Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

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Asian Journal of Plant Sciences 3 (4): 449-454, 2004
ISSN 1682-3974

Changes in Carbohydrate Content and Activities of Acid Invertase, Sucrose Synthase and Sucrose Phosphate Synthase in Broccoli During Short Term Storage at Low Temperature

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Abstract: This study investigated the changes in carbohydrate content and activities of invertase, sucrose synthase and sucrose phosphate synthase in two broccoli cultivar (*Brassica oleracea* L. Cvs. 'Hartland' and 'Sairin') stored at 5°C. Respiration rate rapidly declined after 1 day of storage and gradually decreased at the end of the storage period. Color did not change significantly at the end of the storage period. In both cultivars and portions, the invertase activity increased gradually at the end of the storage time. The acid invertase in cell wall-bound fraction (CWBF) showed a higher activity than that in soluble fraction (SF). Again, the branchlets showed a significantly higher invertase activity than that of florets. In both cultivars, sucrose synthase (SS) activity gradually increased in the florets with some fluctuations in the branchlets at the end of the storage time. Sucrose phosphate synthase activity (SPS) activity did not change markedly in any portion of the two cultivars at the end of the storage period. Sucrose content gradually decreased in both portions of the two cultivars with time. It was negatively correlated with the acid invertase and SS activities in both portions accounting well for the relation between the substrate and the activity. Fructose and glucose content were higher than sucrose in the florets as well as branchlets in both cultivars.

Key words: Broccoli, cultivar, invertase, storage, sucrose phosphate synthase, sucrose synthase, sugar content

INTRODUCTION

Broccoli (*Brassica oleracea* L.) is highly perishable vegetable with shelf life of only a few days under normal postharvest handling at ambient temperatures[1]. Low temperature is extremely important to achieve adequate shelf-life in the perishable produce. Heads of broccoli stored at 5°C can have a storage life of 14 days; storage life at 10°C is about 5 days[2]. The storage life of vegetables has been demonstrated to be inversely related to their respiration rate during storage[3]. The higher the rate of respiration, the faster the produce deteriorates. Lower temperatures slow respiration rates and the ripening and senescence processes, which prolongs the storage life of fruits and vegetable.

Normal postharvest physiology of broccoli during air storage is characterized by the rapid loss of sucrose from florets[4]. However, application of exogenous sucrose was found to increase the longevity of broccoli[5]. Thus sugar (especially sucrose) affect the shelf life of broccoli. Sucrose is the major sugar transported through the phloem from source organs to sink organs. It is broken down in plants either by sucrose synthase (EC 2.4.1.13) or invertase (EC 3.2.1.26). Depending on the physiological activities, invertase is a hydrolyse, cleaving sucrose into glucose and fructose.

Invertase, sucrose synthase and sucrose phosphate synthase are the important sugar metabolizing enzymes in broccoli. Invertase is considered to play a key role in carbohydrate metabolism and in the regulation of sucrose transport in higher plants[6]. Invertase is responsible for the reduction in sucrose level that accompanies the rapid deterioration of harvested broccoli[7]. In plants three forms of invertase have been reported, the acid invertase, which has optimum activity at about pH 5, is located in either the vacuolar or cytosolic compartments (soluble fraction) and the insoluble extra-cellular forms which are referred to cell wall-bound fraction. The hydrolysis of sucrose by cell wall-bound invertase and subsequent import of hexoses into target cells appears to be crucial for appropriate

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metabolism growth and differentiation in plants[8]. In the previous studies it is found that broccoli quality deteriorated rapidly due to high respiration, high invertase and SS activities during storage at ambient temperature[9]. To further understand how respiration and changes in acid invertase, SS and SPS activities influence the postharvest life of two broccoli cultivar stored at low temperature, hence this study was conducted.

MATERIALS AND METHODS

Plant materials: Two broccoli (*Brassica oleracea* L.) cultivars 'Sairin' and 'Hartland' were harvested from a commercially grown crop and transported to the laboratory. Harvested broccoli heads were stored at 5°C for 5 days. After 24 h intervals, the broccoli heads of each variety were taken out from storage and florets were shaved off a razor blade and placed in the individual bag and immediately stored at -30°C until analysis.

Respiration rate measurement: Each broccoli head was weighed and carefully placed in a 6 L glass jar held at 5°C. Carbon dioxide production was measured on intact head at 24 h intervals. Production of CO_2 was measured by taking 10 ml gas sample from the glass jar sealed for 1 h and injected to a TCD gas chromatograph equipped with a 1 m activated charcoal column at 60°C (GC -8 AIT, Shimadzu Co. Ltd.). The results were expressed as mL CO_2 kg^{-1} h^{-1}.

Color assessment: Color changes in broccoli heads were determined with a chromameter (Minolta CR-200), equipped with an 8mm measuring head. The meter was calibrated using the manufacturer's standard white plate. Color changes were quantified in the L, a, b color space. Hue angle ($h° = \tan^{-1}(b/a)$ when a>0 and b>0 or $h°=180° + \tan^{-1}(b/a)$ when a<0 and b>0) was calculated from the a and b values[10]. On each head, three readings were taken from different portions.

Enzyme extraction for invertase: Approximately 5 g of sample from each portion was mixed with 1% of polyvinylpolypyrrolidone (PVPP) and 1 g sea sand. The sample was then homogenized using a cooled mortar and pestle with 5 ml of 0.2 M citrate-phosphate buffer (C-P buffer) at pH 5.0. The resulting homogenate was then filtered through four layers of cotton cloth and the filtrate was centrifuged at 11 000xg for 10 min. The total supernatant was dialyzed with 0.2M C-P buffer (pH 5.0), diluted 40 times for 12 h and the inner solution was designated as soluble fraction. The residual tissues were re-extracted in 5 ml of 0.2 M NaCl C-P buffer for about 24 h with occasional stirring. The supernatant was dialyzed as described above and dialyzed solution was designated as cell wall-bound fraction. All extraction procedures were carried out at 0-4°C followed immediately by the enzyme assays.

Enzyme assays for invertase: The standard assay medium for acid invertase consisted of 0.2 M C-P buffer (pH 5.0), 0.1 ml of 0.5 M sucrose, 0.1 ml of water and 0.1 ml of crude enzyme solution. The blank experiment contained distilled water instead of sucrose. The assay mixture was incubated at 45°C for 15 min. After the reaction mixture was neutralized with 0.1 N NaOH or 0.1 N HCl, a coloring Somogy's copper reagent was added and the mixture was heated for 10 min in boiling water. The amount of reducing sugars was estimated by the method of Somogyi[11]. Soluble protein content was determined by the method of Lowry[12]. Using bovine serum albumin was used as the standard. The enzyme activity was expressed as the amount of glucose produced per min per mg of protein.

Enzyme extraction for SS and SPS: 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 $MgCl_2$, 1 mM EDTA and 1 M DTT using a cooled mortar and pestle. The resulting homogenate was then filtered through four layer of cotton cloth and the filtrate was centrifuged at 11 000x *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. All extraction procedures were carried out at 0-4°C.

Enzyme assays for SS and SPS: SS and SPS activities were assayed at 37°C by the method described by Hubbard[13] with slight modifications. Reaction mixtures (70 µl) contained 50 mM Hepes-NaOH buffer (pH 7.5), 15 mM $MgCl_2$, 25 mM fructose-6-phosphate, 25 mM glucose-6-phosphate and 25 mM UDP-glucose. The mixture was incubated for 30 min at 37°C and the reaction was terminated with the addition of 70 µl of 30% KOH. Enzyme blanks were terminated with KOH at 0 min. Tubes were kept at 100°C for 10 min to destroy any unreacted fructose or fructose-6-phosphate. After cooling, 2 ml of anthrone reagent (150 mg anthrone with 100 ml of 15% H_2SO_4) was added and incubated in a 40°C water bath for 15 min. After cooling, color development was measured at 620 nm. SS was assayed as above but with 25 mM fructose instead of fructose-6-phosphate and in the absence of glucose-6-phosphate. The soluble protein

content was determined by the method of Lowry[12] using bovine serum albumin as the standard. The enzyme activity was measured as micromole of sucrose or sucrose-phosphate produced per min per milligram of protein.

Determination 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. ARI monitor (Hitachi L-3300) was used. Sucrose, glucose and fructose were identified by their retention times and were quantified according to standards.

Statistics: A randomized complete block design was adopted with three replications. The level of significance was calculated from the F-value of ANOVA. The relationship between sugar content and enzyme activities were described by linear correlation analysis.

RESULTS

Weight loss: In both cultivars, weight loss was very high after 2 days of storage. After that, no significant change was observed throughout the experimental period (Fig. 1)

Respiration rate: In both cultivars, respiration rate was initially high and these levels rapidly decreased after 1 day of postharvest storage and slowly decreased at the end of the 5 days of storage (Fig. 2). 'Sairin' heads produced higher rate of CO_2 than 'Hartland'.

Color assessment: In both cultivars, hue angle (used as a measure of degreening) did not differ significantly at the end of the storage period (Fig.3).

Acid invertase activity: In both cultivars and portions, the acid invertase activity (Soluble and cell wall-bound fraction) increased with few fluctuations throughout the storage period (Fig. 4 and 5). 'Sairin' showed a significantly higher activity than 'Hartland'.

Sucrose synthase activity: In both cultivars, sucrose synthase activity gradually increased in the florets

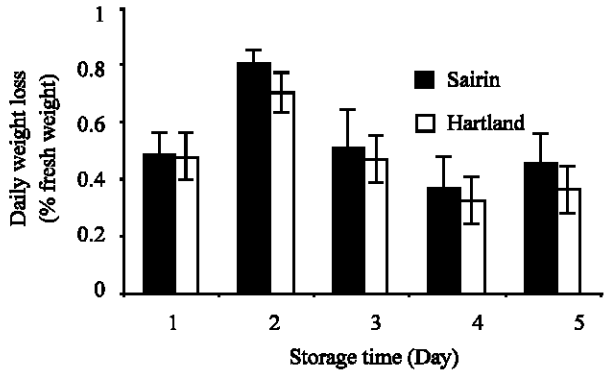

Fig. 1: Weight loss of two broccoli cultivars stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

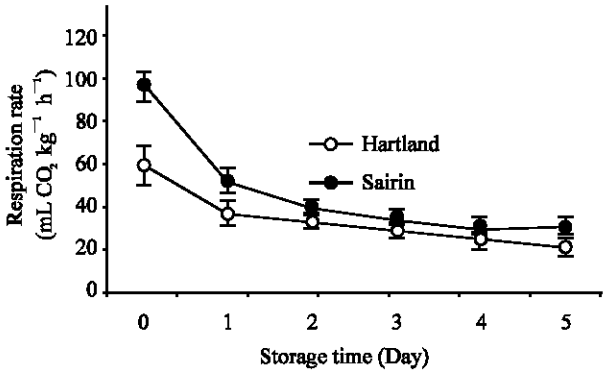

Fig. 2: Changes in respiration rate of two broccoli cultivars stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

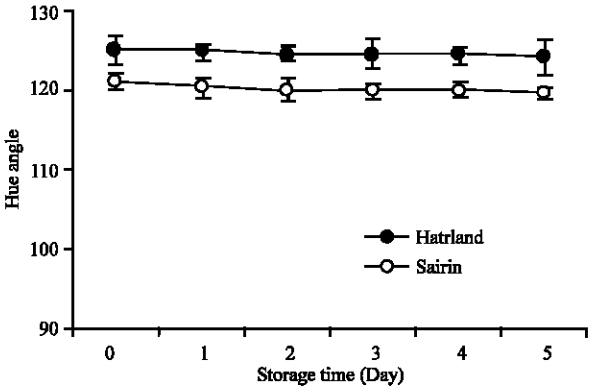

Fig. 3: Color changes of heads of two broccoli cultivars stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

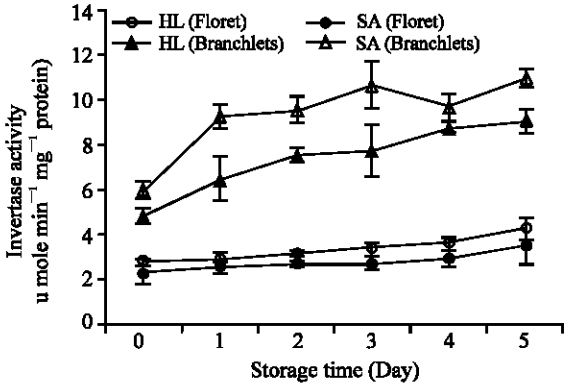

Fig. 4: Changes in acid invertase activity in the soluble fraction in the florets and branchlets of broccoli stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols. HL= Hartland, SA= Sairin

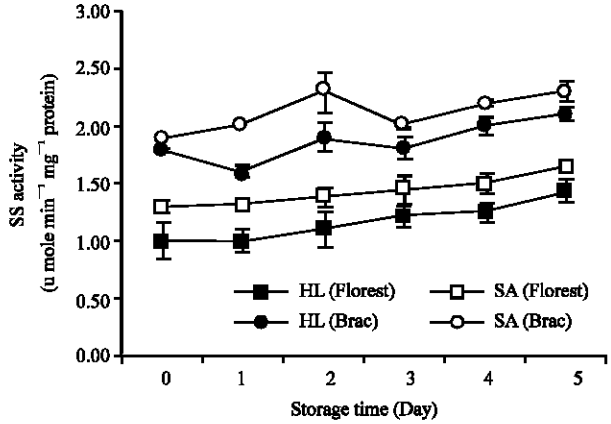

Fig. 6: Changes in sucrose synthase activity in the florets and branchlets of broccoli stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols. HL= Hartland, SA= Sairin

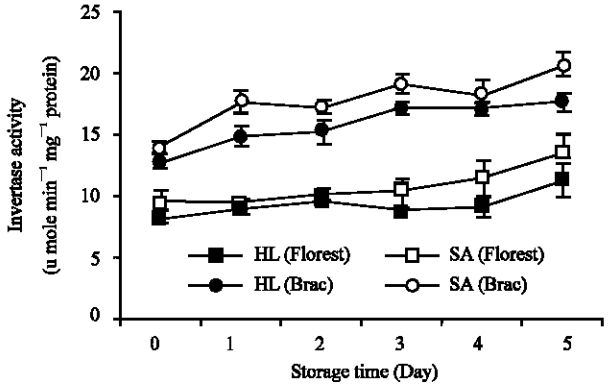

Fig. 5: Changes in acid invertase activity in the cell wall-bound fraction in the florets and branchlets of broccoli stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols. HL= Hartland, SA= Sairin

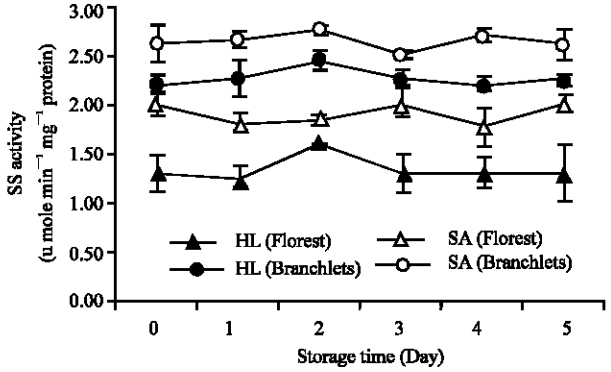

Fig. 7: Changes in sucrose phosphate synthase activity in the florets and branchlets of broccoli stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E which, when absent, is concealed by the graph symbols. HL= Hartland, SA= Sairin

although with some changes in the branchlets at the end of the storage time (Fig. 6). In both cultivars, branchlets showed higher activity than florets.

Sucrose phosphate synthase activity: SPS activity did not change markedly in any portion of the two cultivars at the end of the storage period (Fig. 7). But higher SPS activity was found in the branchlets than florets.

Changes in soluble sugar content: Sucrose content decreased gradually in the florets and branchlets of two cultivars throughout the storage period while there was no remarkable change observed in glucose and fructose (Fig. 8 and 9). A higher amount of soluble sugars (sucrose, glucose and fructose) were observed in the branchlets than florets in both cultivars. Among the three sugars, the level of fructose always remained higher than that of glucose and sucrose in the florets as well as in the branchlets of the two cultivars.

Correlation coefficients (r) between acid invertase activity and sugar content: There was a highly significant negative correlation observed between the acid invertase activity of the soluble and cell wall-bound fraction and

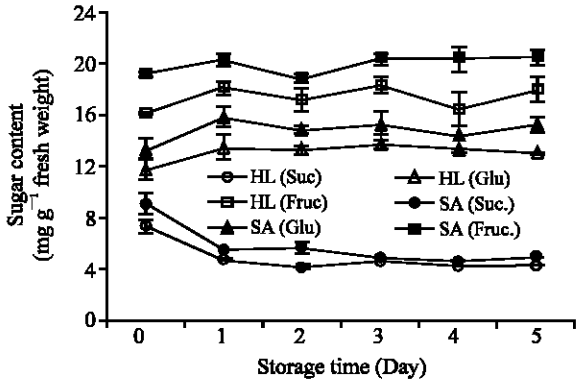

Fig. 8: Changes in soluble sugar contents in the florets of broccoli stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols. HL= Hartland, SA= Sairin

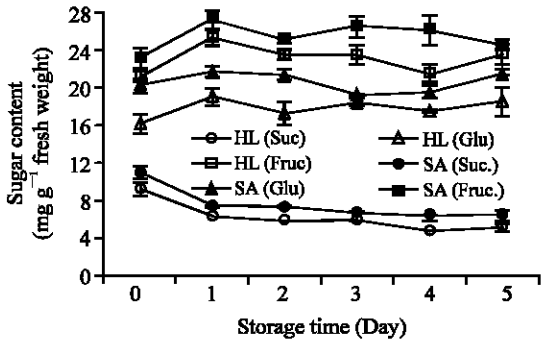

Fig. 9: Changes in soluble sugar contents in the branchlets of broccoli stored at 5°C. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols. HL= Hartland, SA= Sairin

sucrose content in the florets and branchlets of both cultivars. There was also a significant negative correlation found between the SS activity and sucrose content in the florets of both cultivars. No significant correlation was found between the SPS activity and soluble sugars (glucose and fructose) in both portions of each cultivar (Table 1).

Table 1: Correlation coefficients (*r*) between the enzyme activities and sugar content in the florets and branchlets of two broccoli cultivars

			Correlation value (r)			
			Acid invertase			
Sugar content	Cultivars	Portion	SF	CWBF	SS	SPS
Sucrose	Hartland	Florets	-0.688**	-0.557*	-0.596**	-0.263
		Branchlets	-0.963**	-0.963**	-0.537*	0.338
	Sairin	Florets	-0.646**	-0.646*	-0.647**	-0.232
		Branchlets	-0.959**	-0.958**	-0.553*	-0.093
Glucose						
	Hartland	Florets	0.224	0.080	0.163	0.073
		Branchlets	0.369	0.487*	-0.260	0.030
	Sairin	Florets	0.153	0.067	0.141	-0.216
		Branchlets	0.201	0.009	0.263	0.412
Fructose						
	Hartland	Florets	0.257	0.328	-0.184	-0.208
		Branchlets	0.038	0.087	0.484*	0.387
	Sairin	Florets	0.463*	0.433	0.489*	0.001
		Branchlets	0.511*	0.400	-0.090	-0.021

SF = soluble fraction, CWBF = cell wall-bound fraction,
SPS = sucrose phosphate synthase, SS = sucrose synthase
*, ** denote significant at $p<0.05$ and $p<0.01$, respectively, n=18

DISCUSSION

To investigate the effect of low temperature storage on the process of senescence, we stored broccoli at 5°C for 5 days. During for 5 day low temperature storage many physiological and biochemical changes occurred except yellowing of the florets. The changes that were observed include weight loss, respiration, soluble sugar content and invertase, SS and SPS activities. Weight loss was highest after 2 days of storage which is due to moisture loss and loss in reducing substances of the commodity. Respiration rate was initially high for both cultivar. These levels rapidly declined after 1 day of storage and slowly decreased at the end of the storage period (Fig. 2). The high initial respiration rate in both cultivars might be due to the stress imposed by harvest. Hardenberg *et al.*[3] reported that the respiration rate of storage fruit and vegetables in influenced by the storage temperature. From their study it was found that the respiration rate was 40% lower at 13°C than at 24°C, 30% lower at 7°C than 13°C, 45% lower at 3°C than 7°C.

In the present study, acid invertase activity in the soluble fraction was higher in the branchlets than florets and significantly increased until the end of the storage period in both cultivars (Fig. 4). Recent study[7] demonstrated that soluble acid invertase activity steadily increased in broccoli florets and branchlets throughout storage for up to 6 days. 'Sairin' showed the higher invertase activity (SF and CWBF) than 'Hartland'. The higher invertase activity observed in 'Sairin' might be due to the content of anthocyanin, because anthocyanin containing commodity contain higher amount of soluble sugar. Li *et al.*[14] found a positive correlation between anthocyanin and soluble sugar (reducing and non-reducing) contain in the skin of apple. In both cultivars and portions, the SS activity also increased with some fluctuations throughout the storage period. This increased activity may lead to degrade the sucrose level that accompanies the deterioration of harvested broccoli. In many instances, it is assumed that SS is responsible for sucrose degradation[15-17]. Soluble sugars decline substantially during the storage period and concentration was higher in branchlets than florets in both cultivars.

King and Morris[18] reported that soluble sugar concentration (sum of glucose, fructose and sucrose) was lower in floral section than base section (branchlets) in broccoli. Sucrose content change rapidly after 1 day of storage and gradually decreased at the end of the storage period but the ratio of glucose and fructose did not alter markedly in both florets and branchlets during storage period (Fig. 8 and 9). There was a highly significant negative correlation between acid invertase (SF and CWBF) and SS activity and sucrose content in the florets and branchlets in both cultivars (Table 1), suggesting that the decrease of sucrose content was associated with increase in the invertase and SS activities. Such correlation has previously been observed in broccoli florets[9,19] and top portion of asparagus spear [20]. They reported that sucrose is the major sugar which is cleaved by both invertase and SS to the same direction to produce glucose and fructose.

Based on the results obtained, it is found that although the broccoli green color (hue angle) did not change significantly during short term storage at low temperature, some physiological biochemical and enzymatic changes occurred. This result indicate that degreening or yellowing of florets is clearly a relatively late event in the postharvest senescence of broccoli, preceded by major physiological, biochemical and enzymatic changes during storage.

ACKNOWLEDGMENTS

The financial support of the Ministry of Education, Science, Sports and Culture of Japan under scholarship program for foreign students is gratefully acknowledged.

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