CLONING AND CHARACTERIZATION OF A NOVEL ABSCISIC ACID (ABA)-INDUCED HVA22-LIKE PROTEIN FROM *Triticum turgidum* spp. *dicoccoides* IN RESPONSE TO DROUGHT STRESS

by

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Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University

February 2010

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Keywords: Drought stress, HVA22-like protein, wild emmer wheat, *Triticum turgidum* ssp. *dicoccoides,* cellular localization

ABSTRACT

An HVA22-like protein was found to be differentially expressed in root tissue of wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*), under prolonged drought stress conditions. In this study we were able to clone and characterize the open reading frame of *HVA22-like protein* from root tissue of wild emmer wheat accession number TR39477, which was previously shown to be a drought-tolerant genotype. Sequence analysis indicated that *HVA22-like protein* product was a membrane protein and had four hypothetical transmembrane domains. Presence of the protein was shown by expressing it both in *Escherichia coli* and *Saccharomyces cerevisiae* and analyzing with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Localization of the protein in the cell was observed in *S. cerevisiae* utilizing a vector containing green fluorescent protein (GFP) gene. Results obtained using a confocal laser microscope indicated that the transformation of yeast cells with only the empty vector containing GFP gene yielded in a homogenous distribution of the GFP upon induction with galactose whereas the HVA22-like protein tagged with GFP was localized in the cell. Expression of GFP tagged HVA22-like protein was further confirmed with western blot analysis using mouse anti-GFP antibody. The work presented in this thesis was the first study to identify and characterize the *HVA22-like protein* and its protein product from *Triticum turgidum* ssp. *dicoccoides*.

KURAKLIK KOŞULLARI ALTINDA ABA İLE İNDÜKLENEN HVA22-TÜRÜ BİR PROTEİNİN YABANİ BUĞDAYDAN (*Triticum turgidum* ssp. *dicoccoides*) KLONLANMASI VE KARAKTERİZASYONU

Esen Doğan

Biyoloji Bilimleri ve Biyomühendislik Programı

Tez Danışmanı: Doç. Dr. Hikmet Budak

Anahtar Sözcükler: Kuraklık stresi, HVA22-türü protein, yabani buğday, *Triticum turgidum* ssp. *dicoccoides,* hücresel lokalizasyon

ÖZET

Uzun kuraklık koşulları altında, HVA22-türü bir proteinin yabani buğday (*Triticum turgidum* ssp. *dicoccoides)* kök dokusunda farklı olarak ekspres edildiğini göstermiştir. Bu çalışmada, yabani buğdayın kuraklığa dayanıklı olduğu önceden gösterilen TR39477 numaralı çeşidinin kök dokusundan HVA22-türü bir proteinin açık okuma çerçevesi (ORF) klonlanmış ve karakterize edilmiştir. Sekans analizi, protein ürününün dört adet olası transmembran bölgesi (domain) olduğuna işaret etmektedir. Proteinin varlığı hem *E. coli*'de hem de *S. cerevisiae*'de gerçekleştirilen ekspresyonlarla gösterilip SDS-PAGE yöntemi ile analiz edilmiştir. Proteinin hücre içindeki lokalizasyonu yeşil florosan protein (GFP) geni taşıyan bir vektörün kullanılması ile *S. cerevisiae*'de gözlenmiştir. Konfokal lazer mikroskobu kullanılarak elde edilen sonuçlar sadece boş vektör ile transforme edilen maya hücrelerinin galaktoz ile indüklenmesi sonrasında ekspres edilen GFP'nin hücre içinde homojen olarak dağıldığını gösterirken, HVA22-türü protein sekansını barındıran vektörün ekspresyonunun indüklenmesi sonrası GFP ile işaretlenmiş HVA22-türü proteinin hücre içinde lokalize olduğunu göstermektedir. GFP ile işaretlenmiş HVA22-türü proteinin ekspresyonu ayrıca western blot tekniği kullanılarak fare anti-GFP'si ile doğrulanmıştır. Bu tezde sunulan çalışma, HVA22-türü bir proteinin *Triticum turgidum* ssp. *dicoccoides* bitkisinden izole edilip karakterize edilmesini gösteren ilk çalışmadır.

Biricik Anneme ve Babama…

ACKNOWLEDGEMENTS

I would like to thank;

My supervisor Assoc. Prof. Hikmet Budak for giving me the opportunity to work on such an exciting subject and providing me with the resources generously.

Prof. Zehra Sayers, Prof. Selim Çetiner and Assoc. Prof. Batu Erman for their invaluable help and support.

Prof. Hüveyda Başağa, Assist. Prof. Alpay Taralp and Assist. Prof. Devrim Gözüaçık for critical reading of this text.

Prof. Michel J. Koch who made me ask questions, think and dare… and just for being there.

Prof. Vasıf Hasırcı for believing in me, caring for me and bearing with me.

Anastassia Zakhariouta, without whom the long nights of lab work would be unbearable, coming along and shared the good and bad with me all the time. Thank you my dear friend!

Bahar Soğutmaz Özdemir and Burcu Kaplan Türköz for their invaluable support and advices.

Emel Durmaz, Tuğsan Tezil, Özgür Gül and Çağrı Bodur for their technical support and unending patience for my questions.

Emir Yalvaç from Yeditepe University without whom the confocal images would just be a dream.

Last but not the least, my beloved Serkan Çelik for coming into my boat, steering me away from clouds and shining a light into my life.

This project was supported by COST project # FA0604-106 O 839

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ABBREVIATIONS

ABA	Abscisic acid
AGE	Agarose gel electrophoresis
cDNA	Complementary DNA
HVA22-like protein	HVA22-like protein gene
HVA22-like protein	HVA22-like protein gene product
IPTG	Isopropyl β-D-thiogalactoside
ORF	Open reading frame
Kz	*Triticum durum* var. Kızıltan
Kz7LS	Kz 7-day drought stressed leaf
Kz9LS	Kz 9-day drought stressed leaf
Kz-LC	Kz control leaf
Kz7RS	Kz 7-day drought stressed root
Kz9RS	Kz 9-day drought stressed root
Kz-RC	Kz control root
LWC	Leaf water content
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SOC	Super optimal broth
SPAD	Single photon avalanche diode

SWC	Soil water content
TR	*Triticum turgidum* ssp. *dicoccoides* tolerant genotype
TR7LS	TR 7-day drought stressed leaf
TR9LS	TR 9-day drought stressed leaf
TR-LC	TR control leaf
TR7RS	TR 7-day drought stressed root
TR9RS	TR 9-day drought stressed root
TR-RC	TR control root
TS	*Triticum turgidum* ssp. *dicoccoides* sensitive genotype
TS7LS	TS 7-day drought stressed leaf
TS9LS	TS 9-day drought stressed leaf
TS-LC	TS control leaf
TS7RS	TS 7-day drought stressed root
TS9RS	TS 9-day drought stressed root
TS-RC	TS control root
X-gal	5-bromo-4-chloro-3-indolyl β -D-thiogalactoside

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1. INTRODUCTION

There are biotic (plant pathogens, competition with other organisms etc.) and abiotic (drought, high temperature, salinity, cold etc.) environmental stress factors which affect growth and productivity in plants. Drought is the major stress factor for plants which have agronomical value. When the parts of earth that are available to agricultural practices all around the world is classified considering environmental stress factors; drought has the highest affect with 26% fraction. It is followed by mineral stress with 20% and cold and freezing stress with 15%. The other entire stress types sum up to 29% whereas only the areas which make up the 10% of total are not affected with any kind of stress factors (Kalefetoğlu and Ekmekçi, 2005).

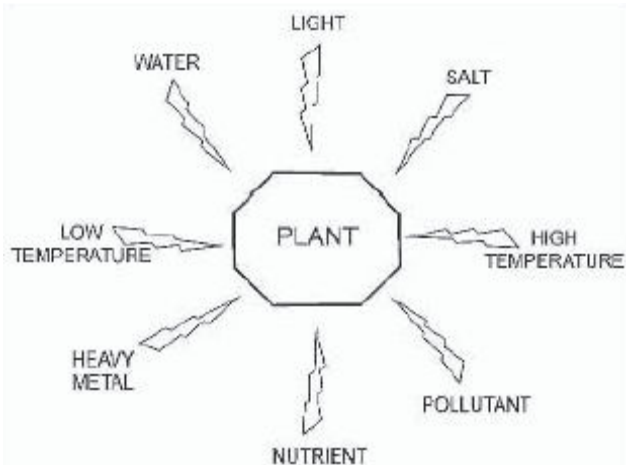


Figure 1.1 Types of abiotic stress factors that have adverse effects on plants. (Adapted from Madhava Rao K.V. et al., 2006)

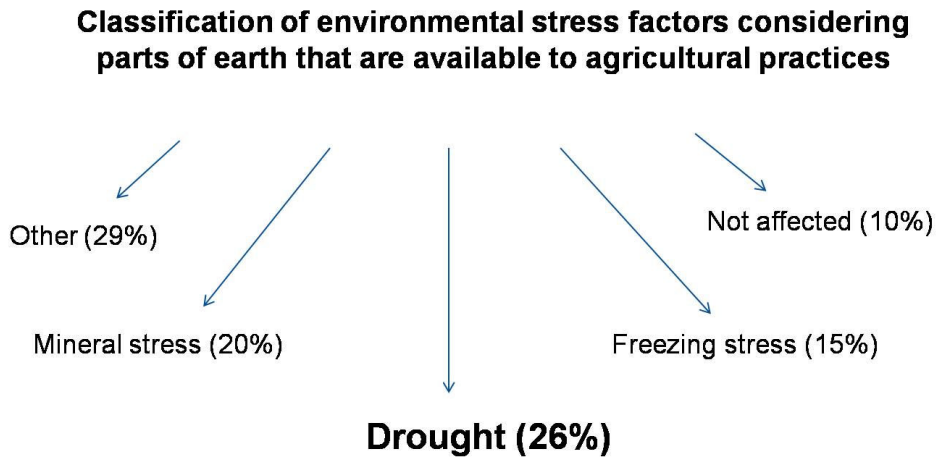


Figure 1.2 Classification of environmental stress factors. Drought is the major stress factor for plants which have agronomical value

As water resources become more limited, use of water for agricultural purposes is also getting more restricted. It is known that Turkey is included among the countries which constitute a risk group regarding global warming and this clearly points out the importance of taking measures against drought in an agricultural perspective.

Considering the fact that amelioration of agricultural areas is impossible either in terms of physical properties or almost impossible in financial terms; it is clear that making plants that have agronomical value more resistant to drought is a more applicable strategy in dealing with the problems that global warming will bring about. From an application point of view, in relation with the dynamics of duration of drought in every target area; it is very crucial that suitable genotypes are determined and selected while optimizing both the available water amount and efficiency of using available water in order to increase the yield.

Wild and primitive wheat types are richer in terms of genetic variation compared to modern wheat types. As a result, wild and primitive wheat genotypes are often utilized in molecular studies and breeding programs. Consequently, it is critical that mechanisms those are responsible for drought resistance and molecules that are effective in these mechanisms to be revealed and studied in wild emmer wheat so that resistance of modern bread wheat and modern durum wheat being the crops which are produced the most in worldwide can be improved (Vasil, 2007). There are studies related to genetic diversity for drought tolerance retained in the tetraploid wild emmer

wheat, which was originated and probably domesticated in Turkey (Luo et al., 2007; Nevo, 1998; Peleg et al., 2005).

Microarray technology is a very common and powerful tool used both in analysis of expression and identification of genes under various abiotic stress conditions. There are different kinds of microarray studies aimed at identifying stress-inducible genes under a variety of conditions, for instance cold, high salinity and drought, in Arabidopsis and rice and also in some other plants (Kawasaki et al., 2001; Seki et al., 2001; Seki et al., 2002; Rabbani et al., 2003; Dubouzet et al., 2003; Maruyama et al., 2004). Construction of cDNA libraries and expression sequence tag (EST) sequencing results are also very promising in terms of discovery of novel genes which are involved in stress response pathways via analysis of transcript profiles (Vij and Tyagi, 2007; Bohnert et al., 2006; Wong et al., 2005)

Although the molecular studies that are aimed at understanding the underlying mechanisms of different defense properties observed in plants against different environmental stress conditions are mostly performed in model plants such as *Brachypodium distachyon* and *Arabidopsis thaliana* due to reasons such as ease of handling of their relatively small genomic content; it would be wise to include plants that have agronomical value since the improvement of those plants is becoming more crucial considering the ever-increasing population of the world and increasing harshness of environmental conditions.

A recent study of our group (Ergen and Budak, 2009) resulted in the construction of subtractive cDNA libraries and sequencing ESTs in order to analyze the expression profile of drought-inducible genes under slow drought conditions in wheat genotypes and cultivar accessions originating from Turkey. An initial screen of 200 genotypes had resulted in selection of 26 of these, which were promising for purposes of screening the drought-inducible genes in terms of displaying contrasting properties either being drought-tolerant or drought-sensitive. This selection was based on both physiological data and phenotypic appearances. Physiological data consisted of leaf water content (LWC) determination, soil water content (SWC) determination and single photon avalanche diode (SPAD) measurements whereas phenotypic differences was observed in terms of change in stature of the plants, wilting, crispiness and paling of the leaves. SPAD values provided a relative chlorophyll content measurement of leaves

which changed during stress treatment and served as an indicator of the level of deterioration caused by water-deficit conditions. Detailed information on basis of SPAD technology and interpretation of SPAD measurements in chlorophyll content determination are given in section 2.4.2 of this text.

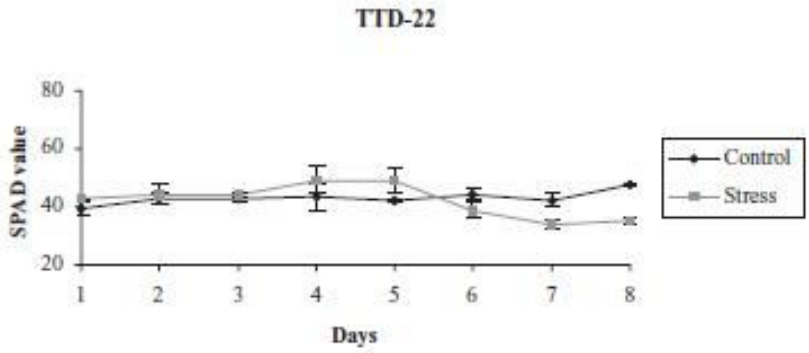


Figure 1.3 Graphical representation of SPAD values of wild emmer wheat sensitive genotype. After a temporary increase, SPAD values displayed a significant decrease for stress group of sensitive genotype of wild emmer wheat. (Adapted from Ergen and Budak, 2009)

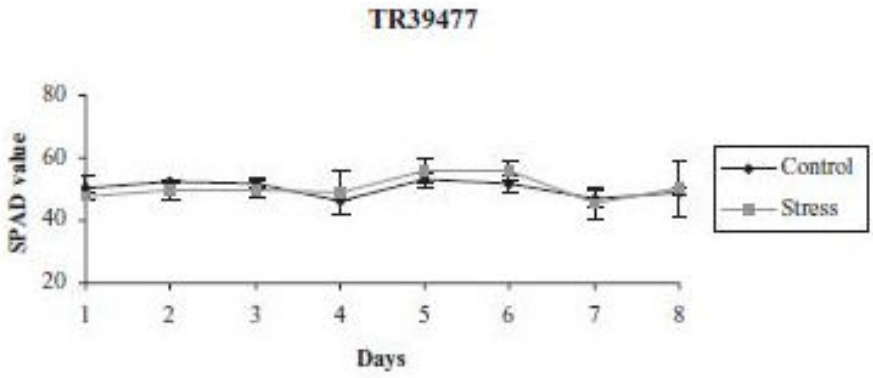


Figure 1.4 Graphical representation of SPAD values of wild emmer wheat resistant genotype. SPAD values did not display a significant difference for control and stress group of tolerant genotype of wild emmer wheat in contrast to sensitive genotype. (Adapted from Ergen and Budak, 2009)

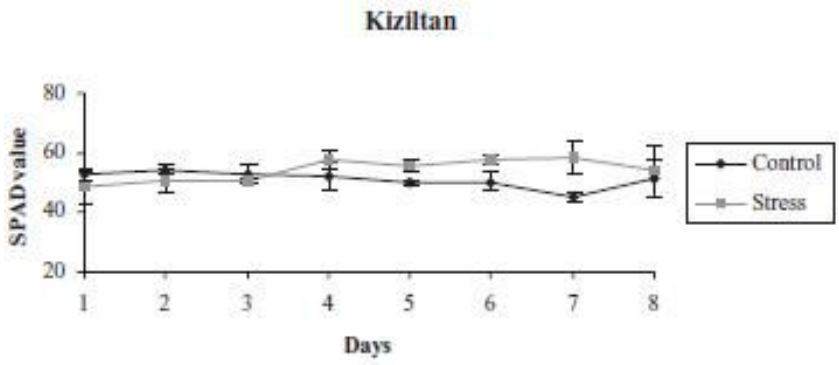


Figure 1.5 Graphical representation of SPAD values of modern durum wheat variety Kızıltan. A temporary increase was observed in the SPAD values of stressed plants of modern durum wheat variety Kızıltan at around fifth day of water shortage. (Adapted from Ergen and Budak, 2009)

A second selection was performed among those 26 contrasting genotypes by repeating the experiment and again considering LWC, SWC, SPAD measurements together with phenotypic deterioration levels under prolonged drought conditions and six cDNA libraries were constructed using seven day stressed leaf and root samples of tolerant (TR39477), sensitive (TTD-22) wild emmer wheat genotypes and modern durum wheat variety Kızıltan out of those 26 genotypes. Reason for selecting the seven day after the beginning of drought stress treatment as a time point for differential expression analysis was that the levels of LWC displayed a significant difference both for resistant and sensitive genotype (86% and 74% respectively). According to results of the study, an HVA22-like protein was found to be differentially expressed at expressed sequence tag (EST) level in root tissue of wild emmer wheat (*Triticum turgidum* spp. *dicoccoides*) tolerant genotype under slow drought conditions.

It is well-established that abscisic acid (ABA) mediated signaling is involved in many stress responses including those to drought stress. HVA22 is one of the proteins whose expression is induced and regulated by ABA which was first isolated from barley (*Hordeum vulgare* L.) (Guo and Ho, 2008). Many homologues of HVA22 have been identified in various organisms such as fungi, plants, animals but not in prokaryotes, implying that HVA22 and HVA22-like proteins play unique role especially in eukaryotes (Shen et al., 2001).

The objective of the present study was to show that an *HVA22-like protein* gene of wild emmer wheat was differentially expressed in root tissue of drought-tolerant genotype (TR39477) under prolonged drought conditions both at transcript level and protein level but it is not expressed in root tissue of cultivated modern durum variety Kızıltan under the same conditions. For that reason the drought treatment experiment was repeated and terminated at the seventh day of slow drought imposition since the subtractive cDNA libraries were constructed using 7-day stressed root and leaf samples for aforementioned three genotypes. A comparison of expression of HVA22-like protein in root and leaf tissues of sensitive genotype was also included.

We were able to clone, to our knowledge for the first time, the full length open reading frame (ORF) of an *HVA22-like protein* from *Triticum turgidum* spp. *dicoccoides.* Its sequence analysis indicated that ORF of *HVA22-like protein* translated into a 219 amino acid protein with an estimated average molecular weight of about 25 kDa and its pI was around 9.6. Further prediction for its structure also indicated that it was indeed a membrane protein as previously shown (Brands and Ho, 2002) and it had four hypothetical transmembrane domains.

In order to show the presence of protein product, expression analyses were done both in *Escherichia coli* and *Saccharomyces cerevisiae.* As expected its expression in *E. coli* retarded the growth of organism dramatically, however we were able to show its presence by SDS-PAGE analysis. Objective of expression studies done in *S. cerevisiae* was to show that HVA22-like protein displayed localization in yeast cells owing to its predicted transmembrane domains and utilized a vector containing GFP gene. Results obtained using a confocal laser microscope indicated that the transformation of yeast cells with only the empty vector containing GFP gene yielded in a homogenous distribution of the GFP protein upon induction with galactose whereas the HVA22-like protein tagged with GFP at its C-terminal was localized in the cell. Expression of GFP tagged HVA22-like protein was further confirmed with western blot analysis using mouse anti-GFP antibody.

2. BACKGROUND

2.1 Wild emmer wheat

Triticum turgidum spp. *dicoccoides* (wild emmer wheat) is wild wheat that is native to Fertile Crescent of the Near East which also covers the southeastern region of Turkey (Zohary and Hopf, 2001). It is annual, monocotyledonous and predominantly self-pollinating tetraploid wheat. It is known to be the sole wild stock in the *Triticum* genus, which is cross-compatible and interfertile with the *turgidum* wheat which is cultivated. Tetraploid wild emmer wheat (4X BBAA) is the progenitor of hexaploid modern bread wheat (*Triticum aestivum* L. spp. *aestivum*, 6X BBAADD) and wild relative of cultivated tetraploid modern durum wheat (*Triticum turgidum* L. spp. *durum*, 4X BBAA).

Figure 2.1 Map showing location of the Fertile Crescent. Dark green line represents the Fertile Crescent in which many of the crops that have agronomical value today are thought to be originated from and cultivated (Adapted from Brown T.A, 2002).

2.2 Plant responses to abiotic stress factors

Upon perception of abiotic stress there might either be production of new gene products together with the modification or degradation of the existing ones (Yamaguchi-Shinozaki and Shinozaki, 2005) as depicted in Figure 2.1. (Ashraf and Foolad, 2007). When the number of genes induced under abiotic stress conditions are taken into account, it is obvious that the discovery of new gene products are important for establishing drought stress tolerance in plants and it is also crucial for improving the crop yields in fields as a practical means (Ergen et al., 2009; Shinozaki and Yamaguchi-Schinozaki, 2007). There are a considerable number of reviews concerning abiotic stress response mechanisms in plants (Wingler et al., 2008; Neill et al., 2008; Nakashima et al., 2009; Bruce et al., 2007) and a more detailed description of responses to drought stress conditions can be found in the following sections of this text.

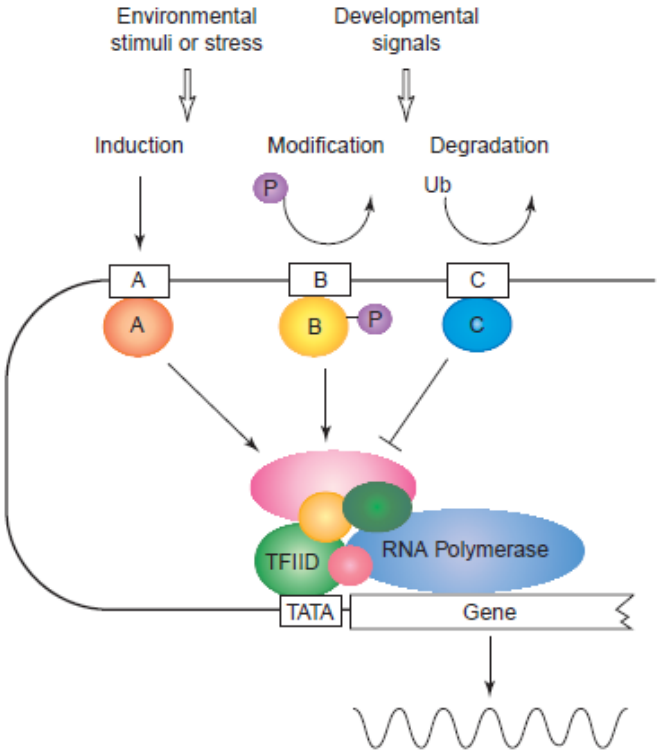


Figure 2.2 Transcriptional regulatory networks involved in environmental stress conditions. A, B and C in rectangular boxes represent the cis-acting factors, circles labeled as A, B and C denotes the transcription factors (Adapted from Yamaguchi-Shinozaki and Shinozaki, 2005).

2.2.1 Involvement of ABA-signaling in response to abiotic stress

Abscisic acid (ABA) is a plant hormone whose production is increased under stress conditions and also under water deficit conditions specifically (Christman et. al, 2005; Chinnusamy et al., 2008). An analogy between ABA and adrenalin can describe the role of ABA in plants as adrenalin in our veins also triggers and drives reactions against stress. Studies related to ABA dates back to early times regarding it as a stress hormone however it might have other functions even when there is no stress condition (Zeevart and Creelman, 1988). For example, it has long been known that ABA is involved in the seed development and germination and it also seems to be involved in embryo growth and differentiation together with accumulation of certain molecules in cotyledon or endosperm (Crouch and Sussex, 1981; Fong et al., 1983; Bray and Beachy, 1985; Vilardell *et al.*, 1990).

An intriguing point of view of ABA involvement in abiotic stress is that since it is a stress hormone which triggers a myriad of responses under various stress responses, inevitably there is cross-talk among some of the responses that are triggered by ABA such as cold, salinity and drought, however there are also some certain differences. (Lachno and Baker, 1986; Shinozaki and Yamaguchi-Shinozaki 2000). There are also indications so as to genes that are encoding putative RNA-binding proteins and an aldose reductase enzyme which is known to be involved in the synthesis of sorbitol in plants are also induced by ABA signaling. (Bartels et al., 1991; Ludevid et al., 1992).

Although it is well-established that there are many genes whose expression is activated by ABA whose production in turn is known to be increased upon sensing drought and other abiotic stress conditions (Shinozaki and Yamaguchi-Shinozaki, 2007); drought tolerance is not restricted to ABA activation of gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005; Shinozaki and Yamaguchi-Schinozaki, 2000). Among the genes which are identified as drought-inducible, some are induced by exogenous ABA treatment whereas some does not respond to same treatment. To date, expression analysis of drought-inducible genes has led to the identification of at least four independent regulatory pathways which play role in controlling gene expression that is responsible for drought stress tolerance in plants. Two of these pathways are

involved in ABA-dependent responses whereas the other two acts independent of ABA signaling (Yamaguchi-Shinozaki and Shinozaki, 2005; Barrero et al., 2006).

2.2.2 Effects of drought on plant physiology

Shortage of water usually results from insufficient rain fall, poor water storage capacity of soil and also when the water uptake of plants cannot compensate for the high rates of transpiration. Effects of drought range from molecular to morphological levels. As mentioned earlier, transcriptional regulation is one of the major responses to drought; expression of some genes might be turned whereas existent expression of certain genes might be ceased (Yamaguchi-Shinozaki and Shinozaki, 2005; Umezawa et al., 2006; Seki et al., 2007). Water deficit conditions can also lead to loss of turgor, change in cell volume, increase in the concentrations of various solutes, disruption of membrane integrity, increased levels of protein denaturation together with denaturation of some other molecular components as well (Bartels and Souer, 2003; Lawlor and Cornic, 2002; Lawlor, 2002; Grifth and Parry, 2002; Parry et al., 2002). A reduction in growth of plants is also observed (Kaya et al, 2006, Harris et al., 2002; Farooq et al., 2009). The extent of water deficit stress condition response of plants depends on the duration of water shortage, severeness of the water shortage as well as on the species, genotype and the developmental status of plants.

It is important to understand the functions of gene products formed under drought conditions in order to have a better evaluation of water deficit tolerance mechanisms of plants and to be able to improve the water tolerance of plants in field. Although there is a considerable number of genes which are characterized and shown to be involved in drought tolerance have been identified, the details of signal transduction and drought perception together with the following establishment of tolerance still waits to be evaluated and resolved (Madhava Rao K.V. et al., 2006; Ingram and Bartels, 1996.).

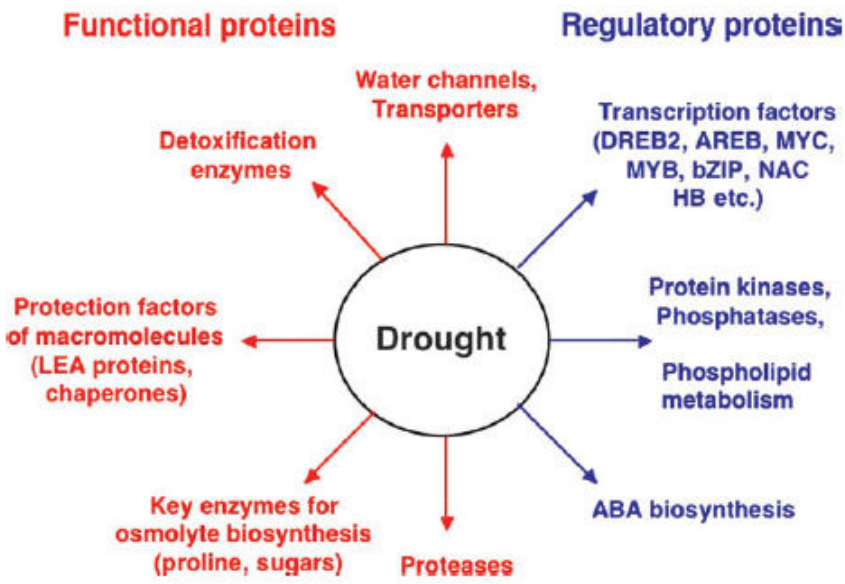


Figure 2.3 Functions of drought stress-inducible genes in stress tolerance and response (Adapted from Shinozaki and Yamaguchi-Schinozaki, 2007).

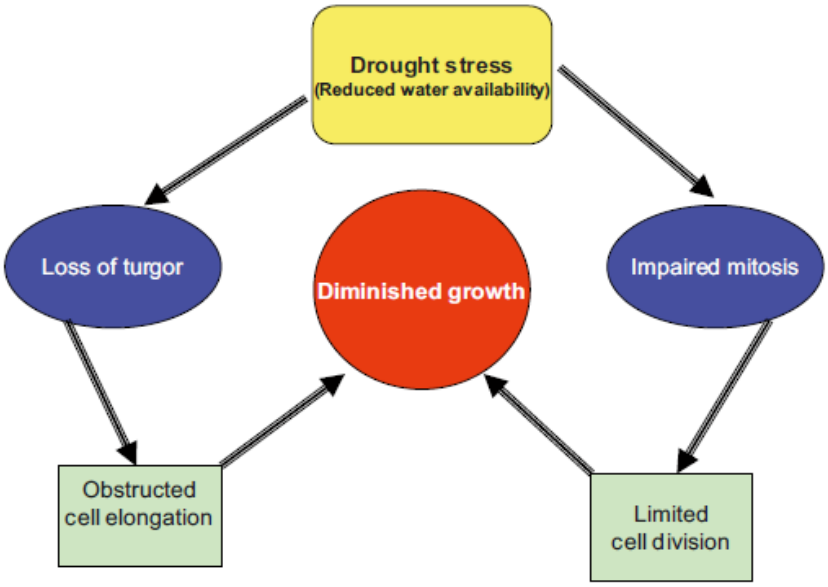


Figure 2.4 Scheme of possible mechanisms playing role in growth reduction in plants under water deficit conditions. (Adapted from Farooq et al., 2009).

It is important to understand the functions of gene products formed under drought conditions in order to have a better evaluation of water deficit tolerance mechanisms of plants and to be able to improve the water tolerance of plants in field. Although there is a considerable number of genes which are characterized and shown to be involved in drought tolerance have been identified, the details of signal transduction and drought perception together with the following establishment of tolerance still waits to be evaluated and resolved (Madhava Rao K.V. et al., 2006; Ingram and Bartels, 1996.).

2.2.2.1 Photosynthesis

Along with the reduction in crop growth and yield, leaf water content, and reduced nutrient uptake, drought stress also causes a decreased rate of photosynthesis upon stomatal closure, which is almost always the first measure of defense upon stress perception (Farooq et al., 2009; Mansfield and Atkinson, 1990; Cominelli et al., 2005; Tezara et al., 1999; Flexas and Medrano, 2002). As the drought conditions get harsher, a decrease in Rubisco activity is observed following stomatal closure (Bota et al., 2004), and there occurs a change in adjustment to CO_2 available in the chloroplast and also in relative total chlorophyll content (Loreto et al., 1995). Possible reason for the decline in photosynthesis rate of a plant under water deficit conditions might be due to the fact that increasing concentration of solutes in the cell results in a higher viscosity of the cytoplasm and they may become toxic hindering the activity of enzymes that are involved in the photosynthetic pathway (Hoekstra et al., 2001).

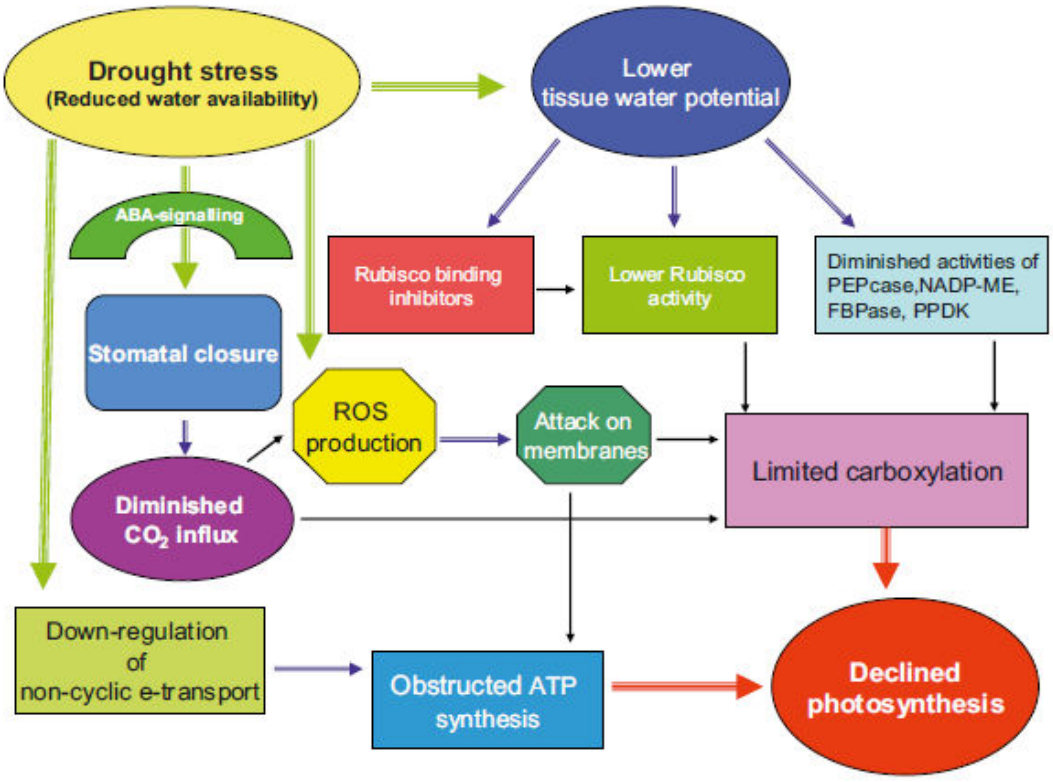


Figure 2.5 Possible mechanisms that result in the decreased rate of photosynthesis in a plant under drought conditions (Adapted from Farooq et al., 2009).

2.2.2.2 Importance of chlorophyll content determination in drought-stress response using single photon avalanche diode (SPAD) measurement

Measuring the chlorophyll content can be useful in diagnosing the health status of a plant (Kumar et al., 2002). Chlorophyll content can change in response to different environmental stresses (biotic or abiotic) (Fanizza et al., 1991; Samdur et al., 2000; Lawson et al., 2001) and it is also dependent on the developmental stage of the leaf (Costa et al., 2001).

Chemical extraction of chlorophylls which requires the destruction of leaf samples and makes use of various solvents in the process was used until newer non-destructive methods was developed. Traditional destructive method is a time consuming one and it also requires spectrophotometric measurements which in turn are converted into concentrations using standard solutions and equations (Arnon, 1949; Lichtenthaler, 1987; Ritchie, 2008). In contrast, newly developed optical methods are non-destructive, easy to perform and also can be used in the field very quickly however they provide a

relative chlorophyll content (i.e. index) rather than measuring absolute chlorophyll amounts in per unit leaf area (Hawkins et al., 2007; Markwell et al., 1995).

A SPAD (Single-Photon Avalanche Diode) meter (also known as a chlorophyll meter) is a device which measures the ratio of light transmitted at 920 nm to that at 650 nm; former is not affected by the leaf chlorophyll content whereas the latter is absorbed by chlorophyll strongly. Consequently a SPAD measurement is correlated to leaf chlorophyll content and it is found to be near-linear (Neufeld et al., 2006). The relationship between the ratio of the absorbance and chlorophyll amount of a leaf is also dependent on species (Bonneville and Fyles, 2006).

However, as the leaves become more injured due to stress, SPAD meter readings become unreliable (Neufeld et al., 2006). Several reasons are held responsible for that deviation of the near-linear relationship between the chlorophyll content and SPAD values. One of them is that chlorophyll amount becomes too low to be detected by SPAD meter accurately (Gratani, 1992). Another reason might be that the spatial distribution of chlorophyll might display variations resulting in additional scattering in the SPAD/total chlorophyll relationship (Castelli et al., 1996; Monje and Bugbee, 1992). Also the presence of necrotic tissue in the later stages of injury further changes the transmittance of light due to loss of cytoplasm and emergence of dead cells throughout the cell. It is known that especially the presence of dead cells and loss of pigments lead to a reduction in absorbance as enhancing the light transmission and also causing an increased scattering (Castelli et al., 1996; Monje and Bugbee, 1992).

2.3 HVA22-like protein and its homologues

HVA22-like protein family identified in various but limited number of plants such as barley and Arabidopsis (Chen et al., 2009; Chen et al.; 2002) and in some other eukaryotes, including mammals, is one of the known drought response proteins which also plays a unique role in eukaryotes but not in any of the prokaryotic organisms probably due to its membrane-associated nature and its role in vesicular trafficking (Guo and Ho, 2008; Shen et al. 2001).

HVA22 was first isolated from barley aleurone layers (Chen et al., 2002; Guo and Ho, 2008). Many homologues of HVA22-like proteins have been identified in

various organisms such as fungi, plants, animals but not in prokaryotes, implying that HVA22 and HVA22-like proteins play unique role especially in eukaryotes (Shen et al., 2001). Recently HVA22-like protein has been shown to be involved in vesicular trafficking and programmed cell death in cereal aleurone cells (Guo and Ho, 2008) together with autophagic internalization (Brands and Ho, 2002; Chen et al., 2009). However there is also evidence that not all HVA22-like protein homologues play role only in drought response in plants and they can also be involved in salt stress response. RNA interference studies performed in Arabidopsis also suggests that some HVA22-like homologues have role in plant reproductive development (Chen et al., 2002).

HVA22-like protein has been shown to have a yeast homolog Yop1p, which interacts with GTPase-interacting protein Yip1p, (Brands and Ho, 2003; Brands and Ho, 2002) and its deletion together with the deletion of Rtn4a, which is known to be a reticulon protein having role in shaping the endoplasmic reticulum, has also been demonstrated to be responsible for the disruption of endoplasmic reticulum membranous networking structure. This finding is supported by the later work of a group displaying the involvement of HVA22 in membranous vesicle formation in autophagy pathway (Chen et al., 2009). HVA22-like protein is also a unique type of ABA-inducible genes considering the fact that it has been shown to be induced by exogenous treatments of cycloheximide which is known to be a chemical that blocks translational elongation (Shen et al. 1993)

One definition of the candidate genes is given as the “transgenic intervention points” (Gutterson and Zhang, 2004) since they can be utilized in agricultural enhancement of crops. In that context, HVA22-like protein is a candidate gene which plays an enhancing role in tolerance under water deficit conditions suggested by the results of two earlier studies (Ergen and Budak, 2009; Ergen et al., 2009). For that reason it is important to investigate that protein family in plants those have agronomical value.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

Triticum turgidum spp. *dicoccoides* accession numbers TR39477and TTD-22 and *Triticum durum* variety Kızıltan used in this study were obtained from a set of seeds characterized in a previous study of our group (Ergen & Budak 2009).

3.1.2 Chemicals and Commercial Kits

A detailed list of chemicals and kits used in the present study is given in Appendix C.

3.1.3 Buffers and Solutions

The growth media, buffers, and solutions used in the present study were prepared according to Sambrook et al., 2001 unless otherwise stated.

3.1.4 Primers

Primers were commercially synthesized in Iontek Company, Istanbul.

3.1.5 Culture Growth Media

a. *Escherichia coli*

LB Broth (Lennox L broth) containing tryptone, yeast extract and NaCl (Sigma) was used for liquid culture preparation of bacterial cells. 20 g of LB Broth was used for preparation of 1 L liquid medium. The liquid medium was autoclaved at 121°C for 15 minutes before using. Ampicillin was added to liquid medium afterwards at a final concentration of 100 µg/ml for selection.

LB Agar (Luria Bertani, Miller) containing tryptone, yeast extract, NaCl and agar (Sigma) were used for preparation of solid medium. 40 g of LB Agar was used for preparation of 1 L solid medium and the solution was then autoclaved at 121°C for 15 minutes. Autoclaved medium was poured to petri plates after cooling down to ~50°C. Ampicillin was added to liquid medium afterwards at a final concentration of 100 µg/ml for selection.

b. *Saccharomyces cerevisiae*

Yeast peptone-dextrose (YPD) agar medium was used for viability test of INVSc1 (Invitrogen, Germany) yeast strain. YPD liquid medium was used in preparation of competent yeast cells.

Complex synthetic minimal medium lacking uracil (SD-Ura) medium was used for both liquid expression cultures and solid medium in plates for propagation of appropriate colonies under selection. Liquid medium contains drop-out medium and either 2 % glucose for regular growth or 0.1% glucose for expression cultures. Solid SD-Ura medium contains 2% glucose.

3.1.6 Equipment

A detailed list of equipment used in the present study is given in Appendix D.

3.2 Methods

3.2.1 Plant growth conditions

Seeds were surface sterilized before pre-germinating them in petri dishes for one week at 4°C in the dark. Seedlings which were at the similar germination stage were transferred to six pots for wild emmer wheat resistant genotype, six pots for wild emmer wheat sensitive genotype and five pots for durum wheat var. Kızıltan (two seeds per pot) which contained a clay : sand mixture (3:2). Mineral composition was adjusted afterwards with the addition of 100 ppm N, 2.5 ppm Fe, 100 ppm P, 20 ppm S and 2.5 ppm Zn and mixed thoroughly before planting the seeds. Total weight of the soil used was 1700g.

Plants were then grown under a natural environment in greenhouse (10–12 hours daylight; temperature 25±3°C). Positions of the pots were changed randomly every 3-4 days and well watered daily for three weeks. Drought stress treatment was started by withholding water from stress treatment pots whereas control group plants were kept with well-watering scheme daily. Treatments were performed in triplicate.

Plant samples were collected at the end of seventh day of drought treatment at the regional midday. The drought-tolerant and drought-sensitive wild emmer wheat genotypes together with modern durum wheat variety Kızıltan were compared in terms of physical appearance based on the SPAD measurements.

3.2.2 Single-Photon Avalanche Diode (SPAD) measurements

SPAD measurements were taken from the leaf tissues for relative chlorophyll content determination starting with the stress treatment applied to stress group plants using SPAD-502 Chlorophyll Meter (Konica, Minolta, Ramsey, NJ, USA). SPAD measurements were also taken for the control group plants until the end of the sample collection which was performed on the seventh day.

3.2.3 Sample collection

Root and leaf samples from both control group plants and stress group plants were collected on the seventh day of treatment and directly frozen in liquid nitrogen and stored at -80°C. 9-day stress root and leaf samples were obtained from the tissue sample collection of our group's previous study (Ergen and Budak, 2009).

3.2.4 Total RNA isolation

200 mg leaf and 300 mg root samples were first ground in liquid nitrogen using autoclaved mortars and pestles. After thorough grinding either 2 ml Trizol® (Invitrogen) or TRI Reagent® (Sigma Aldrich) was added and continued grinding. 1 ml of homogenized sample was then transferred to a microcentrifuge tube using a wide-bore pipette tip. Homogenized samples were kept on ice until all samples were homogenized. All the processed samples were then incubated at room temperature for 10 minutes. 0.4 ml chloroform was added to each sample and the tubes were shaken vigorously and incubated at room temperature for 5 minutes. The samples were centrifuged at 11,000 x g for 15 minutes at 4°C. The upper aqueous phase which contained the RNA was transferred to a clean microcentrifuge tube. Following extraction with chloroform, 0.5 ml isopropanol was added in order to precipitate RNA. The samples were then incubated at room temperature for 10 minutes and centrifuged at 11,000 x g for 10 minutes at 4°C. RNA pellet was washed with 1 ml 75% DEPC-treated ethanol. The samples were vortexed until the pellet was removed from the bottom of the tube. After washing the samples were centrifuged at 7,500 x g for 5 minutes at 4°C. Then the supernatants were discarded carefully and the RNA pellets were air-dried until

there was no liquid in the microcentrifuge tubes. The pellets were then dissolved in 30 µl RNase-free water incubating at 55°C for one hour and by pipetting up and down every 15 minutes to ensure complete dissolution. For quantification of isolated total RNA, NanoDrop spectrophotometer was used. The samples were then stored at -80°C until further use.

3.2.5 First strand cDNA synthesis

First strand cDNA synthesis by reverse transcription was performed with 1.5 µg of total RNA for each sample using Transcriptor High Fidelity cDNA synthesis kit (Roche, Germany) following manufacturer's instructions.

3.2.6 Primer design

Expressed sequence tag (EST) sequence (Accession number: FK827962) which was annotated as HVA22-like protein K from a previous study (Ergen & Budak, 2009) was first translated into its corresponding amino acid sequence before using **B**asic **L**ocal **A**lignment **S**earch **T**ool for protein sequences version (BLASTp). The most significant hit (probability score of $6e^{-25}$) was a complete coding sequence *Receptor expression-enhancing protein 6 mRNA* (Accession number: EU962261) which was amplified from Zea Mays (clone 241353). The sequence was submitted in 2008 and contained 1108 bases. Primers were designed covering the 591 bp region from base 223 to 813 which included a start codon and ended with a stop codon therefore defined a open reading frame which had the potential of coding HVA22-like protein.

Details of the sequences and primer design scheme can be found in Appendix D.

Table 3.1Primer sequences designed for wild emmer wheat resistant and sensitive genotypes and durum wheat Kızıltan HVA22-like protein coding sequence.

Name	Orientation	Sequence
Hva22-like protein	Forward	5'- ATG GCT CTC CTC GCC CC -3'
	Reverse	5'- TTA AGT TTC AGT TCC CGA CAC ACC AGC -3'

For localization experiments, a different set of gene-specific primers were designed with addition of homologous recombination site sequences those were present in the **y**east **e**nhanced **g**reen **f**luorescent **p**rotein (yEGFP) vector to 5' ends of primers given in Table 3.1. Stop codon was removed from the reverse primer sequence in order to be able to tag the protein with GFP.

Table 3.2 Primer sequences designed for localization experiments

Name	Orientation	Sequence
Hva22-like protein-GFP	Forward	5'- ACC CCG GAT TCT AGA ACT AGT GGA TCC CCC ATG GCT CTC CTC GCC CC -3'
	Reverse	5'- AAA TTG ACC TTG AAA ATA TAA ATT TTC CCC AGT TTC AGT TCC CGA CAC ACC AGC -3'

3.2.7 PCR amplification of HVA22-like protein cDNAs

PCR reactions were set up using the reverse transcription products. iTaq DNA Polymerase Kit (iNtRON, South Korea) was used following manufacturer's instructions for seven day and nine day stressed root samples of wild emmer wheat drought-tolerant genotype and modern durum wheat variety Kızıltan. For the rest of the samples Taq DNA Polymerase (Fermentas, Germany) was used again following manufacturer's instructions.

Reactions were run in a gradient PCR machine with an initial denaturation at 94°C for 5 minutes. Thirty cycles were performed with denaturation at 94°C for 1 minute; annealing either at 55°C or 57.3°C or 60°C for 45 seconds and extension at 72°C for 1 minute. Final extensions were done at 72°C for a further 10 minutes. Results were checked at 1% agarose gel by running the gel at 100V for 35 minutes in 1X TBE buffer using 5µl sample from each of the reaction tubes and adding 1 µl of 6X loading dye.

3.2.8 Gel extraction

After analysis of the PCR results by AGE, the remaining PCR products of 45µl which proved to contain the ~660bp amplicon of interest were run this time in 1.5% agarose gel for the purpose of obtaining sharper bands.

The agarose gel fragments with expected molecular weights were excised with a clean scalpel. The bands of interests were purified using QIAquick® Gel Extraction Kit (Qiagen, Germany) following the manufacturer's instructions. cDNA was eluted in 30 µl sterile dH_2O and quantified by measuring the absorbance of samples at 260 nm using NanoDrop spectrophotometer. The samples were stored at -20°C.

3.2.9 Ligation to cloning vector

The PCR products were ligated to the pGEM®-T Easy Vector System I (Promega, Germany) in 10-µl reactions. 3:1 (insert:vector) ratios were prepared in order to obtain proper ligation via T-A cloning. Ligation reactions were performed following manufacturer's protocol. The reactions were incubated overnight at 4°C to maximize the number of transformants as indicated by the manufacturer's protocol.

3.2.10 Preparation of chemically competent cells

From an *E .coli* DH5α strain, single colony was inoculated in 50 ml LB. It was left for growth overnight (~16 hours). An aliquot of 4 ml from the overnight culture was transferred to 400 ml LB medium in a sterile 2 L flask. Cells were grown until an OD_{600} measurement of ~0.375 was reached and the culture was divided into pre-chilled 50 ml falcon tubes. Falcons were incubated on ice for 10 minutes before centrifugation at 2,700 x rpm for 7 minutes at 4°C. Resulting supernatant was discarded and the remaining cell pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% Glycerol, 10 mM PIPES – pH: 7.0). The falcon tubes were then centrifuged again at 1,800 x rpm for 5 minutes at 4°C. Supernatant was discarded and the cell pellet was once again resuspended in 10 ml same ice-cold $CaCl_2$ solution. Then falcons were incubated on ice for 30 minutes and were centrifuged at 1,800 x rpm for 5 minutes at 4°C. Supernatant was discarded and cell pellet was resuspended in 2 ml of ice-cold $CaCl_2$ solution. Resuspended cell pellets were then aliquoted dispensing 200 µl into pre-chilled 2 ml eppendorf tubes and immediately freezing in liquid nitrogen. The

competent cells were then stored at -80°C. A control transformation was also performed.

3.2.11Transformation

Ligation reaction end products (5 µl) was added into 100 µl chemically competent DH5α strain of *Escherichia coli* and kept on ice for 20 minutes after gently mixing the suspension by stirring with the help of the pipette tip. Cells were then subjected to heat-shock at 42°C for 45 seconds and directly returning them into ice afterwards. After a further incubation on ice for 5 minutes, 1 ml SOC medium was added onto each tube and left for incubation at 37°C for 45 minutes.

Because the ligation products were of a TA-cloning procedure, from each tube directly 200 µl of cells was dispensed onto LB plates containing 100 µg/ml ampicillin since pGEM®-T Easy vector contained an ampicillin resistance gene as a selectable marker. 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal were added onto plates and allowed to dry at 37°C for 30 minutes right before spreading for the purposes of blue/white colony screening. Plates were then incubated at 37°C for ~12 hours.

3.2.12 Colony selection

pGEM®-T Easy vector (Promega, Germany) allowed for blue/white selection by utilizing LacZ gene and white colonies were selected as positive clones and used in the following procedures.

3.2.13 Plasmid isolation

For confirmation of the selection of "true" positive colonies, selected white colonies were inoculated in 4 ml of LB medium containing 100 µg/ml ampicillin and incubated overnight (12-16 hours) at 37°C with constant shaking at 270 x rpm. Plasmid isolation was then performed using High Pure Plasmid Isolation Kit (Roche, Germany) following manufacturer's instructions. DNA was eluted in 50 µl elution buffer and quantified using NanoDrop spectrophotometer. Samples were kept at -20°C for further use.

3.2.14 Restriction digestion confirmation

pGEM®-T Easy vector provided EcoRI cut site in order for extraction of the insert in one reaction for downstream application Restriction digestion of the isolated plasmids using EcoRI cut site was also due the fact that original primers lacked specific restriction sites in design.

3.2.15 Sequencing

Selected plasmids were sent to REFGEN Biotechnology Company, ANKARA for commercial sequencing.

3.2.16 Sequence analysis

The sequences obtained were first subjected to the VecScreen algorithm (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) provided in the National Center for Biotechnology Information (NCBI) webpage in order to eliminate the contaminating vector sequences. BLASTP, BLASTN, BLASTX, and TBLASTX algorithms (Altschul et al., 1997; Altschul et al., 1990) were used to analyze the DNA sequences which are all provided at NCBI webpage (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

Protein secondary structure and membrane topology predictions was performed using HMMTOP server v2.0 (http://www.enzim.hu/hmmtop/html/submit.html) and the results were confirmed utilizing membrane topology predictions were performed utilizing TMHMM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Graphical representation of predicted transmembrane helices was shown using and TMRPres2D application (http://bioinformatics.biol.uoa.gr/TMRPres2D/) which provides two dimensional drawings of the given sequence. Protein structure modeling was performed using Modweb algorithm (http://modbase.compbio.ucsf.edu/ModWeb20-html/modweb.html) and the output then was visualized using Pymol software (http://www.pymol.org/).

In order to investigate the functional relationships of HVA22-like protein with other known proteins a conserved domain search was performed using the tool provided in NCBI conserved domain search webpage (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Obtained result was further analyzed using **C**onserved **D**omain **A**rchitecture **R**etrieval **T**ool (CDART) (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi).

3.2.17 Protein Expression Analysis

3.2.17.1 Subcloning into expression vector

3.2.17.1.1 Isolation of expression vector from glycerol stock

Several 4 ml LB medium containing 100 µg/ml ampicillin were inoculated with 200 µl TOPO cells (*E. coli*) containing empty pET22b(+) expression vector and incubated at 37°C overnight by constant shaking at 280 x rpm. Plasmid isolations were performed using High Pure Plasmid Isolation Kit (Roche, Germany) following manufacturer's instructions.

3.2.17.1.2 Restriction digestion of expression vector and cloning vector containing fragment of interest

Both pET22b(+) vector (~1 µg) and the plasmid containing fragment of interest (~1 µg) were digested with 10 units of EcoRI (Fermentas, Germany) at 37°C for ~2.5 hours. In the last 45 minutes for incubation, 1 µl of calf intestinal alkaline phosphatase (CIAP) (Fermentas, Germany) was added into the microcentrifuge tube containing pET22b(+) vector as substrate in order to prevent the possible self-ligation of vector. Results were analyzed with agarose gel electrophoresis.

3.2.17.1.3 Gel extraction

Bands corresponding to 660bp HVA22-like protein coding sequence and to ~5.4kb pET22b(+) vector were extracted using QIAquick® Gel Extraction Kit (Qiagen, Germany) following manufacturer's instructions. Concentrations were determined using NanoDrop spectrophotometer and by agarose gel electrophoresis.

3.2.17.1.4 Ligation

Ligations were performed using a insert:vector ratio of 5:1 corresponding to an amount of 100 ng vector and 55 ng of insert in a total of 10 µl reaction volumes. Proper control ligations were also set up together with the actual ligation reaction. Reaction mixtures were then incubated at 4°C overnight.

3.2.17.2 Transformation of pET22b(+) expression vector construct containing *HVA22-like protein* ORF

Ligation reaction end products (2 µL) were added onto 100 µL chemically competent DH5α strain of *Escherichia coli* and kept on ice for 20 minutes after gently mixing the suspension by stirring with the help of the pipette tip. Cells were then subjected to heat-shock at 42°C for 45 seconds and directly returning them into ice afterwards. After a further incubation on ice for 5 minutes, 1 ml SOC medium was added onto each tube and left for incubation at 37°C for 45 minutes.

Cells were pelleted at 5,000 x g for 3 minutes and resuspended in the remaining medium (~100 µl) after discarding the supernatant. Resuspended cells were then dispensed onto LB plates containing 100 µg/ml ampicillin since expression vector contained an ampicillin resistance gene as a selectable marker. 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal were added onto plates and allowed to dry at 37°C for 30 minutes right before spreading for purposes of blue/white screening. Plates were then incubated at 37°C for ~12 hours.

3.2.17.3 Colony PCR

For confirmation of the true positive colonies colony PCR was performed with the gene-specific primers given in Table 3.1. using the reaction set-up and cycling parameters described in section 3.2.6 of this text at a annealing temperature of 57.3°C.

3.2.17.4 Plasmid isolation

In order to be able to transform the Rosettagami2™ (Novagen, Germany) and BL21(DE3) *E. coli* competent cells for expression studies, inoculations from true

positive colonies of DH5α *E. coli* competent cells containing *HVA22-like protein* ORF in pET22b(+) vector (Novagen, Germany) were performed.

White colonies which were determined to be true positive were inoculated in 4ml of LB media containing 100 μg/ml ampicillin and were then incubated overnight (12-16 hours) at 37°C with constant shaking at 270 x rpm. Plasmid isolation was performed using a commercial plasmid isolation kit (Roche, Germany) following manufacturer's instructions. DNA was eluted in 50 μL elution buffer and quantified using NanoDrop spectrophotometer. Samples were kept at -20°C for further use.

3.2.17.5 Transformation into Rosettagami2™ and BL21(DE3) *E. coli* competent cells

Ligation reaction end products (4 μL) were added into 100 μL chemically competent DH5α strain of *Escherichia coli* and kept on ice for 25 minutes after gently mixing the suspension by stirring with the help of the pipette tip. Cells were then subjected to heat-shock at 42°C for 45 seconds and directly returning them into ice afterwards. After a further incubation on ice for 5 minutes, 1 ml SOC medium was added onto each tube and left for incubation at 37°C for 55 minutes.

Cells were pelleted at 5000 x g for 3 minutes and resuspended in the remaining medium (~100 μl) after discarding the supernatant. Resuspended cells were then dispensed onto LB plates containing 100μg/ml ampicillin since expression vector contained an ampicillin resistance gene as a selectable marker. 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-gal were added onto plates and allowed to dry at 37°C for 30 minutes right before spreading for purposes of blue/white screening. Plates were then incubated at 37°C for ~12 hours.

3.2.17.6 Orientation determination

Restriction digestion with XhoI (Fermentas, Germany) was performed in order to determine the orientation of insert. Reaction mixtures were incubated at 37°C for 3 hours and were then thermally inactivated incubating at 80°C for 20 minutes. Results were analyzed using agarose gel electrophoresis.

3.2.17.7 Culture inoculation

In order to prepare starter cell cultures for the actual expression, 5 ml LB media containing 100 µg/ml ampicillin was inoculated using colonies which were previously determined to contain the HVA22-like protein coding sequence in correct orientation by restriction digestion. Those cultures were then incubated at 37°C overnight by constant shaking at 280 x rpm.

3.2.17.8 Induction with IPTG, growth curve construction and sample collection

In order to determine their density, OD_{600} measurements of starter cultures were taken and diluted to a final OD_{600} value of 0.1 using fresh 50 ml LB media containing 100 µl/ml ampicillin in 250 ml flasks. Cells were grown until an optical density of ~0.6 was reached (~2.5 hours after dilution). Then cells were induced by addition of IPTG at a final concentration of either 0.5 mM or 0.7 mM or 1 mM IPTG. One flask was kept uninduced for control purposes.

Samples (1 ml) were collected by pelleting the cells at 13,200 x rpm for one minute. Supernatants were discarded and pellets were immediately frozen and kept until use for protein extraction at -20°C. Sample collection was performed in one-hour intervals starting with the induction while recording OD_{600} values.

3.2.17.9 Protein extraction

Cell pellets were resuspended thoroughly in 25 µl lysis buffer containing 1 mg/ml lysozyme, 25 mM Tris-HCl pH 8.5, 10 mM EDTA and 50 mM glucose. An equal volume of triton buffer containing 25 mM DTT, 100 mM NaCl, 200 mM $MgCl_2$ and 0.8 % Triton X-100 was then added to resuspended pellets in order to achieve complete lysis and solubilization. Solubilized cells were then centrifuged for 10 minutes at 13,200 x rpm in a tabletop centrifuge at 4°C. Supernatants were transferred into a clean 1.5-ml microcentrifuge tube. 3 µl 6X SDS loading dye was added to 15 µl of those supernatants for SDS-PAGE analysis.

3.2.17.10 SDS-PAGE analysis

SDS-Polyacrylamide gels with a 5% stacking gel concentration and a 12% separating gel concentrations were prepared as described in Sambrook et al., 2001 and 18 µl of samples were loaded into each lane. Gels were stained overnight with Coomassie Blue R-250 at room temperature and destained overnight with 10% acetic acid solution at room temperature.

3.2.17.11 Sequencing

Plasmids with which the expression analyses were performed were sent to Refgen, Ankara for commercial sequencing.

3.2.17.12 Sequence analysis of pET22b(+) vector containing expressed coding sequence

The sequences obtained were first subjected to the VecScreen algorithm (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) in order to eliminate the contaminating vector sequences. Then BLASTN algorithm was used for comparison of the expression vector construct sequence with the cloning vector construct which was previously shown to contain the coding sequence for *HVA22-like protein*.

3.2.18 Cellular Localization

3.2.18.1 Amplification of *HVA22-like protein* with homologous recombination compatible gene-specific primers

PCR reactions were set up using remaining cDNAs obtained as described in section 3.2.4 as templates. In order to obtain blunt-end PCR products which was required for homologous recombination process and for improved accuracy *pfu* DNA polymerase (Fermentas, Germany) was used.

Reactions were run in a gradient PCR machine for determination of the optimum annealing temperature. PCR was started with an initial denaturation at 94°C for 5 minutes. Thirty five cycles were then performed with denaturation at 94°C for 1 minute;

annealing either at 55°C or 57.3°C or 60°C for 1 minute and extension at 72°C for 2 minutes. Final extensions were done at 72°C for a further 15 minutes. Results were analyzed using AGE and subsequent extraction from agarose gel was performed using a commercial gel extraction kit (Roche, Germany) following manufacturer's instructions. Concentration was determined using NanoDrop spectrophotometer.

3.2.18.2 Linearization of yEGFP vector

Linearization of yEGFP vector was achieved by incubating 2 µg of vector with 10U SmaI restriction enzyme (Fermentas, Germany) for ~17 hours at 30°C. Efficiency was analyzed using AGE and subsequent extraction from agarose gel was performed using a commercial gel extraction kit (Roche, Germany) following manufacturer's instructions. Concentration was determined using NanoDrop spectrophotometer.

3.2.18.3 Cloning HVA22-like protein coding sequence into GFP-8His containing yEGFP vector by homologous recombination

Procedure for homologous recombination was performed as described in Drew et al., 2008 without any modifications. Briefly, the gene of interest which included 5' overhangs (35bp) that complemented upstream and downstream sequences to either side of the SmaI site in yEGFP vector was amplified with *pfu* polymerase (Fermentas, Germany) using PCR. Then cloning with homologous recombination was performed by addition of 5 µl of 150 ng/µl PCR product and 3 µl of 25 ng/µl SmaI-linearized yEGFP vector into *S. cerevisiae* competent cells together with 42 µl sterile dH_2O in order to complete the volume to 50 µl. For the expression of only GFP, intact yEGFP vector was added (5 µl of 150 ng/µl) into competent cells by completing the volume to 50 µl with sterile dH_2O.

3.2.18.4 Competent yeast cell preparation and Transformation

Procedure for competent yeast cell preparation and subsequent transformation was performed as described in Drew et al. 2008 without any modifications. Briefly, in a 50-ml capped tube 5 ml of yeast peptone dextrose (YPD) medium was inoculated with the *Saccharomyces cerevisiae* strain INVSc1 (Invitrogen, Germany) with a sterile loop and grown overnight at 30°C with constant shaking at 260 x rpm. Overnight culture was diluted to an OD_{600} of 0.1 in a 250-ml flask containing 50 ml of YPD and grown under the same conditions until an OD_{600} of 0.5-0.6 was obtained. Cells were then spun down at 3,000 x g for 5 minutes at 4°C. Then the supernatant was discarded and cells were resuspended in 25 ml dH_2O. Cells were once again spun down under the same conditions and were resuspended in 1 ml of 100 mM sterile lithium acetate (LiAc). Resuspended cells were then transferred into a 1.5-ml microfuge tube and centrifuged at 8,000 x g for 15 seconds. Supernatant was discarded and the pellet was resuspended in 400 µl of 100 mM sterile LiAc.

Fresh microfuge tubes containing 240 µl of 50% (wt/vol) PEG 3350 (autoclaved) were prepared for transformation and were put on ice. 50 µl aliquots of cell suspension from the previous step were added into each tube and vortexed for 5 seconds to obtain a homogeneous cell suspension. Single-stranded carrier DNA (25 µl of 2 mg ml^{-1}) was added into each suspension and vortexed for 5 seconds. DNA mix products of the homologous recombination cloning protocol (50 µl) were added into each tube, vortexed for 5 seconds and labeled accordingly. Tubes were then incubated at 30°C and exposed to heat-shock for 25 minutes at 42°C. Cells were then removed from the heat block and centrifuged at 8,000 x g for 15 seconds at room temperature and washed in 100 ml of sterile dH_2O to get rid of the remaining medium. Each suspension was then plated onto –URA selective media plates and incubated for ~4 days at 30°C.

S. cerevisiae strain INVSc1 (Invitrogen, Germany) was used as host organism since the yEGFP vector contained URA marker for selection purposes and one of the phenotypic properties of INVSc1 strain was its –URA phenotype.

3.2.18.5 Colony PCR

Colony PCR was performed using Taq DNA polymerase (Fermentas, Germany) following manufacturer's instructions and with cycling parameters as described earlier in section 3.2.17.1 of this text. Yeast cells were burst in a microwave oven at maximum temperature for 4 minutes prior to PCR reaction and used as templates. PCR was started with an initial denaturation at 94°C for 5 minutes. Thirty five cycles were then performed with denaturation at 94°C for 1 minute; annealing either 57.3°C for 1 minute and extension at 72°C for 2 minutes. Final extensions were done at 72°C for a further 15 minutes. Results were analyzed using AGE.

3.2.18.6 Expression and Sample Collection

Procedure for expression was performed as described in Drew et al., 2008 with slight modifications. Briefly, appropriate colonies were picked from the plates according to colony PCR results and each were inoculated in an aerated 50-ml capped tube containing 10 ml –URA selective medium with 2% glucose. Inoculations were also performed for uninduced sample collection. Cultures were then inoculated overnight at 30°C with constant shaking at 260 x rpm. Aliquots of 10 µl from overnight cultures were spotted onto fresh –URA selective plates and allowed to dry at room temperature before transferring them to incubator for growth at 30°C until the colonies started to form. Afterwards, remaining overnight cultures were diluted to an OD_{600} of 0.12 in 50-ml capped tubes containing 10 ml –URA medium with 0.1% glucose and incubated at 30°C with constant shaking at 260 x rpm until an OD_{600} of ~0.6 was reached (ca. 7 hours). Expression of HVA22-like protein-GFP and only GFP were induced by addition of sterile galactose to a final concentration of 2%. Cultures were then left for expression for ~22 hours which was previously found to be the optimum duration for expression of membrane proteins.

Expression was performed twice; one for visualization of cellular localization of the HVA22-like-protein-GFP construct together with only GFP and the other was for detection of construct with anti-GFP using western blotting for confirmation purposes. Cell cultures used for visualization were centrifuged at 13,200 x rpm for 3 minutes at 4°C after ~22 hours of expression and were then used for slide preparation in order to be

analyzed in confocal laser microscopy. Cell cultures used in western blotting were centrifuged at 5,000 x g for 40 minutes at 4°C after ~22 hours of expression and were then kept at -20°C until further use in protein extraction.

3.2.18.7 Visualization of cellular localization using confocal laser microscopy

Slides to be analyzed with confocal laser microscope (Leica, USA) were prepared on ice dispensing 1µl of aliquot from cells which were resuspended in 1 ml –URA selective medium containing 50% glycerol after centrifugation at 13,200 x rpm for 3 minutes at 4°C and immediately covering it with a cover slip and applying nail polish to the edges of the slip in order to prevent evaporation of medium owing to the viscous and sticky nature of the chemical.

3.2.19 Western Blotting

3.2.19.1 Protein extraction from yeast cells

Cell pellets (approximately 2.3 mg wet weight) were removed from -20°C freezer and resuspended in 100 µl distilled water. 100 µl of 0.2 M NaOH was added and cell suspensions were incubated at room temperature for 5 minutes. Cells were then pelleted in a tabletop microfuge at maximum speed for 1 minute and the supernatant was discarded. Pellets were resuspended in 50 µl 2XPAGE sample buffer containing β-mercaptoethanol as reducing agent and then incubated at 40°C for 30 minutes. Prior to pelleting the cells again in a tabletop microfuge at maximum speed for 1 minute, 12 µl of resuspensions was reserved for representation of whole cell protein content. After pelleting the cell suspensions, supernatant was transferred into a clean microfuge tube and 12 µl was used for representation of soluble protein fraction in SDS-PAGE separation. After separation of soluble fraction into a new clean microfuge, the remaining insoluble fraction was dissolved in 50 µl 2XPAGE sample buffer containing β-mercaptoethanol as reducing agent and boiled at 95°C for 5 minutes. From each sample tube 18 µl was used for representation for insoluble fraction.

3.2.19.2 SDS-PAGE and blotting of membranes

SDS-Polyacrylamide gels with a 5% stacking gel concentration and a 12% separating gel concentration were prepared as described in Sambrook et al., 2001. Gels were then blotted on PVDF membranes for 1 hour at 25 mA constant current at room temperature in transfer buffer prepared by mixing 14.41 g Tris base, 3.028 g glycine and 200 ml methanol completing the volume to a final of 1 L by adding distilled water. Blotted membranes were blocked with 5% non-fat dry milk solution on an orbital shaker overnight at 4°C on orbital shaker.

3.2.19.3 Antibody incubation and signal detection

Blocked membranes were incubated with anti-GFP (Roche, Germany) overnight at 4°C on an orbital shaker. Secondary antibody incubation was performed with a dilution factor of 1:3000 using 5% non-fat dry milk solution for 1 hour at room temperature again on an orbital shaker. Following secondary antibody treatment membranes were washed three times for 15 minutes each at room temperature. TBS buffer containing 0.1% Tween20 was used in washing steps.

For signal detection, the membranes were treated with Pierce ECL Western Blotting Substrates (Pierce, Thermo Scientific) and the resulting signals were analyzed on CL-XPosure Film (Pierce, Thermo Scientific).

4. RESULTS

4.1 Greenhouse Experiments

Different types of wheat genotypes differ considerably in their ability to tolerate water scarcity. A variation in response to water deficiency was detected both between two different wild emmer wheat genotypes used in this study and also among wild emmer wheat genotypes and modern durum wheat Kızıltan as well which was in agreement with the previous study of our group (Ergen and Budak, 2009) that constituted the basis of the current study. In agreement with the previous study, the initial general response of the plants to water deficiency was reduction in shoot growth, emergence of pale green leaves and necrotic streaks on both sides of the young leaves. Tolerance to water deficiency determination was restricted to SPAD measurements and leaf symptoms in this study since a comprehensive initial screen comprising 200 different genotypes was already performed by an additional leaf water content, soil water content, proline accumulation levels analyses. That initial screening was also repeated with the most promising 26 contrasting genotypes as mentioned earlier utilizing the same comprehensive physiological and phenotypic data analyses; consequently the current study utilized three different genotypes which were used in construction of cDNA libraries from which a large collection of EST sequencing was performed following slow drought stress treatment.

The experiment carried out in the controlled greenhouse conditions confirmed that tolerant genotype showed the highest tolerance to water-deficit conditions having more green leaves and a more erect stature. Modern durum wheat Kızıltan followed it with a moderate tolerance when compared to sensitive genotype being the most susceptible one under drought stress condition. These findings were also consistent with SPAD values which were collected daily.

4.2 SPAD Measurements

SPAD values were calculated as the average of six measurements from three different individuals for each plant set taken in the afternoon each day following the start of drought stress treatment (Martinez and Guiamet, 2004; Babar *et al.,* 2006). Data was in agreement with the previous work of our group (Ergen and Budak, 2009). A temporary increase present in the SPAD values of wild emmer wheat sensitive genotype can be explained by the increase inflicted upon ratio of transmittance due to emergence of dead cells and necrotic tissue which is absent in the control group of the same genotype (Neufeld et al, 2006; Castelli et al., 1996; Monje and Bugbee, 1992; Yomomato et al., 2002). Therefore the SPAD measurements belonging to control group of sensitive genotype remained nearly constant until the end of the seventh day whereas that of stress group plants showed a significant decrease around fifth day of the water shortage which was also the case in the previous study (Ergen and Budak, 2009). SPAD values of drought-tolerant genotype control group and stress group plants did not differ significantly. Graphs showing the relation between the SPAD values of stress and control group plants from each genotype are represented below.

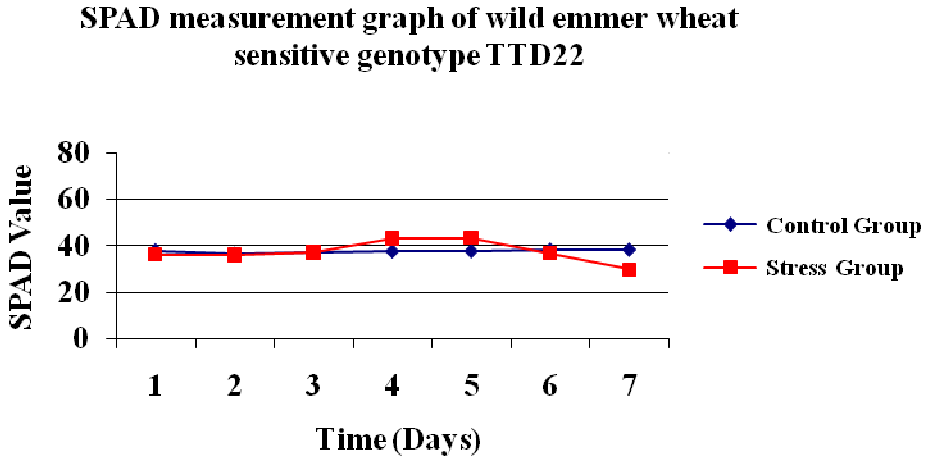


Figure 4.1 A decline in the SPAD values obtained from stress group plants of wild emmer wheat sensitive genotype was observed compared to that of control group of plants.

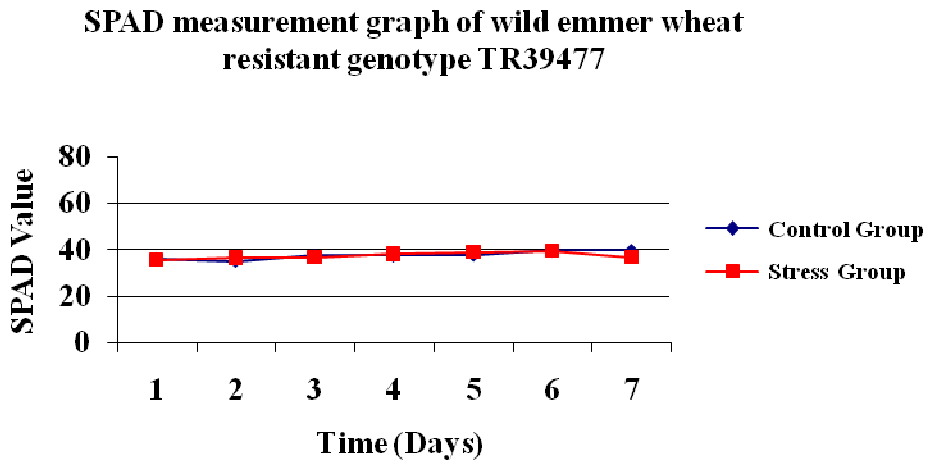


Figure 4.2 SPAD values obtained from stress group plants and control group plants of wild emmer wheat resistant genotype did not show a significant difference.

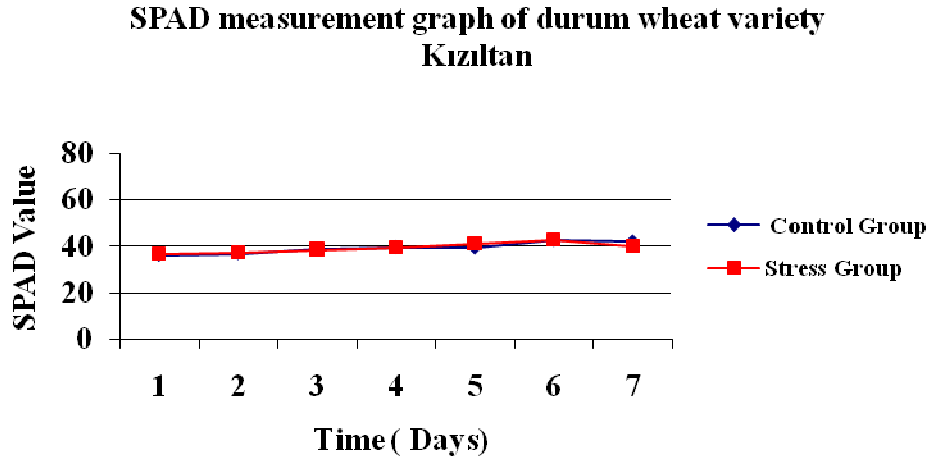


Figure 4.3 SPAD values obtained from stress group plants and control group plants of durum wheat Kızıltan genotype did not show a significant difference.

4.3 Amplification of *HVA22-like protein* using gene specific primers by reverse transcription-PCR

PCR amplification using gene-specific primer pair gave the differentially expressed fragment size of ~700bp although the expected amplicon size was 591bp according to the region which was used from Zea Mays clone in primer sequence design (detailed in Appendix D).

Figure 4.4 shows the agarose gel electrophoresis (AGE) image of gradient-PCR which demonstrates the proper amplification of the fragment of interest. This was achieved best at 57.3°C as annealing temperature. Expected molecular weight was around 660bp and bands with expected sizes were observed only in 9-day stressed samples of wild emmer wheat. The fragments with desired molecular weights were extracted from gels to be purified to be used in subsequent cloning steps. All AGE images were taken after running a 1% agarose gel in 0.5X TBE buffer at 100V for 40 minutes and staining with 0.005% ethidium bromide unless otherwise stated.

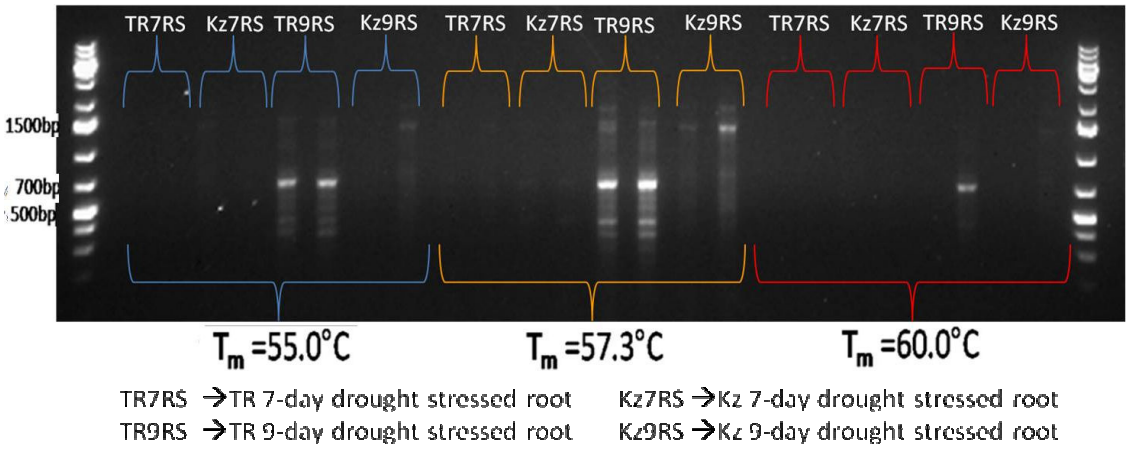


Figure 4.4 AGE gel image of HVA22-like protein amplification by RT-PCR. HVA22-like protein was differentially expressed in 9-day stressed root tissue samples of *Triticum turgidum ssp. dicoccoides* drought-tolerant genotype (TR) and it was absent in samples of 9-day stressed modern durum wheat variety Kızıltan (Kz) root tissue.

PCR amplification of cDNAs synthesized from total RNAs isolated from leaf tissues of resistant and sensitive accessions together with modern durum wheat variety Kızıltan leaf samples displayed differential expression of HVA22-like protein in leaves although it was at very low level compared to levels of expression observed in 9-day stressed root sample of wild emmer wheat resistant genotype. This is in agreement with the fact that ABA is produced in roots and transported to leaves (Zhang and Davies W.J., 1987; Zhang, 2006; Trapeznikov et al., 2003; Sharp and LeNoble, 2002) consequently the induction of HVA22-like protein in roots is higher than that of in leaf tissue.

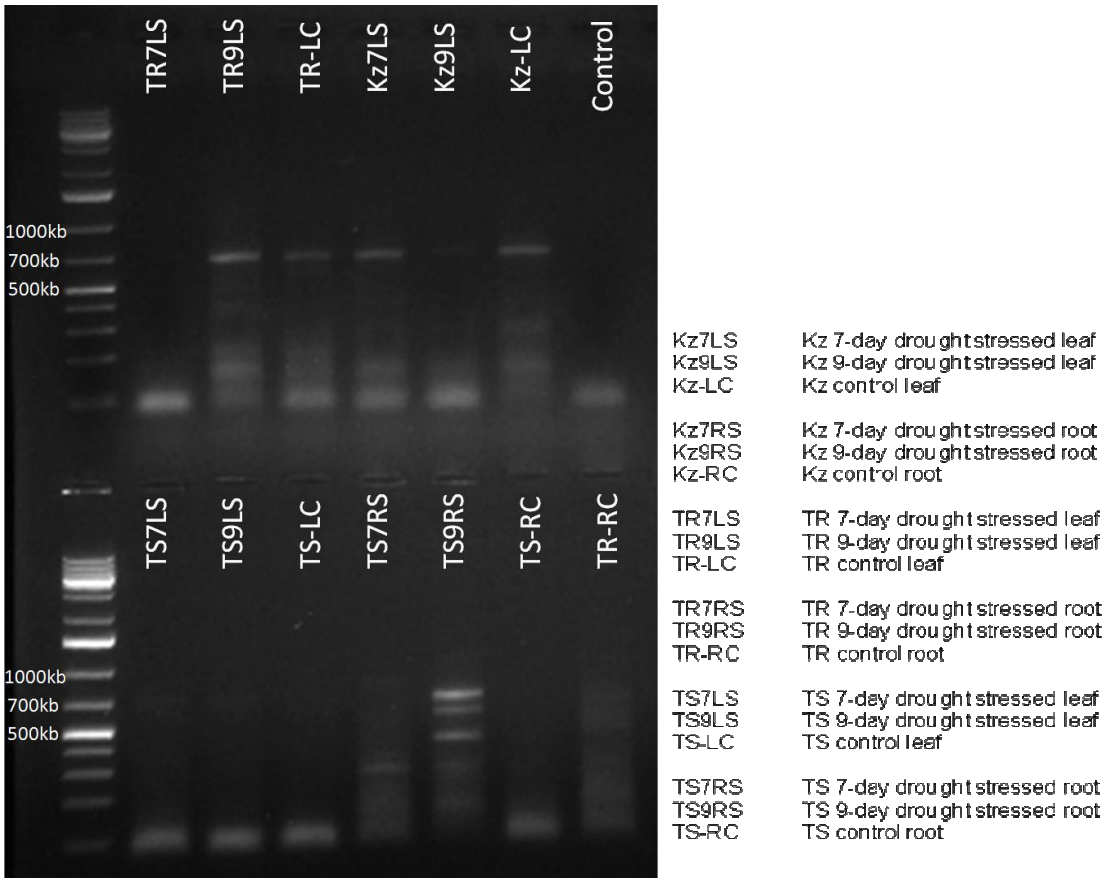


Figure 4.5 AGE gel image of RT-PCR results. *HVA22-like protein* expression was also differential in leaf tissues. (*TR*, drought tolerant wild emmer wheat genotype; *TS*, sensitive wild emmer wheat genotype; *Kz*, modern durum variety Kızıltan; Contro*l*, reaction control with no template)

An important outcome of RT-PCR experiments was that there is no induction of HVA22-like protein in control group root tissue samples of drought-tolerant genotype which further consolidates the claim that the cloned gene of interest in current study is indeed a drought-inducible gene. However lower amounts compared to drought-tolerant genotype; there is also an induction of HVA22-like protein in the water deficit stressed root tissue of sensitive genotype. It suggests that the wild relative still retains the wild variation in its genotype although it does not confer significant amounts of resistance to drought.

4.4 Transformation, Colony Screening, Restriction Digestion Confirmation of insertion

The extracted bands of interest, amplified with gene-specific primers, were ligated into pGEM®-T Easy vector via T-A cloning strategy and transformed to E. *coli* DH5α cells. True white colonies were selected for insertion confirmation of fragment of interest using restriction digestion. Restriction digestion reactions using EcoRI (Fermentas) were performed following plasmid isolation in order to check and confirm that the white colonies truly contain the vectors with the fragment band of interest.

Products of the restriction reactions were separated in 1 % agarose gel at 100V for 35 minutes, as illustrated in Figure 4.6. Observation of the bands with desired molecular weights confirmed that HVA22-like protein coding fragment was cloned into DH5α cells successfully.The concentrations of isolated plasmids were measured prior to restriction digestion using Nanodrop spectrophotometer in order to ensure the correct amount of substrates. Colonies circled in Figure 4.6 confirmed to containing of the fragment of interest and the colony that yielded the proper concentration of plasmid was chosen to be sent for commercial sequence analysis.

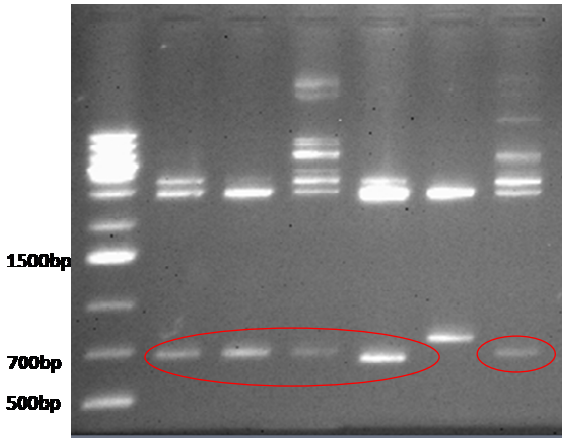


Figure 4.6 AGE gel image of confirmation digestion of successful insertion. Confirmation of insertion was performed with restriction enzyme digestion with EcoRI.

4.5 Sequence Analysis

Isolated plasmids obtained after the first cloning, were sent REFGEN Biotechnology Company, ANKARA to be sequenced using both M13 forward and

reverse primers. Sequence results were first processed with VecScreen algorithm of NCBI in order to remove the vector contamination. This process yielded a 660 bp ORF which translated into a 219 amino acid residue protein hypothetically with a theoretical average molecular weight of ~25kDa and theoretical pI value of 9.6. The insert sequence was then compared to the nucleotide and protein sequences available at the Entrez nucleotide and protein databases using the BLASTN and BLASTP algorithms because the result obtained yielded an open reading frame (ORF) strictly starting with a start codon and ending with a stop codon.

In order to confirm that the sequence being expressed was same as the initially cloned sequence, expression vector containing *HVA22-like protein* clone in it was also sent to be sequenced with the original gene-specific primers. Expressed construct was also sequenced using commercial T7 terminator primer which was included in the pET22b(+) sequence. Results obtained were compared to that of the initial cloning sequence analysis results using nucleotide BLAST algorithm and it was shown that the two sequences shared 98% identity most probably due to technical reasons caused by the fact that two constructs were sent to be sequenced separately. Consequently conditions for both sequencing reactions were not identical.

4.5.1 Conserved domain search and conserved domain architecture retrieval tool

In order to investigate the possible conserved domains present in the HVA22-like protein sequence, tools presented at the NCBI webpage was used as mentioned earlier. The results indicated that the HVA22-like protein from wild emmer wheat had a region called TMPIT-like protein domain and a number of proteins which are considered as members of this family were annotated as “transmembrane proteins induced by tumour necrosis factor alpha”. This result is important since as the previous studies (Shen et al., 2001; Guo and Ho, 2008; Brands and Ho, 2002; Nziengui and Schoefs, 2009) has shown that HVA22-like protein is involved both in initial signal transduction and in establishing the stress responses later on upon drought stress detection. Proteins that have TMPIT-like domain in their structure, such as phosphoinositide PI4, 5P(2) from *S. cerevisiae,* are known to be involved in stress responses (Goffeau et al., 1996; Philippsen et al., 1997; Novo et al., 2009). This result

consolidates that the sequence cloned and expressed in this study is indeed a HVA22-like protein which was not previously studied and shown in wild emmer wheat.

4.5.2 BLASTN

The most significant BLASTN alignment result returned as a *Triticum aestivum* cDNA clone cultivar Chinese Spring with a probability (e-value) of 0.0 and a sequence identity of 97% covering a region of 96% of the sequence (Accession Number: AK331663) . Although this sequence has not been annotated, this result is both meaningful since wild emmer wheat is the progenitor of modern bread wheat as mentioned previously and it is most probable that the gene cloned in the current study is a homologue of the aforementioned sequence.

4.6 Secondary Structure Prediction, Modeling and Membrane Topology

4.6.1 Prediction of secondary structure using HMMTOP v2.0 server

HMMTOP server uses an algorithm for determination of the topology of membrane proteins. It is important to note that it is not an algorithm for predicting whether a protein sequence belongs to membrane proteins or not. HMMTOP server is used with high reliability after determining whether a certain protein is a membrane associated protein either with prior experimental knowledge or by the help of other available algorithms used in predicting the structure or localization of the protein. Since HVA22-like protein family members are known to be transmembrane proteins (Guo and Ho, 2008) HMMTOP server v2.0 was used for the prediction of membrane topology of the HVA22-like protein isolated from wild emmer wheat. Algorithm predicted four transmembrane domains with N-terminus of the protein inside the cell. Results are given in Figure 4.7.

```
N-terminus:  IN
Number of transmembrane helices: 4
Transmembrane helices: 76-93 100-119 134-151 166-183

     seq  MALLAPLDPA TLDKVEEELE RARAAILDGD VAAFLPSKGN GKFLKKFVGP    50
    pred IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII

     seq  VNVRVARKEE KLKVKDEYNN YRDRAAYMFL LFPSTLLLLR WWVWDGCLPA   100
    pred IIIIIIIIII iiiiiiiiii iiiiiHHHHH HHHHHHHHHH HHHooooooH

     seq  LAVQVYQAWL LFLYTSFALR ENVLLVNGSD IRPWWIYHHY LAMLMALVSL   150
    pred HHHHHHHHHH HHHHHHHHHi iiiiiiiiii iiiHHHHHHH HHHHHHHHHH

     seq  TWEIKGQPDC SSKQRGVQLF LRWAIMQGIA MHLQNRYQRQ RLRTRIALGK   200
    pred Hooooooooo oooooHHHHH HHHHHHHHHH HHHiiiiiii iiiiiiiiII

     seq  AKRMDVVAGE TAGVSGTET  219
    pred IIIIIIIIII IIIIIIIII
```

Figure 4.7 Secondary structure prediction of HVA22-like protein. HMMTOP server v2.0 was used to predict the membrane topology of translated amino acid sequence of HVA22-like protein. (Predictions are indicated in red: H= membrane helix, I=inside, i= inside helix t ail, o= outside helix tail)

TMHMM server was used with the same 219 amino acid residue HVA22-like protein for confirmation of the prediction with HMMTOP algorithm prediction and results obtained were in agreement suggesting four potential transmembrane domains for the given sequence.

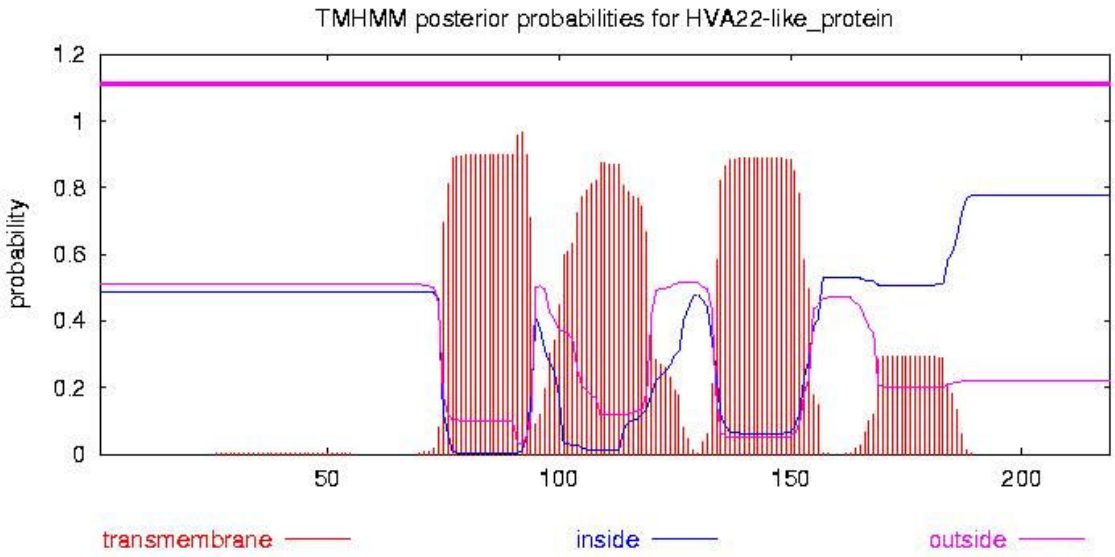


Figure 4.8 Graphical representation of membrane topology prediction. TMHMM program was used to show the graphical representation of predicted membrane topology of translated amino acid sequence of HVA22-like protein.

4.6.2 Graphical representation of predicted membrane topology usingTMRPres2D

Prediction and graphical representation of the cloned sequence was performed using TMRPres2D (**T**rans**M**embrane protein **Re**-**Pres**entation in **2 D**imensions) server. Results obtained were in agreement with the previous number of predicted transmembrane domains and N-terminal of the protein was again shown to be inside the cell.

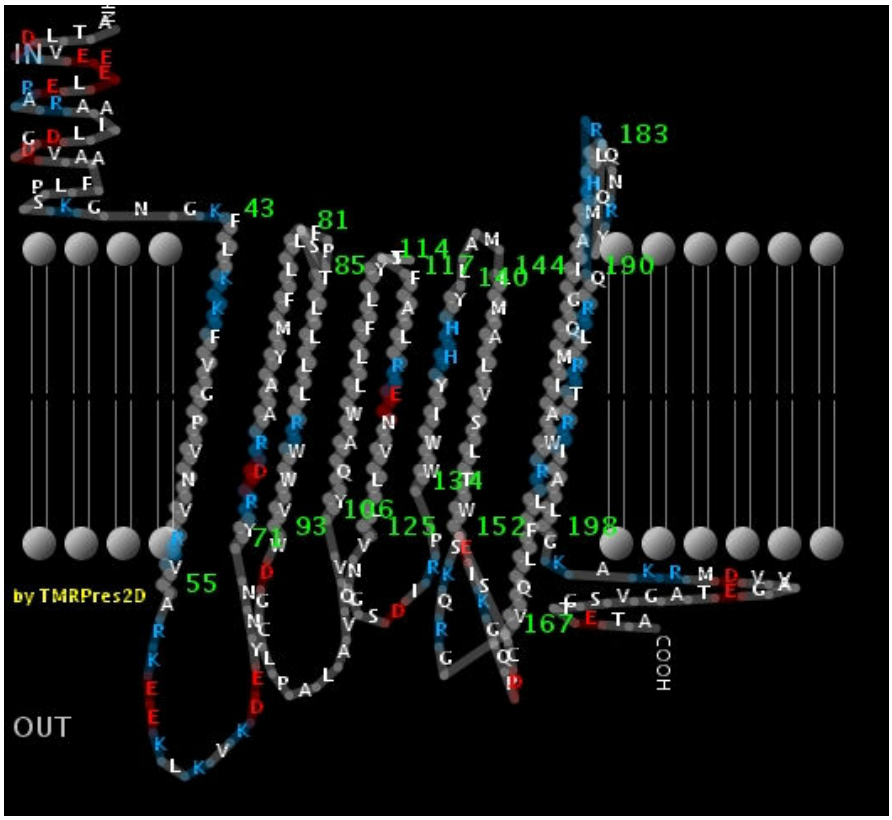


Figure 4.9 Two dimensional depiction of membrane topology prediction. Membrane Topology using TMRPres2D prediction server suggested that the N-terminal of the protein was inside the cell and the C-terminal protruded outwards.

4.6.3 Prediction of three-dimensional structure using Modweb and visualization by Pymol

Another structural prediction was run using Modweb algorithm in order to visualize the possible three dimensional structure of the cloned sequence and the resulting pdb file (protein data bank format) was visualized using a commonly used visualization tool Pymol. Modweb was used for *ab initio* structural modeling of the sequence although template data set based predictions are also available via Modweb server. Predicted three dimensional structure of the cloned HVA22-like protein is given below in Figure 4.10.

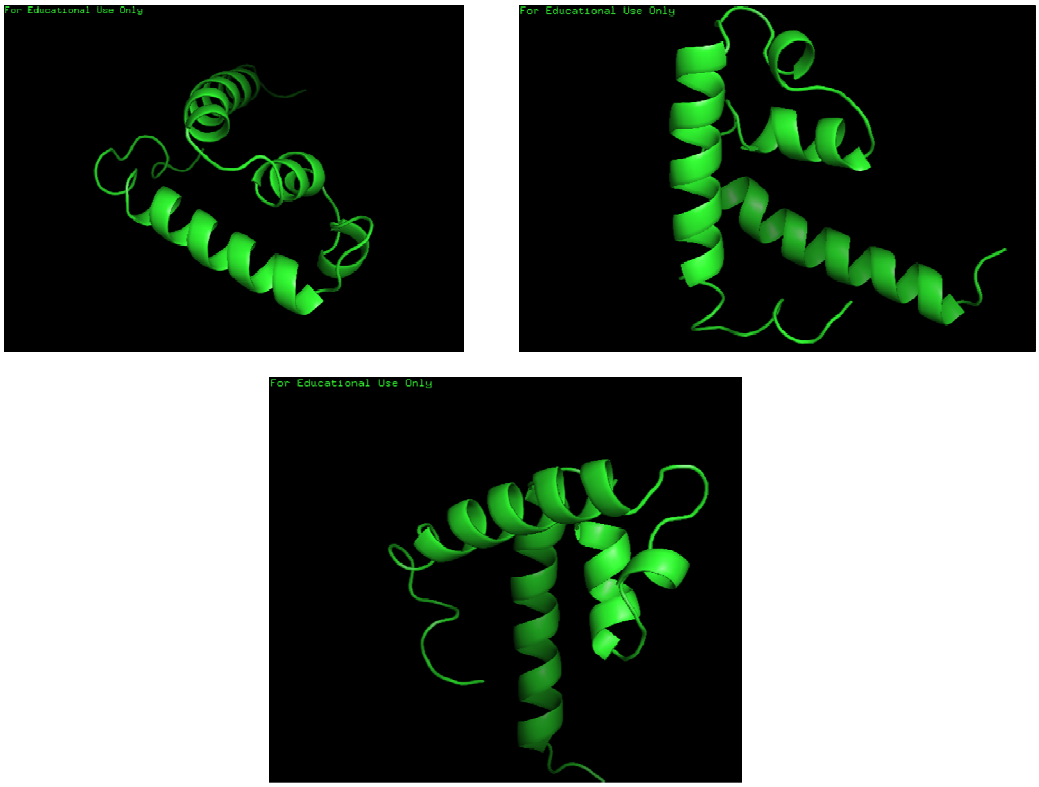
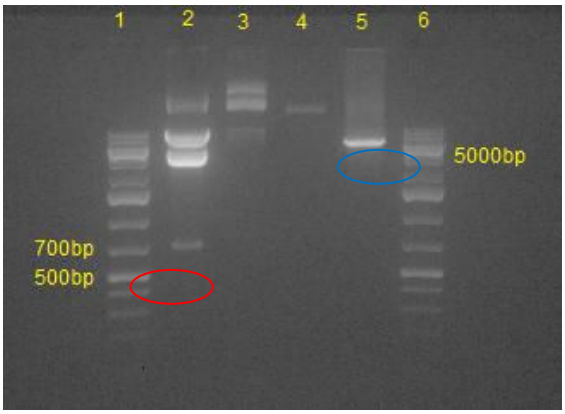


Figure 4.10 Pymol image of HVA22-like protein structure predictions. Structural prediction by Modweb was visualized using Pymol program.

4.7 Protein Expression

4.7.1 Restriction digestion of expression vector and cloning vector containing fragment of interest

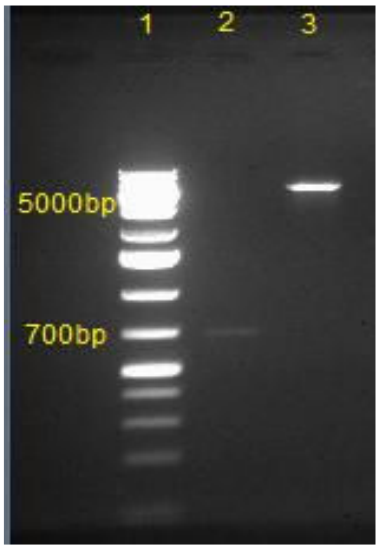
In order to construct the expression vector containing the HVA22-like protein ORF; first the empty expression vector and cloning vector containing fragment of interest was digested with EcoRI restriction enzyme. Digestion with EcoRI provided the compatible ends required for the following ligation reaction. However the lack of two different restriction sites prevented the directional cloning scheme which could be obtained by using two different restriction enzymes. This problem was overcome as explained in ORF orientation determination section (4.7.4). AGE analysis results of the restriction digestions are given in Figure 4.11 below.



Lane 1. Fermentas 1Kb Plus Ladder
Lane 2. Digested pGEM®-T Easy vector containing fragment of interest
Lane 3. Undigested pGEM®-T Easy vector containing fragment of interest
Lane 4. Undigested pET22b(+) vector
Lane 5. Digested pET22b(+) vector
Lane 6. Fermentas 1Kb Plus Ladder

Figure 4.11 AGE gel image of restriction digestion. Gel extraction of HVA22-like protein ORF from cloning vector pGEM®-T Easy with EcoRI digestion (shown in red circle) together with the digestion of expression vector pET22b(+) with EcoRI (shown in blue) for subcloning purposes was performed.

4.7.2 Quality Control of Gel Extraction by AGE



Lane 1. Fermentas 1Kb Plus Ladder
Lane 2. Gel Extract of Digested pGEM®-T Easy Vector Containing Fragment of Interest
Lane 3. Gel Extract of Digested pET22b(+) Vector

Figure 4.12 AGE gel image of gel extraction. Further AGE analysis of extracted digestion products which was shown in red and blue circles in Figure 4.11 confirmed the purity of fragments prior to ligation.

Before being used in ligation, restriction digestion products were extracted from agarose gel (1.5%) after ensuring the efficient digestion by agarose gel electrophoresis. The purity and concentration of the gel extracts were then determined again using 1 µl of samples in agarose gel electrophoresis.

4.7.3 Colony PCR

Colony PCR was performed with the original gene-specific primers in order to determine whether the selection of true positive colonies were successful. As can be seen in Figure 4.12, except two lanes which had technical problems, all analyzed colonies confirmed the successful cloning *of HVA22-like protein* ORF into pET22b(+) expression vector.

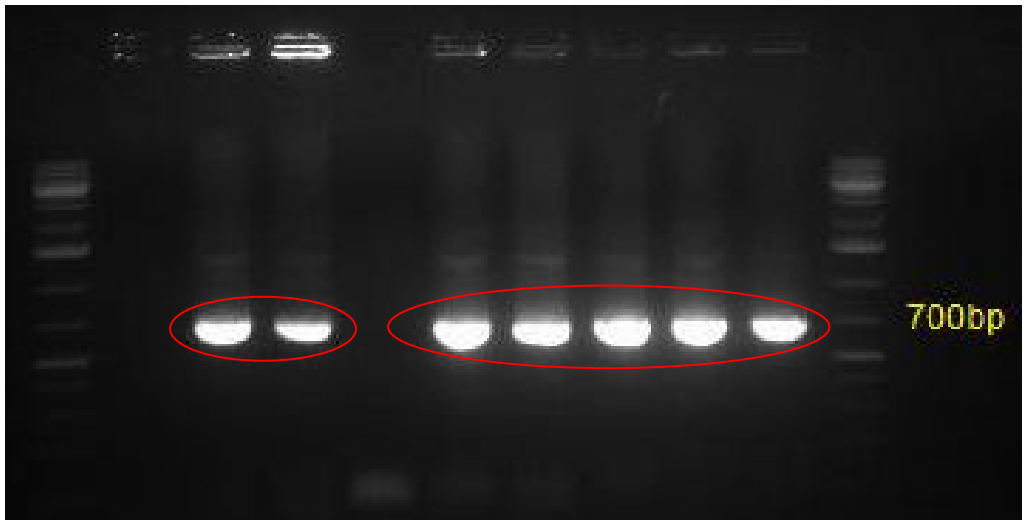


Figure 4.13 Figure 4.13 AGE gel image of colony PCR confirmation. Colony PCR bands of expected size with 660bp clearly indicated the successful cloning of HVA22-like protein ORF into the expression vector pET22b(+).

4.7.4 ORF Orientation determination

Due to the lack of restriction sites at both ends of the original primer pair, the insertion of ORF into expression vector was done using only a single, EcoRI, restriction enzyme. However this prevented the directional cloning which would facilitate the expression of protein once the fragment of interest was inserted. In order to check the orientation of insert for correct direction, so that the expression of the protein product could take place, another restriction enzyme, XhoI, was selected. Original cloned

sequence of the *HVA22-like protein* contained an XhoI cut site, and the expression vector also had XhoI cut site in its multiple cloning site. The fragment that had the correct orientation for expression would yield a 286bp fragment. Fragments with the wrong orientation yielded bands of a larger size (~400bp) since XhoI site corresponded to the farther end of the cloned sequence in reference to the cut site that was present in the original empty pET22b(+) vector. Only one of the colonies had the insert in correct orientation as circled in yellow in Figure 4.14.

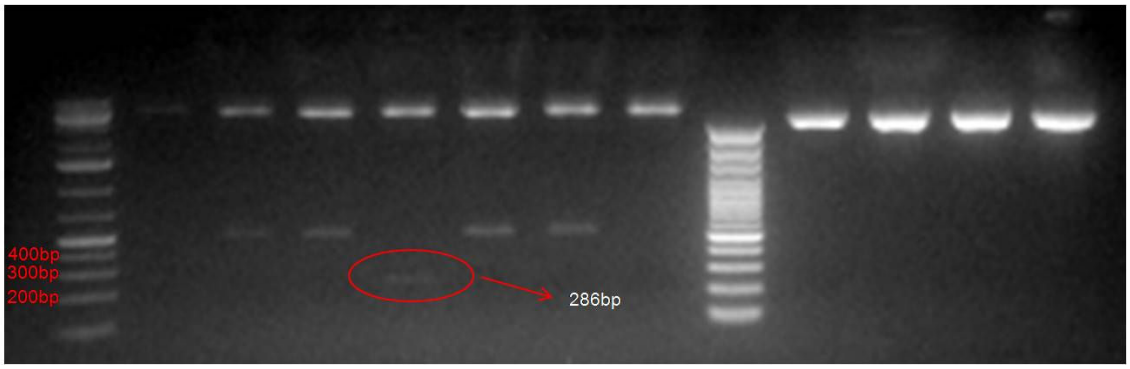


Figure 4.14 AGE gel image of orientation determination of the fragment of interest. ORF orientation determination was performed using restriction digestion by XhoI.

4.7.5 Growth Curve Construction of Rosettagami2™ and BL21(DE3) cells

In HVA22-like protein expression analysis two different strains of *E. coli*, namely Rosettagami2™ and BL21(DE3) strains, were used in order to show that the results were not depended on the bacterial strain. Varying concentrations of IPTG (i.e. 0.5 mM, 0.7 mM, 1 mM) were also used to investigate whether the concentration of IPTG used for induction had any effect in expression.

HVA22-like protein, which was formerly shown to be an exclusively eukaryotic protein with no known prokaryotic homologues to date, retarded the growth of both bacterial strains. This response can be observed from the representative graphs of OD_{600} measurement data collected in various time intervals from growing cultures. Representative graphs also indicated that bacteria could probably have a survival mechanism against the protein's negative effect possibly using its proteases to degrade the protein and eliminating it from the environment since bacterial cells showed a dramatic increase in their cell density after a certain time which is more obvious in

BL21(DE3) cells. In addition, increasing concentration of IPTG, from 0.5 mM to 1 mM, had a further negative effect growth although it was not very significant.

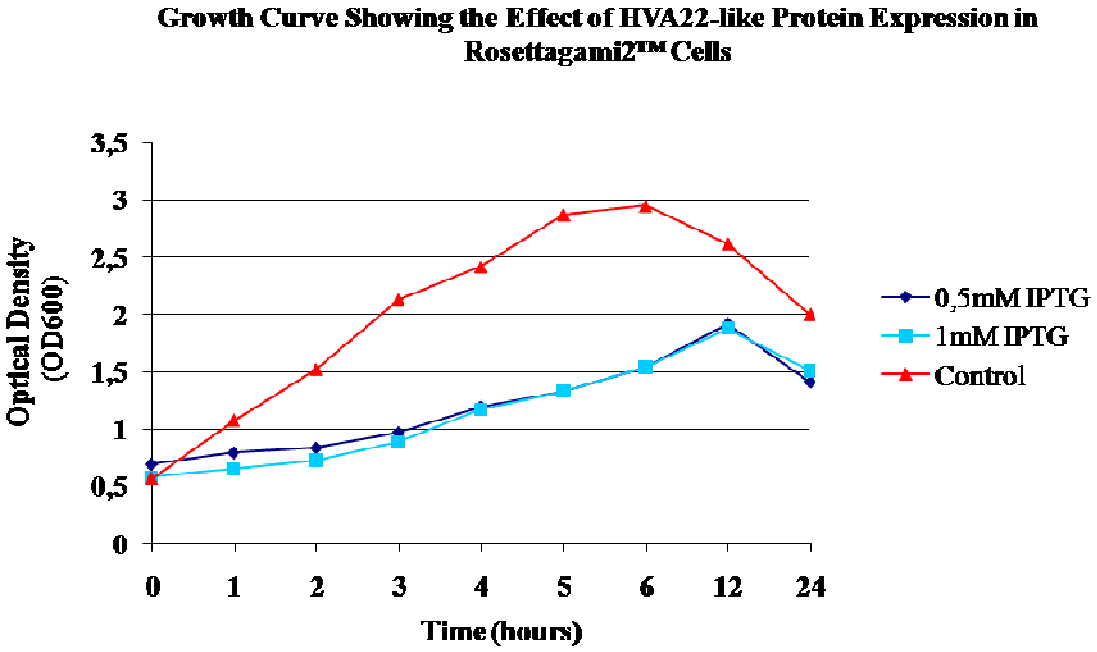


Figure 4.15 Growth curve of Rosettagami2™ cells expressing HVA22-like protein Growth curve constructed using OD600 measurement data obtained from Rosettagami2™ cells expressing HVA22-like protein upon induction with IPTG indicated that the protein had a retarding effect on growth of bacterial cells.

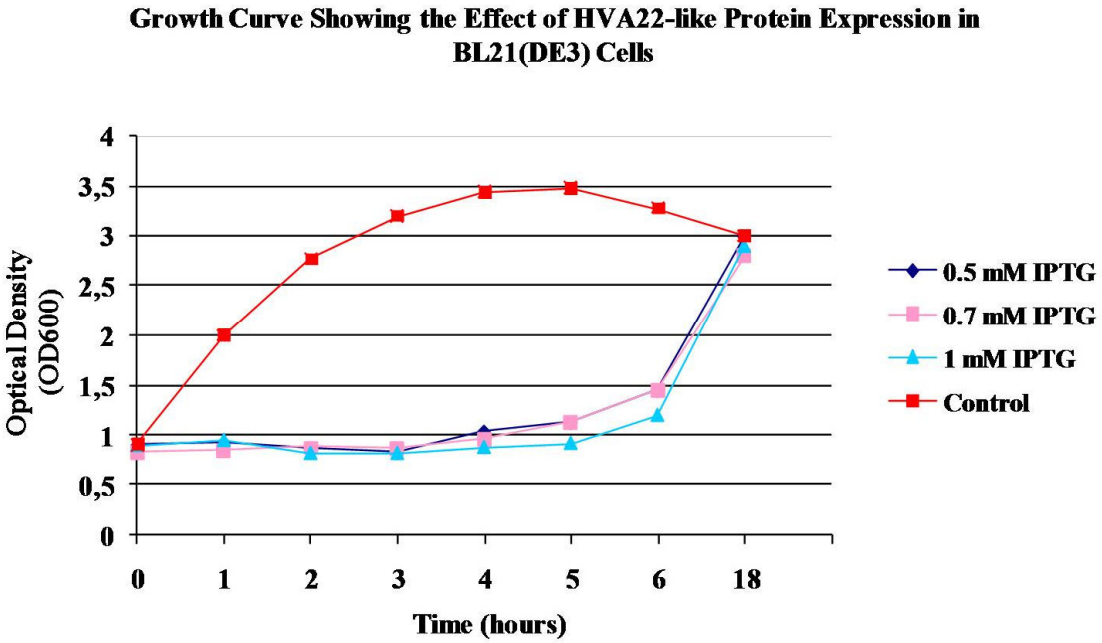


Figure 4.16 Growth curve of BL21(DE3) cells expressing HVA22-like protein. Growth curve constructed using OD_{600} measurement data obtained from BL21(DE3) E.coli cells expressing HVA22-like protein upon induction with IPTG indicated that the protein had a dramatic retarding effect on growth for both bacterial cells.

4.7.6 SDS-PAGE analysis

SDS-PAGE analysis of the samples containing HVA22-like proteins which were extracted from either Rosettagami2™ or BL21(DE3) cells upon induction with varying concentrations of IPTG (0.5 mM, 0.7 mM, 1 mM) showed that there was a production of a protein with a molecular weight of ~25kDa, which was in agreement with the calculated theoretical molecular weight of *in silico* translated HVA22-like protein (~25128 daltons) sequence obtained by commercial sequencing. SDS-PAGE gel images also suggested that there was an elimination of the aforementioned protein product indicated by the diminishing bands of the ~25kDa bands. This interpretation is consistent with the results obtained by construction of growth curves of bacterial cells as mentioned the previous section.

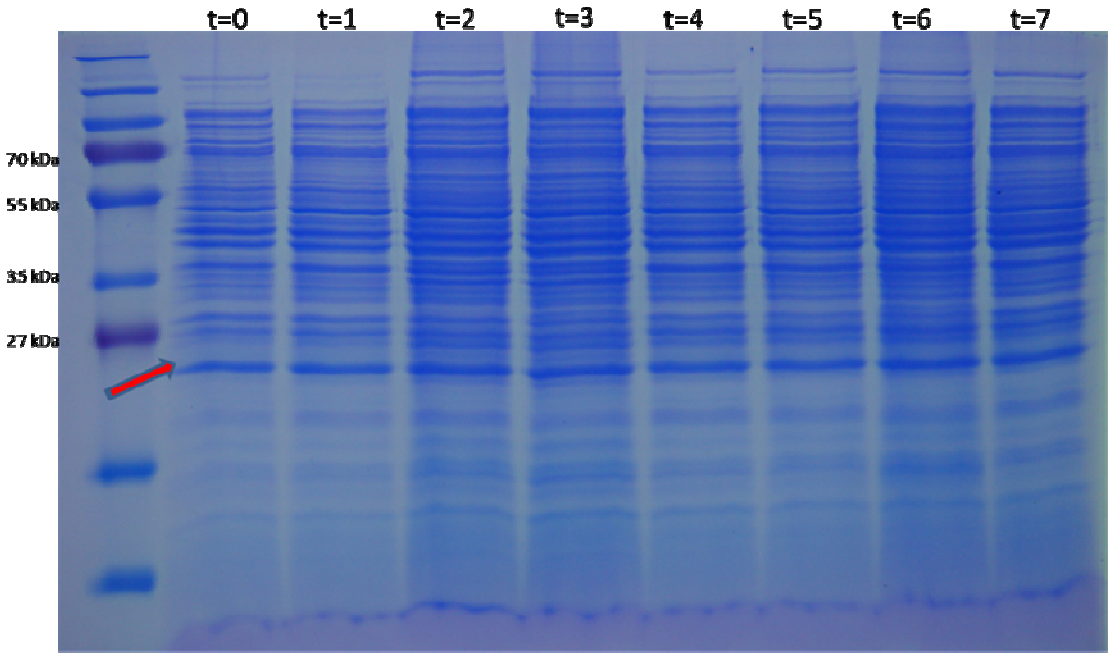


Figure 4.17 SDS-PAGE gel image of Rosettagami2™ cells. SDS-PAGE analyses of the samples containing HVA22-like proteins extracted from Rosettagami2™ cells upon induction with 0.7mM IPTG showed the expression of the protein as indicated with the arrow. Cell pellets were collected in one hour intervals; i.e t=0 just before induction; t=1 one hour after induction, t=2 two hours after induction etc.

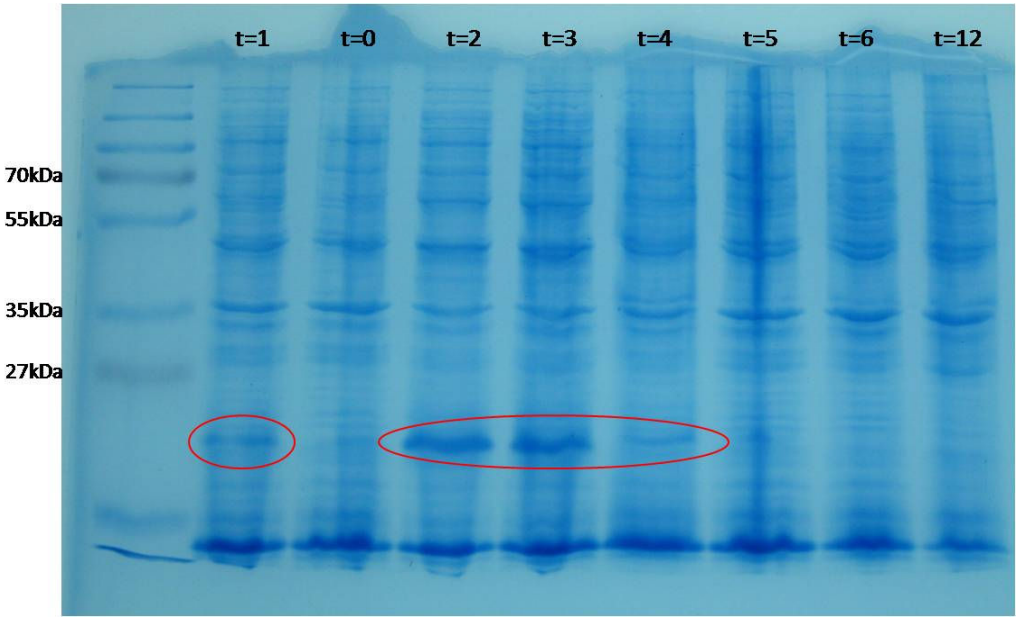


Figure 4.18 SDS-PAGE gel image of BL21(DE3) cells. SDS-PAGE analyses of the samples containing HVA22-like proteins extracted from BL21(DE3) cells upon induction with 0.5mM IPTG showed the expression of the protein as indicated with red

circles. Cell pellets were collected in one hour intervals; i.e t=0 just before induction; t=1 one hour after induction, t=2 two hours after induction etc.

4.7.7 Sequence analysis of pET22b(+) vector containing expressed coding sequence

Comparison of the expression vector construct sequence with the cloning vector construct which was previously shown to contain the coding sequence for *HVA22-like protein* utilizing BLASTN algorithm further confirmed the expression of correct coding sequence with a sequence identity of 98% and with a coverage value of 95%.

4.8 Cellular localization of HVA22-like protein

4.8.1 Amplification of HVA22-like protein ORF with homologous recombination compatible gene-specific primers

Prior to transformation of yeast cells with yEGFP vector consisting of HVA22-like protein open reading frame, PCR amplification was performed using *pfu* polymerase in order to obtain the coding sequence containing homologous recombination sites compatible with the ones present in the yEGFP vector. Three different annealing temperatures were tested using gradient PCR. Expected amplicon size was 720bp and results were checked using AGE.

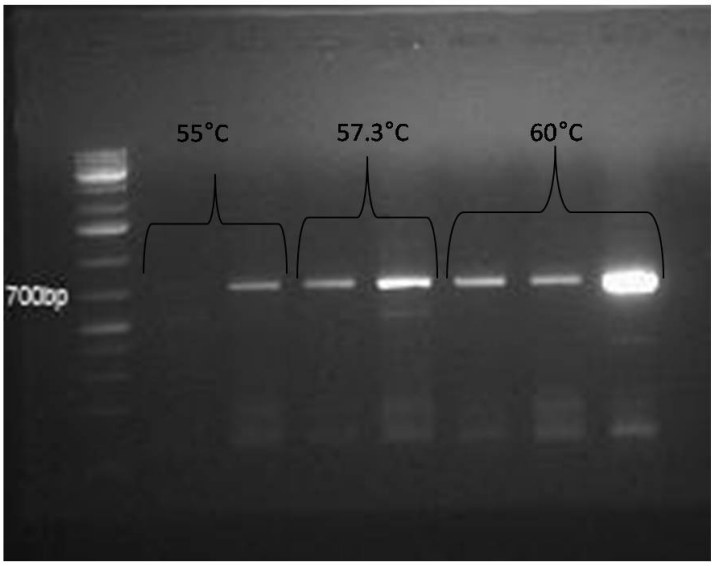


Figure 4.19 AGE gel image of HVA22-like protein amplification with primers designed for homologous recombination. Analysis of PCR by AGE showed the successful amplification of HVA22-like protein coding sequence including homologous recombination sites.

4.8.2 Quality control of gel extraction

After extraction of PCR products of *HVA22-like protein* and restriction digestion product of empty yEGFP vector from agarose gels (1.5%) purity of the gel extracts and relative concentrations were checked using AGE with 1 µl samples. Results indicated that all samples had the required concentration and purity for homologous recombination. Lanes labeled 1-4 depicted in Figure 4.20 contained the amplicons obtained at different annealing temperatures which was explained in the previous section and virtually they all had the same sequence due to being amplified with the same primer pairs.

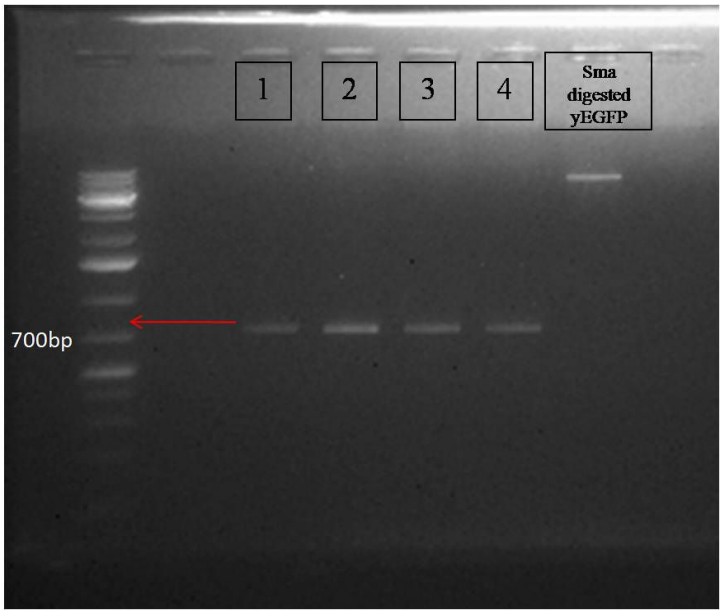


Figure 4.20 AGE gel image of HVA22-like protein amplicon and digested yEGFP vector gel extracts. Bands pointed with an arrow were confirmed to have the proper purity and concentration required for homologous recombination reaction prior to transformation.

4.8.3 Confirmation of successful transformation of yeast cells with colony PCR

Colony PCR was performed using gene-specific primers given in Table 3.2 in order to select the true positive colonies. Bands shown in red circles belonged to true positive colonies. Colonies which yielded the brightest bands were chosen for downstream expression and confocal microscopy visualization analyses.

A very faint bands of expected sizes were observed even where the yeast cells were transformed with empty vector (circled in blue). As mentioned earlier, this was because the *HVA22-like protein* has homologs in diverse organisms and it also has a homologue in yeast cells, namely Yop1p, which was discovered in some earlier studies (Brands and Ho, 2002; Calero et al., 2001). However for ensuring that was the actual reason for obtaining bands where they were not expected, a PCR reaction was set up using the empty vector as template and the primers given in Table 3.2 and results were analyzed using AGE which is presented in Figure 4.22. Results proved that empty yEGFP vector did not contain the HVA22-like protein ORF sequence in its structure.

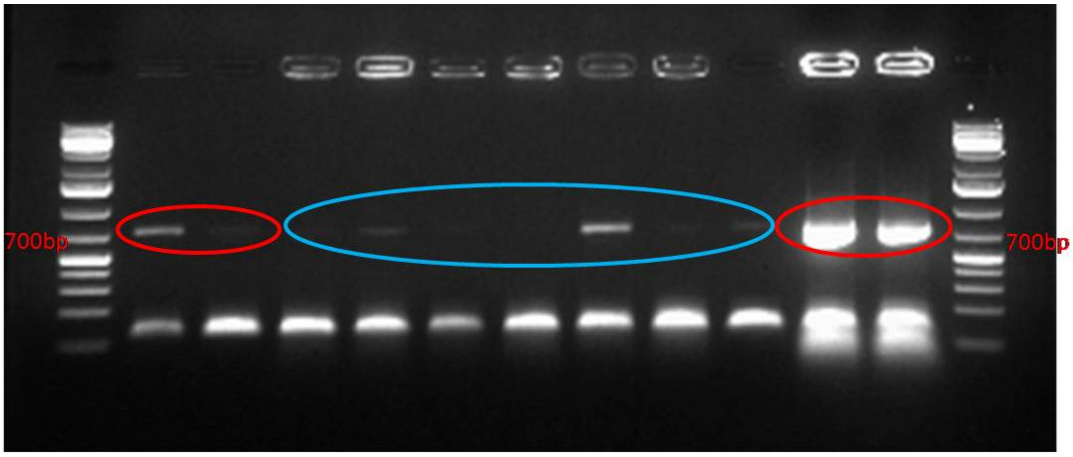


Figure 4.21 AGE analysis of colony PCR. True positive transformant colonies (circled in red) of INVSc1 yeast cells were selected using colony PCR.

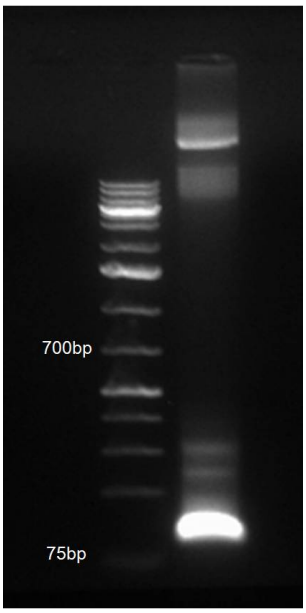
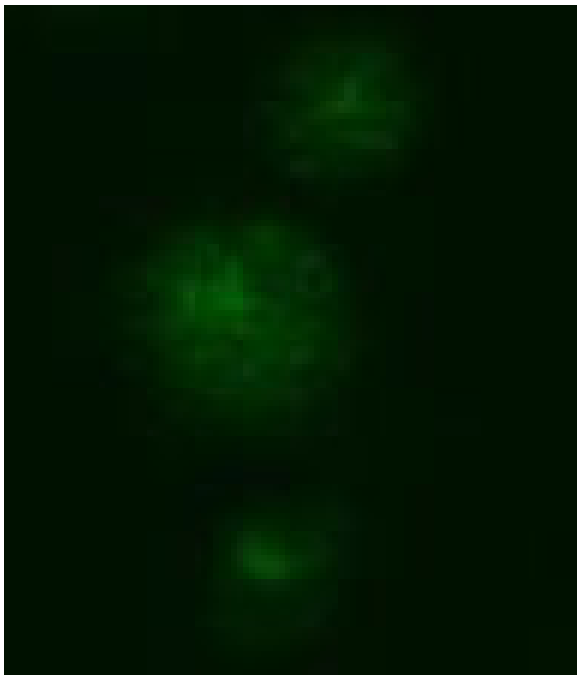


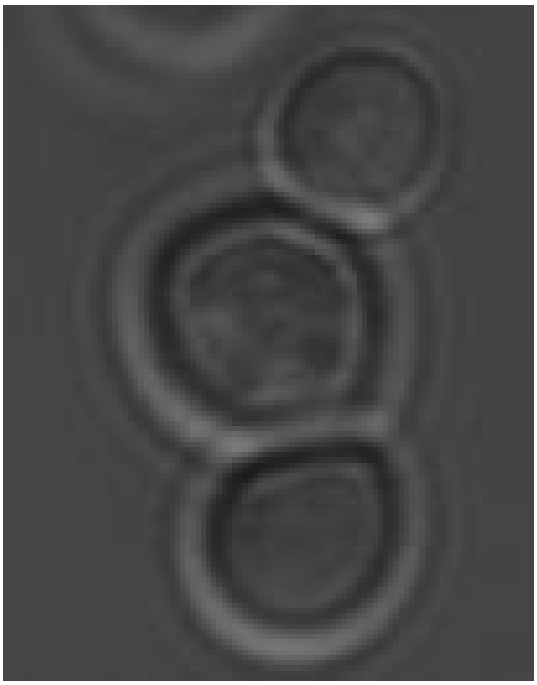
Figure 4.22 AGE gel image of empty yEGFP vector amplification by PCR. AGE analysis of PCR reaction performed using empty yEGFP vector as a template and gene-specific primers for homologous recombination proved that empty yEGFP vector did not have the potential to yield false positive results.

4.8.4 Confocal laser microscopy

Images were obtained by confocal laser miscrocope from two different channels; one was the signal coming from emission of GFP protein only and the other one was the phase contrast image of the yeast cells confirming that the signal coming from GFP channel was not due to a possible artifact caused by slide preparation or any other technical problems. Then the images coming from these two different channels were merged manually to display the localization of GFP only and HVA22-like protein tagged with GFP within the cell. Here it is important to note that C-terminal membrane protein-GFP fusions were fluorescent in all yeast cellular compartments (Huh et al., 2003) in contrast to *E. coli*, in which GFP was shown to be inactively fused to periplasmic segments of inner-membrane proteins (Drew et al, 2002). In our construct, HVA22-like protein is tagged with GFP at its C-terminal.



Signal coming from GFP only (63X Magnification)



Phase contrast image of yeast cells (63X Magnification)

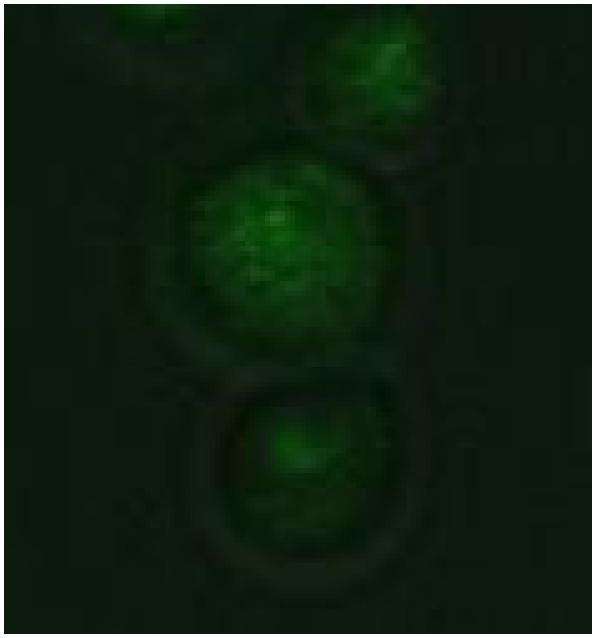
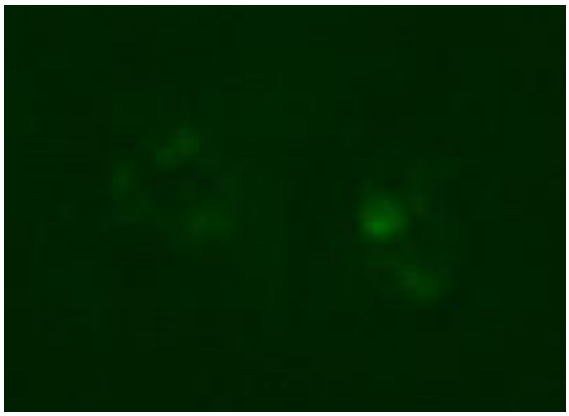


Figure 4.23 Merged image of signal coming from GFP only and phase contrast image of the yeast cells showing the localization of GFP without the HVA22-like protein with a homogeneous distribution throughout the yeast cells (63X magnification).

As seen from the images obtained by confocal laser microscope, expression of empty yEGFP vector without the presence HVA22-like ORF in it yielded a homogeneous distribution pattern of the signal upon excitation with laser at 488 nm and the localization image was captured with emission detection around 505-535 nm.



Signal coming from HVA22-like protein tagged with GFP (63X magnification)

Phase contrast image of yeast cells (63X magnification)

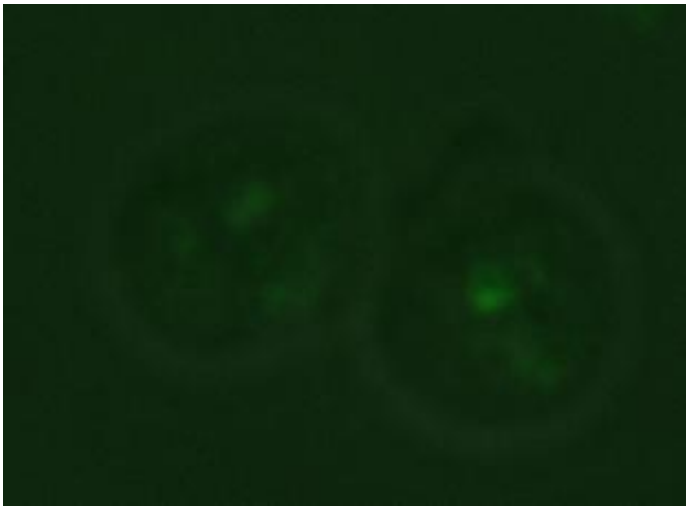


Figure 4.24 Merged image of signal coming from signal coming from GFP-tagged HVA22-like protein and phase contrast image of the yeast cells showing the localization of GFP-tagged HVA22-like protein with a more punctuate distribution throughout the yeast cells (63X magnification).

As seen from the images obtained by confocal laser microscope, expression of vector construct which consisted of HVA22-like ORF tagged with C-terminal GFP

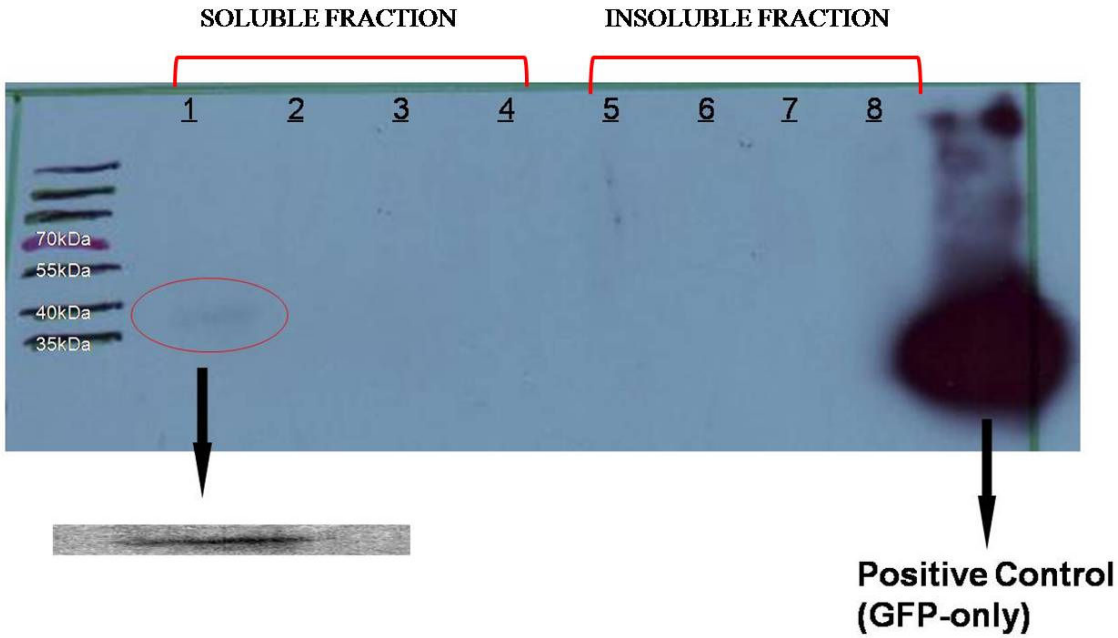
yielded a more localized pattern of the signal upon excitation with laser at at 488 nm and the localization image was captured with emission detection around 505-535 nm.

4.9 Western Blotting

Samples from which the protein extraction was performed were harvested 22 hours after induction as suggested by Drew et al., 2008. Signal detection using ECL western blotting substrates was proved to be successful however the signal coming from HVA22-like protein isolated from yeast cells was very weak as displayed in Figure 4.25. Possible reasons might be inefficient protein extraction from yeast cells due to their robust cell wall structure or the insufficient amount of starting cell amount in extraction process although the former seems more likely. In contrast, purified GFP which was used as a positive control yielded a very strong signal indicating the adequacy of chemicals and materials used in blotting and signal detection steps.

As seen from the Figure 4.25, the only obtained signal was coming from the soluble fraction of induced cell samples expressing HVA22-like protein tagged with GFP. It is most likely that we could not receive any signal from insoluble fraction samples since solubilization of those using 2X SDS sample loading buffer was not efficient and there were too much cell debris remaining which also prevented the proper loading of samples into wells due to their high viscosity and sticky nature that further minimized the effective concentration of extracted protein.

Expected band size of the expressed HVA22-like protein tagged with GFP was ~50kDa since they both have an average molecular weight of ~25kDa. However the observed band had a size of ~40kDa. This is probably due to fact that the mobility of membrane proteins in gels also depends on their shapes. Experimental approach should be used rather than just considering the amino acid composition and the length of the protein for determination of the actual molecular weight of membrane proteins. For example sedimentation equilibrium analytical ultracentrifugation is a preferred technique when one is concerned with the molecular weights of membrane proteins (Fleming, 2008; Lustig et al., 2000).



1st lane – induced HVA22-like protein + yEGFP construct
2nd lane – uninduced HVA22-like protein + yEGFP construct
3rd lane – induced empty vector
4th lane – uninduced empty vector
5th lane – induced HVA22-like protein + yEGFP construct
6th lane – uninduced HVA22-like protein + yEGFP construct
7th lane – induced empty vector
8th lane – uninduced empty vector

Figure 4.25 Western blotting result. Signal detection obtained upon treatment with ECL western blotting substrates indicated a successful blotting and antibody treatment process and also confirmed the expression of HVA22-like protein in yeast cells.

5. DISCUSSION

In order to survive, an organism should have adequate response and defense mechanisms against threats caused by the physical environment in which the organism exists. Some organisms respond to threatening environmental stress conditions by avoidance and ability to move away. However, some organisms, such as plants, should have intricate and well-developed defense mechanisms against those harsh conditions in order to survive due to their lack of ability to move. Although wild progenitors of the modern cultivated crops have such resistance mechanisms and genetic variation suited to those needs, modern crops have lost most of those versatile properties in their genetic make-up in order to acquire properties to provide higher yields by means of agricultural practices.

The aim of this study was to show that an HVA22-like protein was involved in prolonged drought stress in wild emmer wheat. Analyses indicated that it was differentially expressed significantly in root tissue under prolonged (nine days) water-deficit conditions. For this purpose, standard greenhouse experiments were designed and a screening was performed among wild emmer drought-tolerant genotype, drought-sensitive genotype and modern durum wheat variety Kızıltan. This screening was a repetition of an early screening experiment of our group in order to ensure the original work (Ergen and Budak, 2009) which utilized these three genotypes in construction of subtractive cDNA libraries and sequencing of ESTs was in agreement with the current work. For that purpose, data was obtained using a chlorophyll-meter (SPAD meter) for determination of the relative chlorophyll content of the plants which is an important indicator of the health status of the plants during screening process (Kumar et al., 2002). A SPAD meter is a commonly used device in especially experiments conducted under abiotic stress conditions for determination of the detrimental effect levels of the stress of interest (Chubachi et al. 1986; Jiang and Vergara 1986; Yadava, 1986; Zhao et al., 2007).

Following the screening experiments, standard cloning procedures starting with the extraction of total RNA from samples that were obtained from root and leaf tissues of both stress and control group plants were used as described in Sambrook et al, 2001. As a result, an open reading frame (ORF) which belonged to an *HVA22-like protein* whose EST was identified in the early work (Ergen and Budak, 2009) was cloned, characterized and also shown to be existent at the protein level.

After cloning and sequencing the coding region of HVA22-like protein, extensive *in silico* analysis of that sequence was performed utilizing a variety of bioinformatics tools. HVA22 protein which was first isolated from barley was shown to have several homologues in other organisms such as maize and Arabidopsis as well (Brands and Ho, 2002; Shen et al., 2001). HVA22-like protein family members are known to be transmembrane proteins that are thought to play a role in vesicular trafficking besides having a role in drought tolerance (Guo and Ho, 2008; Shen et al., 2001) and our results were consistent with these findings. Secondary structure predictions together with the membrane topology prediction analysis of cloned sequence results predicted HVA22-like protein from wild emmer wheat to have four transmembrane domains. In addition, subcellular localization prediction of HVA22-like protein was performed utilizing Plant-mPloc server used (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) and results suggested that this protein was highly likely to be localized in chloroplast (Chou and Shen, 2008; Chou and Shen, 2007; Shen and Chou, 2006; Chou, 2005). This finding does not necessarily contradict with the earlier works by several different groups in which the HVA22 protein was shown to be localized in endoplasmic reticulum and Golgi (Guo and Ho, 2008). Because, the protein sequence of HVA22-like protein from *Triticum aestivum* is also predicted to be localized both in mitochondria and chloroplast by the same algorithm. All these findings suggest that HVA22-like proteins are most likely to be localized in different compartments of cells and they do not need to be localized at the same specific compartment in different organisms. This makes sense since different members of HVA22-like protein family members were shown to be involved in different kind of stress responses although they are all induced under stress (Chen et al., 2002). Interproscan (http://www.ebi.ac.uk/Tools/InterProScan/) results also yielded an interesting point of view indicating that the translated amino acid sequence of the cloned fragment contains a TMPIT-like protein domain and a number of proteins which

are considered as members of this family are annotated as “transmembrane proteins induced by tumour necrosis factor alpha”. This result is highly intriguing because some of the HVA22-like protein family members are previously shown to play a role in inhibition of programmed cell death (Guo and Ho, 2008) while having a role in vesicular trafficking. Having a predicted TMPIT-like protein domain in our cloned protein sequence also seems to be in agreement with these findings because the proteins those have the TMPIT-like protein domain are said to be induced by tumour necrosis factor alpha and they are also known to be induced under stress conditions. This result increases the possibility of protein product of our cloned sequence having a defensive role under stress conditions as inferred from the previous results (Ergen et al., 2009; Ergen and Budak, 2009; Guo and Ho, 2008).

In protein expression study that was performed utilizing both Rosettagami2™ and BL21(DE3) strains of *E. coli*, we were able to confirm the presence of the protein which had a theoretical molecular weight of ~25kDa. A growth curve for each of the strains was also constructed by the cell density data obtained with a spectrophotometer at OD_{600}. We observed a dramatic retardation in growth of the bacterial cells compared to expected reduced growth rates. This dramatic retardation was probably due to the fact that, HVA22-like protein is an exclusively eukaryotic protein (Shen et al., 2001; Guo and Ho, 2008) and its overexpression in a prokaryotic cell caused a more dramatic effect. However, bacterial cells seemed to have survival mechanism that resulted in the elimination of the protein from the environment possibly with proteosomal degradation which was reflected both by the increased rates of growth of cells after a time point (Figure 4.15 & Figure 4.16) and also the disappearance of the protein bands in SDS-PAGE gel images (Figure 4.17 & Figure 4.18).

When performing confocal laser microscopy imaging in cellular localization experiments green fluorescent emission caused between 505nm and 535nm by the presence of green fluorescent protein (GFP) enabled us to make a comparison between cells expressing the empty vector whose product is only GFP as and cells expressing the HVA22-like protein tagged with GFP at its C-terminal. The results indicated that HVA22-like protein expressed in yeast cells displayed punctuate and network-like distribution within the cells whereas the distribution of the GFP protein by itself was homogeneous. This result confirmed that cloned HVA22-like protein sequence from wild emmer wheat was a membrane associated protein which had predicted

transmembrane domains. Although localization of the HVA22-like protein could be demonstrated to some extent, we were not able show the specific cellular compartment in which it was localized. In order to be able to assign a certain subcellular localization for the HVA22-like protein from wild emmer wheat, a colocalization experiment using specific organellar markers should be performed (Guo and Ho, 2008; Waller et al., 2010).

Here in this work, we identified a novel drought stress response protein for the first time in *Triticum turgidum* ssp. *dicoccoides* and a deeper analysis and understanding of this gene in wild emmer wheat would present rich opportunities for improving the resistance and tolerance of modern bread wheat to water deficit conditions.

6. CONCLUSION

Using standard cloning and expression techniques, this study for the first time and clearly demonstrated that a novel HVA22-like protein is also present in wild emmer wheat and is differentially expressed in root tissue of the wild emmer wheat resistant genotype under prolonged drought stress conditions compared to its modern relative, the durum wheat.

Further functional and structural studies will provide a better understanding of this protein. Since our protein is a drought-responsive protein and does not have any detectable activity using standard assay procedures, transforming this gene into a sensitive genotype of wild emmer wheat under the same drought conditions will be a beneficial plan. In addition, since the successful cloning of *HVA22-like protein* into a vector containing two different tags was achieved, structural characterization studies using biophysical techniques such as dynamic light scattering (DLS) on purified protein products might be another alley for further investigation. Predicted pI value of the protein will also facilitate the purification of the protein.

Barley HVA22 protein was shown to be localized in the Golgi and endoplasmic reticulum, and the subcellular localization prediction performed using Plant-mPloc server suggests that our HVA22-like protein is most probably localized in chloroplast. This finding also could be tested using chloroplast-specific antibodies and the investigation of co-localization could again be visualized using confocal laser microscopy.

In conclusion, utilizing the aforementioned approaches, the cloned HVA22-like protein coding sequence would provide many new opportunities for further experimental investigation of drought stress responses in wild emmer wheat with a complete characterization of protein since to our best knowledge there is no previous study regarding the HVA22-like protein in *Triticum turgidum* spp. *dicoccoides.* Here we show that HVA22-like protein isolated from this species plays a key role in prolonged

drought stress response. Further characterization of this protein will be useful for improvement of wheat genotypes.

7. REFERENCES

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. Basic local alignment search tool. (1990) *J. Mol. Biol.* **215**:403-410.

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. (1997) *Nucleic Acids Res.* **25**:3389-402.

Araus J.L., Ferrio J.P., Buxo R., Voltas J. The historical perspective of dryland agriculture: lessons learned from 10,000 years of wheat cultivation. (2007) *J. Exp. Bot.* **58**:131-145.

Arnon D.I. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. (1949) *Plant Physiol.* **24**:1-15.

Ashraf M., Foolad M.R. Roles of glycine betaine and proline in improving plant abiotic stress resistance. (2007) *Environ. Exp. Bot.***59**:206-216.

Babar M.A., Reynolds M.P., van Ginkel M., Klatt A.R., Raun W.R., Stone M.L. Spectral reflectance to estimate genetic variation for in-season biomass, leaf chlorophyll, and canopy temperature in wheat. (2006) *Crop Sci.* **46:**1046-1057.

Barrero J.M., Rodriguez P.L., Quesada V., Piqueras P., Ponce M.R., Micol J.L., oth abscisic acid (ABA)-dependent and ABA-independent pathways govern the induction of NCED3, AAO3 and ABA1 in response to salt stress. (2006) *Plant Cell Environ.* **29**:1-4.

Bartels D., Engelhardt K., Ronacarati R., Schneider K., Rotter M., Salamini F. An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related Protein (1991) *EMBO J.* **10**:1037-1043.

Bartels, D. and Souer, E. Molecular responses of higher plants to dehydration. In: Plant responses to abiotic stress. (2003) *Topics in Current Genetics*. Berlin; Springer; **4**:9-38.

Bohnert H.J, Gong Q., Li P., Ma S. Unraveling abiotic stress tolerance mechanisms – getting genomics going. (2006) *Curr. Opin. in Plant Biol.* **9**:180-188.

Bonneville M-C., Fyles J. W. Assessing variations in SPAD-502 chlorophyll meter measurements and their relationships with nutrient content of trembling Aspen foliage. (2006) *Commun. Soil Sci. Plan.* **37**:525–539.

Bota J., Flexas J., Medrano H., Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? (2004) *New Phytol.* **162**:671–681.

Brands A., Ho T-H. D. Function of a Plant Stress-Induced Gene, HVA22. Synthetic Enhancement Screen with Its Yeast Homolog Reveals Its Role in Vesicular Traffic. (2002) *Plant Physiology* **130**:1121-1131.

Bray E.A., Beachy. R.N. Regulation by ABA of β-iconglycinin expression in cultured developing soybean cotyledons. (1985) *Plant Physiol.* **79**:746-750.

Brown T.A. Genomes 2 (2002) John Wiley & Sons Inc., USA. 2nd edition

Bruce T.J.A., Matthes M.C., Napier J.A., Pickett J.A. Stressful "memories" of plants: Evidence and possible mechanisms (2007) *Plant Sci.* **173**:603-608.

Calero M., Whittaker G.R., Collins R.N. Yop1p, the yeast homolog of the polyposis locus protein 1, interacts with Yip1p and negatively regulates cell growth. (2001) *J. Biol. Chem.* **276**:12100-12112

Castelli F., Contillo R., Miceli F. Non-destructive determination of leaf chlorophyll content in four crop species. (1996) *J. Agron. Crop Sci.* **177**:275-283.

Chen C-N.N., Chen H-R., Yeh S-Y., Vittore G., Ho T-H.D. Autophagy is enhanced and floral development is impaired in AtHVA22d RNA interference Arabidopsis. (2009) *Plant Physiol.* **149**:1679-1689.

Chen C.N., Chu C.C., Zentella R., Pan S.M., Ho T.H. *AtHVA22* gene family in *Arabidopsis*: phylogenetic relationship, ABA and stress regulation, and tissue-specific expression. (2002) *Plant Mol. Biol.* **49**:633–64

Chinnusamy V., Gong Z., Zhu J-K. Abscisic acid-mediated epigenetic processes in plant development and stress responses. (2008) *J Integre Plant Biol.* **50**:1187-1195.

Chou K.C., Shen H-B. Cell-PLoc: 2008 A package of web-servers for predicting subcellular localization of proteins in various organisms. (2008) *Nature Protoc.* **3**:153-162.

Chou K-C., Shen H-B. Large-scale plant protein subcellular location prediction. (2007) *J. Cell. Biochem.* **100**:665-678.

Chou K-C. Using amphiphilic pseudo amino acid composition to predict enzyme subfamily classes. (2005) *Bioinformatics* **21**:10-19.

Chubachi T., Asano I., Oikawa T. The diagnosis of nitrogen nutrition of rice plant (Sasanishki) using chlorophyll meter. (1986) *Japan J. Soil Sci. Plant Nutr*. **57**:190-193.

Christmann, A., Hoffmann, T., Teplova, I., Grill, E. and Müller, A. Generation of active pools of abscisic acid revealed by in vivo imaging of water stressed Arabidopsis. (2005) *Plant Physiol.* **137:**209-219.

Cominelli E., Galbiati M., Vavasseur A., Conti L., Sala T., Vuylsteke M., Leonhardt N., Dellaporta S.L., Tonelli C. A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. (2005) *Curr. Biol*. **15**:1196-1200.

Costa C., Dwyer L.M., Dutilleul P., Stewart D.W., Ma L.B., Smith D.L. Inter-relationships of applied nitrogen, SPAD, and yield of leafy and non-leafy maize genotypes. (2001) *J. Plant Nut.* **24**:1173-1194.

Crouch M.I., Sussex I.M. Development and storage-protein synthesis in Brassica napus L. embryos in vivo and in vitro. (1981) *Planta* **153**:64-74.

Drew D. et al. Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and PhoA/GFP fusion analysis. (2002) *Proc. Natl. Acad. Sci. USA* **99**:2690-2695.

Drew D., Newstead S., Sonoda Y., Kim H., Heijne G., Iwata S. GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in Saccharomyces cerevisiae. (2008) *Nature Protoc.* **3**:784-798.

Dubcovsky J., Dvorak J. Genome plasticity a key factor in the success of polyploidy wheat under domestication. (2007) *Science* **316**:1862-1866.

Dubouzet J.G., Sakuma Y., Ito Y., Kasuga M., Dubouzet E.G., Miura S., Seki M., Shinozaki K., Yamaguchi-Shinozaki K. OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. (2003) *Plant J.* **33**:751-763.

Ergen N., Budak H. Sequencing over 13,000 expressed sequence tags from six subtractive cDNA libraries of wild and modern wheats following slow drought. (2009) *Plant Cell Environ.* **32**:220-236.

Ergen N.Z., Thimmapuram J., Bohnert H.J., Budak H. Transcriptome pathways unique to dehydration tolerant relatives of modern wheat. (2009) *Funct. Integr. Genomics* **9**:377–396.

Fanizza G., Ricciardi L., Bagnulo C. Leaf greenness measurements to evaluate water stressed genotypes in *Vitis vinifera*. (1991) *Euphytica* **55**:27-32.

Farooq M., Wahid A., Kobayashi N., Fujita D., Basra S.M.A. Plant drought stress: effects, mechanisms and management (2009) *Agron. Sustain. Dev.* **29**:185–212.

Fleming K.G. Determination of membrane protein molecular weight using sedimentation equilibrium analytical ultracentrifugation. (2008) *Curr. Protoc. Protein Sci.* Aug;Chapter 7:Unit 7.12.1-7.12.13

Flexas J., Medrano H. Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. (2002) *Ann.Bot-London* **89**:183-189.

Fong F., Koehler D.E., Smith J.D. Fluridone induction of vivipary during maize seed development. (1983) In: Kruger JE, LaBerg DE, eds. *Third International Symposium on Pre-Harvest Sprouting in Cereals.* Boulder, Co. USA: Westview Press, 188-95.

Goffeau A., Barrell B.G., Bussey H., Davis R.W., Dujon B., Feldmann H., Galibert F., Hoheisel J.D., Jacq C., Johnston M., Louis E.J., Mewes H.W., Murakami Y., Philippsen P., Tettelin P., Oliver S.G.. Life with 6000 genes. (1996) *Science* 274:546-567.

Gratani L. A non-destructive method to determine chlorophyll content of leaves. (1992) *Photosynthetica* **26**:469-473.

Griffiths H., Parry, M.A.J. Plant responses to water stress. (2002). *Ann. Bot.* **89:**801-802.

Guo W-J., Ho T-H.D. An Abscisic Acid-Induced Protein, HVA22, Inhibits Gibberellin-Mediated Programmed Cell Death in Cereal Aleurone Cells. (2008) *Plant Physiol.* **147**:1710-1722.

Gutterson N., Zhang J.Z., Genomics applications to biotech traits: A revolution in progress? (2004) *Curr. Opin. Plant Biol.* **7**:226–230.

Hawkins J.A., Sawyer J.E., Barker D.W., Lundvall J. P. Using relative chlorophyll meter values to determine nitrogen application rates for corn. (2007) *Agron. J.* **99**:1034-1040.

Hoekstra F.A., Golovina E.A., Buitink J. Mechanisms of plant desiccation tolerance. (2001) *Trends Plant Sci.* **6**:431–438.

Huh W.K., Falvo J.V., Gerke L.C., Carroll A.S., Howson R.W., Weissman J.S., O'Shea E.K. Global analysis of protein localization in budding yeast. (2003) *Nature* **425**:68-691.

Ingram, J. and Bartels, D. The molecular basis of dehydration tolerance in plants. (1996) *Ann. Rev. Plant Physiol. Plant Mol. Biol*. **47:**377-403.

Jiang X.X., Vergara B.S. Chlorophyll meter (SPAD-501) to quantify relative cold tolerance in rice. (1986) *Int Rice Res News Lett*. **11**:10-11.

Kalefetoğlu T., Ekmekçi Y., The effects of drought on plants and tolerance mechanisms. (2005) *G.U. J. Sci.* **18**:723-740.

Kawasaki S., Borchert C., Deyholos M., Wang H., Brazille S., Kawai K., Galbraith D., Bohnert H. Gene expression profiles during the initial phase of salt stress in rice. (2001) *Plant Cell* **13**:889–905.

Kumar R.R., Marimuthu S., Jayakumar D. and Jeyaramraja P.R. In situ estimation of leaf chlorophyll and its relationship with photosynthesis in tea. (2002) *Ind. J. Plant Physiol.* **7**:367-371.

Lachno D.R., Baker D.A. Stress induction of abscisic acid in maize (*Zea mays* cultivar LG 11) roots. (1986) *Physiol. Plantarum* **68**:215-221.

Lawlor D.W. Limitation to photosynthesis in water stressed leaves: stomata vs. metabolism and the role of ATP. (2002) *Ann. Bot*. **89:**1-15.

Lawlor D.W., Cornic, G. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. (2002) *Plant Cell Environ.* **25:**275-294.

Lawson T, Craigon J, Tulloch AM, Black CR, Colls JJ and Landon G. Photosynthetic responses to elevated CO2 and ozone in field-grown potato (*Solanum tuberosum*). (2001) *J. Plant Physiol.* **158**:309-323.

Lichtenthaler H.K. Chlorphylls and carotenoids: pigments of photosynthetic biomembranes. (1987) *Method. Enzymol*. **148**:350-382.

Ludevid D., Hofte H., Himelblau E,. Chrispeels M.J. The expression pattern of the tonoplast intrinsic protein y-TIP in *Arabidopsis thaliana* is correlated with cell enlargement. (1992) *Plant Physiol.* **100**:1633-1639.

Luo M.C., Yang Z.L., You F.M., Kawahara T., Waines J.G., Dvorak J. The structure of wild and domesticated emmer wheat populations, gene flow between them, and the site of emmer domestication. (2007) *Theor. Appl. Genet.* **114:**947-959.

Lustig A., Engel A., Tsiotis G., Landau E. M., Baschong W. Molecular weight determination of membrane proteins by sedimentation equilibrium at the sucrose or Nycodenz-adjusted density of the hydrated detergent micelle. (2000) *Biochim. Biophys. Acta* **1464**:199-206.

Madhava Rao K.V., Raghavendra A.S., Janardhan K.R. (eds.) Physiology and molecular biology of stress tolerance in plants (2006) *Springer* , Netherlands Chapter 1: pp.1-14.

Mansfield T.J., Atkinson C.J. Stomatal behaviour in water stressed plants, in: Alscher R.G., Cumming J.R. (Eds.), Stress responses in plants: adaptation and acclimation mechanisms (1990) Wiley-Liss, New York, pp. 241-264.

Marchler-Bauer A. et al., CDD: specific functional annotation with the Conserved Domain Database. (2009) *Nucleic Acids Res.* **37:**(D)205-10.

Marchler-Bauer A., Bryant S.H., CD-Search: protein domain annotations on the fly. (2004) *Nucleic Acids Res.* **32:**(W)327-331.

Markwell J., Oesterman J.C., Mitchell J.L. Calibration of the Minolta SPAD-502 leaf chlorophyll meter. (1995) *Photo. Res.* **46**:467–472.

Martinez D.E., Guiamet J.J. Distortion of the SPAD 502 chlorophyll meter readings by changes in irradiance and leaf water status. (2004) *Agronomie* **24:**41-46.

Maruyama K., Sakuma Y., Kasuga M., Ito Y., Seki M., Goda H., Shimada Y., Yoshida S., Shinozaki K., Yamaguchi-Shinozaki K. Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. (2004) *Plant J*. **38**:982-993.

Monje O.A., Bugbee B. Inherent limitations of nondestructive chlorophyll meters: a comparison of two types of meters. (1992) *Hortscience* **27**:69-70.

Nakashima K., Ito Y., Yamaguchi-Shinozaki K. Transcriptional Regulatory Networks in Response to Abiotic Stresses in Arabidopsis and Grasses. (2009) *Plant Physiol.* **149**: 88-95.

Neill S., Barros R., Bright J., Desikan R., Hancock J., Harrison J., Morris P., Ribeiro D., Wilson I. Nitric oxide, stomatal closure, and abiotic stress (2008) *J. Exp. Bot.* **59**:165-176.

Neufeld H. S., Chappelka A.H., Somers G. L., Burkey K.O., Davison A. W., Finkelstein P. L. Visible foliar injury caused by ozone alters the relationship between SPAD meter readings and chlorophyll concentrations in cutleaf coneflower (2006) *Photosynth. Res.* **87**:281-286.

Nevo E. Genetic diversity in wild cereals: regional and local studies and their bearing on conservation *ex situ* and *in situ*. (1998) *Genetic Resources and Crop Evolution* **45:** 335-370.

Novo M., Bigey F., Beyne E., Galeote V., Gavory F., Mallet S., Cambon B., Legras J-L., Wincker P., Casaregola S., Dequin S. Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast *Saccharomyces cerevisiae* EC1118 (2009) *Proc. Natl. Acad. Sci. USA* **106**:16333-16338.

Nziengui H., Schoefs B. Functions of reticulons in plants: What we can learn from animals and yeasts. (2009) *Cell. Mol. Life Sci.* **66**:584-595.

Parry M.A.J., Andralojc P.J. Khan S., Lea P.J., Keys A.J. Rubisco activity: effects of drought stress. (2002) *Ann. Bot*. **89**:833-839.

Peleg Z., Fahima T., Abbo S., Krugman T., Nevo E., Yakir D., Saranga Y. Genetic diversity for drought resistance in wild emmer wheat and its ecogeographical associations. (2005) *Plant Cell Environ.* **8:**176-191.

Philippsen P., Kleine K., Pohlmann R., Dusterhoft A., Hamberg K., Hegemann J.H., Obermaier B., Urrestarazu L.A., Aert R., Albermann K., et al. The nucleotide sequence of *Saccharomyces cerevisiae* chromosome XIV and its evolutionary implications. (1997) *Nature* **387:**93-98.

Pieper U., Eswar N., Davis FP., Braberg H., Madhusudhan M.S., Rossi A., Marti-Renom M., Karchin R., Webb B.M., Eramian D., et al. MODBASE: a database of annotated comparative protein structure models and associated resources. (2006) *Nucleic Acids Res.* **34**:D291–D295.

Pieper U., Eswar N., Webb B.M., Eramian D., Kelly L., Barkan D.T., Carter H., Mankoo P., Karchin R., Marti-Renom M.A, Davis F.P., Sali A. MODBASE: a database of annotated comparative protein structure models and associated resources. (2009) *Nucleic Acids Res.* **37**:D347-D354.

Rabbani M.A., Maruyama K., Abe H., Khan M.A., Katsura K., Ito Y., Yoshiwara K., Seki M., Shinozaki K., Yamaguchi-Shinozaki K. Monitoring expression profiles of rice

genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. (2003) *Plant Physiol*. **133**:1755-1767.

Ritchie R.J. Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. (2008) *Photosynthetica* **46**:115-126.

Sambrook J., Manniatis T., Fritsch E.F. Molecular cloning: a laboratory manual (2001) Cold Spring Harbor Laboratory Press. 3rd edition.

Samdur M.Y., Singh A.L., Mathur R.K., Manivel P., Chikani B.M., Gor H.K., Khan M.A. Field evaluation of chlorophyll meter for screening groundnut (*Arachis hypogaea* L.) genotypes tolerant to iron-deficiency chlorosis. (2000) *Curr. Sci. Bangalore* **79**:211-214.

Seki M., Narusaka M., Abe H., Kasuga M., Yamaguchi-Shinozaki K., Carninci P., Hayashizaki Y., Shinozaki K. Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. (2001) *The Plant Cell* **13**:61–72.

Seki M., Umezawa T., Urano K., Shinozaki K. Regulatory metabolic networks in drought stress responses. (2007) *Curr. Opin. Plant Biol.* **10**:296-302

Seki M., Ishida J., Narusaka M., et al. Monitoring the expression pattern of around 7000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. (2002) *Funct. Integr. Genomic*s **2**:282–291.

Sharp R.E., LeNoble M.E. ABA, ethylene and the control of shoot and root growth under water stress. (2002) *J. Exp. Bot.* **53**:33-37.

Shen H-B., Chou K-C. Ensemble classifier for protein folding pattern recognition (2006) *Bioinformatics* **22**:1717-1722.

Shen Q., Uknes S.J., Ho T.H. Hormone response complex in a novel abscisic acid and cycloheximide-inducible barley gene. (1993) *J. Biol. Chem.* **268**:23652–23660.

Shen Q., Chen C-N., Brands A., Pan S-M., Ho T-H. D. The stress- and abscisic acid-induced barley gene HVA22: developmental regulation and homologues in diverse organisms. (2001) *Plant Mol. Biol.* **45**:327-340.

Shinozaki K., Yamaguchi-Shinozaki K. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. (2000) *Curr. Opin. Plant Biol.* **3**:217-223.

Shinozaki K., Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance (2007) *J. Exp. Bot.* **58**:221–227.

Spyropoulos I.C., Liakopoulos T.D., Bagos P.G., Hamodrakas S.J. TMRPres2D: high quality visual representation of transmembrane protein models. (2004) *Bioinformatics* **20**:3258-3260.

Tezara W., Mitchell V.J., Driscoll S.D., Lawlor D.W. Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. (1999) *Nature* 401:914–917.

Trapeznikov V.K., Ivanov I.I., Kudoyarova G.R. Effect of heterogeneous distribution of nutrients on root growth, ABA content and drought resistance of wheat plants. (2003) *Plant and Soil* **252:**207-214.

Tusnády G.E., Simon I. The HMMTOP transmembrane topology prediction server. (2001) *Bioinformatics* **17**:849-850.

Tusnády G.E., Simon I. Principles Governing Amino Acid Composition of Integral Membrane Proteins: Applications to Topology Prediction. (1998) *J. Mol. Biol.* **283**:489-506.

Umezawa T., Fujita M., Fujita Y., Yamaguchi-Shinozaki K., Shinozaki K.. Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. (2006) *Curr. Opin. Biotech.* **17**:113-122.

Vasil I.K. Molecular genetic improvement of cereals: transgenic wheat (*Triticum aestivum* L.) (2007) *Plant Cell Rep.* **26**:1133-1154.

Vij S., Tyagi A.K. Emerging trends in the functional genomics of the abiotic stress response in crop plants. (2007) *Plant Biotechnol J.* **5**:361-380.

Vilardell, J., Goday, A., Freire, M.A., Torrent, M., Martinez, C., Torne, J.M., Pages, M. Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. (1990) *Plant Mol. Biol.* **14**:423-432.

Waller J.C., Dhanoa P.K., Schumann U., Mullen R.T., Snedden W.A. Subcellular and tissue localization of NAD kinases from Arabidopsis: compartmentalization of de novo NADP biosynthesis. (2010) *Planta* **231**:305-317.

Wingler A., Roitsch T. Metabolic regulation of leaf senescence: interactions of sugar signalling with biotic and abiotic stress responses. (2008) *Plant Biol.* **10**:50 – 62.

Wong C.E., Li Y., Whitty B.R., Diaz-Camino C., Akhter S.R., Brandle J.E., Golding G.B., Weretilnyk E.A., Moffatt B.A., Griffith M. Expressed sequence tags from the Yukon ecotype of *Thellungiella* reveal that gene expression in response to cold, drought and salinity shows little overlap. (2005) *Plant Mol. Biol.* **58**:561-574.

Yadava U.L. A rapid and nondestructive method to determine chlorophyll in intact leaves. (1986) *HortScience* **22**:1449–1450.

Yamaguchi-Shinozaki K., Shinozaki K. Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. (2005) *Trends in Plant Sci.* **10**:88–94.

Yamamoto A., Nakamura T., Adu-Gyamfi J.J., Saigusa M. Relationship between chlorophyll content in leaves of sorghum and pigeonpea determined by extraction method and by chlorophyll meter (SPAD-502). (2002) J. Plant Nut. **25**: 2295-2301.

Zeevaart, J.A.D., Creelman, R.A. Metabolism and physiology of abscisic acid. (1988) *Annu. Rev.Plant Physiol. Plant Mol. Biol.* **39:** 439-473.

Zhang J. Accumulation of ABA in maize roots in response to root severing. (2006) *New Phytol.* **127**:309-314.

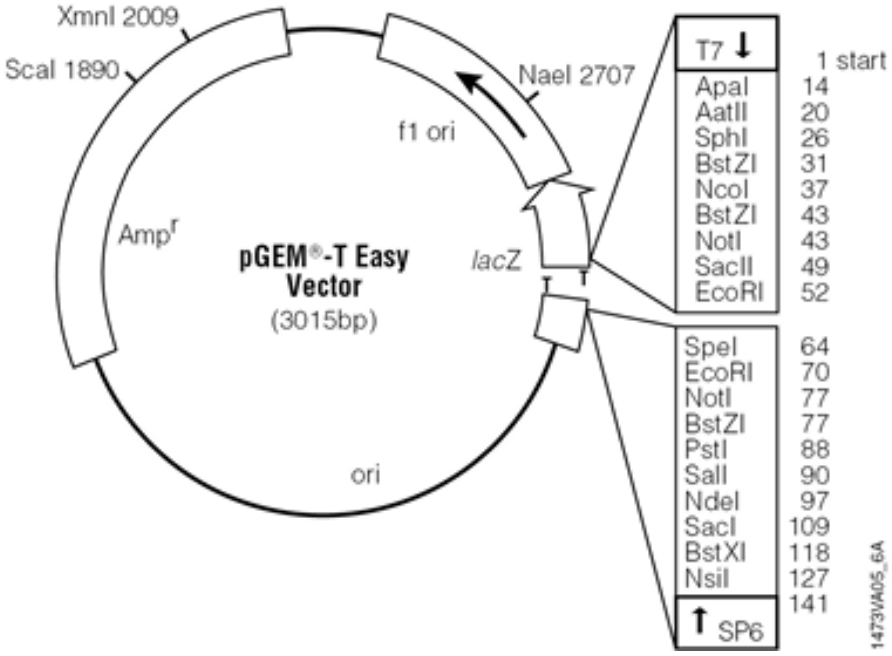
Zhang J., Davies W.J. Increased synthesis of ABA in partially dehydrated root tips and ABA transport from roots to leaves **(**1987) *J. Exp. Bot.* **38**:2015-2023.

Zhao H., Dai T., Jing Q., Jiang D., Cao W. Leaf senescence and grain filling affected by post-anthesis high temperatures in two different wheat cultivars. (2007) *Plant Growth Regul.* **51**:149-158.

Zohary D., Hopf M. Domestication of Plants in the Old World: The Origin and Spread of Cultivated Plants in West Asia, Europe, and the Nile Valley (2001) *OUP Oxford.* 3rd edition.

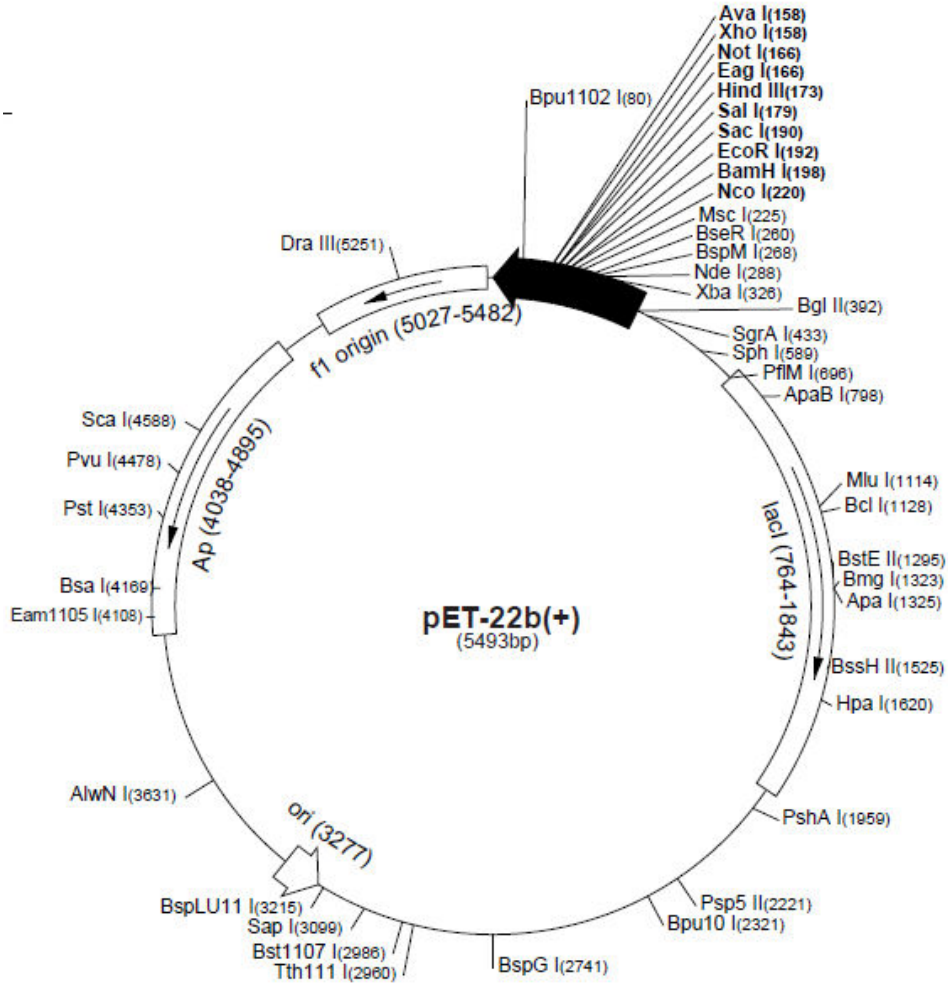
APPENDIX A

VECTOR MAPS AND SEQUENCES

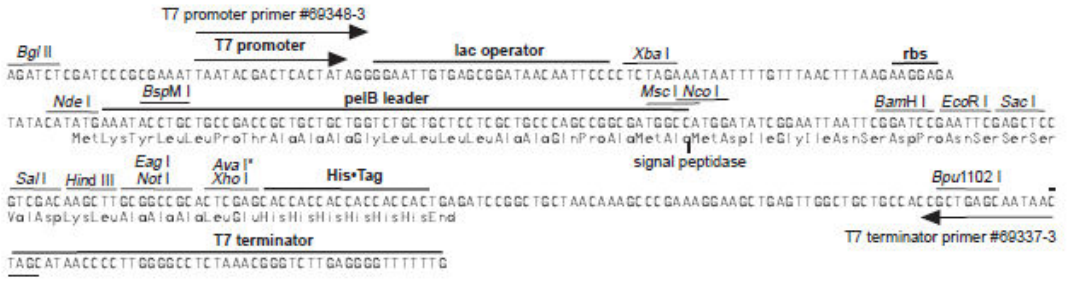


pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

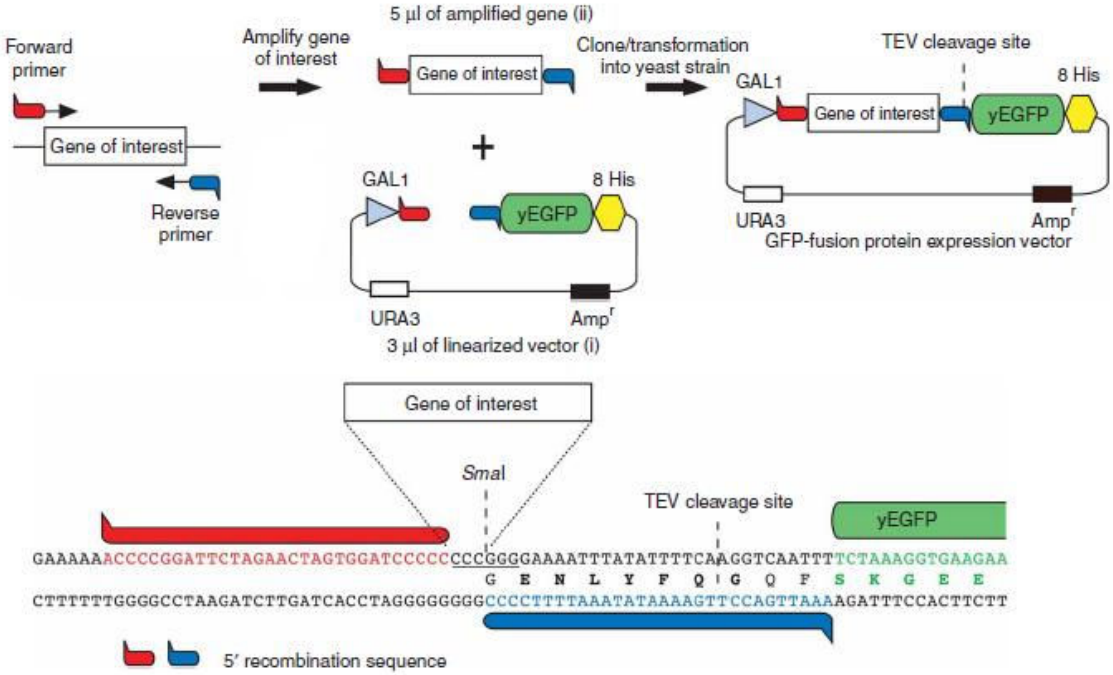


pET-22b(+) sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-225
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
bla coding sequence	4038-4895
f1 origin	5027-5482



pET-22b(+) cloning/expression region

yEGFP vector

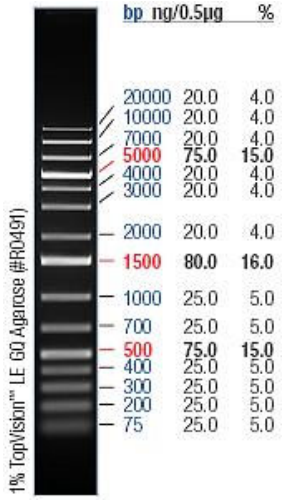


Cloning by homologous recombination into 2 µ Saccharomyces cerevisiae GFP-fusion vector.

APPENDIX B

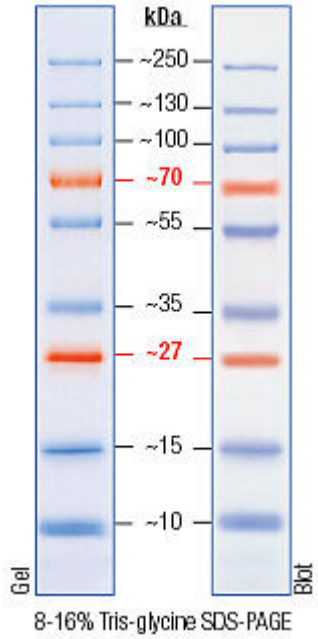
MOLECULAR WEIGHT MARKERS

DNA Molecular Weight Marker, Fermentas 1Kb Plus Ladder



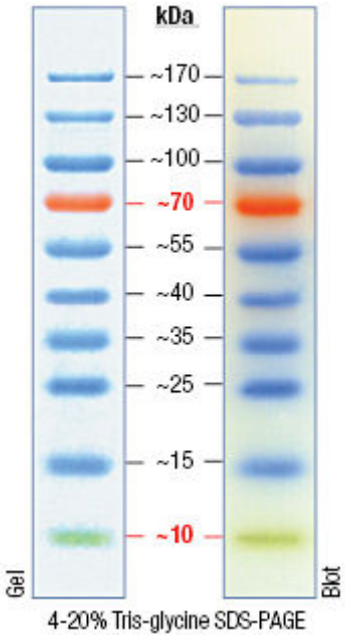
0.5µg/lane, 8cm length gel,
1X TAE, 7V/cm, 45min

Protein Molecular Weight Markers,



8-16% Tris-glycine SDS-PAGE

SDS-PAGE analysis



4-20% Tris-glycine SDS-PAGE

Western Blotting

APPENDIX C

LIST OF EQUIPMENT

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Nüve, OT 032, TURKEY
Balance:	Sartorius, BP 221 S,
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Beckman Coultier ™ Microfuge® 18 Centrifuge, USA
Cassette:	Kodak Biomax MS casette, USA
Confocal Laser Microscope	Leica, Germany
Deep-freeze:	-80ºC, Thermo Electron Corporation, USA
	-20ºC, Bosch, TURKEY
Deionized water:	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
	SCIE-PLAS, TURKEY
Gel documentation:	UVITEC, UVIdoc Gel Documentation System,UK

	BIO-RAD, UV-Transilluminator 2000, USA
Heating block:	Bioblock Scientific, FRANCE
	Bio TDB-100 Dry Block Heating Thermostat, HVD Life Sciences, AUSTRIA
Ice machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
	Nüve EN 120, TURKEY
Laminar flow:	Kendro Lab. Prod., Heraeus, Herasafe HS12, GERMANY
Magnetic stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Micropipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP Multical®, GERMANY
	HANNA, pH213 microprocessor pH meter, GERMANY

Power Supply:	Wealtec, Elite 300, USA
	Biogen, AELEX, USA
Refrigerator:	+4ºC, Bosch, TURKEY
Shaker:	Excella E24 Shaker Series, New Brunswick Sci., USA
	GFL, Shaker 3011, USA
	Innova™ 4330, New Brunswick Sci., USA
Spectrophotometer:	BIO-RAD, SmartSpec™ 3000, USA
	VARIAN, Cary 300 Bio Uvi-visible spec., AUSTRALIA
Speed vacuum:	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	MJ Research, PTC-100, USA
	TECHNE, TC 512, UK
Water bath:	TECHNE, Refrigerated Bath RB-5A, UK
	JULABO, TW 20, USA

APPENDIX D

LIST OF SEQUENCES

1. **TR7RS-293 TR7RS *Triticum turgidum* ssp. *dicoccoides* cDNA similar to HVA22-Like protein K, mRNA sequence (Accession# FK827962)**

Wqeqweqweqwe

dbEST Id: 59260512
EST name: TR7RS-293
GenBank Acc: FK827962
GenBank gi: 197668173

CLONE INFO
DNA type: cDNA

PRIMERS
PolyA Tail: Unknown

SEQUENCE

GACACATCTTGCGGGGAGACTGGCAAGTGTGGCAACCAAGCTCATGTTTCA
ACTCCTAGGTGTGTCTGGCTCTTCTGTCGGAGTTGAATTTGGGTCTTCAGTC
ACGCTAACTTCTTCTGATTGTCCAGGTTGGTCAAGCCCATTTACGATGTAAT
TTGCTGTTGTAGCACCCCTGATGGCCATGTTCTCTACAAAACGAATTTCATC
TTCATGGTTGCTCACGAACTTTGTAAGTTCCTTGGACAAAATATTCAGGATT
CTATCAATTTTTGCTCGATGCTTCCGGAAAAACGGGCGCAGGTATCTTCTGT
A

Entry Created: Aug 30 2008
Last Updated: Aug 31 2008

PUTATIVE ID Assigned by submitter
HVA22-Like protein K

LIBRARY
Lib Name: TR7RS
Organism: Triticum turgidum subsp. dicoccoides
Cultivar: TR 39477
Subspecies: dicoccoides
Tissue type: Roots
Develop. stage: 5 weeks after germination
Lab host: E. coli

Vector: pGemT-Easy
R. Site 1: EcoRI
Description: Suppression-subtractive hybridization was performed using the PCR-Select cDNA Subtraction Kit (Clontech) according to the manufacturer's protocol. Approximately 100ng PCR-amplified cDNA were ligated without further purification into 50ng pGEM-T Easy vector (Promega).

CITATIONS
Title: Constructing and sequencing cDNA libraries from contrasting wild emmer wheats in response to drought stress
Authors: Budak,H.
Year: 2008
Status: Unpublished

2. **Zea mays clone 241353 receptor expression-enhancing protein 6 mRNA (Acccession# EU962261)**

LOCUS EU962261 1108 bp mRNA linear PLN 10-DEC-2008
DEFINITION Zea mays clone 241353 receptor expression-enhancing protein 6 mRNA, complete cds.
ACCESSION EU962261
VERSION EU962261.1 GI:195625097
KEYWORDS FLI_CDNA.
SOURCE Zea mays
ORGANISM Zea mays
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD clade; Panicoideae; Andropogoneae; Zea.
REFERENCE 1 (bases 1 to 1108)
AUTHORS Alexandrov,N.N., Brover,V.V., Freidin,S., Troukhan,M.E., Tatarinova,T.V., Zhang,H., Swaller,T.J., Lu,Y.P., Bouck,J., Flavell,R.B. and Feldmann,K.A.
TITLE Insights into corn genes derived from large-scale cDNA sequencing
JOURNAL Plant Mol. Biol. 69 (1-2), 179-194 (2009)
PUBMED 18937034
REFERENCE 2 (bases 1 to 1108)
AUTHORS Alexandrov,N.N., Brover,V.V., Freidin,S., Troukhan,M.E., Tatarinova,T.V., Zhang,H., Swaller,T.J., Lu,Y.-P., Bouck J., Flavell,R.B. and Feldmann,K.A.
TITLE Direct Submission
JOURNAL Submitted (04-AUG-2008) Ceres, Inc., 1535 Rancho Conejo Blvd., Thousand Oaks, CA 91320, USA
FEATURES Location/Qualifiers
source 1..1108
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/clone="241353"
CDS 223..813
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FPSNSGSKHVYKRYLRPFFLKHQAKIDRFLNILSKELTKFVSSHEDEIRFIENMAI
RGATTAN HIVNGLDQPEETQAVNTIEGPNTTVTEEAGVSGTET"

ORIGIN

```
1    aaaataacca tcctccctag ctttcccgtt ccagccgtcg cgttgagttg ctgccgcctc
61   ccccggtccc cccacctccc gccccggcgc ccctctctct ctctctcttc tgccgcacag
121  agcccaaatc gcggcggatc cctcgctctc cagggagccg ctgcggcgcc gggttttgta
181  caccgacttg tttgcctacc tccagctcct cctcggcgac ccatggctct cctcgccccc
241  gccatctccg gcgaggttgg tctgcggctc ttattggcac cgctaagctc taacatagtc
301  atgcgtacag ccagttgtgc tgttgggatt ggtctacctg tatactccac tttcagagct
361  atagagaaga aggatgaaca agagaaagag cggatgcttt tgtattgggc agcatatgga
421  tcttttagca ttgctgaagt gtttgcagat aagcttcttt caagtgttcc tctctattat
481  catgtgaagt ttgctatcct tgtgtggctc cagttccctt caaacagtgg atcaaagcat
541  gtatacaaaa gatacctgcg cccatttttc ctgaaacatc aagcaaaaat tgataggttt
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721  gaccaacctg aagaaacaca agcggttaat acaattgaag gtccaaatac aactgtgaca
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901  actgttcctg aaaggctaga tctagaggca atgttttagc tttaactaga tattcatata
961  gcaagtaaaa tcggtgttgg acaaatttat tacagtgtaa aaatgacccg gtgtggaatg
1021 cagttcccag caatgttgtc atttttcacg tgggaataat ttgaagtctg cgatgaagaa
1081 tctttctaaa gcaaaaaaaa aaaaaaaa
```

3. *Triticum turgidum* ssp. *dicoccoides* HVA22-like protein Sequencing Results (provided by Refgen, Ankara)

LOCUS
DEFINITION Triticum dicoccoides HVA22-like protein, complete cds.
ACCESSION
VERSION
KEYWORDS
SOURCE Triticum dicoccoides (emmer wheat)
ORGANISM Triticum dicoccoides
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP clade; Pooideae; Triticeae; Triticum.

REFERENCE 1 (bases 1 to 660)
AUTHORS Esen, Doğan, Hikmet Budak
TITLE Cloning and characterization of a novel abscisic acid (ABA)-induced HVA22-liklike protein from *Triticum turgidum* spp. *dicoccoides* in response to drought stress
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 660)
AUTHORS
TITLE
JOURNAL

FEATURES Location/Qualifiers
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/organism="Triticum dicoccoides"
/mol_type="mRNA"
/db_xref="taxon:4565"
CDS 1..660
/codon_start=1
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/db_xref=" "
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YQAWLLFLYTSFALRENVLLVNGSDIRPWWIYHHYLAMLMALVSLTWEIKGQPDCSS
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T"

ORIGIN

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181 aagctcaaag tgaaggacga gtacaacaat tatagggata gggctgccta tatgttcctg
241 ttgttcccat ccactctcct cttactgaga tggtgggtgt gggatgggtg ccttccagca
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541 atgcatctcc agaataggta ccagcgtcaa agattacgca cccgaattgc tctgggaaag
601 gctaaaagaa tggatgtcgt cgctggagaa acagctggtg tgtcgggaac tgaaacttaa
```

4. Primer Design Scheme

Zea mays clone 241353 receptor expression-enhancing protein 6 mRNA

ACCESSION# EU962261

Primers cover a **591 bases** long region as shown in highlight.

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   1 aaaataacca tcctccctag ctttcccgtt ccagccgtcg cgttgagttg ctgccgcctc
  61 ccccggtccc cccacctccc gccccggcgc ccctctctct ctctctcttc tgccgcacag
 121 agcccaaatc gcggcggatc cctcgctctc cagggagccg ctgcggcgcc gggttttgta
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 301 atgcgtacag ccagttgtgc tgttgggatt ggtctacctg tatactccac tttcagagct
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 421 tcttttagca ttgctgaagt gtttgcagat aagcttcttt caagtgttcc tctctattat
 481 catgtgaagt ttgctatcct tgtgtggctc cagttccctt caaacagtgg atcaaagcat
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 661 tttatagaaa atatggctat cagaggggct acaacagcaa accacatcgt aaatggcctc
 721 gaccaacctg aagaaacaca agcggttaat acaattgaag gtccaaatac aactgtgaca
 781 gaagaagctg gtgtgtcggg aactgaaact taagcaaaac tttctatctc tgctcatgat
 841 cccagacgct gcttcaaaat tgttgttgag gtcacccagc ttgtttggat tgtgaatact
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 961 gcaagtaaaa tcggtgttgg acaaatttat tacagtgtaa aaatgacccg gtgtggaatg
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