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Agrobacterium-Mediated Genetic Transformation and Regeneration of Salt Tolerant Transgenic of Sour Orange Rootstock (*Citrus aurantium* L.)

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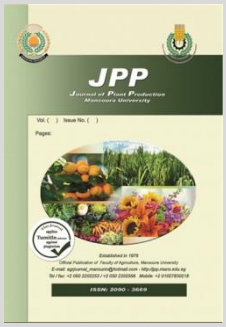

ABSTRACT

Traditional breeding techniques of citrus are complicated and time intensive. In this study, an effective and simple method for the production of sour orange transgenic plants incorporating salt tolerance gene (Pr10a) has been established. Transgenic plants were produced by *Agrobacterium tumefaciens* -mediated gene transfer using epicotyl explants under the selection pressure of phosphinothericin and NaCl as selective agents. Epicotyl segments were excised from *in vitro* seedlings and incubated with *Agrobacterium tumefaciens* EHA105 strain harboring the transgene (Pr10a) under the control of mannopine synthase promoter (p-MAS). In addition, the used dicistronic binary vector contained luciferase (Luc) gene linked to internal ribosome entry site elements in the T-DNA region. MS medium containing 1.5 mg/l 6-benzylaminopurine (BA) and 0.2 mg/l indole butyric acid (IBA) proved optimal for shoot regeneration (97%) from sour orange epicotyl. The number of marker gene (Luc) expressing plants increased as the inoculation time and bacterial density (OD_{600}) increased up to20 min and 0.4, respectively. Under this condition, the transformation efficiency of epicotyl segments was 24.2%. PCR analysis using Pr10a and Luc primers confirmed the existence of the transgenes in transgenic plants where the expected band size of 480 and 837 bp were obtained for both transgenes, respectively. The integration of T-DNA containing Pr10a and Luc genes in the transgenic sour orange was revealed by Southern blot analysis and verified the results of luciferase enzyme activity and PCR analysis of Pr10a and Luc genes. A simple integration pattern for all the transgenes including the single copy integration was predominantly observed.

***Keywords*:** Genetic transformation; Shoot organogenesis; Sour orange; Pr10a; Salinity

INTRODUCTION

Citrus is regarded as one of the most profitable fruit crops worldwide. In Egypt, the total cultivated area of citrus trees is 541723 Feddan (227523.9 ha) with fruit production of about 4.10 million tons (El-Gengaihi *et al*., 2020). Citrus rootstocks are wide varieties, each with its own set of benefits. The tolerance of a rootstock to the prevailing soil, climatic, and disease conditions, as well as its ability to produce great harvests of good quality fruit, are all factors in its selection. Sour orange (*Citrus aurantium* L.) is a commonly used rootstock for all citrus cultivars in the Mediterranean region (Nasser *et al*., 2014), due to its adaptability to various soil conditions, resistance to gummosis and producing high fruit quality.

In the Mediterranean region, salinity is one of existing problems that is expected to worsen as a result of sea level rise and over extraction of coastal aquifer (Vincent *et al*., 2020a). Different agricultural strategies and approaches can be utilized to mitigate and reduce losses due to salinity stress, such as employing optimal irrigation and nutrition systems, using traditional breeding, and conducting genetic transformation to evolve new salt tolerant genotypes. (Munns, 2005; Vincent *et al*., 2020b). Citrus is a salt-sensitive crop because of physiological disruptions, and growth and fruit yield reductions can occur at relatively low salinity levels. (Bernstein, 1969; Bielorai *et al*., 1978; Walker *et al*., 1982; Zekri, 1987). Citrus rootstocks show some differences in their salt tolerance. Sour orange is classified as sensitive (Cooper, 1951) to relatively salt-tolerant rootstock (Zekri, 1987), based on visual leaf burn symptoms and leaf Cl content or growth and water relations measurements.

It is well known that using traditional breeding techniques in citrus is difficult and time consuming due to high heterozygosity and the long juvenility period (Poles *et al*., 2020). So, using of genetic engineering and new plant breeding techniques (Limera *et al*., 2017; Eriksson *et al*., 2018) can overcome these difficulties and novel traits can be incorporated in short time with the maintenance of the characteristics of the original cultivar. Because the majority of citrus species are not natural hosts for *A. tumefaciens* and are resistant to *Agrobacterium*-mediated transformation techniques, their mutual interaction has not evolved to its full potential, as it has in other species. (Singh and Rajam, 2010). The first effort at citrus transformation via direct DNA transfer was reported by Kobayashi and Uchimiya (1989). *Agrobacterium*-mediated transformation has been widely used, with this approach accounting for over 90% of all transgenic plants produced (Gong, and Liu, 2013). Trans-grafting is a recent technique in which a non-genetically modified scion is grafted on a transgenic rootstock, leading to an improved scion performance and non-transgenic fruit yield. (Lusser *et al*., 2012; Limera *et al*., 2017). Additionally, the efficiency of *Agrobacterium*- mediated transformation method is genotype dependent (Poles *et al*., 2020), since some citrus genotypes are regarded easy to transformation (e.g., citranges) (Peñaet *et al*., 1995; Dutt and Grosser, 2009), Duncan grapefruit (Dutt and Grosser, 2009; Orbovi´c, and Grosser, 2015), while other species are reported as recalcitrant (e.g., Clementine) (Cervera *et al*., 2008) and sour orange (Ghorbel *et al*., 2000).

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In this study, we aimed to develop an efficient/successful protocol to obtain transgenic sour orange rootstock with a higher salinity tolerance, which will enable improving vegetative growth, increasing productivity and fruit quality especially under the prevailing salinity condition.

MATERIALS AND METHODS

1. Plant materials and growing conditions

Seeds were taken from mature sour orange fruits and cleaned for 30 minutes under running tap water. The outer cover of the seeds was removed, then the seeds were surface sterilized in 70% ethanol for 30 seconds, then immersed in 50% commercial Clorox (5.25% sodium hypochlorite NaOCl) for 20 minutes before being rinsed three times in sterile distilled water for 5 minutes each time (Abou Elyazid and El-Shereif, 2014). The seeds were cultured for 4 weeks on half strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 15g/l sucrose and solidified with 7g/l agar. All media were adjusted to a pH 5.7 before autoclaving at 101K.Pa, 121ºC for 20 minutes. The cultures were incubated in dark conditions for 3 weeks at 25 °C, before being switched to a 16 h light/8 h dark cycle using cool white fluorescent light (55 µmol m^{-2} s^{-1} photosynthetic photon flux density [PPFD]) for an extra week. Epicotyl explants were grown in 20 mL MS media in sterilized glass Petri dishes (100 x 15 mm). Each Petri dish contained 10 explants and there were three replicates in each treatment. After eight weeks of incubation, the shoot organogenesis percentages were recorded.

2. Agrobacterium tumefaciens strain and vector

Transformation of sour orange was carried out using *Agrobacterium tumefaciens* EHA 105 (Hood *et al*., 1993) carrying a pSoup and pGreen II based dicistronic vector (Figure 1) that contained salt tolerance conferring gene Pr10a under the control of Mannopine Synthase (MAS) promoter (El-Banna *et al*., 2010), and the NOS promoter -*bar* –NOS terminator cassette as a selectable marker gene conferring bialaphos resistance. The *Agrobacterium* strain was grown overnight in 50 ml of Luria-Bertani liquid medium (LB) for agrobacterium containing 50 mg l^{-1} kanamycin and 10 mg l^{-1} rifampicin in shaker incubator at 28 °C and 160 rpm under dark conditions. The *Agrobacterium* suspension of 0.8-1.0 optical density (OD_{600}) was centrifuged at 4000 rpm for 10 min at 4 °C. The pellet was then resuspended in the same volume (50ml) of MS liquid medium containing MES at pH 5.6.

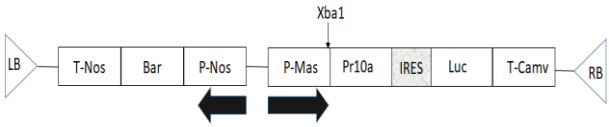

Figure 1. Schematic representation of the T-DNA region of the dicistronic binary vector pGreen II 0229 used in this study. LB = left border; Bar = phosphinothricin acetyle transferase; T-Nos = terminator signals derived from nopaline synthase; P-Nos = promoter sequence derived from nopaline synthase; P-Mas = promoter sequence derived from manopine synthetase; IRES = internal ribosome entry site; Luc = Luciferase gene; T-Camv = terminator of 35S Cauliflower mosaic virus; RB=right border.

3. Bacterial Optical Density (OD_{600})

Agrobacterium suspension resuspended in MS medium were diluted again in MS medium containing 100 µM acetosyringone and a CO8000 cell density meter (WPA, Cambridge, UK) was utilized to measure the OD_{600}. The OD_{600} was adjusted to 0.1, 0.4 or 0.7 and kept for 3 h at 25 °C before incubation with cut epicotyl explants.

4. Inoculation and co-cultivation

Aseptic epicotyls of 4 weeks old *in vitro* seedling were divided into portions (1.0 cm in length). These portions were used as explants for transformation experiments. The epicotyl segments were inoculated with *Agrobacterium* suspension for 15, 20, 30 and 40 minutes in a 9 cm Petri dish and shaked occasionally. Epicotyl segments were blotted dry by filter paper and cultured on MS medium augmented with 1.5 mg/l BA and 0.2 mg/l IBA, sucrose 30 g/l and agar 7 g/l, as recommended by Abou Elyazid (2002). The infected Epicotyl explants were incubated at 25 °C for 3 days under dark conditions. The explants were then washed twice with MS liquid medium supplemented with ticarcillin at 300 mg l^{-1} to remove the agrobacterium and blotted dry on sterilized filter paper. Dry explants were inoculated on MS medium enriched with 1.5 mgl^{-1} BA and 0.2 mgl^{-1} IBA, 30 g l^{-1} sucrose, 7 g l^{-1} agar and 300 mg l^{-1} ticarcillin to eliminate the growth of *Agrobacterium* (Ling et al., 1998) and 5 mg l^{-1} phosphinothericin (PPT) as an agent for the selectable marker gene (bar gene). After two weeks, epicotyl segments were subcultured to MS regeneration medium enriched with 200 mg l^{-1} ticarcillin and 5 mg l^{-1} ppt. The subcultures were performed two weeks intervals on the same MS medium with reduced level of ticarcillin at 100 mg l^{-1}. After six weeks, regenerated shoots were obtained and then inoculated to MS medium containing 100 mg l^{-1} ticarcillin and 5 mg l^{-1} PPT for shoot proliferation. The proliferating shoots were grown on half strength MS containing 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} IBA, 15 g l^{-1} sucrose, and 7g l^{-1} agar for in vitro rooting. In addition, 100 mg l^{-1} ticarcillin, 5 mg l^{-1} ppt, and 300 mg l^{-1} NaCl were added for salt tolerance selection. For subsequent growth of plantlets, they were cultured for 6 weeks on MS medium devoted plant growth regulators (PGRs) and incubated at 25 °C room temperature and 55 µmol m^{-2} s^{-1} PPFD and a photoperiod of 16 h light. The plantlets with 5–6 leaves were transplanted to plastic pots filled with a sterilized mixture of peat moss and vermiculite (1:1; v/v) and were kept for one week in a controlled growth chamber prior to their growth in the greenhouse.

5. DNA isolation and PCR analysis of transgenic plants

Total DNA was extracted from the young leaves of transgenic lines and wild type (WT) plants (control) according to CTAB method described by Doyle and Doyle (1990). An EMPLEN photometer P330 was utilized to estimate the quality and concentration of DNA. PCR was carried out using specific primers for Pr10a gene, Pr10a F (5´-AATCCCG GG A TGGGTGTCACTAGCTATACACATG-3) and Pr10a R (5´-AAAAAGCTTTTAAGCGTAG A CAGAAGGATTGGCG-3´) and Luc F(5´CCTTCCGCATAGAACTGCCT-3´) and Luc R(5´-TCCAAAACAACAACGGCG-3´) for Luc gene. The following protocol was used for PCR: 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 57°C annealing temperature (Ta) for 30 seconds, and 72°C for 1 minute, with a final extension step of 72°C for 10 minutes. Amplification was performed in thermal cycler (MJ Mini Bio RAD), in 25 µl reaction volume containing the following reagents: 5 µl of 10x buffer, 1.0 µl of dNTPs (10 mM), 1.0 µl of $MgCl_2$ (25 mM), 0.5. µl l of each forward and reverse primers (10 pmol), 2.0 µl of DNA (25 ng/µl), 0.25 µl of taq polymerase (5u/µl) and 14.75 d.d. H_2O. The PCR products were

electrophoresed in 1 % agarose gel in TBE buffer at 80 V. Ethidium bromide was utilized to stain the gel and subsequently tap water was used for destaining. The bands were detected using a UV-trans-illuminator, and a UVITEC Gel documentation system, UK was used for photography.

6. Quantitative luciferase assay

Promega luciferase assay Kit (Promega, Madison, USA was used for the monitoring of luciferase gene expression. For quantitative measurements of luciferase enzyme activity, leaf tissue from transgenic sour orang plants were grinded using mortar and pestles in liquid nitrogen to obtain crude protein extracts. The leaves powdered material were suspended in 300 µl of 1X lysis reagent (CCLR, Promega) and vortexed at room temperature. The lysate was incubated at 4°C for one hour then centrifuged at 14000 rpm for 10 minutes at room temperature to remove cell debris. 20µl of cell lysate was mixed with 100 µl of Luciferase Assay Reagent (LAR). Luciferase enzyme activity was measured in a Berthold Luminometer (Lumat LB 9501, Berthold Technologies, Wildbad, Germany).

7. Southern blot analysis

Young leaves of sour orange wild type and transgenic plants were used to istolate total DNA the DNeasy Kit (Qiagen, Valencia, CA). Twenty-five microgram DNA was digested overnight with Fast Digest enzyme XbaI (Fermentas), and the resulting restricted fragments were separated by electrophoresis on a 1.0 % (w/v) agarose gel and then transferred to a Hybond N+-nylon membrane (GE Healthcare) according to the manufacturer's instructions. A PCR fragment of PR10a gene was amplified from the transformation vector using the corresponding primer set and was used as a hybridization probe. The Pr10a amplified fragment was tagged and recognized using PCR-DIG Mix (Roche, Germany) as per manufacturer's instructions**.**

8. Experimental design and data analysis

All experiments were performed in a completely randomized design and repeated twice. Data presented as means ± standard error.

RESULTS AND DISCUSSION

1. Shoot regeneration of sour orange using epicotyl explants

Epicotyl segments developed direct shoots at the cut edges within 20 days of culture onto MS medium containing1.5 mg L^{-1} BA and 0.2 mg l^{-1} IBA. This medium has proved optimal for shoot regeneration from sour orange epicotyl (Figure 2 B). The regeneration percentage reached 97%. There have been several medium formulas mentioned in the literature, but there is no consensus. (Bond and Roose, 1998; Yang *et al*., 2000; Almeida *et al*., 2003). We hypothesized that combining BAP and NAA in the regeneration medium is beneficial for boosting cell division and differentiation from the transgenic competent cell in sour orange. Earlier reports investigated that transgenic cells undergo cell divisions and redifferentiation which leads to transformation events that further enhanced by a combination of BAP and NAA or Kinetin and NAA in the regeneration medium (Ghaderi *et al*., 2018; Ghorbel *et al*., 2000). Similar levels of PGRs have been reported for optimal transformation of Hamlin (Dutt and Grosser, 2009; Mendes *et al*., 2002).

2. Transformation Conditions

In this study the effect of two parameters that influence transformation frequency were investigated. The optical density at wave length 600 nm (OD600) of agrobacterium suspension and the inoculation duration were found to be important variables in improving T-DNA delivery and/or encouraging transgenic plantlet recovery. The transgenic shoots that were grown on MS medium containing PPT as selective agent exhibited healthy growth while no shoots were obtained from the wild type explants (Figure 2 A and C) and the explants showed strong browning after 30 days. Another selection step was performed based on the expression level of the Luc transgene, measuring Luc enzyme activity assay in the transgenic regenerated plantlets (Figure 3).

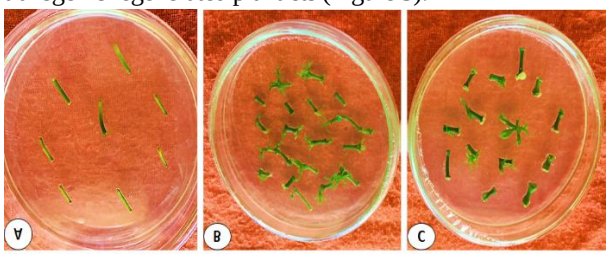

Figure 2. Shoot organogenesis of sour orange epicotyl segments. (A) Wild type explants on MS medium enriched with 200 mg/l ppt; (B) Wild type explants on control MS medium; (C) Transgenic shoots emerged on MS medium enriched with PPT at 200 mg l^{-1} as an agent for transgene selection

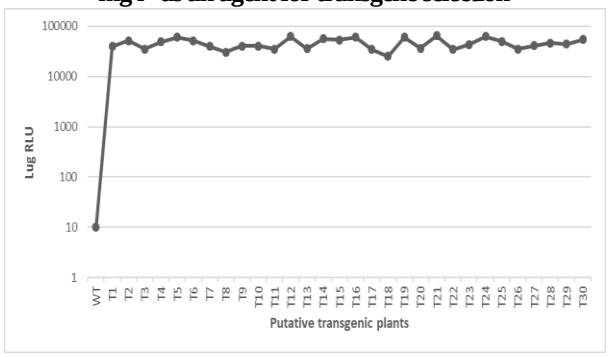

Figure 3. In vitro luciferase gene expression in 30 randomly selected transgenic sour orange plants. Chemiluminescence in leaves extracts measured in a Berthold Luminometer using the Promega kit, WT, wild type plant, T1-T30: putative transgenic plants.

The results revealed that the highest number of plants expressing Luc gene were obtained when the Epicotyl explants were incubated for 20 min in agrobacterium suspension at optical density (OD_{600}) of 0.4, whereas the transformation efficiency dramatically decreased with the increment of both factors (Table 1).

The highest transformation efficiency percentages were recorded at incubation time of 20 min regardless the values of bacterial density. The optical density (OD_{600}) of 0.1 and 0.7 produced transformation percentages of 18 and 12.6, respectively at incubation time of 20 min (Table 1). However, regardless of the bacterial density, the percentages of transformation efficiency reduced drastically with increasing the infection period to 40 minutes. Similar findings were also reported in soybean (Zia *et al*., 2010). Many factors such as bacterial optical density, explant age and duration of infection have been highlighted to be restricted factor for *Agrobacterium* mediated transformation (Kuta and Tripathi, 2005; Sharafi *et al*., 2013a and b; Sharafi *et*

al., 2014). The *Agrobacterium*-mediated transformation methodology was found to be superior and overcome the problems related with the microprojectile bombardment method, which correlated with DNA breakage and subsequently the reduction transformation efficiency (Yong *et al*., 2009). Corredoira *et al*. (2005) reported that an OD600 of 0.6 or higher corresponded to the middle of the exponential phase of growth, whereas OD600 values of 0.3 and 0.9 were associated with the early and late exponential phases, respectively. The optimal stage for infection is when *Agrobacterium* is in its early exponential phase of growth. Reduced OD600 values, which equate to a lower cell density in the medium, had a major impact on transformation. Other woody transformation systems, such as chestnut (Corredoira *et al*., 2005) and almond (Archilletti *et al*., 1995), have also revealed a link between OD600 and transformation efficiency. For genetic transformation of sour orang, an OD600 of 0.4 was optimal. These results confirm previous findings where an OD_{600} of 0.15–0.6, corresponding to mid-log phase was suggested as an optimal optical density for DNA delivery (Dutt and Grosser, 2009).

Table 1. Effects of inoculation time and optical density (OD600) on sour orange transformation efficiency.

	OD_{600}					
	0.1		0.4		0.7	
Inoculation time (min.)	Transgenic plants (No.)*	TE%**	Transgenic plants (No.)	TE%	Transgenic plants (No.)	TE%
15	10.3±0.82	11.4	14.3±0.88	15.9	10.3±1.4	11.4
20	18.0±1.0	20.0	22.0±1.2	24.4	11.3±1.42	12.6
30	7.0±0.57	7.7	10.7± 0.91	11.9	1.3±0.8	1.4
40	5.0±0.60	5.6	1.0±0.60	1.1	0.0	0.0

***The data represent the mean of three experiments ± SE**

**** TE; transformation efficiency percentage as explants with Luc enzyme expression.**

3. Generation of transgenic sour orange lines and validation of transgene integration

Epicotyl explants of sour orange were transformed with *Agrobacterium* strain carrying a dicistronic transformation vector containing the PR10a, and Luc reporter gene. Out of 12 transformation trials using 1,440 sour orang explants, 105 putative transformed plants were regenerated, representing Luc enzyme activity (an example is presented in Figure 3) were successfully regenerated from all experiments using 2 mg l^{-1} PPT for selection Luciferase gene expression was observed by Chemiluminescence in leaves extracts in all of the PPT-resistant plants. Figure 3 shows the Luciferase enzyme activity in the leaves of the transgenic sour orang plants was higher (LUR=35000 - 63210) compared to the non-transformed plants, which did not show any Luc enzyme expression (LUR=10 referred to background). The stable integration of the T-DNA was proved in different line by PCR and Southern blot hybridization. Transgene-specific primer sets were used in PCR analysis (Figure 4). All transgenic lines tested showed the expected fragments of 480 and 834 bp for the PR10a and Luc, respectively, while no fragment was amplified in the non-transformed control DNA samples (wild type). Southern analysis of transgenic sour orange plants revealed a set of unique bands for each of the transgenic lines ranged from 1-2 bands suggesting one or two T-DNA copies present in the transgenic plants, confirming that each line resulted from an independent transformation event (Figure 5). Whereas, no hybridization signal was detected in the wild type (control) plants.

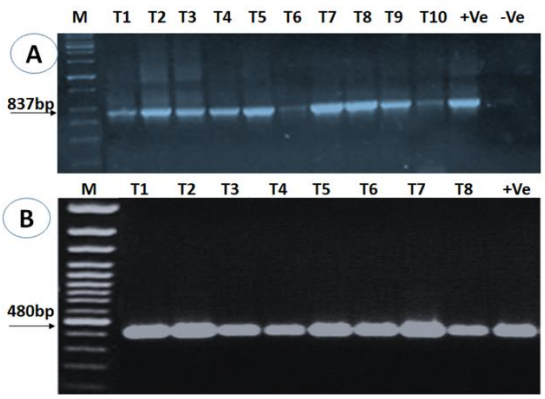

Figure 4. PCR analysis of luciferase (Luc) and PR10a genes in transgenic plants. M :100 bp plus DNA molecular weight marker, -ve: negative control (non-transformed control plants), +ve; positive control (plasmid), T1-T10: transgenic plants. The size of the amplified fragment is 837 and 480 bp for Luc and Pr10a genes, respectively.

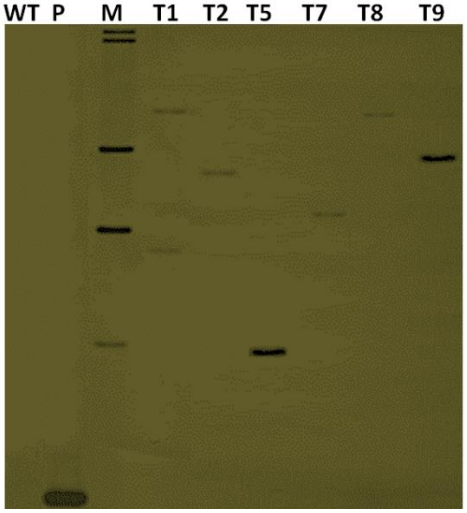

Figure 5. Southern blot analysis for the Pr10a gene in six randomly chosen transgenic plants (lanes 4–9) and a wild type plant (lane 1); P, positive control (plasmid DNA); M, DNA marker.

One of the barriers to successful plant transformation is a lack of understanding of gene expression throughout the selection and regeneration processes. As a result, optimizing transformation efficiency and reproducibility in many laboratories remains a primary concern for researchers. The most prevalent approach for genetic transformation of citrus cultivars is *Agrobacterium*-mediated transformation for incorporation of the T-DNA (Dutt and Grosser, 2009 and 2010). Drought and salinity are abiotic environmental stresses threatening modern agricultural productivity worldwide (Rodziewicz *et al*., 2014; Dresselhaus and Hückelhoven, 2018). Abiotic stress exerts its negative impacts on most plant processes, such as disrupting the ionic and osmotic equilibrium, photosynthesis, or protein synthesis. PR10 proteins have been proved to play multiple roles in plant defense when exposed to abiotic and biotic stresses. Previous reports indicated that heterologous overexpression of PR10 from numerous plant sources caused robust increases in plant tolerance against abiotic stresses in potato and faba bean (El-Banna *et al*., 2010; Hanafy *et al*., 2013). Similarly, SmPR10 from *Salix matsudana* Koidz in transgenic *Arabidopsis thaliana* enhanced plant resistance to NaCl stress (Han

et al., 2017). The overexpression of Peanut AhSIPR10 introduced into banana conferred higher tolerance against salt and drought stresses, and the transgenic plants showed better photosynthetic efficiency under water stress (Rustagi *et al*., 2014). An evidence that overexpression of rice RSOsPR10 resulted in tolerance to drought stress in rice and salt and drought stresses in bentgrass has been also reported (Takeuchi *et al*., 2016). To our knowledge of previous literature, this is the first report of introducing the PR10a gene into sour orange to enhance the drought or salt tolerance of this valuable citrus rootstock. The transgenic rootstocks will be used for trans-grafting of a non-genetically modified (GM) scions of citrus leading to better performance and production of GM-free fruits.

CONCLUSION

One of the main goals of citrus breeding is to develop citrus rootstocks that are more tolerant to biotic and abiotic stresses. Due to various characteristics of citrus such as long juvenility and high heterozygosity, traditional citrus breeding techniques are complicated and time intensive. A reliable and effective sour orange transformation method was established in this study, allowing us to include the salt and/or drought tolerance gene (Pr10a) in a sufficient number of plants. We concluded that T-DNA transfer and gene expression in the sour orange epicotyl segments were impacted by some of the conditions investigated. The maximum percentage of transformation efficiency (24.2%) was obtained upon inoculation of the epicotyl segments with *Agrobacterium* for 20 min at OD_{600} of 0.4. We plan to examine the salt tolerance capacity of sour orange transgenic rootstocks grown under field conditions.

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إستحداث نباتات معدله وراثيا متحمله للملوحه لأصل النارنج بإستخدام الأجروباكتريم

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في هذه الدراسه تم الحصول على نباتات نارنج معدله وراثيا متحمله للملوحه من خلال إستخدام بكتيريا الأجروباكتريم كوسيط لإدخال جين تحمل الملوحه (Pr10a) بإستخدام السويقه الجنينيه العليا الناتجه من زراعة بذور النارنج معمليا (in vitro). تم استخدام الكاشف الجينى لوسيفيريز (Luciferase) والمرتبط بجين تحمل الملوحه للإستدلال على وجود الجين في النباتات الناتجه وذلك في وجود الفوسفينوثريسين (phosphinothericin) وكلوريد الصوديوم كعاملى انتخاب للنباتات المهندسه وراثيا. إزداد معدل التحول الوراثى بزيادة مدة المعامله بالبكتريا (20 دقيقه) والكثافه الضوئيه للمعلق البكتيرى (0.4)، حيث انه تحت هذه الظروف وصلت كفاءة التحول الوراثى لـ 24.2%. أكد تحليل الـ PCR بإستخدام بوادىء جينات Pr10a و الـ Luc وكذلك تحليل الـ southern blot وجود الجين في النباتات المعدله وراثيا.