Regulation of Starch Synthesis in Cassava

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Abstract

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Cassava (*Manihot esculenta* Crantz) is a root crop, one of the world's most important but under-exploited staple food crops and source of income. It is a high starch producer with levels between 73.7 and 84.9% of its total storage root dry weight. Increasingly, there is a need for diverse *novel* starches for both food and non-food applications. In response, *sbeII* encoding starch branching enzyme II was cloned. The relationship between spatial-temporal expression patterns of starch synthesis genes and the plasticity of the storage root development states was examined. To gain further insight into the transcriptional activity of *sbe*, diurnal transcript abundances, sugar and hormone signaling were studied.

Results showed that the transcriptional activity of *sbe* increased with the developmental states of the storage root. Analysis of *sbe* diurnal transcript patterns identified the existence of an endogenous semidian oscillator (12 h) in the storage root cells but its nature and function remains unknown. To elucidate the relationship between *sbe* expression and sugar status; a combination of biochemical, genomic, histological and therapeutic approaches were used. In these analyses, sucrose was identified as the main signal that mediates transcriptional induction of *sbe*. Other identified effectors were abscisic acid (ABA), glucose, glucose-1-phosphate (G-1-P) and turanose. Repeated experimentation located the semidian oscillator upstream of G-1-P/G-6-P but downstream of glucose at the level of hexokinase. The discovery that *sbe* expression is not induced by mannose, mannitol, 3-*O*-methyl-glucose and palatinose, but turanose, uncovered the existence of a sucrose transporter (SUT) and/or sensor. Notably, the induced *sbe* expression profile and level, and the biochemical properties of sucrose and turanose suggested that the SUT might as well as serve a sensor, although the existence of an independent extracellular sensor was not precluded. It was also shown that sucrose and ABA singly or in combination mediate *sbe* expression, and ABA by itself or with sucrose decouples the endogenous semidian oscillator via a bypass mechanism. Furthermore, it was revealed that either sucrose or ABA is not sufficient to promote maximal *sbe* expression, but the dual additive interactive effect is essential. The study also revealed the importance of protein phosphatases, protein kinases and first evidence for plastid *de novo* protein synthesis in the regulation of *sbe* expression. Okadaic acid, which preferentially inhibits type 1 and 2A protein phosphatases, PP1 and PP2A, respectively, abolished *sbe* expression, in the presence or absence of other effectors, i.e., sucrose, glucose, G-1-P, turanose and ABA. Conversely, cantharidin, a potent inhibitor of PP2A, did not, suggesting that sugar and ABA signaling pathways converge at the PP1 level downstream of the sugar signaling pathway. Chloramphenicol, which specifically inhibits plastid *de novo* protein synthesis, with or without sucrose, blocked *sbe* expression but cycloheximide, a cytosolic *de novo* protein synthesis inhibitor, did not, suggesting a role of plastid *de novo* protein synthesis in the regulation of *sbe*. The emerging implications from this study have been summarised into a working regulatory model for *sbe* expression.

In summary, this work has established that *sbe* transcriptional activity depends on the plasticity of the storage root growth and development states, rhythmicity of an endogenous semidian oscillator, interactive effects of sucrose and ABA signaling pathways, plastid *de novo* protein synthesis, and dephosphorylation events.

Key words: ADPG; alternative splicing; amylopectin; amylose; plastid signal

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To my parents and family

"A will to fight till the heights are won is the treasure of success"

CONTENTS

Appendix

List of publications[2]

This thesis is based on the following papers, which will be referred to by their assigned Roman numerals.

I. Expression patterns of the gene encoding starch branching enzyme II in the storage roots of cassava (*Manihot esculenta* Crantz) (2003) **Yona Baguma**, Chuanxin Sun, Staffan Ahlandsberg, Joel Mutisya, Sara Palmqvist, Patrick R. Rubaihayo, Michael J. Magambo, Thomas G. Egwang, Håkan Larsson, Christer Jansson.
*Plant Science **164**, (4): 833-839*

II. Sugar-mediated semidian oscillation of starch synthesis genes in the cassava storage root (2004) **Yona Baguma**, Chuanxin Sun, Mats Borén, Helena Olsson, Sara Palmqvist, Joel Mutisya, Patrick R. Rubaihayo, Christer Jansson.
PNAS. Submitted

III. Profiling starch accumulation in the storage roots of cassava (2004) **Yona Baguma**, Chuanxin Sun, Mats Borén, Joel Mutisya, Helena Olsson, Sara Palmqvist, Linley Chiwona-Karltun, Patrick R. Rubaihayo, Christer Jansson.
Science. Submitted

IV. Evidence for nucleo-plastid signaling during regulation of starch synthesis genes in the cassava storage root (2004) **Yona Baguma**, Chuanxin Sun, Joel Mutisya, Patrick R. Rubaihayo, Christer Jansson.
Manuscript

Additional publications

I. Starch branching enzymes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*): Comparative analyses of enzyme structure and gene expression (2003) Joel Mutisya, P. Sathish, Chuanxin Sun, Lena Andersson, Staffan Ahland*sb*erg, **Yona Baguma**, Sara Palmqvist, Benjamin Odhiambo, Per Åman, Christer Jansson.
*Journal of Plant Physiology **160**, (8): 921-930*

II. Transcriptional regulation of the *sbeIIb* genes in sorghum (*Sorghum bicolor* and barley (*Hordeum vulgare*): importance of the barley *sbeIIb* second intron (2004) Joel Mutisya, Chuanxin Sun, Sara Palmqvist, **Yona Baguma**, Benjamin Odhiambo, Christer Jansson.
Journal of Plant Physiology. Submitted

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Abbreviations

List of selected abbreviations commonly used in the text:

ABA	abscisic acid
ADPG	ADP-glucose
AGPase	ADP glucose pyrophosphorylase
ATP	adenosine triphosphate
cDNA	complementary DNA
dap	days after planting
DBE	starch debranching enzyme
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Fru	fructose
FRK	fructokinase
GBSS	granule bound starch synthase
Glc	glucose
Glc-1-P	glucose-1-phosphate
Glc-6-P	glucose-6-phosphate
HXK	hexokinase
Kb	kilobases
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
Pi	inorganic phosphate
PPi	pyrophosphate
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
sbe	starch branching enzyme genes
SBE	starch branching enzyme
SDS	sodium dedocyl sulphate
SI	sucrose invertase
SPS	sucrose phosphate synthase
SS	starch synthase
SUC	sucrose
SuSy	sucrose synthase
SUT	sucrose transporter
6-dGlc	6-deoxy-D-glucose
3-*O*-mGlc	3-ortho-methylygluco

1. Introduction

1.1. Cassava

Cassava (*Manihot esculenta* Crantz) is a root crop belonging to section fructicosae of family Euphorbiaceae, Dicotyledonae (Jos, 1969). It is one of the world's most important, and under-exploited staple food crops and source of income. It is the third most important source of calories in the tropics consumed by some 600 million people on a daily basis in Africa, Asia, and Latin America. It provides a cheap source of dietary carbohydrate energy (720.1 x 1012 kJ day^{-1}) ranking fourth after rice, sugarcane and maize, and sixth among crops in global production. In Sub-Saharan Africa, it is the leading food security base and a major source of cash incomes for most resource constrained households. A more significant attribute, however, is that cassava withstands unreliable rains and drought conditions, performs well on soils with low fertility, and has high productivity and low labour demands compared to other major food crops. Moreover, cassava is further more significant in communities with a weakened labour force as a result of the ravages of HIV/AIDs. Consequently, the Food and Agriculture Organisation (FAO) of United Nations (UN) has identified cassava as a crop in East Africa that *"will spur rural industrial development and raise incomes for producers, processors and traders, and contribute to the food security of its producing and consuming households"*. Recently, the New Partnership for Africa's Development (NEPAD) has identified cassava as *'A Poverty Fighter in Africa'* and launched a Pan African Cassava Initiative that seeks to tap the enormous potential of the crop for food security and income generation (NEPAD, 2004). In Latin American, the Consortium of Latin America and the Caribbean for cassava research for development (CLAYUCA), a private-public initiative, is promoting cassava as a raw material for animal feed, ethanol and starch industries (Ceballos, 2002). In South East Asia, cassava is being exploited for ethanol for automobile fuel (Klanarong *et al.,* 2003). Overall, in a global perspective, there is a commitment to propel the contribution of cassava for human welfare by integrating biodiversity, biotechnology and breeding strategies under the *"Global Cassava Improvement Plan (GCPI)"*. Together, these initiatives define an evolving and dynamic role of cassava as a catalyst for development.

Cassava is a high starch producer with levels between 73.7 and 84.9% of its total storage root dry weight (Fig. 1). This attribute together with the unique properties of its starch creates demand for particular food and nonfood applications. For example, cassava starch readily gelatinises on cooking with water and the solution remains comparatively fluid after cooling. The excellent clarity of its starch is desirable for transparent gels, its bland flavour in pharmaceuticals, and its resistance to shear stress and freezing in the film-forming industries. Besides, cassava starch is used in baby foods, gari, chips, sago, pappads, paints, corrugated boxes, plastics and the tanning of leather. More recently, cassava has found a speciality in the production of synthetic rice. In spite of its potential, cassava starch remains under-exploited mainly because of considerable fluctuations in starch grades and qualities supplied. In response, a project *"metabolic engineering of starch biosynthesis in cassava"* was developed to contribute to improved starch quantity and quality.

1.2. Starch and its applications

Starch is the most important form of carbon reserve in photosynthetic eukaryotes or their nonphotosynthetic derivatives (apicomplexa parasites or dinoflagellates) (Ball & Morell, 2003). It is a glucose polymer of α-glucans linked by α-1,4 bonds and branched at α-1,6 positions (Ball & Morell, 2003; Ball *et al.,* 1996). It exists in the leaf chloroplasts as transitory starch (i.e. the primary product of photosynthesis) and in the amyloplasts as storage starch (Ball *et al.,* 1996). It is a huge (0.1 > 50 μm in diameter) complex quaternary structure made up of two major glucan polymers: amylopectin (85 - 70%) and amylose (15 - 30%) (Fig. 2). Amylopectin (10^7 - 10^9 Da), by far the major component in leaf starch is composed of intermediate size α-1,4 linked glucans that are clustered together and hooked to longer spacer glucans by α-1,6 linkages, and is responsible for the granular nature of starch (Ball & Morell, 2003). Amylose (10^5 - 10^6 Da), which constitutes between 11% and 37% of storage starch (Shannon & Garwood, 1984), is smaller, essentially linear with less than 1% α-1,6 branches and synthesised within the matrix formed by amylopectin (Ball & Morell, 2003; Buléon *et al.,* 1998; Takeda, Guan & Preiss, 1993). In general, amylopectin is similar to glycogen (10^7 Da) except for its fewer branch points (*ca*. 5% of the total linkages), which are discontinuously arranged resulting into clusters of unbranched chains (Fig. 2c). Other quantitatively minor components of starch include proteins, lipids and minerals. Cereal starches have protein levels between 0.25 and 0.5%, while in potato tuber and cassava, the levels are generally <0.1%. On the other hand, the starch from cereal endosperm contains little or no phosphate (Blennow *et al.,* 2000; Ritte *et al.,* 2000), whereas in dicots, for example potato (Nielsen *et al.,* 1994) and *Arabidopsis* (Yu *et al.,* 2001), approximately 10 and 1 in every 2000-glucosyl residues are phosphorylated, respectively.

As early as 4000 – 3500 B.C, the Egyptians obtained the first starch from wheat. At present, starches are made from many different raw materials such as maize, rice, potato, cassava, barley, sweet potato and wheat. The intact (native) starch granules are used in the manufacture of facial and talcum powders, separators of carbonless copy paper to prevent premature rupturing of ink microcapsules on the bottom sheets and as filler material in the production of thin plastic films. In the food industry, high amylose starches are used as resistant starch in functional foods where they provide a low glycemic index (the rise or fall in blood sugar) and prevent colon cancer. Resistant starch is that fraction of the starch, which escapes human small intestinal digestion and enters the large bowel where it is fermented by the resident microflora. The resulting short chain fatty acids are metabolised and are beneficial in many health conditions. Paste producing starches are used to thicken products such as soups, sauces, gravies and dairy products. In addition to the food industry, there are many industrial uses of starch. Starches with high levels of amylopectin are used in paper coatings and adhesives, as viscosity modifying agents in drilling mud during oil exploration, and as joint compounds for finishing gypsum panel walls in the construction industry. In agrochemicals; starches are used as mulches, for pesticide delivery and seed coatings, while in pharmaceuticals, they are used as diluents, binders and in drug delivery.

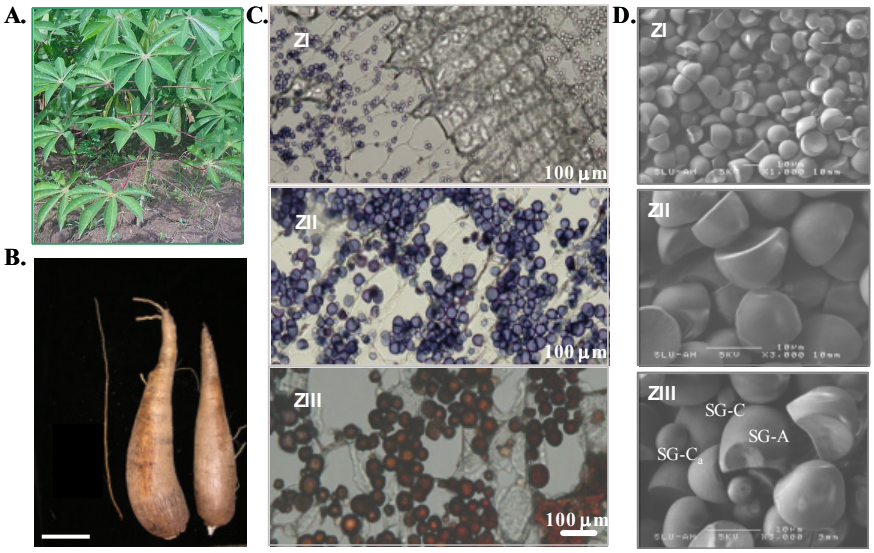

Figure 1. Display of a cassava plant and its starch granule architecture. **A**. Cassava plant. **B**. Cassava storage roots. **C**. Iodine stained map showing cellular distribution of amylose (blue-black stained) and amylopectin (reddish-brown stained fractions). **D**. The scanning electron micrograph of starch granule architecture. Abbreviations: ZI, periderm region; ZII, cortical region; ZIII, parenchyma region; SC-A, starch granule type A; SC-C, starch granule type C; SC-C_a, starch granule type C_a as described (Baguma *et al.,* submitted).

1.3. Starch biosynthesis and the enzymes involved

1.3.1. The overall pathway

The synthesis of α-1,4 glucans consists of three critical steps in the chloroplast and amyloplast (Alisdair, Willmitzer & Trethewey, 2002) (Fig. 3); the supply of glucose-6-phosphate (Glc-6-P) into the plastid, the synthesis of ADP-glucose (ADPG) from Glc-1-P, and the synthesis of starch from ADPG. Briefly, the first committed and rate-limiting step involves the synthesis of ADPG from Glc-1-P and ATP, catalysed by ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.23). Once activated, the nucleotide sugar (ADPG) is transferred by the starch synthase (SS; EC 2.4.1.21) to the nonreducing end of an α-1,4 glucan resulting into the generation of linear α-1,4 glucans. Following this, the linear α-1,4 glucans are used as substrates by starch branching enzyme (SBE or Q-enzyme; EC 2.4.1.18) to introduce α-1,6 interlinear chain linkages resulting into amylopectin. Subsequently, amylopectin is crystallized into starch by the concerted effort of starch debranching enzymes (DBE; EC 2.4.1.41), phosphorylase (P-enzyme; EC: 2.4.1.1) and glucanotransferase (D-enzyme, EC 2.4.1.25) (Ball *et al.,* 1996; Colleoni *et al.,* 1999; James, Robertson & Myers, 1995; Smith, Denyer & Martin, 1997; Sun *et al.,* 1997). Finally, UDP-glucose: protein glucosyltransferase or amylogenin (38 or 45 kDa, EC 3.6.1 category) has been speculated to be involved in the initial priming process of starch synthesis. In subsequent sections, the role and subcellular localisation of each of these enzymes is described.

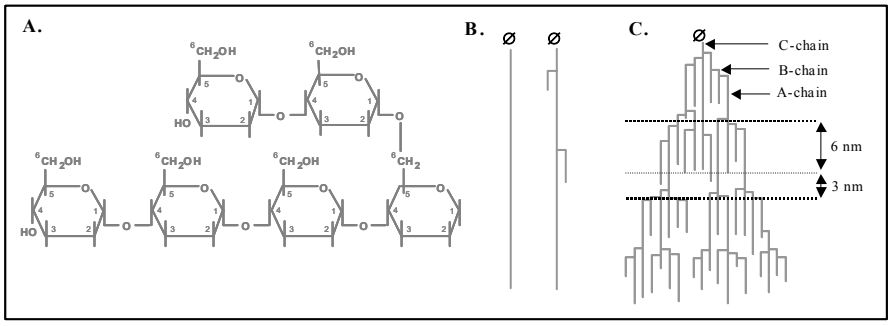

Figure 2. Schematic illustration of starch structure. **A.** Chains of α-1,4- and α-1,6-linked glucosyl residues. **B.** Amylose. **C.** Cluster structure of amylopectin with 3-nm amorphous lamellae (containing the branched regions) and 6-nm semicrystalline lamellae (containing ordered double helices), which alternate with 9-nm periodicity. ∅, reducing end.

1.3.2. The synthesis of ADPG through ADP-Glucose pyrophosphorylase (AGPase)

ADP-glucose pyrophosphorylase catalyses a rate-limiting reaction in prokaryotic glycogen and plant starch synthesis (Smith, Denyer & Martin, 1995). In either case, all share similar molecular size (*ca*. 220 kDa), catalytic and allosteric regulatory properties but differ in higher-order protein structure. As for bacterial AGPase (*glgC*), it is a homotetrameric enzyme encoded by a single gene. Conversely, higher plant AGPase is a heterotetrameric enzyme ($\alpha_2\beta_2$) composed of a pair of large subunits and a pair of small subunits, encoded by different genes (Salamone *et al.,* 2002). At the regulatory level, small effector molecules whose nature reflects the major carbon assimilatory pathway of the organism modulate the enzyme catalytic activity. Indeed, bacterial AGPase are activated by intermediates of glycolysis [e.g. pyruvate, fructose-6-phosphate (Fru-6-P), fructose-1,6-bisphosphate (Fru-1,6-bisP)] and inhibited by AMP. Likewise, blue-green algae and higher plants AGPase are activated by 3-phosphoglyceric acid (3-PGA) and inhibited by Pi, key intermediates in CO_2 assimilation by the C_3 pathway. Structure-function analysis of AGPase indicate that the sequences of the small subunits particularly the lysine residue near the *C*-terminus are highly conserved between species, whereas those of the large subunits are more divergent (Smith-White & Preiss, 1992), suggesting different roles in enzyme function. Actually, the small subunit is crucial for catalytic activity while the large subunit is important for its regulatory properties. In addition, the small subunit by itself is capable of forming a homotetrameric enzyme exhibiting near-normal catalytic properties but with impended allosteric regulatory properties. In contrast, the large subunit, is incapable of forming an active enzyme, but enhances the allosteric properties of the small subunit.

Recently, it has been shown through phylogenetic studies that plant AGPase's are distinctly localised and subdivided into leaf, stem, root and endosperm types. At plastid level, AGPase are further classified into two groups based on subcellular localisation, i.e. plastidial and cytosolic forms (Denyer *et al.,* 1996; Okita, 1992; Smith, 1988; Thorbjornsen *et al.,* 1996).

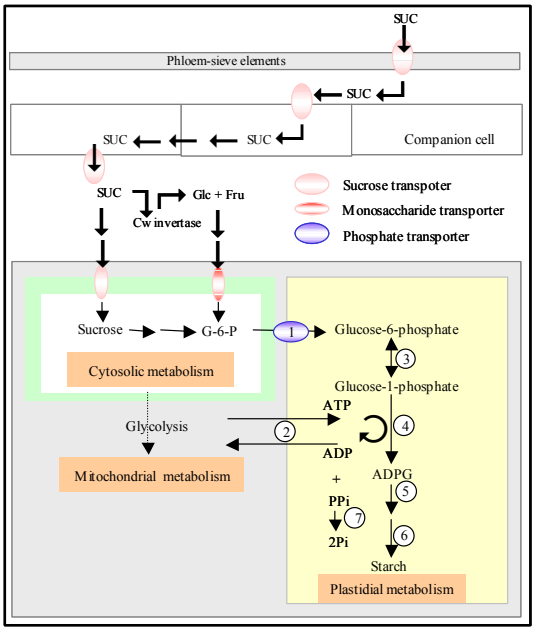

Figure 3. Phloem unloading and plastidial carbon metabolism. 1, G-6-P transporter; 2, amyloplast adenylate transporter; 3, plastidial phosphoglucomutase; 4, ADP-glucose pyrophosphorylase; 5, starch synthases; 6, starch branching enzymes; 7, inorganic pyrophosphatase; Cw invertase, cell wall invertase; Pi, inorganic phosphate; PPi, pyrophosphate.

Extended phylogenetic analysis of the small subunit proteins suggest that the cytosolic type probably evolved from the leaf localised type (Johnson *et al.,* 2003). Such lineage raises the question of a modulating factor between the two spatially localised active types: the photosynthetic and sink storage tissue types. In consonance, it has been shown that photosynthetic tissue has a pool of triose phosphates that accumulate in the chloroplast during the light cycle. Likewise, in sink tissue, 3-PGA pool is generated as an intermediate in the respiratory process of glycolysis and may be quite low whereas Pi peaks during starch accumulation. Taken together, this shows that in sink tissue, Pi inhibition may dominate over 3-PGA activation of AGPase, and phosphate may be the key physiological factor that modulates AGPase activity. This is further corroborated by the fact that maize endosperm AGPase, which is mainly cytosolic, exhibits significant sensitivity to Fru-6-P activation atypical of chloroplast or tuber AGPase (Salamone *et al.,* 2002). Conversely, tissues without an extraplastidial AGPase, glucose-6-phosphate (Glc-6-P) is transported into the amyloplast, where plastidic phosphoglucomutase converts it to Glc-1-P for ADPG synthesis by plastidic AGPase (Fig. 3).

Insight into the role of cytosolic and plastidial AGPase in starch synthesis has been gained through studies involving mutants of maize, *Shrunken-2* and *Brittle-2* (Denyer *et al.,* 1996; Giroux, 1996; Hannah & Nelson, 1976) and barley, *Ris∅16* (Johnson *et al.,* 2003). *Ris∅16* lacks cytosolic AGPase activity but has unaffected

plastidial activity. *Ris∅ 16* exhibits reduced starch content indicating that a cytosolic AGPase is required to achieve the normal rate of starch synthesis. On the other hand, both A- and B-type starch granules are present in *Ris∅ 16* showing that the cytosolic form is not necessary for the synthesis of these two granule types. In barley, the plastidial activity by itself is sufficient for normal starch synthesis, albeit reduced rate of accumulation. In contrast, maize plastidial activity alone is not sufficient for normal rate of starch accumulation (Thorbjornsen *et al.,* 1996). Considering that these conflicting lines of evidence are not a consequence of experimental error, it is deduced that knowledge gained from one species may not directly apply to other allied crops. Moreover, in spite of this progress, more work is required to completely understand the physiological function of the various AGPase isoforms.

1.3.3. The synthesis of amylose by granule bound starch synthase I (GBSSI)

GBSSI belongs to the class of starch synthases. All members appear to share the same basic structure, consisting of a glass domain (substrate-binding site), a typical transit peptide (Harn *et al.,* 1998) and eight motifs (Cao *et al.,* 1999; Vrinten & Nakamura, 2000). The class is classified into three distinctly localised fractions in the plastids: those bound exclusively to the granule (granular-bound starch synthases, GBSS); those with exclusive or nearly exclusive activity in the soluble phase (starch synthases, SS); and ones present in both the granule-bound and soluble phase. In addition, the fractions are further subdivided into four subclasses based upon cDNA and amino acid sequences, i.e. GBSS (60 kDa), SSI (57 kDa), SSII (77 kDa) and SSIII (110-140 kDa). More recently, pea GBSSI has been further subdivided into GBSSIa and GBSSIb isoforms (Edwards *et al.,* 2002). Likewise, in monocots, there is evidence to suggest that SSII further diverged into two subdivisions, SSIIa and SSIIb (Harn *et al.,* 1998).

The synthesis of amylose was first attributed to the major granule-bound starch synthase I (GBSSI) many decades ago (Leloir, De Fekete & Cardini, 1961). Since then, this discovery has been corroborated by several independent studies involving *waxy* mutants with a defective *gbssI* gene product. Such mutants have been identified in various species e.g., rice (Murata, Sugiyama & Akazawa, 1965), maize (Weatherwax, 1922), wheat (Nakamura *et al.,* 1995), barley (Ishikawa, Ishihara & Itoh, 1994), *amf* potato (Hovenkamp-Hermelink, 1987) and *Iam* pea (Denyer, Foster & Smith, 1995). To corroborate the *waxy* phenotype, *gbssI* in potato was antisensed and analyses of the corresponding amylose content in the transgenic lines showed a marked decrease (Visser, 1991). Unexpectedly, despite compelling evidence that GBSSI is the sole enzyme in amylose synthesis, graminea *waxy* mutants were recently shown to accumulate normal starch granules in tissues such as pericarp, leaf, stem and root indicating that another gene(s) controls amylose production. For example, the leaves and stem of *waxy* rice, the leaves and pericarp of *waxy* maize, the pericarp of *waxy* wheat, and the pods, leaves and nodules of *Iam* pea all contain amylose. This observation raised the possibility that although GBSSI has been considered a sole player in the synthesis of amylose, other players are involved. Recently, this was strengthened by the

isolation of a second isoform of GBSSI, designated GBSSII from *waxy* wheat (Nakamura *et al.,* 1998) and pea leaves, designated GBSSIb (Edwards *et al.,* 2002). Indeed, both isoforms were demonstrated to be involved in the synthesis of amylose.

1.3.4. The synthesis of amylopectin by the soluble starch synthases (SS)

Various studies have demonstrated that SSI, SSII and SSIII are involved in amylopectin synthesis, although the role of SSI and SSIIb remains unclear. In pea, studies of *rug5* (Craig *et al.,* 1998), which is closely allied with a defect in *ssII* gene showed altered amylopectin branching pattern with decreased intermediate-sized glucans (dp 15 - 25) and increased short-chain glucans (dp < 10) (Craig *et al.,* 1998; Fontaine *et al.,* 1993). In potato tubers, antisense inhibition of SSII and SSIII singly (Edwards *et al.,* 1999; Lloyd, 1999) or in combination (Lloyd, 1999) resulted into a significant shift from longer to shorter chains. In wheat, elimination of the *ssIIa* gene product (SGP-1) manifested a phenotype with reduced starch content and altered starch structure (Yamamori *et al.,* 2000). Likewise, the fairly recently discovered *sugary–2-like* phenotype in maize mutants defective in SSIIa, demonstrate that SSIIa is involved in starch synthesis. Overall, present understanding indicate that loss of SSII (dicots) or SSIIa (monocots) results in reduced starch content, reduced amylopectin chain length distribution, deformation of the starch granules, altered physicochemical properties of starch and perturbed crystallisation. It has also been shown through studies of the maize mutant, *dull1* (Gao *et al.,* 1998), *Chlamydomonas reinhardtii* mutant, *STA3* (Fontaine *et al.,* 1993) and transgenic potato carrying an antisense SSIII construct (Edwards *et al.,* 1999) that SSIII contributes to amylopectin branch-length distribution.

1.3.5. The role of starch branching enzymes (SBE or Q-enzyme)

Starch branching enzymes (SBE, 40 – 152 kDa) are involved in amylopectin synthesis. They catalyse the hydrolysis of α-1,4 linkage and subsequent formation of α-1,6 glucosidic bond between the cleaved chain and a hydroxyl group on **C**6 of a glucosyl moiety of an α-1,4 glucan template. They belong to the α-amylase family characterised by a catalytic $(\beta/\alpha)_8$-barrel domain (Jespersen *et al.,* 1993; Svenson, 1994). The domain encompasses specific active sites that arise from the inter-connecting β-loops providing for substrate binding and catalytic activity. To date, two classes [referred to as A (SBEII) and B (SBEI)] have been identified based on amino acid sequences and *in vitro* catalytic properties of purified enzymes (Burton *et al.,* 1995). In monocots, SBEII have further been subdivided into SBEIIa and SBEIIb filiations depending on specific catalytic properties, length of amino acid residues in the *N*-terminal domain and *C*-terminal polyglutamic acid repeats (Jobling *et al.,* 1999). Type I SBE, has been identified in maize (Baba *et al.,* 1991), rice (Kawasaki *et al.,* 1993), pea (Burton *et al.,* 1995), cassava (Salehuzzaman, Jacobsen & Visser, 1992), and wheat (Morell *et al.,* 1997). More recently, a *novel* SBEI cDNA, *sbeIc*, was isolated from developing endosperm of wheat (Båga *et al.,* 2000), uncovering the wide diversity of type I SBEs. Type II SBE, has been identified in pea (Bhattacharyya *et al.,* 1990), maize

(Gao *et al.,* 1997) rice (Mizuno *et al.,* 1993), barley (Sun *et al.,* 1998), wheat (Nair *et al.,* 1997), cassava (Baguma *et al.,* 2003) and sorghum (Mutisya *et al.,* 2003). Notably, SBEI and SBEII isoforms exhibit different substrate preferences. SBEII isoforms have lower affinity for amylose than SBEI, implying that SBEI isoforms uses longer glucan chains than SBEII isoforms (Guan & Preiss, 1993; Martin & Smith, 1995). Also, it has been found that SBEI isoforms have greater branching activity and preferentially use amylose as a substrate. Moreover, their protein structure exhibits further contrasting architectural features, i.e. SBEII possess an extended serine rich *N*-terminal domain while SBEI have a lengthy *C*-terminus of *ca*. 100 amino acid residues. The *N*- and *C*-terminal domains have been associated with protein anchoring and regulatory functions, respectively.

It has been shown from several studies that SBE isoforms are differentially and independently expressed during organ/tissue development and within the amyloplast. Genes encoding SBEI are commonly and constitutively expressed in photosynthetic and vegetative tissues while SBEII are preferentially expressed in starch storage compartments. The reverse is rare but common. For example, potato SBEII is predominantly expressed in leaves with very low but detectable levels in the tuber, whereas SBEI is the major isoform in the tuber (Jobling *et al.,* 1999). This disparity is widened by the expression pattern of *sbeII* in monocots like in wheat (Nair *et al.,* 1997), barley (Sun *et al.,* 1998), maize (Gao *et al.,* 1997) and rice (Mizuno *et al.,* 1993) where maximal expression of *sbeII* is attained early in kernel maturation. By contrast, SBEI is strongly expressed in the second half of embryo development in maize (Gao *et al.,* 1996), wheat (Morell *et al.,* 1997) and barley (Mutisya *et al.,* 2003), while maize SBEIIa is more highly expressed in leaves than in endosperm. In pea embryo, both forms are present at comparable levels in the soluble fraction, whereas in potato, SBEI is the most abundant soluble fraction than SBEII (Jobling *et al.,* 1999). Most significantly, whereas both SBEIIa and SBEIIb share similar patterns of action, similar expression profiles, and are both distributed between the granule and stroma, only mutations in SBEIIb cause a high amylose phenotype in cereal grains. This raises the question of function for the different isoforms.

In elucidating the function of the different SBE isoforms, insight has been gained from analysis of starches derived from contrasting *sbe* mutants. In both monocots and dicots, mutational and gene suppression of SBEI cause minimal effects on general starch synthesis and composition in tubers, leaves and endosperm (Ball & Morell, 2003; Blauth *et al.,* 2002; Satoh *et al.,* 2003; Seo *et al.,* 2002). However, it has been shown that loss of BEI protein in rice results into significant changes in the fine structure of amylopectin and physicochemical properties of the resulting starch in the rice endosperm (Satoh *et al.,* 2003). As for SBEII, exclusive elimination of SBEII in potato led to increased levels of amylose (Jobling *et al.,* 1999), although, combined suppression of both SBEII and SBEI markedly increased the amylose content of the resulting starch phenotype (Schwall *et al.,* 2000). This showed that the seemingly obscure function of SBEI in starch synthesis might be reflective of overlapping specificities and complementation between SBEI and SBEII (SBEIIa and SBEIIb). It has also been proposed that the enzyme might not interact with the substrate until SBEII (SBEIIa and SBEIIb) have acted (Ball & Morell, 2003). In spite of these efforts, the question of function

for the different isoforms remains not clearly resolved. Notably, isoform substrate specificities (cleavage and branching junctions), configuration of the substrate molecules (helical structure or interchain) and enzyme complexes during starch synthesis require to be examined.

1.3.6. The role of debranching enzymes (DBE)

Starch debranching enzymes belong to the α-amylase super family (Jespersen *et al.,* 1993). They hydrolyse the α-1,6 glucan branches of amylopectin. The group is subdivided into two classes. The direct DBE, involved in the hydrolysis of α-1,6-linkages of α-polyglucans and the indirect DBE, engaged in hydrolysis of α-1,6-branches by 4-α -glucanotransferase and amylo-1,6-glucosidase. Direct DBE are further subdivided into pullulanase-type or R-enzyme (EC: 3.2.1.41) and isoamylase (EC: 3.2.1.68) (Doehlert & Knutson, 1991; Ishizaki *et al.,* 1983). The defining difference is their substrate specificity in which pullulanases debranch pullulan and amylopectin but not glycogen, whereas isoamylase debranch both glycogen and amylopectin (Nakamura *et al.,* 1996). Moreover, pullulanases generates maltosyl groups, while isoamylase releases maltotriosyls and large oligosaccharides. In cereals, isoamylase is a larger (400 kDa) multimeric enzyme composed of one type of isoamylase subunit (Burton *et al.,* 1995). Conversely, in potato, two distinct subunits define an equally large heteromultimeric enzyme (Ishizaki *et al.,* 1983).

There is good accumulated evidence that DBEs plays a crucial role in starch biosynthesis. This has been accumulated through the analysis of *sugary-1* mutants of maize and rice endosperm (James, Robertson & Myers, 1995; Fujita *et al.,* 2003; Kubo *et al.,* 1999; Zeeman *et al.,* 1998), *sta7* mutant of *Chlamydomonas* (Dauvillée *et al.,* 2001; Mouille *et al.,* 1996), *dbe1* mutant in *Arabidopsis* (Zeeman *et al.,* 1998), which accumulate a water-soluble polysaccharide (WSP), designated phytoglycogen. This analysis led to the *trimming model* (Ball *et al.,* 1996), which asserts that the synthesis of amylopectin, its organisation and incorporation into a starch granule is the result of 'trimming' by DBE of the highly branched glucans synthesised by SS and SBE. This model hypothesises discontinuous synthesis by way of preamylopectin, *a theoretical intermediate of amylopectin*, through the activity of DBEs that results in altered efficiency of the enzyme complex. In spite of this account, the trimming model has some limitations as observed in *Arabidopsis dbe1* mutants, which accumulate starch and phytoglycogen suggesting that the accumulation of phytoglycogen in wild-type is presumably forestalled by the action of a specific isoamylase enzyme, perhaps in concert with other glucan-degrading enzymes. To date, analysis of the *Arabidopsis* genome has revealed that there are three genes encoding isoamylase like proteins (*ISA1*, *ISA2*, and *ISA3*). These are conserved in divergent plants and evidence from potato and *Arabidopsis* indicate that the protein encoded by *ISA1* and *ISA2* are subunits of one heteromultimeric isoamylase protein *in vivo* (Hussain *et al.,* 2003). Loss of *ISA1* and *ISA2* in *Arabidopsis* manifest a phytoglycogen accumulating phenotype suggesting that both are essential in starch synthesis. This leaves *ISA3* with undefined role but presumably might be important in starch degradation. Taking this knowledge into consideration and from the point of view that during

mobilisation of transitory starch, both starch synthesis and degradation occurs concurrently, it seems plausible that *ISA3* is required to debranch glucan structures that arise during starch degradation, while *ISA1* and *ISA2* are essential for amylopectin synthesis. Taken together, the latter prompted the development of a new model *"simultaneous processing"*, where specific isoamylase, in this case *ISA1* and *ISA2*, clears the stroma of WSPs generated by *ISA3* and other starch degrading enzymes, leading to simultaneous stalled phytoglycogen accumulation but increased amylopectin synthesis. In spite of these exciting models, it remains unresolved whether glucan trimming, WSP clearing or some other mechanism explains the mode of action of DBE.

1.3.6. The function of disproportionating enzyme (D-enzyme)

D-enzyme was first reported in potato tuber. Later, it was detected in beans, carrot, peas, spinach, tomato and *Arabidopsis* (Lin & Preiss, 1988; Manners & Rowe, 1969; Okita *et al.,* 1979). The enzyme disproportionate soluble oligosaccharides of at least three glucose residues or amylopectin into maltooligosaccharides (Colleoni *et al.,* 1999; Lin & Preiss, 1988; Takaha *et al.,* 1996). *In vitro* analysis of potato D-enzyme showed that the protein is capable of transferring branched glucans and or producing cyclic glucans (Colleoni *et al.,* 1999; Takaha *et al.,* 1998). An attempt to understand its function in starch biosynthesis through mutation studies in *Chlamydomonas* and *Arabidopsis* showed conflicting roles. In *Chlamydomonas*, the mutants showed significant reductions in total glucopolysaccharide, abnormal starch granule, altered component proportions and atypical amylopectin chain-length distribution relative to the wild type. Conversely, *Arabidopsis* mutants overproduced starch (Critchley *et al.,* 2001) while transgenic potato tubers transformed with antisense D-enzyme constructs showed no effect on starch synthesis and its fine structure (Takaha, 1998). These inconclusive findings suggest additional research to clearly establish the function of D-enzymes in starch synthesis.

1.3.7. Other players and evidence implicating their involvement

RI protein has been reported to be involved in phosphorylation of tuber starch (Lorberth *et al.,* 1998). Recently, ADP-glucose pyrophosphatase, an enzyme likely with dual role in controlling levels of ADPG linked to starch synthesis and other metabolic pathways has been added to the list of enzymes involved in starch biosynthesis (Kleczkowski, 2001). Also, starch phosphorylase (P-enzyme) has been shown to be involved in the catalysis of reversible phosphorylytic cleavage of starch and it seems very likely that P-enzyme is a part of the complex degrading enzymes involved in starch breakdown (Larsson *et al.,* 1996; Lin & Preiss, 1988). It has also been shown that several enzymes involved in starch degradation e.g. α-amylase, β-amylase, D-enzymes, α-glucosidase (maltase), glucan water dikinase (GWD) and α-glucan phosphorylase are equally important players in starch biosynthesis. Most significantly, is the fact that glucan water dikinase (GWD) appears to control the overall rate of starch breakdown with a central rate limiting role in starch breakdown machinery and downstream starch synthesis. A detailed account on the roles, subcellular localisation and regulation of these enzymes has

been recently reviewed (Zeeman, Smith & Smith, 2004). In the same review, the authors present a model for starch breakdown and its connection to starch synthesis. Lastly, sucrose invertase (SI), sucrose synthase (SuSy) and sucrose phosphate synthase (SPS) are crucial in sucrose metabolism and contribute to sink strength in divergent crop species (Hajirezael *et al.,* 2003; Roitsch *et al.,* 2003).

1.3.8. *Amylogenin: the elusive primer*

Despite the intense research in plant carbohydrate metabolism, several intriguing questions still remain. One relates to the primer for starch synthesis, if indeed such a thing exists! The rift in our understanding is further aggravated by the fact that the mechanism underlying the priming of glycogen synthesis in *E. coli* is equally unresolved. Nonetheless, glycogenin has been identified as the priming protein for the initiation of glycogen synthesis in yeast and mammalian cells (Cheng *et al.,* 1995; Roach *et al.,* 1998), but the situation in plants remains a mystery. Along with this evidence, it is expected that protein similar to glycogenin may be involved in the initiation of starch synthesis in plants. As a corollary, a self-glycosylating protein named UDP-glucose:protein glucosyltransferase or amylogenin (38 kDa) first reported in potato (Lavintman & Cardini, 1973) and later in maize (Singh *et al.,* 1995) has been thought to be involved in starch synthesis. One of the main reasons for assuming that amylogenin functions in starch synthesis was its occurrence in starch synthesising endosperm tissue of maize (Singh *et al.,* 1995). However, recent results indicate that this may not be true. The evidence include the covalent nature of amylogenin-glucan glycosidic linkage (β-glucosylarginine instead of α-glucosyltyrosine), its substrate specificity (UDPG and not ADPG) and its subcellular localisation (Golgi and not plastid) bringing into question its function in starch priming, but not necessarily against its function in polysaccharide synthesis.

So, what is amylogenin? It has been revealed through polypeptide functional studies and tryptic peptide sequence matches that amylogenin is closely related to *Pisum sativum* reversibly glycosylated polypeptide (*Ps*RGP1), which is said to be involved in the synthesis of xyloglucan (Dhugga, Tiwari & Ray, 1997). Notably, RGP1 does not interact with ADPG, the principle substrate for starch synthesis in plants. Moreover, RGP1 is localised in the *trans*-cisternae of Golgi dictyosomes and not plastids, the sites for xyloglucan and starch synthesis, respectively. Furthermore, the fact that amylogenin associates with starch synthesising maize endosperm tissue (Singh *et al.,* 1995), does not exclude the possibility that as this tissue cellularises from the liquid endosperm, it also synthesises cell wall polysaccharides. Thus, neither the substrate specificity nor the localisation of amylogenin seems to agree with a role in starch priming.

The question of what primes starch synthesis still remains raising the concern of what could be the other possible primer alternatives? In response, two possibilities have been suggested. First, short maltooligosaccharides (MOS) may prime amylose synthesis (Zeeman, Smith & Smith, 2002). Second, amylopectin-primed amylose synthesis has equally been identified as a plausible model (Ball *et al.,* 1998). The evidence for these models has been accumulated from MOS-accumulating mutants and pulse-chase studies involving either a pulse of

ADP[^{14}C]Glc to isolated starch granules or $^{14}CO_2$ to intact plants, followed by a chase period in unlabeled substrate, for the former and latter, respectively. Furthering our understanding of the mechanism(s) that govern the initiation of starch synthesis is likely to have profound impact on the starch industry.

1.3.10. Modification of starch: opportunities and challenges

Prospects for manipulating starch composition using modern molecular tools have given an impetus to research on starch metabolism. Transgenic plants and starch mutants such as *ae*, *amf*, *sug*, *wx*, *shr1*, *shr2* and *flo* harbouring altered starch content and composition have been identified and or produced in several plants such as barley, maize, pea, rice, oats, potato and wheat, opening such a possibility in cassava and kindling the need to understand the key regulatory steps controlling starch biosynthesis or mobilisation in its storage roots. This section presents the scientific and end-user motivations for development of genetically modified starch crops, current applications and prospects for metabolic engineering of starch in cassava.

In dicots, examples of genetically engineered crops include potato with a 30% increase in starch yield derived from altered AGPase activity (Stark *et al.,* 1992). In addition, an attempt has been made to increase the yield of starch through heterologous expression of *E. coli* PPi in transgenic potato (Geigenberger, 1998). Interestingly, the extent of modification depended upon the potato variety used. In the *Russell Burbank* variety, the modification produced a 30% increase in starch content, but in the *Prairie*, such changes were not recorded. This disparity suggested that there might be a limit to the amount of starch, that potato can accumulate, which the *Prairie* variety had already reached, or the result was reflective of pleiotropic effects of the modified AGPase on other enzymes in potato. Aside modifying AGPase by itself to enhance the rate of starch synthesis, new elegant strategies based on enzyme-substrate kinetics have been designed with spectacular outcome. For instance, increasing the levels of a 'plastidic ATP–ADP transporter', a protein involved in supplying ATP for the reaction, increased the yield of starch in potato (Geigenberger, 2001). Other notable modifications include antisense SBEI in transgenic potato (Flipse *et al.,* 1996) and simultaneous alterations of SS activity also in potato (Edwards *et al.,* 1999; Gao *et al.,* 1998; Lloyd, 1999; Yamamori *et al.,* 2000). The latter affected starch content, glucan chain lengths, granule morphology and properties of the starch. More recently, simultaneous antisense inhibition of the genes for three starch synthases in transgenic potato yielded starch with short-chain amylopectin and very little amylose (Jobbling et al., 2002).

In monocots, *Himalaya 292*–a *novel* barley cultivar - derived from a single nucleotide change in *ssIIa* (Morell *et al.,* 2003), has higher amylose (70%) and NSP than the parent strain. As for oats, 17 transgenic lines with reduced amylose content have been produced by antisense inhibition of *gbssI*. In wheat, altered activity of *sbe* led to higher proportion of A-type granules in transgenic lines (Båga *et al.,* 1999, Davis *et al.,* 2001). Also, in wheat loss of one or two of its *gbssI* gene, remarkably reduce amylose content. More recently, *notch2* isoamylase deficient mutant of barley associated with an apparent increase in phytoglycogen (Burton *et al.,* 2002). For maize, altered AGPase activity led to 18% increase in

starch yield (Giroux, 1996). Also, *su1* maize isoamylase mutants accumulate phytoglycogen instead of amylopectin (James *et al.,* 1995). In rice, EM557 mutant lacking the BEI protein altered the fine structure of amylopectin (Satoh *et al.,* 2003). Likewise, reduction in ISO1 protein activity in rice endosperm markedly altered amylopectin into a water–insoluble modified amylopectin and a water-soluble polyglucan (Fujita *et al.,* 2003).

Beyond the test tube and other laboratory conditions, it is recognised that gene action is influenced by other factors including diurnal functions. For instance, in rice it has been shown that temperature at which a plant develops influence the proportions of amylose and amylopectin produced by affecting the activity of different SSs. Furthermore, there is evidence that altering the rate at which starch is produced affects the fraction composition. At lower rates of production a greater proportion of the starch is converted into amylopectin, because of higher enzyme units per substrate molecule. This raises the concern that attempts to improve starch yield might result in starch with increased amylose content, which would be undesirable for some applications.

While there are significant species-dependent differences among starches, starch in its native form has limited applications. As a consequence, it is often necessary to chemically or physically modify the starches to tailor for various uses. However, if the plant that produced the starch could carry out such modification *in planta* that would be of great advantage. Thus, the quest for *in planta* modifications of starch synthesis has gained momentum. Examples of such *in planta* modifications include starch with increased degree of phosphorylation, high or all-amylose starch, all-amylopectin starch, and starch with defined granule sizes.

Despite the progress made in analysing and manipulating the mechanism of starch synthesis in plants, the interactive roles of the key enzymes involved and their metabolic regulation are not clearly understood. For example, *rug* pea mutant originally intended only to affect starch production, also resulted in wrinkled seeds containing abnormal quantities of lipid and storage proteins. Such unintended occurrences alludes to one cardinal fact that prior to any attempt aimed at generating novel starch, it would be important to make sure that the intended modification has no or negligible effects on other untargeted nutritional qualities.

Finally, one recurring theme while dissecting starch metabolic pathways is that the enzymes involved come in multiple forms, which differ in their physical and chemical properties and in the type of starch that they produce. While this increases the complexity of the process, it nonetheless increases the scope for metabolic engineering. Taken together, the aforementioned aspects in allied crops convincingly demonstrate that modification of starch biosynthesis in cassava is equally possible. Moreover, many of the genes involved in its starch biosynthesis have been cloned and insight into their regulatory properties has been obtained. Thus, prospects for carefully planned modifications are equally possible in cassava.

1.4. The regulation of starch biosynthesis

Regulation of starch synthesis involves a flexible but complex network of highly interactive catabolic and anabolic reactions in the cytosol and plastids. Most interestingly, the plant cells must know when to use sucrose for starch synthesis and or for other metabolic processes. Also, it is well established that different genes and their isoforms diversely located in the plant cells are involved in starch metabolism. Thus, it is important that their activities are well coordinated consistent with their roles. Overall, available evidence suggest that the entire regulatory network for starch synthesis relies on genetic (including developmental cues), cellular metabolic status and environmental factors for its function. In the following sections major regulatory mechanisms of starch synthesis are reviewed.

1.4.1. Developmental regulation

The role of developmental regulation of starch synthesis genes has been established through spatial and temporal analysis studies in a number of crop species. It is governed by the interplay of genetic control, mitotic activity, histodifferentiation and cellular metabolic status (Borisjuk *et al.,* 2002). In barley, *faba* bean, wheat and maize; it has been shown that the pattern of storage starch accumulation correlates well with cell expansion but is spatially distinct from the pattern of mitotic activity. Detail analysis has identified that metabolic status maintains a specific stage of differentiation and or directs the next developmental programme including starch biosynthesis (Wobus & Weber, 1999). Most remarkably, it has been shown that the expression of storage-associated genes is dependent on changes in sucrose levels in a histodifferentiation-dependent manner (Weber, Wobus & Borisjuk, 1997; Visser *et al.,* 1994) with specific-isoform expression profiles. Consistent with this, *sbeII* and *sbeI* genes in pea are expressed early and late in embryo development, respectively (Smith, 1988). Similarly, in maize endosperm, it has been shown that *sbeI* is strongly expressed between 10 and 28 dap (Baba *et al.,* 1991; Gao *et al.,* 1996) whereas, *sbeIIb* is expressed throughout endosperm development (Gao *et al.,* 1996). In contrast, rice *sbeI* is immediately detectable post-pollination (Umemoto, Nakamura & Ishikura, 1994), while *sbeIIb* in rice is detectable at 5 dap with maximal expression between 5 and 15 dap (Mizuno *et al.,* 1992). In sorghum, the onset of *sbeIIb* and *sbeI* expression starts from 10 dap, attains maximum expression between 16 and 22 dap, and declines thereafter (Mutisya *et al.,* 2003). A similar pattern for *sbeI* in wheat has been reported (Morell *et al.,* 1997). In barley, *sbeIIa* and *sbeIIb* predominate during early stages of grain development, while *sbeI* is maximally expressed during late development (Sun *et al.,* 1998; Mutisya *et al*., 2003). Taken together, these findings suggest that the expression profile and expression levels of different *sbe* isoforms is a well-coordinated process that involves the interactive effects of cellular mitotic changes, metabolic signals and genetic control, although the whole mechanism remains to be clearly understood.

1.4.2. Diurnal regulation

Plant metabolic networks involve different gene expression-mediated pathways (Dijkwel *et al.,* 1997; Dunlap, 1999; Halford, Purcell & Hardie, 1999). These expressions manifest as steady-state levels or as day/night recurring cycles called diurnal rhythms. Diurnal rhythms are entrained by light and revolve on an internal circadian clock (Schaffer *et al.,* 2001). Light mediates circadian function through signaling pathway(s) that involve the phytochrome systems, which upon light reception are transported to the nucleus (Kircher *et al.,* 1999; Sakamoto & Nagatani, 1996). In the nucleus, the phytochrome interacts with *PIF3* (*PHYTOCHROME INTERACTING FACTOR 3*) a basic helix-loop-helix transcription factor (Ni, Tepperman & Quail, 1998, 1999) and binds to the G-box element (Giuliano *et al.,* 1988a, 1988b; Martinez-Garcia, Huq & Quail, 2000). The effects of light results in recurring responses with some exhibiting typical overt circadian rhythms (Schaffer *et al.,* 2001). The basic model of a circadian system is composed of three control components: input, oscillator and output (Kondo & Ishiura, 1999). The input, which is triggered by light and temperature, transmits a signal to the oscillator for resetting (Kreps & Simon, 1997). In turn, the cycling of the input pathway trains the clock to a specific schedule sustained by the oscillator (Schaffer *et al.,* 2001). In a coordinated sequence, the oscillator controls the output to overt circadian rhythms.

To date, there are indications that starch metabolism and some of the genes involved are entrained to the circadian rhythm of the plant. Light-responsive elements; G, GT1, and GATA have been identified in promoters of light regulated starch synthesis genes and convincingly shown to be essential for light responsive transcription (Chattopadhyay *et al.,* 1998; Millar & Kay, 1996). *Cis*-acting elements that bind specific transcription factors (Chattopadhyay *et al.,* 1998; Terzaghi, Bertekap & Cashmore, 1997) have also been identified in the *gbssI* promoter of *Snapdragon* (Merida *et al.,* 1999) and *sbe*2-1 in *Arabidopsis*. Besides, diurnal oscillation of AGPase in *Chlamydomonas* (Zabawinski *et al.,* 2001) and *gbssI* in *Snapdragon* (Merida *et al.,* 1999) suggest involvement of circadian mode of regulation. At the level of starch, it has been shown that plants transferred from a typical diurnal regimen to continuous light perturb starch synthesis during subjective night (Li *et al.,* 1992), and in one case, starch was even degraded (Kruger *et al.,* 1983). More recently, work involving micro-array analysis has revealed that several *Arabidopsis* genes encoding starch synthesis/degrading enzymes [e.g. chloroplastic isoforms of α-amylase, β-amylase, glucan water dikinase (GWD), *sbe*2-1] are under the influence of circadian clock. However, there exist conflicting evidence with regard to the relationship between gene expressions, protein level and enzymatic activities of such diurnally regulated genes. For instance, it is not known in the case of α- and β-amylases whether the amounts of protein or enzymatic activities match with gene expression profiles and levels. In all cases, however, entrainment to the circadian mode of regulation may serve to prime the events in starch metabolism along with other key factors such as day length, temperature and nutrient availability. Whatever the significance, little is known about the underlying molecular mechanisms. This requires further studies.

1.4.3. Sugar signaling

Sugar signaling is a well-established phenomenon in a number of plants. Sugar signaling involves three critical steps i.e. sugar/signal sensing, signal transduction, and action at target genes. Sugar sensing might arise from changes in apoplast sugar concentrations, sugar flux through the plasma membrane, fluctuations in intracellular sugar levels, and, from glycolytic, plastidial and or vacuolar sugar species interconversions (Loreti *et al.,* 2001). Whatever the mode of sensing, it is established that sugar signals mediate many metabolic processes in plants including germination, seedling growth, leaf and root development, starch synthesis and senescence, in concert with expression of the relevant genes (Jansson, 2004; Sheen, Zhou & Jang, 1999; Smeekens, 2000). It has been shown that, *cab* and *rbcs* (encoding chlorophyll a/b-binding protein and the small subunit of rubisco, respectively) are repressed by sucrose and glucose (Jang & Sheen, 1994; Rolland, Moore & Sheen, 2002; Smeekens, 2000), while starch synthesis genes (Sun *et al.,* 2003) and the patatin promoter (Smeekens, 2000) are activated by sucrose. Additionally, the expression of α-amylase (Loreti, Alpi & Perata, 2000) is repressed by sucrose. In spite of this evidence, it remains unclear how the sugar effects are sensed and or transduced to the target gene(s), although plant HXK's have been identified and implicated in the process (Guglielminetti *et al.,* 2000; Kaplan & Baker, 1997; Pego & Smeekens, 2000; Rolland, Moore & Sheen, 2002; Sherson *et al.,* 1999). It has been shown that plant HXK's associates with chloroplast, amyloplast, mitochondrial membranes, or with the golgi apparatus (Rolland, Moore & Sheen, 2002) providing further evidence for a role in starch synthesis. Additional evidence indicates a role of fructokinases (Gonzali *et al.,* 2001) and the existence of a glycolysis-dependent pathway (Xiao *et al.,* 2000).

Recently, the existence of a sucrose sensor has been reported (Loreti, Alpi & Perata, 2000). It is speculated that the sensor functions either as an active sucrose carrier or by a metabolic-derived signal. In the first case, the transporting carrier activates the next protein in the signaling pathway. In the second case, metabolites downstream, act as metabolic messengers. In congruence, transporting and none transporting sucrose sensors have been identified (Barker *et al.,* 2000; Loreti, Alpi & Perata, 2000; Smeekens & Rook, 1997). That said, the mode of sucrose transport and the nature of sucrose transporters remain highly controversial. Nonetheless, changes in extracellular pH and membrane potential are implicated. Here, it involves activation at the plasma membrane H^+-ATPase, which in turn generates a negative membrane potential and acidifies the extracellular environment relative to the cytoplasm (Chandran, Reinders & Ward, 2003; Lalonde, Wipf & Frommer, 2004). Most interestingly, sucrose transporters display interactive specificity with the glucosyl hydroxls 3, 4 and 6 of sucrose unravelling the significance of side group orientation or linkage position (Chandran, Reinders & Ward, 2003), and its binding with sucrose is dependent on the presence of a fructosyl moiety for a hydrophobic surface. Further downstream, sugar signals and or sensors, initiate complex signaling networks to target genes interlinked by plant Ser/Thr protein related kinases, *SnRKs* (Rolland, Moore & Sheen, 2002). Specific *cis*-elements e.g. sugar responsive elements (SURE; (Grierson *et al.,* 1994; Sun *et*

al., 2003), SP8 (Ishiguro & Nakamura, 1994), TGGACGG (Maeo *et al.,* 2001), G-box (Giuliano, Hoffman *et al.,* 1988) and B-box (Grierson *et al.,* 1994; Zourelidou *et al.,* 2002) respond to arriving signals and cause gene action in concert with transcription factors such as SUSIBA2 (Sun *et al.,* 2003).

1.4.4. Hormonal signaling

The interactive effects of sugar and hormonal signaling have long been known to modulate plant metabolic processes, including but not limited to seed maturation, dormancy and changes in gene expression (Jansson, 2004; Leon & Sheen, 2003; Sheen *et al.,* 1999). It has been shown that abscisic acid (ABA) enhances sugar induction of *Apl3* and *sbe2.2* gene expression in *Arabidopsis* (Rook *et al.,* 2001). In addition, it has been shown that some *Arabidopsis* ABA-insensitive mutants (*sis5*, sugar insensitive; *sun6*, sucrose uncoupled; *gin6,* glucose insensitive = *abi4*) and ABA synthesis mutants (*sis4*, *gin1* = *aba2*) are equally defective in sugar sensing, and vice verse (Arenas-Huertero *et al.,* 2000; Huijser *et al.,* 2000; Laby *et al.,* 2000). Additionally, ABA-insensitive mutants (*abi1*, *abi2* and *abi3*), unaltered in sugar sensitivity exist, indicating that a specific ABA-related transduction pathway mediates sugar sensing. Nonetheless, it is established that many of the sugar-sensing mutants are altered in their ability to synthesis or transduce ABA signals. ABA signaling involves putative ABA receptors (extracellular or intracellular), cell-surface membrane proteins including ion channels, glycoproteins and membrane trafficking proteins, secondary messengers such as phosphatidic acid, Ca^{2+} and protein phosphorylation/dephosphorylation cyclic cascades. The role of PP1/PP2-mediated ABA signal transduction is of particular interest to this thesis.

The opposition of kinase-catalysed phosphorylation and phosphatase hydrolytic dephosphorylation maintains the steady state phosphorylation levels of most enzymes in cyclic cascades. In this thesis, serine/threonine (Ser/Thr) protein phosphatase type 1 (PP1) and type 2 (PP2) are markedly essential in ABA-mediated *sbe* regulation. PP2 subgroup is further subdivided into PP2A, 2B, and 2C based on whether or not its activity is dependent on divalent cations. PP2B and PP2C activity requires Ca^{2+} and Mg^{2+}, respectively, but PP2A, like PP1 does not. Sequence and structural analyses indicate that PP1, PP2A, and PP2B are tightly related and defined as PPP, whereas PP2C, pyruvate dehydrogenase phosphatase and several Mg^{2+}-dependent Ser/Thr phosphatases form a distinct family, named PPM (Cohen, 1997). Most notably, it has been revealed from *Arabidopsis* sequence database and other genome-wide surveys that several genes encode for proteins related to Ser/Thr phosphatases. At least, 7 genes encode PP1 catalytic subunit, 6 for PP2A catalytic subunit, 70 for PP2C and a handful for other PPP types. Taken together, their effects depend on the manner by which they are regulated. To date, it is well established that multiple regulatory mechanisms are involved (i) at the level of gene expression, (ii) at the level of protein localisation, (iii) at the level of substrate specificity, and (iv) through regulation by the activity of other enzymes. For instance, genes for PP2A catalytic subunits are constitutively expressed but their expression levels are developmentally regulated (for a review, see Luan, 2003). In addition, the noncatalytic domains of PP

functions to localise catalytic activity in the cell while their interactive effects with other regulatory proteins modulate their activity. In this case, it has been clearly established that a bicyclic cascade involving phosphorylase kinase and phosphoprotein phosphatase-1, enzymes that are involved in the glycogen phosphorylase bicyclic cascade, modulate the activity of glycogen synthase. Lastly, it has been shown that PP2A and its regulatory subunit are crucial for plant developmental processes and hormonal signal transduction. For instance, the *rcn1* gene, which encodes a 65 kDa A-subunit of PP2A, is essential for normal root development in *Arabidopsis* (Garbers *et al.,* 1996).

2. Present investigation

Methodological aspects of this work are described in papers I – IV.

2.1. Objectives of this investigation

The specific objectives were to -

i. Clone the gene encoding starch branching enzyme II (*sbeII*) and examine the spatial and temporal expression profiles of starch synthesis genes (**Paper I**);
ii. Elucidate *sbe* regulatory pathways and develop a working regulatory model to guide rational metabolic engineering of starch synthesis (**Paper II, III and IV**); and
iii. Attempt the development of *novel* cassava starches with modified properties (**Laboratory material**).

2.2. The spatial and temporal expression profiles of starch synthesis genes

Previously, cassava cDNA's encoding AGPase (both small and large subunits), GBSSI, GBSSII and SBEI were cloned (Munyikwa, Jacobsen & Visser, 1997; Munyikwa *et al.,* 1994; Salehuzzaman, Jacobsen & Visser, 1992, 1993). Here, a partial cDNA encoding starch branching enzyme II (SBEII) was cloned, apparently derived from a single-copy *sbeII* gene (**Paper I**). Database comparisons of its nucleotide sequences with genebank *sbe* sequences showed a high degree of similarity (<79%) to other plant *sbeII*. It is weakly and constitutively expressed in all tissues during early development, but markedly expressed in the storage root at maturity (**Paper I**). This finding indicated that *sbeII* is expressed in dynamic patterns in developing cassava storage roots. These results differed from its botanical relative potato where *sbeII* is more strongly expressed in the leaf than in the tuber (Larsson *et al.,* 1996). Also, it was found that *sbeII* transcription activity increased starting 90 days after planting (dap) and attained maximum abundance at 360 dap (**Paper I**). A similar shift in spatial and temporal expression profile was observed for *sbeI* in this study. The concerted expression profiles of *sbe* uncovered the existence of a well-coordinated developmental programme that ensures a high transcriptional activity of starch biosynthesis genes in the mature storage root. In addition, the finding that *sbe*

expression and SBE activity prepatterned storage root development together with cassava's phasic development corroborated this finding (**Paper III**). Another key finding in this investigation was that the 360 dap old storage roots had *gbssII* and *gbssI* transcripts, as well as a longer *gbssI* transcript, designated *gbssI'* (**Paper I**). The *gbssI'* transcript was identified as a variant transcript of *gbssI* characterized by the retention of the first three introns. Båga *et al.*, 1999, found that *sbeI* in wheat by itself encodes three preproteins differing in transit peptide sequences, although the putative proteins remain unidentified. Their finding clearly showed involvement of alternative splicing. Similarly, *pvsbe2* of kidney beans (*Phaseolus vulgaris* L.), encodes two SBEs with different subcellular localisation and protein accumulation profiles as well as distinct enzymatic properties (Hamada *et al.,* 2002). Based on these reports, it is possible that *gbssI* and *gbssI'* arise from the same gene and *gbssI'* might be involved in regulating the enzymatic properties of *gbssI* during storage root development. Very likely, *gbssI'*, down-regulates the abundance and activity of GBSSI in fully matured storage root zones.

2.3. The role of developmental and diurnal regulation in *sbe* expression

As described in section 2.2, the transcription and enzymatic activity of *sbe* prepatterned the storage root development states, but the spatial and temporal developmental starch accumulation patterns remained unclear. Following this, a study was designed to examine the relation between the storage root histodifferentiation stages, and *sbe* transcription and endogenous enzymatic branching activity. At 180 dap, *sbe* transcription activity increased from the periphery to the hilum of the storage root (**Paper III**). Interestingly, at 360 dap, greater *sbe* transcription activity relocated away from the hilum to the periphery. Likewise, the same pattern was seen for endogenous enzymatic branching activity, revealing the physiological relevance of the relocation. Developmentally, these findings agreed with the storage root compartmentalisation growth model and provided additional evidence that the pattern of *sbe* transcription activity and its enzymatic activity prepatterns starch accumulation. Furthermore, as previously reported (Borisjuk *et al.,* 2002; Weber *et al.,* 1995), *sbe* transcription activity remained spatially distinct from the pattern of mitotic activity, since no coincidence was observed between maximum *sbe* expression and cellular mitotic activity as deduced from cellular density and size. To further corroborate this evidence, histological studies were performed to map the *in situ* starch granule organisation in the storage root, and establish a relationship between starch granule organisation and mitotic gradient (**Paper III**). The map showed a gradual increase in both cellular and starch granule size (5 – 40 μm) away from the periphery of the periderm to the hilum interphased by characteristic secondary xylem starch-depleted buffer zones (**Paper III**). Similar results were obtained from granule scanning electron microscopy of granule architecture (**Paper III**). Most notably, the iodine stained monographs showed an inward increment in the relative amylopectin content revealing that the storage root of cassava is not a homogenous tissue (**Paper III**). In practice, these results uncovered the existence of distinct starch domains in the storage root of cassava and identified the need to unravel the underlying developmental regulatory mechanism.

Developmental regulation is a multifactor regulatory mechanism that in part depends on the prevailing diurnal functions. Consequently, the relationship between *sbe* transcription activity and diurnal functions was examined (**Paper I** and **II**). Maximal expression levels of *sbe* occurred within 6 h inside the light zone irrespective of the light-dark interface schedules (**Paper I**). Although, it remained unclear whether the observed oscillation was typical of an endogenous oscillator or otherwise. To dissect this question, changes in *sbe* transcript abundance in storage root cells were monitored under prolonged light and or dark conditions. This analysis showed that *sbe* mRNA oscillated in periods of 12 h within a circadian day (**Paper I** and **II**). In turn, this prompted an investigation to establish whether the observed expression pattern was reflective of the endogenous SBE enzymatic activity. The results matched (**Paper II**), indicating a well-coordinated biological phenomenon. This phenomenon was taken to mean that the overt oscillations in the transcription activity of *sbe* are under the regulation of a *bona fide* semi-diurnal regulatory mechanism, driven by an endogenous semidian oscillator, which is premised in the storage root cells of cassava, although its nature and function remains unknown. At the same time, these results suggested that the semidian oscillator might have evolved to synchronize the photosynthetic apparatus with starch biosynthesis machinery, analogous to the circadian rhythm of *cab* genes, which prepares the predawn photosynthetic cell for the approaching light exposure (Kreps & Simon, 1997). In our case, the 12 h rhythm observed for *sbe* expression suggested that the regulation of *sbe* is tightly linked with both photosynthesis (the day pathway) and starch breakdown (the night pathway). During the day, triose-P derived from the Calvin cycle and exiting the chloroplast through the triose-P translocator is the main source of sucrose flux to the storage root cells, while maltose and glucose derived from the breakdown of starch (i.e. transitory starch) and exiting by separate carriers, maltose and glucose translocators respectively, is the main source of carbon for the synthesis of sucrose and storage starch in the storage root cells at night. A similar phenomenon has been reported for sucrose metabolism in spinach leaves (Servaites & Donald, 2002), and is mediated by distinct but well-coordinated regulatory mechanisms. In addition, it has been reported for some plants that circadian clocks affect starch accumulation in leaves (Li, 1992; Merida *et al.,* 1999; Wang, 2001), although it is not known precisely how such mode of regulation functions and how it relates to changes in starch accumulation patterns. Subsequently, this raised the concern on the relation between cellular sugar metabolic status and *sbe* transcription activity. To address this concern, diurnal changes in sucrose accumulation in the storage root were analysed and found consistent with *sbe* transcription and activity profiles (**Paper II**). Again, these data raised the quest for the function-relation of sugars in *sbe* regulation. The key findings from this quest are presented in the next section.

2.4. The interactive effects of sucrose and abscisic acid signaling in *sbe* expression

The interplay between sugar and hormonal signaling networks is known to regulate several metabolic processes during plant growth and development (Leon

& Sheen, 2003). Thus, by considering previously established developmental dependent *sbe* expression profiles and expression levels, it was equally assumed that sugar signaling plays a role in the regulatory mechanism of *sbe* expression in the storage roots of cassava. Subsequently, the effects of exogenously supplied sugars on the expression of *sbe* were studied in metabolically active starch synthesising 360 d-old plants as described (**Paper II**). The transcription activity of *sbe* was strongly induced by sucrose to levels observed in 360-d-old plants. Glucose and fructose had minimal effects. Surprisingly but significantly, turanose a transportable but non-metabolisable sucrose analog substantially induced *sbe* expression, suggesting that sucrose stimulation might be independent of its metabolism into constituent hexoses. On the other hand, poorly metabolisable and or non-metabolisable sugars (i.e. mannose, 3-*O*-methyl glucose, mannitol and palatinose) did not induce *sbe* expression, suggesting that *sbe* transcription is independent of osmotic effects. Most notably, the effects of the two sucrose (Glc[1→2]Fru) analogs {i.e. turanose (Glc[1→3]Fru) and palatinose (Glc[1→6] Fru)} indicated that the stimulatory effect of turanose might have a relation with its transportation into the cell and/or the steric position of hydrogen at positions 2 and 3 in the Fru moiety is important for *sbe* stimulation. Also, this finding suggested that the Fru moiety must be part of a disaccharide to trigger expression since; Fru by itself was not sufficient to induce readily detectable *sbe* expression. Consistent with this hypothesis, it was shown previously that induction by turanose might be associated with its transport across the plasma membrane and the disaccharide-linked fructosyl unit has been identified as essential for a hydrophobic interaction between sucrose and the sucrose transporter, SUT (Chandaran *et al.,* 2003; Loreti *et al.,* 2000). Further analysis of the effects of palatinose and turanose were not sufficient to convincingly neither show nor preclude the likelihood of extracellular and or intracellular sucrose sensing mechanism. To dissect this possibility, an attempt was made to unravel the effects of sucrose and turanose by simultaneously examining their stimulatory capacity in the presence or absence of glucosamine, a potent hexokinase (HXK) inhibitor. In this analysis, the finding that sucrose and turanose-induced *sbe* expression was impaired (sucrose) or abolished (turanose) in the presence of glucosamine suggested that HXK and downstream metabolism of sucrose might be involved (**Paper II**). Broadly, these results were considered to suggest that turanose and or sucrose elicited signal actuates endogenous HXK and in turn, potentiated HXK modulates semidian *sbe* transcription oscillation. Taken together, these findings implied the existence of an intracellular disaccharide sensor and concomitant recruitment of downstream hexose metabolites in the regulatory mechanism of *sbe*. Moreover, the existence of multiple sugar signaling mechanisms involving sucrose as a specific signal (León & Sheen, 2003), signaling at the SUT level (Smeekens & Rook, 1997) and involvement of glycolysis-dependent sensing pathway (Loreti *et al.,* 2001) have been reported previously in a number of crop species suggesting a likelihood of a similar phenomenon in this case.

In practice, these results were interpreted to suggest that *sbe* preferentially recognises a photosynthetic product, sucrose. In proof of concept, sucrose therapeutic studies were conducted in decapitated plants and we found that *sbe* transcript levels were strongly enhanced. This result convincingly revealed the

requisite for sucrose flux from the photosynthetic source to the sink storage root cells to trigger expression (Fig. 4). Furthermore, these data uncovered the existence of both apoplastic (storage root fed) and symplastic (therapeutic fed) modes of sucrose phloem uploading in the growing cassava storage roots. In potato tuber, phloem unloading is predominantly apoplastic during stolon elongation, but primarily symplastic during the initial phases of tuberisation (Viola *et al.,* 2001). In contrast, because cassava storage root concurrently initiates new deposition sites and fill existing ones in a compartmentalised manner, it is possible that apoplastic and symplastic modes of phloem uploading associate with initiation and filling events, respectively.

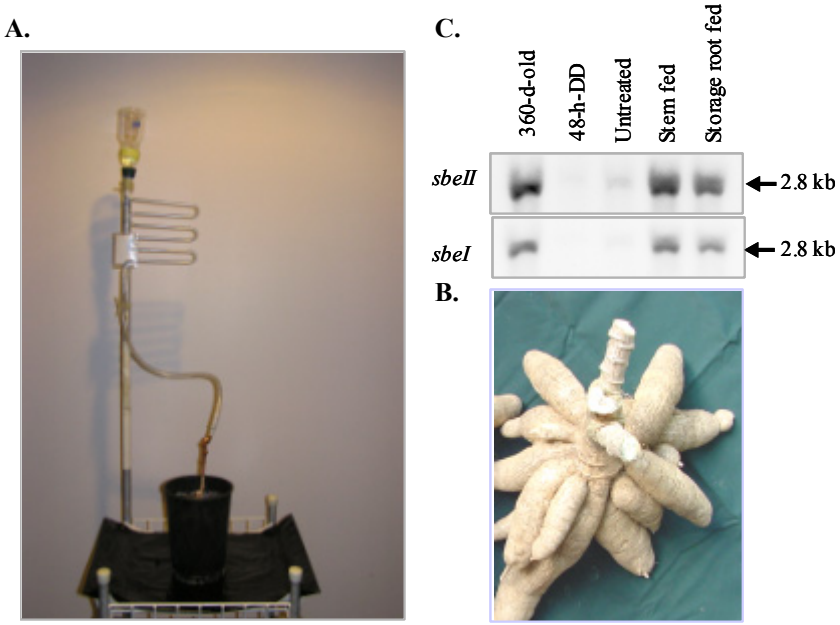

Figure 4. Sucrose import into the cassava storage root. A. Symplastic loading (therapeutic system). B. Apoplastic loading (storage root fed). C. RNA-gel blot analysis of sucrose induce *sbe* mRNA.

Further analysis showed that *sbe* semidian transcript and enzymatic activity oscillation in the isolated discs persisted without any damping through day 4, although in decapitated plants, the rhythmicity was abolished. This comparison between the effects arising from intact and uncoupled storage roots suggested that factors in addition to diurnal function and sugar signaling influence the transcription activity of *sbe* genes. To investigate this clue, it was speculated that hormonal-effect is involved in downstream signaling cascades for *sbe* expression. We found that ABA induced *sbe* expression, strongly enhanced the sucrose-effect, and uncoupled the function of the semidian oscillator (**Paper II**). Also, it was shown that ABA effect very likely is independent of sucrose stimulation since by itself and or in perturbed sucrose signaling background, its ability to induce *sbe* was retained. As a consequence of these results, the role of other regulatory

components on nuclear expression of *sbe* was studied to gain additional insight into the functioning of the entire regulatory network.

2.5. Involvement of plastid *de novo* protein synthesis and protein dephosphorylation in *sbe* expression

Data presented previously provided evidence for the role of plastid signaling in eliciting gene expression in the nucleus (Kropat *et al.,* 2000). As a preliminary approach, a study was performed to investigate whether a similar signaling pathway plays a role in starch synthesis in the amyloplast of cassava. Specifically, the role of plastid *de novo* protein synthesis and protein dephosphorylation on *sbe* transcription activity was investigated. During experimentation, 360 d-old plants were entrained as previously described (**Paper I** and **III**) and subjected to known various inhibitors of *de novo* protein synthesis and protein phosphatases as indicated in **Paper IV**. Clearly, the expression of *sbe* in cycloheximide-treated storage root discs was not affected, indicating that cytosolic *de novo* protein synthesis is not essential for its expression. In contrast, *sbe* expression was completely abolished in chloramphenicol-treated discs suggesting that plastid *de novo* protein synthesis plays a significant role in its regulatory network. Collectively, these results provided the first evidence that nucleoplastidic signaling might be involved in the regulation of *sbe* expression. Indeed, nucleoplastidic signaling, commonly referred to as retrograde signaling has been demonstrated previously in a number of plant signaling systems (for a review see Kropat *et al.,* 2000). The mechanism involves the participation of a number of cytoplasmic regulatory factors that link nuclear sensing to plastid signals in the signaling transduction highway. To explore this possibility, the link between plastidial signaling and cytoplasmic protein dephosphorylation was examined. Here, okadaic acid, which preferentially inhibits type 1 and 2A protein phosphatases, PP1 and PP2A respectively, by mimicking the role of cytosolic phosphoprotein phosphatase inhibitor 1 and 2, abolished the transcriptional activity of *sbe*, in the presence or absence of sucrose. Conversely, cantharidin, a potent inhibitor of PP2A, did not, indicating that PP1 is essential for *sbe* expression. In addition, inhibition of PPI by okadaic acid was considered to suggest that it had delinked nucleocytoplasmic traffic and prevented upstream plastidic signal transduction resulting into abolished *sbe* expression. Hence, on the basis of these results, a nucleo-plastid signaling working model for the regulation of *sbe* was proposed (**Paper IV**). In this model, sucrose signaling via the plastid (amyloplast) was identified as a major route in the storage root of cassava. It was also found that cytosolic and nuclear signaling pathways alone were not sufficient to elicit *sbe* expression without the plastid factor. Likewise, the plastid factor by itself was equally not sufficient to elicit *sbe* expression without the presence of the cytosolic component. Moreover, these findings further revealed that the plastid factor appeared to stimulate nuclear transcription of *sbe*, possibly after it had actuated relevant cytoplasmic regulatory components. In addition, the fact that sucrose-chloramphenicol-treated discs resulted in abolished *sbe* expression but that expression was rescued when the discs were treated simultaneously with ABA showed that the sucrose-stimulated plastid signal functions to activate downstream cytoplasmic signaling components. In spite of these intriguing results, the nature

of the signal generated by the amyloplast, the signal transduction mechanism across the amyloplast membrane, and the mechanism by which the transduced signal is sensed by cytoplasmic regulatory components remain a mystery. However, within the wisdom of these data it was speculated that redox signaling might be involved. Analysis of the regulatory mechanism of the catalytic subunit of AGPase in potato tubers also indicates that this is the case (Tiessen *et al.,* 2002). Tiessen and co-workers showed that when sucrose was supplied to potato tubers, starch synthesis increased, albeit with low levels of phosphorylated intermediates, including 3-PGA (Geigenberger & Stitt, 2000; Tiessen *et al.,* 2002). Similarly, these data demonstrate that sucrose and or its plastid-derived signal is crucial for eliciting nuclear expression of *sbe.*

2.6. A working model for the regulation of *sbe* expression

On the basis of results described in this thesis, a dual control mechanism by sugar and hormonal signaling is proposed for the regulation of *sbe* expression. It is evident that *sbe* is transcriptionally active for only a few hours, producing peak mRNA 6 h after illumination. Its rhythmic expression occurs independently of the presence of light/dark cycles indicating that an intrinsic semidian oscillator transcriptionally regulates its expression. Furthermore, correlation of the effects of ABA and sucrose signaling suggest that a rhythmic signal mediates activation and or synthesis of the essential regulatory downstream transcription proteins. ABA induced signal and or by itself, but independently of the semidian oscillator, actuates downstream transcription regulatory activators that mediate recruitment of basal transcription machinery for *sbe* expression. Likewise, the sucrose-stimulated plastid factor mediates *sbe* expression through similar transcription activators, although, in this case, the semidian oscillator is involved. Consistently, continuous supply of ABA abolished the rhythmic oscillation in *sbe* transcript levels. Conceivably, sucrose exerts its effect through an endogenous semidian oscillator, but ABA does not. Following this, it is logical to surmise that the sucrose-dependent plastid signal arises from either its gated import at the SUT level and or is a consequence of cycling in HXK activity.

Repeated experimental evidence provided additional support for the involvement of HXK as a central semidian oscillator, since glucose by it self sustained oscillation but G-1-P did not. Here, HXK is the link between glucose and G-1-P. Further evidence in support of HXK as the oscillator has been deduced from the effects of turanose. Turanose-induced *sbe* mRNA oscillation was considered to suggest that turanose mediates *sbe* transcription machinery by eliciting a signal at the plasma membrane (SUT and/or a specific sensor), which in turn actuates HXK and downstream HXK-dependent glucose phosphorylation. Arising from this analysis, the possibility for multiple sugar signaling mechanisms, i.e. HXK-mediated signaling pathway and gated sucrose import/perception at the plasma membrane SUT, remained opened. Whatever the mechanism(s), at regulatory mechanistic level, this model asserts that ABA and sucrose signaling pathways initially operate independently but share a common signal transduction junction via the PP1 regulatory step located downstream of the sucrose-signaling pathway. Taken together, it is evident that a gated mechanism at

the sucrose transport level and or HXK cycling events drives the semidian rhythmicity of *sbe* expression.

In practice, a concerted ABA-sucrose mode of action might be linked to storage root developmental phases. Initially, ABA might have a role in storage root differentiation and initiation of the storage root bulking similar to the biphasic ABA pattern in daffodil, *Narcissus pseudonarcissus* (Hunter *et al.,* 2004). During storage root development, ABA might be involved in upregulating *sbe* transcription machinery so as to cope with physiological sugar levels for starch deposition. Ultimately, during senescence, increased ABA levels might associate strongly with increased assimilate mobilisation for deposition of storage reserves including starch. In congruence, this might be a preparedness bypass semidian oscillator mechanism that effectively links arrhythmic supply of mobilised reserves with ultimate carbon metabolism in the storage root organ. In summary, this study has established that transcriptional control of *sbe* genes occurs either at the SUT and HXK levels. Additionally, it has been clearly shown that the transcriptional activity of *sbe* genes in cassava is driven by an endogenous semidian oscillator and interconnected sucrose and ABA signaling pathways, interlinked through unknown PP1-dependent transcription activators. Finally, the study has unlocked evidence for a role of plastid factor(s) in *sbe* expression.

3. Conclusions and perspectives

In this work, a cDNA encoding starch branching enzyme II in cassava was cloned. The spatial and temporal expression profiles were studied and found to strongly correlate with developmental states of the storage root, diurnal functions and cellular metabolic status. *Sbe* transcripts oscillated diurnally with a peak at 6 h after illumination. Repeated analyses of *sbe* diurnal expression profiles in detached storage root slices revealed the existence of a semidian oscillator with a rhythm of 12 h. It was further identified that sucrose is a major signal molecule. Also, this study showed that HXK activity is crucial for the intracellular signaling pathway and that the hexose phosphates, not the phosphorylation step, mediate the signal. Furthermore, it was clearly established that sucrose sensing and/or transport at the plasma membrane level activates HXK. Finally, with regard to the sugar signaling cascade, repeated experimental evidence located the semidian oscillator upstream of G-I-P/G-6-P but downstream of glucose, i.e. at the HXK level.

ABA by itself or with sucrose, induced *sbe* expression, although alone did not maximally drive *sbe* expression compared to its interactive effects with sucrose. Most surprisingly, ABA uncoupled the function of the semidian oscillator observed to control *sbe* expression suggesting the existence of an independent, ABA mechanism bypassing the semidian oscillator. Additionally, it was found that ABA stimulation does not dependent on *de novo* synthesis since fluridon a potent ABA synthesis inhibitor exerted no effect. This analysis also showed that sugar signaling pathway for *sbe* expression in the cassava storage root cells is independent of ABA biosynthesis. However, the inability of the storage root cells to take up fluridon was not excluded. This requires to be investigated further.

In this study, it has also been well established that sugar and ABA signaling pathways converge at a protein phosphatase. In addition, it is very likely that the potentiative effects of ABA on protein phosphatase 1, enhance the sugar signaling pathway, since their combined effect on *sbe* expression was higher than when applied singly.

The role of plastid *de novo* protein synthesis was examined and found essential in the regulatory network of *sbe* expression. This revealed the significance of a plastid factor, but at the same time showed that the plastid factor by itself without the cytoplasmic regulatory component is not sufficient to induce expression of starch synthesis genes in the cassava storage root.

Together, this work showed that starch synthesis in cassava depends on the plasticity of the storage root development states, rhythmicity of the semidian oscillator, sucrose and ABA-mediated interconnected signaling transduction pathways, plastid *de novo* protein synthesis and protein phosphorylation and dephosphorylation cycling.

Finally, in spite of this progress, questions still remain. The most glaring challenge is the lack of knowledge on the nature and function of the endogenous oscillator, as well as the molecular basis underlying the interactive effects between sucrose and ABA signaling. The identity and role of involved transcription activators, plastid *de novo* proteins and phosphatases in starch synthesis is far from clear. Furthermore, the linkage between the plasticity of the storage root developmental states and starch accumulation, and whether it typifies a specific signaling network remains unsettled. These questions need to be addressed to obtain a comprehensive understanding of the whole signaling mechanism.

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