Journal of Experimental Botany, Vol. 57, No. 12, pp. 3243–3258, 2006
doi:10.1093/jxb/erl084 Advance Access publication 22 August, 2006

Journal of Experimental Botany

RESEARCH PAPER

Biochemical and genomic analysis of sucrose metabolism during coffee (*Coffea arabica*) fruit development

Clara Geromel[1,*], Lúcia Pires Ferreira[2,*], Sandra Maria Carmelo Guerreiro[4], Aline Andréia Cavalari[1], David Pot[2,5], Luiz Filipe Protásio Pereira[2,3], Thierry Leroy[5], Luiz Gonzaga Esteves Vieira[2], Paulo Mazzafera[1] and Pierre Marraccini[2,5,†]

[1] *UNICAMP (Universidade Estadual de Campinas), Departamento de Fisiologia Vegetal, IB, CP 6109, 13083-970 Campinas, SP, Brazil*

[2] *IAPAR (Instituto Agronômico do Paraná), LBI-AMG, CP 481, 86001-970 Londrina PR, Brazil*

[3] *EMBRAPA Café, Laboratório de Biotecnologia, CP 481, 86047-902 Londrina, PR, Brazil*

[4] *UNICAMP (Universidade Estadual de Campinas), Departamento de Botânica, IB, CP 6109, 13083-970 Campinas, SP, Brazil*

[5] *Cirad, UMR PIA, Avenue d'Agropolis, F-34398 Montpellier Cedex 5, France*

Received 3 February 2006; Accepted 20 June 2006

Abstract

Sucrose metabolism and the role of sucrose synthase were investigated in the fruit tissues (pericarp, perisperm, and endosperm) of *Coffea arabica* during development. Acid invertase, sucrose phosphate synthase, and sucrose synthase activities were monitored and compared with the levels of sucrose and reducing sugars. Among these enzymes, sucrose synthase showed the highest activities during the last stage of endosperm and pericarp development and this activity paralleled closely the accumulation of sucrose in these tissues at this stage. Carbon partitioning in fruits was studied by pulse–chase experiments with ^{14}C-sugars and revealed high rates of sucrose turnover in perisperm and endosperm tissues. Additional feeding experiments with $^{14}CO_2$ showed that leaf photosynthesis contributed more to seed development than the pericarp in terms of photosynthate supply to the endosperm. Sugar analysis, feeding experiments, and histological studies indicated that the perisperm plays an important role in this downloading process. It was observed that the perisperm presents a transient accumulation of starch which is degraded as the seed develops. Two full-length cDNAs (*CaSUS1* and *CaSUS2*) and the complete gene sequence of the latter were also isolated. They encode sucrose synthase isoforms that are phylogenetically distinct, indicating their involvement in different physiological functions during cherry development. Contrasting expression patterns were observed for *CaSUS1* and *CaSUS2* in perisperm, endosperm, and pericarp tissues: *CaSUS1* mRNAs accumulated mainly during the early development of perisperm and endosperm, as well as during pericarp growing phases, whereas those of *CaSUS2* paralleled sucrose synthase activity in the last weeks of pericarp and endosperm development. Taken together, these results indicate that sucrose synthase plays an important role in sugar metabolism during sucrose accumulation in the coffee fruit.

Key words: *Coffea arabica*, endosperm, gene expression, sucrose synthase, sugar partitioning.

Introduction

Coffee is a very important crop with more than seven millions tons of green beans produced every year on about 11 millions hectares worldwide. After oil, coffee ranks second on international trade exchanges, being responsible for several million jobs in producer and consumer

* These authors contributed equally to this work.
† To whom correspondence should be addressed at Cirad/EMBRAPA, Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia, PBI, CP 02372, 70770–900 Brasilia, DF, Brazil. E-mail: marraccini@cirad.fr

countries. The two main species cultivated throughout the tropical world are *Coffea arabica* (2*n*=4*x*=44), which grows in highlands and represents approximately 70% of world production, and *Coffea canephora* (2*n*=2*x*=22), which grows in lowlands and represents the remaining 30%. Production involving other species, such as *Coffea liberica*, is incipient and consumption limited to local and restricted markets (Söndahl and Lauritis, 1992).

In terms of cup quality, *C. arabica* (Arabica) is appreciated to a greater extent by consumers due to its lesser bitterness and better flavour compared with *C. canephora* (Robusta). What exactly determines cup quality is a complex phenomenon that is far from being understood. Sucrose is one of the compounds in the raw coffee bean that has been implicated as an important precursor of coffee flavour and aroma because it degrades rapidly during roasting, forming anhydro-sugars (such as 1,6 anhydro-glucose) and other compounds like glyoxal (De Maria *et al.*, 1994). Such molecules are then able to react principally with amino acids (Maillard reaction) forming aliphatic acids, hydroxymethyl furfural and other furans, and pyrazine. These compounds are all considered to be essential contributors to coffee flavour, either as volatile (Grosch, 2001) or non-volatile (Homma, 2001) components. The preference for Arabica coffees seems to be related in part to differences in sucrose content (Casal *et al.*, 2000), which range from 5.1% to 9.4% of dry matter in harvested coffee beans of this species, whereas for Robusta these values are always lower, usually ranging from 4% to 7% of dry matter (Ky *et al.*, 2001; Campa *et al.*, 2004).

Despite the importance of sucrose as a precursor of coffee beverage quality, nothing is known about its distribution in tissues nor the role of key enzymes of its metabolism, such as invertases (EC 3.2.1.26, β-fructosidase, β-fructofuranosidase), sucrose phosphate synthase (SPS: EC 2.4.1.14), and sucrose synthase (SUS: EC 2.4.1.13). Invertases, which catalyse the irreversible hydrolysis of sucrose to glucose and fructose, are involved in various aspects of the plant life cycle and the response of the plant to environmental stimuli (Roitsch and González, 2004). By contrast, SPS (UDP-glucose: D-fructose 6-phosphate 2-glucosyl-transferase) appears to function mainly in the direction of sucrose synthesis (UDP-glucose+fructose-P→sucrose-P+UDP) while SUS (UDP-glucose: D-fructose 2-glucosyl-transferase) catalyses a reversible reaction (UDP-glucose+fructose↔sucrose+UDP). Both enzymes are thought to play a major role in sucrose partitioning for energy purposes as well as in metabolic, structural, and storage functions of plant cells (see review by Sturm and Tang, 1999). Sucrose cleavage activity of SUS is also linked to cell wall biosynthesis by providing UDP-glucose for the cellulose synthase complex (Amor *et al.*, 1995) and substrates for starch synthesis in sink organs (see review by Herbers and Sonnewald, 1998).

The present study was conducted to understand sucrose metabolism and its transport during coffee fruit development. Concentrations of reducing sugars and sucrose, and activities of sucrose-metabolizing enzymes, were monitored in the pericarp, perisperm, and endosperm throughout fruit development. To analyse sugar partitioning between these tissues, histological studies and feeding experiments using ^{14}C-labelled sucrose, fructose, and CO_2 were carried out. In addition, two distinct SUS-encoding cDNAs were isolated (designated *CaSUS1* and *CaSUS2*) from mRNA of coffee endosperm. The molecular structure of these sequences and relationships between sucrose/reducing sugar contents, activities of sucrose-metabolizing enzymes, and expression of SUS genes are presented and discussed.

Materials and methods

Plant materials

Fruits and tissues were harvested from 15-year-old plants of *Coffea arabica* cv. IAPAR 59 cultivated under field conditions. Fruits were collected between 13.00 h and 16.00 h, every 4 weeks from flowering (end of September 2002) up to complete maturation (mid-May 2003). After collection, tissues were immediately frozen in liquid nitrogen and stored at −80 °C before being analysed. Fruit tissues (perisperm, endosperm, and pericarp) were separated and used independently to extract total RNA or were analysed for sugars and enzyme activity. The same material was used for the $^{14}CO_2$ and ^{14}C feeding experiments.

Sugar determination, enzymatic analysis and western blotting

Fruits tissues were freeze-dried, ground in a mortar and pestle, and extracted with 80% ethanol in a Polytron homogenizer using 1 ml per 300 mg of tissue. Extraction proceeded for 30 min at 75 °C in cap-sealed tubes and the supernatant was obtained after centrifugation. The extraction was carried out three times with the same volume of ethanol, and the combined supernatants were used for the analysis of sugars. Total soluble sugars (Dubois *et al.*, 1956), sucrose (Van Handel, 1968), and reducing sugars (Somogyi, 1952) were determined in the extracts. For analysis of enzymes the tissues were extracted with 100 mM HEPES, pH 7.0, containing 2 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 2% (w/v) ascorbic acid. The supernatant recovered by centrifugation (27 000 *g* for 20 min) was desalted on PD10 minicolumns (Amersham Biosciences) and the protein content determined with a ready-to-use Bradford (1976) reagent (Bio-Rad). Acid invertase (AI) was assayed by incubating an aliquot of the desalted extract containing 60 μg of protein with 25 mM sucrose in 50 mM citrate-phosphate buffer pH 3.5, at 37 °C, for 1 h (modified from Yelle *et al.*, 1991). SPS was assayed by incubating 60 μg of protein with 25 mM uridine 5′-diphosphoglucose (UDPG), 25 mM fructose-6-phosphate, 30 mM glucose-6-phosphate, 20 mM phenyl-β-glucoside in 50 mM K-phosphate buffer, pH 7.5. The assay was incubated at 37 °C for 1 h and stopped by the addition of 30% (w/v) KOH and boiling for 10 min. Sucrose phosphate content was determined according to Van Handel (1968). SUS activity was assayed in the direction of sucrose synthesis in a reaction containing 50–60 μg of protein, 25 mM UDPG, 25 mM D-fructose, 50 mM 2-(*N*-morpholino)ethanesulphonic acid hydrate (MES), at pH 6. The amount of protein, substrate concentrations, and pH used in this assay were defined in preliminary tests. After 60 min of incubation at 30 °C the reaction was stopped by the addition of 30% (w/v) KOH and boiling for 10 min. Sucrose content was

determined according to Van Handel (1968). Extracts obtained for enzyme analysis were used in western blot experiments. The proteins were separated by 10% (w/v) polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes using a Mini protean electrophoresis apparatus (Bio-Rad). The membranes were probed with a polyclonal antibody towards SUS from *Pisum sativum* using the protocol described by Déjardin *et al.* (1997). The membranes were developed using an anti-rabbit secondary antibody conjugated with alkaline phosphatase.

^{14}C-feeding experiments

Three pulse-chase experiments were carried out to study sucrose metabolism in coffee fruits. In the first experiment, incubation with $^{14}CO_2$ was carried out with fruits at 120±20 DAF with the perisperm, endosperm, and pericarp representing respectively 20%, 28%, and 52% of the fruit fresh weight, or with leaves. Two Eppendorf™ tubes were left inside the plastic bags, one containing a 1 M HCl solution and the other an aqueous solution carrying 10 Mdpm $NaH^{14}CO_3$ (50–62 mCi mmol^{-1}, Amersham Biosciences). By external handling, the tubes were opened and the contents mixed. The branches were left enclosed in the plastic bags from 06.00 h to 10.00 h, the bags opened and the fruits collected the next day (08.00 h). The fruits were separated into endosperm, perisperm, and pericarp, freeze-dried, and extracted as described for the sugar measurements. The radioactivity in each extract was then estimated in a scintillation counter after the addition of scintillation fluid. To eliminate interference by chlorophyll quenching, a known amount of radioactivity was added to each sample and the radioactivity counted again. These data were used to calculate the counting efficiency and thereby to correct the values obtained in the first counting. In other experiments, fruits were harvested, fixed with lanolin in plastic boxes and fed either with 0.9 μCi [U-^{14}C]sucrose (50 μCi mmol^{-1}) or [U-^{14}C]fructose (50 μCi mmol^{-1}) (Amersham Biosciences) and then kept under a 100 W incandescent light positioned 50 cm (approximately 80 μmol photons m^{-2} s^{-1}) over the boxes for 24 h. Endosperm, perisperm, and pericarp tissue were separated and processed as above for radioactivity determination. In this experiment, the ethanolic extracts were reduced in volume in a SpeedVac® (Savant) and the sugars separated by descending paper chromatography using ethyl acetate:pyridine:-H_2O:acetic acid:propionic acid (50:50:10:5:5, by vol.) as solvent. Glucose, fructose, and sucrose were applied over the samples and also in lateral lanes (50 μg) as markers. The chromatograms were developed for 20 h and the sugars revealed with aniline reagent (Walkey and Tillman, 1977). The spots were cut from the chromatograms in thin strips that were then placed in scintillation flasks. After addition of 1 ml methanol and 5 ml scintillation fluid, the radioactivity was determined in a scintillation counter (LS 6500 Scintillation Counter, Beckman) for 10 min. Spots of glucose and fructose were placed together in the same flasks. The lateral control markers were also processed in the same way. As mentioned above, a known amount of radioactivity was added to each sample and the radioactivity counted again in order to correct for quenching interference. In a third experiment, fruits at different maturation stages were collected from the same tree and placed inside a glass flask sealed with a rubber cap containing a tube with an aqueous solution with 4 Mdpm $NaH^{14}CO_3$ (50–62 mCi mmol^{-1}, Amersham Biosciences) to which a few drops of 3 M HCl solution were applied with a syringe. The experiment was carried out under light as described for sugar feeding. The flask was then opened and the fruits left for a further 24 h. Since fruits at different developmental stages were used in this experiment, following incubation each fruit was weighed separately and separated into four groups: pinheads (60 DAF), green 1 (130 DAF), green 2 (165 DAF), and mature (≈ 234 DAF). For each group, endosperm, perisperm, and endocarp were separated and processed as above for radioactivity determination.

Histological studies

Fruits were collected at 40, 60–75, and 205–234 DAF and fixed in neutral formalin buffer (10% (v/v) formol, 0.4% (w/v) NaH_2PO_4, H_2O, 0.65% (w/v) anhydrous Na_2HPO_4). Dehydration was carried out by treating segments sequentially with 30%, 50%, and 70% (v/v) ethanol for 12 h at each concentration. The material was then treated with a plastic resin (Historesin, Leica) according to the protocol of Gerrits and Smid (1983). The polymerized resin blocks were then glued onto wooden blocks with plastic adhesive and 12 μm-thick sections cut on a rotary microtome (American Optical M 820, Phoenix). The sections were mounted on glass slides, heated to 65 °C, followed by staining for 3 min in 0.05% (v/v) toluidine blue in 0.1 M acetate buffer, pH 4.7, and washing in running water for 5 min (O'Brien *et al.*, 1964). Sections were tested for starch with Lugol's iodine solutions according to Johansen (1940). The sections were observed and photographed with a light microscope (Olympus, model BX51).

cDNA isolation and gene cloning

Cloning of *SUS* cDNA sequences was facilitated by the use of EST available from the Brazilian Coffee Genome Project (http://www.lge.ibi.unicamp.br/cafe/). The *CaSUS1* cDNA was isolated from endosperm of fruits at 147 DAF. Total RNA was extracted as described previously (Rogers *et al.*, 1999*a*) and 500 μg were treated with Oligotex dT beads (Qiagen) to purify 2 μg of mRNA which was reverse-transcribed according to the protocol defined in the Marathon™ cDNA amplification kit (BD Biosciences Clontech). Subsequently, a 3′ RACE PCR reaction was performed using the AP1 primer 5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′ from the Kit and the UPC1C3M1 SUS primer 5′-TAT ACT CTG TTT CTC CGT TAC TCT TTT TT-3′ deduced from a cluster formed by the compilation of 250 SUS-encoding ESTs, found within the Brazilian Coffee Genome Project. PCR was performed using a PTC-100 Thermocycler (MJ Research) with Advantage2 *Taq* DNA polymerase according to the supplier (BD Biosciences Clontech) under the following conditions: initial denaturation 94 °C, 1 min; followed by 35 cycles of 94 °C, 30 s; 52 °C, 30 s; and 72 °C, 6 min, and a final extension step of 72 °C, 6 min. A PCR fragment of about 3 kb was obtained, ligated in pTOPO2.1 (Invitrogen) and amplified in *E. coli* TOP10 cells (Invitrogen). A recombinant plasmid was selected, purified using the Qiafilter extraction Kit (Qiagen) and double-strand sequenced with universal and internal primers. The *CaSUS2* cDNA was isolated by the same 3′ RACE PCR reaction as described for *CaSUS1* cDNA isolation, except that mRNAs were from endosperm of fruits at 234 DAF. The UPC4M1 5′-GAA AGC GCT AGA GAA CTC TTG ATC GAG TA-3′ primer used was deduced from a SUS-encoding contig formed by the compilation of 12 ESTs that did not cluster with the *CaSUS1* cDNA. PCR was performed as follows: initial denaturation 94 °C, 1 min, and 35 cycles of 94 °C, 30 s; 68 °C, 6 min, with a final extension step of 72 °C, 6 min. The PCR amplified fragment was treated as previously described. The *CaSUS2* gene was amplified from genomic DNA (10 ng) of *C. arabica* cv. IAPAR 59 using the primers UPC4M1 and REVC7 5′-GGA AGA CTG CCG CGG AGA CCA GAC ATC T-3′ deduced from its corresponding cDNA. PCR conditions used for the *CaSUS2* cDNA amplification were as described for *CaSUS1*, except that 40 cycles were performed. The fragment obtained was cloned in pTOPO2.1 (Invitrogen) and double-strand sequenced.

Probe preparation

The internal probe (probe A: 667 bp) of *CaSUS1* was amplified by PCR using this cDNA as a template, the specific primers SUS10 5′-GTT ATC CTG ATA CCG GTG-3′ and SUS11 5′-GGA TCA AAA ACA TCA ATG CC-3′, and the Advantage2 *Taq* DNA polymerase under the following conditions: initial denaturation step 94 °C, 1 min;

followed by 35 cycles of 94 °C, 1 min; 50 °C, 1 min; 72 °C, 3 min, with a final extension of 72 °C, 6 min. The 3′ probe (probe B: 544 bp) of *CaSUS1* was amplified under the same conditions except that the primers UPC5C3 5′-AGC GAG CTC CTT GCC AA-3′ and REVC5C3 5′-CTT ATT ACA AAA TGA CAT TTG A-3′ were used. The *CaSUS2* probe (probe C: 589 bp) localized in the 3′ region of the *CaSUS2* cDNA was amplified with the primers UPC6 5′-ACT CTG CGG CAA TGG TAA A-3′ and REVC7 as described above, using this cDNA as template. All these probes were purified by ethanol precipitation in presence of 10% (v/v) NaAc pH 5.2, resuspended in water and quantified. Then 50 ng was labelled by random-priming with 50 μCi of [α-^{32}P]dCTP (Amersham Biosciences) according to Sambrook *et al.* (1989).

Northern and Southern-blot analysis

Total RNA (15 μg) was denatured in 12.55 M formamide, 2.2 M formaldehyde, and 20 mM 3-(*N*-morpholino)-propanesulphonic acid (MOPS) buffer, pH 7.0 (also containing 5 mM Na-acetate and 0.1 mM EDTA) at 65 °C for 5 min and fractionated on a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde in MOPS buffer (Rogers *et al.*, 1999*a*). Hybridization with Ultrahyb™ buffer (Ambion) and washing steps were performed according to the manufacturer's recommendations. To ensure that equal amounts of total RNA were loaded, gels stained with ethidium bromide were performed for each northern blot analysis. RNA blots were prepared in duplicate and probed independently with probe A (*CaSUS1*) and C (*CaSUS2*). Genomic DNA was extracted from fresh coffee leaves as described previously (Marraccini *et al.*, 2001). For Southern-blot analysis, 10 μg were digested with restriction endonucleases *Dra*I, *Eco*RI and *Hin*dIII independently, separated on a 0.8% (w/v) agarose gel and finally transferred to Hybond N$^+$ membranes (Amersham Biosciences). Hybridization and washings were carried out as described before (Marraccini *et al.*, 2001).

Multiple alignments and phylogenetic analysis

Phylogenetic analyses were conducted using MEGA version 3.0 (Kumar *et al.*, 2004). Multiple alignments of SUS proteins were obtained by CLUSTALW program (Thompson *et al.*, 1994) followed by manual adjustment. A phylogenetic tree was inferred by the Neighbor–Joining (NJ) method with Poisson distance. Bootstrap analysis was carried out (5000 trials) to assess support for individual nodes.

Results

Coffee fruit growth

Under the field conditions used in this study, fruits of *C. arabica* cv. IAPAR 59 completed their maturation within eight to nine months. Since fruits did not grow during the first two months following anthesis and fecundation (Fig. 2A), producing cherries of very small size (<2 mm), tissue separation was not possible at this stage, which explains the absence of data for sugars (Fig. 2C, D) and enzymatic assays (Fig. 3A, C) at 30 d after flowering (DAF). Between 60 and 90 DAF, a rapid expansion of the perisperm occurred, followed by the development of the endosperm which became detectable at 75 DAF and easily separable from the perisperm at 118 DAF, when perisperm and endosperm were present in equal proportions (Fig. 2B). Afterwards, the perisperm gradually disappeared up to the formation of a thin tissue known as the silver skin membrane, which surrounds the endosperm at the mature stage (Fig. 1C), making its analysis difficult between 205 and 234 DAF. On the other hand, the endosperm turned from a liquid (147 DAF) to the solid state (176 DAF), before dehydration during the month up to harvest (between 205 and 234 DAF). Over the same period, the observed increase of the cherry fresh weight appeared to be related exclusively to the increase in mass of the pericarp (Fig. 2B).

Accumulation of sugars in coffee fruits

Sugar content was measured in each tissue during fruit development. Reducing sugars (glucose and fructose) accumulated during the perisperm expansion phase up to 260 mg g^{-1} dry weight (DW) at 89 DAF (Fig. 2C). An accumulation of sucrose was also observed over this period, albeit to lesser extent (32 mg g^{-1} DW) in this tissue (Fig. 2D). After 89 DAF, both reducing sugars and sucrose

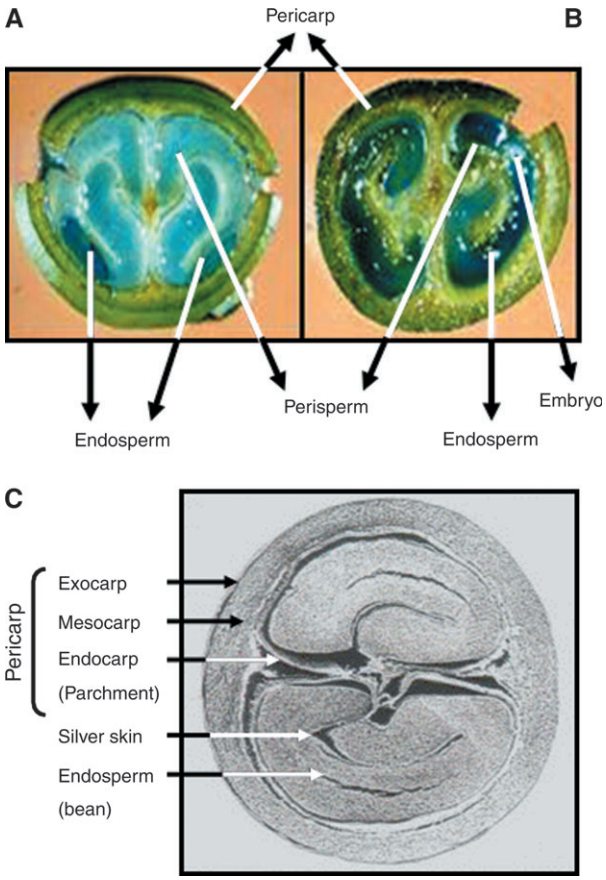

Fig. 1. Cherries of *C. arabica* at 60–90 DAF (A) and 120–150 DAF (B) were sectioned transversely and stained with Evans Blue dye to show the endosperm surrounded by the perisperm (A). After growth (B), the endosperm replaces the space previously occupied by the perisperm. (C) Mature cherry (220–250 DAF) with the endosperm representing the main tissue and the perisperm reduced to the silver skin (courtesy of Nestlé Inc.). The embryo at the distal position can not be observed at this stage.

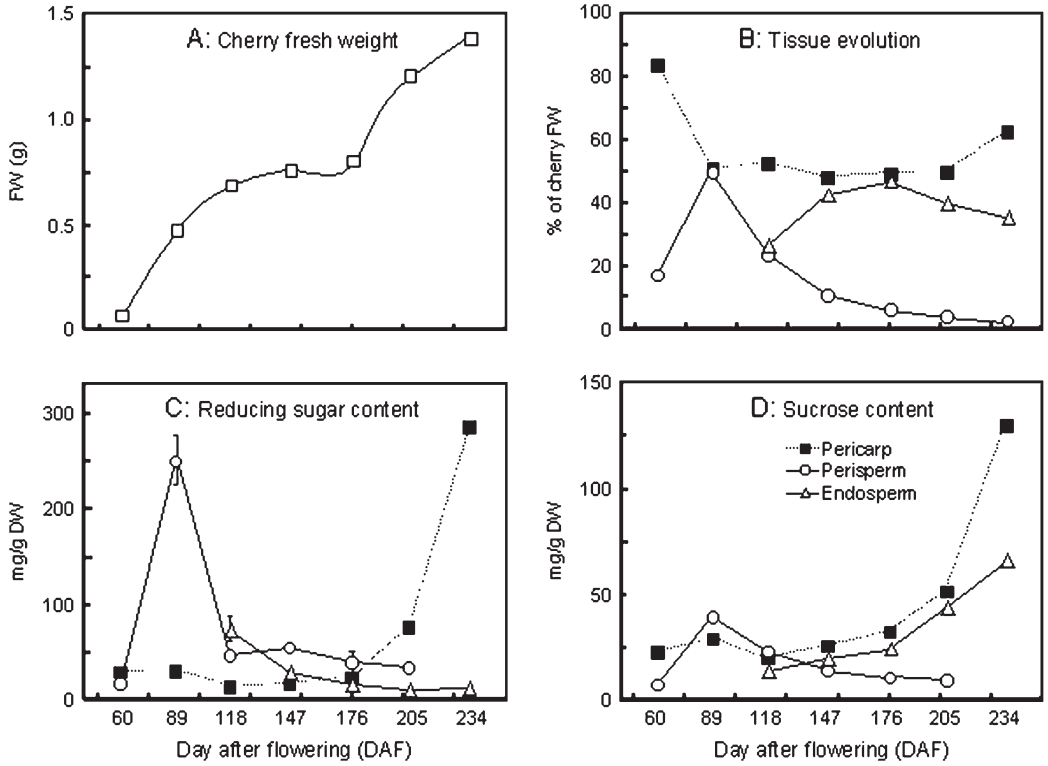

Fig. 2. Weight of tissues, sucrose and reducing sugar contents during *C. arabica* fruit ripening. (A) Cherry fresh weight (FW). (B) Evolution of pericarp (filled squares), perisperm (open circles), and endosperm (open triangles) tissues expressed in percentage of cherry FW. Contents of reducing sugars (C) and sucrose (D) in isolated tissues were expressed as mg g^{-1} dry weight (DW).

decreased steadily in the perisperm as this tissue disappeared, but the reducing sugars/sucrose ratio always remained greater than 1. In the endosperm, the reducing sugars/sucrose ratio was greater than 1 during the two first developmental stages analysed (118 and 147 DAF). Then, during the following stages, the amount of reducing sugars gradually decreased to become almost undetectable in the mature endosperm (Fig. 2C), whereas sucrose accumulated close to 6% of the DW (Fig. 2D). In the pericarp, the reducing sugars and sucrose presented a sudden accumulation after 176 DAF, reaching, respectively, 280 and 180 mg g^{-1} of the DW at maturation (Fig. 2C, D).

Enzymatic activities during coffee fruit development

In order to evaluate the importance of sucrose-metabolizing enzymes during coffee fruit development, AI, SPS, and SUS activities were measured *in vitro* in protein extracts prepared from isolated pericarp (60–234 DAF), endosperm (118–234 DAF), and perisperm (60–205 DAF) tissues (Fig. 3A–C).

AI activity in the perisperm reached a maximum (5.52 μg reducing sugars h^{-1} μg^{-1} protein) at 60 DAF (Fig. 3A), therefore preceding the peak of reducing sugars detected at 89 DAF in this tissue (Fig. 2C). Thereafter, AI activity decreased with development becoming undetectable between 89 and 176 DAF. In the pericarp, AI showed two peaks of similar values (near 4 μg reducing sugars h^{-1} μg^{-1} protein) at 118 and 205 DAF without concomitant changes of reducing sugar content. Whatever the developmental stage analysed, no AI activity was detected in the endosperm. SPS activity remained low in the perisperm between 60 and 118 DAF, but was maximal (5.16 μg sucrose h^{-1} μg^{-1} protein) at 147 DAF and decreased slightly afterwards. In the pericarp, SPS activity was almost negligible during early development, but reached a maximum (6.98 μg sucrose h^{-1} μg^{-1} protein) at 205 DAF and decreased up to maturation. As for the perisperm, maximal SPS activity in the endosperm was observed at 147 DAF, and then decreased gradually towards maturation.

SUS activity was low in the perisperm from 60 to 118 DAF, but increased between 147 and 205 DAF. Activities showed similar patterns in both the pericarp and endosperm, with a small but continuous increase between 60 (pericarp) or 120 (endosperm) and 205 DAF, followed by a sudden increase at harvest time. In addition, activities at 234 DAF presented a similar range (near 23 μg sucrose h^{-1} μg^{-1} protein) and paralleled the sucrose accumulation observed in these tissues (Fig. 2D), as well as being relatively constant over the day (data not shown).

A western blot analysis of endosperm proteins was also carried out using antibodies against the major SUS isoform of pea teguments, probably corresponding to the product of the *PsSUS1* (GB accession number AJ012080) gene (C Rochat, personal communication). Under semi-denaturing

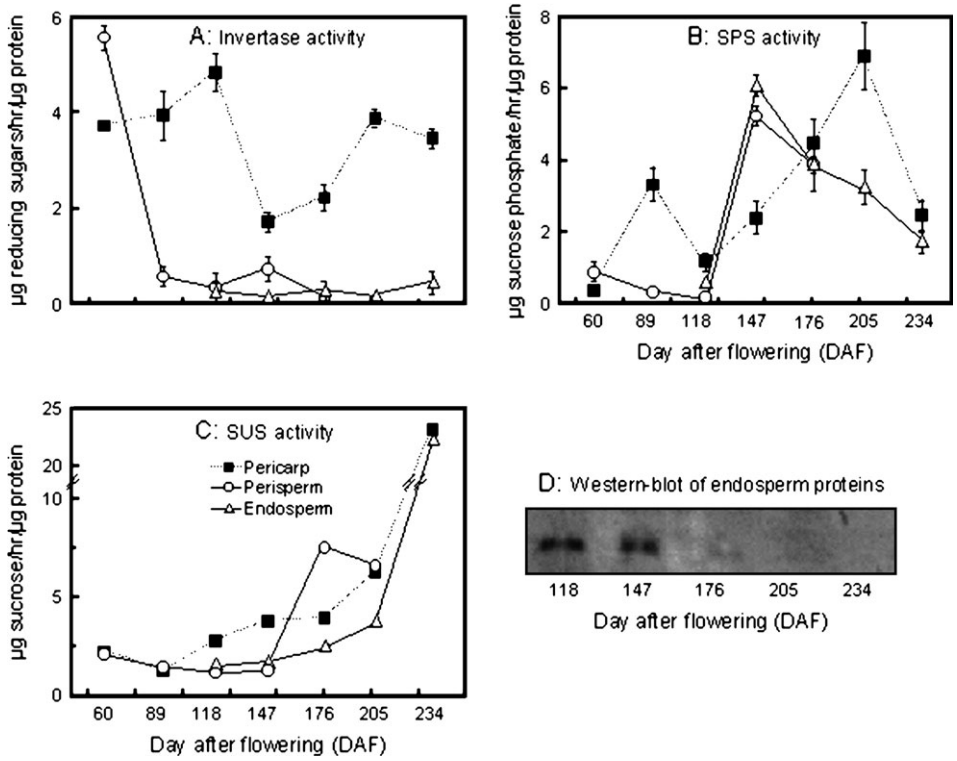

Fig. 3. Enzymatic activities in individual tissues of *C. arabica* fruits under development. The symbols used to represent each tissue are as follows: pericarp (filled squares), perisperm (open circles), endosperm (open triangles). (A) Acid invertase activity was expressed as µg reducing sugars h^{-1} $µg^{-1}$ protein. (B) Sucrose-phosphate synthase and sucrose synthase (C) activities were measured as µg sucrose h^{-1} $µg^{-1}$ protein. (D) Western blot: proteins were extracted from developing endosperm and probed with polyclonal antibodies raised against the abundant SUS isoform from *Pisum sativum* (Déjardin *et al.*, 1997).

electrophoresis conditions, a single SUS isoform was recognized in protein extracts obtained at 118 and 147 DAF (Fig. 3D). A weaker signal was also observed at 176 DAF, but no antibody cross reaction was detected near maturation, at 205 and 234 DAF.

Analysis of ^{14}C distribution in coffee fruits

To analyse sugar metabolism and transfer in coffee cherries, pulse–chase experiments were performed by incubating leaves or green (±120 DAF) fruits with $^{14}CO_2$ and measuring the distribution of radioactivity in the different fruit tissues after 24 h (Fig. 4A). When $^{14}CO_2$ incubations were carried out with fruits, most of the radioactivity remained in the pericarp, but a significant proportion was detected in the perisperm and endosperm. When $^{14}CO_2$ was supplied to leaves, low radioactivity was found in the pericarp, whereas the perisperm accumulated the major proportion of radioactivity. In both experiments, the large accumulation of radioactivity detected in the perisperm reveals the importance of this tissue in photosynthate translocation within coffee cherries. The distribution of radioactivity was also determined in fruits after 4 h of the incubation with $^{14}CO_2$ and a similar situation was observed, although less radioactivity was detected in the fruit tissues (data not shown).

Feeding with ^{14}C-sucrose and ^{14}C-fructose was carried out by applying the labelled compounds to a cut made at the peduncle insertion in the fruit (Fig. 4B–D). In the pericarp, high levels of radioactivity (total and specific) were found in the form of sucrose when labelled sucrose was fed, and as reducing sugar (mainly fructose) when feeding was performed with labelled fructose. Although this experiment did not allow a clear conclusion to be drawn as to the participation of the pericarp in the assimilation process, it did provide information on sugar metabolism in each tissue. For example, the significant accumulation of radioactivity in the perisperm indicates a role for this tissue in the transfer of sugars between the pericarp and the endosperm (Fig. 4B). Besides the transport of labelled sucrose and fructose from the pericarp to perisperm and endosperm, these sugars were readily metabolized to reducing sugars and sucrose, respectively (Fig. 4C, D), indicating the simultaneous synthesis and degradation of sucrose within these tissues. When ^{14}C-sucrose was supplied, the perisperm presented a higher specific radioactivity in the form of sucrose and reducing sugars than for the same sugars found in the endosperm (Fig. 4C). The opposite was observed when ^{14}C-fructose was fed, with the highest sucrose specific radioactivity being found in the endosperm, indicating that active conversion of reducing sugars to sucrose occurred in

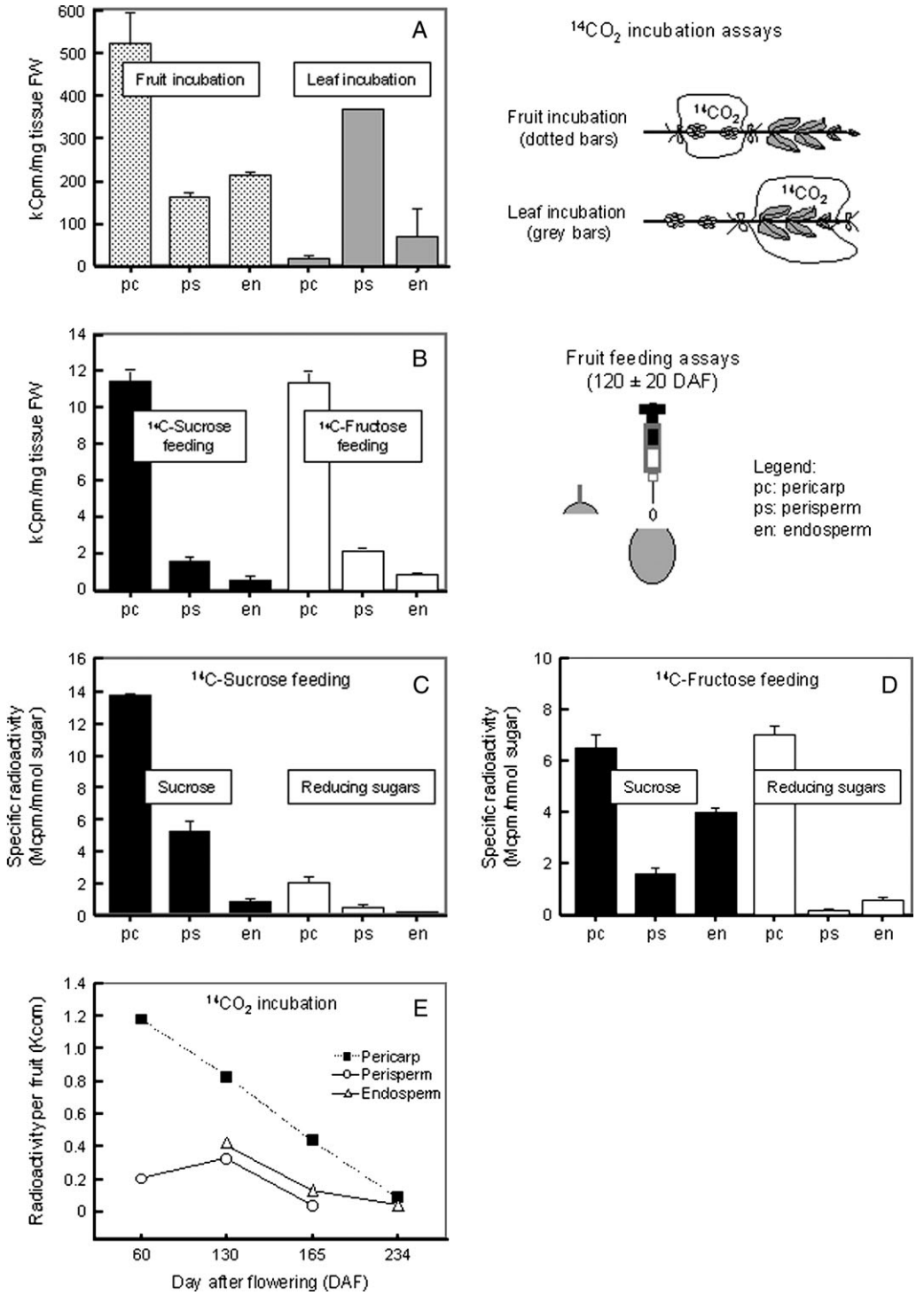

Fig. 4. Analysis of sucrose transport and metabolism in coffee cherries by ^{14}C pulse–chase experiments. (A) Fruits at 120±20 DAF (dotted bars) or leaves (grey bars) were exposed to $^{14}CO_2$ for 24 h and total radioactivity was measured in pericarp (pc), perisperm (ps), and endosperm (en) tissues. A schematic representation of this experiment is also given (right panel of graph A). For feeding experiments (B–D), labelled compounds were applied to a cut made at the peduncle insertion of fruits at 120±20 DAF as shown in the schematic representation given in the right panel of graph B. Fruits were then kept illuminated for 24 h and dissected to measure the radioactivity in each tissue. (B) ^{14}C-sucrose (black bars) or ^{14}C-fructose (white bars) were administered and their distribution was estimated by measuring the total radioactivity. After ^{14}C-sucrose (C) or ^{14}C-fructose (D) feedings, the distribution of the radioactivity in sucrose (black bars) and reducing sugars (white bars) was evaluated in pericarp, perisperm and endosperm tissues. (E) Fruits at different developmental stages were detached and incubated with $^{14}CO_2$ to determine the distribution of radioactivity in each tissue.

this tissue. Such conversions also occurred in the pericarp where the same specific radioactivity was found in sucrose and reducing sugars when ^{14}C-fructose was used in the feeding experiment.

In a third feeding experiment, fruits at different developmental stages were detached and incubated with $^{14}CO_2$ (Fig. 4E). These data show that even during its disappearance with fruit growth, the perisperm retains its role as a transit tissue. Fruit photosynthesis also sharply decreases with the maturation of coffee fruits, as suggested by the gradual decrease of radioactivity in all tissues, mainly in the pericarp.

Histological study of coffee fruits

Tissue organization and evolution was analysed by histological sections of fruits at different developmental stages (Fig. 5A–D). At 40 DAF, the perisperm constituted the main tissue of the fruit and the endosperm was not apparent (Fig. 5A). The latter began to develop internally to the perisperm between 60 and 75 DAF (Fig. 5B). At the mature stage (205–234 DAF), the endosperm filled the entire inner space of the locule while the perisperm was reduced to a few cell layers surrounding the endosperm (Fig. 5C). Throughout development the perisperm always appeared tightly connected to the endocarp by the funicule (pedicel). At the funicule/perisperm boundary, a symplastic continuity was observed with xylem and phloem vascular tissues entering directly in contact with the perisperm. These vascular tissues were not observed within the perisperm or endosperm tissues. Also, no vascular connection was observed between the pericarp and the perisperm. The endocarp is a thick cellulosic tissue (Fig. 1C) which is present from the beginning of fruit formation and seems to provoke the isolation of the perisperm. Therefore, if some vascular connection between pericarp and perisperm truly exists, it could only be through the peduncle. Whatever the developmental stage, the perisperm surface facing the endocarp had an epiderm formed by a cuticle-like layer

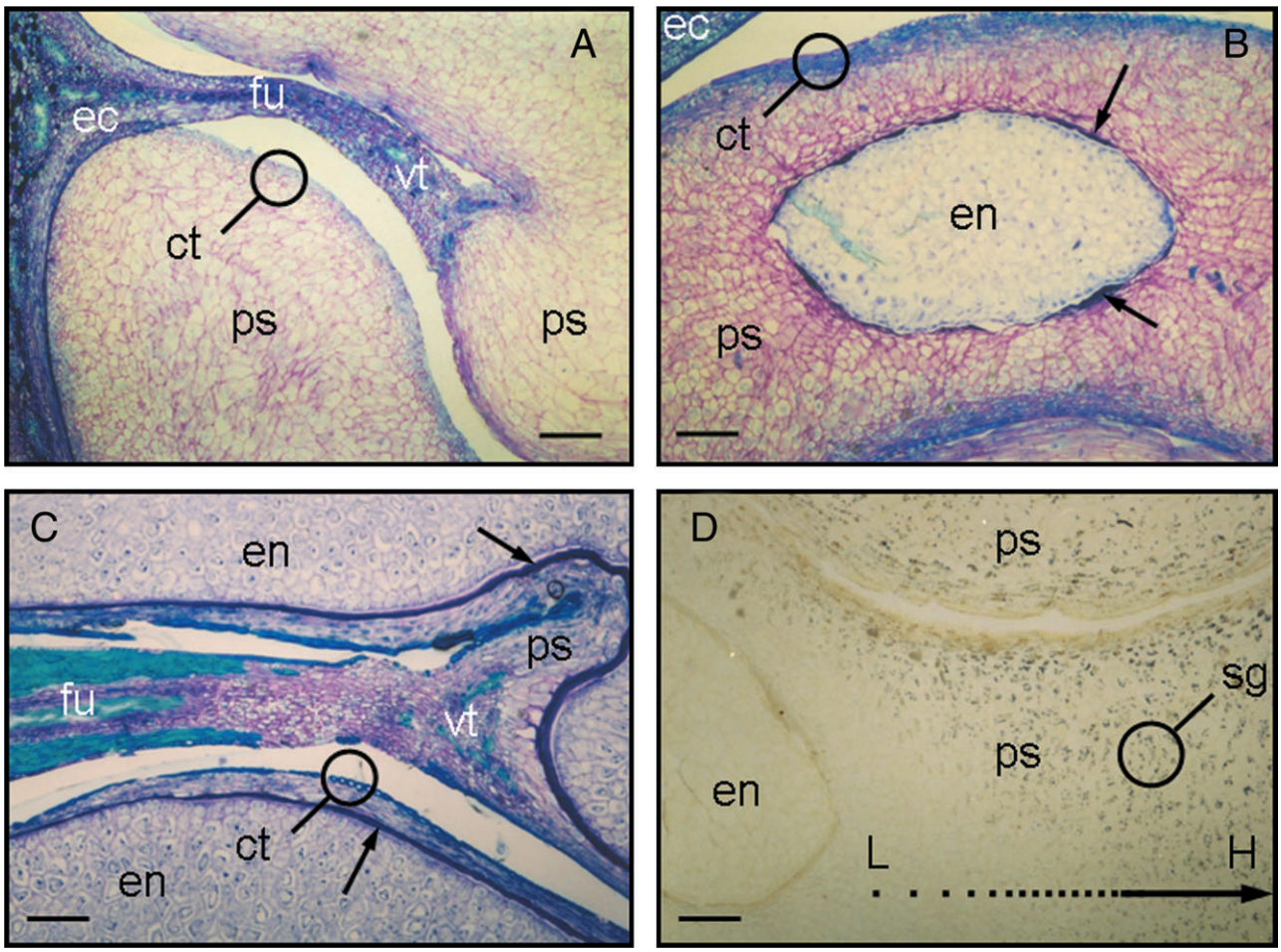

Fig. 5. Histological analysis of coffee fruits at different stages of ripening. Fruits at ≈ 40 DAF (A), 60–75 DAF (B, D), and 205–234 DAF (C) were stained to show the endocarp (ec), perisperm (ps), the endosperm (en), the vascular tissue (vt) located in the funicule (fu) and the cuticle-like layer (ct) of the perisperm. Black arrows show cells at the interface between perisperm and endosperm. (D) Starch granules (sg) revealed by Lugol's iodine coloration are indicated. A dashed arrow indicates the starch gradient in the perisperm from low (L) to high (H) content, respectively in regions close to and distant from the endosperm. Bars represent 200 μm (A, C, D) and 80 μm (B).

(Fig. 5A–C). It could also be observed that perisperm cells in contact with endosperm cells lose their shape, indicating that they are undergoing important changes supposedly ending in cell death (see black arrows in Fig. 5B and C), since at the end of fruit development the perisperm is reduced to a few cell layers (Fig. 5C). Lugol's iodine staining also showed that young perisperm tissue (60–75 DAF) contained starch granules that were no longer observed in the regions close to the growing endosperm (Fig. 5D).

Analysis of CaSUS1 *and* CaSUS2 *cDNA sequences*

The *CaSUS1* cDNA is 2979 bp long containing a 188 bp 5′ untranslated region, a 293 bp 3′ untranslated region including a putative poly(A) addition signal site (TAA-TAA) located 18 bases upstream of a polyA tail of 77 adenosine residues. It also has a single open reading frame coding for a protein of 806 amino acids with a theoretical molecular mass of 92.5 kDa and an estimated isoelectric point (pI) of 6.70. This protein contains a typical SUS motif (pfam00862) in its first 554 amino acid residues and a glycosyl transferase motif (pfam00534) in its C-terminal part (amino acids residues 565–727), confirming that it belongs to the SUS family. At the amino acid level, it shows high homology (95% with 89% of identity) with SUS deduced from the *Sus3*-65 and *Sus4*-16 genes of *Solanum tuberosum* (Fu and Park, 1995). In comparison with other SUS proteins, the CaSUS1 isoform contains a putative phosphorylation site LTRVHSLR (amino acid residues 6–13), with Ser-11 being the probable site of this modification, and two hydrophobic domains of 21 amino acids (motif I: 269–289; motif II 675–695) that may be involved in the binding of this protein to membranes.

The *CaSUS2* full-length cDNA is 2889 bp long and contains 31 bp of 5′ untranslated sequence, 377 bp of 3′ untranslated sequence, a poly A tail of 45 adenosine residues and an open reading frame coding for a protein of 811 amino acids. The predicted molecular mass of CaSUS2 protein is 92.8 kDa with an estimated pI of 6.57. This protein shows high similarity to the SUS2 isoform from potato (AAO67719), CitSUSA from *Citrus unshiu* (BAA88904) and CpSS2 from the resurrection plant *Craterostigma plantagineum* (Hochst.) (CAB38022) with 92%, 91%, and 90% similarity, respectively. The N-terminal putative phosphorylation site of CaSUS2 differed from the CaSUS1 sequence, but retained the Ser-11 amino acid residue. By comparison, these two coffee SUS isoforms showed only 68% of identity and 82% of similarity, therefore demonstrating that they are phylogenetically distant.

Isolation of the SUS2 *gene from* C. arabica

The genomic sequence of *C. arabica* for the *CaSUS2* gene was amplified by a PCR-reaction using primers localized at the extremities of its corresponding cDNA. Sequence analysis revealed that this gene contains 15 exons interrupted by 14 introns, all of them bordered by the 5′-GT/3′-AG consensus. Its structure was strictly identical to that observed for the group of dicot SUSA genes characterized by the split of exons 6 and 12 (Komatsu *et al.*, 2002). Nucleic sequences of the *CaSUS2* gene in common with the *CaSUS2* cDNA appeared to be strictly identical, indicating that this gene truly encodes for the cloned cDNA.

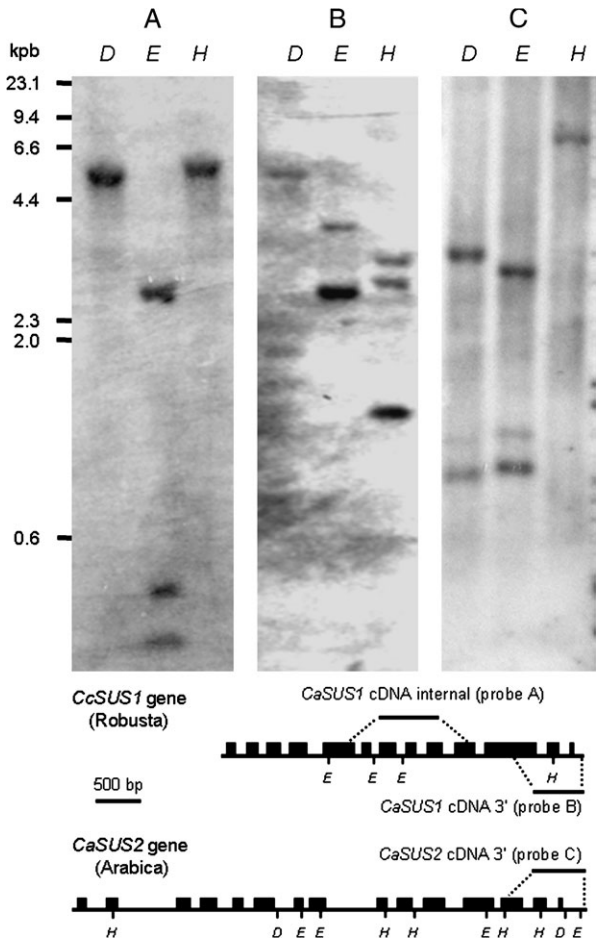

Fig. 6. Southern-blot analysis of *Coffea arabica* genomic DNA hybridized with *CaSUS1* and *CaSUS2* probes. Fifteen micrograms of genomic DNA digested with either *Dra*I (D), *Eco*RI (E), or *Hin*dIII (H) restriction enzymes were fractionated by electrophoresis on 0.8% (w/v) agarose gel, and transferred to a nylon (Hybond-N^+) membrane. Hybridizations were carried out with the probe A corresponding to a 667 bp internal fragment of *CaSUS1* cDNA (A), probe B corresponding to a 544 bp fragment overlapping the 3′ region of *CaSUS1* cDNA (B), and probe C corresponding to a 589 bp fragment overlapping the 3′ region of *CaSUS2* cDNA (C). Schematic organization of *CcSUS1* (Robusta; Leroy *et al.*, 2005) and *CaSUS2* (Arabica, this study) genes are also presented (black boxes=exons), as well the localization of cDNA probes used. Molecular length standards are indicated at the left in kilobases.

Southern blot analysis

The structure and complexity of *CaSUS1* and *CaSUS2* genes in the *C. arabica* genome was examined by Southern blot analysis conducted using the probes A and B of *CaSUS1* cDNA and the probe C of *CaSUS2* cDNA (Fig. 6). In all cases, the hybridization patterns obtained were

different, indicating that the probes were gene-specific under the stringent conditions used. For the internal probe of *CaSUS1*, single bands were observed with *Dra*I (5.2 kb) and *Hin*dIII (5.8 kb) digestions, whereas three bands (2.6, 0.45, and 0.3 kb) were detected with the *Eco*RI digestion (Fig. 6A). These results were those expected from a comparison of the position of the probe with the restriction map of the *CcSUS1* gene from *C. canephora* (Leroy *et al.*, 2005). When using the *CaSUS1* distal probe (Fig. 6B), the *Dra*I fragment at 5.2 kb and the *Eco*RI fragment at 2.6 kb were conserved. This is to be expected considering that no restriction sites for these enzymes are supposed to exist in the *CaSUS1* gene. For the *Hin*dIII digestion, three bands were observed, a strong one at 1.5 kb and two others at 2.8 kb and 3.0 kb since only one *Hin*dIII restriction site is supposed to exist in the *SUS1* gene of *C. canephora*.

For *CaSUS2*, hybridization revealed a fragment of 6.4 kb when the Arabica genome was digested with *Hin*dIII (Fig. 6C). From the *CaSUS2* gene sequence and *Hin*dIII digestion, at least two bands should be expected: one around 400 bp and another greater than 560 bp. With *Eco*RI digestion, three signals were observed: one at 2.7 kb and two others at 1.3 (faint band) and 1.1 kb. For the *Dra*I analysis, three bands were also revealed at 3.2 kb for the higher and 1.2 (faint) and 0.9 kb for the smaller. For these two digestions, only two bands should be expected as the probe overlapped a genomic region with only one *Eco*RI and *Dra*I restriction site.

Expression of SUS genes in individual tissues of coffee fruits

Cherries were collected regularly between 30 DAF to 234 DAF. To analyse the expression of *CaSUS1* and *CaSUS2* genes, total RNA was extracted from entire fruits as well as individually from the pericarp (60–234 DAF), perisperm (60–176 DAF), and endosperm (118–234 DAF). *CaSUS1* transcripts of approximately 2.9 kb were strongly detected in the pericarp, with peaks at 60 and 147 DAF (Fig. 7B), and also at immature stages of perisperm (60–89 DAF) and endosperm (118–147 DAF) development (Fig. 7C, D). *CaSUS1* gene expression was not observed in the later stages of perisperm (118–176 DAF), pericarp and endosperm development (205–234 DAF). As expected, the *CaSUS2* mRNA was also approximately 2.9 kb. *CaSUS2* transcript levels were maximal at later stages of pericarp (205–234 DAF) and endosperm (234 DAF) development (Fig. 7B, D). Weak *CaSUS2* expression was also observed in the early stages (60 and 89 DAF) of perisperm development (Fig. 7C), as well as at 176 DAF. For both experiments, the hybridization pattern of entire fruits with *CaSUS1* and *CaSUS2* probes coincided quite well with those detected individually for each tissue (Fig. 7A).

Because the perisperm tissue was reduced to a thin membrane (silver skin) surrounding the endosperm (bean)

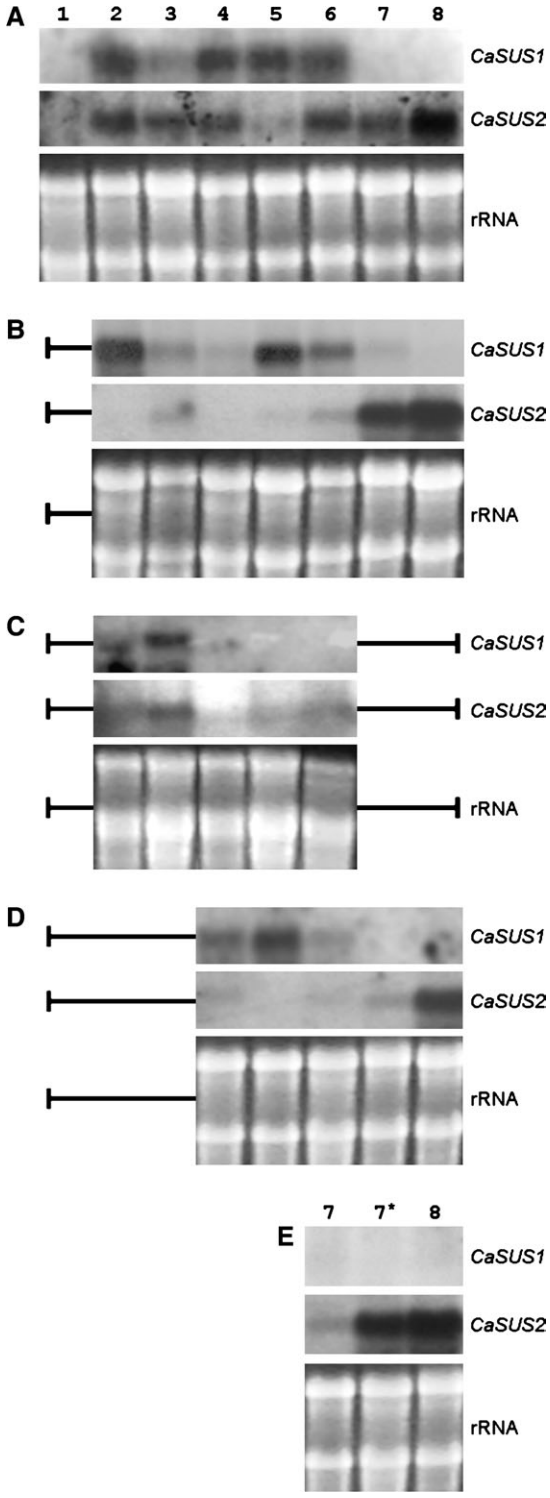

Fig. 7. Expression of *CaSUS1* and *CaSUS2* genes during coffee fruit development. Total RNA (15 µg) isolated from entire fruits (A) or from pericarp (B), perisperm (C), and endosperm (D) at regular developmental stages (lane 1, 30 DAF; 2, 60 DAF; 3, 89 DAF; 4, 118 DAF; 5, 147 DAF; 6, 176 DAF; 7, 205 DAF, and 8, 234 DAF) was separated in a formaldehyde-agarose gel and transferred onto a nylon membrane. (E) Hybridization of total RNA isolated from endosperm at 205 DAF with (lane 7*) or without (lanes 7 and 8) perisperm is also presented. Probes used correspond to probe A (*CaSUS1*) and C (*CaSUS2*). rRNAs stained by ethidium bromide were used to monitor the equal loading of RNA samples.

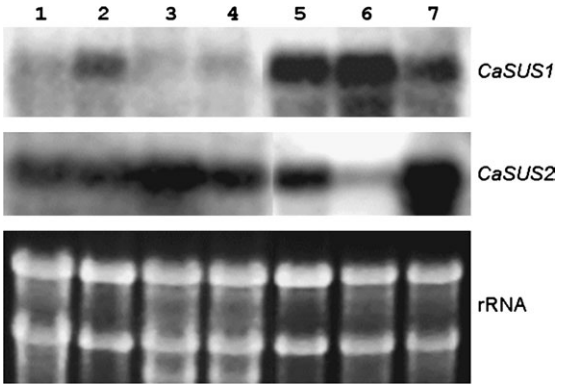

Fig. 8. Organ-specific expression of *CaSUS1* and *CaSUS2* genes. Total RNAs were extracted from young flower buds (lane 1), mature flower buds (lane 2), illuminated (distal) leaves with 10 cm length (lane 3), shaded (proximal) leaves with 10 cm length (lane 4), orthotropic stem (lane 5), entire fruit at 147 DAF (lane 6) and roots (lane 7) of *C. arabica* IAPAR 59. Probes used correspond to probe A (*CaSUS1*) and C (*CaSUS2*). rRNAs stained by ethidium bromide were used to monitor the equal loading of RNA samples.

at 205–234 DAF, it was not possible to extract RNA of sufficient quality for analysis by northern blot (see RNA degradation initiating in perisperm at 176 DAF). However, *CaSUS2* transcripts were strongly detected at 205 DAF in RNA from the endosperm still surrounded by the perisperm membrane (lane 7* in Fig. 7E), but not in RNA extracted from endosperm without perisperm (lane 7 in Fig. 7E), showing that *CaSUS2* transcription also occurred at the later stages of perisperm development.

Expression of SUS genes in coffee tissues

CaSUS1 and *CaSUS2* gene expression was also investigated in various tissues of *C. arabica* (Fig. 8). *CaSUS1* expression was barely detectable in young flower buds and leaves whatever their localization in plagiotropic stems. Transcripts of this isoform were observed in old flower buds and roots, and accumulated to a high level in stems. *CaSUS2* expression was low in flower buds and mature (opened) flowers. High *CaSUS2* gene expression was observed in roots and in light-exposed leaves (positioned at the terminal ending of the branch). *CaSUS2* mRNA levels were moderate in stems and shaded leaves (collected inside the plant) and low in flowers whether young or mature. As an internal control, high expression of *CaSUS1* in entire coffee fruits at 147 DAF and the absence of *CaSUS2* mRNAs at the same time (see lane 5 in Fig. 7A) was confirmed.

Discussion

Sugar partitioning during coffee fruit development

In higher plants, sugar metabolism has been shown to be essential for the control of seed development, mainly through the regulation of the source/sink process (Herbers and Sonnewald, 1998). For example, the high ratio of hexoses (H) to sucrose (S) observed in embryos of *Vicia faba* characterize the phase of intensive cell division (Weber *et al.*, 1998). The transition of the pre-storage phase to the maturation (storage) phase, when cell elongation and differentiation occur, is characterized by a clear switch in carbohydrate state, from a high to low H/S ratio. In sink organs of most plant species, these changes are controlled by the sucrose-cleaving enzymes like invertase, which has several isoforms differing in their biochemical properties and cellular localization, and SUS (Sturm and Tang, 1999). While invertase only functions in the direction of sucrose hydrolysis, SUS is a reversible enzyme capable of degrading and synthesizing sucrose. In this study, high H/S ratios coincided with the expansion phases of fruit tissues, as in the perisperm at 89 DAF, the endosperm between 118–147 DAF, and the pericarp between 205–234 DAF (Fig. 2). In the perisperm, the transition from a high to a low H/S ratio also corresponded to its gradual disappearance and to the beginning of endosperm growth. The same was observed in the endosperm where transition from a high to low H/S ratio coincided with the end of volume increase and the beginning of the storage phase (Rogers *et al.*, 1999*a*). In this regard, high hexose contents should control perisperm and endosperm expansion by creating a hydrostatic pressure gradient, thereby enabling mass flow of water and nutrients into these tissues (Herbers and Sonnewald, 1998).

In the perisperm, the highest concentration of reducing sugars was observed at 89 DAF (Fig. 2C) while maximal AI activity was found at 60 DAF (Fig. 3A). No particular variations of SUS activity were observed at this period. However, in part, these reducing sugars could come from starch degradation, as indicated by the histological study of young (60–75 DAF, Fig. 5D) perisperm (see below), but not apparently from the cleavage of sucrose imported from the leaves or pericarp. Maximal SPS activity in the perisperm and endosperm tissues was detected at 147 DAF, when the perisperm was in decline and the rapid growth phase of the endosperm terminated. Despite the generally held involvement of SPS in sucrose resynthesis, activities were not accompanied by sucrose accumulation in these tissues. However, they did overlap temporally with the synthesis of 11S storage proteins (Rogers *et al.*, 1999*a*), the beginning of polysaccharide accumulation (Redgwell *et al.*, 2003), and α-galactosidase activity (Marraccini *et al.*, 2005), essential for galactomanan deposition in endosperm cell walls. Therefore, SPS activity detected here appeared to be well co-ordinated with the differentiation of the endosperm into storage tissue, as reported during cotyledon development of *Vicia faba* (Weber *et al.*, 1996). Other evidence of a lack of relationship between sugar concentration and enzymatic activities was obtained with the pericarp (Fig. 3A), where maximal AI activity observed at 118 DAF was not followed by reducing sugar accumulation, as well as with the perisperm at 176 and 205 DAF

(Fig. 3C) where no changes of reducing sugar and/or sucrose contents paralleled SUS activity. A similar but inverse situation was observed in the endosperm, where the high level of reducing sugars at 118 DAF was not accompanied by a significant increase of either AI and SUS activities. The degradation and (re)synthesis cycle of sucrose as well as the existence of rapid sugar transfers between the perisperm and endosperm tissues, rather than measurements of enzymatic activities *per se*, could explain these results (Nguyen-Quoc and Foyer, 2001). Such a complex interaction among tissues is also supported by the pulse–chase experiments with ^{14}C-fructose and ^{14}C-sucrose and the specific radioactivity of sucrose and reducing sugars found in the different tissues. In addition, the action of other sucrose-cleaving enzymes, for instance, neutral invertases (Hubbard *et al.*, 1991) and the presence of enzymatic activities in tissues and compartments that are difficult to investigate, cannot be excluded. This might be the case of immature (0–60 DAF) perisperm where maximal AI activity was measured at 60 DAF (Fig. 3A) or with the endosperm which can be identified a few days after anthesis (De Castro and Marraccini, 2006), but can only be easily separated from the perisperm after 118 DAF. Finally, the presence of extracellular (apoplastically localized) invertase, facilitating sucrose transfers out of maternal tissues (Weber *et al.*, 1997; Nguyen-Quoc and Foyer, 2001), could have been missed during tissue isolation.

By contrast, SUS activities detected in the pericarp and endosperm tissues between 176 and 234 DAF (Fig. 2D) were paralleled closely by sucrose accumulation in these tissues (Fig. 3C). In addition, the large accumulation of reducing sugars occurring in the pericarp at 205 and 234 DAF (Fig. 2C), also reported by Marín-López *et al.* (2003), might arise from the relatively high AI activity measured in this tissue (Fig. 3A). It could also result from the degradation of complex polysaccharides since transcripts of polygalacturonase (EC 3.2.1.15), that parallel those of ACC (1-aminocyclopropane-1-carboxylic acid) oxidase, were reported for late stages of pericarp maturation (Cação *et al.*, 2003; Pereira *et al.*, 2005).

The C partitioning and sugar exchanges existing between the perisperm, pericarp, and endosperm tissues of fruits was investigated by pulse–chase experiments with ^{14}C-labelled compounds (Fig. 4). In the experiment with $^{14}CO_2$, incubation of leaves yielded more radioactivity in the perisperm than fruits exposed to $^{14}CO_2$. This indicates that the pericarp retains most of the carbon assimilated *in loco*, while the carbon consumed by the endosperm comes predominantly from the leaves. Vaast *et al.* (2005) estimated that photosynthesis by the pericarp may account for approximately 30% of the total carbon allocated in the fruit. Perhaps this might be due in part to a lack of vascular connections between the pericarp and the other fruit tissues, as mentioned above. In this study, it is also shown that the pericarp contribution to CO_2 assimilation decreased gradually with maturation, since less radioactivity was found in the tissues of mature (reddish) fruits exposed to $^{14}CO_2$ than in younger ones (Fig. 4E). Independently of the pathways used to transport sucrose between the different tissues of coffee fruits, these experiments highlight the existence of intensive exchange of sugars between fruit compartments which occur mainly through simultaneous biosynthetic and catabolic processes of sucrose metabolism.

Coffee fruit histology: role of the perisperm tissue during bean development

Coffee fruits have a quite peculiar tissue organization and development (De Castro and Marraccini, 2006). In *C. arabica*, the perisperm arises from the nucellus and constitutes the predominant tissue occupying the inner space (locule) of fruits until 90–100 DAF. This locule space is delimited by the endocarp (also called the parchment layer; Fig. 1C), which is a hard and cellulose-rich tissue present from the early stages of fruit development. As the fruit grows, this space is rapidly occupied by the endosperm while the perisperm, at this point referred to as the silver skin, becomes a thin tissue reduced to few cell layers surrounding the endosperm.

For the first time, the histological study presented here clearly reveals the connections existing between these tissues. This identified the presence of vascular tissues originating in the central endocarp region and entering directly into contact with the perisperm exclusively by the funicule extremity (Fig. 5A). This situation persists during further fruit development, even when the perisperm is reduced to the silver skin. The epiderm of the perisperm facing the endocarp also contains cell layers showing a modified cell wall structure and probably undergoing death. At the moment, it is not known whether this particular differentiation would favour or impede solute exchanges. Whatever the situation, the continuity observed between vascular tissues of the funicule and the perisperm indicate the symplastic unloading of photosynthates from the sieve tube (Patrick and Offler. 1995) to the perisperm and subsequent diffusion within this tissue.

At early and late developmental stages, a cuticle-like layer was also detected on the surface of the perisperm facing the endocarp, but not on the perisperm side facing the endosperm. By analogy with the structure and function of other plant seeds, this cuticle should form an impermeable barrier to apoplastic transfers by isolating a sector of the seed apoplast from the perisperm and preventing solute delivery by passive (diffusion) mechanisms (see review by Patrick and Offler, 1995). Finally, the large number of plasmodesmata present in endosperm cells (see review by De Castro and Marraccini, 2006) argues for simple diffusion or symplastic transfer of solutes in this tissue.

Histological studies also revealed the presence of starch granules in the perisperm (60–75 DAF; Fig. 5D), but not in the cells closer to the endosperm, suggesting starch

hydrolysis in this particular region. This disappearance of starch could also explain the peak of reducing sugars detected in the perisperm at 89 DAF. When transferred into the endosperm, reducing sugars could provide the energy necessary for cell division and elongation and function as precursors for the synthesis of storage protein and polysaccharides. Previous observations also indicate the function of the perisperm as a tissue capable of providing the endosperm with additional precursors, such as organic acids for the synthesis of chlorogenic acids (Rogers *et al.*, 1999*b*).

The investigation also shows that the role of the perisperm is not limited to transient accumulation of biochemical precursors. Even when reduced to a thin membrane at 205 DAF, the results presented here clearly demonstrate that the perisperm retained the capacity to transcribe genes, such as *CaSUS2* for example (Fig. 7E), and to retain SUS enzymatic activities (Fig. 3C). Taken together with the results of the pulse–chase experiments, these data clearly demonstrate the important function of the perisperm tissue in bean development.

Two isoforms of sucrose synthase are expressed in coffee fruits

The coincidence of SUS activities and sucrose accumulation observed in pericarp and endosperm tissues between 176 and 234 DAF (Figs 2D, 3C) led to the performance of an in-depth study of the molecular characteristics of SUS. The use of EST sequence data available from the Brazilian Coffee Genome Project allowed the isolation of two SUS-encoding cDNAs of similar size, but differing in the

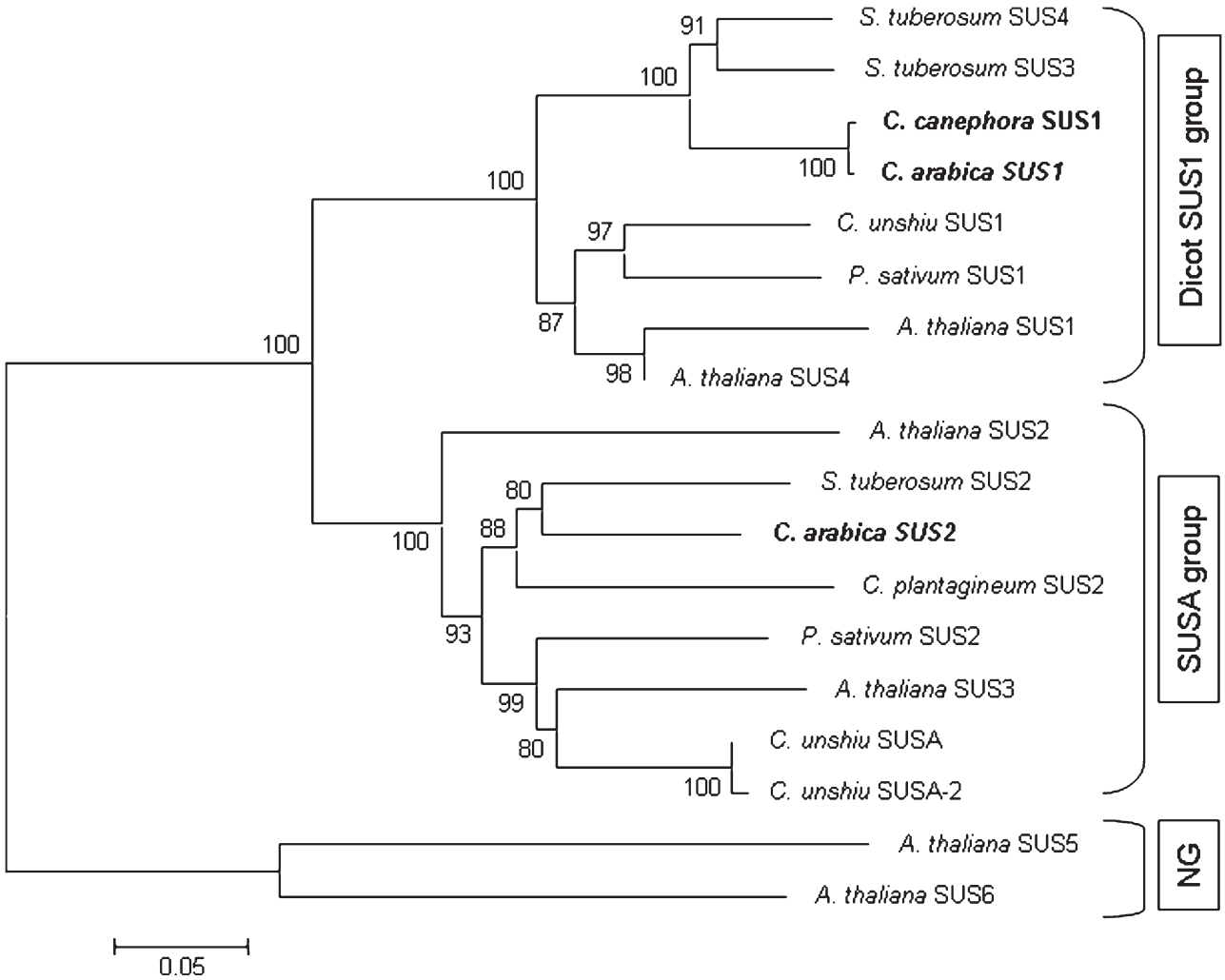

Fig. 9. Comparison of deduced amino acid sequences of plant sucrose synthase. The phylogenetic dendrogram was generated by multi alignment using CLUSTALW based on identity and the Neighbor–Joining method with Poisson correction. Bootstrap values are shown as percentages. EMBL database accession numbers of SUS proteins are as follows: *Arabidopsis thaliana* SUS1 (NP197583), SUS2 (NP199730), SUS3 (NP192137), SUS4 (AAK59464), SUS5 (NP198534), SUS6 (NP177480); *Citrus unshiu* SUSA (BAA88904), SUSA-2 (BAA88981), SUS1 (BAA88905); *Coffea arabica* SUS1 (CAJ32596), SUS2 (CAJ32597); *Coffea canephora* SUS1 (CAI56037); *Craterostigma plantagineum* SS2 (CAB38022); *Pisum sativum* SUS1 (CAA09910), SUS2 (CAA04512); *Solanum tuberosum* SUS2 (AAO67719), SUS3 (U24088), SUS4 (U24087).

primary sequence of the proteins they encode. The deduced amino acid sequence of the *CaSUS1* cDNA showed a high degree of similarity with SUS of the Dicot SUS1 group (Fig. 9) frequently identified in sink organs of other plant species, as with SUS3, SUS4 from potato tubers (Fu and Park, 1995), and SUS1 from pea seed coat (Déjardin *et al.*, 1997) and citrus fruit (Komatsu *et al.*, 2002). By contrast, the deduced amino acid sequence of *CaSUS2* cDNA presented high sequence similarity with SUS of the dicot SUSA group, such as SUSA from *C. unshiu* (Komatsu *et al.*, 2002). This protein also showed relatively high identity (82%) and homology (90%) to the SS2 protein coded by the *CpSS2* (AJ132000) gene from the resurrection plant *Craterostigma plantagineum* (Kleines *et al.*, 1999). At the amino acid level, the SUS1 and SUS2 proteins shared only 68% of identity and 82% of similarity, therefore agreeing with the existence of two differentially expressed and non-allelic loci for this enzyme in coffee. The phylogenetic organization of coffee sucrose synthases into at least two distinct groups was reinforced by the comparison of SUS-encoding genes (Fig. 9). Indeed, the structure of the previously reported *CcSUS1* gene of *C. canephora* (Leroy *et al.*, 2005) clearly differed from the *SUS2* gene of *C. arabica* described here, mainly by the split of the exons 5 and 11 observed in the latter, but not in the former. In this regard, the organization of the coffee *CcSUS1* gene is similar to that of the *AtSUS1* and *CitSUS1* genes, respectively, from *A. thaliana* and *C. unshiu*, whereas that of the *CaSUS2* appeared to be close to the genes belonging to the dicot SUSA group (Komatsu *et al.*, 2002; Baud *et al.*, 2004).

At the nucleic acid level, *CaSUS1* cDNA from *C. arabica* (this study) presented 18 differences over the 2421 bp of the protein coding sequence of the *CcSUS1* gene from *C. canephora* (Leroy *et al.*, 2005). Fifteen of them were silent and did not modify the final amino acid sequence of the SUS1 protein; two others were considered as neutral (F_{532} and R_{663} in *CaSUS1* changed, respectively, by Y and K amino acid residues in *CcSUS1*) and only one led to a radical amino acid change (G_{259} in *CaSUS1* changed E in *CcSUS1*). None of these changes affected the N-terminal phosphorylation site, the putative hydrophobic domains, or the theoretical pI of the proteins and 5′ and 3′ untranslated regions, suggesting that these sequences were highly conserved during evolution.

In comparison with the *CcSUS1* gene sequence published for *C. canephora* (Leroy *et al.*, 2005), the Southern blot analysis performed with the distal probe of the *CaSUS1* revealed additional bands (Fig. 6B, lines E, H). The same was observed when using the *CaSUS2* probe overlapping the 3′ unstranslated region of this cDNA (Fig. 6C). Because *C. arabica* is amphidiploid resulting from a natural cross between diploid species *C. eugenoides* and *C. canephora* (Lashermes *et al.*, 1999), these patterns could characterize sequence divergence in the 3′ regions of independent *SUS1* and *SUS2* genes coming from each parent. Alternatively, they could correspond to hybridization of the probe with more distant SUS-encoding genes. Whatever the possibility, the simple patterns obtained suggest that *CaSUS1* and *CaSUS2* genes were probably unique in the Arabica genome. Sequence alignment of full-length *CaSUS1* cDNA with a partial SUS1 cDNA (AJ575256) previously amplified by RT-PCR using degenerated primers deduced from conserved SUS domains (Marraccini *et al.*, 2003), revealed eight differences. Interestingly, all of them affected the third base of codons, without modifying the amino acid composition of the deduced SUS1 protein. Since both *CaSUS1* cDNA sequences were amplified from the same cultivar (IAPAR 59) of *C. arabica* and verified by double-strand sequencing, this suggests that they originated from different alleles of the *CaSUS1* gene.

CaSUS1 *and* CaSUS2 *genes are differentially expressed and may assume different functions*

Transcripts of *CaSUS1* were abundant in the pericarp at 60 and 147 DAF. They were also high at the immature stage of perisperm and endosperm development and decreased towards the ripening stage. By contrast, mRNA levels of *CaSUS2* were undetectable in young endosperm and barely detectable in young pericarp and perisperm, but increased towards the ripening of pericarp and endosperm tissues. The high *CaSUS2* gene expression observed at 205 and 234 DAF in the pericarp and at 234 DAF in the endosperm overlapped perfectly the peaks of SUS activity and sucrose concentration measured at the latest developmental stages of these tissues. The differential expression of *CaSUS1* and *CaSUS2* genes presented here showed great similarities, respectively, with the expression profiles of *CitSUS1* and *CitSUSA* genes of *C. unshiu* (Komatsu *et al.*, 2002). In this species, the *CitSUS1* gene was expressed early in the development of peel and edible tissues, whereas *CitSUSA* was expressed in the latest developmental stages of these tissues (Komatsu *et al.*, 2002). In addition, sucrose accumulation and SUS activity coincided quite well with the *CitSUSA* expression profile and to a lesser extend that of *CitSUS1*.

In coffee, mRNA levels of *CaSUS1* detected in the pericarp and in the early stages of perisperm and endosperm development were not accompanied by SUS activity or particular alterations of sucrose or reducing sugar contents. Even when SUS enzymatic assays were tested in the direction of sucrose degradation, no SUS activity was detected at the time of *CaSUS1* gene expression (data not shown). However, western blot analysis using antibodies raised against the SUS1 isoform of pea teguments (Déjardin *et al.*, 1997) clearly identified a SUS protein in the endosperm at 118–147 DAF (Fig. 3D), that probably corresponds to the CaSUS1 protein. This is also supported by the phylogenetic dendrogram, where both proteins clustered in the Dicot A group (Fig. 9) and by the perfect

overlapping of the western blot and *CaSUS1* gene expression in the endosperm (Fig. 7D). The absence of SUS activity coincident with *CaSUS1* gene expression could be explained by the necessity of post-translational modifications as shown in soybean, where the binding of ENOD40 peptide A to SUS was required to activate sucrose-cleaving activity at the early stage of root nodule organogenesis (Röhrig *et al.*, 2004).

At the phylogenetic level, the CaSUS2 isoform was classified in the SUSA group of plant SUS (Fig. 9) which also contains the proteins coded by the *AtSUS2* and *AtSUS3* genes of *Arabidopsis thaliana* and *CpSS2* from the resurrection plant *C. plantagineum* (Komatsu *et al.*, 2002; Baud *et al.*, 2004). In the former species, the *AtSUS2* gene was considered as a marker of seed maturation since its maximal expression was detected in seeds at 12 DAF and decreased towards maturation. By contrast, *AtSUS3* transcripts were not detected before 12 DAF, but increased steadily after this time and also paralleled the sucrose accumulation observed during the late-maturation phase (Baud *et al.*, 2002). In addition, *AtSUS3* expression was up-regulated under dehydration, such as in leaves submitted to water deprivation (Baud *et al.*, 2004). This could explain its high expression in the late-maturation phase of seed development where intense desiccation was reported (Baud *et al.*, 2002). Interestingly, the CaSUS2 isoform also had great similarity with the SUS protein of *CpSS2* gene from *C. plantagineum*, whose expression increased in roots submitted to water depletion (Kleines *et al.*, 1999). Although effects of water stress on SUS gene expression were not studied in the present investigation, the endosperm dehydration observed during the latest stages of endosperm development (De Castro *et al.*, 2005) or in roots of field-grown coffee plants (Fig. 8), could explain the high levels of *CaSUS2* transcripts detected in these tissues.

Whatever the situation, the high *CaSUS2* gene expression in pericarp and endosperm reported here argue strongly for an important role of this gene in sucrose accumulation in these tissues. This should be confirmed by comparing SUS activities and *CaSUS2* gene expression in endosperms of Arabica (this study) and Robusta species that differ in their sucrose content (Ky *et al.*, 2001; Campa *et al.*, 2004). Such experiments are underway. In addition, the importance of *CaSUS2* in cup quality through a control of sucrose metabolism is being verified by growing coffee trees under shade and also by reducing the number of fruits on the tree. In both cases it is known that cup quality is improved.

Acknowledgements

We are grateful to Dr Alan C Andrade and Felipe Vinecky (EMBRAPA Recursos Genéticos e Biotecnologia, Brasilia, DF, Brazil) for their help in sequencing of *CaSUS1* and *CaSUS2*. We also thank Dr C Rochat (INRA, Versailles, France) for providing the polyclonal antibody serum against SUS from pea and Dr Ladaslav Sodek for reviewing the text. This project was supported by the Brazilian Consortium for Coffee Research and Development (CBP&D-Café). P Marraccini received financial support (DCSUR-BRE-4C5-008) from the French Embassy in Brazil. C Geromel and AA Cavalari received student fellowships from CAPES-Brazil and FAPESP-São Paulo, respectively, and P Mazzafera received a research fellowship from CNPq. The *CaSUS1* and *CaSUS2* cDNA and the *CaSUS2* gene sequences were deposited in the EMBL/GenBank database under the accession numbers AM087674, AM087675, and AM087676 respectively.

References

Amor Y, Haigler C, Johnson S, Wainscott M, Delmer D. 1995. A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proceedings of the National Academy of Sciences, USA* **92,** 9353–9357.

Baud S, Boutin J-P, Miquel M, Lepiniec L, Rochat C. 2002. An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. *Plant Physiology and Biochemistry* **40,** 151–160.

Baud S, Vaultier MN, Rochat C. 2004. Structure and expression profile of the sucrose synthase multigene family in *Arabidopsis*. *Journal of Experimental Botany* **55,** 397–409.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein–dye binding. *Analytical Biochemistry* **2,** 248–254.

Cação SMB, Galvão RM, Pereira LFP, Vieira LGE. 2003. Identificação e caracterização de genes de poligalacturonase de *Coffea arabica*. III. Simpósio de Pesquisa dos Cafés do Brasil, Porto Seguro, 98–99.

Campa C, Ballester JF, Doulbeau S, Dussert S, Hamon S, Noirot M. 2004. Trigonelline and sucrose diversity in wild *Coffea* species. *Food Chemistry* **88,** 39–44.

Casal S, Oliveira MB, Ferreira MA. 2000. HPLC/diode-array to the thermal degradation of trigonelline, nicotinic acid, and caffeine in coffee. *Food Chemistry* **68,** 481–485.

De Castro RD, Estanislau WT, Carvalho MLM, Hilhorst HWM. 2005. Functional development and maturation of coffee (*Coffea arabica*) fruits and seeds. *Proceedings of the 20th International Scientific Colloquium on Coffee, Bangalore, International Scientific Association on Coffee, Paris*, 619–635.

De Castro R, Marraccini P. 2006. Cytology, biochemistry and molecular changes during coffee fruit development. *Brazilian Journal of Plant Physiology* **18,** 175–199.

De Maria CAB, Trugo LC, Moreira RFA, Werneck CC. 1994. Composition of green coffee fractions and their contribution to the volatile profile formed during roasting. *Food Chemistry* **50,** 141–145.

Déjardin A, Rochat C, Maugenest S, Boutin J-P. 1997. Purification, characterization and physiological role of sucrose synthase in the pea seed coat (*Pisum sativum* L.). *Planta* **201,** 128–137.

Dubois MK, Giller KA, Hamilton JK, Rebers PA, Smith T. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28,** 360–356.

Fu H, Park WD. 1995. Sink- and vascular-associated sucrose synthase functions are encoded by different gene classes in potato. *The Plant Cell* **7,** 1369–1385.

Gerrits PO, Smid L. 1983. A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. *Journal of Microscopy* **132,** 81–85.

Grosch W. 2001. Volatile compounds. In: Clarke RJ, Vitzthum OG, eds. *Coffee: recent developments*. Oxford: Blackwell Science, 68–89.

Herbers K, Sonnewald U. 1998. Molecular determinants of sink strength. *Current Opinion in Plant Biology* **1,** 207–216.

Homma S. 2001. Non-volatile compounds, part II. In: Clarke RJ, Vitzthum OG, eds. *Coffee: recent developments*. Oxford: Blackwell Science, 50–67.

Hubbard NL, Pharr DM, Huber SC. 1991. Sucrose phosphate synthase and other sucrose metabolizing enzymes in fruits of various species. *Physiologia Plantarum* **82,** 191–196.

Johansen DA. 1940. *Plant microtechnique*. New York: McGraw-Hill.

Kleines M, Elster RC, Rodrigo MJ, Blervacq AS, Salamini F, Bartels D. 1999. Isolation and expression analysis of two stress-responsive sucrose-synthase genes from the resurrection plant *Craterostigma plantagineum* (Hochst.). *Planta* **209,** 13–24.

Komatsu A, Moriguchi T, Koyama K, Omura M, Akihama T. 2002. Analysis of sucrose synthase genes in citrus suggests different roles and phylogenetic relationships. *Journal of Experimental Botany* **53,** 61–71.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignments. *Briefings in Bioinformatics* **5,** 150–163.

Ky CL, Louarn J, Dussert S, Guyot B, Hamon S, Noirot M. 2001. Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild *Coffea arabica* L. and *C. canephora* P. accessions. *Food Chemistry* **75,** 223–230.

Lashermes P, Combes M-C, Robert J, Trouslot P, D'Hont A, Anthony F, Charrier A. 1999. Molecular characterization and origin of the *Coffea arabica* L. genome. *Molecular Genetics and Genomics* **261,** 259–266.

Leroy T, Marraccini P, Dufour M, *et al.* 2005. Construction and characterization of a *Coffea canephora* BAC library to study the organization of sucrose biosynthesis genes. *Theoretical and Applied Genetics* **111,** 1032–1041.

Marín-López SM, Arcila-Pulgarin J, Montoya-Restrepo EC, Olivero-Tascón CE. 2003. Cambios físicos y químicos durante la maduración del fruto de café (*Coffea arabica* L. var. *Colombia*). *Cenicafé* **54,** 208–225.

Marraccini P, Rogers WJ, Allard C, André ML, Caillet V, Lacoste N, Lausanne F, Michaux S. 2001. Molecular and biochemical characterization of endo-β-mannanases from germinating coffee (*Coffea arabica*) grains. *Planta* **213,** 296–308.

Marraccini P, Pereira LFP, Ferreira LP, Vieira LGE, Geromel C, Cavalari AA, Mazzafera P, Leroy T. 2003. *Coffea arabica* partial mRNA for sucrose synthase (*sus1* gene). EMBL/GenBank accession number AJ575256.

Marraccini P, Rogers WJ, Caillet V, Deshayes A, Granato D, Lausanne F, Lechat S, Pridmore D, Pétiard V. 2005. Biochemical and molecular characterization of α-D-galactosidase from coffee beans. *Plant Physiology and Biochemistry* **43,** 909–920.

Nguyen-Quoc B, Foyer CH. 2001. A role for 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. *Journal of Experimental Botany* **52,** 881–889.

O'Brien TP, Feder N, McCully ME. 1964. Polychromatic staining of plant cell walls by toluidine blue. *Protoplasma* **59,** 368–373.

Patrick JW, Offler CE. 1995. Post-sieve element transport of sucrose in developing seeds. *Australian Journal of Plant Physiology* **22,** 681–702.

Pereira LFP, Galvão RM, Kobayashi AK, Cação SMB, Vieira LGE. 2005. Ethylene production and ACC oxidase gene expression during fruit ripening of *Coffea arabica* L. *Brazilian Journal of Plant Physiology* **17,** 283–289.

Redgwell RJ, Curti D, Rogers J, Nicolas P, Fischer M. 2003. Changes to the galactose/mannose ratio in galactomannans during coffee bean (*Coffea arabica* L.) development: implications for *in vivo* modification of galactomannan synthesis. *Planta* **217,** 316–326.

Rogers WJ, Bézard G, Deshayes A, Meyer I, Pétiard V, Marraccini P. 1999*a*. Biochemical and molecular characterization and expression of the 11S-type storage protein from *Coffea arabica* endosperm. *Plant Physiology and Biochemistry* **37,** 261–272.

Rogers WJ, Michaux S, Bastin M, Bucheli P. 1999*b*. Changes to the content of sugars, sugar alcohols, myo-inositol, carboxylic acids, and inorganic anions in developing grains from different varieties of Robusta (*Coffea canephora*) and Arabica (*C. arabica*) coffees. *Plant Science* **149,** 115–123.

Röhrig H, John M, Schmidt J. 2004. Modification of soybean sucrose synthase by S-thiolation with ENOD40 peptide A. *Biochemical and Biophysical Research Communications* **325,** 864–870.

Roitsch T, González M-C. 2004. Function and regulation of plant invertases: sweet sensations. *Trends in Plant Science* **9,** 606–613.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Somogyi N. 1952. Notes on sugar determination. *Journal of Biological Chemistry* **195,** 19–23.

Söndahl MR, Lauritis JA. 1992. Coffee. In: Hammerschlag FA, Litz RE, eds. *Biotechnology of perennial fruit crops*. Cambridge: CAB International, 401–420.

Sturm A, Tang G-Q. 1999. The sucrose-cleaving enzymes of plant are crucial for development, growth and carbon partitioning. *Trends in Plant Science* **4,** 401–404.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Van Handel E. 1968. Direct microdetermination of sucrose. *Analytical Biochemistry* **22,** 280–283.

Vaast P, Angrand J, Franck N, Dauzat J, Génard M. 2005. Fruit load and branch ring-barking affect carbon allocation and photosynthesis of leaf and fruit of *Coffea arabica* in the field. *Tree Physiology* **25,** 753–760.

Walkey JW, Tillman J. 1977. A simple thin layer chromatographic technique for the separation of mono and oligosaccharides. *Journal of Chromatography* **132,** 172–174.

Weber H, Borisjuk L, Wobus U. 1997. Sugar import and metabolism during seed development. *Trends in Plant Science Research* **2,** 169–174.

Weber H, Buchner P, Borisjuk L, Wobus U. 1996. Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implication for seed development. *The Plant Journal* **9,** 841–850.

Weber H, Heim U, Golombek S, Borisjuk L, Wobus U. 1998. Assimilate uptake and the regulation of seed development. *Seed Science Research* **8,** 331–345.

Yelle SRT, Chetelat RT, Dorais M, DeVerna JW, Bennett AB. 1991. Sink metabolism in tomato fruit. IV. Genetic and biochemical analysis of sucrose accumulation. *Plant Physiology* **95,** 1026–1035.