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Research Article

Effect of Heat Shock Stress on Protein Threonine Phosphatase Activity

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Abstract Phosphorylation and dephosphorylationare the two conjugate processes carried out by unique enzymes called kinases and phosphatases. These processes serve as an on-off switch in the control of cellular activities in eukaryotic cells. Protein kinases and protein phosphatases are obligate partner of each other. They are substrate specific and control cellular circuitry. The present study shows that protein phosphatases activity is also affected by heat stress. In the present work, the eight days growing seedlings were stressed by heat from 30 C to 70 C. It was observed that the specific activity of protein threonine phosphatase (PThPase) was found maximum at 40 C and further gets reduced upto 70 C. At 70 C the specific activity found to be reduced up to 55%.When peanut seedlings were stressed at 70 C for different time intervals from 0 to 6 hrs then there was an appreciable reduction in the specific activity of PThPase. It continuously decreases upto 6 hrs. When the different parts of seedling stressed at 70 C for 2 hrs then the maximum reduction in specific activity was observed in cotyledon followed by hypocotyl, epicotyl and then roots. The results suggest that the role of PThPase is stress-related cellular processes and likely to inhibit the activity of intracellular protein threonine phosphatases (InPThPases).

Keywords: *Arachis hypogaea* L., Protein threonine phosphatase (PThPases), Protein Kinase, Protein phosphorylation, Heat stress.

Introduction

Protein Serine/Threonine phosphatase enzymes are ubiquitous in all eukaryotes but there is a little work on it and their physiological role in plants remain unknown yet. In nature, plants are exposed to various stresses, which affect their physiology, morphology and development. Among these stresses, the fluctuation of temperature, water status of soil, and intensity of light are the most crucial signals affecting plant growth (Boyer, 1982; Trewavas et. al.,1997). In response to stress, various genes are up-regulated, which can mitigate the effect of stress and lead to adjustment of the cellular processes leading to stress tolerance in plants. Many different kinds of stress can be encountered by a cell or organisms. One important category of stressors is genotoxic agents like ultraviolet and ionizing radiations as well as many chemical mutagen and carcinogens. Organism must also be defined against physiochemical stresses acting mainly through mechanisms other than DNA damage; such stresses include shear, wounding, infection, oxidative, salt and heat shock. Plants can mitigate environmental stress conditions through acclimation. In case of fluctuating stress condition such as high temperatures, maintaining a stress memory enables a more efficient response upon recurring stress (Castellanos et. al., 2020). In plants several studies have been carried out to understand molecular responses to abiotic and biotic stresses. However, the complete circuitry of stress responsive genes that plants utilize in response to those environmental stresses is still unknown. The protein phosphatase 2A has been known to have a crucial role in biotic and abiotic stresses (Khan et. al., 2020)

The real progress will require identification of physiological substrates and purification of native enzymes using conventional and non-conventional protein purifications methods. The interaction of native and modified bovine serum albumin (BSA) with catechin, a flavanoid having vitamins activity has been studied by Arora et. al.(1989). Thus, identification and characterization of PThPase, DSPTPase and PKases that modulates the phosphorylation status and function of key regulatory proteins in cell will provide new insights into the role of PThPase in biological process in plants, including the relief against the environmental stresses such

as thermo-tolerance. Quaitesch et. al. (2000), Lee et.al.(1994) and Mishra et.al.(2002) have reported that a heat shock protein A (HSPA3) which is a thermo-inducible protein as a key regulator may undergo reversible phosphorylation.

Peanut seedlings used in this study were sensitive to the environmental stresses, especially to the heat stress. One of the mechanisms, which the plants have evolved to fight against stress, is the induction of heat shock proteins. Heat shock proteins (Hsps) are responsible for protein folding, translocation, degradation in many cellular processes stabilize proteins and membranes and can assist in protein refolding under stress conditions. Heat shock proteins are known to be expressed in plants not only when they experience high temperature stress but also in response to a wide range of other environmental stresses. Heat shock proteins can play a crucial role in protecting plants against stress by reestablishing normal protein conformation and cellular homeostasis (Wangxia et. al., 2004). On contrary, it has been shown by Joank et. al.(1996) that heat shock does not induce MAP kinase activation. The heat activated MAPKase in tomato cell cultures and plant has been reported by Link et. al., 2002.

Methods and Material

Seed Germination and Preparation of Crude Enzyme

The peanut seeds were purchased from an authorized seed store and washed with double distilled water. The seeds surface was sterilized with 1% HgCl2 and allowed to germinate under aseptic conditions for 14 days (Devi et.al., 2005). The seeds were germinated on autoclaved Whatman filter paper. The whole plant and different parts of plant like root, hypocotyl, epicotyl and cotyledon were crushed manually and the crude enzyme extract was prepared by homogenizing the plant tissue with extraction buffer in (1:3 ratio) at 0-4 C.The extraction buffer was made up of 100 mM-Tris PH 7.5, 50 mM NaCl, 10 mM EDTA, 0.04% beta mercaptoethanol. The homogenate was filtered through four layered cheese cloth. The filtrate was centrifuged at 10000 rpm for 30 minutes. The supernatant was used for enzyme assay.

PThPase Activity

Protein threonine phosphatase activity was assayed by using O-Phospho L- threonine (Sigma). The reaction mixture is composed of 10 µl substrate, 10 µl enzyme 180 µl Tris-HCl (100 mM) (total volume 200µl). Now the reaction mixture is incubated for 30 minutes at 30 C. 10% TCA solution is added to stop the reaction and the reaction is kept at ice for 5 minute and again it is centrifuged at 10000 rpm for 5 minutes in refrigerated centrifuge at 0-5 C, so that the protein is completely precipitated out. 200 µl of supernatant of the reaction mixture was assayed for inorganic phosphate (Pi) release by the malachite green method (180 mg malachite green in 400 mL distilled water and 5.0 gm Ammonium molybdate in 40 mL Conc. HCl, makeup volume 500 mL) (Lanzetta et. al., 1979). 100 µl supernatant is mixed with 1 mL of malachite green solution and the absorbance is measured by UV-visible spectrophotometer (Shimazdu). The similar method is also used for the protein threonine phosphatase (PTPase), protein tyrosine phosphatase (PTPase), protein serine phosphatase (PSPase), by using O-phospho-L-Threonine, O-phospho-L-tyrosine, O-phospho-L-Serine as substrate, respectively. "One unit was defined as the amount of protein in mg that liberated one nano mole of inorganic phosphate (Pi) per minute under assay condition." The protein concentration is measured by the help of method given by Lowry (1951), in which bovine serum albumin is used as the standard substance.

Heat Stress on Peanut

The heat stress is carried out by treating the germinated seedlings through the following steps:

1. Stressed between the temperature range 30 C to 70 C for 2 hrs.
2. Stressed between different time intervals from 0 to 6 hrs at 70 C.
3. Different parts of germinated seedlings stressed at 70 C for 2 hrs.

SDS-PAGE of Crude Enzyme in Stressed and Non-stressed Samples

To deduce changes associated with oxidative stress in peanut seedlings on its protein content sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis was carried out according to the method of Laemmli (1970) using 10% polyacrylamide gel. Coomassie brilliant blue R-250 staining was carried out to visualize protein bands on the gels. The molecular weight of the protein was estimated by comparing the relative mobility of proteins of different molecular size using standard molecular weight marker (97.4-14.3kDa; Standard molecular mass markers: Lysozyme,14.3 KDa; Trypsine inhibitor, 20.1 KDa; Carbonicanhydrase, 29.5 KDa; Ovalalbumin,45.0KDa; Bovine serum albumin, 66.0 KDa; Phosphorylase-b,97.4KDa). All the experiments were replicated atleast thrice with three replicates each and the data was pooled to mean of the values obtained individually (Sharma et. al., 2017).

Results and Discussion

Effect of heat stress on the specific activity of PThPase in 8 day old germinating peanut seedlings:

An attempt was made to examine the effect of heat stress on the specific activity of PThPase. Fig. 1 showed a marked effect of heat stress on the specific activity of PThPase. It was observed that the specific activity of PThPase increases slightly upto 40°C but sharply decreases upto 50°C and then decreases regularly as the temperature increases upto 70°C for 2 hr treatment. At 70°C the specific activity was found to be reduced upto 55%. Further, the 8 day old germinating peanut seedlings which showed highest level of PThPase activity was subjected to heat stress by keeping them at 70°C for the indicated periods varying from 0-6 hrs. Fig. 2 showed that the specific activity of PThPase decreased continuously by increasing the time from 0-6 hrs treatment at 70°C.These results were obtained using the whole seedlings. Hence, it was not possible to observe that which part of the seedlings was most affected.

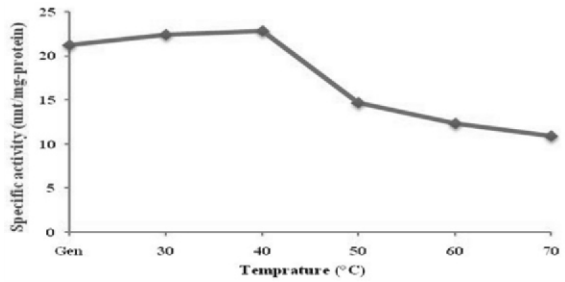

Fig. 1: Effect of heat stress on PThPase applied by subjected the peanut seedlings at different temperatures.

Note: Gen refers to without heat stress seedlings.

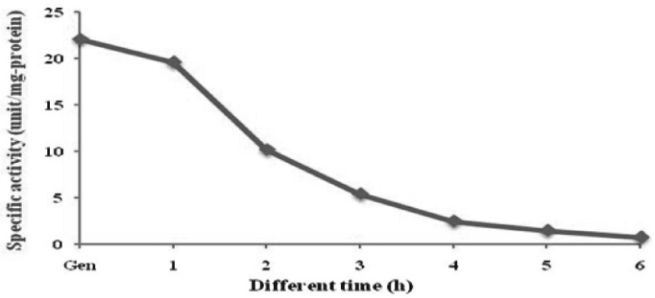

Fig. 2: Effect of heat stress on PThPase applied by subjected the peanut seedlings to heat stress at 70ºC for different time.

Note: Gen refers to without heat stress seedlings.

The different part of the seedlings after allow to return at normal temperature of germination (28±2°C) were excised and processed for PThPase activity determination. Control were not subjected to heat stress, but were kept under normal condition of germination for the stress period. The results of the specific activity of PThPase in different parts of the peanut seedlings after heat stress are given in Fig. 3. The figure reveals that the specific activity of PThPase decreased significantly in hypocotyls and cotyledons during the heat stress at 70°C for 2 hrs.

The 2h stress at 70°C resulted in almost 6.3-fold decrease in the specific activity of PThPase in cotyledons. Similarly, in hypocotyl, epicotyl and root the specific activity of PThPase was reduced upto 4.0 fold, 2.9 and 2.6 fold after 2h heat shock at 70°C, respectively. The results clearly showed that the heat stress had a profound effect on the specific activity of PThPase in growing tissues, cotyledons and hypocotyls, implicating that dephosphorylation of protein were more reduced level in the stressed tissues than unstressed ones. These results further suggest that the protein kinase, especially the heat-activated mitogen activated protein kinase (MAPK) level could be enhanced as these enzymes participate in signaling pathways and that under the unstressed condition there is a precise balance in the MAPKs and PThPase in order to regulate the cellular metabolism.

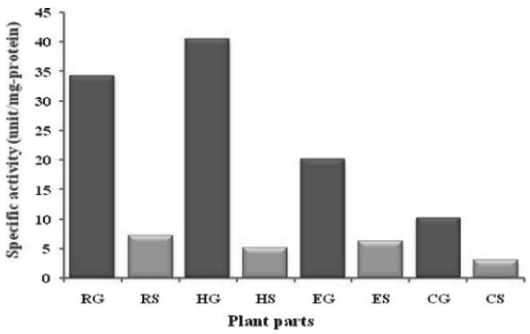

Fig.3: Effect of heat stress on the level of the specific activity of PThPase indifferent parts of peanut seedlings.

Note: Rg=Root general, Rs=Root stress; Hg=Hypocotyl general, Hs= Hypocotyl stress; Eg=Epicotyl general, Es=Epicotyl stress; Cg=cotyledon general, Cs= Cotyledon stress; Mr=marker Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (NaDS- PAGE): A protein band profile (containing PThPase) in different part of germinating peanut seedlings by treating at 70°C for 2 hrs:

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (NaDS- PAGE): A protein band profile (containing PThPase) in different part of germinating peanut seedlings by treating at 70°C for 2 hrs:

NaDS-PAGE profile of protein bands, after heat shock on different part of germinating peanut seedlings at 70°C for 2 hrs, a change in results was observed in protein band using 10% polyacrylamide gel as compared to the unstressed plant parts (Fig. 4).A high molecular weight protein band was visible between 45.0kDa to 66.0kDa marker range in stressed cotyledons, whereas the same protein band was found absent in unstressed cotyledon of germinating peanut seedlings. Another high molecular weight protein band, between 45.0kDa to 120kDa disappeared in stressed roots, hypocotyls and epicotyls, whereas the same protein band was visualized in same unstressed group of germinating peanut seedlings.

A low molecular weight protein band, under 29.0kDa marker range also disappeared in stressed roots, hypocotyls and epicotyls, whereas the same protein band was unchanged in unstressed cotyledon of germinating peanut seedlings while there was no change found in and a low molecular weight protein band of stressed and unstressed cotyledon of germinating peanut seedlings. The effect of heat stress on protein profile, analyzed by NaDS-PAGE but these results did not identify the functionality of proteins affected by the heat stress.

On the basis of results obtained, it may be concluded that heat stress had an intense effect on the specific activity of PThPase. Surprisingly, the heat stress on PThPase differed from oxidative, salt and surfactant stresses in the sense that as a consequence of the heat stress, the specific activity of PThPases slightly increased in roots and epicotyl but decreased significantly in hypocotyls and cotyledons.

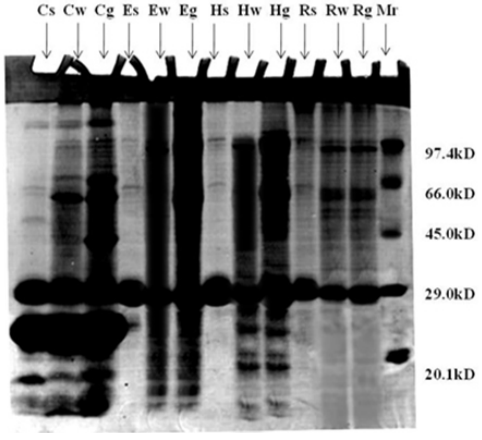

Fig. 4: A comparison of protein bands profile by NaDS-PAGE. Different part of peanut seedlings subjected to heat shock at 70°C for 2h, allowed to cool at room temperature, all parts were excised and soluble proteins were analyzed by NaDS-PAGE using 10 % polyacrylamide gel. Protein bands were stained with CBB R-250.

Note:Rg=Root general, Rw=Root water Rs=Root stress; Hg=Hypocotyl general, Hw=Hypocotyl water, Hs=Hypocotyl stress; Eg=Epicotyl general, Ew=Epicotyl water, Es=Epicotyl stress; Cg=Cotyledon general, Cw=Cotyledon water, Cs=Cotyledon stress.

It was however found to decrease in roots after salt, surfactant and heat stresses. These results have providence that the PThPases serve critical functions in plant responses to stress signals and development.

Future Perspectives

Abiotic and biotic stresses adversely affect plant growth, development and may change eventually in the enzymatic level. Although there are many aspects about the roles of protein threonine phosphatases in stress related responses which are still unknown. The recent advances revealed that protein threonine phosphatases are key components of stress signal transduction pathways. They also balance the function of protein kinases and play positive and dynamic roles in stress signaling. The activity of protein phosphatases surprisingly affected by the heat treatment which affects the cellular signalling pathway.

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