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1 **Developing a robust *in vivo* hairy root system for assessing transgene** 2 **expression and genome editing efficiency in papaya**

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12 **Abstract**

13 Papaya is one of the most important fruits in tropical and subtropical countries. However, genetic
14 improvement has had limited success to date due to time-consuming and complex transformation and regeneration
15 technologies, as well as a lack of reproducible and efficient transient gene expression assays. Here, we report the
16 development of a highly efficient *Rhizobium rhizogenes*-based *in vivo* hairy root system for evaluating transgene
17 expression and activity including CRISPR/Cas gene editing reagents in the Vietnamese papaya cultivar Linhan.
18 To optimize the papaya transformation parameters, we introduced the *R. rhizogenes* strain K599 into papaya
19 hypocotyls at 1-, 5- and 10-mm below the cotyledon nodes by a needle using 5-, 7- and 10-day old seedlings and
20 then monitored the frequency of hairy root formation at 18 days post infection. We found that the age of the
21 seedlings and the distance of the infection site from the cotyledon node were inversely correlated with the efficacy
22 of hairy root induction, being 5-day-old plants and 1-mm distance the best parameters. The established protocol
23 was then employed to investigate transformation frequency using the GUS reporter gene. Of the tested hairy roots,
24 47.22% were positive for GUS staining, which indicates high level of transgene transfer and stability. Finally, we
25 introduced a dual guide RNA CRISPR/Cas9 cassette targeting eukaryotic translation initiation factor isoform 4E
26 (*eIF(iso)4E*) gene into papaya by *R. rhizogenes* and then screened for gene editing events by heteroduplex analysis
27 and Sanger sequencing. Our analysis revealed that 50% of induced roots contained the expected mutations in the
28 *eIF(iso)4E* gene, which makes our system ideal for testing transgene activity prior making stable transgenic papaya
29 lines.

30 **Key message**

31 We developed an efficient procedure for papaya *in vivo* hairy root induction which may be used to validate
32 transgene expression and accelerate CRISPR/Cas-based genome editing studies in papaya.

33 **Keywords:** *R. rhizogenes*, Papaya (*Carica papaya* L.), CRISPR/Cas9, hairy root transformation, K599

34 **Introduction**

35 Papaya (*Carica papaya* L.) is one of the most important fruits of the tropical and subtropical regions
36 (Evans & Ballen, 2012). In Southeast Asia, papaya is cultivated in all countries, especially in Indonesia,

37 Philippines, Thailand, Malaysia and Vietnam (FAO, 2019). The ripe fruit is a rich source of antioxidants and
38 nutrients (carotenes, vitamin C, and flavonoids), B vitamins (folate and pantothenic acid), minerals (potassium and
39 magnesium), and fiber (Ming et al., 2008). The unripe fruit produces large amounts of enzymes, which are
40 extensively used in industry and pharmaceutical companies (Yogiraj et al., 2014).

41 Similar to other tropical fruit species, papaya cultivation is jeopardized by several pathogens, of which
42 papaya ringspot virus (PRSV) is one of the biggest threats. PRSV belongs to the genus *Potyvirus* in the family
43 *Potyviridae* and is transmitted by aphid vectors. PRSV infection affects all stages of papaya plant growth, and
44 results in severely reduced fruit yield including fruit size and quality (Sharma & Tripathi, 2014; Tripathi et al.,
45 2008). It is believed that PRSV is a sole factor in preventing the expansion of papaya production worldwide (Abreu
46 et al., 2015). Until now, the most effective method to control PRSV infection is transgenesis. The first two PRSV
47 resistant genetically modified (GM) papaya cultivars Sun Up and Rainbow expressing the coat protein (CP) of
48 PRSV as transgene were generated in Hawaii, which have been in commercial production since 1998 (Wu et al.,
49 2018). Recently, another GM papaya cultivar Huanong 1 carrying the *Nib* gene of PRSV was created in China (Ye
50 & Li, 2010). However, transgenic papayas exhibit only limited virus resistance that can be broken by PRSV
51 isolates from other geographical regions (Tennant et al., 2001; Wu et al., 2018).

52 Genome editing may be used as an alternative strategy to give rise to durable wide-spectrum virus
53 resistance. Indeed, CRISPR/Cas9-mediated gene editing has been successfully employed to generate potyvirus
54 resistant plants by targeting the viral host factors eukaryotic translation initiation factor 4E (eIF4E) and its isoform
55 eIF(iso)4E in cucumber (Chandrasekaran et al., 2016), *Arabidopsis* and cassava (Pyott et al., 2016; Gomez et al.,
56 2019). The above approach may also be harnessed for papaya. However, genome editing is still in infancy in this
57 species (Brewer & Chambers, 2022) due to complex and inefficient plant transformation and regeneration
58 technologies (Fitch et al., 1993) and the lack of efficient and reproducible transient gene expression systems (Fabi
59 et al., 2014).

60 In this study, we optimized an *R. rhizogenes*-mediated hairy root induction method (Noorda-nguyen et
61 al. 2010) using the GUS reporter gene and the Vietnamese papaya cultivar Linhan, and subsequently developed a
62 pipeline to test the activity of a CRISPR/Cas9 construct targeting the *eIF(iso)4E* gene for further research to
63 generate virus resistant papaya.

64 **Materials and methods**

65 ***Plant materials***

66 Mature seeds of the local papaya cultivar Linhan were provided by the Fruit and Vegetable Research
67 Institute (Ha Noi, Vietnam). Seeds were sown in 7x14 cm (width x height) pots containing clean mixed soil, and
68 then incubated at $25 \pm 2^\circ\text{C}$ with 80% relative humidity under 16-h light/8-h dark photoperiod in a growth chamber
69 (DK-GC 010). Five to ten-day-old seedlings were used for bacterial infection.

70 ***Single guide RNA (sgRNA) design and CRISPR/Cas9 vector construction***

71 Guide RNA sequences, named gRNA1 and gRNA2 were designed by CCTop ([https://cctop.cos.uni-
72 heidelberg.de:8043/](https://cctop.cos.uni-heidelberg.de:8043/)) (Stemmer et al., 2015) to induce targeted mutations at the first and second exons of the
73 *eIF(iso)4E* gene (NCBI: FJ644949.1), respectively (Supplementary Fig. S2a). The forward and reverse
74 oligonucleotides corresponding to each gRNA were annealed to form double-stranded DNA, which were
75 subsequently cloned into the *BsaI* sites of pKSE401 (Addgene: #62202) (Xing et al., 2014) to yield pKSE401-

76 gRNA1 and pKSE401-gRNA2. These single gRNA-CRISPR/Cas9 vectors were then used as templates to amplify
77 the gRNA expression cassettes, AtU6-gRNA1 and AtU6-gRNA2 by *Bsa*I site flanking primers (Supplementary
78 Table S1). The dual gRNA CRISPR/Cas9 construct was generated by assembling the AtU6-gRNA1 and AtU6-
79 gRNA2 PCR fragments into pKSE401 using Golden Gate cloning (Gao et al., 2013), which was then confirmed
80 by Sanger sequencing. The recombinant vector pKSE401-gRNA1-gRNA2 (Supplementary Fig. S2b) was
81 subsequently introduced into *R. rhizogenes* K599 for hairy root induction in papaya.

82 ***Hairy root induction in papaya***

83 The procedure for *R. rhizogenes*-mediated hairy root induction is illustrated in Fig. 1 and Supplementary
84 Fig. S1. Briefly, a single colony of *R. rhizogenes* K599 wildtype and transformed strains carrying the pZY102
85 (Zeng et al., 2004) and pKSE401-gRNA1-gRNA2 binary vector was resuspended into 200 μ l of liquid YEP
86 medium (10 g/L yeast extract, 10 g/L bacto peptone, 5 g/L NaCl, pH 7.0), supplemented with 100 mg/L
87 streptomycin and 15% glycerol. The entire bacterial suspension was spread onto solidified YEP medium
88 containing 100 mg/L streptomycin and then incubated at 28°C overnight in the dark. A needle (26G x ½'') was
89 used to collect bacterial mass and stab through the papaya hypocotyls at different wounding sites (Supplementary
90 Fig. S1e). The infected seedlings were placed in trays with vented humidity domes at 90% relative humidity and
91 kept in the growth chambers for hairy root induction.

92 ***Papaya transplantation***

93 Papaya plants with induced hairy roots (3 - 4 cm) were cut just below the wounding sites and transferred
94 to pots containing vermiculite and perlite at the ratio of 3:1 (v/v) in trays with vented humidity domes (Fig. 1g, h)
95 and watered with 1/10 MS solutions twice a week.

96 ***GUS histological analysis***

97 Papaya hairy roots transformed with *R. rhizogenes* carrying the pZY102 vector were collected and used
98 for GUS histochemical staining as described by (Jefferson et al., 1987). Briefly, hairy roots were incubated in 5-
99 bromo-4-chloro-3-indolyl glucuronide solution at 37°C in the dark for 10 – 12 hours. The *gus* gene expression, as
100 indicated by blue staining in papaya hairy roots, was observed and recorded by a Canon G11 camera.

101 ***Analysis of transgene integration and Cas9-induced mutations***

102 DNA was extracted from papaya hairy roots by the CTAB method (Doyle & Doyle, 1987), which was
103 subsequently used for amplifying the GUS gene or the *eIF(iso)4E* locus using gene specific primers
104 (Supplementary Table S1). PCR was performed as follows: 94°C for 3 min, 35 cycles at 94°C for 30s, 58°C for
105 30s, 72°C for 30s, and final elongation at 72°C for 7 min. The GUS-specific PCR products were separated in 1%
106 agarose gel. The *eIF(iso)4E*-specific amplicons were analyzed for Cas9-induced mutation by heteroduplex
107 analysis using native polyacrylamide gel electrophoresis (PAGE) (Zhu et al., 2014). Briefly, PCR products of wild
108 type (WT) and tested hairy roots were mixed in equal amounts, and incubated at 95°C for 10 min followed by re-
109 annealing at room temperature to form homoduplex and heteroduplex DNA. The samples with induced mutations
110 were detected on 15% native PAGE by differentiated migration of DNA bands as compared to the WT sample. To
111 further characterize the Cas9- induced mutations, the amplicons were ligated into the pJET1.2/blunt cloning vector
112 (Thermo FisherScientific, USA) and up to 10 clones were sequenced by the Sanger method using the ABI3500XL
113 system (Applied Biosystems). Indels at the CRISPR/Cas9 target sites were identified by DNA alignment using
114 MEGA-X software version 10.2.5.

115 **Data analysis**

116 All experiments were performed in three replicates (n = 30). Data was collected and analyzed using one-
117 way ANOVA followed by a post hoc Duncan's multiple-range test in SPSS program version 20 (IBM corporation,
118 USA). Significant differences were indicated at $p < 0.05$.

119 **Results and Discussion**

120 ***Optimizing papaya in vivo hairy root induction***

121 *Injection sites*

122 To optimize the papaya transformation parameters, we first tested how the position of the *R. rhizogenes*
123 injection site affected the efficiency of hairy root induction. To this end, we introduced *R. rhizogenes* K599 cells
124 into the hypocotyls of 5-day-old papaya seedlings at 1-, 5- and 10-mm below the cotyledon nodes by a needle (Fig.
125 1). We then monitored the frequency of hairy root formation at 18 days post infection (dpi). We found that the
126 efficacy of hairy root induction was the highest when the *Rhizobium* was introduced 1 mm below the cotyledons
127 (56.67%, Fig. 2a). This value was significantly reduced to 23.33% and 13.33% for the 5 mm and 10 mm injection
128 sites, respectively. We also noted that no hairy root emerged from the infected seedlings after 18 dpi. Our results
129 indicate that the position of infection site is crucial for efficient hairy root induction, where the closer the site is to
130 the cotyledon node, the higher the efficacy of hairy root formation. It is in line with previous observations made
131 with soybean (Cao et al., 2009; Kereszt et al., 2007). In contrast, the hairy root induction rate was significantly
132 lower at the injection sites close to the cotyledons in pigeon pea (Meng et al., 2019). For cucumber, the highest
133 transformation frequency was associated with infection site 1 cm away from the cotyledons, which decreased in
134 each direction (Fan et al., 2020).

135 *Papaya seedling age*

136 Next, we investigated the impact of plant age on the efficacy of hairy root induction. Five-, seven- and
137 ten-day-old papaya seedlings were used for *R. rhizogenes* injection at 1 mm below the cotyledon nodes, and hairy
138 root formation was recorded at 18 days post infection. We found that 5-day-old seedlings had the highest root
139 induction rate (63.33%), which was significantly lower for 7- and 10-day old plantlets, 43.33% and 36.67%,
140 respectively (Fig. 2b). This result indicates that the seedling age is a key factor in papaya hairy root induction; the
141 younger the seedlings, the higher the efficacy. However, less than 5 day-old plants were not suitable for *in vivo*
142 hairy root induction due to wound-induced hypersensitive response and consequently the low survival rate (data
143 not shown). Previous studies identified similar trends in other crops (Cao et al., 2009; Fan et al., 2020;
144 Tariverdizadeh et al., 2018). In soybean, where 1 to 5-day-old seedlings were used for bacterial infection, the
145 average time for hairy root emergence was found to be shorter for younger seedlings (Cao et al., 2009). Similarly,
146 higher hairy root induction frequency was observed for 5 and 7-day-old cucumber seedlings when compared to
147 older plantlets (Fan et al., 2020). In addition, the highest hairy root induction was observed for the youngest
148 explants (7-day-old) of fenugreek (Tariverdizadeh et al., 2018).

149 ***Reporter gene expression in papaya hairy roots***

150 We then used the above optimized plant transformation system to assess transgene expression in papaya
151 hairy roots. First, *R. rhizogenes* cells harboring the pZY102 vector were injected into 5-day-old papaya seedlings
152 at 1 mm below the cotyledons and subsequently GUS histochemical staining was performed at 18 days post
153 infection using *in vivo* induced hairy roots. In two independent large-scale experiments involving over 100 plants

154 (Table 1), we observed 55% hairy root induction rate in average, which was in line with our preliminary data (Fig
155 2). Importantly, 47.22% of tested hairy roots were positive for *gus* gene, which was confirmed by GUS staining
156 and PCR (Fig. 2c, d).

157 Although *Agrobacterium tumefaciens*-mediated stable papaya transformation was developed over three
158 decades ago, the transformation efficiency has not been improved considerably (Azad et al., 2013; Cabrera-ponce
159 et al., 1996; Cheng et al., 1996; Fitch et al., 1993; Fitch & Manshardt, 1990). To accelerate gene function analysis
160 and transgene expression, an *Rhizobium rhizogenes*-based hairy root induction system was first established in
161 papaya in 2010 (Noorda-nguyen et al., 2010) with approximately 20% efficacy. In this study, we further optimized
162 the *R. rhizogenes*-mediated transformation system (see above), which resulted in over a two-fold increase in hairy
163 root induction (~55%; Fig 2, Table 1). Moreover, we developed a protocol for growing the papaya plants with
164 transgenic hairy roots in soil in the greenhouse, which promotes root development and may open new avenues for
165 transgenic research.

166 ***Application of the hairy root induction system to test gene editing reagents***

167 Finally, we employed the hairy root induction system to assess the efficacy of a genome editing vector
168 pKSE401-gRNA1-gRNA2 (Supplementary Fig. S2b), which targets the *eIF(iso)4E* gene in papaya. After
169 transformation, six hairy root lines were randomly selected for examining CRISPR/Cas9-induced mutations by
170 heteroduplex analysis. We identified three lines (E1, E12 and E14), which showed shifted DNA bands when
171 compared to a WT sample, suggesting targeted mutations at the *eIF(iso)4E* locus (Fig. 3a). Indeed, Sanger
172 sequencing of PCR products amplified from the E1, E12 and E14 lines confirmed Cas9-mediated genome editing
173 including large DNA deletions between the two gRNA target sites (Fig. 3b). Line E1 harbored biallelic mutations
174 of *eIF(iso)4E* with a 1302 bp and 1269 bp DNA lesion, respectively. Interestingly, the 1269 bp deletion was also
175 found in heterozygous form in line E14. In E12, two deletion mutants (-1273 bp and -1281 bp) and a WT allele
176 were detected by sequencing. Taken together, all identified mutations were DNA lesions, indicating that each
177 gRNA was very active and highly specific, which resulted in simultaneous DNA cuts and subsequent deletions
178 between the gRNA1 and gRNA2 target sites.

179 To our knowledge, this is the first report demonstrating the utility of the hairy root induction system for
180 investigating the activity of a CRISPR/Cas9 gene expression cassette in papaya. Generating stable transgenic
181 papaya for validating CRISPR/Cas9 constructs is challenging due to inefficient, complex and time-consuming
182 transformation and regeneration technologies (Azad et al. 2013; Cabrera-ponce et al. 1996; Cheng et al. 1996;
183 Fitch et al. 1993; Fitch & Manshardt 1990). Thus, our highly efficient transgene expression system may be used
184 for rapidly testing and optimizing gene editing reagents to improve agronomic traits including virus resistance in
185 papaya.

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189 **Author contributions:** HC and PD conceived and supervised the study. TH, NN, and PD designed the study. AM
190 and MM designed, and sequence verified the CRISPR/Cas9 target sites. NL designed the CRISPR/Cas vector. TH
191 and NN performed the experiments. TH and NN wrote the manuscript. LN, TB, ND, AM, and PD assisted in

192 writing the manuscript. NN and PD analyzed the data. NP, AM, HC, and PD revised and proofread the manuscript.
193 All authors contributed to the article and approved the submitted version.

194 **Declarations**

195 **Conflict of interest:** Authors declare that they have no conflict of interest.

196 **Data availability**

197 All data generated or analysed during this study are included in this published article [and its supplementary
198 information files]

199 **Additional Information**

200 **Supplementary Fig. S1** Schematic of papaya hairy root induction and optimization. **a** *R. rhizogenes* K599 carrying
201 a binary plant transformation vector. **b** Bacterial infection. **c** Papaya hairy root formation. **d** Molecular analysis of
202 independent papaya hairy root lines. **e** Position of wound site (1, 5 and 10 mm away from cotyledon nodes); red
203 arrow indicates the shoot tip. **f** Age of seedlings used for bacterial infection.

204 **Supplementary Fig. S2** Schematic of the papaya eIF(iso)4E locus and the CRISPR/Cas9 construct pKSE401-
205 gRNA1-gRNA2. **a** Sequence and position of sgRNA1 and sgRNA2 targeting the eIF(iso)4E gene. PAM are
206 highlighted in red. Arrows indicate the position of primers used for genotyping and sequencing. **b** CRISPR/Cas9
207 construct for dual editing the eIF(iso)4E gene. KanR, Kanamycin resistant gene; Cas9, Maize-codon-optimized
208 Cas9 gene; 35S promoter, Cauliflower Mosaic Virus 35S promoter. sgRNA1 and sgRNA2 are expressed under
209 the control of the Arabidopsis U6 promoter (AtU6p). U6ter, Arabidopsis U6 terminator; NLS, Nuclear localization
210 signal; LB/RB – left and right border.

211 **Supplementary Table S1** Sequence of oligonucleotides used in this study

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296 Tables

297 **Table 1** Summary of the transfection experiments with *R. rhizogenes* harboring the pZY102 vector

Replicates	Survival plant (%) ^(*)	Hairy root induction (%) ^(**)	Hairy roots with GUS (%) ^(***)
1 st	90	60	50
2 nd	100	50	44.44
Mean	95	55	47.22

298 ^(*) and ^(**), numbers are calculated as percentage of total number of injected plants

299 ^(***), numbers are calculated as percentage of total number of plants with induced hairy roots

300 Legends to figures

301 **Fig. 1** Procedure of papaya hairy root induction using *R. rhizogenes* K599. **a** 5-day-old seedlings individually
 302 grown in pots containing mixed soil. **b** Collection of *R. rhizogenes* strain K599 from solidified YEP plate for
 303 papaya transformation. **c** Introduction of *R. rhizogenes* K599 into hypocotyl by a needle. **d** *R. rhizogenes* adhesion
 304 at the wound site. **e-f** Representative papaya seedlings at 10 and 18 days after infection. **g** Typical phenotype of a
 305 plant with hairy root prior transferring the upper part into soil. The position of cut site is indicated by a black bar.
 306 **h** Chimera papaya plant with transgenic hairy root in vermiculite. **i** Image of elongated hairy roots at 10-days after
 307 transplanting (10-DAP).

308 **Fig. 2** Optimization of hairy root induction and characterization of transgene expression in induced papaya hairy
 309 roots. **a, b** Effect of injection sites and seedling age on papaya hairy root formation at 18 dpi, respectively.
 310 Statistical analysis was performed using one-way ANOVA post-hoc Duncan's test. Different letters indicate
 311 significant differences at $p < 0.05$ ($n = 10$). **c** Representative image of GUS-stained hairy roots transformed by
 312 pZY102. The red and black arrow indicates transgenic and non-transgenic hairy root, respectively. **d** Testing
 313 pZY102-transformed hairy root lines for the presence of *gus* reporter gene by PCR. WT, wild-type hairy root; H1,
 314 H2.1, H2.2, H3 and H4 independent hairy root lines; +, DNA amplified from the pZY102 vector as positive control.
 315 M, 1 kb DNA ladder (ThermoScientific, USA).

316 **Fig. 3** Identification and characterization of CRISPR/Cas9-induced mutations at the targeted *eIF(iso)4E* locus in
 317 papaya hairy roots. **a** Detection of CRISPR/Cas9-induced mutations in hairy root lines by heteroduplex mobility
 318 assay. Plants were infected with *R. rhizogenes* harboring the pKSE401-gRNA1-gRNA2 gene editing vector. WT,
 319 wild-type hairy root; E1, E5, E8, E9, E12 and E14, CRISPR/Cas9-induced *eIF(iso)4E* mutant hairy roots, red
 320 letters indicate gene edited lines with extra DNA bands compared to WT; red triangles indicate DNA band shifts.
 321 **b** Sequence analysis of the *eIF(iso)4E* locus from the selected hairy root lines after heteroduplex analysis. Cas9
 322 gRNA1 and gRNA2 target sequences are underlined. PAM sequence is highlighted in blue. Δ indicates the size of
 323 identified DNA deletion. “clones” indicate the number of sequenced clones from the corresponding hairy root line.

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