Exploration of high Temperature stress responsive genes in potato response to tuberisation using yeast functional screening

A Report Submitted

In partial fulfillment of the requirements for the award of degree of

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In

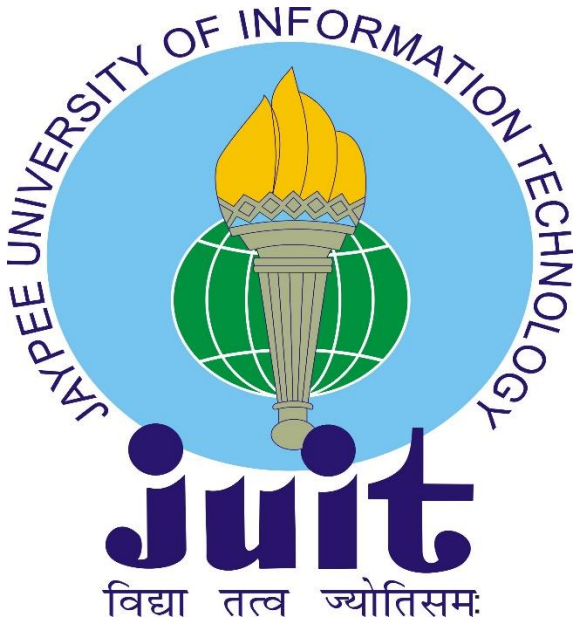
Biotechnology

By

Tashil Sharma

UNDER THE GUIDANCE OF

Dr. Anil Kant Thakur



JAYPEE UNIVERSITY OF INFORMATION AND TECHNOLOGY, WAKNAGHAT

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CERTIFICATE OF ORIGINALITY

This is to certify that the work submitted in this report entitled: **"Exploration of high Temperature stress responsive genes in potato response to tuberisation using yeast functional screening"** submitted by **Tashil Sharma** in partial fulfillment of the requirements for the award of degree of Masters of Technology in Biotechnology, of **Jaypee University of Information Technology, Solan**, has been carried out under the supervision of **Dr. Anil Kant Thakur and Dr. Sundaresha Siddappa**. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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ABSTRACT

Potato (*S. tuberosum*) is extremely sensitive to high temperature, a slight increase in optimum temperature may lead to severe decline in the tuber yield. Although, many improvements and progressions were made in the breeding of thermo-tolerant varieties of potato, the mechanism at molecular level which leads to thermo-tolerance is not clear and also not properly understood. The primary phase to understand the thermotolerance progression and processes is to extract and pinpoint the genes of utmost importance that are involved in the same. In this research methodology yeast-based efficient screening and functional elucidation method was used to identify and then further characterize and categorize genes of Kufri surya which is a heat tolerant variety. Two cDNA expression libraries were constructed from thermally stressed potato plants (35 °C) at 0h, 17 days and 45 days of treatment. No probable candidate genes were identified based on this method because of manual and technical errors in the ongoing process and superior capability of yeast cells to over-express the heterologous potato cDNA sequences to tolerate heat stress. However, the experimentation will be continued in order to extract the results.

CHAPTER 1

INTRODUCTION

Potato is one of the most important food crop in the world majorly grown after rice and wheat which is grown under temperate climatic conditions, billions of worldwide population consume potato, and its global production surpasses millions of tonnes per year [1]. The crop best grows in cool and seasons free of frosts and its performance decline in heat. This makes the crop predominantly susceptible to high temperatures which is well-thought-out to be the most critical and overpowering factor which affects majorly the yield and growth. Potatoes harvests a fibrous rich root system. The growth of roots occurs when soil temperature is between 10 to 35˚C, but most dynamic development of the root system is when soil temperature is between 15 to 20˚C. Leaf progression happens to be at temperatures between 7 to 30˚C, but ideal growth temperature is assumed to be 20 to 25˚C. Same is considered ideal for stolon growth [2]. The tuber part of a potato is an enflamed stolon part. The tuber instigation is triggered by short day lengths (photoperiods) along with the major role of growth hormones. As cool as the soil temperature will be, more swift the tuber initiation and rise in its number will be. This will lead to formation of short stolons and shoots [3]. If the length of the days are longer it will delay the initiation of tuber and this favors growth of shoot and stolon. Tuberisation process is repressed at high temperature which will further reduce photo assimilated segregation of tubers in a significant proportions. Yet, this does not change the fact that average yield of potato at global level is very low compared to the possible yield, which is caused majorly due to the negative aspects of abiotic stresses such as low or high temperatures, salinity and drought [5]. Out of all these abiotic stresses, the stress conditions induced by high-temperature acts as chief environmental limitation that affects the growth, quality of potato and tuber yield [7].

Heat or thermal stress is well-defined as the rise in temperature afar an edge level for a certain period of time adequate in causing damage that cannot be reversed and inhibits plant progression and development. High-temperature stress can cause potential decline in probable yield of tuber by distressing seed tubers ability to sprout, inadequate induction of tubers and its development, reduced rate of photosynthesis and carbon portioning in tubers. Persistent heat stress most oftenly leads to tuber malformation either with heat necrosis of tubers internally or cracks and its progression may be under different genetic controls [2,5,7]. Transient or continuous increase in temperatures can cause variations in various biological conditions such as biochemical, physiological and morpho-anatomical changes in plants, which can further affect plant growth and development and can reduce the yield economically. Such restraints will worsen the conditions in future because of deteriorated environmental changes that cause global warming [4]. It is predicted that upgraded thermotolerance of potato may significantly uplift its production. Using advanced genetic approaches, crop with better quality can be produced and the opposing effect of heat stress can be [6]. Therefore, getting well acquainted with biological reactions of potato plants and studying their response towards high temperature is essential.

Even after quite a few improvements in refinement programs for heat tolerant variety of potato, molecular processes that involve thermo-tolerance is not well understood. The foremost phase on the way to consider the thermo-tolerance process is to recognize the significant genes that are convoluted in it. Crop plants with enhanced stress tolerance have been tried to be achieved using traditional approaches, and till date have met with partial attainment, this is as a result of the problems and difficulties in incorporating tolerance associated with the characteristics from the plant background which is highly diverse. Several micro arrays and variable presentable studies indicated that majority of genes play an important role in abiotic stress

responses in many crops. Unfortunately, the precise and efficient effect of the genes throughout such conditions could not be extracted using these methods, this is because of the complexities in the responses of crops towards different stress conditions. Hence, molecular approaches have been given much importance as it is expected to improve the ability of plants to survive under such stress conditions. Current methodologies projected to focus on gene identification related with abiotic stress resistance, drought and salinity followed by alterations in genetic material of the genes expressed in plants and permitting to endure constricting enforced growth by unfavorable and critical environmental conditions. Henceforth, microbial efficient screening of microorganisms to understand expression and its level is an influential means for gene identification with definite purpose and utilities, which is independent of the parameters of their expression. The use of yeast or E.coli functional screening for expressing plant cDNAs has been used for functional revelation of the genes inducing stress, to comprehend the processes at molecular level to improve stress tolerance, using gene manipulation techniques [21].

CHAPTER 2

LITERATURE REVIEW

The E. coli or yeast screening to assess the expression of plant cDNAs was effectively used to recognize genes that show their involvement in tolerating stress. Functional microbial screening that consists of differentially expressed genes are effective, powerful and rapid tool, to detect stress tolerant genes in plants [8]. Jatropha curcas which is an impending source of biodiesel plant, to develop and escalate its production under stress environments to get advantage in commercial plantations, mining of genes [9]. It was commenced to identify the unique genes expressed in stress conditions in this plant that have the ability to be exploited to boost its tolerance towards trauma. Expression libraries of cDNA were created from roots strained with salt of J. curcas, synchronized under the control and regulation of the yeast system, to identify the genes that are expressed during saline tolerance [10]. Using an imitation model based screening method, approximately, Thousands of transformants of yeast were screened to identify the same that express differential sequences of genes of the plant having more capability in stress tolerance. Full length genes from the same were obtained that confirmed their involvement in tolerance. Along with this, optimization of surroundings for salt stress was done, considerations were salt stress in expression system of yeast were defined, and further three salt hypersensitive yeas strains were isolated through random mutagenesis. Moreover, phenotypes that were sensitive towards salt were demonstrated, and analyzed the expression patterns of the genes obtained from the analysis in both root and leaf tissues after treatments with salt [11].

Similarly, Mobilization of the plant growth hormone abscisic acid has been recognized, though, the transportation process at molecular level is not completely understood. By the use of an improved yeast two-

hybrid system, Arabidopsis cDNA was marked off and had the ability to induce interactions between the receptors of Abscisic acid receptors and protein phosphatase under low concentrations of the hormone [12].

In addition members of the importers as potential candidates for ABA importers were identified. Transport analyses in insect cells and yeast validated the candidate IMPORTING TRANSPORTER 1, which was further characterized and then facilitates uptake of ABA [13]. Remarkably, the inflorescence stems of nrt1.2/ ait1 showed lesser external temperature than compared with those of the WT. Promoter activities were also detected in inflorescence stems, leaves and roots around vascular tissues [14,15]. This information suggested that the function of an importer of ABA at the site of its biosynthesis is significant for the regulatory parameters of stomatal aperture during the process of inflorescence [16].

In another study this method was used to identify, classify and characterize potato genes with ability to provide tolerance against heat stress [18]. Expression libraries using cDNA from heat-stressed potato plants (35 °C) after treatment 2 and 48 h respectively, was constructed. Many genes were recognized on the basis of yeast cells ability to over-express differential cDNA sequences of potato to tolerate thermal stress [21].

Further cross-resistance investigation of the resultant clones in response to other abiotic stresses was also performed that showed that some genes responded to salt, few of them to drought and others to heat/salt/drought stresses. Comparison of the genes were made with reported whole potato transcriptome data which revealed that most of them had fluctuating expression arrangements under drought, salt and heat stresses [19,20]. These arrangements were then confirmed by selecting random genes and then analyzing there expression under various stress conditions using molecular biology techniques. Gene ontology (GO) improvement investigation of all the genes directed that major number is involved in signal transduction, protein folding, various cellular metabolism, response to stress, signifying their promising role in providing

tolerance against heat in potato. This resulted in the genes identification and this can be used as an input through modifications engineering heat tolerance and other broad spectrum abiotic stresses in potato and other plants [21].

The other study using this method was done to confer salt stress tolerance in plant *Salicornia europaea*. Salinity is an ecological aspect that undesirably affects growth and productivity of crops. Halophytes have developed several mechanisms to acclimatize to saline environments. *Salicornia europaea* is one of the plant species which has the ability to resist high salt concentrations. Therefore, it is considered as a decent model to study the salt tolerance mechanism because they do not possess special glands or bladder that secretes salt. This technique was used to identify genes that encodes for special proteins to facilitate tolerance against salt. The genes are predicted to encode novel proteins, unique coiled-protein with unknown function and unique short peptide of 30-35 residues. Use of a synthetic peptide exogenously, reliable to the remains, enhanced *Arabidopsis* ability to tolerate salt. These genes may have applications for enlightening tolerance of crops towards salt by genetic transformation methodologies [22].

Identification of most important heat tolerant genes in plants is important for the swift advancement of its stress-tolerant varieties. Expression library was created from hyper-osmotic stressed potato plants. The transformants of yeast expressing different cDNAs were selected because of their ability to persistence in hyper-osmotic stress conditions [23]. The tolerances of the selected yeast transformants with several abiotic stresses was also studied and compared. Specific potato cDNAs expressed in the tolerant yeast transformants were identified [24,25]. Many genes were found proficient of enhancing hyper-osmotic stress tolerance of yeast. According to the data generated on the basis of relative, twelve genes were carefully chosen, which

could have a prominent effect in providing tolerance to potato with better ability to survive in heat and salt stresses [25].

This strategy was also applied to elucidate functional aspect from another variety that is salt tolerant called Pokkali, library of cDNA was constructed. This approach enabled the yeast cell to grow under the suitability of a cDNA clone under certain defined conditions specifically in context to salt. Using BLAST tool and the generated sequences search the specific gene mannose-1- phosphate guanyl transferase (OsMPG1) was identified from rice. Further using database to analyze the genome sequence more genes of this particular family mentioned above were identified. Some of the genes out of the total were able to functionally counterpart yeast mutant –YDL055C. Detailed transcript profiling of the genes was done under various altered forms of abiotic stresses (oxidative stress, salinity, cold or UV light, drought, and heat stress) [26]. The resultant genes exhibited variance in the expression system under different abiotic stresses, displaying prompt response to multiple stresses. Functional validation of the genes from the particular family was carried out by overexpressing it in the transgenic tobacco, the results showed its association in improving tolerance towards salinity stress [26].

Yeast functional assay has not only been used in studying functional expression and quantitative analysis in plants but also in humans. There are various studies reported for the use of this system in mammals as well. A Mammalian Apoptosis Suppressor that showed dreadful phenotypic characteristics was identified by functional screening in yeast. Then the phenotype was exploited to identify. By exploiting this phenotype, an inhibitor was identified [27]. Further it was seen that the inhibitor suppressed apoptosis induced by other genes or when the cells are deprived of growth factors when overexpressed in mammals. However, it was

seen that the antisense strand of the inhibitor induced apoptosis leading to become a regulator of cell death pathways.

The status of tumor suppressor gene *p53* was tested in patients with breast cancer using this approach. Mutations like missense, point mutations, insertion, deletion, nonsense, splice site mutations etc were observed. After the survival analysis the results showed that mutations in p53 gene conferred worse prognosis with major and adverse risk factors associated [28].

To study the stability and clonality of p53 gene in astrosytic tumor cells of humans, Quantitative analysis of the p53 gene mutations was done using this screening method. In many of the brain tumors conditions mutations in p53 gene was observed and further expanding the clones of tumor cells resulted in progression of tumors [29]. To get rid of this problem yeast based p53 functional system was used for analyzing the alleles of p53 mutants quantitatively. Tumor cells were derived from patients where the cell lines and the tyoe of tumor matched. The assay system showed the mutations and some did not but later on recurrence showed the growth of tumor cells which is the indication that demonstrates that mutant clone can lead to overgrowth of the tumors [30]. In some results *de novo* mutations were also seen that indicated stron selective pressure during *in vitro* establishment of cell lines when compared to *in vivo* tumor growth. This showed the practical utility of p53 functional system in studying clonality processes which then supports the hypothesis of tumor progression in mutant clones *in* [30].

Cytochrome c oxidase subunit was identified as a suppressor of induced cell death was also analysed by yeast-based functional screening. Novel suppressor associated with a particular protein was identified by constructing mammalian cDNA library and using yeast as a system for screening [31]. The overexpression

of the suppressor significantly inhibited the induced apoptosis upto a certain level in yeast and human glioblastoma derived cells. The production of reactive oxygen species was inhibited in yeast and glioblastoma cells indicating a protective effect against cell damage induced by reactive oxygen species and the free radicals generated. The results showed that yeast-based functional screening of human genes for inhibitors in yeast helped in identification of the protein that has an important role in suppressing the toxicity levels in yeast and also has a potential to protect the mammalian cells from induced programmed cell death [31].

Again in one of the studies the p5 status was measured and screened in human cell lines, where the tumor cell lines were extracted from different tumor types and normal cell lines were also taken for functional analysis of different forms of genes in yeast assay [32]. Majority of tumor cell lines expressed the mutated form of *p53* gene. More than fifty percent of cell lines were seen to be transformed in both the alleles and the cDNA of the gene was not amplified. The normal cell lines or the already preserved ones expressed mutant form of *p53* only. The mutants derived were further analyzed for growth that is sensitive to temperature. Wild type of p53 was detected in majority of tumor cells. These results showed that during immortalization and carcinogenesis, the inactivation of *p53* is the most commonly occurring genetic event [32].

Sphingosine Phosphate Lyase gene from mammals was recognized and yeast was used to study its functional expression [33]. This putative mouse gene was similar and somewhat identical to the *C. elegans* SPL gene and that of yeast. Manifestation of the mouse gene in a yeast strain, which carried deletion, of the same suggested that gene can complement the yeast defect functionally when expressed. Using extracts from the sphingosine-resistant transformants the *in vitro* evaluation of enzyme confirmed the activities of genes

encoded by this cDNA cloned into mouse. Expression of genes at different levels of tissues were analyzed using Northern Blotting. Mapping of gene to Chromosome was done using chromosomal localization [33].

CHAPTER: 3

METHODOLOGY

Plant Material

Kufri surya a heat tolerant potato variety from hilly regions was taken for the experimental studies. Phenotyping of contrasting potato cultivars for tuberisation at 24°C night temperature was done. The sampling was done at different durations of 0 hours, 17 days and 45 days.

Figure3.1: Phenotyping of Kufri surya at elevated temperature

Figure 3.2: Tuber formation at elevated temperature

Total RNA Isolation:

Total RNA was isolated from all the three samples using Trizol method. The Quality and quantity of the RNA was analyzed on 1.3% RNA agarose gel and Nanodrop respectively.

Preparation of cDNA Synthesis, using SMARTer™ PCR cDNA Synthesis Kit

SMARTer cDNA synthesis works on ***Switching Mechanism at 5' End of RNA Transcript*** (SMART). "It is a PCR based method that produces high quality cDNA from nanogram quantities of total RNA". It provides increasing yield, lower background and higher specificity. This technology is useful when we have limited amount of starting material.

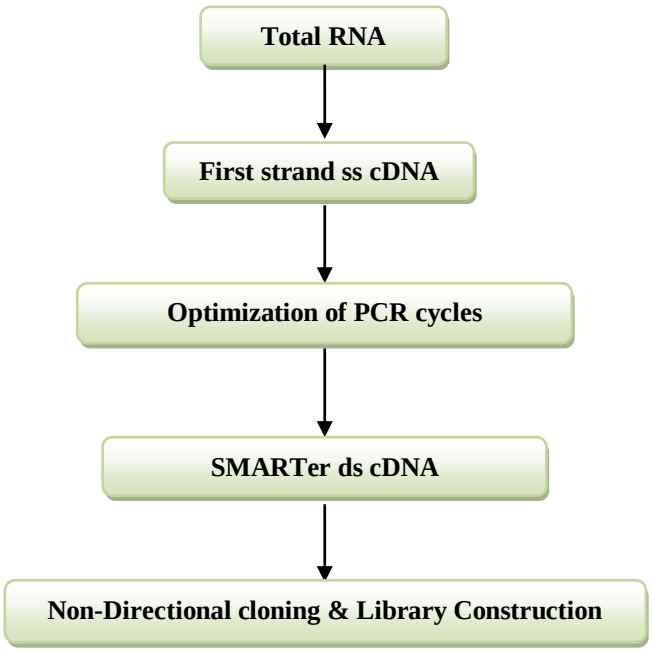

Figure 3.3: Flowchart representing SMARTer cDNA synthesis protocol for Non-directional cloning and library preparation

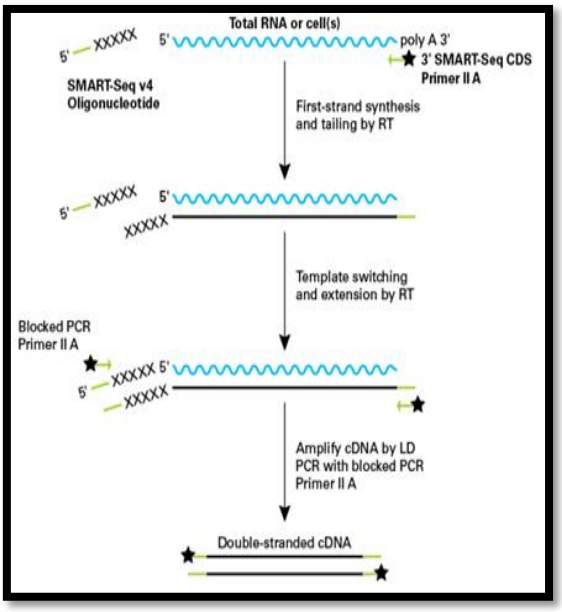

Figure 3.4: Schematic Diagram showing SMARTer cDNA synthesis

cDNA Amplification by LD PCR

The protocol was followed according to the manufacturer's guidelines. The first strand synthesis was then followed by cDNA amplification by LD PCR according to the user manual.

To determine the optimal number of cycles for our samples and conditions, we performed a range of cycles: 15, 18, 21 and 24 cycles.

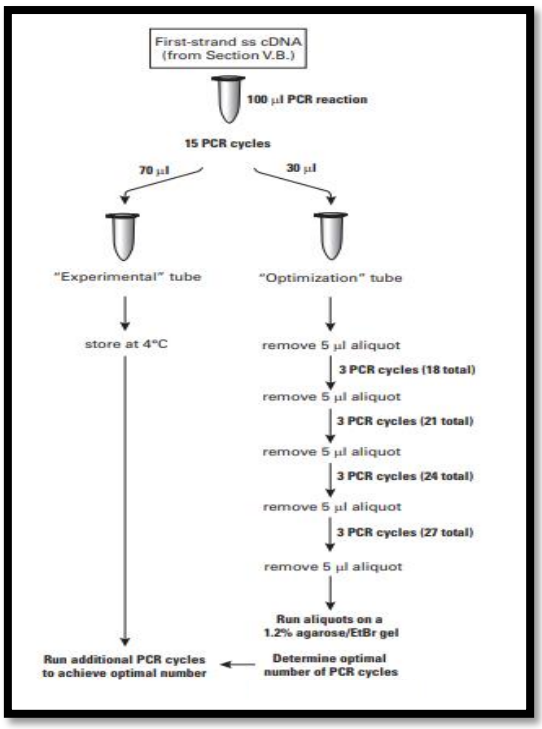

Figure 3.5: Optimizing PCR parameters for cDNA synthesis

- After optimization, the product with optimum cycles was chosen further for doubles strand cDNA synthesis. The guidelines in user manual was followed to get ds cDNA.
- Before using a yeast vector for cloning we cloned the cDNA into the pSMART2IFD linearized vector from the kit itself.
- The set up for the cloning reaction for all the three samples 0 hour, 17 days, 45 days was:

Reagents	**Volume**
5X In-Fusion HD Enzyme Premix	2µl
pSMART2IFD Linearized Vector	2 µl
cDNA	4 µl
ddH_2O	2 µl
Total Volume	10 µl

Table 3.1: Guidelines for In-fusion Cloning Reaction

- Further steps were performed according to the user manual.

Transformation of Recombinant Plasmids into E.coli

- For this Electrocompetent cells HST08 E.coli and cells grown in lab were used.

Figure 3.6: Electrocompetent HST08 E.coli cells

- 100µl of Electrocompetent cells and lab cells were added into the samples from cloning reaction after re-suspending the pellet in 5 µl of water.
- After mixing the samples, Electroporation was performed at 2.5 kV with pulse 1 for electrocompetent cells and heat shock of 1 min was given to lab cells and incubates for 1 hour at 37°C.

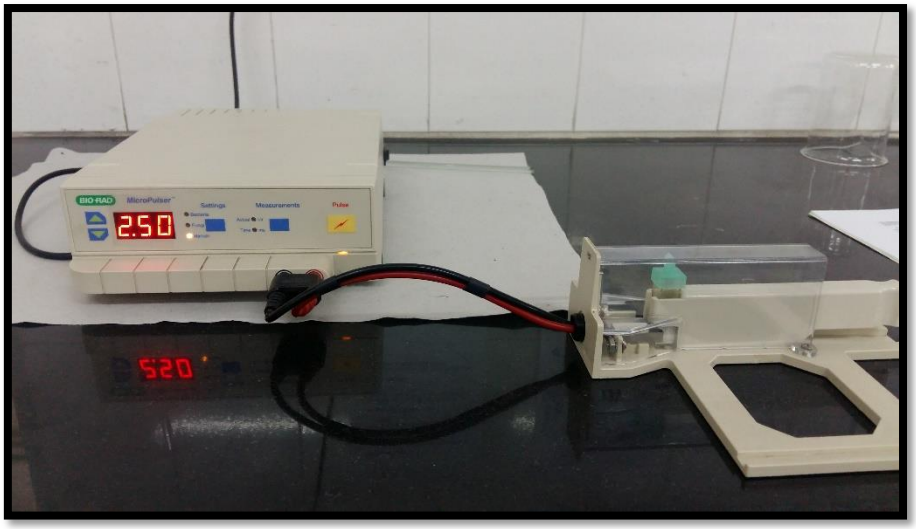

Figure 3.7: Electroporation apparatus

- The cells obtained then were spread onto the ampicillin plates and were incubated at 37°C for overnight.
- After the incubation period, the colonies were picked and streaked onto new plates for future use and also were used pooled into sterile solution of 5ml of LB broth.

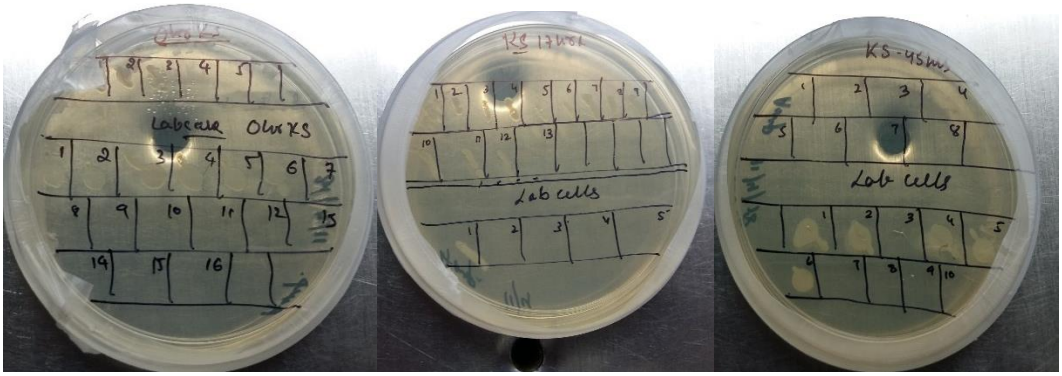

Figure 3.8: Colonies streaked on Ampicillin plates

- The 5ml LB broth tubes were incubated for overnight, and next day were used for plasmid isolation using Nucleospin ® Plasmid kit following the user manual guidelines.

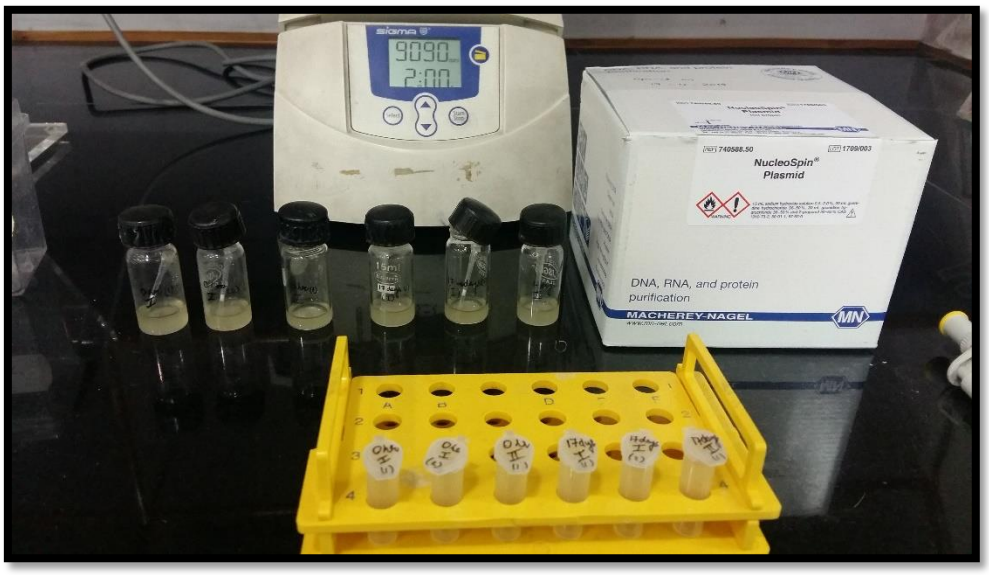

Figure 3.9: Plasmid Isolation using Nucleospin plasmid kit

- The Concentration of isolated plasmid was checked using Nanodrop
- The colonies from the plates were used to perform colony PCR.

Figure 3.10: Samples for performing Colony PCR

- The colonies showing the results were selected further for screening.
- The samples were then stored at -70°C.
- The whole procedure was again followed for cloning and transformation using pYES2 yeast vector.

CHAPTER 4

Results & Discussion

- The quality and quantity of the isolated RNA was checked using Gel Electrophoresis and nanodrop.

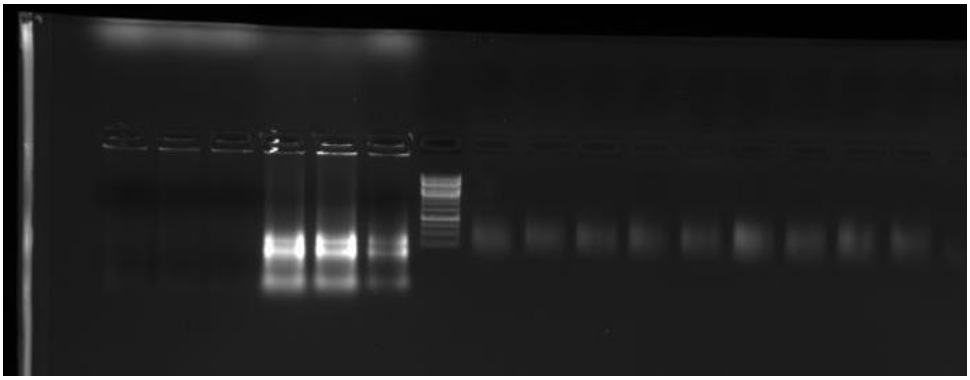

Figure 4.1: Total RNA isolation and quality check

Samples	Concentration	260/280 ratio
Kufri surya (0 hour)	879 ng/µl	1.96
Kufri surya (17 days)	1153.9 ng/µl	1.94
Kufri surya (45 days)	582 ng/µl	1.97

Table4.1: Quantity estimation of isolated RNA using Nanodrop

- After single strand cDNA synthesis, its amplification was done and optimum cycles to carry out amplification was found out to be 24 cycles.

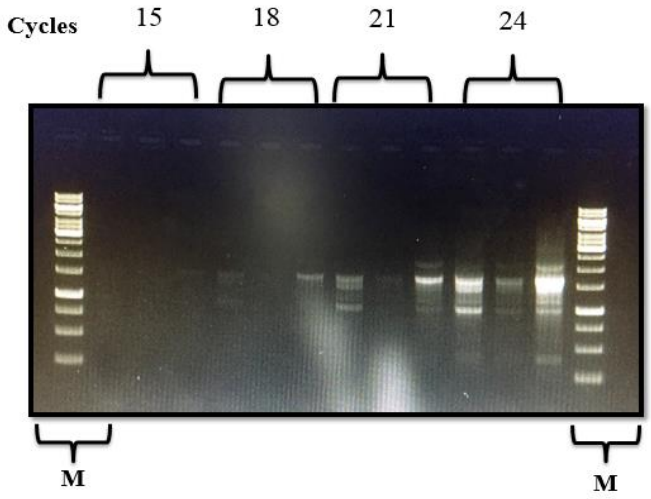

Figure 4.2: Analysis for optimizing PCR parameters: Total RNA subjected to first-strand cDNA synthesis. A range of PCR cycles performed (15, 18, 21 and 24). 5 µl of each PCR product was electrophoresed on a 1.2% agarose/EtBr gel in 1X TAE buffer following the number of PCR cycles. The optimal number of cycles determined in this experiment was 24 cycles. Lane M: 1 kb DNA ladder size markers.

- After getting the Colonies, Colony PCR was performed to check for the positive colonies showing results.
- There were more than 50 colonies we got from the lab cells and the cells used from the kit. Out of which only 9 colonies showed vector insertion.

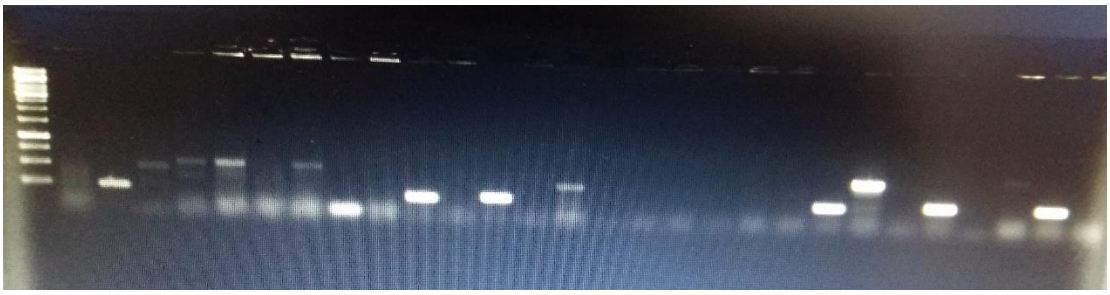

Figure 4.3: Colony PCR yielding 9 potential colonies with vector insert against 1 kb ladder.

- Plasmid was isolated successfully from the 9 samples and its quantity was checked using Nanogram.

Sample	Colonies from Lab cells	Colonies from Kit Cells
0 hour	3	1
17 days	1	3
45 days	1	-

Table 4.2: No. of colonies from each of the samples.

Samples	**Concentration (ng/µl)**	**260/280 Ratio**
Kufri surya 0 hour (lab 1)	786.9	2.12
Kufri surya 0 hour (lab 2)	948.2	2.13
Kufri surya 0 hour (lab 3)	803	2.12
Kufri surya 0 hour (kit 1)	920	2.12
Kufri surya 17 days (lab 1)	641	2.02
Kufri surya 17 days (kit 1)	1101	2.11
Kufri surya 17 days (kit 2)	1157	2.12
Kufri surya 17 days (kit 3)	1166	2.13
Kufri surya 45 days (lab 1)	404	2.07

Table 4.3: Quantity check using Nanodrop

Discussion

The Screening was performed but we could not elucidate any sort of results.When we did the Literature mining, we found in some papers that the vector has to be linearized. So, we linearized the vector using Primer-1: 5’ TTGATACCACTGCTTAGGGCGAGCTTAATATTCCCT 3’ and Primer-2: 5’ TCTCATCGTACCCCGCTCCTCGGTCTCGATTCTACG 3’ (10 µM each). But we could not come to any conclusion as we were not able to see any amplification using PCR.

In some literature the vector was being used as it is because it is said to be already linearized according to the kit. Still, no result could be elucidated. This might be due to any sort of handling errors, inappropriate concentration of sample or chemicals, improper insertion of vector into the cell, contamination etc.

Since, this was a time bound project and also performed in other institutional premises, there were many obstructions while working on the same for several months. There were many technical issues which hindered heat shock process. No availability of some biochemical compounds to carry on the particular process and many more. But, this is not the end to this project, as I have developed keen interest in this project, I will be continuing to work on the same, till I am able to extract possible outcomes out of it.

CHAPTER: 5

CONCLUSION

The approach described in the report will deliver a fast and complete assay system for large scale screening wide range of genes for diverse abiotic stress tolerance in short durations. Through the utility of above mentioned screening strategy, we were not able to isolate genes with already known function in stress tolerance and novel sequences with known or unknown functions in heat stress tolerance from Kufri surya. However, Yeast-based screening method is an efficient method to extract and identify probable genes that can tolerate or show tolerance to various abiotic stresses through various modifications and making use of genetic engineering methodologies. qPCR analysis in potato plants that are exposed to abiotc stresses have shown their confirmation in multiple stress-tolerance mechanisms in many studies. Though the initial steps gave us some hopes of identifying the genes responsible, but proceeding further, we could not elucidate any functional aspect due to manual or technical errors. Since, this work was one of its own kind done for the first time, it needs to be performed again to elucidate any sort of expression. Therefore, the project will be continued to elucidate the expression of those genes. If we are able to identify the genes, they will not only give us a starting point to understand the environment of molecular processes related to response of Potato towards tolerance to high-temperature stress, but will also serve as probable end point target for development of broad-spectrum abiotic and biotic stress-tolerant potato cultivars.

CHAPTER: 6

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