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Trehalose Accumulation in Wheat Plant Promotes Sucrose and Starch Biosynthesis

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Abstract

Seeds of *Triticum aestivum* L. (cv. Sakha 93) were sown in pots and grown under controlled conditions in growth chamber. The plants were irrigated with half strength of Hoaglad solution without or with 10 or 30 µM validamycin A, a potent inhibitor of trehalase. Plants were collected at three different stages of growth (17, 24 and 31 DAP). Validamycin A decreased the activity of trehalase which leads to the accumulation of trehalose in shoot and root of wheat plants. Raising trehalose level in the plant tissues was accompanied by increase in the sucrose content and starch content of the shoot. The increased contents in sucrose and starch were mainly attributed to the increased levels of trehalose. The effect of trehalose on the sucrose degrading enzymes (alkaline and acid invertases and sucrose synthase) showed stimulation of alkaline invertase activity and inhibition of acid invertase and sucrose synthase. The opposite behavior of sucrose degrading enzymes suggests a regulation mechanism controlling the sucrose pool.

Keywords: Trehalose, Trehalose -6-phosphate, Carbohydrate, *Triticum aestivum*, Trehalase, Invertase, Sucrose Synthase, Validamycin.

1. Introduction

Trehalose is a non-reducing disaccharide, formed of two α, D glucose molecules linked by an α,α -1,1 glycosidic linkage. Although there are three possible anomers of trehalose: α, α- trehalose; α, β (neotrehalose) and β, β (isotrehalose); yet only the α, α 1-1 configuration has been isolated from and biosynthesized in living organisms (Elbein *et al*., 2003). Trehalose is widespread through the biological world. It is found in a number of different bacteria (Kaasen *et al*., 1994; Shimakata and Minatagawa, 2000) and fungi (Nwaka and Holzer, 1998). Trehalose was also present in many plants, it has been detected in the resurrection plant *Selaginella lepidophylla* (Adams *et al*., 1990), *Glycine max* (Müller *et al*., 1992), *Arabidopsis thaliana* (Müller *et al*., 2001), *Triticum aestivum* (El-Bashiti *et al*., 2005), *Phaseolus vulgaris* (Gracía *et al*., 2005), *Lotus japonicus* and *Medicago truncatula* (Lopéz *et al*., 2006; 2009).

There are five known naturally occurring trehalose biosynthetic pathways. Only one of these, the *OtsA–OtsB* (TPS –TPP) pathway which involves the intermediate trehalose -6- phosphate (T6P). The recent discovery of trehalose pathway in plants and T6P in particular has a powerful function in metabolic regulation. A direct action of T6P as a signal is the redox activation of ADP-glucose pyrophosphorylase (AGPase), the key enzyme of starch synthesis (Kolbe *et al*., 2005). T6P was reported to inhibit SnRK1 which is a protein kinase involved in the regulation of carbohydrate metabolism (Zhan *et al*., 2009). Altering the trehalose pathway exerts numerous effects on metabolism and development. These effects include embryo development (Eastmond *et al*., 2002), starch metabolism (Kolbe *et al*., 2005), sucrose utilization (Schluepmann *et al*., 2003) and tolerance of abiotic stresses (Almeida *et al*., 2005; Karim *et al*., 2007 and Pilon-Smits *et al*., 1998). Over-expression of genes encoding TPS and TPP is reported to be effective for improving abiotic stress tolerance in tobacco (Holmström *et al*., 1996), potato (Yeo *et al*., 2000), tomato (Cortina and Culiăñez-Maciă, 2005), *Arabidopsis* (Miranda *et al*., 2007), rice (Ge *et al*., 2008) and maize (Jiang *et al*., 2010).

The photosynthetic assimilation of atmospheric CO_2 by leaves yields sucrose and starch as end products of two separated pathways namely sucrose in cytosol and starch in chloroplasts. Whereas starch is an insoluble polyglucose formed in the plastids, fructan is soluble polyfructoses synthesized and stored in the vacuole. Wheat, barley and many other grasses from temperate climates store fructan (Taiz and Zeiger, 2006).

In higher plants, two enzymes are able to catalyze sucrose degradation: invertase (E.C. 3.2.1.26) and sucrose synthase (E.C. 2.4.1.13). Invertases are usually divided into acid, neutral or alkaline groups according to the optimum pH requirements and subcellular localization (Schroeven *et al*., 2008; Tao *et al*., 2010).

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Abbreviations :AGPase (ADP-glucose pyrophosphorylase),**T6P** (Trehalose -6-phosphate),**TPS** (Trehalose phosphate synthase),**TPP** (Trehalose phosphate phosphatase),**DAP** (Days after planting),**SnRK1** (Sucrose nonfermenting related protein kinase).

Sucrose synthase activity has been shown to play a major role in energy metabolism, controlling the mobilization of sucrose into various pathways important for the metabolic, structural, and storage functions of the plant cell (Hesse and Willmitzer, 1996; Tang *et al*., 1999).

Trehalase (EC 3.2.1.28) is a glycosyl hydrolase that hydrolyzes trehalose. It is detected in many prokaryotic and eukaryotic cells. It is the only known pathway of utilization of trehalose (Reguera *et al*., 2011). Trehalase has been detected in various organisms such as *Saccharomyces cerevisiae* (Alizadeh and Klionsky, 1996), *Lentinula edodes* (Murata *et al*., 2001) and *Acidobacterium capsulatum* (Inagaki *et al*., 2001). It is also present in soybean (Aeschbacher *et al*., 1999), *Medicago sativa* (Wolska-Mitaszko *et al*., 2005), *Phaseolus vulgaris* (Gracía *et al*., 2005) and *Triticum aestivum* (Kord *et al*., 2012). This enzyme plays an important role in trehalose metabolism, as it is either directly involved in the assimilation of exogenous trehalose, or it controls its level in the cell.

Validamycin A ($C_{20}H_{35}NO_{13}$) is a non-systemic antibiotic with fungicide action. It is produced from fermentation of *Streptomyces hygroscopicus* variety limoneus. It is most effective against soil borne diseases and is used for the control of *Rhizoctonia solani* in rice, potato and other vegetables (Asano *et al*., 1987). It is a specific competitive inhibitor of trehalase. Treatment with validamycin raised trehalose in plant tissue (Müller *et al*., 1995).

The present work is allotted to study the effect of trehalose on the metabolism of carbohydrates in wheat plant grown under different levels of validamycin A.

2. Materials and Methods

2.1. Plant Material

Seeds of *Triticum aestivum* L. (cv. Sakha 93) was purchased from Agriculture Research Center, Giza, Egypt.

2.2. Design of the Experiment

Seeds of wheat were sterilized by immersing in sodium hypochloride (20 %) (v/v) for 20 min, rinsed with sterilized distilled water, and sown in plastic pots (12 cm × 12 cm), containing 500 g sterilized mixture of perlite and vermiculate (1:1 w/w). Eighteen pots were used. The pots were kept in growth chamber at 25-18 °C with 16 h light and 8 h dark photocycle (5000 Lux) at 70 % relative humidity. Seedlings were grown up to 10 days and irrigated with sterilized tap water. The seedlings were thinned to ten seedlings in each pot and irrigated with half strength of Hoagland solution. The pots were grouped into three sets. The first set of pots irrigated with half strength of Hoagland solution and designated as control. The second and the third sets were irrigated with half strength of Hoagland solution containing 10 or 30 µM validamycin A, respectively. Plants were irrigated twice every week keeping the field capacity at 70 %. Plant samples were collected after 17 days of planting (DAP) when two leaves were fully expanded, after 24 DAP when three leaves were fully expanded and after 31 DAP when four leaves were fully expanded.

The harvested plants of three pots of each set were divided into shoots and roots, ground in liquid nitrogen and used for the determination of carbohydrates (trehalose, sucrose, starch and fructan). The remaining three pots of each set were used in the determination of enzymes (trehalase, alkaline invertase, acid invertase and sucrose synthase).

2.3. Determination of Trehalase Activity

Crude extracts of shoots and roots were prepared according to the method of Müller *et al*. (1992). Extracts were prepared by homogenized 0.1 g of frozen tissue in a mortar with 10 mg/g fresh weight PVP and 1 mL of 100 mM cold sodium citrate buffer pH 5.5, containing 1 mM PMSF, 2 mM EDTA. The crude extract was centrifuged for 15 min at 10,000 g, the supernatant was used to determine enzyme activity.

Trehalase activity was measured by estimating the glucose produced by hydrolysis of trehalose with the glucose oxidase-peroxidase kit (Spainreact) as described by Bergmeyer and Bernt (1974). The reaction mixture contained 100 mM trehalose (Sigma Aldrich Co.), 50 mM sodium citrate buffer (pH 5.5) and 0.25 mL crude extract in a final volume of 1.5 mL. After incubation at 55 °C for 30 min, the reaction was stopped by boiling for 3 min and then the reaction mixture was centrifuged at 5000 g for 10 min. For the analysis, 10 µL of the supernatant was mixed with 1 mL of glucose oxidase-peroxidase kit solution, mixed by vortex and then the mixture was incubated at 37 °C for 15 min. The absorbance of the sample was measured at 470 nm. Enzyme and substrate blanks were subtracted. One unit (nkat) of trehalase activity is defined as the amount of enzyme that hydrolyzes nmol trehalose per second at pH 5.5.

The total soluble protein was determined according to Lowry *et al*. (1951). The specific activity of trehalase was expressed as nmol/mg protein/sec.

2.4. Extraction and Determination of Carbohydrates

2.4.1. Extraction and Determination of Trehalose

Trehalose was extracted according to Ferreira *et al*. (1997). 0.1 g of frozen tissue (shoots or roots) was boiled in 2 mL ethanol, evaporated at 60 ºC, the residue was dissolved in 5 mL of 5 mM H_2SO_4, centrifuged at 10,000 g for 10 min and filtered. Filtrate was heated in boiling water bath for 60 min to hydrolyze sucrose in the extract, since the sucrose retention time is the same as that of trehalose. The pH was adjusted to 7.0, the solution was evaporated and the residue was dissolved in distilled water. The content of trehalose was determined by the method described by Cizmarik *et al*. (2004) using HPLC (Hewlett Packard, HP 1090 liquid chromatograph), Hypersil, 100 × 3 mm, 3 µm column. The separation was carried out at 32 °C using a flow rate of 0.8 ml/ min with acetonitrile: H_2O (85: 15) as mobile phase. The elution was detected with a Diode Array Detector (DAD).

2.4.2. Extraction and Determination of Sucrose

Sucrose was extracted by grinding 0.1 g of tissue, mixing with 10 mg PVP, 80% ethanol. The homogenate

has been incubated at 70 °C for 20 min. After centrifugation at 4,000 g for 10 min, the supernatant was separated and the pellet was re-extracted twice with 80 % ethanol at 70 °C for 30 min. The supernatant was pooled and evaporated. The residue was dissolved in 1 mLof deionized H_2O. The content of sucrose was determined using previously mentioned HPLC method (Cizmarik *et al*., 2004).

2.4.3. Extraction and Determination of Starch

Starch was extracted according to the method of Grotelueschen and Smith (1967). The released glucose from hydrolysis of starch was determined in the supernatant by glucose oxidase peroxidase kit according to the method described by Bergmeyer and Bernt (1974).

2.4.4. Extraction and Determination of Fructan

Fructan was extracted by adding 250 µL HCl (0.2 M) to 250 µL of sugar extract, heated at 80 °C for 10 min. Samples were neutralized by adding 250 µL of 0.2 M NaOH and diluted by deionized H_2O (Sprenger *et al*., 1995). The released fructose was determined by Nelson's method (Clark and Switzer, 1977).

2.5. Extraction and Determination of Invertase Activity

One gram of tissue was homogenized in 10 mL of extraction buffer (50 mM phosphate buffer (pH 7.5), 1 mM 2-mercaptoethanol and 5 mM $MnSO_4$. After centrifugation at 20,000g for 10 min, alkaline invertase was measured according to the method described by Morell and Copeland (1984).

Acid invertase was assayed according to Schellenbaum *et al*. (1998). The specific activity of alkaline invertase and acid invertase were expressed as µmole sucrose/ mg protein/min.

2.6. Extraction and Determination of Sucrose Synthase Activity

Plant extract was prepared by homogenizing 0.2 g of tissue in a mortar with 33% PVP and 1.5 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 1mM EDTA and 20 % (v/v) ethylene glycol (López *et al*., 2009). The plant extract was centrifuged at 20,000 g for 10 min. The supernatant was used for determining enzyme activity. Sucrose synthase activity was measured according to Morell and Copeland (1985). Specific activity of sucrose synthase is expressed as µmole sucrose/ mg protein/min.

2.7. Statistical Analysis

The data of the experiment were subjected to statistical analysis of variance, (One way ANOVA) using SPSS 10.0 software. The significant differences were considered at $P < 0.05$ (Snedecor and Cochran 1989).

3. Results and Discussion

As shown in Table (1), treatment with validamycin A (10 or 30 µM) decreased the activity of trehalase in the shoot and root of the wheat cultivar at the different growth stages as compared to the control. This decrease was more pronounced with the higher validamycin A concentration (30 µM). The drop in the specific activity of trehalase was accompanied by accumulation of trehalose in the treated plants; the level of trehalose was higher with the higher validamycin A concentration.

Several researchers have used validamycin A to raise the level of trehalose in plants. Thus, Müller *et al*. (1995) reported that trehalose increased significantly upon validamycin A treatment in nodules of *Glycine max* and *Vigna uniguiculata*. López *et al*. (2006; 2009) reported that validamycin A was able to increase the level of trehalose in nodules of *Lotus japonicas* and *Medicago truncatula*. Trehalose significantly increased in rice seedling by using validamycin A (Garg *et al*., 2007). Validamycin A was used safely to raise the level of trehalose in plant tissues, since it proved to have no effect on growth (Müller *et al*., 2001; Gracia *et al*., 2005; Qaid, 2010). Validamycin A does not affect nitrogen fixation in nodules and does not cause any visible damage or growth reduction in soybean and common bean (Müller *et al*., 1995; Gracia *et al*., 2005). Pellny *et al*. (2004) and Gomez *et al*. (2006) who reported that trehalose pathway and in particular T6P exerts numerous effects on plant growth and development.

It is clear from the present work that the accumulation of trehalose in the wheat plant is accompanied by increase in the level of sucrose in shoot and root (Figure 1). These results are in agreement with those reported by Bae *et al*. (2005) using *Arabidopsis thaliana* seedlings and by Garg *et al*. (2007) using rice seedlings. However, reduction in sucrose content in response of trehalose accumulation was reported by Müller *et al*. (1998) in nodules of soybean; Wingler *et al*. (2000) in shoots of *Arabidopsis thaliana*; Müller *et al*. (2001) in flowers, leaves and stems of *Arabidopsis* and in nodules of *Lotus japonicas* and *Medicago truncatula* (Lopéz *et al*., 2006; 2009).

From the literature, it is clear that trehalose and T6P accumulate in plants treated with the potent trehalase inhibitor "validamycin A". When the cytoplasmic trehalose level increases, its feedback inhibition of trehalose phosphate phosphatase (TPP) activity enhanced the level of T6P (Schluepmann *et al*., 2004; Brohmann, 2006).

Trehalose and its intermediate (T6P), both have indispensable effect on plant growth and development. T6P was reported to inhibit SnRK1 (sucrose nonfermenting related protein kinase). Zhang *et al*. (2009) found that genes normally induced by SnRK1 were repressed by T6P and those normally repressed by SnRK1 were induced strongly supporting a role of T6P as inhibitor of SnRK1 in vivo. The inhibition of SnRK1 by T6P stops the phosphorylation of sucrose -6-phosphate synthase (SPS) and keeps the enzyme SPS in the active form which leads to increase sucrose synthesis (see Diagram 1).

Table 1. Mean values for specific activity of trehalase and the content of trehalose in shoot and root of *Triticum aestivum* (cv.Sakha 93) at 17, 24 and 31 DAP treated with 10 µM and 30 µM validamycin A. Specific activity (S.A): nmol/mg protein / sec. Trehalose content: (mg/g dry weight). Values are means of three replicates. (*) Significantly different from control (P <0.05).

Treatments	Shoot					
	17 DAP		24 DAP		31 DAP	
	S.A	**Trehalose**	**S.A**	**Trehalose**	**S.A**	**Trehalose**
Control	0.186 ±0.007	0.097 ±0.005	0.299 ±0.006	0.238 ±0.002	0.272 ±0.003	0.346 ±0.015
10 µM	0.130 * ±0.003	0.143* ±0.006	0.178* ±0.005	0.887* ±0.030	0.094* ±0.003	1.057* ±0.042
30 µM	0.054* ±0.002	0.487* ±0.009	0.099* ±0.005	1.777* ±0.090	0.062* ±0.002	4.799* ±0.230
	Root					
Treatments	17 DAP		24 DAP		31 DAP	
	S.A	**Trehalose**	**S.A**	**Trehalose**	**S.A**	**Trehalose**
Control	0.366 ±0.006	0.342 ±0.015	0.310 ±0.006	0.431 ±0.025	0.659 ±0.007	0.564 ±0.030
10 µM	0.278* ±0.005	1.242* ±0.028	0.209* ±0.004	1.554 ±0.042	0.313* ±0.008	1.748* ±0.180
30 µM	0.199* ±0.005	2.976* ±0.090	0.162* ±0.004	3.568* ±0.056	0.254* ±0.005	3.628* ±0.190

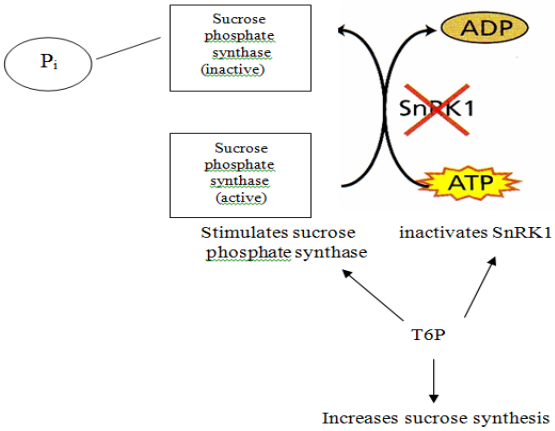

Diagram 1. Effect of T6P on regulation of sucrose synthesis. T6P inhibits SnRK1, thus precludes the phosphorylation of sucrose -6- phosphate synthase thus keeping it in the active form, which means increase in sucrose synthesis. Modified from Taiz and Zeiger (2006).

In the present work, the starch content increased in the shoot of wheat by almost 30% to 50 % over the control in response to trehalose accumulation. However, it decreased in the root of the plant (Figure 1). Our results are in agreement with those reported by other authors. Wingler *et al*. (2000) reported that starch was accumulated in the shoot of *Arabidopsis*, Fritzius *et al*. (2001) found that starch accumulated in shoot of *Arabidopsis* seedlings grown on trehalose. Bae *et al*. (2005) reported that starch was 3-fold greater in the trehalose treated samples than in the control of *A. thaliana* seedlings.

It is interesting to mention here that T6P which acts as a sugar signal is likely generated in the cytosol and possibly transported to the chloroplasts where it could produce trehalose by TPP enzymes in the chloroplasts. A direct action of T6P as a signal is the redox activation of ADP-glucose pyrophosphorylase (AGPase), the key enzyme of starch synthesis (Kolbe *et al.*, 2005; Lunn *et al.*, 2006).

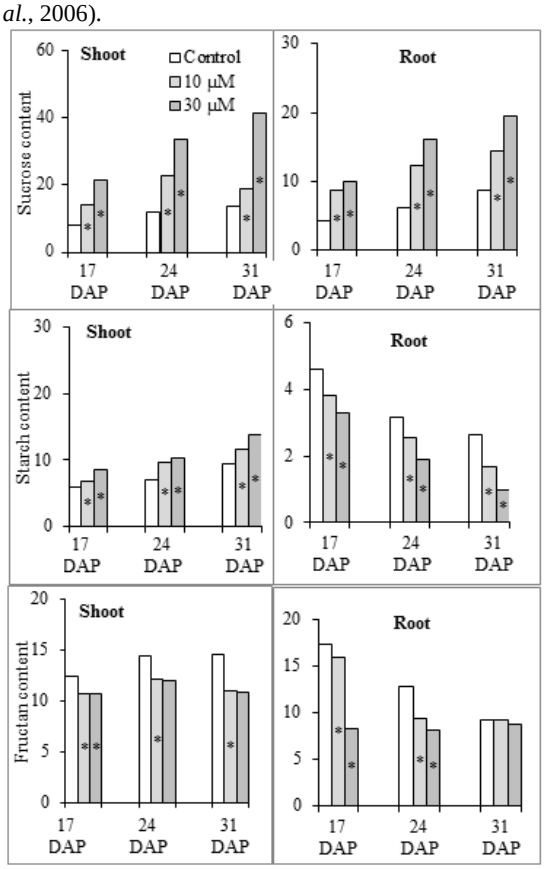

Figure 1. The contents of sucrose, starch and fructan in shoot and root of *Triticum aestivum* (cv.Sakha 93) at 17, 24 and 31 DAP treated with 10 µM and 30 µM validamycin A. (*) Significantly different from control (P <0.05).

Our results show that accumulation of trehalose was accompanied by increases in sucrose and starch contents. The increase was more pronounced with the higher trehalose concentration which was achieved by using the higher validamycin A concentration and also by the increase in plant age.

In the present study, the fructan content did not show remarkable changes in the shoot of wheat cultivar (Figure 1). However, the fructan content of the roots showed significant decreases particularly with the higher concentration of validamycin A (30 µM). As previously mentioned, the accumulation of trehalose raised the sucrose level in the roots, but decreased the starch content. Such decrease is due to the hydrolysis of starch to maintain soluble sugar supply. The same trend happened with the fructan fraction.

In the present work, the analysis of sucrose degrading enzymes showed that the specific activity of alkaline invertase increased in response of increasing the level of trehalose (Figure 2). This takes place in shoot and root of wheat plant using the two concentrations of validamycin and at the three stages of growth. On the other hand decrease in specific activity of acid invertase as well as sucrose synthase were found. It is possible that the activation of some and the inhibition of other sucrose degrading enzymes may have significance in sucrose pool regulation. So that, we can conclude that low amount of trehalose accumulation can alter the soluble carbohydrate pool of the plant.

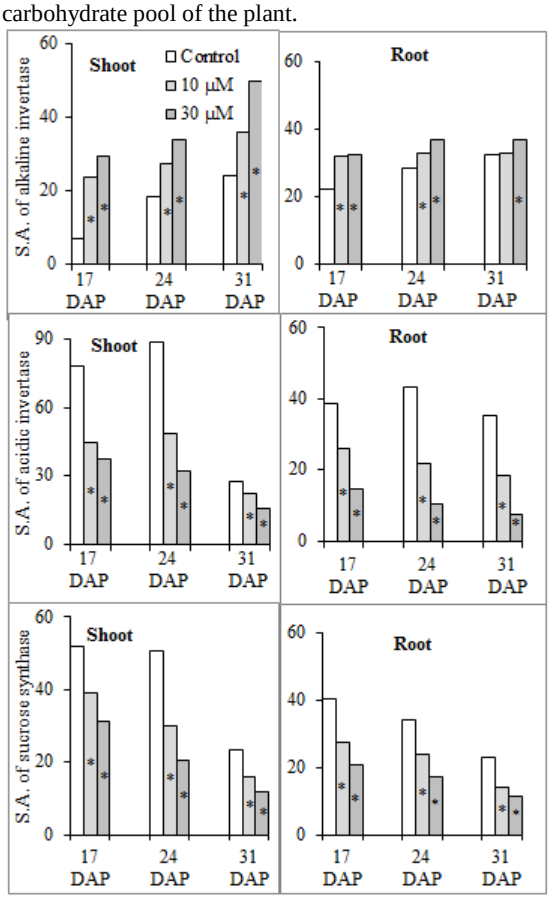

Figure 2. Specific activities of alkaline invertase, acidic invertase and sucrose synthase in shoot and root of *Triticum aestivum* (cv.Sakha 93) at 17, 24 and 31 DAP treated with 10 µM and 30 µM validamycin A. (*) Significantly different from control (P <0.05).

4. Conclusion

This work demonstrates that accumulation of trehalose in wheat plant has considerable potential for raising sucrose and starch contents in shoots. Moreover, the elevation of level of a sucrose degrading enzyme (alkaline inveratse) and decreasing of others (acid invertase and sucrose synthase) suggest that trehalose accumulation plays a role in regulating the sucrose pool.

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