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Agrobacterium tumefaciens-Mediated Transformation of Potato and Analysis of Genomic Instability by RAPD

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Abstract: In order to enhance the system of potato transformation and further regeneration, four potato cultivars were transformed using the *Agrobacterium tumefaciens* harboring β-glucuronidase (GUS) gene.The results revealed that the highest percentages of shoot formation obtained were for Spunta cv. Stem explants resulted in more callus and regeneration after transformation and selection than leaf explants. In all cases the performance was genotype-dependent. The GUS positive expression ranged from 40 % (Hermes) to 92.8 % (Lady Rosetta) for leaf explants and from 33.3 % (Hermes) to 91.6 % (Lady Rosetta) for stem explants. RAPD analysis elucidated the induction of somaclonal variations, which were also found to be genotype-dependent. It occurs more frequently when new plants are formed via leaf explants, while, using stem as explants were genetically stable. Similarity coefficients among the genotypes as well as among the different stages in tissue culture for each cultivar before and after transformation were calculated. Dendrograms were constructed accordingly.

Keywords: *Agrobacterium,* transformation, potato, genomic instability and RAPD

INTRODUCTION

Potato is the most important noncereal food crop. Sterility and tetraploidy in conjunction with a high level of heterozygosity greatly reduce the efeciency of traditional methods for potato breeding. Therefore, an alternative approach for further improvement of commercial potato varieties is genetic transformation. Several protocols for genetic transformation have been successfully employed to generate transgenic plants resistant to herbicides, insects and diseases[5]. However, these procedures have major limitations, such as low frequency of transformation and, more importantly, the occurrence of somaclonal variations at very high rates resulting from harsh tissue culture, thus limiting the production of transgenic plants for commercial applications[2]. The success and efficiency of transformation depends on the tissue culture system, and the methods required vary with species. Responses of potatoes to published regeneration regimes have shown cultivar specificity[21]. Due to the simplicity of the transformation system and precise integration of transgenes, *Agrobacterium* Ti plasmid-based vectors continue to offer the best system for plant transformation[19]. Not all cells subjected to transformation techniques, however, will be modified, so successful DNA transfer is tested by using a specific 'marker' gene. Several methods exist which rely on the co-cultivation of various tissue explants, e.g. leaf and stem segments or tuber discs, with engineered bacteria[16]. Because somaclonal variation is a common phenomenon in plant cell cultures, controlling it is a challenge[3]. Four critical variables for somaclonal variation were reported: genotype, explant origin, cultivation period and the cultural conditions[7]. RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among calli of potato cultivars[3].

The present work had three objectives: 1) Establishment of the optimal conditions for the system of callus induction and regeneration in four commercial potato cultivars, 2) Establishment of the system of transformation in potato using *Agrobactrium*, and 3) Assessment of genomic instability during *in vitro* culture and transformation in potato by RAPD analysis.

MATERIALS AND METHODS

Plant Materials and Bacterial Strain: Four potato cultivars (*Solanum tuberosum*) were used in this study as explant sources for tissue culture and genetic

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transformation with GUS gene namely, Spunta, Nicola, Hermes and Lady Rosetta. The cultivars were obtained from International Potato Center (CIP) Kafr El-Zayat, Egypt. For genetic transformation, *Agrobacterium tumefaciens* strain LBA4404 carrying the pBI121 construct (Fig. 1) was used; kindly provided from Boyce Thompson Institute (Cornell University). This plasmid contains the npt II selectable marker gene and the GUS reporter gene transcribed by the 35S promoter between the T-DNA borders, as described by Jefferson *et al*.[12].

Tissue Culture Procedure of Potato: Tissue culture studies were carried out to establish the suitable conditions for callus induction and regeneration of the four potato cultivars according to Visser[20] and Richard[16].

Transformation of Potato: Potato was transformed as described[20]. Potato stem segments and young leaf explants were soaked in *Agrobacterium* inoculum for 10, 20 or 30 minutes, which was suspended in MS liquid medium. The co-cultured explants were transferred to LC1 medium, nine explants per plate, and cultured for 72 hrs at 19°C in the dark. After three days, the explants were transferred to LC1 medium containing 50 mg/L kanamycin monosulfate and 500 mg/L carbenicillium. Regenerated shoots were transferred after 8 weeks to LC2 medium containing 50 mg/L kanamycin monosulfate and 500 mg/L carbenicillin. After one month the regenerated shoots were transferred to rooting medium, LC3.

Histochemical Assay for GUS: Segments of regenerated shoots and tubers were incubated with the GUS enzyme histological assay substrate 5-bromo-4-chloro-3-indolyl glucuronide in the dark at 37°C for 72 hrs according to Bansal *et al*.[1] then cleared with 70% ethanol for 24-28 hr at 65°C. The insoluble indigo dye deposit produced as a result of GUS activity indicates the success of transformation in potato.

Molecular Marker Analysis: Genome DNA purification kit (Promega part # TM050) was used for DNA isolation, as described in the manufacturer manual, from green leaves, calli, and plantlets for each cultivar. All PCR reactions were carried out in a final volume of 25 µl using Ready-To-Go PCR beads (Amersham Pharmacia Biotech code # 27-9557-01). Fifty pM of each primer (Biolegio) as well as 50 ng of template DNA were added to the reaction mixture and the total volume was adjusted to 25 µl. The sequences of the primers are shown in Table (1). Thermocycling was conducted in a Biometra-UNO II with 94°C for 5 minutes as initial denaturation followed by 40 cycles of 94°C for 1 minute, 38°C for 1 minute and 72°C for 2 minutes. This was followed by a 7 minutes final extension at 72°C[14]. The PCR product was analyzed by electrophoretic separation in 2% agarose gel.

Table 1: Primer sequences used in this study.

Primer code	Sequence (5' to 3')
K1	5' CCTGGGTGGA 3'
K2	5' CCTGGGCCTC 3'
K3	5' CCCGCCTCCC 3'
K4	5' CCGGCCTTAC 3'
K5	5' TTCCCCAAGC 3'
OPB-01	5' GTTTCGCTCC 3'
A18	5' AGGTGACCGT 3'
G5	5'CTGAGACGGA 3'

PCR products (10µl) were loaded onto a 2% (w/v) agarose gel in TBE buffer and were visualized under UV light (305 nm) after staining by ethidium bromide. 50 bp step ladder (Sigma s-7025) size marker was used, ranging from 50 to 3000 bp.

Data Handling and Cluster Analysis: Data were scored for computer analysis on the basis of the presence and absence of the amplified products for each primer. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to generate similarity coefficients which were used to construct a dendrogram by UPGMA (unweighted pair-group method with arithmetical averages) using NTSYS-pc Software[17].

RESULTS AND DISCUSSIONS

Results:

Establishment of Potato Tissue Culture System: Culture conditions were optimized for the four different potato cultivars. Response to callus induction and regeneration was found to be genotype-dependent. Lady Rosetta cultivar gave best response for callus induction while Spunta cultivar regenerated best (Table 2).

Optimization of an *Agrobacterium*-mediated Transformation System for Potato: *Agrobacterium*-mediated transformation for potato was optimized using *β*-Glucuronidase (GUS) as a reporter. The PBI 121 construct harboring the GUS gene driven by the *CaMV* 35*S* promoter was used. Parameters optimized were plant explant and co-cultivation period.

Table 2: Callus induction and regeneration responses of the four potato cultivars *in vitro*.

Cultivars	Callus weight (g) after 2 weeks of culture	Percntage of callus (%) after 2 weeks of cultre	Percentage of plantlets after 5 weeks of culture
Spunta	0.259	80.9	82.6
Lady Rosetta	0.296	92.3	68.0
Nicola	0.223	72.0	60.3
Hermes	0.193	39.9	40.5

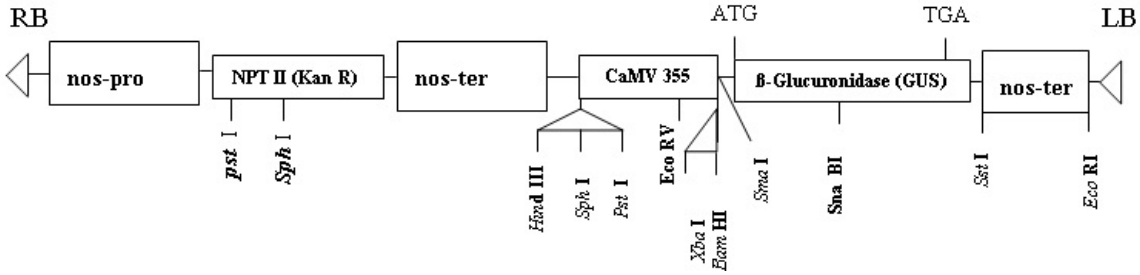

Fig. 1: pBI121 contains the cauliflower mosaic virus 35S promoter (CaMV 35S), coding region of *E.* coli β-glucuroidase gene (GUS) and 3` termination region from nopaline synthase (nos-ter).

The used co-cultivation periods with the optimal bacterial density adversely affected the callus growth and subsequently regeneration. Transformation was conducted on leaf and stem sections of potato cultivars at 10, 20 and 30 min of cocultivation with the bacteria. In the presence of 50 mgl^{-1} kanamycin, un-inoculated explants lost green pigment and died without callus or shoot formation.

Results of potatoes inoculation with PBI121 are shown in Table (3). The leaf explants of potato cultivars were inoculated at 10, 20 and 30 min. Shoots started to develop under selection for 8 weeks after inoculation. The highest percentages of transformed callus and shoot were recorded for Spunta; 36.3, 31.6 and 37.2 % for callus and for shoot were 17.6, 20.2 and 19.6 % at 10, 20 and 30 min. respectively. These percentages were 18.6, 24.7 and 23.2 % (callus) and 11.2, 11.1 and 11.2 % (shoots) for Lady Rosetta followed by 18.4, 20.3 and 23.1 of callus and 7.6, 9.2 and 9.6 % of shoots for Nicola at 10, 20 and 30 min. respectively. In contrast, Hermes recorded the lowest callus and shoot percentage 9.3, 13.7 and 16.5 % (callus) and 3.1, 6.8 and 7.2 % (shoots) at 10, 20 and 30 min. respectively.

The different inoculation times (10, 20 and 30 min) resulted in different percentages of transformation in both callus and shoots for Hermes, where it increased about twice at 30 min compared with 10 min.

As stem explants differed from leaf explants in many aspects. The central portion of inoculated stem explants were bleached and turned brown, while small hard, green knobs of callus developed at one or both cut ends of stem segment. Shoots started to develop under selection 4-6 weeks after inoculation. Percentages of transgenic calli were higher in all cultivars for stem explants than in leaf explants.

For stem explants of both Lady Rosetta and Nicola, a relatively high frequency of shoots (21.8% in both cases), were regenerated. Spunta exhibited less percentages, about 15% at 10 and 20 min inoculation and 18.5% at 30 min while, Hermes on the other hand also recorded the lowest percentages 0.0, 7.7 and 7.9 % at 10, 20 and 30 min times of inoculation (Table 3). The roots were formed after approximately 2-4 weeks from root formation while the microtubers were formed after four weeks.

Histochemical GUS Expression: GUS expression was tested in the four potato cultivars transformed with pBI121. Basal GUS expression of pBI121 construct with CaMV 35S-promoter was observed in all tissues, of stems, leaves, roots and microtubers. Transformed section from stems, leaves, roots and tubers stained intensely with the substrate, 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc), while non-transformed tissue did not.

Untransformed plants never showed staining with x-gluc, even after the assay was extended for several days. The histochemical GUS expression in shoots seems to be conserved in tissues and exhibited strong GUS expression (Fig. 2).

Table 3: Percentages of calli and shoots of four potato cultivars after inoculation with *Agrobacterium tumefaciens* containing the construct PBI121 for 10, 20 and 30 min. and selection.

Cultivator	Time inoculation (min)	Leaves		Stem	
		Callus (%)	Shoot (%)	Callus (%)	Shoot (%)
Nicola	10	18.4	7.6	27.9	20.9
	20	20.3	9.2	28.1	21.8
	30	23.1	9.6	25.0	21.4
Total		21.0	9.1	27.2	21.3
Lady Rosetta	10	18.6	11.2	29.5	21.8
	20	24.7	11.1	29.6	21.1
	30	23.2	11.2	32.1	21.4
Total		21.5	11.2	30.3	21.5
Spunta	10	36.3	17.6	37.5	15.6
	20	31.6	20.2	39.4	15.1
	30	37.2	19.6	37.0	18.5
Total		34.9	18.9	38.0	16.3
Hermes	10	9.3	3.1	13.0	0.0
	20	13.7	6.8	23.1	7.7
	30	16.5	7.2	23.7	7.9
Total		13.6	5.9	20.0	6.0

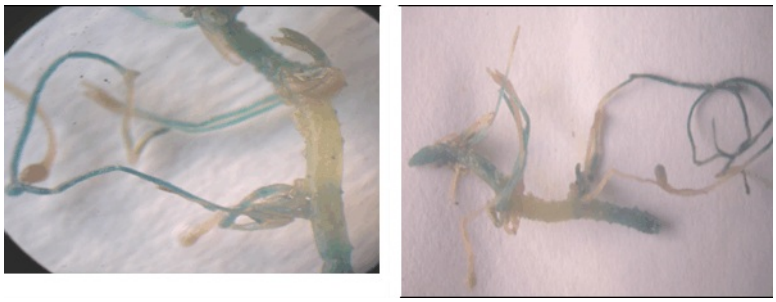

Fig. 2: Histochemical GUS assay of different explants of plant regenerated on Kanamycin.

Results in Table (4) indicate that Lady Rosetta showed the best response with 92.8 % and 91.6 % of GUS-positive transgenic plants for both leaves and stems, respectively at 30 min of inoculation. Spunta showed 90 % and 80 % of GUS-positive transgenic plants, for both leaf and stem explants, respectively, followed by Nicola exhibiting 82.1% and 83.3 % for leaf and stem explants respectively. Hermes showed the lowest response with 40 % and 33.3 % of GUS-positive transgenic plants, for both leaf and stem explants, respectively. The highest percentage of GUS positive transgenic plants after 10 min inoculation was 77.7 % and 60 % for both leaf and stem explants of Spunta, respectively. While, the highest percentage of GUS positive transgenic plants after 30 min inoculation was 92.8 % and 91.6 % for both leaf and stem explants of Lady Rosetta.

Transgenic plants were obtained from both leaf and stem explants. However, higher percentage of GUS expression was observed in transgenic plants derived from leaf explants as compared to stem explants. Percentage of GUS positive transgenic plants obtained from leaf explants ranged from 40 % (Hermes) to 92.8 % (Lady Rosetta), while those obtained from stem explants ranged from 33.3 % (Hermes) to 91.6 % (Lady Rosetta).

RAPD Analysis for Detection of Genetic Polymorphism: The genomes of intact plants (leaves), callus, regenerated shoots and transgenic plants of the four potato cultivars were compared using eight random primers, which generated a total of number of 127 bands, of which 110 turned to be polymorphic bands.

Table 4: Percentage of GUS expressed in transgenic potato plants.

Cultivar	Time of inoculation (min)	GUS positive transgenic plants from leaf explants (%)	GUS positive transgenic plants from stem explants (%)
Nicola	10	58.3	55.5
	20	80.7	71.4
	30	82.1	83.3
Lady Rosetta	10	61.1	52.9
	20	88.8	86.6
	30	92.8	91.6
Spunta	10	77.7	60.0
	20	87.5	80.0
	30	90.0	80.0
Hermes	10	33.3	0.0
	20	42.8	33.3
	30	40.0	33.3

Many faint bands (13%) were not included in the analysis due to the lack of reproducibility across different reactions. Band profiles for individual primers comprised 13 to 28 bands. Examples of the RAPD patterns obtained with the DNA from different cultivars and their calli, shoots and transgenic are shown in (Figs. 3 and 4). Similarity coefficients (Table 5) show the pair-wise similarity for the tested potato genomes. Associations among these genomes revealed by UPGMA cluster analysis are presented in Figure 5. The four cultivars showed a broad genetic base, with the highest genetic similarity estimates (GS) of only 0.4 between the cultivars Nicola and Hermes. The cultivar Spunta showed the least genetic similarity to other cultivars (GS= 0.27). When taking the calli, *in vitro* plantlets and transgenic plantlets of the four calli into consideration (Fig. 6), four main groups could be distinguished showing a major distance between the four potato genomes (Hermes, Nicola, Lady Rosette and Spunta). Within each group, a cultivar, its calli, its *in vitro* plantlets and its transgenic plantlets are grouped together. This observation was expected since the similarity between four main clusters was only about 55% (Table 5). The dendrogram (Fig. 5) and RAPD patterns (Figs. 3 and 4) showed that Hermes and Lady Rosette cultivars have higher genetic similarity estimates (GS) considering their calli, shoots and transformed tissues (GS = 0.64-0.84) and (GS = 0.66-0.81) respectively. Lower GS values were estimated for the cultivars Nicola and Spunta (GS = 0.52-0.69) and (GS = 0.31-0.75) respectively as shown in fig. (6).

The generated dendrogram in Fig. (6) indicated that the transgenic plants obtained from stem explants were very close to their original plant for all four cultivars. While the transgenic plants obtained from leaf explants revealed high somaclonal variation for all four cultivars.

Discussion: Plant transformation has been widely recognized as a tool to improve crops. But the emphasis has been on crops that are amenable to manipulation in tissue culture. In spite of the fact that production of transgenic potatoes by *Agrobacterium*-mediated transformation was achieved in several species of potatoes, the transformation and regeneration of potatoes were relatively low. Moreover somaclonal variation has occurred among regenerated transgenic potatoes[15]. Optimal regeneration conditions should be worked out, on both, stem and leaf explants, of particular genotypes of interest before attempting transformation. Because every genotype has its own special requirements as far as composition in the media, it is virtually impossible to give regeneration and transformation protocol that works well with every genotype.

The present results demonstrated that the efficiency of callus growth rate and shoot formation to be in part genotype dependent. Also, it revealed that the MS medium in combination,

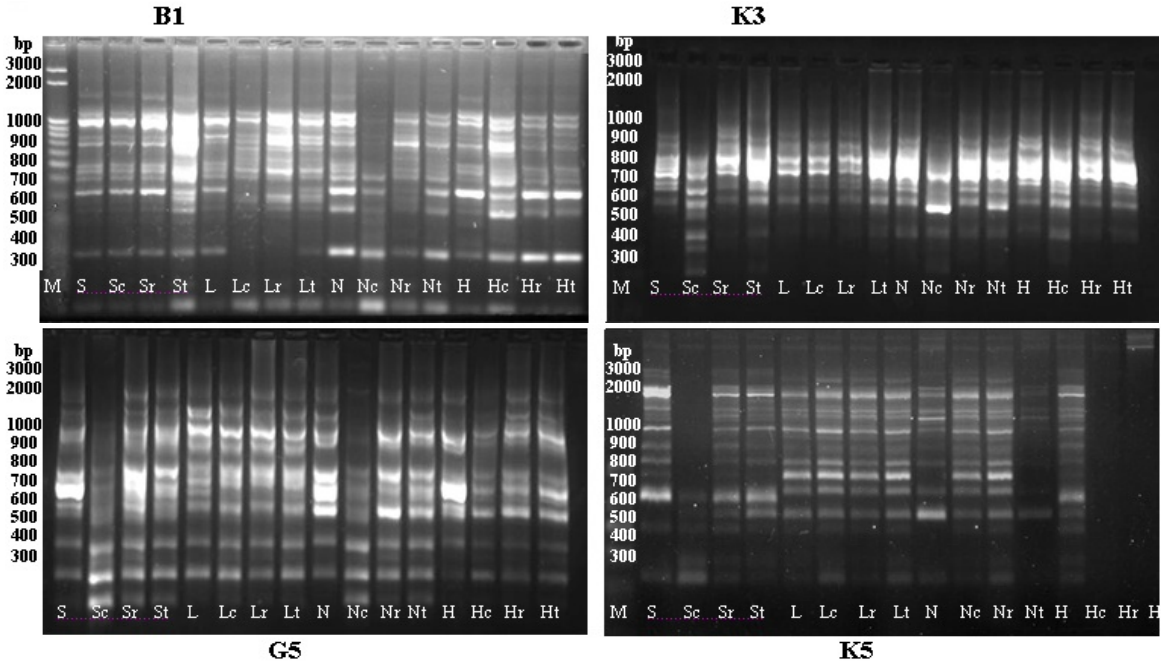

Fig. 3: RAPD patterns for the tested four potato cultivars and their calli, in vitro plantlets & transgenic plant generated by 10-mer random primers (B1, K3, K5 and G5). M= Marker, S= Spunta, Sc, Sr, St =callus, regenerated plantlet, transgenic plantlet for Spunta. L= Lady Rosetta, Lc, Lr, Lt =callus, regenerated plantlet, transgenic plantlet for Lady Rosetta. N= Nicola, Nc, Nr, Nt =callus, regenerated plantlet, transgenic plantlet for Nicola. H= Hermes Hc, Hr, Ht =callus, regenerated plantlet, transgenic plantlet for Hermes.

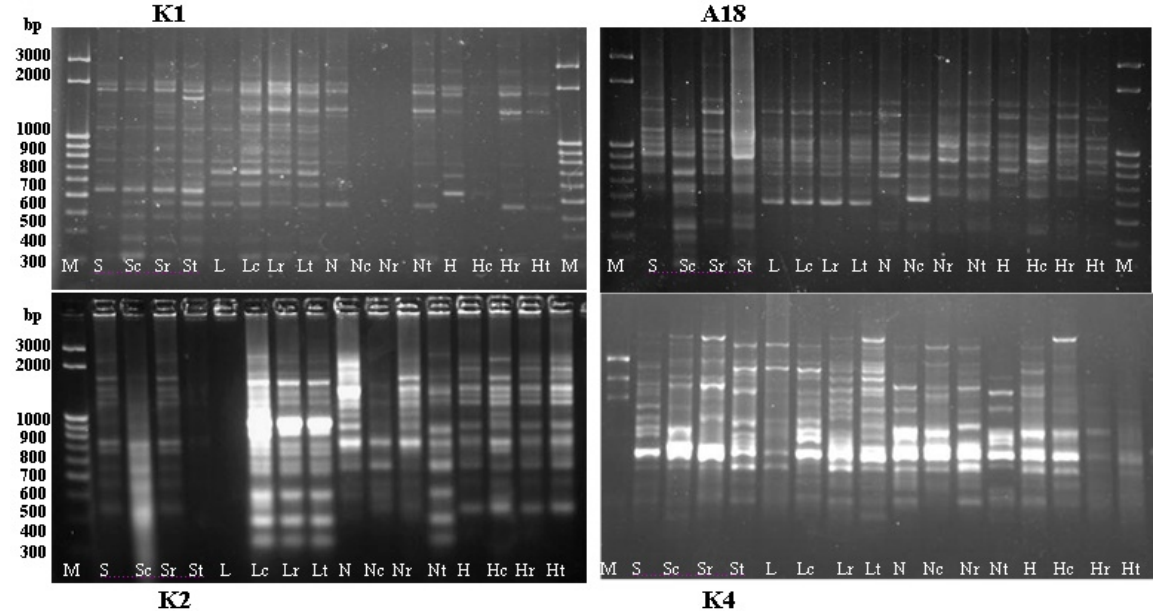

Fig. 4: RAPD patterns for the tested four potatoes cultivars and their calli, in vitro plantlets & transgenic plant generated by 10-mer random primers (K1, A18, K2 and K4, respectively). M= Marker, S= Spunta, Sc, Sr, St =callus, regenerated plantlet, transgenic plantlet for Spunta. L= Lady Rosetta, Lc, Lr, Lt =callus, regenerated plantlet, transgenic plantlet for Lady Rosetta. N= Nicola, Nc, Nr, Nt =callus, regenerated plantlet, transgenic plantlet for Nicola. H= Hermes Hc, Hr, Ht =callus, regenerated plantlet, transgenic plantlet for Hermes.

Table 5: Similarity coefficients among genotypes.

	Ht	Hs	Hc	H	Nt	Ns	Nc	N	Lt	Ls	Lc	L	St	Ss	Sc	S
Ht	1.000															
Hs	0.8387	1.000														
Hc	0.6957	0.7206	1.000													
H	0.7463	0.7206	0.6438	1.000												
Nt	0.5584	0.6000	0.6184	0.5769	1.000											
Ns	0.5676	0.6338	0.6216	0.6216	0.6267	1.000										
Nc	0.4521	0.5143	0.5139	0.4667	0.6000	0.5882	1.000									
N	0.5455	0.5658	0.6053	0.5443	0.5823	0.6901	0.5205	1.000								
Lt	0.5309	0.5500	0.6076	0.5176	0.5476	0.5119	0.4217	0.5714	1.000							
Ls	0.4286	0.4815	0.5375	0.5060	0.5181	0.4471	0.3735	0.4941	0.7973	1.000						
Lc	0.4588	0.4762	0.5119	0.5176	0.5294	0.4598	0.4048	0.4943	0.8133	0.7703	1.000					
L	0.5063	0.5658	0.4878	0.5500	0.5244	0.4524	0.4125	0.4535	0.7467	0.6579	0.7945	1.000				
St	0.4048	0.4568	0.4471	0.4756	0.4819	0.5000	0.4051	0.5125	0.4943	0.4598	0.5294	0.5060	1.000			
Ss	0.5181	0.5750	0.5357	0.5357	0.5172	0.5119	0.4390	0.5465	0.5333	0.4778	0.4945	0.5233	0.7067	1.000		
Sc	0.3659	0.4177	0.4321	0.4500	0.4217	0.4074	0.4000	0.4096	0.2979	0.2935	0.2979	0.3146	0.5000	0.6400	1.000	
S	0.4875	0.5455	0.4878	0.5844	0.4535	0.4578	0.3494	0.4762	0.4333	0.4419	0.4222	0.4471	0.6184	0.7467	0.6250	1.000

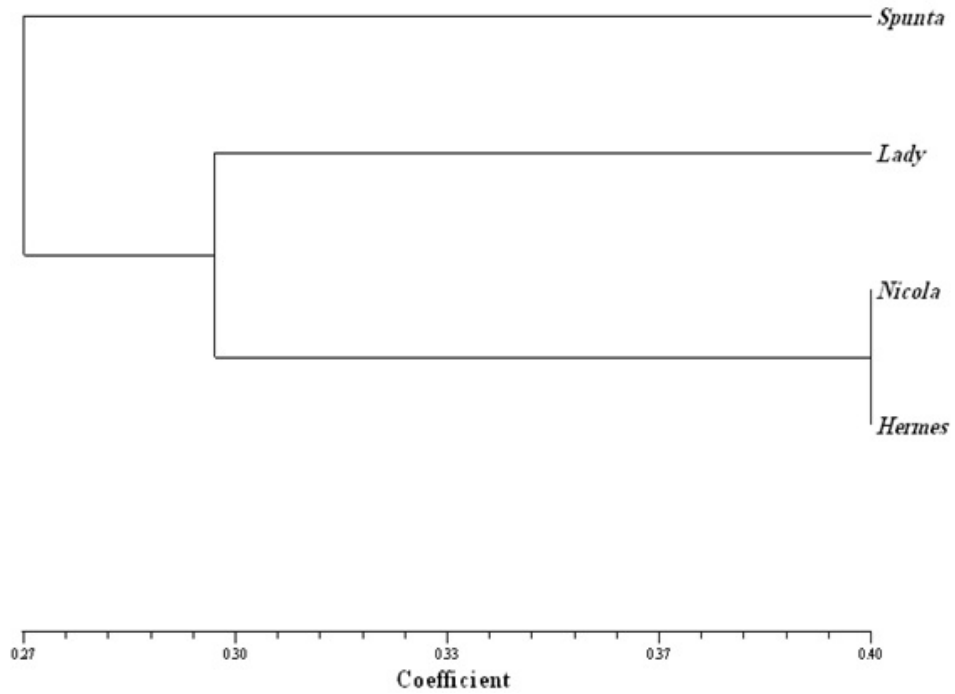

Fig. 5: Dendrogram of four potato cultivars by UPGMA analysis from pairwise comparison of RAPDs.

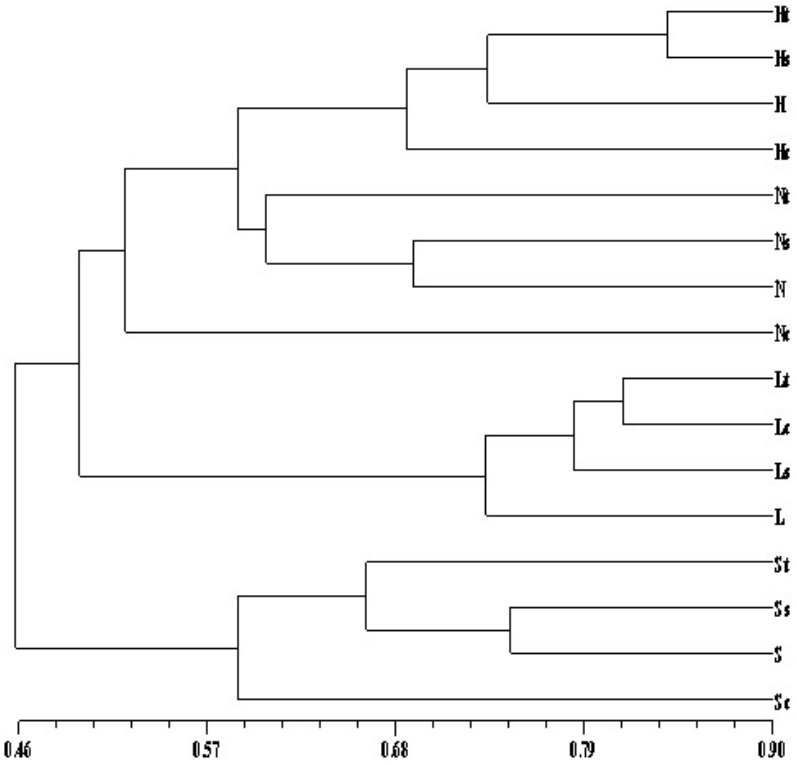

Fig. 6: Dendrogram of four potato cultivars and their calli, shoots, and transgenic plants by UPGMA analysis from pairwise comparison of RAPDs.

with GA_3, BAP, and NAA for callus induction, and supplemented with BAP, and GA_3 for shoot formation, appeared to be suitable for regenerating plantlets from leaf explants. These results are in agreement with a number of published papers[3,21,15].

An efficient transformation protocol has been developed for the transformation of the four commercially important potato cultivars in Egypt: Spunta, Lady Rosetta, Nicola and Hermes using stem and leaf explants. In this study the binary vector pBI121 in *Agrobacterium* have been used. Although, Spunta produced shoots more than Lady Rosetta on kanamycin when leave explants were used, it was noticed from the results that Lady Rosetta gave the highest number of shoots; higher than Spunta when stem explants were used. Hermes was the lowest in transformation frequency for both explants. These results agree with those mentioned by Hobbs *et al.*[10] and Davis *et al.*[4] that transformation efficiency can be cultivar-dependent. The efficiency of plant transformation is dependent on a number of steps. Firstly, genes transferred from *Agrobacterium* to plants during a period of co-culture with plant tissue. Secondly, the transformed cells that have to be selected (generally by exposure to an antibiotic such as kanamycin) and thirdly, the tissue culture step that is required to regenerate the transformed cells into plants. All these processes must be optimized if transformation is to be efficient for a range of cultivars. Many studies suggested that *Agrobacterium tumefaciens* strain and plant genotype play a role in this process in a number of plant species[4,6].

The data also indicated that in all of the studied cultivars, both leaf and stem explants showed approximatly same transformation ability. The highest percentage of selected shoots on kanamycin was obtained from leaf explants of Spunta cultivar while; the highest percentage from stem explants was obtained in Lady Rosetta. De Block[5] found that leaf segments are better to transform, and a number of researchers have claimed that stem segments are the most optimal for use[20]. Such discrepancy was explained by Visser[20] who mentioned that before even attempting transformation of either of these explant types, optimal regeneration conditions should be worked out, both for stem and leaf explants, of the particular genotype of interest. Because every genotype has its own special requirements of the medium composition, it is virtually impossible to give a regeneration and transformation protocol that works well with every genotype.

The GUS positive cases ranged from 33.3 % to 92.8 % for leaf explants and 33.3 % to 91.6 % for stem explants. The bacterial density used for infection was also critical, 10 min infection time or prolonged 15–30 min infection time, adversely affected the callus growth and subsequently regeneration, although the transient GUS activity was high. Saedler *et al.*[18] reported that the observed differences in the expression of the two genes (*GUS* & Kanamycin resistant) might be due to specific methylation of the chimeric CaMV35S *GUS* gene leading to gene, inactivation. However, Matzke *et al.*[13] showed that the lack of correlation of expression of *NPT II* and *GUS* gene might be more likely ascribed to differential regulation of the NOS and CaMV35S promoters by enhancers located.

In this study, the RAPD analysis was successfully used for estimating the genetic relationship between four potato cultivars. Clustering of the four tested potato varieties indicated that the cultivars selected for this study had a broad genetic background. This is further empasized by the grouping of (greater similarity) between each cultivar and its calli, *in vitro* plantlets and transgenic plantlets as compared to other cultivars and their calli, *in vitro* plantlets and transgenic plantlets. Bordallo *et al.*[3] have shown that susceptibility to somaclonal variation is related to genotype. Thus with this broad genetic base of the four studied cultivars, differences in the level of somaclonal variation and success of transformation were expected. These results are in agreement with previous studies that have also shown RAPD analysis to be useful in estimating reliable genetic relationships among potato cultivars[11]. Results presented here have indicated that the RAPD analysis was not only efficient in detecting genotypes and somaclonal variations but also sensitive in detecting the variation on the DNA level among transgenics of the four potato cultivars.

The Hermes and Lady Rosette cultivars showed higher genetic similarity estimates (GS) to their calli, shoots and transformed tissues. Lower GS values were estimated for the cultivars Nicola and Spunta. Cultivars were expected to respond differently to tissue culture and transformation as due to the variation in their genomes. Results indicated that Lady Rosetta showed least genetic variation due to tissue culture and transformation as indicated by the least estimates of variation between the original plants, its callus, shoots and transgenic, where the mean genetic similarity coefficient was 77 %. On the other hand Nicola expressed maximum variation amounting to an average of 60 %.

The present results based on the RAPD analysis showed that, among the transgenic plants analyzed for the four studied cultivars, transgenic plantlets obtained from stem explants revealed higher genetic similarity as compared to those obtained from leaf explants. These results are in agreement with those of Hanisch and Ramulu[8] and Higgins *et al.*[9] who observed that a common problem in potato regeneration/transformation using leaf explants or a long callus phase raises considerably the level of somaclonal variation observed among the regenerates. The variation in the sampling of the internodes on the plant is less important when compared to the leaves.

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