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Agrobacterium tumefaciens-Mediated Transformation in *Citrus* and Characterization of Transgenic Regenerants

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Citrus is one of the most widely grown and economically important fruit crops in the world. Development of an efficient direct regeneration system from leaf explants of citrus has been attempted in this study. The effects of several factors, including culture medium, lighting condition, explant age and genotype on regeneration response were examined based on three parameters, percentage of explants producing shoots, mean number of shoots per explant and shoot forming capacity. Shoot regeneration medium consisted of MT salts plus 0.5 mg/ L BA, 0.5 mg/L Kinetin, 0.1 mg/L NAA, 3% sucrose and 0.8% agar, pH 5.8, proved to be the most effective medium for direct induction of shoots from leaf explants among the three media tested. Highly significant difference in the response of shoot bud regeneration was noted between the two cultivars, with Bingtangcheng being more responsive than Valencia. Culture of explants excised from fully developed leaves resulted in better shoot regeneration capacity compared to undeveloped ones. However, the two lighting conditions used herein did not cause significant difference in shoot regeneration. Phenotypic observation and RAPD analysis confirmed that all the regenerated plants from both genotypes were genetically identical

to their donor plants, suggesting absence of detectable genetic variation in the regenerated plants. The data generated in this study revealed that direct initiation of plants from leaf explants has been successfully accomplished. To our knowledge, this is the first report on direct regeneration of shoots from leaf explants in *Citrus*, which will provide an alternative source for citrus genetic manipulation in the future.

Attempts were made to develop an efficient *Agrobacterium tumefaciens*-mediated transformation system to produce transgenic Valencia sweet orange (*Citrus sinensis* L. Osbeck) plants from leaf discs via direct organogenesis in the current study. Various factors affecting T-DNA delivery including explant age, *Agrobacterium* concentration, immersion time, inoculation and co-cultivation media, co-cultivation period and temperature, kanamycin, acetosyringone and antibiotic concentrations were optimized based on *GFP* gene expression and recovery of kanamycin resistant shoots following cocultivation as indicators. Shoots regenerated on medium containing MT salts supplemented with 0.1 mg/L NAA, 0.5 mg/L BA and 0.5 mg /L kinetin arose directly from leaf margins in the form of protuberances without any callus intervening phase. Leaf explants from 3 months old in vitro seedlings had positive effects on transformation efficiency. Infection in *Agrobacterium* suspension of OD_{600}: 0.6 for 10 min and co-culturing on medium containing 25 mg/L acetosyringone for 3 days at 25 ^{o}C yielded efficient transient *GFP* expression, shoots regeneration response and transformation efficiency. The transformation efficiency (12.33%) and mean number of transformed shoots per explant (5.4) were obtained after transfer of 6-8 weeks on selection medium (MT salts augmented with 0.5 mg/L BA, 0.5 mg/L Kinetin, 0.1 mg/L NAA, 50 mg/L kanamycin and 250 mg/L cefotaxime). Integration of *GFP* gene in transgenic plants was confirmed by PCR analysis. Flow cytometry analysis revealed that all the transgenic

plants produced in this system were of diploid nature and morphologically identical with their donor plants. This protocol in the present study is the first of its kind ever developed to transform and regenerate citrus plants using leaf direct regeneration system. Development of this highly efficient regeneration transformation protocol paved the way for generating a T-DNA mutant collection of citrus that will in turn significantly impacts functional genomic research and gene discovery in Rutaceae and other fruit crops.

Cold is one of the most important environmental factors that adversely affect the growth and productivity of citrus plants. In an effort to induce cold tolerance in a citrus cultivar 'GWZ' (Gailiangcheng orange x Weizhang Satsuma mandarin), *PtrICE* gene was introduced in cell suspensions cultures through transformation mediated by *Agrobacterium*. After about 8 weeks of transfer into selection medium (MT salts + 50 mg/L kanamycin + 400 mg/L cefatoxime), whitish kanamycin resistance calli formed from transformed cells, whereas, non-transformed cells grew slowly in the form of dead brownish layer. Six kanamycin-resistant cell lines were isolated and proliferated. The integration of transgene in all cell lines was confirmed by PCR, and RT-PCR analysis. In comparison to the non-transformed cell lines, the transformed ones exhibited a higher level of antioxidant enzymes (SOD, POD, and CAT) activities under stress conditions, which enabled them to inhibit the production of ROS. In addition, the viability of transformed cell lines determined via TTC reduction assay and Evans Blue staining method was more pronounced compared to non-transformed ones in this study. Over-expression of *PtrICE* gene reduced electrolyte leakage levels (EL), Malondialdehyde (MDA) contents and H_2O_2 accumulation in transgenic cell lines in all stress treatments.

Transgenic cell lines exhibited enhanced tolerance under cold stress is being used for regeneration and future breeding purposes.

Gibberellins (GAs) are endogenous hormones that play a predominant role in regulating plant stature. In this work, *TfGA2-ox2* gene was introduced into callus lines of ‘Bingtangcheng’ Sweet orange (*Citrus sinensis* L. Osbeck) by *Agrobacterium*-mediated transformation with the aim to decrease the amounts of bioactive GA levels in these plants and thereby reducing their stature. To optimize the transformation conditions, several factors were assessed, including co-cultivation period, duration of pre-culture in darkness and the infection time of *Agrobacterium tumefaciens.* PCR analysis confirmed the successful integration of *TfGA2-ox2* gene in kanamycin-resistant calluses. The average percentage of kanamycin-resistant calli on selection medium was 11.71%. Maximum transformation efficiency of resistant callus lines based on polymerase chain reaction detection was 69.23%. This approach may provide an alternative to the application of chemical growth retardants used for reducing the stature of plants.