Expression of *E. coli* *glgB* in Potatoes Generating Tuber Starches with Various Features

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Abstract: Expression of *glgB* in amylose-free mutant leads to accumulation of phytoglycogen of low molecular weight in potato tuber. Performance of *glgB* expression in wild type potatoes is unknown at this point. In this study a *glgB* was isolated from *E. coli* JM 109 and it was expressed in two potato genotypes after function of *glgB* was confirmed. 18 transgenic lines were obtained by PCR and Southern blot screening using *glgB* transformants. RT-PCR showed that *glgB*, driven by 35S promoter, was expressed at different levels in 18 transgenic lines. Starch viscosity and amylose contents were assayed with tuber starches of the 18 lines. Data analysis showed that although starch viscosity of all lines was significantly higher than that in control, genotype of DXY expressing *glgB* showed much higher starch viscosity than genotype of ZHB expressing *glgB* did. Meanwhile, amylose contents were the same as those in control and they were constant in all individuals with ZHB as host expressing *glgB*. However, all individuals with DXY as host expressing *glgB* showed a higher amylose contents than those in the control. The results indicated that starch generation altered along with different genotypes of transformed potatoes hosting gene. Expression of *glgB* led to a higher starch viscosity of various extents.

Background

Genetic improvement on the endogenesis starch is rapidly expanding the application of starch. Regulation of downstream gene expression in the starch biosynthesis pathway generated potato starch with shorter chains and less amylose. Study on starches modified by granule-bounded starch synthase (*GBSSI*) found that the amylopectin cluster and the starch granule remained the same as those in the control, but the structure of the amylopectin cluster changed to form starch with unique potential dynamic values in application (Kozlov et al., 2007).

The potato expressed *E. coli* *glgC* increased tuber starch accumulation through raising ADP-glucose pyrophosphorylase (AGPase) activity (Stark et al., 1992). Inhibition of GBSSI expression led to reduction in the amylose in the potato starch (Kuipers et al., 1994), and starch branch enzyme (SBE) activities in transgenic lines of which SBE A and B were suppressed were less than 1% of those of the control. Amylopectin with high molecular weight was no longer synthesized, thus starch granule changed morphologically, and phosphorus content in the starch increased by 5 times. This starch is potentially valuable for application (Schwall et al., 2000).

Starch branch enzyme and soluble starch synthase (SSS) are closely related to the apparent amylose content and taste quality in rice. SBE1 and SBE3 express specifically the genotype of high palatability (Sun et al., 2011). SBEIIa is essential for transportation of temporary-storage starch in maize leaves along with the cycle of day and night (Yandeau-Nelson et al., 2001). Activity of ADP-glucose pyrophosphorylase in the potato tuber changes with the day-night circle (Tiessen et al., 2002). In addition, the other enzymes involving in starch synthesis including SBE are...
sensitive to factors such as the day-night circle, which limits generation of different kinds of starches (Jobling, 2002).

Expression of *E. coli* *gltB* in amylose-free potato led to generation of tuber starch with low molecular weight and increased branch points (Anne et al., 1996). Amylose is required for formation of alpha 1-6 branch chain in starch; however, much less amyloses were used as substrates in amylose free potato. Branch enzymes, encoded by native potato SBE and *E. coli* *gltB* expressed in potato, work with much less amyloses as substrates. This resulted in an unstable reaction catalyzed by the branch enzymes in tubers since both branch enzymes were sensitive to the day and night cycle.

With a relative constant (not much less in amylose potato) supply of amyloses as substrates, shortage of substrates for alpha 1-6 branch as it is in amylose free potato could be avoided. In other words, branch enzyme activity was increased by expression of *gltB* in wild type potatoes rather than in mutants. Starch molecular weight and other property could be improved, but it is still unknown what aspect of starch was improved when branch enzyme was enhanced in a wild type potato.

In this study, *gltB*, driven by 35S promoter is expressed in the two wild types of potatoes. It was found that *gltB* expression showed a genotype specific effect on starch viscosity. Results showed that the starch tuber starch viscosity in *gltB* transformed lines was 15 time higher than that in the control. Measurement and analysis of the starch indicate that *gltB* can be expressed in different potato genotypes, which result in different starch types with improved starch viscosities as high as 15 times as the control group.

### 1 Results

#### 1.1 *gltB* isolation

With *gltB* specific primer, a 2184 bp product was amplified as shown in Figure 1A. The product was ligated into pGEM-T and sequenced. pG-gltB+ and BL-gltB- were recognized after sequence alignment. With the *gltB* sequence BLAST was done in the Nucleotide database in NCBI. It was found that *gltB* sequence isolated from this study is 99% similar to the *gltB* sequence in the JM109 genome recorded in the database (Welch et al., 2002). Putative polypeptide of the *gltB* is made up of 728 amino acid. After aligning it with other *gltB* sequences from other bacterial strains, it was found that there are 3 specific amino acid mutation sites in the putative polypeptide, which are K39E, V71A and I248V. The sequence was registered in NCBI nucleic acid database with accession number EU44744.

![Figure 1 *gltB* isolation and constructing recombinant plasmids](http://mpb.sophiapublisher.com)

**Figure 1** *gltB* isolation and constructing recombinant plasmids

Digestion of purified pG-gB+ with BamH I, Nco I and Sac I released a 1.3 kb fragment and a 2.2 kb fragment, respectively, as shown in Figure 1B. The same was done with pG-gB-, and the result is shown in figure 1C. All fragments were the same as expected.

1.2 glgB expression in recombinant strains
To ensure glgB with three mutation sites function in glycogen biosynthesis as expected, functional confirmation of the glgB isolates was done by expressing glgB in E.coli. To do so, the glgB was subcloned into downstream of T7 promoter in pET-28c in both 5’-3’ and 3’-5’ directions. Purified recombined plasmids pE-gB+ and pE-gB- were digested with Xba I and Pst I as well as Bam H I. As shown in Figure 1D and 1E, expected fragments were released from pE-gB+ and pE-gB-. It indicated that the recombined bacterial strains BL-EgB+ and BL-EgB-, containing the pE-gB+ and pE-gB- respectively, were constructed.

To find effect of glgB expression on glycogen in the strains, glycogens from BL-EgB+, BL-EgB- and BL-21 were extracted and scanned with 190-1900 nm wavelength coverage. The result was shown in Figure 2A, 2B and 2C. The absorption peaks of glycogen from BL-EgB+ were the same as that from BL-21, but different from those of BL-EgB-. An extra absorption peak appeared between 300 nm and 400 nm in glycogen from BL-EgB-, which is absent in other strains. This indicated that the expression of reverse glgB driven by T7 promoter changed the glycogen structure in BL-EgB-. Therefore, it is concluded that glgB isolate is functional in glycogen biosynthesis.

1.3 glgB expression in potato transgenic lines
To express glgB in potatoes, transformation vector pC-SaS was constructed and transformed into agrobacterial LBA4404 to generate recombinant strains LBA-gB. The recombined pC-gB was extracted from LBA-gB, and it was cut with BamH I and EcoR I. A 1.2 kb and a 940 bp as well as a 2.2 kb fragment were released, respectively, as shown in Figure 3.

26 PCR positive transformants were found from two groups of transgenic lines. Some of those were shown in figure 4A. Southern blot showed that among the 26 PCR positive transformants, 9 were inserted with

Figure 2 Light-absorption features of glycogen from the recombined bacteria BL-gB+ and BL-gB-
Note: A: BL-gB+ ; B: BL-gB- ; C: BL-21

Figure 3 Digestion result of vector pC-gB
Note: M1.DNA molecular standard; 1: Products of BamH I digestion on pC-gB; 2: Products of EcoR I digestion on pC-gB; 3: Recombined plasmid pC-gB; M2: DNA molecular standard
single copy of the recombined \textit{glg}B, and the other 9 with double copies of the recombined \textit{glg}B, as shown in Figure 4B.

RT-PCR result with RNA of the 18 transgenic lines and \textit{glg}B specific primers showed that \textit{glg}B mRNA was expressed in all of 15 transgenic lines. The expression amounts in 4 transgenic lines were much higher than the other 10, as shown in Figure 4C and 4D.

The other 8 lines are in the second category, as starch stained from those lines turned light blue, such as DF2 and DF6 from DXY host, and ZF6 and ZF8 from ZHB host. Typical color results were partially shown in Figure 5.

1.5 Starch viscosity and amylose content of tuber from transgenic lines

Based on results of starch staining, 8 lines with typical blue and light blue, 4 from DXY host and 4 from ZHB host were selected for analysis of starch viscosity and amylose contents. In the line of DXY genotype expressing \textit{glg}B, the highest starch viscosity, 388.06 mPa.s occurred in the line of DF6, which was 12 times higher than the control. Starch viscosity of DF3 and DF6 were much higher than DF5 and DF2, and also significantly higher than the control (p<0.01), as shown in Figure 6.

In the group with ZHB genotype as the host expressing \textit{glg}B, the highest starch viscosity, from the line ZF7 was 212.46 mPa.s, which was 15 times higher than the control; viscosity of ZF7 and ZF8 were significantly higher than that of ZF6 and ZF10, as well as the control group (p<0.01), as shown in Figure 6.

Starch viscosity of the 8 transgenic lines varied between 36.12 mPa.s and 388.06 mPa.s.

Data analysis on amylose content (AC) indicated, as shown in figure 7 that AC in DXY host lines was significantly higher than that in control. Among them, the highest AC was in the line DF6 and it was 1.2 times higher than the control. ACs of the 4 transgenic lines from ZHB host were the same as that in the control.
Figure 6 Starch viscosities of the transgenic lines

Figure 7 Amylose contents (AC) test result in transgenic lines that express glgB

The line of the DXY host was different from that of the ZHB host in both starch viscosity and amylose content. Starch viscosity in DXY host line was much higher than that in ZHB host line. Amylose content in DXY host line was significantly higher than the control, but it was the same between ZHB host line and control. Therefore, glgB expression in different host genotypes led to various responses for starch biosynthesis.

Data analysis showed that there was no significantly correlation between starch viscosity and amylose contents of the transgenic lines. There wasn’t any correlation between amount of RT-PCR products and starch viscosity, either. Difference in starch viscosities between two genotypes host lines and the controls indicates that glgB expression within potato regulates starch biosynthesis through increasing amyllopectin synthesis. This result is in accordance with results obtained when glgB is expressed in amf potato done by Anne et al (1996). Meanwhile, effect of glgB expression in potato on amylose biosynthesis shows genotype specificity.

2 Discussions

In this study, we transformed glgB into two potato cultivars. Two glgB-expressing transgenic groups were obtained. Starch viscosity and amylose content in starches isolated from the two groups were assayed. Data analysis of the assay indicated that two genotypes performed differently in response to glgB expression. This is the first evidence to show crop genotype-specific response to an extraneous gene expression. This means that more than one crop genotypes have to be taken into consideration when a transgenic breeding program is made to improve starch quality of the crop.

In addition, in this study, we found that starch viscosities of all transgenic lines were significantly higher than that in the control, one genotype expressing glgB showed much higher starch viscosity than the other genotype expressing glgB. This finding indicates clearly that starch feature can be modified in wider extent than expected through expression of extraneous genes involved in polysaccharide biosynthesis. Plant starch feature could be subjected to not only endogenesis genes but also extraneous relatives.

Many factors could affect the expression of a target gene transformed into a plant species, such as the location of insertion in host genome, completeness of gene inserted, copy number etc; however, alteration of gene expression involving starch biosynthesis, such as starch branch enzyme is a way to find how starch biosynthesis is regulated. It is particularly important for polyploidy starch plant, such as potato; However,
it can never be neglected that even though glgB is completely inserted and expressed, alteration of starch biosynthesis resulting from glgB may be different in different genotypes of a plant, because initiation and regulation of starch accumulation in the early stage is variable among different genotypes. Therefore, a further study on the transgenic lines is needed to reveal variation in stabilized accumulated starches.

Starches used in this study were isolated from immature tubers, which are different from the full mature tuber; therefore, immature tubers may be different in the starch composition from mature tubers. Though non-transformed genotypes grown under the same condition are regarded as the control in comparing results of starch test in the study, further assessment on the starch features in different transgenic lines is needed when mature tubers are harvested outdoor.

3 Material and Methods

3.1 glgB isolation

Genome DNA was isolated following instruction as described on Molecular cloning Molecular Cloning: A Laboratory Manual (Third Edition) (CSH Press 2001). According to *E. coli* JM109 glgB DNA sequence (AE016768) in Genbank, primer pairs PF1 and PR1 were designed. PF1: 5’-cagttccagctggtggacacaat-3’; and PR1: 5’-cctgtcagctgaagtaagc-3’. With diluted JM109 genome DNA and the primers, 25 µL PCR reaction was set as 10 × PCR buffer solutions (2 µL 10 mM dNTP, 1 µL 1 µmol/L PF1, 1 µL 1 µmol/L PR1, 0.25 µL pfu DNA polymerase). Sterilized DDW was added to fill it up to 25 µL. After mixing them, PCR amplification was performed.

The reaction was carried out as following: denaturation at 95°C for 30 sec, annealing at 52°C for 60 sec, extension at 72°C for 3.5 min, (30 cycles in total) extension at 72°C for 10 min and keeping it at 4°C for the last cycle. PCR products were recovered with a gel recovery kit (Promega).

To set up the ligation reaction of PCR products and vector, a reaction was done with 3 µL recovered PCR products and 1 µL pGEM-T easy vector following instruction of the pGEM-T easy kit. 2 µL ligation reaction products were transformed into *E. coli* supercompetent cells by heat shock. Transformed cells were plated on a LB patri dish. Cells were grown overnight. The white clones were screened. Positive clones were selected by *Nco I* and *Sac I* digestion analysis and confirmed by sequencing (ABI 3730XL DNA Sequencer at the Shenggong, Shanghai, China). Target recombinant bacterial strains BL-gB+ and BL-gB-, containing glgB in 5’-3’ (pG-gB+) and 3’-5’ (pG-gB-) direction, respectively were obtained.

3.2 Construct of pE-gB

Isolate recombinant plasmid pG-gB+ and pE-gB. Cut both DNA with *Nco I* and *Sac I*. Recovery and purify cut fragments. Ligate the fragment with T4 DNA ligase and transform it into the super competent cells of host bacteria BL21. The recombinant plasmid pE-gB+, in which glgB was downstream of the T7 promoter was obtained. Likewise, the recombinated plasmid pE-gB- was obtained. Recombined bacterial strains BL-EgB+ and BL-EgB-, containing pE-gB+ and pE-gB- respectively were proven by digestion analysis with *Nco I* and *Sac I* as well as *BamH I*. Glycogen from recombinated bacterial strains was isolated and measured in accordance with methods used by Haugen et al (1976).

3.3 Construction of pC-gB

Isolate pC-SaS from the *Agrobacterial* LBA4404-SaS, pG-gB from the recombinated bacterial strain BL-gB+. Cut pC-SaS and pG-gB with *Nco I* and *Spe I*. Recovery and purify 11 kb fragment from pC-SaS and 3.2 kb fragment from pG-gB with a recovery kit (Promega). Ligate fragments with T4 DNA ligase (Promega) and transform ligated plasmid into the competent cells of the *E. coli* BL5α. After proving glgB being ligated downstream of 35S promoter in pC-SaS, transform the ligated plasmid into competent cells of the agrobacterial to obtain recombinated *Agrobacterial* LBA-gB containing recombinated pC-gB.

3.4 Transformation of glgB into potato

Cut 1 cm stem segments with axillary bud from genotype DXY and ZHB as the explants, and pre-culture on MS media for 2 days. According to the protocol (Anne et al., 1994), after plantlet in glass tuber grows up to 8~10 cm at height, transfer it into
the peat soil and grow for 30 days under light density of 20 000 Lx, 25°C and with 12 h irradiation per day. Then move and grow plantlets under the same condition but with 8h irradiation per day. Minitubers were harvested after 30 days.

3.5 Screening for glgB transgenic lines
Isolate genome DNA using CTAB method with leaves from two groups of transformants. With glgB primer cloned from the JM109 genome DNA, PCR was done as described above. PCR product was tested with 1% agarose gel electrophoresis.

glgB probe was made as instruction DIG labeled kit (Mylab, Ltd. Beijing). Southern blot was carried out following Molecular Cloning: A Laboratory Manual (Third Edition) (CSH Press 2001). Hybridization signal on HyBond N+Membrane was tested with Dig Hybridization assay kit (Mylab, Ltd. Beijing). Image of signal on the membrane was taken with digital cameral.

3.6 RT-PCR test of glgB expression in transgenic lines
Leaf DNA was extracted as described above from each of the transgenic lines. With glgB prime as above, RT-PCR was done as the instruction of RT-PCR kit (Takara).

3.7 Staining starch from the transgenic lines
Extract starch with method as described by Kuipers et al (1994), 0.02% starch suspension at 20°C was stained with 5% Lugol.

3.8 Assay of amylose contents
Assay of amylose contents was done with the method as described by Kuipers et al (1994).

3.9 Assay of starch viscosity
To measure the dynamic viscosity of tuber starch, the following reactions were made: add 3 g of purified starch (water contents 16%) in ddH2O to make 3% water suspension (w/v) .Heat up suspension to 70°C and incubate at 70°C for 15 min for gelatinization. Add 10 mL the gelatinized suspension to a capillary viscometer in a device kept constant at 60°C. The time required for the suspension to fully pass a 3 mm interstice in the viscometer was recorded as t1. Dynamic viscosity was calculated with the formula η = ηr×t/ηr, ηr is the solvent viscosity, and here ηr = 0.469 mPa.s when ddH2O goes through the 3 mm interstice at 60°C, t0 is the time required for equal volume solvent (ddH2O) to flow through the interstice. All data was analyzed by a statistic tool, DPS ver. 9.50 (Data Processing System, Ruifeng, Hangzhou, China).

Authors’ Contributions
YXL designed the experiment andprimers used, performed data analysis, wrote and revised the manuscript and supervised performance of the project. LJF performed glgB isolation, vector construction and glgB function confirmation in E. coli. FXJ performed glgB transformation into potatoes, screening for transgenic lines expressing glgB and phenotype assay. CXQ, CQ and HQ involved partially in the experiments above.

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