

Synthesis of HSP for Thermo tolerance in Plants

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Abstract

Organisms produce a modest number of highly conserved heat shock proteins in response to environmental stress. The development of thermotolerance is closely tied to the synthesis of these proteins in a wide range of organisms, including bacteria, plants, invertebrates, and vertebrates. The conclusion that heat shock proteins are necessary for stress tolerance and that the principal heat shock protein in plants has no impact on normal growth or physiology is thus directly supported by these data. The results also suggest that an organism's natural environment may have an impact on whether or not it exhibits a heat shock response.

Keywords-Heat shock protein, Thermotolerance, Stress tolerance, Physiology etc.

I. Introduction to Heat Shock Proteins in Plants

Heat shock proteins are a highly conserved set of proteins that play a crucial role in the adaptation and survival of plants under stress conditions, particularly in response to high temperatures^(Abdul-Baki, 1991). These proteins are synthesized in response to thermal stress and assist in maintaining cellular homeostasis by acting as molecular chaperones, ensuring proper protein folding and preventing protein denaturation. Furthermore, heat shock proteins are known to play a key role in thermotolerance, which is the ability of plants to tolerate and survive under high temperature conditions.

Heat shock proteins are categorized into several families based on their molecular weight, including HSP100, HSP90, HSP70, HSP60, HSP40, and small heat shock proteins (sHSPs). The synthesis of heat shock proteins is a crucial aspect of plant thermotolerance^(Coito et al., 2012). Studies have shown that the expression of specific heat shock proteins is induced in response to different temperatures and durations of heat stress, indicating that there may be differences in the means of achieving thermotolerance, depending on the specific conditions of the heat stress^(Ishii et al., 2018).

Regulation of HSP Synthesis in Plants Regulation of HSP Synthesis in Plants Heat shock protein synthesis in plants is regulated by various factors, including heat stress transcription factors, heat shock elements, and other environmental cues. These factors work together to control the expression of heat shock protein genes and ensure the timely synthesis of heat shock proteins in response to thermal stress. Furthermore, studies have shown that the expression of heat shock proteins can be enhanced through genetic engineering techniques, leading to increased thermotolerance in plants^(Pérez-Clemente et al., 2013).

Genetic Engineering for Increased HSP Synthesis and Thermotolerance in Plants

Genetic engineering has been successfully employed to enhance heat shock protein synthesis in plants, resulting in increased thermotolerance. Several plant species have been genetically engineered to overexpress specific heat shock proteins, such as HSP101, HSP70, or small heat shock proteins (sHSPs), leading to improved thermotolerance. These genetic engineering techniques have been shown to increase the levels of heat shock proteins in plants, thereby conferring enhanced thermotolerance.

Table 1: Heat Shock Proteins and their Functions in Thermotolerance

Table 1 Families of Hsps in human beings, their site, and suggested functions (Kregel, 2002).

Hsp Families	Cellular location	Proposed functions
Hsp27 (sHsp)	Cytosol, nucleus	Microfilament stabilization, antiapoptotic
Hsp60	Mitochondria	Refolds proteins and prevent aggregation of denatured proteins, proapoptotic
Hsp70		Antiapoptotic
Hsp72(Hsp70)	Cytosol, nucleus	Protein folding, cytoprotection
Hsp73(Hsc70)	Cytosol, nucleus	Molecular chaperones
Hsp75(mHsp70)	Mitochondria	Molecular chaperones
Hsp78(GRP78)	Endoplasmic reticulum	Cytoprotection, molecular chaperones
Hsp90	Cytosol, endoplasmic reticulum, nucleus	Regulation of steroid hormone receptors, protein translocation
Hsp110/104	Cytosol	Protein folding

Synthesis Process of Heat Shock Proteins

The synthesis of heat shock proteins in plants is a complex process that is regulated by various factors. The accumulation of heat shock proteins in plants plays a vital role in thermotolerance, as they help protect cellular structures and maintain homeostasis under heat stress conditions. Heat stress transcription factors, such as heat shock factors, are key regulators of heat shock protein synthesis in plants (Coito et al., 2012). These transcription factors are activated by heat stress and bind to specific regulatory sequences in the promoter regions of heat shock protein genes. Once bound to the promoter regions, heat stress transcription factors initiate the transcription of heat shock protein genes, leading to the synthesis of heat shock proteins. The expression of heat shock proteins can also be enhanced through genetic engineering techniques. For instance, genetic transformation of plants with different heat shock factors under the control of promoter has been shown to improve the thermotolerance of plants.

The precise mechanism by which heat shock proteins confer thermotolerance is not fully understood (Panwar & Srivastava, 2012). However, it is believed that heat shock proteins play a crucial role in protecting cellular structures and functions under heat stress conditions. These proteins act as molecular chaperones, assisting in protein folding and preventing protein aggregation.

Furthermore, heat shock proteins are involved in the repair and degradation of damaged proteins, ensuring the maintenance of protein homeostasis. In addition to protein protection and maintenance, heat shock proteins also participate in signaling pathways that activate stress responses and regulate gene expression.

Table 2 Location of sHsps types in plant cell and the DNA accession number (after Waters et al., 1996).

Species	Protein	DNA accession number
<i>Chloroplast-localized proteins</i>		
<i>Arabidopsis thaliana</i>	HSP21	X54102
<i>Glycine max</i>	HSP22	X07188
<i>Peunia hybrida</i>	HSP21	X54103
<i>Pisum sativum</i>	HSP21	X07187
<i>Triticum aestivum</i>	HSP26A	X58280
<i>Triticum aestivum</i>	HSP26B	X67328
<i>Zea mays</i>	HSP26	L28712
<i>Mitochondrial-localized protein</i>		
<i>Chenopodium rubrum</i>	HSP23	X15333
<i>Endoplasmic reticulum-localized proteins</i>		
<i>Arabidopsis thaliana</i>	HSP22	U11501
<i>Glycine max</i>	HSP22	X63198
<i>Pisum sativum</i>	HSP22	M33898
<i>Class I Cytocologically localized proteins</i>		
<i>Arabidopsis thaliana</i>	HSP17.6	X16076
<i>Arabidopsis thaliana</i>	HSP17.4	X17293
<i>Arabidopsis thaliana</i>	HSP18.2	X17295
<i>Chenopodium rubrum</i>	HSP18.3	X53870
<i>Daucus carota</i>	HSP18.0	X53852
<i>Daucus carota</i>	HSP17.8	X53851
<i>Glycine max</i>	HSP17.5	M11318
<i>Glycine max</i>	HSP17.6	M11317
<i>Glycine max</i>	HSP18.5	X07160
<i>Helianthus annuus</i>	HSP17.6	X59701
<i>Lycopersicon esculentum</i>	HSP17.8	X56138
<i>Medicago sativa</i>	HSP18.1	X58710
<i>Medicago sativa</i>	HSP18.2	X5871 1
<i>Oryza sativa</i>	HSP16.9	X60820
<i>Oryza sativa</i>	HSP17.4	D 12635
<i>Pisum sativum</i>	HSP18.1	M33899
<i>Triticum aestivum</i>	HSP16.9A	X13431
<i>Triticum aestivum</i>	HSP16.9B	X64618
<i>Triticum aestivum</i>	HSP16.9C	L 14444
<i>Zea mays</i>	HSP17.2	X65725
<i>Class II Cytocologically localized proteins</i>		
<i>Arabidopsis thaliana</i>	HSP17.6	X63443
<i>Glycine max</i>	HSP17.9	X07159
<i>Ipomea nil (Pharbatia nil)</i>	HSP18.8	M99430
<i>Ipomea nil (Pharbatia nil)</i>	HSP17.2	M99429
<i>Lilium longiflorum</i>	HSP17.6	D21816
<i>Lilium longiflorum</i>	HSP16.5	D21818
<i>Pisum sativum</i>	HSP17.7	M33901
<i>Triticum aestivum</i>	HSP17.3	X58279
<i>Zea mays</i>	HSP17.5	X54076
<i>Zea mays</i>	HSP17.8	X54075

Role of Heat Shock Proteins in Thermotolerance

Any protein's ability to form and fold into a three-dimensional structure determines how it will perform. Half of the primary amino acid sequence is necessary for the formation of three-dimensional structure. Herein lies the significance of Hsps' function in the folding of other proteins. According to Morimoto and Santoro (2008), Hsps shield cells from harm and aid in their recovery and survival after being returned to normal development settings. However, Timperio et al. (2008) noted that while the function of Hsp as molecular chaperones under heat stress is undeniable, it is possible that under non-thermal stress, where protein unfolding is not the primary effect, protection from damage may take place in a different manner in addition to ensuring the maintenance of proper protein structure. According to some theories, the general function of Hsps is to act as molecular chaperones that control protein folding, accumulation, localisation, and destruction in all plant and animal species. In situations of heat stress, these proteins act as chaperones to help other proteins refold and avoid their irreversible aggregation. Each of these Hsp groups has a distinct mechanism, and the functions of each are described.

Types of sHsps-

These proteins share a C-terminal alpha-crystallin domain of between 80 and 100 amino acid residues. The breakdown of proteins with improper folding is one of this class' distinctive functions. The sHsp ubiquitin protein and its attached enzymes serve as the model protein. The fact that these sHsps' activity is unrelated to ATP is another feature that sets them apart from other chaperone types. The structure, categorization, and function of sHsps as well as the outcomes of gene transcription in *A. thaliana* were, however, more fully described in this work. These findings showed that plant heat tolerance was influenced by additional variables, including reactive oxygen species and plant growth regulators. The sHsps are unable to refold foreign proteins, but they can bind to partially folded or denatured substrate proteins to stop incorrect protein aggregation or irreversible unfolding. According to recent research, sHsp 18.1 and 16.6 from *Synechocystis* sp. PCC6803 and *Pisum sativum*, respectively, bind to unfolded proteins and facilitate subsequent refolding by Hsp70/Hsp100 complexes. Six diverse Anthophyta species, including C3, C4, CAM, monocot, and dicot species, were found to have a positive qualitative relationship between the accumulation of sHsps in the plastids and thermotolerance of heat shock (from 28 to 40 C). Four species not belonging to the Anthophyta were used individually to produce similar findings. Another study found that the NADH: ubiquinone oxidoreductase (complex I) was protected by the mitochondrial sHsp in *Pyrus pumila* (P. Mill.) K. Koch var. McIntosh apple fruit under heat stress. According to this information, these proteins may play a part in helping plants adapt to heat stress.

According to a recent review (Nakamoto and V'gh, 2007), there are some signs that tiny heatshock proteins play a significant role in membrane quality control and may, therefore, help to maintain membrane integrity, especially under stressful circumstances.

Hsp60 class It is widely acknowledged that this class of Hsp60 proteins, also known as chaperonins, play a crucial role in supporting plastid proteins like Rubisco. According to several research, this class may help numerous proteins that are carried to organelles like chloroplasts and mitochondria fold and aggregate. To stop proteins from aggregating, these Hsps60 attach to various protein types after their transcription but before folding. Plant chaperonins have a limited range of functions, and it is generally accepted that stromal chaperones (Hsp70 and Hsp60) are responsible for bringing newly imported proteins into functional shape in the chloroplast.

Hsp70 class The Hsp70 acts as a chaperone for freshly generated proteins in practically all species to avoid their buildup as aggregates and to ensure appropriate folding during their transport to their final site. The development of cross-adaptation to temperature stress brought on by heat acclimation (HA)- or cold acclimation (CA) pretreatment in grape plants may be greatly aided by Hsp70 and sHsp17.6, which primarily function as molecular chaperone and play a crucial role in protecting plant cells from the harmful effects of heat stress. In a study of the plant *P. sativum*, Lee and Vierling (2000) discovered cooperation in the actions of this class (protein folding) with small heat-shock proteins such sHsp18.1 (prevention of protein aggregation). Hsp70 takes part in guidance complex import (translocon), which involves binding to protein precursors and transferring them through membranes into organelles like chloroplasts. There is some evidence to suggest that Hsp70B, which is located in the stroma of chloroplasts, is involved in photo protection and photosystem II repair both during and after photoinhibition. A more recent work on *A. thaliana* revealed that Hsp70, which is present in the stroma of chloroplasts, is essential for the differentiation of germination-initiating seeds and for their heat tolerance. **Hsp90 class** Given that Hsp90 can bind to Hsp70 in numerous chaperone complexes and plays a significant role in the function and trafficking of proteins, the class that Hsp90 belongs to also includes other classes (Pratt and Toft, 2003). This class also performs another crucial function by controlling cellular signals, such as the activation of the glucocorticoid receptor (GR). Cytoplasmic Hsp90 causes pathogen resistance by interacting with resistance protein (R), the pathogen's signal receptor. According to a study on *A. thaliana* and

two species of tobacco, *Nicotiana tabacum* and *Nicotiana benthamiana*, the interaction between Hsp90 and resistance protein is extremely important for the functioning of the latter. This method is similar to the steroid receptor's controlling mechanism in mammals. According to Thao et al. (2007), rice's innate-immune response and pathogenic resistance depend on Hsp90. In *A. thaliana*, there were some hints that Cytoplasmic Hsp90 adversely inhibited hsf when heat stress wasn't there, but when heat stress is present, this role is momentarily halted, allowing hsf to function.

Hsp100 class,

This class's ability to reactivate aggregated proteins by dissolving inactive protein aggregates and assisting in the degradation of irreparably damaged polypeptides is one of its specialties. The plant required one cytoplasmic member of this class for high heat tolerance, but it wasn't required for germination or growth in the absence of stress. The role of this class is not limited to temperature adaptation; rather, a particular member of the family performs housekeeping tasks that are crucial for chloroplast development. It appears that this session also helps the body return to normal after a really stressful scenario. Principal Hsps that are expressed in high levels during times of stress typically serve comparable purposes by addressing issues with inappropriate folding and aggregation. There have been numerous analyses about Hsps and their significance, and one in-depth analysis of physiological, ecological, and evolutionary issues came to the following conclusions:

1. Hsps expression could happen in a natural environment
2. The expression of Hsps may be associated with stress tolerance.
3. The hsp genes are present in all species, but their patterns of expression differ.
4. The degree of environmental stress is associated with the species' threshold for Hsps expression.

Detailed Review of Heat Shock Protein Research

A significant amount of research has focused on understanding the synthesis and function of heat shock proteins in plants, particularly in relation to thermotolerance. Studies have shown that heat shock proteins are induced in response to heat stress in plants. These studies have also demonstrated that the expression of heat shock proteins is regulated at both the transcriptional and post-translational levels.

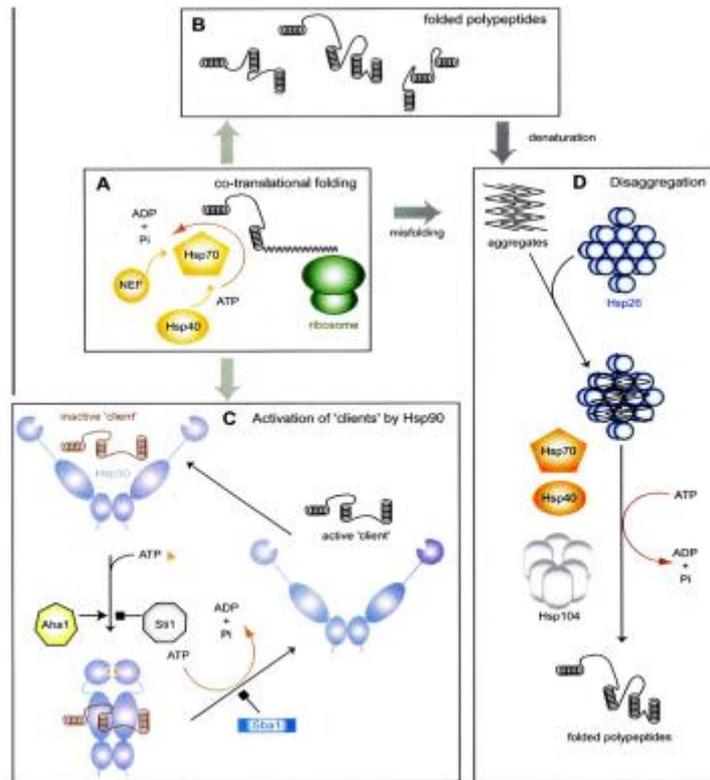
At the transcriptional level, heat shock transcription factors bind to heat shock elements in the promoter regions of heatshock protein genes, activating their expression in response to heat stress. The accumulation of heat shock proteins, under the control of heat stress transcription factors, is known to play a central role in the response to heat stress and acquired thermotolerance in plants and other organisms^(Coito et al., 2012). In plants, the heat shock protein 17 has been identified as an important player in thermotolerance. Heat shock protein 17 is involved in assisting protein folding and preventing protein aggregation, which are essential processes for maintaining cellular homeostasis under heat stress conditions. Another heat shock protein that plays a crucial role in thermotolerance is heat shock protein 70.

Heat shock protein 70 acts as a molecular chaperone and assists in protein folding, ensuring proper folding and preventing misfolding or aggregation of proteins under stressful conditions. Moreover, heat shock protein 90 has also been identified as an important player in enhancing thermotolerance in plants.

Heat shock protein 90 is involved in the formation of signaling complexes that are crucial for stress response pathways. In addition to their role in protein protection and maintenance, small heat shock proteins (sHSPs) have been found to prevent protein aggregation. Furthermore, sHSPs are known to play a role in the folding, intracellular distribution, and degradation of proteins. These findings highlight the importance of heat shock proteins in protecting plants against heat stress and improving thermotolerance.

Analysis of Heat Shock Protein Synthesis Tables Analysis of Heat Shock Protein Synthesis Tables

Figure 1 Simple illustration of part of the chaperone machines that operate in the cytosol: (A) Folding of proteins by Hsp70 is cotranslational, nucleotide exchange factors (NEFs) and Hsp40 facilitate this process. (B) Once protein synthesis is complete. Homologues of Hsp70 promote folding in other cellular compartments. (C) Certain proteins are presented in a largely folded though inactive state, to the Hsp90 chaperosome, the ATP-dependent action of which leads to activation of the substrate protein. Co-chaperones act as adaptors between Hsp70 and Hsp90, with specific co-chaperones acting as inhibitors (e.g. Sti1) or stimulators (e.g. Aha1) of the Hsp90 ATPase. (D) Misfolding and cellular stress lead to aberrant protein conformations, which can lead to aggregation.



Thermotolerance Enhancement Strategies in Plants

The synthesis of heat shock proteins is a crucial mechanism employed by plants to enhance thermotolerance and protect against heat stress. These proteins, including heat shock protein 17, heat shock protein 70, and heat shock protein 90, play essential roles in assisting protein folding and preventing protein aggregation, thereby maintaining cellular homeostasis under heat stress conditions^(Jespersen & Huang, 2015).

This table highlights the regulatory mechanisms that control heat shock protein synthesis in response to heat stress. The up-regulation of heat shock proteins is mainly regulated by heat stress transcription factors, which act as central regulators of HSP expression. These transcription factors activate the expression of heat shock protein genes in response to elevated temperatures, ensuring the production of proteins that aid in protein folding and prevent aggregate formation. Studies also demonstrate the involvement of various transcription factors in the synthesis of heat shock proteins^(Mallick et al., 2020).

Furthermore, genetic engineering techniques have been employed to enhance thermotolerance in plants by increasing the synthesis of heat shock proteins^(Pérez-Clemente et al., 2013). Studies have shown that genetic engineering can successfully increase thermotolerance in plants by enhancing the synthesis of heat shock proteins .

Table 3 Transgenic attempts to enhance plant temperature stress tolerance.

Phenotypes	Function	Plant	Gene
HS tolerant; HS sensitive	TF	Tomato	HsfA1
HS tolerant	TF	Arabidopsis	Hsf3
HT sensitive	HSP	Arabidopsis	Hsp70
Abolished acquired thermotolerance	HSP	Maize	Hsp101
HT tolerant	HSP	Carrot	Hsp17.7
HT tolerant	Fatty acid desaturation	Tobacco	Fad7
HS tolerant	AOS metabolism	Barley	Hvapx1

Source: sample of a larger table of Sung et al. (2003).
Abbreviations: AOS, active oxygen species; HS, heat shock; Hsf, heat-shock factor; HSP, heat-shock protein; HT, high temperature; TF, transcription factor; APX, ascorbate peroxidase; fad7, fatty acid desaturation.

Future Research Avenues in Heat Shock Protein Studies

The synthesis of heat shock proteins for thermotolerance in plants has been extensively studied, but there are still several areas that require further research. Firstly, further investigation is needed to understand the precise mechanisms by which heat shock proteins confer stress tolerance in plants. This includes unraveling the signaling pathways involved in heat stress response and identifying the specific roles of different heat shock proteins in protecting plant cells from damage. Secondly, more research is required to explore the potential of manipulating heat stress transcription factors to enhance the synthesis of heat shock proteins in plants. By

utilizing genetic engineering techniques, researchers can potentially increase the production of heat shock proteins in plants, thereby improving their thermotolerance. Additionally, the use of proteomic approaches can provide valuable insights into the identification and characterization of novel heat shock proteins and their specific functions in thermotolerance. In conclusion, genetic engineering techniques have shown promise in enhancing thermotolerance in plants by increasing the synthesis of heat shock proteins.

II. Conclusion:

Heat Shock Proteins and Plant Thermotolerance

In conclusion, the synthesis of heat shock proteins is an important mechanism in enhancing thermotolerance in plants. Genetic engineering techniques have been successful in increasing thermotolerance in various plant species by enhancing the synthesis of heat shock proteins.

The positive correlation between the levels of heat shock proteins and stress tolerance in plants has been reported by several studies. These studies suggest that the levels of heat shock proteins play a crucial role in protecting plants from heat stress and improving their ability to tolerate high temperatures.

It is still unclear about the precise mechanisms by which these heat shock proteins confer stress tolerance in plants. Further research is needed to unravel the signaling pathways involved in heat stress response and to identify the specific roles of different heat shock proteins in protecting plant cells from damage. Moreover, exploring the manipulation of heat stress transcription factors to enhance the synthesis of heat shock proteins in plants holds potential for improving thermotolerance. Proteomic approaches can provide valuable insights into the identification and characterization of novel heat shock proteins and their specific functions in thermotolerance.

Overall, the synthesis of heat shock proteins in plants is a critical aspect that contributes to their thermotolerance. Utilizing genetic engineering techniques to increase the production of heat shock proteins in plants offers a promising avenue for improving thermotolerance. Overall, the manipulation of heat shock protein synthesis in plants through genetic engineering and proteomic approaches offers promising avenues for enhancing thermotolerance in plants.

Overall, more research is needed to fully understand the mechanisms and potential for enhancing thermotolerance in plants through the synthesis of heat shock proteins. In conclusion, while there have been positive correlations between the levels of heat shock proteins and stress tolerance in plants, the precise mechanisms by which these proteins confer thermotolerance are still not fully understood^(Panwar & Srivastava, 2012). Despite the positive correlations between the levels of heat shock proteins and stress tolerance in plants, the precise mechanisms by which these proteins confer thermotolerance are still not fully understood. Further investigations are required to determine the signaling pathways involved in the heat stress response and to elucidate the specific roles of different heat shock proteins in protecting plant cells from damage.

References

- [1]. Abdul-Baki, A. A. (1991, November 1). Tolerance of Tomato Cultivars and Selected Germplasm to Heat Stress. <https://scite.ai/reports/10.21273/jashs.116.6.1113>
- [2]. Burke, J.J., Hatfield, J.L., Klein, R.P., Mullet, J.E., 1985. Accumulation of heat shock proteins in field-grown cotton. *Plant Physiol.* 78, 394–398.
- [3]. Chang, Y.Y., Liu, H.C., Liu, N.Y., Chi, W.T., Wang, C.N., Chang, S.H., Wang, T.T., 2007. A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. *Plant Physiol.* 143, 251–262.
- [4]. Chen, W.Q.J., Zhu, T., 2004. Networks of transcription factors with roles in environmental stress response. *Trends Plant Sci.* 9, 591–596.
- [5]. Clos, J., Westwood, J.T., Becker, P.B., Wilson, S., Lambert, K., Wu, C., 1990. Molecular cloning and expression of a hexameric Drosophila heat shock factor subject to negative regulation. *Cell* 63, 1085–1097.
- [6]. Coito, J.L., Rocheta, M., Carvalho, L.C., & Amâncio, S. (2012, May 7). Microarray-based uncovering reference genes for quantitative real time PCR in grapevine under abiotic stress. <https://scite.ai/reports/10.1186/1756-0500-5-220>
- [7]. Dat, J.F., Foyer, C.H., Scott, I.M., 1998. Changes in salicylic acid and antioxidants during induction of thermotolerance in mustard seedlings. *Plant Physiol.* 118, 1455–1461.
- [8]. Ishii, A., Kawai, M., Noda, H., Kato, H., Takeda, K., Asakawa, K., Ichikawa, Y., Sasanami, T., Tanaka, K., & Kimura, Y. (2018, February 8). Accelerated invagination of vacuoles as a stress response in chronically heat-stressed yeasts. <https://scite.ai/reports/10.1038/s41598-018-20781-8>
- [9]. Pérez-Clemente, R.M., Vives-Peris, V., Zandalinas, S.I., López-Climent, M.F., Muñoz, V., & Gómez-Cadenas, A. (2013, January 1). Biotechnological Approaches to Study Plant Responses to Stress. <https://scite.ai/reports/10.1155/2013/654120>
- [10]. Panwar, J., & Srivastava, J.P. (2012, January 1). Pulse productivity : physiological constraints /. <https://scite.ai/reports/10.5962/bhl.title.152699>
- [11]. Zhu, Y., Zhu, G., Q. G., Zhu, Z., & Zuo-yi, L. (2013, October 15). A Comparative Proteomic Analysis of Pinelliaternata Leaves Exposed to Heat Stress. <https://scite.ai/reports/10.3390/ijms141020614>
- [12]. Jespersen, D., & Huang, B. (2015, February 1). Proteins associated with heat-induced leaf senescence in creeping bentgrass as affected by foliar application of nitrogen, cytokinins, and an ethylene inhibitor. <https://scite.ai/reports/10.1002/pmic.201400393>
- [13]. Mallick, B., Kumari, M., Pradhan, S.K., Parmeswaran, C., Naresh, P., & Acharya, G. (2020, June 22). Genome Wide Analysis and Characterization of Heat Shock Transcription Factors (Hsfs) in French Bean (*Phaseolus Vulgaris* L.). <https://scite.ai/reports/10.21203/rs.3.rs-36054/v1>