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Full Length Research Paper

Cloning and expression of pineapple sucrose-phosphate synthase *gene* during fruit development

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A 1132-base pairs (bp) polymerase-chain-reaction product of sucrose-phosphate synthase (SPS) (EC 2.3.1.14) from pineapple (*Ananas comosus* cv. Comte de paris) fruit was cloned and nominated as *Ac-SPS1*. The sequence encodes a putative 377 amino acids protein containing two serine conserved features that had been found in other plant *SPS genes*: the presence of a 14-3-3 protein special binding domain and an activated site of osmosis stress, which can been activated by phosphorylation and dephosphorylation. The Neighbour-joining tree revealed that *Ac-SPS1* belonged to the first kind of sucrose phosphate synthase *gene*. The results indicated that, the *Ac-SPS1* expression was low in the earlier period of fruit growth, then, increasing from 20 days after anthesis and gradually a falling on 40 days, reached the peak with the highest value around 70 days. The SPS activity and sucrose content reached their maximum 80 days after anthesis. It proved that the accumulation of sucrose was correlated with SPS activity and mRNA content and it maximally occurred at 10 d after SPS mRNA and activity had reached its maxima. These results indicated that *Ac-SPS1 gene* played a key role in sucrose accumulation during the pineapple fruit development and transcriptional activation with increase in Ac-*SPS1* expression might be important regulatory events of sugar during pineapple fruit maturation.

Key words: Pineapple fruit, sucrose phosphate synthase, *gene* cloning, expression.

INTRODUCTION

Changes that occurred in the content of fruit sugar and sugar metabolism directly affect the quality of fruits (Islam et al., 1996; Zhang et al., 2004). Furthermore, sucrose is the main component of the fruit sugar and that is the main transporting form of carbohydrate, which determines the flavor of fruit, quality and yield (Najeh et al., 1992; Hyacinthe et al., 1999). In plants, enzymes that are closely connected with the accumulation of sucrose are invertase (Ivr), sucrose synthase (SS) and sucrose phosphate synthase (SPS). However, SPS is considered to be one of the key enzymes that regulate the sucrose synthesis pathway. Due to its importance, leaf SPS has been exhaustively studied, and some of its inhibitors and activators identified (Doehlert and Huber., 1983); moreover, several full-length clones have been isolated (Komatsu et al., 1996; Li et al., 2003; Sonnewald et al., 1993) and expressed in *Escherichia coli* (Sonnewald et al., 1993; Komatsu et al., 1999; 2002) and even transgenic plants are now available (Worell et al., 1991; Galtier et al., 1993). SPS is regulated by at least two mechanisms, one allosteric, which activates the enzyme by binding metabolites like glucose 6-phosphate and is inhibited by

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Abbreviations: FW, Fresh weight; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcription-PCR; **SPS,** sucrose phosphate synthase; **UDP,** uridine diphosphate; **Pi,** inorganic phosphate; **EDTA,** ethylene diamine tetraacetic acid; **Ivr,** invertase; **SS,** sucrose synthase.

inorganic phosphate (Pi) (Doehlert and Huber, 1983) and the other which regulates by covalent protein phosphorylation (Stitt et al.,1988; Walker and Huber, 1989).

During fruit maturation, as in the case of tomato (Dali et al., 1992), peach (Vizzotto et al., 1996), citrus (Komatsu et al., 1999) and sugarcane (Lingle, 1999), there is a direct correlation between sucrose accumulation and SPS activity but the mechanism of its regulation is unknown. It could involve protein synthesis, as happens with several other enzymes (Gray et al., 1994), allosteric and covalent regulation (Doehlert and Huber, 1983; Stitt et al., 1989; Walker and Huber, 1989). In order to follow the accumulation of *SPS* mRNA during the developing pineapple fruit, we set out to isolate a partial but conserved *SPS* sequence. To our knowledge, the results shown here are the first attempt to isolate pineapple fruit SPS *gene* and to follow its expression during development.

MATERIALS AND METHODS

Plant materials

Pineapple (*Ananas comosus* cv. Comte de Paris) fruits from pineapple germplasm plantation were used in all experiments in South Subtropical Crop Research Institute (SSCRI), Zhanjiang, Guangdong and China. Plants were planted in March, 2005. Five uniform fruits were randomly sampled every 10 days throughout fruit development from April to June, 2006. These fruits were transferred to the laboratory within a half hour. Tissues were frozen immediately in liquid nitrogen after sampling and stored at -80˚C.

Extraction of RNA and synthesis of cDNA

Total RNA from intact fruits at different developmental stages were obtained using the method proposed by Lopez-Gomez and Gomez-Lim (1992). First-strand cDNA synthesis was done essentially as described by Sambrook et al. (1989) using AMV-reverse transcriptase, total RNA as template, and poly-dT (16) as primer.

Amplification by polymerase chain reaction (PCR) and cloning

Based on the available databank sequences, the primers *SPS*-F (5'-GGAGCTTGGTCGGGATTCTG ATA-3') and *SPS*-R (5'-CAGGAACATCAGA CTGCTTGTG-3') were designed in such a way that, they would hybridize to a conserved 1100-base pair region within the *SPS* open reading frame. The conditions for PCR amplication were as follows: 38 cycles at 85℃/60 s, 52℃/360 s and 72℃/120 s, 25 µl reaction volume, 1 U Taq, 10 µl of the RNA/DNA template. PCR products obtained after the PCR were purified with the High Pure PCR purification kit (Roche Diagnostics) and cloned into pGEMTeasy using the pGEMTeasy Vector System (Promega, Madison, WI, USA).

Ac-SPS1 expression analysis by semi-quantitative RT-PCR

Total RNA from fruits at different developmental stages were extracted, respectively. Equal amount of RNA were used as Semi-quantitative RT-PCR templates for the *SPS gene*-specific amplification in order to study the expression of *Ac-SPS1*. The sequences for the specific primers SPS-F (5'-GGAGCTTGGTCGGGATTC TGATA) and SPS-R (5'-CAGGAACATCAGACTGCTT GTG-3'), meanwhile, rRNA *gene* (325bp) was amplified as control in the same tube using the primers rRNA-F (5'-AGCCCCGGGTTA CTATGTG-3') and rRNA-R (5'-TCGTTTACGGCGT GGACTACC-3') designed according to sequence of pineapple rRNA *gene* (Accession AY787142). The amplification conditions were the same as Ac-*SPS1*. Equal amount of PCR product was fractionated on 1.2% agarose gel, stained with ethidium bromide and visualized under UV light.

Enzyme extraction and assay

The extraction of enzyme was optimized to avoid interference by phenolics, tannins and artifacts due to differences of tissue composition during each developmental stage. Fruit tissues at different times were frozen in liquid N_2 and homogenized in 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 20 mM cysteine and 1% polyvinylpyrrolidone. After centrifugation at 10,000 rpm for 10 min, the supernatant was dialysed against 20 mM Tris-HCl (pH 7.0), 4 mM EDTA, and 5 mM cysteine. The SPS activity was then measured at saturated substrate conditions with 50 mM Hepes (pH 7.5), 5 mM fructose-6 phosphate, 15 mM glucose-6 phosphate, 10 mM UDP-glucose and 15 mM $MgCl_2$, and at limiting substrate conditions plus Pi (selective assay) with 50 mM Hepes (pH 7.5), 3 mM fructose-6 phosphate, 9 mM glucose-6 phosphate, 10 mM UDP-glucose, 15 mM $MgCl_2$ and 10 mM Pi. The reactions were in boiling water for 10 min. The sucrose was assayed by the thiobarbituric acid method (Percheron, 1962).

Carbohydrate assay

Frozen fruit fresh tissue (1.0 g) was homogenized with 5 mL deionized water, incubated at 80˚C in water for 15 min, cooled and centrifuged at 10,000 rpm for 15 min. The residue was redissolved with 4 mL deionized water and centrifuged at 10,000 rpm for 15 min, combined the two clear supernatant and add water to 10mL. Individual sugars were quantified by injecting a 10 µL aliquot of sample, filtered through 0.45 µm filter into a high performance liquid chromatograph (HPLC) (made in PE Corp., America) equipped with an automatic pump, an automatic injector, an analysis column (Series200, 250 × 4.6 nm, 5µm), a differential refractometer (PE200), and a reporting integrator. The mobile phase was degassed $CH_3CN:H_2O$ = 70:30 (V/V), at a flow rate of 1.0 mL/min and 35℃. Peak height was used to quantify contents of soluble sugars by comparing them with peak height of standard solution.

RESULTS

Total RNA analysis

Several conventional RNA extraction methods have been tried out with the pineapple fruit without much success. Due to the high polysaccharide, polyphenolics and protein of the pineapple fruit, the main problems encountered using conventional methods were low yields, DNA contaminating and some RNA degradation. Total RNA was isolated from pineapple fruit using the method proposed by Lopez-Gomez and Gomez-Lim (1992). The quality of total RNA was checked by the spectrophotometer and agarose gel electrophoresis (Figure 1). The ratio of OD_{260}/OD_{230} was over 2.0, and A_{260}/A_{280} about 1.8. Furthermore, the bands of 5.8S and 5S were

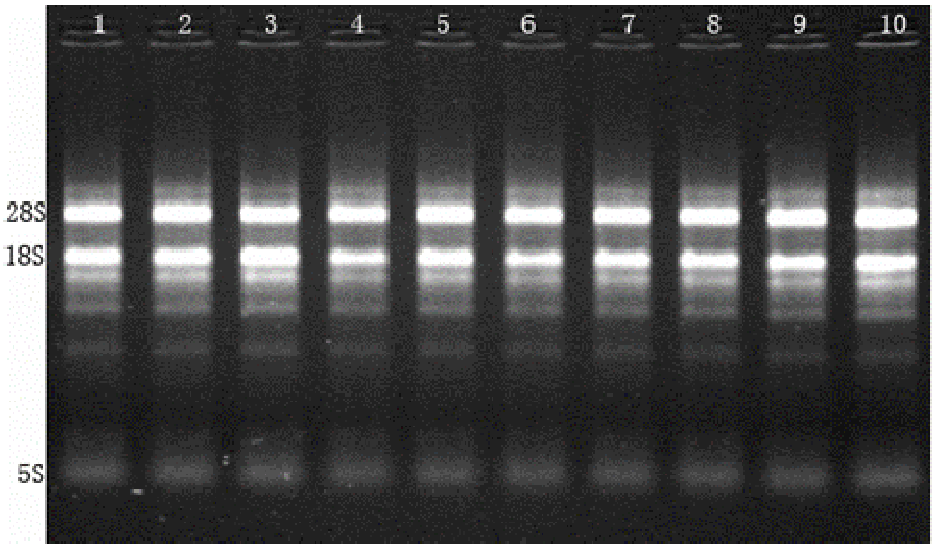

Figure 1. Agarose gel electrophoresis of total RNA from pineapple fruits. Lanes 1 - 7: the total RNA of different stages, lanes 8 - 10: The total RNA of different parts.

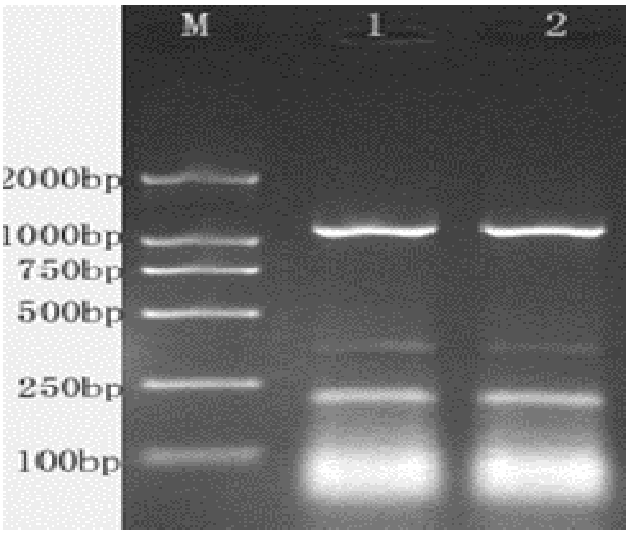

Figure 2. PCR products amplified from pineapple fruits. Lane M: Marker DL2000; lanes 1 and 2: SPS gene.

obvious in electrophoresis analysis. The lanes were not stained by polysaccharides, protein and poly-phenolics. The method greatly improved the extraction quality.

Cloning of SPS from pineapple fruits

Some sequences for the amplification of the SPS *gene* have already been described (Nascimento et al., 1997; Klein et al., 1993). Those of the published primers were actually tested, but showed a poor PCR ampilcation of the pineapple SPS *gene*. Based on the sequence homology search carried out in Genebank, two consensus primers were designed. Using the designed SPS-F and SPS-R primers, an intense and reproducible 1100-bp band was obtained by PCR amplification (Figure 2). Furthermore, the about 400 and 200 bp bands were blurry, repeating amplification result was steady. However, with a view to coding amount of SPS *gene*, the three bands were recovered and cloned into the pGEM-T vector (Promegat) and sequenced.

Sequence analysis of pineapple SPS

All sequences were assembled and edited using the Lasergene software package version 4.03 (DNASTAR, Madi-son, WI). Similarity searches were performed with the BLAST program (Altschul et al., 1997) (www.ncbi. nlm.nih.gov) using the default settings, respectively. 1132bp sequence has high homology to the other plants of *SPS gene*. Whereas, about 400bp and 200bp sequences were found coding unknowing proteins. From Figure 3, the predicted amino acid sequence displayed two serine conserved features: the presence of 14-3-3 protein special binding domain and an activated site of osmosis stress. The site activity can be changed by phosphorylation and dephosphorylation.

Alignments of deduced SPS amino acids

The deduced amino acid sequences of *Ac*-S*PS1* cDNA were aligned with additional plant *SPS* (Figure 4) and a phylogenetic tree (Figure 5) was constructed using DNAman software. As shown in Figure 4, the pineapple fruit SPS has high amino acid sequence homology to the

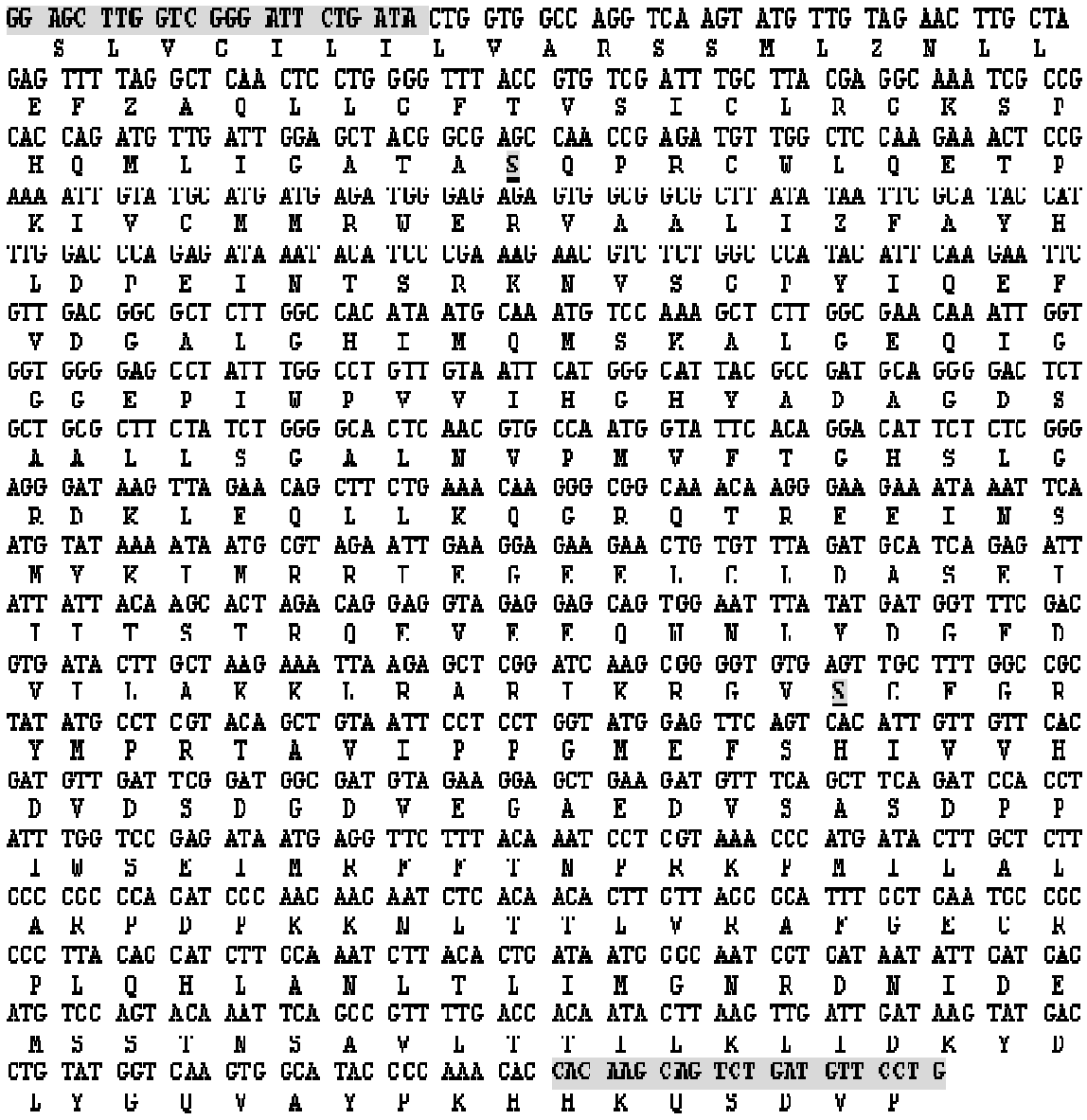

Figure 3. The nucleotide sequence and deduced amino acid sequence of *Ac-SPS1* cDNA from pineapple. The shadow parts are the sequence of primers.

SPS of leaves of rice (*Oryza sativa*), sugarcane (*Saccharum officinarum*), tomato (*Solanum tuberosum*), peach fruit (*Prunus persica*), and potato (*Solanum tuberosum*) found in the GeneBank. Especially, the deduced amino acid of *Ac-SPS1* had 84% identity with bamboo (*Bambusa oldhamii*) and perennial ryegrass (*Lolium perenne).* Moreover, the homology of candiate regions containing two serine conserved features has been found in other plant *SPS genes*. Therefore, the cDNA of SPS was successfully cloned from the pineapple fruit. Figure 5 revealed that, the family of SPS was distinguished with two major groups. *Ac-SPS1* belonged to the first group of sucrose phosphate synthase *gene*. Furthermore, the *Ac-SPS1* was alone in the first group showed the evolution relationship was far.

Semi-quantitative RT-PCR of PinSPS *gene*

Semi-quantitative RT-PCR was performed to investigate the expression pattern of *Ac-SPS1* mRNA in various different developmental stages. From the results (Figure 6), the *Ac-SPS1* mRNA was detected during all developmental stages (20, 30, 40, 50, 60, 70 and 80 days), clearly up-regulated from 20 days and gradually down-graduated on day 40 after anthesis, reached a maximum value around day 70 after anthesis.

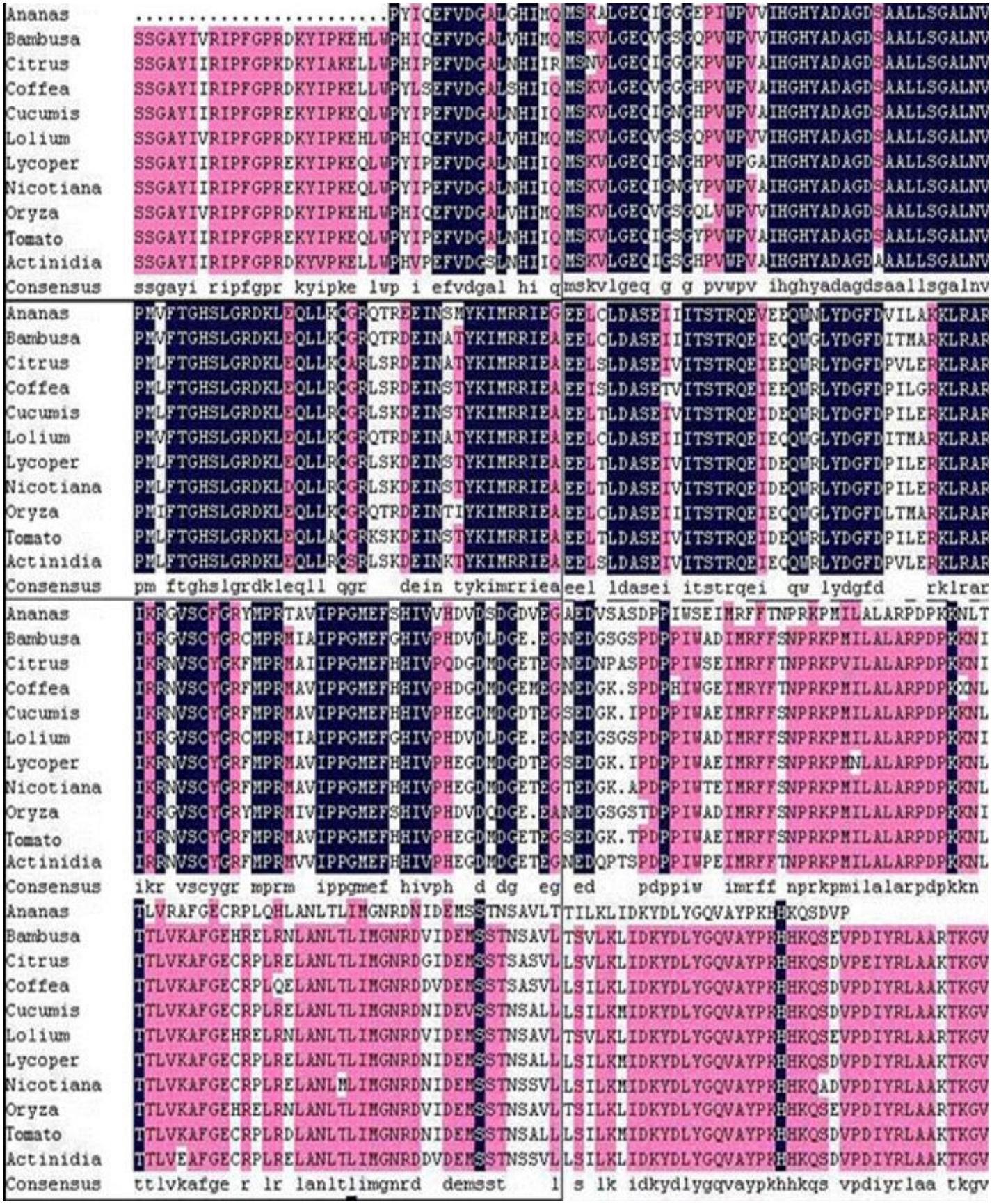

Figure 4. Comparison of amino acid sequences among *Ac-SPS1* and its homologous proteins in different organisms.

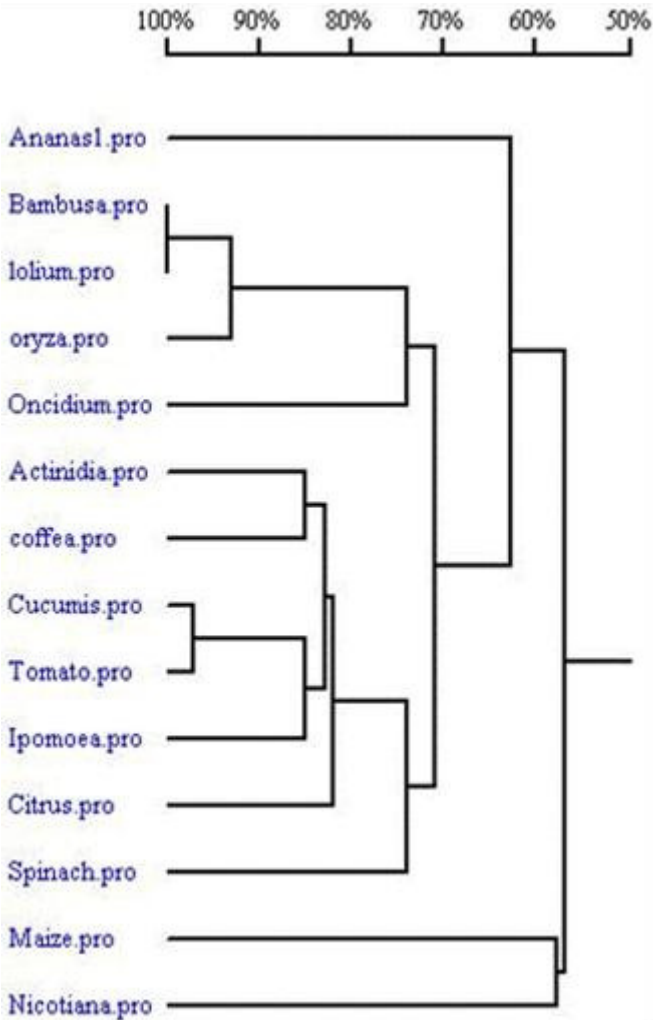

Figure 5. Phylogenetic analysis of the homologous *Ac-SPS1* protein.

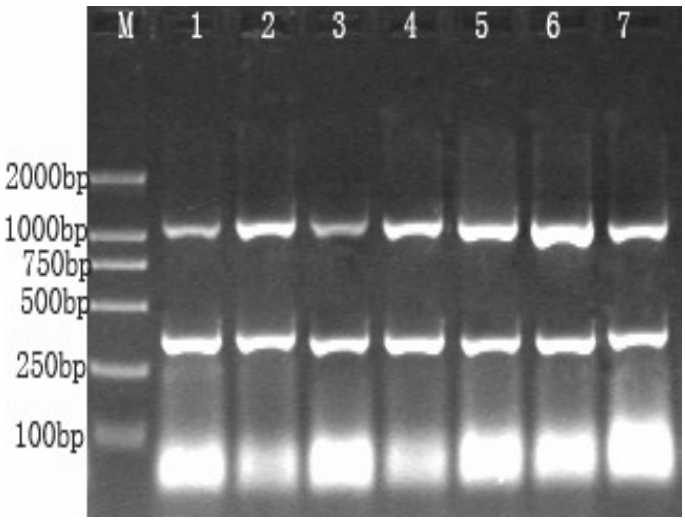

Figure 6. *Ac-SPS1* expression in pineapple fruit different stages by semi-quantitative RT-PCR. M: Marker DL2000, 1-7: Different development stages (20, 30, 40, 50, 60, 70, 80 days).

Sucrose content and SPS enzyme activity analysis

Figure 7a correlates Ac-*SPS1* expression profile with the variation in sucrose content and enzyme activity during fruit development. In the developmental period, days 20 - 40 after anthesis, the variation in SPS mRNA content was associated with SPS activity. After that, there was a much-pronounced increase in mRNA accumulation, days 40 - 70 after anthesis, reaching a maximum value around day 70, and SPS activity, days 40 - 80 after anthesis, reaching its maximum on day 80 after anthesis. It is important to note that the SPS enzyme activity correlated with the mRNA accumulation over the time period.

During the early period of fruit development, two peaks appeared in sucrose phosphate synthase activity and reached the highest value (18.52 µmol/g h FW) when the fruits were in maturity. Meanwhile, the sucrose content in the fruit early development was low and decreased to the lowest level on 40 days, thereafter, there are rapid accumulation of sucrose until the fruit harvested reached its peak when its content was 60.41 mg/g FW, 59.48% of total sugar (data not shown), which showed that 'Comte de paris' fruit mainly belongs to the sucrose accumulation type. As SPS is expected to be involved in sucrose synthesis. Figure 7b also shows a rapid growth in the sucrose content around day 30. The appearance of sucrose correlated directly with the SPS mRNA accumulation and enzyme activity, and sucrose content reached the maximum 10 d after the maximum mRNA content occurred and SPS enzyme activity was achieved. That is to say, SPS is very important for the accumulations of sucrose in the 'Comte de paris' fruit.

These data suggested that the expression of *Ac-SPS1* played a role in the sucrose accumulation. Furthermore, the pattern of change in amount of *Ac-SPS1* mRNA as assessed by the semi-quantitative RT-PCR was similar to the curve of change in *SPS* activity of all developmental stages of fruits.

DISCUSSION

By multiple sequence alignment, *Ac-SPS1 gene* has a high homology with other plants between 80 and 83%. The homology of monocotyledon and dicotyledon *SPS genes* were not obviously different, but can still been distinguished from Phylogenetic tree (Figure 5). Based on strict criteria of the chromosome differences, *SPS* can be divided into four families (A, B, C, D) (Castleden et al., 2004). So far, D family was only found in the gramineae plants, while A, B, C families widely distributed in the monocotyledonous and dicotyledonous plants (Castleden et al., 2004). By cluster analysis, the cloned pineapple *Ac-SPS1* belongs to A group. Sucrose phosphate synthase *gene* is a single group, which the evolution of *SPS genes* have certain relationship.

SPS cDNA clones from the source organ have been isolated and characterized in maize, spinach and

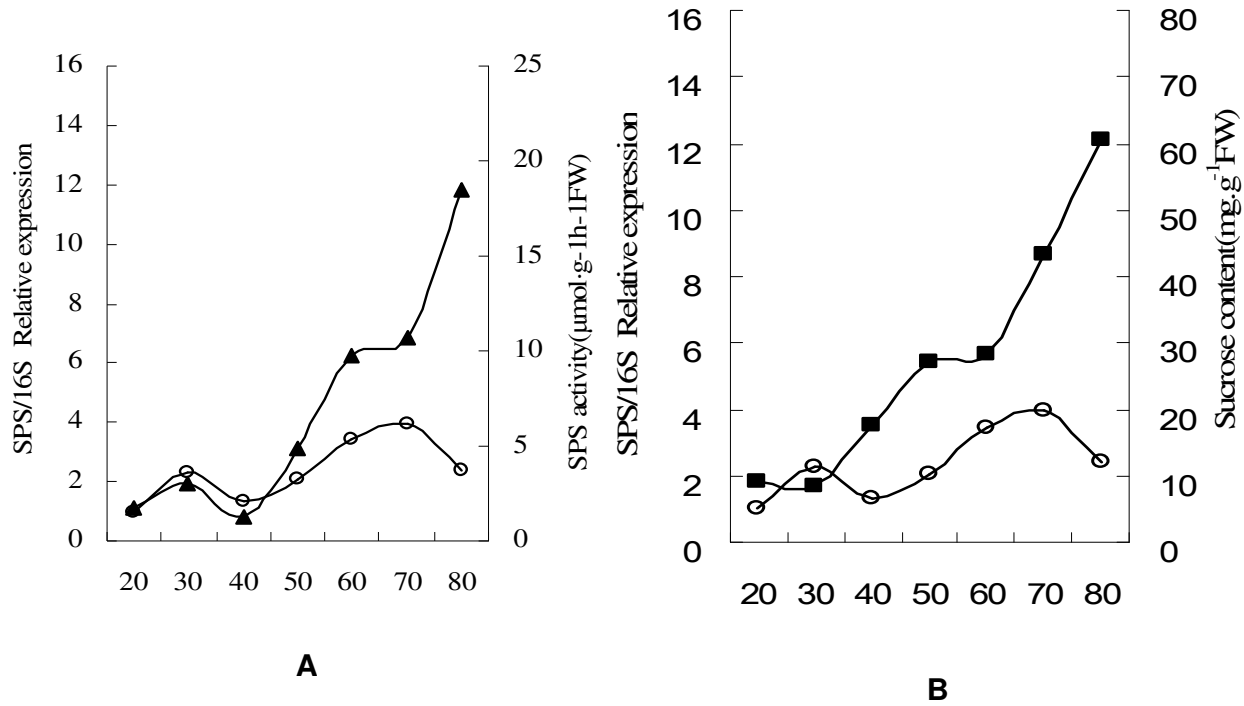

Figure 7. Variation of pineapple fruit SPS activity (▲), *gene* expression (○) and sucrose content (■) during development.

Creterostigma plantagium (Klein et al., 1993; Ingram et al., 1997). To elucidate the role of SPS in the control of photosynthesis, leaf carbon metabolism and growth, several transgenic plants expressing the SPS *gene* have been established and analyzed for one generation and for the following generation (Galtier et al., 1993). On the other hand, the role of SPS in sucrose composition and accumulation has been investigated in sink tissues, such as in fruit, during the fruit development or ripening (Nascimento et al., 1997; Langenkamper et al., 1998). From Figure 7, accumulation of transcripts of *Ac-SPS1* coincided with the increase in SPS activity during the development stage, when sucrose accumulation occurred after 10 days, suggesting that *Ac-SPS1 gene* might have an important role to determine the sucrose accumulation in pineapple fruit. It is noticeable that pineapple fruit sucrose accumulation is concerned with the mRNA accumulate and SPS enzymatic activity. These correlations were also found in citrus ((Komatsu et al., 1999; 2002).

We have shown an increase in SPS mRNA levels as fruit matured. Previous studies have consistently demonstrated that the increase in sucrose concentration in fully grown fruit was correlated to SPS activity (zhang et al., 2008). While an unknown physiological signal promotes this change, it can also be moderated by low temperature and water deficit (Ingram et al., 1997; Langenkamper et al., 1998). Photohormones have also been shown to regulate SPS activity, for example ethylene in kiwifruit (Langenkamper et al., 1998) and auxin in eggplant (Lee et al., 1997). Previous work has shown that the initial increase in sugars is associated with an increase in maximal SPS activity, but not with increased activation (Macrae et al., 1992). An increase in activation state was instead found as fruit develops into the maturity and major sugar synthesis occurred. In this report, we demonstrated that an increase in SPS activity was regulated by the increased mRNA levels.

The results showed SPS activity was consistent with its transcription. Meantime, it indicated that, the accumulation of sucrose is also affected by the regulation of other *genes*. Furthermore, single *gene* expression is not sufficient to explain their relationship, which is consistent with Zhang Xiu-mei (Zhang et al., 2008) and Davies and Robinson (1996) research findings. The results obtained provided additional evidence that the accumulation of fruit sugar was comprehensively regulated by *Ac-SPS1 gene* and other *genes* that were related to sugar metabolism.

These results taken together do not rule out some regulation through translational modification as happens for several other enzymes and other fruits (Gray et al., 1994), may be important regulatory events during pineapple fruit ripening.

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