensity_{GFP} / Control\ Intensity_{RuBisCo}}$$

Intensity values in Equation 2 were calculated as the integrals of the signal strength on the gel image (Adobe Photoshop enhanced SDS-PAGE image, Figure 4) using software ImageJ.

Genomic DNA extraction and PCR

Total genomic DNA was isolated from SDS-PAGE positive and fluorescently active leaves (8 dpi) using a modified CTAB method (Doyle and Doyle, 1990). A forward mGFP primer (5'- GGACGACGGGAACTACAAGA-3') and a reverse mGFP primer 5'- AAAGGGCAGATTGTGTGGAC-3') were utilized for polymerase chain reaction (PCR). The PCR program was run at standard cycle conditions: initial denaturation at 95 ºC for 6 minutes; 30 cycles of 95 ºC for 30 seconds, 54 ºC for 30 seconds, and 72 ºC for 1 minute, and finally a 10-minute elongation at 72ºC. The products (320 bp) were analyzed by ethidium bromide-stained agarose gel electrophoresis.

RESULTS and DISCUSSION

Comparison of the vacuum-assisted experimental setup versus classic syringe infiltration

Before further experiments, first, we wanted to test our vacuum-assisted experimental setup for its competence, and we used the classical syringe infiltration technique for comparison. Two main criteria (i.e., the infiltration medium uptake and damage to tissues) were taken under consideration. For each method, a sample infiltration medium containing *A. tumefaciens* EHA105 armed with pBIN-mGFP5-ER binary vector was used. Figure 2 shows the results of this comparative analysis. In syringe infiltration, despite multiple entry points, the medium could only penetrate a small area (dark green spots) of the leaf, and for each entry point tissues that lie in the vicinity were damaged due to the application. While in our experimental setup, the medium could penetrate almost the entire intercellular space of the leaf. Almost an entire branch could be infiltrated using our experimental setup.

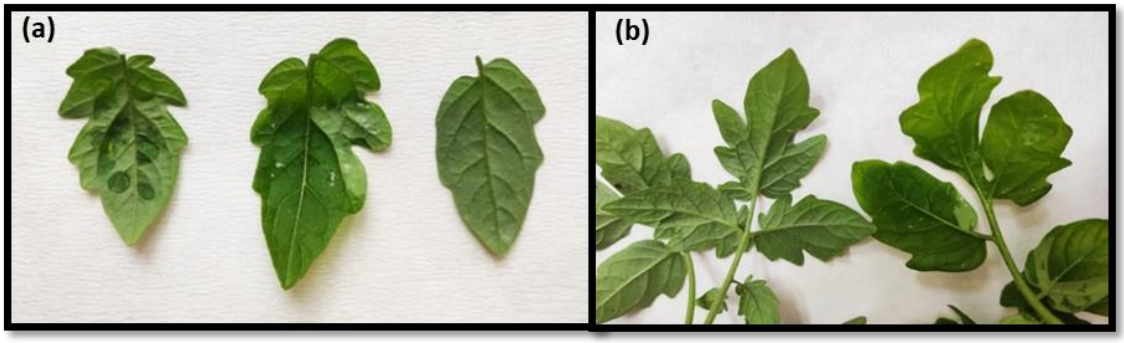

Figure 2. Comparison of two different agroinfiltration methods for the infiltration medium (*A. tumefaciens* strain EHA105 harboring pBIN-mGFP5-ER binary vector) uptake and tissue damage; (a) Uptake performance of both methods on single tomato leaf. Despite multiple entry points for the syringe infiltration method only a limited area was successfully infiltrated. In contrast, almost entire leaf was successfully infiltrated. For the syringe infiltration method vicinity of the entry points were damaged due to application; from left to right, syringe-infiltrated, vacuum-infiltrated, and untreated (control), (b) Leaf uptake performance of our experimental setup on an entire branch; from left to right, untreated (control) and vacuum-infiltrated.

In this study, we also tried to determine the ideal vacuum time. Three different vacuum durations (one, two, and six minutes) were tested. In our setup, leaves of tomato seedlings were not properly infiltrated when exposed to a 200 millibar vacuum in infiltration liquid for one and two minutes (data not shown). However, when the vacuum duration was increased to six minutes, leaves of an entire branch were successfully infiltrated (Figure 2-b). Criteria such as leaf density and leaf size affect the optimum time. Therefore, it should be considered that the vacuum time may vary in different genotypes. For this reason, optimization of the vacuum duration should be done before each study.

Transient expression of green fluorescent protein in tomato leaves

Tomato plants, infiltrated with four different infiltration mediums (each with different *Agrobacterium* strain: AGL1, C58C1, EHA105, and LBA4404), were able to express the reporter gene (mGFP5-ER) transiently. The resulting emission was detected by fluorescence microscopy and quantified using Equation 1. Table 1 shows the measurements and Figure 3 shows "Calculated Fluorescence (*CF)* values" for leaves expressing mGFP5-ER protein. Area *(A)*, measured fluorescence *(f),* integrated density *(ID)* data (Table 1) were obtained via the infiltration of leaves with different bacterial strain at different dpi (3 to 12). Data were measured using ImageJ software. According to ANOVA results, LBA4404 strain was found to be significantly ($p \leq 0,05$) different from the control group based on the 3-12 dpi CF values.

Table 1. Comparison of transient expression response of different *Agrobacterium* strains at different dpi (3 to 12).

Introduced *Agrobacterium* strain	DPI	A	*f*	ID	CF	Introduced *Agrobacterium* strain	DPI	A	*f*	ID	CF
		M*	M	M	M			M*	M	M	M
AGL1	3	19	56	36.680	34.371	**EHA105**	3	27	101	81.842	70.792
	4	21	96	270.766	181.902		4	1	83	30.927	30.927
	5	38	85	188.708	155.865		5	30	94	193.953	152.645
	6	22	94	140.159	140.159		6	23	102	88.739	73.053
	7	13	66	23.003	23.003		7	12	63	65.654	54.255
	8	12	59	14.395	12.339		8	12	29	41.546	24.860
	9	17	25	43.948	30.027		9	23	77	26.500	21.781
	10	18	36	33.252	25.108		10	15	82	48.591	41.137
	11	-	-	-	25.108		11	16	69	80.300	49.798
	12	12	32	9.896	7.681		12	19	59	82.030	75.701
Introduced *Agrobacterium* strain	DPI	A	*f*	ID	CF	Introduced *Agrobacterium* strain	DPI	A	*f*	ID	CF
		M*	M	M	M			M*	M	M	M
LBA4404	3	37	93	128.368	95.908	**C58C1**	3	27	101	81.842	70.792
	4	44	97	203.034	166.205		4	37	93	128.368	95.908
	5	34	99	261.887	211.125		5	37	99	269.345	215.501
	6	15	78	102.762	97.999		6	12	49	63.908	56.845
	7	12	115	140.570	117.103		7	14	57	20.171	14.612
	8	16	109	74.158	47.760		8	18	98	178.829	154.342
	9	16	54	38.452	33.174		9	21	69	28.393	23.355
	10	11	43	65.403	50.077		10	12	80	124.833	98.608
	11	16	109	237.886	192.252		11	13	48	19.372	14.290
	12	19	103	133.445	117.436		12	10	34	12.089	9.209
CONTROL	3	6	14	10,078	7.832						

Area *(A)*, measured fluorescence *(F)*, integrated density (*ID*), Calculated Fluorescence (*CF*)

* Values are mean (M) of 3 different plants. Means were separated by using Tukey's multiple range post hoc test.

Fluorescence emission was peaked at the 5-dpi due to steady accumulation of mGFP5-ER in the cells. Then, in the following days the emission was steadily declined, except for the plant treated with LBA4404 strain. When the mean values of *CF* values are evaluated amongst the four-strain, LBA4404 was found to be the most effective strain for vacuum-assisted transient expression in tomato leaves. Efficiency of LBA4404 was followed by C58, AGL1, and EHA105 in declining order.

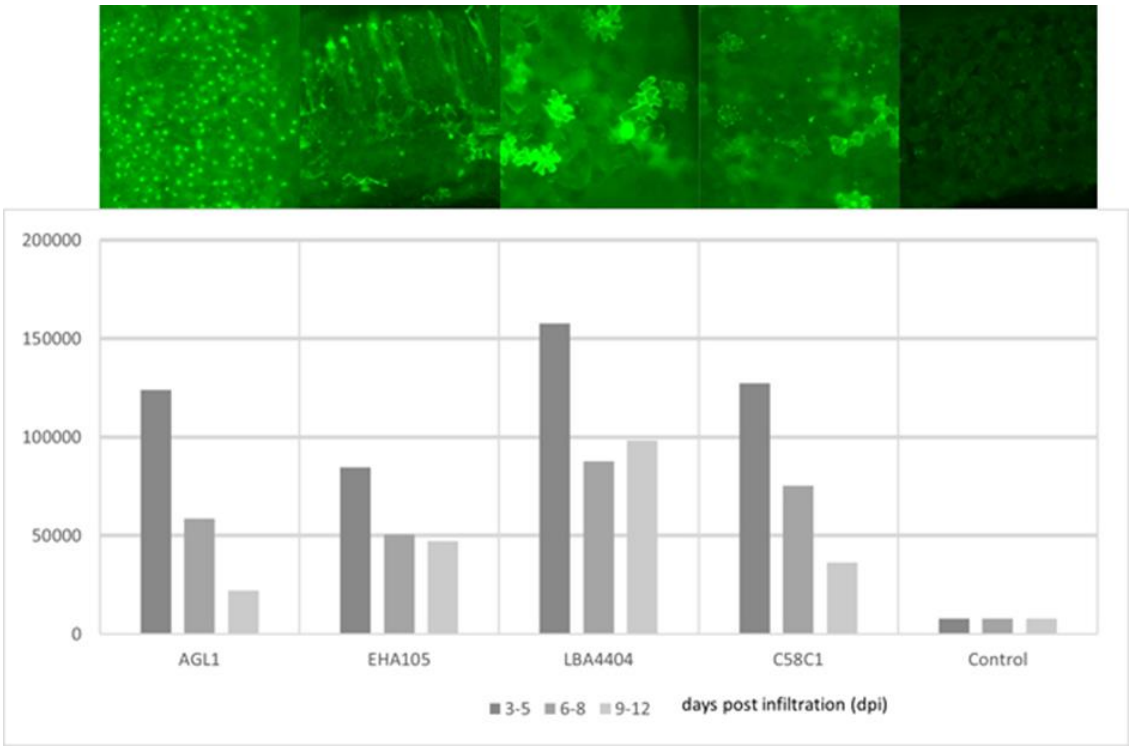

Figure 3. Transient expression of green fluorescent protein in tomato leaves. On the top: Sample fluorescence microscopy images of the leaves infected with different *Agrobacterium* strain and untreated leaf. Using these images and ImageJ software calculated fluorescence (CF) was calculated for different periods; 3-5 days, 6-8 days, and 9-12 days. On the bottom: the graph shows the calculated CF levels of infiltrated and untreated leaves. Plants transfected by strain LBA4404 showed the highest fluorescence, followed by strains C58 and AGL1. Strains EHA105 was found to be the least effective strain for transient expression in the tomato.

Confirmation of transient GFP expression

In addition to our fluorescence microscopy assay, the performance of different *Agrobacterium* strains in our experimental setup was evaluated by SDS-PAGE analysis followed by quantification through image recognition. Pure mGFP5 protein weights 26.8 kDa, considering the localization signals, mGFP5-ER was calculated to be 29.85 kDa. This analysis granted us insight; the most efficient strain to use for recombinant protein production in vacuum-assisted agroinfiltration of tomato plants. Table 2 shows the relative expression of mGFP-ER to RuBisCo.

Table 2. For each *A. tumefaciens* strain, RuBisCo and mGFP-ER values was derived from the integrals of the signal strength on the gel image using ImageJ software. Relative GFP amount (RGA) was calculated using Equation 2.

Strain-DPI	*RuBisCo*	*mGFP-ER*	*RGA*
AGL1-4	26.499	2.186	1.82
AGL1-8	30.446	2.561	1.85
EHA105-4	24.087	1.510	1.28
EHA105-8	25.877	1.748	1.49
LBA4404-4	30.220	3.144	2.29
LBA4404-8	20.509	2.557	2.74
C58C1-4	29.197	2.152	1.62
C58C1-8	26.872	1.775	1.45
Control	23.461	1.066	1.00

Raw RuBisCo and mGFP-ER values derived from gel image. Signal strength plots (See Figure 4-a and 4-c) were used to calculate the integrals of RuBisCo and mGFP-ER (26.9 kDA). Then using Equation 2, raw RuBisCo

and mGFP-ER signal data were transformed into relative GFP amount. Figure 4-b shows RGA (relative GFP amount) ratio for tomato leaves each transfected with a different *Agrobacterium* strain. The GFP and RuBisCo expression on two separate days (4 and 8 dpi) was taken into consideration. For all strains tested, GFP accumulation in the cells was found to be higher on day four compared to day eight except for LBA4404 (also supported with fluorescence microscopy assay). According to data in Table-2 for LBA4404 at 8 dpi, the normalized signal corresponds to approx. 28-30 kDA range was 2.74 times more than the signal for the control sample. However, EHA105 had a signal which was found to be only 1.28 times stronger than the control signal on average. Thus, in concordance with the results of fluorescence microscopy assay, LBA4404 was found to be the most effective strain, followed by AGL1, C58C1 and EHA105.

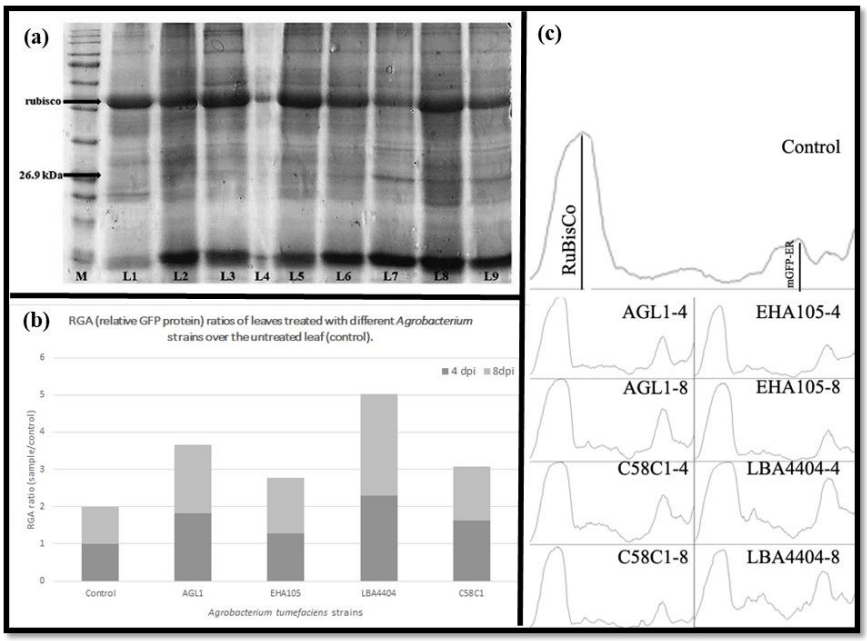

Figure 4. Relative Expression of mGFP-ER; (a) SDS-PAGE gel image, arrows indicate RuBisCo and mGFP-ER (26.9 kDA) bands, respectively, (b) RGA ratios of tomato leaves transfected with a different *A. tumefaciens* strains over non-transfected leaves. (c) Signal strength plots for all leaves (treated / untreated) at 4 and 8 dpi, were generated using ImageJ software and SFS-page gel image. Marked regions in the control plot corresponds to RuBisCo and mGFP-ER, respectively, LBA4404 was found to be the most effective strain for recombinant protein production; followed by AGL1, C58C1 and EHA105.

Finally, to further support the efficiency of our experimental setup, PCR analysis was performed on DNA samples extracted from leaves 12 dpi. There were two reasons behind this choice: first, we opted to wait as much as possible to eliminate the possibility of the amplification of surface contaminant DNA (untransformed). Second, our fluorescence microscopy assay results showed that only a residual fluorescence emission was present at 12th dpi for all the transfected plants. PTGS (post-translational gene silencing) was most likely the reason behind this observed phenomenon. Nevertheless, we wanted to confirm whether the transiently delivered gene was present at that given time. Figure 5 shows the results for PCR analysis. The fragment (320 bp) targeting the GFP gene was detected on DNA samples.

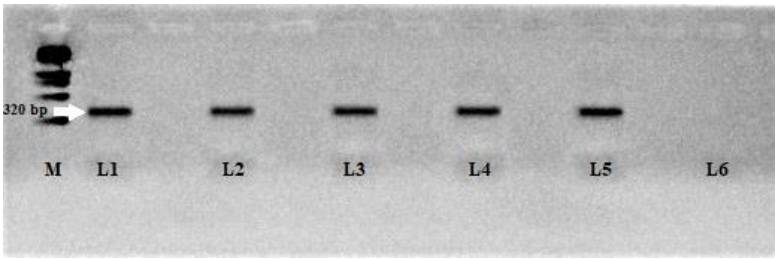

Figure 5. Verification of the presence of mGFP5-ER gene in tomato leaves on the day 12. The results for PCR assay; the expected product (320bp) was detected on all leaves tested. M: GeneRuler 1 kb DNA Ladder (Thermo Scientific), leaves transfected with strain; L1: AGL-1, L2: C58C1, L3: EHA105, L4: LBA4404, L5: plasmid DNA (positive control), L6: water (negative control).

CONCLUSION

The transitory expression of a reporter gene, modified green fluorescent protein (mGFP), in tomato seedlings is accomplished in this study using a vacuum-infiltration approach. It is revealed that the entire leaf can be infiltrated by applying a mere 200 millibar vacuum for 6 minutes, resulting in transient expression. The results were demonstrated by PCR-based detection of the T-DNA and detection of mGFP both analytically with SDS-page analysis and visually by the images acquired by fluorescence microscopy. Furthermore, different *Agrobacterium tumefaciens* strains were tested for their transformation efficiency, and LBA4404 was found to be the most effective strain to be used in this identified vacuum-assisted transient expression setup.

Conflict of interest declaration: The authors declared that for this research article, they have no actual, potential, or perceived conflict of interest.

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REFERENCES

Beihaghi, M., Marashi, H., Bagheri, A., Sankian, M. 2018. Transient expression of CCL21as recombinant protein in tomato. Biotechnology Reports, 17: 10–15.

Chetty, V. J., Ceballos, N., Garcia, D., Narváez-Vásquez, J., Lopez, W., Orozco-Cárdenas, M. L. 2013. Evaluation of four Agrobacterium tumefaciens strains for the genetic transformation of tomato (Solanum lycopersicum L.) cultivar Micro-Tom. Plant Cell Reports, 32(2): 239–247.

Doyle, J. and Doyle, J. 1990. Isolation of DNA from fresh tissue. Focus, 12(13): 13–15.

Haseloff, J., Siemering, K. R., Prasher, D. C., Hodge, S. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proceedings of the National Academy of Sciences of the United States of America, 94(6): 2122–2127.

Heidari-Japelaghi, R., Valizadeh, M., Haddad, R., Dorani-Uliaie, E., Jalali-Javaran, M. 2020. Production of bioactive human IFN-γ protein by agroinfiltration in tobacco. Protein Expression and Purification, 173: 105616.

Hellens, R. P., Allan, A. C., Friel, E. N., Bolitho, K., Grafton, K., Templeton, M. D., Karunairetnam, S., Gleave A. P., Laing, W. A. 2005. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods, 1(1): 13.

Hu, D., Bent, A. F., Hou, X., Li, Y. 2019. Agrobacterium-mediated vacuum infiltration and floral dip transformation of rapid-cycling Brassica rapa. BMC Plant Biology, 19(1): 246.

Khoeyi, Z. A., Seyfabadi, J., Ramezanpour, Z. 2012. Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae, Chlorella vulgaris. Aquaculture International, 20(1): 41–49.

Kimura, S., Sinha, N. 2008. Tomato (Solanum lycopersicum): A model fruit-bearing crop. Cold Spring Harbor Protocols, 3(11): pdb.emo105.

Leuzinger, K., Dent, M., Hurtado, J., Stahnke, J., Lai, H., Zhou, X., Chen, Q. 2013. Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. Journal of Visualized Experiments, (77).

Liu, P. F., Wang, Y., Ulrich, R. G., Simmons, C. W., Vandergheynst, J. S., Gallo, R. L., Huang, C. M. 2018. Leaf-Encapsulated Vaccines: Agroinfiltration and Transient Expression of the Antigen Staphylococcal Endotoxin B in Radish Leaves. Journal of Immunology Research, 2018.

Mamedov, T., Cicek, K., Gulec, B., Ungor, R., Hasanova, G. 2017. In vivo production of non-glycosylated recombinant proteins in Nicotiana benthamiana plants by co-expression with Endo-β-N-acetylglucosaminidase H (Endo H) of Streptomyces plicatus. PLoS ONE, 12(8), e0183589.

Mardanova, E. S., Blokhina, E. A., Tsybalova, L. M., Peyret, H., Lomonossoff, G. P Ravin, N. V. 2017. Efficient Transient Expression of Recombinant Proteins in Plants by the Novel pEff Vector Based on the Genome of Potato Virus X. Frontiers in Plant Science, 8: 247.

Mariashibu, T. S., Subramanyam, K., Arun, M., Mayavan, S., Rajesh, M., Theboral, J., Manickavasagam, M., Ganapathi, A. 2013. Vacuum infiltration enhances the Agrobacterium-mediated genetic transformation in Indian soybean cultivars. Acta Physiologiae Plantarum, 35(1): 41–54.

Norkunas, K., Harding, R., Dale, J., Dugdale, B. 2018. Improving agroinfiltration-based transient gene expression in Nicotiana benthamiana. Plant Methods, 14(1): 71.

Piscitelli, C., Lavorgna, M., De Prisco, R., Coppola, E., Grilli, E., Russo, C., Isidori, M. 2020. Tomato plants (Solanum lycopersicum L.) grown in experimental contaminated soil: Bioconcentration of potentially toxic elements and free radical scavenging evaluation. PLOS ONE, 15(8): e0237031.

Quinet, M., Angosto, T., Yuste-Lisbona, F. J., Blanchard-Gros, R., Bigot, S., Martinez, J. P., Lutts, S. 2019. Tomato Fruit Development and Metabolism. Frontiers in Plant Science, 10: 1554.

Shamloul, M., Trusa, J., Mett, V., Yusibov, V. 2014. Optimization and Utilization of Agrobacterium-mediated Transient Protein Production in Nicotiana. Journal of Visualized Experiments, (86).

Sheludko, Y. 2008. Agrobacterium-Mediated Transient Expression as an Approach to Production of Recombinant Proteins in Plants. Recent Patents on Biotechnology, 2(3): 198–208.

Sparkes, I. A., Runions, J., Kearns, A., Hawes, C. 2006. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nature Protocols, 1(4): 2019–2025.

Van Eck, J., Keen, P., Tjahjadi, M. 2019. Agrobacterium tumefaciens-mediated transformation of tomato. In Methods in Molecular Biology, 1864: 225–234).

Veillet, F., Perrot, L., Chauvin, L., Kermarrec, M. P., Guyon-Debast, A., Chauvin, J. E., Nogue, F., Mazier, M. 2019. Transgene-free genome editing in tomato and potato plants using Agrobacterium-mediated delivery of a CRISPR/Cas9 cytidine base editor. International Journal of Molecular Sciences, 20(2): 402.

Wroblewski, T., Tomczak, A., Michelmore, R. 2005. Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. Plant Biotechnology Journal, 3(2): 259–273.

Young, J. L., & Dean, D. A. 2015. Electroporation-mediated gene delivery. Advances in genetics, 89: 49-88.

Yamamoto, T., Kashojiya, S., Kamimura, S., Kameyama, T., Ariizumi, T., Ezura, H., Miura, K. 2018. Application and development of genome editing technologies to the Solanaceae plants. Plant Physiology and Biochemistry, 131: 37–46.

Zhao, H., Tan, Z., Wen, X., Wang, Y. 2017. An improved syringe agroinfiltration protocol to enhance transformation efficiency by combinative use of 5-azacytidine, ascorbate acid and tween-20. Plants, 6(1): 444.