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A comparative study of cultivar differences in sucrose phosphate synthase gene expression and sucrose formation during banana fruit ripening

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ABSTRACT

Sucrose phosphate synthase (SPS) (E.C.2.4.1.14) is the key enzyme for sucrose formation in banana. A comparative analysis of expression profiles of *SPS* was carried out in association with sucrose metabolism patterns during fruit ripening in three banana cultivars, Cavendish (AAA), Kanthali (AB) and Monthan (AAB). Analysis of the transcript and protein accumulation patterns of the *SPS* gene during ripening revealed differential timing in expression of the gene which correlated well with variation in sucrose metabolism patterns in the three cultivars. The expression levels of *SPS* increased considerably during early ripening in Cavendish after exogenous application of ethylene, while in Kanthali ethylene treatment only moderately increased *SPS* expression during postharvest ripening. In Monthan, expression of *SPS* was very low throughout ripening and ethylene treatment did not stimulate the expression of the gene in this cultivar. Analysis of a promoter fragment of *SPS* in the three cultivars revealed a putative reverse GCC-box ERE motif. DNA–protein interaction studies demonstrated the role of this regulatory element in differential transcriptional regulation of *SPS*. Overall our results provide information about sucrose metabolism patterns at the physiological and molecular levels during fruit ripening in three natural banana cultivars.

1. Introduction

Sucrose phosphate synthase (SPS) is the key enzyme involved in the conversion of starch to sucrose in photosynthetic organs. SPS activity and its regulation have been extensively studied in leaf tissues with identification of some of its activators and inhibitors (Doehlert and Huber, 1983). cDNA clones encoding *SPS* have been isolated from several plant species (Worrell et al., 1991; Hesse et al., 1995; Komatsu et al., 1999). An increase in SPS activity has been reported during fruit ripening in fruit such as tomato (Miron and Schaffer, 1991), *Prunus* species (Hubbard et al., 1991), banana (Nascimento et al., 1997) and kiwifruit (Langenkamper et al., 1998), suggesting a physiological function for SPS in sucrose metabolism during fruit ripening.

Degradation of starch and accumulation of sucrose are some of the major changes that occur during ripening in banana fruit and such changes confer sweetness on the fruit during the postharvest period (Areas and Lajolo, 1981). Strong biochemical evidence is now available to indicate the function of SPS in the conversion of starch to sucrose during ethylene-mediated ripening in banana fruit (Hubbard et al., 1990).

Although expression patterns of *SPS* have previously been studied during ripening of banana (cv Nanicao) (Nascimento et al., 1997), corresponding knowledge about the expression levels of *SPS* during fruit ripening is not yet available in various other cultivars of banana which display distinct ripening behaviours and ethylene biosynthesis (Roy Choudhury et al., 2008a). Therefore, the objectives of the present study were to investigate the expression profile of the *SPS* gene and changes in enzyme activity of SPS along with the patterns of sucrose metabolism during ethylene-mediated postharvest ripening in three banana cultivars, Cavendish, Kanthali and Monthan. To further understand the transcriptional regulation of *SPS* in response to ethylene in the banana cultivars, promoter fragments of the *SPS* gene were isolated from the selected cultivars and the function of a putative reverse GCC-box ethylene responsive element, present in the promoter, was investigated in connection with ethylene-regulated expression of *SPS* during the various stages of ripening.

2. Materials and methods

2.1. Plant materials and ethylene treatment

The banana cultivars Cavendish (AAA group), Kanthali (AB group) and Monthan (ABB group) were obtained from plants grown in soil from August to April at the Bose Institute Experimental field.

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The average temperature was ~32 and ~18 °C during the light and dark periods, respectively. The relative humidity ranged between 70% and 97%.

To study the sucrose metabolism pattern in banana fruit during ripening in harvested unripe bananas (preclimacteric stage), hands were cut from a bunch 80 d post-anthesis (DAP) for each cultivar to avoid heterogeneity due to differences during development. Banana hands from each cultivar was separated into individual fruit and kept at room temperature (25 °C) until the fruit were fully ripe (15 d after harvest). Different ripening stages of the cultivars were previously studied by daily measurements of ethylene biosynthesis and ACC oxidase activity to characterize the time of climacteric ethylene production (8, 10 and 12 d after harvest for Cavendish, Kanthali and Monthan, respectively), as well as to identify the preclimacteric, climacteric and postclimacteric ripening stages (Roy Choudhury et al., 2008a). To avoid differences in ripening behaviour of fruit among different hands of the same cultivar, fruit from the same hand were used as a sample group in each experiment for each cultivar. Again, for each cultivar, pulp tissues were collected from the various stages of ripening, tissues frozen in liquid N_2 and stored at −80 °C for isolation of RNA, protein extracts, starch and sucrose. All experiments were repeated at least three times with five replicates in each case.

Ethylene treatment of preclimacteric bananas (day 0 after harvest) from each cultivar was given by following the protocol described by Roy Choudhury et al. (2008a). Fruit were treated with 100 μL L^{-1} ethylene for 24 h at 25 °C in the dark and then allowed to ripen in air until fully ripe.

Potassium permanganate ($KMnO_4$) was used as an ethylene absorbent for an ethylene inhibition treatment (Scott et al., 1970). Ethylene-treated banana fruit were placed in a sealed airtight container along with 15 g of potassium permanganate enclosed in a paper bag for 16 h at 25 °C in the dark. Fruit were then ripened in air at 25 °C until fully ripe. Samples were harvested every 24 h after ethylene treatment or both ethylene and potassium permanganate treatments. Representative samples from the respective preclimacteric, climacteric and postclimacteric stages of the cultivars were stored at −80 °C for the extraction of total RNA, proteins and nuclear extracts.

2.2. RNA isolation, RT-PCR and molecular cloning of banana SPS

Total RNA was isolated from pulp tissues of banana fruit by modification of the SDS phenol method as described previously (Roy Choudhury et al., 2008a). First strand cDNA was synthesized from 5 μg of total RNA using Thermoscript reverse transcriptase (Life Technologies Inc.) following the manufacturer's instruction. The cDNA clones for *SPS* were isolated from ripe pulp tissue of the three banana cultivars by PCR amplification with the gene specific oligos (SUCP5: 5′-ATGCGGATCCGCTGATGCAGGTGATTCT GC-3′ and SUCP3: 5′-ATGCGGATCCGCGGCATTCACCAAAAGCTTT-3′) made from the banana *SPS* sequence (U59489). The reaction for RT-PCR comprised 30 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min. The amplified cDNAs were cloned in pBluescript cloning vector (Stratagene) for sequence verification. *SPS* sequence from Cavendish banana was subsequently submitted to Gene Bank databases (EF158798). The partial cDNA sequence of *SPS* (561 bp) from Cavendish (EF158798) showed a high degree of sequence identity (96–99%) with the *SPS* cDNA fragment from Kanthali and Monthan fruit both at the nucleotide and amino acid levels, respectively. For semiquantitative RT-PCR analysis, a first cycle of 5 min at 94 °C, 45 s at 57 °C and 1 min at 72 °C was followed by 45 s at 94 °C, 45 s at 57 °C and 1 min at 72 °C for 24 cycles. The conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol. We also performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination in each set of reactions. Equal amounts of PCR products were loaded on 1% agarose gels for transcript profile analysis.

2.3. Preparation of protein extracts and western blotting

Protein extracts were prepared by homogenizing 5 g of pulp tissues by following the protocol of Roy Choudhury et al. (2008b). Immuno-blotting was carried out using anti-banana SPS polyclonal antibody (1:1000 dilution) as described previously (Roy Choudhury et al., 2008b).

2.4. Measurement of SPS enzyme activity

Fresh fruit pulp tissue (~2 g) was homogenized in a cold mortar and pestle in extraction buffer containing 25 mM Hepes/KOH (pH 7.5), 5 mM $MgSO_4$, 15 mM KCl, 2 mM DTT and 5 mM βME. Equal amounts of protein were utilized for the SPS assay. The reaction was carried out in 50 μL reaction mixture containing ~10 μg protein extract and 100 mM fructose 6-P, 100 mM UDPG, 40 mM Hepes/KOH (pH 7.5) and 10 mM $MgSO_4$. Reaction mixtures were incubated at 25 °C for 15 min and then terminated by mixing with 50 μL of 30% KOH. After cooling the tubes, 1 mL anthrone reagent was added and the reaction mix was incubated at 40 °C for 20 min. SPS activity was then measured spectrophotometrically at 620 nm (Vassey, 1989). Five replicates were assayed for each sample for each cultivar.

2.5. Estimation of starch and sucrose content

Starch content in the sample was measured spectrophotometrically at 630 nm by the anthrone reagent according to Hodge and Hofreiter (1962). Quantitative estimation of sucrose was performed by using 30% aqueous KOH and anthrone solution (Van Handel, 1968).

2.6. Cloning of SPS promoter region

Total DNA from young banana leaves was isolated by the method of Henry (1997) with some modifications. The promoter region of *SPS* from Cavendish was isolated by genome walking as described previously (Roy Choudhury et al., 2008c). For isolation of promoter fragments from Kanthali and Monthan, genomic PCR was performed with the promoter-specific primers, GF1: ATGCAAGCTTATCTGACGCTTAACGTACGC and GF2: ATGCGGATCC-CGCCATGATCGAACTGACAAGC. The PCR fragments were cloned, sequenced and aligned to compare the promoter regions among the cultivars. The conserved regulatory elements within the promoter of different cultivars were identified by PLACE database (Higo et al., 1999) and PlantCARE database (Lescot et al., 2002).

2.7. Gel mobility shift assay and South-western blot analysis

Nuclear protein extracts were isolated from banana pulp of different cultivars as described previously (Roy Choudhury et al., 2008a). The 28 bp synthetic dephosphorylated oligonucleotides containing a dimeric reverse GCC-box (5′-AAC**ACCGCCG**-GAGGAAC**ACCGCCG**GAGG-3′) *cis*-element and a mutated version of dimeric reverse GCC-box (mGCC box: 5′-AAC**ACAGCC**-***A***GAGGAAC**ACAGCCA**GAGG-3′) were 5′ end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase and used as the probe. The gel mobility shift assay and South-western blot were performed by following the protocol as described previously (Roy Choudhury et al., 2008a).