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Abstract

Temperature affects the growth and productivity of plants, but its effect depends on nature of plant species i.e. warm season or cool season plants. Increased temperature, increases rate of photosynthesis and respiration but only up to certain levels of increased temperature; there after it will reduce both photosynthesis and respiration. Increased temperature is not only a problematic factor for vegetative growth but also for reproductive development like flowering, pollen development, fruit set and finally total yield of the crop plants. The negative effect of high temperature is becoming a major problem because of predicted increase of 2°C of earth surface temperature by 2050. In present days due to increasing threat of global warming to horticultural crop production, research on high temperature stress in relation to plant productivity becomes important and urgent. Exposure to high temperature causes reduced yields in tomato (*Solanum lycopersicum L.*). The level of proline content of anthers plays important roles in acquiring heat tolerance in tomato but this proline transport to anthers is seriously impaired by heat stress leading to increased level of proline accumulation in leaves (source) instead of developing pollen grains (sink) during heat stress. We made an attempt to develop tomato plants expressing the proline transporter to anthers gene *LePROT1* under the control of HSP 18.2 promoter from Arabidopsis to increase supply of proline to developing anthers of tomato plants under heat stress. Here we got total six transformed plants after confirmation by PCR. We treated all transformed plants with high temperature at 38°C for 2 hours along with controls at 3 days before anthesis and then grew at normal temperatures after heat treatment. All transformed plants performed far better than control plants; pollen stainability averaged 93.98% in transformed plants and 81.1% in untransformed control plants, pollen germinability averaged 91.55% in transformed plants and 74.6% in untransformed control plants. There was a significant difference in yield between untransformed control plants and plants transformed with *LePROT1* gene. All these differences in pollen stainability, germinability and in total yields were positively correlated with proline content in their developing anthers at 3 days before anthesis stage. Proline quantity is significantly different between untransformed control plants (860.87 μM/g FW) and plants transformed with *LePROT1* gene (2117.74 μM /g FW). In this same experiment we also did attempt to screen drought and salt tolerance in plants transformed with *LePROT1* and untransformed control plants in early seedlings stage by artificial invitro stress

treatments. Seeds from T_1 generation plants and control plants were germinated and allowed to grow on artificial medium supplemented with 50g/l PEG to induce drought stress conditions and grown at 38°C for one week. Plants transformed with *LePROT1* gene showed higher growth rate than untransformed control plants in terms of root length. Transformed plants had higher root length (9.19 cm) than control plants (6.66 cm) and there was no significant difference between control and transformed plants in hypocotyl length under drought stress. This increased root length in transformed plants than control plants is in accordance with their higher proline accumulation. Untransformed control plants accumulated less proline content (6.82 µg /g FW) and transformed plants accumulated more proline (11.94 µg/g FW) under artificial drought conditions. Seeds from T_1 generation plants and control plants were germinated and allowed to grow on artificial salt stressed medium supplemented with 100 mM NaCl to induce salt stress conditions and grown at 38°C for one week. Plants transformed with *LePROT1* showed significant differences in both root length (9.42 cm) and hypocotyl length (4.74 cm) than the root length (6.16 cm) and hypocotyl length (1.66 cm) of untransformed control plants. These differences in root and hypocotyl lengths of control and transformed plants was positively correlated with their proline content i.e. control plants accumulated less proline (17.3 µg/g FW) than *LePROT1* transformed plants (25.16 µg/g FW). Proline content in all three treatments i.e. heat stress, salt and drought stress is higher in heat stress and then followed by salt and drought stresses.

Plants can acquire inducible environmental stress resistance by remodeling of membrane fluidity. Plants ability to adjust membrane lipid fluidity, achieved by changing levels of unsaturated fatty acids through fatty acid desaturase genes, is a feature of stress acclimation. Modification of membrane fluidity results in an environment suitable for the function of critical integral proteins. Membrane fatty acids play main important role in tolerance to high temperature. Plastid omega-3 fatty acid desaturase catalyzes the conversion of dienoic fatty acids (16:2 and 18:2) to trienoic fatty acids (16:3 and 18:3) in glycerolipids which are the main constituents of chloroplast membranes. Exposure to high temperature causes reduced yields in tomato (*Solanum lycopersicum L.*). We produced transgenic tomato plants that express the transcript of double-stranded RNA (dsRNA) of tobacco plastid omega-3 fatty acid desaturase gene *Nt FAD7* to induce post transcriptional gene silencing. The steady state messenger-RNA level of targeted gene was low in transformed plants. Under the heat stress

these transformed plants silenced for this gene showed higher number of viable pollen grains (92.79%) and higher yields (90 fruits/ 6 plants) when compared to the viable pollen (74.6%) and yield (25 fruits) untransformed plants. These results indicated that post transcriptional gene silencing of omega 3 fatty acid desaturase gene is useful to increase tolerance to high temperature in plants. In this experiment trienoic fatty acids quantity in transformed plants was from 10 to 45% less than the trienoic fatty acid composition of control plants and correspondingly increased dienoic fatty acids in transformed plants. Transformed seedlings growing under heat stress at 32°C for one week performed far better than the untransformed seedlings in terms of hypocotyl length (3.6 cm) than the control plant hypocotyl length (1.5 cm). These results indicate that down-regulation of the transcript level in the NtFAD7 by introduction of NtFAD7 dsRNA constructs is useful to decrease the trienoic fatty acid contents of the vegetative tissues in higher plants and give tolerance for higher temperature. Therefore, it is reasonable to conclude that the accumulation of proline through proline transporter in relation to heat stress under the control of heat inducible promoter and decrease of trienoic acid formation through gene silencing could be useful methods to increase heat tolerance in tomato and it could be a solution to increase crop yields under this present arena of global warming.

Key words

ABA: Abscisic Acid

CaMV 35S: Cauliflower Mosaic Virus 35 S Promoters

CBF: C- Repeat Binding Protein Factor

CDPKs: Calcium Dependent Kinases

C-HT-L: Control Plant Leaves Treated In High Temperature

CPKs: Calcium Dependent Protein Kinases

CTAB: Cetyl Trimethylammonium Bromide

DAs: Dienoic Acids

DEPC: Diethyle Pyro Carbonate

DGDG: Digalactosyldiacylglycerol

DMSO: Dimethyl Sulfoxide

dNTPs: Deoxyribonucleotide Triphosphate

DREB: Dehydrative Responsive Element Binding

Ds RNA: Double Stranded RNA

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic Acid

ERF: Ethylene Responsive Factor

FAD7: Fattyacid Desaturase 7

FAME: Fatty Acids Methyl Esters

FAO: Food and Agricultural Organisation

GC-MS: Gas Chromatography-Mass Spectrometry

GHS: Gradual Heat Shock

GUS: Beta-Glucuronidase

H_2O_2: Hydrogen Peroxide

HSF: Heat Shock Factors

HSPs: Heat Shock Proteins

HTS: High Temperature Stress

KCM: Potassium Calcium Magnesium Chloride Solution

LEA: Late Embryogenesis Abundant Proteins

LePROT1: Lycopersicon Esculantum Proline Transporter1

MAPK: Mitogen Activated Protein Kinase Kinase

MGDG: Monogalactosyldiacylglycerol

MLV-RT: Moloney Murine Leukemia Virus Reverse Transcriptase

MQ H_2O: Milli Que Water

MV: Methyle Viologen

NOS: Nopaline Synthase

O.D: Optical Density

ORF: Open Reading Frame

OXO: Oxalate Oxidase

P5CS: Δ^1-Pyrroline-5-Carboxylate Synthase

PEG: Polyehtylene Glycol

PG: Phosphatidylglycerol

PLD: Phospholipase D

PLFA: Phospholipid Fatty Acids

POFs: Proteins Of Unknown Functions

PS-II: Photosystem II

$P^{TH\text{-}ASGS}$: Plasmid Containing Antisense Gus Sense gene fragments

ROS: Reactive Oxygen Species

SAMDC: S- Adenosyl Methionine Decarboxylase

SAR: Systemic Acquired Resistance

SHS: Short Heat Shock

SOD: Superoxide Dismutase

STET: Sucrose Triton EDTA TRIS Buffer

TAs: Trienoic Acids

TE: TRIS EDTA BUFFER

T-HT-L: Transformed Plants Leaves Treated In High Temperature

TSB: Transformation and Storage Buffer

Project 1

Engineering of *LePROT1* gene against high temperature, salt and drought stresses

1 INTRODUCTION

Tomato (*Solanum lycopersicum L.*) is the second most important vegetable crop next to potato due to the high value of its fruit in terms of versatility, making it suitable for both fresh consumption and in numerous types of processed products (Rick, 1988). Present world production is about 100 million tonnes of fresh fruit produced on 3.7 million hectares. Tomato production has been reported from 144 countries (FAOSTAT Database, 2004). Tomato and other crops production is influenced by different types of stresses during plant growth and development. Plant stress can be defined as any factor that is present at a level more or less than the normal requirement of the plant that could be lead to the death of the plant. Everywhere in the world, environmental stresses represent the most limiting factors for agricultural productivity. Besides plant-specific physiological traits, a large proportion of the annual crop yield is lost due to pathogens (biotic stress) or the detrimental effects of abiotic stress conditions including extremes in temperature, drought, or salinity. In many cases, both biotic and abiotic factors contribute to the severity of disease and yield loss. All the present day cultivated plants have been selected on the basis of competition and their performance under certain environmental conditions. In addition, the existing crop varieties underwent man-made selection for traits such as yield, size, taste, etc. However, all plant life is presently challenged by rapid environmental changes. In plants, biotic and abiotic stresses can trigger the generation of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide. These destructive reactive compounds can cause protein denaturation, DNA mutations, and lipid peroxidation. However, at low levels, ROS may also function as signaling molecules. Hydrogen peroxide produced by cytosolic or membrane-bound NADPH oxidases are implicated as a signal in defense responses against pathogens and herbivores, stomatal closure, and the regulation of cell expansion and plant development. When plants experience this stress during their lifecycle they will reduce the total yields depending on the developmental stages of plants. In all types of plant stresses, abiotic stress is one of the most important stresses that can influence the plant growth and yield.

1.1 Abiotic stress

Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment. The non-living variable must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of the

organism in a significant way. Abiotic stress is the most harmful factor concerning the growth and productivity of crops worldwide (Ji-Ping, 2007). Plants respond and adapt to these abiotic stresses in order to survive in unfavourable environmental conditions. Abiotic stress induces various biochemical and physiological responses in plants (Bray et al., 2000). Accumulation of various substances, such as sugars, sugar alcohols and proline, is observed during these stresses in various plants. These molecules are thought to function in osmotic adjustment. A plant hormone, abscisic acid, is produced under stress conditions and plays important roles in response to and tolerance against abiotic stress. Abiotic stress causes the crop loss higher than any other factor and, because of it, most major crops are reduced in their yield by more than 50% from their potential yield (Wang, 2007). It has also been speculated that this yield reduction will become worse with the dramatic climate changes expected in the future (Lane and Jarvis, 2007). Abiotic stresses like drought, oxidative, salt and high temperature stresses are well known as abiotic factors that have detrimental affect on plant growth and yield.

1.1.1 Drought stress

Drought is defined as the limitation of water availability over a prolonged period of time. Water is arguably the single most important constituent of a plant cell, comprising more than 90% of the fresh weight of most herbaceous plants. Water availability is one of the major limitations to plant productivity (Boyer, 1982). Drought is one of the most serious world-wide problems for agriculture. Agricultural regions affected by drought can experience yield losses up to 50% or more. Crop plants which can make the most efficient use of water and maintain acceptable yields will be at an advantage. Plants cannot maintain their cellular water content at a fixed point, constantly losing water to the surrounding environment through evapotranspiration. Land plants cannot prevent water loss to the environment; and the problem is exacerbated since gas-exchange is required for other beneficial processes, such as CO_2 fixation. Developing crops that are more tolerant to water deficits, while maintaining productivity, will become a critical requirement for enhancing agriculture in the twenty-first century. Understanding how plant cells tolerate water loss is a vital prerequisite for developing strategies that can impact agricultural and horticultural crop productivity and survival under these conditions of decreasing water availability. Significant work has been accomplished in this area.

1.1.1.2 Ability to survive in water-limited environments

Plants have developed various mechanisms to adapt their growth to limited water availability conditions. Some species of xerophytic and xeromorphic plants avoid drought-induced damage by maintaining a high cellular water content (such as cacti) or by establishing extremely deep rooting systems. Other species, including both mesophytic and xerophytic plants escape drought-induced damage by rapidly completing their life cycles (i.e. ephemeral plants). Avoiding drought is an extremely important adaptation for survival in a water-limiting environment.

1.1.1.3 Molecular responses to dehydration

1.1.1.3.1 Abscisic acid (ABA)

ABA is important molecule involved in dehydration stress. The level of ABA increased in plants in response to dehydration stress which in turn activates other gene cascades involved in protection against low water availability. ABA reduces water loss via transpiration by closing stomata of plant cells. In Arabidopsis plants under dehydration stress causes prompt synthesis of detectable ABA with in 2 h of stress and it reaches to maximum level after 10 h of dehydration stress (Kiyosue, 1994). Therefore, the treatment of plants with exogenous ABA has been used to mimic dehydration responses by this way we can make plant resistant to dehydration.

1.1.1.3.2 MAP Kinases

MAPK signaling pathway seems to play an important role in the molecular response to dehydration in plants. Characterization of multiple MAPK proteins revealed how they are activated and how signals are transmitted to the specific targets that are ultimately responsible for protection against dehydration damage. Plant cell senses dehydration through a change in the fluidity of the cell membrane by activation of MKK4, reducing the fluidity of cell membrane (Sangwan, 2002). In *A. thaliana* the levels and activities of the MAP Kinases, ATMPK4 and ATMPK6 are rapidly increased by a variety of stresses including dehydration (Ichimura, 2000).

1.1.1.3.3 Phosphatases

Phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolyzing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group. The important role of phosphatases in dehydration stress signal transduction has been established using the ABA-insensitive *Arabidopsis* mutants, *abi1* and *abi2*. These mutants display pleiotropic

phenotypes affecting seed dormancy, stoma regulation, and signal transduction during water stress (Koornneef, 1984; Merlot and Giraudat, 1997). Constitutive or conditional enhanced expression of *activated disease resistance 1* gene *(ADR1)* conferred significant drought tolerance in Arabidopsis (Chini, 2004).

1.1.1.3.4 Calcium signalling

In plants, a temporary increase in cytosolic [Ca+2] has been reported in response to a diverse range of abiotic and biotic stimuli (Kiegle, 2000) and is the sign of the early responses to dehydration (Sanders, 1999). The effect of calcium signalling depends on the Ca+2 wave which depends on the Ca+2 channel, the cellular location, and the dynamics of the change in [Ca+2] and its availability for downstream signalling pathways at the time the change in [Ca+2] occurs (Sanders, 1999). *Ca+2 dependent Kinases (CDPKs)* class of *Arabidopsis* enzymes, CPK10 (*AtCDPK1*) and CPK11 (*AtCDPK2*), are induced within 10 minutes upon dehydration stress (Urao, 1994).

1.1.1.3.5 Heterotrimeric G proteins

Heterotrimeric G protein is involved in stomata opening and water deficit-induced phospholipase D activity. In guard cells, ABA signalling requires G protein in response to dehydration stress (Frank, 2000). In *Arabidopsis* plants with a mutant allele of the *Gα* gene (*GPA1)*, ABA fails to inhibit the closure of stomata (Wang, 2001).

1.1.1.3.6. Phospholipid signalling

Phospholipase activity generates phosphatidic acid, which acts as a secondary messenger to activate downstream targets. Phospholipids signalling in various stress in plants is well studied (Munnik, 2001). In *A. thaliana,* transcriptional levels of *Phospholipase D genes (PLD)* like PLDδ is induced after 3 h of onset of water stress, while *PLD*α is constitutively expressed during drought stress (Sang et al., 2001). In *Arabidopsis phospholipase C (PLC), AtPLC2 gene* is rapidly activated upon dehydration and its expression is independent of ABA (Hirayama, 1997).

1.1.1.4 Transcriptional studies on the drought tolerance

Drought triggers high-level expression of many genes which are involved in drought tolerance. A number of transcription factors that regulate the expression of several genes related to stress have been discovered. One such class of transcription factors is DREB/CBF, which binds to drought

responsive *cis*-acting elements. DREBs belong to the ERF family of transcription factors consisting of two subclasses, i.e. DREB1/CBF and DREB2 that are induced by cold and dehydration, respectively. Apparently DREBs are also involved in biotic stress signaling pathway (Agarwal, 2006). Trans-activation activity of ATMYB2, a drought-inducible Myb-related protein in *Arabidopsis,* acts as a transcriptional activator; the C-terminal acidic region of ATMYB2 can function as a transcriptional activation domain during drought tolerance (Urao, 1996). In Arabidopsis homeodomain-leucine zipper gene *Athb-12* as well as their other homologous leucine zipper genes accumulates especially during drought stress (Lee Y.-H, 1998). Molecular responses of plant against drought stress are shown as a flow chart in figure 1.

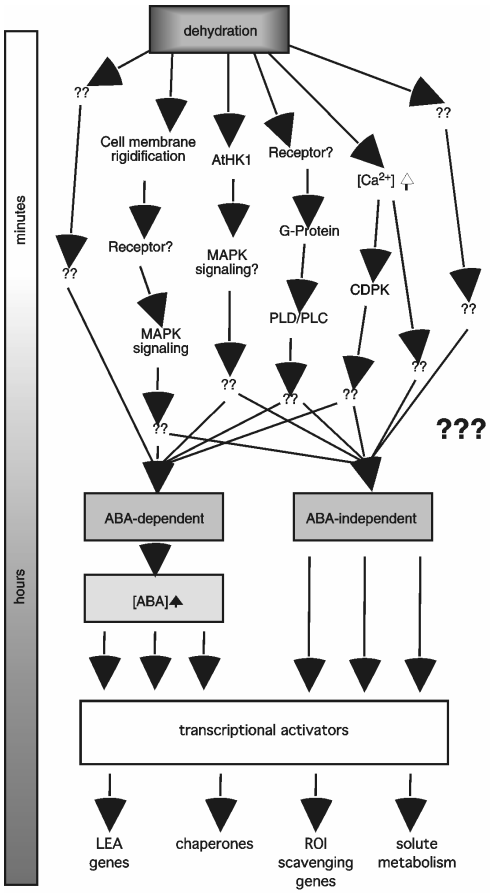


Fig. 1 Flow chart of drought inducible genes and related transcriptional factors (Dorothea and Eric, 2003)

1.1.2 Oxidative stress

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Plants, like all aerobic organisms absolutely require oxygen for normal growth and development and oxygen is a double-edged sword in terms of concentration. Continuous

exposure to oxygen can result in cellular damage and ultimately death. This is because molecular oxygen is continually reduced within cells to several forms of Reactive Oxygen Species (ROS), in particular the superoxide free radical anion (O^{-2}) and hydrogen peroxide (H_2O_2) which react with various cellular components to bring about acute or chronic damage sufficient to result in cellular death.

In response to increased ROS, the expression of genes encoding antioxidant proteins are induced, as well as that of genes encoding proteins involved in a wider range of cellular rescue processes. Environmental stresses increase ROS generation; there would be evolutionary pressure for selection of ROS signalling mechanisms that induce genes encoding antioxidant and cellular defence proteins. This defence role for ROS and such proteins may be one reason why the induction of many cellular defence/rescue genes is a common response to several environmental stresses and oxidative stress; and helps to explain acclimation and cross-tolerance, in which previous exposure to the same stress or to a different stress induces tolerance to subsequent exposure (Bowler and Fluhr, 2000). Biotic and abiotic environmental stresses not only enhance ROS generation via non-specific mechanisms, but also trigger defence signalling mechanisms that, if successful, start with induction of ROS generation, continue with induction of defence responses, and in the end removal of ROS to restore redox balance and cell survival.

1.1.2.1 ROS generation

In plants both electron transport and enzymatic sources are responsible to generate ROS. ROS production is increased by various stresses like excessive light energy, wounding, ozone, drought, UV-irradiations, pathogen challenge, low and high temperatures, and heavy metals (Dat, 2000; Bray, 2002; Neill, 2002; Vranova, 2002). The initial work on ROS generation in plants via NADPH oxidase-like enzymes started with plant-pathogen interactions, focussing on the "oxidative burst" (Doke, 1983). The oxidative burst, leading to the generation of H_2O_2, is typically induced in plants and cell cultures following challenge with either pathogens or elicitor molecules derived from them, and numerous studies have provided evidence for NADPH oxidase being the source of H_2O_2 (Lamb and Dixon 1997; Bolwell, 1999). Recent data implicate NADPH oxidase as also being the source of H_2O_2 generated during drought and ozone exposure, or following ABA treatment.

1.1.2.2 Removal of ROS

ROS produced during plant metabolic reaction under stress can be removed either via non-enzymatic or enzymatic mechanisms. Non-enzymatic antioxidants include vitamin C (ascorbate), vitamin E (tocopherol), glutathione, flavonoids, alkaloids, and carotenoids (Bray, 2000). In plants Enzymatic ROS scavenging mechanisms include SOD (superoxide dismutase), that is present in many cellular compartments, catalase, located in peroxisomes, and the ubiquitous ascorbate-glutathione cycle. SOD catalyses the dismutation of superoxide to H_2O_2, and is thus one of the primary mediators of H_2O_2 production from intracellular sources of superoxide. Antioxidant activity was increased by drought stress in several plant species (Gogorcena, 1995). When plants exposed to high light intensity also led to an accumulation of transcripts which codes for SOD (Mishra, 1995). In tobacco over-expression of pea chloroplastic Cu/Zn SOD results in increased resistance to methyl-viologen-induced membrane damage (Allen, 1995). Antisense silencing of catalase in tobacco generated enhanced levels of H_2O_2 in response to both abiotic and biotic stresses (Willekens, 1997). Transgenic tomato plants over expressing CaKR1(Hyeon, 2007) produced lower levels of free oxygen radicals, such as superoxide and hydrogen peroxide, and showed enhanced resistance to salinity and oxidative stress (Eun Soo Seong, 2007). Transgenic tobacco (*Nicotiana tabacum*) line BS1-31 accumulated mannitol approximately at the concentrations of 100 mM in chloroplasts and was identical to the wild type in phenotype and photosynthetic performance. The presence of mannitol in chloroplasts resulted in an increased resistance to methyl viologen (MV)-induced oxidative stress, documented by the increased retention of chlorophyll in transgenic leaf tissue following MV treatment (Shen, 1997). Transgenic Arabidopsis plants that constitutively expressed 23 different proteins of unknown function (POFs) four of which specific to Arabidopsis and other 18 are not specific but expressed in Arabidopsis in oxidative stress studies, confer tolerance to oxidative stress (Song Luhua, 2008).

The transgenic tobacco plants expressing wheat *oxalate oxidase (OxO)* gene showed increased tolerance to MV or high light-induced oxidative stress in both short-time and long-time tests by measuring their maximal photochemical efficiency of PSII (F_v/F_m), ion leakage and malondialdehyde (Wan, 2009). Mechanism that regulates redox balance in plants under oxidative stress is shown in figure 2.

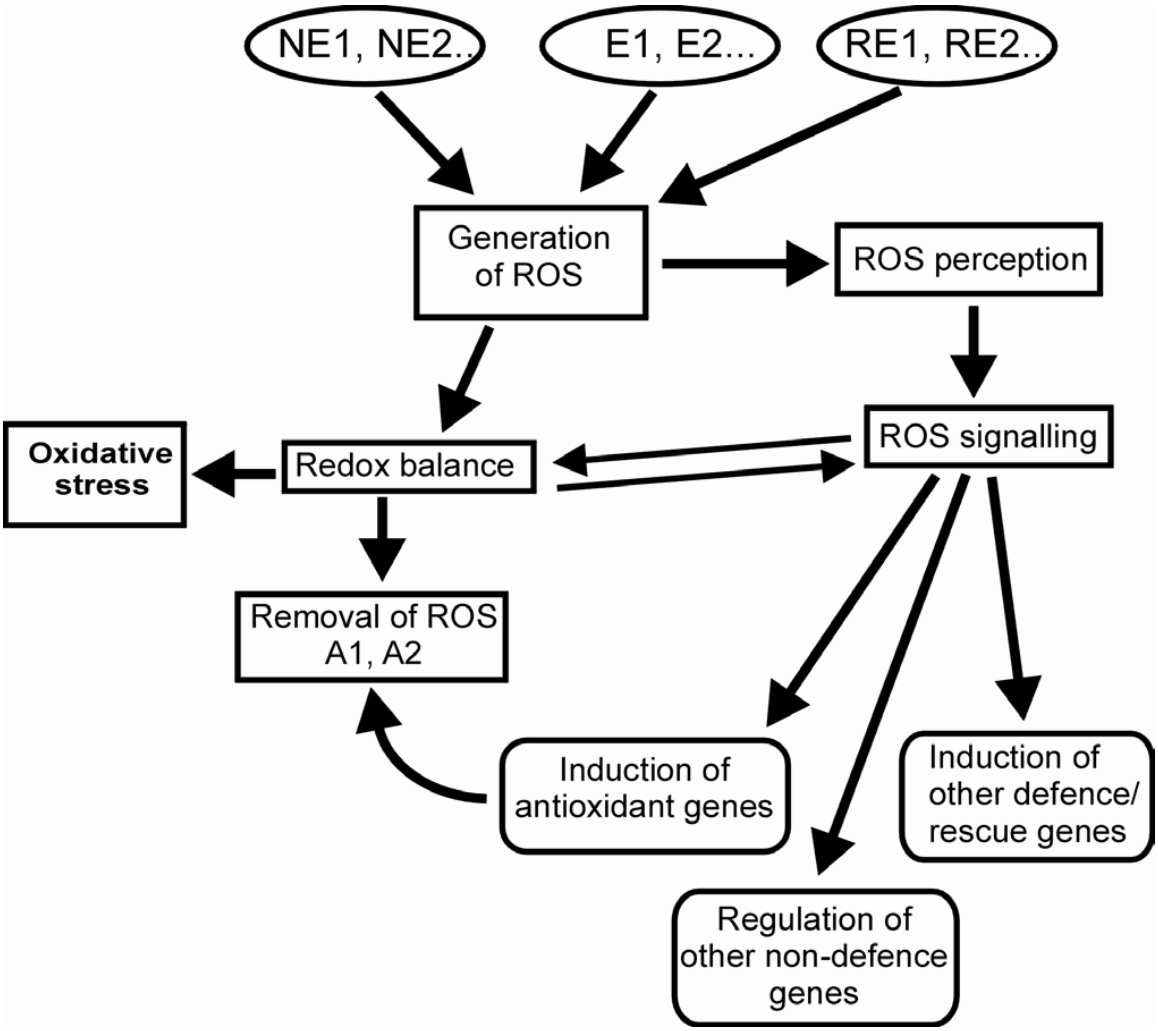


Fig. 2 Outline of the mechanisms that regulate redox balance in plant cells. NE1, NE2: non-enzymatic sources of ROS; E1, E2: enzymes generating ROS as side-products; RE1, RE2: dedicated ROS generating enzymes; A1, A2: non-enzymatic and enzymatic antioxidants (Radhika desikan et al., 2003)

1.1.3 Salt stress

Soil salinity is a major abiotic stress that adversely affects crop productivity and quality. The problem of soil salinity is increasing due to irrigation, improper drainage, seawater in coastal areas, and salt accumulation in arid and semi-arid regions. Salinity is detrimental to plant growth as it causes nutritional constraints by decreasing uptake of phosphorus, potassium, nitrate and calcium, ion cytotoxicity and osmotic stress. Under salinity, ions like Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the function of these proteins.

1.1.3.1 Plant adaptations to NaCl stress

Ion and osmotic homeostasis is necessary for plants to be salt tolerant. Intracellular ion homeostasis requires determinants that control toxic ion uptake and facilitate their compartmentalization into the vacuole (Zhu, 2003). Coordination between roots to shoot that restricts ion movement to the aerial part of the plant minimizes salt load to the shoot meristem and metabolically active cells. Establishment of ionic and osmotic homeostasis after salt stress is not only essential to prevent cellular death but is also required for the physiological and biochemical steady states necessary for growth and completion of the life cycle (Bohnert, 1995). Osmotic balance in the cytoplasm is achieved by the accumulation of organic solutes that do not inhibit metabolic processes, called compatible osmolytes. These are sugars (mainly sucrose and fructose), sugar alcohols (glycerol, methylated inositols), complex sugars (trehalose, raffinose, and fructans), charged metabolites (glycine betaine) and amino acids such as proline (Chen and Murata, 2002). These solutes also function to protect cellular structures through ROS scavenging (Zhu, 2001). Plants exposed to salt stress increase the synthesis of several proteins like late embryogenesis-abundant (LEA) proteins in vegetative tissues. These LEA proteins have properties similar to chaperones for the maintenance of protein structure during an osmotic stress episode (Ingram and Bartels, 1996). Transgenic oat plants expressing barley *HVA1,* a barley *(Hordeum vulgare L.)* group III LEA protein gene*,* an osmotic stress tolerance gene, show an increase in tolerance to salt stress conditions (200 m*M* NaCl) for yield traits including number of days to heading, panicle length, number of spikelets/panicle, number of tillers/plant, number of kernels/panicle, 1000-kernel weight, and kernel yield/plant (Hesham Oraby, 2005).

1.1.4 Heavy metal stress

In agricultural soils, heavy metal pollution is an increasing problem because of soil amendment with municipal sewage sludge and intense use of phosphate fertilizers, which contain Cd as a contaminant. In acidic soils heavy metal transport into plant root is increased in acid soils. Plant micronutrients like Fe, Mn, etc and other heavy metals cause phytotoxic effects, which include reduction of root growth, reduction in chlorophyll content and photosynthesis, inhibition of enzyme activities at high concentrations. So many plant species have developed tolerance at the level of genetics to survive in metal rich soils and in heavy metal polluted soils (Andres, 2001). Heavy-metal-tolerant plants respond to pollution either by transporting heavy metal out of plants cells or by accumulating heavy metals in certain plant parts where it can not interfere with normal

plant metabolic activities (Hall 2002). Heavy metal tolerance mechanisms of plants were shown in fig. 3.

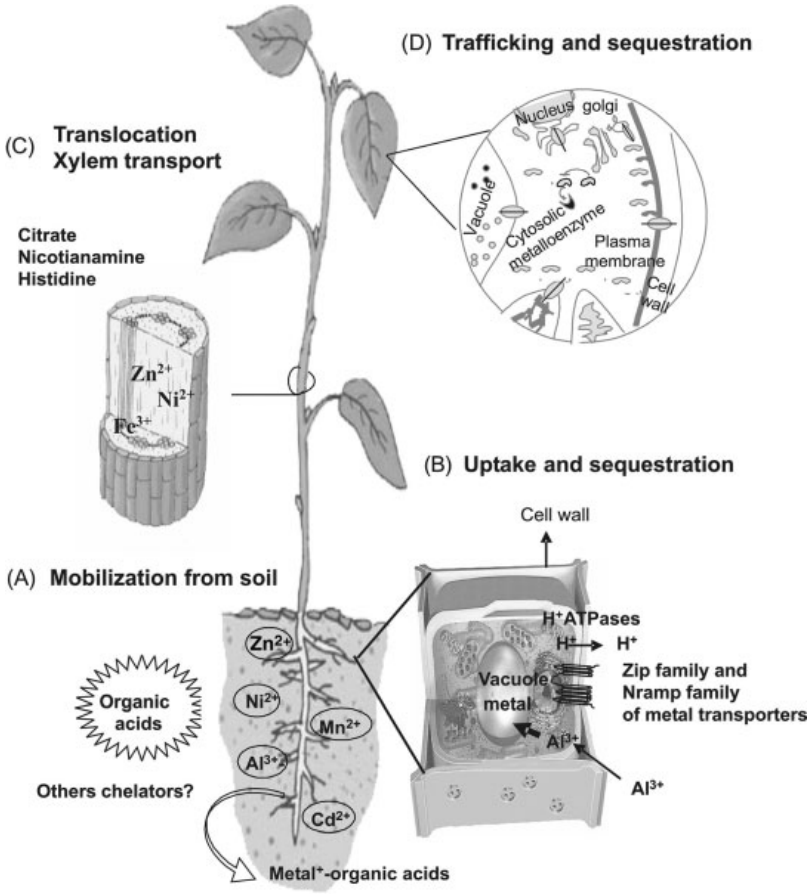


Fig. 3 Mechanisms involved in metal accumulation by plants (Plant abiotic stress book by Blackwell publishers page No. 150)

1.1.5 Cold stress

About two thirds of the world's landmass is annually subjected to temperatures below the freezing point and about half of it suffers from temperatures below – 20°C (Larcher, 2001). Ability of the plant to survive freezing temperatures is an essential trait. Many plants from temperate regions, for instance, are able to sense low, non-freezing temperatures and activate mechanisms that lead to an increase in freezing tolerance, a phenomenon known as cold acclimation (Smallwood and Bowles, 2002). Low temperature may impose stress on a plant in a two-fold manner: by the effects of low temperature alone, and by dehydration of the cells and tissues when cellular water freezes. Low temperatures above the freezing point are detrimental to many plants of the tropics and subtropics which can not acclimatize to cold. This kind of damage has been termed 'chilling' (Sakai and Larcher, 1987) and results primarily from loss of function

of biomembranes connected with a decrease of their fluidity and an inactivation or at least deceleration of the membrane-bound ion pumps. Low temperature stress problems were in fig. 4.

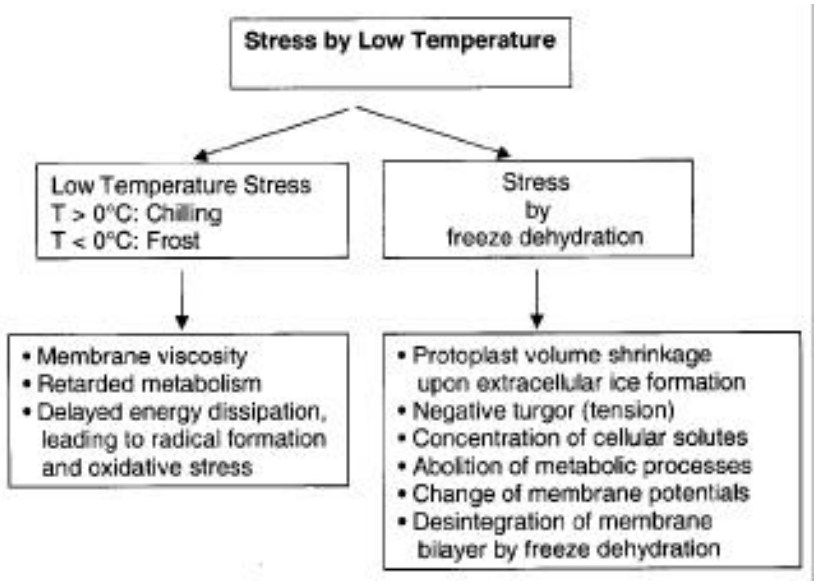


Fig. 4. Cold stress problems in problems in plants. (Plant abiotic stress book by Blackwell publisher page no.230)

1.1.5.1 Cold tolerance in plants

Cold tolerance engineering to make plant resistant to cold stress is a difficult task because cold tolerance is controlled by a group of genes involved in this process. For decades, understanding the mechanisms of chilling and freezing tolerance has been the major goal of investigators studying abiotic stress responses.

1.1.5.2 CBF pathway

A recent important advance made with *Arabidopsis* has been the discovery of a stress response CBF pathway. The *Arabidopsis* CBF cold response pathway has a central role in cold acclimation, the process whereby plants increase in freezing tolerance in response to low nonfreezing temperatures.

The CBF transcription factors recognize the cold- and dehydration-responsive DNA regulatory element designated the CRT (C-repeat)/DRE (dehydration-responsive element) (Baker, 1994). CRT/DRE elements are present in the promoters of many cold- and dehydration-responsive genes of *Arabidopsis*, including those designated *COR (cold-regulated)* (Thomashow, 1999). The *CBF1-3* genes are induced within 15′ when plant's exposed to low non-freezing temperatures. *COR* genes that contain the CRT/DRE-regulatory element were induced after 2 h when plants

were subjected to low temperature (Liu, 1998). Expression of the *CBF* genes results in an increase in freezing tolerance (Gilmour, 2004) and appears to have a role in chilling tolerance as well (Zhang, 2004).

The chilling sensitivity of plants is closely correlated with the degree of unsaturation of fatty acids in the phosphatidylglycerol of chloroplast membranes. Plants with a higher levels of cis-unsaturated fatty acids are resistant to chilling, whereas plants with lower levels of cis-unsaturated fatty acids are not resistant to cold. The chloroplast enzyme glycerol-3-phosphate acyltransferase seems to be important for determining the level of phosphatidylglycerol fatty acid unsaturation.

Transgenic tobacco (*Nicotiana tabacum* cv *SR1*) plants engineered by the introduction of a chloroplast ω-3 fatty acid desaturase gene (*FAD7* gene) isolated from *Arabidopsis* under the control of cauliflower mosaic virus 35s promoter (Over expression) shown increased levels of 16:3 and 18:3 fatty acids and decreased levels of hexadecadienoic and linoleic acids. The low-temperature- induced chlorosis was much reduced in the plants transformed with *fad7* when compared to wild type plants (Kodama, 1994). Transgenic tobacco plants expressing chloroplast-specific *ω-3-fatty acid desaturase gene (FAD7)* under the control of a cold-inducible promoter (*cor15a*) from *Arabidopsis* exposed to low temperature for prolonged periods like 45 days at maximum 3.5^0c. Chloroplast membrane ultrastructure was unaffected in *corl5a-FAD7* transgenic plants where as in wild type plants a loss of granal stacking and disorganization of the thylakoid membrane under prolonged exposure to cold temperatures (Khodakovskaya, 2006).

1.1.6 Heat stress

Heat stress is one of the most important among the abiotic stresses. Heat stress is defined as where temperatures are hot enough for sufficient time that they cause an irreversible damage to plant metabolism or development. The concept of heat stress response had accidentally come into light by the pioneer work of the Italian developmental biologist Ritossa (1962). She increased temperature of growth chamber with cultures of *Drosophila* by mistake and observed occurrence of developmental and extraordinary changes in gene action pattern of polytene chromosome in salivary glands of drosophila, then led to the discovery of production of heat shock proteins (HSPs) in response to heat stress. The problem of high temperature is becoming a major problem because of the predicted increase of earth surface temperature by 2°C by the year 2050.

1.1.6.1 Problems of heat stress

Plants suffer the ups and downs of temperature of their environment, while animals often regulate their temperature, either by movement or by changes in metabolism. Therefore, global warming may affect plants more than animals and there are indications that plants experience substantial damage from high temperature stress. In tropical climates, excess of radiation and high temperatures are often the most limiting factors affecting plant growth and final crop yield. High temperatures can cause considerable pre- and post-harvest damages, including scorching of leaves and twigs, sunburns on leaves, branches and stems, leaf senescence and abscission, shoot and root growth inhibition, fruit discoloration, damage, and reduced yield (Guilioni, 1997; Ismail and Hall, 1999; Vollenweider and Gunthardt- Goerg, 2005). Heat stress has been reported as one of the most important causes of reduction in yield and dry matter production in many crops (Giaveno and Ferrero, 2003). Estimates range up to a 17% decrease in crop yield for each degree Celsius increase in average growing season temperature (Lobell and Asner, 2003).

1.1.6.1.1 Influence of high temperature on plant processes

High temperature stress will influence major plant process during their growth.

1.1.6.1.2 Effects of heat stress on plant Vegetative stages

The plant morphological features depend on the stage of development; however it depends on the genitical background of the cultivars and its location. High temperature stress is one of the environmental factors that limit plant growth (Levitt, 1980; Boyer, 1982; Frova, 1997).

High soil temperatures can reduce plant emergence. The maximum threshold temperatures for germination and emergence are higher for warm-season than for cool-season annuals. For example tomato seeds usually germinate within 5 to 10 days when kept in the optimum temperature range of (21 to 27°C). Germination is delayed by lower temperatures and accelerated by higher temperatures up to a certain limit. Temperatures below 10°C or above 35°C are detrimental to germination. The strength of hydrophobic interactions is strongly temperature dependent. Membranes self assemble from amphipathic lipids as a result of hydrophobic interactions. Therefore, their properties will depend critically on temperature. Membrane components suited to one temperature will be inappropriate at other temperatures. In grapes, heat stress makes chloroplasts in the mesophyll cells become round in shape, the stroma lamellae become swollen, and the contents of vacuoles form clumps, whilst the cristae are disrupted and mitochondria become empty (Zhang, 2005). Heat stress produces malformed and mosaic leaves

in mulberry (Kuniaki, 1996). In wheat, development of leaves was linearly related to shoot meristem temperature and the time between appearances of successive leaves on the main culm in wheat (Sayed, 1996). High temperature stress reduces the rate of biomass allocation to leaves, stems, and roots when plants were grown at 30/26°C (day/night temperature, 12/12hr) in snap bean (Hide, 2006). Heat stress makes *Salvia splendens* plants able to attain more rapid canopy and are able to maintain healthier leaves and supply assimilates to reproductive development and root growth and to accumulate higher biomass to compensate with heat stress loss (Natarajan, 2006). High day temperatures can cause damage to components of leaf photosynthesis, reducing carbon dioxide assimilation rates compared with environments having more optimal temperatures. Sensitivity of photosynthesis to heat mainly may be due to damage to components of photosystem II located in the thylakoid membranes of the chloroplast and membrane properties (Al-Khatib and Paulsen, 1999). Photosynthesis is one of the physiological processes that are most sensitive to high temperature stress (Berry and Björkman, 1980). Inhibition of photosynthesis by high temperature stress is a common occurrence for plants in tropical and subtropical regions and the temperate zones where plants are exposed periodically to high temperatures (Larcher, 1995). It is well documented that high temperature stress causes damage to photosynthetic electron transport (Berry and Björkman, 1980). Heat stress severely damages the mesophyll cells and increases permeability of plasma membrane (Zhang, 2005). With the onset of high temperature regime, *Zygophyllum qatarense* produces polymorphic leaves and tends to reduce transpirational water loss by showing bimodal stomatal behavior (Sayed, 1996). Rice plants grown continuously at 34°C increase accumulation of biomass and faster increase in leaf area than the plants grown at normal temperature at 28°C (Baker, 1990). Heat stress severely reduces the capacity of plants to keep sufficient water level in tissues when there is sufficient moisture availability regardless of ambient temperature (Machado and Paulsen, 2001) and heat stress causes more water loss during day time than in night time. In tomato, heat stress changes the leaf water relations and hydraulic conductivity of roots (Morales, 2003). High temperature during day time increases the transpiration rate of plants leading to water deficiency and decrease in water potential, influencing a lot of physiological processes in plants. Basipetal assimilate transport to roots of heat stresses tomato plants is reduced (Dinar, 1985). When strawberry plants are treated with gradual heat shock (GHS) and short heat shock treatments (SHS), GHS reduces the total protein content of the plants, and SHS increases the activity of peroxidase enzyme (Hatice, 2004). In spinach, when plants treated with high temperature 35°C, significantly reduces the CO_2 assimilation rate when compared to normal temperature (Yunlai, 2007). Tomato plants

grown at 25 - 30°C have more net assimilation ratio than plants grown at other temperatures (Khavari, 1980). High temperatures reduced growth of tubers more than of shoots in potato (Krauss, 1984). Photosynthetic rates were increased slightly at the higher temperature but limited to certain range of temperatures. Heat stress increases accumulation of leaf sucrose and decreases starch accumulation in mature leaves but does not affect glucose. High temperature stress leads to decrease in starch levels in leaves of tomato and callose formation is found on phloem sieve tube plates of leaf petioles exposed to 72 h of high temperatures. The possible reason for this is an increase in starch hydrolysis and inhibition of starch formation (Dinar 1985).

1.1.6.1.3 Effect of heat stress on reproductive process

Plant reproduction is highly vulnerable to environmental conditions such as temperature and, consequently, planet warming may have significant consequences on the reproductive phase with serious implication in agricultural crops. Sexual reproduction process in many cultivated vegetables like tomato is more sensitive to high temperature than vegetative process; therefore plant flowering and fruit set processes are more vulnerable to high temperature (Sato, 2002, Raja Reddy and Kakani 2007). High night temperatures during reproductive development can reduce yields of cowpea *Vigna unguiculata* (L.) (Abdelbagi, 1999). Temperature stress during kernel development affects maize *(Zea mays)* grain growth and yield stability by changing the concentration of hormones (i.e. imbalance in reproductive tissues and in developing kernels) (Nordine Cheikh, 1994). Heat stress during meiosis in wheat can reduce yield by causing abnormal ovary development, complete absence of an embryo-sac accompanied by reduced nucellus development and small embryo-sacs that contain the full complement of cells, but which results in reduced pollen tube growth and seed set (HS Saini*,)*. High temperature stress (HTS) in *Brassica napus* plants grown at high temperature for 1 or 2 weeks after the initiation of flowering, produced seedless, parthenocarpic fruit or aborted on the stem, pollen viability and germinability were slightly reduced and its development was abnormal (Lester, 2004). Heat stress during vegetative development of brassica plants resulted in reduction in the number of flowers, decreased seed yield and led to a decrease in total yield (Morrison, 2002). Ground nut plants grown under high temperature showed strong negative correlation in fruit number, fruit set and pollen production and viability than when plants were grown at normal temperature (Varaprasad, 1999). Wheat plants were subjected to high temperature before anthesis, i.e. between ear

emergence and anthesis resulting in reduction of final grain weight due to size reduction of the carpel during high temperature stress (Calderini, 1999).

High-temperature treatment (day/night, 32/26 °C), of pepper plants reduced the percentage of pollen germination, pollen tube growth and fertilization resulting in flower abscission and finally leading to reduced fruit-set and yield (Leah, 2002). Flower bud abortion occurs in pepper when plants were grown at a 34°C/28°C day/night temperatures (Rylski, 1982). Canola or rapeseed (*Brassica napus* L. cv. Westar) plants grown under high temperature conditions (32°C/26°C; day/night) showed closed flower buds and protruding stigmas. The stamens were reduced in size and the anthers showed abnormal microsporogenesis. The gynoecium, although normal in appearance, did not set seeds and ovule development was aberrant (Polowick, 1988). When chili pepper plants were subjected to heat stress (38/30 °C day/night) immediately after anthesis for 5 or 10 days, fruit growth, seed yield, and seed quality were adversely affected moreover the stage of development at which the plants exposed to high temperatures is an important factor in fruit and seed growth and in seed quality (Pagamas, 2008). When sweet cherry (*Prunus avium* L.) plants were grown under high temperature showed a decreased in stigma receptivity, pollen adhesion, support for pollen tube penetration and its germination (Hedhly, 2003). High temperature stress more than 34°C/21°C day night temperature led to limited carbohydrate supply to the developing flowers and finally led to flower abscission (Rylski, 1982). Pepper plants grown under high temperature (25/35 °C, day/night) showed abscission in a small portion of the flower bud only and reduce uptake of sucrose from source leaf to developing flower buds, fruits and roots of the heat stressed plants (Aloni, 1994). According to Erickson (2002) in bell pepper (Capsicum annum L.) flower buds at <2·5 mm in length, corresponding to microspore mother cell meiosis to tetrad dissolution, and flowers that reached anthesis during the high temperature exposure had reduced fruit set when exposed to 33°C for 48 or 120 h. Flower buds at <2.5 mm in length also had reduced pollen viability when exposed to 33 °C for 120 h and microspores produced during high temperature remained small and clumped without a thick exine. Combination of high day (32 °C) and night temperature (28 °C) had no effect on normal vegetative growth but it affected fruit set, number of seeds per fruit and led to flower abortion (Firon, 2006).

1.1.6.1.3.1 Effect of temperature on flower drop

High temperature stress induces flower abscission by limiting the supply of nutrients to the sink tissues leading to reduced fruit set in tomato. Possible reason for the flower abscission is limited supply of nutrient import, increased production of ethylene by reproductive organs (Aloni, 1994). According to (Roberts, 2002) Auxins play an important role in reducing flower abscission during high temperature in tomato by diffusing down the pedicel and preventing the separation of cells from abscission layer.

1.1.6.1.3.2 Effect of temperature on microsporogenesis

In general, early pollen development stage in plants seems to be sensitive to abiotic stress. Tomato microsporogenesis shows maximum sensitivity to extremely high temperature at the stage of pollen mother cell meiosis (Iwahori, 1965). Heat stress exclusively at or around anthesis showed strong effect and indicated that male reproductive development is generally more vulnerable to damage than female reproductive development. Heat stress at or just before anthesis decreased seed set and reduced pollen viability. In addition, pre-fertilization stages are more sensitive to heat stress than post-fertilization stages. Heat stress caused a reduction in the number of pollen grains, impaired their viability and germinability, caused reduced fruit set and markedly reduced the numbers of seeds per fruit, starch accumulation at 3 days before anthesis and soluble sugar concentration at anthesis (Firon, 2006). Heat stress in tomato decreased the number of pollen grains produced per anther, viability and germination of pollen grains produced (Pressman, 2006). Microscopic investigation of anthers of heat stressed plants showed disruption of development in the pollen, endothecium, epidermis, and stomium. This disruption was reduced, but still observable in plants relieved from high temperature for 10 days before anthesis *(*Sato, 2006). When tomato plants were exposed to 40°C for 3 hours in two consecutive days impaired tapetum functionality and its degeneration failure led to inhibition of microsporogenesis (Iwahori, 1965).

1.1.6.1.3.3 Effect of temperature on anther dehiscence, pollination and fertilization

Anther dehiscence process is defined as rapid swelling of pollen grains, which causes the theca to bulge, disrupting the septum and resulting in theca dehiscence (Matsui, 2000). High temperature stress disturbs the process of release of pollen, pollen grain number, transfer of pollen to the surface of stigma and finally the process of fertilization by controlling the growth of pollen tube. Heat treatment 9 days before anthesis resulted in indehiscent anthers and contained abnormal pollen grains in heat-sensitive genotypes of *Phaseolus vulgaris* (Porch, 2001). Heat stress reduced the number of pollen tubes penetrating the stigma (Gross and Kigel, 1994). Temperature stress affects the rigidity of the pollen tube wall. Pollen grains of *Antirrhinum* pronounced broadening and "bloating" of the distal portion of the tube at 30°C, and extensive bursting of the bloated tubes at 35°C and almost same effects like marked swelling of pollen tube tips, branching, and bursting have also been observed when pollen grains are cultured at temperatures higher than 35-40°C in several plants (Vasil and Bose, 1959; Vasil, 1962). The exposure of tomato flowers to 40°C at after pollination stage for 18 h led to ovary abortion and endosperm degeneration due to inhibition of pollen tube growth and failure of fertilization (Iwahori, 1966). High temperature negatively regulates the activity of SAMDC which is involved in the biosynthesis of polyamines spermidine and spermine by reducing its availability to germinating pollen grains (Song, 2008). In vitro incubation of pollen grains at high temperature showed reduced respiratory activity over time (Karapanos, 2007).

1.1.6.1.3.4 Effect of temperature on fruit-set

The fruit-set process is extremely important in most of the crops because it is directly related to their yield and productivity. The fruit set process was influenced by a number of biotic and abiotic stresses among which high temperature is the most important. Temperature is a key factor for fruit set in tomato (Rylski, 1979). Peet (2002) demonstrated that mean daily temperature at 29°C reduces the fruit number, fruit weight and number of seeds per fruit in comparison to normal mean daily temperature of 25°C. Tomato during high day temperatures shows style exertion as a dominant factor which affects the fruit-set by reducing the chances of perfect fertilization (Fernandez- Munoz, 1991). High night temperatures reduce fruit-set in tomato by showing detrimental effect on functionality of female reproductive organs (Peet, 1996). A lower percentage of fruit-set in tomato under higher temperatures is because of degeneration of endosperm in 24 to 96 h after pollination or fertilization (Iwahori, 1966).

1.1.6.1.3.5 Effect of temperature on fruit maturation and development

Fruits were sensitive to elevated temperature in their later stages of maturation and temperature also affected the rates of fruit growth (Adams, 2001).

1.1.6.1.4 Mechanism of heat tolerance:

Plants respond to increased temperature by manifesting different mechanisms for surviving under elevated temperatures, including long-term evolutionary phenological and morphological adaptations and short-term avoidance or acclimation mechanisms such as changing leaf orientation, transpirational cooling, or alteration of membrane lipid compositions. Plants tend to adopt shorter life cycle in response to high temperature like escaping mechanism. The primary stress signals (e.g., osmotic or ionic effects, changes in temperature or membrane fluidity) would trigger downstream signaling processes and transcription controls to boost secondary signals.

1.1.6.1.5 Plant resistance mechanism to heat stress

Plants respond to increased heat stress by several mechanisms. These mechanisms includes synthesis of specialized proteins called heat shock proteins (HSPs), by changing membrane lipids, osmoprotectants, late embryogenesis abundant proteins and factors involved in signaling cascades and transcriptional control, which act significantly to counteract the stress effects.

1.1.6.1.5.1 Heat shock proteins:

HSPs are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures. This increase in expression is transcriptionally regulated. The dramatic upregulation of the heat shock proteins is a key part of the heat shock response and is induced primarily by heat shock factors (HSF). It is well established that direct relationship and an important component involved in tolerance against high temperature in plants is the synthesis of chaperons or HSP (Vierling, 1991). HSP101, is required for heat acclimation in plants, yeast and bacteria (Hong and Vierling, 2000; Lee, 2007). HSP21 protein protects PSII from temperature-dependent oxidative stress and helps in maintaining photosynthetic system under high temperature (Inbal, 2005). High accumulation of glycine betaine in transformed plants gives high level of resistance to plants through the synthesis of HSP- 70 protein (Alia, 1998*)*.

1.1.6.1.5.2 Membrane lipids

Membrane lipid saturation is considered an important element in high temperature tolerance. Alteration of the unsaturation level of fatty acids could be a useful method for enhancing the tolerance of plants to environmental stresses especially temperature (Somerville and Browse, 1991). Plastid omega 3 fatty acid desaturase (*FAD7*) catalyzes the conversion of dienoic fatty acids (16:2 and 18:2) to trienoic fatty acids (16:3 and 18:3) in glycerolipids which are the main constituents of chloroplast membranes. Transgenic tobacco plants low in trienoic fatty acids could be produced by co- suppression of the plastid omega 3 desaturase genes (*FAD7*), and the transgenic plants exhibited high temperature tolerance by increasing the rate of photosynthesis (Murakami, 2000). Heat treatment increased relative quantities of linolenic acid (among galactolipids) and *trans*- 3-hexaldecanoic acid (among phospholipids), when compared with the wild type (Behl, 1996).

1.1.6.1.5.3 Osmoprotectants

Osmoprotectants or compatible solutes are small molecules that act as osmolytes and help organisms to survive at extreme osmotic and heat stress. These molecules includes proline, glycine betaine etc. Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes (Santoro, 1992) and functions as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989). Marine red macroalga *Gracilaria tenuistipitata* var. *liui* Zhang exposed to 35°C, decreased the specific growth rate after 3 days and increased the free proline levels after 2 days, but did not affect the free amino acid levels (Yuan, 1999). *Pseudomonas putida* infected sorghum plants treated with high temperature showed that *Pseudomonas putida* strain P6 can induce temperature tolerance in sorghum seedlings through induction of high molecular weight protein synthesis, causing accumulation of proline as an osmolyte and helping seedlings maintain higher membrane integrity under temperature stress. This is the first ever report on implicating microorganisms in alleviating high temperature stress effects in plants (Center for dryland agriculture journal). Sunflower plants grown at 27-33°C showed a threefold increase in proline content over absolute control (Amutha, 2007). Treatment of salt-tolerant tobacco suspension cultured cells with high temperature (40°C) and NaCl (170 or 340 mM*)* resulted in a transient overproduction of proline accompanied by an increase in their thermotolerance (Kuznetsov, 1997).

1.1.6.1.5.4 Responses in reproductive stage

Heat tolerant tomato genotypes grown under high temperature maintains higher yield by showing lowest number of flowers shed, stigma tube elongation and antheridial cone splitting when compared to the susceptible genotypes (Lohar, 1998)

1.1.6.1.6 Strategies for high temperature tolerance in tomato

Recent advances in the field of biotechnology and genetic engineering bring us a lot of solutions to engineer plants to make them resistant to pests, diseases and different abiotic stresses. These are the possible strategies against high temperature tolerance in tomato.

1.1.6.1.6.1 Changing in composition of membrane fatty acid composition

The chloroplast membrane of higher plants contains an unusually high concentration of trienoic fatty acids. Plants grown in colder temperatures have a higher content of trienoic fatty acids. Alteration of the unsaturation level of fatty acids could be a useful method for enhancing the tolerance of plants to environmental stresses especially temperature (Somerville and Browse, 1991). Murakami et al. (2000) produced temperature tolerant tobacco plants which are low in trienoic fatty acids by co-suppression of the plastid omega 3 desaturase gene. Transformed plants were able to better acclimate to higher temperatures.

1.1.6.1.6.2 Increasing of proline supply to anthers

Proline is one of the well known osmoprotectants that is expressed in plants during stress. According to Sato (2006), tomato plants growing under moderately increased temperatures above optimal temperature failed a successful fruit-set due to disruption of proline translocation during the narrow window of male reproductive development. Based on this study, an increase in proline supply to the developing pollen grains could be a solution against high temperature.

1.1.6.1.6.3 Increasing supply of carbohydrates to the developing pollen grains

Carbohydrates are one of the major energy sources in the process of pollen grain development and maturation which leads to the production of functional pollen grains for effective fruit-set to get better yield in tomato. The effect of heat stress on pollen viability was associated with alterations in carbohydrate metabolism in various parts of the anther during its development. According to Peet (2002), continuous exposure of the plants to high temperatures (32/26 °C) prevented the transient increase in starch concentration 3 days before anthesis and led to a

decrease in concentration of soluble sugars in the anther walls and the pollen grains. Based on this study, an increased supply of carbohydrates 3 days before anthesis to the developing anthers could be a good solution against low fruit-set and yields in tomato.

1.1.6.1.6.4 Down regulation of *STYLE 2.1* gene

In tomato, during heat stress exertion of style and stigma above the level of antheridial cone is a commonly observable phenotype shown by plants. This character leads to poor fruit-set in tomato due to limited chances of fertilization by self pollen grains because of differences in height between stigma and anther cone (Fernandez- Munoz 1991). *STYLE 2.1* genes is involved in regulation of length of the growing style in tomato plants (Kai et al., 2007). Silencing of this *STYLE 2.1* gene under the control of conditionally expressed promoters like HSP promoter could be a useful strategy to have very good fruit-set under high temperature in tomato.

1.1.6.1.6.5 Reduction of flower abscission

High temperature induces flower abscission in tomato. This leads to indirect effect of less fruit-set in tomato (Levy, 1978). Tomato flower abscission may be due to the restricted supply of assimilates specially sucrose to the developing flowers (Donar and Rudich, 1985). According to Aloni (1994), flower abscission is related to increase of ethylene production by flowers. According to Roberts (1984), auxins have been found to reduce flower abscission in tomato by diffusing down the pedicel and preventing separation of cells in abscission layer (Wien, 1989). Reducing production of ethylene by flowers by silencing genes responsible for ethylene production and by increasing production of auxins in flowers by over expressing related genes could be a useful strategy to reduce flower abscission during high temperature.

Based on above, all strategies in the present study were carried out with the following

1.2 Objectives

1. Increasing the supply of proline to anthers by expressing tomato proline transporter gene (*LePROT1*) under the control of conditional *HSP18.2* promoter.
2. To see the effect of drought stress coupled with heat stress on transformed plant with *LePROT1* gene.
3. To see the effect of salinity stress coupled with heat stress on transformed plant with *LePROT1* gene.

2 Materials and methods

2.1 Isolation of proline transporter (*LePROT1*)

A 1382 bp of open reading frame of Tomato proline transporter *LePROT1* gene (AF014808.1) was used to generate pBI 121 vectors Plasmid for transform purpose. This open reading frame of proline transporter1 was PCR amplified in a 20µl tube from 5 µl of 10 ng cDNA as template, 3 µl of 4 pmol of corresponding primers: 5'- CGGGATCCAGTTTGCAATGGGAAATAAC-3' (*BamH1* restriction site underlined and 5'- GGAATTCTATCTTGGAACCCTTATAACTAC-3' (*EcoR1* restriction site underlined), 1.8 µl dNTPs 2.5 mM, 1x Taq polymerase buffer with $MgCl_2$, 0.2 µl Taq DNA polymerase 1 U (New England Biolabs) and final volume made upto 20 µl with MQ H_2O. The amplifications were conducted with an initial denaturation at 94°C for 4 min and then followed by 36 cycles of 1 min at 94°C, 1 min at 53°C, 1 min at 72°C, plus 7 min final extension at 72°C and final storage at 4°C.

2.1.1 Isolation of GUS gene

A 1004 bp of DNA fragment of GUS was prepared in 20 µl tubes by PCR from 5 µl of 10 ng pTH1-ASGS (Dr. Hamada from Japan) as a template with 3 µl of 4 pmol of corresponding primers: 5'-GGAATTCGTGTGATATCTACCCGCTTCG-3' (EcoR1 site underlined) and 5'-CGGGATCCGTTTTTCACCGAAGTTCATGC-3' (BamH1 site underlined), 1.8 µl dNTPs 2.5 mM, 1x Taq polymerase buffer with $MgCl_2$, 0.2 µl Taq DNA polymerase 1 U (New England Biolabs) and final volume made upto 20 µl with MQ H_2O. PCR amplification were conducted at with an initial denaturation at 94°C for 4 min and then followed by 36 cycles of 1 min at 94°C, 1 min at 67°C, 1 min at 72°C, plus 7 min final extension at 72°C and final storage at 4°C. The amplification products were loaded on 1% agarose gel with ethidium bromide for staining and separated by electrophoresis.

2.1.2 Restriction digestion and purification of *LePROT1* and GUS fragments

The expected 1382 bp PCR fragment of *LePROT1* and 1004 bp GUS were used as template for restriction digestion. Chemicals and their concentrations were given in table 1. Restriction digestions were conducted at 37 °C for 3 h and restricted products were separated by 0.5% agarose gel electrophoresis. Separated products of both GUS and *LePROT1* were eluted by gel elution kit (Qiagen). In brief, 10 µl of capture buffer type 2 is added for each 10 mg of gel slice

and incubated at 60°C until the agarose is completely dissolved. Samples were centrifuged briefly to collect the liquid at the bottom of the tube. 600 µl of capture buffer sample was transferred onto the assembled GFX Microspin column in collection tube. Samples were incubated at room temperature for 1 minute and then centrifuged at 16000g for 30 sec. Flow through solution was discarded and 500 µl of wash buffer type1 was added to the GFX column. Samples were centrifuged at 16000g for 30 sec for proper column washing. 10 to 50 µl of elution buffer type 4 was added to the center of GFX column and incubated for 1 min at room temperature. Samples were spinned at 16000g for 1 min to recover purified DNA and then stored at store the purified DNA a 4°C for short term storage and -20°C for long term storage.

Tab. 1 Chemical concentration used in restriction digestion of *LePROT1* and GUS fragments.

Chemical concentration *(LePROT1)*	Final volume	Chemical concentration (GUS)	Final volume
PCR product 100 ng	4 µl	PCR product 100 ng	4.8 µl
Buffer 1 x	1.1 µl	Buffer 1x	1.1 µl
EcoR1 5 U	0.5 µl	*EcoR1* 5 U	0.5 µl
BamHI 5 U	0.5 µl	*BamHI* 5 U	0.5 µl
MQ H_2O	3.9 µl	MQ H_2O	3.1 µl
Total	10 µl	Total	10 µl

2.1.3 Vector construction

Isolated open reading frame fragment of tomato *LePROT1* and GUS were ligated into the *BamHI* and *EcoRI* sites pBI 121 plasmid based vector under the control of HSP 18.2 promoter with the terminator sequence from the nopaline synthase gene. Chemicals and their concentrations used in ligation reactions were given in table 2. Ligation reaction mixture was incubated at 4°C overnight and next day reaction was terminated at 70°C for 10 min. The required quantities of incert DNA (*LePROT1* and GUS) were calculated by using this formula.

$$ng\ of\ incert \equiv \frac{ng\ of\ vector\ x\ Kb\ of\ insert}{kb\ of\ vector\ size}\ x\ \frac{incert}{vector}$$

Vector to incert ratio = 3:1

Tab. 2 Chemicals and their concentrations used in ligation reaction.

Chemical concentration	Final volume
Vector 75 ng/ µl	1 µl
LePROT1 17 ng/ µl	0.5 µl
GUS 20 ng/ µl	0.2 µl
Buffer 1 x	1 µl
Ligase 3 U/ µl	1 µl
MQ H_2O	6.3 µl
Total volume	10 µl

2.1.4 Competent cell preparation

A 10 µl glycerol stock of an *E. coli* DH5α strain containing no plasmids was allowed to thaw at room temperature and added to 40 ml of liquid L.B media. This culture was incubated at 37ºC for 1 hr, then transferred to an incubator-shaker, at 37ºC, shaking at 200 rpm for over night until an OD_{600} of 0.5-0.6 was reached. Bacterial cultures were chilled out on ice for 10 to 15 min. The cells were pelleted by centrifugation at 8000 rpm for 1 min at 4ºC, then resuspended in one-half volume (20 ml) of sterile cold TSB (Transformation and storage buffer) solution (Tab. 3) and incubated on ice for 25 min. After another centrifugation step as above, the resulting cell pellet was resuspended in one-tenth volume (4 ml) of sterile cold TSB solution to yield the final competent cell suspension. Competent cells were stored at 4ºC for up to 3 days. Transfer 1.6 ml of the competent cell suspension to sterile cryo-storage tubes, and add 0.4 ml of sterile 100% glycerol to give a final concentration of 20% glycerol, and then mix together. The glycerol stocks are placed at -4ºC, -20ºC and -70ºC separately for later use.

Tab. 3 chemical composition of TSB buffer

Chemical	Volume	Final concentration
L.B. pH 6.1	12 ml	-
PEG 4000	5 ml	9%
MgCl2. 6H2O	200 µl	11 mM
MgSO4.7H2O	200 µl	11 mM
DMSO	1 ml	5.4%

2.1.5 Bacterial transformation

Competent cells were transformed with the plasmid DNA by standard method of $CaCl_2$ treatment. Transformation reactions were conducted in 200 µl eppendorf tubes with 10ng of ligated plasmid or 0.1 ng of whole plasmid, 20 µl of KCM (Tab. 4) 5 x, 5 µl of 0.45 m $MnCl_2$ in pre-chilled Eppendorf make up volume to 100µl with mQ water and 100 µl of -80°C stored competent bacterial solution defrozen on ice were added. Eppendorf tubes were incubated on ice for 20min, at ambient temperature for 10 min. Bacterial cells were grown on shaker at 37°C and 200 rpm for 1 hour after the addition of 1 ml L.B. Cells were pelleted in centrifuge at 2000 rpm for 2 min. Pellet was resuspended in 200 to 300 µl of L.B. normal and Plated on agar plate containing appropriate antibiotic to select transformed cells and incubate Petri plate upside down at 37°C. Transformed cells will appear on next day.

Tab. 4 chemical composition of KCM (Potassium, calcium and magnesium chloride) solution

Chemical	Volume	Final concentration
KCl	3.78 g	0.5 M
$CaCl_2.2H_2O$	2.21 g	0.15 M
MgCl2. 6H2O	5.08 g	0.25 M

2.1.6 Confirmation of bacterial transformation

Transformation of bacterial cells was confirmed by PCR through plasmid amplification. Plasmid mini preparations were conducted by inoculating 15 ml L.B falcon tube containing kanamycin antibiotic with bacterial colony and incubated on shaker for over night at 37°C and 200 rpm speed. Bacterial cells were pelleted in centrifuge at maximum speed for 1 min and pellet was resuspended in 200 µl STET (Tab. 5) buffer and mixed well with out any clumps. Bacterial lysis reaction was carried out by boiling the eppendorf tubes for 1 min after the addition of 20 µl lysozyme enzyme. Bacterial pellet was removed carefully after centrifuge at 12000g for 10 min. Bacterial plasmid DNA was purified by incubating plasmid at 37°C for 30 min after the addition of 1 µl of RNAse to solution and centrifuged at 12000g for 10 min with 200 µl of chloroform. Plasmids DNA were precipated in 200 µl of ice-cold isopropanol solution for 30 min and pelleted by centrifuge at 12000g for 15 min. Plasmid DNA pellet was washed with 0.5 ml of 95% ethanol by centrifuge at 12000g for 1 min. Pellet was air dried for 10 min and dissolved in 30 µl TE buffer and store at 4°C.

Tab. 5 Composition of STET buffer for 10 ml

H_2O	8.95 ml
Sucrose	0.8 g
Triton X - 100	50 µl
EDTA (50 mM pH 8.0)	1 ml

Transformed bacteria were confirmed by PCR by using isolated plasmid for Kanamycin resistance gene with their corresponding primers 13

5'- GAGGCTATTCGGCTATGACT-3' and GAGCGGCGATACCGT-3' and rest of chemical concentration were like used in paragraph 2.1. PCR reaction was carried out in 20 µl tubes at initial denaturation at 94°C for 5 min, then 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.30 min and final extention at 72°C for 6 min and final storage at 4°C. Amplified positive products of 600 bp size were observed in agarose gel electrophoresis.

2.1.7 Agrobacterium transformation

Agrobacterium strain LA 4104 containing Ti plasmid was grown for over night at 28°C and 250 rpm in 100 ml L.B medium with rifampicin and kanamycin as selective markers until the culture grows to an O.D of 0.5 to 1.0 at 600λ. Bacterial cell suspension was pelleted in centrifuge at 3000g for 5min at 4°C and pellet was resuspended in 1 ml of 20mM of ice-cold calcium chloride solution. Agrobacterium cells were freezed after the addition of 1 µg of vector plasmid DNA containing both *LePROT1* and GUS genes and immediately cells were thawed by incubating in water bath for 5 min at 37°C. Bacterial suspension was incubated in 1 ml L.B at 28°C and 250 rpm for 2 to 4 h. Bacterial cells were pelleted in centrifuge for 30 sec and plated on agar plate containing 50mg/litre kanamycin and incubate at 28°C. Transformed colonies will appear in 2 to 3 days.

2.1.8 Confirmation of transformed agro bacterium

Positive colonies of agrobacterium grown on agar plates containing selective markers were confirmed by procedure explained in paragraph 2.1.6

2.1.9 Micro tom tomato transformation

Seeds of tomato, (*Solanum Lycopersicum* L.) cv Micro-Tom were surface sterilized for 10 min in a 50% Naocl and washed three times with sterilized water for 10 min to remove left over Naocl. Put seeds in Petri plate for pregermination and after transfer germinated seeds to magenta containing MS medium (A). After one week take cotyledenary leaves from germinated seedlings and cut top and bottom parts of leaves, put them in Petri plate containing media B in upside down manner for preconditioning in dark at 25°C. Prepare agrobacterium containing desired plasmid pBI 121 with both *LePROT1* and GUS genes in L.B containing 30 mg/l rifampicin and selectable antibiotic in construct. Transformed bacterium was grown at 250 rpm over night. Centrifuge the bacterial culture at 3000 rpm and resuspend the pellet in L.B without antibiotic until final concentration of 10^7cells (O.D. of 0.1 to 0.2). After 24 to 48 h cotyledons in B are co-cultivated with agro bacterium for 20 min with gentle shaking on shaker i.e. like 50 to 100 rpm. Remove bacterial solution and maintain cotyledons in plate B in dark at 25°C. After 24 hours transfer the explants to regeneration medium C and put them in chamber at 25 °C with 16 hours light and 8 hours dark. After one week transfer explants to Petri plate containing medium D keep explants for one week in this medium and then transfer explants in every 20 days to new Petri plate containing media D with same photo period i.e. 16 h light and 8 h dark. After approximately 6–8 weeks, shoots were excised and transferred to Medium E for rooting and selection. Chemical and hormonal concentrations of growth medium are given in table 6.

Tab. 6 Chemical and hormonal concentrations in used in micro-Tom tomato regeneration.

COMPONENT	A	B	C	D	E
MS	1 X	1 X	1 X	1 X	1 X
Sucrose	3%	3%	3%	3%	3%
Agar	0.6%	0.6%	0.6%	0.6%	0.6%
pH	5.8	5.8	5.8	5.8	5.8
Zeatin mg/lt		0.7	0.7	0.7	
IAA mg/lt		0.35	0.35	0.35	0.35
IBA mg/lt.					0.1
2.4.D mg/lt		0.1			
Cefotaxime mg/lt			200		200
Vancomycin mg/lt			50		
Carbencilin mg/lt				500	
Kanamycin mg/lt				100	50

2.2 DNA extraction

DNA was extracted from the transformed plants using the CTAB method. Leaves of transformed plants were harvested into Eppendorf and ground to the powder with liquid nitrogen in mortar and pestle. 600 µl of the CTAB-buffer (200 mM Tris-HCl (pH 7.5), 2 M NaCl, 50 mM EDTA and 2% N-cetyl-N, N, N- trimethylammonium bromide) was added to each eppendorf and mixed by inversion. Samples were incubated at 60°C for 1 hour, cooled on ice and extracted once with 600 µl of chloroform/isoamylalcohol (24:1). The aqueous phase was obtained after centrifugation at room temperature (10 min at 12000 rpm). Supernatant was transferred to new eppendorf tube and was mixed well with 400 µl of ice cold isopropyl alcohol. Samples were incubated at -20°C for 1hour. DNA pellet was collected after centrifugation at room temperature (10 min at 12,000 rpm), rinsed once with 75% ethanol (650 µl), dried (10 min at 60°C) and resuspended in 200 µl of 1mM Tris-HCl (pH 8.0) and 0.1mM EDTA. Samples were stored at -20°C.

2.2.1 Confirmation of transformed plants

DNA was extracted from leaves of transformed plants using the CTAB-method described above and used as template for the PCR reaction. Transformed plants were confirmed by PCR for Kanamycin resistance gene with their corresponding primers 13 pmol of 5'-

GAGGCTATTCGGCTATGACT-3' and 5'-GAGCGGCGATACCGT-3' and rest of chemical concentration were like used in paragraph 2.1. PCR reaction was carried out in 20 µl tubes at initial denaturation at 94°C for 5 min, then 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.30 min and final extention at 72°C for 6 min and final storage at 4°C. Amplified positive products of 600 bp size were observed in agarose gel electrophoresis.

2.2.2 Plant growth conditions

Untransformed control plants and plants transformed with *LePROT1* first were grown under normal ambient temperature and then transferred to heat chamber for heat treatment. Heat treatment was given for 2 h at 38^0C at 3 days before anthesis stage. Plants after heat treatment were transferred to growth chamber under normal growth conditions and flower buds collected after 12 h of heat treatment for pollen staining, germinability check and for proline quantification.

2.2.3 Pollen Staining

To determine the percentage of stainable pollen from both *LePROT1* transformed and control plants after heat stress, flowers at 3 days before anthesis were sampled 12 h after heat treatment. One anther was removed from each flower was placed on microscopic slide and scraped it to release pollen by adding 1% ACETIC-ORCEIN and 50% glacial acetic acid solution. This method enables us to record the percentage of non-viable (stained green) and viable (stained purple) pollen grains under the light microscope. This procedure was repeated for three times for each anther and average results were calculated.

2.2.4 Pollen germination

Pollen of plants transformed with construct containing the *LePROT1* gene after heat treatment was analyzed for their germinability at 22^0C on pollen germination medium containing sucrose 10%, boric acid 100 mg/l, calcium nitrate 300 mg/l, magnesium sulphate 200 mg/l, potassium nitrate 100 mg/l and 1% phytagel along with untransformed control plant. This method enables us to record the number of non-germinable and germinable pollen grains with aniline blue staining and the fluorescence was examined with UV light filter under microscope. This procedure was repeated for three times for each anther and average results were calculated.

2.2.5 Productivity

Total productivity in terms of number of fruits of heat stress treated transformed plants and an untransformed control plant was recorded.

2.2.6 Proline quantification

proline content from the anthers of both transformed and control plants after heat treatment was quantified according to Bates et al (1973) as follows 1 g of fresh anthers at 3 days before anthesis stage and leaves from both control and transformed plants were powdered in a cold mortar with liquid nitrogen until pulverization and homogenized with 5 mL of sulphosalicylic acid 3%. Then, the homogenate is filtered through whatman no. 2 filter paper. Two ml of the filtrated sample are mixed with 2 ml of glacial acetic acid and 2 ml of acid-ninhydrin in test tubes. After agitation, the sample is incubated at 100 °C for 1 h, and this produces the coloured complex formation. After one hour, reaction is stopped by putting the tubes in an ice bath. 4 mL toluene is added to each tube, and vortexed for 15-20 s. Then, organic and inorganic phases are separated, obtaining the cromophore dissolved in toluene. The maximum absorbance of the chromophore when dissolved in toluene is attained at a wavelength of 520 nm. Thus, the organic phase of each sample and standard containing the chromophore is collected, and absorbance at 520 nm is measured spectrophotometrically. The proline concentration was determined from a standard curve and proline content is calculated on a fresh weight basis (µg proline $(g\ FW)^{-1}$).

2.2.7 Plant growth under drought stress

T_0 generation seeds were germinated on MS media supplemented with normal MS salts and 50 g/l PEG along with untransformed control plant seed and allowed to grow them in three replicates for one week under heat stress at 38°C. Parameters like root length and hypocotyl length were recorded under heat stress.

2.2.8 Proline quantification under drought

Proline quantity from the seedlings grown under drought stress conditions was quantified according to Bates et al 1973 as mentioned in paragraph 2.2.6.

2.2.9 Plant growth under salt stress

T_0 generation seeds were germinated on MS media supplemented with normal MS salts and 100 mM NaCl along with untransformed control plant seed and allowed to grow them in thee

replicates for one week under heat stress at 38°C. Parameters like root length and hypocotyl length were recorded under heat stress.

2.3 Proline quantification under salt stress

Proline quantity from the seedlings grown under salt stress conditions was quantified according to Bates et al 1973 as mentioned in paragraph 2.2.6

2.4 Statistical analysis

All data were compared between the transformed plants and control plants in temperature treatments and differences were tested by Student t-test.

3 Results

3.1 Isolation of *LePROT1*

Open reading frame of tomato *LePROT1* gene was isolated by PCR by using cDNA as template with the help of the primers designed on the open reading frame sequence of tomato LeProT (Proline transporter1 AF014808.1) with added restriction sites. We isolated 1382 bp size of *LePROT1* ORF (Fig. 4)

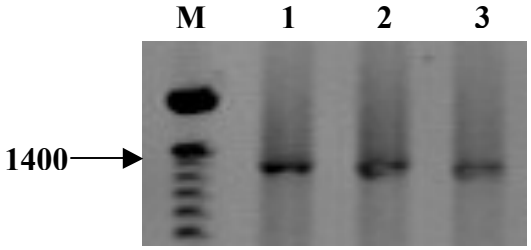


Fig. 4 Isolated ORF of *LePROT1* in wells from 1, 2 and 3 along with molecular marker (M).

3.1.1 Vector construction

Isolated fragments of tomato *LePROT1* and *GUS* were cloned into the *BamHI* sites of the pBI121 vector under the control of Arabidopsis HSP 18.2 promoter with nopaline synthase gene as the terminator sequence (Fig. 5).



Fig. 5 vector structure for chimeric gene expression which contains 1382 kb of *LePROT1* cDNA; HSP 18.2 is HSP 18.2 promoter sequence. GUS is a 1.0-kbp fragment of the ß-glucuronidase gene. NOS is the terminator sequence from the nopaline synthase gene. Restriction sites shown are for *BamHI* and *EcoRI.*

3.1.2 Micro Tom transformation

Micro Tom tomato was transformed with the ligated vector by cotyledenary leaf disc transformation method with transformed *Agrobacterium*. Transformed plants were maintained on MS medium supplemented with kanamycin as selective marker (Fig. 6).

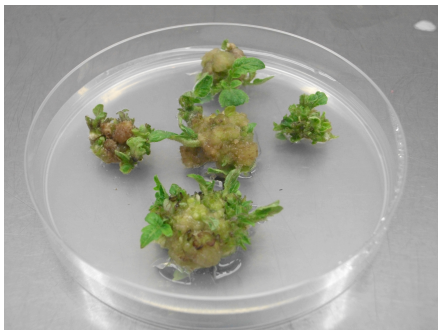


Fig. 6 Kanamycin resistant callus explants transformed with LePROT1 gene on MS medium.

3.1.3 Confirmation of transformed plants

Plants transformed with *LePROT1* gene were confirmed by PCR for kanamycin resistance gene (Fig. 7). We got total six transformed plants after confirmation and they were maintained for further experiments.

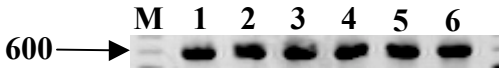


Fig. 7 Kanamycin confirmed plants in lines from 1 to 6 along with marker (M).

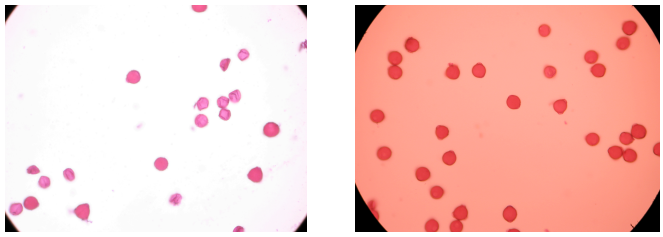
3.1.4 Pollen analysis

We analyzed pollen from the plants transformed with *Le PROT1* and control plants after heat treatment for their viability in terms of number of viable and non viable pollen grains (Table. 7) with aceto-orcein stain. Significant difference was found for pollen stainability between control and all transformed plants (*P≤ 0.05 after t Student test). We found pollen viability of transformed plant (Fig. 8B) is better when compared to the pollen viability of control plants (Fig. 8A) after heat treatment.

Tab.7. Percentage of stainable pollen grains (SPG) and non-stainable (NSPG) pollen grains of plants transformed with LePROT1 gene and control (C) plants with their standard error of the means (SEM)

Plant	% SPG ± SEM	% NSPG ± SEM
C	81.1 ± 1.8	18.9 ± 0.6
1	95.4 ± 1.2	4.6 ± 0.3
2	94.3 ± 2.1	5.8 ± 0.3
3	92.5 ± 4.0	7.5 ± 1.2
4	91.6 ± 2.1	8.4 ± 0.3
5	96.6 ± 2.3	3.4 ± 0.6
6	93.5 ± 2.6	6.5 ± 0.6

Fig. 8 Pollen staining of control plant (A) and Plant transformed with *LePROT1* (B)



A **B**

3.1.5 Pollen germinability

Pollen collected from the plants transformed with *LePROT1* and untransformed control plants after heat treatment was germinated on in vitro germination media to see their ability to germinate at normal conditions. Significant difference was found for pollen germinability between control and all transformed plants (*P≤ 0.05 after t Student test). Pollen from transformed plants germinated better than the pollen from control plants (Tab. 8 and Fig. 9)

Tab. 8 Percentage of germinated pollen grains (GP), Non-germinated pollen grains (NGP) of plants transformed for *LePROT1* gene with their standard error of the means (SEM) and control (C) plants at 22^0C.

Plant	GP ± SEM	NGP ± SEM
C	74.6 ± 1.2	25.3 ± 0.6
1	95.8 ± 1.2	4.2 ± 0.3
2	96.1 ± 1.1	3.9 ± 0.3
3	92.8 ± 1.8	7.2 ± 0.3
4	86.7 ± 1.5	13.3 ± 0.3
5	87.4 ± 2.01	12.6 ± 0.7
6	90.5 ± 1.8	9.5 ± 0.6

Fig. 9 Germinated pollen of control plant (A) and plant transformed with *LePROT1* (B)



A **B**

3.1.6 Proline quantification under heat stress

Proline content of both T_1 transformed and control plant androecium at 3 days before anthesis and leaves from both control and transformed plants was quantified after heat treatment and leaves from both control and transformed plants. Proline content of transformed plants was 2 to 3 folds higher than the proline content of control plants (Tab. 9).

Tab. 9. Proline content of anthers from plants transformed with *LePROT1*, control and proline content from leaves of control (CHT-L) and transformed plants (THT-L) after heat stress expressed in µM/g FW with their standard error of the means (SEM).

Plant	Conc. in µM/g FW
C	860.87 ± 85.62
C-HT-L	2242.48 ± 77.02
T-HT-L	1068.76 ± 19.15
1	1263.66 ± 41.31
2	2516.42 ± 58.78
3	2879.93 ± 45.07
4	1210.6 ± 82.73
5	1291.81 ± 12.48
6	3543.96 ± 184.44

3.1.7 Productivity

Productivity of transformed plants is compared with the productivity (Tab. 10) of control plants in terms of fruit number per plant from the first three trusses which were grown under high temperature.

Table 10. Productivity of transformed plants transformed with *LePROT1* and untransformed control plants in terms of number of fruits per plants after heat stress.

Plant	Fruit Number
C	7
1	15
2	13
3	11
4	12
5	11
6	10

3.1.8 In vitro drought stress

Seeds of the plants transformed with *LePROT1* gene and control plants were germinated on MS media supplemented with 50g/l PEG to induce drought stress. We recorded root length (R.L) and hypocotyl length (H.L) of both transformed and control plants under heat stress. We found significant differences in root length between transformed and control plants but not in shoot length (Fig. 10 and table 11).

Table 11 Root and Hypocotyl length in centimeters (Cm) of plants transformed with *LePROT1* and untransformed control plant under drought stress (50mg/l PEG) after heat stress with their standard error of the means (SEM).

Plant	R.L (Cm)	H.L (Cm)
C	6.66 ± 0.66	4.20 ± 0.28
1	8.73 ± 0.14	4.00 ± 0.28
2	10.00 ± 1.52	4.66 ± 0.16
3	9.50 ± 0.28	4.33 ± 0.44
4	9.00 ± 0.57	4.33 ± 0.33
5	8.96 ± 0.54	4.33 ± 0.16
6	9.00 ± 0.28	4.00 ± 0.50

T **C**
50 mg/l PEG **50 mg/l PEG**

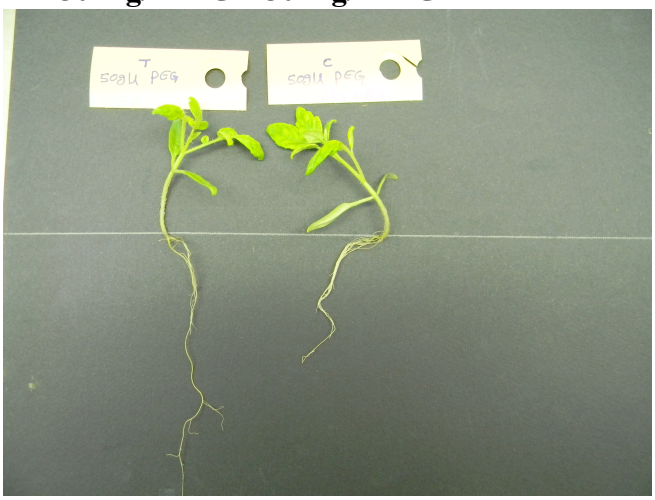


Fig. 10 Root and shoot length of *LePROT1* transformed (T) and control (C) plants under drought stress (50 mg/L)

3.1.9 Proline content under drought stress

Plants transformed with *LePROT1* and untransformed control plants grown under heat stress on MS media supplemented with 50mg/l PEG were used to quantify proline content under drought conditions. There is significant difference in proline content between control and transformed plants (Tab. 12).

Table 12 Proline content of plants transformed with *LePROT1* and untransformed control plant under drought stress (50mg/l PEG) after heat stress with their standard error of the means (SEM).

Plants	Proline content µg/g FW
C	6.82 ± 0.22
1	9.70 ± 0.22
2	14.78 ± 0.55
3	14.07 ± 0.38
4	10.84 ± 0.48
5	11.45 ± 0.34
6	10.85 ± 0.25

3.2 In vitro salt treatment

Seeds of the plants transformed with *LePROT1* gene and control plants seeds were germinated on MS media supplemented with 100mM Nacl to induce salt stress. We recorded root length (R.L) and hypocotyl length (H.L) of both transformed and control plants under heat stress. We found significant differences in both root and hypocotyl length between transformed and control plants (Fig. 11 and Table 13).

Table 13 Root and hypocotyl length centimeters (Cm) of plants transformed with *LePROT1* and untransformed control plant under salt stress (100mM) after heat stress with their standard error of the means (SEM).

Plant	R.L (Cm)	H.L (Cm)
C	6.16 ± 0.44	1.66 ± 0.16
1	10.1 ± 0.44	5.06 ± 0.34
2	8.66 ± 0.66	4.83 ± 0.49
3	9.50 ± 0.28	4.73 ± 0.29
4	8.00 ± 0.28	4.00 ± 0.25
5	9.16 ± 0.72	4.63 ± 0.38
6	11.1 ± 0.92	5.20 ± 0.55

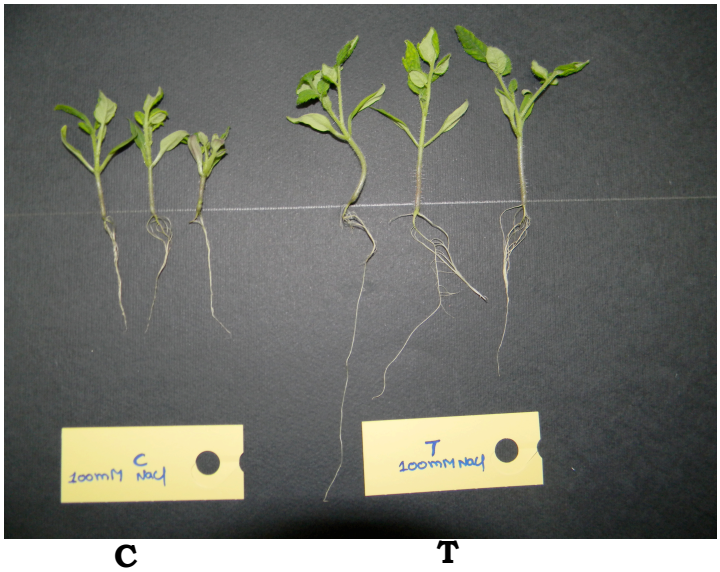


Fig. 11 Root and hypocotyl length of *LePROT1* transformed (T) and control (C) plants under salt stress (100mM Nacl)

3.2.1 Proline content under salt stress

Plants transformed with *LePROT1* and untransformed control plants grown under heat stress on MS media supplemented with 100mM Nacl were used to quantify proline content under drought conditions. There is significant difference in proline content between control and transformed plants (Tab. 14)

Table 14. Proline content of plants transformed with *LePROT1* and untransformed control plant under salt stress (100mM) after heat stress with their standard error of the means (SEM).

Plants	Proline content µg/g FW
C	17.33 ± 0.94
1	27.42 ± 1.27
2	23.94 ± 0.86
3	22.84 ± 0.66
4	22.61 ± 1.52
5	25.64 ± 1.82
6	28.52 ± 2.98

4 Discussion

Increase in temperature will lengthen the effective growing season in areas where agricultural potential is currently limited by cold temperature stress. Thus, increased temperature will cause a poleward shift of the thermal limits to agriculture. Global warming impact will be greater in the northern than southern hemisphere because there is more high-latitude area cultivated in the northern hemisphere. Increased temperature would also affect the crop calendar in tropical regions. Temperature is an important climatic factor which can have profound effects on the yield of crops. Climate change is a major concern in these days because of global warming effect. Global warming is predicted to increase the frequency and severity of 'heat waves' in temperate zones (Semenov and Halford, 2009). Researchers are engaged in understanding its impact of increased temperature due to global warming on growth, yield of crops, and also identifying suitable management options to sustain the crops. Heat stress is one of the main abiotic stresses that limit plant biomass production and productivity, especially in tropical and subtropical countries (Boyer 1982).

Plants usually accumulate some compatible solutes in response to water and salt stress for osmotic adjustment of plant cells during these stresses. These compatible solutes are neutral organic compounds non toxic to the cell, that does not inhibit enzymatic activity even at high concentrations (Aspinall and Paleg, 1981). Proline appears to be the most widely distributed osmolyte accumulated under stress conditions not only in plants but also in eubacteria, protozoa, marine invertebrates and algae (Delauney and Verma, 1993). The accumulation of proline in wilted plant tissue was first observed by Kemble and MacPherson (1954) working with ryegrass. Free proline content increases under water and osmotic stress (Bhaskaran et al., 1985) and in salt, freezing stress (Pérez-Alfocea et al., 1994). Accumulation of compatible solutes like proline is often regarded as a basic strategy for survival and protection of plants under abiotic stress (Shabala and Cuin, 2006). Proline is considered as an inert compatible osmolyte that protects subcellular structures and macromolecules under osmotic stress (Hare and cress, 1997). Transport of amino acids is regulated not only by endogenous but also by environmental signals in plants. Water deficit, like drought and salt stress, affects long-distance transport and results in massive changes in partitioning of carbon and nitrogen. Meristem, developing tissues, and reproductive organs usually import amino acids to support growth and development. Salinity is one of the major abiotic factors limiting global agricultural productivity, rendering an estimated one-third of the world's irrigated land unsuitable for crops (Frommer et al., 1999). Higher amount of salt in

the soil adversely affects plant growth and development. Plant physiological process such as seed germination, seedling growth, vegetative growth, flowering and fruit set are adversely affected by high salt concentration, ultimately causing diminished economic yield and also quality of produce. Proline accumulation is a common metabolic response of higher plants under salinity stress. In this present experiment *LePROT1* transformed plants survived better than the control plants when grown under 100 mM NaCl coupled with high temperature stress at 38^0C. This positive performance of *LePROT1* transformed plants over untransformed control plants is in close accordance with their quantity of proline accumulation i.e. transformed plants accumulated more proline when compared to untransformed control plants under salinity stressed condition coupled with high temperature at 38^0C (Tab. 14). There was significant difference between transformed plant and untransformed plants in both root and hypocotyl length under salt stress (Tab. 13 and Fig. 11) and also in their proline content. This increased proline content under salt stress in transformed plants is only because of efficient transport of proline by *LePROT1* gene when compared to untransformed control plants. These results were in accordance with Shen et al. (2002) in which Arabidopsis plants transformed with a proline transporter from halophyte Atriplex hortensis L. confers resistance to salt stress by accumulating more proline in roots than control plant roots under salt stress conditions, whereas there is no significant difference in proline quantity under non-stressed growth conditions. Akihiro ueda et al. (2001) showed that in barley plants grown at 200 mM NaCl salt concentration, transcriptional level of proline transporter *HvProT* was very low in leaves and did not increase under salt stress, whereas in roots expression of proline transporter was observed after 30 min under salt stress. The key enzyme of proline biosynthesis Δ^1-pyrroline-5-carboxylate synthetase (P5CS) was expressed later than *HvProT* under salt stress and *HvProT* mRNA was strongly expressed in root cap cells under salt stress. Here, tomato plants transformed with LePROT1 showed higher root and hypocotyl length than untransformed control plants. This better performance of transformed plants under salinity coupled with high temperature stress is because of more availability of proline than control plants. Nanjo et al (1999) silenced *P5CS1* in Arabidopsis resulting in inhibition of the bolting process, i.e. the fast stem elongation occurring immediately after flower transition. Silenced plants showed bushy appearance. This works shows importance of proline in stem elongation in plants.

This proline accumulation in combination of both salt and high temperature stress is transient, but sufficient to counteract salt stress under high temperature stress, because of transmethylation of proline to form derivatives like N-methylproline, prolinebetaine, hydroxyprolinebetaine

(Vladimir and Nina, 1997). These proline transmethylated derivatives have capacity to protect plant cells under severe stress conditions (Solomon et al. 1994). Proline transporter transports both proline and glycine betaine, so plants transformed with proline transporter had more tolerance towards salinity stress coupled with heat stress. So it is reasonable to conclude that conditional expression of proline transporter in tomato makes plants tolerant to both salinity and high temperature.

Water deficit (drought) is one of the most common environmental stresses that affects growth and development of plants (Aslam et al., 2006). This drought and heat stress mostly occurs together and affects growth and yield of crops. Present experiment was carried by inducing artificial drought conditions with PEG 60 mg/l under heat stressed conditions (38^0C). Plants transformed with *LePROT1* showed significant difference in root length but not in hypocotyl length (Tab. 11 and Fig. 10). This character i.e. increased root length may be the possible reason for higher survival rate for transformed plants than untransformed control plants. This increased survival rate of transformed plants is positively correlated with their proline content (Tab. 12), i.e. transformed plants accumulated more proline than the control plants. These results are in agreement with Akihiro Ueda et al., (2008) in which they transformed Arabidopsis with barley proline transporter (HvProT) under the control of either the CaMV35S promoter or a root cap promoter and they found growth retardation and biomass reduction in Arabidopsis expressing proline transporter under the control of CaMV35S probably because of lack sufficient proline in these plants. Differently, plants transformed with proline transporter under the control of root cap promoter showed increased growth by enhancing more root elongation when compared to wild type plants. This positive growth in root cap promoter transformed plants is because of accumulation of 2 to3 folds more proline in root tip region when compared to wild type plants. These results had shown the importance of proline transporters in drought tolerance during vegetative growth cycle of plants. In maize plants growing at lower water potentials, proline deposition per unit length increased up to 10-fold in the apical region of the growth zone compared to roots at high water potential (Gary and Robert, 1991). Osmotic pressure in root tip cells under low water potential was adjusted by increasing concentration of proline in cytoplasm through active transport of exogenous proline instead of de novo proline biosynthesis in root tip cells (SHEN Yi-Guo et al 2002). Maize plants growing under low water potential showed increased proline concentrations in the primary root growth zone particularly high toward the apex, where elongation of root occurs. This increased proline deposition plays an important role in the maintenance osmotic adjustment and root elongation at low water potentials (Gary and

Robert, 1991). Proline has been shown to function as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes which include the prevention of protein aggregation and stabilization of M4 lactate dehydrogenase during extreme temperatures (Rajendra kumar et al, 1994). Our data such as root and hypocotyl length and proline content in transformed plants demonstrated that active LePROT1 could increase proline content in transformed plants and consequently promote salt and drought tolerance in transgenic tomato plants.

High temperature stress mostly influences the reproductive process of the plants than vegetative process. High temperature stress did not affect the reproductive development of the plants such as number of flowers and pollen grains produced per plant (Sato 2006). Tomato plants growing under high temperature accumulates more quantity of proline produced in leaves in response to heat stress. This high proline accumulation in heat stressed plants may be due to the lack of sufficient proline transporter from source to sink i.e. flower buds. In this present experiment proline quantity in control plants leaves was 2 folds (Table 9) higher than the proline content in the leaves of transformed plants, this might be due to the efficient transport of produced proline by proline transporter gene in transformed plants that was expressed under heat inducible promoter when compared to the untransformed control plants. These results are in close accordance with Kuo et al., (1986) where they quantified proline in different tomato tissues grown under high temperature and they found proline content in leaves was higher than the proline content in anthers and pistils. Heat stress significantly reduced fruit set (Tab. 10) between plants transformed with *LePROT1* gene and control plants. The reduction of fruit set under heat stress was likely to be caused by reduction in number of viable and germinable pollen grains; this agrees with the previous reports of N. Firon et al (2006) in which several tomato cultivars were grown under heat stress, in growth chambers (day/night temperatures of 31/25 °C) or in greenhouses (day/night temperatures of 32/26 °C), or under control (day/night temperatures of 28/22 °C) conditions. In the present experiment pollen grain viability (Table 7 and Fig. 8) and germinability (Table 8 and Fig. 9) of plants transformed with *LePROT1* was higher when compared to the untransformed control plants. This might be due to the sufficient availability of proline in transformed plants than the control plants. These results were in agreement with Kuo et al., 1986 who got higher germination of pollen grains collected from hot season planted plants after the addition of proline in pollen germination medium because these pollen grains lacks sufficient quantity of proline to germinate. Dashek and Harwood (1974) shown proline involves

in pollen tube growth i.e. tube elongation in their autoradiography experiments with radiolabelled proline. This might be the reason of higher fruit set in transformed plants in this present experiment. According to Walton et al (1998) and Hare and Cress (1996) *ProT1* was expressed in all organs of the plants, highest levels were found in the floral stalk phloem that enters the carpels and were down-regulated after fertilization. This is consistent with the evidence that proline synthesis and degradation play an important role in flowering and seed set. Pollen grains need to accumulate enough reserves to be viable. Pollen viability and germinability of plants under heat stress was affected by a lot of factors, like starch accumulation, accumulation of late embryogenesis abundant proteins (LEA), in these factors proline accumulation in developing pollen grains is one of the factors that affect pollen viability (Zhang and Croes, 1983; Lansac et al., 1996). Proline has been proposed to provide energy to sustain metabolically demanding programs of plant reproduction. According to Atkinson et al (1977) the oxidation of one molecule of proline yields 30 ATP equivalents, so proline seems well suited to sustain high energy-requiring processes.

In tomato, proline concentration in reproductive organs was found to be six to ten times higher than in the rest of the plant (Schwacke et al., 1999). Since a developing anther is a sink organ, most amino acids, including proline, are transported from source organs to sink organs. Sink–source translocation requires trafficking between symplastic and apoplastic environments and vice versa, because nutrients produced in the symplast of source tissues (i.e. most likely the photosynthetic leaves) have to cross a plasma membrane for translocation via phloem. After translocation to sink organs (i.e. developing flower buds, anther and microspores), nutrients move to a symplastic environment, because most nutrients supplied to developing microspores will be carried out through tapetum cells. Tapetum is a cell layer which takes a crucial role in supplying nutritional storage during pollen development and degrades around the time of pollen mitosis in a programmed cell death fashion (Scott et al., 2004). Prior to and during degradation, tapetum cells supply various nutrients such as amino acids, lipids and carbohydrates. Therefore, proline has to pass a plasma membrane at least a few times before reaching pollen grains through proline transporter gene. In the current experiment, it was observed that the proline content of the anthers was significantly different between control and transformed plants at 3 days before anthesis stage and this positively reflected in their pollen grain production (Table 9). Therefore, it is reasonable to conclude that the accumulation of proline through proline transporter in relation to heat stress in developing anthers is part of the reason for the higher pollen viability, germinability and fruit

set in transformed plants than in control plants and of the increased tolerance to drought and salt during early seedling growth in tomato.

Project 2

Engineering of *FAD7* in tomato against high temperature

1 INTRODUCTION

Plant membranes contain different types of lipid molecules and their composition will change according to external environmental cues. Fatty acids are crucial components of cellular membranes, suberin, and cutin waxes that provide structural barriers to the environment (Beisson et al. 2007). Plants can acquire inducible stress resistance by remodeling of membrane fluidity (Iba, 2002), through lipase activity of a-linolenic acid (Grechkin 1998), and as modulators of plant defense gene expression (Kachroo et al. 2001).

1.1 Role of fatty acids in plant abiotic stress

Plants can acquire inducible stress resistance by remodeling of membrane fluidity (Iba, 2002). Plants ability to adjust membrane lipid fluidity is achieved by changing levels of unsaturated fatty acids through fatty acid desaturase genes is a feature of stress acclimation. Modification of membrane fluidity results in an environment suitable for the function of critical integral proteins, such as the photosynthetic machinery during stresses.

1.1.1 Low temperature stress

Phase transition from a liquid crystalline to a gel-like in plant membranes is common process in plants at low temperatures. This phase transition reduces the fluidity and ion leakage and deactivation of membrane proteins. Quantity of saturated phosphatidylglycerol (PG) content in chloroplast membranes may be related to the phase transition temperature and in low-temperature adaptability of plants (Iba 2002). A mount of saturated phosphatidylglycerol (PG) with saturated fatty acids (16:0, 18:0) is less than 20% and it is more than 40% in chilling sensitive plants (Murata et al., 1992). Phase transition temperature in the chilling sensitive plants is higher than the phase transition temperature for chilling-resistant plants. Polyunsaturated fatty acid level maintenance in chloroplast lipids makes plants survival to low temperature stress and normal chloroplast membrane formation under chilling stress (Iba 2002). Seedlings of Arabidopsis *FAD5* mutant which contains high levels of palmitic acid (16:0) due to lack of active chloroplast ω-9 fatty acid desaturase and *FAD6* which contains high levels of palmitoleic acid (16:1) and oleic acid (18:1) due to lack of active ω-6 fatty acid desaturase became chlorotic, decreased number of thylakoid membranes, smaller chloroplasts and contains 50% chlorophyll content than wild-type under chilling stress (Vijayan and Browse, 2002). Long term cultivation of Arabidopsis *FAD2*

mutant, which is deficient in an endoplasmic reticulum (ER) localized ω-6 fatty acid desaturase at 6^0C resulted in withering and death when compared to wild type plants due to less quantity of polyunsaturated fatty acids in extra chloroplast membrane (Miquel et al 1993).Trienoic fatty acids (TAs), hexadecatrienoic acid (16:3) and linolenic acid (18:3), are the major polyunsaturated fatty acid species in membrane lipids. Transgenic tobacco (*Nicotiana tabacum* cv *SR1*) plants engineered by the introduction of a chloroplast ω-3 fatty acid desaturase gene (*FAD7* gene) isolated from *Arabidopsis* under the control of cauliflower mosaic virus 35s promoter (Over expression) shown increased levels of 16:3 and 18:3 fatty acids and decreased levels of hexadecadienoic and linoleic acids. The low-temperature- induced chlorosis was much reduced in the plants transformed with *fad7* when compared to wild type plants (Kodama, 1994).

1.1.2 High temperature

High temperature tolerance in plants can be achieved by reducing the level of trienoic fatty acids in plant chloroplast membranes. Iba 2002 produced high temperature (36^0C) tolerant tobacco plants with low in trienoic fatty acids and high in dienoic fatty acids in their chloroplast membranes by co-suppressing the ω-6 desaturase and transformed plants shown thermo stable photosynthetic machinery proteins in transformed tobacco plants but not in wild type plants. Temperature depended membrane lipids unsaturation is shown in fig.1.

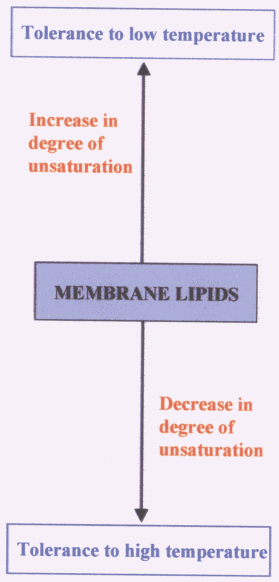


Fig.1 Schematic representation of the influence of saturation levels of membrane lipids on the temperature sensitivity of plants (Anil Grover et al, 2000)

1.1.3 Salt and drought stress

The lipid bilayer has been firmly established as the universal basis for cell-membrane structure. The lipid bilayer of cell membranes serves as a barrier to the passage of most polar molecules because of it interior hydrophobic nature. This barrier function is crucially important as it allows the cell to maintain concentrations of solutes in its cytosol that are different from those in the extracellular fluid and in each of the intracellular membrane-bounded compartments. Cell membrane transports inorganic ions and small water-soluble organic molecules across the lipid bilayer through specialized proteins called transmembrane proteins, these proteins transfers specific ion or molecule or a group of closely related ions or molecules (Albert's et al., 2003). Transgenic tobacco cells and plants over-expressing ω-3 fatty acid desaturase gene containing higher level of trienoic fatty acids have shown increased tolerance to salt and drought stress conditions (Zhang et al. 2005). Fresh water cyanobacter mutant Synechocystis which lack of ω-6 and ω-3 desaturase activities shown reduced recovery of photosynthetic machinery under salt stressed conditions due to absence polyunsaturated fatty acids (Allakhverdiev et al. 1999). Daklma et al. 1995 analyzed the fatty acid composition of leaf lipids in drought-stressed rape (*Brassica napus*) plants and found decreased 18:3 fatty acids monogalactosyldiacylglycerol (MGDG), mainly in the chloroplast and decreased 18:2 fatty acids in phospholipid fractions. Gigon et al 2004 found that drought tolerance capacity is depends on the capacity to maintain polar lipid content and stable lipid composition, and increase the digalactosyldiacylglycerol (DGDG) to MGDG ratio, and fatty acid unsaturation.

1.1.4 Heavy metal

When plants exposed to heavy metals shows rapid inhibition of the growth of above and below ground parts, the onset of senescence, and decreased photosynthetic activity. The cellular responses that occur when plants are exposed to heavy metals include changes in the amounts of various lipids and lipid fatty acid composition. Pepper (*Capsicum annuum*) seedlings cultured in solution with cadmium have lower MGDG, a decreased MGDG to DGDG ratio, and enhanced accumulation of phospholipids fatty acid composition of the leaves changed with exposure, but no major composition changes were detected in the roots. Lipid unsaturation in leaves (18:2 + 18:3) was decreased (Jemal et al. 2000). Verdoni et al. 2001 found alterations in the fatty acid composition of chloroplast lipids but not the fatty acid composition of the roots of tomato plants growing in heavy metal contaminated soils. Maize (Zea mays) seedlings exposed to Cu+ have decreased unsaturation of total fatty acids in the roots, however compositional changes in polar

fatty acids showed a general trend of increased unsaturation Chaffai et al. 2007. These responses suggest that the 18-C fatty acid classes may have specific roles in maintaining membrane function such that plant growth can occur under heavy metal stress.

1.1.5 Pathogen stress

Chloroplast oleic acid (18:1) levels in Arabidopsis are critical in inducing pathogen defence through programmed cell death and systemic acquired resistance (SAR) mechanism Kachroo et al. 2001). In soy bean Oleic (18:1) and linoleic (18:2) acid levels regulate fungal development, seed colonization and mycotoxin production by Aspergillus spp. (Xue et al, 2006). Based on the role of fatty acids in different stress in plants this present experiment was carried out with following objectives.

1.2 Objectives

1. To see the effect of *FAD7* gene silencing on reproductive process of tomato.
2. To see the differences in seedling growth of silenced and control plants at normal and high temperature.

2 Materials and methods

2.1 Construction of vector

Isolated sense fragment of tobacco fatty acid desaturase was cloned into the *XbaI* and *EcoRI* sites and anti sense fragment was cloned into *BamHI* and *SacI* restriction sites of pTH1-ASGS (Gifted from Dr. T. Hamada, Ishikawa Prefectural University, Japan) vector separated by 1Kb GUS fragment to form loop in silencing mechanism, under the control of *CaMV35s* constitutive promoter. The constructed vector was confirmed by restriction digestion of vector for cassette.

2.1.1 Competent cell preparation

A 10 µl glycerol stock of an *E. coli* DH5α strain containing no plasmids was allowed to thaw at room temperature and added to 40 ml of liquid L.B media. This culture was incubated at 37ºC for 1 h, then transferred to an incubator-shaker, at 37ºC, shaking at 200 rpm for over night until an OD_{600} of 0.5-0.6 was reached. Bacterial cultures were chilled out on ice for 10 to 15 min. The cells were pelleted by centrifugation at 8000 rpm for 1 min at 4ºC, then resuspended in one-half volume (20 ml) of sterile cold TSB (Transformation and storage buffer) solution (Tab. 1) and incubated on ice for 25 min. After another centrifugation step as above, the resulting cell pellet was resuspended in one-tenth volume (4 ml) of sterile cold TSB solution to yield the final competent cell suspension. Competent cells were stored at 4ºC for up to 3 days. Transfer 1.6 ml of the competent cell suspension to sterile cryo-storage tubes, and add 0.4 ml of sterile 100% glycerol to give a final concentration of 20% glycerol, and then mix together. The glycerol stocks are placed at -4ºC, -20ºC and -70ºC separately for later use.

Tab. 1 chemical composition of TSB buffer

Chemical	Volume	Final concentration
L.B. pH 6.1	12 ml	-
PEG 4000	5 ml	9%
MgCl2. 6H2O	200 µl	11 mM
MgSO4.7H2O	200 µl	11 mM
DMSO	1 ml	5.4%

2.1.2 Bacterial transformation

Competent cells were transformed with the plasmid DNA pTH1-ASGS by standard method of $CaCl_2$ treatment. Transformation reactions were conducted in 200 µl eppendorf tubes with 10ng of ligated plasmid or 0.1 ng of whole plasmid, 20 µl of KCM (Tab. 2) 5 x, 5 µl of 0.45 m $MnCl_2$ in pre-chilled Eppendorf make up volume to 100µl with mQ water and 100 µl of -80°C stored competent bacterial solution defrozen on ice were added. Eppendorf tubes were incubated on ice for 20min, at ambient temperature for 10 min. Bacterial cells were grown on shaker at 37°C and 200 rpm for 1 h after the addition of 1 ml L.B. Cells were pelleted in centrifuge at 2000 rpm for 2 min. Pellet was resuspended in 200 to 300 µl of L.B. normal and Plated on agar plate containing appropriate antibiotic to select transformed cells and incubate Petri plate upside down at 37°C. Transformed cells will appear on next day.

Tab. 2 chemical composition of KCM (Potassium, calcium and magnesium chloride) solution

Chemical	Volume	Final concentration
KCl	3.78 g	0.5 M
$CaCl_2.2H_2O$	2.21 g	0.15 M
MgCl2. 6H2O	5.08 g	0.25 M

2.1.3 Confirmation of bacterial transformation

Transformation of bacterial cells was confirmed by PCR through plasmid amplification. Plasmid mini preparations were conducted by inoculating 15 ml L.B falcon tube containing kanamycin antibiotic with bacterial colony and incubated on shaker for over night at 37°C and 200 rpm speed. Bacterial cells were pelleted in centrifuge at maximum speed for 1 min and pellet was resuspended in 200 µl STET (Tab. 3) buffer and mixed well with out any clumps. Bacterial lysis reaction was carried out by boiling the eppendorf tubes for 1 min after the addition of 20 µl lysozyme enzyme. Bacterial pellet was removed carefully after centrifuge at 12000g for 10 min. Bacterial plasmid DNA was purified by incubating plasmid at 37°C for 30 min after the addition of 1 µl of RNAse to solution and centrifuged at 12000g for 10 min with 200 µl of chloroform. Plasmids DNA were precipated in 200 µl of ice-cold isopropanol solution for 30 min and pelleted by centrifuge at 12000g for 15 min. Plasmid DNA pellet was washed with 0.5 ml of 95% ethanol

by centrifuge at 12000g for 1 min. Pellet was air dried for 10 min and dissolved in 30 µl TE buffer and store at 4°C.

Tab. 3 Composition of STET buffer for 10 ml

H_2O	8.95 ml
Sucrose	0.8 g
Triton X – 100	50 µl
EDTA (50 mM pH 8.0)	1 ml

Transformed bacteria were confirmed by PCR by using isolated plasmid for Kanamycin resistance gene with their corresponding primers 13 pMol 5'-GAGGCTATTCGGCTATGACT-3' and 5'-GAGCGGCGATACCGT-3' and rest of chemical concentration were like used in paragraph 2.1.8. PCR reaction was carried out in 20 µl tubes at initial denaturation at 94°C for 5 min, then 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.30 min and final extention at 72°C for 6 min and final storage at 4°C. Amplified positive products of 600 bp size were observed in agarose gel electrophoresis.

2.1.4 Agrobacterium transformation

Agrobacterium strain A.t.LBA 4404 containing Ti plasmid was grown for over night at 28°C and 250 rpm in 100 ml L.B medium with rifampicin and kanamycin as selective markers until the culture grows to an O.D of 0.5 to 1.0 at 600λ. Bacterial cell suspension was pelleted in centrifuge at 3000g for 5min at 4°C and pellet was resuspended in 1 ml of 20mM of ice-cold calcium chloride solution. Agrobacterium cells were freezed after the addition of 1 µg of pTH1-ASGS plasmid DNA and immediately cells were thawed by incubating in water bath for 5 min at 37°C. Bacterial suspension was incubated in 1 ml L.B at 28°C and 250 rpm for 2 to 4 h. Bacterial cells were pelleted in centrifuge for 30 sec and plated on agar plate containing 50mg/litre kanamycin and incubate at 28°C. Transformed colonies will appear in 2 to 3 days.

2.1.5 Confirmation of transformed agro bacterium

Positive colonies of agrobacterium grown on agar plates containing selective markers were confirmed by procedure explained in paragraph 2.1.3.

2.1.6 Micro tom tomato transformation

Seeds of tomato, (*Solanum Lycopersicum* L.) cv Micro-Tom were surface sterilized for 10minutes in a 50% NaoCl and washed three times with sterilized water for 10 minutes to remove left over Naocl. Put seeds in Petri plate for pregermination and after transfer germinated seeds to magenta containing MS medium (A). After one week take cotyledenary leaves from germinated seedlings and cut top and bottom parts of leaves, put them in Petri plate containing media B in upside down manner for preconditioning in dark at 25°C. Prepare agrobacterium containing desired plasmid pTH1-ASGS in L.B containing 30 mg/l rifampicin and selectable antibiotic in construct. Transformed bacterium was grown at 250 rpm over night. Centrifuge the bacterial culture at 3000 rpm and resuspend the pellet in L.B without antibiotic until final concentration of 10^7cells per ml (O.D. of 0.1 to 0.2). After 24 to 48 h cotyledons in B are co-cultivated with agro bacterium for 20 min with gentle shaking on shaker i.e. like 50 to 100 rpm. Remove bacterial solution and maintain cotyledons in plate B in dark at 25°C. After 24 hours transfer the explants to regeneration medium C and put them in chamber at 25 °C with 16 h light and 8 h dark. After one week transfer explants to Petri plate containing medium D. Keep explants for one week in this medium and then transfer explants in every 20 days to new Petri plate containing media D with same photo period i.e. 16 h light and 8 h dark. After approximately 6–8 weeks, shoots were excised and transferred to Medium E for rooting and selection. Chemical and hormonal concentrations of growth medium are given in table 4.

Table 4 Chemical and hormonal concentrations in used in micro-Tom tomato regeneration.

COMPONENT	A	B	C	D	E
MS	1X	1X	1X	1X	1X
Sucrose	3%	3%	3%	3%	3%
Agar	0.6%	0.6%	0.6%	0.6%	0.6%
pH	5.8	5.8	5.8	5.8	5.8
Zeatin mg/lt		0.7	0.7	0.7	
IAA mg/lt		0.35	0.35	0.35	0.35
IBA mg/lt.					0.1
2.4.D mg/lt		0.1			
Cefotaxime mg/lt			200		200
Vancomycin mg/lt			50		
Carbencilin mg/lt				500	
Kanamycin mg/lt				100	50

2.1.7 DNA extraction

DNA was extracted from the transformed plants using the CTAB method. Leaves of transformed plants were harvested into Eppendorf and ground to the powder with liquid nitrogen in mortar and pestle. 600 µl of the CTAB-buffer (200mM Tris-HCl (pH 7.5), 2M NaCl, 50mM EDTA and 2%N-cetyl-N, N, N- trimethylammonium bromide) was added to each eppendorf and mixed by inversion. Samples were incubated at 60°C for 1 hour, cooled on ice and extracted once with 600 µl of chloroform/isoamylalcohol (24:1). The aqueous phase was obtained after centrifugation at room temperature (10 min at 12000 rpm). Supernatant was transferred to new eppendorf tube and was mixed well with 400 µl of ice cold isopropyl alcohol. Samples were incubated at -20°C for 1hour. DNA pellet was collected after centrifugation at room temperature (10 minutes at 12,000 rpm), rinsed once with 75% ethanol (650 µl), dried (10 min at 60°C) and resuspended in 200 µl of 1mM Tris-HCl (pH 8.0) and 0.1mM EDTA. Samples were stored at -20°C.

2.1.8 Confirmation of plants

DNA was extracted from leaves of transformed plants using the CTAB-method described above and used as template for the PCR reaction. Transformed plants were confirmed by PCR for

Kanamycin resistance gene with their corresponding primers 13 pmol 5'-GAGGCTATTCGGCTATGACT-3' and 5'- GAGCGGCGATACCGT-3' and 1.8 µl dNTPs 2.5 mM, 1x Taq polymerase buffer with $MgCl_2$, 0.2 µl Taq DNA polymerase 1 U (New England Biolabs) and final volume made upto 20 µl with MQ H_2O. PCR reaction was carried out in 20 µl tubes at initial denaturation at 94°C for 5 min, then 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.30 min and final extention at 72°C for 6 min and final storage at 4°C. Amplified positive products of 600 bp size were observed in agarose gel electrophoresis.

2.1.9 GUS expression

The putative transformants (both root and leaf samples) were histochemically detected for the presence of the GUS gene following the procedure of Jefferson et al. (1987). The samples from the putative transgenic plants and untransformed control plants were incubated in a solution containing the stocks of 2 mM X-glucuronide in DMSO, 100mM Tris HCl (pH 7.0), 50 mM NaCl, 2 mM potassium ferricyanide and 0.1 % (v/v) triton X-100 pipetted in required quantities to make gus staining solution overnight at 37°C and cleared in 75% ethanol for 4 h to remove the chlorophyll and clean the tissue. The tissues were inoculated on a glass slide and observed under microscope for blue staining.

2.2 RNA isolation

Total RNA was extracted from the untransformed control and *FAD7* transformed plants using TRIZOL reagent (Invitrogen). 1 mL of TRIZOL was added to each eppendorf containing 50 to 100 mg leaf tissue and pulverised with mortar and pestle. Samples were incubated in room temperature for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, extracted once with 200 µl of chloroform, mixed by inversion and again incubated in room temperature for 2 min. The aqueous phase obtained after centrifugation (10 min at 12000g at 4°C) was mixed by inversion with 500 µl of isopropanol, and incubated in room temperature for 10 min. Samples were centrifuged (15 min at 12000g at 4°C) and the RNA pellet was rinsed twice with 75% ethanol (1000 µl), dried at room temperature and resuspended in 20 µl of DEPC H_2O. Samples were stored at -20°C.

2.2.1 RNA quantification

The RNA concentration was estimated by spectrophotometer with absorbance at 260 and 280nm. In brief 5 µl of extracted RNA was added to 995 µl of DEPC water. Absorbance of machine was adjusted to zero with blank DEPC water and reading taken at 260 and 280 nm. The ratio of (A260/280) should be 1.8 for good quality RNA. The concentration of RNA was calculated by using this formula.

$$\text{RNA [µg/ml]} = \frac{\text{O.D at A260 x 40 RNA µg/ml x1000}}{\text{1 O.D x 5}}$$

2.2.2 cDNA synthesis

cDNA was synthesized from extracted RNA by PCR amplification. PCR amplifications were performed in 20 µl total volume, containing 0.2 µg of RNA as template with 2.5 mM oligo $(dT)_{12}$ M primer, 1 mM dNTPs in DEPC water, 1x MLVRT buffer, 10 mM DTT and 10 U M-MLVRT enzyme (Promega). PCR amplifications were conducted at with initial denaturation at 65°C for 5 min and then followed by 60 min at 37°C, 5 min at 95°C and final cycle 5 min at 4°C. Note: MLVRT enzyme was added after 10 min at 37°C.

2.2.3 Amplification for actin

Actin amplifications were conducted by using synthesized 50 ng of synthesized cDNA as template, 3 µl of 4 pmol of actin primer 5'- GAY TCT GGD GAT GGK STS AS -3' and 5'- ATY TTC ATG CTR CTD GGA GC -3', 2 µl of 1 X buffer, 2 µl of 2.5 mM dNTPs, 0.2 µl of 1 U Taq DNA polymerase and final volume made upto 20 µl with MQ H2O. PCR amplification were conducted with an initial denaturation at 94°C for 4 min and then followed by 36 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, plus 7 min final extension at 72°C and final storage at 4°C.

2.2.4 Confirmation of transgene silencing

Silencing of transformed *FAD7* was confirmed by amplification of cDNA as template. Amplification were conducted on 50 ng of cDNA, 3 µl of 1.25 mM primers for sense fragment (GGAATTCAAAGAAAGGTTCTCACTTTGA (*EcoRI* site was underlined) and GCTCTAGAAAGTGATCCTCCTTCATGCT WITH (*XbaI* site was underlined) and for anti sense fragment (CGGGATCCAAAGAAAGGTTCTCACTTTGA (*BamHI* site was underlined) and CGCGAGCTCAAAGTGATCCTCCTTCATGCT (*SacI* site was underlined), 2 µl of 1x

buffer, 0.2 µl of 1U Taq DNA polymerase and final volume made upto 20 µl with MQ H_2O. PCR amplification were conducted with an initial denaturation at 94°C for 4 min and then followed by 36 cycles of 1 min at 94°C, 1 min at 67°C, 1 min at 72°C, plus 7 min final extension at 72°C and final storage at 4°C.

2.2.5 Plant growth conditions

Untransformed control plants and plants transformed with *FAD7* RNAi construct were subjected to heat treatment for 2 h at 32^0C at flowering stage. Plants after heat treatment were transferred to growth chamber under normal growth conditions and flower buds collected after 12 hours of heat treatment for pollen staining, germinability check.

2.2.6 Pollen staining

To determine the percentage of stainable pollen from both *FAD7* RNAi construct transformed and control plants after heat stress, flowers were sampled 12 h after heat treatment. One anther was removed from each flower was placed on microscopic slide and scraped it to release pollen by adding 1% ACETIC-ORCEIN and 50% glacial acetic acid solution. This method enables us to record the percentage of non-viable (stained green) and viable (stained purple) pollen grains under the light microscope. This procedure was repeated for three times for each anther and average results were calculated.

2.2.7 Pollen germination

Pollen of plants transformed with *FAD7* RNAi construct after heat treatment was analyzed for their germinability at 22^0C on pollen germination medium containing sucrose 10%, boric acid 100 mg/l, calcium nitrate 300 mg/l, magnesium sulphate 200 mg/l, potassium nitrate 100 mg/l and 1% phytagel along with untransformed control plant. This method enables us to record the number of non-germinable and germinable pollen grains with aniline blue staining and the fluorescence was examined with UV light filter under microscope. This procedure was repeated for three times for each anther and average results were calculated.

2.2.8 Productivity

Productivity of T_2 transformed plants along with the productivity of control plants which were grown under high temperature in terms of fruit number per plant from the first three trusses was recorded.

2.2.9 Seed germination

Seeds from the T_3 generation plants transformed with a silencing construct for the *FAD7* gene were germinated at 32^0C (high temperature) for four days to see the differences in their germination at high and normal temperature. Seed germination data was recorded and their means were compared at two different temperatures.

2.3 Seedling growth

Seeds from the T_3 generation plants transformed with a silencing construct for the *FAD7* gene were tested at 32^0C (high temperature) and 26^0C (normal temperature) for one week to see the differences in their growth. Seedlings growth i.e. roots length and hypocotyl lengths were recorded at two different temperatures.

2.3.1 Fatty acid quantification

2.3.1.1 PLFA extraction

1 g of tissue was collected from three genotypes of *FAD7* transformed plants and untransformed control plants was collected after treatment and ground in liquid nitrogen to a fine powder using a mortar and pestle. The powdered tissue was transferred to a test tube for the extraction of the trienoic linolenic acid from the total plant lipids. PLFA was extracted with tree step extraction using a modified procedure by Bligh and Dyer (1959) and by Zelless (1992). The lipids are extracted from the leaves by adding solutions of Phosphate-Buffer (pH 7.4) Methanol, Chloroform in this ratio 0,8:2:1 and incubated for a minimum of 2 h and then extracted again with same solutions but with different concentrations Chlo-Meth-P-buffer 2:2:1,8), then samples were incubated for over night. After one night the falcone tubes were centrifuged for 20 min a 4000 rpm to separate the solvent from the leaves, and the phases were separated by using separating funnel. The two phases of samples was dried by rotary evaporation prior to storage at -20°C. The total lipid was fractionated on a silic acid column SPE (3ml/500 mg silica Sep-pak VacTM , Waters) with Acetone-Chloroform-Methanol solutions into neutral lipids, glycolipids and phospholipids and sample was dried by rotary evaporation prior to storage at -20°C. The phospholipids were then subjected to a mild alkaline methylation for transesterifyng the fatty acid into methyl esters.

2.3.1.2 PLFA analysis

Fatty acid methyl esters were separated and quantified by gas chromatography-mass spectrometry (GC/MS). Sample were dissolved in dichloromethane, CH_3Cl_3, containing nonadecanoic acid methyl ester (C19:0) as internal standard. Samples were injected into GC/MS (Shimadzu GCMS-QP5050), equipped with column VF-5ms, 30m 0.25 mm, 0,25 mm internal diameter), with helium as carrier gas. The GC-MS was programmed from 89°C for 2 min and raised at 6 min to 280°C and it was held for 5 min. Fatty acid were identified by relative retention times compared with standards (BAME 24 47080-U; Supelco, Inc. e FAME 37 47885-4; Supelco, Inc.). PLFAs are quantified by using internal standard. Quantification of FAMES was accomplished by using varying concentrations of C19 and allowed peak areas to be converted to a molar basis. Individual fatty acid methyl esters (FAME) were identified and expressed as percentage of total amount of fatty acids (% abundance) found in a given sample. Standard nomenclature rules were followed when referring to different fatty acids (Frostegard et al., 1993) in the form of A:BωC, where A designates the total number of carbons, B the number of double bonds, and C the distance of the closest unsaturation from the aliphatic end of the molecule. The suffixes "i-," "a-,"and "me-" refer to iso-anteisomethyl branching, and mid-chain methyl branching, respectively, with cyclopropyl rings indicated by "cy"(Kates,1986).

2.4 Statistical analysis

All data were compared between the transformed plants and control plants in temperature treatments and differences were tested by Student t-test.

3 Results

3.1 Transgenic plants harboring dsRNA construct

We introduced the NtFAD7 dsRNA construct into tomato plants to produce efficiently plants with low trienoic fatty acid content. The binary construct, pTH1-ASGS (Fig. 2), which was designed to produce a dsRNA transcript it was obtained from Dr.Hamada. The dsRNA was transcribed under the control of *CaMV 35S* promoter in pTH1-ASGS. All the eleven T_0 transformed plants obtained were confirmed by PCR for kanamycin and GUS (Fig. 3) with their respective primers.

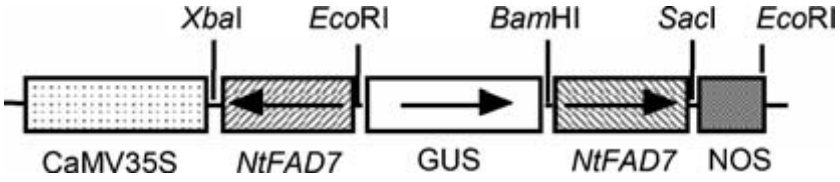


Fig. 2 Structure of chimeric gene for expression of the *NtFAD7* dsRNA: The *NtFAD7 dsRNA* construct, *pTH1- ASGS*, contains 0.5-kbp fragments of the NtFAD7 cDNA in antisense and sense orientation. CaMV35S is the CaMV 35S promoter sequence. GUS is a 1.0-kbp fragment of the ß-glucuronidase gene. NOS is the terminator sequence from the nopaline synthase gene. Restriction sites shown are for *XbaI, BamHI, EcoRI* and *SacI*.

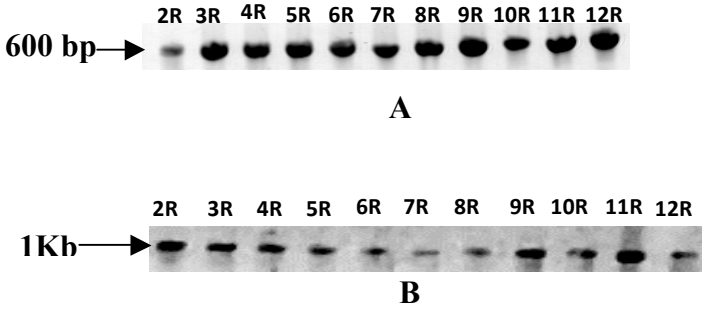
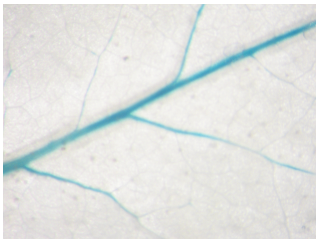


Fig. 3 PCR Confirmation of transformed plants. (A) Confirmation of transformed plants for Kanamycin gene. (B) Confirmation of transformed plants for GUS gene.

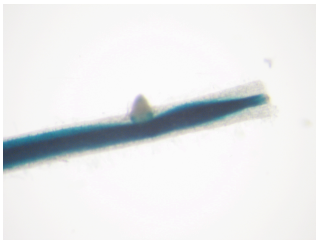
3.1.1 GUS EXPRESSION

We did histochemical GUS assay to see the GUS gene expression in the root and leaves samples of T_1 transformed plants. All the eleven transformed plants showed positive result for their GUS

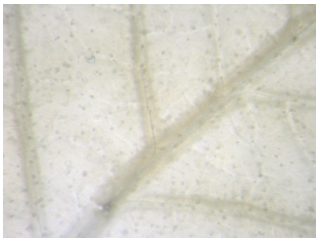
expression. It indicates that the NtFAD7 dsRNA construct is transcribed normally in tomato plants as like normal genes. GUS expression level was similar in all transformed plants. (Fig. 4)



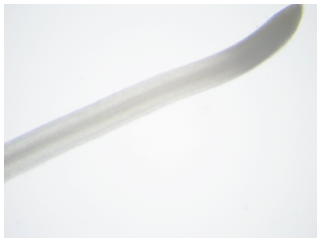
A



B



C



D

Fig. 4 Histochemical analysis of GUS activity in transformed plants Plate (A) and (B) expression of GUS activity in transformed plant leaf and root. Plate (C) and (D) GUS activity expression in control plant leaf and root.

3.1.2 Gene expression analysis

Total RNA from all transformed lines with *FAD7* and control plants was isolated with TRIZOL and their quantities are as follows in table 5 and fig. 5.

Tab. 5 RNA concentrations (ng/µl) of *FAD7* transformed plants and untransformed control plants.

Plant	Concentration of RNA ng/µl	Plant	Concentration of RNA ng/µl
Control	5.07	7R	3.42
2R	5.69	8R	7.57
3R	4.44	9R	8.36
4R	7.83	10R	3.14
5R	6.89	11R	8.20
6R	3.77	12R	7.14

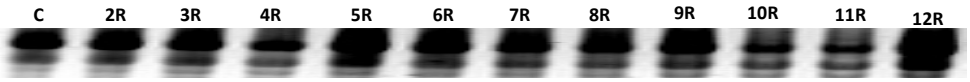


Fig. 5 Isolated RNA from plants transformed with *FAD7* lines from 2R to 12R and control plant (C)

cDNA Synthesis

We used total isolated RNA (Fig. 5) from both transformed and control lines to synthesize cDNA for silencing confirmation studies.

Amplification with Actin

Equal quantity of c-DNA from all the transformed lines and control plants was used to amplify to see the level of expression in transformed and control plants (Fig. 6).

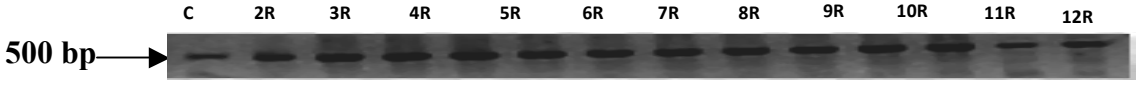


Fig. 6 Amplified actin house keeping gene from plants transformed with *FAD7* lines from 2R to 12R and untransformed control plant (C).

RT-PCR

We used synthesized cDNA to amplify *FAD7* gene by using the respective primers in both sense and anti- sense orientation. We got amplification in control plant whereas no amplification in transformed lines (Fig. 7). These results indicated that transformed gene effectively silenced the endogenous *FAD7* gene in transformed plant.

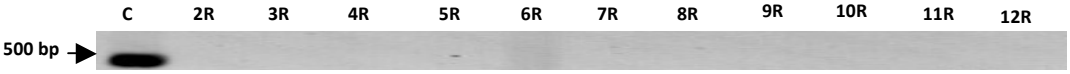


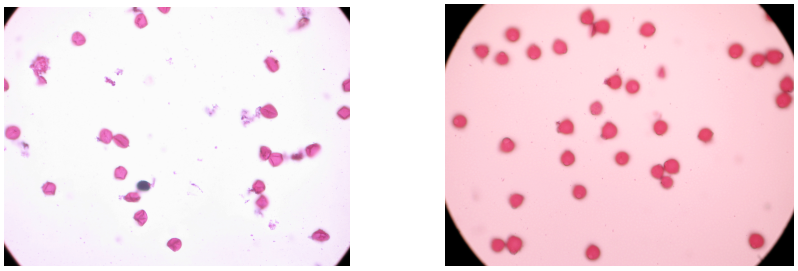
Fig. 7 Amplification of *FAD7* gene from transformed plants from lines 2R to 12R and control plant (C).

3.1.3 Pollen analysis

we analyzed pollen of transformed T_2 and control plants after heat stress for their viability in terms of number (table. 6) with aceto-orcein stain, we found pollen quality of transformed is better when compared to the pollen quality of control plants (Fig. 8).

Tab. 6 Percentage of stainable pollen grains (SPG), non-stainable pollen grains (NSPG) of plants transformed for *FAD7* gene with their standard error of the means (SEM) and control (C) plants.

Plant	SPG (% ± SEM)	NSPG (% ± SEM)	Plant	SPG (% ± SEM)	NSPG (% ± SEM)
C	45.74 ±2.33	54.25 ± 1.52	7R	84.55 ± 2.40	15.44 ± 2.02
2R	91.35 ± 0.88	8.64 ± 0.33	8R	57.89 ± 0.66	42.10 ± 2.33
3R	94.82 ± 4.17	5.17 ± 1.52	9R	96.61 ± 1	3.38 ± 0.66
4R	92.30± 2.88	7.69 ± 0.33	10R	96.87 ± 2.33	3.125 ± 0.33
5R	98.79 ± 5.04	1.20 ± 0.33	11R	95.55 ± 2.40	4.44 ± 0.33
6R	84.55 ± 2.33	4.92 ± 0	12R	96.77 ± 2.30	3.22 ± 0.33



A **B**

Fig. 8 Pollen staining of Control plant (A) and Plant transformed with *FAD7* silencing construct (B).

3.1.4 Seed germination and seedling growth

Seed germination from transformed plants is more when compared to control plants under heat stress (Tab. 7).Germinated seedlings from the plants transformed with *FAD7* were grown on MS media at normal and high temperature conditions. It was shown that there was no genotype × treatment interaction both for root length and hypocotyl length. Whereas differences between genotypes were not found at 26^0C, transformed plants performed better than controls at 32^0C although the differences were significant only for hypocotyl length (Table 8 and Fig. 9)

Table 7 Seed germination percentage of transformed and control plants at 32^0C.

Plant	Seed germination%
Control	66.66 ± 1.45
2R	51.66 ± 1.66
4R	93.33 ± 0.66
5R	95.55 ± 0.57
6R	96.66 ± 0.66
7R	100 ± 0.00
8R	98.33 ± 0.33
10R	88.33 ± 0.33
11R	100 ± 0.00
12R	100 ± 0.00

Note: 3R and 9R genotypes data was not collected because of fungal contaminations during germination.

Table 8 Root length (RL) and Hypocotyl length (HL) in centimeters (Cm) of plants silenced for *FAD7* gene and control plant (C) at different temperatures with their standard error of the means (SEM). Means followed by the same lowercase letter in the same column are not significantly different for P≤ 0.05 according to Duncan multiple range test.

Plant	R.L ± SEM (Cm)	H.L ± SEM (Cm)			
	26^0C	32^0C	26^0C	32^0C	
C	6.3 ± 0.6	3.5 ± 0.5	3.6 ± 0.4	1.5 ± 0	e
2R	5.5 ± 0.2	4.6 ± 0.1	3.5 ± 0.7	2.5 ± 0.2	c-d-e
3R	3.5 ± 0.7	5.1 ± 0.1	5.8 ± 0.2	3.8 ± 0.1	c-a-b
4R	3.3 ± 0.7	5.1 ± 0.1	5.3 ± 0.1	3.1 ± 0.1	c-d-e
5R	6.3 ± 0.4	5.8 ± 1.3	3.6 ± 0.4	3.3 ± 0.1	c-a-b
6R	6.1 ± 0.4	5.8 ± 0.1	3 ± 0.2	2.3 ± 0.1	d-e
7R	3.7 ± 0.4	5.6± 0.2	5.6 ± 0.4	2.9 ± 0.2	c-d-b
8R	5.6 ± 0.4	4.0 ± 0.2	3.5 ± 0.2	2.6 ± 0.1	c-d-e
9R	6 ± 0.2	5.1 ± 0.1	4.1 ± 0.4	3.6 ± 0.1	a-b
10R	5.8 ± 0.4	4.6 ± 0.1	3.6 ± 0.4	3.1 ± 0.1	c-a-b
11R	6 ± 0.2	5.1 ± 0.3	3.1 ± 0.1	2.7 ± 0.1	c-d-e
12R	6.8 ± 0.6	5.5 ± 0.2	4.5 ± 0.2	3.6 ± 0.1	a



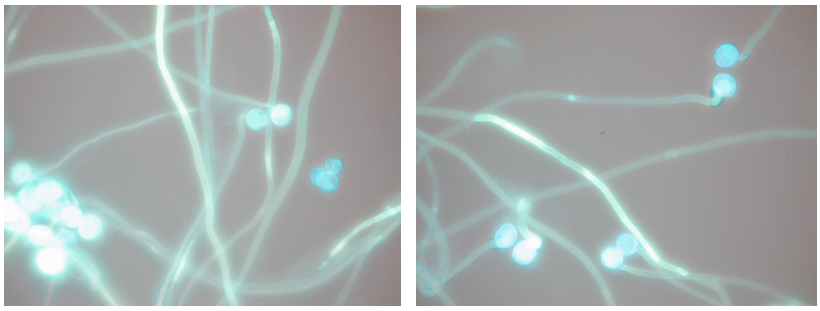
Fig. 9 Seedling growth of plants transformed with *FAD7* (T) and control (C) plants at 32^0C

3.1.5 Pollen germination

Pollen collected from the plants transformed with *FAD7* gene silencing construct after heat treatment was germinated on in vitro germination media to see their ability to germinate at normal conditions. Significant difference was found for pollen germinability between control and all transformed plants (*P≤ 0.05 after t Student test). Pollen from transformed flower (Fig. 10A) germinated better than the pollen from control plants (Tab. 9 and Fig 10B).

Table 9 Germinated pollen grains (GP), and Non-germinated pollen grains (NGP) of plants silenced for *FAD7* gene and control plant (C) at different temperatures with their standard error of the means (SEM).

Plants	% GP ± SEM	% NGP ± SEM
	22^0C	22^0C
C	74.7 ± 1.5	25.2 ± 0.4
2R	90.8 ± 1.1	9.1 ± 0.6
3R	91.4 ± 1.4	8.5 ± 1.2
4R	91.6 ± 1.1	8.3 ± 0.3
5R	92.9 ± 1.1	7.0 ± 2.1
6R	93.2 ± 2.0	6.7 ± 0.6
7R	92.8 ± 1.7	7.1 ± 0.3
8R	90.5 ± 0.8	9.4 ± 0.3
9R	96.1 ± 1.2	3.8 ± 0.3
10R	92.0 ± 0.8	7.9 ± 0.7
11R	93.6 ± 1.5	6.3 ± 0.3
12R	95.8 ± 1.1	4.1 ± 0.6



A **B**

Fig. 10 germinated pollen grains of Control (A) and *FAD7* transformed plants (B) after heat treatment.

3.1.6 Fatty acid quantification

Fatty acid content was estimated from both silenced plants and control plants (Tab. 10) with GC-MS with standard protocol. Results shown that trienoic fatty acids quantity in transformed plants was from 10 to 45% less than the trienoic fatty acid composition of control plants.

Table 10 Percentage of different fatty acids composition of control plant (C) and plants silenced for FAD7 gene with their standard error means (SEM).

Plant	% 16:0 ± SEM	% 18:0 ± SEM	% 18:2 ± SEM	% 18:3 ± SEM
C	35.0 ± 2.5	1.4 ± 0.8	19.3 ± 0.2	44.2 ± 3.1
2R	34.7 ± 2.4	3.9 ± 1.0	12.7 ± 1.2	40.8 ± 4.9
4R	54.9 ± 1.2	5.5 ± 1.3	15.7 ± 0.4	23.7 ± 0.5
8R	42.4 ± 8.2	2.7 ± 0.3	18.3 ± 3.7	36.4 ± 4.9
12R	54.6 ± 2.1	4.8 ± 0.3	16.4 ± 1.2	24.1 ± 0.6

3.1.7 Productivity

We compared the productivity of T_2 transformed plants with the productivity of control plants in terms of fruit number per plant (Table. 11) from the first three trusses which were grown under high temperature. We found productivity of transformed plants higher when compared to the productivity of control plants. We found significant difference in yield between plants transformed with *FAD7* and untransformed control plants.

Tab. 11 Productivity of *FAD7* transformed plants along with the productivity of control plants.

PLANT	FRUIT NUMBER	PLANT	FRUIT NUMBER
C	25 ± 0.96	7R	28 ± 0.33
2R	76 ± 1.21	8R	30 ± 0.90
3R	21 out of 2 Pls ± 0.57	9R	48 ± 0.61
4R	32 ±0.69	10R	58± 0.70
5R	31 ± 0.80	11R	90 ±1.59
6R	47± 0.81	12R	43± 0.86

Note: Fruit number of 3R plant came from two plants only other plants were died because of fungal wilt.

4 Discussion

Biomembranes, which are the boundaries between inner and outer environments and also between cellular compartments, provide the major sites for the perception and propagation of different abiotic and biotic stresses signals, leading to alleviations of deleterious stress effects. Plant cell membranes contains different type of lipid molecules as building blocks and these lipid molecules and their derivative also serves as bioactive agents to mediate stress signalling in plant cells (Wang, 2004). Plant cell membrane contains more polyunsaturated fatty acids such as dienoic fatty acids (DAs) and Trienoic fatty acids (TAs) to the 70% of total lipids (Harwood 1980). Plants have capacity to change their lipids and fatty acids composition of their membranes according to the different environmental stress stresses like high temperature (Murakami et al., 2002), low temperature (Murata et al,. 1992), wounding (Takumi et al., 1999), and pathogen invasion **(**Yaeno et al. 2004). Omega-3 fatty acid desaturase has been considered to be the rate-limiting enzyme for the production of trienoic fatty acids (TAs) (Iba *et al*. 1993). Therefore, the control of the expression of omega-3 fatty acid desaturase genes by genetic techniques is one of the most effective methods for the modification of the content of dienoic fatty acids and trienoic fatty acids. Saturation/unsaturation of thylakoid membrane lipids was considered to be extraordinarily associated with temperature stress, of which saturation of thylakoid membrane lipids by catalytic hydrogenation increases the thermal stability of membranes (Thomas *et al*. 1986). In this research the fatty acid content in tomato plants that were silenced for *FAD7* under the control of *35S-CaMV* promoter was measured along with non-silenced plants (control). The results show that individual fatty acids do not differ significantly among transformed plants; however transformed plants shown up to 40% reduction in trienoic fatty acids to that of the control and all treatments produced increase of stearic acid (Dienoic fatty acid) significantly greater than other fatty acids (Tab. 10). This reduction in quantity of trienoic fatty acids in transformed plants is because of silencing of FAD7 gene which involves in conversion of dienoic fatty acids to trienoic fatty acids. The reduction of trienoic fatty acids in transformed plants are in agreement with the observed phenotype. Growth analysis of WT and transgenic lines was investigated under heat stress. The hypocotyl length of transformed plants was higher than the hypocotyl length of control plant (Tab. 8 and fig. 9). These results indicated that the level of TAs was important for the growth of tomato at high temperature. This is consistent with the results reported previously (Murakami et al. 2000; Liu et al. 2006). This increased hypocotyl length in transformed plants may be because of low availability of growth regulator jasmonic acid because

linolenic acid i.e. trienoic fatty acid is precursor for jasmonic acid synthesis in octadecanoid pathway, so transformed plants with low trienoic fatty acids contains low jasmonic acid than control with more trienoic fatty acids and shown increased hypocotyl length. These results are in agreement with Kumari and sudhakar, (2003) there they found high concentration of JA inhibits root growth and stem elongation and reduces its fresh and dry weight, as well as protein content and proline accumulation in ground nut. Plants transformed with *FAD7* showed decreased level of trienoic fatty acids and increase heat tolerance by increased pollen stainability (Tab. 6 and fig. 8), germinability (Tab. 9 and Fig. 10) and finally in production (Tab. 10) whereas we found contrast results in untransformed control plants ; this is not really in agreement with the results of Mlchele McConn et al 1996 in which they developed Arabidopsis triple mutant line for fad3-2 fad7-2 fad8 genes which is defective in producing trienoic acids i.e. 0.1% in total fatty acid profile of the plants and it shown less pollen viability and no seeds under normal condition. It should be noted that in some species high-temperature tolerance does not correlate with the degree of lipid saturation, suggesting that factors other than membrane stability might limit the growth at high temperature (Wang et al 2010). The possible reason for these seedless fruits results may be the complete malfunction of all gene which involves in trienoic fatty acid production and their insufficient availability towards pollen and seed development, where as in the present experiment we silenced only *FAD7* and we left rest two enzymes i.e. fad3-2 and fad8 untouched, so there is availability of trienoic fatty acids towards pollen and seed development to complete plant life cycle. We demonstrated that silenced *FAD7* gene in tomato reduced the level of TAs and increased the level of DAs under high-temperature stress and exhibited high-temperature tolerance and its importance in reproductive development of the plants. Fatty acids indirectly involved in seed germination process in many plants through production of jasmonic acid and its signalling properties in different physiological process during seed germination. In this present experiment we germinated seeds of transformed plants and untransformed control plants under heat stress at 32^0C (Tab. 7) and we found rate of germination in transformed plants is high even though we have differences among transformed plants but still it is higher than the germination percentage of untransformed control plants. These results are in close agreement with Corbineau et al., 1988 where they found reduced seed germination in sunflower seeds treated with jasmonic acid and methyl jasmonate by reducing oxygen uptake by germinating seeds. Similar results were found in different plants species like *Amarantus caudatus* (Bialecka and Kepcznski, 2003), cocklebur (Nojavan and Ishizawa, 1998), tobacco (Preston et al., 2002) and rapeseed and flax (Wilen et al., 1994).

Conclusions

Temperature affects the growth and productivity of plants, but its effect depends on the nature of plant species, i.e. warm season or cool season plants. Increased temperature is not only a problematic factor for vegetative growth but also for reproductive development like flowering, pollen development, fruit set and finally total yield of the crop plants. Exposure to high temperature causes reduced yields in tomato (*Solanum lycopersicum* L.). In the present experiment, tomato plants expressing the proline transporter gene *LePROT1* under the control of HSP 18.2 promoter from Arabidopsis showed increased supply of proline to developing anthers of tomato plants under heat stress. These transformed plants showed higher resistance to heat stress when compared to untransformed control plants. These transformed plants performed far better than untransformed plants in terms of their pollen stainability, germinability, yield and seedling growth in artificial drought and salt stress induced medium under heat stress.

Plants can acquire inducible environmental stress resistance by remodeling of membrane fluidity. In the present experiment, transgenic tomato plants that express the transcript of double-stranded RNA (dsRNA) of tobacco plastid omega-3 fatty acid desaturase gene *NtFAD7* to induce post transcriptional gene silencing produced decreased levels of trienoic fatty acids when compared to untransformed control plants. These transformed tomato plants performed far better than the untransformed control plants in terms of pollen stainability, germinability, yield and seedling growth under heat stress. Therefore, it is reasonable to conclude that the accumulation of proline through proline transport in relation to heat stress under the control of heat inducible promoter before anthesis and decrease of trienoic acid formation through gene silencing could be useful methods to increase heat tolerance in tomato and they could be a solution to increase crop yields under this present arena of global warming.

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ACKNOWLEDGEMENT

I sincerely bow to his omnipresence……

I feel immense pleasure in expressing my regards to Professor Andrea Mazzucato, Department of Agro Biology and Agro Chemistry (DABAC), chairman of my advisory committee for his guidance, Valuable suggestion and sustained interest throughout the course of my research work.

I wish to extend my sincere thanks to Dr. Stefania Astolfi and Silvia Rita Stazi and Anita Maienza for their invaluable help in quantification experiments. We are very grateful to Prof. T. Hamada (Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Japan) and Proff Jean-Philippe Galaud (Surfaces Cellulaires et Signalisation chez les Vegetaux, CNRS-Universite´ Paul Sabatier, Pole de Biotechnologie vegetale, Tolosan, France) for their kind gift of plasmid pTH-ASGS and plasmid $p^{GIND\ 01}$ used in present work.

Mere words can't express my warmest thanks to Proff. G.P. Sorressi, Irene Olimpieri, Maurizio Enea Picarella, Pietro Mosconi, Luigi Sellei, Fabrizio Ruiu and Enrico Santangelo for their encouragement, cooperation, enduring patience and support which remain biggest source of inspiration for me.

I feel lacunae of words to express my sincere appreciation for my family members, for their love, affection and heartiest blessing. Of course, I have no words to acknowledge love, affection, power of determination and spiritual support received from my grand parents and parents. Blessings encouragement and inspiration from them have helped in building my educational carrier and reaching to this level.

Last but never the least; words are small trophies to express my deep sense of indebtedness and affection to K. Premchand, M. Krishna Reddy, Vinod Kumar Yadav, Salvo Licitra, Sandro Manusia, Maurizio Benelli, Carlo Renzi, Mario pelegrini, Xacobe, Elena Lozano, Riccardo Bertoldi, Mattia Sbaraglia and Tiziana Proietti.

Lastly my thanks are due to all those people who helped me directly and indirectly making this work possible.