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A Sucrose Synthase Gene from Broccoli: cDNA Cloning, Sequencing and its Expression During Storage

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Abstract: The rapid senescence of broccoli head after harvest at ambient temperatures is accompanied by rapid drop of sucrose from florets. To understand such biochemical changes, we isolated a cDNA for Sucrose Synthase (SS) from broccoli branchlets. The cDNA clone, BoSS (*B. oleracea* sucrose synthase) (AB212623) encodes an mRNA of 608 bp. The deduced amino acid sequence is highly homologous to SS from *Arabidopsis*, cotton, mungbean and pea. Transcripts of BoSS were present in both branchlets and florets throughout the storage period. The SS enzyme activity of both portions gradually increased throughout the storage duration except for a transient decrease on the 48 h of storage. However, transcripts were only consistent with enzyme activity up to 24 h of storage. The initial increase in enzyme activity and transcripts level after 24 h of storage caused about 50% decline of sucrose content.

Key words: Broccoli, florets, gene expression, storage, sucrose synthase

INTRODUCTION

Sucrose synthase (sucrose-uridine-diphosphate-glucosyltransferase; EC 2.4.1.13), is an important enzyme in carbohydrate metabolism of plants that catalyzes the reversible conversion of sucrose in the presence of uridine-diphosphate into uridine-diphospho-glucose and fructose. It is found in all plant tissues with high level in sink tissues[1]. Biochemical and physiological studies indicate that sucrose synthase acts only in the breaking down of sucrose *in vivo*[1]. Sucrose synthase genes have been isolated primarily from starch-storing plants, such as maize[2], rice[3], barley[4], potato[5], mungbean[6] and pea[7]. This gene has also been isolated from sucrose or hexose-storing plants such as tomato[8] and sugar beet[9]. Two or three genes encoding sucrose synthase isoforms have been isolated from monocot species such as maize[10], barley[11], wheat[12] and rice[13,14] and dicot species such as *Arabidopsis*[15,16], potato[17], tomato[18] and carrot[19] with contrasting sequence feature and/or expression pattern. The expression of SS genes are cell-specific, developmentally regulated or regulated by tissue carbohydrate status[20,21] and have also been significantly modulated in response to changing sugar supply[22,23].

Broccoli kept at room temperature after harvest senesced rapidly and sucrose level decreased rapidly[24]. Within the first 6 h after harvest, the sucrose content of the florets dropped to less than half the level present at harvest[25]. Sugar level changes are the initial signal in controlling the onset of developmental senescence that has severe effect on the shelf life of green vegetables such as broccoli, lettuce and chinese cabbage[26]. However, external supply of sucrose to the plants was reported to delay senescence symptom and can also increase the longevity of broccoli[24,27].

There are two alternative biochemical pathways for sucrose degradation in plant. One involves hydrolysis by invertase followed by phosphorylation via hexokinase and fructokinase and the other route, which is unique to plants, involves a UDP-dependent cleavage of sucrose that is catalyzed by sucrose synthase.

The function and expression of sucrose synthase have been studied previously in many plants during growth stage[8], fruit development[28], starch synthesis[9], endosperm development[29], seed formation and cell elongation[30] and nodule formation[31]. However, to our knowledge isolation and expression analysis of sucrose synthase gene in broccoli during storage have not yet been studied.

In this study, a sucrose synthase cDNA (designated BoSS, partial clone) was isolated from broccoli branchlets. The patterns of expression of the SS gene and changes of the enzyme activity and sugar content in florets and branchlets during storage at 20°C have also been discussed.

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MATERIALS AND METHODS

Plant material: Broccoli heads were harvested from Agricultural Experiment Station, Miki-Branch, Kagawa, Japan in March 2005. Right after harvest the heads were brought to the laboratory and stored at 20°C in perforated plastic bag for 5 days. At intervals of 24 h storage, the florets and branchlets were removed separately from broccoli head with a sterile razor blade, frozen immediately in liquid nitrogen and stored at -80°C until needed for assay of sucrose synthase, extraction of total RNA and sugar determination.

Extraction and assay of SS: Approximately 5 g sample from each portion was mixed with 1% of PVPP and 1 g sea sand. The sample was then homogenized with 10 mL of 0.2 M K-P buffer (pH 7.8) containing 10 mM ascorbate, 15 mM $MgCl_2$, 1mM EDTA and 1M DTT using a cooled mortar and pestle. The resulting homogenate was then filtered through four layers of cotton cloth and the filtrate was centrifuged at 11, 000 x *g* for 20 min. The total supernatant was dialyzed with 40 times diluted 0.2 M K-P buffer (pH 7.8) for 16 h and the inner solution was used as the crude enzyme. SS activity was assayed at 37°C by the method described by Hubbard[32] with slight modifications. The enzyme activity was measured as micromole of sucrose or sucrose- phosphate produced per min per milligram protein.

Determination of soluble sugars by HPLC: About 4 g of broccoli sample (for each portion) was mixed with 1 g sea sand and homogenized in a cooled mortar and pestle. Ten mL of distilled water was added to the homogenate and was centrifuged at 11, 000 x *g* for 10 min. The mixture was filtered through a cellulose nitrate membrane filter (0.5 μm pore size). Soluble sugars were analyzed by high performance liquid chromatography (HPLC) using stainless steel column (10.7 mm ID x 30 cm) packed with silica gel (gel pack C 610). The filtered water was pumped through the column at a flow rate of 1.0 mL min^{-1}. The pressure was adjusted to 14-15 kg cm^{-2} and the temperature to 60°C. A RI monitor (Hitachi L-3300) was used. Sucrose, glucose and fructose were identified by their retention times and were quantified according to standards.

RNA isolation: Total RNA was extracted according to the Hot Borate method of Wan and Wilkins[33].

Amplification of poly (A)+ RNA by RT-PCR: The first stand cDNA was synthesized from 5 μg of total RNA by reverse transcriptase with Oligo-(dT) primer according to the instruction of SUPER SCRIPT™ First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed in a total volume of 25 μL containing 1 μL of the first strand cDNA product, 10x PCR buffer, 1.5 mM $MgCl_2$, 200 μm dNTPs, 1 unit Taq DNA polymerase (Promega) and 400 μM primers. The primers 5′-TTGGGATACCCTGACACCGG-3′ as upstream primer and 5′-ACAACACGGTAGAGTCCAGG-3′ as the downstream primer were designed and synthesized on the basis of amino acid domains (LGYPDTG and PGLYRVV, respectively) conserved with various SS genes. The PCR procedure started with 10 min at 95°C and carried out 35 cycles of 30 sec at 95°C, 30 sec at 54°C and 30 sec at 72°C and 10 min at 72°C with ASTEC Program Temperature Control System PC-700. The PCR products were confirmed by agarose gel electrophoresis.

Cloning and Sequencing of cDNA: The amplified cDNA was ligated to the plasmid pT7 Blue (Novagen) according to the instructions of DNA Ligation Kit Ver. 2.1 (TaKaRa Bio Inc, Japan) and cloned into XL1-Blue (Stratagene, La Jolla, CA, USA). The cloned cDNA was sequenced using the Big Dye Terminator Ver. 3.1 Cycle Sequencing Kits (Applied Biosystems) and ABI 3100 Genetic Analyzer (Applied Biosystems) at the Gene Research Centre of Kagawa University, Miki, Kagawa, Japan.

Sequence data analysis: Sequence and homology analysis and phylogenetic tree construction were performed using computer software Bioedit[34].

Preparation of the dioxigenin (DIG)-UTP labeled PCR probe: Dig labeling PCR probe was prepared according to the instruction of PCR DIG labeling Mix (Roche) using cloned SS cDNA as a template.

RNA gel blot analysis: Ten μg of total RNA was subjected to electrophorosis on a 1% agarose (Type 11) gel containing 20x MOPS and 37% formaldehyde. After electrophorosis for 30 min, RNA was visualized with ethidium bromide under UV light to confirm equal loading of RNA in each lane. RNA was transferred to a positively charged nylon membrane Hybond™ N+ (Amersham Biosciences) by capillary action with 20x SSC. The membrane was dried and exposed under UV to fix RNA. The membrane was pre-hybridized at 50°C with 20x SSPE, 100x Denhardt's solution, formamide and 10% SDS for 3 h. Hybridization was performed at 50°C using the gene specific DIG labeled PCR probe for 24 h using the same pre-hybridization buffer. After hybridization, membrane was washed twice with 2x SSPE containing 0.1% SDS for 10 min at room temperature, once with 1x

SSPE containing 0.1% SDS for 15 min at 65°C and once with 0.2x SSPE containing 0.1% SDS for 10 min at 65°C. For anti-DIG antibody conjugation, the membrane was also washed with Buffer A containing maleic acid and Tween 20 at room temperature and was blocked with 10% blocking reagent in maleic acid buffer for 30 min. Subsequently the membrane was incubated with Anti-Digoxygenin-AP, Fab fragments (Roche) in blocking buffer for 30 min. Hybridization signals were detected by color reaction using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-Ntroblue Ttrazolium chloride (NBT) as the substrates. Levels of transcripts were measured using computer software Image J[35].

RESULTS

Isolation and identification of cDNA clone: A cDNA clone, termed BoSS (*B. oleracea* sucrose synthase) (AB212623) was isolated, sequenced. The cloned cDNA consisted of 608 nucleotides (Fig. 1). The cDNA shared high homology with SS genes from other plants (Table 1). The partial cDNA, BoSS had 89, 80, 83% identity at nucleotide level and 92, 88, 87% identity at deduced amino acid level with *Arabidopsis* (NM122090), cotton (U73588) and mungbean (Q01390), respectively.

A phylogenetic tree of plant sucrose synthase was made using deduced amino acid sequences by multi-alignment analysis using Bioedit software (Fig. 2). The amino acid sequence of BoSS (AB 212623) and *Arabidopsis* SS (NM122090) show a high degree of similarity and strongly clustered in a subgroup belonged to dicot.

Soluble sugars content: Sucrose content decreased gradually both from the florets and branchlets throughout the storage period while there was no remarkable change observed in glucose and fructose content except in the branchlets where fructose content decreased until 72 h after harvest (Fig. 3). Among the three sugars, the level of fructose and glucose remained higher than that of sucrose in the florets as well as in the branchlets.

Sucrose synthase activity: Figure 4 shows the SS activity in both florets and branchlets portion of broccoli head. The SS activity of both portions gradually increased throughout the storage duration except for a transient decrease on the 48 h of storage.

Expression of SS gene: Expression of BoSS was investigated by RNA gel blot analysis of total RNA prepared from florets and branchlets of broccoli head. The DIG-labeled 608 bp cDNA was used as a probe for gel blot analysis to determine the level of transcripts for SS. Transcripts of BoSS were detected in both portions throughout the storage period. However, the transcripts were only consistent with enzyme activity up to 24 h of storage. BoSS transcript levels were higher in the florets than in the branchlets (Fig. 5).

Table 1: Percentage of nucleotide and deduced amino acid homology between SS from broccoli and other plants in database

Plant (Accession No.)	Nucleotide	Amino acid
Arabidopsis (NM122090)	89	92
Cotton (U73588)	80	88
Mung bean(Q01390)	83	87
Feba bean (AF315375)	81	87
Alfalfa (AF049487)	83	86
Pea (AJ012080)	82	86
Soybean (AF030231)	80	87
Sugar beet (AY457173)	80	83
Carrot (Y16091)	81	82
Rice-SS (Z15028)	82	81
Potato U24087)	80	81
Bamboo(AF412037)	84	81
Sugarcane (AF263384)	81	80

Broccoli SS (AB212623) is calculated as 100%

DISCUSSION

The most important feature of broccoli postharvest senescence is sepal yellowing due to chlorophyll degradation[36]. Ethylene has an important role in this chlorophyll breakdown[37,38]. In addition, sucrose concentration has been reported as one of the factors controlling chlorophyll breakdown in broccoli[39]. It was further suggested that the sensitivity of plant tissue to ethylene is influenced by the sugar level in harvested broccoli florets, which indicates that the decline in sugars can raise the ethylene sensitivity and accelerate plant senescence after harvest[40]. The decline of sucrose may be caused by its cleavage by either invertase or sucrose synthase. To determine the changes in the activities and gene expression of SS in relation to sucrose content of broccoli during storage, we isolated a cDNA for sucrose synthase from broccoli branchlets. The isolated clone, BoSS had high sequence similarity to *Arabidopsis* (NM122090), cotton (U73588), mungbean (Q1390) and pea (AJ012080) cDNA.

Transcripts of BoSS were present in both branchlets and florets with high expression in the latter. The SS enzyme activity of both portions gradually increased throughout the storage duration except for a transient decrease on the 48 h of storage. However, transcripts were only consistent with enzyme activity up to 24 h of storage. The initial increase in enzyme activity and transcripts of BoSS after 24 h of storage caused about 50% decline sucrose.

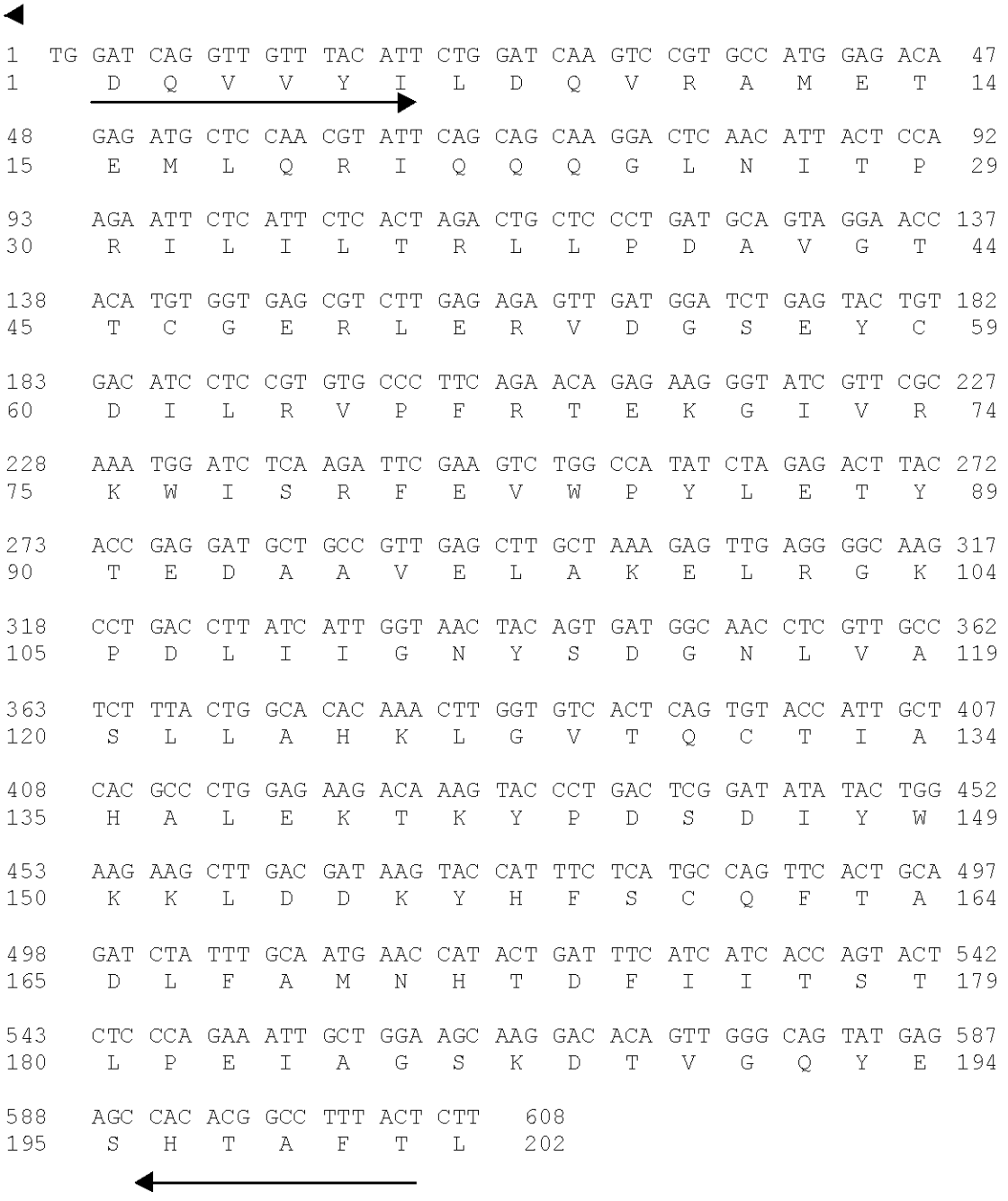

Fig. 1: Nucleotide sequence and deduced amino acid sequence of the cDNA clone corresponding to BoSS. The predicted amino acid sequence is given in single letter code for each amino acid. The arrow indicate the positions of degenerate primers (→sense, antisense←) used for RT-PCR. Numbering refers to total nucleotide (upper) and amino acid (lower) residues on each line

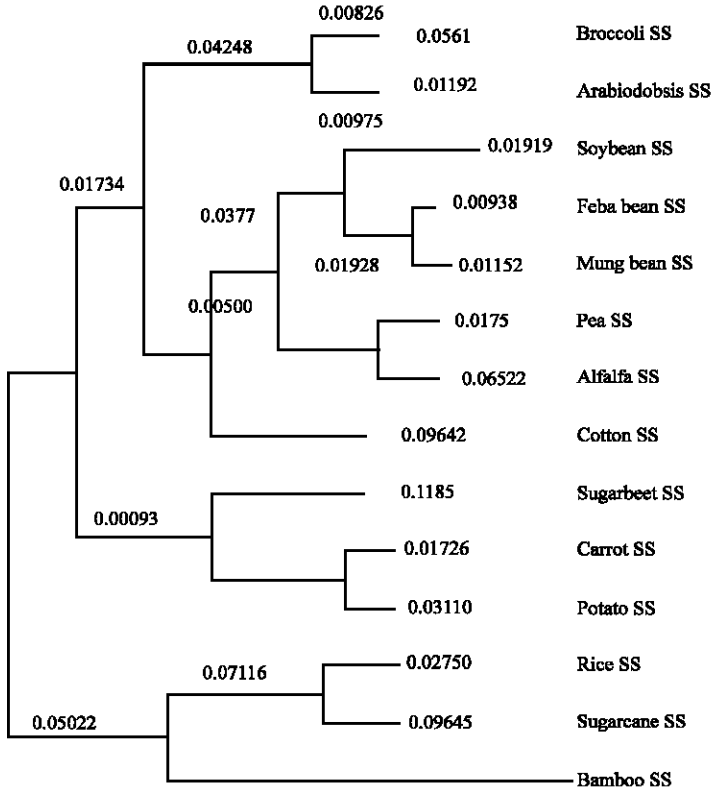

Fig. 2: Phylogenetic tree of the alignment of BoSS deduced amino acid sequence with other SS in the database. Protein sequence were aligned using UPGMA and phylogenetic tree was constructed using Bioedit Software

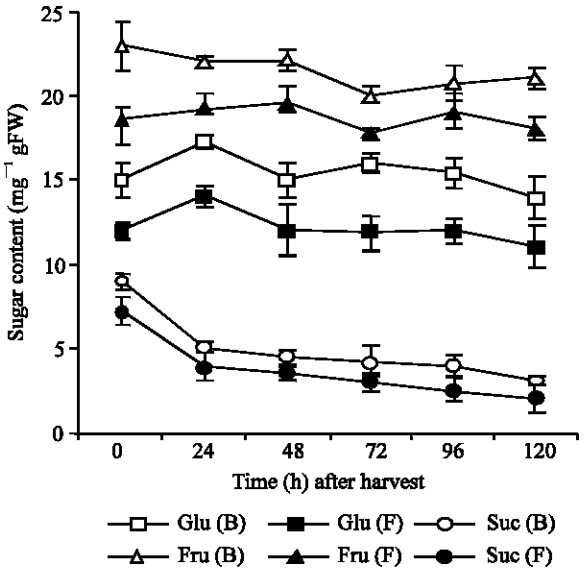

Fig. 3: Changes in sugar content in the florets (F) and branchlets (B) of broccoli stored at 20°C. Each point represents the mean of three replications. Vertical bars indicate SE which, when absent, is concealed by the symbol

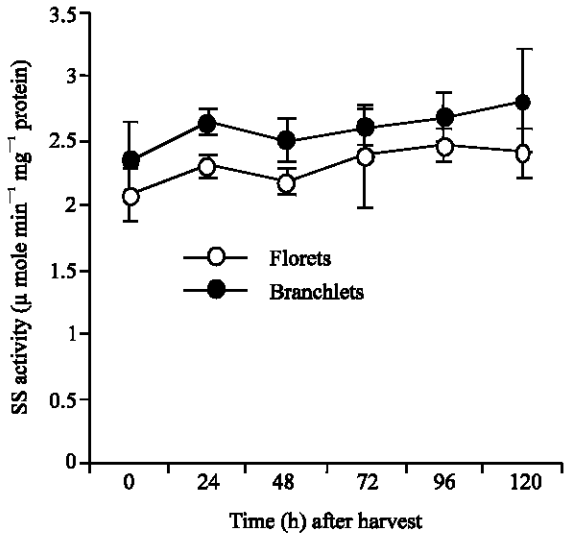

Fig. 4: Changes in sucrose synthase activity in the florets and branchlets of broccoli stored at 20°C. Each point represents the mean of three replications. Vertical bars indicate SE which, when absent, is concealed by the symbols

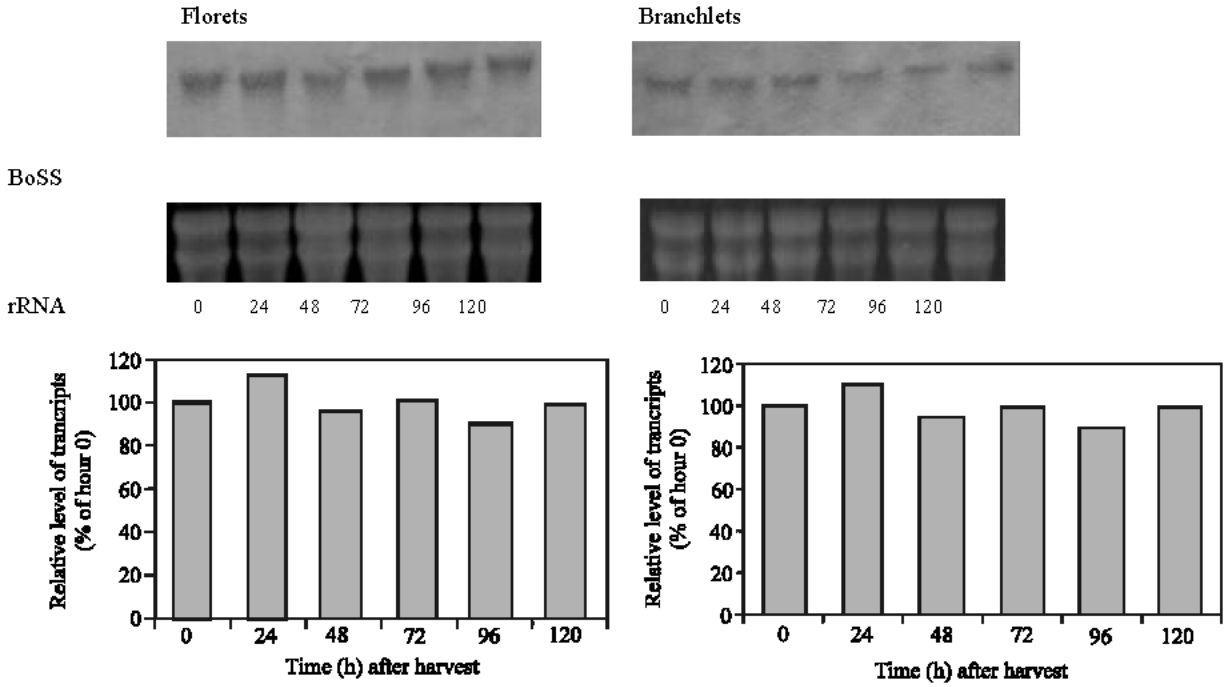

Fig. 5: RNA gel blot analysis of total RNA (each 10 μg) isolated from broccoli florets and branchlets and separated by electrophorosis on 1% agarose, 37% formaldehydegel, including 20x MOPS and was transferred to Hybond N^+ membrane. After blotting on the membrane RNA was hybridized to DIG-labeled BoSS cDNA as probe. The bottom panel is a representative from ethidium bromide stained gels showing rRNA. Relative level of transcripts (%) of each band was measured from color precipitation of NBT and BCIP appeared on the membrane using Image J software

Although, after 48 h of storage enzyme activity increased and sugar level decreased continuously but the level of transcripts were not changed corresponding with the enzyme activity. This inconsistency between SS activity and gene expression might be explained by posttranscriptional regulation or encoding multigene family or sugar level is regulated by other harvest related genes such as asparagine synthetase and β-glactosides[41].

It may be concluded from this study that rapid decline of sucrose from broccoli head after harvest is a consequence of the enzyme activity of sucrose synthase. Regulation of this enzyme activity might have an important aspect to delay the senescence of this perishable product after harvest. In this point of view, this study is a preliminary research for further characterization and regulation of the function of SS gene in broccoli during storage.

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The nucleotide sequence data reported in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB212623.

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