

Molecular Cloning of Sucrose Isomerase Gene and *Agrobacterium*-Mediated Genetic Transformation of Potato (*Solanum tuberosum* L.) Plants

Hemaid I. A. Soliman

Plant Genetic Resources Department, Desert Research Center, El-Matariya 11753, Cairo, Egypt.
Tissue Culture and Biotechnology Labs., Maryout Research Station, Desert Research Center, Alexandria, Egypt
Email: hahemaid@yahoo.com

Abstract—Potato (*Solanum tuberosum* L.) is one of the most common and important food sources on the planet, and they essential as a staple dietary item for much of the world's population. Potatoes contain carbohydrates, which lead to high blood sugar. Palatinose (isomaltulose, 6-O- α -D-glucopyranosyl-D-fructose) is a functional isomer of sucrose its non-cariogenicity low calorific value and it is an ideal sugar substitute to use in food production. The sucrose isomerase(*pall*) gene that is obtained from *Erwinia rhapontici* is one of the most common genes that can convert sucrose into palatinose. In present study, *pQE-30-pall* construct was successfully transformed and expression into *E. coli*. Sucrose isomerase (*pall*) gene was cloned and overexpressed into a plant expression vector *pBinAR-pall* contains sucrose isomerase gene (*pall*) fused to proteinase inhibitor II signal sequence under *CaMV-35S* promoter and *Octopine Synthase (OCS)* terminator. Expression of the protein was verified by western blot assay. Also, expression of the *pall* gene within the apoplast of transgenic tubers under control of a tuber-specific patatin class I B33 promoter instigated quantitative conversion of sucrose into palatinose. Tuber extracts from potato cv. Désirée were analyzed for their soluble carbohydrate composition using HPLC.

Keywords—Potato; *Agrobacterium*; Sucrose isomerase; Palatinose; B33 promoter; Expression; HPLC.

Abbreviations: SP- signal peptide of the proteinase inhibitor II gene ; MS- Murashige and Skoog medium; BAP- Benzyl Amino Purine; ZR- Zeatin Riboside; IAA- Indole Acetic Acid; IBA-3-Indole Butyric Acid; TE- Transformation Efficiency; OCS- polyadenylation signal of the octopine synthase gene; ER- Endoplasmic Reticulum; B33- promoter of the class I patatin gene B33; *pall*: *Erwinia rhapontici* sucrose isomerase gene; EcoRI, Asp718, BamHI, SalI, HindIII- restriction cleavage sites.

I. INTRODUCTION

Potato (*Solanum tuberosum* L.) is a staple food and is considered the most important economic tuber crop around the world (Joseph *et al.*, 2015). Sucrose substitutes vary greatly in degree of sweetness, volume, texture and stability under different conditions and sweetener is not a perfect substitute for sucrose in all applications. In some cases high amounts of sucrose over long periods was found to cause cancer diseases (Price *et al.*, 1970). Some microbes contain Isomaltulose synthase (*Pall*), catalyzes the isomerization of sucrose to produce isomaltulose (palatinose, α -D-glucopyranosyl-1, 6-D-fructofuranose) and trehalulose (α -D-glucopyranosyl-1, 1-D-fructose), as the main products with residual amounts of glucose and fructose (Börnke *et al.*, 2001; Zhang *et al.*, 2003; Watzlawick and Mattes, 2009). Isomaltulose is a naturally occurring isomer of sucrose (α -D-glucopyranosyl-1,2-D-fructofuranose) that is valued as an acariogenic sweetener (Takazoe, 1989). Palatinose is a nutritional sugar it is digested more slowly than sucrose and has health advantages for diabetics and nondiabetics (Lina *et al.*, 2002). Also, it is the first non-cariogenic sugar, similar physico-chemical properties as sucrose (taste, texture, and mass) but shows a slower rate of release of monosaccharides into blood (Goda and Hosoya, 1983; Minami *et al.*, 1990). They described the report on the cloning and characterization of a bacterial sucrose isomerase (*pall*) gene from *Erwinia rhapontici* which catalyses the conversion of sucrose into palatinose and its function has been tested by heterologous expression in *Escherichia coli*. This enzyme is strictly substrate-specific toward sucrose and the reaction is essentially irreversible. The yield of palatinose formed from sucrose ranged from 85 and 15% for trehalulose, respectively (Cheetham, 1984). Expression of a chimeric sucrose isomerase (*pall*) gene within the apoplast of transgenic tobacco plants and accumulated considerable amounts of non-cariogenic sucrose isomer palatinose (Xuguo *et al.* 2016). However,

conversion of sucrose into the non-metabolizable isomer palatinose caused severe growth retardations in these plants most likely due to the depletion of a carbohydrate source for sink development (Börnke *et al.*, 2002).

In this study, description the cloning and characterization of a chimeric sucrose isomerase (*pall*) gene from *Erwinia rhapontici* and introduced *pall* gene with different promoter into potato plants were described. In addition to, Expression of the *pall* gene which conversion of sucrose into palatinose within the apoplast of transgenic tubers was studied.

II. MATERIALS AND METHODS

Isolation and cloning of sucrose isomerase gene.

The coding region of the sucrose isomerase (*pall*) was cloned by polymerase chain reaction (PCR). Genomic DNA from *E. rhapontici* was isolated by a standard protocol and used as a template. Amplification was carried out using the following specific gene primers 5'-GGGATCCTCACCGTTCAGCAATCA3' and 5'-GTCGACCTACGGATTAAGTTTATA-3', which were obtained from sucrose isomerase sequence (GenBank Acc. No.: AF279281) and signal peptide of proteinase inhibitor II gene (Keilet *et al.*, 1986), which was fused via a linker with the sequence ACC GAA TTG GG to the *Erwinia rhapontici* sucrose isomerase gene, which comprises the nucleotides 109 to 1803. Thus, a signal peptide of a plant protein, which is required for the uptake of proteins into the endoplasmic reticulum (ER) was fused N-terminally to the sucrose isomerase sequence.

Plasmid Construction and bacterial strain.

The *Erwinia rhapontici* sucrose isomerase gene and expression vector pQE-30 (Qiagen Inc. Valencia, CA) were digested with restriction enzymes *Bam*HI/*Sal*I

respectively. The digested products were separated using agarose electrophoresis and the bands were extracted. The purified sucrose isomerase gene (*pall*) and the linear vector were ligated overnight at 16°C with T4 DNA ligase followed by transformed into *E. coli* JM109 competent cells. The transformation mixture was plated on (Luria Broth) LB agar plates containing ampicillin (100 µg/mL⁻¹). The plates were incubated for 16h at 37°C. The desired recombinant plasmid pQE-30-*pall* was confirmed by PCR and restriction enzyme digestion with *Bam*HI/*Sal*I and DNA sequencing (Invitrogen).

Construction of the sucrose isomerase overexpression construct the coding region of the *pall* gene, ranges from codons 109 to 1,803bp was amplified from *Erwinia rhapontici* by PCR. The *pall* gene fused to proteinase inhibitor II signal sequence (SP) was inserted in sense orientation between the CaMV-35S promoter and Octopine synthase (OCS) terminator within a binary vector plasmid pBinAR by the restriction endonuclease Asp718 (Höfgen and Willmitzer, 1990; Börnke *et al.*, 2001). This plasmid is a derivative of the binary vector pBin19 (Bevan, 1984). The *A. tumefaciens* strain LBA4404 harbouring recombinant binary vector plasmid pBinAR-*pall* was maintained on LB medium (Chilton *et al.*, 1974) supplemented with 25 mg L⁻¹ rifampicin and 50 mg L⁻¹ kanamycin and incubated overnight at 28°C in an incubator shaker at 90 rpm/min before using in transformation (Fig. 1).

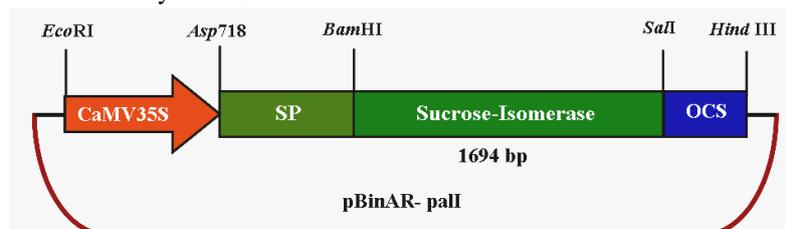


Fig.1: Structure of *pall* expression construct used to transformed potato. The *pall* coding region ranging from 109 to 1,803bp was inserted between the CaMV-35S promoter and OCS terminating region of vector pBinAR using *Bam*HI and *Sal*I restriction sites, respectively. The signal sequence for the proteinase inhibitor II (SP) was inserted in front of the *pall* coding region.

The 35S promoter was removed from the pBinAR vector using the restriction endonucleases EcoR I and Asp718. A fragment with a length of about 1526 by comprising the tuber-specific promoter of the class I patatin gene (B33-promoter) of potato and inserted into the pBinAR vector by EcoR I and Asp718. This resulted in the plant expression

vector pBin33-Kan. The *pall* gene was introduced in the sense orientation into the plant transformation vector pBin33-Kan by Asp718/*Xba*I between the patatin B33 promoter and the octopine synthase polyadenylation signal (Fig. 2).

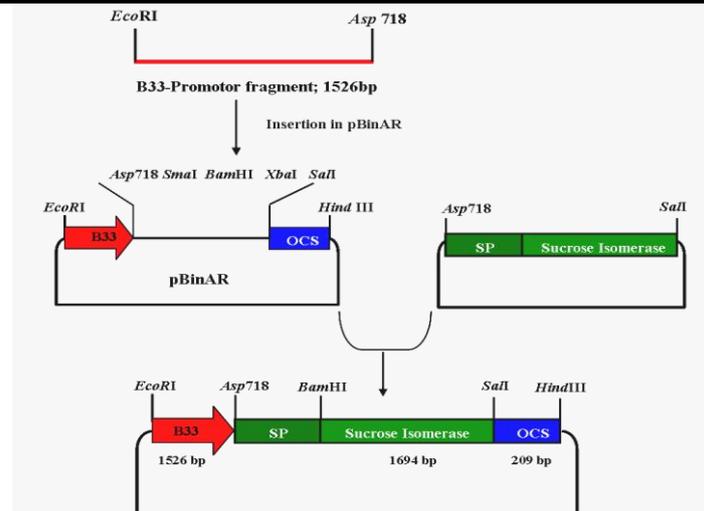


Fig.2:Schematic representation of the construct pBin33, which mediates the expression of sucrose Isomerase from *E. rhapontici* of transgenic Potato tubers. The gene for the Sucrose-Isomerase, merged with the signal peptide of the proteinase inhibitor I gene (Schaewen et al., 1990), the tubers-specific B33 promoter of Patatin class I gene was between ASP 718 / EcoRI fragment and the OCS terminator cloned into the vector pBin19. The obtained construct pBin33 was then used for the production of transgenic potato plants.

In vitro regeneration and tuber formation of potato (*Solanum tuberosum* L.)

Leaf explants were collected from 4 to 6 week-old *in vitro* grown potato (*Solanum tuberosum* L.) cv. Désirée and cultured on MS (Murashige and Skoog, 1962) medium supplemented with 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose and different growth regulators IAA (0.05, 0.1, 0.5 mg L⁻¹) in combination with zeatin riboside (1.0, 2.0, 3.0, 4.0, 0.5 mg L⁻¹) for six weeks for direct regeneration. The cultures were incubated at 25±2.0°C with a 16/8 h light/dark photoperiod provided by cool-white fluorescent lamps (40-50 μmol m⁻²s⁻¹). The shoots regenerated were cultured on MS medium containing 100 mg/l myo-inositol, 1.0 mg L⁻¹ BAP, 0.5 mg/l kinetin and 2.5 g L⁻¹ phytigel with different concentrations 5%, 6%, 7%, 8%, 9% and 10% of sucrose for *in vitro* tuberization in potato. The plantlets were incubated under dark condition at 25±2.0°C. Microtubers were harvested after eight weeks of incubation. Data were recorded on mean number of shoots/explant, mean length of shoots/explant (mm), regeneration percentage, microtuber formation per explant, percent of explant formed microtuber, number of microtuber/explant and average weight of microtuber/explant.

Agrobacterium-mediated transformation of potato.

The leaf explants were immersed in the *Agrobacterium* suspension containing either the binary vector pBinAR-pall or the binary vector pBin33-Kan for 20 min. After infection, the leaf explants were placed on MS medium-free hormone at 25±2°C for co-cultivation of 48h. And then, the cultures were transferred on shoot induction medium (MS plus 100 mg L⁻¹ kanamycin and 300 mg L⁻¹ cefotaxime to inhibit further bacterial growth) for six weeks and then cultures were incubated at 25±2°C

with a 16/8 h light/dark photoperiod provided by cool-white fluorescent lamps. Shoots were transferred to MS medium 1.0 mg L⁻¹ BAP, 0.5 mg L⁻¹ kinetin, 10% sucrose with 100 mg L⁻¹ kanamycin and 300 mg L⁻¹ cefotaxime at 25±2.0°C under dark for micro tubers formation. Transformation frequency was calculated by multiplying the percentage of explants that produced plants by the percentage of plants that were confirmed to be transgenic by PCR.

Expression of pall gene in *E. coli*.

E. coli XL-1 Blue cells were transformed with recombinant plasmid pQE-30-*pall* extracted from JM 109. The bacteria cells were grown in 5 ml LB liquid medium at 37°C with 100 μg/mL⁻¹ ampicillin for 4-6 h at 220 rpm till to an absorbance of 0.6 at 600 nm. Expression of the fusion protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) with a final concentration of 0.5 mM for 5 h at 37°C. These samples were harvested by centrifugation at 13,000 rpm for 1 min. The pellet was resuspended in 1 ml of 30 mM HEPES-KOH (pH 7.5) for preparation of palatinose. The suspension was centrifuged at 15,000 rpm for 2 min at 4°C, and the supernatant was used for enzyme measurements. The expression of *pall* protein was analyzed by SDS-PAGE. For conversion of sucrose into palatinose by *E. Coli* cell suspension, the *E. coli* cells (1 g wet wt.) expressing the *pall* gene were washed two times with 50 mmol L⁻¹ PBS (pH 6.0). The cell pellets were resuspended in the same solution at the desired concentrations. Reactions were conducted in 50 ml flasks containing 10 ml 550 g L⁻¹ sucrose solution at 30 °C and shaken at 150 r/min for about 5 h. Aliquots of the reaction mixture were sampled and analyzed for the amounts of palatinose formed. The reactions were

terminated by heating the flasks for 10 min in a boiling water bath.

Antibody preparation and western blot assay.

The sucrose isomerase (*pall*) gene subcloned in expression vector pQE-30 which resulted pQE-*pall* and introduced into *E. coli* XL-1Blue. The protein over expressed in *E. coli* was purified to form antibodies against sucrose isomerase (*pall*) by immunization rabbit. The plant proteins were separated by SDS-PAGE and transferred to the porablot and incubated with antibodies against the *pall* protein from transgenic potato plants.

HPLC analysis

Reaction products were filtered through 0.22 μ m membrane filters before HPLC analysis (Agilent 1200, USA system equipped with a refractive index detector). The samples were diluted 10-fold and 20 μ l of diluted sample was injected onto a Rezex RCM-Monosaccharide Ca^{++} column (Phenomenex, USA) for measurement of the sugar composition. The mobile phase was water with a flow rate of 0.5 ml/min at 80 °C. Glucose, fructose, sucrose and palatinose were used as standard sugars. HPLC analysis of soluble carbohydrate composition of tuber extracts was carried out as previously described (Börnke *et al.*, 2002). The samples extracted with 80% ethanol at 80°C for one hour.

Gel preparation and electrophoresis

All plasmid, restriction digestion and amplified PCR products were loaded onto 1.5 % agarose gel. The purity and concentration of amplified product was checked from the band in agarose gels. Concentration of the DNA was estimated using a 1Kb DNA Ladder.

Statistical analysis

The experiments were three replicates for each treatment, each treatment contain 30 Jars (ten jar for each replicate), and four explants were cultured in Jar. Analysis of variance (ANOVA) was applied to data using Costat Software (2006). The differences among means for all treatments were tested for significance at 5% level by Duncan

(1955). All values are reported as means \pm standard deviation.

III. RESULTS AND DISCUSSION

In vitro regeneration of potato (*Solanum tuberosum* L.) plants.

The study of the effect of growth regulators on the shoot induction of potato cv. Désirée after six weeks is shown in table 1. The highest value of mean number of shoots per explant (39.65) and mean length of shoots per explant (33.5 mm) was recorded on MS medium containing 0.1 mg L⁻¹ IAA and 3.0 mg L⁻¹ ZR and also this treatment had the highest regeneration rates (82 %) after six weeks compared to other treatments (Fig.3 A and B). The mean number of shoots and regeneration percentage was increased in parallel with increasing concentration of ZR up to 3.0 mg L⁻¹ and then mean number of shoots per explant (Fig.3 C and D) while mean length of shoots per explant was decreased under 4 and 5 mg L⁻¹ ZR treatment. Shoots were cut and transferred to the root induction medium containing 1 mg L⁻¹ IBA. After rooting, the plantlets were transferred to greenhouse for acclimatization and grown in soil. These results agree with Gustafson *et al.* (2006) that used NAA in combination with trans-zeatin, the highest mean number of shoots per explant (27.3) and regeneration rate (71%) of *Solanum tuberosum* L. cv. Shepody. Webb *et al.* (1983) investigated the regeneration of shoots from potato leaf discs cultured on different concentrations of NAA and BA. While, Park *et al.* (1995) reported that regenerated shoots from callus developing from edges of leaf explants of four North Dakota potato genotypes on MS medium containing 3.5 mg L⁻¹ IAA and 3.0-4.0 mg L⁻¹ ZR. Also, Gustafson *et al.* (2006) obtained 67% regeneration frequency in stem explants of potato from MS medium supplemented with 0.1 mg L⁻¹ IAA, 0.1 mg L⁻¹ ZR. While, shoot regeneration from leaf petioles explants of commercial cultivars was obtained on MS medium supplemented with 3.0 mg L⁻¹ BA, 2.0 or 0.5 mg L⁻¹ IAA and 1.0 mg L⁻¹ GA3 (Yee *et al.* 2001).

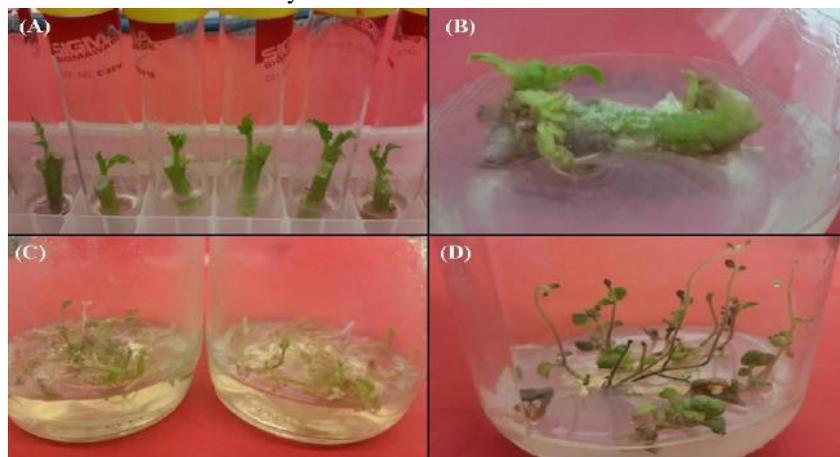


Fig.3: *In vitro* regeneration of shoots from leaf explants of cv. Désirée. Leaf explants were collected from 4 to 6 week-old *in vitro* grown potato (A); shoots regenerated directly from explants on MS medium containing 0.1 mg L⁻¹ IAA and 3.0 mg L⁻¹ ZR (B); Rooting is concomitant with shoot elongation. Some 4-6 weeks after the subculture plantlets (cv. Désirée) have a well development root system (C); Branching by activation of axillary buds also starts, but at higher internodes (D).

Microtuber formation of potato (*Solanum tuberosum* L.) cv. Désirée.

The effect of sucrose on microtuber production was presented in **Table 2**. The results showed that the percentage of plants producing microtuber increased with the increase of sucrose concentrations. The highest number of microtuber was (5.25) detected under 10% sucrose whereas the lowest number was (2.35) at 5% sucrose. Also, the highest average weight of microtuber was (397 mg) observed on 10% sucrose as shown in **Figure 4**. The present investigation was tended to find out the effect of sugar level on microtuberization and found that microtuberization increased with the increasing sugar level and the optimum concentration was 10% sucrose which was similar to observed previously by *Saha et al.* (2013). Potato tubers are modified shoots closely associated with

stolons from which they develop (**Fig. 4**). Tubers and stolons differ by planes of cell division which in stolons promote elongation while, in tubers increase their thickness. Signal that the plant is competent to produce tubers generated in leaves is transmitted to other plant parts by the phloem system. Signal induces a change in the plane of the cell elongation and division. Cell division plates become parallel to the elongation axis of stolons promoting radial growth. At the sub cellular level, transition in the plane of cell divisions is connected with the arrangement of microtubules (*Efstathioset al.*, 2012). Under *in vitro* conditions, the change in the microtubules arrangement in the subapical zone of stolon outgrowth can be observed on the tuberization medium after 50 days (*Sanz et al.*, 1996).

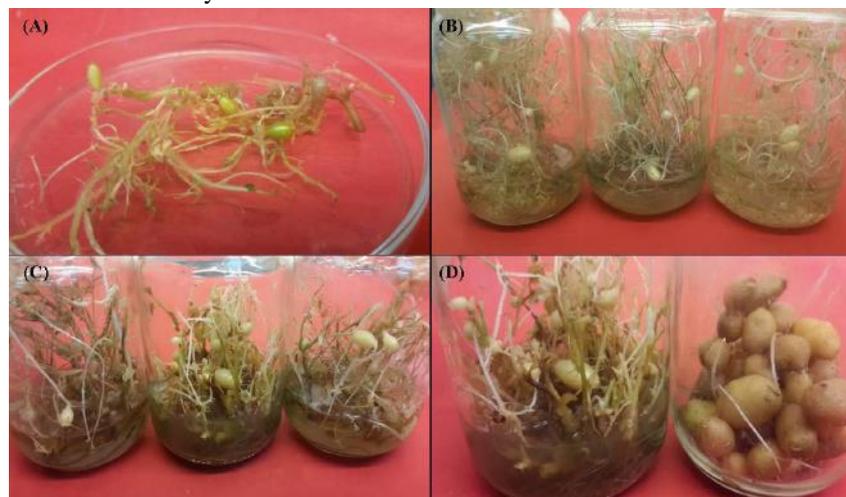


Fig. 4: Microtuber formation of cv. Désirée. Tubers usually form on the tip of stolons, tuber bearing shoots (A); Microtuber formation at 5% sucrose (B); Microtuber formation at 8% sucrose (C); Biggest size of microtuber production under dark condition due to application of MS + 10% sucrose + 1.0 mgL⁻¹BAP and 0.5 mg L⁻¹ kinetin (D).

Table.1: The effect of plant growth regulators on adventitious shoot from leaf of potato (*Solanumtuberosum* L.) cv. Désirée after six weeks.

Growth regulators (mgL ⁻¹)		Mean number of shoots/explant	Mean length of shoots/explant (mm)	Regeneration (%)
ZR	IAA			
1.0	0.05	12.45±0.65 ^k	11.0±0.59 ^j	20
1.0	0.1	19.50±0.87 ^h	12.5±0.46 ⁱ	38
1.0	0.5	23.48±0.93 ^g	17.4±0.63 ^g	45
2.0	0.05	15.47±0.69 ^j	10.2±0.57 ^k	28
2.0	0.1	17.15±1.21 ⁱ	13.5±0.28 ^h	34
2.0	0.5	19.42±0.82 ^h	11.0±0.49 ^j	39
3.0	0.05	27.55±0.96 ^f	19.5±0.92 ^f	65
3.0	0.1	39.65±0.58 ^a	33.5±0.49 ^a	82
3.0	0.5	34.26±0.85 ^b	25.5±0.94 ^b	79
4.0	0.05	29.83±0.79 ^e	20.8±0.85 ^e	68
4.0	0.1	31.75±0.68 ^d	23.6±0.73 ^d	74
4.0	0.5	33.38±0.88 ^c	24.8±0.59 ^c	76

Each treatment had 5 replications (plates) with 10 explants per replication. Values followed by the same letter are not significantly different from each other.

Table.2: Effect of different sucrose concentrations on in vitro microtuber production of potato (*Solanum tuberosum*)cv. Désirée.

Avg. wt. (mg) of microtuber	No. of microtuber per explant	Percent (%) of explant formed microtuber	No. of culture Showing microtuber	No. of explant culture	Sucrose concentration
220±0.95 ^f	2.35±0.34 ^f	40	12	30	5%
253±0.78 ^d	2.60±0.29 ^e	67	20	30	6%
289±0.83 ^a	3.25±0.54 ^d	77	23	30	7%
340±1.03 ^e	3.75±0.42 ^c	80	24	30	8%
365±0.55 ^c	4.25±0.39 ^b	90	27	30	9%
380±0.71 ^b	5.25±0.56 ^a	97	29	30	10%

Each treatment had 5 replications (plates) with 10 explants per replication. Values followed by the same letter are not significantly different from each other.

Expression of sucrose isomerase (*pall*) gene in *E. coli*.

Cloning of the *pall* gene from *Erwinia rhaponticiby* PCR using specific primer; 5'-GGGATCCTCACCGTTCAGCAATCA3' and 5'-GTCGACCTACGGATTAAGTT TATA-3'. The amplified *pall* genes were inserted into the expression plasmid pQE-30 using *Bam*HI and *Sal*I recognition sites into the sequences (underlined), respectively. The recombinant plasmid pQE-30-*pall* was transformed into bacteria (JM 109) and restriction endonuclease digestion with *Bam*H I/*Sal* I (Fig.5). The recombinant expression vector plasmid pQE-30-*pall* extracted from JM 109 and then transformed into *E. coli* XL-1Blue cells. To confirm the gene expression of protein product, the *pall* gene was expressed in *E. coli* under the control of an IPTG-inducible promoter. Enzymatic activity was assayed by incubation of a crude cell extract prepared from the expressor strain with sucrose solution and by subsequent sugar analysis via HPLC. Chromatograms of bacterial extraction indicated the presence of additional peaks in the reaction mixture. In comparison with the standards, the results indicated this major peak of palatinose (Fig. 6). This clearly demonstrates the sucrose isomerase activity of PalI gene to convert sucrose to palatinose. The conversion of sucrose into palatinose drastically affected the sucrose content of *E. coli*. These results were agreed with (Börnke *et al.*, 2001). The appearance of palatinose in the bacterium extract indicated the sucrose isomerase activity of the recombinant PalI protein and the ability of the *pall* gene to convert sucrose to palatinose. Glucose and fructose as by-products of the reaction has been described previously (Cheetham, 1984). He also mentioned that the optimum pH for isomerase activity was between 6.0 and 6.5 and optimum temperature was 30°C, which is in good agreement with the finding that the enzyme is localized to the periplasmic space of *E. rhapontici* cells.



Fig.5: Cloning of the *pall* gene from *Erwinia rhaponticiby* PCR (A). Plasmid pQE-30 digested with *Bam*HI and *Sal*I (B). Lane *pall*: *pall* gene, Lanes (1-13): samples from selected colonies, Lane M: DNA marker (1kb plus DNA ladder).

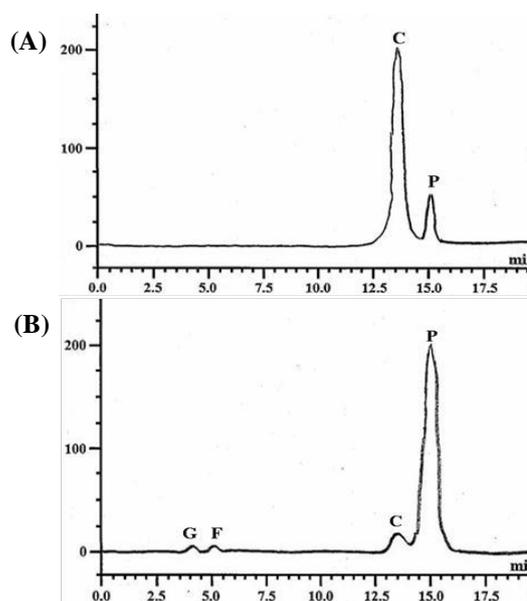


Fig.6: HPLC analysis of the *E. coli* (pQE-30-*pall*) culture supernatant: The palatinose peak was assigned from culture supernatant after 45 min. (A) and after 150 min. (B) comparison to a standard. S; sucrose, P; palatinose, G; glucose, F; fructose.

Transformation of potato (*Solanum tuberosum* L.)cv. Désirée.

Binary vectors pBinAR-pall and pBin33-Kan.

A. tumefaciens colonies transformed with pBinAR-pall or pBin33-Kan were analyzed by colony PCR using *pall* gene-specific primers. The expected band size of 1803 bp was observed in selected colonies. This confirmed the presence of the vector in the colonies. Presence of the pBinAR-pall or pBin33-Kan plasmid was verified in

PCR-positive colonies by digestion with *Asp718/XbaI*. A 1804 bp fragment was released from DNA extracted from the colonies (Fig.7). These results confirm successful cloning of the expression vector pBinAR-pall or pBin33-Kan into *A. tumefaciens*.

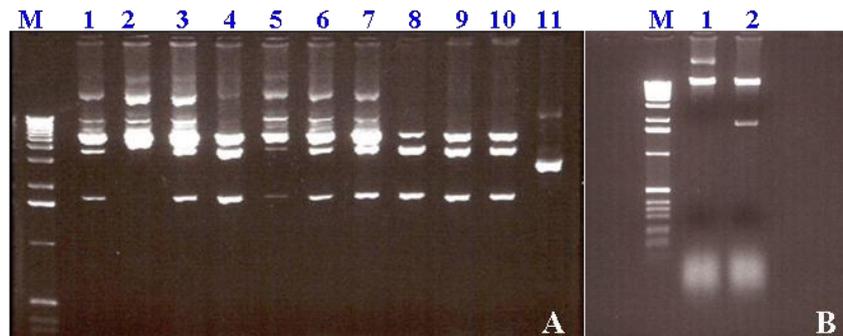


Fig.7: *A. tumefaciens* colonies transformed with pBinAR-pall or pBin33-Kan were analyzed by colony PCR using *pall* gene-specific primers. (A): pBinAR-pall digested with *Asp718/XbaI* to release the vector DNA (upper band) along with the insert DNA (1,803 bp, lower band) and (B): pBin33-Kan digested with *Asp718/XbaI*. Lane M: DNA marker (1kb plus DNA ladder).

Agrobacterium-mediated transformation.

Leaves excised from 4 to 6 week-old *in vitro* grown potato cv. Désirée explants and incubated with *Agrobacterium* strain LBA4404 carrying either the binary vector pBinAR-pall or the binary vector pBin33-Kan for 20 min. Explants were co-cultivated with *Agrobacterium* for 48 h on the MS medium-free of hormones. After co-cultivation in the dark, the explants were transferred to the shoot induction medium containing 0.1 mg L⁻¹ IAA and 3.0 mg L⁻¹ ZR with 300 mg L⁻¹ cefotaxime and 100 mg L⁻¹ kanamycin to select for transformed cells for six weeks. And then cultures were incubated at 25±2°C with a 16/8 h light/dark photoperiod provided by cool-white fluorescent lamps. Shoots were transferred to a rooting medium containing 1 mg L⁻¹ IBA and 50 mg L⁻¹ kanamycin and then the plantlets were transferred to greenhouse and grown in soil. At this time, the transformation efficiency was evaluated for further analysis with *Agrobacterium* harboring the binary vector pBinAR-pall. For *in vitro* tuber formation, the transgenic potato shoots were cultured on MS medium containing 1.0 mg L⁻¹ BAP, 0.5 mg L⁻¹ kinetin, 10% sucrose with 50 mg L⁻¹ kanamycin and then shoots were incubated under dark condition at 25±2.0°C. Transgenic potato plantlets were checked by PCR and Western analyses. Potato leaf discs were transformed using *Agrobacterium*-mediated gene transfer under potato promoter gives rise to tuber-specific expression (Rocha-Sosa *et al.*, 1989). This result is also in accordance with Sarker and Mustafa (2002) where histological *GUS* assay

showed the expression of *GUS* gene in the leaf tissues of transformed shoots. While, recent study, Veale *et al.* (2012) used *In vitro* potato explants were infected with *Agrobacterium* LBA4404 strain harbouring the binary vector pSPUD5 carrying the *cryIIa1* gene under the transcriptional control of the (ocs) promoter and the *ntpII* gene, cultured on the pre-culture medium with 50 µM acetosyringone.

PCR-detection

To confirm the stable transformation in the genome, DNA was isolated using genomic DNA isolation kit (Bio Basic, Canada) from putative transformed plants of high dose (100 mg L⁻¹) kanamycin exposure plants. After transformation with *Agrobacterium* strain LBA4404 carrying either the binary vector pBinAR-pall or the binary vector pBin33-Kan contains sucrose isomerase gene (*pall*) fused to the signal peptide of proteinase inhibitor II gene (SP), isolated DNA was quantified and amplified by PCR using the specific primers. The quality of the plant DNA was confirmed by a positive control PCR reaction. Sixty putatively transformed plants were tested for *pall* gene. PCR analysis revealed that putative potato transformed plants displayed expected 1803 bp size band (size 1694 bp of *pall* gene and size 109 bp of signal peptide of proteinase inhibitor II gene) as shown in (Fig.8). The genomic DNA from non transformed control potato plants did not appear any band in PCR reaction. Transformation efficiency (TE) was calculated as percentage number of PCR positive events compared with

the total number of regenerated plants infected with *A. tumefaciens* carrying the binary vector pBinAR- *pall*. Analysis of variance showed that the highest observed 32.8% against *pall* gene in potato cv. Désirée. This result is in agreement with the findings of [Beaujean et al. \(1998\)](#), [Gustafson et al. \(2006\)](#) and [Molla et al. \(2011\)](#).

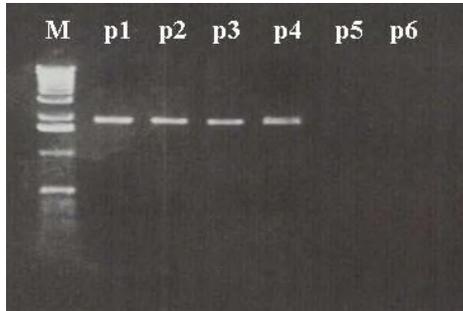


Fig.8: PCR analysis revealed that putative potato transformed plants displayed expected 1803 bp size band (size 1694 bp of *pall* gene and size 109 bp of signal peptide of proteinase inhibitor II gene). Lane M: DNA marker (1kb plus DNA ladder), Lanes (p1-p4): transformed potato plants, Lanes (p5-p6): nontransformed plants.

shoots were screened for expression of *pall* protein *in vitro* plantlets by Western Blotting assay using a polyclonal antibody raised examined leaves of the *pall*-expressing potato. Total proteins were descriptively isolated by SDS-PAGE and blotted the protein with antibodies specific for *pall*-proteins. Our results showed that *pall* protein was expressed in all samples of transgenic potato plants compared to the nontransgenic potato control. This is in contrast to results obtained with constitutive expression of *pall* in transgenic potato plants. The detection of *pall* protein can be performed using any part of potato plantlets, which was expressed mainly under the regulation of the CaMV-35S promoter. In addition the antibody obtained will provide a basic detector of genetically modified potato plants. [Rui-juan et al. \(2016\)](#) used western blot assay for detection PMI protein in genetically modified rice and showed that PMI was expressed in all samples except anther, indicating the constitutive expression of PMI in genetically engineered rice.

HPLC analysis

Sucrose isomerase expression under the control of the tuber-specific patatin class I B33 promoter leads to *in vivo* conversion of sucrose into palatinose, tuber extracts from potato cv. Désirée were analyzed for their soluble carbohydrate composition using HPLC. The chromatograms assay indicated that there was an additional major peak that was not present in the control and compared to the standard this peak could be set to palatinose (Fig.9). An additional minor peak eluted close to the sucrose signal in extracts from transgenic tubers. Quantitative analysis of non-structural carbohydrates of transgenic tubers has shown accumulation of palatinose in the range of 2.4 mol g⁻¹ FW to 19.5 mol g⁻¹ FW while, sucrose and glucose content was only 1.4 mol g⁻¹ FW and 0.48 mol g⁻¹ FW, respectively, whereas the non-transgenic tubers contained 16.8 mol g⁻¹ FW sucrose and 4.85 mol g⁻¹ FW glucose. The conversion of sucrose into palatinose drastically affected the sucrose and glucose content of transgenic tubers. These results indicate almost quantitative conversion of sucrose into palatinose via *pall* expressing potato tubers. These results are compatible with [Rocha-Sosa et al. \(1989\)](#), they have the sucrose isomerase gene expression under the control of the tuber-specific patatin class I B33 promoter in transgenic potato plants. This protein was combined with the signal peptide of the proteinase inhibitor II, which governs secretion of the enzyme into the apoplasmic space ([Von Schaeuwen et al., 1990](#)). The patatin class I B33 promoter it seems inactive during early tuberization ([Taubberger et al., 1999](#)). The apoplasmic localization of the sucrose isomerase leads to accumulation of palatinose. This indicates the presence of sucrose within the apoplast even in later stages of tuber development which have been interpreted as a result of

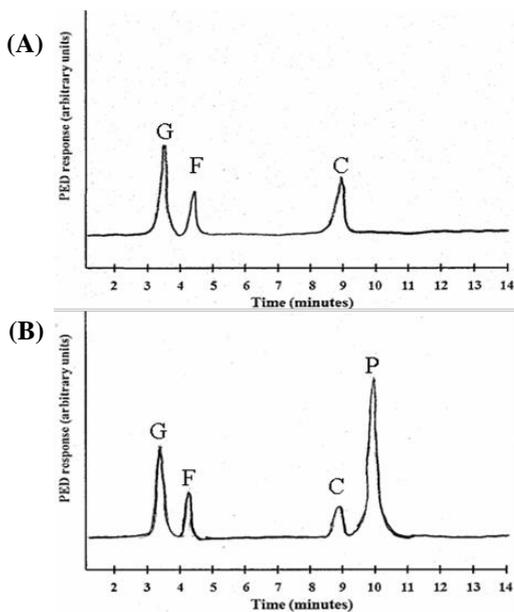


Fig.9: HPLC analysis of soluble carbohydrates of tuber extracts of potato cv. Désirée (A) tuber extract from nontransgenic plants, and (B) tuber extract from transgenic plants. S; sucrose, P; palatinose, G; glucose, F; fructose, PED; pulsed electrochemical detector.

Expression of sucrose isomerase (*pall*) gene in transgenic potato plants.

Potato transgenic plants were transformed with *Agrobacterium* harbouring recombinant binary vector plasmid pBinAR- *pall* contains sucrose isomerase gene (*pall*) fused to proteinase inhibitor II signal sequence (SP) under CaMV-35S promoter and Octopine synthase (*OCS*) terminator ([Börnke et al., 2002](#)). 60 regenerated potato

leakage of recipient receptor cells rather than an obligatory step in phloem unloading (Oparka *et al.*, 1992). This is consistent with the results obtained from transgenic potato tubers expressing yeast invertase within apoplast where a reduction in sucrose content was observed accompanied by increased palatinose content (Sonnewald *et al.*, 1997; Hajirezaei *et al.*, 2000).

IV. CONCLUSION

The results demonstrate that *in vitro* regeneration on leaf explants cv. Désirée, in a single step regeneration procedure on MS medium supplemented with 0.1 mg L⁻¹ IAA and 3.0 mg L⁻¹ ZR and shoots regenerated directly from explants without an intervening callus stage. Microtuber production formed on MS medium containing 10% sucrose with 1.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ kinetin under dark condition. Also, we were able to genetically modified potato plants to produce non-cariogenic, low-calorie sucrose isomer palatinose. Palatinose production was achieved by expression of a sucrose isomerase isolated from *Erwinia rhapontici*. Sucrose isomerase overexpression construct contains the coding region of the *pall* gene fused to proteinase inhibitor II signal sequence under CaMV-35S promoter of screening for expression of *pall* protein *in vitro* plantlets by Western Blotting using a polyclonal antibody also, our use tuber-specific patatin class I B33 promoter leads to *in vivo* conversion of sucrose into palatinose with potato cv. Désirée tubers, tuber extracts from potato were analyzed for their soluble carbohydrate composition using HPLC.

ACKNOWLEDGEMENTS

The authors are thankful to the Tissue culture and Biotechnology Labs., Marout Research Station, Desert Research Center, Egypt.

REFERENCES

- [1] Beaujean, A., Sangwan, R.S., Lecardonne, A. and Sangwan-Norree, B.S. 1998. *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced internodal explants: an efficient protocol of transformation. *J Exp Bot* 49:1589-1595.
- [2] Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* 12: 8711-8721.
- [3] Börnke, F., M. Hajirezaei, and U. Sonnewald. 2001. Cloning and characterization of the gene cluster for palatinose metabolism from the phytopathogenic bacterium *Erwinia rhapontici*. *J. Bacteriol.* 183:2425–2430.
- [4] Börnke, F., M. Hajirezaei, and U. Sonnewald. 2002. Potato tubers as bioreactors for palatinose production. *J Biotechnol.*,96 (1):119-24.
- [5] Cheetham, P.S.J., 1984. The extraction of a novel isomaltulose-synthesizing enzyme from *Erwinia rhapontici*. *Biochem. J.* 220, 213–220.
- [6] Chilton, M.D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P., Nester, E.W. 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc Natl Acad Sci USA* 71: 3672–3676.
- [7] Duncan, D.B. 1955. Multiple range and multiple F tests. *Biometrics* 1:1-42.
- [8] Efstathios, R., Bjorn, K., Marian, O., Wouter, K., Harro, J. B., Richard, G.F., Christian, W.B. 2012. The effects of auxin and strigolactones on tuber initiation and stolon architecture in potato. *Journal of Experimental Botany*, Vol. 63, No. 12, pp. 4539–4548. doi:10.1093/jxb/ers132.
- [9] Goda, T., Hosoya, N. 1983. Hydrolysis of palatinose by rat intestinal sucrose-isomaltase complex. *J Japanese Soc. Nutr. Food Sci.*, 36: 169-173.
- [10] Gustafson, V., Mallubhotla, S., MacDonnell, J., Sanyal-Bagchi, M., Chakravarty, B. Wang-Pruski, G., Rothwell, C., Audy, P., DeKoeper, D., Siahbazi, M. and Regan, S. 2006. Transformation and plant regeneration from leaf explants of *Solanum tuberosum* L. cv. ‘Shepody’ *Plant Cell, Tissue and Organ Culture*, 85: 361–366.
- [11] Hajirezaei, M.R., Takahata, Y., Trethewey, R.N., Willmitzer, L., Sonnewald, U., 2000. Impact of elevated cytosolic and apoplastic invertase activity on carbon metabolism during potato tuber development. *J. Exp. Bot.* 51, 439–445.
- [12] Höfgen, R., Willmitzer, L. 1990. Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum*). *Plant Sci.* 66, 221–230.
- [13] Joseph, N., Anbazhagan, M. and Srinivasan, S. (2015). *In vitro* growth of potato plant (*in vitro* tuberization) *INT J CURR SCI* 2015, 17: E 29-36.
- [14] Keil, M., Sanchez-Serrano, J., Schell, J. and Willmitzer, L. 1986. Primary structure of a proteinase inhibitor II gene from potato. *Nucleic Acids Res.* 14: 5641-5650.
- [15] Lina, B. A. R., D. Jonker, and G. Koziarnowski. 2002. Isomaltulose (Palatinose): a review of biological and toxicological studies. *Food Chem. Toxicol.* 40:1375–1381.
- [16] Minami, T., Fujiwara, T., Ooshima, T., Nakajima, Y. and Hamada, S. 1990. Interaction of structural isomers of sucrose in the reaction between sucrose and glucosyltransferases from mutans streptococci. *Oral Microbiol. Immunol.* 5:189-194.
- [17] Molla, M.M.H., Nasiruddin, K.M., Al-Amin, M., Haque, M.S. and Maniruzzaman (2011). *Agrobacterium*-mediated Transformation in

- Potato. *Thai Journal of Agricultural Science*, 44(2): 93-102.
- [18] Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plantarum* 15, 473–497.
- [19] Oparka, K.J., Viola, R., Wright, K.M., Prior, D.A.M., 1992. Sugar transport and metabolism in the potato tuber. In: Farrar, J.F., Gordon, A.J., Pollock, C.J. (Eds.), *Carbon Partitioning Within and Between Organisms*. BIOS Scientific Publishers, Oxford, pp. 91–114.
- [20] Park, Y.D., Ronis D.H., Boe, A.A., Cheng, Z.M. 1995. Plant regeneration from leaf tissues of four North Dakota genotypes of potato (*Solanum tuberosum* L.). *Journal* 72, 329-338.
- [21] Price, J.M., Biava, C.G., Oser, B.L., Vogin, E.E., Steinfield, J. and Ley, H.L. 1970. 'Bladder tumors in rats fed cyclohexylamine or high doses of a mixture of cyclamate and saccharin'. *Science* 167, 1131-1132. DOI: 10.1126/science.167.3921.1131.
- [22] Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratmann, M., Schell, J., Willmitzer, L., 1989. Both developmental and metabolic signals activate the promoter of a class I patatin gene. *EMBO J.* 8, 23–29.
- [23] Rui-juan, R., Peng-cheng, W.U., Jin-ping, L.A.N., Han-fu, W.E.I., Jian, W.E.I., Hao CHEN, Jianan SHI, Yu-jie, H.A.O, Li-juan, L.I.U, Shi-juan DOU, Li-yun LI, Lin, W.U., Si-qi, L.I.U, Chang-cheng, Y.I.N., Guo-zhen, L.I.U. 2016. Western blot detection of PMI protein in transgenic rice. *Journal of Integrative Agriculture*, 15(4): 726–734.
- [24] Saha, D., Rana, R.S., Sureja, A.K., Verma, M., Arya, L., Munshi, A.D. 2013. Cloning and characterization of NBS-LRR encoding resistance gene candidates from Tomato Leaf Curl New Delhi Virus resistant genotype of *Luffa cylindrical* Roem. *Physiol Mol Plant Pathol.*, 81:107–117.
- [25] Sanz, M.J., Mingo-Castel, A., Van Lamieren, A.A.M., Vreugdenhill, D 1996. Changes in the microtubular cytoskeleton precede in Vitro tuber formation in potato. *Protoplasma* 191, 46-54.
- [26] Sarker, R.H., Mustafa, B.M. 2002. Regeneration and *Agrobacterium*-mediated genetic transformation of two indigenous Potato varieties of Bangladesh. *Plant Tissue Culture*, 12(1): 69-77.
- [27] Sonnewald, U., Hajirezaei, M.R., Kossmann, J., Heyer, A., Trethewey, R.N., Willmitzer, L., 1997. Increased potato tuber size resulting from apoplastic expression of a yeast invertase. *Nat. Biotech.* 15, 794–797.
- [28] Takazoe, I. 1989. Palatinose—an isomeric alternative to sucrose, p. 143–167. In T. H. Grenby (ed.), *Progress in sweeteners*. Elsevier, Barking, United Kingdom.
- [29] Tauberger, E., Hoffman-Benning, S., Fleischer-Notter, H., Willmitzer, L., Fisahn, J., 1999. Impact of invertase over-expression on cell size, starch granule formation and cell wall properties during tuber development in potatoes with modified carbon allocation patterns. *J. Exp. Bot.* 50, 477–486.
- [30] Veale, M. A., Slabbert, M.M. and Van Emmenes, L. 2012. *Agrobacterium*-mediated transformation of potato cv. Mnandi for resistance to the potato tuber moth (*Phthorimaea operculella*). *South African journal of Botany* Vol. 80: 67-74.
- [31] Von Schaewen, A., Stitt, M., Schmidt, R., Sonnewald, U., Willmitzer, L., 1990. Expression of a yeast derived invertase in the cell wall of tobacco and Arabidopsis plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic plants. *EMBO J.* 9, 3033–3044.
- [32] Watzlawick, H., Mattes, R. 2009. Gene cloning, protein characterization, and alteration of product selectivity for the trehalulose hydrolase and trehalulose synthase from *Pseudomonas mesoacidophila*, MX-45. *Appl. Environ. Microbiol.* 75, 7026–7036.
- [33] Webb, K.J., Osifo, E.O. and Henshaw, G.G. 1983. Shoot regeneration from leaflet discs of six cultivars of potato (*Solanum tuberosum* subsp, *tuberosum*). *Plant Science Letters* 30: 1-8.
- [34] Xuguo, D., Sheng, C., Yixin, A., Jing, W. 2016. Enhancing the Thermostability of *Serratia plymuthica* Sucrose Isomerase Using B-Factor-Directed Mutagenesis. *journal.pone.* 11(2): 1-16. doi: [10.1371/journal.pone.0149208](https://doi.org/10.1371/journal.pone.0149208).
- [35] Yee, S., Stevens, B., Coleman, S., Seabrook J.E.A., Li, X.Q. 2001. High efficiency regeneration *in vitro* from potato petioles with intact leaflets. *American journal of potato research* 78, 151-157.
- [36] Zhang, D.H., Li, N., Lok, S.M., Zhang, L.H. and Swaminathan, K. 2003. Isomaltulose synthase (PalI) of *Klebsiella* sp. LX3 – Crystal structure and implication of mechanism. *J. Biol. Chem.* 278, 35428–35434.