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Genetic Transformation of *Citrus sinensis* L. with an *antisense ACC oxidase* Gene

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ABSTRACT

This work was carried out to optimize the conditions for highly effective embryogenic callus induction from mature seeds, plantlet regeneration and genetic transformation of *Citrus sinensis* L. by *Agrobacterium tumefaciens* strain EHA105 (pCAMBIA 1305.1). Embryogenic calli could be successfully induced from mature seeds employing the MT medium supplemented with 500 mg/l malt extract. The percentage of embryogenic callus induction was 85. With the same medium, the high proliferation rate of embryogenic callus was achieved. The liquid MT medium containing 500 mg/l malt extract in combination with 50 mg/l lactose could be used as the embryoid development medium. Somatic embryos, however, could be regenerated with normal shoots and roots in the MS medium, with the regeneration percentage of 60. The delivery of an *antisense ACC oxidase* gene into the species *C. sinensis* mediated by *Agrobacterium tumefaciens* strain EHA 105 was successful by co-cultivating explants with the strain EHA 105 for 10 min, following that by eliminating the bacterium with 200 mg/l cefotaxime, and subsequently selecting transformed embryoid with 20 mg/l hygromycin. Verified histochemically by GUS assay, putative transformants showed the percentage of *gus* gene expression of 100. Molecular analysis using PCR confirmed the integration of the *antisense ACC oxidase* gene into plant genome.

Keywords: *Citrus sinensis*; *antisense ACC oxidase*; *Agrobacterium tumefaciens*; Cefotaxime

1. Introduction

Citrus is the most widely grown fruit crop throughout the world [1]. It is the number one fruit of the world on account of its high nutritional value. A large number of the production of fruits and fruit products, and the citrus industry is considered to be a major fruit industry [2]. Harvested fruits which are usually stored before they reach the market for fresh consumption [3] are subjected to biotic and abiotic stress conditions, especially stress from excess ethylene production during the degreening process of citrus fruits [4-8]. Even though citrus fruit is non-climacteric and produces very low amounts of ethylene at mature green state [9], a diurnal low temperature treatment (5˚C) of attached and mature-green grapefruit (*Citrus paradisa* Macf.) as well as harvested tangerine (*C. reticulata* Blanco.) fruits were found to enhance ethylene production and the yellowing of the citrus peel [10]. Furthermore, ethylene treatment during the degreening process of citrus fruits is involved in ethylene production as well as enhances chlorophyllase activity [11-13] and the synthesis of carotenoids [13]. Ethylene applied also enhances heat damage to flavedo tissue of cured citrus fruits [6], increases the appearance of chilling injury symptoms, stem-end rot decay and the content of volatile off-flavors in the juice head space and fruit internal atmosphere during postharvest storage [14]. Attempts to produce transgenic citrus with ethylene-resistant traits by introducing an *antisense ACC oxidase* gene into plant genome to block ethylene biosynthesis has been widely made in order to overcome the stress caused by ethylene [15,16] as this method is powerful and feasible.

This study was therefore carried out to optimize the conditions for highly effective embryogenic callus induction from mature seeds, plantlet regeneration and genetic transformation of *Citrus sinensis* L. by *Agrobacterium tumefaciens* strain EHA105 (pCAMBIA 1305.1).

2. Materials and Methods

2.1. Embryogenic Callus Induction and Plantlet Regeneration

Mature seeds of *C. sinensis* were initially washed with mild detergent and rinsed with tap water for 3 times. They were subsequently surface-sterilized in 70% (v/v)

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ethyl alcohol for 5 minutes and 20% (v/v) sodium hypochlorite with 2 drops of Tween-20 for 10 minutes. After being rinsed 3 times with sterile distilled water, seeds were cultured on Murashige and Tucker (MT) medium [17] supplemented with varied concentrations of malt extract (0, 200, 300, 400 and 500 mg/l), 50 g/l sucrose and 8 g/l agar, pH 5.8, kept at 25˚C ± 2˚C in dark condition for 8 weeks to determine the optimal concentration of malt extract for embryogenic callus induction. For embryogenic callus proliferation, embryogenic calli were monthly subcultured using the same medium and conditions for 3 months.

For plantlet regeneration, embryogenic calli (1.0 g fresh weight) were transferred to a 250 ml flask containing 50 ml liquid MT medium amended with 500 mg/l malt extract and 5% (w/v) lactose, pH 5.8. The cultures were maintained on a rotary shaker with a continuous shaking (100 rpm) at 25˚C ± 2˚C under a long photoperiod (16 h light: 8 h dark) with light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 12 weeks for embryoid development. After that, embryoids in the cotyledonary stage were selected and rinsed with sterile distilled water for 3 minutes, dried with sterile tissue papers. Then they were cultured on solid MT medium supplemented with 500 mg/l malt ex- tract and kept at 25˚C ± 2˚C under a long photoperiod (16 h light: 8 h dark) with light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 12 weeks.

2.2. Effect of Antibiotics on Embryoid Regeneration

To determine the effect of antibiotics on embryoid regeneration, embryoids developing in the torpedo shape, sized 0.5 cm, were cultured on the MT medium supplemented with cefotaxime concentrations of 0, 100, 200, 300, 400, 500 and 600 mg/l. The effective concentrations of hygromycin were also determined. The concentrations tested were 0, 10, 15, 20 and 25 mg/l. All kinds of antibiotics were added to the medium after autoclaving. The cultures were maintained at 25˚C ± 2˚C under a long photoperiod (16 h light: 8 h dark) with light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 5 weeks.

2.3. *Agrobacterium*-Mediated Transformation

Agrobacterium tumefaciens strain EHA105 (pCAMBIA-1305.1) were used for the establishment of the transformation. The plasmid pCAMBIA1305.1 carried GUS gene and hygromycin-resistant (*hpt*II) gene, each expressed under the CaMV35S promoter. The bacterial strain was cultured in Luria Broth (LB) liquid medium supplemented with 100 mg/l kanamycin and maintained on a reciprocal shaker at 28˚C for 48 hours until OD_{600} = 1.5 - 1.8.

Embryoids in the torpedo stage were used as explants for transformation in this experiment. The explants were soaked in *Agrobacterium* suspension for 0, 5, 10, 15, 20 and 25 minutes. Then they were cocultivated on the MT medium for 3 days. After cocultivation, the explants were washed thoroughly in sterile distilled water containing 300 mg/l cefotaxime for 15 minutes. Explants were subsequently transferred to the MT medium supplemented with 500 mg/l malt extract, 200 mg/l cefotaxime and 15 mg/l hygromycin for 6 weeks.

2.4. Histochemical GUS Assay

The histochemical assay for GUS gene expression was performed using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as a substrate [18]. Briefly, putative transformants were transferred to a 1.5 ml microtube containing X-gluc and subsequently incubated overnight at a temperature of 37˚C.

2.5. PCR Analysis

Total genomic DNA was extracted from embryoids of transformed plantlets and nontransformed control plantlets by the CTAB method [19]. The primer sequences for PCR were as follows: NOS forward sequence (F)5'-GAATCCTGTTGCCGGTCTTG-3', reverse sequence (R)5'-TTATCCTAGTTTGCGCGCTA-3' to yield a 180 bp fragment. The DNA was denatured at 94˚C for 4 min, followed by 35 cycles of amplification (1 min at 92˚C; 1 min at 55˚C; 2 min at 72˚C). The final incubation at 72˚C was extended to 4 min, and the reaction material was cooled and kept at 4˚C. The PCR products were visualized by running the completed reaction on a 2% agarose gel containing ethidium bromide.

2.6. Statistical Analysis

Statistical significance was accepted at $p < 0.05$. All results were analyzed by One-way ANOVA using the Statistical Package for Social Sciences v17.0 software (SPSS Inc. IL, USA).

3. Results

3.1. Embryogenic Callus Induction and Plantlet Regeneration

The development of seeds into friable calli was distinct 4 weeks after being cultured on the MT medium supplemented with malt extract. Differences in callus induction percentage were recorded (**Figure 1**). The maximum callus induction percentage at 85 was obtained in the medium containing 500 mg/l malt extract. Moreover, the development of calli into embryogenic calli showing embryoids in the globular stage was evident in week 8 only in the presence of 500 mg/l malt extract. It was found

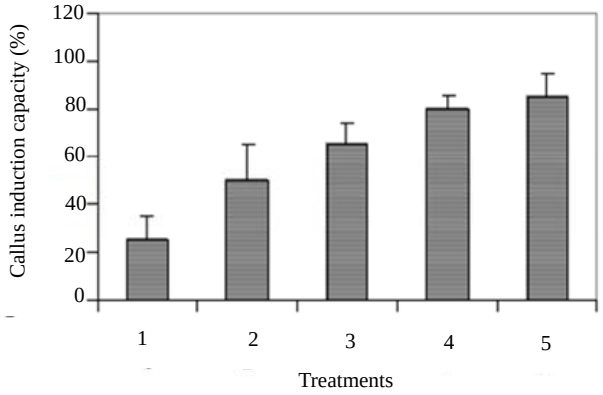

Figure 1. Callus induction percentage under different conditions: 1) 100 mg/l; 2) 200 mg/l; 3) 300 mg/l; 4) 400 mg/l and 5) 500 mg/l.

that embryogenic callus induction under high concentrations of malt extract was more effective than that under low concentrations.

The development of embryogenic calli into plantlets was obtained in the MT medium supplemented with 500 mg/l malt extract and 5% lactose. After 6 weeks of culture, embryoids in the heart stage were seen and finally developed into the torpedo stage in week 8. After 12 weeks of culture, embryoids in the cotyledonary stage were obtained (**Figure 2**).

3.2. Effect of Antibiotics on Embryoid Regeneration

Antibiotics used in the study strongly reduced regeneration capacities of *C. sinensis* embryoids. In the presence of 100 - 600 mg/l cefotaxime and 10 - 25 mg/l hygromycin, a slight inhibitory effect was observed. The highest dose of cefotaxime that yielded surviving embryoids was 200 mg/l (**Figures 3(a)** and **4(a)**). The lowest dose of hygromycin that completely inhibited embryoid growth was 20 mg/l (**Figures 3(b)** and **4(b)**). All of the embryoids turned brown and finally died in five weeks after they were transferred to the selective medium.

3.3. Agrobacterium-Mediated Transformation

Differences in levels of GUS activities in embryoids after being cocultivated for 0 - 25 minutes were detected (**Figure 5(a)**). The optimal cocultivation time for the maximum GUS activities was 10 min. *Agrobacterium*-mediated transformation of *C. sinensis* yielded a maximum percent expression (100%) (**Figure 5(b)**). To determine the integration of T-DNA fragments in hygromycin-resistant plantlets, polymerase chain reaction (PCR) analysis was carried out. It was found that the size of amplified fragment was 180 bp for NOS, whereas non-transformed control plantlets did not show any expected band size (**Figure 6**).

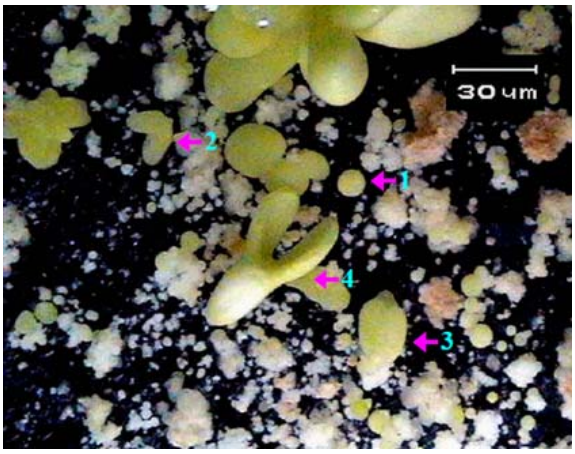

Figure 2. Embryoids in different stages: 1) globular stage, 2) heart stage; 3) torpedo stage; and 4) cotyledonary stage.

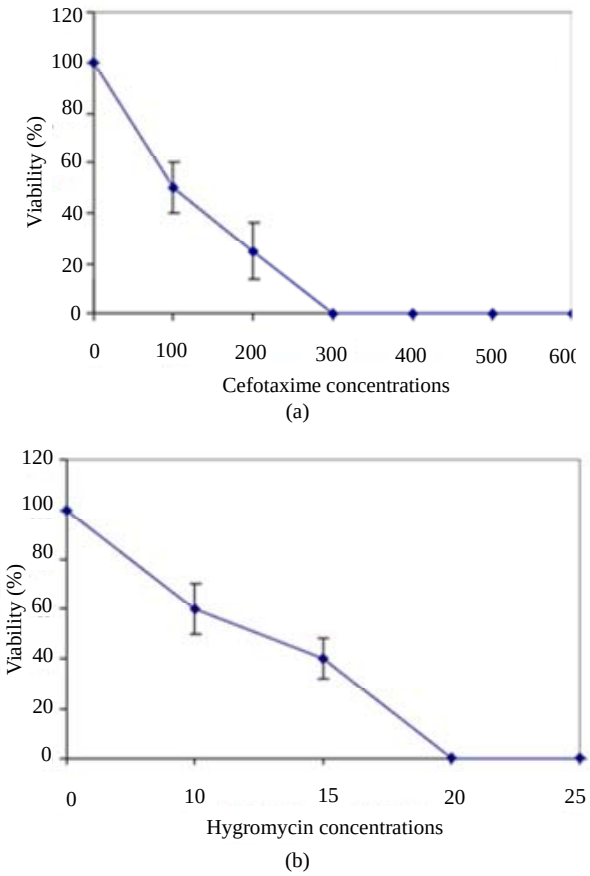

Figure 3. Effect of different concentrations of cefotaxime (a) and hygromycin; (b) on *C. sinensis* embryoid growth.

4. Discussion

Addition of malt extract in the medium is essential to embryoid induction in citrus. In this study, the optimal concentration of malt extract for embryogenic callus induction was 500 mg/l. The results were in agreement with the finding reporting that inducing ovules derived

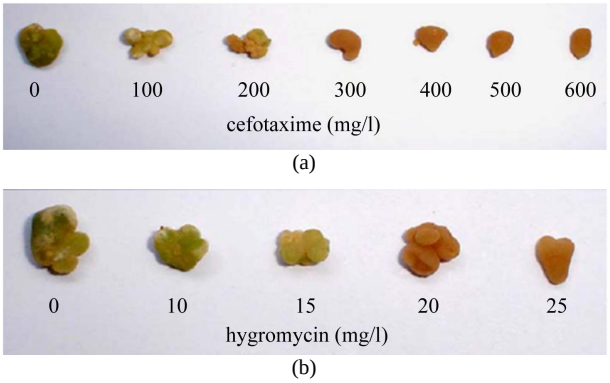

Figure 4. Embryoids on the MT medium containing different concentrations of cefotaxime (a) and hygromycin (b).

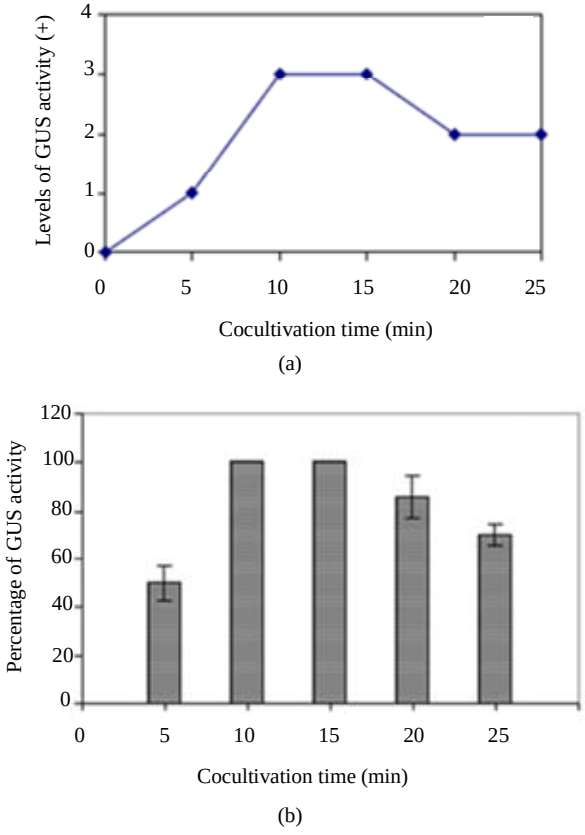

Figure 5. Levels (a) and percentages (b) of GUS expression in embryoids after being cocultivated for 0 - 25 min.

from 8 week seeds of *C. sinensis* into somatic embryos was obtained in the MT medium supplemented with 500 mg/l malt extract [20]. Another report also claimed that addition of 500 mg/l malt extract in the Murashige and Skoog (MS) medium could induce the development of *C. sinensis* style tissues into somatic embryos [21].

A selective agent is crucial for selection of the transformants and for avoiding development of undesirable numbers of the escapes. It was suggested that hygromycin is an excellent selective agent and needs to be opti-

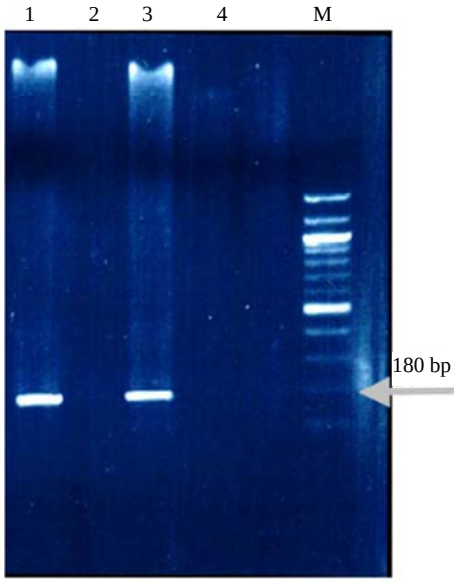

Figure 6. PCR analysis in transformed embryoids in *C. sinensis* using primers to detect NOS; lane M: 100 bp ladder, lane 1: transformed plantlets; lane 2: nontransformed control plantlets; lane 3: pCAMBIA1305.1 (positive control), and lane 4: negative control.

mized for each plant species [22]. In this report, *hpt*II encoding resistance to hygromycin was used in the production of transgenic citrus. Hygromycin are aminoglycoside antibiotics which cause harmful death to plant cells by inhibiting transcription and translation.

In order to eliminate *A. tumefaciens* after cocultivation, addition of antibiotics in the medium is required. In this study, cefotaxime showed strong inhibition of the regeneration potential in *C. sinensis*. However, it was found that the concentration of 200 mg/l did not inhibit the regeneration of explants. Our findings are in agreement with the finding reporting that cefotaxime concentration of 200 mg/l did not inhibit the regeneration of explants in *Malus sylvestris* var. "Delicious" [23].

In this study, a number of transformed *C. sinensis* were produced using *Agrobacterium*-mediated transformation system. The results confirmed that embryoids can be used as explants for this transformation system. Moreover, our findings showed that the CaMV35S promoter was useful for *C. sinensis* transformation.

5. Conclusions

In summary, this report described the use of *A. tumefaciens* strain EHA105 (pCAMBIA 1305.1) to transfer screenable and selectable marker genes into *C. sinensis* and showed molecular evidence of primary transgenic plants which demonstrated stable integration of transgenes. We confirm that embryoids of *C. sinensis* are the suitable target tissues for *Agrobacterium*-mediated transformation.

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REFERENCES

[1] Z. N. Yang, I. L. Ingelbrecht, E. Louzada, M. Skaria and T. E. Mirkov, "*Agrobacterium*-Mediated Transformation of the Commercially Important Grapefruit Cultivar Rio Red (*Citrus paradisi* Macf.)," *Plant Cell Reports*, Vol. 19, No. 12, 2000, pp. 1203-1211. doi:10.1007/s002990000257

[2] H. C. Chaturvedi, S. K. Singh, S. K. Sharma, A. K. Sharma and S. Agnihotri, "Citrus Tissue Culture Employing Vegetative Explants," *Indian Journal of Experimental Biology*, Vol. 39, 2001, pp. 1080-1095.

[3] L. González-Candelas, S. Alamar, P. Sánchez-Torres, L. Zacarías and J. F. Marcos, "A Transcriptomic Approach Highlights Induction of Secondary Metabolism in Citrus fruit in Response to *Penicillium digitatum* Infection," *BMC Plant Biology*, Vol. 10, 2010, p. 194. doi:10.1186/1471-2229-10-194

[4] I. L. Eaks, "Physiological Studies of Chilling Injury in Citrus Fruits," *Plant Physiology*, Vol. 35, No. 5, 1960, pp. 632-636. doi:10.1104/pp.35.5.632

[5] W. C. Cooper, G. K. Rasmussen and E. S. Waldon, "Ethylene Evolution Stimulated by Chilling in Citrus and *Persea* sp.," *Plant Physiology*, Vol. 44, No. 8, 1969, pp. 1194-1196. doi:10.1104/pp.44.8.1194

[6] S. Ben-Yehoshua and I. L. Eaks, "Ethylene Production and Abscission of Fruit and Leaves of Orange," *Botanical Gazette*, Vol. 131, No. 2, 1970, pp. 144-150. doi:10.1086/336525

[7] H. Hyodo, "Ethylene Production by Albedo Tissue of Satsuma Mandarin (*Citrus unshiu* Marc.) Fruit," *Plant Physiology*, Vol. 59, No. 1, 1977, pp. 111-113. doi:10.1104/pp.59.1.111

[8] J. B. Biale, R. E. Young and A. J. Olmstead, "Fruit Respiration and Ethylene Production," *Plant Physiology*, Vol. 29, No. 2, 1953, pp. 168-174. doi:10.1104/pp.29.2.168

[9] Y. Aharoni, "Respiration of Oranges and Grapefruit Harvest at Different Stages of Development," *Plant Physiology*, Vol. 43, No. 1, 1968, pp. 99-102. doi:10.1104/pp.43.1.99

[10] W. C. Cooper, G. K. Rasmussen and D. J. Hutchison, "Promotion of Abscission of Orange Fruits by Cycloheximide as Related to the Site of Treatment," *Bioscience*, Vol. 19, No. 5, 1969, pp. 443-444. doi:10.2307/1294480

[11] A. C. Purvis and C. R. Barmore, "Involvement of Ethylene in Chlorophyll Degradation in Peel of Citrus Fruits," *Plant Physiology*, Vol. 68, No. 4, 1981, pp. 854-856. doi:10.1104/pp.68.4.854

[12] E. E. Goldschmidt, M. Huberman and R. Goren, "Probing the Role of Endogenous Ethylene in the Degreening of Citrus Fruit with Ethylene Antagonists," *Plant Growth Regulation*, Vol. 12, No. 3, 1993, pp. 325-329. doi:10.1007/BF00027214

[13] E. E. Trebitsh, E. E. Goldschmidt and J. Riov, "Ethylene Induces de Novo Synthesis of Chlorophyllase, a Chlorophyll Degrading Enzyme, in Citrus Fruit Peel," *Proceedings of the National Academy of Science of the USA*, Vol. 90, No. 20, 1993, pp. 9441-9445. doi:10.1073/pnas.90.20.9441

[14] R. Porat, B. Weiss, L. Cohen, A. Daus, R. Goren and S. Droby, "Effects of Ethylene and 1-Methylcyclopropene on the Postharvest Qualities of 'Shamouti' Oranges," *Postharvest Biology and Technology*, Vol. 15, No. 2, 1999, pp. 155-163. doi:10.1016/S0925-5214(98)00079-9

[15] A. J. Hamilton, G. W. Lycett and A. J. Grierson, "Antisense Gene That Inhibits the Synthesis of the Hormone Ethylene in Transgenic Plants," *Nature*, Vol. 364, 1990, pp. 284-287. doi:10.1038/346284a0

[16] P. W. Oeller, M. W. Li, L. P. Taylor, D. A. Pike and A. Theologis, "Reversible Inhibition of Tomato Fruit Senescence by Antisense RNA," *Science*, Vol. 254, No. 5030, 1991, pp. 437-439. doi:10.1126/science.1925603

[17] T. Murashige and D. P. H. Tucker, "Growth Factor Requirement of *Citrus* Tissue Culture," *Proceedings of 1st Citrus Symposiums*, Vol. 3, University of California, Riverside, 1969, pp. 1155-1161.

[18] R. A. Jefferson, "Assaying Chimeric Genes in Plants: The GUS Gene Fusion System," *Plant Molecular Biology Reporter*, Vol. 5, No. 4, 1987, pp. 387-405. doi:10.1007/BF02667740

[19] J. J. Doyle and J. L. Doyle, "A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue," *Phytochemical Bulletin*, Vol. 19, 1987, pp. 11-15.

[20] J. Kochaba, P. Spiegel-Roy and H. Safran, "Adventive Plants from Ovules and Nucelli in *Citrus*," *Planta*, Vol. 106, No. 3, 1972, pp. 237-245. doi:10.1007/BF00388101

[21] F. Carmini, M. C. Tortorici, F. Pasquale, F. G. Crescimanno and F. Pasquale, "Somatic Embryogenesis and Plant Regeneration from Undeveloped Ovules Stigma/Style Explants of Sweet Orange Navel Group (*Citrus sinensis* (L.) Osb.)," *Plant Cell, Tissue and Organ Culture*, Vol. 54, No. 3, 1998, pp. 183-189. doi:10.1023/A:1006113731428

[22] K. Datta, R. Valazhahan and N. Oliva, "Overexpression of the Cloned Rice Thaumatin-Like Protein (PR-5) Gene in Transgenic Rice Plants Enhanced Environmental Friendly Resistance to *Rhizoctonia solani* Causing Sheath Bright Disease," *Theoretical and Applied Genetics*, Vol. 98, No. 6-7, 1999, pp. 1138-1145. doi:10.1007/s001220051178

[23] S. Sriskandarajah, B. P. Goodwin and J. Spiers, "Genetic Transformation of Apple Scion Culture 'Delicious' via *Agrobacterium tumefaciens*," *Plant Cell, Tissue and Organ Culture*, Vol. 36, No. 3, 1994, pp. 317-329. doi:10.1007/BF00046089

