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

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***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-alpha-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*(*palI*) 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- palI construct wassucceffuly transformed and expression into* E. coli. *Sucrose isomerase* (*palI*) gene *was cloned and overexpressed into a plant expression vector* pBinAR-*palI contains sucrose isomerase gene (palI) 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 palI 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 potatocv. Désiréewere 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; palI: *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 (*PalI*), 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 diabeticsand 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 onthe cloning and characterization of a bacterial sucrose isomerase (*palI*) 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 (*palI*) gene within the apoplasm 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 cloningand characterization of a chimeric sucrose isomerase (*palI*) gene from *Erwinia rhapontici* and introduced palI gene with different promoter into potato plants were described.In addition to, Expression of the *palI* gene which conversion of sucrose into palatinose within the apoplast of transgenic tubers was studies.

## II. MATERIALS AND METHODS

**Isolation and cloning of sucrose isomerase gene.**

The coding region of the sucrose isomerase (*palI*) 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 (Keil*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 *BamHI/SalI* respectively. The digested products were separated using agarose electrophoresis and the bands were extracted. The purified sucrose isomerase gene (*palI*) 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 (Luratia Broth) LB agar plates containing ampicillin (100$\mu$g/mL$^{-1}$). The plates were incubated for 16h at 37°C. The desired recombinant plasmid pQE-30- *palI* was confirmed by PCR and restriction enzyme digestion with *BamHI/SalI* and DNA sequencing (Invitrogen).

Construction of the sucrose isomerase overexpression construct the coding region of the *palI* gene, ranges from codons 109 to 1,803bp was amplified from *Erwinia rhapontici* by PCR. The *palI* 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-palI was maintained on LB medium (Chilton *etal*.,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).

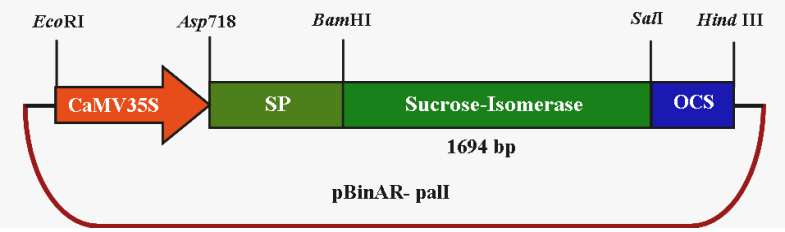


*Fig.1: Structure of palI expression construct used to transformed potato. The palI coding region ranging from 109 to 1,803bp was inserted between the CaMV-35S promoter and* OCS *terminating region of vector pBinAR using BamHI and Sal I restriction sites, respectively. The signal sequence for the proteinase inhibitor II (SP) was inserted in front of the palI 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 vectorby EcoR I and Asp718. This resulted in the plant expression vector pBin33-Kan.The palI gene was introduced in the sense orientation into the plant transformation vector pBin33-Kan by *Asp*718/*Xba*I between the patatin B33 promotor and the octopine synthase polyadenylation signal (Fig. 2).

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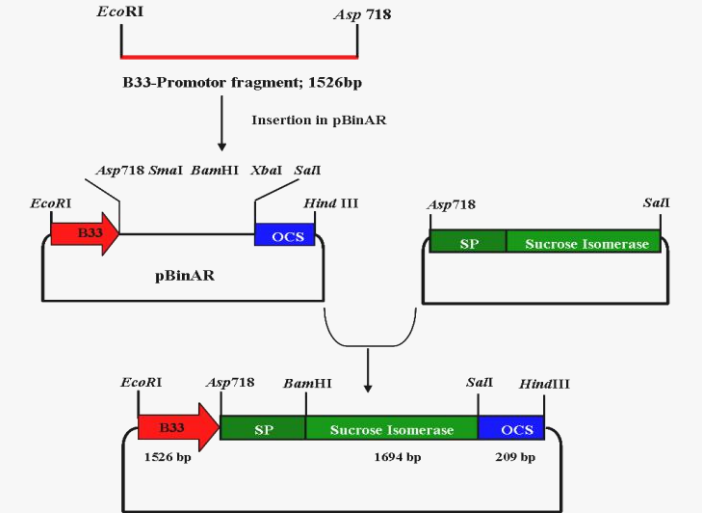


*Fig.2:Schematic representation of the construct pBin33, which mediates the expression of sucrose Isomerase from E. rhapontici of transgenic Potato tubers. The gene for the Sucrose-Isomerase, merged with the signal peptide of the proteinase inhibitor I gene (Schaewen et al., 1990), the tubers-specific 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 pBin33was then used for the production of transgenic potato plants.*

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

Leaf explants were collected from 4 to 6 week-old *in vitro* grown potato (*Solanumtuberosum* L.)cv. Désiréeand cultured on MS (Murashige and Skoog, 1962) medium supplemented with 100 mg $L^{-1}$, myo-inositol, 30 g $L^{-1}$ sucroseand different growth regulators IAA(0.05, 0.1, 0.5 mg $L^{-1}$)in combination with zeatinriboside (1.0, 2.0, 3.0, 4.0, 0.5 mg $L^{-1}$) for six weeksfor 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 $m^{-2}s^{-1}$). The shoots regenerated were cultured MS medium containing 100 mg/l, myo-inositol, 1.0 mg $L^{-1}$ BAP, 0.5 mg/l kinetin and 2.5 g $L^{-1}$ phytagel 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 wereharvested after eight weeks of incubation. Data wererecorded 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-palI or the binary vector pBin33-Kan for 20 min. Afterinfection, 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 with100 mg $L^{-1}$ kanamycin and 300 mg $L^{-1}$ cefotaxime at 25±2.0°C under darkfor 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 *palI* gene in *E. coli*.**

*E. coli* XL-1Blue cells were transformed with recombinant plasmid pQE-30- *palI* extracted from JM 109. The bacteria cells were grown in 5 ml LB liquid medium at 37°C with 100 $\mu$g/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 at37°C. These samples were harvested by centrifugation at 13.000 rpm for 1min. 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 *palI* 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 *palI* 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 (*palI*) gene subcloned in expression vector pQE-30 which resulted pQE- *palI* and introduced into *E. coli* XL-1Blue. The protein over expressed in *E. coli* was purified to form antibodies against sucrose isomerase (*palI*)by immunization rabbit. The plant proteins were separated by SDS-PAGE and transferred to the porablot and incubated with antibodies against the *palI* protein from transgenic potato plants.

**HPLC analysis**

Reaction products were filtered through 0.22 µm membrane filters before HPLC analysis (Agilent 1200, USA system equipped with a refractive index detector). The samples were diluted 10-fold and 20 µl 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 loadedonto 1.5 % agarose gel. The purity and concentration of amplified product waschecked from the band in agarose gels. Concentration of the DNA wasestimated 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 ± standard deviation.

## III. RESULTS AND DISCUSSION

***In vitro* regeneration ofpotato (***Solanum tuberosum* L.) **plants**.

The study of the effect of growth regulators on the shoot induction of potato cv. Désiréeafter 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 1mg $L^{-1}$IBA. After rooting, the plantlets were transferred to greenhouse for acclimization and grown in soil. These results agreement 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 3.5mg $L^{-1}$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 MSmedium 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, 2.0or 0.5mg $L^{-1}$IAA and 1.0 mg $L^{-1}$ GA3 (Yee *et al*. 2001).

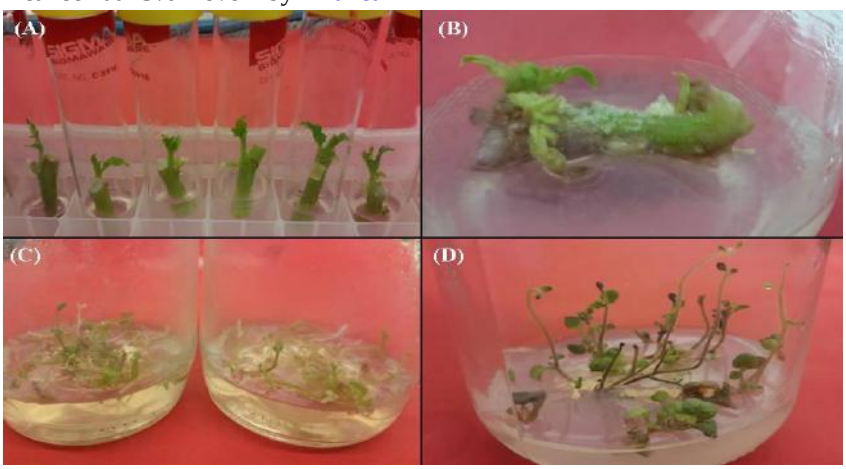


*Fig.3:In vitro regeneration of shoots from leaf explants of cv. Désirée.Leaf explants were collected from 4 to 6 week-old in vitro grown potato (A); shoots regenerated directly from explants on MS medium containing 0.1 mg $L^{-1}$ IAA and 3.0 mg $L^{-1}$ ZR (B); Rooting is concomitant with shoot elongation.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

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**Microtuber formation ofpotato (***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 (Efstathios*et 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).

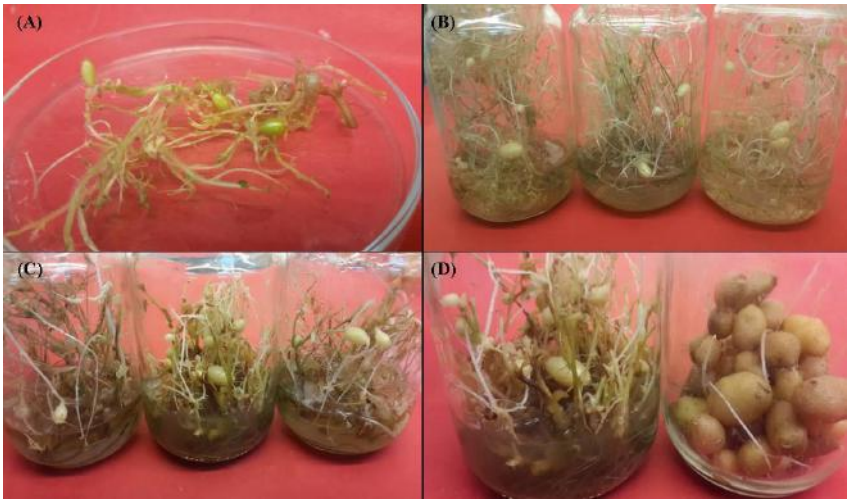


*Fig. 4: Microtuber formation of cv. Désirée. Tubers usually form on the tip of stolons, tuber bearing shoots (A); Microtuber formation at 5% sucrose (B); Microtuber formation at 8% sucrose (C); Biggest size of microtuber production under dark condition due to application of MS + 10% sucrose + 1.0 mgL$^{-1}$BAP 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 (*palI*) genein *E. coli*.**

Cloning of the *palI* genefrom *Erwinia rhapontici*by PCR using specific primer; 5′-GGGATCCTCACCGTTCAGCAATCA3′ and 5′-GTCGACCTACGGATTAAGTT TATA-3′. The amplified*palI* genewas inserted into the expression plasmid pQE-30using *Bam*HI and *Sal*I recognition sites into the sequences (underlined), respectively. The recombinant plasmid pQE-30- *palI* 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- *palI* extracted from JM 109 and then transformed into *E. coli* XL-1Blue cells.To conform thegene expression of protein product, the *palI* gene was expressed in *E. coli* under the control of an IPTG-inducible promoter.Enzymatic activity was assayed by incubation ofa crude cell extract prepared from the expressor strain withsucrose 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 sucroseisomerase activity of the recombinant PalI proteinand the ability of the palI 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.

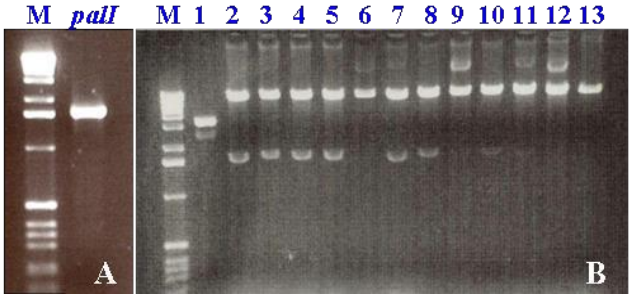


*Fig.5: Cloning of the palI genefrom Erwinia rhaponticiby PCR (A) .Plasmid pQE-30 digested withBamHI and Sal (B). Lane palI: palI gene, Lanes (1-13): samples from selected colonies, Lane M: DNA marker (1kb plus DNA ladder).*

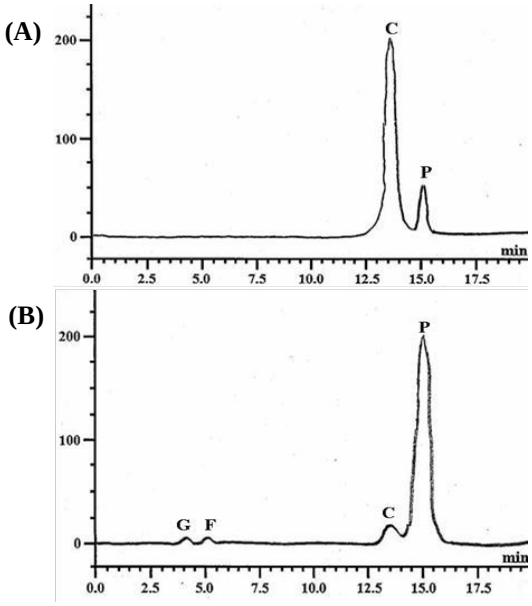


*Fig.6: HPLC analysis of the E. coli (pQE-30- palI) culture supernatant: The palatinose peak was assigned from culture supernatant after 45 min. (A) and after 150 min. (B) comparison to a standard.S; sucrose, P; palatinose, G; glucose, F; fructose.*

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

**Binary vectorspBinAR-palIand pBin33-Kan.**

*A. tumefaciens* colonies transformed with pBinAR-palIor pBin33-Kan wereanalyzed by colony PCR using palIgene-specific primers. The expectedband size of 1803 bp was observed in selected colonies. This confirmed the presence of the vector in the colonies.Presence of the pBinAR-palIor pBin33-Kan plasmid was verified in PCRpositive colonies by digestion with *Asp*718/*Xba*I. A 1804 bp fragment wasreleased from DNA extracted from the colonies (Fig.7). These results confirm successful cloning of the expression vector pBinAR-palIor pBin33-Kan into *A. tumefaciens*.

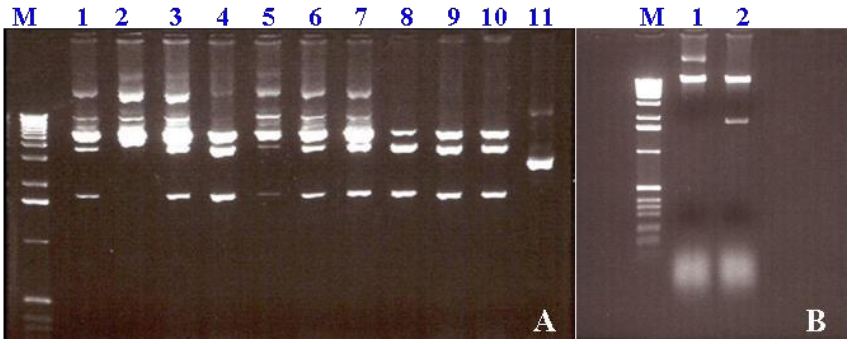


*Fig.7: A. tumefaciens colonies transformed with* pBinAR-*palIor pBin33-Kan wereanalyzed by colony PCR using palIgene-specific primers. (A):* pBinAR-*palI digested with* Asp*718/*Xba*I to release the vector DNA (upper band) along with the insert DNA (1,803 bp, lower band) and (B): pBin33-Kan digested with* Asp*718/*Xba*I. Lane M: DNA marker (1kb plus DNA ladder).*

***Agrobacterium*-mediated transformation**.

Leaves excised from 4 to 6 week-old *in vitro*grown potatocv. Désiréeas explants and incubated with *Agrobacterium* strain LBA4404 carrying either the binary vector pBinAR-palI 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 1mg $L^{-1}$ 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-palI. 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 patatin 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 *cry1Ia1* gene under the transcriptional control of the (ocs) promoter and the *nptII* 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- *palI* or the binary vector pBin33-Kan contains sucrose isomerase gene (*palI*) 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 *palI* gene. PCR analysis revealed that putative potato transformed plants displayed expected 1803 bp size band (size 1694 bp of *palI* 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- *palI*. Analysis of variance showed that the highest observed 32.8% against *palI* gene in potatocv. Désirée. This result is in agreement with the findings of Beaujean *et al*. (1998), Gustafson *et al*. (2006) and Molla *et al*. (2011).

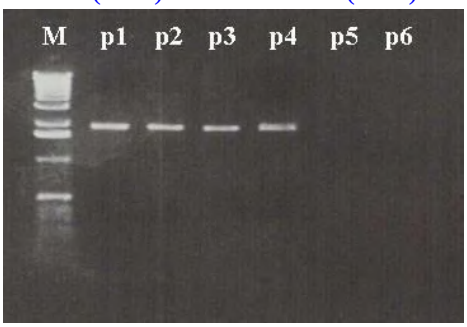


*Fig.8: PCR analysis revealed that putative potato transformed plants displayed expected 1803 bp size band (size 1694 bp of palI gene and size 109 bp of signal peptide of proteinase inhibitor II gene). Lane M: DNA marker (1kb plus DNA ladder), Lanes (p1-p4): transformed potato plants, Lanes (p5-p6):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

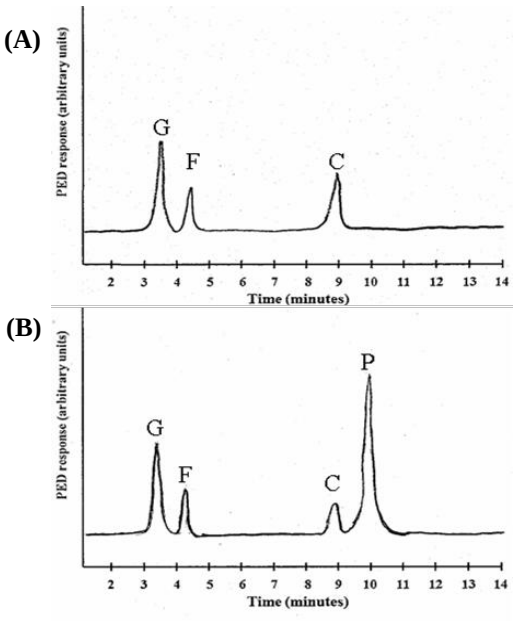


*Fig.9: HPLC analysis of soluble carbohydrates of tuber extracts of potato cv. Désirée (A) tuber extract from nontransgeic plants, and (B) tuber extract from transgenic plants. S; sucrose, P; palatinose, G; glucose, F; fructose, PED; pulsed electrochemical detector.*

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

Potato transgenic plants were transformed with *Agrobacterium*harbouring recombinant binary vector plasmid pBinAR- *palI* contains sucrose isomerase gene (*palI*) fused to proteinase inhibitor II signal sequence (SP) under CaMV-35S promoter and Octopine synthase (*OCS*) terminator (Börnke *et al*., 2002). 60 regenerated potato shoots were screened for expression of *palI* protein *in vitro* plantlets by Western Blotting assay using a polyclonal antibody raised examined leaves of the palI-expressing potato. Total proteins were descriptively isolated by SDS-PAGE and blotted the protein with antibodies specific for *palI*-proteins. Our results showed that palI 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 *palI* in transgenic potato plants. The detection of *palI*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 potatocv. Désiréewere 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 *palI* expressing potato tubers. These results are compatible withRocha-Sosa *etal*. (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 Schaewen *et al*., 1990).The patatin class I B33 promoter it seems inactive during early tuberization (Tauberger *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

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 *palI* gene fused to proteinase inhibitor II signal sequence under CaMV-35S promoterof screening for expression of *palI* protein *in vitro* plantlets by Western Blotting using a polyclonal antibodyalso, 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 potatowere analyzed for their soluble carbohydrate composition using HPLC.

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