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

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Abstract

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

Key message

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

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

Introduction

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

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

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

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

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

Materials and methods

Plant materials

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

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

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

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

Hairy root induction in papaya

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

Papaya transplantation

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

GUS histological analysis

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

Analysis of transgene integration and Cas9-induced mutations

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

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

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

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

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

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

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

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

Declarations

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

Data availability

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

Additional Information

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

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

Supplementary Table S1 Sequence of oligonucleotides used in this study

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Tables

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

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

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

Legends to figures

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

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

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