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ORIGINAL ARTICLE

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# Expression of *Escherichia coli* branching enzyme in caryopses of transgenic rice results in amylopectin with an increased degree of branching

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**Abstract** Physiochemical properties of starch are dependent on several factors including the relative abundance of amylose and amylopectin, and the degree of branching of amylopectin. Utilizing *Agrobacterium*-mediated transformation, a construct containing the coding region of branching enzyme of *Escherichia coli*, under transcriptional control of the rice (*Oryza sativa* L.) starch-branching enzyme promoter was introduced into rice cv. Nakdong. To enhance *glgB* expression, the first intron of rice starch-branching enzyme and the matrix attachment region (MAR) sequence from chicken lysozyme were included in the expression vector. Eleven independent transgenic rice plants were generated. Southern blot analysis indicated that the copy number of *glgB* integrated into transgenic rice varied from one to five. High-performance liquid chromatographic analysis of starch from transgenic lines revealed that amylopectin from transgenic lines exhibited greater branching than that of non-transgenic rice. The A/B1 ratio in amylopectin increased from 1.3 to 2.3 and the total branching ratio, A + B1/B-rest, increased from 6 to 12 in transgenic rice. The observed increase in the short-chain fractions with a degree of polymerization between 6 and 10 is expected to have a significant effect on retrogradation. Our study demonstrates that amylopectin branching can be altered in vivo, thus changing the physicochemical properties of starch.

**Keywords** Amylopectin · Branching degree · Starch-branching enzyme · *Oryza*

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**Abbreviations** DP: Degree of polymerization · SBE: Starch-branching enzyme · MAR: Matrix attachment region · TP: Transit peptide

## Introduction

The properties of rice starch, which are important in food production and for industrial use, vary with the relative accumulation of two major components, amylose and amylopectin. Rice is a vital source of human nutrition and thus quality traits of the grain are of utmost concern. Additionally, innovative industrial uses for rice starch create a demand for the continued development of starches that exhibit specific characteristics (Ellis et al. 1998). Starch synthesis in plants is a complex biochemical process. With respect to plant metabolism, starch is considered to be either reserve starch or transitory starch. Accumulation of transitory starch is diurnal as the carbohydrate is synthesized in chloroplasts, metabolized and then transported nocturnally to the cytosol for sucrose synthesis (Geiger and Servaites 1994). Reserve starch is synthesized in heterotrophic storage organs.

Plant starch is comprised of 20–30% amylose and 70–80% amylopectin (Martin and Smith 1995). Amylose, primarily a linear array of α-(1,4)-linked glucosyl residues, is associated with amorphous regions of the starch granule. Intra- and inter-chain hydrogen bonding gives rise to secondary helical structures, which define in part the physicochemical properties of starch (Imberty et al. 1988). Amylopectin is a macromolecule consisting of short chains of α-(1,4) residues with non-randomly linked α-(1,6) branches (Kainuma 1988). This large highly branched molecule is radially oriented in the starch granule, forming concentric crystalline and amorphous regions. Branch chains in amylopectin form clusters at 9-nm intervals along the axis of the molecule. Adjacent branches within clusters form double helices, which pack together to form crystalline arrays.

Four enzymes, ADP-glucose pyrophosphorylase (EC 2.7.7.23), starch synthase (EC 2.4.1.21), starch-branching enzyme (SBE; EC 2.4.1.28), and starch-debranching enzyme (EC 2.4.1.41) are involved in starch biosynthesis (Martin and Smith 1995). The enzyme catalyzing the formation of amylose is granule-bound starch synthase (GBSS I) while amylopectin is synthesized by the combined actions of soluble starch synthase (SSS) and starch-branching enzymes (SBEI and SBEII). Starch-branching enzymes I and II are expressed in a coordinate fashion with granule-bound starch synthase and ADP-glucose pyrophosphorylase, respectively, during endosperm development (Gao et al. 1996; Morell et al. 1997).

The degree of branching is determined by the ratio of branching enzyme to debranching enzyme (Hizukuri 1986). Cooking and textural characteristics of rice depend not only on the ratio of amylose, but also on the degree of amylopectin branching. Short chains of glucose with a degree of polymerization (DP) of 6–9 have been shown to inhibit retrogradation (Lu et al. 1996). Moreover, the inhibition of retrogradation in a whole-rice system and in isolated amylopectin was demonstrated when external chain length was decreased from DP 13.9 to DP 11.58 (Yao et al. 2003). In order to produce a modified starch in rice seed, the gene encoding *Escherichia coli* glycogen-branching enzyme (*glgB*) was introduced into rice cultivar Nakdong under transcriptional control of the seed-specific rice SBEI (*SbeI*) promoter. Targeting of the heterologous protein was achieved by fusion of the *SbeI* gene transit peptide (TP) to the coding sequence of the *glgB* gene. Expression of *E. coli* branching enzyme in transgenic rice resulted in a marked increase in the degree of branching of the amylopectin, with high numbers of short branches.

## Materials and methods

### Construction of expression vector

The oligonucleotides used to construct the plant expression vector are shown in Table 1. The rice *SbeI* promoter region with the amyloplast localization signal (SBEI TP) was amplified by polymerase chain reaction (PCR) from plasmid pSBE1 (Kawasaki et al. 1993) using the primers XbhSb, SbBast, BastSb and Salint2 (Table 1). The SBEI TP coding sequence included the first intron of *SbeI*. Subsequent cloning was facilitated by addition of restriction sites to the primer sequences. The amplified *SbeI* promoter with *Xho*I and *Bam*HI restriction sites was cloned into the corresponding sites of pBluescript SK(+), resulting in an intermediate vector (pBSBP). The coding sequence of the *E. coli* glycogen-branching enzyme (*glgB*) was obtained by PCR from genomic DNA using the primers Ncoglg and glgNot (Table 1). The resulting product was cloned into the pGEM-T vector, generating pGEM *glgB*. PCR-amplified oligonucleotide, which encodes SBEI TP, was subcloned into the *Bam*HI–*Nco*I site of pGEM *glgB*, generating pTSTG40. After excision with *Bam*HI–*Not*I, the *SbeI* TP and *glgB* coding region were cloned into corresponding sites of pBSBP, yielding pBSTG70. This cloning procedure resulted in the creation of two amino acids, methionine and glycine, at the junction of *SbeI* TP and the *glgB* start codon. The insert from pBSTG70 was excised with *Xho*I and *Not*I and ligated into the same sites of pSBG700MAR, generating the plasmid pSG70 M (Fig. 1). This final vector contained the *SbeI* promoter and TP, the *E. coli glgB* coding region, and the 3′ region of the potato proteinase inhibitor gene. Additionally, the cassette containing the cauliflower mosaic virus 35S promoter, the *bar*-coding region and the 3′ region of the nopaline synthase gene (*nos*) were included in the vector. The expression cassette is flanked by the 5′-matrix attached region (MAR) of chicken lysozyme gene (Fig. 1).

### Production of transgenic rice plants

Rice (*Oryza sativa* L. cv. Nakdong) was transformed by *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli. Callus induction, co-cultivation with *A. tumefaciens*, and the selection of transformed calli were carried out as described (Jang et al. 1999). Regenerated plants were transferred to soil and grown to maturity. Transgenic lines were selected on the basis of resistance to a phosphinothricin-based herbicide. Phosphinothricin acetyl transferase, encoded by the *bar* gene, detoxifies phosphinothricin herbicides (Duan et al. 1996).

**Table 1** Oligonucleotides used in PCR to create the plasmid pSG70 M. *Lower-case letters* denote added restriction enzyme sites; *+1* denotes the translation start site of the corresponding gene

| Primer | Oligonucleotide sequence | Location and application |
|---|---|---|
| XbhSb | 5′-aatctagaaactcgagCCAGCCAGGCGATCGTGT-3′ | −2,269 to −15 (amplification of *SbeI* promoter) |
| SbBast | 5′-ttggatccttctgcagTGGCCGTGGAGGCGACTC-3′ | −32 to −15 (amplification of *SbeI* promoter) |
| BastSb | 5′-aggatccaactgcagGAGTCGCCTCCACGGCCA-3′ | −32 to −15 (amplification of *SbeI* TP) |
| Salint2 | 5′-ttgtcgacctccTCCACAACAGTCACC-3′ | +2,521 to +2,536 (amplification of *SbeI* TP) |
| glgseq | 5′-AACATTGCCACCCCCGCC-3′ | +187 to +204 (DNA sequencing) |
| Ncoglg | 5′-aaccatgggcATGTCCGATCGTATCGAT-3′ | +1 to +18 (amplification of *glgB*) |
| glgNot | 5′-aagcggccgcTCATTCTGACTCCCGAACC-3′ | +2,169 to +2,181 (amplification of *glgB*) |
| RTglgB | 5′-CAACCGTATTCTTGGTGAGC-3′ | +1,326 to +1,345 (RT–PCR) |

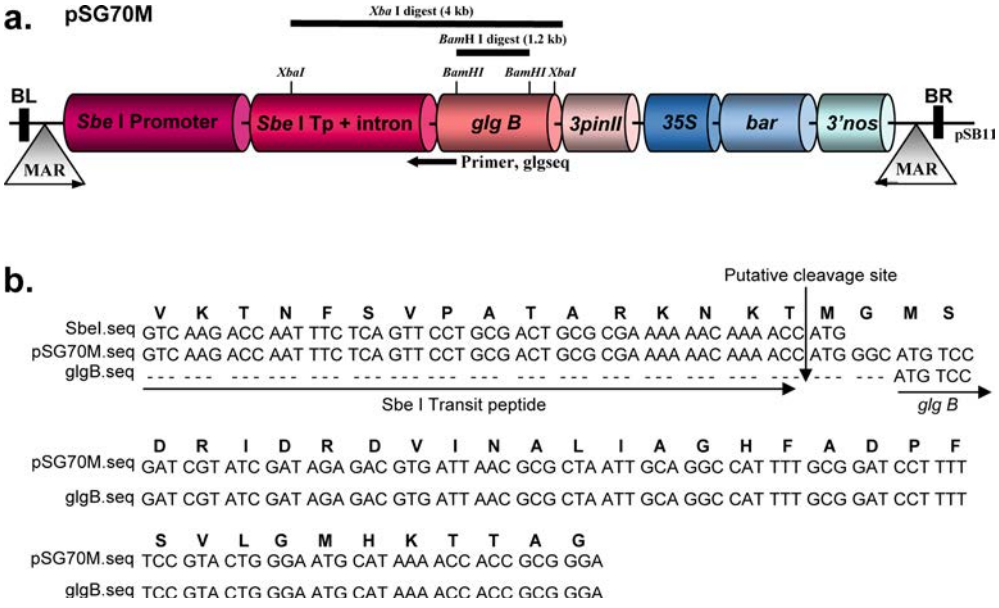


**Fig. 1a,b** The pSG70 M plasmid used for rice (*Oryza sativa*) transformation (**a**) and nucleotide sequence analysis of the junction between the starch branching enzyme I (*SbeI*) transit peptide (TP) and the *glgB* coding region (**b**). **a** pSG70 M contains the *SbeI* promoter, including its TP and first intron, followed by the *E. coli* glycogen-branching enzyme (*glgB*) coding region. Additionally, the plasmid contains the 3′-region of the potato proteinase inhibitor II gene (*3pinII*), and the *bar* cassette, which is comprised of the cauliflower mosaic virus 35S promoter (*35S*), the *bar* coding region, and the 3′-region of the nopaline synthase gene (*3′*nos). The entire expression cassette is flanked by the 5′-matrix attachment region (*MAR*) of the chicken lysozyme gene (Mlynarova et al. 1994). **b** Nucleotide and amino acid sequence alignment of the *SbeI* TP which is translationally fused with *glgB* in the plasmid pSG70 M. The junction region between the *SbeI* TP and the *glgB* coding region was confirmed by DNA sequencing

## Southern blot hybridization

Genomic DNA was isolated from leaf tissue of 11 transgenic rice plants and 1 non-transformed control plant. Genomic DNA (10 μg) was sequentially digested with restriction enzymes *BamH*I and *Xba*I, separated on 0.9% agarose gels, and then blotted onto Hybond-$N^+$ membrane. The membrane was hybridized overnight at 65°C with a $^{32}$P-labeled DNA probe, using a random primer labeling kit (Takara Mirus Bio, Madison, WI, USA). After hybridization, the membrane was consecutively washed once in 2× SSC (0.3 M NaCl, 50 mM sodium acetate, pH 7.0) containing 0.1% (w/v) SDS at 65°C for 15 min, once in 1× SSC with 0.5% (w/v) SDS at 65°C for 15 min, and finally in 0.5× SSC containing 0.5% (w/v) SDS at 65°C. Hybridizing bands were detected by autoradiography, using intensifying screens for signal enhancement.

## RNA isolation and reverse transcription–polymerase chain reaction (RT–PCR) analysis

To minimize ribonuclease (Rnase) contamination, sterile, disposable polypropylene tubes were used for RNA isolation. One gram of frozen immature seeds was pulverized under liquid nitrogen and extracted with 10 ml of buffer containing 4 M guanidium isothiocyanate, 25 mM Na-citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. After phenol:chloroform extraction, nucleic acids were precipitated by adding 1/10 vol. of 3 M sodium acetate (pH 4.8) and 2.5 vol. of ice-cold ethanol. Precipitated nucleic acids were recovered by centrifugation at 12,000 *g* for 25 min and dissolved in RNase-free water. An equal volume of 4 M LiCl was added and the suspension left on ice overnight. Precipitated RNA was recovered by centrifugation as described above and subjected to an additional round of LiCl precipitation. After brief air-drying the RNA pellet was dissolved in RNase-free water and stored at −80°C. The RNA content was estimated by measuring the ratio $A_{260}$:$A_{280}$ using a spectrophotometer. To monitor the expression of *glgB* in transgenic rice, RT–PCR reactions were carried out using SuperScript TM II kit following the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was treated with one unit of DNase I (Invitrogen) for 15 min at room temperature to remove residual DNA. The primers, RT *glgB* and *glg*Not (Table 1) were used in the RT–PCR reaction. Products of RT–PCR were separated by electrophoresis on a 1% (w/v) agarose gel, and then blotted onto a Hybond-$N^+$ membrane for confirmation using a transgene-specific probe.

## Fractional distribution and ratios of debranched starch polymers in transgenic rice

To analyze the fractional distribution and ratios of debranched starch polymers in transgenic rice, starch (20 mg) isolated from transgenic and non-transgenic mature seeds was gelatinized by incubating with 500 μl of 0.3 N NaOH at 40°C for 2 h. After adjusting the pH

to 6.2–6.4 with 0.6 N HCl, the starch solution was debranched with 250 units of *Pseudomonas* isoamylase (EC 3.2.1.68; Sigma, St. Louis, MO, USA) at 37°C overnight. The resulting glucosyl polymers were precipitated by adding 4 vol. of ethanol, centrifuged and the pellet dried. After gelatinization by treating with 40 µl of 0.3 N NaOH at 4°C overnight, the glucosyl polymers were separated by high-performance gel-filtration chromatography on a Superose-12 column using 0.02 M NaOH, 0.2 N NaCl buffer. Elution of the glucosyl polymers of varying molecular mass was monitored using a differential refractive index detector. The polymers were divided into four distinct groups according to their molecular mass (Kim et al. 1995).

### Chain length distribution profile of amylopectin

The chain length distribution of amylopectin from transgenic and non-transgenic rice seeds was analyzed as described by Kortstee et al. (1996).

## Results

### Molecular analysis of transgenic rice plants

Transformation of embryogenic calli of the rice cultivar Nakdong with the expression vector pSG70 M resulted in the production of 11 independent transgenic lines as determined by resistance to phosphinothricin. To verify the integration of *glgB* and to estimate the number of copies of *glgB* in these transgenic lines, we performed Southern blot analysis (Fig. 2). Genomic DNA isolated from young leaf tissues of the 11 transgenic lines and 1 non-transgenic rice plant was sequentially digested with *Xba*I and *Bam*HI. The restricted DNA was transferred to a nylon membrane and probed with a 1.2-kB *Bam*HI fragment that contained the coding region of *glgB*. Two hybridization bands of 4.0 and 1.2 kb were detected in each transgenic line but no hybridization occurred in the non-transgenic plant (Fig. 2). The sizes of the hybridizing bands indicated that no major rearrangement of the transgene had occurred (Fig. 1). Comparing the intensity of hybridization bands representing *glgB* to that of bands corresponding to one, three, and five genome equivalents of pSG70 M, we determined that the copy number of *glgB* integrated into rice genome varied approximately from one to five (Fig. 2).

To demonstrate the expression of the introduced *glgB* gene in transgenic rice, we isolated total RNA from three independent transgenic lines 70M3-11, 70M3-85 and 70M3-114 and performed RT–PCR analysis. These transgenic lines were chosen because each contained a different copy number of *glgB*. The primers used for RT–PCR were designed to amplify an 860-bp fragment of the *glgB* carboxy-terminal region. As expected we detected an 860-bp transcript from the reaction containing RNA from each of the selected transformants (Fig. 3). The RT–PCR product co-migrated with an internal control DNA fragment obtained using pSG70 M plasmid DNA as a template for PCR analysis (Fig. 3). Under identical conditions, no RT–PCR product was generated using RNA from a non-transformed control rice plant. Hybridization analysis using an isotope-labeled *glgB* coding region as a probe revealed strong hybridization with the 860-bp fragment (data not shown).

### Analysis of fractional distribution and ratios of debranched starch of transgenic rice

To determine the changes in the degree of branching and chain length distribution in transgenic rice, isolated starch from transgenic seed was treated with isoamylase

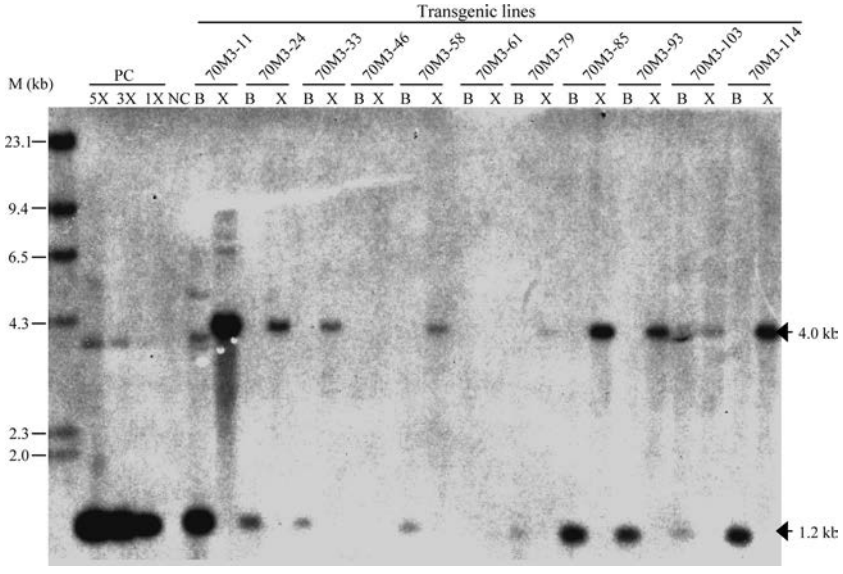


**Fig. 2** Southern blot analysis of transgenic rice plants. Genomic DNA from leaf tissues of eleven independent lines were digested with *Bam*HI (*B*), *Xba*I (*X*) and hybridized with the 1.2-kb *glgB* coding region. *PC*, *Bam*HI-digested pSG70 M; *NC*, genomic DNA from an non-transformed control plant; *1X*, *3X*, and *5X* denotes one, three, and five genome equivalents of pSG70 M; *M*, lambda DNA digested with *Hind*III as size marker

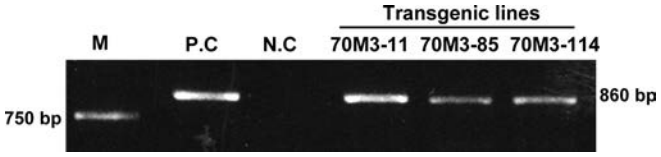


**Fig. 3** RT–PCR analysis of *glgB* expression in transgenic rice. Total RNA isolated from one non-transformed plant and three transgenic plants was used in RT–PCR reactions. The sizes of the RT–PCR products are shown. *M*, Marker; *P.C*, positive control using DNA from pSG70 M; *N.C*, non-transformed control plant

and the cleaved side-chains were analyzed by high-performance liquid chromatography The starch polymers were resolved into four distinct groups denoted fractions I, II, III and IV, based on molecular mass (Fig. 4). According to the model for amylopectin structure proposed by Hizukuri (1986), side chains are designated A, B1, B2, B3 and Bn depending on degrees of polymerization (DP) and branching (DB). Glucosyl polymers with an average DP of 12–16 are designated A chains. The B1 chains, which carry an A or another B chain, have an average DP of 20–24 and the B2, B3 and Bn chains which extend in two or more clusters have an

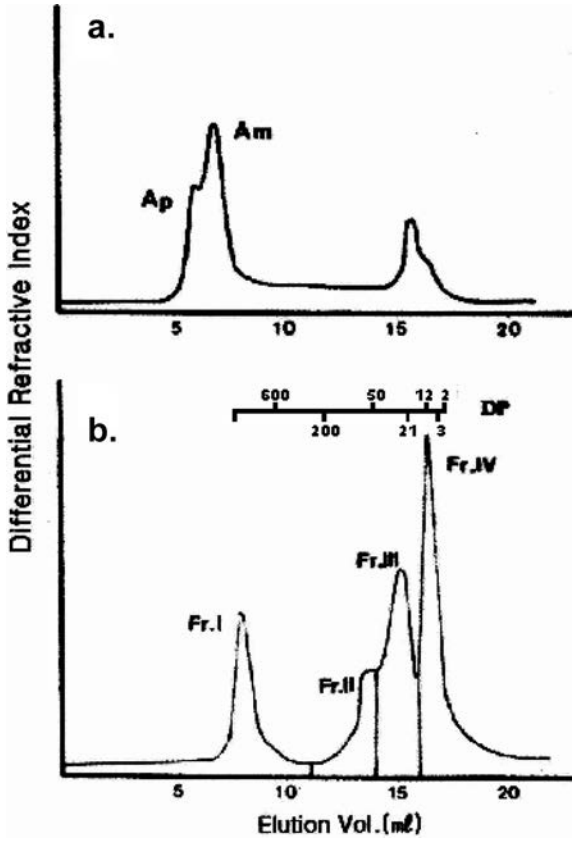


**Fig. 4a,b** Elution profiles of native (**a**) and debranched (**b**) rice starch. Native starch and debranched starch were fractionated on a Superose-12 column with 0.02 N NaOH buffer at a flow rate of 0.25 ml $min^{-1}$. The degree of polymerization (*DP*) was calculated according to the elution volume of standard dextran. *Ap*, Amylopectin; *Am*, amylose

average DP of 45, 74 and 104, respectively. In our experiment, fraction IV, with a DP less than 21, was denoted as A chains and fraction II, with a DP from 21 to 50, was denoted as B1 chains. Fraction II, with a DP of more than 50, was designated as B-rest. Fraction I, having a DP greater than 600, was regarded as amylose. Compared to the non-transgenic control, the degree of branching of starch in transgenic rice increased with copy number of *glgB* gene integrated. The A/B1 ratio in amylopectin increased from 1.3 to 2.3 in transgenic rice containing more than 5 copies of the gene (Table 2). The total branching ratio, A + B1/B-rest, increased from 6.0 to 12 in transgenic rice containing a high copy number of *glgB* genes (Table 2). Analysis of chain length distribution of amylopectin revealed significant changes in transgenic rice when compared to that of non-transgenic plants (Fig. 5). There was a marked increase in the proportion of short chain with DP ≤ 10 in transgenic lines 70M3-85 and 70M3-114 (Fig. 5).

## Discussion

Modification of rice starch with increased branching was attained by the insertion of multiple copies of the *glgB* gene derived from *E. coli*. The increase in A/B1 and A + B1/B-rest ratios in transformed plants is indicative of increased branching. In this study, a higher degree of branching was observed in transformants containing multiple copies of *glgB*. In one transgenic line, the A/B ratio was 2-fold greater than that of non-transformed plants. This observed increase was significantly greater than shown in previous studies (Krohn et al. 1994; Kortstee et al. 1996). The *E. coli glgB* gene has previously been expressed in diploid amylose-free (2n = 2×) potato (Kortstee et al.1996; Flipse et al. 1996) and Russet Burbank (2n = 4×) potato (Krohn et al. 1994). Under control of the GBSS promoter and TP, expression of the bacterial gene increased branching of amylopectin in amylose-free potato by 25% (Kortstee et al. 1996). In contrast, only a slight increase in amylopectin branching was noted in wild-type potatoes transformed with *glgB* under transcriptional control of the patatin promoter and Rubisco small-subunit TP (Krohn et al. 1994). Ostensibly the extent of branching of amylopectin in these transgenic plants could be due to differences in the promoter activity. In contrast to the patatin promoter, a significant increase in degree of branching was observed when potato GBSS promoter and rice SBEI promoter were employed to express *glgB* in the respective plant species. Since both GBSS and SBEI promoters are endogenous and involved in starch synthesis, their use to drive the transcription of *E. coli glgB* would presumably result in a higher level of expression of the transgene.

The construct utilized in this study to deliver *glgB* into rice genome was fundamentally different from that used to transform potato in two respects. First, in our experiment the *glgB* gene in the construct was preceded

**Table 2** Fractional distribution and ratios of debranched starch polymers. *A* denotes fraction IV (Fig. 4) with a DP less than 21, *B1* is fraction III with a DP from 21 to 50, and *B1-rest* is fraction II with a DP from 50 to 200. Fraction I, having a DP greater than 600, is regarded as amylose

| Rice (copy No.) | Fraction I | A | B1 | B-rest | A/B1 | B1/B-rest | A + B1/B-rest |
|---|---|---|---|---|---|---|---|
| Non-transgenic | 14.1 | 38.5 | 35.1 | 12.3 | 1.1 | 2.9 | 6.0 |
| 70M3-11 (5) | 13.5 | 47.6 | 31.3 | 7.6 | 1.5 | 4.1 | 10.4 |
| 70M3-24 (1) | 11.7 | 44.8 | 31.9 | 11.6 | 1.4 | 2.8 | 6.6 |
| 70M3-79 (2) | 15.2 | 41.0 | 32.3 | 11.6 | 1.3 | 2.8 | 6.3 |
| 70M3-85 (5) | 14.6 | 47.0 | 28.6 | 10.0 | 1.6 | 2.9 | 7.6 |
| 70M3-114 (5) | 15.9 | 54.4 | 24.1 | 5.6 | 2.3 | 4.3 | 14.0 |

by the first intron region of the *SbeI* gene. Intervening sequences have been shown to enhance the transcription of eukaryotic genes (Hamer and Leder 1979; Vasil et al. 1985). Inclusion of the first *Adh*1 intron enhanced transcription and increased the steady-state level of the mRNA (Callis et al. 1987). Among the nine introns present in *Adh*1, the inclusion of the first intron significantly elevated transcription when compared with the use of downstream introns (Callis et al. 1987). Other studies have identified intervening sequences that are efficacious in enhancing expression of transgenes (Clancy et al. 1994; Chaubet-Gigot et al. 2001; Clancy and Hannah 2002; Le Hir et al. 2003). The mechanism by which these intervening sequences enhance transcription has not been fully determined but is generally thought to involve co-transcriptional or post-transcriptional events (Bourdon et al. 2001; Clancy and Hannah 2002). In addition to introns, our construct employed MAR sequences, which have been shown to elevate transgene expression, reduce variability in expression (Mlynarova et al. 1994) and stimulate position-independent regulation and expression (Phi-Van and Strarling 1996). In addition, alleviation of gene silencing in $T_o$ and subsequent generations (Vain et al. 1999; Allen et al. 2000; Mankin et al. 2003), reduction in copy number variability among transformants (Mylnarova et al.1994), and correlation of gene expression with copy number (Vain et al. 1999) are attributes ascribed to MAR sequences. Successful transformation events for long-term agronomic improvement require stability in gene expression, and inclusion of intron and MAR sequences could aid in this effort.

In vivo modification of starches using genetic engineering holds potential for both enhancing nutritional qualities and for obviating post-harvest modifications often necessary for utilization of this complex carbohydrate. The rice *sbeI* mutant synthesizes amylopectin that exhibits a marked increase in short chain branches, DP ≤ 10, at the expense of long chain branches, DP > 37 (Satoh et al. 2003). Rice starch from mutated plants exhibited a lower gelatinization temperature and other physiochemical properties that were distinctive from those of wild-type plants (Satoh et al. 2003). Increasing the short chain branches minimizes the chance of retrogradation (Gidley and Bulpin 1987). Glucosyl side chains similar in length to that produced by the *sbeI* mutant are generated by *E. coli glgB*, which assimilates polymers with a DP from 5 to 15 both in vivo and in vitro (Binderup et al. 2002). In this study we have shown that the expression of *E. coli glgB* in rice results in an increase in the short-chain fractions with DP between 6 and 10, which should have a significant effect on retrogradation. Further studies are required to examine if this modified starch exhibits altered physiochemical properties that are desirable for human consumption. Current and future research will bring to light a more complete picture of the complex and intriguing mechanisms of starch synthesis, facilitating modification of the system to produce in vivo specific compounds for food and industrial consumption.

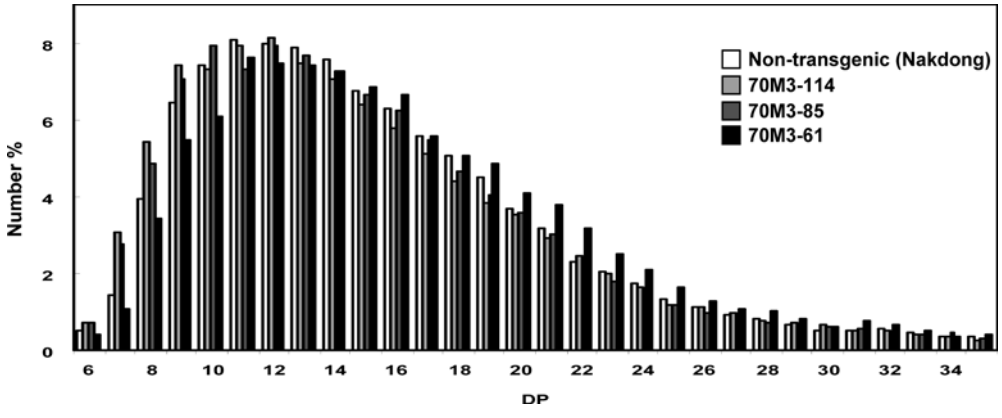


**Fig. 5** Effect of expressed *E. coli* branching enzyme on chain length distribution of amylopectin from non-transgenic and transgenic rice seeds. Amylopectin was debranched with isoamylase and separated on a Dionex series with a Carbopac PA1 column and guard column. A Dionex PED amperometric detector was used to detect the reducing sugars. Glucose and maltose–maltoheptose were used as standards. The amount of chain of a certain length is given as a percentage of the total number of chains (Kortstee et al. 1996)

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