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The Sucrose Starvation Signal Mediates Induction of Autophagy- and Amino Acid Catabolism-Related Genes in Cowpea Seedling

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ABSTRACT

In higher plants, autophagy is bulk degradation process in vacuole necessary for survival under nutrient-limited conditions and plays important roles in senescence, development and pathogenic response, etc. Cowpea is one of the most important legume crops in semi-aride region, which is highly tolerant to drought stress. Changes of photoassimilate status by drought stress and/or sink-source balance appeared to affect autophagy and senescence of leaf in cowpea. Accordingly, we focused on roles of sucrose signal in autophagy and amino acid recycling in cowpea. Effects of starvation stress on the expression of autophagy-related genes (*ATGs*) and amino acid catabolism-related genes in cowpea [*Vigna unguiculata* (L.) Walp] were examined by Reverse transcription-polymerase chain reaction (RT-PCR) and anti-ATG8i specific antibody. Sucrose starvation stress enhanced the expression levels of *VuATG8i*, *VuATG8c* and *VuATG4* in cowpea seedlings. The expressions of amino acid catabolism related genes, such as *asparagine synthase* (*VuASN*1), *proline dehydrogenase*1 (*VuProDH*) and *branched chain amino acid transaminase* (*VuBCAT*2), are also up-regulated under the sucrose starvation. In contrast, high sucrose condition suppressed autophagy and the expressions of *ATGs*. These results indicate that sucrose starvation stress stimulates both autophagy and amino acid catabolism by regulation of *ATGs* and *VuBCAT*2. It is conceivable that sucrose starvation stress enhances autophagy in cowpea, possibly via branched chain amino acid level regulated by the starvation-induced BCAT.

Keywords: Amino Acid Catabolism; ATG; Autophagy; BCAT; Cowpea; Starvation Stress

1. Introduction

Recent studies revealed that higher plants have various pathways of protein degradation systems such as ubiqutin-proteasome, autophagy, and vacuolar processing enzyme cascade [1,2]. Among them, autophagy is non-specific and bulk protein degradation system of intracellular components and is induced under nutrient starvation. Molecular genetic and biochemical studies with autophagy-deficient mutants of budding yeast have unveiled a set of novel genes, autophagy related genes (*ATGs*) [3]. Among *ATG* proteins, *ATG*8 is an ubiquitin-like protein that is first processed by the cysteine protease *ATG*4, removing the carboxyl terminal arginine residue and exposing a glycine residue at the carboxyl terminal end [4]. Through an ubiquitin conjugation-like mechanism, processed *ATG*8 is covalently conjugated to phosphatidyl ethanolamine (PE) on an autophagosome membrane [5]. Molecular genetic studies using mutants of autophagy-related genes (*ATGs*) unveiled that autophagy plays es-

sential roles in growth, senescence, nutrient translocation and stress responses of higher plants [2,3]. In yeast and animal cells, autophagy is negatively regulated by a nutrient sensor kinase, target of rapamycin (TOR), which is stimulated by branched amino acids and by AMP-regulated kinase (AMPK) which is a plant sucrose non-fermenting 1-related kinase (SnRK) homolog involved in sugar sensing [6]. It has been shown that the conserved TOR and SnRK signaling pathways in higher plants play important roles in growth, metabolisms and stress responses including autophagy in response to nutrient and enegy levels [7].

In contrast to budding yeast, which has a single *ATG*8 gene, the *Arabidopsis thaliana* genome encodes nine *ATG*8 (*AtATG*8*a-i*) orthologs [8]. The expressions of *ATG*8*s* and *ATG*4 are enhanced under sucrose starvation in plants, possibly generating intracellular amino acid pools and bioenergentic resource [9]. It is well known that status of nutrient conditions have pleiotropic effects

on stress-related phytohormonal signals [10]. Our previous study indicated that the expression of *GmATG*8*c* and *GmATG*8*i* in soybean at seed maturation stage transiently increased in senescing leaf at pod-filling stage [11]. At the late pod-filling stage, increases in nutrient-up take capacity and synthesis of storage proteins of developing seed possibly exert unknown inter- and intracellular signals to the leaf, leading to acceleration of autophagy and senescence, possibly via nutrient sensing of carbohydrates and nitrogen fluxes from sink organ to source organ. These observations suggest that the autophagy system in plants is regulated in more complex manner than in budding yeast.

Cowpea [*Vigna unguiculata* (L.) Walp] is one of major legume crop widely grown in semi-arid regions of north Africa, which has significant tolerance to drought stress [12]. Previous studies revealed that drought stress on cowpea at the seed filling stages accelerated both leaf senescence and seed maturation by enhancing nitrogen and carbohydrates translocation from senescing leaves to developing seeds [13]. These observations suggest that cowpea has significantly efficient systems of protein degradation in leaves and nutrient translocation to seeds. Resultantly, cowpea cultivation shows little or marginal reduction of the seed yield even under drought stress, compared to well-watered condition [14]. However, regulation system of autophagy and amino acid metabolism in cowpea leaves under drought stress are not yet clarified. As a candidate factor regulating autophagy and senescence, we focused on sucrose signal, which is affected by sink-source balance and reduction of photoassimilate under drought stress. Thus, a set of cowpea *ATG* homologs, *VuATG*8*s* and *VuATG*4, were analyzed to address the relationships of autophagy and amino acid catabolism of cowpea in response to the sucrose starvation condition. Furthermore, it was examined whether sucrose starvation enhanced the expression of *VuBCAT*2 involved in intracellular BCAA levels which possibly regulates autophagy induction via target of rapamycin (TOR) cascade.

2. Materials and Methods

2.1. Plant Materials

Seeds of cowpea (*Vigna unguiculata* (L.) Walp. cv. IT98K-205-8) were sown in the sand filled in plastic box (16 cm side) inside shading incubator set at 25°C. Chemical compound fertilizer, mamekasei (N:P_2O_5:K_2O = 3:10:10) (2 g·box^{-1}) and lime (3 g·box^{-1}) were well mixed with the soil before sowing. After 5 days of seeding, for RNA and protein analysis, each seedling (about 2 g) was dipped in liquid nitrogen for a while and then all were stored at −80°C until analysis.

2.2. Treatments of Sucrose Starvation and Sucrose-Rich Medium

Five-day-old cowpea seedlings were used for analyzing of protein. When the length of the hypocotyls reached between 6 and 8 cm, cotyledons of the seedlings were removed to stop of the supply of nutrients. The seedlings were incubated at 25°C in a nutrient-rich, starvation or starvation-protease inhibitor medium, and harvested at intervals of 0, 12 and 24 hours as described previously [15]. The nutrient-rich medium contained 10 mM MES-KOH, pH 7.4, 3% sucrose, 10 mM KNO_3, 2 mM of $MgSO_4$ and 10 mM KH_2PO_4. The starvation medium contained 10 mM MES-KOH [pH 7.4], 10 mM KCl, 2 mM $MgSO_4$, and 10 mM KH_2PO_4. Additionally, Starvation-protease inhibitor medium was supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor), 10 μg·ml^{-1} leupeptin (a cysteine protease inhibitor), 100 μM E64-d (a membrane permeable cysteine protease inhibitor) (PEPTIDE Inc., Osaka, Japan) and 10 mM quinacrine for alkalization of vacuolar pH.

2.3. Protein Preparation and Immunoblot

To prepare protein samples for immunoblot, 5-day-old seedlings (about 1 g fresh weight) treated in nutrient-rich or starvation medium for 12 or 24 hour were harvested and homogenized on an ice-cooled mortor with liquid nitrogen and mixed with 3 mL of 1xTBScontaining 10 mM EDTA, 5% glycerol, 1% Triton X-100, 0.2% *β*-mercaptoethanol, 1 mM PMSF, 10 μg·mL^{-1} leupeptin, and 1 mM benzamidine. After centrifugation of the crude extracts at 5000 × *g* for 5 min at 4°C, the resulting supernatant was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% acrylamide gel. Protein concentrations were determined with BioRad Protein Assay Kit (BioRad, Hercules, CA) using bovine serum albumin as standard. Immunoblot was carried out as described previously with minor modifications [16]. After SDS-PAGE with 15% acrylamide gels applied with each 50 μg protein per lanes, separated polypeptides were electro-transferred to PVDF membranes (Millipore, Billerica, USA) in 25 mM Tris base containing 0.05% SDS and 20% methanol at 10 V·cm^{-1} for 2 hours. After blocking with 1 × TBS containing 3% skim milk (TBS-milk) for 1 hour, the PVDF membrane was incubated in TBS-milk with anti-*GmAtg*8*i* specific antibody (dilution, 1/1000) and 0.05% Tween20 for 2 hours at 4°C and then in TBS-milk with horse raddish peroxidase-conjugated anti rabbit antibody (dilution, 1/5000 [v/v], GE Healthcare Bio-Sciences Ltd., Piscataway, NJ) for 1 hour (Okuda *et al.*, 2011). Immuno-reactive signals were visualized by ECL Plus Kit (GE Healthcare Bio-Sciences Ltd.) and Fluor-Chem.

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3. Results

3.1. Sucrose Starvation Induces Autophagy and the Expression of *ATGs*

A phylogenic analysis of deduced amino acid sequences of *ATG*8c and *ATG*8i homologs of *Arabidopsis thaliana*, soybean and cowpea showed that *AtATG*8i, *GmATG*8i and *VuATG*8i (tentative consensus number, TC11912) are classified into a higher plant specific clade of *ATG*8 subfamilies while yeast *APG*8, *AtATG*8c, *GmATG*8c and *VuATG*8c (TC3559) are grouped into another clade of *ATG*8 subfamilies (**Figure 1(a)**). The predicted amino acid sequence of *VuATG*8i has similarities of 92% and 69% to those of *GmATG*8i and *AtATG*8i, respectively. The predicted amino acid sequences of *VuATG*8c has similarities of 92% and 87% to those of *GmATG*8c and *AtATG*8c, respectively. VuATG4 (FG847544) was identified to have the highest similarites of 86% and 55% in deduced amino acid sequences to *GmATG*4 (Glyma18-g48380) and *AtATG*4a (At2g44140), respectively.
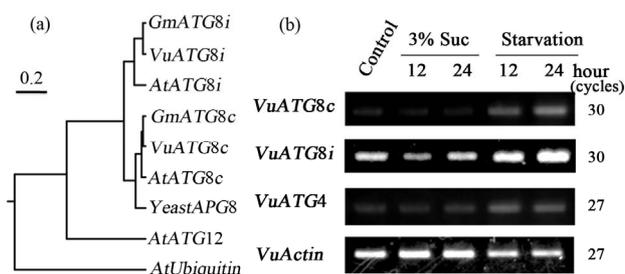
Figure 1. Effects of sucrose starvation on expression of autophagy-related genes. (a) A phylogenic tree of ATG8 homologs and ubiquitin-related proteins from cowpea and other organisms was constructed by ClustalW (UPGMA). The genes used were follows: *VuATG8c, VuATG8i* and *VuATG4* (Table 1); *GmATG8c* (Glyma15g11510), *GmATG8i* (Glyma-02g01180); *AtATG8c* (At1g62040), *AtATG8i* (At3g 15580), *AtATG12* (At1g54210), *AtUbiquitin* (At4g05050); budding yeast *APG8* (AY692870). Tentative consensus (TC) numbers of cowpea genes and gene index numbers of soybean and *Arabidopsis* were referred to TGI database (http://compbio. dfci.harvard.edu/tgi/plant.html) and Phytozome ver 9.0 (http://www.phytozome.net/), respectively. (b) The expression profiles of cowpea *ATG* homologs in response to nutrient conditions are shown. Nutrient treatments and semi-quantitative RT-PCR was carried out at indicated numbers of thermal cycles as described in Materials and Methods. *VuActin* was used as a standard.

It was examined whether the expressions of *VuATG*8c,i and *VuATG*4 are regulated in response to various nutrient conditions. Sucrose starvation treatment on cowpea seedlings significantly enhanced the expression levels of *VuATG*8c and *VuATG*4 at 12 and 24 hour, while 3% sucrose had little or marginal effect on the expression of those genes (**Figure 1(b)**). In all conditions, the expression levels of *VuATG*8i were relatively higher than those of *VuATG*8i and *VuATG*4. In contrast to *VuATG*-8c and *VuATG*4, 3% sucrose treatment transiently suppressed the expression of *VuATG*8i at 12 hour. Sucrose starvation treatment slightly enhanced the expression levels of *VuATG*8. Previous studies with *Arabidopsis* and soybean revealed that the upregulation of *ATG*8s and *ATG*-4 mRNA were accompanied with induction of autophagy in response to sucrose starvation stress [9, 15,17]. The present data that sucrose starvation induced *VuATG*-8c,i and *VuATG*4 is consistent to the previous observation on autophagy induction in Arabidopsis and soybean.

3.2. Induction of Amino Acid Catabolism-Related Genes and a bZIP Transcription Factor

Recently, low energy stresses such as reduced photosynthesis and/or sucrose starvation appeared to induce daramatic change of amino acid catabolism for asparagine

Table 1. Gene specific oligo DNA primers used for RT-PCR and cloning.

Gene name	Accession No.	TC*	Primer sequences
VuATG8c	AB779661	TC3559	F-GTTGGATCCTCACAGATC ATGGCCAAAAGC
			R-ATGCGTCGACTATAGACA ACAGAACTTGCA
VuATG8i	AB779662	TC11912	F-AACGGATCCATGGGAGG AAGTTCAAGTTTC
			R-GAAGGTGCACCAGAACA GAACAATGAGTGA
VuATG4	AB779663	FG847544	F-GGCGGATCCCCGGTTGTC TGCATTGAAGAT
			R-CAATGCCATGGATAAGA GTTGCCAATCATC
VuASN1	AB779664	TC7410	F-TGGGGTTTAGAAGCCAGA GTACCA
			R-CAACTCCTAAGGGAGAA ACCTCCA
VuProDH	AB779665	TC15194	F-TACCGGATCCATGCCAAA GAGAGGTTGCATGCTC
			R-CCAAGTCGACATTAAAA AACAGCAGCTTTTAGCCT
VuBCAT2	AB779666	TC3413	F-GGAGGGATCCGCTCCGCT CAACTTGCACGT
			R-CAATCGTCGACACCCTTG CTTAATCTACTTCA
VubZIP53	AB779667	TC2820	F-TCCCGGATCCTTGTCGGA AATGGCATCGATTC
			R-TAAAGTCGACGGGGATG AAATGGATCAACGCAG
VuActin	AF143208	-	F-GCGTGATCTCACTGATGC CCTTAT
			R-AGCCTTCGCAATCCACAT CTGTTG

*TC: tentative consensus number.

biosynthesis by inducing the expression of *asparagine synthase* (*AtASN*1), *proline dehydrogenase*1 (*AtProDH*), and *branched chain aminoacid transaminase* (*AtBCAT*2) [18,19]. Among cowpea homlog genes amino acid catabolic-related enzymes, *VuASN*1 (TC7410)*, VuProDH* (TC15194) and *VuBCAT*2 (TC3413) were identified to have the highest similarites of 82%, 62% and 62% in deduced amino acid sequences to *AtASN*1 (At3g47340), *AtProDH* (At3g30775) and *AtBCAT*2 (At1g10070), respectively. Heterodimers of *bZIP*1 *and bZIP*53 have been identified to be crucial transcriptional regulators in Asn, Pro and BCAA under sucrose starvation [20]. Among bZIP homolog genes in cowpea ESTs, *VubZIP*53 (TC-2820) was identified to have the highest similarites of 51% and 26% in amino acid sequences to *bZIP*53 (At3g-62420) and *bZIP*1 (At5g49450), respectively.

Sucrose starvation treatment significantly enhanced

the expression of *VuASN*1*and VuBCAT* at 12 and 24 hour, while 3% sucrose treatment had little or marginal effects on that of *VuASN*1 and *VuBCAT* (**Figure 2**). On the other hand, 3% sucrose treatment gradually repressed the expression of *VuProDH* at 12 and 24 hour, while sucrose starvation treatment slightly induced *VuProDH*. Sucrose starvation and 3% sucrose treatmens both had little or marginal effects on the expression of *VubZIP*53, even though *VubZIP*53 is supposed to be a cowpea ortholog of *AtbZIP*53 which is induced under low energy stress [20].

3.3. Effects of Sucrose Starvation on ATG8 Protein Levels in Cowpea Seedlings

To analyze status of autophagy induction in cowpea seedling under various nutrient conditions, we analyzed the profiles of *ATG*8*i*-related protein in response to 3% sucrose and sucrose starvation treatment by anti-*GmATG*8*i* specific antibody [15] (**Figure 3**). The previous studies revealed that sucrose starvation in the presence of leupeptin and E-64d significantly increases *ATG*8*i* protein levels because degradation of autophagosomes composed of *ATG*8 proteins in vacuole is efficiently inhibited by vacuolar proteases, leupeptin and E-64d [15]. Combination of sucrose starvation and protease inhibitors, leupeptin and E-64d, significantly increased the immunoreactive signals of *ATG*8*i* protein at 12 and 24 hour, compared to sucrose starvation in the absence of leupeptin and E-64d (**Figure 3**). The significant difference of *ATG*8*i* levels in sucrose-starved cowpea seedlings between the presence and the absence of protease inhibitors indicates that sucrose starvation stimulated autophagy leading to translocation of *ATG*8-associating autophagosomes to vacuole. 3% sucrose treatment increased immunorective signals of *ATG*8*i*-related protein in cowpea seedling at 12 and 24 hour, while 3% sucrose treatment had little or marginal effects
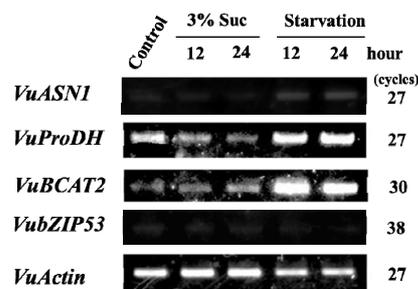
Figure 2. Effects of nutrient conditions on the expression of amino acid catabosim-related genes and *bZIP*. The expression of *VuASN1*, *VuProDH*, *VuBCAT2* and *VubZIP*53 was analyzed. Nutrient treatments and semi-quantitative RT-PCR was carried out at indicated numbers of thermal cycles as described in Materials and Methods. *VuActin* was used as a standard.

on the expression of *VuATG8i* (**Figures 1(b)** and **3**). It is supposed that the apparent increase of *ATG8i* protein levels under 3% sucrose resulted from reduction of degradation rate of *ATG8* proteins by suppression of autophagy.

4. Discussion

In higher plants, autophagy appeared to be induced by various environmental stresses to enhance nutrient capture, amino acid recycle and translocation of carbohydrates and nitrogen [21]. Several *ATG* mutants of *Arabidopsis thaliana* show early senescence phenotypes or high susceptibility to starvation stress [22,23]. It is reasonable that acceleration of autophagy and nitrogen metabolism in response to change of nutrient status play pivotal roles in senescence and nutrient translocation. The present data indicates that sucrose nutrient signals on cowpea seedlings have pleiotropic effects on autophagy and amino acid catabolism. Starvation signals enhanced the expressions of *ATGs* and amino acid catabolism-related genes (**Figures 1(b)** and **2**). Thus, the expression of amino acid catabolism-related genes in response to sucrose starvation signal is regulated possibly by similar mechanisms to the expression of cowpea *ATGs*. In the other hand, sucrose feeding suppressed *ATG8* degradation mediated by autophagy (**Figure 3**). It is possible that sucrose and amino acids play pivotal roles in the nutrient signal cascades regulating autophagy. Recently, It has been reported that low energy stress on *Arabidopsis* plant transcriptionally and translationally activates heterodimer *bZIP* trasnription factors, *bZIP*1 and *bZIP*53, which regulate the expression of *ASN*1, *ProDH* and *BCAT*2, via the promoter regions containing G-box, C-box and ACT motifs [20]. The expression of cowpea *ZIP*53 ortholog, *VubZIP*53, was not induced by sucrose starvation (**Figure 2**). However, the possibilities are not excluded that unidentified *bZIP* type transcription factors forming a heterodimer with *VubZIP*53 is induced by sucrose starvation or that *VubZIP*53 is translationaly activated by

sucrose starvation. Recent studies unveiled that branched amino acid levels regulate autophagy by inactivation of TOR, which phosphorylates a crucial regulator complex, *ATG*1-*ATG*13 at upstream of autophagy induction [6]. It was reported that plant BCAT isozymes with different sub-cellular localization are regulated in complex manner by environmental stresses [24]. BCATs catalyse the final transamination step for synthesis of BCAA in plants and bacteria. The enzymes are also involved in the degradation of BCAA to provide an alternative carbon source under stress conditions in plants, animal and bacteria. Barley *BCATs* and *Arabidopsis BCAT*2 are induced by drought stress and sucrose starvation, respectively [19, 20]. The observation that sucrose starvation enhanced the expression of *VuBCAT*2 prompted us to propose a hypothesis that an increased degradation of BCAA by BCAT results in inactivation of cowpea TOR, in tern, leading to induction of autophagy via *ATG*1-*ATG*13 cascade.

Regarding to drought tolerance of cowpea, drought stress enhances leaf senescence and seed maturation drastically in seed filling stage. Then, drought treatment transiently caused increase in sugar contents in leaves and then significantly enhanced sucrose transport from leaves to seeds [13]. In aspects of photoassimilate and sink-source balance, drought stress on cowpea caused significant reduction of photosynthesis by stomata closure and developing seeds accelerates uptake of carbohydrate from source organs, leading to occurrence of severe low energy stress in leaf tissues mimicking sucrose starvation. Our previous study indicates that increased uptake of photoassimilate by sink organs involved in autophagy induction, nitrogen translocation and leaf senescence of soybean at the pod filling stage [11]. In *Arabidopsis*, ASN1 mediated the biosynthesis of asparagine, which is used to store and transport nitrogen especially under stress conditions where carbon is limited [25]. In *Arabidopsis*, ProDH degrades the compatible osmolyte, proline, which is recycled to support carbon, nitrogen and energy demand under carbohydrate limitation [20]. *ProDH* is up- and down-regulated by hyposmotic condition and dehydration stress, respectively according to the role of proline as osmolyte [18]. Therefore, the expression of *BCAT*2, *ASN*1 and *ProDH* can be assumed to be the indicator of dramatic change of amino acid catabolism for carbon supplies and nitrogen remobilization. Based on the present results of sucrose starvation experiments with cowpea seedling, it is conceivable that drought stress on cowpea at the seed filling stage, promotes asparagine biosynthesis and proline degradation via reduction of photoassimilate. Resultantly, drought stress accelerates nutrient translocation from senescing leaf to developing seed, rather than proline biosynthesis
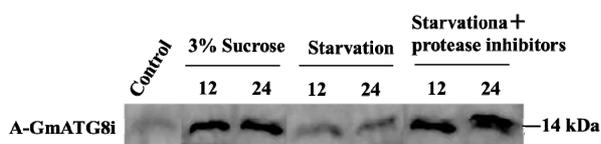
Figure 3. Immunoblot of *ATG8i*-related proteins in cowpea seedlings under various nutrient conditions. Cowpea seedlings were treated in 3% sucrose and sucrose starvation in the absence or the presence of protease inhibitors. Extracted proteins were subjected to SDS-PAGE and immunoblot by anti-*ATG8i* specific antibody as described in Materials and Methods. The immunoreactive signals of *ATG8i*-related polypeptides were detected by ECL at a relative molecular mass with about 14 kDa as indicated.

for maintaining drought tolerance of the leaf. To address mechanisms of rapid senescence of cowpea leaf at the seed filling stage in response to drought stress, we need to analyse *ATGs* and amino acid catabolism-related genes in cowpea plant at the seed filling stage.

The present study reveal that sugar starvation signal on cowpea seedling stimulates both autophagy and amino acid catabolism possibly by transcriptional regulation of *VuATGs*, *VuASN*1, *VuProDH* and *VuBCAT*2. It still remains to be clarified how the sugar starvation signal regulates autophagy and the expression of those genes via *bZIP*53-like transcription factors and/or TOR-ATG1-*ATG*13 cascade. Increasing evidence suggests that the network of various nutrient signals, such as sugars and amino acids, regulate plant development, senescence and primary metabolisms. Therefore, identifications and molecular dissection of the signaling molecules involved in sugar signaling are important for research of autophagy and amino acid catabolism in higher plant.

5. Acknowledgements

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