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An experimental comparison of different transformation procedures assessed in tomato cv Rio Grande with yeast *HAL 1* gene

[Maya Rio Grande *HAL 1* geninin domateslere farklı gen transformasyon koşullarının incelenmesi]

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ABSTRACT

Objective: Tomato (*Solanum lycopersicon L.*) is one of the most important vegetable crops and a genetic model for improving other dicotyledonous crop plants. In basic and practical studies for tomato improvement, successful transformation is essential. The aim of this study was to assess and compare different transformation procedures in tomato cultivar Rio Grande transformed with the *Agrobacterium tumefaciens* strain LBA4404 containing pPM7 carrying the *HAL1* and *NPTII* gene.

Methods: Three different transformation procedures were carried out in this project. One of them was tissue culture dependent and the others were *in planta* transformation protocols i.e. *in vitro* fruit injection method in which fruits are infiltrated with *Agrobacterium* suspension for different incubation periods and *in vivo* fruit / flower injection method which involves simple injecting bacterial culture into intact fruits and flowers attached to the mature plants.

Results: The tissue-culture approach in tomato showed transformation efficiency from 10-13% with apical meristems yielding best results. In the *in planta in vitro* fruit-injection method, the percentage of seed germination on selection medium was higher for treated seeds as compared to control showing transformation efficiency in the range of 34 - 42%. During *in planta in vivo* fruit / flower injection method, out of 1100 seeds assayed, only 8 were found to be resistant to antibiotic with transformation rate 0.0018%. Transformed plants were selected on 100 µg/ml kanamycin and final selection of transformed plants was made on the basis of PCR.

Conclusion: Among different transformation procedures depicted in tomato *(Solanum lycopersicon L.)*, *in vitro* fruit injection *method* was found to be easier and economical way of getting transformed plants as compared to conventional tissue culture based transformation methods.

Key Words: *In planta* transformation, Solanaceae, *HAL 1* gene, Genetics.

Conflict of Interest: The authors declare no conflict of interest.

ÖZET

Amaç: Domates *(Solanum lycopersicon L.)* en önemli sebzele bitkilerinden biridir ve diğer dikotiledon bitkilerin gelişiminde genetik modeldir. Temelde ve pratik uygulamalarda domatesin gelişiminde başarılı bir transformasyon gereklidir. Bu çalışmanın amacı, Rio Grande kültivatöründen elde edilen, *HAL1* ve *NPTII* genelerini taşıyan pPM7 içeren LBA4404 *Agrobacterium tumefaciens* sujunun domatese farklı transformason koşullarının incelenmesidir.

Metod: Bu çalışmada 3 farklı transformasyon koşulu incelenmiştir. Bunlardan biri doku kültürü bağımlı ve diğerleri bitki içi transformasyon protokollerine dayalı yöntemlerdir. Bunlar için farklı inkübasyon periyotlarında *Agrobacterium* süspansiyonu meyve içine *in vitro* şartlarda enjekte edilmiştir. Diğer yöntemde *in vivo* şartlarda bakteri kültürü olgun bitikilerin meyve ve yapraklarına enjekte edilmiştir.

Bulgular: Domateste doku kültürü yaklaşımda apikal meristemlerin gelişiminde % 10-13 ile en iyi sonuçlar elde edilmiştir. Bitki içi *in vitro* meyveye enjeksiyon metodu, kontrollerle karşılaştırıldığında diğer muamele edilmiş tohumlara gore tohum gelişimi %34-42 oranında daha yüksek tarnsformasyon verimliliği göstermiştir. Diğer meyve ve yaprak enjeksiyonu süresince, 1100 tohum ölçümünde sadece 8 tohumda antibiyotiğe karşı direnç gözlenmiştir ve transformasyon oranı %0.0018 olarak bulunmuştur. Transforme bitkiler 100 µg/ml kanamisin ortamında seçilmişlerdir ve transforme bitkiler için son ayrım PCR temelli metotla yapılmıştır.

Sonuç: Uygulanan transformasyon metotları arasında domatesin *(Solanum lycopersicon L.)* meyvesine, *in vitro* enjeksiyon yöntemi daha kolay olduğu ve transforme bitki elde ediliminin diğer konvansiyonel yöntemlere nazaran ekonomik olarak daha uygun olduğu bulunmuştur.

Anahtar Kelimeler: Bitki içi tranformasyon, Solanaceae, *HAL 1* geni.

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Genetic transformation of plants occurs naturally. Scientists have been able to carry out controlled plant transformation with specific genes since the mid-1970s. Now a day, it has become a routine experiment in the field of recombinant DNA technology [1]. Different methods have been employed in plant transformation such as polyethylene glycol, electroporation, biolistic and *Agrobacterium*-mediated transformation. *Ag*-mediated transformation of tomato has been used since 1980s, when McCormick and his collaborators performed transformation assays of leaf discs from various cultivars [2]. Since then, several reports on genetic transformation of tomato have been published. Transformation efficiencies ranging from 10 to 30% have been obtained with several tomato cultivars. Many parameters affecting transformation efficiency in tomato have been tested which is basically determined by the explant genotype [3-7].

However, tissue culture - transformation and regeneration of higher plants remain tedious, time consuming and often costly. Even for species for which this procedure has been greatly simplified, such as *Arabidopsis thaliana, Solanum lycopersicon L. or Medicago truncatula* [8-10], it can take several months to produce transgenic plants suitable for analysis. Therefore an efficient and reliable protocol that would avoid or at least minimize regeneration and tissue culturing is highly appreciated.

There has been a continuous effort to find alternative methods for plant transformation that are more efficacious and economical than the standard tissue culture approaches currently utilized. Alternative techniques would be successful for species that are recalcitrant to tissue culture. Additionally, it is likely that they would be less genotype-dependent, since tissue culture response is strongly influenced by genotype. These methods are called *in planta* because genes are generally delivered into intact plants. One example of this approach is the transformation method developed for *Arabidopsis*. In this method, *Arabidopsis* flowering plants are sprayed, infiltrated or dipped into an *Agrobacterium* suspension and some of the harvested seeds are transgenic [11,12]. The overall efficiency is low but the simplicity of method makes it extremely attractive. Vacuum infiltration of young Chinese cabbage (*Brassica rapa L.*) plants resulted in the recovery of two transgenic plants after screening 20,000 seeds harvested from 30–50 plants [13].

This study was aimed to develop an easy, rapid and efficient protocol for genetic transformation which would avoid or at least minimize tissue culturing in tomato (*Solanum lycopersicon L.*). This study also compares the efficiencies of different transformation procedures used for tomato (*Solanum lycopersicon L.*) with yeast *HAL1* salt tolerant gene to increase their salt tolerance and also carries out the molecular analysis of transformed plants. Three different transformation procedures were carried out in this project.

- Transformation through tissue culturing.
- *In planta* transformation using *in vitro* fruit injection method.
- *In planta* transformation using *in vivo* fruit and flower injection.

Materials and Methods

Bacterial strain and plasmid construction

The *Agrobacterium tumefaciens* strain LBA4404 containing pPM7 was used for transformation experiment. The *HAL1* expression cassette was constructed from the plasmid pMOG180 as described by Gisbert et al [14].

Transformation through tissue-culturing

Tissue-culture transformation of tomato was based on the protocol derived from Khoudi et al. [15] with some modifications. The cotyledon, apical meristems and hypocotyl explants cut from one week old seedlings were dipped in *A. tumefacians* culture for 10 minutes and shifted to co-cultivation medium. After 2 days, explants washed with diluted cefotaxime (50 mg/l) were shifted to regeneration MS [16] selection medium (MS containing 0.5 mg/1 Zeatin and 0.5 mg/l IAA (SM1) or MS containing 1 mg/1 Zeatin and 0.5 mg/1 IAA (SM2) supplemented with 50 µg/ml kanamycin sulphate and 100 µg/ml cefotaxime. Rooting of the transgenic shoot was obtained on half MS medium.

In planta transformation using in vitro fruit injection method

Fresh, healthy and mature Rio Grande fruits were purchased from the local market, rinsed thoroughly with tap water and dried with blotting paper. *Agrobacterium* was grown in Luria Broth medium supplemented with rifampicin 40 mg/l and kanamycin 50 mg/l until an O.D.= 0.8 was achieved. Surface sterilization of fruits was done by washing with 0.1% aqueous mercuric chloride solution for 1 minute followed by rinsing thrice with autoclaved distilled water. One ml sterile hypodermic syringes were used to evenly inject the *Agrobacterium* culture throughout the fruit. Fruits were incubated at 28°C for 6, 10, 24 and 48 hours incubation periods. For each incubation period, three replications with total of 12 fruits were selected and placed in already cleaned and autoclaved 500 ml beaker inside the laminar flow hood.

In order to observe the effect of *Agrobacterium* treatment on the germination of seeds compared to control (untreated), seeds were collected from *Agrobacterium* injected fruits and were inoculated on selection medium (half MS medium containing 100 mg/l kanamycin) under sterile conditions. Seeds from each incubation period were inoculated in three replications. Each replication contained approximately 40 seeds per fruit per flask. Germination

percentage was noted after 15 days of seeds inoculation while the survival rate of seedlings on selection medium was noted after 40 days.

In planta transformation using in vivo fruit and flower injection

Agrobacterium tumefaciens strains LBA4404 containing plasmid pPM7 was grown for two days in LB medium until an O.D. = 0.80 was reached. Medium was supplemented with rifampicin 40 mg/l and kanamycin 50 mg/l. Five ml of *A. tumefaciens* suspension was placed in sterile 25 ml falcon tube and centrifuged at 4,000 rpm for 10 minutes. For flower treatment, the pellet obtained was resuspended in 4 ml of pollen germination medium (20% sucrose with 100 ppm H_3BO_3 and 300 ppm $CaCl_2.2H_2O$) to make a thick solution where as for injecting the fruits, the pellet was suspended in 4 ml normal saline solution (0.9% NaCl).

Treatment of flowers and fruits

Mature greenhouse grown tomato plants were selected for this experiment. Twenty flowers were taken as the starting experimental material with approximately five flowers per plant. A drop of prepared bacterial solution with pollen germination medium was applied directly to the stigmas of mature emasculated flowers of tomato Rio Grande cultivar grown in green house. Furthermore, the bacterial solution mentioned above was also injected into the ovary of mature flowers to ensure the complete approach of *Agrobacterium* strain to the germ line of tomato plant. In control plants, a mock treatment of pollen germination medium without bacteria was applied to the stigma before pollination. The control and treated flowers were allowed to pollinate under natural conditions and developed into mature fruits which were harvested.

Three different stages of fruits were selected for in planta transformation treatment.

- Mature red tomato fruit
- Immature small sized green tomato fruit
- Immature medium sized green tomato fruit

Twenty five intact fruits present on plant were chosen for this experiment. Two ml of bacterial saline solution was injected into the fruits at these stages. The control fruits were injected with saline solution without bacterial suspension. This treatment to flowers and fruits was repeated three times after every 48 hours. When reached to maturity, the ripened treated and control fruits were harvested.

Screening of seeds recovered from inoculated plants

Almost 600 seeds were obtained from the fruits of injected flowers as some of the flowers were not able to survive after bacterial treatment. Similarly, the numbers of seeds obtained from the injected fruits were 700. Seeds from control and treated plants were incubated on germination medium (half strength MS medium with 100 mg/l kanamycin). After selection of the transformants obtained from three different transformation protocols, the control and the kanamycin- resistant seedlings were individually transferred to small pots containing soil. Acclimatization was done for 15 days in growth room 25±2°C, 16 h of photoperiod, illumination of 45 μE m^{-2} s^{-1} and 60% relative humidity. Once being hardened, the plants were shifted to green house. Extensive care was taken till they reached to maturity.

Molecular analysis

For extraction of genomic DNA from the seedlings, simplified CTAB method of Doyle and Doyle, [17] was used. Polymerase chain reactions for the detection of *NPTII* and *HAL 1* genes were performed following the standard method of Taylor, [18]. The forward and reverse primers for *NPT II* gene were 5`-AAGATGGATTGCACGCGGTTC-3'and 5`-GAAGAACTCGTCAAGAAGGC GA -3`respectively, amplifying a fragment of 781 bp in pPM7 whereas the forward and reverse primers for *HAL 1* gene were 5'- CTACCATGGATTTCAAAGATTTAGGATTGCATG-3'and 5'TTTCTGCAGTTTTTCACTATTCTGTTTGATTG-3' respectively, amplifying a fragment of 888 bp [19]. Southern hybridisation was based on a non-radioactive digoxigenin method, using the protocol of the DIG HighPrime DNA Labeling and Detection kit (Roche, Mannheim,Germany). Twenty micrograms of genomic DNA isolated from *HAL 1* transgenic plants were digested with *Hind III* restriction enzyme, blotted onto positively-charged nylon membranes by capillary transfer method and overnight hybridized with the corresponding random primed digoxigenin-labeled (DIG) *HAL 1* gene probes in hybridization buffer following a standard procedure (Sambrook et al. [20]. Hybridization and immunological detection on X-ray film using the CSPD substrate was carried out according to the manual of the DIG DNA Labeling and Detection kit (Roche).

Segregation analysis of T1 halotolerant transgenic tomato plants and salt stress treatments

T1 seeds obtained from selfed *HAL 1* transgenic plant were checked for Mendelian segregation pattern by germinating them on half MS medium with kanamycin at a concentration of 100 mg/l. After scoring the Mendelian pattern, young green healthy seedlings were shifted to pot and again confirmed by PCR analysis. Positive T1 halotolerant seedlings were then irrigated with saline water of 150 mM consecutively for eight weeks to monitor their growth under salt stressed conditions.

Results

Effect of regeneration medium on tissue-cultured transformation

The transformation efficiency was assessed by shifting the

Table 1. Effect of regeneration media on the efficiency of transformation

Type of regeneration Media¥	Percentage of explants showing regeneration		
	Apical meristems	Hypocotyls	Cotyledons
SM1 (0.5mg/l IAA & 0.5 mg/l Zeatin)	8±0.957	1±0.427	1±0.38
SM2 (0.5mg/l IAA & 1.0 mg/l Zeatin)	13±0.816*	3±0.540*	2±0.173

¥ Regeneration medium contains MS salt with 50 mg/l kanamycin and 100 mg/l cefotaxime.
* Represents significant differences in data between the two media used ($p<0.05$ following t-test).
Data given represents the average of three experiments performed independently.

Table 2a. Transformation rate of *HAL 1* gene into tomato plant by *in vitro* fruit injection method

Treatment⁜	No. of seeds inoculated	Germination on selection medium	Antibiotic sensitive seedlings	Survival rate on selection medium*	Transformation %#
Control	100	5	5	0	0^{d}
6 Hours	100	20	18	2	0.8 ± 0.12^{d}
10 Hours	100	20	16	4	3 ± 0.46^{c}
24 Hours	100	60	10	50	30 ± 1.53^{b}
48 Hours	100	78	8	70	42 ± 1.03^{a}

⁜ Each treatment was carried out in 3 replicates.
* Survival rate on ½ MS medium containing kanamycin after 40 days.
# Transformation percentage is calculated on the basis of seedlings giving positive PCR results.
The superscript letters are LSD rank orders.

Table 2b. Analysis of variance for stable transformation in kanamycin resistant seedlings

Source of Variation	df	SS	MS	F	p
Incubation period	4	11657.749	2914.437	4093.311	0.0000
Error	10	7.120	0.712		
Total	14	11664.8669			

explants on two different regeneration media. Regeneration efficiency of explants is shown in Table 1. The t-test had shown a significant level of effect ($T=7.55$, $P=0.0006$, $T=5.99$, $P=0.0019$) between these two regeneration media when using apical meristems and hypocotyls as an explants. The significant value of P ($P<0.05$) shows that maximum number of transformants was obtained on SM2 medium with apical meristems regenerating in a better way. Fig. 1a and 1b shows the development of putative transgenic shoot from the apical meristem surviving on

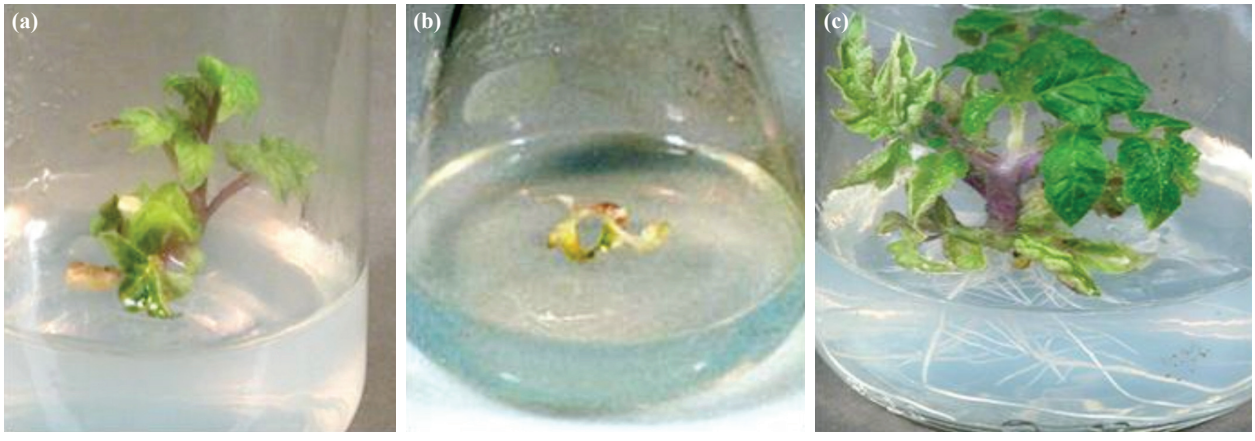

Figure 1. Five weeks old *HAL 1* transgenic shoots **(a)** obtained from apical meristem in comparison with control **(b)** on MS regeneration-selection medium. Rooting response of *HAL 1* **(c)** transformed plant is also shown in the figure.

selection medium as compared to control apical meristem which died on selection medium. Cotyledons and hypocotyls showed the minimum transformation events. When the transgenic shoots were elongated to 2-4 cm, the developed shoots were cut off and cultured on rooting medium i.e. half strength MS medium. The rooting response of transformed plants was found to be 50% and has been shown in Fig. 1c.

Seeds germination on selection medium from Agrobacterium injected in vitro fruits

Seeds germination percentages varied significantly when inoculated on selection medium (Table 2a). Percentages of germination ranged from 3 to 42% for seeds from injected fruits. The germination percentage was high for 48 hours incubation period (Fig. 2) followed by 24 hours while 6 and 10 hour incubation periods had no prominent impact on stable transformation efficiency. The survival rate was 0% for control seedlings on selection medium. Further increase in incubation treatments had no significant impact on the germination and transformation efficiency. Different incubation periods showed highly significant ($P<0.01$) impact on the stable transformation in germinating seeds as shown by Analysis of variance test (Table 2b).

Fruits were injected at three different stages. Small sized green fruits could not resist the harmful effects of the bacteria. Such fruits do not reach the maturity stage and fall off the plant shortly. The other two injected stages i.e. mature red tomato fruit and immature medium sized green tomato fruits were able to survive the treatment, but at maturity, irregularities in some of the fruits shape were observed. Some of the treated fruits also showed decreased size as compared to control. However, almost 700 seeds were harvested from injected fruits. From twenty five injected flowers, only seventeen flowers were found healthy reaching the maturity stage. Rest of the flowers withered due to bacterial treatment. Fruits produced from these flowers also showed abnormal and stunted development. About 600 seeds were harvested from this treatment and analyzed for transformation events. Out of 1100 seeds assayed, only 18 were found to be resistant to antibiotic (Table 3). The transformation rate was minimum i.e. 0.0018% and was recorded on the basis of molecular analysis of these transformed plants giving positive PCR results.

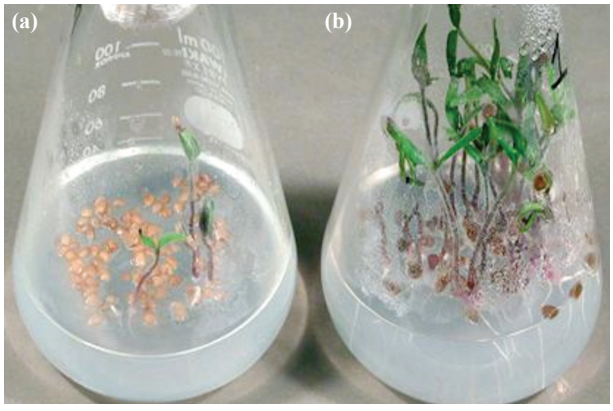

Figure 2. (a, b) Germination of 48 hours incubation period treated seedlings grown on half MS medium containing 100 mg/l kanamycin in comparison with control.

Table 3. Transformation rate of *HAL 1* gene into tomato plants by *in vivo* fruit and flower injection method

Total seeds to assay (from flowers and fruits)	1100
Number of survivors¥	18
Survivor rate (%)	0.016
Number of transformants*	2
Transformation rate (%)	0.0018

¥ The numbers of survivors were determined based on kanamycin-resistance.
*The number of transformants was determined on the basis of positive PCR results.

Molecular analysis of transgenic plants

For molecular analysis of transformed plants, the PCR products were analyzed and the amplified product of *NPTII* (781 bp) and *HAL 1* (888 bp) genes were observed in DNA collected from transformed (T1) plants (Fig. 3). No amplification in PCR product was observed in genomic DNA obtained from plants raised from untreated seeds. Amplified product was also obtained from plasmid DNA isolated from *Agrobacterium tumefaciens* harbouring plasmid pPM7. Southern blot analysis of randomly selected transgenic plants revealed stable integration and complete insertion of the *HAL1* T-DNA cassette into the plant genome. Single T-DNA insertion event was observed in majority of the transgenic lines tested (Fig. 4).

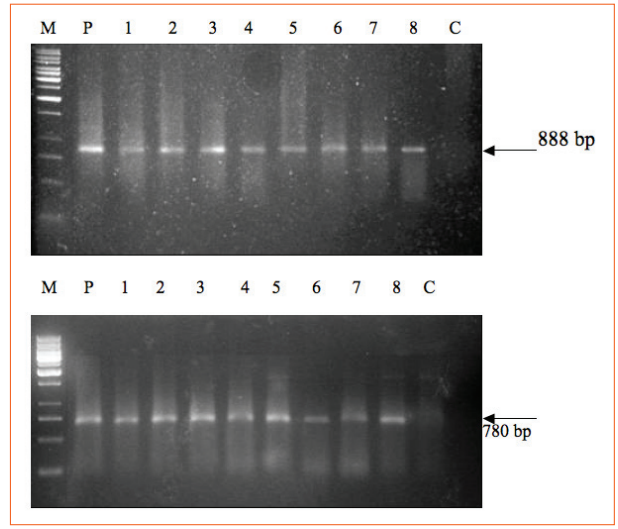

Figure 3. PCR analysis to detect the presence of the *HAL 1* gene **(a)** and *NPT II* gene **(b)** in transgenic tomato plants transformed with pPM7. Lane 1- 8 represents transgenic plants obtained from three independent experiments. Lane P represents plasmid PM7. Lane C corresponds to non-transformed control plantlets. Lane M corresponds to 1 kbp Marker (Fermentas).

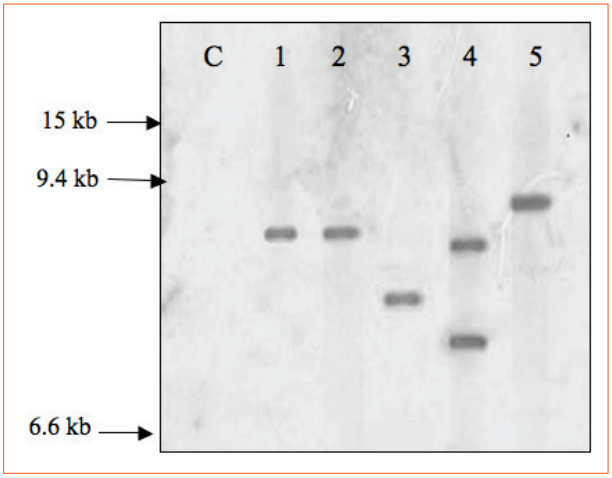

Figure 4. Detection of *HAL1* gene in transgenic tomato plants by Southern hybridization. Lane 1-5 represents independent transgenic tomato plants randomly selected from three different transformation procedures. C is control DNA from untransformed plants.

Segregation and salt tolerant analysis of first generation

Once the PCR positive plants were screened from these three different transformation procedures, the transformants were shifted to small pots containing a mixture of soil and peat moss. Mendelian segregation pattern of the *NPT II* gene in the second generation of the green house grown *HAL I* transgenic plants was studied by germinating these T1 seeds on half MS medium containing selection agent Kanamycin at a concentration of 100 mg/l under sterile conditions. *NPT II* gene (neomycin phosphotransferase II gene) is used in selection of transformed organisms. This gene codes for the aminoglycoside 3'-phosphotransferase enzyme, which inactivates by phosphorylation a range of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin. The tolerance to kanamycin of *HAL 1* progenies gave 3:1 ratio, confirming that *NPT II* was inherited in a simple Mendelian fashion. *HAL 1* seedlings (T1 generation) showed better performance when grown under 150 mM salt stress as compared to control plants. As shown in Figure 5, transgenic *HAL 1* plant exhibited improved appearance (increased number of leaves, increased height and increased number of nodes) than their non-transgenic counterpart. Indeed, transgenic plants continued to grow healthy under salt stress as compared to control which showed wilting symptoms at an early stage. Total chlorophyll determinations also showed that transgenic plants retained more chlorophyll than the control lines.

Discussion

Plant transformation is a key methodology that has allowed the transfer and expression of novel genes for the improvement of economically important plant species. This technique also explores deeper questions about the functions of plant genes. This report examines different transformation techniques in tomato cv Rio Grande using *Agrobacterium tumefaciens* pPM7 *HAL 1*. Our results showed the transformation efficiency in the range of 8-13% for tissue culture approach with apical meristems giving optimum results. We have used Agrobacterium tumefaciens strain LBA4404 and found that transformation frequency showed drastic variations according to explant type and the bacterial strain. For example, transformation experiments with LBA4404 hypocotyls combination revealed only 3% of the transformed shoots. Similarly, experiment with LBA4404 cotyledon combination resulted with no appreciable success. This might be due to the type of strain that affect explants. Similar results had also been reported by Oktem et al. [21] who found no kanamycin resistant calli with hypocotyls or cotyledons infected with LBA4404 strain but using EHA105 *Agrobacterium tumefaciens* strain gave them positive results. In view of these findings, the *Agrobacterium* strain seems to be the most critical factor in our experimental system. Hypocotyls and cotyledons are not worth the extra effort, unless the plant material is limiting.

For many plant species, low transformation efficiency, instability of transgene expression, somaclonal variation and inability to regenerate whole plants are common problems encountered during tissue culturing. A variety of *in planta* transformation procedures have been developed for *Arabidopsis*, including seed transformation, transformation by application of *Agrobacterium* to cut shoots, transformation by infiltration or application of Agrobacterium to flowers [22,23]. In contrast, few *in planta* approaches have been developed for other plant species as well [24-26].

The two *in planta*, infiltration-based transformation methods reported here have been used successfully to transform tomato *(Solanum lycopersicon L.)* and may also be suitable for other species. The transformation frequency in the second method ranges from 30-42% and represents one of the novel methods of transformation. Increase in number of transformants with increase in incubation period has been observed. 48 hour incubation period showed the maximum number of transformants followed by 24 hour period. This indicates that most of the T-DNA insertion events in fruit seeds occur at two day incubation treatment. The difference in transformation values between 48 and 24 hours incubation periods was more prominent (10%) as compared to the difference between 6 and 10 hours incubation periods (2%). Further increase in incubation period slows down the T-DNA insertion events resulting in lower transformation frequency. This finding can be explained by the reduction in activity of *Agrobacterium* with the length of post inoculation period. Desfeux et al. [27] while working on *in planta* transformation of Arabidopsis suggested that *Agrobacterium* persists for a limited period at levels high enough to achieve reasonable rates of transformation in newly forming flower buds.

Screening of resistant seedlings is one of the most important step employed in the *in planta* protocols. Discrimination between resistant and control seedlings is not always absolute at fourth week as 5% of control seedlings still survive on selection medium. However, control seedlings totally die out in the eighth week on the selection medium. It is not possible to screen resistant seedlings for 8 weeks on antibiotic containing medium, as after 8th week, resistant seedlings also begin to fade. Consequently, it is recommended that selection of resistant plants should be done at 4rth week, recognizing that the genotypes selected at this time may include a low number of non-transformed plant escapes. Escapes are one of the common problems reported at screening level. Cervera et al. [28] was of the view that these escapes are not due to the inefficiency of kanamycin selection medium but owing to the protection of non transformed cells from the selection agent by the surrounding transformed cells or the remaining bacteria in the explants.

The transformation frequency in the second *in planta* approach (*In planta* transformation using *in vivo* fruit and flower injection) ranges up to 0.0018%. This represents one of the lowest transformation rates. The results obtained are consistent with Qing et al. [29] who obtained 2 transformants out of 20,000 seeds harvested from pakchoi infiltrated plants. Similarly, Clough and Bent, [30] reported the transformation rate as 0.1-3% in *Arabidopsis* plants transformed by using floral dip method. Another report was also represented by Yang et al. [31] who obtained 33 individual kanamycin resistant plants from 75,000 seeds of *Arabidopsis thaliana*.

One reason for such low *in-vivo in planta* transformation rate might be the bacterial inoculation period. The plants were treated with *Agrobacterium* suspension in greenhouse in November when the temperature of the surroundings is not more than 15°C. This temperature is not suitable for bacterial activity for treated plants. This low temperature might prevent the T-DNA insertion events in the injected fruit and flower parts. So, one can repeat the same experiment at different temperatures at the time of injection so as to check whether surrounding temperature plays a crucial role in transformation events or not. Similar experiments can also be conducted by starting with a large number of parent plants so that the seeds analyzed could be in the range of 20,000-30,000.

The physiological state of the plants, the infiltration medium, the concentration of *Agrobacterium*, and the vacuum treatment time, can all affect the *in planta* transformation frequency and one can conduct the *in planta* transformation methods both *in-vitro* and *in-vivo* fruit/flower injection method by studying these parameters. It might therefore be possible to increase the efficiency and simplify the protocol of *in planta* tomato transformation by improving those parameters and factors. This opens up the possibility for transformation of species recalcitrant to tissue culture and/or plant regeneration

Conclusion

Comparing the results of tissue culture transformation with *in planta* transformation protocols, it seems logical to conclude that the *in-vitro* fruit injection transformation method is much easier, effective and economical way of getting transformed plants. *In planta in-vitro* fruit transformation simply avoids the use of expensive hormones such as zeatin, different media formulations, large infrastructure and highly skilled practitioners as used in our tissue-cultured experiments.

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Conflict of Interest

There are no conflicts of interest among the authors.

References

[1] Christou P, McCabe DE, Swain WF, Russell DR. Legume transformation. In: Verma DPS, editor. Control of plant gene expression, CRI Press, Boca Roton, Florida., USA. 1993. pp. 547-64.

[2] McCormick S, Niedermeyer J, Fry J, Barnson A, Horsch R, et al. Leaf disk transformation of Lycopersicon esculentum Mill. using *Agrobacterium tumefaciens*. Plant Cell Rep 1986; 5:81-4.

[3] Cortina C, Culianez-Macia FA. Tomato transformation and transgenic plant production. Plant Cell Tissue Organ Cult 2004; 76(3):269-75.

[4] Qiu D, Diretoo G, Tavarza R, Giuliano G. Improved protocol for *Agrobacterium* mediated transformation of tomato and production of transgenic plants containing carotenoids biosynthetic gene CsZCD. Sci Hort 2007; 112:172-5.

[5] Yasmeen A. An improved protocol for the regeneration and transformation of tomato cv Rio Grande. Acta Physiol Plant 2009; 31(6):1271-7.

[6] Pramesh H, Fakrudin B, Kuruvinashetti MS. Genetic transformation of local variety of tomato using gus gene, an efficient genetic transformation protocol for tomato. J Agri Tech 2010; 61:87-97.

[7] Safdar N. Apical meristems: potent and competent explants for improved miniprep transformation method in tomato. Minerva Biotechnol 2011; 234:93-102.

[8] Che P, Lall S, Nettleton D, Howell SH. Gene Expression Programs during shoot, root and callus development in Arabidopsis tissue culture. Plant Physiol 2006; 141(2):620-37.

[9] Jabeen N, Mirza B, Chaudhry Z, Rashid H, Gulfraz M. Study of the factors affecting Agrobacterium - mediated gene transformation in tomato *Lycopersicon esculentum* Mill. cv Rio Grande using rice chitinase CHT-3 gene. Pak J Bot 2009; 4150:2605-14.

[10] Trieu AT, Burleigh SH, Kardailsky IV, Maldonado-Mendoza IE, Versaw WK, et al. Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. Plant J 2000; 22:531-41.

[11] Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, et al. The maternal chromosome set is the target of the T-DNA in the *in planta* transformation of *Arabidopsis thaliana*. Genetics 2000; 155:1875-87.

[12] Chung MH, Chen MK, Pan SM. Floral spray transformation can efficiently generate Arabidopsis transgenic plants. Transgenic Res

2000; 9(6):471-6.

[13] Liu F, Cao MQ, Yao L, Li Y, Robaglia C, et al. *In planta* transformation of Pakchoi *Brassica campestris* L. ssp Chinesis by infiltration of adult plants with *Agrobacterium*. Acta Hortic 1998; 467:187-93.

[14] Gisbert C, Rus AM, Bolarin MC, Lopez-Coronado JM, Arrillaga I, et al. The yeast *HAL 1* gene improves salt tolerance of transgenic tomato. Plant Physiol 2000; 123:393-402.

[15] Khoudi H, Nouri-Khemakhem A, Gouiaa S, Masmoudi K. Optimization of regeneration and transformation parameters in tomato for the improvement of its salinity and drought tolerance. African J Biotechnol 2009; 822:6068-76.

[16] Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiol 1962; 15:472-93.

[17] Doyle JJ, JL Doyle. Isolation of plant DNA from fresh tissue. Focus 1990; 12:13-5.

[18] Taylor GR. Polymerase chain reaction: basic principles and automation. In: McPherson MJ, Quirkie P, Taylor GR editors. PCR: A Practical Approach. IRL Press, Oxford; 1991. p. 1-14.

[19] Safdar N, Yasmeen A, Mirza B. An insight into functional genomics of transgenic lines of tomato cv Rio Grande harbouring yeast halotoelrance genes. Plant Biol 2011; 134:620-31.

[20] Sambrook J., Fritsch E.F., Maniatis T. Molecular cloning. A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press, New York, USA, pp. 1989; 1-808.

[21] Oktem HA, Bulbul Y, Oktem E, Yucel M. Regeneration and *Agrobacterium* mediated transformation in tomato (*Lycopersicon esculentum* Mill.). Turk J Bot 1999; 23:345-8.

[22] Dehestani A, Ahmadian G, Salmanian AH, Jelodar NB, Kazemitabar K. Transformation efficiency enhancement of *Arabidopsis* Vacuum infiltration by surfactant application and apical Inflorescence removal. Trakia J Sci 2010; 8(1):19-26.

[23] Harrison SJ, Mott EK, Parsley K, Aspinall S, Gray JC, et al. A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation. Plant Methods 2006; 2(19):12-9.

[24] Ahmad M, Mirza B. An efficient protocol for transient transformation of intact fruit and transgene expression in Citrus. Plant Mol Bio Rep 2005; 23:419a-k.

[25] Bratic A, Majic DB, Miljuš-djukic JD, Jovanovic ZS, Maksimovic VR. In planta transformation of buckwheat *Fagopyrum esculentum* moench. Arch Biol Sci 2007; 59(2):135-8.

[26] Hasan M, Khan AJ, Khan S, Shah AH, Khan AR, et al. Transformation of tomato *lycopersicon esculentum* Mill. with Arabidopsis early flowering gene apetalai API through Agrobacterium infiltration of ripened fruits. Pak J Bot 2008; 401:161-73.

[27] Desfeux C, Clough SJ, Bent AF. Female reproductive tissues are the primary target of *Agrobacterium* transformation by the Arabidopsis floral-dip method. Plant Physiol 2000; 123:895-904.

[28] Cervera M, Juarez J, Navarro A, Pina JA, DuranVila N, et al. Genetic transformation and regeneration of mature tissues of woody fruit plants by passing the juvenile stage. Trans Res 1998; 7:51-7.

[29] Qing MC, Fan L, Lei Y, Bouchez D, Tourneur C, et al. Transformation of Pakchoi *Brassica campestris* L. ssp Chinesis by Agrobacterium infiltration. Mol Breed 2000; 6:67-72.

[30] Clough SJ, Bent AF. Floral dip: A simplified method for *Agrobacterium* - mediated transformation of *Arabidopsis thaliana*. The Plant J 1998; 16:735-43.

[31] Yang SX, Zhao YX, Zhang Q, He YK, Zhang H, et al. *HAL 1* mediates salt adaptation in *Arabidopsis thaliana.* Cell Res 2001; 11:142-8.