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Rapid and efficient in vitro regeneration of transplastomic potato (*Solanum tuberosum* L.) plants after particle bombardment

Md Jakir HOSSAIN[1], Emre AKSOY[1], Neslihan Zahide ÖZTÜRK GÖKÇE[1], Faiz Ahmad JOYIA[2], Muhammad Sarwar KHAN[2], Allah BAKHSH[1,*]

[1]Department of Agricultural Genetic Engineering, Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University, Niğde, Turkey

[2]Center of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad, Faisalabad, Pakistan

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Abstract: In contrast with traditional nuclear gene transformation, transplastomic technology has opened a new horizon for the transgenic plant research that offers several beneficial aspects including the convenient use of transgene stacking and the generation of high expression levels of foreign proteins. However, this technology has been well adopted and established in tobacco, the introduction and adoption of cost-effective, swift, and reproducible protocol for in vitro regeneration of transplastomic potato is challenging and laborious. The present research aimed to develop such prompt and efficient protocol to instigate and revive the regeneration potential with the combinations of different plant growth regulators (PGRs). Leaves and internodal explants from four potato cultivars were transformed with chloroplast transformation vector via particle bombardment and cultured on MS media supplemented with suitable PGRs and selection agents. Leaf explants of cultivar Kuroda induced highest (92%) number of calli where cultivar Sante produced the highest (85.7%) transplastomic shoots. Thidiazuron was found more proficient (41%) for shoot regeneration. Finally, within only seven weeks, we got 21 spectinomycin resistant shoot, and 16 of those showed integration of target genes into the plastome in PCR screening.

Key words: Particle bombardment, growth regulators, in vitro regeneration, transplastomic approach, rapid and cost effective

1. Introduction

Plastid genome engineering to produce viable transplastomic plants remains a blessing for the trait development endeavors of crop plants. Plastids seem to be an appropriate compartment for the accumulation of certain proteins or their biosynthetic (fatty acid and amino acid) products and photosynthesis with thousands of copies of the genome measuring about 155-kb where every plant cell harbor 10 to 100 plastids (Yu et al., 2017). The first effective chloroplast transformation was recorded in *Chlamydomonas* (Boynto et al., 1988) and this technique is routine and stable only in tobacco (*Nicotiana tabacum*; Svab and Maliga, 1993). Hopefully, reproducible plastid transformation protocols have also been identified in tomatoes (*Solanum lycopersicum*; Ruf et al., 2001), potatoes (*Solanum tuberosum*; Valkov et al., 2011), lettuce (*Lactuca sativa*; Ruhlman et al., 2010), Arabidopsis (*Arabidopsis thaliana*; Yu et al., 2017; Ruf et al., 2019) and soybean (*Glycine max*; Dufourmantel et al., 2004). Engineering plastid genome to produce transplastomic plants facilitate high rates of protein expression, the capability of expressing multiple proteins from polycistronic mRNAs, transgene stacking in operons, and the lack of epigenetic effects allowing stable transgenic expression and gene containment due to lack of pollen transmission (Wani et al., 2015; Hossain and Bakhsh, 2020). Transplastomic plants have been widely used to manufacture therapeutics, vaccines, antigens, and industrial enzymes, and for the development of various agronomic characteristics, including biotic and abiotic stress tolerance. Except tobacco, low transformation efficiencies have been reported for several species (Skarjinskaia et al., 2003; Zubko et al., 2004; Nguyen et al., 2005; De Marchis et al., 2009), and multiple reasons are attributed to this low regeneration of transplastomic shoots. Relatively lack homologous recombination system, nonoptimal homology and length of flanking sequence, the promoter that drives the expression of the selectable marker gene, the type and nature of explants, or the regeneration protocol is the mentionable causes those are basic hindrance for the sufficient regeneration of transplastomic plants. Additionally, technical factors restricting this approach

* Correspondence: abthebest@gmail.com

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include the lack of efficient in vitro regeneration and cereal transformation protocols, reduced transgene expression in nongreen plastids, a limited number of selection markers, a restricted range of transformable plants and low access of inducible gene expression (Valkov et al., 2009, Ahmad et al., 2016).

Despite recent developments in many species, potato plastid transformation is still constrained by low frequencies of transformation and low expression of transgenes (Nguyen et al., 2005; Gargano, 2005) compared to the nuclear genetic transformation (Bakhsh 2020; Dangol et al., 2020). Transgene expression in potato with the plastid rrn and the bacterial trc promoters ranged from 1% to 20%, but due to its low efficacy, GFP concentration achievable with the prokaryotic promoter was lower than with rrn one (Nguyen et al., 2005). Generation of transplastomic line is a long and complex process involving organelle sorting to accomplish homoplasmic shoots during repeated cell divisions in-vitro (Bock, 2001; Maliga, 2004). Therefore, the implementation of an appropriate method for transplastomic potato selection and regeneration is essential. Besides, the transformation of potato plastid has been attempted to date with vectors designed for tobacco. However, vectors with homologous potato flanking sequences could be constructed and tested to analyze the impact of increasing homology on the efficiency of plastid transformation as the full potato ptDNA sequence is available (Gargano et al., 2005).

Several methods have employed to deliver transgenes into the plastid genome and biolistic transformation is the most widely used methods for plastid transformation. In vitro potato regeneration is an effective method for the mass propagation of potato plantlets by callus induction from both the leaf and internodal explants. Trustworthy induction of callus and subsequent regeneration of potatoes by plant growth regulators enables year-round production of young and large plantlets without seasonal dependence. Integration of gene of interest into the plastome for the development of stable transplastomic lines has emerged as an outstanding technology in both basic and applied research (Ruf et al., 2019). The efficiency of plastid transformation is predictable through direct organogenesis. For example, under optimized conditions, plastid transformation and regeneration in lettuce, through morphogenesis, is as effective as in tobacco. However, the efficiency of plastid transformation is not highly predictive in plant species where regeneration requires somatic embryogenesis (Verma et al., 2008). Callus based in vitro regeneration system of potato comprises with three-stage procedure. The necessary steps involved are callus induction, the development of de novo outgrowth of shoots, and the rooting and adaptation to ex vitro conditions. This technique will allow the additional potato breeding programs to select new, improved potato lines with improved agronomic characteristics (Laboney et al., 2013, Haque et al., 2009).

For the efficient selection of transplastomic event, *aadA* (aminoglycoside-3"-adenylyltransferase) is routinely used selection marker that encode resistance to the translation-blocking antibiotic spectinomycin. Spectinomycin binds to chloroplast ribosomes (16S rRNA) and blocks translation on 70S plastid ribosomes of the prokaryotic form, inhibits the translation of plastid protein that readily bleaches the tissue and fails to produce functional chloroplasts and stops greening and shot regeneration in tissue culture cells**.** The *aadA* protein modifies the antibiotic by adenylation when the plastid genome is transformed with the *aadA* gene and prevents binding to chloroplast ribosomes that allow greening (Wirmer and Westhof, 2006; Wilson, 2014). To date, several studies have been conducted to produce stable transplastomic potato to achieve several desirable traits and different PGR combinations were applied for the in vitro regeneration (Sidorov et al., 1999; Nguyen et al., 2005; Valkov et al., 2011, 2014; Scotti et al., 2011, 2012; You et al., 2019).

In this study, we aim to recapitulate a standard regeneration protocol for the transplastomic potato through the optimization of the selection/regeneration procedure and by using homologous transformation vectors. We constructed a potato chloroplast transformation vector, delivered it to plant cell via particle bombardment, regenerated transplastomic shoots on selection media containing appropriate PGRs combination to revamp the physical damage oriented low regeneration proficiency. In-vitro callus induction, plantlet regeneration, and rooting of four potato cultivars have been reported where internodal, and leaf segments were used as explants. Therefore, this study's prime concern was to optimize a significant and rapid in vitro regeneration protocol to accomplish transplastomic potato from four potato cultivars.

2. Materials and methods

2.1. Plant material

Sterilized leaves and internodal explants from four potato cultivars (Kuroda, Simply Red, Challenger and Sante) were used as plant materials for the transformation experiment. Internodes and leaves from mentioned cultivars were treated with 70% ethanol for 1 min and then washed into autoclaved distilled water for 2–3 times. After that, intermodal segments were treated with 0.1% $HgCl_2$ and 2–3 drop Tween-20 mixture for 2 min and leaves were treated with 10% bleach and 2–3 drops Tween-20 mixture for 5 min with some modification as described by Çimen (2020) for citrus. Both explants were washed 2–3 times with sterilized distilled water and dried on sterilize filter paper.

2.2. Description of potato chloroplast transformation vector

Potato chloroplast transformation vector (PCTV1) was constructed at the Center of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad (Faisalabad, Pakistan). The vector harbors potato chloroplast genome sequences from *trnA- trnI* region to help precise transgene integration into potato chloroplast genome via homologous recombination. It also harbors FLARE-S as marker genes, which were amplified along with its promoter and terminator sequences from pMSK49 vector whose details can be found in Khan and Maliga (1999). The FLARE-S marker gene confers resistance to spectinomycin as well as streptomycin.

2.3. Transformation of potato explants using gene gun

Gold particles of around 0.6 µm diameter were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and coated with pPCTV1 as described by Joyia (2012). Biolistic transformation was carried out with those DNA coated gold particles under a partial vacuum (25 mmHg) with a Dupont PDS-1000/He Biolistic gun available at CABB following methodology described by Khan and Maliga (1999). Plates containing bombarded explants were placed at 27 ± 1 °C in dark for 48 h and then shifted to regeneration medium containing spectinomycin as selective agent.

2.4. In vitro regeneration of bombarded potato tissues with appropriate selection agent

Forty-eight hours after the bombardments, explants were placed on regeneration medium (bombarded side in contact with the medium) for the first round of selection to facilitate the preferential replication of plastids containing transformed ptDNA copies. Regeneration medium was supplemented with BAP 2 mg/L, NAA 0.2 mg/L, GA3 0.1 mg/L, and Thidiazuron (TDZ) 2 mg/L and antibiotic (Spectinomycin-50 mg/L for selection of transformed cells) that induced the division of cells of explants and the formation of embryogenic callus. To evaluate the performance of different cytokinines (Trans-zeatin, Trans-zeatin riboside and Thidiazuron) on shoot induction especially for the axillary and adventitious shoot proliferation and somatic embryogenesis, two more media were prepared where TDZ was replaced with 2 mg/L of Trans-zeatin (TZ) and Trans-zeatin riboside (TR) respectively. Unbombarded explants were cultured on the same media with and without antibiotics as negative and positive controls, respectively. Petri dishes were placed under white, fluorescent lamps in the culture room (1900 lux) with a dark period of 16 h/8 h at 26 ± 2 °C. After 4–5 weeks of incubation, bombarded explants on regeneration and selection medium, the calli were formed. The percentage of callus induction from the total number of bombarded explants was calculated as given below.

% age of callus induction = Number of calli induced / Total number of explants bombarded ×100

Five to six weeks later, shoot primordia started to emerge from the well-developed calli and those were transferred to a medium where MS was supplemented with spectinomycin 50 mg/L and NAA 0.1 mg/L. The numbers of putative transgenic shoots were recorded. Percentage (%) of shoot proliferation was calculated as given below.

% age of shoot proliferation = Number of shoot regenerated / Total number of calli ×100

2.5. Screening of putative transgenic potato by PCR

For the confirmation of transgene integration into the potato chloroplast genome, putative transplastomic shoots were analyzed by PCR. Before conducting the PCR, genomic DNA from putative transplastomic plants was isolated by the method described by Zhang et al. (2000) with some modifications. The genomic DNA was then subjected to PCR assays. PCR analysis of transgenic plantlets was carried out following the procedure as described by Saiki et al. (1990) using gene specific primers (Table 1). The PCR analysis was conducted using gene-specific primer pairs. A total of 50 µL PCR reaction mixtures in a 0.2 mL PCR tube were prepared. DNA from untransformed leaf and plasmid DNA of chloroplast transformation vector were used as negative and positive controls, respectively. All the reactions were made using polymerase Taq DNA (Fermentas, USA). A reaction volume of 50 µL contained: (i) Reaction buffer (ii) dNTPs (100 µM) (iii) primers (30 pmol) (iv) 50 mM MgCl2 for Taq polymerase (v) suitable volume of template DNA solution containing 50–100 ng of template DNA (vi) suitable volume of sterile DNAase/RNAase free water to generate a total of 50 µL. The PCR profile was as follows: Denaturation at 95°C for 5 min, followed by annealing temperature at 55.8 °C (for *aadA*

Table 1. Primers name, sequences, product size and annealing temperature information used in the study.

Primer	Sequence (5'-3 ')	Product size(bp)	Annealing temperature (°C)
AadA-F	GGCTCCGCAGTGGATGGCGGCCTG	540	56
AadA-R	GGGCTGATACTGGGCCGGCAGG		
gfp-F	AGCAGATCATATGAAGCGGCACG	398	54
gfp-R	CGTAAGGGCAGATTGTGTGGAGA		

gene) for 15 s. Annealing temperature 56.3 °C for 15 s was maintained for GFP gene. The extension was repeated at 72 °C for 30 s. The final extension was performed for 7 min at 72 °C. PCR amplification was conducted using Mastercycler Gradient PCR (Eppendorf AG 22331, Hamburg, Germany). Once the plants were confirmed for transgene integration by PCR, they were subjected to further rounds of selection.

2.6. Calculation of transformation efficiency

Transformation efficiency for transplastomic potato cultivars was calculated based on the data recorded during different cultural conditions and results obtained from the PCR. It revealed information regarding the incorporation of desired genes into the chloroplast genome. Percentage of transformation efficacy (% MTE) was calculated as stated below:

% age of transformation efficiency = Number of transplastomic plants regenerated / Total number of explants bombarded ×100

2.7. Statistical analysis

In vitro regeneration data of studied cultivars were recorded and calculated with standard statistical tools. Statistically significant differences between the means of the data were calculated by ANOVA (5%). To measure whether the means are significantly different from each other or not Tukey HSD test was applied. Mean and standard error (mean ± SE) of data were calculated from 3 replications (n = 3). All statistical analysis was done by Statistix 8.1 software.

3. Results

3.1. Screening and selection of putative transformants on selection medium

Around 100 explants (internodes and leaf) from every potato cultivar (Kuroda, Simply Red, Challenger and Sante) were bombarded with plastid transformation vector (PCTV1) bearing FLARE-S as a marker gene. Forty-eight hours after the bombardments, explants were shifted to the regeneration medium supplemented with suitable growth regulators and antibiotics for the selection of transformed cells. Both callus induction and plant regeneration from explants required the appropriate combinations and concentrations of plant growth regulators in the culture media. We cultured our explants on three types of medium (RM_1, RM_2 and RM_3) where RM_1 was used for callus induction and RM2 and RM3 was utilized for shoot regeneration. TDZ was replaced by TZ and TR in RM2 and RM3. Spectinomycin-50 mg/L was added for the selection of transformed cells. Callus induction was observed after 3–5 weeks, and mean percentage of callus induction was calculated. The unbombarded explants (used as control) and bombarded explants which were not transformed, due to selection pressure; those were turned to dark color and were found to get dead within two weeks of culture due to selection pressure (Figure 1B). No callus and shoots were developed from such nontransformed explants. Spectinomycin selection was continued on the selection medium which induces greening. Since spectinomycin prevents the greening of wild-type cells, only spectinomycin-resistant cells formed visible green cell clusters. Among the bombarded potato cultivars, Kuroda revealed the highest callus induction percentage from internodes (89.0%) and leaves (92.0%) (Table 2). Callus induction proficiency of cultivar Kuroda depicted higher in number compared with cultivar Simply Red (internodes - 87.0%, leaf explants - 82.0%). Lower callus induction proficiency from the leaf (74.0%) and internodes (71.66%) explants of cultivar Challenger was recorded compared with cultivar Kuroda and Simply Red (Table 2). Among the four potato cultivars, internodal explants of cultivar simply Red induced more calli in number than leaf explants that were recorded as an exception because all cultivars induced more calli from their leaf explants. Callus color and callus morphology data were recorded where calli were manifested several colors from studied cultivars. More prevalence of light brown (LB) color of calli was recorded during the experiments. Besides this, yellowish green (YG), light green (LG), green (G), dark green (DG), and brown (B) color calli were found from studied potato cultivars. Internodal explants from all cultivar induced light brown (LB) color calli, and leaf explants induced green (G) color calli. From internodal explants, cultivar Kuroda and Sante induced relatively light brown (LB) calli whereas cultivar challenger and Red induced brown (B) and light green (LG) calli. Contrarily, from leaf explants, cultivar Kuroda and Challenger induced yellow green (YG) and dark green (DG) calli respectively and cultivar Simply Red and Sante induced green (G) color of calli. As for as callus morphology is concerned, compact (C), granular (G), and friable (F) type of calli were induced and recorded properly during this current experiment. Explants from potato internodal segments induced more compact and granular type calli. Cultivar Kuroda and Simply Red produced compact type calli and cultivar Challenger and Sante induced granular and compact type calli, respectively. When leaf tissues were used as explants, Cultivar Kuroda and Sante induced granular calli whereas cultivar Simply Red and Challenger induced friable type of calli. In sum, from internodal and leaf tissues, Kuroda induced a total of 181.0 calli that was the highest number among all cultivars studied. From cultivar Simply Red and Sante, 169.0 and 166.0 calli were induced, respectively. Therefore, cultivar Challenger induced the lowest number (145.6) of calli. Later, embryogenic growth was observed in developed calli after 2–3 weeks of callus formation. The,

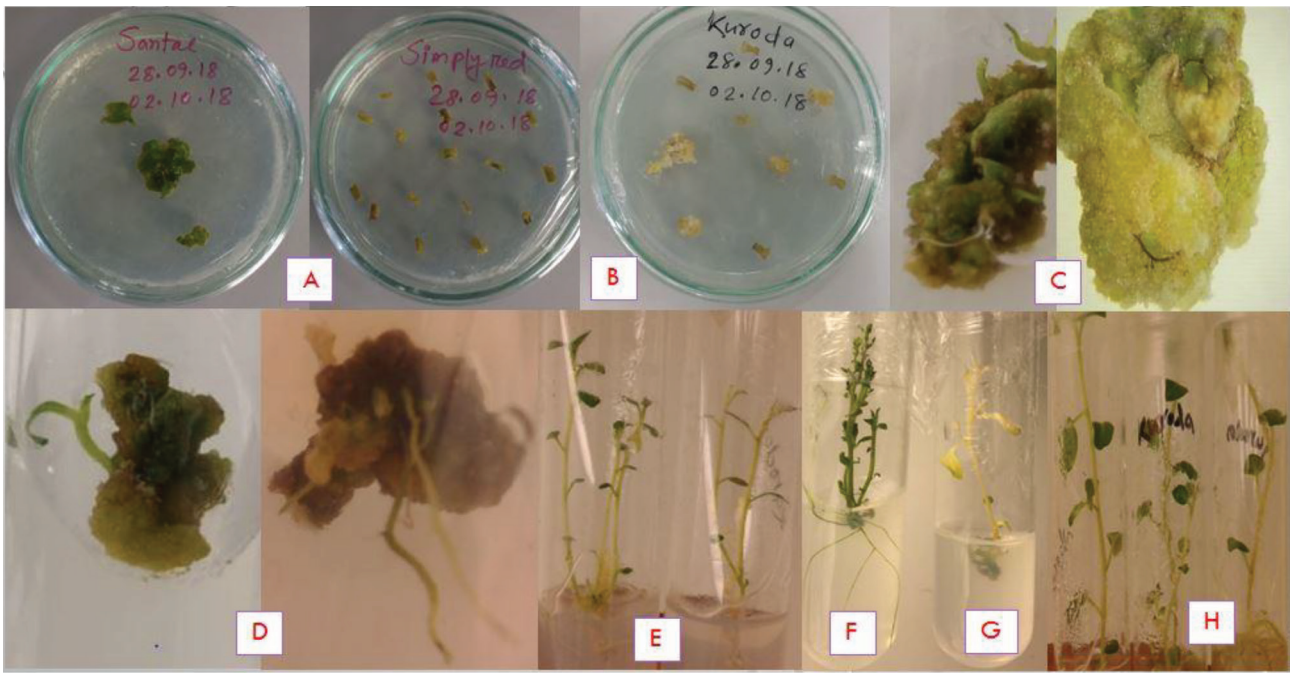

Figure 1. The different step of selection, callus induction, regeneration and plantlet production on regeneration selection medium. A. Bombarded explants on selection media (leaf and internodes). B. Blackish discoloration of nontransformed explants denotes its decayed condition because of selection pressure, initiation of callus formation from transformed explants. C. Fully developed mature calli. D. Emergence of microshoot from calli. E. Multiplication and culture of microshoot with presence of selective agent. F. Second round of selection where spectinomycin resistance shoots were capable to create roots against selection pressure. G. Died of nontransformed shoot because of selection pressure and there is no root was formed. H. Spectinomycin resistant and PCR positive transplastomic shoots.

shoot formation was noticed from those embryogenic cells, and the number of transplastomic candidate shoots were recorded. Three cytokinins (TDZ, TR and TZ) showed various responses in cases of shoot regeneration proficiencies and days required. Concerning shoot formation, TDZ produced the highest percentage of shoot formation (41%) requiring 49 days, whereas TR resulted in 40.3% shoot formation within the same number of days. TZ's shoot production ranges were recorded as 17% to 39.6% within 49 days (Table 2). Shoot production proficiencies also varied depending on cultivar types. Cultivar Kuroda and Sante produced a higher number of shoots, whereas cultivar Kuroda and Challenger respectively yielded the lowest number of shoots. The transformation efficiency was calculated after six weeks. The bombarded explants of different cultivars showed varying responses on regeneration media containing selection agent (Table 2). After that, the total number of shoots produced by every cultivar was calculated and recorded. Cultivar Sante and Challenger 9.6% shoot regeneration efficiency (%) followed by cultivar Simply Red (8.8%). On the other hand, cultivar Kuroda produced the lowest (6%) shoot regeneration efficiency (Table 2). Proliferated shoots were subcultured for several rounds of selection to achieve homoplastomic condition on MS media supplemented with 50 mg/L spectinomycin. After successive selection, cultivar Sante produced the highest number of spectinomycin resistant shoots (43.7%) followed by cultivar Challenger (42.8%). Cultivar Simply Red and Kuroda produced 33.3% and 28.0% spectinomycin resistant shoots, respectively (Table 2).

3.2. Confirmation of transgene integration by PCR

The integration of transgene was confirmed by polymerase chain reaction (PCR) (Figures 2 and 3). Among the spectinomycin resistant shoots, cultivar Sante produced the highest number of PCR positive shoots (85.7%), and cultivar Kuroda produced the lowest number of positive shoots (66.6%). Although the cultivar Kuroda produced a smaller number of shoots, its percentage of PCR positive shoots were same to cultivar Challenger. Cultivar Simply Red and Challenger showed an equal number of PCR positive shoots. However, their Spectinomycin resistant shoots were different in numbers, and their PCR positive shoots percentages were recorded as 80% and 66 %, respectively. Finally, from four cultivars, we got a total of 16 PCR positive shoots.

4. Discussion

An efficient protocol for the selection of transplastomic cells and in vitro regeneration after particle bombardment is a

Table 2. In vitro regeneration of transplastomic potato with different hormonal combinations along with selection pressure.

Potato cultivars	PGR: BAP 2 mgl^{-1}, NAA 0.2 mg l^{-1}, GA3 0.1 mgl^{-1}TDZ 2 mgl^{-1}	Callogenesis						Total number of calli	Effects of 3 cytokinins on shoot proliferation				Total shoot regenerated from different cultivars	Shoot regeneration (%)	Number of Sp^r shoots	% of Sp^r shoots	Number of PCR positive shoots	% of PCR positive shoots	Total PCR positive shoots
		Induction (%)		Color		Morphology													
		Internodes	Leaf	Internodes	Leaf	Internodes	Leaf		Cytokinins	Shoot regeneration percentages (%) in different required days									
										36	42	49							
Kuroda		89.0 ± 0.57a	92.0 ± 0.57a	LB	YG	C	G	181.0	TDZ	33.3 ± 1.73ab	38.6 ± 0.88ab	41.0± 1.52a	11 ± 1.15a	6.0 ± 1.52a	3.0 ± 0.57a	27.2 ± 1.09b	2.0 ± 0.33b	66 .6 ± 1.52b	16 ± 0.57
									TR	20.0 ±0.72 c	34.6 ±1.20ab	40.3 ± 0.88ab							
									TZ	17.0 ± 0.58 c	35.0 ± 1.63ab	39.6 ± 1.76ab							
Simply Red		87.0 ± 1.15a	82.0 ± 1.15b	B	G	C	F	169.0	TDZ	40.0 ± 0.68ab	37.2 ± 1.50 ab	40.6± 1.20ab	15 ± 1.52a	8.8 ± 0.23a	5.0 ± 0.30a	33.3 ± 1.15b	4.0 ±0.57a	80 ± 1.15a	
									TR	33.3 ± 1.15ab	35.6 ±0.95 ab	39.3 ± 0.60ab							
									TZ	20.0 ±1.56 c	35.0 ±1.15 ab	39.0 ± 1.15ab							
Challenger		71.6 ± 0.33c	74.0 ± 1.15c	LG	DG	G	F	145.6	TDZ	32.9 ± 2.34b	39.3 ± 0.33ab	39.6 ± 1.20ab	14 ± 0.57a	9.6 ± 0.51a	6.0 ± 0.83a	42.8 ± 1.10a	4.0 ± 1.00a	66 ± 1.52b	
									TR	20.0±0.78 c	38.6 ± 1.85ab	39.3± 2.33ab							
									TZ	17.0± 0.93c	37.2 ± 0.92ab	38.1± 2.52 ab							
Sante		80.0 ± 0.57b	86.0 ± 1.15b	LB	G	C	G	166.0	TDZ	40.0 ± 1.50ab	35.6 ± 0.56ab	40.0 ±1.52 ab	16 ± 1.73a	9.6 ± 0.75a	7.0 ± 0.95a	43.7 ± 1.45a	6.0 ± 0.83a	85.7 ± 1.83a	
									TR	33.3 ± 1.50ab	34.6 ± 1.20ab	39.6 ±1.20 ab							
									TZ	20.0 ± 1.15c	33.3± 0.88ab	38.0± 2.08ab							

Spectinomycin resistant: Spr. Every regeneration selection media was supplemented with 50 mg/L Spectinomycin. Yellowish green: YG, light green: LG, green: G, dark green: DG, light brown: LB, brown: B, compact: (C), granular: (G), friable: (F). TZ: Trans-zeatin, TR: Trans-zeatin riboside, TDZ: Thidiazuron. Mean of different values shown by different letters in the same columns are statistically different determined using Tukey's HSD test at the 0.05 level of significance. Results and standard error (mean ± SE) were calculated from 3 replications (n = 3).

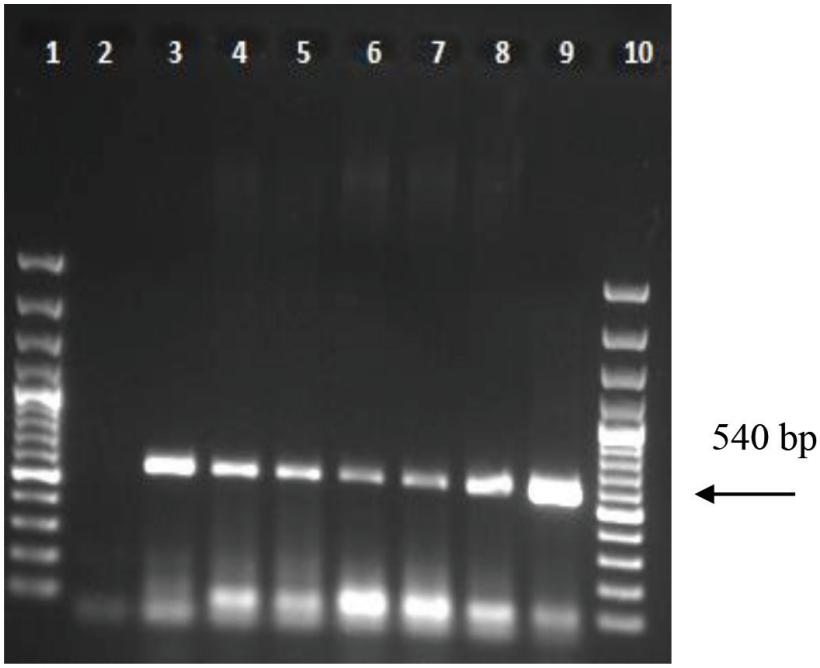

Figure 2. PCR analysis of transplastomic shoots. A. PCR analysis of putative transplastomic shoots showed the amplification of the expected internal fragment (540 bp) of *aadA* gene. Lanes 1 and 10: 100bp plus ladder (Thermo Fisher Scientific), Lane 2: negative control, Lane 3: PCTV plasmid used as positive control, Lanes 4 to 9: representative transplastomic shoots from cultivars.

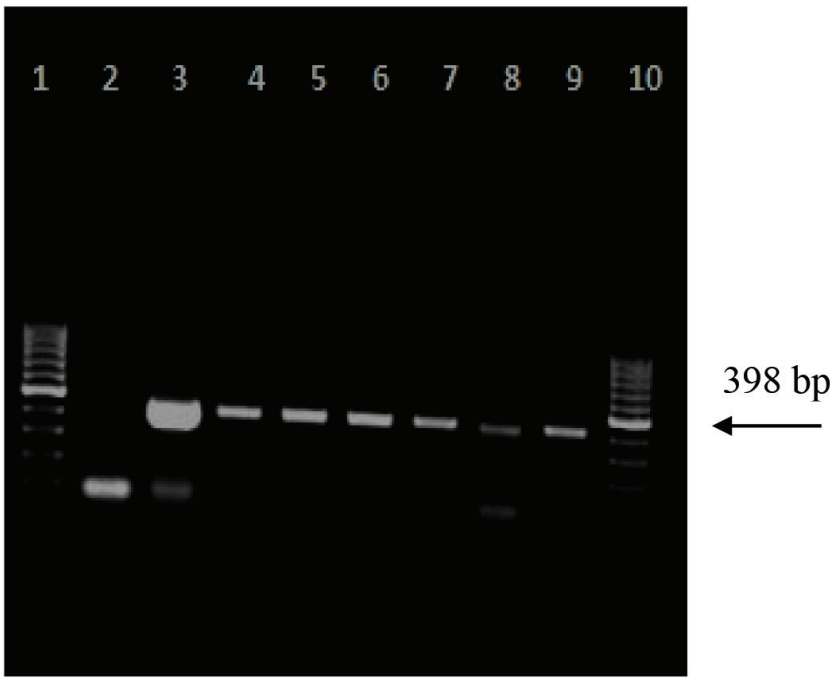

Figure 3. PCR assay to amplify green fluorescent protein gene (*gfp*) from transplastomic shoots during second round of selection on spectinomycin. Lanes 1 and 10: DNA GeneRuler mix and 100 bp ladder (Thermo Fisher Scientific), Lane 2: negative control, Lane 3: plasmid DNA PCTV as positive control, Lanes 4–9: representative transplastomic shoots from cultivars.

prerequisite for implementing the plastid transformation strategy in potato. Generating viable transplastomic potato lines in laboratory conditions is quite challenging. Many factors are primarily responsible for poor in vitro regeneration of plant cells after particle bombardment, especially when chloroplast transformation is attempted. One of the several reasons is the relative sizes of chloroplasts/plastids and gold particles (microcarriers). The average size of a mature chloroplast is 4–5 µm and the smallest available size of gold particles 0.6 µm. The size of immature/undifferentiated plastids in calli is even smaller ranging 20 mµ–1.5 µm which is merely double of the size of smallest gold particles. Then, the particles are accelerated to very high velocity for bombardment. Such a high momentum of metal particles causes substantial damage to the chloroplasts after particle bombardment on explants (Russel et al., 1992; Mookkan, 2018). In this study, we contemplated establishing reliable and effective potato plastome engineering protocol through the incorporation of the marker gene into the potato plastome. We recovered plastid transformants with the two-step regeneration protocol utilized by Nguyen et al. (2005) and this requirement for a callus phase for recovery of transplastomic potato is similar to that reported for tomato by Ruf et al. (2001). However, according to Nguyen et al. (2005), only one month on callus induction medium was required for potato compared to about six months for tomato before shoot regeneration whereas in our experiment well-developed calli were formed within induction 3–5 weeks. In comparison, Sidorov et al. (1999) documented potato plastid transformation with a single step procedure on which they used a noncommercial genotype (FL1607 breeding line) based on its responsiveness in in vitro. They reported 6.67% (1 transformant out of 15 shots) and 2.85% (1 from 35 shots) regeneration performance from two vectors (pZS197 vector and vector targeting the *rps12-rrn16* intergenic region, respectively). These findings are much lower than the regeneration proficiencies (ranges between 66.00% and 85.7%) of four potato cultivars used in our study. On the other hand, Nguyen et al., 2005 used Désirée, a commercially important cultivar and a two-step process tissue culture procedure was adopted to produce transplastomic potato on which they recorded 5.5 % (1 out of 18 shots) and 4.0% (1 out of 25 shoots) from pZS197 and pMSK18 vectors, respectively. However, Nguyen et al. (2005) reported integration of *aadA* and *gfp* in pMSK18 transplastomic potato plants by PCR as a proof of transplastomic event. Adopting the same genotype and underlying regeneration protocol previously published by Nguyen et al. (2005), Valkov et al. (2011) roughly doubled (11 shoot per 100 bombardments/one shoot every nine bombardments) the efficiency of transformation compared to previous results from Sidorov et al. (1999) and Nguyen et al. (2005). This finding obtained by Valkov et al. (2011) is similar to our current findings regarding the cultivars Challenger and Sante.

During in vitro regeneration in *Arabidopsis*, Yu et al. (2017) used leaf tissue as explants and obtained eight events on five bombarded plates in the *acc2-1* mutant background and four events on four bombarded plates in the Slavice accession (approximately 100-fold enhanced). This transformation efficiency is comparable to the transformation efficiency obtained in tobacco by Maliga and Tungsuchat-Huang (2014) where four to five transplastomic events per bombardment was achieved. Khan et al. (2015) conducted an experiment to produce transplastomic tobacco to confer enhanced tolerance to salinity and drought in plants on which nine transgenic clones were recovered from 35 bombardments on selection and regeneration medium.

When homologous potato sequences replaced tobacco-flanking sequences in transformation vectors, a drastic increase in transformation efficiency was reported to be about one shoot per bombardment. The biolistic approach was employed with species-specific vectors that allowed up to 1–2 shoots per bombardment to boost the frequencies of plastid transformation. On the contrary, when the recombination of flanking sequences originating from the petunia plastome was used, the tobacco transformation efficiency decreased by more than tenfold (DeGray et al., 2001). Using homologous flanking sequences in a transformation event is an essential factor that can improve the frequency of transformation, and Scotti et al. (2011) ensured this in potato. Another study by Valkov et al. (2014) on potatoes reported one transplastomic shoot per bombardment. This efficiency is comparable to that commonly obtained in tobacco production. Nonetheless, our decreased efficiency compared to Valkov et al. (2014) may be due to the disparity in vector size, flanking sequences, and genetic variation between the cultivars under analysis (Valkov et al., 2011). Svab and Maliga (1993) and Langbecker et al. (2004) got higher lines of tobacco transplastomic. Svab and Maliga conducted high-frequency plastid transformation and regeneration in tobacco, 1993 on that experiment they achieved 84 spectinomycin resistant clones from 79 bombarded leaf cultures. DNA gel blot analysis of 50 clones confirmed 40 (80%) *aadA* integration into the plastid genome. This finding (85.7 %) correlates our present findings. On the other hand, plastid transformation was much lower than tobacco in Arabidopsis, potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*). For instance, one transplastomic line was achieved from 40 (2.5%) or 151 (0.66%) bombardments in *Arabidopsis*, from 35 bombardments (2.85%) in potato, from 25 (4.0%) bombardments in oilseed rape, from 20 (5.0 %)

bombardments in tomato (Sidorov et al., 1999; Ruf et al., 2001).

It is essential for the regeneration strategy of transplastomic plants to identify transplastomic events. It took 5 to 12 weeks for the RLD (reduced number of long days) ecotype to regenerate in 1998 (Sikdar et al., 1998). The use of spectinomycin-sensitive knockout lines *acc2* and the dicistronic operon vector pATV1 shortened the period for transplastomic event detection to 3 to 5 weeks. The use of the *acc2* knockout lines shortened scoring because spectinomycin effectively prevented the proliferation of nontransformed cells, allowing the identification of green cell clusters resistance to spectinomycin. Spectinomycin resistance may be due to the plastid genome integration of *aadA*, nuclear genome integration of *aadA*, and fortuitous expression of an upstream promoter or spontaneous *rrn16* gene mutation (Svab and Maliga, 1993). gfp, encoded in the second ORF, is expressed only in chloroplasts, allowing confocal microscopy to rapidly classify transplastomic clones in a limited number of heteroplasmic cells (Yu et al., 2017). Our current research also coincides with these current studies, and we hope our spectinomycin resistant shoots are likely due to the plastome incorporation of *aadA* (Figure 2), which decreased the transplastomic event detection to 5 to 7 weeks only.

Although transplastomic technology offers considerable advances, it has not yet entered the market. Integration and transmission of chloroplast expression vectors into the chloroplast genome is a relatively straightforward, yet lengthy method. Besides, the recovery of homoplasmic plant lines is another big obstacle for this technology (Ahmad et al., 2016). Current attempts to streamline this process include introducing high throughput cloning methods for chloroplast expression vector construction (Gottschamel et al., 2013; Vafaee et al., 2014) and finding new selectable markers (Bellucci et al., 2015). Nevertheless, it has proven extremely difficult to extend the transplastomic technology to field crops.

5. Conclusion

Considering the hurdles and drawbacks regarding the introduction of efficient and prompt in vitro regeneration protocol for transplastomic potato, we intended to optimize a swift and effective protocol of in vitro regeneration after biolistic gene delivery method. Once our vector was integrated into the chloroplast genome through homologous recombination, we critically examined our findings and contrasted them with existing studies that helped us judge and compare our regeneration efficiency. As the development of homoplasmic transplastomic lines is a lengthy process and it can take 7–12 months, utilizing the current regeneration protocol, we got our desired transplastomic shoots within only 36 to 49 days that is outstanding achievement ever. On the other hand, we achieved 85.7% regeneration proficiency with our studied protocol that is also remarkable in terms of existing findings (0.6 % or 1.2%). We hope our studied protocol can minimize the bombardment mediated damage oriented low rate of in vitro regeneration.

Author's contribution

The research project was designed by AB and FAJ who secured funding from the Scientific and Technological Research Council of Turkey (TÜBİTAK) as principal investigator. MJH did all experiments, collected data, and analyzed it. MJH and AB composed initial draft of the manuscript which was further reviewed critically by all coauthors. EA, NZOG, FAJ and MSK participated in bilateral project activities. All authors read the manuscript and presented in its current form.

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