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Synthesize a transfer vector containing the gene encoding ADP-glucose pyrophosphorylase enzyme to improve the starch content in *Manihot esculenta* Crantz

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Abstract

Starch is polysaccharide which plays important role in the metabolism of living organisms. In addition, starch is raw materials in the food and materials industry. Increasing the starch content of crops based on improving the activity of some genes encoding enzymes plays an important role in the process of biosynthesis and accumulation of starch, which is one of the research are of interest. Like in bacteria, the process of starch biosynthesis in plants occurs by using ADP-Glc as raw materials to made long link chain α-1.4-glucoside. ADP-Glcpyrophosphorylase (AGPase) has an important role in the biosynthesis of ADP-Glc molecule, and therefore AGPase is a critical enzyme of glycogen synthesis in bacteria and starch in plants. In this study, the gene encoding for AGPase enzyme (symbol Lan1_opt), size 1.5 kb originating from the glgC gene coding for the enzyme AGPase of E. coli has been optimised. This gene had G336 mutation replacing aspartic acid by glycin in order to decrease the affinity of the inhibitor. It has been combined with gene transfer plant vector and was transformed into tobacco plants, operated under the control of a 35S promoter. The integration of the Lan1_opt gene into plant genome was confirmed by PCR technique showing the expression of AGPase proteins in the genomic tobacco plants. Moreover, AGPase enzyme activity in leaves of this genomic tobacco plants was higher than 21%-43% compared with the AGPase enzyme activity in leaves of tobacco plants control. Our data provided the gene encoding AGPase (Lan1_opt) was an effective strategy to strength the process of starch biosynthesis in plants.

Keywords: Transfer vector, gene encoding, ADP-glucose pyrophosphorylase enzyme, *Manihot esculenta* Crantz

Introduction

Cassava (Manihot esculenta Crantz) is the root crops that is growing in tropical regions of Africa, Asia and Latin America, provide food for 500 million people[1]. Beside, cassava is used for many different purposes in industrial starch, such as cosmetics, pharmaceuticals, especially biofuels[2]. Along with economic development, the amount of starch needed for the industry is increasing remarkably in developing countries. To meet the starch used, the scientists have applied for technical scientific advances mainly transgenic techniques in plant breeding. The synthesis of starch in plants needed 3 enzymes are ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.23), starch synthase (SS; EC 2.4.1.21) and starch branching enzyme (SBE; EC 2.4.1.28)[3]. To improve starch content, we can enhance the activity of three enzymes. The rate of starch synthesis is determined mainly by the activity of AGPase, the first enzyme in the pathway[4]. AGPase catalyses the first step in the biosynthesis of starch in the plastids by converting Glc1P and ATP into ADPGlc and PPI[5]. The enzyme AGPase has been successfully transferred into a number of crops such as cassava[6], potatoes[1,7,8], wheat[9], rice[9,10]. In this research, we designed a vector carrying AGPase gene for control the synthesis of starch in cassava and firstly were expressed in tobacco plants. The result is a prerequisite for transferring AGPase genes into cassava.

Materials and methods

Nicotiana tobacco K326 cultivars was supported by Plant Resources Center, the Institute of Agricultural Science Vietnam.

Vector cloning pUCIDT-AMP contain the gene encoding the enzyme carries AGPase (named Lan1_opt) were synthetically by the company IDT (Integrated DNA Technologies, USA).

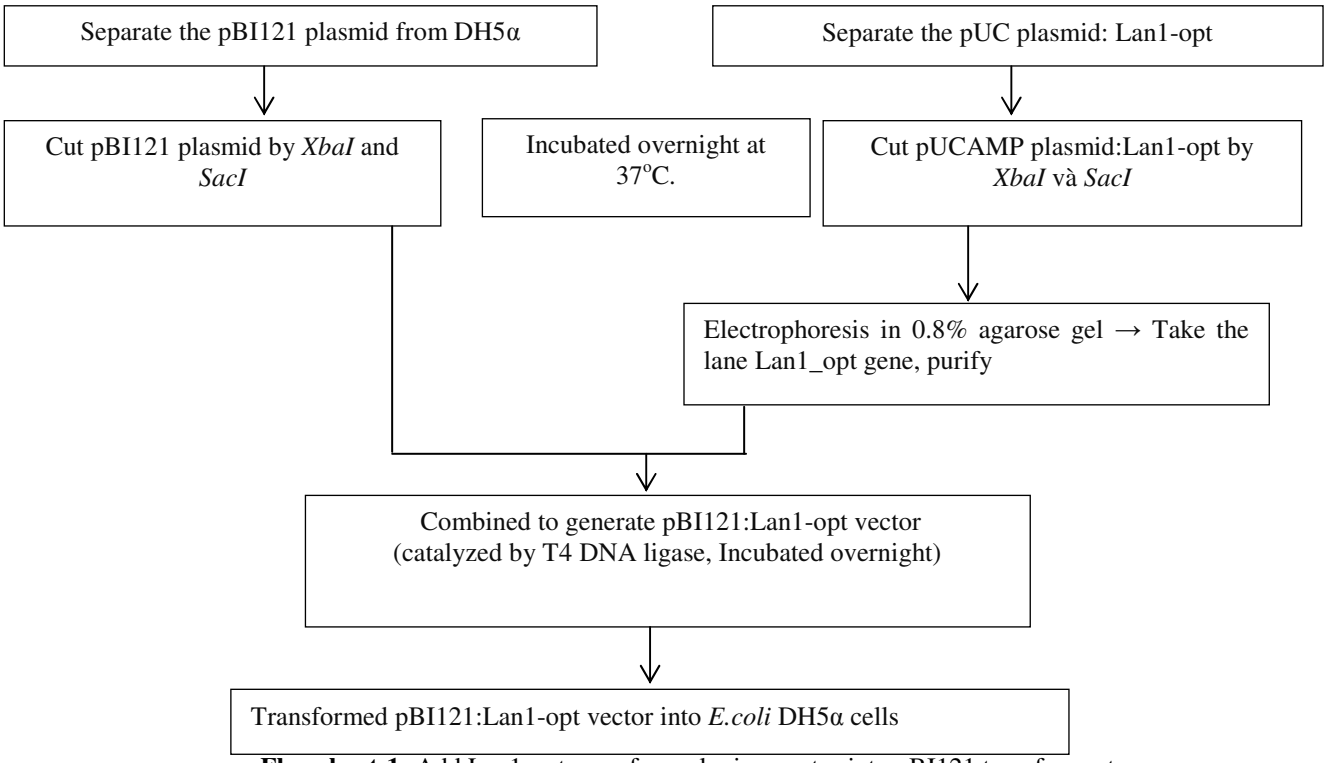
Plant transformation vectors Vector pBI121, E. coli (DH5α) and Agrobacterium tumefaciens CV58C1 strains were supported by Plant Resources Center, the Institute of Agricultural Science Vietnam.

Taq-polymerase, PCR buffer, primer were purchased from Invitrogen; EDTA, SDS, Trypton, Tris-HCl, glycerol, NaCl, ethanol, restriction enzyme, kit plasmid, bacto peptone, yeast extract were purchased from Fermentas, South Korea. The antibiotic kanamycin, rifampicin, cefotaxime, carbenicillin were purchased from Merck.

Synthetized genes encoding AGPase enzyme: Based on the genetic structure of glgC (code NC_000913.3) encoding AGPase enzymes in E. coli has been published in GenBank and characteristics of the AGPase enzyme in plants, we modified the sequence of the glgC gene by creating the G336D mutation.

Nucleotide segment encodes for signal peptide chain protein in chloroplasts are derived from the rbcS gene encoding the small section of ribulose 1.5 bisphosphate carboxylase enzyme in Arabidopsis is attached to the 5' position, and the sequence coding for the tail of c-myc and KDEL is attached to the 3' position of the DNA segment. Recognised location of XbaI restriction enzyme is attached on5' position and the recognised location of SacI is added on 3' position of the gene. AGPase encoding gene (Lan1_opt) is purchased from Integrated DNA Technologies (USA) and is cloned in the vector pUCIDT-AMP.

Design transgenic plant vector carrying pBI121 structure: Lan1_opt: Lan1_opt target gene was amplified by transforming the vector pUCAMP: Lan1_opt into E. coli DH5α.



Flowchart-1: Add Lan1-opt gene from cloning vector into pBI121 transfer vector.

Table-1: Ingredients of culture medium *E.coli* after transformation vector carrying the target gene LAN1-opt to gene amplification.

Sequence number	Ingredient	Volume
1	LB buffer	20 ml
2	Xgal (40mg/ml)	20µl
3	IPTG (100mM)	20µl
4	Carbe (Ampi) (50mg/ml)	20µl
	Total volume	20060 µl

Cloning: Choose 3 white colonies. Cultured in liquid LB (1 colony + 5ml LB + 5µlCarbe / tube), overnight. Cut plasmid and checked gene target LAN1-opt.

Table-2: Ingredients reaction for cutting plasmid *pUCAMP: LAN1-opt*

Sequence number	Ingredient	Volume
1	Plasmid	5µl
2	XbaI	1µl
3	SacI	1µl
4	Y Buffer (Yellow)	1µl
5	Rnase	1µl
	Total volume	9µl

Table-3: Ingredients for reaction cut plasmid pBI121

Sequence number	Ingredient	Volume
1	Plasmid pBI121	92µl
2	XbaI	8µl
3	SacI	8µl
4	Y Buffer (Yellow)	12µl
	Total volume	120µl

Table-4: Ingredients in coupling reaction pBI121plasmid with gene Lan11-opt

Sequence number	Ingredient	Volume
1	Plasmid pBT:Lan1-opt	10µl
2	Plasmid pBI121	10µl
3	Buffer T4	4µl
4	T4 ligase	1µl
5	H_2O	6 µl
	Total volume	30µl

DNA plasmids were transformed into E. coli variable cells (DH5α) by heat shock method[11]. DNA plasmid was extracted and cleaned by the method of Sambrook et al.[12]. Recombinant DNA was checked by PCR with specific primers G336 F / G336KDEL R.

Recombinant vector pBI121 transgenic/AGPopt were transformed into *A. tumefaciens* C58/pGV2260 by methods of electrical impulses for transfer gene into plants.

Transfer the gene into tobacco plants via Agrobacterium bacteria: *Nicotina tabacum* K326 cultivars grown to be 3-4 leaves are used to conduct gene transfer experiments.

Regenerated shoots and gene transfer: The part of leaves about 1 cm^2 after cutting were put on GM's medium sensors (MS + sucrose 30g/l + Agar 8 g/l + BAP 1 mg/l). After two days on the medium sensors, leaves were lifted out and soak in the suspension of bacteria containing the gene transfer vector carrying *Lan1_opt* gene. After 10 minutes of soaking, the leaves were co-cultured with bacteria in the GM medium without antibiotic (BAP 1 mg/l). After 2 days, the leaves was transferred to GM medium containing (BAP 1 mg/l) supplemented with bactericidal antibiotic cefotaxime 500mg/l and selective antibiotic kanamycine 50mg/l for regenerating. After about 4 weeks, the shoots appeared and then were transferred into the GM medium without BAP.

Regenerated root: After 3-4 weeks the shoots were developed about 2-3 cm then were cut and transferred to rooting RM medium (MS + sucrose 30g/l + Aga 8g/l + IBA 0,1mg/l) with selective and bactericidal antibiotic.

The gourd land and the net house: After 3-4 weeks the plants had the roots compleltely development with 3-4 leaves. They were ready for the gourd husk: sand (1: 1). Plants had grown to have 4-5 leaves and then they were transfered to grow in 40% of the cultivated land, 30% of the smoking husk or humus and and 30% animal droppings item which were previously mixed well and clean handling of the disease in natural conditions.

Confirmation of the Lan1_opt gene expression in tobacco plants: The presence of the Lan1_opt gene in tobacco plants was confirmed by PCR analysis with primers G336 F/R G336KDEL.

Confirmation of the expression of genes by analyze the AGPase enzyme activity using the spectrophotometry method (measuring absorbance of NADPH at the wavelength 340nm).

AGPase activity per fresh tissue weight were calculated following the formula

$$AFW = \frac{Abs340}{6.22.L}.\frac{V1.V3}{V2}.\frac{1}{m.t}$$

where: The extinction coefficient of NADPH is 6.22 $mM^{-1}cm^{-1}$ at 340 nm, Abs_{340} - the absorbance at 340 nm of sample compared with control so với đối chứng, A_{FW} - AGPase activity per fresh tissue ($U.g^{-1}$), L - Optical path long (cm), has value 1.0 for most of case, m - fresh tissue weight (g), t - reaction time (min), V_1 - Extracts volume (ml), V_2 - the added volume of extracts to create the reaction mixture (ml), V_3 - final volume of mixture reaction (ml)

Then, the AGPase per gram of protein

$$Apr = \frac{AFW.m}{V1.Cpr}$$

where: Apr: Anzyme AGPase activity per gram of protein ($U.g^{-1}$), Cpr: protein concentration in extract ($mg.ml^{-1}$).

Results and discussion

The biosynthesis of gene encoding AGPase: The expression DNA recombinant in plants (eukaryotes) and bacteria (Prokaryote) has many differences, in which the expression of target gene in the plant depends on many factors like the genetic code, promotor, terminator and another conditioner. AGPase in E. coli bacteria is the single gene. It has specific activity higher hundreds of times than in plants[13]. In bacteria, AGPase was activated by fructose 1.6-bisphosphate-(FBP) and was inhibited

by adenosine monophosphate (AMP)[14]. Mutant glycine-336 (G336D) provided for AGPase enzyme has higher activity with activator FBP or not required an activator, affinity to substate (ATP and glucose-1-phosphate) higher, decreasing affinity with the AMP inhibitor[9]. So, changes of the genetic code of artificial transferred gene into the plants would boost the performance of AGPase enzyme.

The design of gene encoding AGPase (Lan1_opt) were followed as: GlgC gene of E. coli bacteria (NC_000913.3, GenBank) was the G336D mutation, as results of changed the glycin by aspartic acid, and a number of different nucleotide sequences were designed for the plant which does not affect the process of translation. The structure of the gene after mutation G336D was attached at the top of the 5' segment 171-bp coding for signal peptide sequences (including 57 amino acid) which have the function of leading protein to the chloroplast. This protein was derived from the rbcS gene encoding the small segment of ribulose 1.5 bisphosphate carboxylase enzyme in Arabidopsis, also the top 3' was added the c-myc tail (11 amino acids) and KDEL sequence (4 amino acid) with 48 bp to detect recombinant protein by Western blot method. In addition, the recognised location of XbaI restriction enzyme was added to the 5' position and recognised the location of SacI was added to the 3' position of Lan1_opt gene to facilitate the cutting and combine genes in the design process of plant transformation vector.

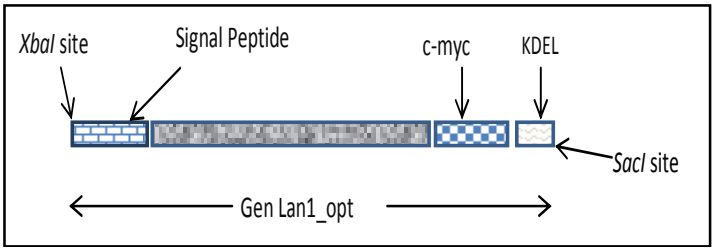


Figure-1: Schematic structure of synthetic Lan1_opt gene

Lan1_opt gene had a size of 1527 bp (Figure-1), it was synthesised by Integrated DNA Technologies (USA). The nucleotide sequence was shown in Figure-2. Lan1_opt gene was cloned in a pUCIDT-AMP vector shown in Figure-3.

Recombinant vector containing Lan1_opt gene: Vector design process showen in Figure-4. *pBI121* vector was used to transfer into plant cells through *Agrobacterium tumefaciens*. The model described in Figure-5.

00481 .....GCT CTAGAATGGC AAGCATGATT TCTAGTAGTG CAGTAACTAC TGTCAGTAGA
00561 GCTTCTACAG TGCAAAGTGC TGCAGTCGCA CCATTTGGTG GTTTGAAAAG TATGACTGGA TTCCCGGTGA AGAAGGTCAA
00641 CACCGATATT ACCTCAATCA CAAGCAATGG GGGGAGGGTT AAGTGCATGG TGTCACTTGA AAAGAATGAT CACCTTATGC
00721 TCGCTAGACA GCTACCTTTG AAAAGCGTTG CTCTCATCCT TGCCGGTGGC AGGGGGACAA GGCTAAAGGA CCTTACTAAC
00801 AAAAGGGCAA AGCCCGCAGT ACATTTTGGC GGAAAATTTA GAATTATCGA TTTTGCTCTT AGCAACTGCA TCAATAGTGG
00881 AATTAGAAGA ATGGGAGTTA TCACACAATA TCAGTCCCAT ACTCTTGTCC AACATATTCA AAGGGGATGG TCATTCTTCA
00961 ATGAGGAAAT GAACGAATTC GTGGATTTAC TACCAGCACA ACAGAGAATG AAAGGGGAGA ACTGGTACAG GGGCACTGCA
01041 GATGCCGTGA CTCAGAATCT GGATATCATA AGGCGATACA AGGCAGAGTA TGTTGTTATA CTGGCCGGAG ACCATATCTA
01121 TAAACAAGAT TATAGCAGAA TGCTTATCGA CCATGTGGAG AAAGGTGCTC GTTGTACAGT GGCTTGTATG CCAGTACCTA
01201 TAGAGGAAGC ATCAGCTTTT GGGGTTATGG CAGTCGACGA AAATGACAAA ATTATTGAAT TTGTCGAGAA ACCTGCCAAT
01281 CCACCAAGTA TGCCGAACGA TCCCTCAAAA TCATTGGCCA GCATGGGTAT CTACGTTTTT GATGCTGATT ATCTATATGA
01361 GCTTCTTGAA GAGGACGACA GAGATGAAAA CAGTTCACAT GATTTTGGAA AAGATCTGAT TCCAAAAATT ACTGAAGCTG
01441 GGTTGGCCTA CGCTCATCCA TTTCCACTTT CATGCGTTCA GTCTGATCCT GATGCTGAAC CGTATTGGAG GGATGTGGGG
01521 ACACTGGAAG CATACTGGAA AGCAAATTTA GATCTCGCAA GCGTTGTTCC AGAGCTTGAT ATGTACGATC GTAATTGGCC
01601 TATTCGAACC TACAACGAAT CCCTCCCTCC TGCTAAGTTT GTACAGGATA GGTCAGGAAG TCATGGCATG ACATTGAATT
01681 CCCTTGTCAG CGATGGTTGC GTGATATCCG GATCAGTGGT AGTACAAAGC GTTTTATTCA GCCGTGTAAG AGTTAATTCT
01761 TTTTGTAATA TTGATAGTGC CGTTCTCCTT CCAGAAGTTT GGGTTGGAAG GTCTTGCAGA TTGAGGAGAT GTGTCATTGA
01841 TAGGGCATGT GTTATTCCAG AAGGTATGGT TATCGGCGAG AATGCAGAAG AGGACGCTCG TCGATTTTAT CGAAGCGAAG
01921 AAGGTATCGT ATTAGTAACT CGTGAGATGC TAAGGAAATT AGGTCATAAG CAAGAAAGGG AACAAAAGCT TATAAGCGAA
02001 GAAGACCTTA ATAAAGATGA ACTGTAGGAG CTCG

Figure-2: The nucleotide sequence of the gene structure Lan1_opt

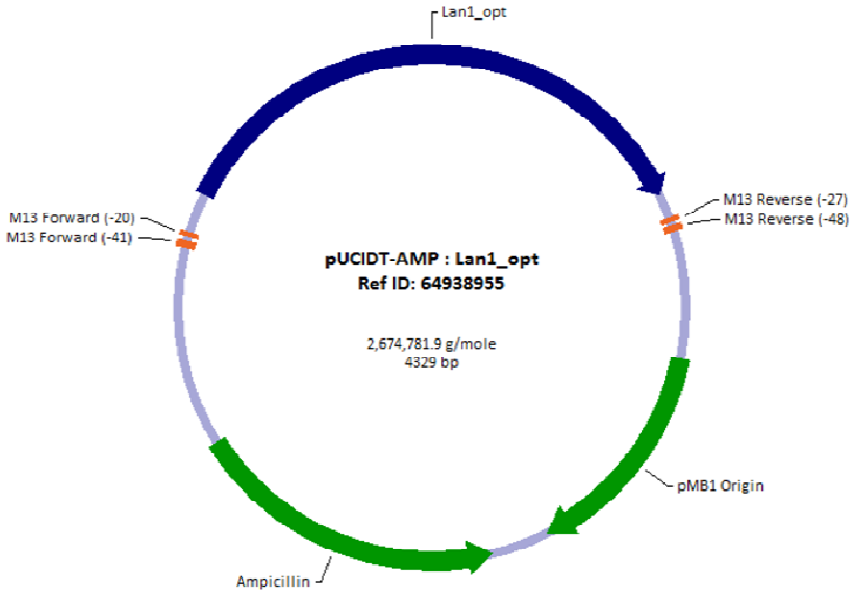


Figure-3: Carrying vector Lan1_opt gene

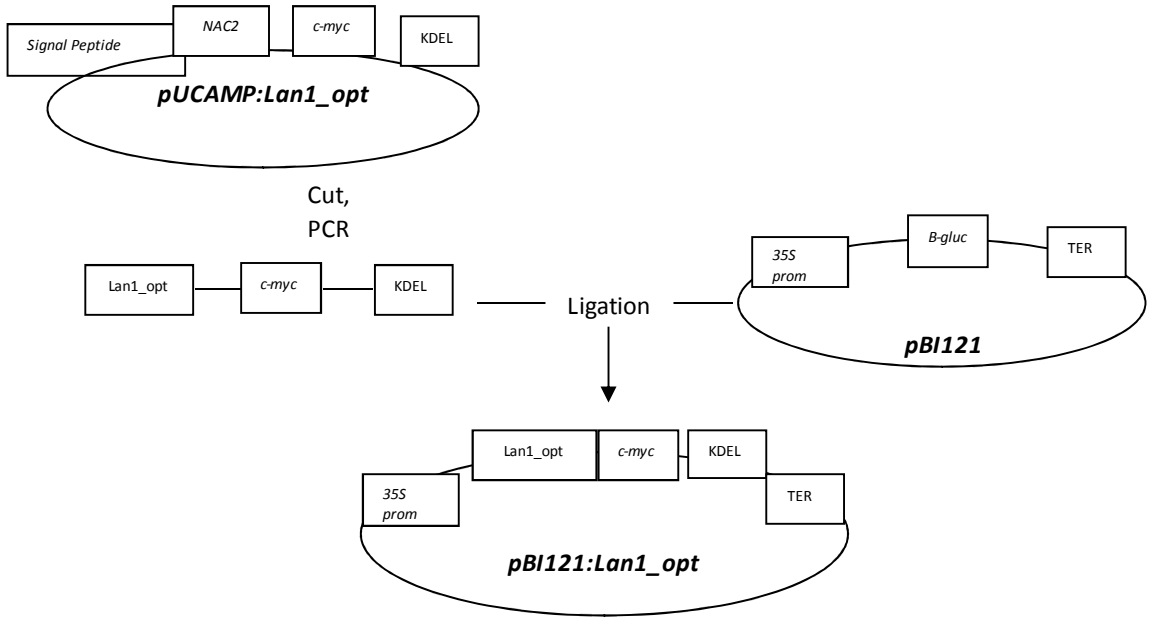


Figure-4: Schematic design pBI121: Lan1_opt vector

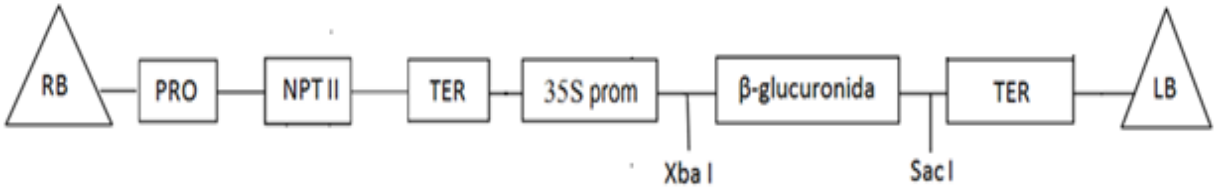


Figure-5: Model pBI121 vector

The pUC: Lan1_opt and pBI121 vectors were processed simultaneously by both *Xba*I and *Sac*I enzyme.

The *gus* gene in Lan1_opt fragment gene and pBI121 vector was removed. Lan1_opt fragment gene and pBI121 vector were purified (Figure-6) and were used for the reaction reassembling to generate recombinant pBI121: Lan1_opt vector.

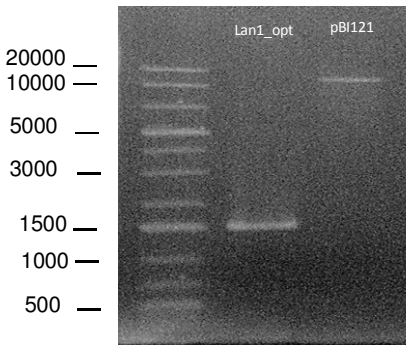


Figure-6: Electrophoresis of pUCAMP: Lan1_opt vector products by *XbaI* and *SacI* in cutting reaction

The Lan1_opt fragment gene and pBI121 vector were combined to forming recombinant pBI121: Lan1_opt vector under catalytic of T4-DNA ligase enzyme and this recombinant pBI121: Lan1_opt vector was cloned in E. coli DH5α. The recombinant vector was confirmed by PCR using primers G336 F (ATG GCA AGC ATG ATTTCTAGTAGT) và G336KDEL R (CAG TTC ATC TTT ATT AAG GTC). This vector was checked by cutting using XbaI and SacI restriction enzyme.

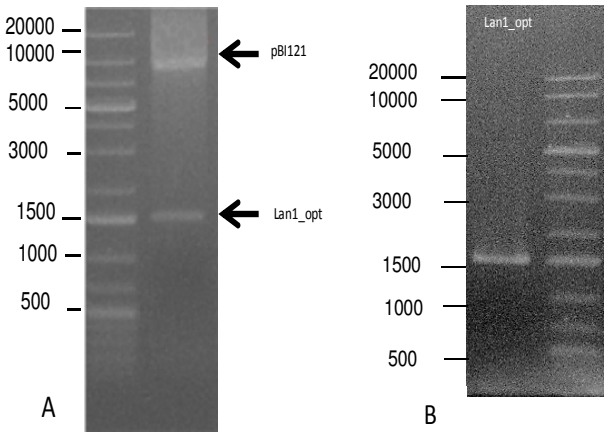


Figure-7: Gel electrophoresis of *pBI121* and *Lan1_opt* vector after cutting reaction by *XbaI* and *SacI* (A) and gel electrophoresis of colony-PCR products with specific primers G336 F and G336 KDEL R from *pBI121: Lan1_opt* vector (B)

The recombinant pBI121:Lan1_opt vector were cut with XbaI and SacI and run on 0.8% agarose gel electrophoresis (Figure-7A) showed two lanes corresponding to two segment genes; one about 1500 bp size is corresponded to Lan1_opt gene (appended with c- myc and KDEL segment) and the second segments with larger size is the vector pBI121 1kb. We also performed colony-PCR reactions with specific primers G336 F and G336KDEL R, the results in Figure 7B showed that the gene-sized segment product obtained corresponding to the theoretical calculations (1,527 kb).

Thus genetic structure Lan1_opt has been incorporated successfully into pBI121 vector using in transgenic plants.

The plasmid from the bacterial line is checked and selected to transform into *Agrobacterium* and transform into tobacco plants.

Transform genes into tobacco plants: Transgenic tobacco plants were selected on medium containing antibiotics kanamycine and were checked transgene by PCR with specific primers G336 F and G336KDEL R.

We used simple PCR technique to confirm the presence of a target gene in the genome of the plant. We randomly selected 9 of 42 generated transgenic tobacco lines, for each transgenic tobacco lines 3 weeks olds as raw materials, using 200 ng of total extracted DNA as a template for PCR's *Lan1_opt* genes with specific primers AGP336F/R. The pBI121/Lan1_opt transgene vector were used as the positive control and total DNA extracted from non-transgenic tobacco leaves were used as the negative control for the reaction. The electrophoresis image (Figure 10) showed that the specific band did not appear in the transgenic plant lane 1, 8 other samples had bands consistent with theoretical calculations at about 1500bp, which is correspondent to *Lan1_opt* gene. Thus it can be concluded that *Lan1_opt* gene were successfully transfered into these groups of plants.

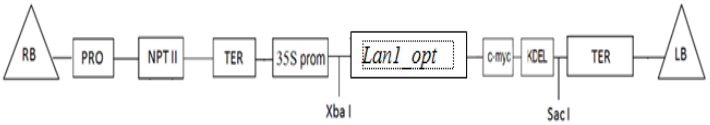


Figure-8: Model *pBI121:Lan1_opt* vector

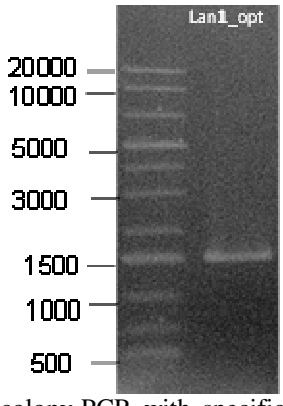


Figure-9: Product colony-PCR with specific primers G336 F and G336KDEL R12 plasmid isolated from *Agrobacterium* line

Evaluate the expression of genes Lan1_opt encoding for AGPase enzymes through the AGPase activity: The unit of measurement of AGPase activity enzyme is the enzyme unit.

One enzyme unit was defined as the amount AGPase enzyme metabolise 1 µM ADP-glucose into glucose 1-phosphate for 1 hour at pH 7.5 and 30°C. The principle of chemical reactions with the participation of AGPase were:

Firstly, AGPase being present in plant tissue extracts catalysed the ADP-Glc and PPi into Glc-1-P and ATP. Second, after the reaction was stopped by boiling, phosphoglucomutase (PGM) was added to the reaction mixture; PGM catalyzed Glc-1-P into Glc-6-P. Lastly, glucose-6-phosphate dehydrogenase (G6PDH) is added, G6PDH catalyzed the Glc-6-P in the presence of NADP + into 6-phosphogluconic acid, simultaneously, NADP is converted to NADPH and NADPH generated was equal the amount of Glc-6-P oxidised[15]. By measuring absorbance at 340 nm of NADPH, we determined the amount of ADP-glucose is metabolised by the action of APGase.

$$\text{ADP-glucose} + \text{PPi} \xrightarrow{\text{AGPase}} \text{ATP} + \text{Glc-1-phosphate}$$

$$\text{Glc-1-phosphate} \xrightarrow{\text{PGM}} \text{Glc-6-phosphate}$$

$$\text{Glc-6-phosphate} + NADP^+ + H_2O \xrightarrow{\text{G6PDH}} \text{6-phosphogluconic acid} + NADPH + H^+$$

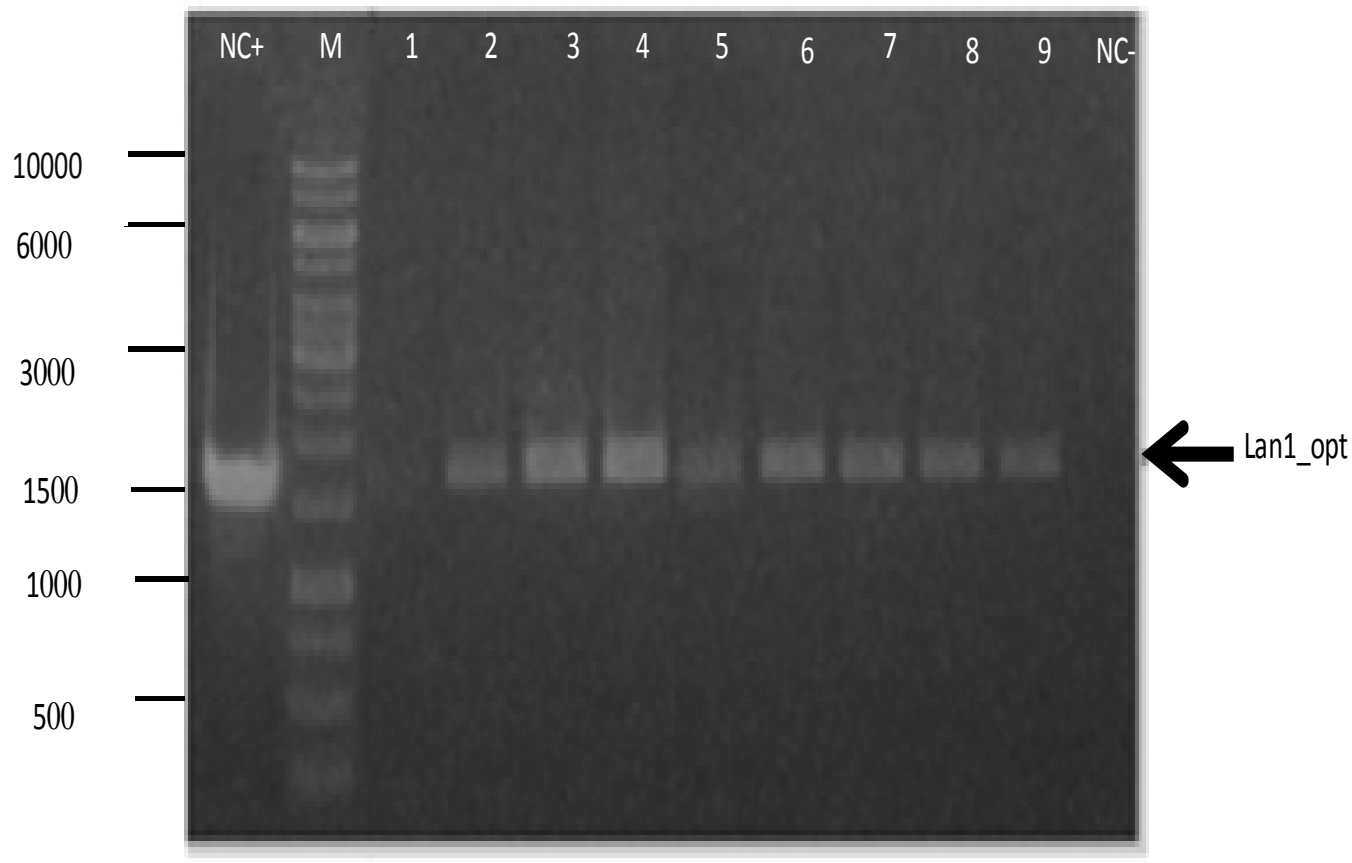


Figure-10: Gel electrophoresis of PCR products of the transgenic tobacco lines.
M: marker 1kb; 1-9: transgenic tobacco plants; NC +: non-transgenic control. The transgenic tobacco plants which had positive results are being planted and continue monitoring to analyse the possibility of transgene expression.

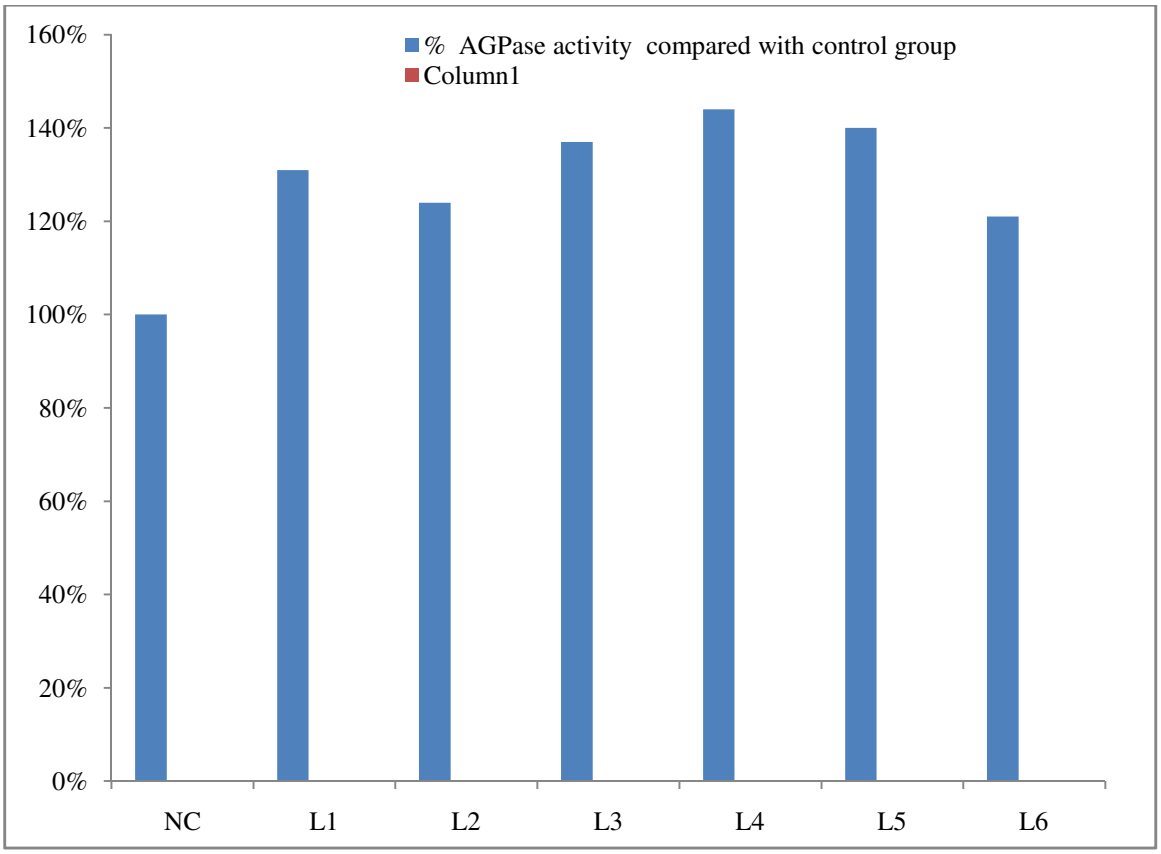


Figure-11: AGPase enzyme activity in leaves of *Lan1_opt* transgenic tobacco plants compared with non-transgenic control plants.

The results showed that the *Lan1_opt* transgenic tobacco plants had AGPase activity in leaves increased from 21-43% compared to controls. In 6 transgenic tobacco plants analysing, the 4th plant had AGPase activity highest (43% compared to control).

Conclusion

We have synthesized successfully gene encoding AGPase Lan1_opt gene, which had the mutation G336D based on the glgC gene sequence in GenBank (NC_000913.3). Lan1_opt gene had 1527 bp of size. It was combined successfully into a pBI121 vector and transferred into the tobacco plant. AGPase enzyme activities in transgenic tobacco plants increased from 21% to 43% compared with non-transgenic tobacco plants, which initially showed its contribution to increasing the efficiency of starch synthesis.

Acknowledgment

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