Identification and Evaluation of TaOSCAs in Response to Calcium Stress in Wheat

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Thesis submitted to the National University of Sciences and Technology Islamabad in partial fulfillment of the requirements for degree of MS in

Plant Biotechnology

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Dedications

To

My family and my friends without them my success would not be possible.

My mentor

Dr Azmat Ullah Khan, who guided & motivated me throughout my academic career.

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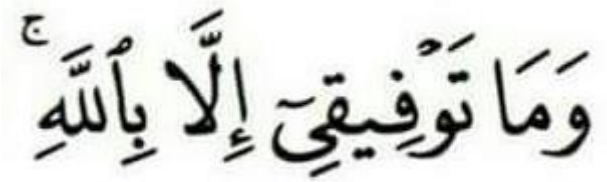
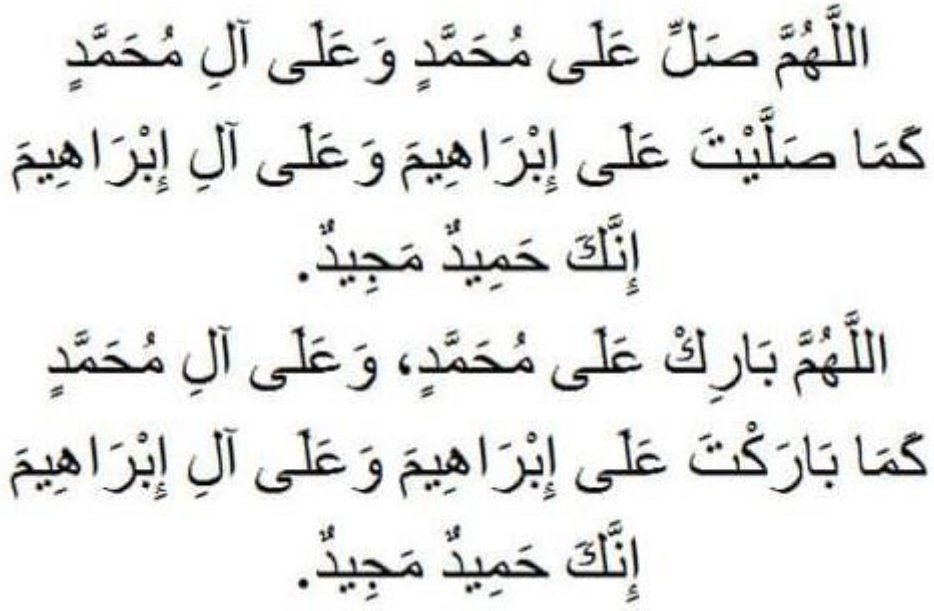
All praises & glory to **Almighty Allah (S.W.T)**, the most beneficent and merciful. Because of His help and blessings that I was able to complete my research work.


وَمَا تَوْفِيقِيٓ إِلَّا بِٱللَّهِ ۚ

"My Success is Only by Allah"

Durood-o-Salaam to our beloved Prophet **MUHAMMAD** ﷺ (PBUH), the last prophet of Allah and the prophet who had brought us from the darkness to the lightness.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كَمَا بَارَكْتَ عَلَى إِبْرَاهِيمَ وَعَلَى آلِ إِبْرَاهِيمَ
إِنَّكَ حَمِيدٌ مَجِيدٌ.

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List of Abbreviations

ACT	**Actin**
BLAST	Basic local alignment search tool
Bp	Base pair
cDNA	Complementary DNA
Ca-ClC	Calcium-dependent Chloride
CSCs	Ca^{2+}- permeable stress gated cation
DACC	Depolarization activated cation
DEPC	Diethyl Pyrocarbonate
DTT	Dithiothreitol
dCT	Delta CT
DNA	Deoxy Ribonucleic Acid
dNTPs	Deoxynucleotide Triphosphate
FAO	Food and Agriculture Organization
HACC	Hyperpolarization activated cation
HEDTA	Hydroxyethyl Ethylenediamine
IRSA	Indus River System Authority
IWGSC	International Wheat Genome Sequencing Consortium
MES	2-(N-morpholino) ethanesulfonic acid
mg/L	Milligram/Liter
mL	Milli Liter
mM	Milli Molar
MEGA	Molecular evolutionary genetic analysis
MSU	Michigan State University Rice Genome Annotation

	Project
OSCAs	Hyperosmolality-gated calcium permeable channels
PGSB	Plant Genome and System Biology
qRT-PCR	Quantitative real time polymerase chain reaction
RAP	Rice Annotation Project
ROS	Reactive oxygen species
Rpm	Revolution per Minute
UV	Ultraviolet
USDA	United States department of agriculture
VICC	Voltage independent cation channels
µM	Micro Molar

Abstract

Wheat is having ascendancy order in grains production around the world. As highly dominating and most widely grown cereal in the world. It is also an important source of carbohydrates and essential amino acids. Calcium being an essential macromolecule for the plants, is very important for different biological mechanisms as involved in the cellular membranes formation, acting as counter-cation for organic and inorganic anions, act as obligate intracellular messenger for the coordination of environmental changes and important role in signaling of developmental process. In present research work, BLAST searches were conducted in different databases for the retrieval of OSCAs gene family of selected wheat variety. Identification of OSCAs was done and phylogenetic analysis were conducted in economically important members of *Poaceae, Brassicaceae* and *Malvaceae* family. Plants form selected wheat variety were grown under different treatments (Calcium deficiency, normal and toxicity) by using hydroponic system to determine the effects of calcium deficiency and toxicity on plant phenotype. Furthermore, the plants (individually shoots & roots) were used to check the expression level of OSCAs. Insilco studies revealed, there were 10 OSCAs (*TaOSCA.1, TaOSCA.2, TaOSCA.3, TaOSCA.4, TaOSCA.5, TaOSCA.6, TaOSCA.7, TaOSCA.8, TaOSCA.9, TaOSCA.10*) exist in *Triticum aestivum*. Phylogenetic analysis revealed high evolutionary relatedness among OSCAs in different plant family members. Phenotypical analysis was resulted chlorosis in leaves & low growth in plants those were facing calcium deficiency (0mM Ca). Similarly, growth was inhibited in plants those were facing toxicity (8mM Ca). Expression analysis of all TaOSCAs was resulted, during calcium stress conditions TaOSCAs were regulated. 7 (*TaOSCA.1, TaOSCA.2, TaOSCA.4, TaOSCA.5, TaOSCA.7, TaOSCA.9, TaOSCA.10*) out of 10 have shown regulation indicating the presence of these protein transporters across cells of roots and shoots in wheat. Expression analysis further revealed the presence of these proteins may

working as channels or carrier for the transport of calcium. OSCAs work as mechanoreceptors, identification of TaOSCAs can be further used for the development of improved wheat variety.

1. Introduction

Highly dominating and most widely grown cereal crop in the world is wheat (*Triticum aestivum*). As cereals are the most fundamental and essential members of the well-known grass family that having long stalks and rich in starch due to which these are used as raw material for many other products. (Sarwar et al., 2013)

Due to the importance of grains, wheat has an ascendancy order in production around the globe and almost has 20% major role in feeding the world. Wheat is not only a staple food for human but also important for livestock feed. Other than this, it is also important source of essential amino acid (Phenylalanine, glutamic acid and proline etc.), minerals (calcium, manganese, iron, phosphorus, folate, and selenium etc.), vitamins, beneficial phytochemicals and dietary fiber components (Shewry, 2009)

In World wheat market during 2015-2016, total production was 737.2 million tones, in 2016/17 it was 761.4 million tones, in 2017/18 it was 760.0 million tones, in 2018/19 it was 731.6 million tones and currently in Feb 2020 it was recorded as 763.4 million tones (FAO, 2019/20). 263.4 million tones are contributed by major countries of Asia like China (Mainland, India, Pakistan, Bangladesh and Nepal etc.). Among all of them, wheat production of Pakistan was 25.2 million in 2018 and 25.6 million in 2019 (FAO, July 2019) (USDA, 2019) (http://www.fao.org/worldfoodsituation/csdb/en/) 10/01/2020.

Wheat is grown in Rabi season, word Rabi derived from Arabic meaning “spring” that is the time period from November to April in South Asia. In Pakistan like all other Rabi crops, wheat is sown in mid of November after monsoon and harvested in April or May (https://agrinfobank.com.pk, May 2013). Wheat is grown in all provinces of Pakistan and contribute 19.8% to the GDP (Ali et al., 2017). About 80% of the farmers cultivate a total of about 9.00 million hectares (ha) that is 40% of the cultivated land. Wheat cultivated area that

is contributed by Punjab is 6.5 million hectares, Sindh having 1.16 million hectares, KPK 0.75 million hectares and Baluchistan 0.38 million hectares (USDA, 2019).

Figure 1.1 represent the yearly yield produced wholly from 2009 to 2018 in Pakistan showing increase in production because of increase in demand. (FAO, 2019).

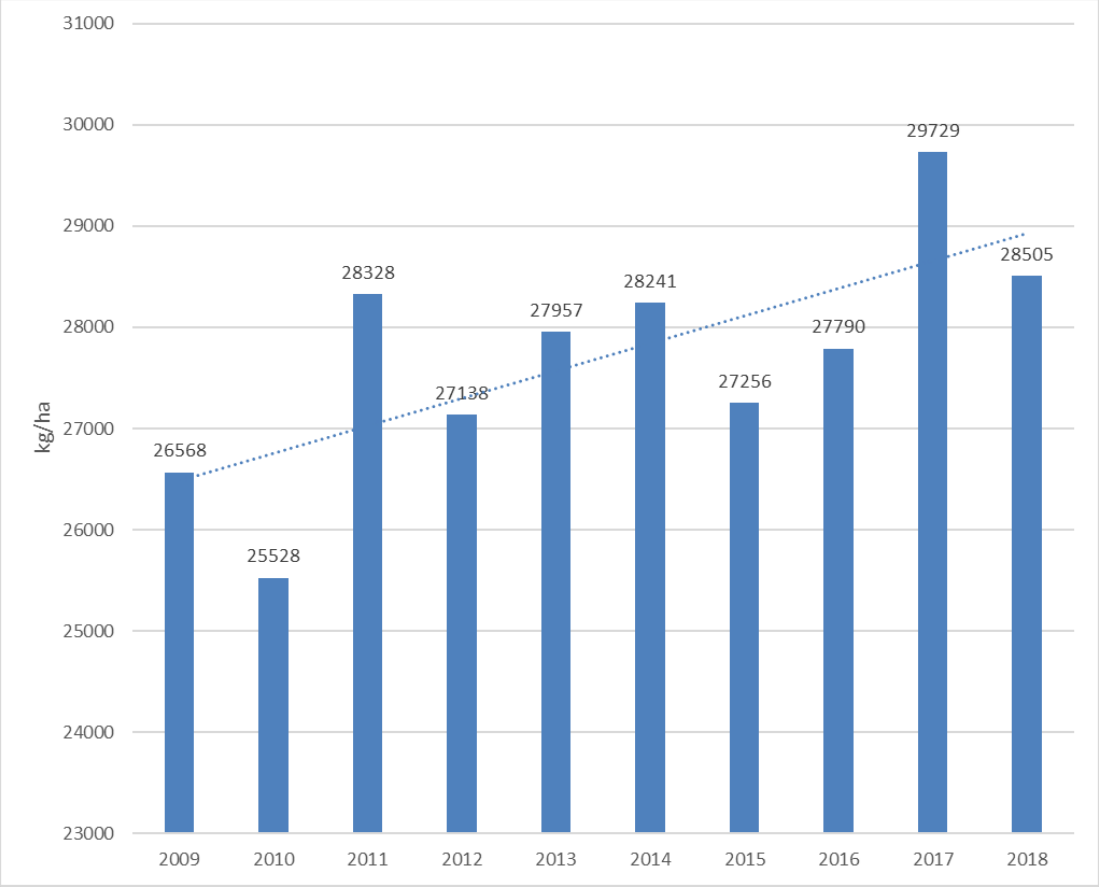


Figure 1.1: Timeline of wheat yield in Pakistan

1.1. Classification & Origin of modern Wheat:

There are many other species having genus *Triticum* (*T. aethiopicum, T. durum, T. urartu T. spelta* and many others*)* but most widely grown is *Triticum aestivum* (Common wheat). Wheat belongs to *Poaceae* (Grass family also known as *Gramineae*), Order-*Cyperales*, Subclass-*Commelinidae*, Class-*Liliopsida* (Monocotyledons) Division-

Magnoliophyta (Flowering plants), Super Division-Spermatophyta (Seed plants), Subkingdom-Tracheobionta (Vascular plants) and belongs to kingdom plantae (USDA, Plants classification report).

Present wheat (*Triticum aestivum)* is evolved by proceeding of two polyploidization between *Triticum urartu* (AA genome) and Aegilops speltoides-related species (BB genome) 500,000 (0.5 million) years ago. Hybridization gave *Triticum turgidum spp. deccocoides* and other related species. Among them *Triticum turgidum spp. durum* having genome AABB produced that further hybridized with *Aegilops tauchii* that gave DD genome 10,000 years ago and forming hexaploid *Triticum aestivum* having AABBDD genome (Feldman etal., 1995; Huang etal., 2002). Wheat is an allohexaploid having 21 pairs of chromosomes composed of 7 homologous chromosomes (A1, B1, D1, A2, B2, D2, A3, B3, D3, A4, B4, D4, A5, B5, D5, A6, B6, D6, A7, B7, D7) (Haider,2012). As in given figure 1.2 is given.

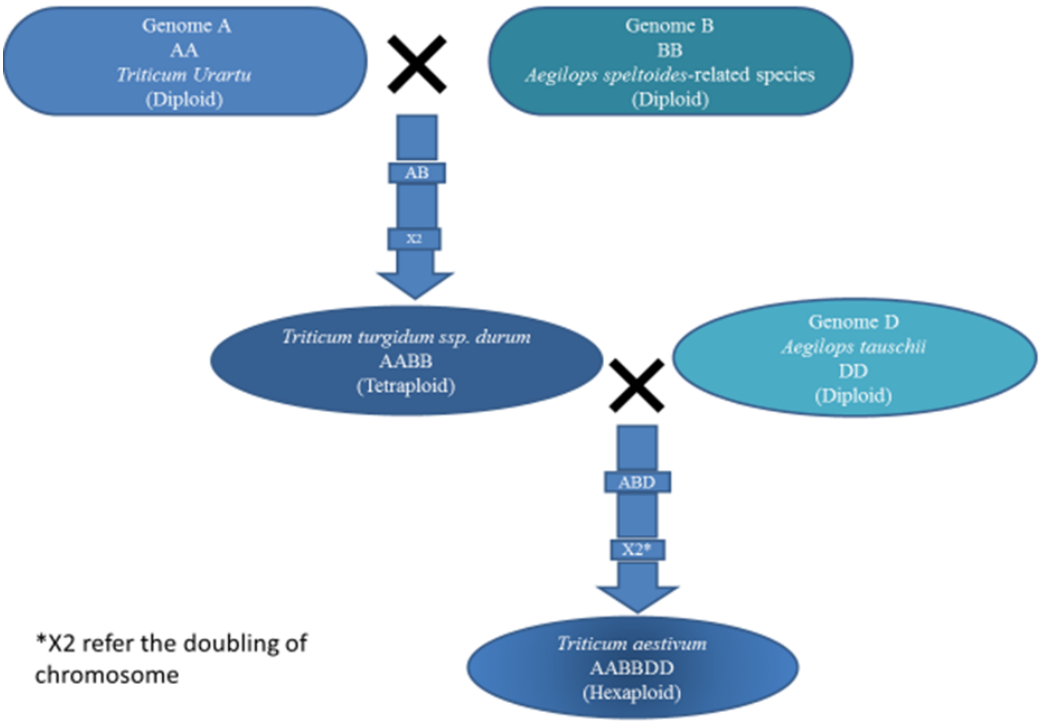


Figure 1.2: Evolution of modern hexaploidy (*Triticum aestivum*) through domestication

1.2. Genome Sequence and Databases:

Wheat genome has been sequenced and extensive genome was released in 2012 (Brenchley et al.,2012) This information provides an opportunity to have novel insights into origin of wheat (IWGSC, 2014). All possible data is available on URGI (Unité de Recherche Génomique Info) (URGI,2020), UniProtKB (UniProtKB, 2020), EnsemblPlant (EnsemblPlant, 2020 and Plant Genome and System Biology (PGSB, 2020).

1.3. Biotic and Abiotic stresses:

Plants species must face a wide range of environmental stresses having huge impact on lowering the crop productivity. These stresses maybe biotic or abiotic contributing individually damaging role on crops. Fighting and resistance of the plants against these stresses is a complicated phenomenon. Occurrence of these stresses faced by plants at different stages of plant growth and development. As there is chance that one or more stresses can affects the plant. To manage these biotic and abiotic stresses plants have number of metabolic and physiological responses (Subramanyam et al., 2006)

1.4. Project aims and outline:

According to the estimation of United Nations world's population will be about 9.1 billion by 2050. More urbanization and such increase in population demanding 70% more wheat production (Peng et al.,2011). Also, the demand for the food is increasing about 1% each year in developing country as well that is an alarming situation. According to FAO GIEWS, below the average of rain fall that was coupled with low irrigation water availabilities had lowering the 9 % yield than the previous year in Pakistan. Furthermore, in December 2018 drought alert was indicated in Sindh and Baluchistan by Pakistan Meteorological Department and Indus River System Authority (IRSA) estimated 40% low irrigation causing low vegetation in all over the country concerns for the yield prospects

(FAO, 2019). As wheat has played very important role throughout the history of humanity by meeting food demands. As time passing there is a need of developing new and high yield wheat varieties that will have to fight with environmental changes and stresses (Shiferaw et al., 2013). Wheat genome can be used for the characterization of genes by the usage of different techniques. There is a huge space for the annotation of the wheat genes. For example, using Quantitative PCR we can check the expression of different genes undertaking *Brachipodium* as model organism.

Membrane system of all living plants have diversity of proteins (channels & carrier transporter) responsible for the movement of ions or other molecules across the cell. These transporters are widely distributed all the living systems and can be found in plasma membrane, mitochondria, membranes of chloroplast, endoplasmic reticulum or Golgi apparatus. There are huge number of calcium regulating channels among all living systems involved in the regulation of inter/intra cellular transportation of calcium ions e.g., as Calcium-dependent Chloride Channel (Ca-ClC), Calcium-dependent Protein Kinase and Calcium-permeable Stress-gated Cation Channel (CSC families etc. Among them Putative hyperosmolality-gated calcium permeable channels (OSCAs) are the largest protein family involved in the transportation of calcium ions. Previously this family of protein was characterized as osmosensor in *Arabidopsis* and then identify and expression profiling was done in rice, *Brachipodium* and among majority of plants. As comprehensive information about OSCAs among different genera of plants is present but identification and evaluation of this family is insufficient in wheat crop. Present information can be used for the manipulation of wheat plant to produce wheat having improved traits. Based on all available information it is hypothesized that OSCAs may also be present in wheat with some putative function. Therefore, the current research was conducted to validate this hypothesis.

1.5. Research Objectives

Following given are the aims and objectives of current research.

1) To identify *TaOSCAs* orthologues in wheat plant.

2) To conduct Phylogenetic analysis of *OSCAs* in *Poaceae*, *Brassicaceae* and *Malvaceae*

3) Evaluation of wheat *OSCAs* under Calcium stress via hydroponic system.

2. Literature Review

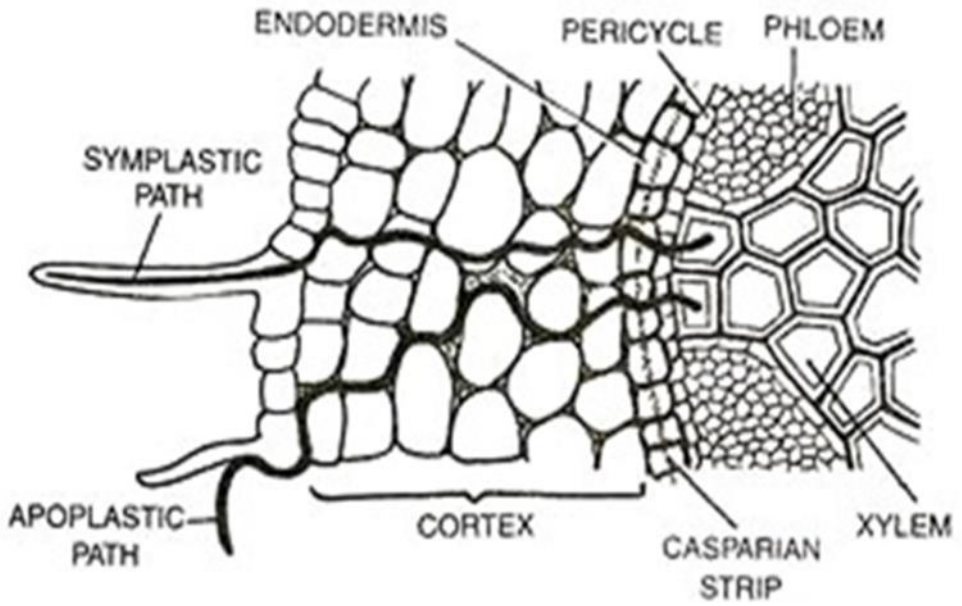
An extraordinary and magnificent attribute of cellular life is the synthesis of biomolecules and other substances by taking up of materials from the environment (air, soil or artificial supplementation). Absorption, supply and synthesis of these chemical compounds is necessary for the development and growth is coined as nutrition and the chemical compounds required are considered as nutrients. The process in which these nutrients are converted into cellular components or used for energetic purposes are called metabolic processes (K.Mengel et al, 2001). Deficiency of any of the nutrient can affect plant normal metabolic process and cause diseases like chlorosis, hidden hunger (nutrient deficiency without any morphological clues), mottling (spotting), necrosis (death of plant tissue) and stunting (shorter height) etc. (McCauley., 2011). There are about 20 essential nutrients those are required for the proper growth and development. Some of them are required in large amounts given the name macronutrients and those required in lesser quantity are called as micronutrients. As carbon (C), oxygen (O), hydrogen (H), nitrogen (N), potassium (K), phosphorous (P), calcium (Ca) magnesium (Mg) and sulfur (S) are considered as macronutrients. Remaining are the micronutrients like iron (Fe), molybdenum (Mo), copper (Cu), manganese (Mn), boron (B), zinc (Zn) and chlorine (Cl). Some of the element like cobalt (Co), sodium (Na), nickel (Ni) and silicon (Si) are not essential elements for some of the plants but not for all (Arnon and stout, 1939) (K.Mengel et al.,2001).

As these nutrients have major role in plant growth, as carbon, hydrogen and oxygen are the major constituents for the formation of carbohydrates, lipids, proteins and nucleic acid. Similarly, nitrogen and phosphorus are major constitutes of nucleotide formation that is structural and functional unit of DNA or RNA. Majority of all other micronutrients are act as cofactor/activator for different enzymes those are involved in major biochemical process like manganese that is a micronutrient activates the enzymes involved in the formation of

chlorophylls and chlorine with other micronutrients as ionic form essential for osmosis and ionic balance. So, given information results that these elements are compulsory for healthy growth of plants.

2.1. Nutrients and their uptake from the soil

In the environment of land maximum of the nutrients are dissolved in soil, ion exchange is a process in which these nutrients are made possible to reach the root cells. All essential elements are dissolved in water reach to root epidermal layer than finally entered the plant body towards xylem bundles with the help of cytoplasm of cells those are linked by plasmodesmata (symplast) or through spaces between the adjacent cells (apoplast pathway) because of the difference in water potential that is lower in soil as compared to plant body. Basic nutrients are provided by soil for the development of the plant. As many of the nutrients are limited in the soil because of the change in pH that leads towards physical and chemical reactions or it can be limited due to soil erosion also. Texture of the soil is also important factor that can also have a contribution in limiting the availability of the essential elements in soil. Similarly, effect of the climate, high moisture causing leaching of essential nutrients. Furthermore, temperature is also an important factor because in low or cold temperature the uptake of nutrients is reduced. Another phenomenon is the interaction of the elements can also affect the soil availability of the nutrients. For example, high level of calcium can cause decrease in uptake of boron (Adriano, 2001).



Pathways (Apoplast & Symplast) showing uptake of sap (Water with nutrients) from soil through root hairs (Adopted from Manisha & Kumar)

Figure 2.1: Ultrastructure of the root showing different pathways (Symplast & apoplast) whereby calcium must pass and have to reach the xylem for the transport of upper area of the plant.

2.2. Uptake of Calcium and its role

Calcium is considered as essential macronutrient and very important divalent cation Ca^{2+} for both plants and animals. In animals, it is structural part of bones and teeth, having major role in muscle contraction, involved in the nerve impulse, clotting of the blood, homeostatic balance in body cells and involved in the regulation of the heartbeat (Piste et al., 2012). Similarly, in plants calcium is required as various structural role for the formation of cell wall and membranes. In vacuole, it also acts as counter-cation for the organic and inorganic anions as well. Furthermore, calcium in ionic (Ca^{2+}) form present in cytoplasm act as obligate intracellular messenger for the coordination of environmental changes and involved in signaling of developmental process (Marschner, 1995). Naturally, deficiency of the calcium is rare because of the presence of high concentration of calcium carbonate and gypsum in

agricultural soil but beyond a critical concentration calcareous soils can exist that can restrict the growth of the plant. By following apoplast or symplast pathway, calcium is delivered to lower and aerial parts of plant by xylem bundles that is coming from roots via soil solution (White, 2001). Appropriate concentration that is required for plant in natural habitats is in between 0.1 and 5.0% (Marschner, 1995). Given values consider for both calcium availability in environment and contrasting calcium requirement of different plant species. Plant species are divided into two classes by ecologists i.e. calcifuges and calcicoles. Calcifuges are the plant species those occur on acid soils with low calcium and calcicoles are the plant occur on calcareous soils. Concentrations of calcium in both classes differ in natural habitats. Calcifuges grow better in soil having low calcium level and less responsive in higher concentration of the calcium. Similarly, calcicole plants can sustain low calcium in their natural habitat. They inhibit the growth by inducing calcium deficiency (Lee, 1999).

2.2.1. Degree of low calcium:

Abnormal and low concentration of calcium can be harmful for young leaves as they become distorted and converted to abnormally dark green, tips of the leaves become dry or brittle and, in the result, died. Furthermore, stems become weak and higher probability of poor germination (McCauley, 2011). As the calcium, part of plant cell walls and membranes, microscopic observations also done as deficiency of the calcium causing swelling of primary cell wall that become diffuse and eventually dissolve because calcium has the ability to bound pectin molecules found in the cell wall together with magnesium that having role in stability of the cell wall. Also, cells become separated that exhibiting necrosis of the cells (Bussler, 1963). In wheat roots, deficient calcium concentration beyond normal limit causing decline in growth having fall in cell elongation (Burstrom, 1952). Prolonged deficiency of calcium causing inhibition of root growth. Similarly, deficiency of calcium greatly affects

shoots and leaves. Low calcium causing reduction in the length of epidermal cells and low growth longitudinally in the plants.

2.2.2. Degree of high calcium:

High concentration of the calcium in soil don't have major effect on the plant as in most of the cases plants can manage and survived. But beyond a critical concentration, it can compete with other cations like magnesium and potassium causing their deficiencies (Buechel, 2018). Reduction in both of cations causing low growth of the plant with necrosis and chlorosis. Magnesium being a central atom in chlorophyll molecules, causing yellowing of leaf margins, yellowish-green patches will appear in case of leaves in wheat in prolonged toxicity of calcium (McCauley, 2011).

It seems fair the importance of the calcium existing in soil, in cell wall (being part of pectin and protein molecules), ion binding, plant and cells growth and interaction with other cations.

2.3. Transport for calcium in cellular membranes:

Calcium either is taken by plant body through apoplast or symplast pathway while enter plant body must cross through different protein transporters (channels and carrier) when enter or exit through cells. There are number of channels and carriers those are involved in the influx or efflux or the calcium. For example, high calcium concentration gradient requires energized active transportation of calcium that maybe catalyzed by Ca^{2+}-ATPases and H^{+}/Ca^{2+}-antiporters. Because these transporters involved in the maintenance of low calcium level in cytoplasm (Sze et al., 2000) (Hirschi. 2001).

Similarly, number of different channels that have permeability for calcium influx also been identified. Classification of these channels have been done on the voltage-dependence for example depolarization activated cation channels (DACC), voltage independent cation

channels (VICC) and hyperpolarization activated cation channels (HACC) (White, 2000) (Sander et al., 2002) (Miedema et al., 2001). Several of the calcium-permeable channels are exiting in the membranes of plastid, tonoplast (membrane of vacuole), in cisternae (membrane of endoplasmic reticulum) and majorly in plasma membranes of most of the cells.

2.4. OSCAs (Hyperosmolality-Gated Calcium permeable channels):

Studies have revealed that salt stress can induce ionic stress and osmotic stress in plants. Prolonged or severe stresses causing number of morphological changes and physiological drought that is harmful for the plant as limiting the growth and development in plants. Hypo or hyper concentration of calcium in plant body is due to insufficient information about membrane transporters existing throughout the plasma membranes and other cellular organelles. As majority of the crops are sensitive to salts, water or drought stresses. Because of these stresses and sudden change in solute concentrations causing plant membrane to open the channels and transporters for homeostasis. Continues adjustment and sudden changes causing the plant membrane to damage. In plants, animals and protists calcium is the major regulator of osmotic stress. During osmotic pressure calcium concentration increases inside the cell that is attain by calcium pumps/carrier or it may be done by calcium channels/gates. This is because of the importance of the calcium increased inside the cytoplasm called calcium signature (Knight et al., 1997). Analysis of the previous studies reveled that the hyperosmolality-gated calcium-permeable channels (OSCA) family is the largest protein family that has calcium-dependent channel for the transportation of calcium. These channels are also acting as osmosensors, when the plants are in abiotic stress like having role during drought and salinity responses (Yuan et al., 2014). Another study revealed about the similarity of these channels to TMEM channels (protein channels/ pore opening channel formed by dimers in plasma membrane involved in the translocation of Ca^{2+} and Cl^{-} ions. As OSCA and TMEM has maximum evolutionary conserved regions by showing these are like

each other (Murthy et al., 2018) (Zhang et al., 2018). Furthermore, one member of OSCA protein family indicated a role for CSCs as osmosensors. As CSCs (Ca^{2+}- permeable stress gated cation channel families) are the group of family having four different categories (Medrano-soto et al., 2018). On the other hand, CSCs have much of the similar properties to TRP channels (transient receptor potential), those are found in animals having major role as mechanosensor/osmosensors (Pedersen & Nilius, 2007; Hou et al., 2014). However, members of OSCAs protein family don't have any similarity with the TRPs (Zhang et al., 2018).

2.5. Diversity of OSCAs gene family among different genera:

Hyperosmolality-gated calcium-permeable channels (OSCA) family is encoded by number of different OSCAs genes responsible for calcium trafficking across different plant species. As 21 OSCA genes have been identified in soybean, 15 in *Arabidopsis thaliana*, 12 in maize and 11 genes in rice (Cao et al., 2020). Similarly, 35,21 and 22 OSCA genes have been identified in *Gossypium hirsutum*, *Gossypium arboreum* and *Gossypium raimondii* genomes (Xiu et al., 2019). 16 of the OSCA genes have also been identified in *Pyrus bretschneideri* commonly known as pear (Gu et al., 2018). By the application of different techniques, potential role of each protein has been identified. For example, OSCA1.1 has potential function in the involvement of magnifying dehydration and salt induced osmotic stress response in plants (Xiu et al., 2019).

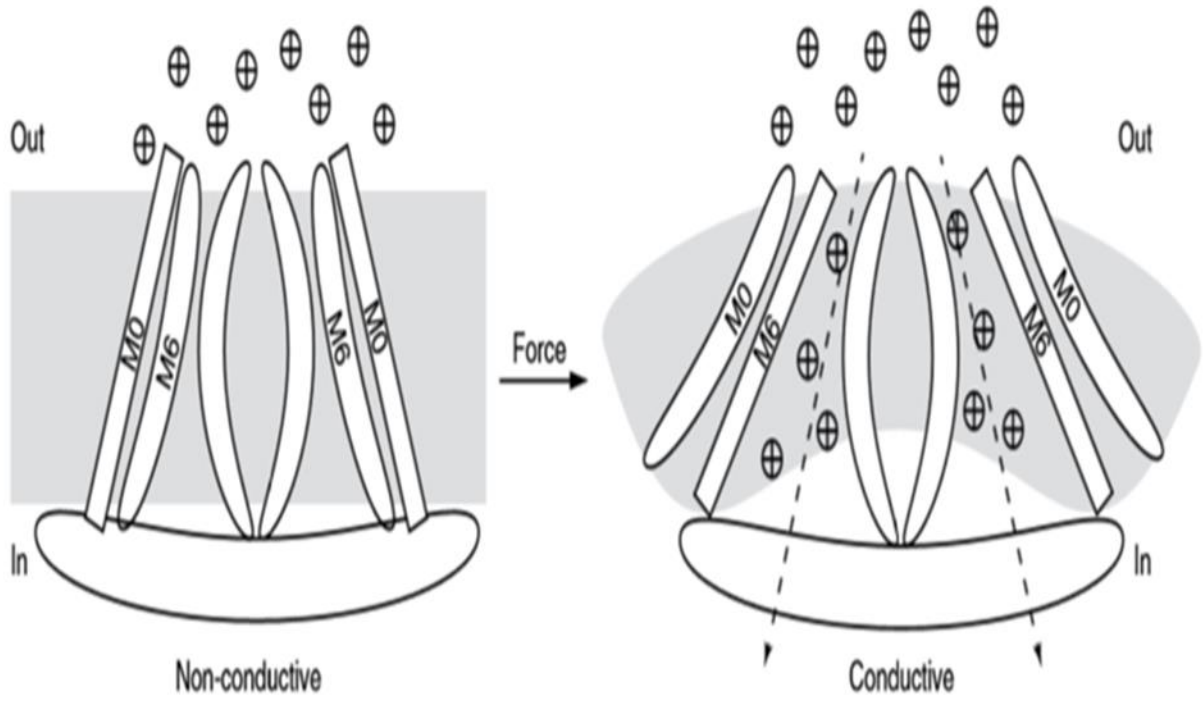
2.6. Potential role of OSCAs gene family members among different species:

A study suggested in *Arabidopsis thaliana*, different members were acting as mechanically activated ion channels those convert external forces to electrical signals means they act as osmosensor. By using forward genetic screen strategy, scientists identified OSCA1(a member out of 15) in *Arabidopsis thaliana* as, it may involve in upstream of ABA indicating the role of this member in calcium-mediated osmotic signaling in the guard cells. By the application

of different drought and salinity stress on seedlings, OSCA1 were identified as novel gene encoding a protein of 772 amino acids with the help of fine mapping. Scientists also gave the possible location of OSCA1 in plasma membrane acting as membrane channel having over expression during the high Ca^{2+} concentration externally. It was also reported that, increasing level of Ca^{2+} externally my increase the expression of OSCA1. As different biotic and abiotic stimuli set off these hyperosmolality-gated channels for Ca^{2+} uptake inside the cells and confirmation of these channels as osmosensor (Yuan et al., 2014).

2.6.1. OSCAs in Arabidopsis thaliana:

In another study, by the usage of cryo-electron microscopy scientists revealed the structure of two homologue of the OSCA in *Arabidopsis thaliana*. They concluded that OSCA channels exist in symmetric dimers having structural similarity with the mammalian TMEM16 family of the proteins (calcium-activated chloride channel). Scientists also suggested during the stress or tension conformation changes occur as, the cross-sectional area of the transmembrane expanded and these ion channel pore open and transported Ca^{2+} inside the cells (Zhang et al., 2018).



Shown the activation of AtOSCA1.1 channel in the plasma membrane change in osmolality causing movement of M0 and M6 helix of AtOSCA1.1 causing opening to transfer calcium ions inside the cell (Zhang et al., 2018)

Figure 2.2: Shown the activation of AtOSCA1.1 channel in the plasma membrane change in osmolality causing movement of helices resulting in opening and closing of calcium channels.

2.6.2. OSCAs in Oryza sativa

By using RGAP (Rice Genome Annotation Project), scientists identify 11 putative OSCA genes in *Oryza sativa* L. ssp. Japonica. To find out the evolutionary information, they constructed phylogenetic tree among different sub species of *Oryza sativa* and concluded the 11 members shared same structures with their homologs. For structural characterization of this family, four standard architectures were given among OsOSCAs as, DUF221 domain, transmembrane helices (TM) region, a coiled-coil region and the low-complexity region were most common among OsOSCAs. In some of the members transmembrane helices (TM) region were not present suggested deletion event also occurred in genomes of subspecies of

Oryza sativa. Further DUF211 was identified as having seven transmembrane domain regions of these calcium-dependent channel. Also shown homology to the anoctamin (TMEM16) in mammals and salt taste chemo-sensation transmembrane channel like protein (TMC) in *C.elegans* or TMCs of the hair cells of the inner ear. Expression analysis of the OsOSCAs were also performed by using qRT-PCR suggesting 5 out of 11 genes were expressed in all tissues indicating universal role of this family. Other members having medium or no expression in some region showing tissue-specific expression patterns. 10 of the genes involved in developmental process and seed imbibition. Further analysis was done showing the role of OsOSCAs in circadian clock as well. 9 of the OsOSCAs members were also shown down or upregulation under applying abiotic stresses (NaCl, PEG, ABA and drought) (Li et al., 2015).

2.6.3. OSCAs in Zea maize:

In case of *Zea maize*, 12 OSCAs were identified and given name as ZmOSCAs. After having phylogenetic analysis, these 12 members were classified into 4 classes and has DUF221 domain conserved in all classes randomly located on 1,3,5,6, and 8 chromosomes encoded the polypeptides of 249-810. It was also revealed under abiotic stresses (NaCl, PEG and ABA) (ABA having very important signaling hormone produced when plant faces different abiotic stresses) 11 out of 12 (except ZmOSCA3.1) ZmOSCAs genes were upregulated. As remaining (ZmOSCA3.1) gene was down regulated under PEG treatment. Further by using Yeast Two-Hybrid system and Biomolecular fluorescence interaction of the protein was also estimated because during stress proline content was also alter resulting two genes may involve in stress response. The interaction of protein was later confirmed by Yeast Two-Hybrid system. Over expression of one of the members (ZmOSCA2.4) among ZmOSCAs in *Arabidopsis thaliana* was estimated having enhancement of plant tolerance against the drought stress (Cao et al., 2020).

2.6.4. OSCAs in Gossypium spp:

Analysis revealed 35,21 and 22 OSCA genes in *Gossypium hirsutum*, *Gossypium arboreum* and *Gossypium raimondii* genomes. Why this high number of OSCAs is possibly due to plant evolution (chromosome doubling, chromosome rearrangement or due to gene loss) and given the name GhOSCAs gene family. Through phylogenetic analysis it was disclosed that the distribution of the OSCAs gene family was earlier than the monocotyledons and dicotyledons divisions which show the significant role of the OSCAs in different biological process. There were also conserved regions (conserved motifs) existed in some of the members of GhOSCAs that indicate the functional similarities in a single group. Further analysis was reported as the cis-regulatory element of the GhOSCAs involved in number of different mechanisms like low-temperature response, ABA-response, stress and defense response, heat response and many other different biological pathways. Expression profiling of the genes were also done under salt and drought stress that resulted tissue specific expression in cotton plant. Most of the genes were responsive to these stresses some were upregulated; some gave down regulation and little of them didn't show any response in some regions but perform function in other regions of the plant. By using VIGS (virus induced gene silencing), scientists produced plants having knockout one of them member of GhOSCAs (GhOSCA1.1) showing higher rate of water loss on leaves instead of wild types. This result shows the involvement of the genes in drought resistance as well. By using RT-qPCR further expression was measured showed that the member of the GhOSCAs may improve salt and drought tolerance in *Gossypium* plant species (Xiu et al., 2019).

2.6.5. OSCAs in Pyrus bretschneideri:

16 of the OSCAs genes were recognized in *Pyrus bretschneideri* (Pear) and given the name PbOSCAs gene. Comparison between different species scientists suggested OSCAs and

OSCAs-like genes expressed across in different taxa like angiosperms, algae, moss, yeast and mammals. Phylogenetic analysis resulted four different clades and showing conserved domains having activity for Ca^{2+} transport. Study suggested that 16 of the OSCA-like genes were present on 8 of the 17 chromosomes. As comparisons of the genes between same species suggested the duplication of the genes could be found in pear. Expression profiling of PbOSCAs is done by using qRT-PCR showing 14 out of 16 genes were expressing in roots as gave idea of having critical role in roots as osmosensors. Some of the other genes were also expressed in leaves, fruits and pollen. It was also given that the PbOSCAs genes were also involved in reproduction. Genes were highly responsive in roots in treated roots indicating as osmosensor involved in osmotic response (Gu et al., 2018).

3. Material & Methodology

3.1. Insilco identification of *TaOSCAs* & phylogenetic analysis:

Phylogenetic trees of *Poaceae* and other plant family were made by using MEGA 7.0 Software having Maximum Likelihood method and the Bootstrap value was kept 1000. The data of protein sequences of all OSCAs members of *Oryza sativa* has taken from The Rice Annotation Project (RAP-DB) and further cross checked by Aramamnon a plant membrane protein database and Rice Genome Annotation Project (MSU-RGAP). Protein sequences of all OSCAs of *Brachipodium* that was subjective plant has taken from Aramamnon. By using *Brachipodium*, all protein members have been blasted on UniProt database to find out the homologue sequences in other *Poaceae* family members. Data has been retrieved for ten *Poaceae* members from UniProt database. Muscle alignment is done by Clustal W and tree has been made.

3.2. Primer designing:

For the purpose of primers, all OSCAs nucleic acid sequences were retrieved from International Wheat Genome Sequencing Consortium URGI data base (IWGSC-URGI Wheat) and further confirmed by EnsemblPlants. Primers for OSCAs genes were designed by using NCBI primer blast server. Primers were set to required size base pairs in between 18-23. The GC content was set to minimum 50 and maximum 60% and melting temperature was kept in between 55 & 65. The product size of the designed primers was kept in between 130-150. As FASTA format of all OSCAs gene sequences were loaded on NCBI Primer Blast server for the retrieval of primers. Further confirmation of primers, blast analysis was performed to check specificity of each primer pair.

3.3. Physiology Experiments: Experimental evaluation of Wheat plants under Calcium stress

In current study wheat plants were full-grown hydroponically to determine the consequences of Calcium toxicity/deficiency on plant composition. Lombnaes and Singh (2013) methodology was used for growing plants hydroponically creating some modifications per the treatments.

3.3.1. Phase 1: Surface sterilization & germination of *T. aestivum* seedlings

Sterilization of the seeds were done by using given series of steps

a) Seeds were immersed in 1% bleach solution for the duration of 15 minutes and were put on the roller for equivalent mixing of seeds with bleach in a falcon tube.
b) On removing bleach solution, washing of seeds was done by using double distilled water to remove any traces of bleach. Washing was performed in Laminar flow hood supporting sterilized conditions.

Setting of seeds for germination is given below once sterilized:

a) All seeds were put into a double distilled water filled falcon tube. Falcon tube was placed on the slow moving shaker overnight in dark to break seed dormancy.
b) By placing sterilized filter paper that was previously soaked in distilled water on tray, sterilized seeds were placed on the tray with their indented line face down by using sterilized tweezers.
c) Transparent bags were used to seal out the trays for maintaining the sterilized conditions. Prepared trays were placed in the dark cupboard at ASAB Glass House at room temperature for 5 days.

3.3.2. Phase 2: Preparation of Lombnaes media preparation

Hydroponics setup was designed for the growth of wheat seedlings in Lombnaes media (Singh, 2003). Control media was used for seedlings growth for first 14 days. Preparations of stock solutions were made by using final concentrations (Table) for media preparation in sterilized equipment & glassware. HEDTA & MES were added as powdered form at the end while preparing media. One liter of Lombnaes media was added in each hydroponic pot. Media was changed every 48 hours intervals. Chemical nutrients are given in table 3.1.

Table 3.1: Compositions of stock solutions for Lombnaes media

Stock solutions (500ml each)	**Final Concentration in media**
HEDTA	100µM
MES	1mM
10 mM MnCl2.4H2O	0.6µM
1M KNO3	1mM
1M Ca(NO3)2.4H2O	2mM
80mM KH2PO4	80µM
0.5 M MgSO4.7H2O	0.5mM
75 mM Fe(NO3)3	75µM
0.9M NaOH	0.9mM

Table 3.2: Final concentration of micronutrients in Lombnaes media

Micronutrient stock solutions (250ml each)	**Final concentration (µM)**
80 mM ZnCl2	8
0.5M CuCl2	2
0.5M NiCl2.6H2O	0.1
0.5M Na2MoO4.2H2O	0.1
0.5M H3BO3	10

3.3.3. Phase 3: Exporting germinated seedlings to hydroponic pots

Well grown germinated seedlings were transferred to bottles (hydroponic pots) having 1 liter of Lombnaes media. Bottles were secured with dark tape and gap was created into the top of the bottle. Cotton plug was utilized to keep the seedlings contained in bottle. Seeds were put in such a way that the roots would project underneath in media solution and the shoots would just over the cotton plug. For assurance of appropriate air circulation in each bottle, a clear thick 2-3mm tube was emerged into the bottle. For homogenous aeration across all replicates, one end of the tube related to air compressor while other end of the tube was fed in bottle until it reaches the bottom.

3.3.4. Phase 4: Harvesting and Grouping of plants followed by treatments

Growing plant on standard Lombnaes media for 14 days, first harvesting was done by collecting 9 plants. Morphology changes were noted by taking pictures prior to harvesting and fresh weights of root and shoot is measured and preserved at -80°C dividing remaining into three groups according to the conditions were imposed like control, toxicity and deficiency as Fig 3.1.

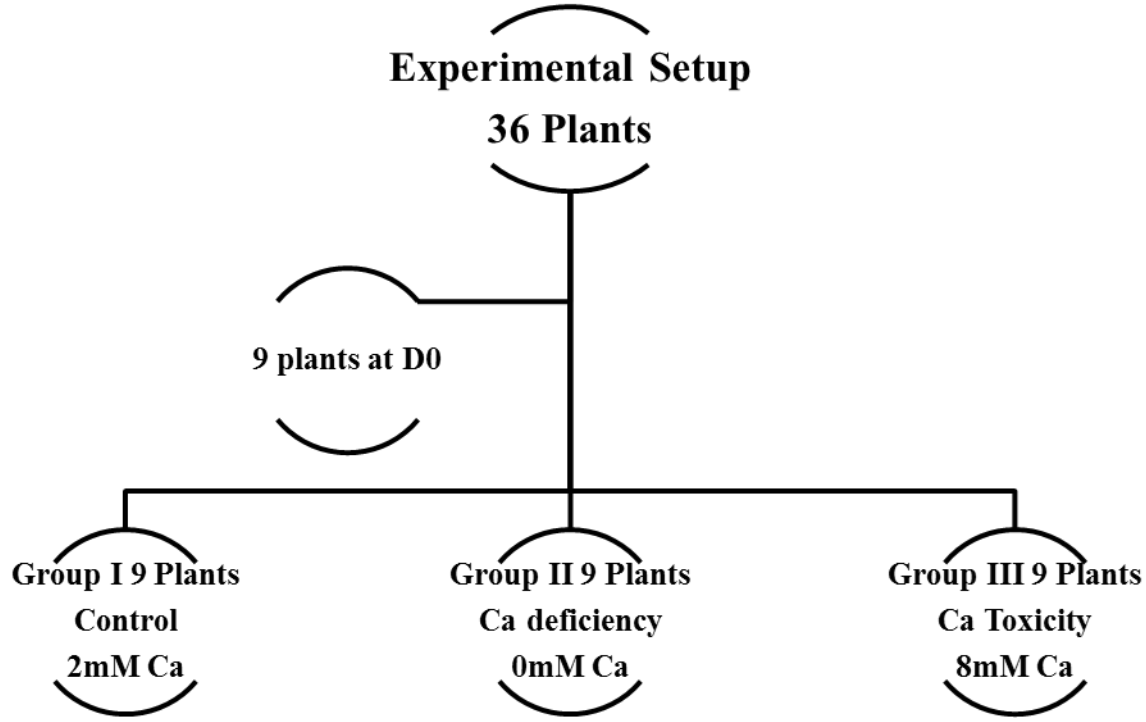


Figure 3.1: Groups division & harvesting plan out before and after the application of calcium

All remaining plants (Group I, Group II and Group III) were grown for 21 days on Lombnaes media having treated concentrations of Calcium (control, toxicity and deficiency. Media solution was changed after 48 hours all the way from beginning. Three plants from each group were harvested after the interval of 7^{th}, 14^{th} and 21^{st} day as in given chart flow and preserved at -80°C for future purpose.

Pictures of roots and shoots were taken followed by measuring the weights as well. The Student's T-test was applied to identify the differences between fresh weights of plants grown in different groups.

3.4. Extraction of RNA from *T. aestivum*

For maximum yield of total RNA from harvested plants (Roots and Shoots) Novel Plant RNA Mini Kit was used. In this sample was ground in liquid nitrogen and filtered by

usage of filter column to remove cell debris. Due to presence of binding buffer and chaotropic (agent that remove hydrogen bonding) salt, the lysate of RNA was bounded to glass fiber matrix in the spin column. DNase I treatments were done for the removal of DNA residues and other contaminants were washed by ethanol contained wash buffers. Finally, purified total RNA was eluted by using RNase-free water. For the future usage, all extracted RNA samples were preserved at -80°C.

3.5. Evaluation of amplicons by using Agarose gel electrophoresis

To check the integrity of RNA samples 1% (w/v) agarose/TAE (1mM EDTA 40mM, Tris/acetate, pH 8.00,) was used. On melting agarose in TAE buffer, 5.0 µL of Ethidium bromide was added in the solution for the detection of RNA. Prior to loading on the gel 1.5 µL DNA loading dye (Bioline, MA, USA) was mixed with 5µL of extracted RNA. Similarly, same amount of loading dye is added for PCR products. For the assessment of product size, 3µL of 100bp DNA ladder (New England, BioLabs Inc) was loaded along the PCR products. Gels were run on gel electrophoretic apparatus (WEALTEC ELITE 300 PLUS) at 80V, 120mA for the duration of 40 mins in 1X TAE buffer and were visualized under UV light in Gel Doc.

3.6. First strand cDNA synthesis:

Extracted RNA templates were used to synthesize first strand complementary DNA. For this WizScript cDNA kit was used. Standard protocol is given:

i. Following first reaction mixture was prepared in PCR tube

Table 3.3: Reagents for cDNA synthesis for first reaction

Reagent	**Volume**
Oligo dT20	1µL
dNTPs mix (2.5mM)	1µL
Template RNA	Total RNA <5µg
RNase free H_2O	Up to 10µL

i. Incubation of prepared mixture was done at 65°C for 5min and cooled immediately on ice

ii. Preparation of second reaction mixture was done by followings:

Table 3.4: Reagents for cDNA synthesis for second reaction

Reagent	**Volume**
First reaction mixture	10 µL
10X reaction Buffer	2 µL
RNase inhibitor 40U/µL	0.5 µL
RTase 200U/µL	1µL
DTT 100mM	1µL
RNase free H_2O	Up to 20 µL

iii. By mixing all reagents incubation was done at 42°C for 60 min

iv. Reaction was stopped by incubating tubes at 70°C for 10 min & cooling on ice

v. Storage of all synthesized first strand cDNA was done at -20°C .

3.7. Primer Specificity and Optimization

Before moving to qPCR, each TaOSCA primers were checked for specificity by using Gradient PCR. For the OneTaq 2X Master mix (NEW ENGLAND BioLabs Inc) with standard buffer was used and a total of 25µL reaction mixture was prepared.

i. As given below reaction mixture was prepared:

Table 3.5: Reagents for qualitative PCR for amplification of the TaOSCAs

Reagent	**Volume**	**Final concentration**
OneTaq 2X Master Mix	12.5µL	1X
10µM Forward Primer	1 µL	0.2 µM
10µM Reverse Primer	1 µL	0.2 µM
Template cDNA	2 µL	< 1000 ng
Nuclease Free H_2O	Up to 25 µL	

ii. Reaction mixture was gently mixed and quick spin was applied for the collection of liquid at bottom of the tube.

iii. The reaction mixture was then immediately transferred to a preheated thermocycler(94°C)

For routine PCR all possible thermocycling conditions were applied as initial denaturation was done at 94°C for 1 min. A total of 35 cycled PCR was used. On which each PCR cycle was consisted of three steps as 94°C for 1 min, 57°C (variable for different genes) for 1 min and 72°C for 1 min and 30 sec. Final extension of 68°C for 7 min is applied for stopped the reaction. PCR products were then preserved at -20°C.

3.8. Real time PCR

Quantitative real time PCR was used to study the expression of *TaOSCAs* in treated and control plant samples because of its more popularity, sensitivity and economical usage. Already prepared cDNA was used as template for the reaction. To check the expression of the *TaOSCA* 5X HOT FIREPol EvaGreen (Solis BioDyne) qPCR Mix plus (ROX) was used. EvaGreen DNA binding dye had more superior alternative to SYBER Green I for the Real time PCR. As the standard dye was FAM and internal passive reference dye was ROX that was used to normalize the fluorescent reporter signals those were generated in qPCR.

i. Reaction and their final concentrations are given below.

Table 3.6: Reagents for Real Time PCR for expression profiling of the TaOSCAs

Reagent	**Volume**	**Final concentration**
5X HOT FIREPol EvaGreen qPCR Mix plus	4µL	1X
10pmol/µL Forward Primer	1µL	500nM
10pmol/ µL Reverse Primer	1 µL	500nM
Template cDNA	2 µL	10ng/ µL
PCR H_2O	Up to 20 µL	

ii. Gently mixing all components, 95°C temperature (initial activation) was required for the activation of HOT FIREPol DNA Polymerase for the duration of 12 min.

iii. 40 cycles were applied in which each cycle having 95°C for 30sec as denaturation, 60°C (Variable for different *TaOSCAs*) for 40 sec as annealing and elongation was done at 72°C for 20sec

Data was collected at the completion of reaction for all *TaOSCAs* along with actin as housekeeping gene. Further melt curve analysis was also done for all *TaOSCAs* for the confirmation and to avoid any multiple peaks in graph.

3.9. Pfaffl method for relative expression:

Relative expression and statistical analysis of the genes was measured by Pfaffl method and further applied student t-test to find out the significance difference between the expression of the TaOSCAs in wheat root and shoot individually.

Table 3.7: qPCR plan out for expression profiling of the TaOSCAs

Ca def 0mM D7	Ca def 0mM D14	Ca def 0mM D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	
Ca def D7	Ca def D14	Ca def D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	
Ca def D7	Ca def D14	Ca def D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	
Ca def D7	Ca def D14	Ca def D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	
Ca def D7	Ca def D14	Ca def D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	
Ca def D7	Ca def D14	Ca def D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	
Ca def D7	Ca def D14	Ca def D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	
Ca def D7	Ca def D14	Ca def D21	Ca toxicity D7	Ca toxicity D14	Ca toxicity D21	control D0	control D7	Control D14	Control D21	SDW	

The table 3.7 represent the 96-well qPCR plan out in which all treated cDNA plant samples (0mM Ca, 2mM Ca and 8mM Ca) harvests (Day 7, Day 14 and Day 21) were used for expression analysis.

Red blocks indicate empty wells-will not matter in case of PCR strips

SDW=sterile distilled water

Brown box are for TaOSCA.1 gene (use primer pair 1)

Green boxes are for TaOSCA.2 gene (use primer pair 2)

Purple boxes are for TaOSCA.3 gene (use primer pair 3)

Blue boxes are for housekeeper actin (use primer pair 4)

3.10. Statistical Analysis

Student t-test is applied for the evaluation of the *TaOSCAs* in control and treated samples.

4. Results

10 homologues of *Triticum aestivum* OSCAs were identified in wheat as given in table (Table 4.1) and given the name TaOSCAs (TaOSCA1-TaOSCA10). Sequences of all the TaOSCAs were retrieved by considering *Brachypodium distachyon* as a query plant because already OSCAs were identified in *Brachypodium* and sequences were obtained from Aramemnon database. As *Brachypodium* was also monocot plant and belong to family Poaceae. Sequences were retrieved from other members from other Poaceae members (Table 4.2) for phylogenetic analysis. Similarly, OSCAs sequences were also retrieved for economically important members of *Brassicaceae* (Appendix 7.5) and *Malvaceae* (Table 7.4).

Table 4.1: OSCAs among Triticum aestivum (Family Poaceae)

Triticum aestivum	**Accession Numbers**	**Sequence Length**	**Database**
TaOSCA1	A0A341UPE2, A0A341VTF9, A0A341VFP4	698, 696, 797	UniProt
TaOSCA2	A0A1D5S1G0, W5ALB6, A0A1D5SJ36	764, 764, 764	UniProt
TaOSCA3	W5DRS8, W5ECH3, W5ESQ4	745, 745, 745	UniProt
TaOSCA4	A0A1D5WPV8, A0A077S7X4, A0A341TL42	709, 709, 717	UniProt
TaOSCA5	A0A341TRA1, A0A341UEV9, A0A341U0Z9	766, 766, 766	UniProt
TaOSCA6	A0A1D6RTK9, A0A1D5W6V7, A0A1D5W6V4	768, 768, 761	UniProt
TaOSCA7	A0A341QB59, W5C1X8, W5BA01	698, 725, 725	UniProt
TaOSCA8	A0A0F7J1N5, A0A0F7IRW9, W5A3G2	802, 805, 801	UniProt
TaOSCA9	A0A341NQF4, W5A4Z3, W5AGL0	767, 767, 766	UniProt
TaOSCA10	W5G3A3, W5FL46, A0A341V1R0	777, 779, 804	UniProt

Table 4.2: OSCAs of reference plant (*Brachypodium distachyon*)

Brachypodium distachyon	**Accession Numbers**	**Sequence length**	**Database**
BdOSCA.1	Bradi4g03360.1	795	Aramemnon
BdOSCA.2	Bradi2g39230.1	768	Aramemnon
BdOSCA.3	Bradi1g10130.1	740	Aramemnon
BdOSCA.4	Bradi2g60810.1	707	Aramemnon
BdOSCA.5	Bradi1g12670.1	762	Aramemnon
BdOSCA.6	Bradi2g40490.1	786	Aramemnon
BdOSCA.7	Bradi1g58380.1	729	Aramemnon
BdOSCA.8	Bradi3g34540.1	794	Aramemnon
BdOSCA.9	Bradi2g26180.1	767	Aramemnon
BdOSCA.10	Bradi4g00860.1	764	Aramemnon

4.1. Phylogenetic Analysis

For the determination of the evolutionary relatedness among OSCAs from the members of family Poaceae like *Triticum aestivum, Brachypodium distachyon, Leersia perrieri, Panicum hallii, Sorghum bicolor, Setaria italica, Zea mays, Dichanthelium oligosanthes, Hordeum vulgare* and *Aegilops tauschii.* The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-16623.89) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 106 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 348 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 Phylogenetic tree 4.1. Further phylogenetic trees were also constricted among the members of Brassicaceae and Malvaceae Phylogenetic tree 4.2 & Phylogenetic tree 4.3.

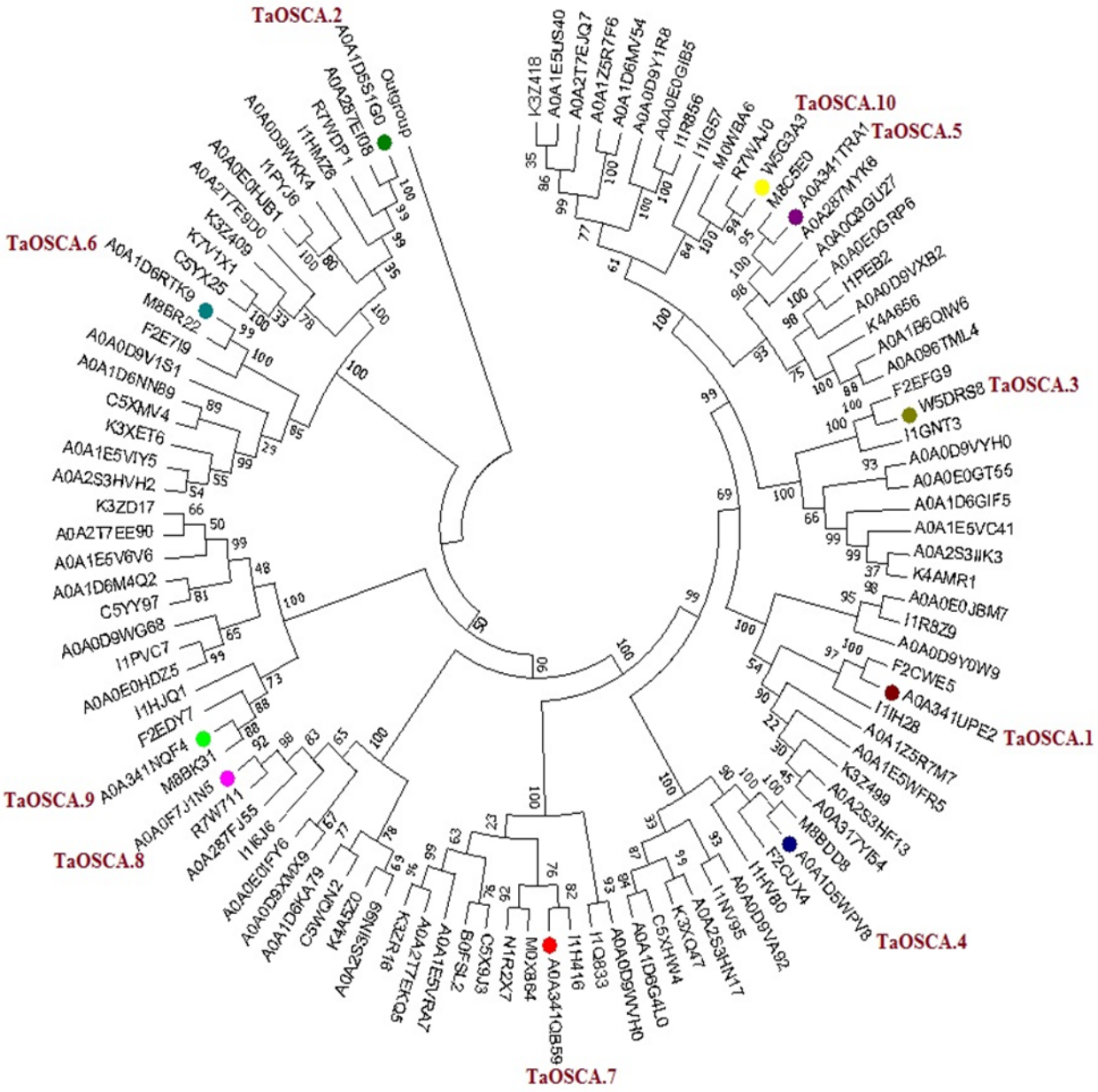


Figure 4.1: Molecular phylogenetic analysis of Poaceae family by Maximum Likelihood method. Gap open penalty was kept at 10

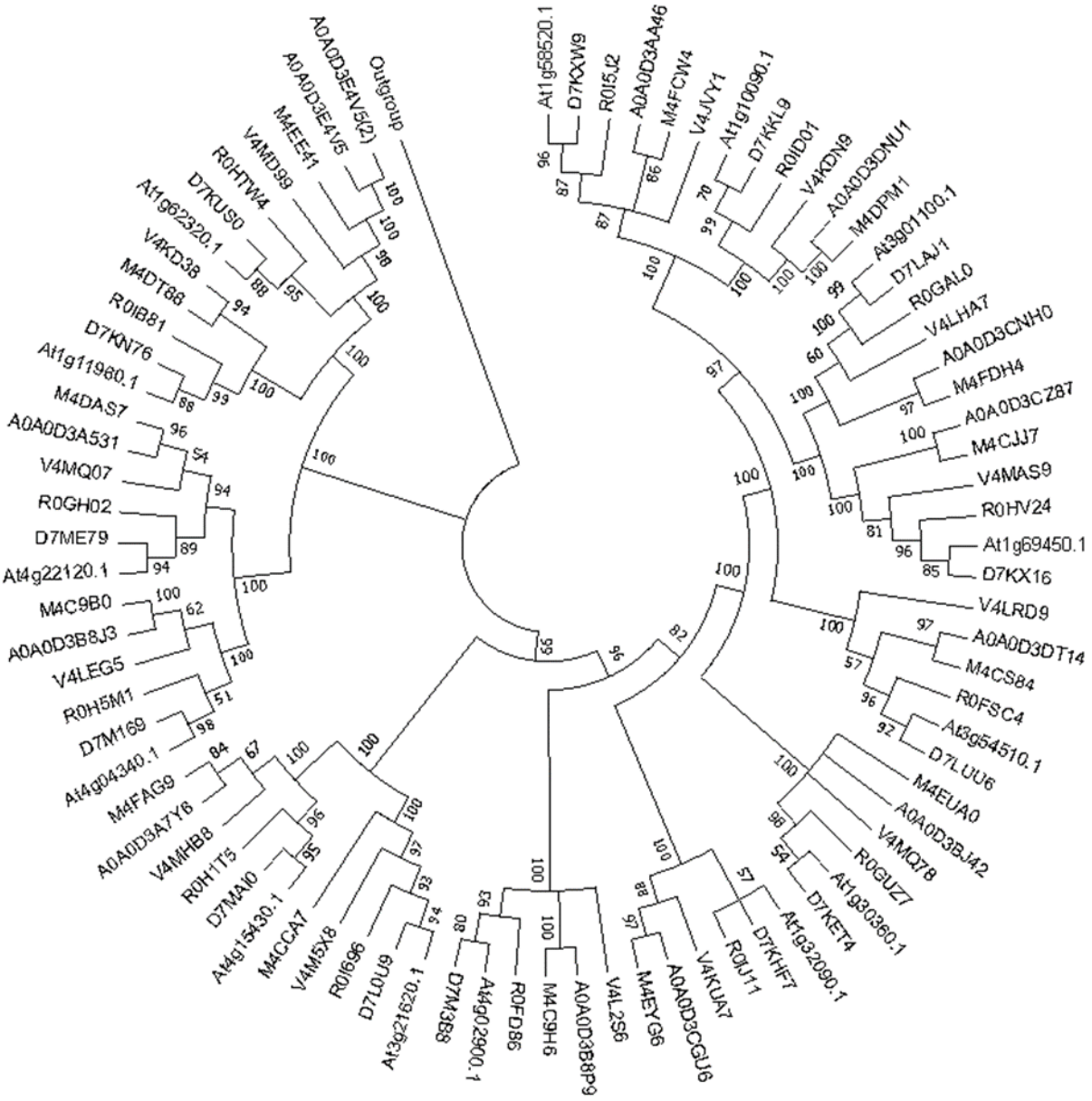


Figure 4.2: Molecular phylogenetic analysis of Brassicaceae family by Maximum Likelihood method. Gap open penalty was kept at 10

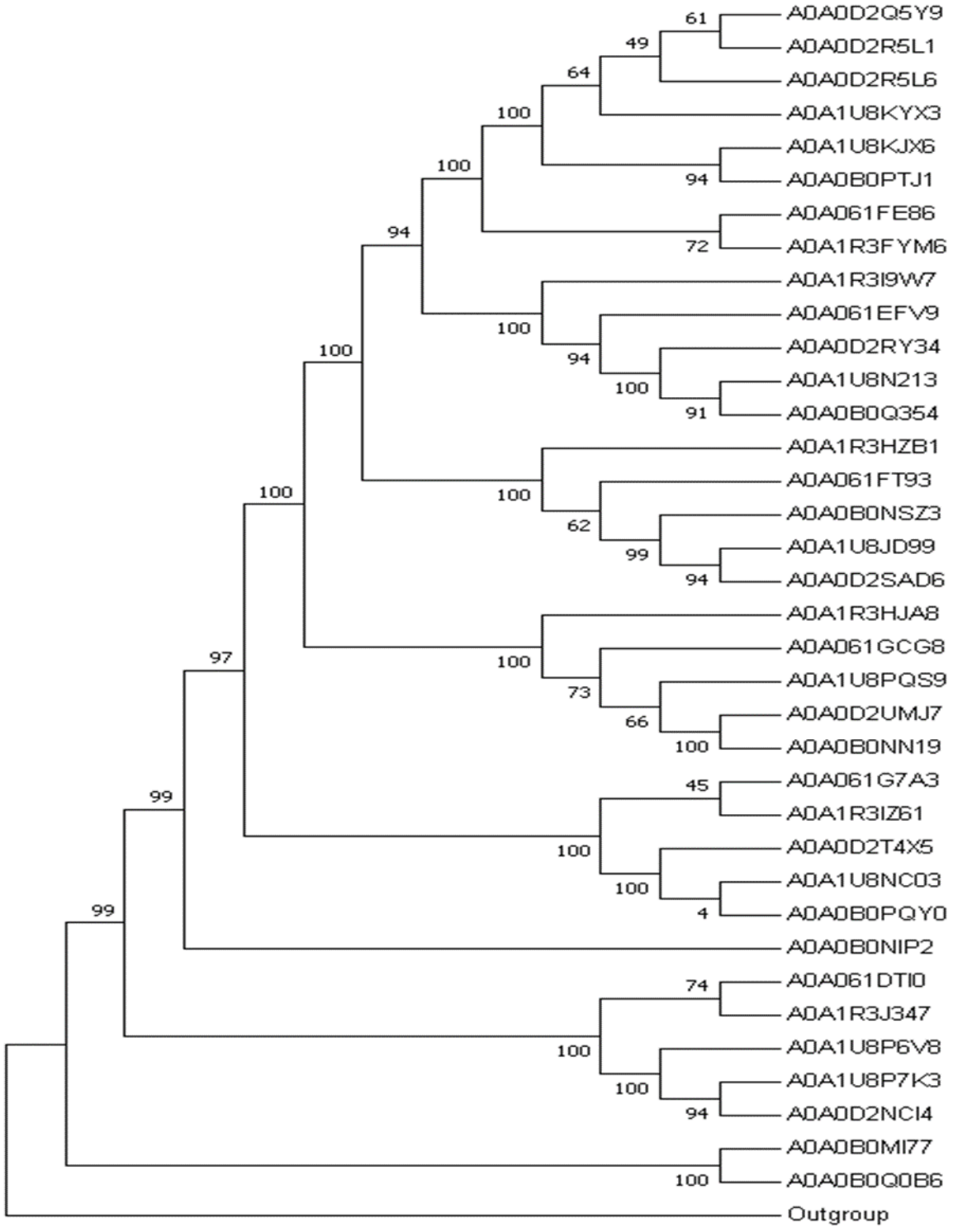


Figure 4.3: Molecular phylogenetic analysis of Malvaceae family by Maximum Likelihood method. Gap open penalty was kept at 10

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-18406.79) is shown Phylogenetic tree 4.2. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 84 amino acid sequences from accession numbers of *Arabidopsis thaliana, Capsella rubella, Eutrema salsugineum, Arabidopsis lyrate, Brassica oleracea* and *Brassica rapa*. All positions containing gaps and missing data were eliminated. There was a total of 503 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

The evolutionary history was inferred in the members of Malvaceae family by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-13728.22) is shown Phylogenetic tree 4.3. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 37 amino acid sequences form *Theobroma cacao, Corchorus capsularis, Gossypium hirsutum, Gossypium raimondii,* and *Gossypium arboreum*. All positions containing gaps and missing data were eliminated. There was a total of 544 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

4.2. Growth of wheat plants under calcium:

Phenotypically analysis was conducted on the plants those were grown hydroponically on Lombanaes media. Plants were observed grown on control media for first 14 days and harvested and given the name D^0. Plants were further split into three groups (Control 2mM Ca, deficiency 0mM Ca and toxicity 8mM Ca). Plants were grown on given treatments and harvested at Day 7, D14 and D21. Wheat plants were closely analyzed for the appearance of symptoms associated with the calcium deficiency and toxicity accordance with the normally provided calcium concentration. Following given figures represent the morphological symptoms appeared before and after given the treatment. As deficiency (0mM Ca) of calcium in the media causing chlorosis in the leaves and having low growth in the plant as comparison to the plants growing on normal (2mM Ca) calcium concentration. Stunted growth in the roots was also observed with low volume of roots in 0mM Ca treated plants from Day 7 – D21 as comparison to the control plant growing at 2mM Ca. Growth was severely inhibited in the plants those were treated with toxicity (8mM Ca) of calcium. In Fig 4.4, 4.5 & 4.6.

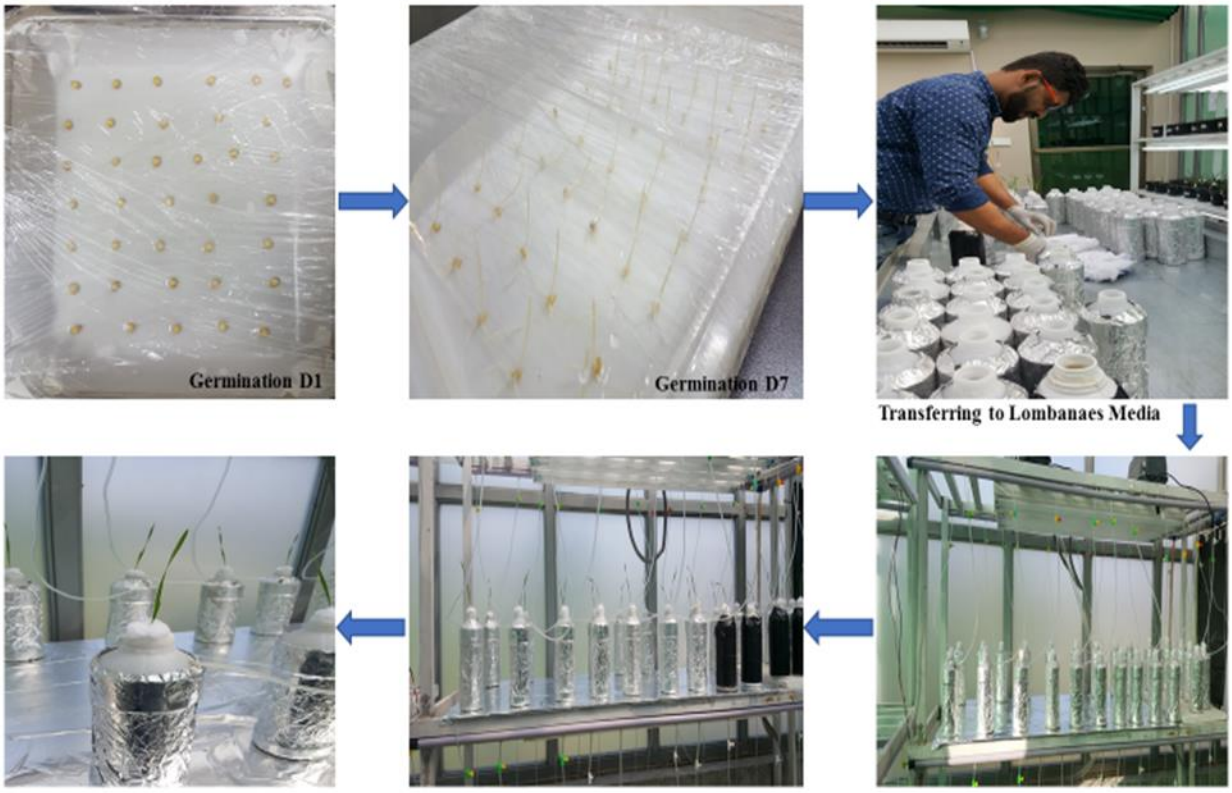


Figure 4.4: Growing of seeds on tray followed by transfer of seedlings to control media

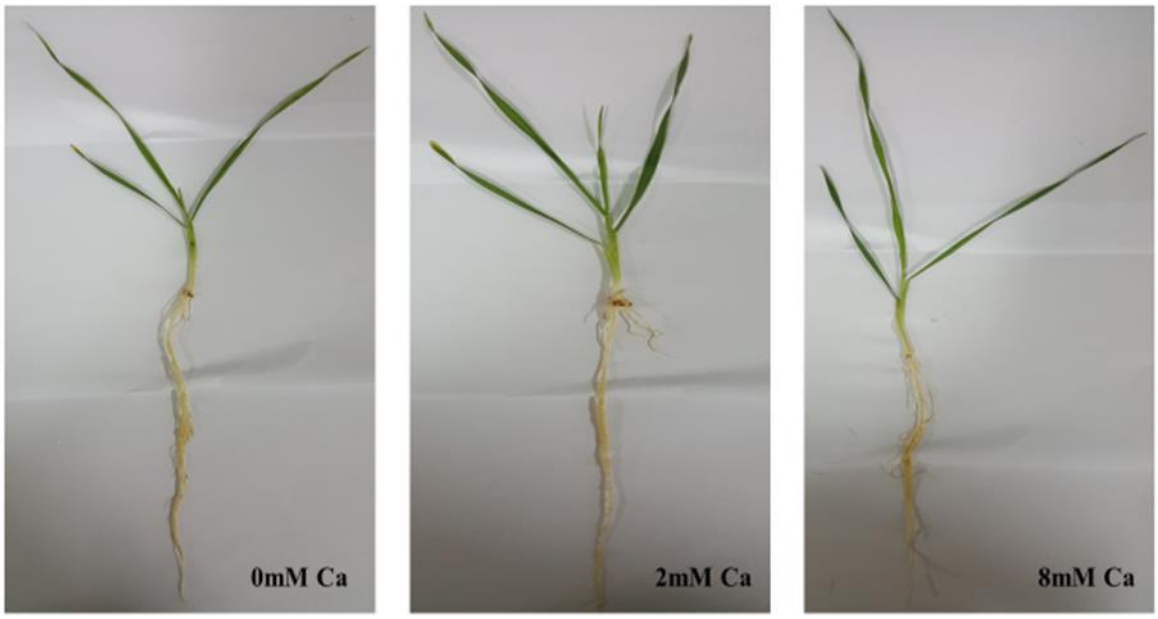


Figure 4.5: Day 7 harvest, showing phenotypically analysis at different conditions 0mM Ca, 2mM Ca & 8mM Ca

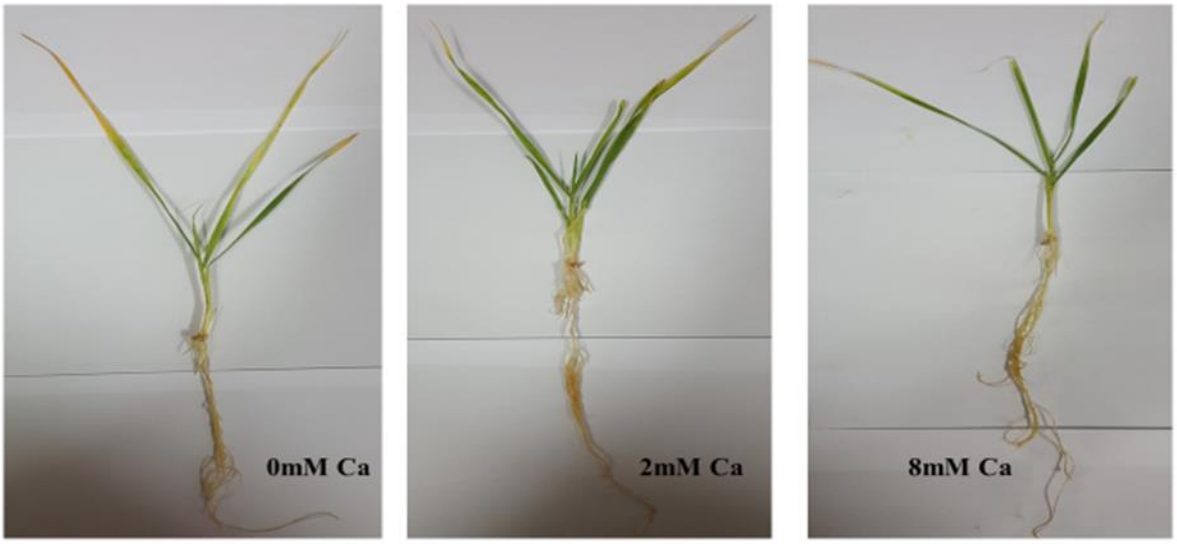


Figure 4.6: Day 14 harvest, showing phenotypically analysis at different conditions 0mM Ca, 2mM Ca & 8mM Ca

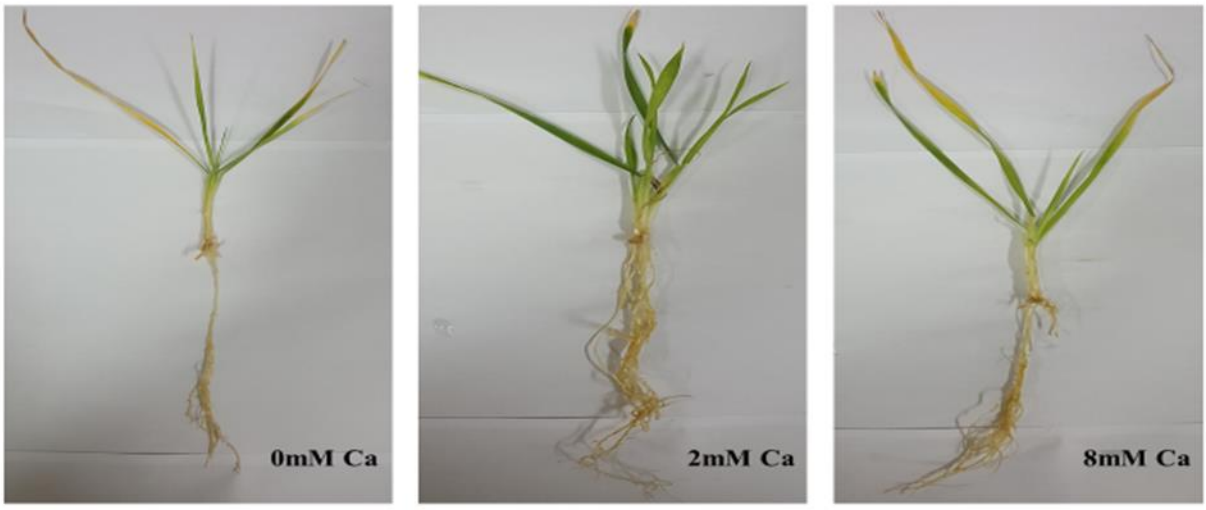


Figure 4.7: Day 21 harvest, showing phenotypically analysis at different conditions 0mM Ca, 2mM Ca & 8mM Ca

4.3. Fresh Weight Analysis:

At each harvesting fresh weight of each root and shoot and whole plant were measured and recorded. Significant differences in shoot fresh weight, root fresh wight and whole plant fresh weight were dictated with the help of student's t test in given figures 4.8, 4.9 & 4.10.

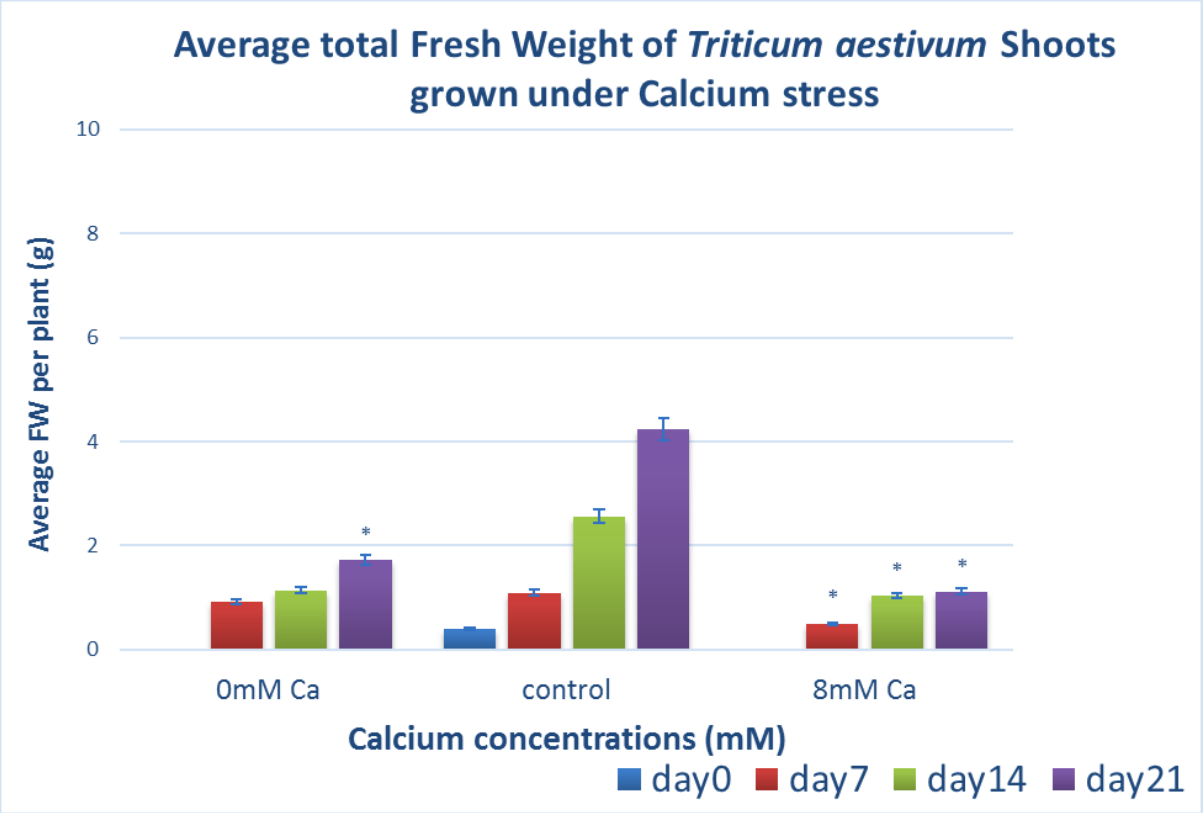


Figure 4.8: Significant differences * in shoot fresh weight were dictated with the help of student's t test, where, P<0.05, Control= 2 mM Calcium

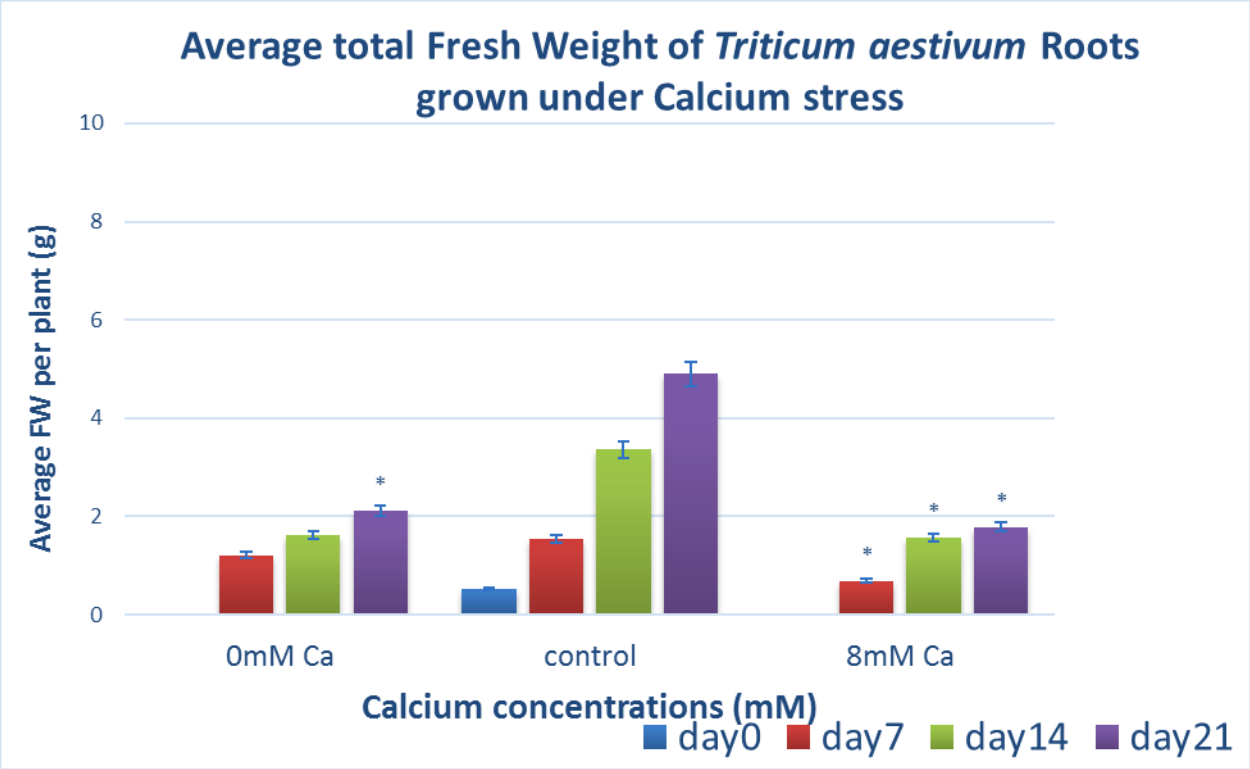


Figure 4.9: : Significant differences * in root fresh weight were dictated with the help of student's t test, where, P<0.05, Control= 2 mM Calcium

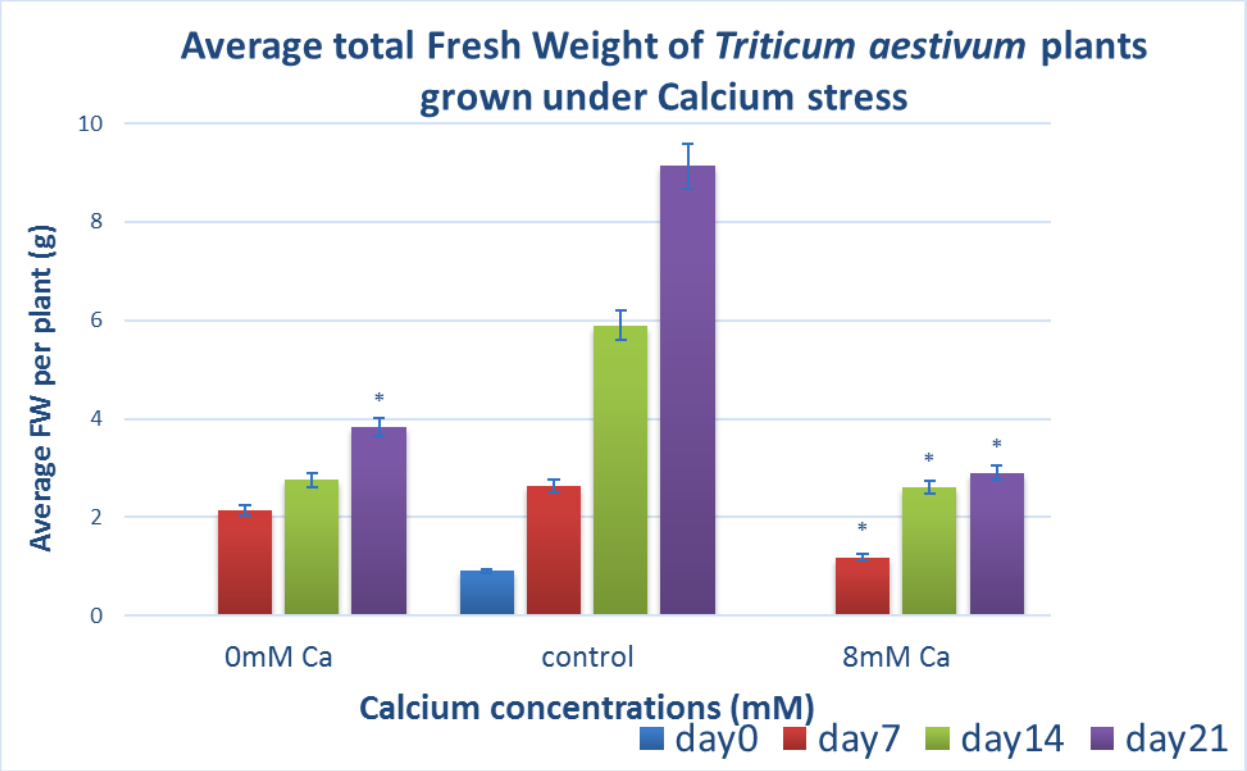


Figure 4.10: Significant differences * in whole plant fresh weight were dictated with the help of student's t test, where, P<0.05, Control= 2 mM Calcium

4.4. Primer synthesis:

Following given primers were synthesized by using NCBI Primer Blast Software and Primer3Plus. As actin was housekeeping gene/normalizer or reference gene previously reported was used in this study.

Table 4.3: Primers list

Sr No	Forward	Length	Reverse	Length
MTR1	TGCACCCAAGTATGACACCG	20	GAGGCACCAGCAGACTTGAA	20
MTR2	GAAGGACACGCTGGAGCA	18	GCTACAATCACCTCCTCCG	19
MTR3	CTCGGCTACTTGGTGTACCG	20	TTGATGGTGAACACGCCCAG	20
MTR4	TTCATTCGCCACAATCCCCT	20	CCGCCTCCCTCTCAGTTTTA	20
MTR5	CATCTGCCTTGGTGTCTTTGG	21	GGAAAGTCCCGAATAACGGC	20
MTR6	TACCGCCATCAGGAGGCAA	19	ATTTCTCATCGTCGTCGCC	19
MTR7	ACTTGCCACACACGCTTCTA	20	AGCTTTTCAGGCTTCAGGCA	20
MTR8	GAGCCAAGCCTGAACCTCAA	20	GCTGATGCTCCGATTTGTCG	20
MTR9	CACTGCAACGAGCAAATGA	19	ACAGTGGGATTCTTCTCTTCTTCA	24
MTR10	GCGTATTGCCAGTTCCCTGA	20	CTGGAGGGTCTCTTTGCCG	19
Actin	ACCTTCAGTTGC CCAGCAAT	20	CAGAGTCGAGCACAATACCAGTTG	24

4.5. RNA Extraction:

By using RNA Plant mini kit RNA was successfully extracted from all the harvested plants at different intervals as in Fig 4.11. For future use, all the extracted RNA samples were preserved at -80^{o}C in Ultra-refrigerator.

4.6. Primer optimization and specificity:

After synthesizing cDNA from all the extracted RNA samples optimization and specificity of the all primers were done by using conventional PCR and qPCR to remove any possibility of any primer dimer formation or nonspecific binding of the primers. Gel image Fig 4.12 and Sharp peaks were indicated the specific amplification with the help of melt curve analysis as given Fig 4.13.

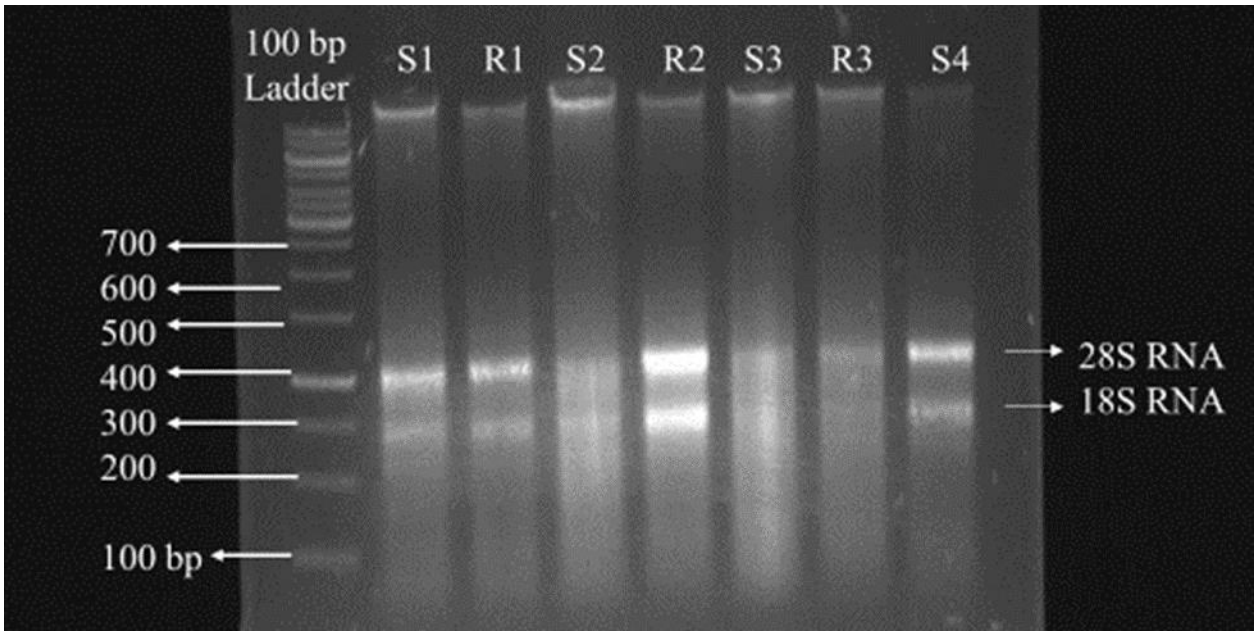


Figure 4.11: The results of total RNA extraction protocol on wheat roots and shoots (S1=shoot, R1=Root), electrophoresed on 1% agarose gel (W/V) agarose /TAE (40 mM Tris/acetate, pH 8.0, 1 mM EDTA)

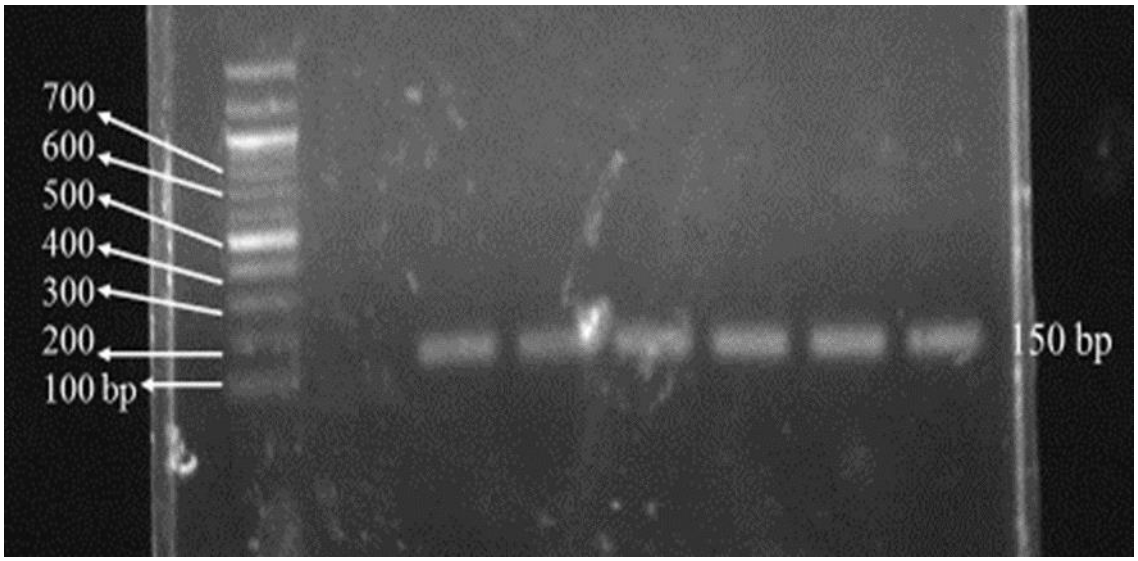


Figure 4.12: The result of amplicon(Shoot) by using MTR1 primer, electrophoresed on 1% agarose gel

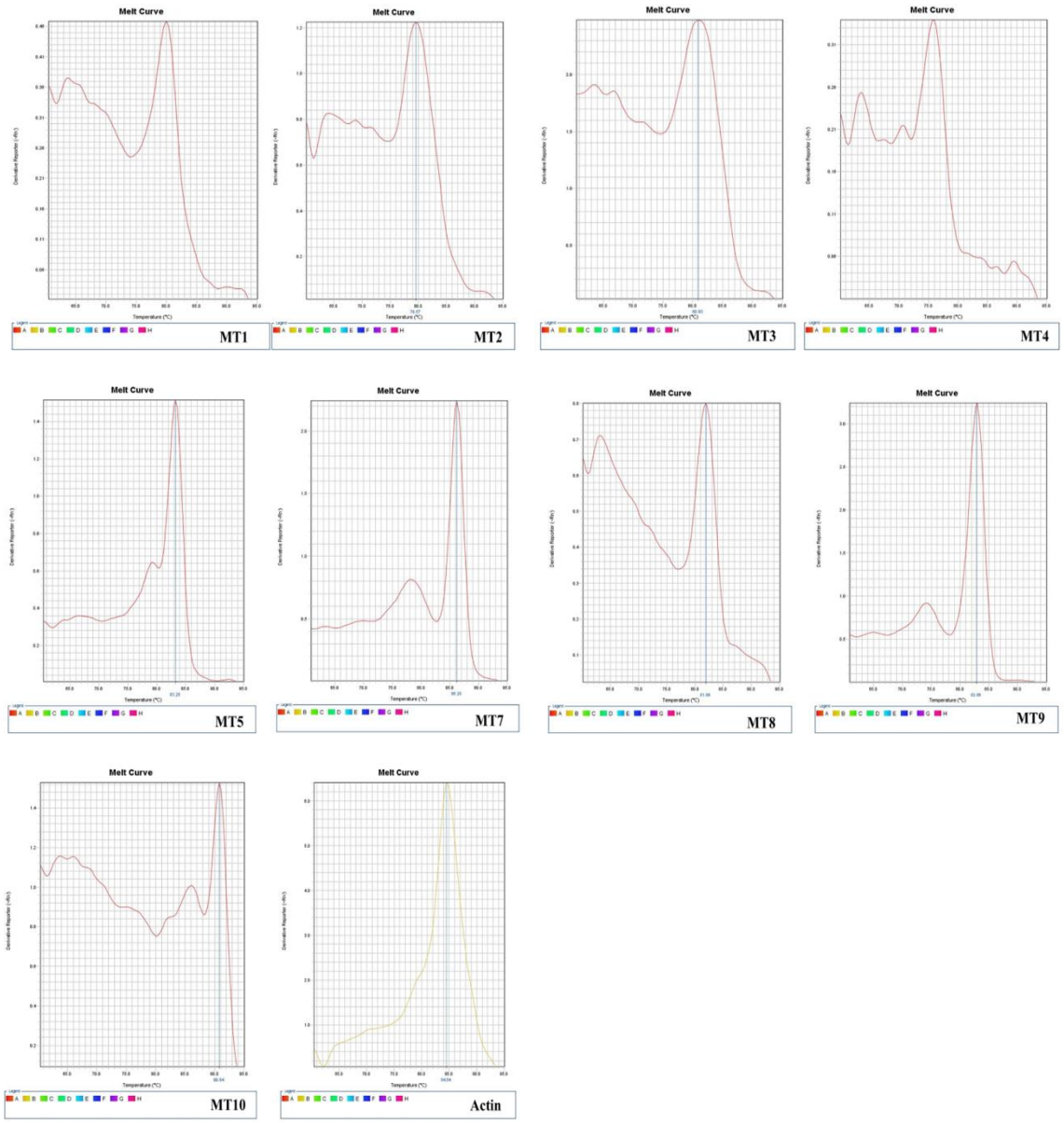


Figure 4.13: Melt Curve analysis of MTR1, MTR2, MTR3, MTR4, MTR5, MTR7, MTR8, MTR9, MTR10 Actin, by using qPCR

4.7. Expression Profiling of TaOSCAs:

In present study, Applied Biosystems 7500 Fast Real time PCR system was used to determine the Ct values and efficiencies and with the help of Pfaffl method expression of the genes were analyzed. Out of 10 genes, 7 were successfully identified and evaluated against specific treatments applied to the plants. These 7 genes were separately showing response against deficiency and toxicity of the calcium. These stress responsive genes were validated both in

roots and shoots in the plants in different time interval (D7, D14 & D21) under the baseline of D0.

Expression analysis of the TaOSCA.1 separatly in root and shoot has been measured under the base line of the D0 plant. Under calcium deficiency, expression of the TaOSCA.1 with reference to control plant at D7, D14 and D21 increased, similarly in case of toxicity of the calcium has shown more expression as compared to the plants those were facing 0mM of the calcium fig 4.14.

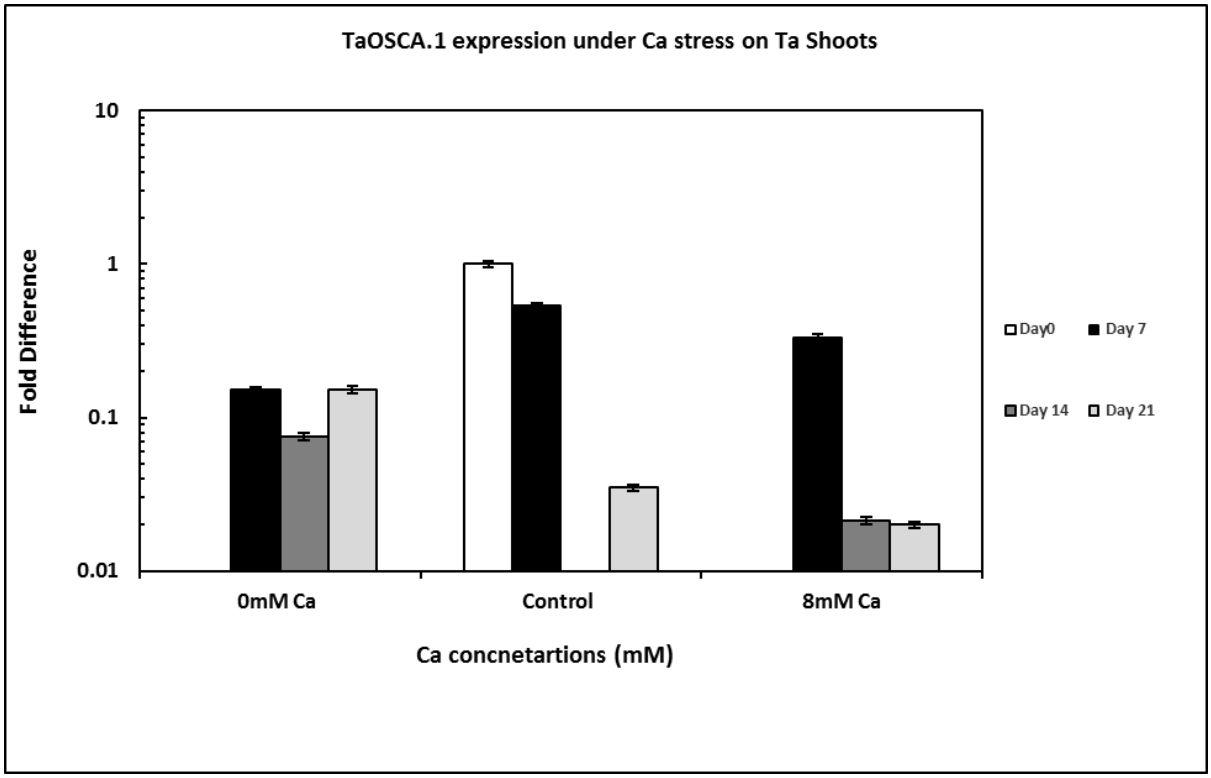


Figure 4.14: Expression profiling of TaOSCA.1 in shoots

Similarly the expression of the TaOSCA.1 in roots was also measured. Expression of the TaOSCA.1 in root was much similar to the expression of the shoot. But analysis suggested there were more expression in roots at 8mM of the Ca but it stays only for D7 but after this the expression was low enough that might posibility these transporter may stop working during toxicity but in case of deficiency there were almost equal expression at D14 and D21

in tha plants those were held at 0mM of the Ca which clearly indicating the plant is trying to maintain the inner balance of the calcium concentration fig 4.15.

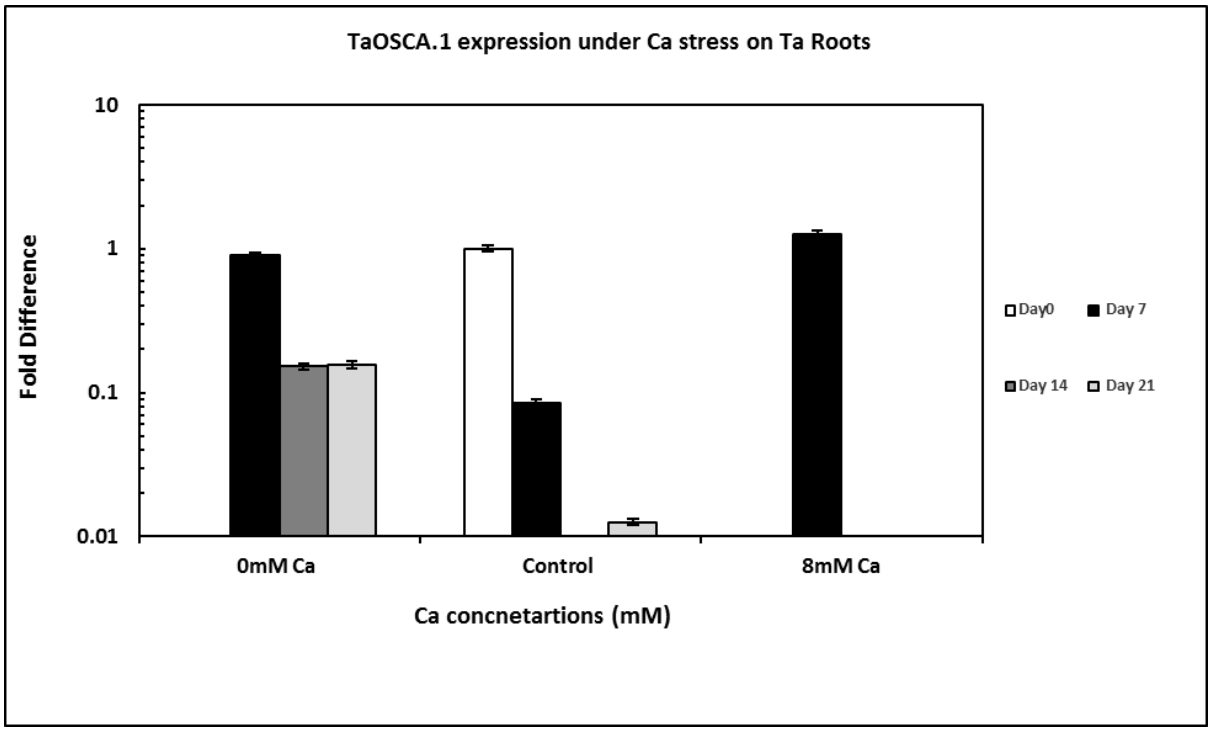


Figure 4.15: Expression profiling of TaOSCA.1 in roots

Expression of the TaOSCA.2 in case of the shoot indicated initially the expression was much higher in plants those were treated with 0mM and 8mM calcium at D7 but than in case of deficiency the expression was high at D14 but in case of toxicity the expression of the TaOSCA.2 remains constant for D14 and D21 fig 4.16.

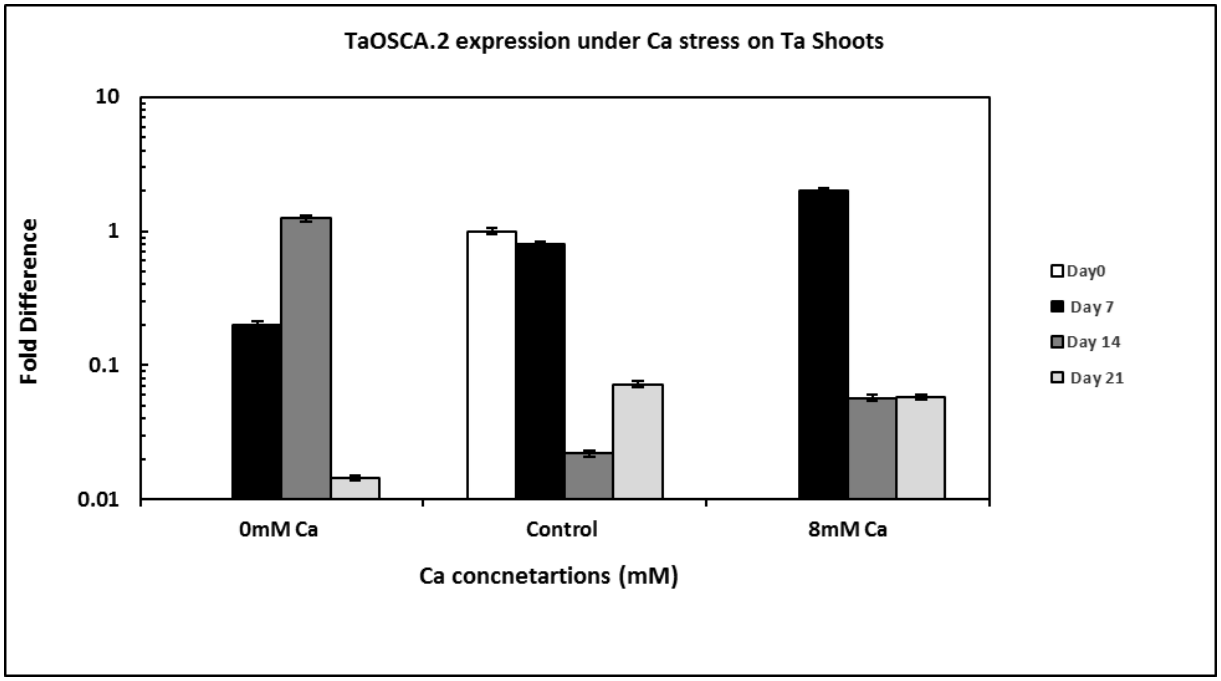


Figure 4.16: Expression profiling of TaOSCA.2 in shoots

In case of roots the expression was shown both during deficiency and toxicity but the expression was high in case of toxicity but it stay only for D7 but after it start decreasing and than stopped as in Fig 4.17.

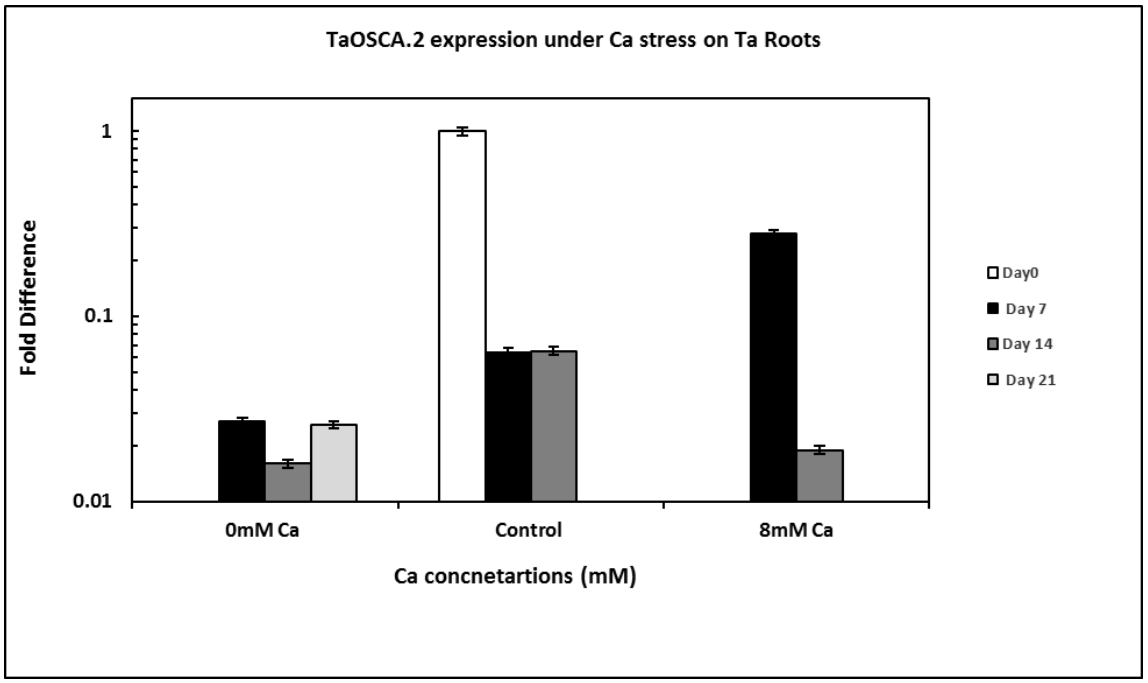


Figure 4.17: Expression profiling of TaOSCA.2 in roots

TaOSCA.4 has shown expression in both deficiency and toxicity but only for initial days. The expression was high in deficiency of the calcium but then remain approximately equal. But during toxicity the expression was decreased down to the lowest as Fig 4.18.

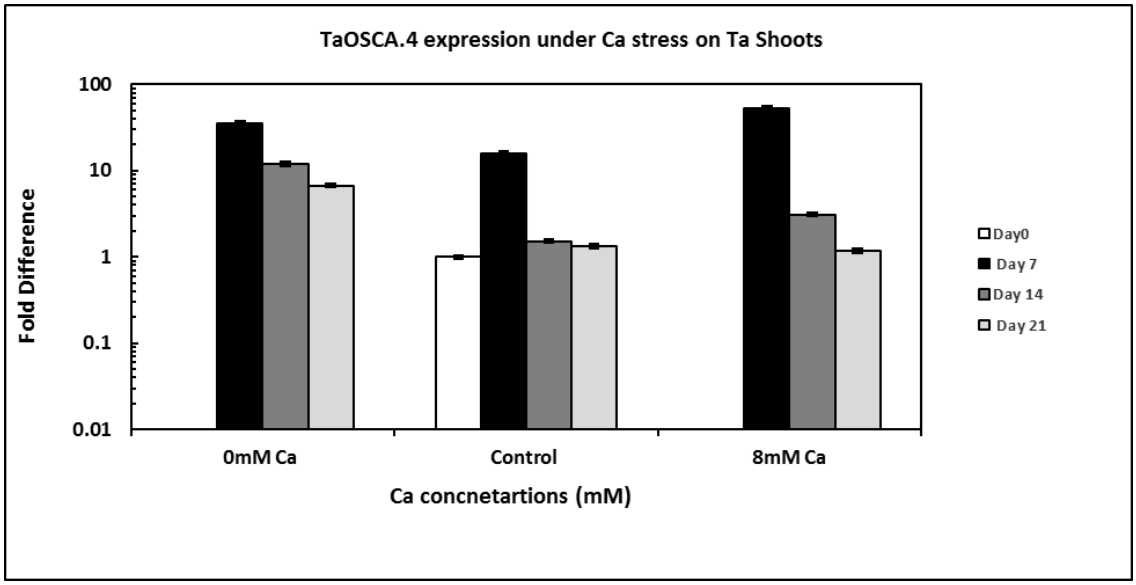


Figure 4.18: Expression profiling of TaOSCA.4 in shoots

In case of the root the expression and the behavior of the TaOSCA.4 was much similar as indicated in Fig 4.19.

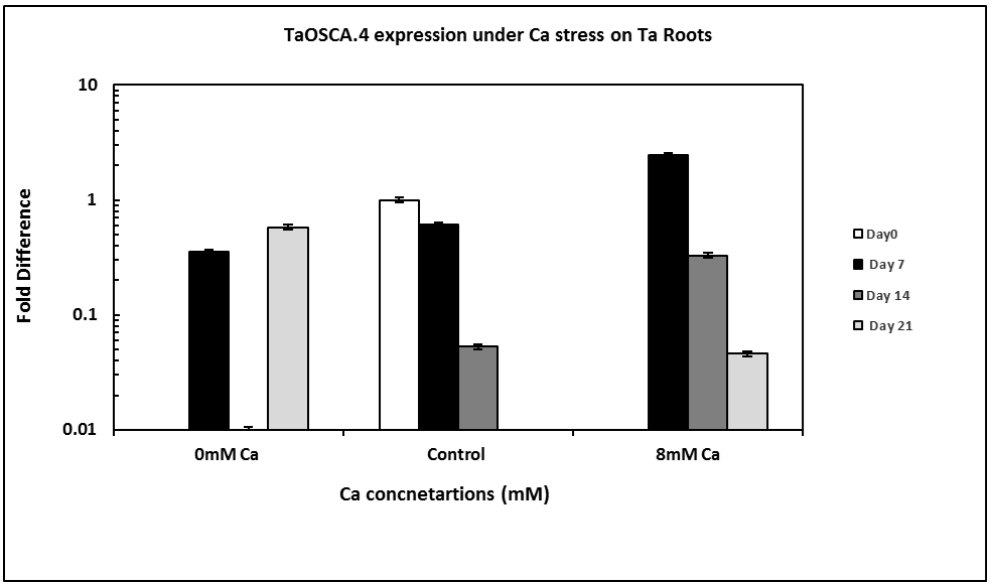


Figure 4.19: Expression profiling of TaOSCA.4 in roots

TaOSCA.5 was also evaluated both in shoots and roots. The expression was much higher in case of shoots both in deficiency and toxicity irrespective the expression was low in case of roots indicate the importance of TaOSCA.5 as in Fig 4.20 & Fig 4.21.

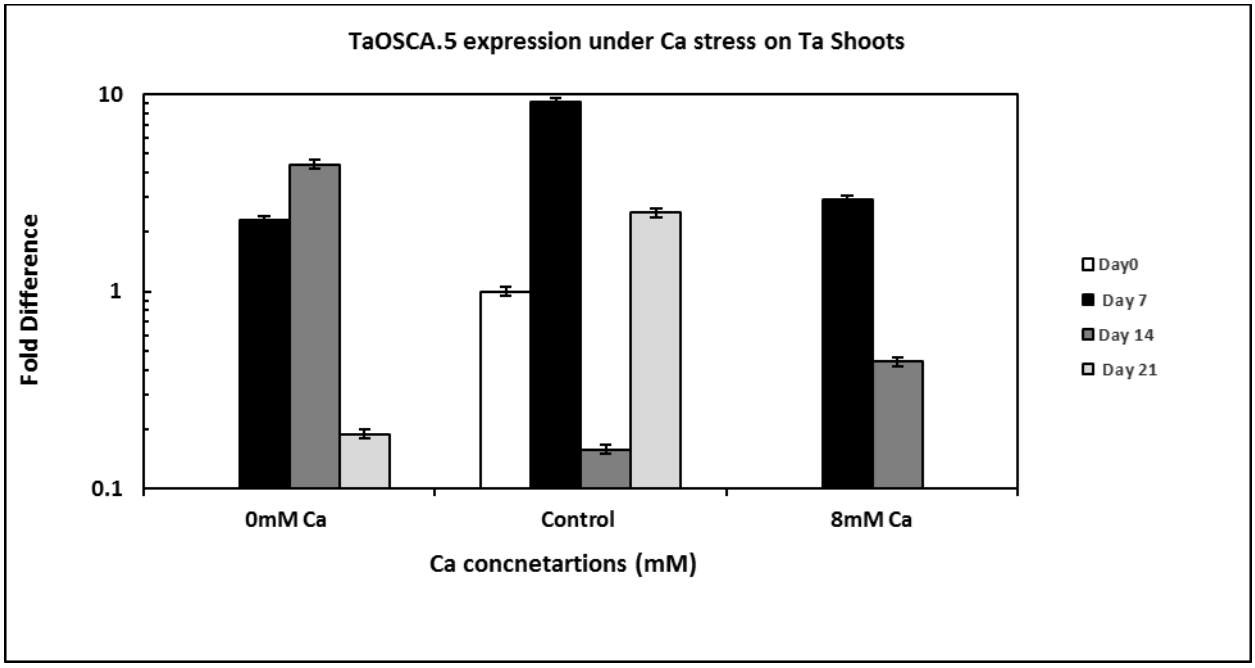


Figure 4.20: Expression profiling of TaOSCA.5 in shoots

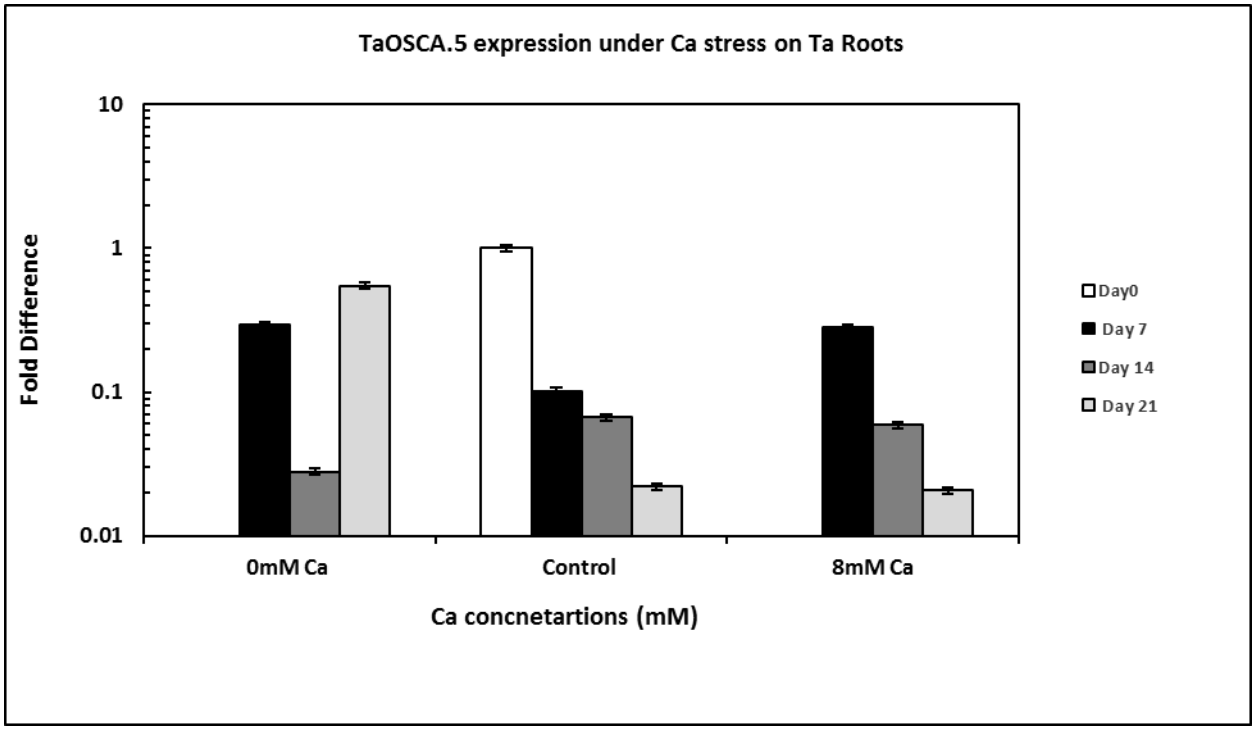


Figure 4.21: Expression profiling of TaOSCA.5 in roots

Shoots have shown much higher TaOSCA.7 expression as compared to roots. As Fig 4.22 indicated the expression was much higher initially during the toxicity but decreased down at D21. Similarly in case of roots Fig 4.23 indicated the expression was one fold higher in deficiency.

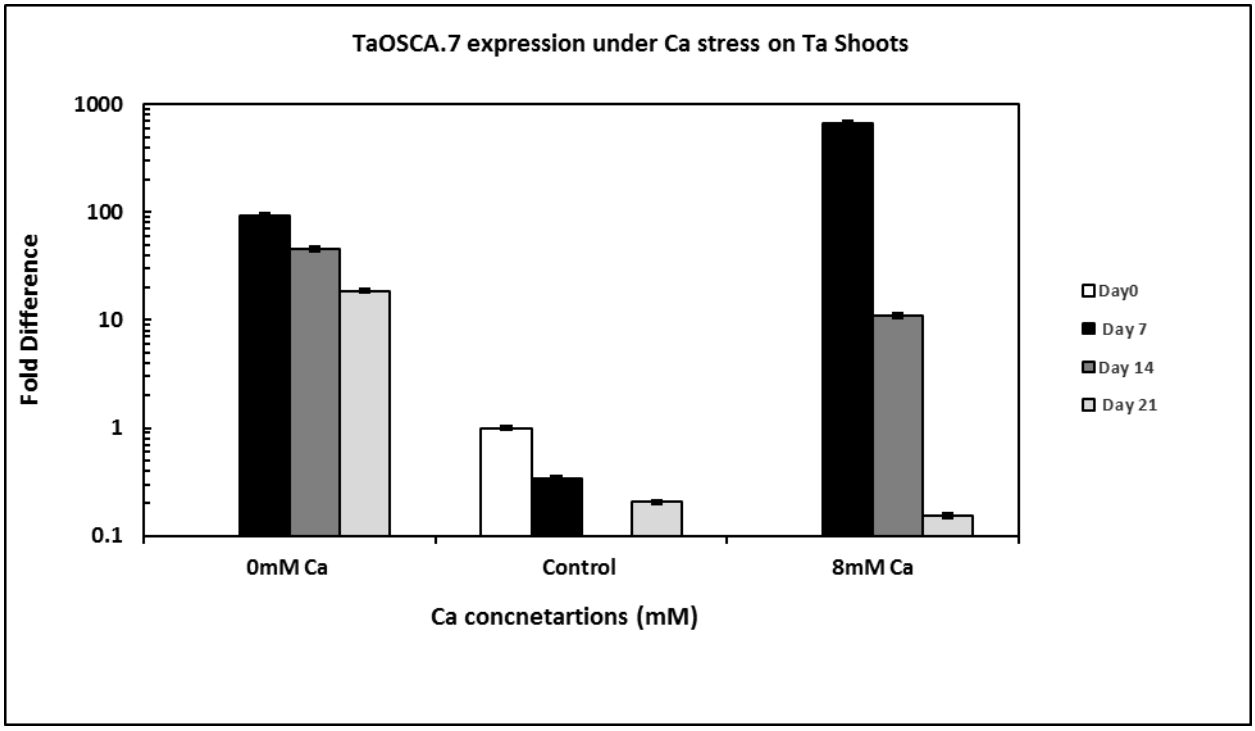


Figure 4.22: Expression profiling of TaOSCA.7 in shoots

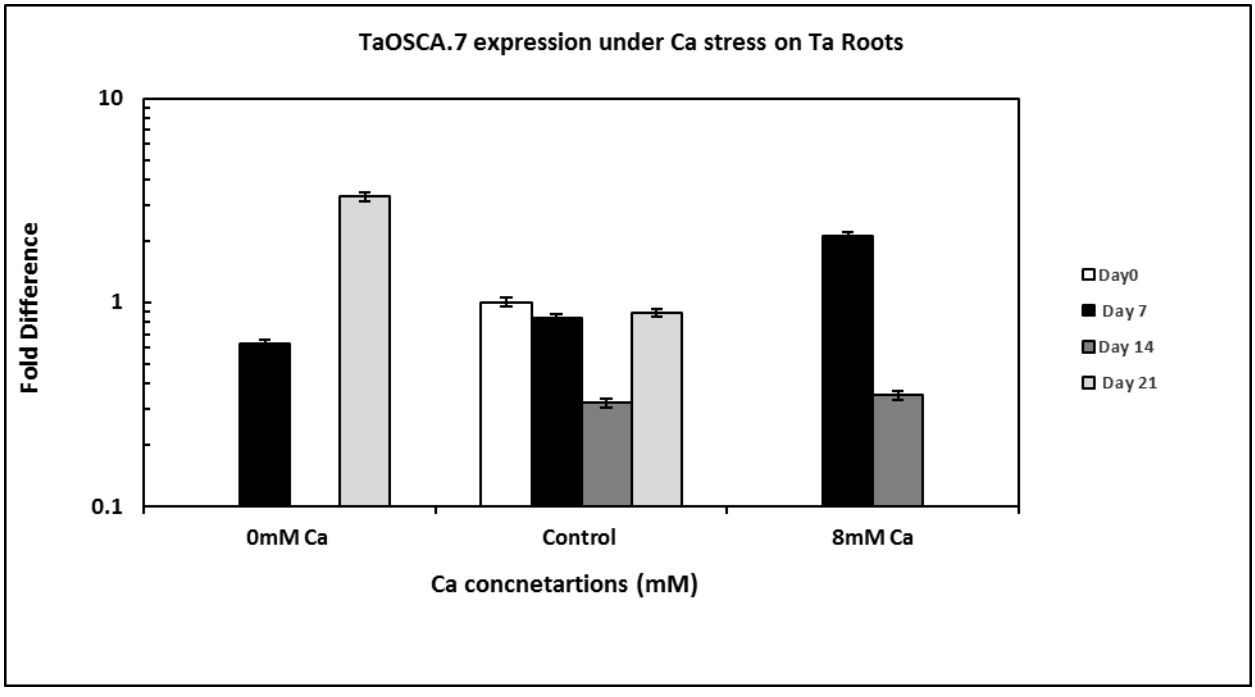


Figure 4.23: Expression profiling of TaOSCA.7 in roots

Expression of the TaOSCA.9 in shoots were much higher in contrast to roots. As it was shown Fig 4.24 especially during the toxicity but it was higher in comparison to control and deficiency. In case of roots the expression was much lower during toxicity as in Fig 4.25.

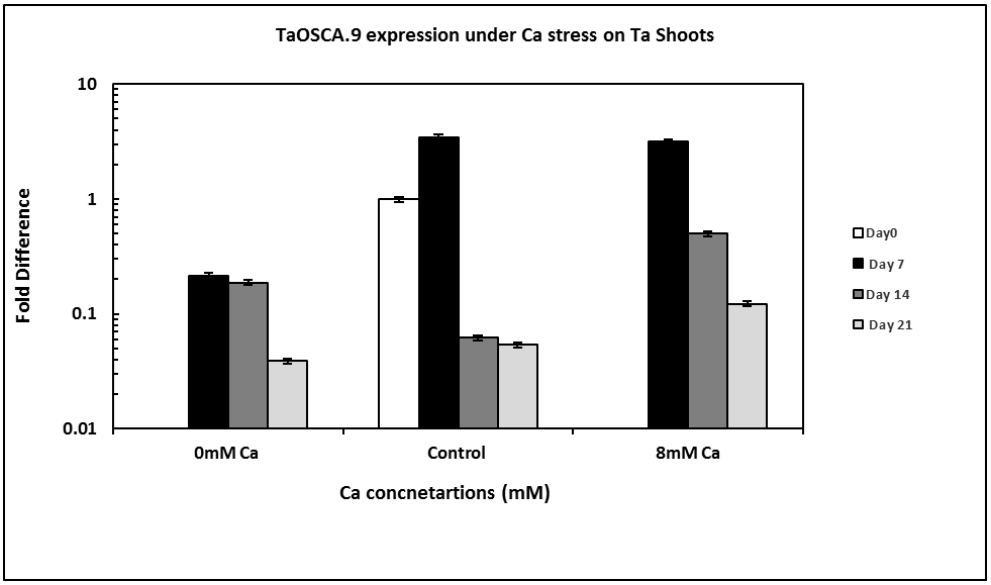


Figure 4.24: Expression profiling of TaOSCA.9 in shoots

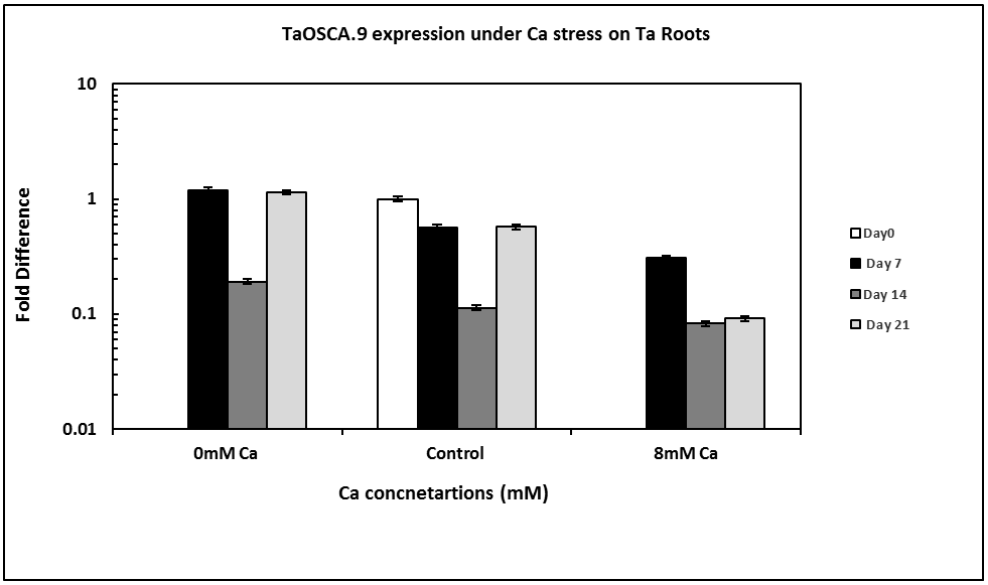


Figure 4.25: Expression profiling of TaOSCA.9 in roots

In comparison to the roots, TaOSCA.10 has shown expression as toxicity of the media causing increase in expression leades towards sudden decrease down Fig 4.26. But in case of roots deficiency of the media causing increase and activity of the TaOSCA.10 as in Fig 4.27.

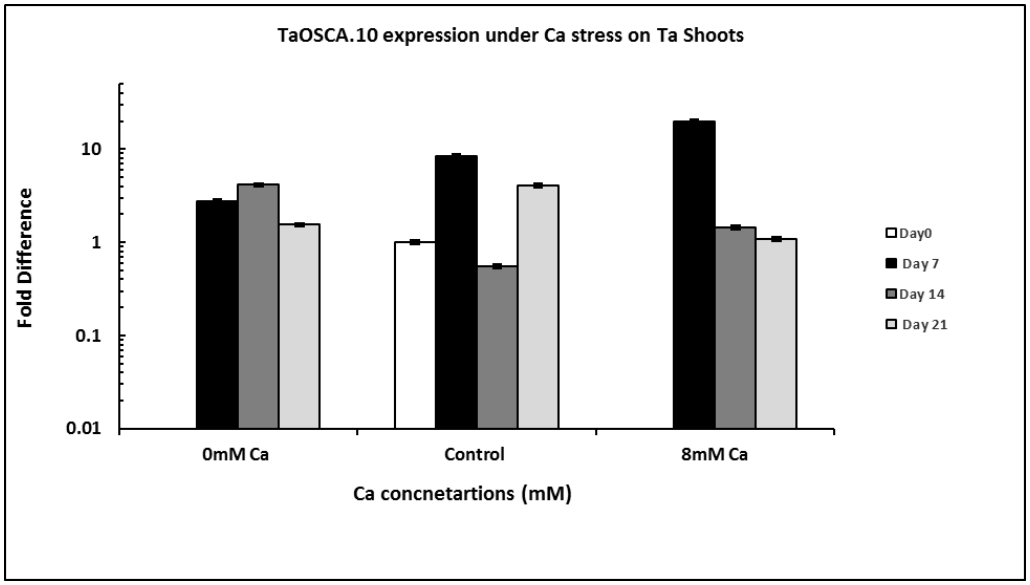


Figure 4.26: Expression profiling of TaOSCA.10 in shoots

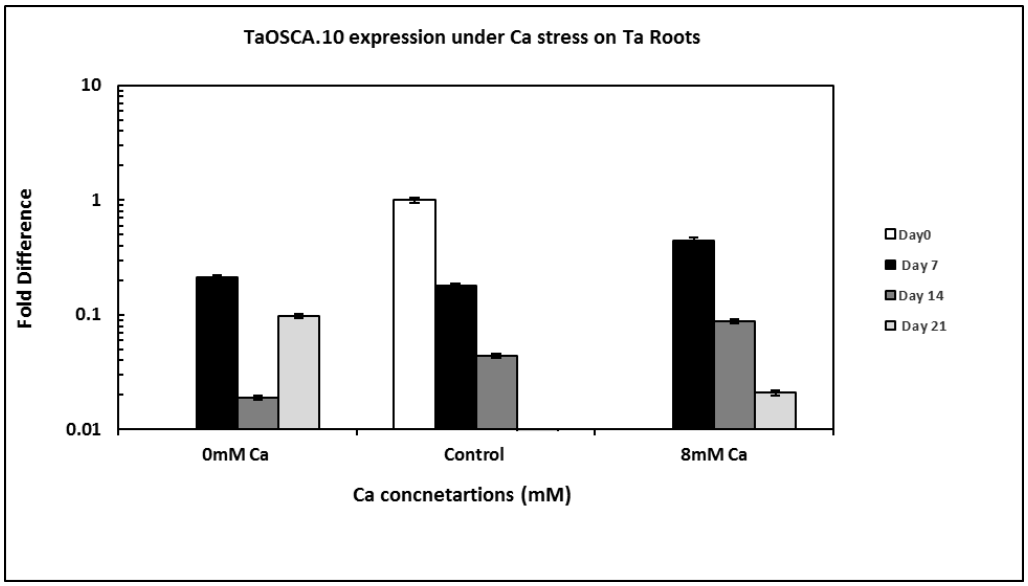


Figure 4.27: Expression profiling of TaOSCA.10 in roots

Fig 4.23-4.36 Graphs showing the relative expression of TaOSCA.1, TaOSCA.2, TaOSCA.4, TaOSCA.5, TaOSCA.7, TaOSCA.9, TaOSCA.10, both in Shoot and root under calcium stress.

5. Discussion

Because of major cereal crop bread wheat is having a lot of attention for the world and extensive research is going on in Pakistan as well for the development of better and improved verity of wheat crop. Number of databases like International Wheat Genome Sequencing Consortium (IWGSC) provides information about wheat genome. IWGSC used to separating the chromosome with the help of flow-cytometric sorting and constructed libraries with 500 bp target size (Marcussen et al., 2014).

Calcium is very important essential part of nutrient for the normal development and growth of the plant. Deficiency and higher concentration above optimum range can cause low crop yield. Calcium being a macronutrient is compulsory for the plant as having role in cell wall and cell membranes, prevention against salinity, drought and component of number of cellular signaling pathways (Marchner, 2011). As majority of the regions of Pakistan has high calcium but beyond its limit has vigorous effect on the plants leads towards necrosis and chlorosis of the plants.

Different studies have shown that in high degree of calcium in the soil causing accumulation of calcium inside the cell leads towards the activation of specific channels or transporters in the plants. Number of different types of calcium channels were identified and evaluated against different abiotic and biotic stress conditions. These channels exist all over the cell in plasma membrane, in the membranes of chloroplast, vacuole and other major organelles. Comparative genomic studies have much importance in identifying, evaluating and arranging genes for these transporters. As like wheat other species like *Brachypodium*, rice, maize, sorghum and barley belong to Poaceae family. Initially, as *A.thaliana* has been sequenced and annotated and used for the identification of number of genes in wheat (Kaul et al., 2000). But *A.thaliana* is dicot specie and do not share higher resemblance to the wheat. Further the rice

genome database helped for comparative studies for other cereal crops. OSCAs previously identified in *A.thaliana* and then in rice. As sequence analysis of the wheat suggested a lot of chromosomal rearrangements and duplication other than rice new model plant *Brachypodium distachyon* was used. This is because of much closely relatedness to the bread wheat. As Brachypodium distachyon is a monocot, wild annual grass belonging to family Poaceae Vogel et al., 2010). Furthermore, the Brachypodium has been successfully sequenced and genome and much of the genes are annotated and available on different data bases like Aramemnon etc. Brachypodium getting more popularity as model organism because of its small genome size, small size, lack of seed-shattering, self-fertility and having very short life cycle of about 11-18 weeks (Draper et al., 2001).

In present study, *Brachypodium distachyon* OSCAs sequences were retrieved from Aramemnon database as this database included all the information related to the proteins (transporters, channels) those exist in the membranes of the plants. *Brachypodium distachyon* was used as reference for blast in URGI and 10 OSCAs genes were predicted in wheat and used for primer synthesis. Similarly, available sequences of 10 *Brachypodium* OSCAs were used for blast in UnirProtKB (UniProkKB 2019) to identify OSCAs in wheat and retrieved protein sequences as well. OSCA proteins sequences were also retrieved for other members of *Poaceae, Brassicaceae* and *Malvaceae* families. With help of these retrieved protein sequences, phylogenetic trees were constructed by using MEGA 7.0 using Maximum likelihood method (kumar et al., 2016). Evolutionary relatedness of the OSCAs in among all families (Poaceae, Brassicaceae and Malvaceae) was measured and phylogenetic trees were shown.

Wheat seedlings were grown on Lombanaes media for growth and further for physiology. Lombanaes media was supplemented by different known concentrations of the chemicals and was used for the plant development for first fourteen days. Further the experiment was

divided into three sections. In experiment I, the plants were grown under deficiency of the calcium, experiment II was grown under normal calcium concentrations and experiment III, the plants those were grown had to face the toxicity of the calcium in hydroponic system. Plants were harvested by following Day 7, Day 14 and D21 from each experiment and measured the fresh weight of the plant immediately. Physiological symptoms show both in shoots and roots in deficiency and toxicity of the plants as deficiency of the calcium affected most causing chlorosis and low volume of the roots.

Expression analysis of the TaOSCAs were done by using Real time PCR. As it was believed during calcium stress conditions TaOSCAs in wheat plant must be regulated. Seven out of ten TaOSCAs have shown regulation against calcium stress as TaOSCA.1, TaOSCA.2, TaOSCA.4, TaOSCA5, TaOSCA7, TaOSCA9 and TaOSCA.10 represent clearly the presence of these protein transporters across the cells of roots and shoots in wheat. Expression analysis revealed the presence of these proteins in the roots and shoots may working as channels or carrier for the transport of calcium.

5.1. Conclusion & future recommendations:

Current study was aimed at the identification of the TaOSCAs in wheat. In silico analysis have shown, there were 10 TaOSCAs in wheat. Phylogenetic analysis was revealed among economically important species of families Poaceae, Brassicaceae and Malvaceae showed evolutionary relatedness. Application of the stress conditions (deficiency and toxicity) has detrimental effects on the growth and development of the plant after having phenotypic analysis. Expression analysis of 7 TaOSCAs (TaOSCA.1, TaOSCA.2, TaOSCA.4, TaOSCA5, TaOSCA7, TaOSCA9 and TaOSCA.10) were performed both in roots and shoots in response to calcium stress indicating their role in regulating and translocating calcium in wheat.

As majority of the OSCAs work as mechanoreceptors, there is availability of the wheat whole genome, but it requires functional characterization further and need to be annotated. Full length of the TaOSCAs can be used for the sequencing and characterized. Further available information can be very helpful to studying the interaction of the TaOSCAs through different strategies like Yeast-Two hybrid systems. Study can be further extended and helpful in the manipulation of the genes for the development of improved wheat verity. If we can manipulate these protein members and check the interactions, we can enhance the wheat plant to withstand with calcium and drought stress conditions.

6. References

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barley grown in chelatorbuffered nutrient solution and its effect on uptake of Cu, Fe, and

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7. Appendix

Table 7.1: Accession numbers and sequence length from OSCAs in *Brachypodium*

Accession Numbers	Sequence length
Bradi4g03360.1	795
Bradi2g39230.1	768
Bradi1g10130.1	740
Bradi2g60810.1	707
Bradi1g12670.1	762
Bradi2g40490.1	786
Bradi1g58380.1	729
Bradi3g34540.1	794
Bradi2g26180.1	767
Bradi4g00860.1	764

Table 7.2: Accession numbers and sequence length from OSCAs in *Triticum aestivum*

Accession Numbers	Sequence Length
A0A341VFP4	797
A0A1D5SJ36	764
W5ESQ4	745
A0A341TL42	717
A0A341U0Z9	766
A0A1D6RTK9	768
W5BA01	725
A0A0F7IRW9	805
A0A341NQF4	767
A0A341V1R0	804

Table 7.3: OSCAs in Family Poaceae

Family Poaecae	OSCA.1	OSCA.2	OSCA.3	OSCA.4	OSCA.5	OSCA.6	OSCA.7	OSCA.8	OSCA.9	OSCA.10
Leersia perrieri	A0A0D9V1S1	A0A0D9WKK4	A0A0D9WG68	A0A0D9XMX9	A0A0D9Y1R8	A0A0D9VXB2	A0A0D9VYH0	A0A0D9Y0W9	A0A0D9VA92	A0A0D9WVH0
Panicum hallii	A0A2S3HVH2	A0A2T7E9D0	A0A2T7EE90	A0A2S3IN99	A0A2T7EJQ7	A0A2T7EJQ7	A0A2S3IIK3	A0A2S3HF13	A0A2S3HN17	A0A2T7EKQ5
Brachypodium distachyon	I1HND1	I1HMZ6	I1HJQ1	I1I6J6	I1IG57	A0A0Q3GU27	I1GNT3	I1IH28	I1HV80	I1H416
Sorghum bicolor	C5XMV4	C5YX25	C5YY97	C5WQN2	A0A1Z5R7F6	A0A1B6QIW6		A0A1Z5R7M7	C5XHW4	C5X9J3
Setaria italica	K3XET6	K3Z409	K3ZD17	K4A5Z0	K3Z418	K4A656	K4AMR1	K3Z499	K3XQ47	K3ZR16
Zea mays	A0A1D6NN89	K7V1X1	A0A1D6M4Q2	A0A1D6KA79	A0A1D6MV54	A0A096TML4	A0A1D6GIF5	A0A317YI54	A0A1D6G4L0	B0FSL2
Dichanthelium oligosanthes	A0A1E5VIY5	A0A1E5VIY5	A0A1E5V6V6	A0A1E5VIY5	A0A1E5US40	A0A1E5US40	A0A1E5VC41	A0A1E5WFR5	A0A1E5US40	A0A1E5VRA7
Hordeum vulgare	F2E7I9	A0A287EI08	F2EDY7	A0A287FJ55	M0WBA6	A0A287MYK6	F2EFG9	F2CWE5	F2CUX4	M0X864
Aegilops tauschii	M8BR22	R7WDP1	M8BK31	R7W711	R7WAJ0	M8C5E0	R7WE63	R7WAJ0	M8BDD8	N1R2X7

Table 7.4: OSCAs in Family Malvaceae

Family Malvaceae	**OSCA.1**	**OSCA.2**	**OSCA.3**	**OSCA.4**	**OSCA.5**	**OSCA.6**	**OSCA.7**	**OSCA.8**	**OSCA.9**	**OSCA.10**
Theobroma cacao	A0A061DTI0	A0A061DTI0	A0A061DTI0	A0A061G7A3	A0A061FE86	A0A061FE86	A0A061FE86	A0A061EFV9	A0A061FT93	A0A061GCG8
Corchorus capsularis	A0A1R3J347	A0A1R3J347	A0A1R3J347	A0A1R3IZ61	A0A1R3FYM6	A0A1R3FYM6	A0A1R3FYM6	A0A1R3I9W7	A0A1R3HZB1	A0A1R3HJA8
Gossypium hirsutum	A0A1U8P6V8	A0A1U8P6V8	A0A1U8P7K3	A0A1U8NC03	A0A1U8KYX3	A0A1U8KJX6	A0A1U8KYX3	A0A1U8N213	A0A1U8JD99	A0A1U8PQS9
Gossypium raimondii	A0A0D2NCI4	A0A0D2NCI4	A0A0D2NCI4	A0A0D2T4X5	A0A0D2Q5Y9	A0A0D2R5L6	A0A0D2R5L1	A0A0D2RY34	A0A0D2SAD6	A0A0D2UMJ7
Gossypium arboreum	A0A0B0MI77	A0A0B0Q0B6	A0A0B0NIP2	A0A0B0PQY0	A0A0B0PTJ1	A0A0B0PTJ1	A0A0B0PTJ1	A0A0B0Q354	A0A0B0NSZ3	A0A0B0NN19

Table 7.5: OSCAs in Family Brassicaceae

Family Brassicaceae	OSCA.1	OSCA.2	OSCA.3	OSCA.4	OSCA.5	OSCA.6	OSCA.7	OSCA.8	OSCA.9	OSCA.10	OSCA.11	OSCA.12	OSCA.13	OSCA.14
Arabidopsis thaliana	At4g04340.1	At4g22120.1	At1g11960.1	At1g62320.1	At3g21620.1	At4g15430.1	At4g02900.1	At1g32090.1	At1g58520.1	At1g10090.1	At3g01100.1	At1g69450	At3g54510.1	At1g30360.1
Capsella rubella	R0H5M1	R0GH02	R0IB81	R0HTW4	R0I696	R0H1T5	R0FD86	R0IJ11	R0I5J2	R0ID01	R0GAL0	R0HV24	R0FSC4	R0GUZ7
Eutrema salsugineum	V4LEG5	V4MQ07	V4KD38	V4MD99	V4M5X8	V4MHB8	V4L2S6	V4KUA7	V4JVY1	V4KDN9	V4LHA7	V4MAS9	V4LRD9	V4MQ78
Arabidopsis lyrata	D7M169	D7ME79	D7KN76	D7KUS0	D7L0U9	D7MAI0	D7M3B8	D7KHF7	D7KXW9	D7KKL9	D7LAJ1	D7KX16	D7LUU6	D7KET4
Brassica oleracea	A0A0D3B8J3	A0A0D3A531	A0A0D3E4V5	A0A0D3E4V5	A0A0D3A7Y6	A0A0D3A7Y6	A0A0D3B8P9	A0A0D3CGU6	A0A0D3AA46	A0A0D3DNU1	A0A0D3CNH0	A0A0D3CZ87	A0A0D3DT14	A0A0D3BJ42
Brassica rapa	M4C9B0	M4DAS7	M4DT88	M4EE41	M4CCA7	M4FAG9	M4C9H6	M4EYG6	M4FCW4	M4DPM1	M4FDH4	M4CJJ7	M4CS84	M4EUA0

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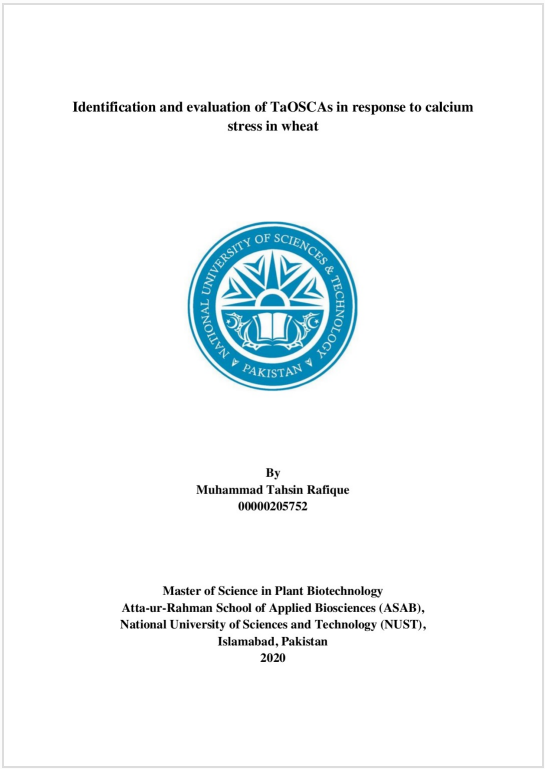

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