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CHARACTERISATION AND ROLE OF *ISOAMYLASE1* (*MEISA1*) GENE IN CASSAVA

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ABSTRACT

The current concept for starch biosynthesis in plants is that amylopectin, the major fraction of starch, is synthesised by the concerted actions of ADP-Glc pyrophosphorylase (AGPase), soluble starch synthase (SS), starch-branching enzyme (BE), and starch-debranching enzyme (DBE). We have isolated a cDNA clone of *Isoamylase1* gene, a member of DBE family from cassava (*Manihot esculenta* Crantz) storage root. The cloned cDNA fragment sequence (764 bp) showed high identity of 90% to *Rcisa1*, and 81% identity to *Psisa1*, *Stisa1* and *Atisa1*. The deduced protein sequence showed highest (92%) identity with *RCISA1*. The comparative sequence analysis confirmed the cloned fragment to be *M. esculenta isoamylase1* gene (*Meisa1; accession number GU229751*). Genomic analysis revealed occurrence of at least two copies of the *Meisa1* gene. Highest *Meisa1* transcript expression levels were detected in fibrous root followed by in the stem and least detected in the leaf and petiole. Analysis of the temporal expression pattern in the storage root showed initial and maximum expression at 90 days after planting (DAP), and declined thereafter to undetectable levels by 180 DAP. The results implicate a major role of *Meisa1* in storage root differentiation and early starch granule initiation.

Key Words: *Isoamylase*, *Manihot esculenta*, starch

RÉSUMÉ

Dans le concept actuel de biosynthèse de l'amidon dans les plantes, l'amylopectine qui est la principale fraction de l'amidon, est synthétisée par des actions conjuguées de l'ADP-Glc pyrophosphorylase (AGPase), l'amidon soluble de synthase (SS), l'amidon branchant les enzymes (BE) et celui débranchant les enzymes (DBE). Nous avons isolé un clone d'ADNc du gène Isoamylase1, un membre de la famille de DBE de la racine de stockage du manioc (*Manihot esculenta* Crantz). Un clone d'ADNc de gène Isoamylase1 a été isolé à partir de la racine de stockage du manioc (*Manihot esculenta* Crantz). Le fragment de la séquence d'ADNc (764 bp) cloné a montré une identité élevée de 90% de Rcisa1 et 81% d'identité de Psisa1, Stisa1 et Atisa1. La séquence de protéine déduite a montré une identité avec RCISA1 la plus élevée (92%). L'analyse comparative des séquences a confirmé que le fragment cloné était le gène M. *Esculenta* de l'*Isoamylase1* (*Meisa1; numéro d'accession GU229751*). L' analyse du génome a révélé une occurrence d'au moins deux copies du gène de Meisa1. De niveaux élevés d'expression d'écriture ont été aussi détectés dans la racine fibreuse ensuite dans la tige et moins détectable dans la feuille et la pétiole. Analyse de l'expression du profil temporel dans la racine de stockage a montré une expression initiale et maximale de 90 jours après la plantation (JAP), et qui, ensuite est retombée à des niveaux indétectables 180 JAP. Les résultats impliquent un rôle majeur du *Meisa1*dans la différenciation de racine de stockage et l'initiation précauce de granules d'amidon.

Mots Clés: *Isoamylase, Manihot esculenta*, amidon

INTRODUCTION

Cassava (*Manihot esculenta*, Crantz) is a major starchy staple crop for half billion people in the tropical and sub-tropical parts of the world (IFAD and FAO 2000). Cassava starch has wide uses in food and non-food applications based on physico-chemical characteristics (Nuwamanya *et al*., 2009), which are influenced by the proportions of amylopectin and amylose. Starch is a huge complex structure made of α-glucan monomer units stored in the chloroplast as transient starch and amyloplast as storage starch. The glucan molecule is a heterogeneous mixture of highly branched amylopectin and less branched amylose fractions. These fractions have α-1, 4-linked glucose linear units with α-1, 6 branches; the length and number of branches vary between and within cultivars, and among species (Patron and Keeling, 2005).

Starch biosynthesis is mediated by a multiple of enzymes including ADP glucose pyrophosporylase, starch synthase, starch branching, and starch debranching enzymes. In higher plants, two debranching enzymes namely isoamylase and pullulanase have been reported (Nakamura *et al*., 1996). They differ in their substrate specificity; isoamylase catalyses amylopectin, glycogen and phytoglycogen whereas pullulanase catalyzes amylopectin and pullulan. However, both enzymes hydrolyze α-1, 6 branch points in their respective substrates (Nakamura *et al*., 1996). *Isoamylase1* genes have been cloned from a number of crop species e.g. barely (Sun *et al*., 1999), sweetpotato (Kim *et al*., 2005), bean (Takashima *et al*., 2007) and their roles validated. However, little is known about the nature, characteristics and role of isoamylase in cassava.

Mutants that have deficiency in *Isoamylase1* gene such as maize (*Sugary1* or *su1*; James *et al*., 1995), rice (*Sugary1* or *sug-1*; Nakamura *et al*., 1996), Chlamydomonas (*sta7*; Mouille *et al*., 1996,), Arabidopsis (*dbe*; Zeeman *et al*., 1998) and Barely (*notch2*; Burton *et al*., 2002) have altered number and spatial distribution of branches in amylopectin. The mutant accumulates semi-crystalline water soluble polysaccharides called phytoglycogen. Complementation of rice S*ugary1* mutant by wheat *Isoamylase1* restored amylopectin synthesis and granule structure in rice endosperm (Kubo *et al*., 2005). On contrary, antisense inhibition of rice *Isoamylase1* restored *Sugary1* mutant phenotype (Fujita *et al*., 2003). In general, isoamylase enzymes are involved in amylopectin biosynthesis in concert with other starch biosynthesis enzymes.

To understand the role of *isoamylase1* in cassava starch metabolism, *Isoamylase1* cDNA was cloned for the first time and examined its genomic copy number, spatial and temporal expression patterns.

MATERIALS AND METHODS

Plant material. Cassava cultivar 92/00057 was grown in the BioCenter phytotron, the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. Plant samples were collected from leaf, petiole, stem, fibrous root and storage root at mid-day, the peak period for starch biosynthesis enzymes *SbeI* and *SbeII* (Baguma *et al*., 2003) and used for genomic and transcript analysis.

RNA gel blot analysis. Total RNA was isolated from different cassava plant parts using the extraction buffer described by Salehuzzaman *et al*. (1994) with addition of 50 mM of ß marcaptoethanol. 50 µg of total RNA was electrophoresed on denatured agarose gel and blotted onto nylon membranes (Hybond-N, Amersham) according to Sambrook *et al.*'s (1989) method. The membranes were hybridized at 42 ^{o}C in 6 X SSC, 50 % formamide, 5 X Denhadt's solution, 0.5 % SDS, 150 µg ml^{-1} denatured salmon sperm and 1 - 2 ng ml^{-1} of probe *Meisa1* or *SbeII* (Baguma *et al*., 2003). The probes were labeled using α- [^{32}P]-dCTP- Rediprime II Random Prime labeling system according to manufacturer's instructions (Amersham Bioscience, Uppsala Sweden). The membranes were washed as described by Baguma *et al*. (2003) and exposed to X- ray films (Fuji, Japan) for seven to fourteen days depending on signal intensity.

Isolation of *Isoamylase1* gene. First strand cDNA was synthesized from 3 µg total RNA extracted from storage root using Moloney Murine Leukemia Virus (MMLV)-RT and oligo(dT)$_{18}$ primer, following manufacturer's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). PCR amplification of *Isoamylase1* gene was conducted using degenerate primers designed from multiple alignments of predicted *Isoamylase1* amino acid sequences of five plant species (*Pisum sativum* (gi DQ092413), *Ipomoea batatas* (gi DQ074643), *Phaseolus vulgaris* (gi AB300052), *Arabidopsis thaliana* (gi NM129551), and *Solanum tubersom* (gi AY132996), using the program T and coffee (Geneva, Switzerland) and the DePict 1.0 web interface (http://www.cs.fiu.edu/~giri/bioinf/DePiCt1.0/WebVersion/depict.htm). The resulting primers were AARGGKGARTTYTAYAAYTA and YTCKTCYTTYTTRTCCCA for sense and antisense, respectively. The amplification consisted of 35 cycles of denaturing (95°C, 1 min), annealing (50°C, 2 min) and extension (72°C, 2 min). The PCR product of the expected size was cloned into the pCR®2.1-TOPO® vector using the TOPO TA cloning® kit (Invitrogen, Carlsbad, USA). Plasmid was isolated based on Wizard Minipreps columns (Promega, USA) and analyzed for the insert as described by Sambrook *et al.* (1989).

DNA sequencing was performed on ABI3730XL sequencer (Applied Biosystem, Macrogen Inc. Seoul, Republic of Korea). Nucleotide sequence was used in a BLAST search for similarity with other published sequences in NCBI database (http://www.ncbi.nlm.nih.gov/Blast.cgi). Using Clustalw2 (http://www.ebi.ac.uk/Tools/clustalw2/) identity of the cloned sequence was compared with other similar gene sequences. The cloned nucleotide sequence was deduced into protein using EBI (http://www.ebi.ac.uk/Tools/emboss/transeq/) and similarly compared to other sequences in the database using Protein blast in NCBI database.

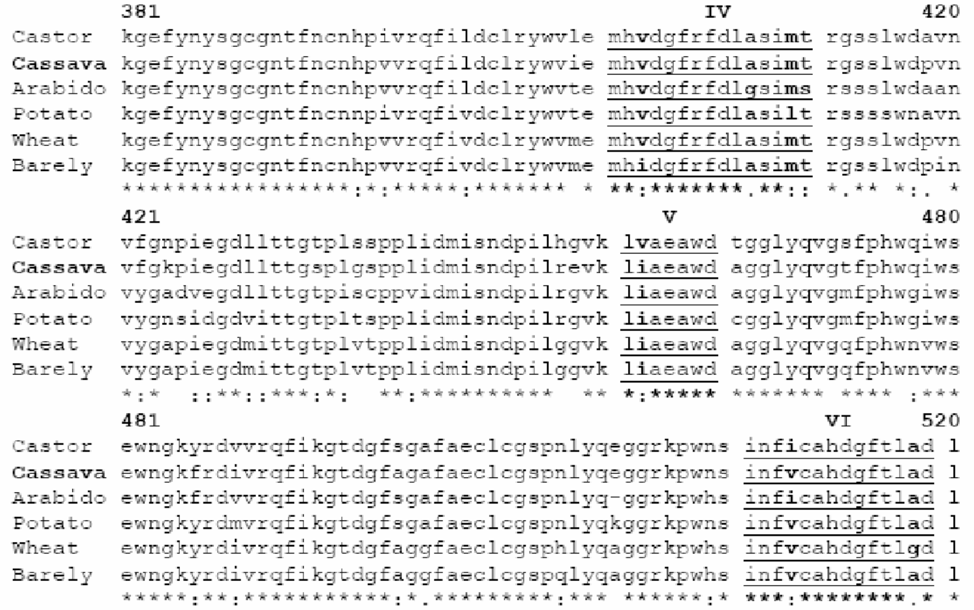
DNA gel blotting. Nuclear genomic DNA was extracted from the upper most twigs of cassava leaves using DNeasy® Plant Mini Kit (QIAGEN). 15 µg genomic DNA was digested overnight with *EcoRV* and *HindIII* (non-cutters), and *NcoI* which cuts once in the probe (Sambrook *et al.*, 1989). The gel was blotted onto nylon membranes (Hybond-N, Amersham) as described by Sambrook *et al.* (1989), hybridized and exposed to X-ray film for three to seven days depending on signal intensity.

RESULT AND DISCUSSION

***Isoamylase1* gene in cassava.** Sequence analysis of the cloned cDNA fragment contained 764 bp harboured within the open reading frame of *isoamylase1*, hereafter named *Meisa1* (accession number; GU229751). The nucleotide BLAST analysis showed high identity ranging between 81 and 90% to *isoamylase1* cDNAs of *Ricinus communis* (XM_002529854.1), *Pisum sativum* (DQ092413.1), *Solanum tuberosum* (AY132996.1) and *Arabidopsis thaliana* (NM 129551.3). The deduced protein sequence showed 92% identity with *R. communis RCISA1*. *Meisa1* has typical conserved motifs of α-amylase super family (Jesperson *et al*., 1993; Beatty *et al*., 1999) as illustrated in Figure 1. A multiple alignment of *Meisa1* with predicted amino acid sequences of isoamylase isoforms from other plant species showed strong similarity with *Isoamylase1* compared to other isoforms (Table 1).

The comparative sequence analysis confirmed the cloned fragment to be *M. esculenta isoamylase1* gene. This is further confirmed by the low sequence identity between *Meisa1* and other isoforms of isoamylases (Table 1). At the protein level, three of the six conserved motifs of the α-amylase family typical of starch hydrolysing enzymes (Jesperson *et al*., 1993; Beatty *et al*., 1999) were observed suggesting that the cloned fragment is part of the gene that is involved in branching or debranching activities.

The similarity of *Isoamylase1* between cassava and castor bean (*R. communis*) confirmed their close relatedness compared with potato, pea and Arabidopsis. The high level of similarity between castor bean and cassava could have an evolutionary bearing since the two species belong to the same family, *Euphorbeaceae*. This is consistent with previous comparisons between castor bean genes and ESTs from other available *Euphorbeaceae* species, which showed that cassava shares the highest sequence similarity with castor bean (http://castorbean.jcvi.org). Given the high synteny of castor bean and cassava genome, and



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        381                                 IV              420
Castor  kgefynysgcgntfncnhpivrqfildclrywvle mhvdgfrfdlasimt rgsslwdavn
Cassava kgefynysgcgntfncnhpvvrqfildclrywvie mhvdgfrfdlasimt rgsslwdpvn
Arabido kgefynysgcgntfncnhpvvrqfildclrywvte mhvdgfrfdlgsims rssslwdaan
Potato  kgefynysgcgntfncnnpivrqfivdclrywvte mhvdgfrfdlasilt rssswnavn
Wheat   kgefynysgcgntfncnhpvvrqfivdclrywvme mhvdgfrfdlasimt rgsslwdpvn
Barely  kgefynysgcgntfncnhpvvrqfivdclrywvme mhidgfrfdlasimt rgsslwdpin
        *****************:*:*****:******* * **:*******.**:: *.** *:. *
        421                               V                480
Castor  vfgnpiegdllttgtplsspplidmisndpilhgvk lvaeawd tgglyqvgsfphwqiws
Cassava vfgkpiegdllttgsplgspplidmisndpilrevk liaeawd agglyqvgtfphwqiws
Arabido vygadvegdllttgtpiscppvidmisndpilrgvk liaeawd agglyqvgmfphwgiws
Potato  vygnsidgdvittgtpltspplidmisndpilrgvk liaeawd cgglyqvgmfphwgiws
Wheat   vygapiegdmittgtplvtpplidmisndpilggvk liaeawd agglyqvgqfphwnvws
Barely  vygapiegdmittgtplvtpplidmisndpilggvk liaeawd agglyqvgqfphwnvws
        *:*  ::**::***:*:  **:**********  ** *:***** ******* **** :***
        481                                                VI     520
Castor  ewngkyrdvvrqfikgtdgfsgafaeclcgspnlyqeggrkpwns inficahdgftlad l
Cassava ewngkfrdivrqfikgtdgfagafaeclcgspnlyqeggrkpwns infvcahdgftlad l
Arabido ewngkfrdvvrqfikgtdgfsgafaeclcgspnlyq-ggrkpwhs inficahdgftlad l
Potato  ewngkyrdmvrqfikgtdgfsgafaeclcgspnlyqkggrkpwns infvcahdgftlad l
Wheat   ewngkyrdivrqfikgtdgfaggfaeclcgsphlyqaggrkpwhs infvcahdgftlgd l
Barely  ewngkyrdivrqfikgtdgfaggfaeclcgspqlyqaggrkpwhs infvcahdgftlad l
        *****:**:***********:*.*********:*** ******:* ***:********.* *
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Figure 1. Multiple protein sequence alignments of isoamylase-type debraching enzymes from representative database sequences of Castor (*Ricinus comminus*, AC XM002529854), Cassava (*Manihot esculenta*, AC GC229751), Arabido (*Arabidopsis thaliana,* AC NM 129551.3), Potato (*Solanum tuberosum*, AC AY132996.1), Wheat (*Triticum aestivum*, AC AF548380), Barely (*Hordeum vulgare*, AC AAM46866. Conserved sequences are designated with * while mismatch is denoted by a dot. Motifs designated as IV, V & VI are conserved in the *Meisa1.*

the fact that castor bean has only one reported isoform of *isoamylase1*, there is high likelihood that only one isoform of isoamylase exists in the cassava genome. This study only identified *isoamylase1* as is the case in castor bean.

Copy number of *Meisa1* gene. Restriction digest with *EcoRV* and *HindIII* that do not cut the probe produced two and four bands, respectively whereas *NcoI* which cuts once produced six bands (Fig. 2). This suggests that *Meisa1* gene occurs in at least two copies in cassava genome. Starch granule bound synthase1 (*GbssI*) has been reported to occur in two copies in cassava genome (Salehuzzaman *et al*., 1993). The occurrence of two or more copies of *Meisa1* and *GbssI* genes could be due to the ploidy level of cassava genome (Awoleye *et al.*, 1994). However, *SbeI* (Salehuzzaman *et al*., 1992) and *SbeII* (Baguma *et al*., 2003) have been reported to occur as single copies. These two genes could also be a consequence of duplication of the same ancestral gene. This further supports the phenomenon for the existence of allelic variants in highly heterozygous allotetraploid cassava genome (Awoleye *et al.*, 1994).

Spatial and temporal expression of *Meisa1*. Using the probe developed against *Meisa1*, it was possible to detect its transcript profile in different parts of the cassava plant. The transcripts were detected in tissues of all plant parts studied. The expression of *Meisa1*, as exhibited by the levels of transcript expression, differed in different plant parts and stage of development of the cassava plant (Fig. 3). The expression of *Meisa1* was first detected at 90 days after planting (DAP), and this was the time when highest expression was observed in the fibrous root followed by the stem, the storage root and least in the leaf and petiole (Fig. 3A). Similarly, the expression of *SbeII* followed the same pattern with maximum expression in fibrous root and stem and detectable levels in petiole. At 270 days after planting, *Meisa1* transcript expression was highest in fibrous root followed by the leaf and least in the stem (Fig. 3B). In contrast, *SbeII* expression was highest in the storage root

TABLE 1. Protein identity score of isoamylase isoforms from *M. esculenta* (*Meisa1*), *R. communis* (*Rcisa1*), *A. thaliana* (*Atisa1*, *Atisa2* and *Atisa3*) and *S. tuberosum* (*Stisa1*, *Stisa2*, *Stisa3*)

	Meisa1	*Rcisa1*	*Stisa1*	*Atisa1*	*Stisa2*	*Atisa2*	*Stisa3*	*Atisa3*	AminoAcid length
Meisa1	100								255
Rcisa1	92	100							795
Stisa1	87	72	100						793
Atisa1	87	71	72	100					783
Stisa2	38	29	28	28	100				878
Atisa2	34	26	28	26	52	100			882
Stisa3	55	42	44	42	29	29	100		766
Atisa3	56	42	44	42	31	31	67	100	764

Protein ID· *Meisa1*, GU229751, Rcisa1, EQ 974162; *Atisa1*, NP181522; *Atisa2*, NP973751; *Atisa3*, NP192641; *Stisa1*, AAN15317; *Stisa2*, AAN15318; *Stisa3*, AAN15319

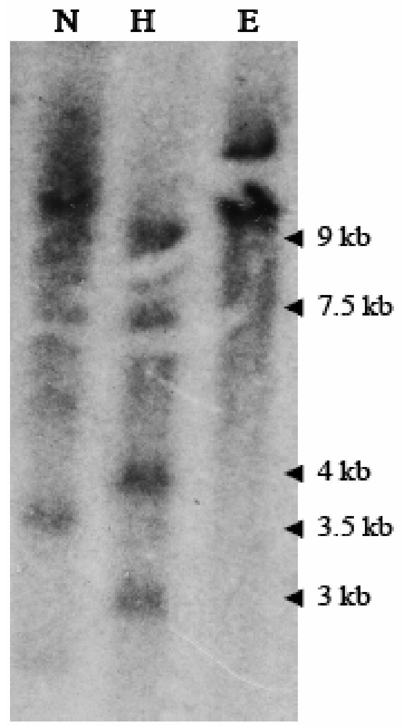

Figure 2. *Meisa1* gene copy number in the cassava genome. Genomic DNA was digested with three restriction enzymes, *NcoI* (N), *HindIII* (H) and *EcoRV* (E) with one (N) or no (H and E) cleavage sites in the probe.

followed by petiole and weakly expressed in the fibrous root. Very high levels of *Meisa1* transcript were detected at 90 days after planting while very low levels observed at 120 days after planting and no detectable level at 180, 240 and 270 days after planting (Fig. 3C) in the storage root. Overtime (90 to 270 days after planting), the expression of the *Meisa1* is limited to a very narrow window, a time that corresponds with tuber initiation.

The observed tissue specific expression pattern of *Meisa1* was similar with previously reported patterns from other starch metabolizing genes in cassava (Salehuzzaman *et al.*, 1992; Salehuzzaman *et al.*, 1994; Baguma *et al.*, 2003). *Meisa1* declined with increased developmental stage of the storage root (Fig. 3C) whereas *SbeI*, *SbeII*, gbssI and *gbssII* (Baguma *et al.*, 2003) increased progressively. The association of *Meisa1* with cassava plant tissues predominantly packed with transient starch suggests a possible role in granule initiation and tuberization. This is

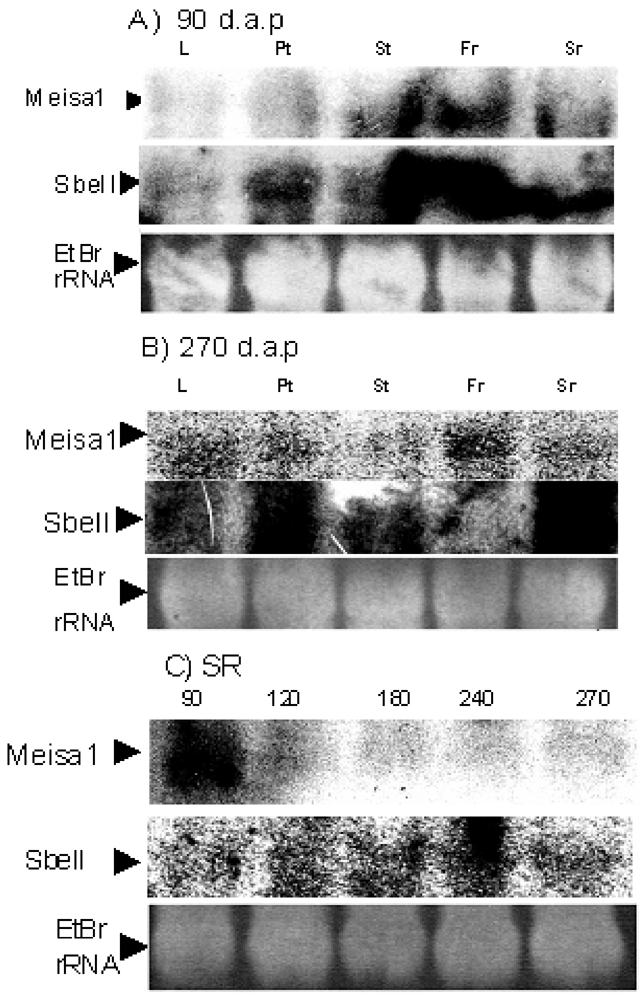

Figure 3. Expression pattern of the cassava *Isomylase* gene (*Meisa1*) and starch branching enzyme II (*SbeII*) in cassava. A. Tissues specific expression at 90 DAP. B. Tissues specific expression at 270 DAP. C. Temporal expression in the storage root of cassava. EtBr; Ethidium Bromide, rRNA; 28S ribosome RNA as internal control for equal loading. L, leaf; Pt, petiole; St, stem; Fr, fibrous root, Sr, storage root.

consistent with previous results where isoamylases have been implicated in granule initiation (Bustos *et al.*, 2004) and γ-rays irradiation produced small granule sized mutants presumably associated with gene mutation of one of isoamylase isoforms (*Iso1* or *Iso2*) (Ceballos *et al.*, 2008). Also, agreeable with the trimming model for starch synthesis (Ball *et al.*, 1996; Myers *et al.*, 2000) that asserts that starch synthesis starts from amylopectin (short branched chains) later on the chains are elongated into amylose and finally compacted into complete starch granules.

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