Hybrid 'Sinta' Papaya Exhibits Unique ACC Synthase 1 cDNA Isoforms

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Five ripening-related ACC synthase cDNA isoforms were cloned from 80% ripe papaya cv. 'Sinta' by reverse transcription-PCR using gene-specific primers. Clone 2 had the longest transcript and contained all common exons and three alternative exons. Clones 3 and 4 contained common exons and one alternative exon each, while clone 1, the most common transcript, contained only the common exons. Clone 5 could be due to cloning artifacts and might not be a unique cDNA fragment. Thus, there are only four isoforms of ACC synthase mRNA. Southern blot analysis indicates that all five clones came from only one gene existing as a single copy in the 'Sinta' papaya genome. Multiple sequence alignment indicates that the four isoforms arise from a single gene, possibly through alternative splicing mechanisms. All the putative alternative exons were present at the 5'-end of the gene comprising the N-terminal region of the protein. 'Sinta' ACC synthase cDNAs were of the *capacs 1* type and are most closely related to a 1.4 kb *capacs 1*-type DNA (AJ277160) from Eksotika papaya. No *capacs 2*-type cDNAs were cloned from 'Sinta' by RT-PCR. This is the first report of possible alternative splicing mechanism in ripening-related ACC synthase genes in hybrid papaya, possibly to modulate or fine-tune gene expression relevant to fruit ripening.

Keywords: ACC synthase, *Capacs 1*, *Carica papaya*, Papaya

Introduction

1-Amino-cyclopropane-1-carboxylate synthase (ACC synthase, EC 4.4.1.14) catalyses the committed step in ethylene biosynthesis in higher plants (Yang and Hoffmann, 1984). As a regulatory enzyme, ACC synthase has a short half-life and

exists *in vivo* as a dimer (Li and Mattoo, 1994). At present, the most extensive studies regarding the molecular mechanisms of ACC synthase-regulated fruit ripening are on tomato. The marked increase of ACC and ethylene production is attributed to the expression of two ACC synthase genes, *LE ACS2* and *LE ACS4* (Olson *et al.*, 1991; Rottman *et al.*, 1991; and Lincoln *et al.*, 1993). In transgenic tomatoes with an antisense ACC synthase gene, the hampered production of ACC synthase leads to very low levels of ethylene generation, and the tomato fruit will not ripen without constant exposure to external sources of ethylene for at least 6 days (Oeller *et al.*, 1991). Consequently, the transgenic tomato exhibited superior post-harvest characteristics, especially in the transport and storage of the fruit.

Such post-harvest characteristics are ideal for papaya, a climacteric fruit with a short shelf life. The quality of the fruit available for local consumption is decreased by rough handling of the fruits at all stages of transport from harvest to the retail market. Papayas with a controlled ripening phase could be developed for the local and export markets resulting in decreased transport and storage costs and are less prone to losses due to spoilage and disease.

To this end, ripening-related *Carica papaya* ACC synthase (*capacs*) cDNAs from different cultivars have been cloned. The Australian cultivar 2001 (Mason and Botella, 1997) and Philippine Solo (Laurena *et al.*, 2002) express at least two types of *capacs* cDNAs: *capacs 1* which is highly expressed at the onset of ripening and gradually decreases during ripening and *capacs 2* which is not detected at the mature green stage but dramatically increases at the onset of ripening and remains at a high level during the various stages of ripening. The cDNA library from 30% ripe fruit of the cultivar Solo Sunrise from Hawaii contains *capacs 1*-type cDNA (Neupane *et al.*, 1998). In addition to published cDNA sequences, four papaya ACC synthase cDNA sequences are accessible through the GenBank sequence database. When compared by phylogenetic analysis, three were identified as *capacs 1*-type and one as *capacs 2*-type (Fig. 1). These sequences indicate that in papaya, the ACC synthase gene and its expression in response to ripening is cultivar-dependent.

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Fig. 1. Phylogenetic relationships of ripening-related papaya ACC synthase nucleotide sequences generated by the Align X Program of the Vector NTI suite. Two clusters of ACC synthase genes were dubbed *capacs 1* and *2* by Mason and Botella (1997), which stands for *Carica papaya* ACC synthase.

Table 1. ‘Ole’ primers used in cloning ACC synthase by RT-PCR (Mason and Botella, 1997)

Primer	DNA sequence	Degeneracy
Ole 2 (F)	gcT cTA gAT AYT Tyg Ayg gNT ggA Arg c	64
Ole 4 (R)	Gcg AAT TcR Tcc ATR TTN gcR AAR cA	64
Ole 5 (F)	ccT gAT cAR Atg ggN YTN gcN gAR AA	512
Ole 6 (R)	cTc Tgc Agc RAA RcA Nac NcK RAA ccA	266

Recent reports show that alternative splicing mechanism may explain the presence of protein isoforms, or in general, protein diversity in plants, especially for proteins with regulatory functions (Kazan, 2003). Further, the estimated 5% of alternatively spliced genes in *Arabidopsis*, obtained by computational analyses may be an underestimation. Alternative splicing might therefore also explain the presence of isoforms of ACC synthase genes in various plants.

To assist efforts in improving the post-harvest characteristics of a locally-developed hybrid papaya ‘Sinta’ using antisense technology, we studied the ACC synthase cDNA expressed in 80% ripe pericarp of the ‘Sinta’ fruit. We hereby report the cloning and characterization of four *capacs 1*-type ACC synthase cDNAs which are encoded by a single-copy gene. This indicates that the four cDNAs may have been brought about by three independent alternative splicing events.

Materials and Methods

Cloning the ACC Synthase by RT-PCR Total RNA from 100 mg 80% ripe ‘Sinta’ papaya fruit was prepared using the RNEasy Plant Mini-Kit (Quiagen, Castsworth, USA). Total RNA was visualized and estimated in a 1% nondenaturing agarose gel. First-strand cDNA was prepared using the Pharmacia First Strand cDNA Synthesis kit using a gene-specific primer (OLE-4) and 5 µg total RNA.

Ripening-related ACC synthase cDNAs were fished out by polymerase-chain reaction (PCR) using two sets of gene-specific primers: OLE 2 & 4 and OLE 5 & 6 (Table 1 and Fig. 2A) using the following 25 µl reaction mix: 3 µl cDNA solution, 0.15 mM each dNTP, 0.75 mM each primer, 1.5 mM MgCl₂, and 0.75 unit Taq Polymerase in TE buffer pH 8.9.

The amplicons were cloned using the pGEM-T Easy cloning kit (Promega, Madison, USA) and transfected to *E. coli* strain DH 5-α. Plasmid minipreps were prepared from transformed bacterial clones. Preliminary characterization of inserts was done by diagnostic digestion of plasmid minipreps with Eco RI.

Sequencing Insert DNAs (amplicons) within the cloning vectors were sequenced using Applied Biosystems PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit following the manufacturer’s instructions. Sequenced samples were run in an Applied Biosystems automatic sequencer (model 373A).

Southern Blotting and labeling Genomic DNA was extracted from 4 g of papaya leaf using a modified CTAB protocol (Doyle and Doyle, 1990). Ten µg DNA were digested each with Bam HI, Eco RI, Hind III, Kpn I, Pst I, and Pvu I, run on a 0.8% agarose gel, and blotted onto nylon membrane. The blot was probed with the labeled DNA and detected by chemiluminiscent reaction using the DIG High-Prime Labeling and Detection kit (Boeringer Mannheim, Indianapolis, USA). Probes were produced from unique clones amplified by PCR, cleaned, and labeled with digoxigenin-dUTP.

Sequence analysis Relative homologies between nucleotide and polypeptide sequences were done using pairwise BLAST. Nucleotide sequences were edited and combined using the Vector NTI software (Informax, Inc., 1998), while phylogenetic analysis and multiple sequence alignments were obtained using the Clustal W algorithm of the Align X software (Informax, Inc., 1998).

Results and Discussion

Cloning of ACC synthase cDNAs by RT-PCR High quality total RNA (Fig. 2B) and OLE 4 primer were used for

Fig. 2. Fishing out the ACC synthase cDNAs by RT-PCR using OLE primers. (A) Schematic diagram of the position of OLE primers in the ACC synthase gene. Indicated are positions in the resulting polypeptide based on the sequence of Neupane *et al.*, 1998. OLE 2&4 are in bold, while OLE 5&6 are boxed. (B) Total RNA extracted from 80% ripe papaya fruit. (C) PCR of OLE 4-primed cDNA using OLE 2&4 primer pair using different $MgCl_2$ concentrations. The annealing temperature used was 52°C. Lane 1: negative control, lane 2: reaction with 1.0 mM, lane 3: 1.5 mM, lane 4: 2.0 mM. Reaction containing 1.5 mM was chosen due to the higher MW bands that were generated in the reaction. (D) Four reamplification reactions using OLE 5&6 primers. Two strong bands of approximate MW 1.1 and 1.4 kb and no fragments lower than 1.0 kb were generated. These amplicons were inserted into pGEM-T Easy.

reverse transcription and were amplified using the OLE 2 and 4 primer pair. Optimization of $MgCl_2$ and dNTP concentrations and reaction buffer for PCR gave rise to sharp and abundant amplification products of approximately 500, 700, and 900 bp as major bands and 1.2 kb and higher as minor bands (Fig. 2C). A reamplification step using nested primers OLE 5 and 6 was done to remove nonspecific amplification products, resulting in clear, thick amplicons of approximately 1.1 and 1.4 kb (Fig. 2D).

Characterization of cloned ACC Synthase cDNAs Eco RI digestion of plasmids from 19 minipreps revealed five clone types as shown by their digest profiles (Fig. 3). All inserts had internal Eco RI sites. Clone types were arranged according to frequency and size. Approximately 60% of the clones contained insert type 1 with aggregate MW of 1.1 kb, the expected amplicon size for OLE 5 and 6 primer pair. Clone

types 2, 3, 4 had higher aggregate molecular weights, 1.4, 1.2, and 1.2 kb, respectively, while clone type 5, with two visible bands, had a total MW lower than the expected amplicon. There were three clones each for types 2 and 3 and one clone each for type 4 and 5.

DNA sequencing The sequences of the five cDNAs (Fig. 4) confirm their identity as ACC synthase genes as they code for polypeptides which contain most of the 12 conserved residues between ACC synthases and aminotransferases (Huang *et al.*, 1991), including four invariant residues present in all ACC synthases (Zarembinski and Theologis, 1994). All five contain the active site motif SLSKDMGPGFR. Phylogenetic analysis groups the five cDNAs with *capacs 1*-type genes. No *capacs 2* was isolated from 'Sinta'.

In comparison to ACC synthase genes of other papaya cultivars, the clones from 'Sinta' show 94-99% homology to

Fig. 3. Diagnostic digest of plasmid minipreps using Eco RI. (A) Enzyme restriction was able to differentiate between five types of inserts, designated clones 1-5. Representative digests for clones 1,2,3, 4, and 5 (separate gel) are indicated by labeled arrows. The pGEM-T Easy plasmid shows up as a constant band of 3 kb for all the digests. (B) Simulated Eco RI digest of the sequenced clones produced by Vector NTI.

whole *capacs 1*-type sequences and 70-82% homology for short segments of *capacs 2*. Phylogenetically, 'Sinta' genes are closest to pACCS1A of Eksotika fruit (Fig. 5).

All five cDNAs have restriction sites for the following enzymes: Eco RI, Nsi I, Bst XI, and Sac I (except for clone 5). Theoretical restriction digest profiles for Eco RI agree well with the experimental data. The fifth clone, which occurs as a single clone, appears to be a cloning artifact from clone 2 to which it has 100% homology as it has no Ole 5 primer sequence at the 5' end. Clone 5 appears to be due to a recombination event of a pGEM-T Easy vector containing clone 2. Such events, leading to deletions in the vector inserts have been documented, especially from bacteria transformed with linearized plasmids, which is possible for the pGEM-T Easy vector. In vivo recyclization of in vitro ligation products explains many of the aberrant DNA molecules that are encountered during gene cloning (Conley *et al.*, 1986).

Southern Blot hybridization analysis All four unique cDNA clones were bound to the same bands in the Southern blots (Fig. 6), with some bands exhibiting different signal intensities. The cDNA clones hybridized to 1-3 bands only per restriction lane, a strong indication that the ripening-related ACC synthase is a single copy gene. The *capacs* genes isolated from other papaya cultivars such as Sunrise Solo

(Neupane *et al.*, 1998) and cv. 2001 (Mason and Botella, 1997) are also single copy genes. Additionally, the Southern blot hybridization data gave evidence that the *capacs* cDNAs were not amplified from contaminating gDNA, as the probes hybridized to both high (>40 kb) and low (<2 kb) molecular weight bands for digests of Pst I, Pvu I, and Hind III. As there is no restriction site in the cDNA for these enzymes, the restriction sites are probably found in the intron regions of the gDNA.

Multiple sequence alignment Multiple sequence analysis of all five *capacs* cDNAs of 'Sinta' revealed regions of high homology interrupted by three segments of 90-111 bp (Fig. 7). These sequences are flanked by evolutionarily conserved splice site sequences, viz, AG:GU(A)AGU at the 5' donor site and CAG:G at the 3' acceptor site (Padgett *et al.*, 1986). As there appears to be only one gene coding for the four unique cDNAs, the sequence alignments indicate that the cDNA clones may be products of three independent alternative splicing events, viz; four constitutive exons (1,3,5, and 7) are present in all four clones, while three alternative exons (2,4, and 6) occur in the 5' end and are AT-rich sequences. Clone type 1, the most dominant of the clones, contains only the constitutive exons. Clones 3 and 4 contain one alternative exon each. Clone 2, the longest, contains all constitutive and

		1		50		100
		P D Q M G L A E N Q V I T Y R N P I H T Y T Y T Y T F L S K V D S H				
type 2	(1)	CCTGATC AGATGGGTCTTGGCG AGAATCAGGTAATTA CATACAGAAACCCTA TACATACATATACAT ATACATACACTTTT AAAGCAAGGTGATT CTC				
type 1	(1)	-----GT-C-----A-----				
type 3	(1)	-----GT-A-C-GA-----				
type 4	(1)	-----GT-G-----A-T-----				
		101		150		200
		L L I W L M Q L <u>F</u> N L I H E W L L K N P E A S I C T A Q G A A				
type 2	(101)	ATCTGCT GATCTGGTTGATGCA GCTTTGCTTTAATTT AATTCAC GAGTGGC TGCTGAAAAACCC A GAAGCC TCCATTG TAC AGCACAAGGAG CAG				
type 1	(28)	-----A-----				
type 3	(29)	-----C-----C-----C-----C-----				
type 4	(101)	-----				
		201		250		300
		E F R D I A I F Q D Y H G L A E F R E V K E K N K K K K W V F N Y				
type 2	(197)	CTGAATT CAGAGATATAGCTAT CTTTCAAGATTATCA TGGCTTGGCTGAATT CAGAGAGGTAAAAGA AAAAAACAAAAAAA AAAATGGGTGTTAA TTA				
type 1	(104)	-----A-----				
type 3	(109)	-----C-----				
type 4	(197)	-----				
		301		350		400
		D L I M T I C I K P K L M F W V Q A V A K F M G K V R R N R A S F				
type 2	(297)	TGATTTA ATTATGACAATTGT ATTAAACC AAAATT GATGTTTGGGTTCA GGCTGTTGCAAGTT TATGGGAAAGTGAG AAGAAACAGAGCTTCA TT				
type 1	(164)	-----G-----C-----				
type 3	(209)	-----G-----C-----				
type 4	(257)	-----				
		401		450		500
		D P D R I V M S <u>E</u> <u>A</u> T G A H E M I A F C L A D P G D A F L V P T				
type 2	(396)	TGACCCCT GATCGGATTGTATG AGTGAGGAGCAACT GGAGCTCATGAAATG ATTGCTTTCTGTTTG GCTGATCTGGCGAT GCATTCTTGGTTCCA CT				
type 1	(210)	-----C-----				
type 3	(309)	-----C-----				
type 4	(303)	-----C-----				
		501		550		600
		<u>E</u> Y <u>P</u> G L F N T L Y R Y M Y I Y I F M G K I S S A F I F L T F M				
type 2	(496)	CCTTAATT ATCCAAGTAATTAA CACTCTCTACAGATA CATGTACATTATAT TTTTATGGGTAAAT AAGCTCGGCTTTTAT TTTCTTAACATTAT GA				
type 1	(310)	-----				
type 3	(409)	-----				
type 4	(403)	-----				
		601		650		700
		T N E I G I A C R F D R N L R W R T G G K L I P V V C E S S N D Y Q				
type 2	(596)	CTAATGA AATGGGATGCTTGC AGGTTTGTATAGAAAT TTGAGATGGAGAACG GGAGGCAAACTCATT CCAGTTGTCTGTGAA AGCTCAAAACGATTAC CA				
type 1	(326)	-----G-----T-----C-----				
type 3	(425)	-----G-----T-----G-----C-----				
type 4	(419)	-----G-----T-----				
		701		750		800
		I T I E A L K A A Y E P A Q E A D I K V K G L F I T N P S <u>E</u> <u>L</u> G*				
type 2	(696)	GATCACC ATAGAAGCCTTGAAA GCTGCTTATGAACCC GCACAAGAAGCTGAC ATCAAGGTAAGGGT TTGTTTCAACCAAC CCATCAAAACCCACTG GGA				
type 1	(399)	-----C-G-----A-AC-----G-----C-----A-----				
type 3	(498)	-----C-G-----G-----C-----C-----G-----C-----C-----				
type 4	(492)	-----C-G-----				

Fig. 4. Nucleotide and deduced amino acid sequences of the four unique ACC synthase cDNAs from papaya cv. 'Sinta'. The active site is underlined and highly conserved residues found in aminotransferases are highlighted with gray. Residues that were found to play important structural and functional roles are boxed. Four residues found to be invariant among ACC synthases have asterisk.

alternative exons.

While there is so far no documented case of alternative splicing in ACC synthase genes, Peck and Kende (1998) have described the presence of two ACC synthase cDNA isoforms (1.6 kb and 1.9 kb) in etiolated pea seedlings sprayed with auxin. Based on 5' RACE and oligonucleotide-directed RNase H mapping, the shorter transcript was the result of an alternative promoter and not alternative splicing. Attempts to express the 1.6 kb in *E. coli* were not successful indicating that the shorter transcript was not functional or active. This "two transcript conserved response" has been observed in suspension-cultured tomato cells treated with fungal elicitors (Spanu *et al.*, 1993), flooded tomato roots (Olson *et al.*, 1995), wounded tomato fruit (Li *et al.*, 1992), organ-specific expression in carnation flowers (ten-Have and Woltering, 1997) and auxin-treated hypocotyls of winter squash (Nakagawa *et al.*, 1991). Kazan (2003) recently noted that alternative splicing might play a significant role in generating protein diversity in plants especially proteins with regulatory functions. Kazan (2003) gave examples of alternatively spliced stress-associated plant genes which include plant disease resistance genes in flax (Ayliff *et al.*, 1999) and an alternative oxidase gene in rice under salt stress (Kong *et al.*,

2003).

Genomic sequences of ACC synthase from other plants document the presence of introns within the ACC synthase gene, which are similar to the alternative exons of Sinta *capacs 1*, are located at the 5' half of the gene. Three introns are present in *Arabidopsis ACC2* gene (Liang *et al.*, 1992); two introns in *Ps-ACS 1a* from pea hypocotyl (Peck and Kende, 1998); three introns in *LE-ACS3* of tomato, each 90-230 nt in length (Yip *et al.*, 1992, Kawakita *et al.*, 1994, and Olson *et al.*, 1995); two introns in *LE-ACS7*, ~100 nt in length (Shiu *et al.*, 1998), three introns in *Md-ACS1* from apple (Harada *et al.*, 1997), four introns in zucchini *CP-ACC1A* and *1B* (Huang *et al.*, 1991), and *LE-ACS 1A* and *1B*. The introns of the *LE-ACS 2* and *4* genes, 324 and 302 bp in length, respectively, are located at the same positions as the first putative exon in 'Sinta' *capacs* clones.

The phylogenetic relationships of the ACC synthase sequences for *capacs 1* were as expected among cultivars that are known to contain a large proportion of Hawaiian germplasm, namely, Philippine Kapoho and Sunrise. *Capacs* from hybrids with a parent from a Hawaiian cultivar formed another group. The *capacs* from Eksotika, a product of the Malaysian cultivar Subang-6 backcrossed several times with

		801		850		900
		P I I T K D T L E A L V T F T N H K N I H L V C D* E I S A A T V F				
type 2	(796)	CCAATTA TTACCAAGGACACAT TAGAAGCTCTAGTCA CCTTCACCAACCACA AGAACATTTCATCTGG TGTGTGATGAGATAT ATGCTGCTACCGTCT TCA				
type 1	(499)	A-----TA---G-C-----C-----T-----				
type3	(598)	A-----C-----C-----				
type4	(592)	-----G-----				
		901		950		1000
		S Q P E F T S I A E I I E E D K I C C N R D L I H I I Y S L S K* D				
type 2	(896)	GCCAGCC CGAATTCACCAGCAT AGCCGAGATAATTGA AGAAGATAAAATTTG TTGCAATCG TGAT CTCATCCACATCATT TACAGTTTATCCAAA GA				
type 1	(599)	-----G-----G---T-----				
type3	(698)	-----				
type4	(692)	-----				
		1001		1050		1100
		M G F P G F K V G I V Y S Y N D A V V S C A R K M S S F G L V S S				
type 2	(993)	CATGGGA TTCCCTGGATTTAAA AGTGGGCATTGTGTA TTCATACAATGATGC AGTGGTGAGTTGTGC TCGTAAGATGTCGAG CTTCGGCCTAGTATC TTC				
type 1	(699)	-----G- -T-----				
type3	(795)	-----G- -T-----				
type4	(789)	-----G- -C-----				
		1101		1150		1200
		Q T Q Y L I A S M L A D D E F V D K F I V E S R K R L A M R H S F				
type 2	(1093)	GCAAACC CAGTATCTGATTGCAT CCATGTTAGCAGACG ATGAATTTGTAGACA AATTTATTGTAGAGA GCAGAAAGAGGCTGG CAATGAGACATAGTT TT				
type 1	(798)	-----				
type3	(894)	-----				
type4	(888)	-----				
		1201		1250		1300
		F T Q R L A Q V G I N C L K S N A G L F V W M D L R R L L K E Q T				
type 2	(1193)	TTCACAC AAAGACTTGCTCAAG TAGGCATTAACTGTT TAAAAAGCAATGCTG GTCTTTTGTGTGGA TGGATTTCGCTAGAC TGCTGAAAGAACAGA CAT				
type 1	(898)	-----				
type3	(994)	-----				
type4	(988)	-----C-----				
		1301		1350		1400
		F E A E M V L W R V I I N E M K L N V S P G S S F H C S E P G W F R*				
type 2	(1293)	TTGAAGCA GAAATGGTGTATTGG AGAGTAATTATAAAC GAAATGAAACTCAAT GTATCTCCTGGTTG TCTTTCCACTGCTCA GAACCTGGCTGGTTC CG				
type 1	(998)	-----A-				
type3	(1094)	-----C-				
type4	(1088)	-----TA-				
		1401		1420		
		V C F A A E				
type 2	(1393)	AGTTTGT TTTGCTGCAGAG				
type 1	(1098)	G--C--C- -C-----				
type3	(1194)	G--C--C- -C-----				
type4	(1188)	A--A--C- -T-----				

Fig. 4. Continued.

Fig. 5. Phylogenetic relationship of 'Sinta' papaya *capacs* cDNAs (referred to as clones 1-5) nucleotide sequences to *capacs* from other papaya cultivars as generated by the Align X Program of the Vector NTI suite.

Sunrise (Chan, 1987), grouped with the genes from 'Sinta', a product of an inbred derived from the native 'Morado' and another inbred derived from a Hawaiian strain. It is interesting to note that the *capacs* 1-type gene from Tainung grouped with the *capacs* 2-type genes from Philippine Solo and Eksotika, an indication, perhaps, of its genetic distance from the Hawaiian strains. The close relationships among the

capacs 1-type genes support the observation that the genetic variability in available breeding lines of papaya is limited (Stiles *et al.*, 1993). Even so, papayas have circumvented this limitation by evolving distinct means of regulating ethylene generation either by differential gene expression, as in the case of Philippine Solo and cv. 2001, or, as in the case of 'Sinta', by producing at least four isoforms of a single gene.

Fig. 6. Southern Blot hybridization bands of the 5 cloned ACC synthase cDNAs onto Sinta papaya gDNA digested with restriction enzymes (A) Clone 1 (B) Clone 2, (C) Clone 3, (D) Clone 4, and (E) Clone 5. Lane designations: (1) EL markers, (2) Lambda EcoRI digest, (3) Bam HI, (4) Eco RI, (5) Hind III, (6) Kpn I, (7) Pst I, and (8) Pvu I.

Fig. 7. Diagrammatic representation of the multiple sequence alignment of the five 'Sinta' papaya ACC synthase cDNAs showing probable alternative splicing in the 'Sinta' papaya ACC synthase gene. Included in the diagram are the common restriction sites. Numbering follows the sequence of clone 2. Forward and reverse arrows refer to OLE 5 and 6 primers, respectively. Fragments in gray represent sequences common to the first four clones (constitutive exons), while those in black, with hatched squares and hatched diagonal lines represent alternative exons. Fragments are numbered at the bottom.

Our results further show that the ripening-related ACC synthase type in papaya may differ with the cultivar. While in the hybrid 'Sinta' only the *capacs 1* isoforms were detected in the ripe stage, in the Australian cultivar 2001 (Mason and Botella, 1997) and Philippine Solo (Laurena *et al.*, 2002), *capacs 2* was the predominant form during ripening and *capacs 1* was highly expressed at the mature green stage or onset of ripening. We are now studying the presence of the isoforms of *capacs 1* at different stages of ripening in the hybrid papaya 'Sinta' fruit and in different tissues of the papaya plant.

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