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Full Length Research Paper

Optimization of regeneration and transformation parameters in tomato and improvement of its salinity and drought tolerance

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As part of our efforts to improve tomato tolerance to abiotic stress, we have undertaken this study to introduce two candidate genes encoding: a sodium antiporter and a vacuolar pyrophosphatase, previously shown to enhance drought and salt tolerance in transgenic *Arabidopsis* plants. First, we evaluated the potential of primary leaves from three to four week-old in *vitro*-grown tomato seedlings as alternative explants to cotyledons for tomato transformation. Our results demonstrated that primary leaves are three times more efficient then cotyledons in terms of regeneration percentage, productivity, and transformation frequencies independently of the medium and genetic construct used. Second, primary leaves were used to introduce the genes of interest using *Agrobacterium*-mediated transformation. Many transgenic tomato plants were easily recovered. The presence of the transgenes and their expression were confirmed by PCR and RT-PCR analysis. The transformation frequencies for primary leaf explants ranged from 4 to 10% depending on the genetic construct used. The time required from inoculation of primary leaves with *Agrobacterium* cells to transfer of transgenic tomato plants to soil was only 2 months compared to 3 to 4 months using standard tomato transformation protocols. The transgenic tomato plants obtained in the current study were more tolerant to salinity and drought stress than their wild-type counterparts.

Key words: ***Agrobacterium***-mediated transformation, cotyledon, primary leaf, Regeneration, tomato.

INTRODUCTION

Tomato (***Lycopersicum esculentum***) is the second most important vegetable crop in the world after potato. It is being cultivated in many regions of the world: in the Americas, southern Europe, the Middle East and North Africa, India, China, Japan and Southeast Asia. Tomato is consumed in many forms such as raw vegetable, added to other food items or as processed products such as paste, whole peeled, diced, juice, sauces and soups. It is a valuable source of health promoting compounds such as the antioxidant lycopene whose consumption is known to reduce the incidence of many types of cancer (Rao and Agarwal, 2000; Pohar et al., 2003). In addition to these characteristics, tomato is one of the most important model systems for basic and applied research and can be easily propagated using either seeds, or clonally by tip or shoot cuttings. Recently, tomato was used as bio-reactor in biopharming for the production and oral delivery of vaccines (Jiang et al., 2007) and as functional food for cancer prevention (Butelli et al., 2008). During the recent years, tomato has become the subject of new areas of intensive research such as: functional genomics, proteomics and metabolomics.

Tomato was first introduced in Tunisia around the year 1600 by the first Andalous who came from Spain and settled in the Medjerdah valley and the Cap Bon region. Presently, Tunisia is one of the most important tomato producers in the Mediterranean region. Tomato is the source of revenue for approximately 10,000 small farmers and the origin of 32 industries specialized in vegetables transformation. It is being cultivated in irri-gated areas where salinity of water and soil, especially during summer period, are major constraints limiting productivity and quality of tomato. Addressing these pro-

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blems by using classical breeding programmes remains a challenge for breeders since tolerance to salinity is a very complex character involving many genes with small effects (Cuartero et al., 2006). Fortunately, with the advent of genetic engineering tools it became possible to introduce a single gene in plants to enhance abiotic stress tolerance, at least to some extent, without adverse effects on the final product quality (Park et al., 2005; Zhang and Blumwald, 2001; Jia et al., 2002; Lee et al., 2003). The establishment of an efficient ***in vitro*** regeneration and transformation protocol is a prerequisite for the genetic improvement of tomato. Although ***Agrobacterium*-**mediated tomato transformation was achieved more than twenty years ago (McCormick et al., 1986), the yield in transgenic tomato plants using reasonable number of explants is still low for many recalcitrant genotypes. Many research groups using tomato as experimental system reported difficulties in transforming some genotypes (Sun et al., 2006). The efficiency of ***Agrobacterium*-**mediated gene transfer to tomato cells is influenced by many factors, the most important are: variety/genotype (Plastira and Perdikaris, 1997), explants type and orientation (Duzyaman et al., 1994; Plastira and Perdikaris, 1997; Bhatia et al., 2004; Bhatia et al., 2005), plant growth regulators (Elbakry et al., 2001; Gubiš et al., 2004), selection system used (Briza et al., 2008), addition of feeder cells or aceto-syringone, ***Agrobacterium*** density, duration of infection (Murray et al., 1998), and effect of antibiotics on regeneration of tomato (Ling et al., 1998). It is clear from these reports that there is no universal protocol for tomato transformation and regeneration therefore optimization of these two factors is always needed. The most frequently used explants for ***Agrobacterium***-mediated tomato transformation are cotyledons (Hamza and Chupeau, 1993; Fray and Earle, 1996; Ling et al., 1998; Hu and Philips, 2001; Park et al., 2003; Qiu et al., 2007).

Rio Grande is one of the most planted tomato cultivars in Tunisia owing to its agronomic qualities (resistance to fungal and bacterial diseases). In order to enhance its tolerance to abiotic stress, through genetic engineering, we used cotyledon explants for transformation with two gene constructs previously shown to enhance drought and salinity tolerance in ***Arabidopsis*** plants (Brini et al., 2007), but obtained poor results in terms of number and quality of regenerated tomato plants. Here, we tested an alternative strategy for transformation and regeneration of Rio Grande using the primary leaves from three to four week-old ***in vitro*** culture seedlings as explants instead of cotyledons and the same gene constructs. Our results demonstrated that it is worthwhile to use primary leaves instead of cotyledons for Rio Grande transformation since organogenesis and transformation frequencies were substantially improved. Also the genes of interest were successfully transmitted to progeny and improved abiotic stress tolerance in transgenic tomato plants. To our best knowledge, this is the first demonstration of the supperiority of young leaves over the most popular cotyledon explants for tomato transformation and regeneration.

MATERIALS AND METHODS

Plant material preparation

The fresh market cultivar of tomato, Rio Grande, was used in this study. Seeds were surface sterilized by immersing in 50% (V/V) commercial bleach for 20 min with occasional agitation followed by five rinses in deionised sterile water. Sterile seeds were germinated on GM medium (Table1) which consisted of MS medium (Murashige and Skoog, 1962) containing 30 g /L sucrose and solidified with 4 g/L phytagel (Sigma). They were placed in growth chamber at 23°C under 16 photoperiod provided by cool white fluorescent light. Cotyledons from 8 d old seedlings and primary leaves (0.5 to 2 cm long) from three week-old seedlings were aseptically excised and both ends were cut. They were then pre-cultured upside-down for 2 d on PC medium (Table 1).

Agrobacterium infection and plant regeneration

Two genetic constructs in the pCB 302.2 binary vector (Xiang et al., 1999) were used in this study: Construct 1 contained the wheat pyrophosphatase ***TVP1*** gene and Construct 2 contained the wheat sodium antiporter ***TNHX1*** gene. Both genes were put under the control of the duplicated 35S promoter (Figure 2a) and were previously shown to enhance drought and salinity tolerance in transgenic ***Arabidopsis*** plants as described in Brini et al. (2007).

Agrobacterium tumefaciens (strain LBA 4404) cells, harbouring one of the above-mentioned constructs, were grown for 2 days at 28°C (until OD_{600} reached 0.5 - 1) in LB medium, consisting of 1% tryptone, 1% NaCl and 0.5% yeast extract supplemented with 100 mg/L kanamycin (Invitrogen). ***Agrobacterium*** cells were collected by centrifugation for 10 min at 3000 rpm and then re-suspended in MS liquid medium to a final OD_{600} = 0.05. Pre-cultured cotyledons and primary leaf explants were immersed into ***Agrobacterium*** solution for 30 min with occasional agitation, blotted dry on sterile filter paper to remove excess of bacteria and returned upside-down to the same pre-culture medium (PC) (Table 1) for another 2 days. At the end of the co-cultivation period, the explants were washed with sterile distilled water containing 500 mg/L cefotaxime (Unimed Laboratories, Tunisia) for 5 times, blotted dry on sterile filter paper. Equal numbers of each type of explants were transferred upside-down (25 explants/Petri dish) to one of the following sequence media for regeneration and shoot elongation: Shoot regeneration medium I (SRI) for two weeks followed by shoot elongation I (SEI) or Shoot regeneration medium II (SRII) for two weeks followed by shoot elongation medium II (SEII) (Table 1). Well-elongated shoots (approximately 3 cm) were excised from the rest of the explants and transferred into Magenta boxes containing the rooting medium (RM, Table 1). Plantlets with well established root system were transferred to soil and kept in growth chamber for acclimatization. After hardening, plants were transferred into bigger pots and kept in greenhouse to produce fruits.

Shoot regeneration data were recorded 45 days after initiation of culture. Due to the fact that the pCB 302.2 vector contains the ***bar*** gene for selection and that the regeneration of Rio Grande in the presence of glufosinate ammonium was very poor, selection pressure was omitted during regeneration steps. To evaluate the morphogenetic capacity and transformation competence of the two types of explants, regeneration percentages and productivity were determined by calculating the following ratios: The percentage of regeneration (number of explants producing shoots) x 100/ number of total explants) and the productivity rate (P) (number of well developed shoots with root system x 100/ number of total explants).

Transformation frequency was calculated as follows: (The

Table 1. Compositions of tissue culture media used in this study for Rio Grande transformation and regeneration.

Component	GM	PC	SRI	SEI	SRII	SEII	RM
Basal solution	MS	MS	MS	MS	MS	MS	MS
Sucrose (g)	30	30	30	30	30	30	30
Myo-inositol (mg)	100	100	100	100	100	100	100
Thiamine HCl (mg)	0.1	0.1	1	1	1	1	0.1
Nicotinic acid (mg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine-HCl (mg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Indole-3-acetic acid (IAA) (mg)	-	-	0.1	0.1	0.2	0.1	0.2
6-benzylaminopurine (BAP) (mg)	-	1	-	-	2.5	0.5	-
Zeatin (mg)	-	-	1	0.2	-	-	-
1-naphtalene acetic acid (NAA) (mg)	-	1	-	-	-	-	-
Carbenicillin (mg)	-	-	300	300	300	300	-
Phytagel (g)	4	4	4	4	4	4	4

GM, germination medium; PC, pre-culture medium; SRI, shoot regeneration medium I; SEI shoot elongation medium I; SRII, shoot regeneration medium II; SEII, shoot elongation medium II; RM, rooting medium.

Table 2. Effect of explant type and genetic construct on regeneration of tomato cv. Rio Grande.

Explant	Constructs		Mean
	Construct 1	Construct 2	
Cotyledon	16 ± 3.26 (170)	25 ± 7.7 (150)	20.83 ± 3.42 a
Primary leaf	87.6 ± 1.6 (84)	96.6 ± 4.7 (70)	92.17 ± 2.56 b
Mean	51.8 ± 16 a	61.17 ± 16.134 a	

Genetic construct 1: harbours the wheat pyrophosphatase ***TVP1*** gene, Genetic construct 2: harbours the wheat sodium antiporter ***TNHX1*** gene.
Values followed by the same letters are not significantly different at α = 5 %. Numbers between parentheses represent total number of explants used.

number of RT-PCR-positive plants) x 100 / total number of primary leaf explants used for transformation.

Statistical analysis of regeneration data

There were three replications each consisting of one Petri dish with 25 explants. An analysis of variance was conducted using explants types x construct combinations as total number of treatments. Thus, a total number of 3 x 2 x 2 treatments were analysed and compared. Each experiment was repeated two to three times. Regeneration percentages were analysed using SPSS version 13 and significant differences between means were assessed by the Tukey's test at 1 and 5% level of significance.

PCR screening of putative transgenic plants

Genomic DNA was isolated from leaf tissue of greenhouse-grown putative transgenic and non-transgenic tomato plants using a rapid DNA extraction method. Briefly, two leaf discs (40 mg) were grinded in 1.5 ml tubes in the presence of 400 µl of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). After centrifugation at 13000 rpm for 10 min, the supernatant was transferred to a new tube and DNA was precipitated with 0.6 V isopropanol, washed with 70% ethanol and re-suspended in 50 µl of sterile water from which 100 ng of DNA was used for PCR amplifications. PCR amplifications were performed in a 25 µl total volume, using 0.15 mM of each dNTP, 2.5 µl of 10x ***Taq*** DNA polymerase buffer, 1.5 mM $MgCl_2$, one unit of ***Taq*** DNA polymerase, 2.5 µM of ***bar*** gene specific primers (f; 5'-GTCTGCACCATCGTCAACC-3' ; r; 5'-GAAGTCCAGCTGCCAGAAAC-3') or ***TVP-1*** gene primers (f; 5'-GTTCTTTACATCACCATC-3'; r; 5'-CTCAACCATCTTGAGAGC-3'). The PCR conditions were as follows: 94°C, 5 min for initial denaturation, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C and 1 min 30 s at 72°C. A 10 min extension period at 72°C was also used at the end of the amplification cycles. The amplified fragments were visualised on 1% agarose gels.

RT-PCR expression analysis

Total RNA was isolated from approximately 200 mg leaf tissue of transgenic tomato lines and non-transformed control plants using the Trizol method (InVitrogen) and following the manufacturer instructions. RNA was treated with RNase-free DNase to remove any contaminating DNA. Reverse transcription reactions were performed for 1 h at 37°C using MML-Reverse transcriptase (InVitrogen) and oligo-dT. First strand cDNA was used as template for PCR amplifications using the same set of primers and conditions described above for screening of putative transgenic plants. Control amplifications in the absence of the reverse transcriptase were also performed to rule out any amplification caused by the presence of

contaminating DNA.

PCR-identification of T1 transgenic tomato plants and salt-stress treatment

T1 seeds obtained from four ***TVP-1*** and five ***TNHX-1*** selfed T0 plants and seeds from non-transgenic tomato plants were sown directly in soil in small pots (one seed per pot). After germination, young seedlings were allowed to grow for four weeks and subjected to PCR analysis. Confirmed PCR-positive T1 seedlings were than irrigated with saline water of 200 mM NaCl by placing them into a container to allow capillarity uptake for 15 days. In another experiment, confirmed PCR-positive ***TVP-1*** plants and non-transgenic tomato plants were cultured hydroponically in a solution of 200 mM NaCl for three consecutive months to monitor root growth under saline conditions.

RESULTS AND DISCUSSION

Optimisation of regeneration conditions and production of stable transgenic tomato plants

Cotyledons are the most frequently used explants in tomato transformation because of the availability of seeds, reproducibility of sterilisation and germination conditions and the possibility of controlling the developmental stage (Sigareva et al., 2004). Several studies have demonstrated that cotyledons and hypocotyls were superior to leaves for promoting shoot organogenesis in tomato (Hamza and Chupeau, 1993; Ling et al., 1998; Plastira and Perdikaris, 1997). Based on these reports we used cotyledon explants to transform Rio Grande with two gene constructs previously shown to confer salt and drought tolerance in ***Arabidopsis*** plants (Brini et al., 2007). Surprisingly, cotyledon explants did not give satisfactory results despite the use of established and published tomato transformation protocols. In fact, although cotyledons were able to regenerate shoots, the majority of them presented abnormalities and only few normal plants were recovered. Therefore, we decided to test the utility of primary leaves as an alternative explants for the ***in vitro*** regeneration and transformation of Rio Grande. Reporter genes such as ***GUS*** or ***GFP*** could be used to compare the amenability of cotyledon and primary leaves for transformation but we preferred to perform our investigation directly with agronomically relevant genes (***TNHX1*** and ***TVP1***).

In a preliminary experiment, we compared the effect of two media (Table 1), differing only in their growth hormone compositions, on the regeneration of Rio Grande and found that the medium which contains zeatin at 1 mg/L for shoot regeneration and 0.2 mg/L for shoot elongation combined with 0.1 mg/L IAA (SRI and SEI, Table 1), gave the best results in terms of regeneration percentages, productivity and morphological quality of the regenerated plants compared to the medium containing BAP and IAA (SRII, SEII, Table 1) (data not shown). Hence, it was used for the rest of the experiments to compare organogenesis capacity and transformation competence of the two types of explants (cotyledons and primary leaves). The behaviour of the two types of explants was already different on the shoot regeneration medium. In fact, after a two week period on SRI, many differentiated shoots and shoot primordia were visible on primary leaf explants (Figure 1a) whereas we observed only a high rate of callus proliferation and formation of roots in the case of cotyledon explants. Furthermore, statistical analysis revealed a significant difference at α = 1 % between regeneration means of primary leaf and cotyledon explants (Table 2). No significant difference was detected between the regeneration means of the two types of gene constructs at α = 5%, which indicates that the genetic construct does not account for the differences in the organogenesis capacity of the two types of explants. Primary leaves were three times more efficient then cotyledons in terms of percentages of regeneration (Table 2). Productivities (P) were calculated for both types of explants and were found: 0.64 for primary leaves and 0.15 for cotyledons. Although many shoot primordia were initiated on cotyledon explants, only few normal plants were recovered (4 out of 170 cotyledon explants), hence our main focus for the following analysis was tomato plants derived from primary leaves. Molecular analysis was performed on greenhouse tomato plants (T0) derived from different primary leaf explants to ensure that they belong to independent transformation events. They were subjected to PCR analysis using ***bar***, ***TVP1*** and ***TNHX1*** specific gene primers. Figure 2b illustrates an example of DNA amplifications of the expected 440 bp fragment from the ***bar*** gene sequence. To confirm the transgenic nature of tomato plants, RT-PCR technique was used. T0 plants from both constructs expressed the corresponding selectable marker gene RNA as shown in Figure 2c. The gene of interest (***TVP1***) was also expressed as illustrated in Figure 2d. No amplifications were obtained from RNA samples in the absence of the reverse transcriptase which rules out the possibility of contaminating genomic DNA sequences.

It has been long assumed that since ***Agrobacterium***-mediated transformation of plant cells commonly occurs at very low frequency, the use of selectable marker genes that confer antibiotic and/or herbicide resistance is required for identification of transformants (Bevan et al., 1983; Hererra-Esterella et al., 1983). The presence of selectable marker is not acceptable for transgenic plants in the field due to biosafety concerns. Therefore, many approaches were followed to remove selectable marker genes after the development of transgenic plants or to replace them by positive selection markers (Hohn et al., 2001). Despite their success, these approaches are tedious and costly. The best solution proposed relies on the efficient transformation of tissue explants and subsequent selection of transformed shoots by polymerase chain reaction (de Vetten et al., 2003). This solution was successful and resulted in the generation of transgenic

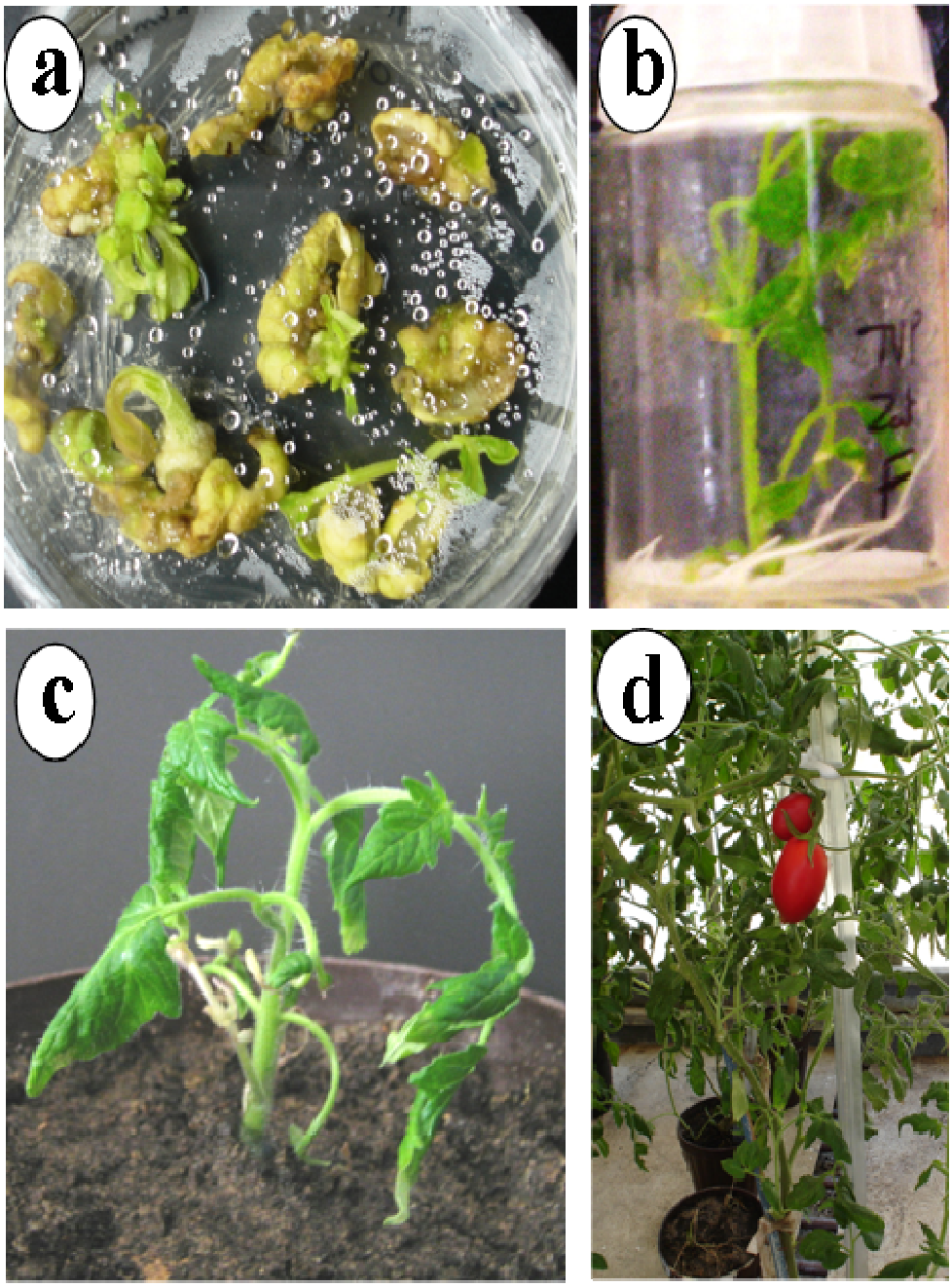

Figure 1. Regeneration of transgenic tomato plants from primary leaf explants. (a) Direct shoot differentiation from primary leaf explants; (b) a transgenic plantlet on rooting medium; (c) rooting shoot being transferred to soil for acclimation; (d) an adult transgenic plant in greenhouse producing fruits.

tobacco plants that transmitted faithfully the transgene to subsequent generations (T1, T2 and even T3) (de Vetten et al., 2003; Jia et al., 2007; Li et al., 2009). In the present study we adopted similar strategy for tomato and did not use any selection pressure. Many transgenic T0 tomato plants were easily identified using PCR analysis. In a typical experiment, transformation of primary leaf tissues with construct 1 (70 explants) and construct 2 (50 explants), generated a total of 54 and 30 regenerated tomato plants, respectively. Based on the number of positive plants determined following PCR and RT-PCR analysis, the estimated transformation frequen-cies for T0 generation were 14% (10 confirmed trans-genic T0 lines out of 70 explants) for construct 1 and 30% for construct 2 (15 confirmed transgenic T0 lines out of50 explants). Transgenic tomato lines (T0) were maintained in green-

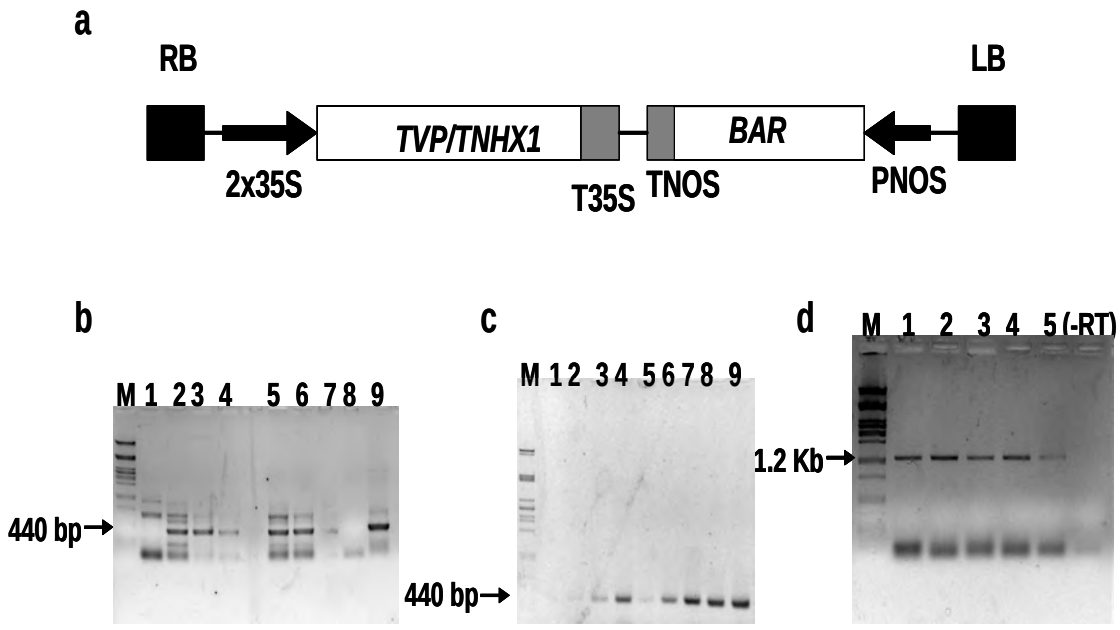

Figure 2. Molecular analysis of transgenic tomato plants. (a) Linear map of the T-DNA regions of the plasmids used for tomato transformation. RB: right T-DNA border, LB: left T-DNA border, 2x35S: duplicated CaMV (35S) promoter, T35S: 35S terminator, PNOS: nopaline synthase promoter, TNOS: nopaline synthase gene terminator, ***BAR***: selectable marker gene for glufosinate resistance (b) PCR results using specific ***bar*** oligonucleotide primers (product 440 bp). M: molecular DNA size marker, Lane 1: non-transformed tomato plant, Lanes 2-6: ***bar***-positive tomato plants from construct 2, Lane ***7***: ***bar***-positive tomato regenerant from construct 1, Lane ***8***: negative control (water absence of DNA), Lane 9: positive control plasmid pCB. (c) RT-PCR results using ***bar*** gene specific primers. Lane1: non-transformed tomato plant, Lanes 2-4: tomato regenerants from construct 2, Lanes 5-9: tomato regenerants from construct 1. (d) RT-PCR results using ***TVP1*** gene specific primers. Lanes 1-5: ***TVP1***-positive tomato plants, Lane 6: amplification using total RNA in the absence of reverse transcriptase.

house until maturity, selfed and produced fruits which contained viable seeds. The seeds of four ***TVP-1*** and five ***TNHX-1*** selfed T0 plants were germinated in pots and plantlets were subjected to PCR analysis using the same conditions as described for the T0 plants to check for the transmission of the transgenes. Three and five T0 lines harbouring ***TVP-1*** and ***TNHX-1***, respectively, transmitted the bar gene to T1 progeny which ruled out the possibility that these plants are escapes. Therefore, the transformation frequencies calculated based on PCR-analysis of T1 plants was 4% for construct 1 and 10% for construct 2. These results confirm further the efficiency of the non-selection approach and prove that concerns and criticisms related to obtaining transgenic plants under no selection pressure are unnecessary. Generation of escapes and chimeras is not unique to this strategy and even following strict selection pressure we can not claim preventing these from occurring. Therefore, further studies aiming at reducing rates of escapes and chimeric plants rather than more criticisms are needed independently of the strategy used; based on selection or not.

The protocol described herein for Rio Grande allowed a significant decrease in the time required for the generation of transgenic tomato plants. Following this protocol, transgenic plants were produced within 2 months, starting from the date of sowing seeds to prepare the explants, compared to 3 to 4 months with standard protocols for tomato transformation. Our time frame for Rio Grande trans-formation and regeneration is comparable to that described by Dan et al. (2006) for the model tomato MicroTom (Table 3). It is probable that the elimination of the selection step in this study speeded up the procedure and contributed significantly to the reduction of the trans-formation cycle through the promotion of regeneration.

Studies that targeted leaves as explants for tomato transformation and regeneration are less abundant than those describing use of cotyledon explants. In these reports, growth chamber or greenhouse grown-plants were used as the main sources of leaf explants. This required further sterilisation steps prior to transformation and regeneration (McCormick et al., 1986; Davis et al., 1991; Agharbaoui et al., 1995). It is well established that leaf explants are more advantageous for tomato trans-formation since they produced the highest rate of normal transgenic plants with the highest percentage of diploids compared to other types of explants, including cotyledons (Ellul et al., 2003; Sigareva et al., 2004). The only impediment to the use of leaves was their low regeneration

Table 3. Comparison of the time required for Rio Grande transformation, using primary leaves, with other established tomato transformation protocols.

Stages of protocol	Standard tomato Protocol (Dan et al., 2006)	MicroTom Protocol (Dan et al., 2006)	RioGrande Protocol (Present work)
Explant			
Preparation (d)	6	7	21
Pre-culture (d)	1	0	2
Co-cultivation(d)	2	2	2
Shoot induction (d)	28-35	21-28	14
Shoot elongation (d)	28-42	14-21	13
Rooting (d)	21-28	21-28	18
Transformation cycle	3-4 months	2-3 months	2 months and 10 ddays

capacity. In fact, Sigareva et al. (2004) found that leaf explants were the least regenerable and this was independently of the tomato genotype used. The difference between their results and ours is probably due to the age of leaf explants and tomato genotypes used. We used primary leaves from 3 - 4 week-old plants whereas they used 6 week-old aseptically-grown plants as source of leaf explants. Therefore, different types of explants of different ages need to be tested for each given tomato genotype in order to determine the most appropriate explants type for transformation experiments.

Phenotype of T1 transgenic tomato plants subjected to salinity stress

The vacuolar sodium antiporter and pyrophosphatase genes from diverse species were successfully overexpressed and shown to confer salinity and drought tolerance in many model and cultivated plants (Park et al., 2005; Zhang and Blumwald, 2001; Jia et al., 2002; Lee et al., 2003). In the present study, we introduced two wheat genes encoding these vacuolar proteins and evaluated their potential to confer salinity tolerance in tomato. To this end, confirmed T1 transgenic tomato plants were subjected to salt stress provided by a 200 mM NaCl solution. Transgenic T1 tomato plants exhibited a better appearance than their non transgenic counterparts. Indeed after only 15 days of salt-treatment, while wild type tomato plants showed yellowing of their leaves, transgenic T1 plants were healthy and green (Figure 3a and b). Total chlorophyll determinations also showed that transgenic plants retained more chlorophyll than their wild-type counterparts (data not shown). In fact, at 200 mM , ***TNHX1*** and ***TVP-1*** plants retained 4 and 7 times more chlorophyll, respectively, than their wild type counterparts. This phenotype is likely due to enhanced capacity of transgenic tomato plants to sequester sodium in their vacuoles which prevents its toxicity in the cytosol and the damage to the photosynthesis apparatus. Furthermore, transgenic tomato plants overexpressing the pyrophosphatase gene exhibited a more robust root system compared to wild type and ***TNHX1*** tomato plants (Figure 3c and d); such phenotype was previously described by Park et al. (2005). This holds true for tomato plants grown in pots as well as those grown continuously in a hydroponic culture during a three months period. The robustness of the root system could provide a better tolerance to drought stress as was previously shown (Park et al., 2005).

Conclusion

We described a simple and efficient protocol for the production of sufficient numbers of transgenic tomato plants using reasonable number of explants from the Rio Grande tomato cultivar. Use of primary leaves as explants instead of cotyledons and omission of the selection pressure saves time, energy and consumables and may contribute to insure the genetic stability sought in many applications of genetic engineering. The transfer and expression of the vacuolar sodium antiporter and pyrophosphatase genes enhanced salt and drought tolerance in transgenic tomato plants. Future experiments are being planned to use this optimized protocol for the production of transgenic tomato lines harbouring useful genes.

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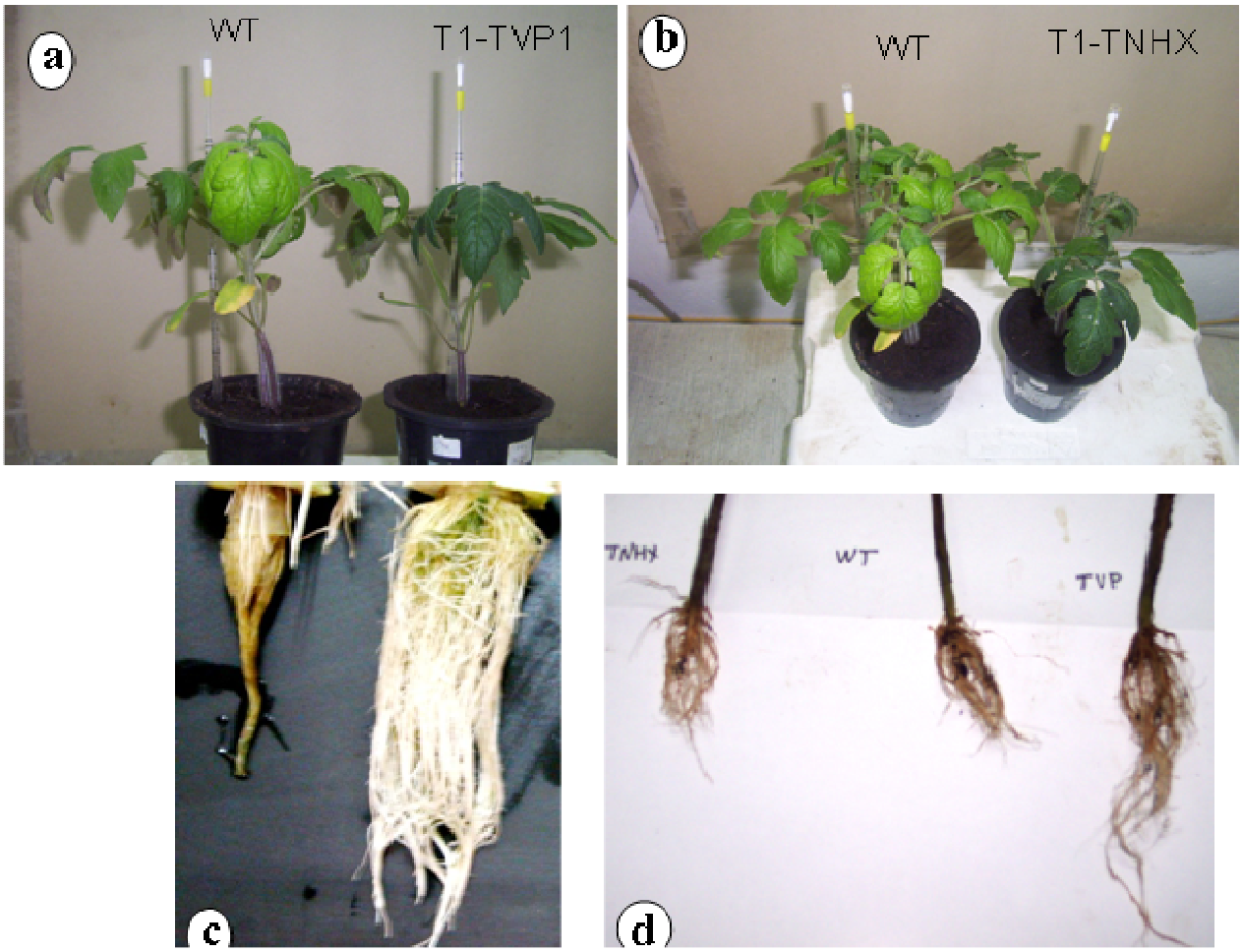

Figure 3. Phenotype of transgenic tomato plants subjected to salt-stress. Phenotype of wild type and T1 transgenic plants harbouring the pyrophosphatase gene (a) and the sodium antiporter gene (b) after 15 days of continuous salt stress with 200 mM Na Cl. (c) Root system growth of a wild type plant (left) and T1 transgenic plant harbouring the pyrophosphatase gene (Wright) grown in hydroponic culture for 3 months in the presence of 200 mM NaCl. (d) Root system growth of a T1 transgenic plant harbouring the sodium antiporter gene (left), wild type plant (middle) and T1 transgenic plant harbouring the pyrophosphatase gene (Wright) grown in pots and irrigated by capillarity u

REFERENCES

Agharbaoui Z, Greer AF, Tabaeizadeh Z (1995). Transformation of the wild tomato ***Lycopersicon chilense*** Dun. by ***Agrobacterium tumefaciens .*** Plant Cell Rep. 15: 102-105.

Bevan MW, Flavell RB, Chilton MD (1983). Achimaeric antibiotic resistance gene as a selection marker for plant cell transformation. Nat. 304:184-187

Bhatia P, Ashwath N, Senaratna T, Midmore D (2004). Tissue culture studies of tomato (***Lycopersicon esculentum***). Plant Cell Tiss. Organ. Cult. 78: 1-21.

Bhatia P, Aswath N, Midmore D (2005). Effect of genotype, explant orientation, and wounding on shoot regeneration in tomato. ***In Vitro*** Cell. Dev. Biol. Plant. 41: 457-464.

Brini F, Hanin M, Mezghani I, Berkowitz GA, Masmoudi K (2007). Overexpression of wheat Na^+/H^+ antiporter ***TNHX1*** and H^+-pyrophosphatase ***TVP1*** improve salt and drought-stress tolerance in Arabidopsis thaliana plants. J. Exp. Bot. 58: 301-308.

Briza J, Pavingerová D, Prikrylova P, Gazdova J, Vlasák J, Niedermeierová H (2008). Use of phosphomannose isomerase-based selection system for ***Agrobacterium***-mediated transformation of tomato and potato. Biol. Plant. 52: 453-461.

Butelli E, Titta L, Gorgio M, Mock HS, Mantos A, Peterek S, Schijjlen EGWM, Hall RD, Bovy AG, Luo J, Martin C (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat. Biotechnol. Doi: 10.1038/ntb.1506; 2008.

Cuartero J, Bolarìn MC, Asìns MJ, Moreno V (2006). Increasing salt tolerance in tomato. J. Exp. Bot. 57: 1045-1058.

Dan Y, Yan H, Munyikwa T, Dong J, Zhang Y, Armstrong C (2006). MicroTom-a high-throughput model transformation system for functional genomics. Plant Cell Rep. 25: 432-441.

Davis ME, Lineberger RD, Miller AR (1991). Effects of tomato cultivar, leaf age, and bacterial strain on transformation by ***Agrobacterium tumefaciens***. Plant Cell, Tiss. Organ Cult. 24: 115-121.

De Vetten N, Wolters AM, Raemakers K, Van Der Meer I, Ter Stege R, Heeres E, Heeres P, Visser R (2003). A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nat. Biotechnol. 21: 439-442.

Duzyaman E, Tanrisever A, Gunver G (1994). Comparative studies on regeneration of different tissues of tomato ***in vitro***. Acta Hortic. 366: 235-242.

Elbakry AA (2002). Effects of genotype, growth regulators, carbon source, and pH on shoot induction and plant regeneration in tomato.

In vitro Cell. Dev. Biol. Plant. 38: 501-507.
Ellul P, Garcia-Sogo B, Pineda B, Rios G, Roig LA, Moreno V (2003). The ploidy level of transgenic plants in ***Agrobacterium***-mediated transformation of tomato cotyledons (***Lycopersicon esculentum*** L.Mill.) is genotype and procedure dependent. Theor. Appl. Genet. 106: 231-238.
Fray A, Earle ED (1996). An examination of factors affecting the efficiency of ***Agrobacterium***-mediated transformation of tomato. Plant Cell Rep. 16: 235-240.
Gubiš J, Lajchová Z, Faragó J, Jureková Z (2004). Effect of growth regulators on shoot induction and plant regeneration in tomato (***Lycopersicon esculentum*** Mill.) Biologia Bratislava. 59: 405-408.
Hamza S, Chupeau Y (1993). Re-evaluation of conditions for plant regeneration and ***Agrobacterium***-mediated transformation from tomato (***Lycopersicon esculentum*** Mill.). J. Exp. Bot. 44: 1837-1845.
Hererra-Estrella L, Depicker A, Van Montagu M, Schell J (1983). Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nat. 303: 209-213.
Hohn B, Levy AA, Puchta H (2001). Elimination of selection markers from transgenic plants. Curr. Opin. Biotechnol. 12: 139-143.
Hu W, Phillips GC (2001). A combination of overgrowth-control antibiotics improves ***Agrobacterium tumefaciens***-mediated transformation efficiency for cultivated tomato (***L. esculentum***). ***In Vitro*** Cell. Dev. Biol. Plant, 37: 12-18.
Jia Hongge, Liao Mingjun, Verbelen Jean-Pierre, Vissenberg Kris (2007) Direct creation of marker-free tobacco plants from agroinfiltrated leaf discs. Plant Cell Rep. 26: 1961-1965.
Jia GX, Zhu ZQ, Chang FQ, Li YX (2002). Transformation of tomato with the BADH gene from Atriplex improves salt tolerance. Plant Cell Rep. 21: 141-146.
Jiang XL, He ZM, Peng ZQ, Qi Y, Chen Q, Yu SY (2007). Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. Transgenic Res. 16: 169-175.
Lee JT, Prasad V, Yang PT, Wu JF, David TH, Charng YY, Chan MT (2003). Expression of ***Arabidopsis*** CBF1 regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. Plant Cell Environ. 26: 1181-1190.
Li Baochun, Xie Claire, Qiu Hui (2009) Production of selectable marker-free transgenic tobacco plants using a non-selection approach: chimerism or escape, transgene inheritance, and efficiency. Plant Cell Rep. DOI: 10.1007/s00299-008-0640-8.
Ling HQ, Krislet D, Ganal MW (1998). Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in ***Agrobacterium***-mediated transformation of tomato (***Lycopersicon esculentum*** Mill.). Plant Cell Rep. 17: 843-847.
McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986). Leaf disc transformation of cultivated tomato (***Lycopersicon esculentum***) using ***Agrobacterium tumefaciens***. Plant Cell Rep. 5: 81-84.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.
Murray SL, Vuuren RJ, Berger DK, Van Vuuren RJ (1998). Tomato transformation is influenced by acetosyringone and ***Agrobacterium tumefaciens*** cell density. J. Southern Afr. Soc. Hortic. Sci. 8: 60-64.
Murray SL, Vuuren RJ, Berger DK, Van Vuuren RJ (1998). Tomato transformation is influenced by acetosyringone and ***Agrobacterium tumefaciens*** cell density. J. Southern Afr. Soc. Hortic. Sci. 8: 60-64.
Park S, Li J, Pittman JK, Berkowitz GA, Yang H, Undurraga S, Morris J, Hirschi KD, Gaxiola RA (2005). Up-regulation of a H^+-pyrophosphatase (H^+-PPase) as a strategy to engineer drought-resistant crop plants. Proc Natl. Acad. Sci. USA. 102: 18830-18835.
Park SH, Morris JL, Park JE, Hirschi, KD, Smith RH (2003). Efficient and genotype-independent ***Agrobacterium***-mediated tomato transformation. J. Plant Physiol**.** 160: 1253-1257.
Plastira VA, Perdikaris AK (1997). Effect of genotype and explant type in regeneration frequency of tomato ***in vitro***. Acta Hort. 447: 231-234.
Pohar KS, Gong MC, Bahnson R, Miller EC, Clinton SK (2003). Tomatoes, lycopene and prostate cancer: a clinician's guide for counselling those at risk for prostate cancer. World J. Urol. 21: 9-14.
Qiu D, Diretto G, Tavarza R, Giuliano G (2007). Improved protocol for ***Agrobacterium*** mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene CsZCD. Sci. Hortic. 112: 172-175.
Rao A, Agarwal S (2000). Role of antioxidant lycopene in cancer and heart disease. J. Am. College Nutr. 19: 563-569.
Sigareva M, Spivey R, Willits MG, Kramer CM, Chang YF (2004). An efficient mannose selection protocol for tomato that has no adverse effect on the ploidy level of transgenic plants. Plant Cell Rep. 23: 236-245.
Sun HJ, Uchii S, Watanabe S, Ezura H (2006). A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol. 47: 426-431.
Xiang C, Han P, Lutziger I, Oliver DJ (1999). A mini binary vector series for plant transformation. Plant Mol. Biol. 40: 711-768.
Zhang HX, Blumwald E (2001). Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat. Biotechnol. 19: 765-768.