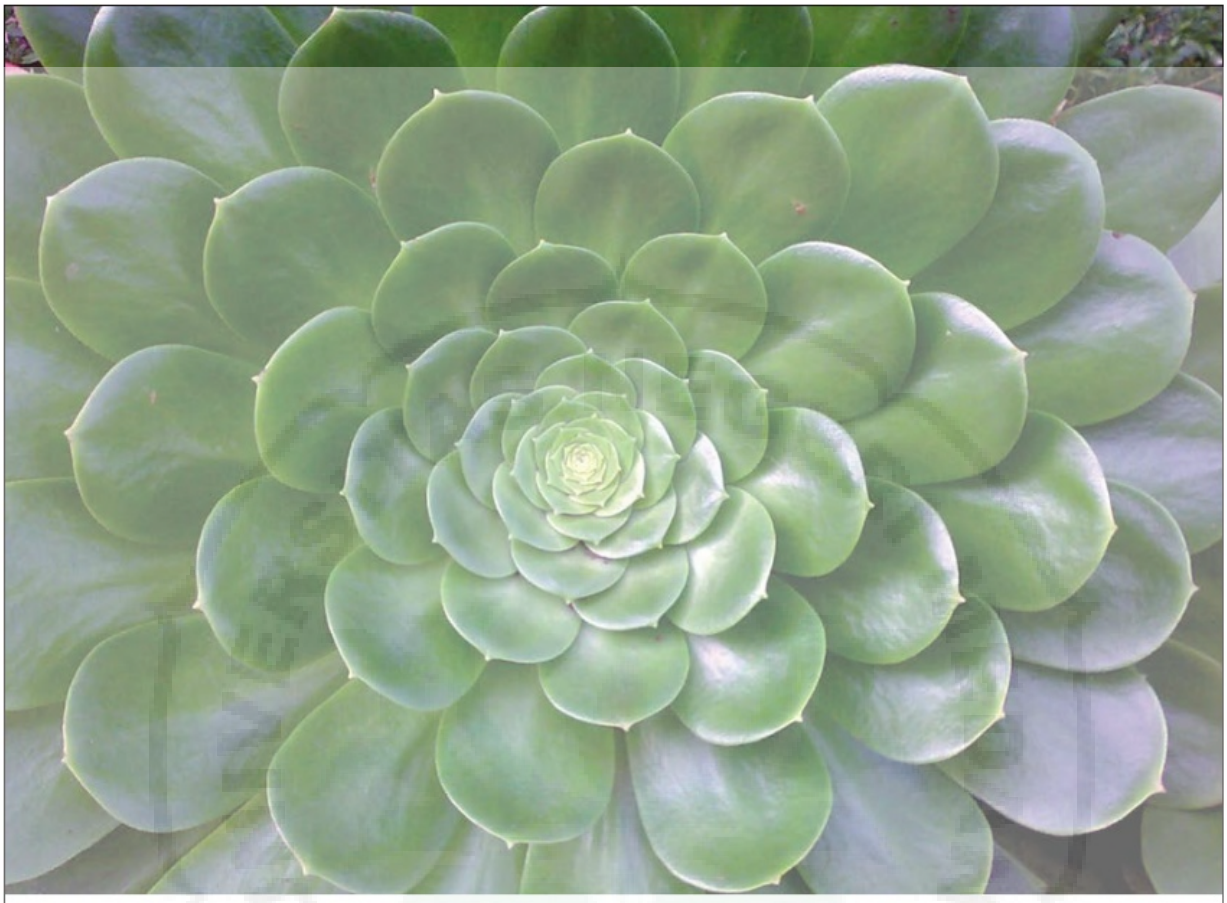
P5CS and HSP 81-2 Gene Expression Profile of Banana (Musa acuminata) in vitro Culture Under Salt Stress Condition

by Diky Setya Diningrat



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Research Article

P5CS and HSP 81-2 Gene Expression Profile of Banana (*Musa acuminata*) *in vitro* Culture Under Salt Stress Condition

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Abstract

Background and Objective: Banana (*Musa acuminata* spp.) is a fruit as a source of staple food of Asia. *Musa acuminata* cv Barangan is a type of banana that live in low-lying areas and most widely consumed by an Indonesian people. Bananas are thought to have resistance to salinity stress by knowing the defense mechanisms against stress is expected banana can be used as an alternative crop for marginal land. Banana (*Musa* spp.) is mesophytic plant that intolerant to high salinity. The presence of proline and Heat Shock Protein (HSP) compounds are an indicator that the plant is under stress conditions. The purpose of this study was to evaluate the banana plant defense mechanisms against the state of high salinity. **Methodology:** In this study will be observed accumulation of proline produced by the activity of banana plantlets after being treated in the form of salinity stress condition. In this study was observed as well, Heat Shock Protein 81-2 (HSP 81-2) and delta-1-pyrroline-5-carboxylate synthase (P5CS1) gene expression profiles of plantlets were treated by salinity stress condition. To achieve the study objectives, *Musa acuminata* Barangan cultivar culturing *in vitro* carried out. Banana shoots were cultured in MS medium with BAP with additional 25, 50, 75 and 100 mM NaCl. Proline analyzed with ninhydrin methods. The RNA was isolated from control (0 mM NaCl) and treated plantlets. The cDNA made from isolated RNA to be used for qRT-PCR analysis. Transcript levels determination was validated and confirmed using quantitative real-time PCR (qRT-PCR). **Results:** The results of this study are as follows, proline accumulated by plantlets treated with NaCl, HSP 81-2 and P5CS1 genes expressed by all plantlets with different levels. The HSP 81-2 highest expressed by shoots and roots of plants with 75 mM NaCl treatment. Likewise, the highest proline accumulation occurred in this treatment. On the whole of the roots and shoots treated by NaCl, HSP 81-2 gene expression is higher than the P5CS1 gene expression. Results from this study may answer the purpose of the study itself. **Conclusion:** *Musa acuminata* cv Barangan plant has defense mechanisms against the state of high salinity. The expected contribution of this study is that *Musa acuminata* cv Barangan can be used as plant resistant to soil with high salt content conditions to resolve the problem of exploitation of critical marginal land.

Key words: Barangan, proline, qRT-PCR, heat shock protein, defense mechanism

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Environmental stresses, such as drought, salinity and extreme temperature are 3 abiotic stress most often found on agricultural land, so that all three types of stress is considered to be the cause of crops/agricultural commodities declining productivity (Xiong and Ishitani, 2006). Plants affected by salinity, drought and extreme temperatures can suffer osmotic stress which leads to turgor pressure loss, cell membrane damage, disruption of protein synthesis and enzyme activity (Hasegawa *et al*., 2000). Among 3 abiotic stresses, salinity is the most important environmental stresses that cause major problems in agricultural production in dry or semi dry tropical areas, in areas exposed to sea water intrusion or in areas subjected to irrigation (Shannon, 1992). Excess salt in the soil will cause ion imbalance, symptoms, mineral deficiencies, osmotic stress, ion toxicity and oxidative stress in plants. As a result, it will also affect the regulation of gene expression, protein synthesis, as well as protein, fats and pigments metabolism in plants (Rai *et al*., 2011)

Plants resistance mechanisms against salinity also indicated by the formation of several compounds (Proline, polyols, trehalose, glycine betaine, etc.) to stabilize proteins and cellular structures and/or to maintain cells turgidity through osmotic pressure regulation and redox metabolism (Rai *et al*., 2011). Proline triggered by stress condition will act as osmoregulator, protein structural stabilizator and cells's protector. Proline is coded by delta-1-pyrroline-5-carboxylate synthase (P5CS) gene. One of the target proteins in stress signaling pathway is Heat Shock Protein (HSP), which is synthesized not only because of the high temperatures but by various stresses (salt, oxidative). Protein can be found in the cytoplasm and organelles (Nucleus, mitochondria, chloroplasts and endoplasmic reticulum). The HSP role is to protect plants from stress and also important in homeostasis (Wang *et al*., 2004). The HSP90 plays a role in the folding and activation of proteins involved in signal transduction and regulate the cell cycle (Krishna and Gloor, 2001). The HSP 90s mediate plant abiotic stress signaling pathways (Liu *et al*., 2006a) and mainly acts as salinity regulator (Liu *et al*., 2006b). Protein with molecular weight ranging from 82-90 kD are included into the HSP 90 family. Based on transcriptome analysis of bananas cultured in 100 mM salt stress, HSP 81-2 gene was considered as top 10 genes found in this condition (Kusdianti *et al*., 2014).

Banana (*Musa* spp.) is mesophytic plant that intolerant to high salinity, in which a study proved that banana production will decreased up to 50% in soil with high salt levels (Shapira *et al*., 2009). Research to obtain banana which is tolerant to stress, biotic or abiotic stress, have been conducted previously. Some study use meristematic culture to assess banana tolerance to salinity (Ikram-Ul-Haq *et al*., 2012; Bidabadi *et al*., 2012). Study about salinity stress (50, 100 and 150 mM Na Cl) to banana cv Barangan culture has been done previously. Tolerance to salinity stress in 100 mM concentration was detected in a form of browning. Multiplication was still occured but growth was slightly inhibited in terms of height compared to shoots in 150 mM concentration (Ilmawati, 2013). From transcriptome data of banana (*Musa acuminata*) cultured in 100 mM salt stress (Widiyanto *et al*., 2013), HSP 81-2 and P5CS1 gene primer were designed (Kusdianti *et al*., 2014) and were subsequently validated in banana shoots culture (*Musa acuminata*) to see the genes expression pattern. The purpose of this study was to observe HSP 81-2 and P5CS1 gene expression profile in banana shoots cultured in NaCl stressed-medium.

MATERIALS AND METHODS

Plant material and experimental treatment: Shoots of banana (*Musa acuminata*) cv Barangan were cultured *in vitro* in MS medium (Murashige and Skoog, 1962) added with 22.2 μM BAP (Fig. 1). Treatments were additional of 25, 50, 75 or 100 mM NaCl. As a control, several shoots were planted in the medium without the addition of NaCl (K). Culture periode was 3-4 weeks.

Proline analysis: Proline content in shoots and roots (Fig. 1) samples were analyzed with ninhydrin methods according to Bates *et al*. (1973). Extracts were measured its absorbance using a wavelong 520 nm spectrophotometer

RNA preparation: The RNA was isolated from control (K) and treatment shoot and root (Fig. 1) which has been grinded

Fig. 1: Shoots of banana (*Musa acuminata* cv Barangan) were cultured *in vitro*, a: Shoot and b: Root

Table 1: Gene primer sequences

Gene name	Primer sequence 5'-3'
18S rRNA (Brunner *et al*., 2004)	F:AATTGTTGGTCTTCAACGAGGAA/ R:AAAGGGCAGGGACGTAGTCAA
Delta-1-pyrroline-5-carboxylate synthase (P5CS1)	F:TGACTGCATTATTGCCAAGG/ R:AATCCTTCGACACCAACAGG
Heat Shock Protein 81-2 (HSP 81-2)	F:GCGTTCCTCCAGATATTCCA/ R:CCACCAAGCACAATGATGAC

(1.5 g) using Pine Tree Method (Chang *et al*., 1993). Pellet was re-suspended in RNAase free water (DPEC), depending on the size of the pellet. The RNA quality and quantity was checked and determined with a spectrophotometer.

Validation and confirmation: Quantitative real-time PCR (qRT-PCR) was used to determine transcript levels (Diningrat *et al*., 2015). Total RNA was extracted from control (K) and treatment as described above. The cDNA synthesis first-strand was performed using thermo scientific reverst first aid strand cDNA synthesis kit (Deepa *et al*., 2014). Transcript levels were analyzed by quantitative real-time PCR using SYBR Green qPCR Master Mix according to the manufacturer's manual (Longo *et al*., 1990). The selected gene was delta-1-pyrroline-5-carboxylate synthase (P5CS1) and Heat Shock Proteins 81-2 (HSP 81-2) (Widiyanto *et al*., 2013) in which these were used to observe expression profile and 18S rRNA was used as a reference (Table 1). The PCR reaction involved these following steps: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 20 sec. Three biological replicates were included in the qRT-PCR assay.

RESULTS AND DISCUSSION

After cultures were 3-4 weeks old, shoots and roots proline analysis and gene expression analysis were carried out. Proline analysis results (Fig. 2) shows the same pattern between proline content in the shoots and roots. When treated with 25 and 50 mM NaCl, proline content in shoots and roots was lower than in the control. In the treatment of 75 and 100 mM NaCl, proline content was increased. The highest increment in shoots occurred in 100 mM (210 mg L^{-1}), while in the root, 75 and 100 mM showed similar results (125 mg L^{-1}) which was higher than the control and three other treatments.

Proline content analysis in shoots and roots of control culture showed that proline content in shoots was higher than in the roots, in which similar results also occured in 25 mM. After receiving higher NaCl treatment, 50 and 75 mM NaCl, proline content in the shoots were lower than in the root. In 75 mM NaCl, proline content in roots is much higher than in

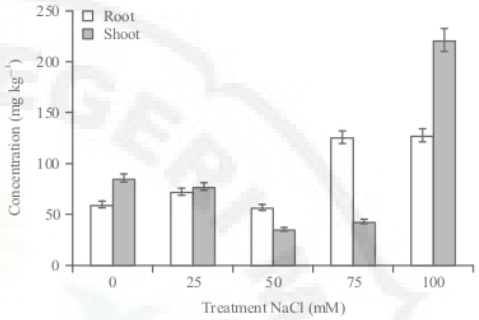

Fig. 2: Proline content in control (K) and NaCl treatment

shoot. Proline content increased in 75 mM because this concentration experiencing stress compared to two prior NaCl concentrations. Similarly, in 100 mM treatment, proline content was much higher as the stress was even more severe. In 75 mM proline content was higher in roots because contact with medium was firstly happen in roots, whereas in 100 mM contact was occurred otherwise. Proline contents in roots for 75 and 100 mM were in the same amount but 100 mM treatmen has high proline content in its shoots (210 mg kg^{-1}). This shows that plantlets which undergo NaCl stress of 100 mM produced high number of proline in its roots or shoots as a form of defense against NaCl stress. In 100 mM treatment, proline content was much higher than the other four treatments and the proline was more accumulated in shoots (210 mg kg^{-1}).

The P5CS1 and HSP 81-2 gene expression profiles in the control and treatment culture showed a different pattern, as well as profiles between shoots (Fig. 3) and roots (Fig. 4). Figure 3 shows that P5CS1 was down-regulated in 25 and 50 mM in which it were down regulated more than 0.5 times that of control. In 75 mM this gene was slightly down-regulated (0.03 times). The P5CS1 gene was up-regulated 0.37 times in 100 mM. Proline is produced only in 100 mM NaCl, These datas suggested that higher NaCl concentration in the medium resulted in increasing proline production. Figure 3 also shows that in shoots, HSP 81-2 gene was up-regulated more than 1.5 times that of controls in all 4 treatments. The HSP 81-2 gene expression was at its highest

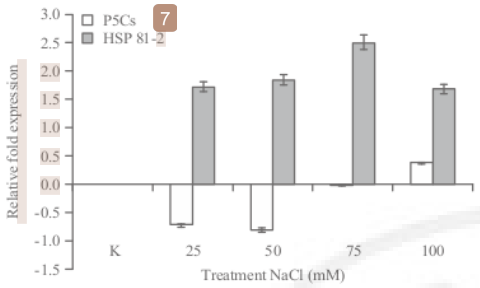

Fig. 3: P5CS1 and HSP 81-2 gene expression profile in shoots, control (K) and NaCl treatment

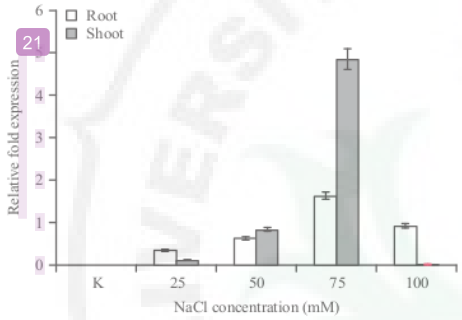

Fig. 4: P5CS1 and HSP 81-2 gene expression profile in roots, control (K) and NaCl treatments

in the 75 mM (2.5 times) and declining in 100 mM (1.68 times). These data indicates that HSP 81-2 gene was much more expressed than P5CS genes, with 75 mM as it's highest. Figure 4 illustrates that with NaCl concentrations increment in the medium, either HSP 81-2 or P5CS1 genes showed the same pattern. The HSP 81-2 and P5CS1 genes in the roots were up-regulated and its expression was increased from 25, 50 and 75 mM until it was decreased in 100 mM NaCl. In 75 mM, HSP 81-2 (4.85 times) and P5CS1 (1.6 times) gene expression were its highest compared to control and other treatments. In 25 mM, proline is higher than protein 81-2, although higher NaCl concentration will resulted in more heat shock protein 81-2 production then proline's and it will sharply decline in 100 mM. Coping mechanism for NaCl stress on the roots involves either heat shock protein 81-2 or proline, although a heat shock proteins 81-2 in 100 mM was very little produced.

Plants coping with stress by producing higher proline as its defense response. Proline content was increased with increasing NaCl concentration i.e., increasing Na^+ ions in growing tissue. This is an indicator of salt stress (Ikram-Ul-Haq *et al*., 2011). Proline accumulation is the first response to salt stress that contributes to the regulation of osmotic pressure (Ranganayakulu *et al*., 2013). Salinity stress resulted HSP 81-2 and P5CS1 gene expression in both roots and shoots. This occurs in *Helianthus tuberosus* L. which the genes induced P5CS2 1.9-2.1 times as a response to NaCl stress (Huang *et al*., 2013). Pospisilova *et al*. (2011) study showed an increase in proline content in transgenic tobacco plants which subjected to drought stress. Rice subjected to salt stress expressing HSP 90 (Hu *et al*., 2009), HSP 90 (OsHSP 93.04-OsHSP 85.88) and HSP 70 (OsHSP 71.10, OsHSP 71.18, OsHSP 72.57, OsHSP 72.90 72897.5) (Ye *et al*., 2012). In *Sueda salsa* (Chenopodiaceae), salinity will induce HSP 70 formation (Li *et al*., 2011). Banana plantlet expressed more heat shock protein 81-2 rather than proline to cope with NaCl stress. The number of these proteins can replace the function of proline, which acts as "Chemical chaperones" (Xu *et al*., 2013).

CONCLUSION

In conclusions, based on the results of the study above we can be summarized as follows, (1) Proline accumulated by plantlets treated with NaCl and HSP 81-2 and P5CS1 genes expressed by all plantlets with different levels, (2) HSP 81-2 highest expressed by shoots and roots of plants with 75 mM NaCl treatment. Likewise, the highest proline accumulation occurred in this treatment, (3) On the whole of the roots and shoots treated by NaCl, HSP 81-2 gene expression is higher than the P5CS1 gene expression, (4) *Musa acuminata* cv Barangan plant has defense mechanisms against the state of high salinity and (5) The expected contribution of this study is that *Musa acuminata* cv Barangan can be used as plant resistant to soil with high salt content conditions to resolve the problem of exploitation of critical marginal land.

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