TREHALOSE METABOLISM IN WHEAT AND IDENTIFICATION OF TREHALOSE METABOLIZING ENZYMES UNDER ABIOTIC STRESS CONDITIONS

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

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Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a non reducing disaccharide of glucose that occurs in a large variety of organisms, ranging from bacteria to invertebrate animals, where it serves as an energy source or stress protectant. Until recently, only few plant species, mainly desiccation tolerant 'resurrection' plants, were considered to synthesize trehalose. Although most plant species do not appear to accumulate easily detectable amounts of trehalose, the discovery of genes for trehalose biosynthesis in *Arabidopsis* and in a range of crop plants suggests that the ability to synthesize trehalose is widely distributed in the plant kingdom. In this study, three wheat cultivars (*Triticum aestivum L.*) Tosun, Bolal (stress tolerant) and Çakmak (stress sensitive) were analysed for the presence of trehalose. Using gas chromatography-mass spectrometry (GC-MS) analysis, trehalose was unambiguously identified in extracts from seeds and seedlings of three different wheat cultivars (Bolal, Tosun and Çakmak).

The trehalose amount was quantified by high performance liquid chromatography connected with refractory index detector. Effects of drought and salt stress on trehalose contents of wheat cultivars were studied at seedling level and trehalose analysis was achieved both on shoot and root tissues. It was found that trehalose had accumulated under salt and drought stress conditions in all wheat cultivars. The highest trehalose accumulation was detected in roots of Bolal cultivar under drought stress condition. Furthermore, trehalose metabolizing enzymes; trehalose-6-phosphate synthase (TPS) and trehalase enzyme activities were measured in roots and shoots of Bolal and Çakmak cultivars under control, salt and drought stress conditions. The most interesting results that we found that TPS activity sharply increased under stress conditions. The activity of TPS in roots under drought stress condition was the highest and reached to 3-4 times of its activity under control condition. The increase in the activity of TPS showed parallelism with trehalose accumulation under stress condition. Trehalase activity in Bolal cultivar decreased under both salt and drought stress conditions, however there was no significant change in trehalase activity of Çakmak variety.

Key words: Wheat, trehalose, trehalose-6-phosphate synthase, trehalase, stress protection, drought, salt.

ÖZ

BUĞDAYDA TREHALOZ METABOLİZMASI VE TREHALOZ METABOLİZMASINDAKİ ENZİMLERİN ABİYOTİK STRES KOŞULLARINDA BELİRLENMESİ

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Trehaloz (α-D-glukopayranozil-1,1-α-D-glukopayranozid), iki glukoz ünitesinden oluşan ve indirgemeyen bir disakkarittir. Trehaloz, enerji kaynağı ve stres koruyucu olarak bakterilerden omurgasız hayvanlara kadar birçok organizmada bulunur. Yakın zamana kadar sadece birkaç bitki türünün, -kuraklığa dayanıklı bitkilerin- trehaloz sentezlediği düşünülüyordu. Birçok bitki türü kolayca ölçülebilen trehaloz miktarını biriktirmiş gözükmemesine rağmen, Arabidopsis ve tahıl bitkilerinde trehaloz biyosentezi genlerinin bulunması trehaloz sentezleyebilmenin bitki aleminde yaygın olduğunu göstermiştir.

Bu çalışmada 3 buğday çeşitinde –2 ekmeklik (*Triticum aestivum L.*) Tosun, Bolal (strese dayanıklı), ve 1 durum (*Triticum durum*) Çakmak (strese dayanıksız)-

trehaloz miktarı analiz edilmiştir. Gaz Kromatografi-Kütle Spektroskopi (GS-MS) analizini kullanılarak, trehaloz 3 farklı buğday çeşitinin (Bolal, Çakmak, ve Tosun) tohumlarında ve fidelerinde belirgin bir şekilde teşhis edilmiştir. Trehaloz miktarı refraktorü indeks detektörüne bağlı HPLC ile ölçülmüştür. Tuz ve kuraklık stresinin buğday çeşitlerinin trehaloz içeriği üzerindeki etkisine fide seviyesinde bakılmıştır ve trehaloz analizi hem gövde hem de kök dokularında yapılmıştır. Trehalozun tuz ve kuraklık stresi koşullarında tüm buğday çeşitlerinde biriktiği bulunmuştur. En yüksek trehaloz birikimi kuraklık stresi koşullunda Bolal çeşitinin köklerinde tespit edilmiştir.

Bunun yanında, kontrol, tuz ve kuraklık stresi koşullarında Bolal ve Çakmak çeşitlerinin kök ve gövdelerinde trehaloz-6-P sentaz ve trehalaz enzim aktivite tayinleri yapılmıştır. Bulduğumuz en ilginç sonuç TPS aktivitesinin stres koşullarında aniden yükselmesidir. En yüksek TPS aktivitesine kuraklık stresi altında köklerde rastlanmıştır ve bu aktivite kontrol koşullarındaki TPS aktivitesinin 3-4 katı kadardır. Stres koşullarında TPS aktivitesinin yükselmesi ile trehaloz birikimi paraleldir. Bolal çeşitinde trehalaz aktivitesi tuz ve kuraklık stresi koşullarında düşmüştür, fakat Çakmak çeşitinde trehalaz aktivitesinde belirgin bir değişiklik olmamıştır.

Anahtar kelimeler: Buğday, trehaloz, trehaloz-6-P sentaz, trehalaz, stres koruyucu, kuraklık, tuz.

To my parents and my wife

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

.

LIST OF FIGURES

FIGURE

LIST OF ABREVIATION

ABREVIATIONS

ABA	Abscisic acid
LEA	Late embryogenesis-abundant
SEM	Standard error of main
EA	Enzyme Activity
SA	Specific Enzyme Activity
EDTA	Ethylenediaminetetra-acetic acid
PMSF	Phenylmethylsulfonyl Fluoride
MES	2-[N-morpholino]ethane sulfonic acid
PVP	Polyvinylpyrrolidone
TPS	Trehalose-6-phosphate synthase
TPP	Trehalose-6-phosphate phosphatase
Tre-6-p	Trehalose-6-phosphate
UDP-glucose	Uridine diphosphate glucose
Mip	Major intrinsic protein
SOD	Superoxide dismutase
PLC	Phospholipase C
ROS	Reactive oxygen species
PDH	Proline dehydrogenase
NR	Nitrate reductase

CHAPTER 1

INTRODUCTION

1.1 Wheat

Wheat is the most important crop grown in the world. It is an excellent feed for livestock; but because of its importance as a human food, only a small part of the total wheat production is used as feed for livestock. Three species of wheat are of commercial importance. These are *Triticum aestivum*, common bread wheat; *Triticum durum*, pasta product wheat; and *Triticum compactum*, pastry flour wheat (Klein and Klein, 1988).

Wheat is the major cereal crop grown in Turkey and its consumption is the highest per person around the world. Around 75% of the total wheat area is located in the Central Plateau and in the transitional regions connecting the Plateu to the coasts (Kün, 1988).

1.1.1 Wheat and Wheat Biotechnology

Wheat is the most widely used cultivated and important food crop in the world. It is the basic crop for about 35% of the human population, accounting for 29% of caloric intake. With global production at more than 584 million metric tones, wheat accounts for the largest share of cereals market (FAO, 1996). The popularity of wheat is based largely on its high nutritive value (>10% protein, 2,4% lipids, and 79% carbohydrates), and the versality of its use in the production of a wide range of food products.

1.1.2 History of Wheat

The foundation of crops first domesticated during the Neolytic age more than 10,000 years ago included primitive forms of wheat. The modern hexaploid bread wheat (*Triticum aestivum* L.) evolved later and became abundant about 8000 years ago. The 25 or so species of the genus *Triticum* are divided into three groups; diploid, tetraploid, and hexaploid, according to their chromosome number. The diploid einkorn wheat *T. monococcum* with the AA genome, has no economic value, and is grown only occasionally as animal feed. The allotetraploid emmer wheat *T. turgidum* var. durum with the AABB genome grows best in warmer climates and is prized for making pasta. The allohexaploid common or bread wheat, *T. aestivum*, is grown in cool climates with moderate rainfall such as North America, Europe China, India and Australia. It has the genome constitution of AABBDD (2n = 6x = 42), formed through hybridization of *T. urartu* (AA) with unknown diploid B genome (possibly *Aegilops speltoides*), and subsequent hybridization with a diploid D genome, *T. tauschii* (Figure 1.8). The AA, BB, and DD genomes of wheat are closely related, and its 21 chromosomes have been classified into seven homologous groups, each composed of three functionaly similar chromosomes. The polyploid nature of the wheat genome makes it very suitable for the incorparation of alien genes. *T. aestivum* and *T. turgidum* var. *durum* account for most of the commercial production and uses of the wheat (Vasil and Vasil, 1999). Among the food crops, wheat is one of the most abundant sources of energy and proteins for the world population. 95% of wheat grown today is the hexaploid type, used for the preparation of bread and other baked products, nearly all of the remaining 5% is durum (tetraploid) wheat.

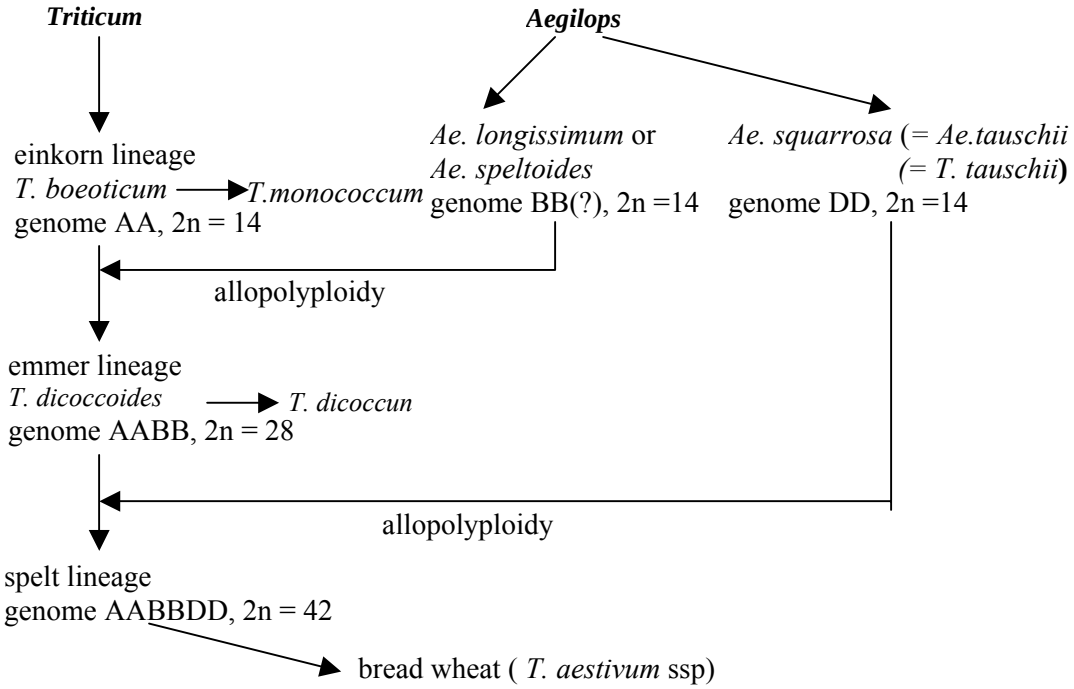

Figure 1.1: Evolution of modern hexaploid wheat (*T. aestivum*)

1.1.3 Wheat Biotechnology

In recent years biotechnology is emerging as one of the latest tools of agricultural research. Together with traditional plant breeding practices, biotechnology is contributing towards the development of novel methods to genetically alter and control plant development, plant performance and plant products. The term biotechnology is composed of two words bio (Greek *bios*, means life) and technology (Greek *technologia,* means systematic treatment). Biotechnology involves the systematic application of biological processes for the beneficial use. One of the areas of plant biotechnology involves the delivery, integration and expression of defined genes into plant cells, which can be grown in artificial culture media to regenerate plants. Thus biotechnological approaches have the potential to complement conventional methods of breeding by reducing the time taken to produce cultivars with improved characteristics. Conventional breeding utilizes domestic crop cultivars and related genera as a source of genes for improvement of existing cultivars, and this process involves the transfer of a set of genes from the donor to the recipient. In contrast, biotechnological approaches can transfer defined genes from any organism, thereby increase the gene pool available for improvement. The improvement of wheat by biotechnological approaches primarily involves introduction of exogenous genes in a heritable manner, and secondarily, the availability of genes that confer positive traits when genetically transferred into wheat (Patnaik and Khurana, 2001)

The genetic improvement of wheat has received considerable attention over the years from plant breeders with the purpose of increasing the grain yield and to minimize crop loss due to unfavourable environmental conditions, and attack by various pests and pathogens. In the early 60's, conventional breeding coupled with improved farm management practices led to a significant increase in world wheat production thereby ushering in the green revolution. Subsequently, the targets of genetic improvement shifted to reducing yield variability caused by various biotic and abiotic stresses and increasing the input-use efficiency (Pingali and Rajaram, 1999). With this change in the global food policy in the last few decades, biotechnology offered a possible solution firstly, by lowering the farm level production costs by making plants resistant to various

abiotic and biotic stresses, and secondly, by enhancing the product quality (*i.e.* by increasing the appearance of end product, nutritional content or processing or storage characteristics). The introduction of foreign genes encoding for useful agronomic traits into commercial cultivars has resulted in saving precious time required for introgression of the desired trait from the wild relatives by conventional practices and alleviating the degradation of the environment due to the use of hazardous biocides. In recent years, wheat improvement efforts have therefore focused on raising the yield potential, quality characteristics, resistance to biotic stresses and tolerance to abiotic stresses depending on the regional requirement of the crop (Patnaik and Khurana, 2001, Vasil, 1994).

1.2 Environmental Stresses

World crop production is limited by environmental stresses. About 20% of the land is affected by mineral stress, 26% by drought stress and 15% by freezing stress (Blum, 1986).

Environmental stresses are of two main types; biotic stresses including infection or competition by other organisms, and abiotic stresses. Six abiotic stresses have long been known to give rise to resistance adaptations.

Another stress type, oxidative stress has been shown to occur in plants exposed to drought, to air pollutants such as ozone and sulphur dioxide, UV light, herbicides and to chilling temperatures, particularly in combination with high light intensities. Reactive oxygen species (ROS) are generated during chemical and environmental stresses, including chilling and freezing, drought, desiccation, flooding, herbicide treatment, pathogen attack and ionising radiation. These ROS attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturation and DNA mutation (Bowler, Van Montagu and Inzé, 1992).

Environmental Stresses

A. Biotic: Infection and/or competition by other organisms

B. Abiotic (Physicochemical stress):

Light: High intensity, low intensity

Temperature: High, low (chilling, freezing)

Water: Deficit (drought), excess (flooding)

Radiation: IR, visible, UV, ionizing (X-ray and γ-ray)

Chemical: Salts, ions, gases, herbicides, heavy metals

Mechanical factors: Wind, pressure

1.3 Responses to Changing Environment

Organisms are continually exposed to environmental stresses that influence their development, growth and productivity. Stress-relieving genes that are transcribed might encode enzymes involved in a particular metabolic pathway, regulatory proteins or proteins with specific protective properties. Stress responses enable the organism to adapt to an unfavourable situation – often by changing the metabolic flow.

With the exception of flooding, the major abiotic stresses all result in water-deficit stress (Fig.1.2). The cell membrane serves as an impermeable barrier to macromolecules and most low molecular mass substances. When the extracellular solute concentrations are altered or extracellular ice forms, there is a flux of water from the cells, causing a decrease in turgor and an increase in concentrations of intracellular solutes, putting a strain on membranes and macromolecules. Minor limitations in water availability cause a reduced photosynthetic rate, but further reductions lead to a complete inhibition of photosynthesis. Under conditions in which photosynthesis is

impaired, but chloroplasts are exposed to excess excitation energy, there is photoreduction of oxygen and concomitant production of reactive oxygen intermediates, such as superoxides and peroxides, which damage membranes and enzymes (Holmberg and Bulow, 1998).

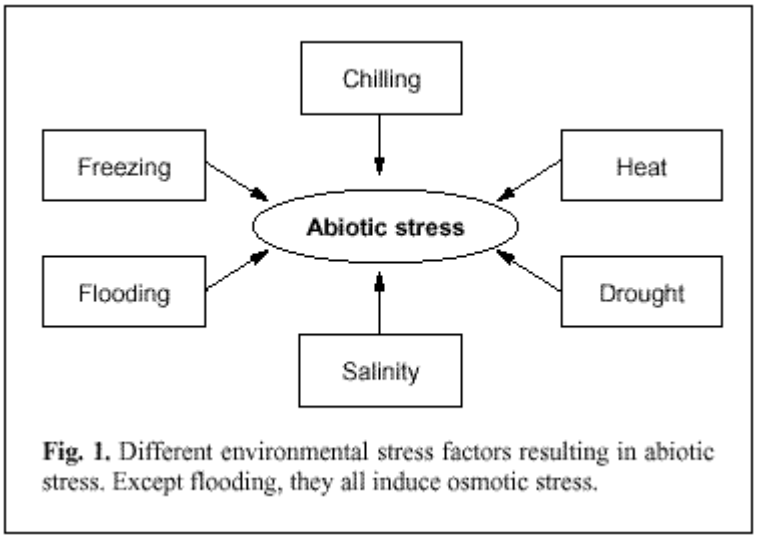

Fig. 1. Different environmental stress factors resulting in abiotic stress. Except flooding, they all induce osmotic stress.

There are several biochemical functions involved in the response of the plant cell to osmotic stress, such as ion exclusion, ion export, cell wall modifications, osmotic adjustments and osmoprotection. Furthermore, plant cells contain antioxidant enzyme systems, such as peroxidases and superoxide dismutases, which scavenge reactive oxygen intermediates (Holmberg and Bulow, 1998).

1.4 Adaptations to Environmental Stresses

Environmental stresses come in many forms; the most prevalent stresses have in common their effect on plant water status. The availability of water for its biological roles as solvent and transport medium, as electron donor in the Hill reaction and as evaporative coolant is often impaired by environmental conditions. Although plant species vary in their sensitivity and response to the decrease in water potential caused by drought, low temperature, or high salinity, it may be assumed that all plants have

encoded capability for stress perception, signaling, and response. First, most cultivated species have wild relatives that exhibit excellent tolerance to abiotic stress. Second, biochemical studies have revealed similarities in processes induced by stress that lead to accumulated metabolites in vascular and nonvascular plants, algea, fungi and bacteria. These metabolites include nitrogen-containing compounds (proline, other amino acids, quaternary amino compounds and polyamines) and hydroxy compounds (sucrose, polyols and oligosaccharides). Accumulation of any single metabolite is not restricted to taxonomic groupings, indicating that these are evolutionary old traits. Third, molecular studies have revealed that a wide variety of species express a common set of gene and similar proteins when stressed. Although functions for many of these genes have not yet been efficiently assigned, it is likely, based on their characteristics, that these proteins play active roles in the response to stress (Bohnert *et al.*, 1995).

Learning about the biochemical and molecular mechanisms by which plants tolerate environmental stress is necessary for genetic engineering approaches to improve crop performance under stress. By investigating plants under stress, we can learn about the plasticity of metabolic pathways and the limits to their functioning. Also questions of an ecological and evolutionary nature need investigation. Are these genes that confer salt tolerance on halophytes and/or drought tolerance on xerophytes evolutionary ancient genes that have been selected against in salt and drought-sensitive plants (glycophytes) for the sake of productivity? Or have some species obtained novel genes in their evolutionary history that have enabled them to occupy stressful environments? How will the introduction of genes conferring stress tolerance into highly productive species affect crop productivity in the field? (Bohnert *et al.*, 1995).

Pathways involving familiar compounds may be exploited to produce stress-tolerant plants. Some of these may not require obtaining genes from tolerant species; overexpressing or altering regulatory features of an endogenous gene or a gene from a different, but stress-intolerant, species might be sufficient. Relevant examples are the engineered overexpression of genes for enzymes that increase putative osmoprotectant compounds such as proline, polyols, or fructans. For example, spinach and other

chenopods, which are only moderately water stress tolerant, accumulate glycine-betaine and related compounds and genes responsible for this accumulation could be utilized for transfer. Finally manipulation of enzymes in the proline metabolic pathway might also be an effective approach to judge from the positive correlation between proline accumulation and water stress tolerance (Bohnert *et al.*, 1995).

1.5 Osmotic Stress and Osmoprotectants

Plants respond to various types of water stress, such as drought, high salinity, and low temperature, by a number of physiological and developmental changes. During water stress, plant cells can undergo changes in concentrations of solutes, in cell volume and in the shape of cell membranes, as well as disruption of gradients in water potential, loss of turgor, disruption of membrane integrity and the denaturation of proteins.

A reduction in the cellular water potential to below the external water potential, resulting from a decrease in osmotic potential allows water to move into the cell. Compatible osmolytes are potent osmoprotectants that play a role in counteracting the effects of osmotic stress. The osmotic potential inside the cell is lowered by the accumulation of compatible solutes in the cytosol. It has been suggested that compatible osmolytes do not interfere with normal biochemical reactions and act as osmoprotectants during osmotic stress (Yoshu *et al*., 1997). These compounds tend to be uncharged at neutral pH, and are highly soluble in water (Ballantyne and Camberlin, 1994). The accumulation of compatible solutes may help to maintain the relatively high water content necessary for growth and cellular function. It might be considered preferable for these natural compounds to have the capacity to form hydrogen-bonds with free water without any interaction with macromolecules, even at low water contents (Rudolph and Crowe, 1985).

Compatible solutes include some amino acids (e.g., proline), sugar alcohols (e.g., pinitol), other sugars (e.g., trehalose) and quaternary ammonium compounds (e.g., glycine betaine), and they can accumulate at high levels without the disruption of protein functions. Accumulation of compatible solutes occurs preferentially in the cytosol under water stress (Matoh *et al*., 1987). Hydrophilic, glycine-rich proteins are the most effective osmoprotectants, they have some characteristics to avoid crystallisation even in high concentration.

Several other kinds of environmental stresses such as drought, metals, cold and freeze also result in the accumulation of organic solutes in many plant species. This suggests small organic solutes have other functions than osmotic adjustment. Numerous studies in the past have shown that small organic solutes are not only non-toxic, but also protect the enzymes at least of the same species *in vitro* against NaCl, heat, dilution, hydroxyl, cold and freeze. In addition, small organic solutes had been demonstrated to stabilise biological membranes. We may conclude that small organic solutes not only act in osmotic adjustment to some extent, but also protect the enzymes and other macromolecules and maintain the membrane integrity against the biologically unfavourable consequences of stress-induced thermodynamic perturbation. Coordination of different osmoprotectants would result in the optimum integration of economization and effectiveness. Two mechanisms are thought to lie behind the activity of these substances:

• The ability to raise the osmotic potential of the cell, thus balancing the osmotic potential of an externally increased osmotic pressure.
• The ability to stabilize membranes and/or macromolecular structures (Holmberg and Bulow, 1998).

1.5.1 Physiological and Biochemical Aspects of Osmotic Stress in Plants

Plant productivity is strongly influenced by dehydration stress induced by high salt, drought, and low temperature, which are generally termed as osmotic stress conditions. Plants respond to these stresses by displaying complex, quantitative traits

that involve the functions of many genes. These responses lead to a wide variety of biochemical and physiological changes such as the accumulation of various organic compounds of low-molecular weight, generally known as compatible solutes or osmolytes, synthesis of late-embryogenesis–abundant (LEA) proteins, and activation of several detoxification enzymes. Although, different plant species have variable thresholds for stress tolerance, and some of them can successfully tolerate severe stresses and still complete their life cycles, most cultivated crop plant species are highly sensitive and either die or suffer from productivity loss after they are exposed to long periods of stress. It has been estimated that two thirds of the yield potential of major crops are lost because of unfavorable environmental conditions. Thus understanding and improvement of the tolerance mechanisms will be an important step for the production of stress tolerant species by genetic engineering approaches (Bajaj *et al.*, 1999).

Under water stress conditions, plant cell lose water and decreases turgor pressure. The plant hormone ABA increases as a result of water stress, and ABA has important roles in the tolerance of plants to drought, high salinity, and cold.

Mesembryanthemum crystallinum (ice plant) is native to Namibian Desert of southern Africa and is adapted to growth in high sodium and under drought and low-temperature conditions. Three mechanisms have been identified to confer stress tolerance in ice plant: induced polyol biosynthesis, regulation of ion uptake and compartmantation, and facilitated water uptake. (Bohnert *et al.*, 1995).

Induced Polyol Synthesis

Accumulation of polyol, either straight-chain metabolites such as mannitol and sorbitol or cyclic polyols such as myo-inositol and its derivatives, is correlated with tolerance to drought and/or salinity, based on polyol distribution in many species, including bacteria, yeast, marine algae, higher plants, and animals. Polyols seem to function in two ways that are difficult to separate mechanistically: osmotic adjustment and osmoprotection. In osmotic adjustment, they act as osmolytes, facilitating the

retention of water in cytoplasm and allowing sodium sequestration to the vacuole or apoplast. Alternatively, protection of cellular structures might be accomplished through interactions of such osmolytes, often termed compatible solutes, with membranes, protein complexes or enzymes (Bohnert *et al.*, 1995).

Regulated Ion Uptake and Compartmentation

A second mechanism that protects the ice plant against water stress is the regulation of ion uptake and compartmentation (Adams *et al.*, 1992). The ice plant takes up sodium when it is available and deposits it in a gradient along its axis, with the highest amounts in the youngest tissues. This gradient parallels the increase in D-pinitol. Particularly high accumulations of sodium and pinitol have been observed in a morphological specialization of the ice plant, the epidermal bladder cells, which are developmentally preformed but increase in size dramatically when plants are salt stressed. The ability of the ice plant to use the sodium as an osmoticum confined to vacuoles in growing parts of the plant (compensated by D-pinitol accumulation in the cytoplasm) is in contrast to glycophytic plants, which attempt to limit sodium uptake or partition sodium into older tissues that serve as storage compartments (Cheeseman, 1988).

Facilitated Water Permeability

A third mechanism for stress protection appears to be regulation of facilitated water permeability. This involves the increased synthesis of aquaporins or water channels, which are the transcript of *mip* (major intrinsic protein) gene whose abundance changes under salt stress (Chrispeels and Agre, 1994). Transcripts of *mip* genes are found predominantly in cells especially involved in water flux, that is the root epidermis, and regions surrounding strands of xylem cells in roots.

1.5.2 Molecular Aspects of Osmotic Stress in Plants

Water deficit elicits complex responses beginning with stress perception, which initiates a signal transduction pathway(s) and is followed by changes at cellular, physiological, and developmental levels. The set of responses observed depends on severity and duration of the stress, plant genotype, developmental stage, and environmental factors providing the stress. In recent years, efforts have turned toward isolation of genes that are induced during water deficit in order to study the function of drought induced gene products and the pathways that lead to gene induction. Changes in gene expression are fundamental to the responses that occur during water deficit, and they control many of the short and long-term responses.

Functions of many gene products have been predicted from the deduced aminoacid sequence of the genes. Genes expressed during stress anticipated to promote cellular tolerance of dehydration through protective functions in the cytoplasm, alteration of cellular water potential to promote water uptake, control of ion accumulation, and further regulation of gene expression. Expression of a gene during stress does not guarantee that a gene product promotes the ability of the plant to survive stress. The expression of genes may result from injury or damage that occurred during stress. Other genes may be induced, but their expression does not alter stress tolerance. Yet others are required for stress tolerance and accumulation of these gene products is an adaptive response (Bray, 1993).

A number of genes that respond to drought, salt and cold have recently been described (Ingram and Bartels, 1996, Shinozaki and Yamaguchi-Shinozaki, 1996, Bray, 1997). The mRNAs of water stress-inducible genes decrease when the plants are released from stress conditions, which show that these genes respond to water stress or dehydration. Expression patterns of dehydration–inducible genes are complex. Some genes respond to water stress very rapidly, whereas others are induced slowly after the accumulation of ABA (abscisic acid). Most of the genes that respond to drought, salt, and cold stress are also induced by exogenous application of ABA (Shinozaki and Yamaguchi-Shinozaki, 1996, Bray, 1997). Dehydration triggers the production of ABA,

which in turn induces various genes. Several genes that are induced by water stress are not responsive to exogenous ABA treatment. This indicates the existence of both ABA-independent and ABA-dependent signal transduction cascades between the initial signal of drought or cold stress and the expression of specific genes (Shinozaki and Yamaguchi-Shinozaki, 1997).

1.5.3 Functions of Osmotic Stress Inducible Genes

A variety of genes have been reported to respond to water stress in various species, and the functions for many of the proteins they encode have been predicted from sequence homology with known proteins. Genes induced during water-stress conditions are thought to function not only in protecting cells from water deficit by production of important metabolic proteins but also in the regulation of genes for signal transduction in the water-stress response (Table 1.1). Thus, gene products are classified into two groups. The first group includes functional proteins that probably for stress tolerance: water channel proteins involved in the movement of water through membranes, the enzymes required for the biosynthesis of various osmoprotectants (sugars, proline, and glycine-betaine), proteins that may protect macromolecules and membranes (LEA proteins, osmotin, antifreeze protein, chaperone, and mRNA binding prteins, proteases for protein turnover (thiol proteases, Clp protease, and ubiquitin), the detoxification enzymes (glutathione-S-transferease soluble epoxide hydrolase, catalase, superoxide dismutase (SOD), and ascorbate peroxidase) and transport proteins (Na^+/H^+ antiporters). The second group contains proteins involved in the regulation of signal transduction and gene expression that probably function in stress response: protein kinases, transcription factors, PLC (phospholipase C), and 14-3-3 proteins, which are signaling molecules (Figure 1.3), (Shinozaki and Yamaguchi-Shinozaki, 1997).

1.5.4 Regulation of Gene Expression by Osmotic Stress

Most water-stress-inducible genes respond to the treatment with exogenous ABA, whereas others do not, which are expressed under drought or cold conditions. Therefore, there are not only ABA dependent pathways but also ABA-independent

pathways involved in the water-stress response. Analysis of the expression of ABA-inducible genes revealed that several genes require protein biosynthesis for their induction by ABA.

In the activation of stress-inducible genes under dehydration conditions, four independent signal pathways are supposed to be functional (Shinozaki and Yamaguchi-Shinozaki, 1996): Two are ABA-dependent (pathways I and II) and two are ABA-independent (pathways III and IV) (Kasuga *et al.*, 1999).

Table 1.1: Proteins expressed under osmotic stress conditions in plants and their suggested functions.

Name	Function
Osmotin	Antifungal
LEA and RAB proteins	Desiccation protection
HS proteins	Heat shock protection
ASI	Amylase/subtilisin inhibitor
WGA	Lectin
MA16	RNA regulation
Oleosins	Oil body stabilization
TSW12	Lipid transfer protein
PEP carboxylasse	CAM metabolism
salT	Na^+ accumulation
7a clone	Ion chanel
Ca^{2+}-ATPase	Ca^{2+}homeostasis
Aldose reductase	Sorbitol synthesis
Methyl transferase	Pinitol
Betaine-aldehyde	Betaine synthesis
Pyrroline-5-carboxylase reductase	Proline synthesis

ABA dependent pathway I requires protein synthesis to activate MYC/MYB and/or bZIP, which bind to DNA regions other than ABREs (ABA-responsive element). ABA-dependent pathway II activates bZIP, a transcription factor that turns on the gene expression through binding to ABREs. ABA-independent pathway IV induces gene expression through activation of DREBP (drought-response-element-binding protein), which binds to the DRE (drought response element) motif and leads to induction of cold–and drought-induced genes. ABA-independent pathway III, is not yet well understood (Bajaj *et al.*, 1999), but there are several drought-inducible genes that do not

respond to either cold or ABA treatment. These genes include rd19 and rd21, which encode a Clp protease regulatory subunit (Figure 1.3), (Shinozaki and Yamaguchi-Shinozaki, 1997).

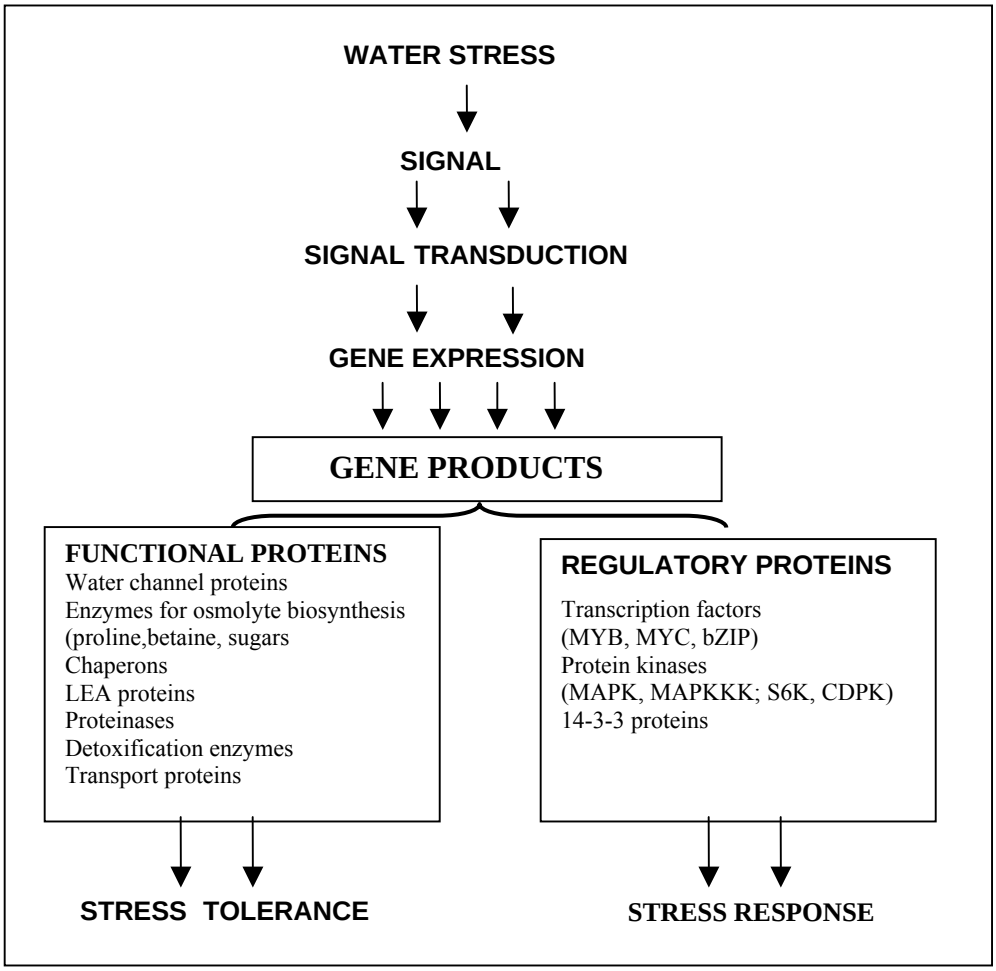

Figure 1.3: Water stress-induced gene products functional in stress tolerance and stress response (Shinozaki and Yamaguchi-Shinozaki, 1997).

1.6 Salt Stress

The most important factors limiting plant productivity are environmental stress, of which salinity and drought are the most serious (Boyer, 1982). Salinity affects more than 40% of irrigated land, especially the most productive areas of the world.

Crop plants are very sensitive to NaCl: the 0.15 M concentration found in animal fluids is very toxic to many crops, such as fruit trees, cereals,, and horticultural plants. Only barley, cotton, and sugarbeet are slightly more tolerant (Downton, 1984).

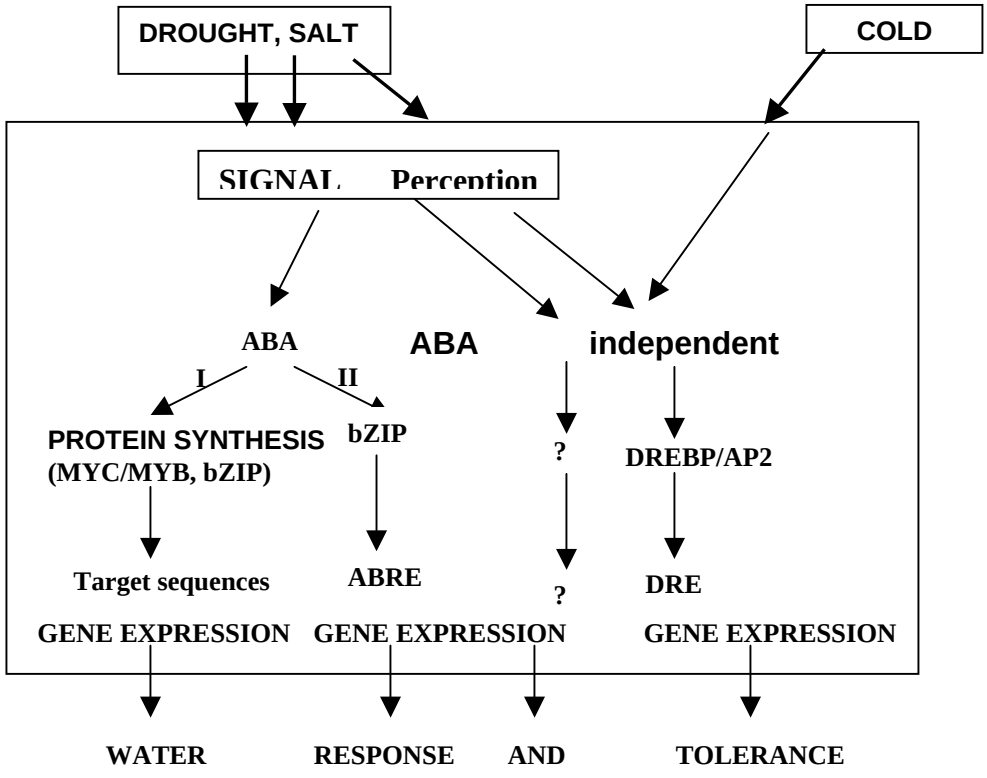

Figure 1.4: Signal transduction pathways in water stress response (Shinozaki and Yamaguchi-Shinozaki, 1997).

The transfer of genes to crop plants that can improve salt tolerance (halotolerance genes) has been carried out by classical breeding with some success. Salt-tolerant relatives can be crossed with crop plants, but then long backcrosses are needed to recover useful agronomical features together with the halotolerance genes.

Genetic engineering may provide a general and rapid method to improve salt tolerance in crop plants. The technologies for the transfer of the desired genes are available even for the more refractory cereals. But, the major problem with this approach is the isolation of the suitable halotolerance genes to be transferred. These genes could be components of the normal adaptation of either crop or halophytic plants to salt stress, and their constitutive overexpression in transgenic plants may improve salt tolerance. On the other hand, halotolerance genes could be isolated from nonplant sources, such as bacteria (Serrano and Gaxiola, 1994).

1.6.1 Adaptations of Plants to Salt Stress

The deleterious effect of salt on plant cells has two components: Osmotic stress and ion toxicity. The osmotic component is not specific for NaCl and results from the dehydration and loss of turgor induced by external solutes.

Turgor is an essential factor for plant cell growth, which is based on cell wall loosening and a turgor-driven increase in volume. Dehydration often leads to irreversible destructive events in proteins and cellular membranes. Ion toxicity results from both the increase in concentration of normal intracellular ions (mostly K^+) during water loss and the uptake of Na^+ and Cl^-. Excess of K^+ caused by cell shrinkage and the uptake of external NaCl result in toxicity to many intracellular enzymes (Serrano and Gaxiola, 1994).

In order to achieve salt tolerance, three interconnected aspects of plant activities are important. First, damage must be prevented or relieved (detoxification). Second, homeostatic conditions must be reestablished in the new, stressful environment. Third, growth must continue, although at a reduced rate (Zhu, 2001).

Detoxification

The nature of the damage that high salt concentrations inflict on plants is not entirely clear. The integrity of cellular membranes, the activities of various enzymes,

nutrient acquisition and function of photosynthetic apparatus are all known to be prone to the toxic effects of high salt stress. An important cause of damage is reactive oxygen species (ROS) generated by salt stress. Plant subjected to salt stress display complex molecular responses including the production of stress proteins and compatible osmolytes. Many of the osmolytes and stress proteins with unknown functions probably detoxify plants by scavenging ROS or prevent them from damaging cellular structures. These organic compounds accumulate at high cytoplasmic concentrations to restore cell volume and turgor. Many of them stabilize compact protein structure by increasing the surface tension of water, as opposed to increasing the interface upon denaturation (Yancey *et al.*, 1982, Serrano and Gaxiola, 1994).

Homeostasis

Another strategy for achieving greater tolerance is to help plants to re-establish homeostasis in stressful environments. Various ion transporters are the terminal determinants of ionic homeostasis. Because Na^+ inhibits many enzymes, it is important to prevent Na^+ accumulation to a high level in cytoplasm and other organelles other than the vacuole. So Na^+ entry should be prevented or reduced (Amtmann and Sanders, 1999). An important goal of salt tolerance studies is to determine which transporters function in Na^+ entry into plant cells and to find a way to block Na^+ influx and thus to achieve increased salt tolerance. Any Na^+ entering into cells can be stored in the vacuole or exported out of the cell. Na^+ compartmentation is an economical means of preventing Na^+ toxicity in the cytosol because the Na^+ can be used as an osmolyte in the vacuole to help to achieve osmotic balance. Many naturally salt tolerant plants (halophytes) rely on this strategy (Zhu, 2001).

Growth regulation

Salt stress like many other abiotic stresses, inhibits plant growth. Slower growth is an adaptive feature for plant survival under stress because it allows plants to rely on multiple resources (*e.g.* building blocks and energy) to fight stress. In nature the extent of salt or drought tolerance often appears to be inversely related to growth rate. One cause of growth rate reduction under stress is inadequate photosynthesis owing to

stomatal closure and subsequently limited carbon uptake (Zhu, 2001). Also there is an important relation between stress and the cell division. For example in *Arabidopsis thaliana*, cyclin dependent protein kinase inhibitor *CDPK1* is activated by ABA, which is accumulating under water and salt stress. These stresses probably also influence cell division through transcriptional and/or posttranscriptional regulation of other components of the cell cycle machinery. Because of the important roles of several hormones in regulating cell elongation, cell expansion is inhibited under stress by reducing the concentrations of the growth promoting hormones such as auxin, cytokinin, and gibberellins (Wang *et al.*, 1998).

1.7 Drought Stress

Drought is the lack of available moisture, which adversely affects crop productivity; it is also the failure of available water for irrigation to raise a crop. Drought has been defined as "the inadequacy of water availability, including precipitation and soil moisture storage capacity, in quantity and distribution during the life cycle of the crop to restrict expression of its full genetic yield potential". The drought resistance is thus defined as "the mechanisms causing minimum loss of yield in a water deficit environment relative to the maximum yield in a water constraint free management of the crop".

Few cultivated plants exhibit true drought tolerance in the sense that they can continue active development under conditions of low water availability. Most plants that withstand water stress do so by avoidance mechanisms. Some plants that are evolved in xerophytic environments where the water stress high, developed structural and functional characteristic that reduce water loss. Plants native to arid regions tend to have root systems that either spreads laterally to take up water from a large area or have roots that grow vertically to tap deep water tables. Also some plants adapt to water stress by dropping their leaves and can regrow a new canopy when adequate water is available. The thick fleshy leaves, and complete absence of leaves are the main adaptation forms in arid environment where water stress is prevalent.

A variety of other water conservation measures have been evolved by plants. In water limited conditions grain and forage grasses, curled leaf formations were observed to prevent transpiration. Also plants that possess crassulacean acid metabolism (CAM) keep their stomata closed during the day, and open them only at night. One of the early responses to water deficit is decreased leaf area due to reduced cell expansion, which is extremely sensitive to water limitation. Also a decrease in photosynthesis rate is the result of drought stress (Blum, 1990).

1.7.1 Genes for Resistance to Drought Stress

Production of transgenic plants carrying certain genes that provide protection under drought conditions are very popular and transgenic plants were produced for improved drought and salt tolerance (Table 1.2) (Khanna-Copra and Sinha, 1998).

Table 1.2: Transgenic plants carrying osmoprotectant compounds against drought and salt stress

Transgenic Overexpressing	Plant	Claim
Mannitol	Tobacco	Tolerance to salinity
Glycine-betaine	Tobacco chloroplast	Marker for osmoprotectant
Fructant	Tobacco	Resistance to drought stress
Proline	Tobacco	Tolerance to osmotic stress
LEA	Rice	Tolerance to water deficit and salinity

Proline, a compatible solute that produce protection against denaturation of enzymes caused by high temperature. The genes related to proline synthesis, proline transport and accumulation are; Δ^1-Pyrolline-5 carboxylate Synthatase (*P5CS*), and proline dehydrogenase (*PDH*) genes. They are involved in synthesis and degradation of proline. Salt stress and dehydration induce the expression of the gene for *P5CS* and down regulates PDH.

Fructans are polyfructose molecules that are produced in only 155 flowering plant species, including wheat and barley. It functions mainly as a storage carbohydrate

but being soluble may help plants survive periods of osmotic stress induced by drought or cold, by variying the degree of polymerization of the fructan pool.

Osmotic stress induces the accumulation of a set of low-molecular weight proteins known as dress proteins in plant tissues such as LEAs and dehydrins. LEA proteins first characterized in cotton as a set of proteins that are highly accumulated in the embryos at the late stage of seed developments (Baker *et al.*, 1988, Khanna-Copra and Sinha, 1998).

Transgenic tobacco plants were synthesized and accumulated the sugar alcohol mannitol by introducing a bacterial gene *mtL* that encodes mannitol 1-phosphate dehydrogenase. The mannitol overproducing tobacco exhibited an increased ability to tolerate high salinity in terms of maintenance of leaf and root growth. (Tarczynski *et al.*, 1993).

1.8 Osmoprotectants: Structural and Functional Features

Under water deficit, many organisms accumulate intracellular low molecular weight compounds to levels sufficient to maintain equal water potential with the external conditions. These compounds may contribute towards osmotic adjustment, besides providing protection to macromolecules such as enzymes and proteins electrolytes and temperature. Plant cells generally accumulate the inorganic ions, which are most commonly present in the environment, but these become detrimental to cellular biochemistry at high concentrations and must be sequestered in the vacuole. To keep the cytoplasm osmotically balanced, the organism usually accumulates specific types of organic molecules, termed compatible solutes. They serve the primary function of maintaining osmotic balance and can accumulate to high concentrations without impairment of normal physiological function (Bartels and Nelson, 1994).

Table 1.3: The complexity of stress adaptation: Major targets for the engineered stress tolerance (Cushman and Bohnert, 2000).

Class of target	Examples	Possible mode(s) of action
Osmoprotectants	Aminoacids (proline,ectoine) Dimethyl sulfonium compouunds (glycine-betaine, DMSP) Polyols (mannitol, d-ononitol, sorbitol) Sugars (sucrose, trehalose, fructan)	Osmotic adjustment; protein/membrane; reactive (OH) scavenging
Reactive oxygen Scavengers	Enzymatic (catalase, Fe/Mn superoxide dismutase, ascorbate peroxidase, glutathione cycle enzymes, glutathione S-transferase, glutathione peroxidase, gamma-glutamylcycteine synthase, alternative oxidase)	Detoxification of reactive oxygen species
Stress proteins	Late embryogenesis abundant proteins (LEA)	Unknown, protein stabilization, water binding/slow desiccation rates, chaperones, protein/ membrane stabilization, ion sequestration
Heat shock proteins	Various heat-, cold-, salt-shock proteins in several subcellular compartments	Reversal/preventation of protein unfolding, translational modulation
Ion/proton transporters	High-affinity K^+channels, plasma membrane, pre-vacuolar, vacuolar and organellar proton ATPases and ion transporters (H^+/ATPase, Na^+/H^+ antiporters)	K^+/Na+ uptake and transport, establishment of proton gradients, removal and sequestration of (toxic) ions from the cytoplasm and organelles.
Membrane fluidity	Fatty acid desaturases	Increased amounts of dieoic and fluidity, chilling tolerance.
Water status	Aqaporins or water channels (solute facilitators, urea, glycerol, CO_2, possibly others and including ions	Regulation of AQP amount differentially in tonoplast and plasma membrane, regulation of membrane location, stomatal behaviour
Signaling components	Homologs of histidine kinases (AtRR1/2), MAP kinases (PsMAPK, HOG), Ca^{2+}-dependent protein kinases, SNF1/kinases, protein phosphatases (ABI1/2), CNA/b signaling systems, Ca^{2+}-sensors (SOS3), inositol kinases	Ca^{2+}-sensors/phosphorylation mediated signal transduction
Control of transcription	Transcription factors, ERBP/AP2 (DREB, CBF), Zinc finger TF (Alfin 1), Myb 8AtMyb2, CpMyb10)	Upregulation/activation of transcription
Growth regulators	Altered biosynthetic pathways or conjugate levels for abscisic acid, cytokinins and/or brassinosteroids	Changes in hormone homeostasis

ABI, abscisic acid insensitive; AP2, APETELA2; AQP, aquaporin; AMPK1, AMP-activated protein kinase; Atmyb, *Arabidopsis thaliana* myeloblastosis (helix-loop-helix) transcription factor; AtRR1, *A.* thaliana two-component response regulators; CBF, C-repeat/DRE binding factor; CNA/B, calcineurin A/B; cpMyb, *C. plantagineum* myeblastosis (helix-loop-helix) transcription factor; DMSP, dimethylsulfoniopropionate; DREB, dehydration-responsive element (DRE) binding protein; HOG, highosmolarity glycerol; PaMAPK, *Pisum sativum* mitogen activated protein kinase; SNF1, sucrose non-fermenting 1; TF, transcription factor.

Characteristically, these types of molecules are not highly charged, but are polar, highly soluble, and have a larger hydration layer (the number of water molecules surrounding and solvating each molecule) than denaturing molecules like urea or inorganic ions like KCl. Compatible solutes are strong water structure formers (Galinski, 1993) and such molecules will be preferentially solubilized in the bulk water of the cell rather than in the hydration shell of proteins where they could interact with the macromolecule; they may then interact with contact with small, highly charged molecules, which preferentially solubilize in the water of the hydration sphere where they may interact electrostatically with the macromolecule, causing damaging effects at high concentration (Arakawa and Timasheff, 1985, Wiggins 1990, Galinski, 1993).

Above some osmoprotectant compounds have been summarized with their predicted functions in the protection mechanisms of plants under water deficit (Table 1.3).

1.8.1 Mannitol

Mannitol is functional as a sugar alcohol. Sugar alcohols may contribute to tolerance at the cellular level by adjustment of the cytosolic osmotic potential when the concentration of electrolytes is lower in the cytosol than in the vacuole. These compounds may also protect membranes and proteins in the presence of high concentrations of electrolytes (Khanna-Chopra and Sinha, 1998, Shen *et al.*, 1997). *mtlD* gene encoding mannitol-1 phosphate dehydrogenase, isolated from *E.coli* and transferred to tobacco plant. These plants showed increased tolerance to high salinity relative to control plants (Tarczynski *et al.*, 1993). Also *Arabidopsis* plants transformed with the same gene and transgenic seeds accumulating mannitol germinated in the presence of high salt (Thomas *et al.*, 1995).

1.8.2 Proline

Proline, being an amino acid, is an effective molecule that accumulates in many organisms from bacteria to plants. Its concentration reaches to high levels under water

deficit, high temperature, freezing, heavy metals and high environmental salinity (Delauney and Verma 1993, Yancey *et al.*, 1982). Proline is a highly water soluble aminoacid and is accumulated in leaves of many halophytic higher plants grown in saline environments (Stewart and Lee, 1974, Briens and Larher, 1982). Proline protects membranes and proteins against the adverse effects of high concentration of inorganic ions and temperature extremes. It's also functional as a protein-compatible hydrotope, and as a hydroxyradical scavenger (Smirnoff and Cumbes, 1989).

In bacteria, exogenously supplied proline works as an osmoprotectant under highly saline culture conditions (Csonka, 1989, Csonka and Hanson 1991). Accumulation of proline is facilitated when the concentrations of less compatible solutes are reduced and cytosolic water volume is increased (Cayley et al, 1992).

1.8.3 Glycine-betaine

Glycine-betaine is accumulated in the cells of a number of halophytes and bacteria as an adaptive response to saline or water stress conditions (Bajaj *et al.*, 1999, Robinson and Jones, 1986). Glycine-betaine is synthesized from choline in two steps, first being converted by choline monooxygenase to betaine aldehyde and then further oxidized by betaine aldehyde dehydrogenase. Salinity induces both enzyme activities (Weretilnyk and Hanson, 1990), suggesting that the pathway is coordinately regulated.

Lilius *et al.*(1996) introduced the *E. coli betA* gene encoding choline dehydrogenase into tobacco. The transgenic plants were more tolerant to salt as measured by dry weight between transgenic and wild-type plants at 300 mM NaCl. Also the gene *codA* from *Arthrobacter globiformis,* which converts choline to glycine-betaine, was introduced to *Arabidopsis.* Resultant plants accumulated glycine-betaine and showed enhanced tolerance to salt and cold stress (Hayashi *et al.*, 1997, 1998)

1.8.4 Ononitol/pinitol

These are cyclic sugar alcohols and are stored in a variety of species which are constantly exposed to saline conditions, and accumulate in tolerant species when exposed to saline environments (Paul and Cockburn,1989). *Mesembryanthemum crystallinum*, a facultative halophyte, accumulates these compounds only when stressed and the proposed synthetic pathway consists of methylation of myo-inositol to the intermediate ononitol, followed by epimerization to pinitol. An inositol methyltransferase cDNA was prepared from this halophyte and cloned under the constitutive promoter and the activity of the gene were observed in transformed tobacco plants. The overexpressed compounds were detected by HPLC and NMR spectroscopy (Vernon *et al.*, 1993).

1.8.5 Polyamins

Polyamines are small, ubiquitous, nitrogenous cellular compounds that have been implicated in a variety of stress responses in plants. Polyamines accumulate under several abiotic stress conditions including salt and drought. Cultivars demonstrating a higher degree of salt tolerance contained higher levels of polyamines (Galtson *et al.*, 1997). Also exogenous application of polyamines gave protection to oat leaves under osmotic stress (Besford *et al.*, 1993).

1.8.6 Late Embryogenesis-abundant (LEA) Proteins

LEA proteins are the low molecular weight proteins known as dress proteins. They are highly accumulated in the embryos at late stage of seed development. LEA proteins are classified into three major groups based on their common aminoacid sequence domains (Baker *et al.*, 1988). The group 3 LEA proteins are functional in stress tolerance based on the correlation of LEA proteins and tissue dehydration tolerance in dehydrated wheat seedlings (Ried and Walker-Simmons, 1993). In rice seedling, the levels of group 2 LEA proteins and group 3 LEA porteins were higher in roots and induced by ABA and salt in salt-tolerant varieties compared to salt-sensitive varieties. The function of HVAI protein, which is a group 3 LEA protein, was

investigated in rice by introducing *HVAI* gene from barley aleurone and embryo during late seed development. Resultant transgenic rice plant exhibited high levels of HVAI protein in leaf and root (Hong *et al.*, 1988).

The protective function of compatible solutes under water stress have been shown in many publications and engineering of sensitive plants with increased osmolyte content is a promising strategy for protecting plants against dehydration stress. Transgenic plants carrying genes encoding enzymes involved in the production of mannitol, proline, fructans, trehalose and glycine-betaine, show marginal to significant reduction of dehydration stress as shown inTable 1.4 (Bajaj *et al.*, 1999).

1.8.7 Trehalose

Trehalose being a non-reducing disaccharide of glucose is found in bacteria, fungi, and some plant species. Plants that produce trehalose are highly tolerant to desiccation stress. The yeast trehalose 6-phosphate synthase gene (*TPS1*) was introduced to tobacco and trehalose-accumulating plants exhibited multiple phenotypic alterations and improved drought tolerance (Romero *et al.*, 1997). Also bacterial trehalose 6-phosphate synthase (*otsA*) and trehalose 6-phosphate phosphatase (*otsB*) were introduced to tobacco by Pilon-Smits *et al.* (1998). The leaves of transgenic plants were larger and showed better growth in terms of dry weight under drought stress.

1.8.7.1 Trehalose Biosynthesis and Stress Protection

Trehalose is a soluble, non-reducing disaccharide of glucose. Three isomers exist: α,α–trehalose, α,β–trehalose and β,β-trehalose. Of these, only α,α-trehalose (1-O-(α-D-glucopyranosyl)-α-D-glucopyranoside) (Fig.1.5) is found in biological material. Trehalose is a relatively small molecule that is osmotically active. It does not pass freely across biological membranes. Trehalose is synthesized in cells from metabolites of glucose. It thus can function in cells as a less chemically reactive store of the reactive compound glucose. Trehalose has been shown to act as protectant in response to different stress conditions in a large number of microorganisms. This is achieved by two

major mechanisms, the protection of membranes and the protection of proteins. An important property of trehalose is that it associates with biological membranes via hydrogen bonds formed between the hydroxyl groups of the sugar and the phosphate of head groups of the membrane phospholipids. By means of this association trehalose effectively protects biological membranes during desiccation and freezing by replacing the water that normally associates with the bilayer. This process maintains the fluidity of membranes by keeping the bilayers in the liquid crystalline state, thus preventing the transition to the gel phase, with the consequent loss of membrane structural and functional integrity, that

Table 1.4: Stress responses of transgenic plants overexpressing various genes involved in stress tolerance (Bajaj *et al.*, 1999).

Gene	Gene product	Performance of transgenic plants
Genes encoding enzymes that synthesize osmoprotectants		
BetA	choline dehydogenase (glycine-betaine synthesis)	Increased tolerance to salt
CodA	choline oxidase (glycine-betaine synthesis)	Salt tolerance in seedlings and increased germination under cold
IMTI	Myo-inosito lO-methyltransferase (D-ononitol synthesis	High performance under salt and drought, high photosynthesis rate
MtID	Mannitol-1phospate dehydrogenase Mannitol synthesis)	Better growth under high salinity
otsA	Trehalose-6 phospahate synthase	Increased dry weight and more
otsB	Trehalose-6-phosphate phosphatase (Trehalose synthesis)	efficient photosynthesis under drought stress
p5cs	Δ^1-Pyrolline-5 carboxylate synthatase (Proline synthesis	Enhanced growth under salt stress
SacB	Fructosyl transferase (Fructan synthesis)	Better growth under osmotic stress
TPS1	Trehalose 6-phosphate synthase (trehalose synthesis)	Increased drought tolerance
Adc	Arginine decarboxylase (Putracine synthesis)	Minimized chlorophyll loss under drought stress
Odc	ornithine decarboxylase (Putracine synthesis)	Tolerance to high salt stress
LEA or LEA related genes		
HVA	group 3 LEA proteins	Increased tolerance to water deficit and salt stress
COR15a	cold-induced gene	Increased freezing tolerance
Regulatory genes		
CBF1	Transcription factor	Increased cold tolerance
DREB1A	Transcription factor	Increased salt, drought, cold tolerance
Oxidative-stress related genes		
Nt107	Glutathione s-transferase (to reduce free radicals)	Enhanced growth under salt and cold stress
Sod	Cu/Zn superoxide dismutase	Protection under chilling and high-light stress
	Fe superoxide dismutase	Protect plants against ozone damage
	Fe superoxide dismutase	No good response to salt stress
	Mn superoxide dismutase	Reduced cellular damage under under oxidative stress
	Mn superoxide dismutase (to reduce free radical)	Increased tolerance to freezing and water deficit
msFer	Ferritin (Iron storage)	Increased tolerance to oxidative damage induced by iron excess or paraquat treatment

would otherwise occur when water is removed. Trehalose is more efficient at protecting dry membranes than other disaccharides; this may in part be due to the fact that it does not readily crystallize but vitrifies instead. Trehalose is one of the most effective molecules to prevent fusion between dehydrated membrane vesicles. Trehalose has also been assigned a role in prevention of oxidative damage to membranes.

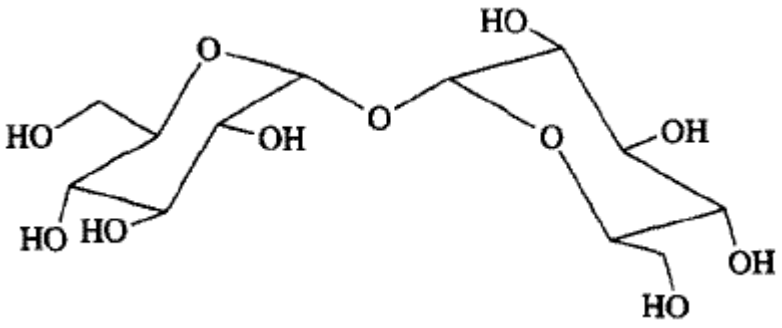

Fig.1.5 The chemical structure of α,α-trehalose (1-O-(α-D-glucopyranosyl)-α-D-glucopyranoside).

Proteins can also be protected by physiological concentrations of trehalose during desiccation, heat-shock or freezing by several mechanisms. First, trehalose replaces accessible "bound" water, probably by hydrogen bonding between hydroxyl groups of the sugar and polar groups of the protein. Second, non-reducing sugars such as trehalose do not participate in the "browning" or Maillard reaction that reducing sugar such as glucose undergo with free amino groups of proteins when the protein solutions are hydrated; indeed, trehalose can actually inhibit this "browning" reaction (Behm, 1997). Furthermore, trehalose remains stable at elevated temperatures and at low pH. These protective properties of trehalose are clearly superior to those of other sugars, such as sucrose, making trehalose an ideal stress protectant (Wingler, 2002).

In yeast, for example, adverse conditions, such as heat, cold or water stress correlate with the accumulation of high concentrations of this non-reducing

disaccharide. In plants a clear role of trehalose in stress tolerance, in particular drought, has been demonstrated for cryptobiotic species, such as the desiccation-tolerant *S. lepidophyll*a. During its dehydration, trehalose accumulates to a level of 12% of the plant dry weight, and acts to protect proteins and membrane structures. Upon rehydration, *S. lepidophylla* regains complete viability and trehalose levels decline.

In higher vascular plants, accumulation of trehalose under adverse conditions is rare **(**Müller, J. et al., 1995a). It has been suggested that in most plant species sucrose has taken over the role of trehalose as a preservative during desiccation. However, in a few desiccation-tolerant angiosperms trehalose is present in relatively large amounts. For example, the resurrection plant *M. flabellifolius* accumulates trehalose up to 3% of its dry weight, although this level is only slightly increased upon drought stress. Whereas sucrose increases from 3 to almost 6% of the dry weight. The combined accumulation of sucrose and trehalose might be sufficient to protect the plant against the adverse effects caused by desiccation (Goddijn and Dun, 1999).

The observation that trehalose can be used to preserve biological structures has been obtained from *in vitro* studies. Trehalose can stabilize dehydrated biological structures, such as lipid membranes or enzymes, more effectively than other sugars (Paiva and Panek, 1996). Because of these specific properties, trehalose has been selected as a target molecule for genetic engineering of plants, both for cost-effective large-scale production of this compound and for engineering drought-tolerance in crops (Serrano et al., 1999). In tobacco, introduction of TPS1 gene, encoding trehalose-6-phosphate synthase from yeast (Romero et al., 1997). The transgenic tobacco plants were assessed for drought tolerance and, although the trehalose concentration was <5 mM in the cytosol, both improved water retention and desiccation tolerance were demonstrated. Again, these results cannot be explained by osmotic adjustments facilitated by trehalose, and appear to be caused by the osmoprotective properties of trehalose itself (Holmberg and Bulow, 1998).

A recent study with rice plants has shown that trehalose may indeed promote resistance to salt stress (Garcia et al., 1997). The results of this study indicate that during osmotic stress trehalose might be more important for rice than proline.

Recently, a cotton EST clone with homology to the *Arabidopsis* gene that encodes TPS has been found to be upregulated under conditions of water stress, indicating that trehalose biosynthesis is specifically induced under these conditions. Although the significance of this finding remains to be elucidated, it contributes towards other circumstancial evidence that trehalose metabolism in higher plants does play a role in the acquisition of stress tolerance (Goddijn and Dun, 1999).

Although the Arabidopsis TPP and TPS genes have been demonstrated to be expressed in all tested organs (Blazquez et al., 1998; Vogel et al., 1998, 2001; Eastmond et al., 2002), trehalose contents in Arabidopsis are close to the detection limit (<1 mg g^{-1} DW; Müller et al., 2001). This apparent lack of trehalose accumulation is probably due to the activity of an Arabidopsis trehalase. After inhibition of trehalase activity by addition of the trehalase inhibitor validamycin A to the growth medium, the content of trehalose in sterilely grown Arabidopsis plants did indeed increase to easily detectable amounts (to about a sixth of the sucrose content; Vogel et al., 2001). The identity of trehalose in these Arabidopsis plants was confirmed by GC–MS analysis. Metabolic profiling using GC–MS analysis has also led to the identification of trehalose in potato (Roessner et al., 2000). These findings suggest that the ability to synthesise trehalose is a common phenomenon in higher plants (Wingler, 2002).

1.8.7.2. Trehalose Metabolism in Plants

In spite of the fact that its biosynthesis is similar to that of sucrose, its evolutionary origin is probably more ancient because it is present in all kingdoms. The absence of reducing ends renders trehalose highly resistant to heat, pH and Maillard's reaction (a reaction between carbohydrates and amino acids that results in discolouration during the processing of potatoes). Moreover, trehalose has a strong stabilizing effect on

biological structures, forming a glass-like structure after dehydration. Because of these characteristics, trehalose is predicted to become a useful stabilizer in foods and an additive in cosmetics and pharmaceuticals (Paiva and Panek, 1996).

The biosynthesis and degradation of trehalose and trehalose-6-phosphate are in many ways similar to that of sucrose (Fig. 1.6). The building blocks of trehalose are UDP-glucose and glucose-6-phosphate, which are linked by the enzyme trehalose-6-phosphate synthase (TPS). Subsequently, the resulting molecule trehalose-6-phosphate (t6p) is dephosphorylated into trehalose by the enzyme trehalose-6-phosphate phosphatase (TPP), although unspecific phosphatases are also able to dephosphorylate t6p. In *E. col*i, the two enzymatic activities involved in trehalose biosynthesis are encoded by OtsA (TPS activity) and OtsB (TPP activity). This is in contrast to the situation in the yeast *Saccharomyces cerevisia*e, where a trehalose synthase complex is involved in the formation of trehalose. In addition to a TPS *(TPS*1) and a TPP *(TPS*2) protein, this complex contains a regulatory subunit encoded by *TSL1* that has a homologue named *TPS3* (Goddijn and Dun, 1999).

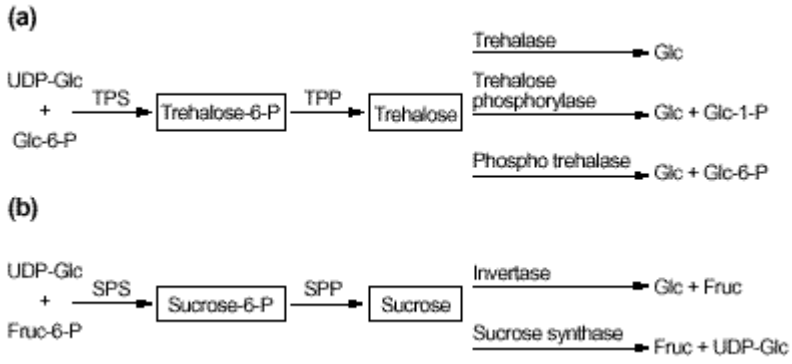

Fig.1.6 A comparison between the enzymatic reactions involved in the biosynthesis and the degradation of (a) trehalose and (b) sucrose. Trehalose is formed by the action of trehalose-6-phosphate synthase (TPS) followed by a trehalose-6-phosphate phosphatase (TPP). The catabolism of trehalose can occur in a variety of ways: in *Euglena gracilis* and *Pichia fermentans* by trehalose phosphorylase; in *E. coli* by phosphorylation and subsequent hydrolysis by trehalose-6-phosphate hydrolase; and in plants, fungi, animals and bacteria via the enzyme trehalase. Abbreviations: SPS, sucrose-6-phosphate synthase; SPP, sucrose-6-phosphate phosphatase.

1.8.7.3 The Role of Trehalose Biosynthesis in the Regulation of Carbon Metabolism

It is unlikely that trehalose contents in plants—other than resurrection plants—are high enough to be directly involved in stress protection. The observation that most of the trehalose formed in Arabidopsis is simultaneously being degraded by trehalase raises the questions of the function of trehalose biosynthesis. In yeast, the trehalose biosynthetic pathway plays an important role in the regulation of carbon metabolism: The precursor of trehalose, trehalose-6-phosphate (T6P), prevents an uncontrolled influx of glucose into glycolysis (Thevelein and Hohmann, 1995). This effect can, at least in part, be explained by an inhibition of hexokinase activity by T6P (Blazquez et al., 1993). Since hexokinase acts as a sugar sensor in yeast and probably also plants, it was suggested that T6P may be involved in the regulation of plant metabolism (Goddijn and Smeekens, 1998). This view was recently supported in a study by Eastmond et al. (2002) who reported that an Arabidopsis mutant with an insertion in the TPS1 gene is impaired in embryo maturation in the phase of storage reserve accumulation (Table 1.5). In contrast to yeast, T6P does, however, not inhibit the activity of hexokinase (Eastmond et al., 2002). The synthesis of T6P may also play a role in the regulation of photosynthetic carbon metabolism: Transgenic tobacco plants expressing the *Escherichia coli* TPS gene exhibit enhanced rates of photosynthesis per unit leaf area, whereas photosynthesis is reduced in plants expressing the E. coli TPP gene (Paul et al., 2001). Furthermore, transgenic plants expressing the E. coli or yeast TPS genes show a variety of other phenotypic effects, including stunted growth and an inhibition of leaf expansion (Goddijn et al., 1997), suggesting additional developmental functions of T6P formation.

While the precise mechanisms of T6P action remain largely unresolved, possible targets of trehalose itself in the regulation of carbon metabolism have been identified (Table 1.5). Similar to sucrose, trehalose induces enzymes involved in the accumulation of storage carbohydrates in photosynthetic tissues. In barley, externally supplied trehalose induces the activity of sucrose: fructan-6-fructosyltransferase, an enzyme of fructan biosynthesis (Müller et al., 2000). In Arabidopsis, trehalose strongly induces the

Table 1.5 Evidence for a role of trehalose biosynthesis in the regulation of carbon metabolism.

Active molecule	Regulated process/pathway	Method	Reference
Trehalose-6-P	Sugar accumulation during drought stress	Transgenic tobacco plants expressing the E. coli TPS gene	Pilon-Smits et al. (1998)
Trehalose-6-P	Photosynthetic capacity	Transgenic tobacco plants expressing the E. coli TPS or TPP genes	Paul et al. (2001)
Trehalose-6-P	Embryo maturation	Arabidopsis mutant with disruption in TPS1	Eastmond et al. (2002)
Trehalose	Fructan biosynthesis in leaves	Feeding of trehalose to barley leaves	Müller et al. (2000)
Trehalose	Carbohydrate contents in roots and nodules	Treatment of soybean plants with the trehalase inhibitor validamycin A	Müller et al. (1995b)
Trehalose	Sucrose metabolism in roots	Feeding of trehalose to soybean plants	Müller et al. (1998)
Trehalose	Starch biosynthesis in cotyledons and leaves	Feeding of trehalose to Arabidopsis seedlings	Wingler et al. (2000)
Trehalose	Starch biosynthesis in cotyledons and leaves	Complementation of starch biosynthetic mutants by trehalose feeding	Fritzius et al. (2001)

expression of ApL3, a gene encoding a large subunit of ADP-glucose pyrophosphorylase, which is an important enzyme in starch biosynthesis. This induction of ApL3 expression leads to increased ADP-glucose pyrophosphorylase activity, an over accumulation of starch in the shoots and decreased root growth (Wingler et al., 2000; Fritzius et al., 2001).

So far, it is not clear to what extent endogenously formed trehalose is involved in the regulation of metabolism. It is possible that trehalase activity normally keeps cellular trehalose concentrations low in order to prevent detrimental effects of trehalose accumulation on the regulation of carbon metabolism. Such a role of trehalase may be of particular importance in interactions of plants with trehalose-producing microorganisms. In support of this hypothesis, expression of the Arabidopsis trehalase gene and trehalase activity were found to be strongly induced by infection of Arabidopsis plants with the trehalose-producing pathogen *Plasmodiophora brassicae* (Wingler, 2002).

1.8.7.4. Enzymes and Genes Taking Role in Trehalose Metabolism

A suitable way for the identification of functional TPS and TPP homologous genes from plants turns out to be the expression of cDNA libraries in corresponding yeast mutants followed by screens for complementing mutants. These screens for TPP and TPS homologs are straightforward since *S. cerevisiae* Tps mutants cannot grow on glucose and since Tpp mutants are thermosensitive (Blazques et al., 1993; Thevelein and Hohmann, 1995). In that way, an *Arabidopsis* TPS (AtTPS 1) has been identified by complementation of a TPS-deficient yeast mutant restoring growth on glucose. Trehalose-6-phosphate synthase isologs have been found in the expressed sequence tag (EST) libraries of *Arabidopsis thaliana*, *Oryza sativa* and *Gossypium hirsutum* and through the *Arabidopsis* genome sequencing effort (Table 1.6). In addition, isologs of TPS, or at least cDNA fragments of genes exhibiting homologies to TPS, are found in tobacco and *Myrothamnus flabellifolia*. In *Selaginella lepidophylla* a complete cDNA of a TPS isolog is known (Table 1.6). This gene shows also homologies to TPP. Since many other plant TPS isologs show these homologies to TPP, it is possible that they

encode a bipartite enzyme activity. Functional TPP activities have been identified in *Arabidopsis* by complementation of TPP-deficient yeast mutants with expressed cDNAs of *Arabidopsis*. Two independent TPP clones were identified that showed TPP activity also in in vitro enzyme assays when expressed as transgenes in yeast. The picture that emerges is that higher plants express several TPS and TPP genes that probably belong to gene families (Table 1.6) (Müller, J. et al., 1999).

Trehalase activities have been identified in many plant tissues including pollen (Müller et al., 1995a). A trehalase strongly stimulated in soybean nodules has been purified and characterized (Müller et al., 1992; and Aeschbacher et al., 1999). This trehalase has a predicted molecular weight of 56 kDa and is a glycoprotein. Like the enzyme found in Lilium pollen, soybean trehalase has broad pH- and high temperature-optima. A trehalase with similar characteristics was also found in sterile soybean cell and tissue cultures. Its activity is stimulated in sterile roots upon treatment with auxins (Müller et al., 1995b). Using degenerate primers from microsequenced peptides of the purified soybean trehalase, a complete cDNA has been isolated by ReverseTranscriptase-PCR. The gene encoding this trehalase, GMTRE1, is expressed at a low but constitutive level. Interestingly, GMTRE1 appears to be a single gene (Aeschbacher et al., 1999). A cDNA fragment showing 95% homology to GMTRE1 at the amino acid level has been identified in *Medicago truncatul*a. This cDNA is, therefore, probably derived from the GMTRE1 homolog of *Medicago truncatula*. In potato, a sequence strongly resembling trehalases has been patented and recently published in the database (Table1.6). An trehalase isolog, T19F06.15, has been identified through the *Arabidopsis* genome sequencing effort. This gene exhibits strong homologies to GMTRE 1, although the overall homology at the amino acid level is only 59%. However, the homologous regions extend along the entire length of the protein and occur at positions conserved among many known trehalases from other organisms. Thus,

Table 1.6 Overview of plant genes of trehalose biosynthesis and degradation identified in microorganisms[a] (Müller et al., 1999).

AC #	Homology	Organism	Gene name	Identity	Predicted polypeptide size	Expression	Activity
A52426	Trehalase	*Solanum tuberosum*	Unnamed	Complete mRNA	581aa	Unpublished	?
AC002343	Trehalase	*Arabidopsis thaliana*	*T19F06.15*	Complete gene	557aa	this work	?
AF124148	Trehalase	*Glycine max*	*GMTRE1*	2123 bp complete mRNA	557aa	Constitutive at low level	Trehalase
Z97344	TPS	*Arabidopsis thaliana*	Unnamed	Complete gene	865aa	?	?
AC003671	TPS	*Arabidopsis thaliana*	*F1707.18*	Complete gene	826aa	?	?
AC004473	TPS	*Arabidopsis thaliana*	*T13D8.4*	Complete gene	861aa	?	?
Y08568	TPS	*Arabidopsis thaliana*	*AtTPS1*	2970 bp complete mRNA	924aa	Constitutive at low level	Complements yeast *Tps2*
U96736	TPS	*Selaginella lepidophylla*	Unnamed	3223 bp complete mRNA	994aa	?	?
AF056946	TPS	*Gossypium hirsutum*	Unnamed	323 bp cDNA fragment	–	Upregulated during water deficit?	?
D40048	TPS	*Oryza sativa*	Unnamed	Expressed sequence tag	–	?	?
AF007779	TPP	*Arabidopsis thaliana*	*AtTPPB*	1436 bp complete mRNA	374aa	Induced in flowers	TPP
AF007778	TPP	*Arabidopsis thaliana*	*AtTPPA*	1739 bp complete mRNA	385aa	Induced in flowers	TPP
AL022605	TPP-like	*Arabidopsis thaliana*	*T19P19.160*	Complete gene	267aa	?	?
AL033545	TPP-like	*Arabidopsis thaliana*	*F7K2_170*	Complete gene	377aa	?	?

[a] References can be obtained under the GenBank accession numbers (AC #).

it is likely that this gene encodes a functional trehalase from *Arabidopsi*s. There is some unpublished work which indicates that T19F06.15 expression correlates well with trehalase activity in various tissues of *Arabidopsi*s. Thus, it is also possible that in *Arabidopsis* a single gene is responsible for expressing the trehalase activity (Müller et al., 1999).

All genes needed to produce and degrade trehalose from UDP-glucose and glucose-6-phosphate, two common precursors in plants, are thus expressed in higher plants. Therefore, it has been suggested that trehalose metabolism is an endogenous metabolism not only in *Selaginella* and *Myrothamnu*s, but generally in higher plants (Müller et al., 1999; and Goddijn and Dun, 1999).

1.8.7.5 Regulation of Trehalose-Synthesizing Enzymes

Evidence that the enzyme TPS in plants binds to 14-3-3 proteins was recently presented. An affinity chromatography experiment was carried out using yeast 14-3-3 proteins linked to a column, thereby retaining phosphorylated proteins, which were eluted specifically by competition with a phosphopeptide. Subsequent peptide sequencing identified several proteins among which were TPS and SPS, indicating that these proteins contain phosphorylated sites that are able to interact with 14-3-3. Although the implications of this interaction and its physiological relevance have not been elucidated, it further stresses the similarities between sucrose and trehalose metabolism. It is tempting to speculate that a regulatory control mechanism is necessary for proteins playing a crucial role in plant development, such as nitrate reductase (NR), which is also subject to regulation via phosphorylation and binding to 14-3-3.

The observed interaction of 14-3-3 with plant-derived TPS2 protein opens a completely new avenue of research. To date, no reports are available on the over- or under-expression of plant-derived TPS2 genes in transgenic plants. It should be kept in mind that over-expression studies will probably be complicated by the fact that plants can regulate TPS2 activity in a similar way to that shown for SPS and NR. This

drawback favours the use of heterologous enzymes to study the impact of modulating trehalose metabolism in plants (Goddijn and Dun, 1999).

1.9 Aim of this Study

Protective function of trehalose under stress conditions in yeast have been studied, however there are no study concerning trehalose content of wheat. Therefore, in the present study first of all it is aimed to determine the trehalose content of different Turkish wheat cultivars (*Triticum aestivum L.*). In this respect, the experiments have been conducted on seeds and seedlings under control and stress conditions, mainly drought and salt stresses. Secondly, for understanding the protective mechanism of trehalose, the characterization of the trehalose synthesizing and degrading enzymes will be studied under drought and salt stress conditions at seedling level.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1 Plant Material

In thıs study, all experiments were performed on two bread wheat (*Triticum aestivum L.)* Bolal and Tosun (stress tolerant) and one durum wheat (*Triticum durum*) Çakmak (sensitive) cultivars. The seeds were provided by the Turkish Ministry of Agriculture.

2.1.2 Chemical Materials

The chemicals used in this study were purchased from Merck Chemical company (Deisenhofen, Deutschland) and Sigma Chemical Company (N. Y., USA). The radioactive material (Uridine diphospho-D-[U-^{14}C] glucose) with specific activity of 331 mCi/mmol, was ordered from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, England).

2.2 Methods

2.2.1 Growth of Plants

The seeds were surface sterilized by immersion in sodium hypochloride (40% (v/v)) for 20 minutes, rinsed with distilled water, and transferred into plastic pots (8 cm

diameter) filled with perlite. Seeds were watered with sterile tap water, and grown in a growth chamber at 25°C with 16 hours light and 8 hours dark photo cycle (5000 lux) at 70 % relative humidity. The plants were watered three times per week.

2.2.2. Stress Application for Carbohydrate Analysis

Stress treatment were achieved on 10 days of seedlings, watering was cut of for drought stress, and the sterile tap water was replaced with a solution containing 2 % NaCl for the salt stress application. The control plants were grown in sterile tap water. Samples of the roots and shoot tissues of control, drought stressed, and salt stressed plants were harvested after 13, 15, and 20 days and subjected to various procedures for analysis. Carbohydrate analysis were carried out on seeds, shoot and root tissues of Çakmak, Tosun and Bolal cultivars.

2.2.3. Stress Application for Enzyme Assay

Drought stress treatment were started on the 7^{th} day of growth and watering was cut off completely. Plants were harvested on the 15^{th} day of growth. For salt stress, at the end of 7^{th} day of growth the plants were moved to solution containing 2% NaCl and continue to grow for 8 days under same physiological conditions. Enzyme analysis were carried out on both shoot and root tissues of Çakmak and Bolal cultivars.

2.2.4 Carbohydrate Analysis

The trehalose contents of the seeds and seedlings were determined by using high performance liquid chromatography (HPLC). The qualitative test was carried out by GC-MS.

2.2.4.1 Trehalose Extraction from Seeds and Seedlings for HPLC

Before trehalose extraction, the seeds were crushed by coffee machine then ground more by liquid nitrogen in mortar. The trehalose extraction was carried according to (Ferreira et al., 1997). By boiling of 40 mg of seeds in 2 ml of ethanol and 100 mg of seedlings in 2 ml ethanol. Ethanol was then evaporated and the residue dissolved in 5 ml of the mobile phase (5 mM H_2SO_4) of the HPLC (LKB, BROMMA, 2150 HPLC PUMP). This solution was then centrifuged at 10,000 rpm for 10 min in a microcentrifuge and filtered through 0.2 μm milipore filter. Then the extract was incubated in boiling water for one hour to hydrolyze the sucrose in the extract, because the sucrose retention time is the same as that of trehalose. Then, sample of this extract was analyzed by using monosaccharide column (Phenomenex, REZEX CAL, 300 × 7.8 mm, S/No. 40450) at flow rate of 0.5 ml/min and detected by refractory index detector (KNAUER, DIFFERENTIAL-REFRACTOMETER). Trehalose content was determined by comparing its chromatogram with that of different concentration of commercial trehalose.

2.2.4.2 Carbohydrate Extraction for GC-MS

The carbohydrate analysis by GC-MS was carried out according to a procedures modified from (Garcia et al., 1997). Samples were harvested at the time mentioned above and ground to a fine powder in liquid nitrogen with a precooled mortar and pestle. One gram of powdered material was transferred to Corex tubes (DuPont) containing 10 μg mL^{-1} phenyl β-D-galactoside as an internal standard, and was placed in an 80°C water bath for 10 min. Insoluble material was removed by centrifugation at 12000 xg for 10 min in sigma centrifuge (Sigma, Laboratory Centrifuges, 3K30). The supernatants were collected in fresh tubes and the pellets were washed three times in 80% ethanol and centrifuged as before, and each wash and the supernatants were pooled with the first supernatant. The extracts were then concentrated to a volume of 0.5 mL, using a rotary evaporator, transferred to crimp-top vials, and dried to a residue at 60°C in oven (GRIFFIN INCUBATOR).

2.2.4.2.1 Carbohydrate Derivatization

Trimethylsilyl derivatives of sugars, polyols, and acids were prepared according to procedure of (Garcia et al., 1997). Typically, 0.015 mL of 2-dimethyl-aminoethanol and 0.4 mL of pyridine containing 30 mg mL^{-1} methoxyamine HCl were added to the crimp-top vials containing the dried extracts. Vials were capped and placed in an 80°C water bath and were incubated for one hour. After the reactions were cooled to room temperature (26-27°C), 0.4 mL of hexamethyl disilazane and 0.02 mL of trifluoroacetic acid were added and the vials were capped and incubated at room temperature for one hour. The insoluble debris were removed by centrifugation; the supernatant from each vial was carefully transferred to fresh crimp-top vials and sealed.

2.2.4.2.2 Carbohydrate Identification by GC-MS

A gas chromatogragh (Agilent 6890 series, GC system) equipped with a mass selective detector and a 30-m methylpolysiloxane column (0.32-mm i.d., 0.25-μm film) was used for analysis. The operating conditions were as follow: injector 100°C, detector 290°C, oven temperature 100°C for 3 min, ramped 5°C min^{-1} to 250°C and will be held for 1 min, ramped 20°C min^{-1} to 260°C and held for 1 min, ramped to 290°C and held for 13 min; flow 1.4 mL min^{-1}; and a split ratio of 30:1. Trimethylsilyl-derivatized compounds were identified by a gas chromatogragh equipped with a quadrupole mass selective detector (Agilent 5973-MSD). Based on the identification of the most abundant solutes, mixed standards were prepared and run each time the machine will be used. These standards were used to verify the retention times and derivatization efficiencies of all major sugars, polyols, and acids under investigation.

2.2.5 Preparation of Crude Extract

Pre-weighted amounts of shoots and roots were ground with liquid nitrogen by using mortar and pestle. The powders were then suspended in ice cold suspension

solution containing 0.1M citrate (Na^+), pH 3.7, 1 mM PMSF, 2 mM EDTA and insoluble polyvinylpyrrolidone (10 mg/ g dried weight). For 1g dry weight of suspension culture 2 ml of extraction buffer was used. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 31,500 rpm (48,000g) for 30 minutes at 4°C in Sorval Combi Plus with T-880 type rotor. The supernatant was used for the enzyme assays.

2.2.6 Analytical Methods

2.2.6.1 Protein Determination

The protein concentration was performed according to Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. Bradford reagent (5X) was diluted 5 times before use.

After each extraction, standard curve was prepared. To find protein concentration of sample, 10 µl of sample were diluted with 490 µl distilled water in a test tube and 5ml 1X Bradford reagent was added. The tubes were mixed, and left at room temperature for at least 10 minutes. The color formation was measured with Schimadzu UV-1201 spectrophotometer against blank solution, which was prepared from 500 µl of distilled water and 5 ml of Bradford reagent.

2.2.6.2 Trehalase Enzyme Assay

Trehalase enzyme activity was measured by discontinuous assay using glucose oxidase-peroxidase kit (Bicon). The enzyme assay is based on the measurement of glucose produced by hydrolysis of trehalose as shown below:

$$\text{Trehalose} + H_2O \xrightarrow{\text{Trehalase}} 2\ \text{glucose}$$

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2\ H_2O_2 + \text{Phenol} + \text{4-amino-antipyrine} \xrightarrow{\text{Peroxidase}} \text{red chinonimin} + 4\ H_2O$$

The hydrolysis of trehalose is resulted in 2 glucose formation. Glucose is converted to red chinonimin by glucose oxidase peroxidase. Red chinonimin is a colored compound that gives maximum absorbance at 546 nm.

The reaction mixture is composed of 10 mM trehalose, 50 mM MES (K^+), pH 6.3 and 0.2 mg/ml crude extract in a final volume of 1 ml. It was incubated at 37°C for 30 minutes. The reaction was started by the addition of trehalose to the reaction mixture, which was preincubated at 37°C for 10 minutes, then the mixture was immediately vortex mixed and at zero time the first aliquot was taken. At 5, 10, 20 and 30 minutes 100 µl of samples were taken from the reaction mixture and immediately put thermostat at 100°C for 3 min to stop the reaction. Precipitates were removed by centrifugation at 8700 rpm for 10 minutes in microcentrifuge. For the analysis, 10 µl of the supernatant was mixed with 1 ml of glucose oxidase-peroxidase kit solution, mixed by vortex and then the mixtures were incubated at 37°C for 15 minutes. The absorbance of the sample was measured at 546 nm in Schimadzu UV-1201 spectrophotometer against blank

solution. The increase in the absorbance against time was assumed to be equal to the amount of glucose formed and was plotted by using Microsoft Excel. Glucose at the level of 5.55 μmol/ml was used to calculate the concentration of glucose in each sample.

The calculation of trehalase enzyme activity is given below:

$$\frac{\Delta A/\Delta t \text{ Sample}}{A_{546} \text{ Standard}} \times \frac{\text{standard conc.}}{2} = \text{EA (glucose produced min}^{-1}\text{)}$$

$\Delta A/\Delta t$ Sample = initial rate; it is the slope of OD_{546} vs time curve.

A_{546} Standard = the absorbance of commercial standard at OD_{546} nm.

Concentration of standard = 5.55μmol/ml

Dividing by 2 = Hydrolysis of 1 mole of trehalose produce 2 moles of glucose.

One unit of trehalase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of trehalose/min at 37°C at pH 6.3.

2.2.6.3 Trehalose-6-phosphate Synthase Assay

Trehalose-6-phosphate synthase (TPS) activity was measured according to a modified procedures of (Vandercammen et al., 1989). The assay mixture containing 6 μl UDP-[U-^{14}C] glucose (10 μCi/ml), 10 mM glucose-6-phosphate, 1mM EDTA, 50 mM KCl, 10 mM magnesium acetate and 25 mM Hepes, pH 7.1. The assay was performed in a total volume of 0.3 ml and was started by the addition of the enzymic preparation (less than 0.2 mg protein). At zero time, 5,10, 15 and 20 min of incubation, a 50-μl portion of the mixture were mixed with 500 μl of a solution containing 10% activated charcoal, 10% ethanol and 10 mM trehalose. This mixture was centrifuged for 10 min at 2000 x g. A 250-μl portion of the supernatant was then mixed with glycogen and ethanol at final concentrations of 0.4% and 66%, respectively. After centrifugation for 10 min at 2000 x g, the radioactivity in an aliquot of the supernatant was determined (Vandercammen et al., 1989).

The radioactivity was measured by using scintillation cocktail. The scintillation cocktail was prepared by dissolving 0.02 gm POPOP (1,4-bis[2-phenyloxazolyl)]benzene) and 0.4 gm PPO (2,5-Diphenyl-oxazole) in 100 ml toluene. The cocktail mixture was left on the magnetic stirrer for overnight till blue colour appeared. The supernatant (625 µl) was taken and put on glass filter paper and let to dry overnight at room temperature. The dried filter paper was incubated in the scintillator tube containing 5 ml of cocktail solution for two hours. Samples were counted in scintillation counter (LKB, WALLAC, 1209 RACKBETA, LIQUID SCINTILLATION COUNTER). By this way, the radioactivity coming from trehalose-6-phosphate and trehalose which contain ^{14}C can be measured (Figure 2.1).

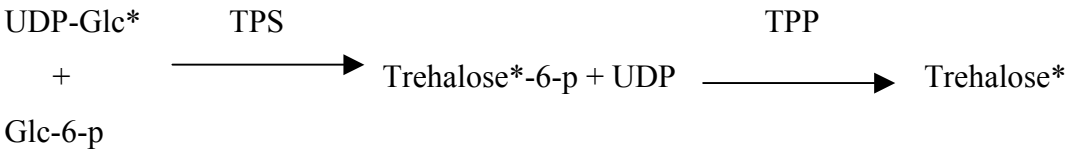

Figure (2.1) Pathway of the radioactive material [^{14}C] by the action of TPS and TPP. (*) Radioactive label [^{14}C].

CHAPTER 3

RESULTS

3.1 Trehalose Contents of Seeds

Trehalose was extracted from seeds according to the procedures in section (2.2.4.1) and trehalose identification was done by HPLC analysis. Figure 3.1 shows the trehalose contents of the seeds of different wheat cultivars. The trehalose content was the highest in Bolal cutivar (2.73 mg/g dry weight), and was the lowest in Çakmak cultivar (2.43 mg/g).The results are average of three different samples. The rough data for all of the species are given in (Appendix A).

3.2 Trehalose Contents in Seedlings

Trehalose contents in seedlings of different cultivars were measured under control, salt and drought stress conditions. We observed that trehalose contents under control condition was the lowest in Çakmak cultivar. The trehalose contents in Bolal and Tosun cultivars were approximately same under control condition (Figures 3.2-3.7).

3.2.1 Effect of Salt Stress on Trehalose Contents of Seedlings

Trehalose content was highly affected under salt stress condition. The amount of trehalose increased sharply in all cultivars during stress period. The highest amount was obseved in the root of Bolal cultivar after 10 days of stress (5495 μg/g fresh weight),

while the least amount was observed in the shoot of Çakmak cultivar (2296.5 μg/g fresh weight) (Figures 3.2-3.7).

Also differences in trehalose contents in different cultivars under salt stress were analysed statistically by One-way ANOVA test with respect to control (Table 3.1).

Table (3.1) One-way ANOVA test of trehalose contents in roots and shoots of different cultivars under salt stress with respect to control (Confidence intervals, 95%).

Time (days)	Bolal (root)	Tosun (root)	Çakmak (root)	Bolal (shoot)	Tosun (shoot)	Çakmak (shoot)
3	0.75	0.060	0.170	0.069	0.125	0.004*
5	0.101	0.010*	0.002*	0.091	0.003*	0.003*
7	0.000*	0.003*	0.049*	0.119	0.012*	0.014*
10	0.001*	0.000*	0.002*	0.013*	0.005*	0.000*

In the table, P-values were given for each sample at different time intervals. Cells with stars (*) indicate the P-values<0.05 meaning the significant difference.

3.2.2 Effect of Drought Stress on Trehalose Content of Seedling

Trehalose contents increased under stress conditions and became maximum by increasing the stress time. This increase was observed in all cultivars, but the highest increase was observed in the root of Bolal cultivar after 10 days of drought stress conditions which was 6250 μg/g fresh weight, while the least trehalose content was observed in the shoot of Çakmak cultivar which was 2715.5 μg/g fresh weight (Figures 3.2-3.7).

Figures 3.5-3.7 show the trehalose content in the shoots of different cultivars. In roots, the trehalose content increased significantly under stress conditions. Also, we observed that trehalose contents were reached to maximum on the 10^{th} day of drought stress in all cultivars.

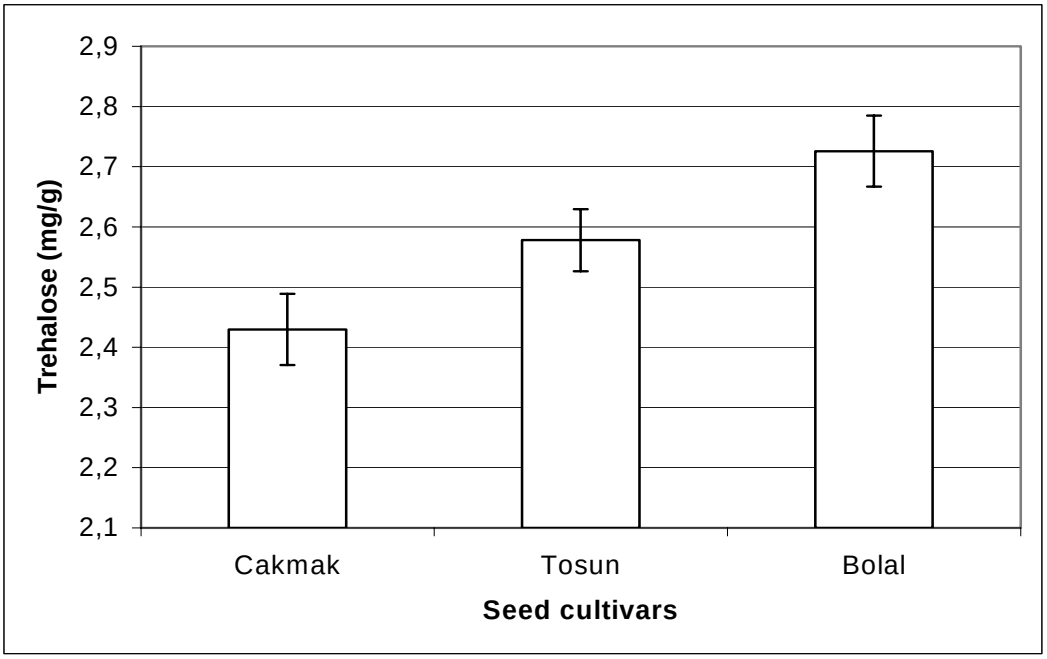

Figure (3.1) Trehalose contents in seeds of different cultivars. Mean values ± SE are given for three independent samples.

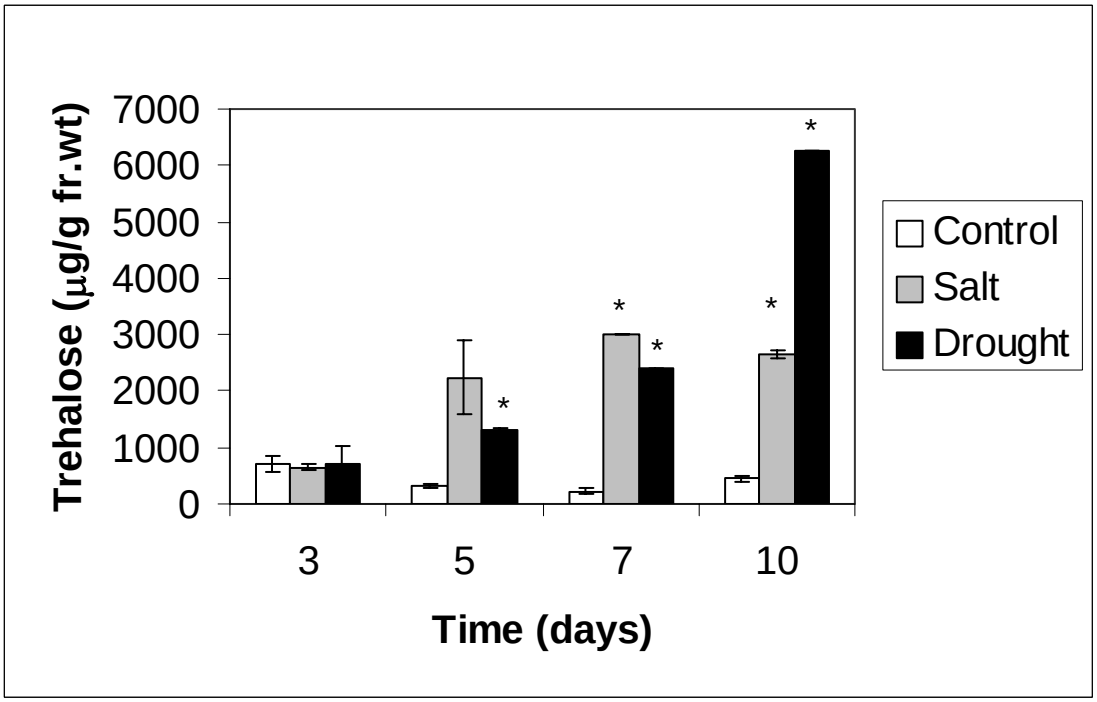

Figure (3.2) Trehalose contents in the roots of Bolal cultivar under control, salt (2% NaCl) and drought stress conditions. Mean values ± SE are given for two independent samples. (*), Significantly different from control ($P < 0.05$).

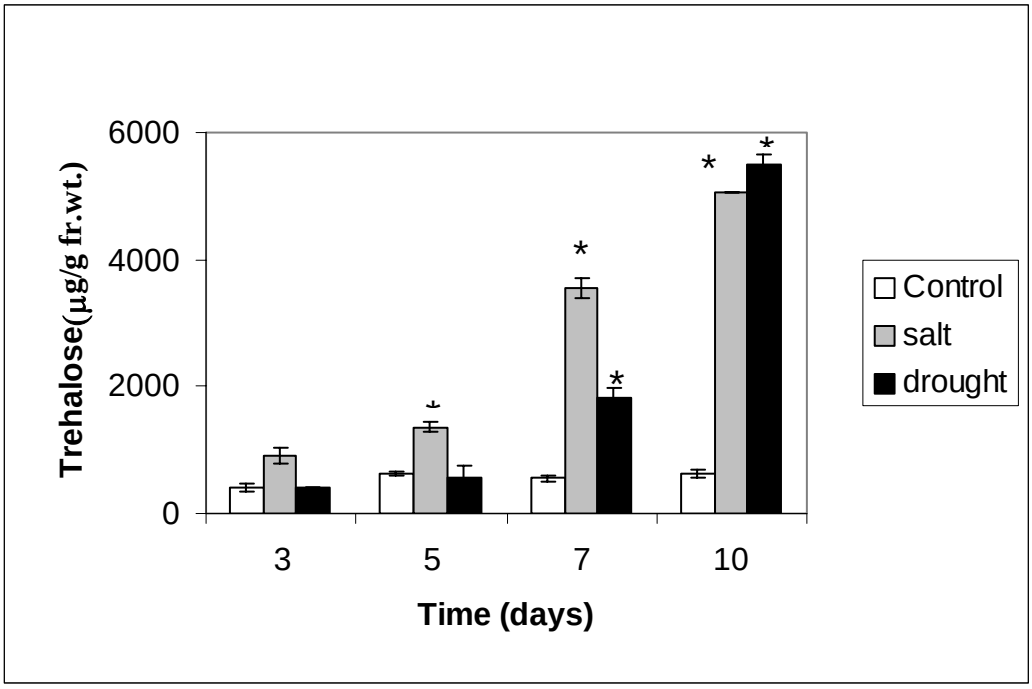

Figure (3.3) Trehalose contents in the roots of Tosun cultivar under control, salt (2% NaCl) and drought stress conditions. Mean values ± SE are given for two independent samples. (*), Significantly different from control ($P < 0.05$).

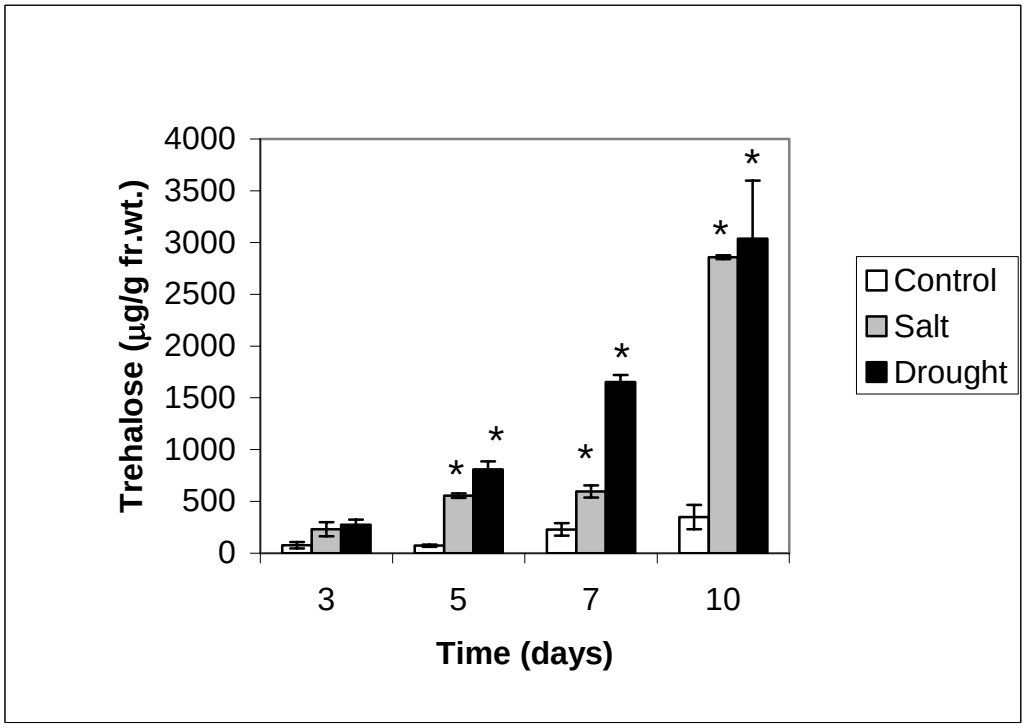

Figure (3.4) Trehalose contents in the roots of Çakmak cultivar under control, salt (2% NaCl) and drought stress conditions. Mean values ± SE are given for two independent samples. (*), Significantly different from control ($P < 0.05$).

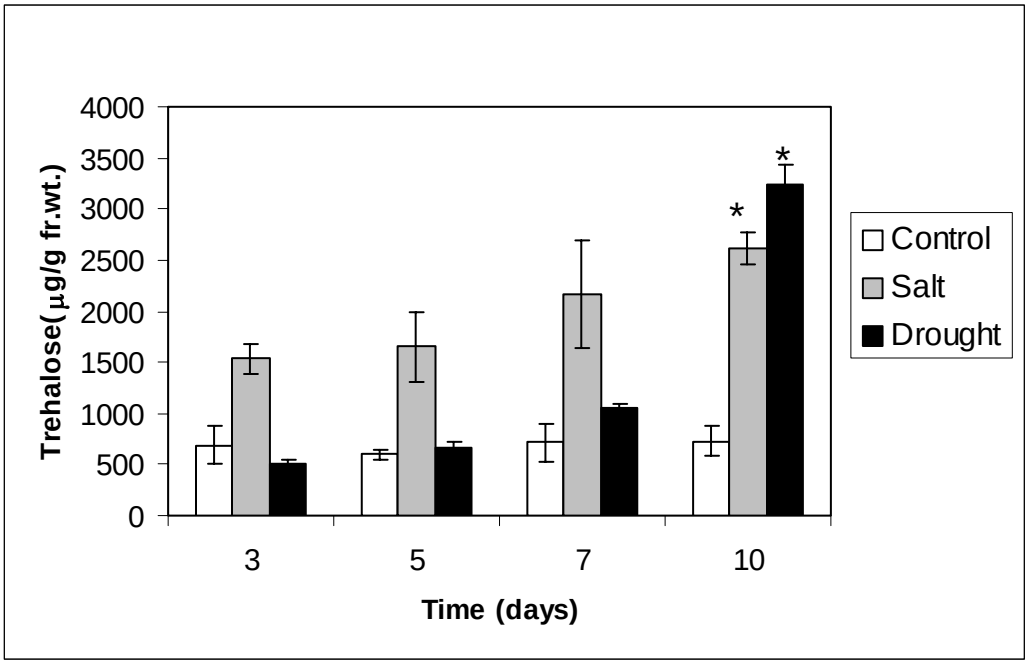

Figure (3.5) Trehalose contents in the shoots of Bolal cultivar under control, salt (2% NaCl) and drought stress conditions. Mean values ± SE are given for two independent samples. (*), Significantly different from control ($P < 0.05$).

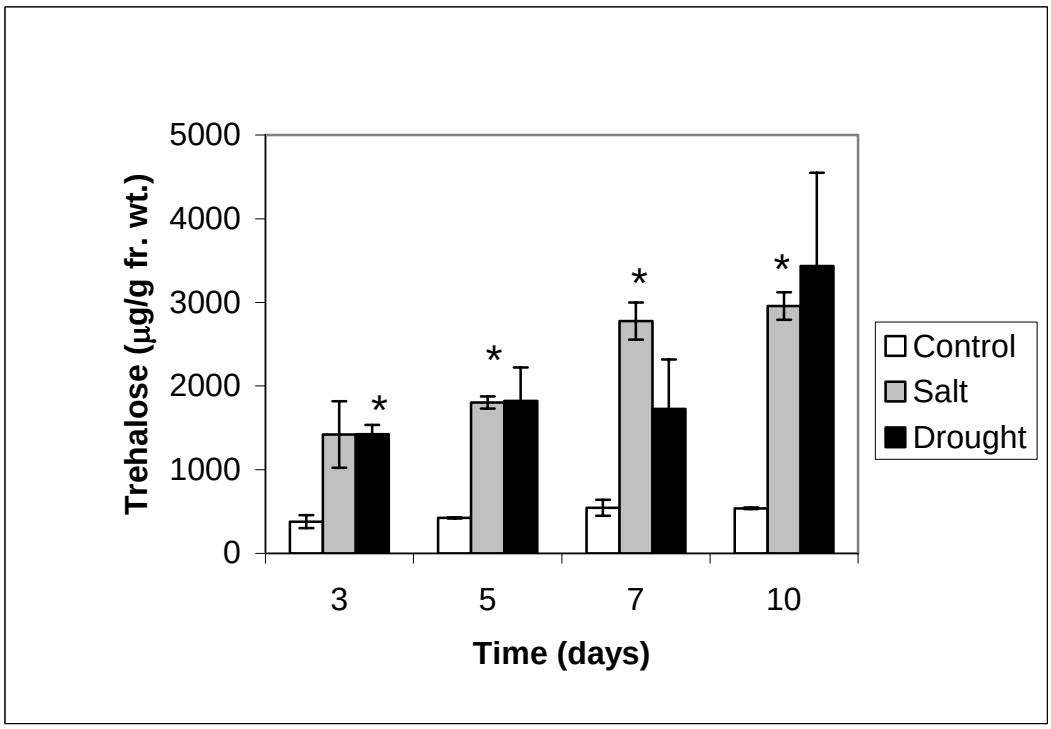

Figure (3.6) Trehalose contents in the shoots of Tosun cultivar under control, salt (2% NaCl) and drought stress conditions. Mean values ± SE are given for two independent samples. (*), Significantly different from control ($P < 0.05$).

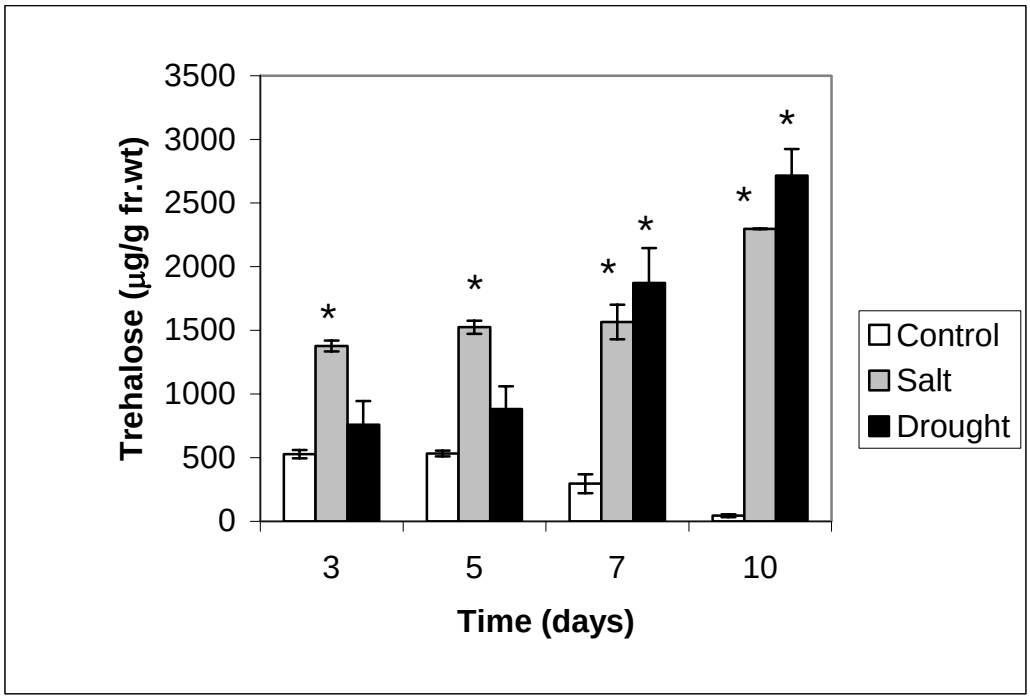

Figure (3.7) Trehalose contents in the shoots of Çakmak cultivar under control, salt (2% NaCl) and drought stress conditions. Mean values ± SE are given for two independent samples. (*), Significantly different from control ($P < 0.05$).

Also differences in trehalose contents in different cultivars under drought stress were analysed statistically by One-way ANOVA test with respect to control (Table 3.2).

Table (3.2) One-way ANOVA test of trehalose contents in roots and shoots of different cultivars under drought stress with respect to control (Confidence intervals, 95%).

Time (days)	Bolal (root)	Tosun (root)	Çakmak (root)	Bolal (shoot)	Tosun (shoot)	Çakmak (shoot)
3	0.969	1.000	0.081	0.446	0.016*	0.347
5	0.005*	0.795	0.010*	0.566	0.073	0.192
7	0.001*	0.014*	0.004*	0.216	0.187	0.031*
10	0.000*	0.001*	0.043*	0.010*	0.122	0.006*

In the table, P-values were given for each sample at different time intervals. Cells with stars indicate the P-values<0.05 meaning the significant difference.

Table (3.3) The comparison of the trehalose contents of three cultivars under salt and drought stress conditions by one-way ANOVA analysis (Confidence intervals, 95%).

Time (days)	3	5	7	10
Root contol (m±SD)	395.2±316	338.3±276.3	357.8±173	475.2±140.5
Root salt (m±SD)	(598.7±342,8)ns	(1385,2±841,8)ns	(2385±1574)ns	(3524.7±1323.5)*
Root drought (m±SD)	(466±231.6)ns	(892.7±366.6)*	(1960±383)*	(4927±1681)*
Shoot control (m±SD)	532.3±154.5	518.0±88.6	518.5±210.1	438.7±354.5
Shoot salt (m±SD)	(1445±84.3)*	(1661.7±139.6)*	(2168.8±606.8)*	(2623.7±330.3)*
Shoot drought (m±SD)	(897.2±473.8)ns	(1118.8±618.6)ns	(1548.8±441)*	(3130.2±364.4)*

m, mean; SD; standard deviation; ns, non-significant; *, significant at $P < 0.05$.

3.3 Identification of Trehalose by GC-MS

The carbohydrate was extracted from the plant and derivatised according to the procedures mentioned in section 2.2.4.2. Figure 3.8 & 3.9 are the chromatograms of the standard (100 μg trehalose) and sample, respectively. The retention time of the trehalose is 50.11 min as it is shown in Figure 3.8. There is a peak in the chromatogram of the sample with retention time of 50.10 min (Figure 3.9), which is the same as that of trehalose. Despite the extreme complexity of the plant chromatogram, this peak was unambigously identified as trehalose by comparison with the trehalose mass spectrum (Figure 3.10).

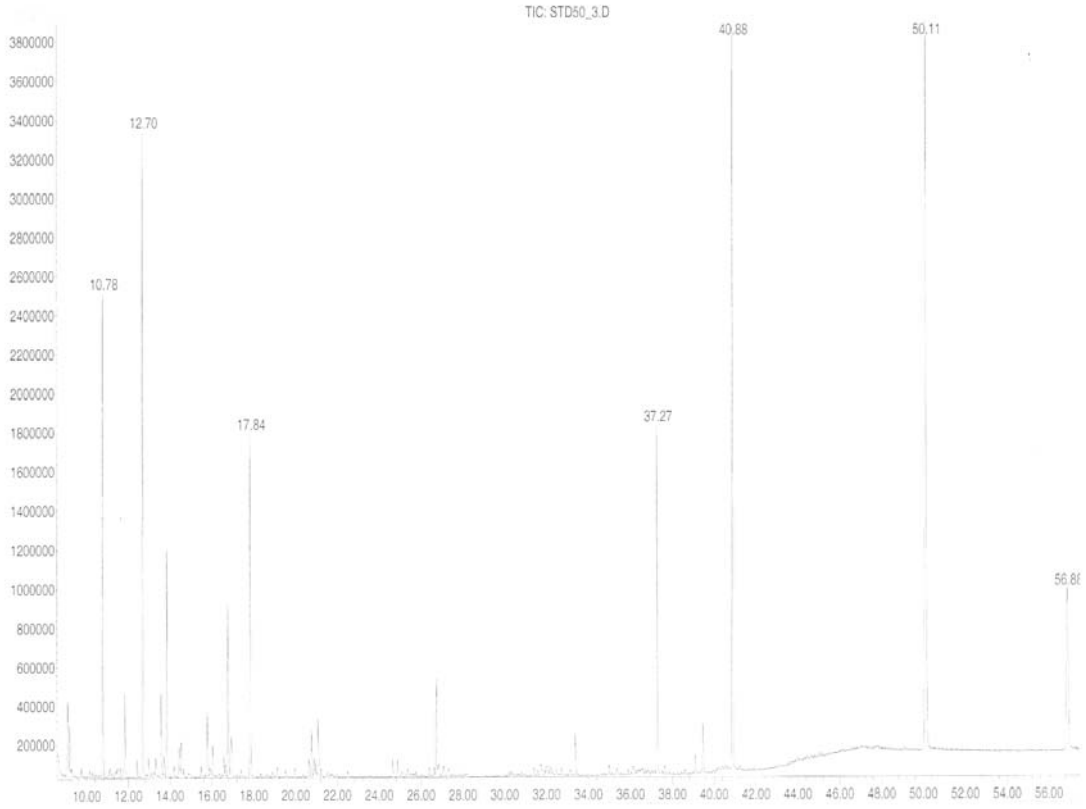

Figure (3.8) GC-MS chromatogram of trehalose standard with retention time 50.11.

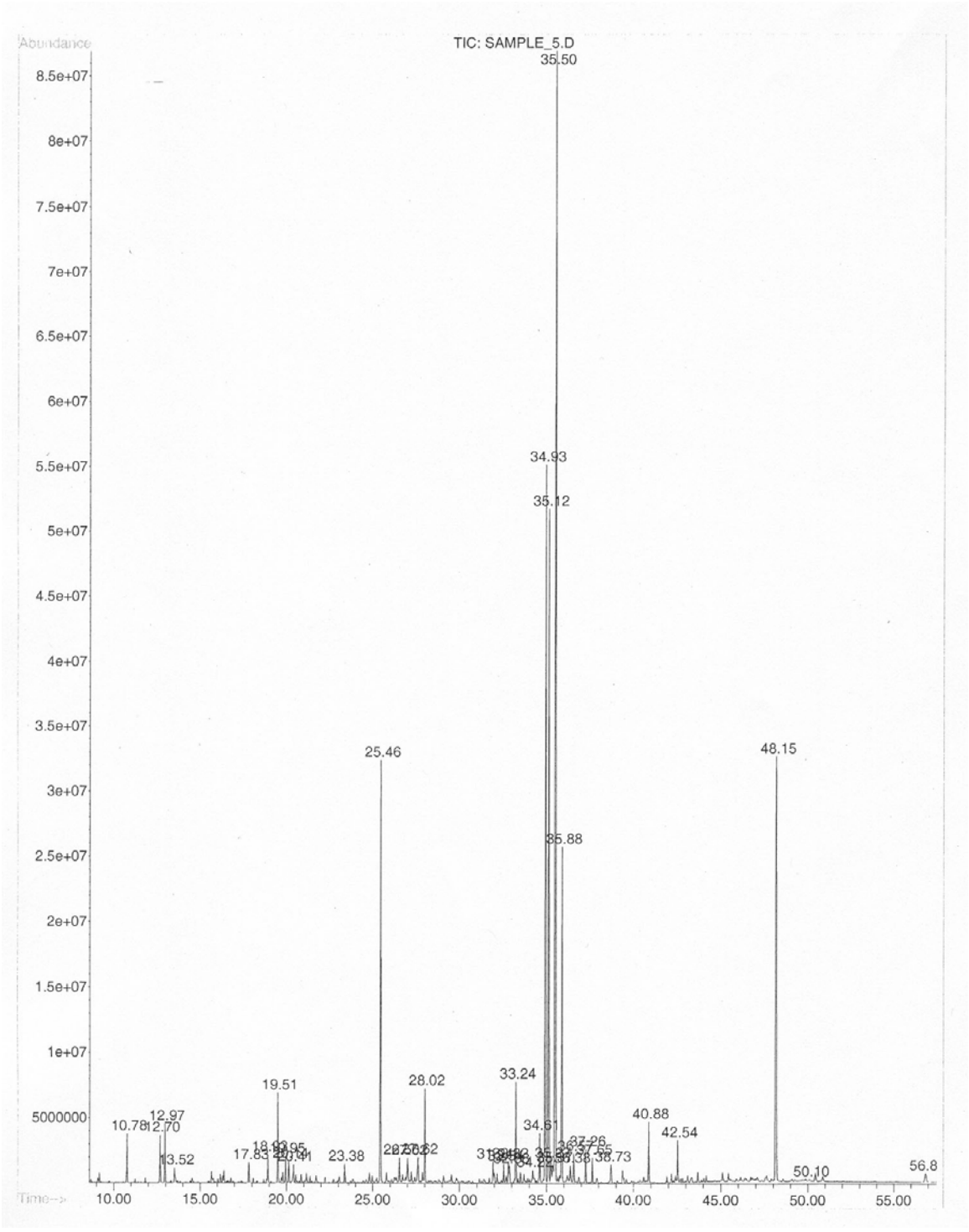

Figure (3.9) GC-MS chromatogram of one representative sample (Bolal root / 7 days drought stress).

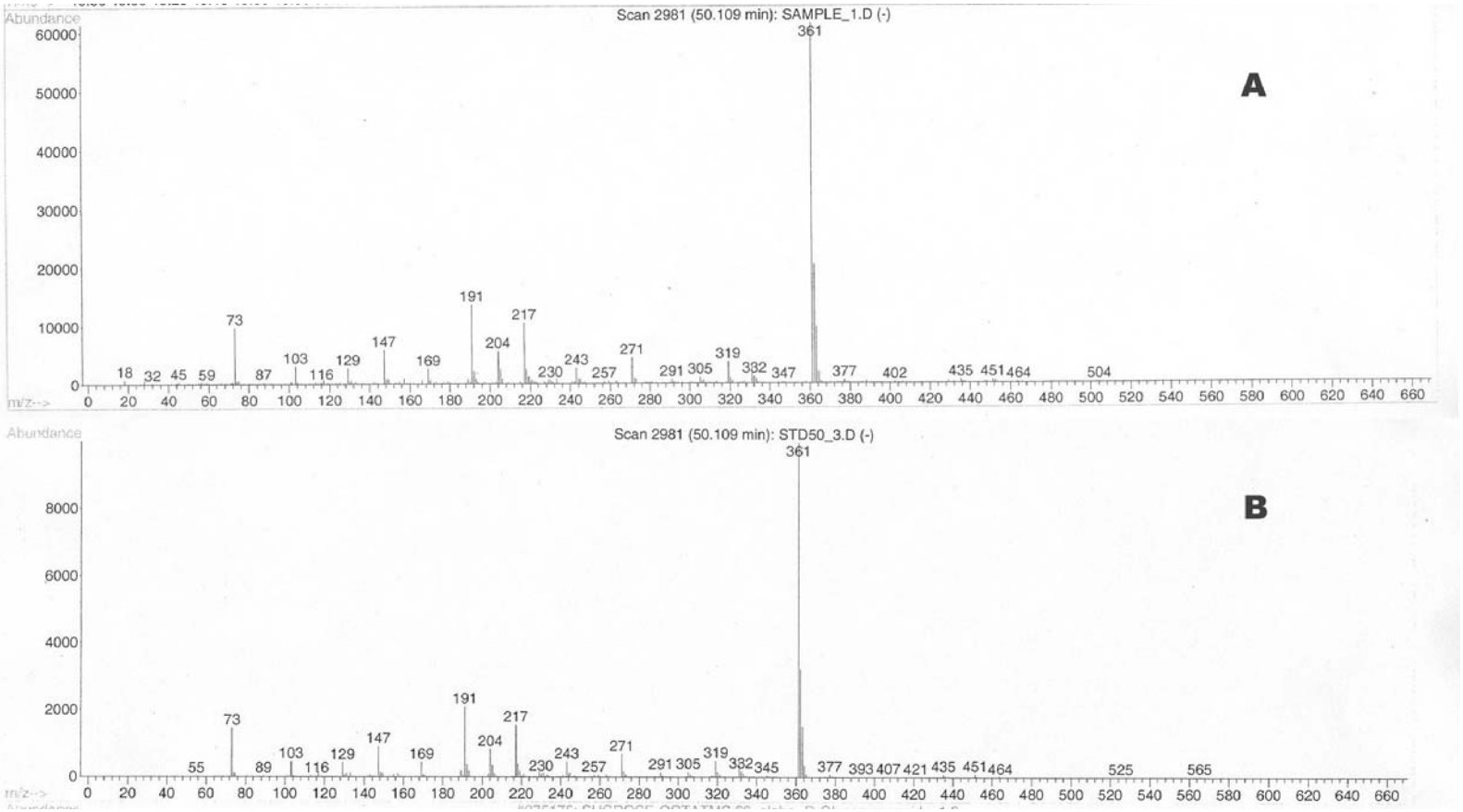

Figure (3.10) Mass spectra of the trehalose peak (retention time 50.109 min) identified by GC-MS in wheat plants (A) and trehalose standard (B).

3.4 Enzymes in Trehalose Metabolism

3.4.1 Trehalose-6-phosphate Synthase

Trehalose-6-phosphate synthase (TPS) is the first enzyme which involves in the trehalose formation in plants as explained in section 1.8.7.2. TPS actvity was measured according to the procedure mentioned in section 2.2.3.4 by using UDP-{U-^{14}C} glucose. The enzyme activity was recorded as increase in the radioactivity that comming from trehalose-6-phosphate and trehalose which produced by catalytic effect of TPS. A representative activity measurement curve for trehalose-6-phosphate synthase is given in Figure 3.11. The experiments were repeated for 3-4 times. The rough data for all of the species under control and stress conditions are given in (Appendix B).

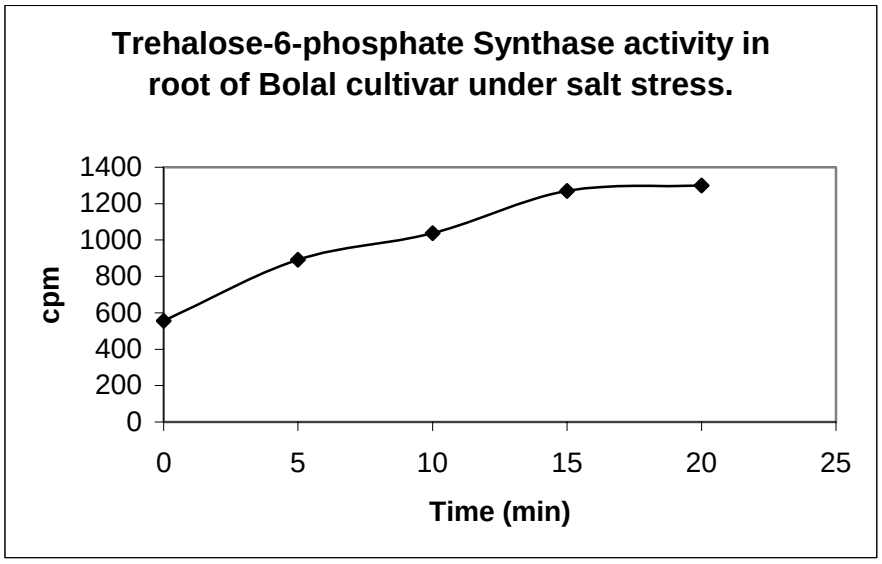

Figure (3.11) Representative curve for measuring TPS activity. Protein concentration= 0.0843 mg/ml; Temperature = 30 ºC; pH= 7.1; Slope= 45.838 cpm/min; SA= 543.78 cpm/min/mg protein.

The specific activity (SA) of TPS for each sample was found by dividing the slope of its curve by the protein concentration of that sample. The enzyme specific activity increases under stress conditions in both Bolal and Çakmak seedlings as shown in Figures 3.12 and 3.13 and Tables 3.4 and 3.5. Each column in Figures 3.12 and 3.13 is representing the mean of at least three different experiments as it is shown in Tables 3.4 and 3.5.

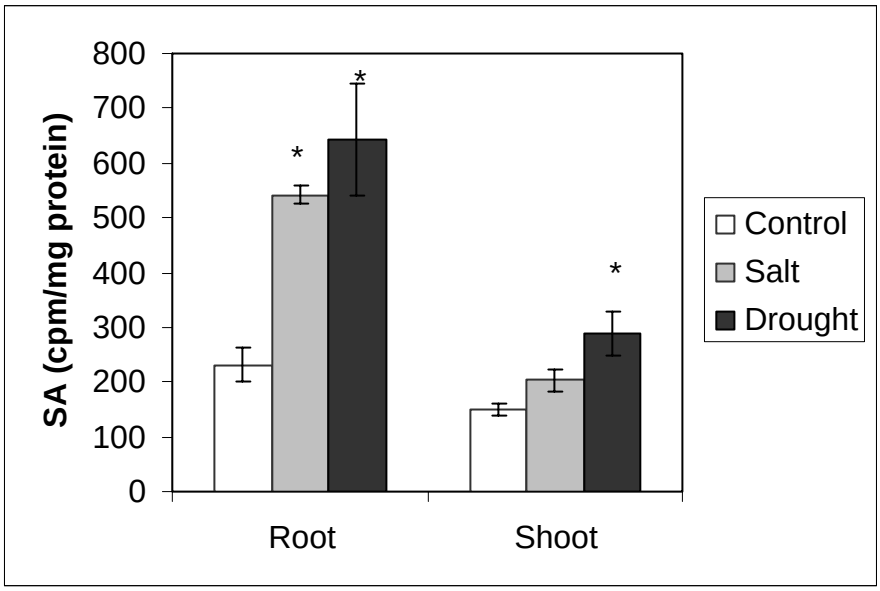

Figure (3.12) Specific Activity of Trehalose-6-phosphate synthase in cpm/mg protein in Bolal root and shoot tissues under control, salt and drought conditions (salt stress by using 2% NaCl; stress duration is 8 days). Mean values ± SE are given for 3-4 independent samples. (*), Significantly different from control ($P < 0.05$).

Table (3.4) The specific activities (SA) of TPS in at least three different samples, their means and their standard error of mean (SEM) in roots and shoots of Bolal cultivar.

Bolal/root	SA1	SA2	SA3	SA4	Mean	SEM
Control	171.3	258.6	264.7	NA	231.5	30.17
Salt	515.1	568.2	543.8	NA	542.4	15.33
Drought	306.9	449.3	800.8	676.2	602.8	111.0
Bolal/shoot						
Control	179.7	133.6	155.8	132.3	146.6	11.17
Salt	150.0	198.7	228.3	240.6	210.5	20.15
Drought	260.5	369.5	240.6	NA	300.1	40.06

NA: not available.

The SA increases sharply in the roots of both Bolal and Çakmak cultivars and was the maximum under drought stress condition, which is a good reflection of trehalose contents under those different conditions. The Change in the SA in shoot of Çakmak was not significant. Also, the enzyme activities were higher in roots than those of shoots in both of Bolal and Çakmak.

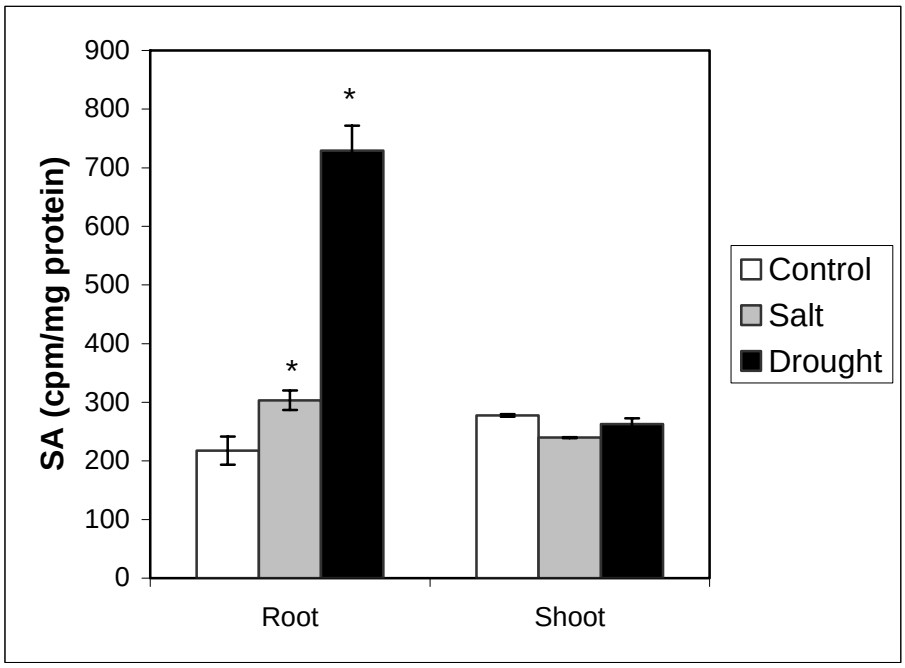

Figure (3.13) Specific Activity of Trehalose-6-phosphate synthase in cpm/mg protein in Çakmak root and shoot tissues under control, salt and drought conditions (salt stress by using 2% NaCl; stress duration is 8 days). Mean values ± SE are given for 3-4 independent samples. (*), Significantly different from control ($P < 0.05$).

Table (3.5) The specific activities (SA) of TPS in at least three different samples, their means and their standard error of mean (SEM) in roots and shoots of Çakmak cultivar.

Çakmak/root	SA1	SA2	SA3	SA4	Mean	SEM
Control	177.8	260.4	214.4	NA	217.5	23.90
Salt	285.3	288.7	336.4	NA	303.5	16.50
Drought	820.0	700.7	626.0	770.9	729.4	42.27
Cakmak/shoot						
Control	274.6	281.7	276.9	NA	277.7	2.09
Salt	238.9	239.2	241.6	NA	239.9	0.85
Drought	247.9	291.4	254.5	257.0	262.7	9.76

NA: not available.

Also differences in the specific activity of Trehalose-6-phosphate synthase in roots and shoots of Bolal and Çakmak cultivars under salt and drought stress conditions were analysed statistically by One-way ANOVA test with respect to control (Table 3.6).

Table (3.6) One-way ANOVA test of specific activity of TPS in roots and shoots of Bolal and Çakmak cultivars under salt and drought stress condititions with respect to control (Confidence intervals, 95%).

Stress	Bolal (root)	Çakmak (root)	Bolal (shoot)	Çakmak (shoot)
Salt(2% NaCl)	0.001*	0.042*	0.057	0.000*
Drought(8 days)	0.019*	0.000*	0.012*	0.254

In the table, P-values were given for each sample under different stress conditions. Cells with stars indicate the P-values<0.05 meaning the significant difference.

3.4.2 Trehalase

Trehalase is the trehalose degrading enzyme in the plants as explained in section 1.8.7.2. Trehalase actvity was measured according to the procedures mentioned in section 2.2.5.2. The enzyme activity was found by drawing $O.D_{546nm}$ versus time and finding its slope. A representative curve shown in Figure 3.14. The experiments were repeated for 2-4 times. The rough data for all of the species under control and stress conditions are given in (Appendix C). By substituting the slope value in the equation in section 2.2.5.2, the enzyme activity can be found. One unit of trehalase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of trehalose/min at 37°C at pH 6.3. The specific activity can be found by dividing the enzyme activity by the protein concentration in the sample.

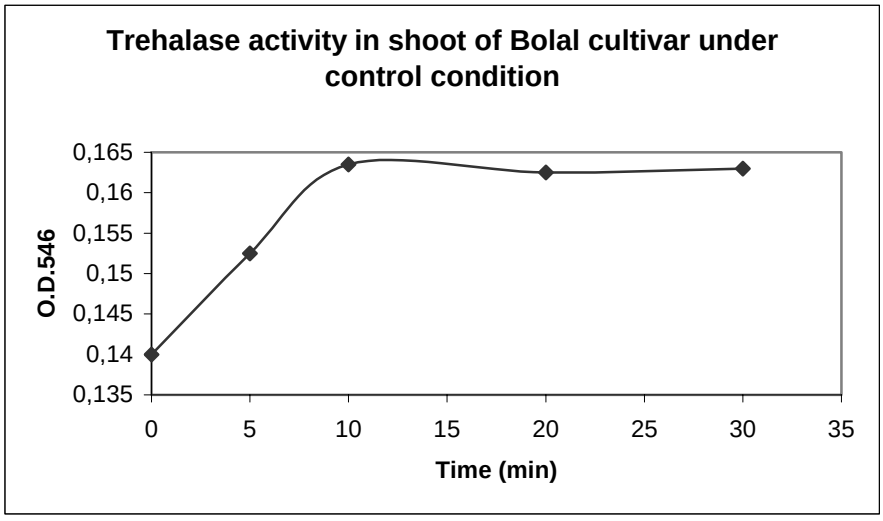

Figure (3.14) Representative curve for measuring Trehalase enzyme activity. [Protein] = 0.1805 mg/ml; [Trehalose]= 10 mM; Temperature= 37 ºC; pH= 6.3. Slope= 0.0024; $O.D._{standard}$ = 0.2425; SA= 0.1522 μmol trehalose/min/mg protein.

The trehalase specific activity (SA) was found to be the highest under control conditions in both root and shoot tissues of Bolal cultivar (Figure 3.15 and Table 3.7). In Çakmak cultivar, there was no significant change in the enzyme activity under the different stress conditions (Figure 3.16 and Table 3.8).

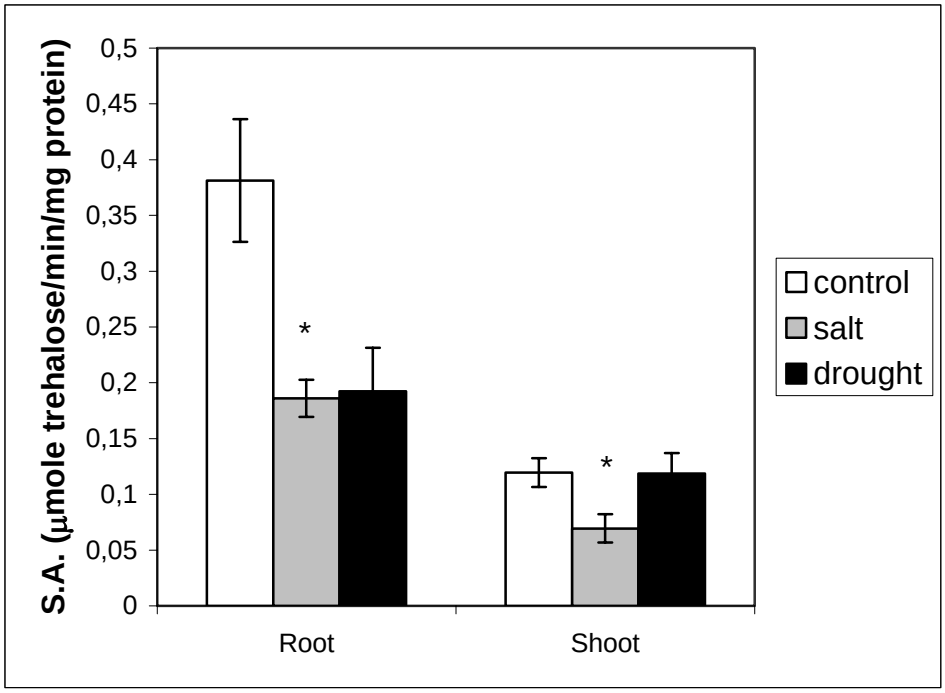

Figure (3.15) Specific Activity of Trehalase enzyme in µmol trehalose/min/mg protein in Bolal root and shoot tissues under control, salt and drought conditions (salt stress by using 2% NaCl; stress duration is 8 days). Mean values ± SE are given for 2-4 independent samples. (*), Significantly different from control ($P < 0.05$).

Table (3.7) The specific activities (SA) of Trehalase in at least three different samples, their means and their standard error of mean (SEM) in roots and shoots of Bolal cultivar.

Bolal/root	SA1	SA2	SA3	SA4	Mean	SEM
Control	0.4914	0.3322	0.3204	NA	0.3813	0.0551
Salt	0.2171	0.1608	0.1803	NA	0.1861	0.0165
Drought	0.2314	0.1535	NA	NA	0.1925	0.0390
Bolal/shoot						
Control	0.1277	0.0999	0.1522	0.0985	0.1195	0.0128
Salt	0.0777	0.0444	0.0864	NA	0.0695	0.0128
Drought	0.1242	0.1619	0.0713	0.1169	0.1186	0.0186

NA: not available.

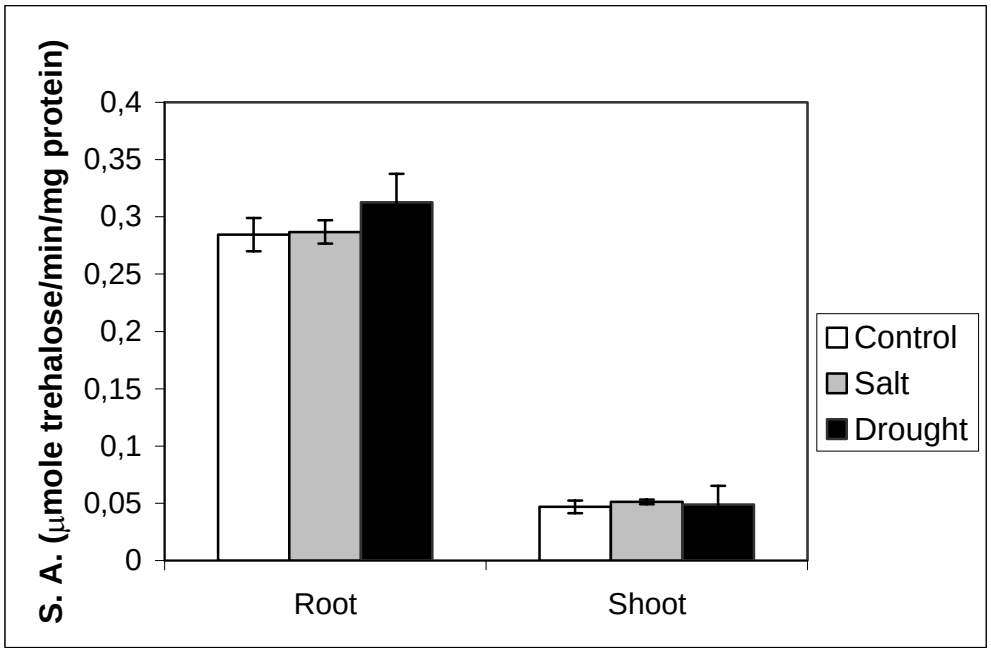

Figure (3.16) Specific Activity of Trehalase enzyme in μmol trehalose/min/mg protein in Çakmak root and shoot tissues under control, salt and drought conditions (salt stress by using 2% NaCl; stress duration is 8 days). Mean values ± SE are given for 2-4 independent samples.

Table (3.8) The specific activities (SA) of Trehalase in at least three different samples, their means and their standard error of mean (SEM) in roots and shoots of Çakmak cultivar.

Çakmak/root	SA1	SA2	SA3	Mean	SEM
Control	0.2989	0.2700	NA	0.2845	0.0145
Salt	0.3052	0.2699	0.2850	0.2867	0.1022
Drought	0.2880	0.3374	NA	0.3127	0.0247
Çakmak/shoot					
Control	0.0367	0.0486	0.0554	0.0469	0.0055
Salt	0.0500	0.0486	0.0554	0.0513	0.0021
Drought	0.0470	0.0216	0.0782	0.0489	0.0164

NA: not available.

Also differences in the specific activity of trehalase in roots and shoots of Bolal and Çakmak cultivars under salt and drought stress conditions were analysed statically by One-way ANOVA test with respect to control (Table 3.9).

Table (3.9) One-way ANOVA test of specific activity of trehalase in roots and shoots of Bolal and Çakmak cultivars under salt and drought stress condititions with respect to control (Confidence intervals, 95%).

Stress	Bolal (root)	Çakmak (root)	Bolal (shoot)	Çakmak (shoot)
Salt (2%NaCl)	0.027*	0.903	0.043*	0.491
Drought (8days)	0.091	0.428	0.967	0.911

In the table, P-values were given for each sample under different stress conditions. Cells with stars indicate the P-values<0.05 meaning the significant difference.

CHAPTER 4

DISCUSSION

4.1 Trehalose Contents of Seeds and Seedling

Until recently, most higher plants, such as wheat and *Arabidopsis*, were not considered to synthesize trehalose (Müller et al., 1995a). However, the discovery of an *Arabidopsis* TPS gene, *At*TPS1 (Blazquez et al., 1998), and of two TPP genes, *At*TPPA and *At*TPPB (Vogel et al., 1998), then the confirmatory information about the presence of trehalose in *Arabidopsis* (Müller et al., 2001; Vogel et al., 2001), suggested that higher plants have the potential for trehalose synthesis.

As far as we know there is no such a study in wheat seeds. Figure 3.1 shows the trehalose contents of the seeds of different wheat cultivars. The trehalose content was the highest in Bolal cutivars (2.73 mg/g dry weight), and was the lowest in Çakmak cultivars (2.43 mg/g).

Here, it is shown that trehalose does occur in the seedlings of different wheat cultivars (Figures 3.2-3.10). This confirms results of other studies in which chromatographic techniques were used for measuring trehalose in plants. For example, trehalose was found in tobacco plants grown hydroponically in the presence of validamycin A (Goddijn et al., 1997), and in a salt stressed rice plant (Garcia et al., 1997). In *Arabidopsis*, a compound that increased in the presence of validamycin A was tentatively identified as trehalose (Müller et al., 2001). To provide unambiguous evidence that trehalose occurs in plants it was, however, necessary to identify trehalose using GC-MS or NMR analysis. Recently, trehalose was identified by GC-MS analysis

in soil-grown potato tubers (Roessner et al., 2000) and in axenically grown Arabidopsis plants (Vogel et al., 2001). In the present study, different wheat cultivars grown under sterile conditions (axenically grown) were used to determine trehalose by GC-MS analysis in order to be sure that microorganisms were not the source of trehalose. Unless axenically grown wheat plants contain seed-borne microbial endophytes, an involvement of microorganisms in the formation of the trehalose found in this study can be excluded, and therefore concluded that trehalose is an endogenous substance in wheat.

Recently, a genetic approach has been used to establish that *At*TPS1 is essential in *Arabidopsis* (Eastamond et al, 2002). Disruption of *At*TPS1 leads to an embryo-lethal phenotype. Embryo morphogenesis of *Arapidopsis tps1* is normal but the development of this mutant retarded and stalls early in the phase of cell expansion and storage-reserve deposition, at the torpedo embryonic stage. The expression of genes that are involved in storage-reserve deposition is also blocked in these mutants, demonstrating that *At*TPS1 and its products are required for both late embryo development and metabolism (Eastamond et al, 2002). It is not known how *At*TPS affect both development and metabolism, but sugar signaling is strongly implicated. At the onset of storage-reserve deposition in wildtype seeds, the supply of sucrose to the embryo increases dramatically, in line with its 'sink status' (Eastamond et al, 2002). This increase is not only required to support the rapid deposition of storage reserves but may also induce this process through sugar signaling (Wobus and Weber, 1999). It is particularly interesting that *Arabidopsis tps1* embryos abort at the torpedo embryonic stage when sucrose increases because this phenotype is paralleled in the *S. cereviseae tps1*Δ mutant, which only exhibits a null phenotype when grown on high sugar concentrations (Thevelein and Hohmannn, 1995). In *S. cereviseae*, the defect can be overcome by restricting the influx of glucose into glycolysis (Thevelein and Hohmannn, 1995). Reducing the availability of sucrose to the embryos of *Arabidopsis tps 1* mutants growing in *in vitro* culture partially complements their phenotype, allowing morphological progression into maturation (Eastamond et al, 2002). However, the embryos could not be induced to germinate precociously (Eastamond et al, 2002). These results suggest that trehalose and its metabolites play a

central role in the regulation of sugar metabolism during storage-reserve deposition in developing and mature seeds in wheat.

4.2 Trehalose Biosynthesis under Stress Conditions

Trehalose has been shown to stabilise proteins and membranes under stress conditions, especially during desiccation. By replacing water through hydrogen bonding to polar residues, trehalose prevents the denaturation of proteins and the fusion of membranes. In addition, trehalose forms glasses (vitrification) in the dry state, a process that may be required for the stabilisation of dry macromolecules (Crowe et al., 1998). Furthermore, trehalose remains stable at elevated temperatures and at low pH and does not undergo Maillard browning with proteins. These protective properties of trehalose are clearly superior to those of other sugars, such as sucrose, making trehalose an ideal stress protectant.

Trehalose has been shown to act as protectant in response to different stress conditions in a large number of microorganisms (Wiemken, 1990). In yeast, for example, adverse conditions, such as heat, cold or water stress correlate with the accumulation of high concentrations of this non-reducing disaccharide (Mackenzie et al., 1988; DeVirgilio et al., 1994). In plants a clear role of trehalose in stress tolerance, in particular drought, has been demonstrated for cryptobiotic species, such as the desiccation-tolerant *S. lepidophylla*. During its dehydration, trehalose accumulates to a level of 12% of the plant dry weight, and acts to protect proteins and membrane structures. Upon rehydration, *S. lepidophylla* regains complete viability and trehalose levels decline.

In higher vascular plants, accumulation of trehalose under adverse conditions is rare (Müller et al., 1995a). However, in a few desiccation-tolerant angiosperms trehalose is present in relatively large amounts. For example, the resurrection plant *M. flabellifolius* accumulates trehalose up to 3% of its dry weight, although this level is only slightly increased upon drought stress. Whereas sucrose increases from 3 to almost 6%

of the dry weight (Drennan et al., 1993; Bianchi et al., 1993). The combined accumulation of sucrose and trehalose might be sufficient to protect the plant against the adverse effects caused by desiccation.

The observation that trehalose can be used to preserve biological structures has been obtained from *in vitro* studies. Trehalose can stabilize dehydrated biological structures, such as lipid membranes or enzymes, more effectively than other sugars (Colaco et al., 1995). Because of these specific properties, trehalose has been selected as a target molecule for genetic engineering of plants, both for cost-effective large-scale production of this compound and for engineering drought-tolerance in crops (Goddijn and Pen. 1995).

In our experiments, trehalose accumulation was observed under drought and salt stress conditions in all wheat cultivars, which reflects the protection properties of trehalose molecule against stress conditions (Figures 3.2-3.7). The amount of trehalose in Çakmak cultivar, which is known as sensitive cultivar, under control and stress conditions was the least. The trehalose contents in the seedlings of Bolal and Tosun cultivars, which are stress tolerant cultivars, under control and stress conditions are higher than that of Çakmak cultivar. These results are strongly support the protective function of trehalose.

4.3 Effect of Stress Conditions on Trehalose Metabolizing Enzymes

4.3.1 Trehalose-6-Phosphate Synthase

Here we studied TPS activity under stress conditions. Trehalose-6-phosphate synthase (TPS) is the first enzyme which involve in the trehalose formation in plants as explained in section 1.8.7.2. TPS gene was first cloned from *Arabidopsis thaliana* (AtTPS1) and expressed in *Saccharomyces cerevisiae* mutant deficient in trehalose synthesis. Their results indicated that AtTPS1 is involved in the formation of trehalose in *Arabidopsis* (Vogel et al., 2001). We found that the enzyme specific activity increases

under stress conditions in both Bolal and Çakmak seedlings as shown in figures 3.12 & 3.13 and tables 3.4 & 3.5. The SA increases sharply in the roots of both Bolal and Çakmak and was the maximum under drought stress condition, which is a good reflection of trehalose contents under those different conditions. The Change in the SA in shoot of Çakmak was not significant. Also, the enzyme activities were higher in roots than those of shoots in both of Bolal and Çakmak.

4.3.2 Trehalase

So far, it is not clear to what extent endogenously formed trehalose is involved in the regulation of metabolism. It is possible that trehalase activity normally keeps cellular trehalose concentrations low in order to prevent detrimental effects of trehalose accumulation on the regulation of carbon metabolism. Such a role of trehalase may be of particular importance in interactions of plants with trehalose-producing microorganisms. In support of this hypothesis, expression of the *Arabidopsis* trehalase gene and trehalose activity were found to be strongly induced by infection of *Arabidopsis* plants with the trehalose-producing pathogen *Plasmodiophora brassicae* (Brodmann et al., 2002).

Trehalase is the trehalose-degrading enzyme in the plants as explained in (Section 1.8.7.2). The Trehalase specific activity (SA) was the highest under control conditions in both root and shoot of Bolal cultivar comparing with stress treatments.. However under drought conditions, there was no significant change trehalase activity in shoot (Figure 3.15 and Table 3.7). In Çakmak cultivar, there was no significant change in the enzyme activity under the different conditions (Figure 3.16 and Table 3.8).

Trehalase is ubiquitous in higher plants and single-copy trehalase genes have been identified and functionally characterized from soybean (*Glycine max*) and *Arabidopsis* (Aeschbacher et al., 1999; Muller et al., 2001). It is likely that trehalase is the sole route of trehalose breakdown in plants as trehalose accumulates in the presence of the specific trehalose inhibitor validamycin A (Müller et al., 2001). Trehalase activities in cell and tissue cultures of gymnosperm *Picea* and of a series of mono- and

dicotyledonous plants including three wheat callus lines was described (Kendall et al., 1990). Since cell and tissue cultures are propagated under sterile conditions, the trehalose is obviously of plant origin in all these cases. Therefore, it can be safely concluded that trehalose activity is present in most of higher plants across all major taxonomic groups (Müller et al., 1995a).

CHAPTER 5

CONCLUSION

In this study, trehalose was detected, identified and quantified in different wheat cultivars under control and stress conditions. The presence of trehalose in axenically grown wheat plants was identified and confirmed by GC-MS analysis. The amount of trehalose was found to be accumulated under stress conditions. Trehalose amount in Bolal and Tosun wheat cultivars (tolerant cultivars) was higher than its amount in Çakmak cultivar (sensitive cultivar) under both control and stress condition. Furthermore, the enzyme activities of TPS and trehalase were measured under control, salt and drought stress conditions in crude extracts prepared from roots and shoots of Bolal and Çakmak cultivars.

The results of trehalose contents and enzyme activities were parallel with each other. When trehalose contents increased under stress conditions, TPS activity induced and trehalase activity decreased.

Since trehalose metabolism has only recently been discovered in higher plants, very few information is available about its role in physiology and development. Studies on trehalose biosynthesis in other organisms, such as *E. coli* and yeast, where the pathway has been analyzed several decades ago, initially will give direction to the researches in plant systems. Observations in yeast indicating that enhanced trehalose levels coincide with increased tolerance to adverse environmental conditions and the control of glucose influx into glycolysis suggest a wide variety of promising applications.

This study showed the importance of the trehalose as osmoprotectant compound in wheat species under salt and drought stress conditions. The accumulation of trehalose in wheat under abiotic stresses was found to be tissue and species specific.

In long term the overexpression of trehalose biosynthetic genes in wheat may seem to be promising for improvement of abiotic stress tolerant transgenic wheat plants.

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APPENDEX A

TREHALOSE CONTENTS IN SEEDS AND SEEDLINGS OF DIFFERENT CULTIVARS

1. Trehalose contents in seeds of different cultivars in mg/g.

Seed	Exp.1	Exp.2	Exp.3	Mean	SEM
Cakmak	2,311	2,489	2,489	2,429667	0,05933
Tosun	2,578	2,489	2,667	2,578	0,051384
Bolal	2,844	2,667	2,667	2,726	0,059

2. Trehalose contents in root of Bolal cultivar under control conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	559	857	708	149
5	286	349	317,5	31,5
7	170	287	288,5	58,5
10	400	500	450	50

3. Trehalose contents in root of Bolal cultivar under salt stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	692	611	651,5	40,5
5	1580	2900	2240	660
7	3020	2996	3008	12
10	2737	2598	2667,5	69,5

4. Trehalose contents in root of Bolal cultivar under drought stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	417	1029	723	306
5	1235	1352	1293,5	58,5
7	2384	2395	2389,5	5,5
10	6250	6250	6250	0

5. Trehalose contents in root of Tosun cultivar under control conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	333	470	401,5	68,5
5	600	648	624	24
7	509	601	555	46
10	557	696	626,5	69,5

6. Trehalose contents in root of Tosun cultivar under salt stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	1024	800	912	112
5	1287	1430	1358.5	71.5
7	3710	3395	3552.5	157.5
10	5049	5049	5049	0

7. Trehalose contents in root of Tosun cultivar under drought stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	409	394	401.5	7.5
5	409	740	574.5	165.5
7	1688	1985	1836.5	148.5
10	5655	5335	5495	160

8. Trehalose contents in root of Cakmak cultivar under control conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	45	107	76	31
5	63	83	73	10
7	170	290	230	60
10	231	467	349	118

9. Trehalose contents in root of Cakmak cultivar under salt stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	165	300	232.5	67.5
5	535	579	557	22
7	654	536	595	59
10	2840	2875	2857.5	17.5

10. Trehalose contents in root of Cakmak cultivar under drought stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	222	325	273.5	51.5
5	885	735	810	75
7	1722	1586	1654	68
10	2470	3600	3035	565

11. Trehalose contents in shoot of Bolal cultivar under control conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	503	875	689	186
5	555	642	598.5	43.5
7	533	894	713.5	180.5
10	882	580	731	151

12. Trehalose contents in shoot of Bolal cultivar under salt stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	1393	1685	1539	146
5	1317	1997	1657	340
7	2685	1643	2164	521
10	2457	2778	2617.5	160.5

13. Trehalose contents in shoot of Bolal cultivar under drought stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	462	555	508.5	46.5
5	723	585	654	69
7	1000	1093	1046.5	46.5
10	3036	3429	3232.5	196.5

14. Trehalose contents in shoot of Tosun cultivar under control conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	460	300	380	80
5	416	430	423	7
7	450	642	546	96
10	530	550	540	10

15. Trehalose contents in shoot of Tosun cultivar under salt stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	1022	1818	1420	398
5	1732	1875	1803.5	71.5
7	2556	3000	2778	222
10	3120	2794	2957	163

16. Trehalose contents in shoot of Tosun cultivar under drought stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	1534	1316	1425	109
5	1420	2222	1821	401
7	1136	2319	1727.5	591.5
10	4550	2315	3432.5	1117.5

17. Trehalose contents in shoot of Cakmak cultivar under control conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	496	560	528	32
5	555	510	532.5	22.5
7	370	222	296	74
10	32	56	44	12

18. Trehalose contents in shoot of Cakmak cultivar under salt stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	1333	1419	1376	43
5	1473	1576	1524.5	51.5
7	1429	1700	1564.5	135.5
10	2293	2300	2296.5	3.5

19. Trehalose contents in shoot of Cakmak cultivar under drought stress conditions in μg/g fresh weight.

Time (days)	Exp.1	Exp.2	Mean	SEM
3	572	944	758	186
5	703	1060	881.5	178.5
7	1600	2145	1872.5	272.5
10	2926	2505	2725.5	210

APPENDIX B

THE ROUGH DATA OF TREHALOSE-6-PHOSPHATE SYNTHASE ENZYME ASSAY

1. Trehalose-6-phosphate synthase assay in root of Bolal cultivar under control conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	614	0	582.6	0	656.9
7	723.8	5	659.9	5	787.6
11	753.2	10	705.3	10	910.3
15	850.5	15	711.8	15	1073.4
20	795.1	20	725.8	20	1200
[protein]	0.0876 mg/ml		0.04745 mg/ml		0.10366 mg/mll

2. Trehalose-6-phosphate synthase assay in root of Bolal cultivar under salt stress conditions.

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	492.8			0	555.1
6.5	694.3	5.5	745	5	891.3
12	754.2	10.5	1007.1	10	1037
16.5	856.9	15	1166.2	15	1270.5
20	1067.5	20	1166.2	20	1299
[protein]	0.05 mg/ml		0.0521 mg/ml		0.084295 mg/mll

3. Trehalose-6-phosphate synthase assay in root of Bolal cultivar under drought stress conditions.

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	539.7	0	547.2	0	608
5	747.2	5	1214.6	5	1326.4
10	816	10	1940.1	10	2081.8
15	909.8	15	2342.9	15	2402.7
20	901.8	20	2850.4	20	2965.2
[Protein]	0.0627mg/ml		0.14235 mg/ml		0.1712662 mg/ml

4. Trehalose-6-phosphate synthase assay in shoot of Bolal cultivar under control conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)	Time (min)	Exp.4 (cpm)
0	583.6	0	692.8	0	502.8	0	593.1
10	999.2	5	987.6	6	622	5.5	887.9
20	1662	10	1114.8	11	886.9	10	984.6
		15	1318.4	15	941	15	1169.7
		20	1475	20	902.3	20	1264.5
[Protein]	0.3 mg/ml		0.3 mg/ml		0.2027 mg/ml		0.3 mg/ml

5. Trehalose-6-phosphate synthase assay in shoot of Bolal cultivar under salt stress conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)	Time (min)	Exp.4 (cpm)
0	536.2	0	611	0	599.5	0	814.5
10	986.2	5.5	841	5.5	924.8	5	1042
20	1338.9	10	1153.2	10.5	1192.2	10	1220.1
		15	1494.5	15	1389.2	15	1265.5
		20	1481	20	1505.5	20	1603.3
[Protein]	0.3 mg/ml		0.3 mg/ml		0.22776 mg/ml		0.1620162 mg/ml

6. Trehalose-6-phosphate synthase assay in shoot of Bolal cultivar under drought stress conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	639.9	0	629.4	0	711.3
4.5	1163.7	7	1532.4	5	804.5
8.5	1389.2	10	1648.7	10	884.3
15	2346.8	15	1782.4	15	1059.9
20	2242	20	2337.8	20	1237.6
[Protein]	0.3 mg/mll		0.3 mg/mll		0.1676025 mg/mll

7. Trehalose-6-phosphate synthase assay in root of Cakmak cultivar under control conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	566.2	0	649.9	0	441.4
5	640.4	10	697.8	5	530.7
10	665.4	15	754.7	10	647.9
15	691.8	20.5	940.3	15	727.9
20	782.1			20	807
[Protein]	0.0452 mg/ml		0.05 mg/ml		0.0866 mg/ml

8. Trehalose-6-phosphate synthase assay in root of Cakmak cultivar under salt stress conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	538.2	0	589	0	450
6	681.8	4.5	734.2	5	570.6
11	873.9	10	781.1	10	628.9
15.5	984.6	15	829.5	15	624
20	1059.5	20	834.5	20	695.3
[Protein]	0.104 mg/ml		0.0521 mg/ml		0.05318 mg/ml

9. Trehalose-6-phosphate synthase assay in root of Cakmak cultivar under drought stress conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)	Time (min)	Exp.4 (cpm)
0	685.8	0	543.1	0	546.7	0	525
5.5	1967.6	5	1002.6	5	852	5	1121.8
8.5	2620.4	10	1515	10	1172.7	10	1636.3
15	3398	15	2213	15	1413.2	15	2011
20	3472.4	20	2647.8	20	1531.5	20	2279.9
[Protein]	0.17 mg/ml		0.1387 mg/ml		0.1 mg/ml		0.129 mg/ml

10. Trehalose-6-phosphate synthase assay in shoot of Cakmak cultivar under control conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	658.9	0	739.7	0	628
3	1090.4	5	1162.3	5	1285
10	1605.8	10	1516	10	1458.6
15	1936.7	15	2030.4	15	1795.9
20	2404.8	20	2107.3	20	2108.8
25	2638.4				
30	3061.6				
[Protein]	0.3 mg/ml		0.3 mg/ml		0.3 mg/ml

11. Trehalose-6-phosphate synthase assay in shoot of Cakmak cultivar under salt stress conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)
0	711.8	0	574.6	0	531.2
5	1124.8	5	838	5	719.3
10	1745.5	10	1098.4	10	1256.1
15	1673.1	20	1365.3	15	1394.7
20	2128.2			20	1886.7
[Protein]	0.3 mg/ml		0.219 mg/ml		0.3 mg/ml

12. Trehalose-6-phosphate synthase assay in shoot of Cakmak cultivar under drought stress conditions

Time (min)	Exp.1 (cpm)	Time (min)	Exp.2 (cpm)	Time (min)	Exp.3 (cpm)	Time (min)	Exp.4 (cpm)
0	580.1	0	664.9	0	588.5	0	706.8
6	1495	5	1790.9	5	970.2	5	1091.9
10	1353.9	10	1828.3	10	1136.8	10	1442
15	1748	15	2304.4	15	1270	15	1875.3
20	2046.4	20	2593.3	20	1526	20	1900.7
[Protein]	0.3 mg/ml		0.3 mg/ml		0.3 mg/ml		0.3 mg/ml

APPENDIX C

THE ROUGH DATA OF TREHALASE ENZYME ASSAY

1. Trehalase assay in root of Bolal cultivar under control conditions.

a. Experiment (1): [Protein]= 0.13 mg/ml; $O.D._{Standard}$=0.265.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.029	0.021	0.025
5	0.04	0.057	0.0485
10	0.09	0.081	0.0855
20	0.071	0.08	0.0755
30	0.087	0.099	0.093

b. Experiment (2):[Protein]= 0.0455 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.097	0.121	0.109
5	0.119	0.121	0.12
10	0.122	0.123	0.1225
20	0.135	0.121	0.128
30	0.143	0.138	0.1405

c. Experiment (3):[Protein]= 0.04225 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$.	$O.D._{546}2$	Average
0	0.08	0.09	0.085
5	0.092	0.075	0.0835
10	0.084	0.095	0.0895
20	0.105	0.105	0.105
30	0.11	0.103	0.1065

2. Trehalase assay in root of Bolal cultivar under salt stress conditions.

a. Experiment (1): [Protein]= 0.13 mg/ml; $O.D._{Standard}$=0.118.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.104	0.107	0.1055
5	0.09	0.115	0.1025
10	0.105	0.125	0.115
20	0.107	0.107	0.107
30	0.167	0.125	0.146

b. Experiment (2): [Protein]= 0.094 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.14	0.15	0.145
5	0.16	0.164	0.162
10	0.146	0.166	0.156
20	0.17	0.185	0.1775
30	0.178	0.178	0.178

c. Experiment (3): [Protein]= 0.0546 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.118	0.109	0.1135
5	0.116	0.12	0.118
10	0.122	0.121	0.1215
20	0.12	0.122	0.121
30	0.122	0.135	0.1285

3. Trehalase assay in root of Bolal cultivar under drought stress conditions.

a. Experiment (1): [Protein]= 0.07 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.073	0.11	0.0915
5	0.08	0.113	0.0965
10	0.105	0.107	0.106
20	0.117	0.102	0.1095
30	0.107	0.11	0.1085

b. Experiment (2): [Protein]= 0.07215 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.145	0.172	0.1585
5	0.2	0.197	0.1985
10	0.189	0.187	0.188
20	0.184	0.197	0.1905
30	0.201	0.201	0.201

4. Trehalase assay in shoot of Bolal cultivar under control conditions.

a. Experiment (1): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.25.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.034	0.034	0.034
5	0.05	0.042	0.046
10	0.065	0.066	0.0655
20	0.088	0.089	0.0885
30	0.1	0.1	0.1

b. Experiment (2): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.25.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.13	0.132	0.131
5	0.126	0.125	0.1255
10	0.147	0.152	0.1495
20	0.15	0.152	0.151
30	0.152	0.152	0.152

c. Experiment (3): [Protein]= 0.1805 mg/ml; $O.D._{Standard}$=0.2425.

Time(min)	$O.D._{546}1$	$O.D._{546}1$	Average
0	0.14	0.14	0.14
5	0.16	0.145	0.1525
10	0.18	0.147	0.1635
20	0.165	0.16	0.1625
30	0.163	0.163	0.163

d. Experiment (4): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.12	0.121	0.1205
5	0.135	0.138	0.1365
10	0.13	0.142	0.136
20	0.13	0.145	0.1375
30	0.14	0.14	0.14

5. Trehalase assay in shoot of Bolal cultivar under salt stress conditions.

a. Experiment (1): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.25.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.052	0.05	0.051
5	0.049	0.057	0.053
10	0.063	0.066	0.0645
20	0.069	0.072	0.0705
30	0.09	0.067	0.0785

b. Experiment (2): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.25.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.072	0.087	0.0795
5	0.082	0.088	0.085
10	0.085	0.09	0.0875
20	0.088	0.08	0.084
30	0.08	0.087	0.0835

c. Experiment (3): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.15	0.159	0.1545
5	0.166	0.167	0.1665
10	0.17	0.17	0.17
20	0.17	0.173	0.1715
30	0.171	0.177	0.174

6. Trehalase assay in shoot of Bolal cultivar under drought stress conditions.

a. Experiment (1): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.097	0.14	0.1185
5	0.096	0.145	0.1205
10	0.142	0.14	0.141
20	0.138	0.139	0.1385
30	0.135	0.142	0.1385

b. Experiment (2): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.218	0.229	0.2235
5	0.251	0.233	0.242
10	0.266	0.24	0.253
20	0.24	0.257	0.2485
30	0.24	0.246	0.243

c. Experiment (3): [Protein]= 0.1848 mg/ml; $O.D._{Standard}$=0.1475

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.124	0.124	0.124
5	0.115	0.142	0.1285
10	0.116	0.145	0.1305
20	0.138	0.128	0.133
30	0.139	0.132	0.1355

d. Experiment (4): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.213	0.213	0.213
5	0.237	0.243	0.24
10	0.234	0.23	0.232
20	0.23	0.23	0.23
30	0.23	0.251	0.2405

7. Trehalase assay in root of Cakmak cultivar under control conditions.

a. Experiment (1): [Protein]= 0.05525 mg/ml; $O.D._{.Standard}$=0.252.

Time(min)	$O.D._{.546}1$	$O.D._{.546}2$	Average
0	0.037	0.042	0.0395
5	0.04	0.036	0.038
10	0.057	0.058	0.0575
20	0.065	0.066	0.0655
30	0.068	0.064	0.066

b. Experiment (2): [Protein]= 0.04 mg/ml; $O.D._{.Standard}$=0.257.

Time(min)	$O.D._{.546}1$	$O.D._{.546}2$	Average
0	0.056	0.056	0.056
5	0.06	0.061	0.0605
10	0.066	0.066	0.066
20	0.065	0.064	0.0645
30	0.064	0.07	0.067

8. Trehalase assay in root of Cakmak cultivar under salt stress conditions.

a. Experiment (1): [Protein]= 0.092 mg/ml; $O.D._{.Standard}$=0.257.

Time(min)	$O.D._{.546}1$	$O.D._{.546}2$	Average
0	0.166	0.117	0.1415
5	0.16	0.17	0.165
10	0.167	0.168	0.1675
20	0.17	0.163	0.1665
30	0.161	0.165	0.163

b. Experiment (2): [Protein]= 0.02 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.062	0.06	0.061
5	0.061	0.062	0.0615
10	0.065	0.067	0.066
20	0.066	0.066	0.066
30	0.068	0.07	0.069

c. Experiment (3): [Protein]= 0.06045 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.099	0.104	0.1015
5	0.097	0.115	0.106
10	0.118	0.115	0.1165
20	0.13	0.128	0.129
30	0.13	0.13	0.13

9. Trehalase assay in root of Cakmak cultivar under drought stress conditions.

a. Experiment (1): [Protein]= 0.04 mg/ml; $O.D._{Standard}$=0.265.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.088	0.075	0.0815
5	0.092	0.089	0.0905
10	0.092	0.092	0.092
20	0.091	0.096	0.0935
30	0.097	0.096	0.0965

b. Experiment (2): [Protein]= 0.05106 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.057	0.068	0.0625
5	0.073	0.084	0.0785
10	0.076	0.077	0.0765
20	0.073	0.084	0.0785
30	0.08	0.079	0.0795

10. Trehalase assay in shoot of Cakmak cultivar under control conditions.

a. Experiment (1): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.265.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.022	0.02	0.021
5	0.02	0.025	0.0225
10	0.023	0.021	0.022
20	0.03	0.028	0.029
30	0.032	0.028	0.03

b. Experiment (2): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.01	0.01	0.01
5	0.01	0.012	0.011
10	0.016	0.021	0.0185
20	0.013	0.011	0.012
30	0.018	0.018	0.018

c. Experiment (3): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.023	0.023	0.023
5	0.03	0.03	0.03
10	0.033	0.03	0.0315
20	0.032	0.03	0.031
30	0.03	0.035	0.0325

11. Trehalase assay in shoot of Cakmak cultivar under salt stress conditions.

a. Experiment (1): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.25.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.04	0.044	0.042
5	0.045	0.041	0.043
10	0.047	0.054	0.0505
20	0.05	0.047	0.0485
30	0.048	0.052	0.05

b. Experiment (2): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.257

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.016	0.015	0.0155
5	0.025	0.026	0.0255
10	0.025	0.024	0.0245
20	0.03	0.028	0.029
30	0.03	0.029	0.0295

c. Experiment (3): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.2255.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.04	0.04	0.04
5	0.04	0.04	0.04
10	0.049	0.049	0.049
20	0.045	0.05	0.0475
30	0.044	0.045	0.0445

12. Trehalase assay in shoot of Cakmak cultivar under drought stress conditions.

a. Experiment (1): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.266.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.026	0.03	0.028
5	0.036	0.036	0.036
10	0.04	0.034	0.037
20	0.035	0.035	0.035
30	0.038	0.039	0.0385

b. Experiment (2): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.257.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	average
0	0.067	0.072	0.0695
5	0.069	0.073	0.071
10	0.07	0.073	0.0715
20	0.075	0.079	0.077
30	0.08	0.07	0.075

c. Experiment (3): [Protein]= 0.2 mg/ml; $O.D._{Standard}$=0.266.

Time(min)	$O.D._{546}1$	$O.D._{546}2$	Average
0	0.045	0.044	0.0445
5	0.055	0.05	0.0525
10	0.05	0.074	0.062
20	0.083	0.065	0.074
30	0.077	0.07	0.0735

VITA

Personal Information

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Title of Ph.D. Thesis: Comparative Studies on Yeast and Wheat Trehalose Enzyme Systems.

Expected graduation: before June, 2003.

- 1996–1998: Master of Science in Biology, Middle East Technical University, Graduate School of Natural and Applied Sciences, Ankara, Turkey. (Cumulative Grade Point Average: 3,25/4,0).

Title of Master Thesis: Photoelectrochemical Hydrogen Production By Using Bacteriorhodopsin Immobilized in Polyacrylamide Gel.

- 1987 – 1991: Bachelor Degree of Science in Biology (Zoology branch), Cairo University, Cairo, Egypt. (With General Grade: Good)

Professional Skills

Growing bacteria in small scale and large scale by using sophisticated fermenters; Using photobioreactors for H_2 gas production; Protein purification and characterization techniques; Filtration chromatography methods; Native- and SDS-PAGE techniqes; Western and southern blotting; Plant transformation techniques; Carbohydrate analysis by HPLC and GC-MS; Spectrophotometric methods; Protein determination by several methods; familiar with use of radioactive materials.

Other Skills

Competent with all common Microsoft-DOS based software,Word, Excel, Power point program, and INTERNET/ WWW information services.

Professional Experiences and Previous Employment

- 1997: Student assistant in "Biochemistry" and "Plant Physiology" laboratories at Biology Department, METU.
- 1998: Teaching assistant in "Biochemistry" laboratory at Biology Department, METU.

Department, METU.

- 1998-2003 :Project assistant at Institute of Natural and Applied Sciences, METU.

- 2001- :Teacher in Pakistan Embassy Study Group-Ankara. Teaching Chemistry and Biology of pakistan secondary school system and International General Certificate of Secondary School Education (IGCSE British system).

Details of Research Projects

- Biohydrogen Production By Using Bacteria.
- Genetic manipulation of crop plants against osmotic stress (AFP-01-08-DPT 2001 K121060).
- The role of osmoprotectants under salt and drought stress conditions in different wheat cultivars (METU-BAP-2000-07-02-03).

Publications

Yücel M., **T. El-Bashiti**, İ. Eroğlu, V. Sediroğlu, L. Türker, U. Gündüz, "Photoelectrochemical hydrogen production by using bacteriorhodopsin immobilized in polyacrylamide gel" Hydrogen Energy Progress XIII , Proceedings of the 13th World Hydrogen Energy Conference, Beijing-China, June 12-15, 2000, eds. Mao, Z. Q. and Veziroğlu, T.N., Volume 1, 396-401.

Zabut, B., Sharif, F. A., **Bashiti, T**. 2002 "Photoproduction of Hydrogen by *Rhodobacter sphaeroides O.U. 001* in a Column Photoreactor: effect of *Halobacterium halobium*" The Journal of Islamic University, Gaza, Volume 10, pp 21-32.

Scholarships

-February 2000-2002 PhD scholarship from Arab Student Aid International (ASAI, P.O. BOX 10, FANWOOD, NJ 07023,

USA).

-1997-2002 scholarship of the Turkish Prime Ministry.

Languages Arabic: Native language

English: Fluent

Turkish: good