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Agrobacterium rhizogenes Generates Transgenic Hairy Roots in *Carica papaya* L.: A New Approach for Studying and Improving Resistance to the Root-rot Pathogen, *Phytophthora palmivora*

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ABSTRACT

Phytophthora palmivora is an oomycete pathogen of *Carica papaya* L. that has a wide host range and causes especially severe root- and fruit-rot diseases. *P. palmivora* flourishes on papaya during the rainy season when fungicide treatments are often washed away. There are no varieties of *C. papaya* L. with resistance to *P. palmivora.* Genetic engineering with significant resistance genes has the potential to improve *C. papaya*'s tolerance to *P. palmivora* and to better understand the interactions between the host plant and this pathogen. However, there must first be an efficient *Agrobacterium rhizogenes*-mediated transformation protocol to produce rapid regeneration of transformed roots to evaluate the functional roles of the defense-related genes and proteins in papaya. In this study, we report the production of *in vivo* composite *C. papaya* L. plants by direct injection of cultures of *A. rhizogenes* transformed with the binary vector, pCNL65, into the cotyledonary nodes of papaya seedlings. Hairy roots were also produced by inoculating *in vitro* hypocotyl and cotyledon pieces of tissue culture plants with *A. rhizogenes*. Both procedures produced hairy roots within 1-3 weeks. Approximately 20% of the roots derived from inoculated seedlings tested positive for beta-glucuronidase (GUS) activity. This method of papaya transformation has the potential for the rapid evaluation of candidate genes involved in plant – pathogen interactions, particularly those involving roots.

Keywords: function of genes, oomycete, plant transformation, plant-pathogen interaction
Abbreviations: GUS; beta-glucuronidase; **IBA**, indole-3-butyric acid; **OD**, optical density; **PCR**, polymerase chain reaction; **YMB**, yeast extract mannitol broth

INTRODUCTION

Predicted disease resistance genes and translation products were identified from the recently published papaya (*Carica papaya* L.) genome sequence (Ming *et al*. 2008) and compared to *A. thaliana*, *Oryza sativa*, and *Populus trichocarpa* (Porter *et al*. 2009). The majority of cloned resistance genes are members of the NBS-LRR superfamily, however, *C. papaya* L. maintains only a limited number of these genes. Papaya has been proposed as a model system for the study of gene function due to its small genome size and relatively low number of genes. Fifty-four NBS encoding R genes have been identified and classified from *C. papaya* (Porter *et al*. 2009). A large number of MAPK and MAPK kinases were predicted, indicating that signal transduction pathways in papaya are highly conserved. Homologs of other conserved signaling components, such as *RAR*1, *NDR*1, *EDS*1, *PAD*4, which are known to be recruited by R genes, were identified in the papaya genome. The broad defense mechanism defined as systemic acquired resistance (SAR) has also been examined and a papaya homolog of the key regulator, NPR1, has been cloned (Zhu *et al*. 2003). Several transcription factors which interact with NPR1 proteins were also identified along with a large number of downstream PR genes. It is hoped that the candidate genes identified in the papaya genome might be incorporated into a hairy root transformation project to allow for the *in planta* evaluation of the functional roles of these disease resistance genes and proteins, for example *Phytopthora* resistance.

Agrobacterium rhizogenes is a soil-borne bacterium responsible for hairy root disease. It induces root formation in plants through the *rolA-D* genes located within the transfer DNA (T-DNA) of its root-inducing (Ri) plasmid (Chilton *et al*. 1982). *A. rhizogenes* transfers and integrates its T-DNA into the host genome through the same mechanism as *Agrobacterium tumefaciens* and induces transformed adventitious root formation that is quite rapid compared to the rate of root formation by plants regenerated from transformed callus. In addition, *A. rhizogenes* can be engineered to transform plants with transgenes using the binary vector or co-integration method. Plant transformation using *A. rhizogenes* can induce the growth of transgenic hairy roots *in vivo* to produce composite plants, a term derived from the fact that transformed roots are induced on a non-transformed plant (Boisson-Dernier *et al*. 2001), or *in vitro* to regenerate plants from hairy roots cultures. True transgenic plants can be obtained by regeneration from *A. rhizogenes*-transformed hairy roots as has been reported for *Medicago trunculata* (Crane *et al.* 2006).

Hairy root cultures have been used to study nodulation and nitrogen fixation, for the production of plant secondary metabolites and for studying interaction with other organisms, such as mycorrhizal fungi or nematodes (Alpizar *et al.* 2006). Hairy root cultures incorporating candidate genes involved in *Phytophthora* resistance, are potentially useful for the characterization of gene function and their involvement in signal transduction pathways. *Phytophthora palmivora* is a soil-borne pathogen that initially attacks lateral roots and spreads to the taproot of papaya. Papaya roots are particularly susceptible in the three month period after seed-

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Short Communication

ling emergence. Infected plants exhibit yellowing of the leaves, premature defoliation and eventual death (Ko 1994). In this study, we investigate the application of *A. rhizogenes* for the production of composite plants by direct injection of cultures of the bacterium into the cotyledonary nodes of papaya seedlings.

MATERIALS AND METHODS

A. rhizogenes transformation

Competent cells of *Agrobacterium rhizogenes* strain A4 were transformed with the binary plasmid pCNL65. This plasmid contains the neomycin phosphotransferase gene (*nptII*) and also a marker gene, (*uidA*), for beta-glucuronidase (GUS). Briefly, 10 µL of pCNL65 (~700 ng) was added to electrocompetent cells, frozen in liquid nitrogen, thawed for 5 minutes at 37°C, resuspended in 1 mL of YMB, and shaken for 3 hours at 28°C. The bacterial culture was plated on YMB plates containing 100 µg/mL kanamycin to select for transformed colonies. Single colonies were picked, grown in liquid YMB with 50 µg/mL kanamycin, and analyzed by PCR for the presence of the neomycin phosphotransferase gene, *nptII* (Fitch *et al*. 1990).

Inoculation

A bacterial colony culture was grown to an OD_{600} of ~0.6 for inoculations. *In vitro* grown *C. papaya* of 'Kapoho' and 'Kamiya' cultivars were used. Explant pieces of approximately three week-old hypocotyls and cotyledons, and leaves and stems from 6-month-old tissue culture plants were inoculated with the bacterial culture for 15 minutes then placed on MS media (Caisson Laboratories, Utah, USA) without growth regulators for 3 days in the dark at 22°C. Explant tissues were then washed three times in sterile water and transferred to agar-solidified half-strength MS medium containing 2% sucrose, 100 mg/L kanamycin and 250 mg/L cefotaxime. Tissues were subcultured every two weeks until roots emerged. For *in vivo* soil-grown plants (Sunshine 4 potting mix), approximately 5-10 µL of a bacterial culture was injected into the cotyledonary nodes using a hypodermic syringe. Injected plants were then incubated in sealed petri plate bags under 12/12 hr light/dark conditions.

Analysis

Samples collected from inoculated materials were tested for beta-glucuronidase activity (GUS assay) which is indicative of the transfer of T-DNA from pCNL65 (Jefferson 1987). Testing was carried out at 51 and 68 days post-injection for *in vivo* transformed roots. *In vitro* plant material was tested after hairy roots had formed, approximately 3 weeks post-inoculation. PCR was used to determine if the T-DNA from either the Ri plasmid (*rolC*) or pCNL65 (*nptII* and GUS gene, *uidA*) was present in the adventitious roots of *in vivo*-transformed plants. DNA was extracted from adventitious roots using the method of Dellaporta (1994). The primers used to amplify a 491 bp fragment of the *nptII* gene were 5′-ACTGAAGCGGGAAGGGACTG-3′ and 5′-GCGGCGATACC GTAAAGCAC-3′. Primers specific for *rolC* were 5′-TGTGACA AGCAGCGATGAGC-3′ and 5′-AAACTTGCACTCGCCATGCC-3′. To ensure there was no inhibition of the PCR reaction primers based on a papaya actin partial sequence (accession AY906938), were also used. The actin primers, amplified a 200 bp fragment in all samples. The *nptII*, *rolC* and actin primers were used at 1 µM. A total volume of 50 µL was prepared for use in a Bio-Rad iCycler® thermocycler. The conditions were 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 45 s with a final extension at 72°C for 10 min (McCafferty *et al*. 2008).

RESULTS AND DISCUSSION

Inoculation of papaya tissue-cultured plants and injection of soil-grown plants with *A. rhizogenes* induced root formation within 1-3 weeks. Some of the emerging adventitious roots were "hairy" in appearance while others were smooth (**Fig. 1**). It appeared that growth of the hairy roots may have reached a plateau around 2 months post-inoculation. Timing of root formation is in line with the findings of Cabrera-Ponce *et al.* (1996) who reported the induction of roots from inoculated papaya leaf disks in 1-4 weeks. Tissue culture-roots emerged earlier (1 week) than did the injection-induced roots on soil-grown plants (2.5 weeks). In previous work with papaya, Consoli *et al.* (1995) investigated different strains of *A. rhizogenes* and inoculation methods. They reported that direct wounding, with a needle, was most efficient even though the A4T strain of *A. rhizogenes* that they used to infect *Centrosema pubescens* was not able to infect papaya.

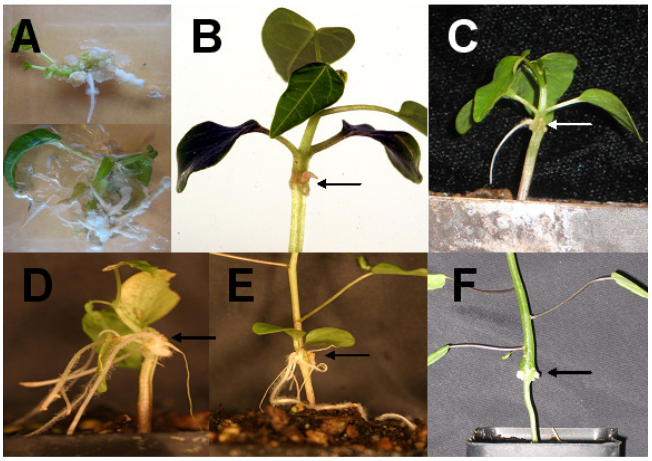

Fig. 1 *A. rhizogenes*-induced hairy roots in *C. papaya* L. plants. (**A**) Tissue culture papaya shoots with *A. rhizogenes*-induced hairy roots. (**B**) 'Kapoho' with hairy root emerging 17 days post-injection. (**C**) 'Kamiya' with hairy root 16 days post-injection. (**D, E**) 'Kamiya' hairy roots 49 days post-injection. (**F**) 'Kapoho' plant with hairy roots removed 68 days post-injection. Site of injection is indicated by arrow.

In the case of tissue culture plants, we found that the hypocotyls were more easily transformed than either the cotyledonary node or mature leaves and stems (**Fig. 1**). Consoli *et al.* (1995) also found that papaya hypocotyl sections were most responsive to infection. Hu and Du (2006) reported that although most plant organs such as hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, or tuber could be used to induce hairy roots, it was important to select the optimum explant for a given species. They also stated that the age of the material is most critical, with juvenile stages being best.

In vivo injected plants had a high rate of hairy root growth, but the number of emerging roots varied (**Table 1**). It appears that there was a difference between the two cultivars tested, with 'Kapoho' forming fewer hairy roots than 'Kamiya'. Expression of binary T-DNA via histological GUS assay was examined 5-8 weeks post-inoculation. Approximately one-third (~30%) of roots tested stained blue while cotyledons, which were used as a negative control, showed no staining (results not shown). PCR analysis of DNA from adventitious root material of *in vivo* grown plants gave positive results using the papaya actin primers. This indicates that there should be no false negatives. Positive results were also obtained with primers for *rolC*. However, PCR analysis did not indicate the presence of the binary T-DNA (*uidA* and *nptII*) (results not shown). This result suggests that although the Ri plasmid (T-DNA) has successfully integrated into the plant, the binary plasmid has been lost. This may be due to inefficient binary T-DNA integration into the plant chromosome and subsequently chimeric expression of transgenes in the hairy roots since only five of the 24 hairy roots tested for GUS activity stained blue (**Table 1**). Limpens *et al.* (2004) pointed out that one of the drawbacks with producing composite plants is that not all roots are transformed and even those that are transformed can be chimeric. Other researchers have also reported problems in detecting binary plasmids in adventi-

Table 1 Hairy root formation in *C. papaya* explants and intact seedlings after injection with *A. rhizogenes* A4. Testing was carried out at 51 and 68 days post-injection for *in vivo* transformed roots. *In vitro* plant material was tested after hairy roots had formed, approximately 3 weeks post-inoculation.

Plant material	№ of explants/plant	Plants with hairy roots	№ of hairy roots/plant	Hairy roots positive in GUS assay
'Kapoho' tissue culture hypocotyls (~ 2 cm)	70	31 (44%)	1-6	-
'Kapoho' cotyledons	25	9 (36%)	1-3	-
'Kapoho' soil-grown seedlings inoculated at the cotyledonary nodes	6	4 (66%)	0-6	2/6
'Kamiya' soil-grown seedlings inoculated at the cotyledonary nodes	16	15 (93 %)	0-10	3/18

tious roots. For example, McAfee *et al.* (1993) were unable to detect T-DNA in adventitious roots by Southern blotting and suggested that either there was no T-DNA transfer or it was present at too low a level to detect.

The 'Kapoho' plants injected with *A. rhizogenes* exhibited an abnormal growth phenotype (**Fig. 1F**). Secondary growth of the hypocotyl below the injection site appears to be inhibited compared to the region above the injection site. Injected plants were no longer able to stand upright outside of the bags. Increased growth of the plant in covered bags may be responsible or have contributed to this observation. What is more interesting is the possibility that *A. rhizogenes*' *rol A-D* genes may have contributed to this change in growth of the plant region below the injection site. In their evaluation of the application of hairy roots to plant genetic engineering, Hu and Du (2006) summarized visible morphological alterations including asymmetrical growth and reduced internode length, which is in line with our observations.

This is to the best of our knowledge, the first report of *A. rhizogenes* strain A4 induction of hairy roots in papaya and of the direct induction of hairy roots on potted plants. The transformation methods reported will need to be optimized in order to produce more abundant hairy roots that stably express binary T-DNA. Alteration of the culture media may promote more root growth. Li and Leung (2003) found that there was a synergistic action with the addition of indole butyric acid (IBA) to media for radiata pine. It might be interesting to screen different strains of *A. rhizogenes* to determine if a particular strain would be better for papaya transformation. It would also be beneficial to optimize plant regeneration from transformed roots. This would allow the effects of genes and promoters at the whole plant level to be assessed and analysis of any resulting progeny. Using *A. rhizogenes* to induce hairy roots in papaya has been shown to be a viable technique and with some refinements, it could prove a useful tool for the study of the papaya/*Phytopthora* interaction.

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REFERENCES

Alpizar E, Dechamp E, Espeout S, Royer M, Lecouls AC, Nicole M, Bertrand B, Lashermes P, Etienne H (2006) Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants for studying gene expression in coffee roots. *Plant Cell Reports* **25**, 959-967

Boisson-Dernier A, Chabaud M, Garcia F, Becard G, Rosenberg C, Barker DG (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Molecular Plant-Microbe Interactions* **14**, 695-700

Cabrera-Ponce JS, Vegas-Garcia A, Herrera-Estrella L (1996) Regeneration of transgenic papaya plants via somatic embryogenesis induced by *Agrobacterium rhizogenes*. *In Vitro Cellular and Developmental Biology – Plant* **32**, 86-90

Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempé J (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. *Nature* **295**, 432-434

Consoli L, Gaziola SA, Vieira MLC (1995) Plant transformation mediated by *Agrobacterium rhizogenes*: Optimization of the infection process. *Brazilian Journal of Genetics* **18 (1)**, 115-119

Crane C, Wright E, Dixon RA, Wang Z-Y (2006) Transgenic *Medicago truncatula* plants obtained from *Agrobacterium tumefaciens*-transformed roots and *Agrobacterium rhizogenes*-transformed hairy roots. *Planta* **223**, 1344-1354

Dellaporta S (1994) Plant DNA miniprep and microprep: versions 2.1-2.3. In: Freeling M, Walbot V (Eds) *The Maize Handbook*, Springer-Verlag, New York, pp 522-525

Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya *via* microprojectile bombardment. *Plant Cell Reports* **9**, 189-194

Hu Z-B, Du M (2006) Hairy root and its application in plant genetic engineering. *Journal of Integrative Plant Biology* **48 (2)**, 121-127

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* **5**, 387-405

Ko W-H (1994) Phytophthora fruit rot and root rot. In: Ploetz RC, Zentmeyer GA, Nishijima WT, Rohrbach KG, Ohr HD (Eds) *Compendium of Tropical Fruit Diseases*, APS Press, St. Paul, MN, pp 61-62

Li M, Leung DWM (2003) Root induction in radiata pine using *Agrobacterium rhizogenes*. *Electronic Journal of Biotechnology* **6 (3)**, 251-258

Limpens E, Ramos J, Franken C, Raz V, Compaan B, Franssen H, Bisseling T, Guerts R (2004) RNA interference in *Agrobacterium rhizogenes*-transformed roots of *Arabidopsis* and *Medicago truncatula*. *Journal of Experimental Botany* **55**, 983-992

McAfee BJ, White EE, Pelcher LE, Lapp MS (1993) Root induction in pine (*Pinus*) and larch (*Larix* ssp.) using *Agrobacterium rhizogenes*. *Plant Cell, Tissue and Organ Culture* **34**, 53-62

McCafferty HRK, Moore P, Zhu YJ (2008) Papaya transformed with the *Galanthus nivalis* GNA gene produces a biologically active lectin with spider mite control activity. *Plant Science* **175**, 385-393

Ming R, Hou S, Feng Y, Yu Q, Dionne-Laporte A, Saw JH (2008) The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature* **452**, 991-996

Porter B, Paidi M, Ming R, Alarm M, Nishijima W, Zhu YJ (2009) Genome-wide analysis of *Carica* reveals a small NBS resistance gene family. *Molecular Genetics and Genomics* **281**, 609-626

Zhu YJ, Qiu X, Moore PH, Borth W, Hu J, Ferreira S, Albert H (2003) Systemic acquired resistance induced by BTH in papaya. *Physiological and Molecular Plant Pathology* **63**, 237-248