

Agrobacterium-mediated transformation of the lemon cultivars Frost Eureka and Frost Lisbon (*Citrus limon*) and bitter orange rootstock Flying Dragon (*Poncirus trifoliata*)

Abstract

Plant genetic manipulation through genetic transformation is an alternative method to improving HLB susceptible citrus cultivars. This transformation incorporates a foreign gene of interest into the plant genome, and through regeneration of transformed cells, a transgenic host-resistant plant is created. *In vitro* seedling-derived epicotyl segments of Frost Eureka, Frost Lisbon, and Flying Dragon were used as explants in *Agrobacterium*-mediated transformations. Epicotyl segments were treated during inoculation with spermidine (SPD), a polyamine known to regulate hypersensitive response (HR)-induced gene expression. The transformed *Agrobacterium* was utilized to infect the germinating seedlings consequently transferring the spinach defensin genes SoD-2 and SoD-7 into plant seedlings. Of the 667 Frost Eureka transformed explants, there were 76 SPD and 102 non-SPD shoots regenerated. From 500 transformed explants of Frost Lisbon, 3 SPD and 6 non-SPD shoots regenerated; lastly, 260 SPD and 223 non-SPD shoots were regenerated from 434 explants of Flying Dragon. Transgenic shoots were verified after testing for the expression of β -glucuronidase (GUS) reporter genes by histochemical GUS staining and fluorescence microscopy on the shoots. Of the regenerative shoots, Frost Eureka had 8 SPD and 9 non-SPD GUS-positive shoots, and Flying Dragon had 7 SPD and 14 non-SPD GUS-positives. Frost Eureka and Flying Dragon explants treated with spermidine have not provided a positive correlation in the attempt for enhancement in GUS activity in regenerative shoots. As indicated by GUS staining, spinach defensin genes have thus far been successfully transferred to the regenerated shoots, and will be further monitored and examined during their growth process to ensure gene stability.

Introduction and Objectives

Citrus is one of the most economically demanding fruit crops worldwide. Threatening citrus production is the destructive citrus disease Huanglongbing (HLB), commonly known as citrus greening, which occurs in many citrus growing areas of the world. This disease is caused by *Candidatus Liberibacter asiaticus*, a bacterium vectored by the Asian citrus psyllid, *Diaphorina citri*. Symptoms of an infected plant include yellowing of leaves and shoots, twig dieback, production of small bitter fruits, and fruits that are oddly shaped and contain the characteristic green patches when ripe. The infected plants then become the source of inoculum; the psyllid vector feeds on it and disperses the pathogen further in the field. There is no cure for this disease to date. Furthermore, there is no naturally occurring genetic resistance known in the citrus family that can be used for conventional breeding purposes.

By genetically modifying lemons with genes encoding spinach defensins, we will be able to successfully develop citrus-greening-disease-resistant lemons. *Agrobacterium*-mediated transformations was the system used to transfer and integrate our genes of interest into the plant genome. Transformations were performed on seedling-derived epicotyl segments as explants. The *Agrobacterium* strain EHA105 containing spinach defensins SoD-2 and SoD-7 genes was pre-treated with and without spermidine in an attempt to enhance the transformation efficiency through increased *vir* gene induction (Kumar, S. V. et al. 2005).

Methods

Plant Transformations

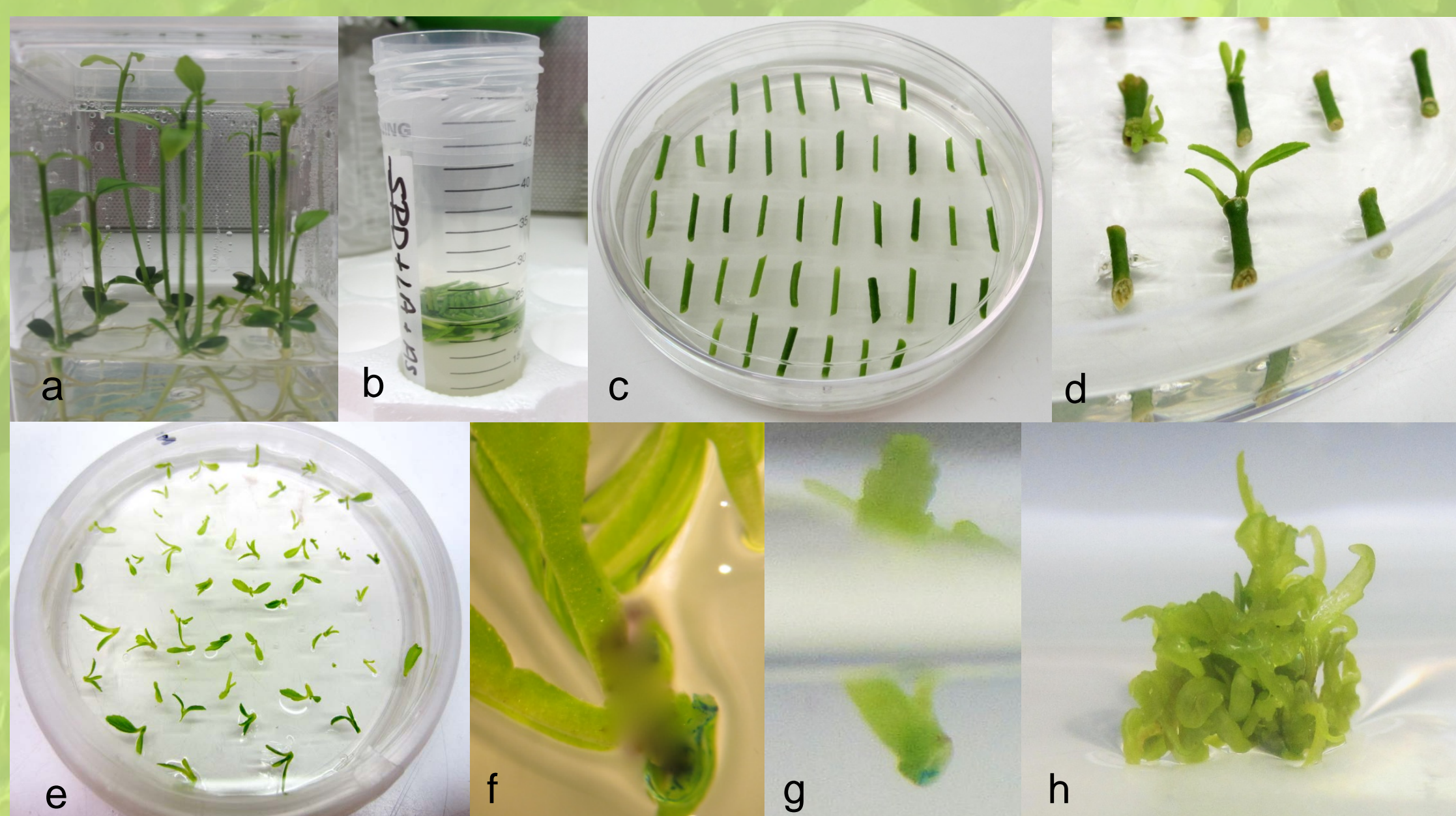


Figure 1. Production and analysis of transgenic plants from epicotyl segments of lemons using *Agrobacterium*-mediated transformation method. a) *In vitro* production of seedlings on half strength Murashige and Skoog (MS) medium (Murashige and Skoog). b) Inoculation of epicotyl segments with *Agrobacterium* strain EHA 105 containing spinach defensins SoD-2 and SoD-7 genes, with and without spermidine. c) Explants placed horizontally on co-cultivation medium (DBA3) (Deng et al. 1992) containing lipoic acid, for *Agrobacterium* growth. d) Explants regenerated on selective (DBA3) (Deng et al. 1992) containing lipoic acid. e) Histochemical GUS staining of shoots performed on transgenic shoots to localize the GUS reporter genes. f) GUS-positive shoots transferred to shoot elongation medium such that the g) basal portion of the shoot is in contact with the medium to promote h) further growth of transgenic shoot.

Results

Table 1. Influence of polyamines on T-DNA transfer to plant cells in Tobacco leaf rings based on GUS activity (Kumar, S.V. et al. 2005)

Treatment of <i>Agrobacterium</i> cells with polyamines	% GUS positive explants	GUS foci/explant
MS (-ve control) ^a	—	—
MS (+ve control) ^b	70 ± 11.31	4.5 ± 0.70
MS + 1 mM PUT	89.5 ± 2.12	17 ± 1.41
MS + 1 mM SPD	94.5 ± 6.36	30.5 ± 6.36

a. Untransformed control leaf explants.

b. Leaf explants transformed with *Agrobacterium* without polyamine pre-treatment.

GUS positive explants have increased approximately 24.5% with the use of spermidine (SPD) in *Agrobacterium* gene transfer.

Table 2. Results of *Agrobacterium*-mediated transformation with and without spermidine treatment on citrus explants

Citrus Variety	Total Explants	Non-SPD Explants	SPD Explants	Non-SPD Shoots	SPD Shoots	GUS + Non-SPD	GUS + SPD
Frost Eureka	667	358	309	102	76	9	8
Frost Lisbon	500	249	251	6	3	-	-
Flying Dragon	434	192	247	223	260	14	7

GUS staining revealed there has been no significant difference of GUS activity between SPD and Non-SPD shoots in Frost Eureka and Flying Dragon. Shoots are being regularly monitored to observe gene silencing of both SPD and Non-SPD regenerated transgenic shoots. Due to an extensive seedling production and regeneration period, Frost Lisbon and other *in vitro* epicotyls of these varieties have yet to be tested.

Future Research

PCR technique using gene specific primers will be conducted to verify successfully transformed seedlings and gene expression will be confirmed through the use of western blots. Viable transformants will also be subjected to selection media to eliminate escapes. Further experimental observations to verify resistance to HLB will be made under greenhouse and later field conditions.

Conclusion

- The current study may provide a favorable alternative method to improve citrus cultivars allowing them to overcome disease and environmental stresses. Since this study is in its early stages, it cannot be concluded that spermidine is not effective nor that lemons have been successfully genetically modified.
- This experience taught me the importance of teamwork and the essence of independence to accomplish goals. I have also learned that I have the patience and motivation needed to carry out careful research, something I wish to continue after receiving my bachelor's degree.

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