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# PLANT TRANSCRIPTION FACTORS NETWORKING OF PYRROLINE-5-CARBOXYLATE (P5C) ENZYME UNDER STRESS CONDITION: A REVIEW

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## Abstract

In proline metabolism up regulation of proline synthesis from glutamate and down regulation of proline catabolism are the regulatory steps to control the proline levels. All this steps are regulated by different transcription factors of different genes encoding the key regulatory enzymes P5C of proline metabolism. Expressions of different genes during transcription are regulated by transcription factors (TFs) that, binding to different TF binding sites in the promoter region. The analysis of *cis*-regulatory element (CRE) in a given promoter considered as the functional tool to understand the signal transduction proline metabolic chain under stress. CRE was recognized by TFs. A total of 24 different classes of TFs were detected to have a binding site in the promoter regions.

***Key words:*** Transcriptional factors, proline, osmotic stress,

## Introduction

Accretion of proline is a quite common metabolic process under the circumstances of low water potential in plants specifically in abiotic and biotic stress conditions. It act as osmoprotectant. It protect the cells from reactive oxygen species (ROS) and maintains redox equilibrium in undesirable environment. Under stress condition proline worked as chemical chaperone, which regulates the glutathione (GSH) pool via sustaining the redox potential of $NADPH/NADP^+$ (Fig. 1). Through the ROS signaling pathway, proline showed apoptosis and autophagy in plant species. Parallel it plays an imperative role in fertilization process in plants, predominantly at the flowering stage. It regulates the activation of specific genes, which control the pollen and embryo developmental process (Mattioli *et al*., 2009; Sharma and Verslues, 2010; Zouari *et al*., 2016). Protective mechanism of proline engrosses the stabilization of stress responsive proteins and anti-oxidative enzymatic system (Table 1). In spite all molecular aspects of proline biosynthesis during stress conditions, their accumulation are still the make a big question mark. Emphases are focus on the untouched research area of transcription factors of key regulatory enzymes of proline metabolism. This review article will try to cover the role of different transcriptional factors and their role in the regulatory activity of an important metabolic P5C enzyme of proline biosynthetic pathway.

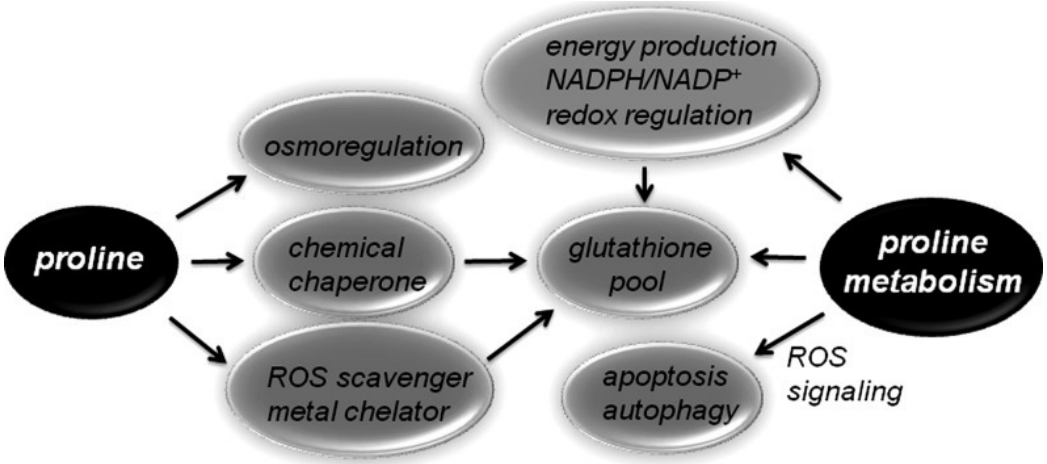


**Fig. 1:** Proline functions in stress protection

**Table 1:** Consequence and outcomes of proline in plants under abiotic stress

| S.No. | Function of proline in stress | Stress conditions | Consequence and outcomes |
|---|---|---|---|
| 1 | Osmolyte function | Abiotic (eg. drought, salt, and temperature etc.) and Biotic | Mitigation of water stress, Balance turgor pressure, Excellent-cryo-protectant (increase the freeze tolerance in yeast and plants) |
| 2 | Chemical chaperone | Oxidative stress | Prevent protein aggregation. Enhanced and stabilized the activity of redox enzymes Increase the activity of antioxidant (eg. superoxide dismutase, catalase and GSH or ascorbate (ASC)-GSH) Mitigate ROS. |

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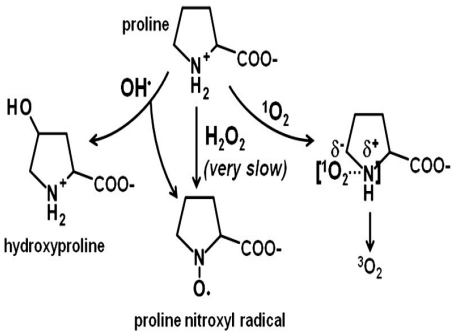
|---|---|---|---|
| | | | Proline is categorized as a weak stabilizer of protein folding and ranks lower in ability to induce protein folding. Hence it stabilize proteins structures by driving burial of the peptide backbone and protein folding. |
| 3 | Metal chelator | Metal stress<br>(Adversely affect the cellular redox balance via the production of free radicals. Hydroxyl radical (OH•) formed by the reduction of $H_2O_2$ by transition metal ions. Such metal ions act as a potent oxidizing agent of biological macromolecules in the cell) | Act as a metal chelator. Proline can protect enzymes from zinc and cadmium-induced inhibition by forming proline-metal complexes. |
| 4 | ROS scavenger | The five-member ring of proline, pyrrolidine, has a low ionization potential that effectively quenches $^1O_2$ most likely through a charge transfer mechanism in which molecular oxygen returns to the ground triplet state ($^3O_2$)<br>  <br>**ROS-scavenging mechanisms of proline** | Free and polypeptide-bound proline can react with $H_2O_2$ and OH• (pH 7–8) to form stable free radical adducts of proline and hydroxyl proline derivatives as (e.g., 4-hydroxyproline and 3-hydroxyproline) |
| 5 | Proline metabolism and ROS signaling | Proline metabolism leads to increased endogenous ROS.<br>ROS (*e.g.*$H_2O_2$): an important physiological signaling molecule that triggers adaptive and survival responses by regulating cell death, proliferation, and apoptosis. | Increases in Proline dehydrogenase (ProDH) and P5CR activities along with down-regulation of P5CDH.<br>Predicted to increase proline-P5C cycling and ROS levels in mitochondria by coupling proline oxidation to reduction of the ETC. |
| 6 | Energy homeostasis and $NADP^+$/NADPH | Changes in proline metabolic flux can also impact stress tolerance.<br>Under different stress conditions, decreased Calvin cycle activity, which is responsible for significant decreases in the $NADP^+$/NADPH.<br>Without sufficient levels of $NADP^+$ available for electron transfer, photosynthetic cells under stress conditions produce more $^1O_2$<br>Light exposure, however, promotes *P5CS* expression, leading to increased proline biosynthesis and $NADP^+$ levels, which ultimately diminishes $^1O_2$ production in the chloroplast.<br>All these observations suggest a link between enhanced proline synthesis and photoinduced oxidative stress | Proline-P5C cycle maintain $NADP^+$/ NADPH levels in the cytosol and drive the oxidative pentose phosphate pathway.<br><br>Increased flux through the oxidative pentose phosphate pathway would support purine nucleotide biosynthesis during stress recovery |
| 7 | GSH pool<br>Glutathione (GSH) is an antioxidant in plants, animals, fungi, and some bacteria and archaea. | GSH preventing cellular damage caused by reactive oxygen species (ROS) such as free radicals, peroxides, lipid peroxides, and heavy metals<br>up-regulation of endogenous proline biosynthesis leads to increased total GSH | Higher GSH levels in the *P5CS* transgenic increases phyto-chelatin synthesis and the formation of metal-thiolate complexes in the vacuole, thereby protecting against heavy metal stress.<br>Gene: *ProDH1* or p53-inducible gene [PIG6]: encodes ProDH.<br><br>Up-regulation of ProDH by p53 increases mitochondrial superoxide ($O_2^{\bullet-}$) production through complex III, leading to cytochrome c release and caspase 9 activation. |

**Enzymes and proteins specificity of in proline metabolism:**

**Enzymatic specificity:** It has been observed that in the plant cells under stress condition, intracellular level of proline found to be rise by 100 folds. In proline metabolism, glutamic acid act as a signaling/ precursor molecule and Δ1-pyrroline-5-carboxylate (P5C) synthetase (P5CS) and P5C reductase (P5CR) are the two key regulatory enzymes of proline metabolic pathways, which maintain the redox balance under the low water potential (osmotic stress) condition. Conversion of proline back to glutamate (proline catabolic pathway) is catalyzed by proline dehydrogenase

(PRODH) and P5C dehydrogenase (P5CDH) (Table 2). Activity of PRODH is regulated by the two isomeric genes PRODH1or ERD5 (Early Responsive to Dehydration) and PRODH2, which were reported in *Arabidopsis.* PRODH1 is the chief dominated form of PRODH pathway and extensively articulated in plants as compare to *PRODH2*. These two forms responded divergent physiological characters in different stress condition. Expression of PRODH1 gene decline sharply in cold temperature stress, drought and salt stress compared to PRODH2. In contrast to PRODH1, expression level of PRODH2 is up four times during salt stress. The P5CDH is the second enzyme of the proline catabolic pathway. It has been noticed that activity of P5CDH is up-regulated by exogenous proline.

**Table 2:** Regulatory functions of core enzymes of proline metabolic pathway in plants under stress

| S. No. | Core enzymes | Features | Existing Isomeric forms | Regulatory functions |
|---|---|---|---|---|
| 1. | Δ1 - Pyrroline – 5- Carboxylate Synthetase (P5CS) or P5C synthase | ❖Bi-functional enzyme in higher eukaryotes: Adenosine tri-phosphate (ATP) and an NADPH dependent<br>❖Mono functional enzymes in bacteria and yeast<br>❖Shows glutamate kinase (GK) and c-glutamyl phosphate reductase (GPR) activities.<br>❖In primitive organisms such as bacteria and yeast, GK and GPR are mono functional enzymes | In plants two isoforms of *P5CS exited:*<br>***a) P5CS1*:**<br>➢ Localized in the chloroplasts.<br>➢ Required for stress-induced proline accumulation.<br>➢ *P5CS1*expression is up-regulated in response to drought and salt stress.<br>➢ *P5CS1* is expressed most highly in shoot tissue but not in dividing cells (Strizhov *et al*., 1997; Yoshiba *et al*., 1999)<br>***b) P5CS2:***<br>➢ localized in the cytosol<br>➢ Essential for embryo and seedling development<br>➢ *P5CS2* is expressed more abundantly in actively dividing callus and cell suspension culture (Strizhov *et al*., 1997)<br>➢ *P5CS2* can be induced by biotic stress/ pathogen response (Fabro *et al*., 2004) and may be more actively involved in plant-pathogen interaction<br>➢ *P5CS2* has little or no transcriptional up-regulation under abiotic stress (Strizhov *et al*., 1997; Zhang *et al*., 1995; Szekely *et al*., 2008, Abraham *et al*., 2003)<br><br>These two isomeric forms are encoded by the two genes i.e. Arabidopsis, *At* P5CS1 and *At* P5CS2 (Szekely *et al*., 2008) | ❖Stimulated salt tolerance: proline accumulation raised 10–18-fold<br>❖ Enhanced the chlorophyll content<br>❖ Reduced the lipid per-oxidation level, and promotes the plant for oxidative stress protection.<br>❖Increased ionic homeostasis<br>❖Catalyzed NADPH-dependent reduction of glutamate to c-glutamate-semi-aldehyde (GSA), which then spontaneously cyclized into D1- pyrroline-5-carboxlyate (P5C)<br>❖Disruption in *P5CS1* leads to significantly lower proline accumulation in plants during stress, resulting in hypersensitivity to salt stress and high levels of ROS.<br>❖Disruption of *P5CS2* does not significantly impact proline accumulation but impairs development of seedlings and fertile plants. |
| 2. | Δ1 - Pyrroline – 5- Carboxylate Reductase (P5CR) or P5C reductase | ❖Synthesis of proline under stress: cytoplasm and the chloroplast.<br>❖Metabolic reaction of proline synthesis is catalysed mainly by P5CR (Lehmann *et al*., 2010), which localized in cytosol and articulated in chloroplasts.<br>❖ P5CR activity is specific for heat and salts stress.<br>❖ Activity of P5CR in water stress condition is still unclear. | Existing isomeric form of P5CR: Two<br><br>Reported in the leaves of *Spinacea oleracea* (Murahama *et al*., 2001): P5CR1 and P5CR2<br><br>Encoded by two distinct genes P5C reductase 1 and 2 (PYCR1 and PYCR2) respectively<br><br>Both the two forms were identified by anion exchange chromatography. | Rate of proline synthesis and its accumulation increases many folds<br><br>Increases water use efficiency (WUE) and relative water (RWC) content in plants under stress<br><br>Showed their effect on high spikelet fertility |

| | | | | |
|---|---|---|---|---|
| | | ❖ P5CR is encoded by single gene. Transcript levels of P5CR are developmentally regulated. Showed highest expression in root tips, shoot meristem, guard cells, hydathodes, pollen grains, ovule and developing seeds under stress (Szoke *et al*., 1992; Hua *et al*., 1997). | Both are the homopolymers, having 10- 12 subunits with the apparent molecular weight 310kDa.<br><br>They showed good affinity with NADPH as compared to NADP. Activity of P5CR1 and P5CR2 is highly sensitive to free ATP and $Mg^{++}$ ions | |
| 3. | Proline dehydrogenase ((ProDH/ PRODH) and Δ1 - Pyrroline–5-Carboxylate Dehydrogenase/ P5C dehydrogenase (P5CDH) | ProDH also referred to as proline oxidase<br><br>Showed catabolic pathway in proline metabolism.<br><br>Catabolic site of ProDH is mitochondria.<br><br>Activity of ProDH and P5CDH: well conserved in eukaryotes and bacteria (gram positive and negative).<br><br>In eukaryotes localization of ProDH is inner membrane of the mitochondria, whereas, P5CDH restricted only in mitochondrial matrix.<br>In Gram-positive bacteria: ProDH binds peripherally to the cytoplasmic membrane, whereas P5CDH is cytosolic<br><br>In Gram-negative bacteria, ProDH and P5CDH are united into a single protein known as proline utilization A (PutA) | *P5CDH encoded by* single copy of gene<br><br>Encoded protein of P5CDH is tetrameric (60kD subunits), restricted in mitochondria (Deuschle *et al*., 2001). | ProDH: catalyzes oxidation of proline into P5C<br><br>P5CDH: catalyzes the intermediate P5C to glutamate<br><br>Expression of P5CDH: in all the tissues except flowers.<br><br>Low water potential: up-regulates the expression of P5CDH (Sharma and Verslues, 2010)<br><br>SRO5 (a mitochondrial protein, potentially important in ROS regulation): down regulated the expression of the P5CDH gene (specifically in salinity stress) |

**Protein specificity:**

Similar to the enzymes, there are certain special kinds of proteins (intra/ intercellular), which are essential for the metabolic pathway of proline under stress conditions but there concrete details and their specific role in metabolic pathway yet to be a big question mark infront of the scientist. The multifarious compartmentation of proline metabolism is little bit clear in the mitochondria, but till date very poor knowledge we have about proline transporter proteins specific to the chloroplast. Recently Szekely *et al*., 2008; Szabados and Savoure, 2010 have submitted some data supporting proline synthesis in the chloroplast of stressed plants. Basic Amino Acid Carrier 1 (BAC1) and BAC2 act as Intracellular Transporter protein in plants under stress. Expression of *BAC2* induced 10–15 times under osmotic stress condition. Arabidopsis Proline Transporters (ProTs) act as intercellular transporter protein, localized in plasma memberane. It helps in intercellular movement of proline, GABA and glycine betaine in the plant cell under salt stress. Three isomeric form of ProTs have been identified as ProT1, ProT2 and ProT3 (Rentsch *et al*., 1996; Grallath *et al*., 2005). Expression of all the three *ProT*s are highly tissue specific in plants under stress. *ProT2 showed* peak expression in roots specifically in root apex zone (Ueda *et al*., 2008). Similarly *ProT1* expression level elevated many folds stems and flowers (Grallat *et al*., 2005). LHT1 and AAP5 are other intercellular transporter proteins. Again they are highly conserved in in their expression (Hirner *et al*., 2006; Rentsch *et al*., 2007; Svennerstam *et al*., 2008).

**Transcription factors (TFs) and their role in P5C enzyme gene regulation**

Eukaryotic gene expression is regulated in a combinatorial manner by transcription factors (TFs) that, binding to different TF binding sites (TFBS) in the promoter region. The analysis of *cis*-regulatory elements (CREs) in a given promoter may therefore represent a useful tool to

understand the signal transduction chain underlying the response to a particular stress (Table 3). Fichman *et al*. (2015) analyzed 1,000 bp up-stream-translation start site (TSS) of *AtP5CS1*, *AtP5CS2*, *AtP5CR*, and *AtOAT* genes via using a specific database for Arabidopsis gene sequences. In all the cases an impressive number of putative CREs were recognized by different TFs classes. Interestingly, a multiple sequence alignment analysis of the 5′ regulatory region of 48 plant *P5CS1* genes showed a high degree of divergence. A higher homogeneity was found for *P5CS2* genes, and the comparison of *A. thaliana* and *A. lyrata* promoters allowed the identification of several CREs known to be recognized by HD-HOX, AP2/EREBP, MYB, WRKY, and bZIP TFs. Concerning *P5CR*, 27 plant sequences were analyzed but, due to their high diversity, no conserved TFBS were identified. Several unique predicted elements were found in *AtP5CR*, including putative bZIP, HD-HOX, MYB and C2C2(Zn) DOF binding sites (Fichman *et al*., 2015). It has been reported that genes coding for the enzymes of the glutamate pathway are the putative target of many transcription factors.

**Table 3:** List of Transcription factors (TFs) reconcile proline accumulation in plant under stress

| S.No | Super family of TFs | | Key role | | | Stress tolerance |
|---|---|---|---|---|---|---|
| 1 | WRKY | TFs targeting: W-box (TTGACC/T) WRKY of super family | plant defense signaling Abiotic stress response (Banerjee and Roy choudhury, 2015) Regulation of proline metabolism | Wheat TaWRKY10 (Wang *et al*., 2013).<br><br>AtWRKY57 (Jiang *et al*., 2016) | Over expressed in tobacco<br><br>Over expression of OsP5CS1 in Transgenic rice | salt and drought<br><br>hyper-osmotic conditions |
| 2 | CaMTAs<br><br>(Calmodulin binding transcription activators) | In P5CR: two W-box in the promoter of OsP5CR<br><br>DNA cis-element that binds to CaMTA: (G/A/C)CGCG (C/G/T) | Role of Calmodulin (CaM)/ sensor protein:<br><br>Biotic and abiotic stress signaling (Yoo et al., 2005) | A. thaliana: CaM binding protein-AtMYB2 complex | Up regulate of AtP5CS1 genes | enhancing salt tolerance |
| | | Microarray data data analysis of CaMTA1 Under drought, salt, and cold stress | AtP5CS1 and AtP5CS2 genes-expression up-regulated (Pandey *et al*., 2013) | | TFBS for CaMTAs in promoter of rice OsP5CS1 and OsP5CS2 genes | Enhanced stress tolerance in roots, but not in leaves (tissue and organ specific) |
| 3 | MYB factors (largest TF families in plants) | Classified into three subfamilies (Based on the presence of one, two, or three repeats in their DNA-binding domain)<br><br>MYB- related group<br><br>MYBR2R<br><br>MYBR1R2R3 | Various abiotic stresses (salt, drought, cold, and excessive light) | Over-expression of MYB2 | reported in Arabidopsis (Yoo *et al*., 2005); Wheat (Mao *et al*., 2011); Rice (Yang *et al*., 2012) | induced proline accumulation |
| | | | | Over-expression of OsMYB48-1 | reported in rice: shown higher expression levels of both OsP5CS1 and OsP5CS2 | Induced proline accumulation in drought (Xiong *et al*., 2014). |
| 4 | PHR1 and PHL1 | | Regulates Pro metabolism under phosphate starvation (induced Pro accumulation) | Phosphate starvation leads P5CS1 activation in Arabidops | | Specifically in salt or ABA (Lehmann *et al*., 2010) |

**Transcription factors and ABA dependent and independent signal transduction pathways in proline accumulation under stress**

Further studies reveal that abscisic acid (ABA)-dependent and independent signaling pathways play a leading role in osmotic-dependent proline accumulation in plant under abiotic stress conditions. In Arabidopsis, ABA-independent *P5CS1* expression has been shown under cold and osmotic stress, while under the same conditions *P5CR* expression did not correlate to proline content. Studies show that bZIP and bHLH family of TFs play an important role in signal transduction pathway of proline biosynthesis. Recently AtbHLH112 was found which act as a transcriptional activator in regulatory steps of P5C enzyme. In ABA-independent stress-responsive gene induction, Dehydration-responsive elements (DRE), DRE-related motifs such as C-repeats (CRT) and low-temperature responsive elements are considered to be the major CREs. TFs belonging to the ERF/AP2 family able to bind DRE/CRT elements were termed DREB1/CBF and DREB2. In particular, the DREB1-type genes are involved in cold-responsive pathways, whereas DREB2-type genes play a role in osmotic-responsive pathways. Experimental results explain that the over-expression of either DREB1 or DREB2 genes improved plant tolerance to drought, salt and freezing. Another class of plant-specific TFs, namely the NAC (NAM, ATAF, and CUC) proteins, is involved in the ABA independent pathway under stress. NAC proteins are a wide family, with almost 110 members in Arabidopsis and 151 members in rice. In several cases, the over-expression of NAC genes resulted in increased drought/salt tolerance and higher free proline levels. Apetala2/Ethylene Responsive Factors (AP2/ERF) are the additional super-family of TFs characterized by the AP2 DNA binding domain. Based on the number of repeated of AP2/ERF they are again classified into three sub-families as: ERF, AP2, and RAV. Studies showed that the over-expression of ERF members is positively correlated with increased osmotic stress tolerance due to proline accumulation.

**Table 3:** Regulatory genes and their role in ABA signaling pathways in proline accumulation

| S. No. | Signaling pathway in proline accumulation | Regulatory genes | | Tolerance level of Proline accumulation |
|---|---|---|---|---|
| 1. | ABA dependent pathway | Abscisic acid-responsive elements (ABREs), belonging to the G-BOX family (ACGTGG/TC) | GmbZIP110 gene in transgenic Arabidopsis | Increased (Xu *et al*., 2016) |
| | | | Over expression of TabZIP60 in transgenics | Increased (Zhang *et al*., 2015) |
| | | | Over-expression of bHLH proteins | Increased (Liu *et al*., 2014, 2015) |
| 2. | ABA independent pathway | Dehydration-responsive elements (DRE): C-repeats (CRT) and low-temperature responsive elements | DREB1 genes (cold-responsive) | Increased (Lata and Prasad (2011) |
| | | | DREB2 genes (osmotic-responsive) | shown tolerance to drought, salt and freezing |
| | | NAC (NAM, ATAF, and CUC) proteins | Over expression of NAC genes | Increased: Shown tolerance against drought/salt Liu *et al*., 2013 |
| 3. | APETALA2/ethylene responsive factors (AP2/ERF): a node between abiotic and biotic signaling pathway | ERF, AP2, and RAV (three specific families) | Over expression of ERF | Increased: shown tolerance in the direction of osmotic stress (Rong *et al*., 2014; Wang *et al*., 2015) |

## Conclusion

In plants, intracellular proline levels have been found to increase by >100-fold during stress. Glutamate appears to be the main precursor in stress-induced proline accumulation in plants. In the metabolic pathway of proline, the final product i.e. proline, and the intermediate substrate, pyrroline-5-carboxylate (P5C), are the regulatory steps as they maintain redox balance in plants cell under osmotic stress condition (da Rocha *et al*., 2012). The transcriptional up-regulation of proline synthesis from glutamate and down-regulation of proline catabolism during stress are thought to control proline levels. All this steps are regulated by transcription of different genes encoding the key enzymes. (Stines *et al*.,1999). However, as post translational regulation of these enzymes has been little explored. Still there are numbers of proteins which act as intra/ intercellular transporter in the plant cell under stress, are needed for proline metabolism but there details and their specific functions yet to be unidentified and unclear.

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