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Standardisation of an Agroinfiltration Protocol for Eggplant Fruits and Proving its Usefulness by Over-expressing the SmHQT Gene

Prashant Kaushik*
Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València, 46022 Valencia, Spain
* Correspondence: prakau@doctor.upv.es; Tel.: +34-963-877-000

Abstract: Eggplant is a fruit vegetable of family Solanaceae, and eggplant fruits are of different shape and sizes that render them as an ideal system for metabolic engineering. Here, we have developed an agroinfiltration protocol for the transient expression of a gene in the eggplant fruit using GUS bearing; pCAMBIA1304 vector. Thereafter, to prove the effectiveness of the developed protocol, we have used the eggplant hydroxycinnamoyl CoA-quinate transferase (SmHQT), which is the central enzyme studied to increase the chlorogenic acid content, in a gene construct with the specific promoter in a plant transformation vector (pBIN19). Also, in our cassette, we also co-expressed the P19 protein of Tomato bushy stunt virus (native promoter) to overexpress the protein. Overall, using the protocol, the chlorogenic content was increased by more than two folds in the transgenic tissues.

Keywords: agroinfiltration; eggplant; fruits; agrobacterium; SmHQT

1. Introduction

Phenolic acids are among the most common phenolic compounds produced by different plant species [1,2]. Eggplant (*Solanum melongena* L.) belongs to Solanaceae and contains high concentrations of phenolic acids, which are beneficial for human health and development. Phenolics of eggplant flesh have proved useful for the protection against several diseases like diabetes, cancer and arthritis [3–5]. Increasing the content of these phenolic compounds especially chlorogenic acid is among the major breeding objectives for eggplant. In the eggplant flesh, the chlorogenic acid forms an ester as 5-caffeoylquinic acid, and it makes up to 90 percent of total phenolics found in the eggplant flesh. [6]. Moreover, it also forms more and lesser spotted esters such as 3-caffeoylquinic acid and 4-caffeoylquinic acid [7,8]. Increases in the concentrations of these phenolic compounds take place in response to environmental stress and under insect pest and pathogen infestations [9,10].

Nevertheless, cultivated eggplant has far less phenolic acids than its several wild relatives [3]. Therefore, several breeding approaches were undertaken in the past and are still ongoing to improve the phenolic acid content of cultivated eggplant, e.g. introgression breeding and backcrossing [11]. Unfortunately, all these techniques can not completely get rid of unwanted genes such as those are associated with the wild species, e.g. prickles, bitterness, alkaloids, etc. [12]. These genes are not uncommon to wild relatives and landraces, which use these defense mechanisms against predators and diseases [13]. Therefore, the use of highly precise genome editing approaches and the use of transgenics technology cannot be overlooked, as they restrict the introduction of external DNA to a specific gene sequence or fragment of interest. With transgenic methods, only the specific sequence will be delivered in the genome so that it precisely expresses only the phenotypes associated with that gene [14–16].

Transgenic plants are routinely used to understand the molecular genetic function of a gene [17]. The plant transformation method based on *Agrobacterium tumefaciens* is amongst the most popular techniques of plant transformations and is widely applied to several commercially important crop plants, like maize and tomatoes [18,19]. Even with lots of new techniques adding, agrobacterium-based methods are more popular than any other technique used for the transgene delivery in plants. This is because this method is comparatively cheap and has a high transformation efficiency [20]. Further, agroinfiltration is a method of injecting agrobacterium with a gene of interest along with a T-DNA vector in the plant tissues, which allows the transient expression of a gene for various purposes. Compared to large time investment in stable transformation, agroinfiltration provides a rapid assay and can further help to elucidate its functions via studies on its function or involvement in the different pathways [21].

In plants, there is an immense potential to mass produce recombinant proteins (e.g. enzymes) via their different organs, like leaves, roots, fruits, etc. [22,23]. Plants provide a low-cost system for protein production because they are easily transformed and moreover the transformed protein can maintain an appropriate structure via post-translational

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48 modifications [24]. Also, the plant with a transient protein is ready for the evaluation within a few days as compared
49 to the months reserved by the stable transformation method. Also, with stable transformation method, there can even
50 be a high probability of bias because of chromosomal positions and epigenetic mechanisms by the constructs [25].
51 Overall, agroinfiltration has become a method of popular choice for assigning a gene function. This method is well
52 established in several fruit-bearing plants like tomato, strawberry, melon and cucumber [26–29].

53 The chlorogenic acid synthesis pathway is known in eggplant, and the enzymes are also mapped on eggplant genetic
54 map, and their genetic inheritance is know [30]. The hydroxycinnamoyl CoA-quinate (HQT) is the central enzyme
55 studied to increase the chlorogenic acid content in Solanaceae and other families. The function of eggplant transferase
56 (SmHQT) enzyme is the esterification of transferase 4-coumaroyl CoA and quinic acid to form CGA, and further to
57 provide the entry molecule of the flavonoid pathway [31,32]. Furthermore, HQT is well studied in other solanaceous
58 vegetables; in the case of tomato, over-expression of HQT resulted in the overproduction of chlorogenic acid by
59 around two-fold [33]. In contrast, the suppression of HQT gene resulted in the reduction of chlorogenic acid content
60 by 90% [34].

61 In eggplants, there is no established protocol to follow for the agroinfiltration assays. Therefore, the objectives of this
62 study were to establish and standardise an effective agroinfiltration protocol for the eggplant fruit and thereafter by
63 applying that protocol to the study the expression pattern in the eggplant genome transiently over-expressing of
64 SmHQT gene. In our cassette, we also co-expressed the P19 protein of tomato bushy stunt virus, which is well used
65 for the characterisation and native expression desired protein in the plant tissues. P19 prevents the post-transcriptional
66 gene silencing (PTGS) of the infiltrated leaves which could result from plant response to the pathogenic, and in
67 consequence, the agroinfiltrated tissue can keep on expressing the desired protein product [35,36].

68 **2. Methodology and Outcomes**

69 *2.1. Development of eggplant HQT gene construct with the specific promoter in a plant transformation vector*

70 Genomic DNA was isolated from eggplant sample and used for amplification of the HQT gene along with its specific
71 native promoter. Primers were designed and synthesised for specific amplification of the SmHQT fragments. The gene
72 was amplified as two fragments; fragment-1 contained the promoter region and exon1 while fragment-2 contained the
73 second exon. Both fragments were combined in cloning protocol. The complete gene was then cloned in a pUC based
74 cloning vector (pBlueScript; at *Hind*III and *Bam*HI restriction sites) and sequence confirmed.

75 PCR calibration

76 The fragments (FI-593bp and FII-874bp) were PCR amplified separately and assembled to obtain a full-length
77 SmHQT gene (1467bp). The full-length PCR product was then gel purified before being used for the restriction
78 digestion and ligation.

79 **Table 1.** PCR conditions.

Component	Amount
Template (gDNA; 50ng)	1.0 µl
Forward Primer (100ng/µl)	2.0 µl
Reverse primer (100ng/µl)	2.0 µl
10X Assay Buffer	5.0 µl
dNTPs (10mM)	2.0 µl
ChromTaq (3U/µl)	0.5 µl
Water	37.5 µl
Total reaction volume	50.0 µl

80 **Table 2.** PCR cycle conditions:

94°C	94°C	Ta°C*	72°C	72°C
5 min	30 sec	40 sec	1min	10 min
		35 cycles		

81 *Ta°C – Fragment I (32% GC): 50°C Ta°C – Fragment II (49% GC): 52°C

82 Cloning and sequencing

83 The optimised gene was then cloned in the cloning vector (pBlueScript KS+ vector) at *Hind*III/*Bam*HI sites. Probable
84 clones were screened by restriction digestion and were further confirmed by sequencing.

85

86 **Table 3.** Digestion conditions for PCR product and vector:

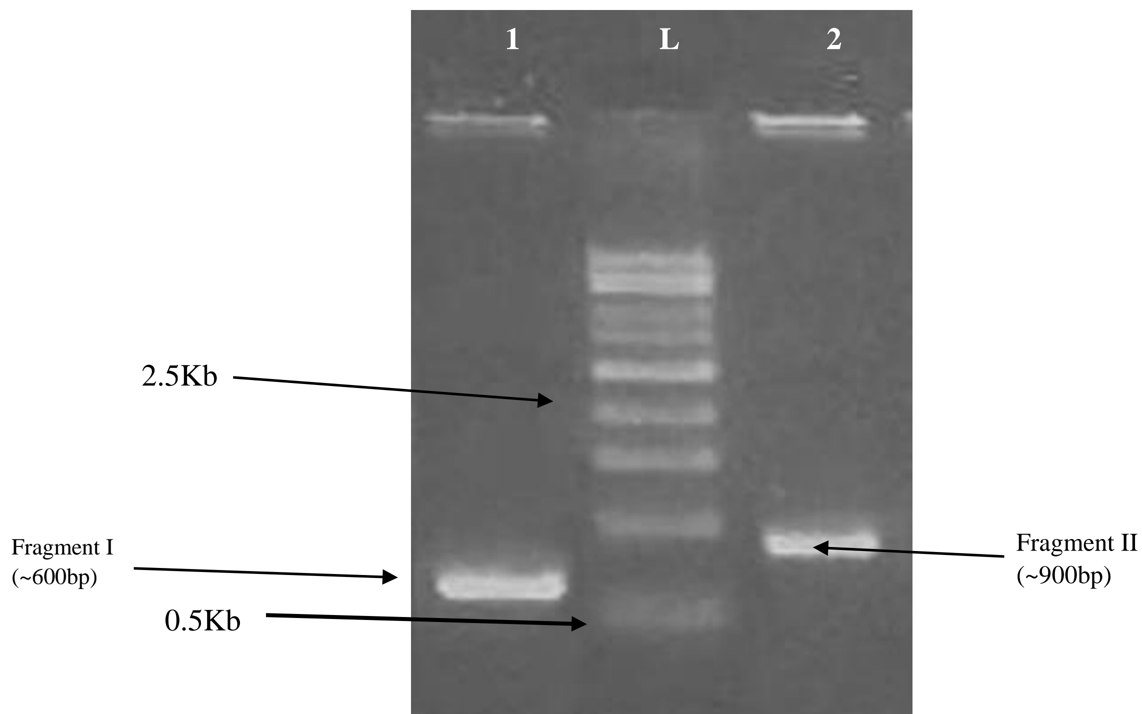
Component	Amount
DNA	4 µg
*Hind*III	36 units
*Bam*HI	36 units
10X Assay buffer	40 µl
Water	X* µl
Total reaction volume	400 µl

87 *Based on the final volume of the reaction. The reaction was incubated at 37°C for 2 hrs.

88 Table 4. Ligation of insert into the vector.

Component	Amount
Vector	100 ng
Insert	100 ng – 150 ng
Chromous Quick Ligase (Cat# -MFE14; Chromous Biotech Pvt. Ltd, Bangalore, India)	1 µl
2X Chromous Quick Ligase assay buffer	10 µl
Water	X* µl
Total reaction volume	20 µl

89 *Based on the final volume of the reaction. Reaction was incubated at room temperature for 30 min.



90

91 **Figure 1.** PCR fragment I and II loaded on a 1% agarose gel.

92 *Transformation in competent cells*

93 The 10 µl of the ligation reaction mix was transformed into 100 µl of DH5α competent cells. Incubated at 4°C (ice)
94 for 30 min and heat shock was given at 42°C for 45 sec. Quick chilled on ice for 2 min and volume made-up to 1ml
95 with LB broth. Incubated at 37°C for 1 hr in shaking incubator for recovery. After that, cells were pelleted at 3000
96 rpm for 3min; Supernatant discarded and cells plated on LB-agar plates with ampicillin; X-gal and IPTG (AXI) plate.
97 Plates incubated overnight at 37°C.

98 AXI Plate: To 100ml of LB add 100 µl ampicillin (Initial concentration: 100mg/ml), 120 µl X-gal (Initial
99 concentration: 100mg/ml), 24 µl IPTG (Initial concentration: 1M).

100 Screening for clones

101 Probable clones were screened by restriction digestion and checked for the release of the gene. Positive clones were
102 sequence confirmed and processed further for Sub-Cloning in the expression vector (pBIN19, Addgene) (Figure S1).

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103 *Sub-Cloning of Sequence Confirmed SmHQT in pBIN19*

104 The pBS+SmHQT clone was restriction digested (*Hind*III/*Bam*HI); released gene was then cloned into pBIN19
105 at (*Hind*III/*Bam*HI). Further, these probable clones were screened and confirmed by restriction digestion.
106 Briefly, the Clone DNA (4 µg of plasmid DNA is required to discharge at least 1 µg of insert) was taken and
107 digested with *Hind*III/*Bam*HI.

108 Table 5. Conditons of sub-cloning.

Component	Amount
DNA	4 µg
*Hind*III	36 units
*Bam*HI	36 units
10X Assay buffer	40 µl
Water :	X* µl
Total reaction volume	400 µl

109 *Based on the final volume of the reaction

110 The released insert was gel eluted and ligated to pBIN19 vector at *Hind*III/*Bam*HI sites. 10 µl of the ligation
111 reaction mix was transformed into 100 µl of DH5α competent cells. Incubated at 4°C (ice) for 30 min and heat
112 shock given at 42°C for 45 sec. Quick chilled on ice for 2 min and volume made up to 1ml with LB. Incubated
113 at 37°C for 1 hr in a shaking incubator. After that, cells were pelleted at 3000 rpm for 3min; Supernatant
114 discarded and cells plated on classical kanamycin plate. Plates incubated overnight at 37°C.

115 Screening for clones:

116 • Probable clones were screened by restriction digestion and checked for the release of the gene.

117 • The SmHQT gene was finally sequence confirmed and used further for all agroinfiltration experiments.

118 *2.2. Development of a p19 construct for using in co-infiltration experiments*

119 Overlapping PCR-based technique was used for development of p19 construct with its native promoter. The
120 gene was synthetically synthesized following recursive PCR based protocol described elsewhere [37]. The
121 optimised genes were then cloned in a cloning vector (pEASY-blunt vector) and amplified; sequence confirmed.
122 The sequence-confirmed gene was sub-cloned in the pBIN19 vector and confirmed by sequencing.

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123 Cloning and sequencing

124 The optimised gene was then cloned in the cloning vector (pUC57 vector; Addgene, Spain) at *Hind*III/*Bam*HI
125 sites. Probable clones were screened by restriction digestion and further confirmed by sequencing.

126 **Table 6.** Digestion of PCR product and vector:

Component	Amount
DNA	4 µg
*Hind*III (Cat# -REN011A;Chromous Biotech Pvt. Ltd, Bangalore, India)	36 units
*Bam*HI (Cat# -REN004A;Chromous Biotech Pvt. Ltd, Bangalore, India)	36 units
10X Assay buffer	40 µl
Water :	X* µl
Total reaction volume	400 µl

127 *Based on the final volume of the reaction. Reaction was incubated at 37°C for 2 hrs.

128 **Table 7.** Ligation with vector:

Component	Amount
Vector	100 ng
Insert	65 ng – 70 ng
Chromous Quick Ligase (Cat# -MFE14; Chromous Biotech Pvt. Ltd, Bangalore, India)	1 µl
2X Chromous Quick Ligase assay buffer	10 µl
Water	X* µl
Total reaction volume	20 µl

129 *Based on the final volume of the reaction. Reaction was incubated at room temperature for 30 min.

130 Transformation in competent cells

131 • 10 µl of the ligation reaction mix was transformed into 100 µl of DH5α competent cells.

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132 • Incubated at 4°C (ice) for 30 min and heat shock given at 42° for 45 sec.

133 • Quick chilled on ice for 2 min and volume made-up to 1ml with LB broth.

134 • Incubated at 37 °C for 1 hr in shaking incubator for recovery.

135 • The cells were then pelleted at 3000rpm for 3min; Supernatant discarded and cells plated on AXI plate.

136 • Plates incubated overnight at 37 °C.

137 AXI Plate: To 100ml of LB add 100 µl ampicillin (Initial concentration: 100mg/ml), 120 µl X-gal (Initial
138 concentration: 100mg/ml), 24 µl IPTG (Initial concentration: 1M).

139 Screening for clones:

140 • Probable clones were screened by restriction digestion and checked for the release of the gene.

141 • Positive clones were sequence confirmed and processed further for Sub-Cloning in the expression
142 vector (pBIN19).

143 Sub-cloning of sequence confirmed p19 in pBIN19

144 • The pUC57+p19 clone was restriction digested (*Hind*III/*Bam*HI); released gene was then cloned into
145 pBIN19 at (*Hind*III/*Bam*HI).

146 • Probable clones were screened & confirmed by restriction digestion.

147 The release of the insert from sequence confirmed pBS clone and subcloning in pBIN19:

148 • Clone DNA (4 µg of plasmid DNA is required to release at least one µg of insert) was taken and
149 digested with *Hind*III/*Bam*HI

Component	**Amount**
DNA	4 µg
*Hind*III	36 units
*Bam*HI	36 units
10X Assay buffer	40 µl
Water :	X* µl
Total reaction volume	400 µl

150 *Based on the final volume of the reaction.

151 • The released insert was gel eluted and ligated to pBIN19 vector at *Hind*III/*Bam*HI sites.

152 • 10 µl of the ligation reaction mix was transformed into 100 µl of DH5α competent cells. • Incubated
153 at 4°C (ice) for 30 min and heat shock given at 42°C for 45 sec.

- Quick chilled on ice for 2 min and volume made up to 1ml with LB.
- Incubated at 37 °C for 1 hr in a shaking incubator.
- The cells were then pelleted at 3000rpm for 3min; Supernatant discarded and cells plated on Kanamycin plate.
- Plates incubated overnight at 37 °C.

Screening for clones

- Probable clones were screened by restriction digestion and checked for the release of the gene.
- The p19 gene was finally sequence confirmed and used further for all Agroinfiltration experiments (Figure S2).

2.3. Use of a control construct (GUS bearing) and agroinfiltration in eggplant fruit

- pCAMBIA 1304 was procured and used for standardisation of agroinfiltration protocols.
- Vector details are attached to the supplementry (Figure S3).

Observation:

- The plates showed mixed colonies indicating contamination and hence competent cells were prepared further using the freeze-thaw protocol.

Preparation of freeze/thaw competent cells and transformation

Agrobacterium tumefaciens strain GV3101 was streaked on an LB agar plate in the presence of Rifampicin (20mg/ml stock) and Gentamycin (50mg/ml stock) and incubated at 28ºC for 24-48Hrs. After that, a single colony was picked from an LB-agar plate, and 5ml starter culture is prepared by growing in at 28ºC for 24-48Hrs, in a shaker incubator. The 100ml of LB was inoculated with 1% of inoculums of overnight grown agrobacterium culture (in presence of antibiotics) and grown to an O.D of 0.7. Further, the culture was spun down with 4ºC for 10 minutes at 5000 rpm. The pellet was resuspended in 10ml of ice-cold sterile 20mM $CaCl_2$ and spun at 4ºC for 10 minutes at 5000 rpm. The cell pellet was re-suspended in 1ml of ice-cold sterile 20mM $CaCl_2$, and 100µl aliquots were made. Around 1µg plasmid DNA was added to the cells and the cells/DNA mix was frozen on dry ice for 5 minutes. The frozen cells/DNA mix was thawed to room temperature for 5-10 minutes and the mix was transferred to a tube containing 2ml LB medium and incubated with shaking at 28ºC for 2-4 hours. The cells were pelleted at 3000rpm for 5 mins and plated on selective LB-agar plates. The LB agar plates were made with Rifampicin; Gentamycin and Kanamycin. Plates incubation was at 28ºC for 3-4 days.

Observation:

- The plates showed individual colonies and the same were replica plated for future use.
- The colonies were screened by PCR for confirmation and once confirmed for the presence of plasmid DNA, were further sub-cultured for use in agroinfiltration experiments.

Agroinfiltration of eggplant fruits

- Loop full culture of agrobacterium clone harbouring the recombinant plasmid was inoculated in 5ml LB broth containing the respective antibiotics and grown at 28ºC for overnight.

190 • The overnight grown culture was sub-culture in a fresh 5ml LB broth and allowed to grow to an O.D of 1.6
191 at 600nm.

192 • The cells were then pelleted at 6000rpm for 5 mins at room temperature.

193 • The cell pellet was re-suspended in infiltration medium (sterile water) to retain the O.D at 1.6.

194 • The final agrobacterium suspension was used for agroinfiltration of Eggplant fruits.

195 • A 2ml syringe with a needle was used to inject the Eggplant fruits at 10-15 spots and allowed to grow for
196 3 to 10 days after infiltration (DAI).

197 • The fruit samples were harvested from 3 days onwards and screened for positive expression of the GUS
198 gene by X-Gluc staining.

199 • 1mM X-Gluc was prepared according to standard protocol, and the fruit sections were stained for 30mins
200 at 37ºC and visualised under a light microscope (LYZER LT-1610X).

201 • The fruits 3 DAI showed best X-Gluc staining, as compared with the 7 DAI and 10 DAI fruits.

202 • Also, post 5 DAI the fruits started to show yellowing in the fruit colour. And hence the 3 DAI was finalised
203 for use on the SmHQT gene studies.

204 *2.2. Agroinfiltration of SmHQT+p19 in Eggplant fruits*

205 • For the agroinfiltration experiment of SmHQT, the pBIN19 clones harbouring the SmHQT gene and p19
206 gene respectively were transformed in agrobacterium using the above-mentioned freeze/thaw protocol.

207 • The agrobacterium is harbouring the pBIN19+SmHQT (Figure S4), and pBIN19+p19 clones, respectively,
208 were screened by colony PCR using vector-specific primers.

209 • The PCR positive colonies were replica plated and sub-cultured for further use in agroinfiltration
210 experiments.

211 • The agroinfiltration was performed as per the standardised protocol mentioned above, and the fruits were
212 harvested at 3 DAI.

213 • Thereafter, fruits were harvested and stored in -80ºC for further studies.

214 Note. The agrobacterium cultures harbouring only the pBIN19+SmHQT and pBIN19+p19 were used at a ratio
215 of 1:1 for all agroinfiltration experiments.

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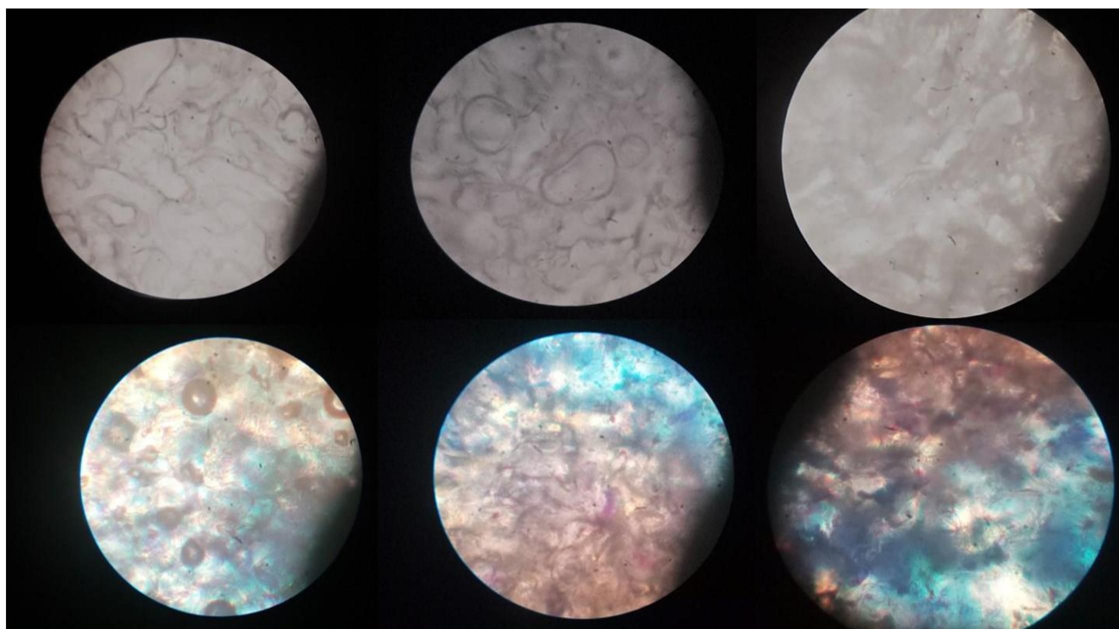
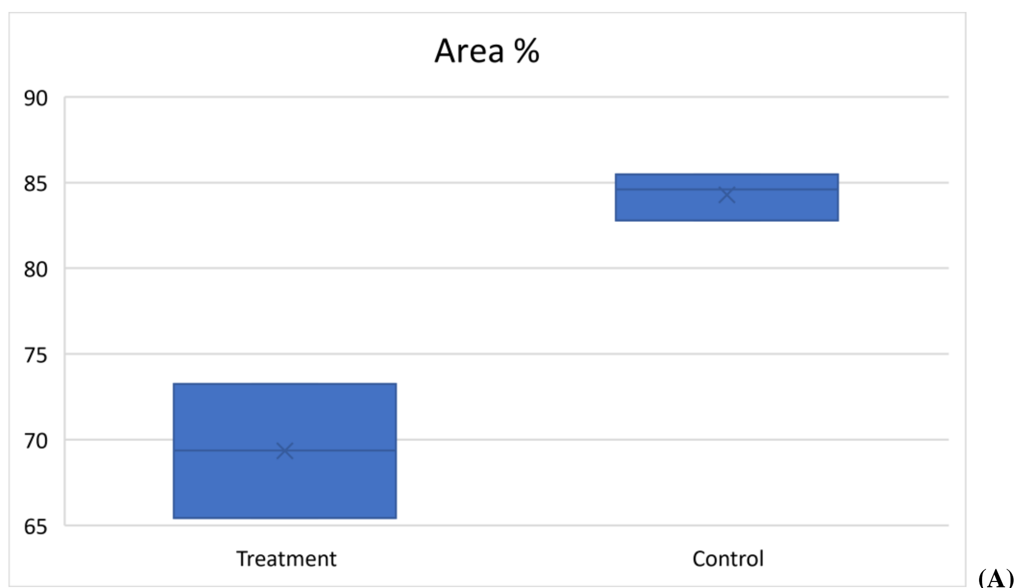
216 

217 **Figure 2.** Comparison of control (above) and transgenic (below) fruit slices via X-Gluc staining.

218 *2.3. High-performance liquid chromatography analysis*

219 For the confirmation of the transgenic tissues HPLC based analysis was performed based on the already
220 standardised protocol for the estimation of chlorogenic acid content in the eggplant defined elsewhere [3].
221 Briefly, The lyophilised fruit tissues (0.1 g) were agitated with a 2ml methanol:water (80:20, v/v) along with
222 0.1% (w/v) of 2,3-tert-Butyl-4-hydroxyanisole (BHT) [30]. After that, the mixture was filtered through 0.2-µm
223 size membrane filters. High-performance liquid chromatography (HPLC) system was used to compare the
224 control and the transgenic fruit samples. The assessment of CGA content was performed on to a 1220 Infinity
225 LC Technique (Agilent Technologies, Santa Clara, CA, USA) [30]. The outcomes were computed by the
226 OpenLAB CDS ChemStation Edition package (Agilent Technologies) following the manufacturer guidelines.
227 The results of HPLC analysis are presented in the Figure 4. Percentage of peak area in the chromatogram was
228 determined based on the chlorogenic acid peak area and the total peak area of other phenolic acids
229 (hydroxycinnamic acid conjugates). The total area increased for other hydroxycinnamic acid conjugates and
230 thereby, resulting in an overall lower percentage of area (Figure 4A).The transgenic showed more than two
231 times of chlorogenic acid content, whereas, area under chlorogenic acid curve was lesser than the control (Figure
232 4 B). While, the peak area also increased in the transgenic sample (Figure 4 C).

233 

(A)

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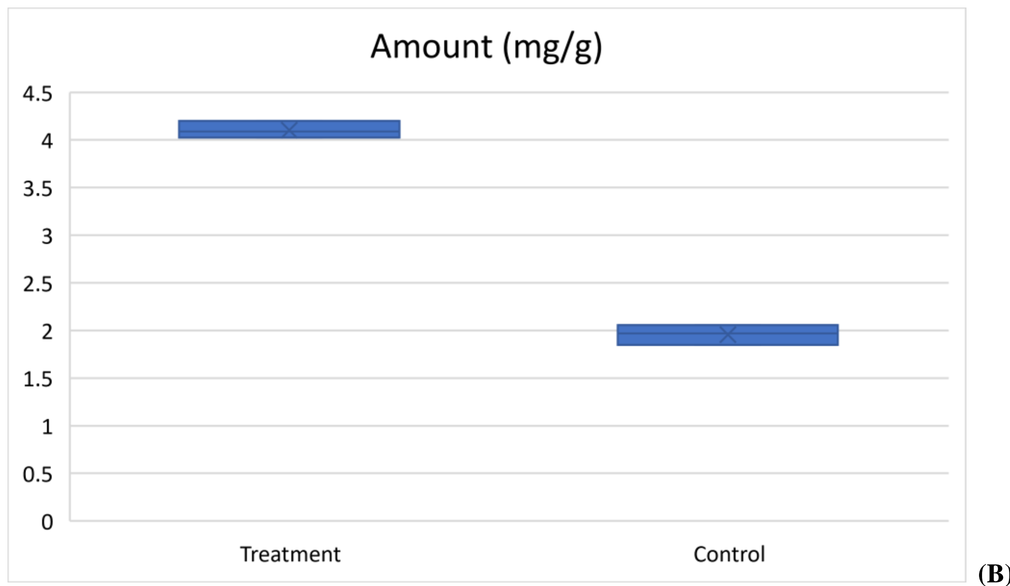

235 (B)

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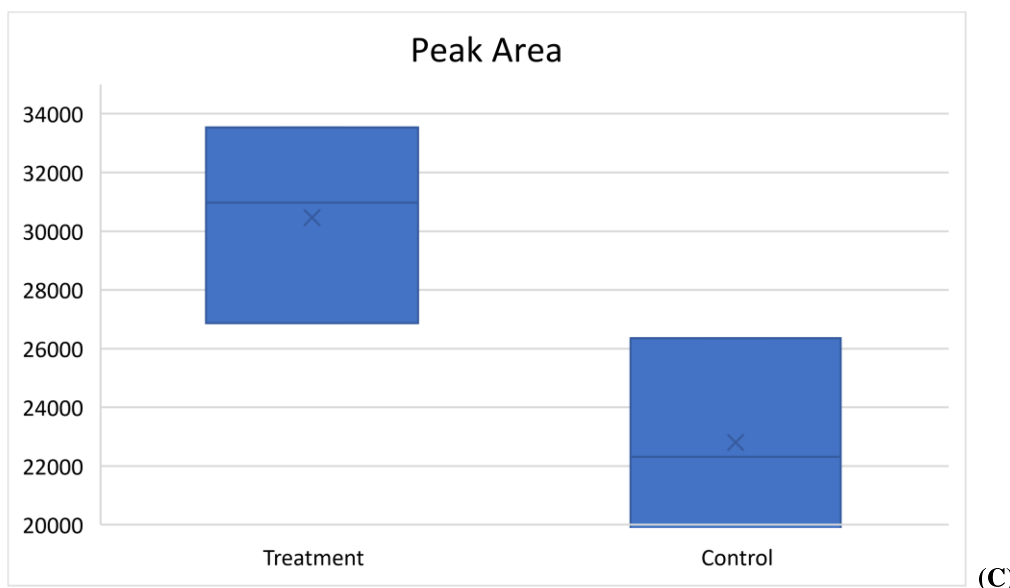

237 (C)

238 **Figure 3.** HPLC analysis based difference among control vs treatment, for area (A, %), amount (B, mg/g) and
239 peak area (C).

240 ## 3. Conclusions

241 Agroinfiltration, a method developed here is user-friendly, and this transient gene expression system designed
242 for the eggplant fruits will be useful in swiftly and precisely identifying the fruit tissue-specific gene functions
243 along with protein production. Further, if this technique is coupled with plant omics tools, e.g. with
244 transcriptomics (RNA-seq), it will provide the detailed information of gene activity, and with metabolomics, an
245 overall shift in the fruit metabolism because of the insert of a particular gene. Also, this method could apply to
246 other members of family Solanaceae those were never discovered from the genetic transformation viewpoint.
247 To avoid the traditional long breeding cycle and to transfer the gene of interest rapidly without even worrying
248 about the unnecessary genomic region linked to a gene, the genetic engineering methods are of great choice. In
249 planta, if we have to study the gene function of a gene at the specific organ of the plant, the agroinfiltration
250 methods are used. In eggplant, there is not any standard agroinfiltration protocol to measure the gene expression.

Although, eggplant produces several fruits that is a system to mass produce a specific protein. The biosynthetic pathway of chlorogenic acid is well known in eggplant. While the hydroxycinnamoyl CoA-quinate (HQT) is the central enzyme studied to increase the chlorogenic acid content in Solanaceae and other families. The function of eggplant transferase (SmHQT) enzyme is the esterification of 4-coumaroyl CoA and quinic acid to form CGA, and further to provide the entry molecule of the flavonoid pathway. In our study, we have developed the agroinfiltration method for the eggplant fruit, and successfully agroinfiltrated the eggplant fruits. Further, we have tested the efficiency of our protocol with natively over-expressing the smHQT gene in the eggplant fruit's flesh.

Supplementary Materials: Figure S1: Vector map pBIN19, Figure S2: Representation of vector pBIN19 with p19 (protein of tomato bushy stunt virus), Figure S3: Vector details of pCAMBIA 1304 used for the standardization of agroinfiltration protocols, Figure S4: Representation of vector pBIN19+SmHQT construct.

Conflicts of Interest: The author declare no conflict of interest.

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