UNIVERSITI PUTRA MALAYSIA

# *INTROGRESSION OF HEAT SHOCK PROTEIN GENES FOR DEVELOPMENT OF HEAT TOLERANT CHILLI (Capsicum annuum L.) GENOTYPES THROUGH MARKER-ASSISTED BACKCROSSING*

USMAN MAGAJI

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# INTROGRESSION OF HEAT SHOCK PROTEIN GENES FOR DEVELOPMENT OF HEAT TOLERANT CHILLI (*Capsicum annuum* L.) GENOTYPES THROUGH MARKER-ASSISTED BACKCROSSING

**By**

**USMAN MAGAJI**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy**

**April 2018**

# DEDICATION

To my loving parents; Late Alhaji Wa`alamu Magaji, Hajiya Fatima Abubakar Kurfi and Alhaji Aminu Abubakar

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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**Supervisor:** **Professor Mohd Rafii Yusop, PhD**
**Institute:** **Institute of Tropical Agriculture and Food Security**

Domestic production of chilli is insufficient (52% self-sufficiency level) and can hardly meet 70% of local demand due to some factors including the poor performance of local varieties under high temperatures above 42 ºC. The optimum growing temperature for chilli ranged from 20 to 30 ºC. Climate change especially high temperature is projected to negatively impact future agricultural production worldwide. According to reports, Malaysia will lose about 10% of major staples food by 2030 due to climate change, which is projected to rise average temperature by 0.3 ºC to 4.5 ºC and this warmer climate will cause a rise in sea level, and this will reduce crop yield. Development of improved heat-tolerant chilli varieties will contribute to self-sufficiency in chilli in Malaysia. Backcrossing together with simple sequence repeat marker strategy was adopted to improve popular Malaysian Kulai 907 (*Capsicum annuum* L.) for heat tolerance. The use of molecular markers in backcross breeding and selection contributes significantly to overcome the main drawbacks such as increase linkage drag and time consumption, in the conventional breeding approach and to speed up the genome recovery of the recurrent parent. The approach was adopted to introgress heat shock protein gene(s) from AVPP0702, a heat-tolerant variety, into the genetic profile of Kulai 907, a high-yielding chilli but heat sensitive. Introgression of heat shock proteins (Hsps) genes has shown considerable success in improvement of crop plants such as maize and rice against heat stress. However, no study has been reported on the introgression of Hsps genes in chilli for the improvement of chilli heat tolerance. The main objective of this study was to develop heat tolerant variety of chilli with high yielding potential and while the specific objectives were; to introgress Hsp genes from heat tolerant (AVPP0702) to high yielding Kulai 907 variety, to identify polymorphic molecular markers for heat tolerant characteristics and recipient parent genome recoveries (RPG) and to validate the backcross progenies for heat tolerance (Hsp loci). Local Kulai 907 variety was used as the recurrent parent and AVPP0702 was used as the donor parent. The parents were grown on seed trays and parental screening was carried out with 252 simple sequence repeat markers (SSR). DNA of young fresh leaves was extracted using CTAB method. Out of the 252 SSR markers, 27% showed clear polymorphism between heat sensitive and tolerant parent. Sixty-eight markers appeared to be polymorphic and used to estimate the recovery of the recurrent parent in the backcross generations; $BC_1F_1$, $BC_2F_1$, $BC_3F_1$ and $BC_3F_2$. The average RPG of the selected four $BC_1F_1$ plants was 80.75% which were used to produce the $BC_2F_1$ generation. $BC_1$-$P_7$ plant was the best in $BC_1F_1$ generation having the highest recovery 83.40% and positive to Hsp-linked markers (Hsp70-u2 and AGi42). After three successive generations of backcrossing, the average genome recovery (RPG) of the recurrent parent in the selected plants in $BC_3F_1$ population was 95.37% and $BC_3F_2$ population was 97.90%. Hsp gene expression analysis was carried out on $BC_1F_1$, $BC_2F_1$, $BC_3F_1$ and $BC_3F_2$ selected genotypes with high recovery of the recurrent parent. The Hsp genes are found to be up-regulated with more than 10.9-, 18.4-, 8.8- and 22.2-fold increase when exposed to heat treatment. The pattern of Hsp expression in the backcross generations was similar with the donor parent (up-regulated). This confirms the successful introgression of stress responsive gene (Hsp) into Kulai 907 variety. Twelve improved heat-tolerant chilli genotypes, namely; $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_2$, $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_9$, $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_{11}$, $BC_1$-$P_7$-$P_{10}$-$P_3$-$P_5$, $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_{13}$, $BC_1$-$P_7$-$P_{10}$-$P_3$-$P_4$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_7$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_9$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_{14}$, $BC_1$-$P_7$-$P_{10}$-$P_3$-$P_{16}$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_{15}$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_{18}$ were selected from the $BC_3F_2$ population that had homozygous Hsp alleles from AVPP0702 and recurrent genome recovery of Kulai 907 (average RPG 97.9%). Most of the morphological and agronomical traits were recovered in the selected improved-heat tolerant genotypes from Kulai 907 such as plant height (75.94 cm), number of days to 50% flowering (56.5%), number of fruits (91.6), stem length (22.3 cm), stem diameter (6 cm), fruit length (13.3 cm) and weight (17.5 cm) and total fruit yield per plant (862.3 g).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**INTROGRESI GEN PROTEIN RENJATAN HABA UNTUK PEMBANGUNAN VARIETI CILI (*Capsicum annuum* L.) KETAHANAN HABA MELALUI KACUKBALIK BANTUAN PENANDA**

Oleh

**USMAN MAGAJI**

**April 2018**

**Penyelia: Profesor Mohd Rafii Yusop, PhD**
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Pengeluaran domestik cili adalah tidak mencukupi (tahap kecukupan 52%) dan ianya sangat sukar untuk memenuhi 70% permintaan tempatan disebabkan beberapa faktor termasuk prestasi varieti tempatan yang rendah di bawah suhu tinggi di atas 42 ºC. Suhu pertumbuhan yang optima untuk cili adalah dari 20 hingga 30 ºC. Perubahan iklim terutama suhu tinggi dijangka memberikan impak negatif terhadap pengeluaran pertanian masa depan di seluruh dunia. Menurut laporan, Malaysia akan kehilangan kira-kira 10% daripada makanan ruji menjelang 2030 ekoran perubahan iklim, yang dijangkakan berlaku peningkatan suhu purata sebanyak 0.3 ºC hingga 4.5 ºC dan iklim panas ini akan menyebabkan kenaikan paras laut, dan ini akan mengurangkan hasil pengeluaran tanaman. Pembangunan varieti maju cili toleran haba akan menyumbang kepada tahap kecukupan cili di Malaysia. Kacukbalik bersama dengan strategi penanda jujukan berulang mudah telah digunakan untuk pembiakbakaan ketahanan terhadap haba ke atas varieti cili popular di Malaysia, varieti Kulai 907 (*Capsicum annuum* L). Penggunaan penanda molekul dalam pembiakbakaan kacukbalik dan pemilihan telah memberi sumbangan untuk mengatasi kekangan utama seperti hambatan pautan dan tempuh masa yang diperlukan dalam kaedah pembiakbakaan konvensional, dan untuk mempercepatkan pemulihan genom induk penerima. Kaedah ini digunakan dengan mengintrogresi gen protein renjatan haba dari AVPP0702, satu varieti yang toleran haba ke dalam profil genetik Kulai 907 yang berhasil tinggi tetapi sensitif kepada haba. Introgresi gen protein renjatan haba (Hsps) telah menunjukkan hasil yang memberasangkan dalam pembiakbakaan tanaman seperti jagung dan padi terhadap tekanan haba. Walau bagaimanapun, tiada kajian yang telah dilaporkan berkaitan penggabungan gen Hsps ke cili untuk penambahbaikan toleransi ketahan haba. Objektif utama kajian ini adalah untuk membangunkan genotip toleran haba serta mempunyai potensi hasil tinggi, dan objektif spesifik adalah; untuk mengintrogresi gen-gen Hsp dari varieti toleran haba (AVPP0702) kepada varieti Kulai 907 yang memberikan penghasilan tinggi, untuk mengenalpasti penanda molekul polimorfik bagi ciri toleran haba dan pemulihan genom induk penerima (RPG), untuk mengesahkan progeni kacukbalik yang toleransi haba (lokus Hsp). Varieti tempatan Kulai 907 telah digunakan sebagai induk penerima dan AVPP0702 sebagai induk penderma. Induk-

induk tersebut telah ditanam dalam bekas percambahan biji benih dan induk tersebut disaring menggunakan 252 penanda berulang jujukan mudah (SSR). DNA dari daun muda yang segar diekstrak menggunakan kaedah CTAB. Dari 252 penanda SSR, 27% telah menunjukkan polimorfik yang jelas antara induk sensitif dan toleran haba. Enam puluh lapan penanda memberikan polimorfik dan telah digunakan untuk menganggarkan pemulihan induk penerima dalam generasi kacukbalik $BC_1F_1$, $BC_2F_1$, $BC_3F_1$ dan $BC_3F_2$. Purata RPG untuk empat pokok $BC_1F_1$ terpilih adalah 80.75% yang telah digunakan untuk menghasilkan generasi $BC_2F_1$. $BC_1$-$P_7$ adalah pokok generasi $BC_1F_1$ terbaik yang mempunyai pemulihan tertinggi iaitu 83.40% dan positif kepada penanda-pautan Hsp (Hsp70-u2 dan AGi42). Selepas tiga generasi kacukbalik, purata RPG bagi pokok terpilih populasi $BC_3F_1$ adalah 95.37% dan $BC_3F_2$ adalah 97.90%. Analisis pengekspresan gen Hsp telah dijalankan ke atas genotip terpilih $BC_1F_1$, $BC_2F_1$, $BC_3F_1$ dan $BC_3F_2$ yang mempunyai pemulihan induk berulang yang tinggi. Gen Hsp didapati menunjukkan regulasi-keatas yang melebihi 10.9-, 18.4-, 8.8-, dan 22.2-ganda peningkatan apabila didedah kepada rawatan haba. Corak ekspresi Hsp dalam generasi-generasi kacukbalik tersebut adalah sama dengan induk penderma (regulasi-keatas). Ini mengesahkan kejayaan introgresi gen ketahanan haba (Hsp) ke varieti cili Kulai 907. Dua belas genotip maju cili toleran haba iaitu $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_2$, $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_9$, $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_{11}$, $BC_1$-$P_7$-$P_{10}$-$P_3$-$P_5$, $BC_1$-$P_7$-$P_{10}$-$P_1$-$P_{13}$, $BC_1$-$P_7$-$P_{10}$-$P_3$-$P_4$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_7$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_9$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_{14}$, $BC_1$-$P_7$-$P_{10}$-$P_3$-$P_{16}$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_{15}$, $BC_1$-$P_7$-$P_{10}$-$P_4$-$P_{18}$ telah dipilih daripada populasi $BC_3F_2$ yang mempunyai alel homozaigus Hsp dari AVPP0702 serta mempunyai pemulihan ciri morfologi dan agronomi cili Kulai 907 (purata RPG 97.9%). Kebanyakan ciri-ciri morfologi dan agronomi telah pulih dalam genotip toleran haba terpilih dari Kulai 907 seperti ketinggian pokok (75.94 cm), bilangan hari hingga 50% berbunga (56.5%), bilangan buah (91.6), panjang batang pokok (22.3 cm), diameter batang (6 cm), panjang (13.3 cm) dan berat buah (17.5 cm), dan jumlah hasil buah per pokok (862.3 g).

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I certify that a Thesis Examination Committee has met on 10 April 2018 to conduct the final examination of Usman Magaji on his thesis entitled "Introgression of Heat Shock Protein Genes for Development of Heat Tolerant Chilli (*Capsicum annuum* L.) Genotypes Through Markerassisted Backcrossing" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| ATP | Adenosine Triphosphate |
| AVRDC | Asian Vegetable Research and Development Centre |
| bp | Base pair |
| cm | Centimeter |
| cDNA | Complementary Deoxyribonucleic Acid |
| CMSI | Cell Membrane Stability Index |
| CVMV | Cucumber Mosaic Virus |
| ºC | Degree centigrade |
| DEPC | Diethylpyrocarbonate |
| EC | Electrical Conductivity |
| et al | et alia |
| gm | Grams |
| h | hours |
| HSC | Heat Shock Cognate |
| HSD | Honest Significant Difference |
| HSE | Heat Shock Element |
| HSF | Heat Shock Factor |
| HSP | Heat Shock Protein |
| kDa | Kilo Dalton |
| Kg | Kilogram |
| µ | micro |
| µS | microSiemans |
| MAB | Marker-assisted backcrossing |
| ml | millilitre |
| Mol | mole |
| MPa | Megapascal |
| % | Percentage |
| PD | Percentage Damage |
| PCR | Polymerase Chain Reaction |
| qRT-PCR | Quantitative Real Time PCR |
| RNA | Ribonucleic Acid |
| SE | Standard Error |
| Sec | Seconds |
| spp | Species |
| $\sigma^2$ | Variance |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Introduction

Chilli (*Capsicum annuum* L.) varieties are among the high-valued important vegetable widely cultivated mostly as a spice crop (Naik *et al*., 2017). *Capsicum* belongs to the family Solanaceae together with other vegetable such as tomato, potato, eggplant, tobacco and petunia. Chilli has multiple uses and functions due to its large variability and great geographical distribution worldwide. It can be consumed fresh, cooked or dried. Its production is seasonal due to lack of appropriate varieties and techniques. Optimum day temperatures for chili pepper growth range from 20-30 ºC (Naik *et al.*, 2017) and day time temperatures rise above 30ºC year round in Malaysia (Sabiha *et al*., 2015). Such conditions are the important factors limiting the production of chili. Though chili is an important spice crop, still Malaysian domestic production cannot meet the 70% demand due to the poor performance of local varieties under high temperatures (DOSM, 2014). According to DOSM (2014), the self-sufficiency level in chilli is 52% and import dependency ratio is 52.9% (45, 000 metric tonnes per year).

Plant growth and development is the product of the interaction between the genotype (genetic potential) and the environment in which the plant grows (Blum, 2018). Plant growth and development depends on biochemical processes (e.g. photosynthesis) that in turn depends on factors in the environment in order to proceed optimally (Blum, 2018). When the environmental condition is less than the optimum requirement (20-30 ºC) chilli plant experiences stress which adversely affects its growth and development and ultimately, its productivity and economic value (Pessarakli, 2016). The common abiotic stresses that plants may be exposed to include; heat, drought, salinity, and mineral toxicity (Zinn *et al*., 2010; Hall, 2011). Among the abiotic stresses, temperature increment (transient 10 – 15 ºC above ambient) due to changing climatic conditions is a serious threat which affects crop production (Jones *et al.*, 1999). Heat stress occurs when temperatures are high enough (above 40 ºC) to cause irreversible damage to plant function (Hall, 2011). Heat stress affects various physiological and metabolic processes in plants such as uptake of water and ions, translocation of solutes, photosynthesis and respiration, and produce inactivation of enzymes, accumulation of unprocessed peptides, and is detrimental in terms of growth and productivity (Larkindale and Vierling, 2008; Frank *et al.*, 2009; Snider *et al*., 2009; Saha *et al*., 2010; Ahmed and Hassan, 2011). A single hot day (above 38 ºC) can be fatal to reproductive success for many plant species including chilli (Kelly *et al.*, 2010). Most physiological processes of plants remain normal at temperatures ranging from approximately 0 to 40 ºC. Well-known responses of plants to extreme temperatures are cold acclimatization and vernalization (Sheldon *et al*., 2000). The molecular mechanisms of these responses have recently been determined through intensive studies (Sharma *et al*., 2005; Kuwabara and Imai, 2009). Under increased temperature (above 30 ºC) flowering asynchrony, decreased pollen fertility and, abscission of flower buds, flowers and fruits are more common in chilli (Barnabas *et al*., 2008; Hedhly *et al*., 2009; Craufurd and Wheeler, 2009; Thakur *et al*., 2010). The commercial vegetable growers are quite aware about the importance of new varieties due to having high yield potential with uniformity in maturity, having tolerance to abiotic and biotic stresses, and better quality as compared to the standard open pollinated varieties (Wilson *et al*., 2012). Suhana *et al* (2014) reported that hybrid chilli 'Ch5' exhibited better performance, with heaviest mean yield per plot (13317 kg) and fruit weight (15.86 g) compared with the MC12 indicating good prospect for commercial cultivation. A hybrid tomato resistant to *Helicoverpa armigera* damage recorded higher yield per plant compared with the local variety (Degri and Sani, 2015).

Stress resistance is an inherent part of all cultivar development programs. Typical response to environmental stress conditions is established by the induction of a set of stress proteins that protects the organism from cellular damage. A basic response of plants to heat stress that is very common is the heat shock proteins (Hsps) expression, which is known as a mechanism in plant tolerance to heat stress (Feder and Hoffman, 1999). Incorporation of heat shock proteins has shown considerable success in improvement of crop plants against heat stress. In breeding heat-tolerant maize, a 45 kDa Hsp was found in $F_2$ population produced from a cross between ZPBL 1304 (tolerant to heat) that produced a 45 kDa Hsp and ZPL 389 line that was not tolerant to heat and does not produce this protein. These $F_2$ plants become more resistant to heat stress (Ristic *et al*., 1998, Ristic *et al*., 1991). In whatever way, screening genetic materials against high temperature depends on field and whole-plant techniques, which as a result of environmental interaction effect their efficiency is less and sensitive (Hall, 2011). Over expression of a rice chloroplast sHsp (Oshsp26) gene showed more tolerance to high temperature and oxidative stresses in *E. coli* (Lee *et al*., 2000). Research have been intensified in determining efficient, suitable and accurate strategies that allow screening large number of genetic materials at the same time so as to breed chilli for heat tolerance in hot and humid areas (Gajanayake *et al*., 2011). However, no work has yet been reported on the incorporation of Hsps in chilli for the improvement of chilli tolerance to heat.

Ancient manual breeding approach contributed substantially to the hereditary change of chilli germplasm in the most recent century (Reddy *et al*., 2014). Ancient manual breeding has additionally been utilized to purposefully grow new heat tolerant genotypes (Driedonks *et al*., 2016). For instance, an assortment of broccoli has an enhanced head quality on account of early development, since this attribute counteracts hot days after the fact in season to influence the warmth touchy blossom start formative stage (Farnham and Bjorkman 2011). In potato breeding a hereditary pick up was gotten after three cycles of intermittent determination for warm resilience prompting solid increment in yield up to 37.8 % (Benites and Pinto, 2011). Albeit regular "yield" breeding has prevailed with regards to creating heat-tolerant lines, a definitive hereditary and physiological base of the changes stay hazy. This keeps the advancement of molecular or different biomarkers, which would help germplasm screening for enhanced heat resistance and take into account productive breeding of the intricate characteristic. Another disadvantage of ancient manual breeding is that the projects are frequently in view of crossing moderately propelled starting material, which has just been utilized as a part of the specific breeding zones particularly identified with the market segment that is focused on. This suggests the potential pick up in heat tolerance level is constrained by the low genetic diversity (Paran and Van

Der Knaap, 2007). Backcross breeding approach can be employed to introduce a specific trait, such as heat tolerance, from one line, often an unimproved line, to another line that is typically an elite breeding line (Hain and Lee, 2005). The backcrossing process can often be accelerated using marker-assisted backcrossing, also known as background selection using molecular markers (Byrne and Richardson, 2005).

Molecular markers are effective in cultivar identification for protecting proprietary rights as well as authenticating plant cultivars. Molecular markers of necessity are applied based on two basic chemical procedures - protein and DNA markers. There are many DNA markers used which include Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSRs), Amplified Fragment Length Polymorphism (AFLP), and Single Nucleotide Polymorphism (SNPs) (Dhaliwal *et al.,* 2013). In general, improved varieties have better yield characteristics and suffer less from different stresses than the traditional ones. Therefore, no research have been reported so far for the incorporation of Hsps in chilli to improve or develop heat tolerant genotypes.

## 1.2 Justification

Malaysian average temperature is projected to increase from 0.3 to 4.5 °C due to greenhouse emission (Alam *et al*., 2011) and the warmer temperature will cause a rise in sea level and thus will reduce crop yield (Alam et al., 2011). With a population of about 31.19 Million, Malaysia is ranked 26$^{th}$ largest greenhouse gas emitter in the world (Alam *et al*., 2011; Hosseini *et al*., 2013) and its likely to increase due to the growth rate of the emission. This will lead to fluctuation in rainfall and ultimately reduce crop yield and increase the risk of drought (Chong and Mathews, 2001). In Malaysia, the estimated annual production of chilli for 2016 was 43,738 metric tonnes (Mt) planted over 4,020 ha cultivated land area (DOA, 2016). While the estimated total chilli consumption is 62,380 Mt (DOSM, 2014), indicating that domestic supplies is insufficient and can hardly meet 70% of the raising demand, thus Malaysia imports approximately 45, 000 metric tonnes per year. Moreover, recently chilli output in Malaysia dropped drastically from 47, 015 Mt in 2015 to 43, 738 Mt in 2016 (DoA, 2016) due to El Nino, leading to the shortage of supply. El Nino is a complex series of climatic changes that occurs irregularly and affects sea surface temperature in most tropics and subtropics. To increase production there is the need to improve/produce varieties having high yielding potential as well as tolerance against high temperature that will be suitable for lowland cultivation. Under this context, it is imperative to identify and incorporate Hsps in chilli for the development of heat tolerant as well as high yielding chilli variety. The experience on the possibility of exploiting the hybrid vigor in chilli has shown considerable promise and as such manoeuvres to control Hsp genes production help in breeding chilli genotypes tolerance to high temperature.

## 1.3 Objectives

**The main objective of this study was**:

To develop heat tolerant variety of chilli with high yielding potential

**The Specific objectives were:**

i. To introgress heat shock protein genes from AVPP0702 variety, donor to Kulai 907, recipient parent
ii. To identify polymorphic molecular markers for chilli heat tolerant characteristics and recipient genome
iii. To validate the backcross progenies for heat tolerance with the polymorphic molecular markers (Hsp loci) and to determine the percentage recovery of Kulai 907 genome in $BC_3F_2$ population.
iv. To determine the homozygous lines that are positive to Hsp genes and phenotypic performance of the $BC_3F_2$ improved heat-tolerant chilli lines similar with Kulai 907.

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