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Molecular And Biochemical Analysis Of Water Stress Induced Responses In Grape

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MOLECULAR AND BIOCHEMICAL ANALYSIS OF WATER STRESS INDUCED RESPONSES IN GRAPE

By

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Water stress affects vine productivity, disease tolerance, and enological characteristics of grape. Florida Hybrid Bunch grape are developed through hybridization of local grape spp with *Vitis vinifera.* These cultivars are mostly grown in southeast region of United States. Water deficit conditions resulted due to failure of rains in the region has developed concern among Florida grape growers to increase water use efficiency of grape. The goal of this research is to identify genes and proteins differentially expressed in response to water stress and to correlate these changes with enological characteristics. Investigating transcripts and proteins will allow us to correlate them and confirm the involvement of specific genes responding to stress. Florida hybrid bunch 'Suwannee' grape plants were maintained under green house conditions. Water stress was induced by withholding irrigation. The leaf samples were collected from both irrigated and stressed plants at 5, 10, 15 and 20 day interval. We generated over 200 Subtractive Hybridization PCR products from control and water stressed leaf tissues.

Cloning, sequencing and transcript analysis revealed that, 54 genes related to drought and defense regulated pathways out of 125 characterized transcripts. Proteins were extracted from leaf tissue with trichloroacetic acid /acetone and separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The proteins were sequenced in LC/Mass Spectrophotometer. The most important differentially expressed genes include sucrose synthase, actin, isoprene synthase, ABF3, SNF1 related protein kinase, WRKY type transcription factors, AP2, ASR2, glyoxalase I and, cytochrome b which play significant role in cell permeability, transportation, photosynthesis and, maintenance in osmotic stress. We have found that ribulose bisphosphate carboxylase and phosphoribulokinase, which play major role in photosynthesis, were suppressed in response to water stress in Florida hybrid bunch. The results suggested that water stress affects expression of cDNAs associated with defense and drought regulated functions. Such profiling studies will be used to explicate specific pathways disconcerted by water deficit treatments, and in the identification of varietal differences.

Keywords: 2D electrophoresis, DDRT, genomics, grape, proteomics, subtractive hybridization.

DEDICATION

I would like to dedicate this research to my daughters Keyura and Chaquayla.

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CHAPTER I

INTRODUCTION

Problem Statement

Grapes (*Vitis* Species) are among the most important fruit crops in the world (Tinlot and Rousseau, 1993). The grape industry is valued for fresh fruit and wine at $2.9 billion, making it the highest value fruit crop in the USA (USDA, 2002). The major products made from grapes are wine 50-55%, raisins - 25-30%, table grape - 10-15%, juice and jelly - 6-9%, (USDA 2002). Grape cultivation worldwide is ranking second in fruit production (FAOSTAT 2007). The United States are the fourth largest wine producers in the world and the third in wine consumption. Among all states, Florida ranking the third in wine consumption, but it imports majority of the wine products because its grape industry has been limited by various diseases and drought (Mortensen and Andrews, 1981).

The genus *Vitis* is divided into two subgenera: 1) *Euvitis* Planch; 2n=38 (the bunch, *Vitis vinifera* L*)* and 2) *Muscadania* Planch; 2n=40 (muscadine grape, *Vitis rotundifolia* L.)(Goldy *et al*., 1988). Majority of grape varieties grown in Florida are muscadines (*Vitis rotundifolia*) and Florida hybrid bunch grape. *Vitis vinefera* (commonly known as California Bunch) are not grown in this region because they are susceptible to Pierce's Disease (PD). Muscadines are primarily used as fresh fruit, but

also make good juice and sweet wines of local importance in the Southeastern US (Goldy, 1992). Muscadines are great source of germplasm resistant to insects, diseases and environmental extremes that are not found in viniferas (Rogers and Rogers, 1978). Florida hybrid bunch grapes are developed through hybridization of local grape spp. with *Vitis vinifera*. Collectively, these hybrid varieties are considered a distinct race (termed "Florida Hybrid Bunch Grape") because of their combination of quality and PD resistance.

Abiotic stresses account to heavy losses for grape production and affect important aroma, flavor and color constituents by altering metabolite composition (Okamoto *et al.,* 2001). Water stress is the major environmental stress, contributing most significantly to the reduction in potential yield and quality (Flowers and Yeo, 1995). Water stress delays ripening and introduces undesired flavors in the wine, and alters nutraceutical composition in berry (Kawasaki et al., 2000). Phenolics and flavonoids in wines contribute to the health benefits such as reducing the risk of cardiovascular disease, cancer and degenerative diseases (Goldberg, 1995). In arid regions of the west where crop production is completely dependant on irrigation, crops with high water use efficiency are in great demand. Drought tolerant grape cultivars are needed to maintain the commercial production of grapes in the times of increasing water demands by growing human population. High wine quality appears to require adequate water supply early in the season, followed by moderate stress that limits further growth but allows ripening of the fruit (Reisch and Pratt, 1996). Grape production under regulated irrigation conditions have been shown to improve the aroma, flavor, and color by altering metabolic composition thereby improving wine quality, and human health benefits

(Powers, 2004). Commercial production of grapes in Florida requires cultivars with minimal water consumption, disease tolerance and value added products with high wine quality. Identification of highly adaptable water-efficient genotypes in Florida hybrid bunch is required to maximize commercial returns under strict water regulations. Greater efforts need to be made with identification and utilization of increased water use efficient Florida hybrid bunch grape for high value added products. Investigation into the cellular and molecular biology of water stress is needed to understand the role of various metabolites in berry development, stress tolerance as well as their influence on fruit, juice and wine characteristics.

Justification

Grape responses to water deficit are governed by a combination of molecular and biochemical signal transduction processes, which coordinately act to determine tolerance or sensitivity at the whole-plant level (de Souza et al., 2005). Very little is known about the underlying processes that confer the adaptation processes of the plant to stress tolerance and in particular, the relation of water stress to improved wine characteristics in grape berries. Most of the stress-response traits are complex and are influenced by multiple genes and extensive genotype-environment interactions (Bohnert et al., 1995). It has been known in higher plants that, water stress activates the protection response by increasing the synthesis of specific transcripts and proteins (Ingram and Bartels, 1996). The key factor in stress response is actual delivery of the inducer into cells. The expression of genes in response to stress involves transcription of the gene to mRNA molecules but, that mRNA translated into a protein that must be targeted to a specific

cellular location before it is active in performing its designated function. The function of gene production in cellular metabolism is in response to the water stress in generating metabolites such as osmoprotectants, which protect the cell against water loss (Daniels et al., 1994). Advances in genomics, informatics, and functional genomics have made it feasible to gain a complete understanding of how many genes become integrated to affect this tolerance. The comprehensive genomic approach would elevate our knowledge of the biological phenomena involved during the resistance to drought, thus allowing significant improvement of viticulture practices during vine development and berry ripening (Tattersall et al., 1997). These strategies are linked since molecular biology tools can generate considerable information on berry growth and vine response to environment. The comprehensive functional genomic will provide a basis to develop proteomic approaches which may be envisaged as a continuation of integrated functional analysis of genes. Indeed, protein translation, protein stability and post-translational modifications may play a significant part to alter the final enzymatic activity resulting from gene expression (Vilardell et al., 1994). This research was focused to initiate qualitative analysis of changes in mRNA and protein levels following water stress in grape. This study involves identification and characterization of genes in response to water stress, and functional annotation. **This research is the first to study the water stress induced transcripts and proteins in Florida Hybrid Bunch grape.**

Objectives of the Research

The major goal of this study is to identify molecular and biochemical components associated with water stress response in Florida Hybrid Bunch grape. Specifically this study is carried out to identify cDNA transcripts in leaf tissue correlating with the differential expression of regulating genes in response to metabolic stress.

The specific objectives were to:

1. Identify and isolate cDNA transcripts expressed differentially in response to water stress in Florida Hybrid bunch grape,
2. Study differentially expressed proteins in response to water stress,
3. Sequence and characterize the water stress induced genes,
4. Functionally annotate genes and proteins using BLAST and infer their role in stress response.

CHAPTER II

LITERATURE REVIEW

Water Stress in Plants

The impact of abiotic stresses on crop productivity is remarkable and causes 2/3 of all yield reductions in agriculture (Flowers and Yeo, 1995). Plants execute various physiological and metabolic responses in their leaves, roots, and seeds to sustain with water stress (Bohnert *et al*., 1995). Most of the stress-response traits are influenced by multiple genes and extensive genotype-environment interactions. Earlier studies on molecular responses in *Arabidopsis thaliana* revealed several genes involved in water stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu *et al*. 1997). The molecular mechanism of drought response has been extensively investigated in agricultural and horticultural crops and many biochemical pathways and numerous genes involved in water stress were identified (Zhu, 2002). Water stressed plants utilize a protection-based mechanism that activates the synthesis of specific transcripts and proteins during dehydration (Ingram and Bartels, 1996).

Water Stress in *Vitis vinifera*

Grape production for raisins and wine is one of the largest and most important agricultural commodities in the US. In the year 2000, grapes had a crop value of 3 billion dollars, and was the number one fruit and sixth in overall crop value behind corn, soy,

hay, wheat, and cotton. Furthermore, it is well established that the consumption of wine at moderate levels has undeniable health benefits including reducing the risk of cardiovascular disease, stroke and cancer. Both phenolics and flavonoids in wines contribute to these health benefits (Pretorius and Hoj, 2005). Grape production efficiency is hampered each year by abiotic stresses like drought, freezing temperatures, and soil salinity (Vitrac et al., 2005). Grapes derived from regulated irrigation have increased levels of phenolics and anthocyanins, resulting in the production of a superior quality wine (Pinelo et al., 2005). However, the molecular and biochemical basis for this phenomenon is poorly understood. Very little is known about biochemical and molecular basis of water stress tolerance is available on Florida Hybrid Bunch (FH) grapes. Therefore, it is crucial to customize the application of comprehensive system of genomics to analyze changes in response to water stress and in turn relate to aroma, flavor, and color of wine as well as nutraceutical characteristics of grape juice.

Physiological and Biochemical Studies

Plants exhibit primary responses to stress by altered physiological and biochemical composition. Physical and biochemical responses to water stress were demonstrated in crops like maize, rice, and peanut using polyethylene glycol and mannitol, both in whole plant and cell cultures (Venkateswarlu and Ramesh, 1992; Venkateswarlu *et al*., 1993; Zheng *et al*., 2004). The basis of developing resistance in plants for water stress is to maintain the cell membrane stability. Several studies were made to correlate the relationship of cell membrane stability with the accumulation of metabolites such as amino acids and the relative water content of the tissues after

imposition of water stress (Stines et al., 1999). Simultaneous changes in physiological responses suggest that induced proteins play an important role in these responses (Kumar et al., 2004). These proteins suggest a strong co-relation between the expression of genes and the level of stress tolerance in different genotypes. Several studies on decease in osmotic potential in grapevines due to water deficiency in leaf tissues contribute to osmotic adjustment however they lacked quantitative analysis of the traits involved in the mechanism (During, 1984).

Functional Genomics

Functional genomics is a rapidly developing technology that allows the identification of large sets of genes that influence a particular biological process. The gene discovery phase is followed by the investigation of specific functions of the individual genes and, the definition of their structural characteristics. Eventually, the objective is to address 'whole genome' analysis, through which the complete nucleotide sequence of a genome will be determined. All structural genes in that sequence are identified to define their functions. In addition, regulatory mechanisms for all genes will be determined during normal growth and development or to environmental stresses, together with the complex interactions that occur in genetic and cellular networks (Breyne and Zabeau, 2001).

Functional genomics technologies represent a fundamental shift from hypothesis-based approaches for the investigation of a particular biological process. In this approach, only one or a few genes or proteins are examined that involves the collection and analysis of data relating to large numbers of genes or proteins. It is well known that

genes or proteins seldom act alone, and functional genomics therefore addresses the complexity of cellular processes (Aharoni and Vorst, 2001). Most of the plant processes are mediated by large numbers of genes, the technology must necessarily be focused on large scale profiling of genes, mRNAs, proteins and metabolites that participate in cellular processes. Given that there are more than 25,000 genes in plants, the precise definition of these processes requires high throughput data collection, structural and functional analyses. In a functional genomics approach, the scientist allows no bias to influence the search for genes that might be involved in the plant's response to water stress, but instead attempts to define all genes that are up- or down- regulated when the stress is imposed (Lander, 1999). This will allow the identification of entire pathways and networks involved in the response, and will almost certainly reveal a number of unexpected responses. Studies involving identification and functional annotation of specific genes in response to a treatment have been carried out in various crop plants. Among the various genomics approaches, candidate gene approach, mapping, differential expression of genes, microarray were proven to be the most promising approaches for functional identification of significant transcripts. Identification of candidate gene approach allows locating a particular region of genes generating polymorphism within or in regulatory sequences of the genes (Gebhardt et al., 2007). Subsequent mapping and analysis of these alleles will lead to variation. Differences in the expression of the candidate genes can be measured using northern blots and analyzing enzymatic activities. Microarray technology permits expression monitoring of thousands of genes at the same time (Seki et al., 2001). This methodology gathers genes on high density filters that are responsive to water stress on DNA chips and analyzes gene expression. This would

allow the determination of the function of a maximum number of genes specifying the crop traits. The water use efficiency is a key determinant of productivity and quality in agriculturally important crops. Relatively small changes in water-use efficiency will have dramatic effects on crop yields and hence on world food production. As a result, plant breeders and agronomists are continuously seeking to improve this trait in crop species. Water use efficiency in higher plants is a complex trait that is influenced by multiple genes and extensive genotype-environment interactions, but is amenable to investigation using new high throughput gene discovery techniques developed in functional genomics programs (Cushman and Bohnet, 2000).

Genetic mapping and marker-assisted selection

Mapping the grapevine genome is facilitated by the use of existing crosses in breeding programs. Markers heterozygous only in one parent will segregate 1:1, while doubly heterozygous markers will have more complex inheritance patterns (Cipriani et al., 1994). A genetic map was developed in a cross between two site-specific hybrid cultivars (Lodhi et al., 1995) and additional linkage mapping efforts are underway in California and in France. Ultimately, it will be possible to combine a significant amount of the genome information generated by the individual groups into a single genome map for grapes. With the abundant availability of molecular markers, significant progress has been made in their use for early selection of desirable phenotypes. In long-cycle vegetative propagated crops such as grapes, Marker-Assisted Selection (MAS) is ideally suited. Once important genes are tagged with a marker, pre-selection of very young seedlings can take place. Pyramiding of multiple genes for a single trait can also be

accomplished with molecular markers. These maps are able to provide necessary guide for the physical mapping and cloning of genes associated with important horticultural traits.

Fingerprinting, genotype identification, and diversity in *Vitis vinifera*

To understand the potential values agronomic traits of the many molecular markers, it is essential that we define the major ones. Isozymes are based on multiple forms of an enzyme which differ in electrophoretic mobility. More than twenty isozyme polymorphisms have been identified in grape (Paterson, 1996). Restriction fragment length polymorphisms (RFLPs) are detected using restriction enzymes that cut genomic DNA molecules at specific nucleotide sequences, yielding DNA fragments variable in size (Staub et al., 1996). Polymerase chain reaction (PCR) is designed to amplify DNA in an automated, cyclic procedure which results in exponential increases in the quantity of a specific sequence of DNA. Selection of a DNA fragment for amplification is a result of primer-annealing, in which a primer binds to complementary single-stranded genomic DNA present in the reaction (Bowers et al., 1996). A commonly used PCR analysis is based on random amplified polymorphic DNA (RAPD). These markers are based on the occurrence of an inverted pair of 9-11 base repeats within a distance of between 200 and 2000 base pairs. This is a single primer reaction which amplifies one or several segments of DNA. Amplified fragment length polymorphisms (AFLP) are based on selective amplification of restriction enzyme-digested DNA fragments (Lin and Walker, 1996; Qu *et al.,* 1996). Sequence Tagged Site (STS) markers, useful in anchoring loci between crosses, have been developed. The most important of these is the microsatellite or simple

sequence repeat (SSR) marker (Paterson, 1997). Since the bases flanking the repeat are conserved, while the length of the repeat varies greatly, SSR-specific primers can be readily designed. So far more than 40 SSR loci have been identified in *Vitis*. Additionally, many other STS markers have been developed for use, including cleaved amplified polymorphic sequences (CAPs), Sequence characterized amplified regions (SCARs), allele-specific associated primers (ASPS), and expressed sequence tags (ESTs).

Only a few molecular markers have been used in grapes due to the difficulty in distinguishing among similar groups of cultivars. These markers include isohyets, RAPDs and, microsatellites (Lamboy and Alpha, 1998) contributing in understanding diversity within grapevine germplasm collection, relatedness of cultivars from different regions and, in the identification of multiple genotypes within homogeneous cultivars.

Gene cloning

A number of important genes have already been cloned from grapes for example, the gene for stilbene synthase responsible for resveratrol production (Hain et al., 1993). While there are several approaches which can be used to clone genes, positional cloning based on genomic linkage maps provide venues for the cloning of additional genes, but has not yet been utilized in grapes. This approach is opening doors to a greater understanding of the genetics of other crops and, with the small genome size of grapes.

Functional Genomics of *Vitis vinifera*

The genome wide analysis in *Vitis* is being carried out by International Grape Consortium to identify and characterize all the genes and proteins. The long term goal of

the grape consortium research was to develop comprehensive genomic tools to facilitate the genetic engineering of improved abiotic stress tolerance traits in *Vitis vinifera*. The specific studies were carried out to accomplishing this goal include; 1) extensive gene discovery through large-scale expressed sequence tag (EST) sequencing and mRNA expression profiling using oligonucleotide microarray-based expression monitoring in roots, leaves, and fruits of grapevines exposed to multiple abiotic stresses; 2) global mRNA expression profile data will be complemented by protein expression analyses using state-of-the-art proteomics methodologies and, 3) identification of specific metabolites and metabolite profiles in grapevines and fruit following abiotic stress that confer desirable aroma, flavor and color quality characteristics and improved health benefits. Metabolite profiles from grape juice of well-watered and water-deficit-treated vines were compared with quantitative data from mRNA and protein expression patterns using comprehensive bioinformatics systems to store and analyze data sets. The project has produced over 45,000 grape (*Vitis vinifera*) ESTs from a range of tissues and cultivars, with nearly 19,000 distinct ESTs covering an estimated two-thirds of the grape genes. The EST primary BLAST matches for 2,479 ESTs from Chardonnay berry tissue and 2,438 from leaf tissue were classified into 80 functional categories to estimate the abundance of transcripts with predicted cellular roles (Pellerone et al., 2001). A high degree of specialization was found with 36% of the leaf transcripts involved in photosynthesis, compared to 3% in the berry; and 18% of the berry transcripts in the disease/defense category, compared to 7% in the leaf. The grape project at the center for plant conservation Genetics, Lismore, Australia is also producing grape ESTs from different tissues to advance gene discovery in the area of dormancy, bud burst, berry

development and vine physiology (Ablett *et al*., 2000). Over 145,000 ESTs from various tissues of grapevines, which include water stress, disease resistance and aroma related sequence entries available in gene bank from *V. vinifera* L *and V. shuttleworthii* (ESTAP www.vbi.vt.edu/`estap) are developed at Virginia Bioinformatics Institute (Blacksburg, VA) in collaboration with University of Nevada (Reno, CA) and S.R. Noble Foundation (Ardmore, OK). Microarray analysis of *V. vinifera* cv. Shiraz developing berries has revealed the expression patterns of several categories of genes. Recently, *Vitis* Affymetrix Gene chip was created on the available EST dataset and made available to the research community.

A genetic engineering strategy has a much higher potential for success than strategies relying on single stress adaptive transgenes because multiple adaptive genes would be over expressed. Earlier studies on *Vitis vinifera* ESTs showed enhanced expression of transcription factors such as CBF/DRE family, related to defense and signal transduction in berry in response to environmental stimuli (Ablett *et al*., 2000). Till date, over 215,949 ESTs with total of 14, 572 unigenes from various tissues of bunch grape and other wild relatives of muscadine grapevines are available in gene banks from *V. vinifera* L and *V. shuttleworthii*. *Vitis* Affymetrix Gene chip was created on the *Vitis* EST dataset and made available to the research community. Studies on *Vitis vinifera* ESTs from different tissues showed enhanced expression of transcription factors related to defense and signal transduction occurred in berry, and photosynthesis related factors in leaf tissue (Scott *et al*., 2000). Several studies revealed significant degree of genetic diversity among the traits related to phenolics, anthocyanins, water stress in bunch grape using molecular markers such as AFLP, SNP (Siles *et al*., 2000; Adam-Blondon *et al*.,

2004; Fanizza *et al*., 2003 and Faes *et al.,* 2004). Molecular mapping of grape genome using PCR based markers in linkage analysis are underway in various laboratories (Thomas *et al*., 1993; Bourquin *et al.,* 1993; Lodhi *et al*., 1995; Diablo *et al.*, 2000; Riaz *et al.* 2004).

Inter-relationship of Water Stress and Disease Tolerance in Grape

Recent studies on water stress demonstrate a complex network of defense pathways and signal interactions that also determine the disease resistance and abiotic stress tolerance. Several mitogen activated protein kinase (MAPK) genes were reportedly induced either by pathogen infection or environmental stresses. MAPK plays an important role in osmoregulatory pathway in eukaryotes. Rice lines infected by blast fungus exhibited reduced levels of defense gene expression and increased levels of disease susceptibility. Grape production is diminishing by the diseases such as Anthracnose (*Elsinoe ampelina/Sphaceloma ampelinum*), Black rot (*Guignardia bidwelli/Phyllosticta viticola*), Downy mildew (*Plasmopara viticola*) and Pierce's disease (*Xyllella fastida*). Studies on transcriptional responses to *Xylella* infection revealed a massive re-direction of gene transcription, with up-regulation of transcripts for phenylpropanoid and flavonoid biosynthesis, ethylene production, ABA-responsive transcripts, adaptation to oxidative stress, and homolog of pathogenesis related (PR) proteins while these responses were not observed on non-inoculated plants under moderate drought stress (Choi *et al*., 2006). This strongly suggests a synergistic interaction between drought stress and disease, as drought stressed plants exhibited a stronger physiological and transcriptional response to the pathogen. Hence, identification

and functional characterization of *Vitis* genes related to disease resistance and abiotic stress tolerance will also greatly enhance our understanding of host defense mechanisms and facilitate the development of novel strategies for reducing biotic and abiotic stresses. Apparent synergistic interaction between water deficit and disease also was observed as the stressed plants exhibited a stronger physiological and transcriptional response to the pathogen. Transcriptional responses to *Xylella* infection included a massive re-direction of gene transcription, with up-regulation of transcripts for phenylpropanoid and flavonoid biosynthesis. These results confirmed the microarray analysis, as synergistic increases or decreases in gene expression were evident for some groups of genes, in particular those relating to flavonoid biosynthesis. Paterson (1996) located markers for traits such as flower sex, *Botrytis* rot and powdery mildew resistance. Statistical procedures can be used to analyze for markers linked to loci affecting quantitatively inherited traits and this process has resulted in our identification of markers for the *V. cinerea* source of resistance to powdery mildew. There are also reports of markers for genes affecting nematode resistance and hypersensitivity to powdery mildew originating in *V. rotundifolia*.

Inter-relationship of Water Deficit Stress and Nutraceutical Components, and Wine Characteristics in Grape Berries

Deficit irrigation practices alter the metabolite composition of berry and can improve the flavors and wine characteristics of the grape. Water deficit stress for 10 days before harvest increased the level of several major amino acids in Chardonnay grape berries and also improved the wine quality (Okamoto *et al.,* 2001). Genes associated

with functional roles in the flavonoid, anthocyanin pathway and aroma biosynthesis were preferentially expressed in the skin and seed in response to water deficit (Deluc *et al*., 2006; Grimplet *et al*., 2006). Several differentially expressed genes were identified in bunch grape ca. Cabernet Sauvignon shoots which are both up and down regulated in response to water deficit stress (Cushman *et al.,* 2004; Da Silva *et al*., 2005). In addition, certain metabolites, such as resveratrol and stilbene compounds were accumulated in leaves and berries in response to abiotic and biotic stresses (Borie *et al*. 2004). Resveratrol (3, 5, 4-trihydroxystilbene) is a phytoalexin, reported in grapes (Creasy and Coffee, 1998; Jeandel *et al.,* 1991), has been associated with reduced cardiovascular disease and reduce cancer risk (Jang *et al*., 1997).

Functional Genomics of Water Stress

The expression of the genes in response to water stress involves not only transcription of the gene to mRNA molecules but mRNAs translation into a protein and its further location to a target tissue. Analysis of gene expression patterns needs to be accompanied by a better understanding of the metabolic processes within a cell. Thus, functional analysis technologies include not only the examination of genes that are activated in response to the stress (transcriptomics), but also the corresponding proteins (proteomics) and the changes in metabolites (metabolomics) that accompany changes in gene activity (Bohmert et al., 2000). Overlapping expression pattern of the genes for various isoforms of alcohol dehydrogenase gene family members has been reported in *Vitis vinifera* (Tesniere and Verries 2001).

Strategies for improving water stress tolerance in Grape

There are several different approaches to developing more stress tolerant *V. vinifera* plants including: a) adapting cultural practices, b) selecting for more tolerant germplasm, c) making hybrids of *V. vinifera* with more tolerant native North American species, and d) using genetic engineering technology to develop more hardy genotypes. Breeding for stress tolerance has proven difficult and has not provided desirable outcomes. Breeding specific characteristics takes considerable time for *V. vinifera* (Boquet et al., 1981). Consequently, clones are vegetatively propagated to prevent loss of desirable grape and wine qualities. Grape hybrids that are cold tolerant than *V. vinifera* have been developed at Cornell University, but wine made from these grapes is inferior to premium quality wines made from *V. vinifera* grapes grown in the major wine producing regions of the world. Thus, the genetic modification of specific premium quality *V. vinifera* clones by recombinant DNA technology is viewed as the most attractive option for improving stress tolerance. A genetic engineering strategy which includes the over-expression of transcriptional activators such as the CBF/DRE family has a much higher potential for success than strategies relying on single stress adaptive transgenes because multiple adaptive genes would be over expressed (Bhatnagar-Mathur et al., 2007). Studies of comparative metabolite profiling and determining the genetic basis of the factors responsible for improving the quality of wine produced from drought-stressed plants would be beneficial to enhance the value added products of grape.

Transcript profiling

Transcript profiling includes mass sequencing of short segments of clones into DNA libraries. These sequences are known as 'expressed sequence tags' or ESTs, and the number of plant EST sequences in public databases has increased exponentially over the last few years. Isolation of genes through differential screening and suppressive subtraction hybridization and differential display RT PCR were found to be most efficient means to identify genes of known function associated with water stress in *Arabidopsis*, rice, barley, cotton, wheat, (Diatchenk*o et al.,* 1996; Gao *et al*., 2003; Ji *et al*., 2003; Saluzzi *et al*., 2002) and in non-climacteric fruits like strawberry (Manning, 1998). Rapid increases in mRNA levels were found using differential screening in grape berries during ripening. Most of these homologues were reported to involve in cell wall structure or stress related responses or they may accumulate as part of the ripening developmental program (Davies and Robinson, 2000; Christopher and Robinson, 2000). A relationship between fruit ripening and changes in mRNA levels has been demonstrated in grape berries by Boss et al. (1996), who showed that the accumulation of transcripts of genes in the flavonoid synthesis pathway was related to anthocyanin production in the berry skin during ripening. In many other fruit, the considerable changes that occur during ripening are also largely the result of changes in gene transcript levels. Microarray analysis of *Vitis vinifera* 'Shiraz' developing berries has revealed the expression patterns of several categories of genes (Waters *et al*., 2004).

Differential display

Differential display RT PCR has been powerful technique to identify most regulated genes in cDNA for a specific tissue in the organism to a given response (Liang and Pardee, 1992). Several transcripts were identified using DDRT PCR for various responses in plants. Genes linked to nematode resistance were identified using this method. (Oberschmidt *et al.,* 2003). A novel ozone induced genes have been identified using DDRT PCR (Sharma and Davis 1995). Two strawberry cDNA sequences similar to pyruvate decarboxylase gene were identified using DDRT (Delue et al., 1999). Several ABA induced genes were identified and cDNA libraries were constructed in barley in response to drought stress (Tommasini *et al*., 2008). Seven cDNAs (pCa-DIs for *Capsicum annuum* drought induced) have been isolated that are rapidly induced when hot pepper plants are subjected to water stress in *Capsicum annum*. Transcripts were identified in sunflower in response to water stress (Liu and Baird, 2003, Torres et al., 2006) and in peanut, (Jain et al., 2002) through differential display RT PCR method.

Subtractive hybridization

Subtractive hybridization (SSH) is a powerful technique that enables to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. This technique has been employed in several crops for isolating genes that are low abundant and up regulated during several physiological, abiotic and biotic stress responses. In *Arabidopsis* cDNA fragments were isolated using subtractive hybridizations to understand molecular control of pollen development. Rubinelli et al., (1998) have isolated cDNAs representing 13 genes. Sequence analysis of

full length cDNAs showed that one of the novel genes, *ATA7*, encodes a protein related to lipid transfer proteins. Another gene, *ATA20*, encodes a protein with novel repeat sequences and a glycine rich domain that shares a predicted structure with a known cell wall protein. The full length *ATA27* cDNA encodes a protein similar to the BGL4 glucosidase from *Brassica napus.* These studies demonstrate that subtractive hybridizations can be used to identify previously unknown genes, which should be valuable tools for further study of pollen and anther development and function. A new nitrate-induced gene OsRab5a in rice was isolated using suppression subtractive hybridization (SSH) method. The gene OsRab5a encodes a small GTP-binding protein of Rab family. This protein has a higher expression in root, weak expression in shoot, flower and grain, and visually not in stem and leaf. Comparison of genomic organization and tissue distribution showed well-conserved genomic organizations and similar expression patterns between OsRab5a gene in rice and Rha1 gene in *Arabidopsis* during evolution (Wang et al., 2002). Several subtracted libraries were developed in various agronomically important crops. Two subtracted cDNA libraries were constructed by reciprocal subtractive hybridization between immature (low sucrose-accumulating) and maturing (high sucrose-accumulating) inter nodal tissue in sugarcane (Carson *et al*., 2002). A stress-responsive gene Gdi15 from groundnut, which is homologous to flavonol 3-O-glucosyltransferase involved in anthocyanin biosynthesis was isolated using subtractive hybridization (Gopalakrishna *et al*., 2001). Suppression subtractive hybridization (SSH) technique followed by the differential hybridization screening was employed to identify rarely transcribed flower maturation-inducible genes in *Dianthus caryophyllus* (Ok et al., 2003). To understand the molecular basis of salt stress response,

the salt tolerant upland rice variety IAPAR 9 was used to identify the genes differentially expressed in NaCl-treated roots and untreated ones by subtractive suppression hybridization method (Wu et al., 2005).

Proteome Analysis (Proteomics)

The proteome is the complete complement of proteins that are present in a particular tissue under particular conditions. Protein extracts from specific tissue are resolved by two-dimensional (2D) gel electrophoresis to identify and characterize over 1,000 proteins (Park, 2004). The identification of individual protein spots on the 2D gel is based upon a combination of amino acid composition, peptide mass spectrometry fingerprinting, NH_2-terminal sequence, molecular mass and pI data (Gorg et al., 2004). Another component of proteome analysis is the investigation of protein-protein interactions. There are also procedures that allow progressively build up a picture of multiple interacting proteins that form transcription factor complexes and control the expression of genes critical for responses to water stress in addition to other cellular processes (Szanics et al., 2006). The identification of individual protein spots on the 2D gel through appropriate protein and nucleotide databases will allow us to progressively build up a profile of proteins that form transcription factor complexes that are critical for responses to biotic and abiotic stresses.

Metabolite Analysis (Metabolomics)

Metabolomics is the high throughput study of the complete complement of metabolites in a particular tissue under defined conditions, and can again be applied to

defining a plant's response to water stress (Bhalla et al., 2005). The evolution of metabolomics is based on the argument that metabolite profiles are the ultimate reflection of gene expression at the biochemical level, and that metabolites are closer to cellular function than either mRNA transcripts or proteins (Tesniere and Verries 2001). Thus, metabolite profiles theoretically provide a more objective measure of the final metabolic activities of the cell. For example, enzymes that catalyse the production of sugars, sugar alcohols and other osmoprotecting metabolites can be the key to a successive response to the stress. Through minimizing water loss from cells, it can be ensured that the adapted plant survives the stress conditions. Metabolite profiles are determined by extraction of the tissue with aqueous or organic solvents, separation of components by gas or liquid chromatography, and the identification of individual metabolites through on-line mass spectrometric analysis and database searching (Bajic et al., 2005). There is accumulating evidence that plant cells possess a surprising level of plasticity that enables them to quickly compensate for changes in gene expression. Metabolomics technologies have the potential to define in detail the regulation of biochemical networks in response to environmental challenges, such as severe water stress (Nikiforova et al., 2005). Finally, when candidate genes are identified, their functional involvement to stress needs to be confirmed. The functional analysis of candidate genes is therefore a crucial component of the more general functional genomics technology.

CHAPTER III

MATERIALS AND METHODS

This research was carried out in Plant Biotechnology laboratory at the Centre for Viticulture and Small Fruit Research CESTA, Florida A & M University, Tallahassee FL.

Plant Material

Florida Hybrid Bunch grape 'Suwannee' obtained from National clonal germplasm Repository USDA, Davis, CA was used to study the molecular and biochemical responses to induced water stress.

Experimental Methods

Water Stress Treatment

The selected two year old plants were grown in five gallon pots under greenhouse conditions. Plants were subjected to water stress by withholding irrigation for 20 days. Water stress was monitored by measuring soil moisture content at 5, 10, 15 and 20 day intervals during plant growth. At least six replica measurements were done in each experiment using quick draw soil moisture probe Series 2900F1 (Soilmoisture Equipment Corp, Santa Barbara CA).

Collection of Samples

Leaf tissues were collected at 5, 10, 15 and 20 days after water stress treatment from irrigated control and water stressed plants. Tissue samples were immediately frozen in liquid nitrogen and stored at -80ºC until used for RNA and protein studies. Care was taken during sampling to avoid RNase contamination using RNAZAP and by wearing gloves.

Section A. GENOMICS

Isolation of total RNA and mRNA

Total RNA from leaf tissues was isolated by SDS buffer extraction followed by modified LiCl precipitation (Lopez and Gomez, 1992). Total RNA obtained from each tissue was used to isolate mRNA using oligotex Direct mRNA midi/maxi kit (Catalogue number 72041) following the procedures described by manufacturer's protocol (Qiagen, CA). The purity of mRNA was determined by the peaks in the image system. All glass ware, pestle and mortar, and plastic ware were treated with 0.1% Diethyl Pyrocarbonate (DEPC) in distilled water overnight, autoclaved and oven dried before use. Stock solutions and buffers were prepared using 0.1%DEPC treated water and then autoclaved.

Isolation of RNA from Leaf tissue of control and water stressed grape plants

Two g of frozen tissue was ground to a fine powder with liquid nitrogen using mortar and pestle in presence of 2% insoluble polyvinylpyrollidone (PVPP). Powdered sample was transferred to a 50 ml sterile centrifuge tube, to which 20 ml pre-warmed

extraction buffer at 65°C was added. Five molar NaCl (0.1 volume) and 1% ß-mercaptoethanol to extraction buffer was added just before use. The sample tubes were vortexed for 1 min and incubated on ice for 5 min. Equal volume of chloroform: isoamylalcohol (24:1) was added, shook for 5 min and centrifuged at 14000 g for 15 min at 4°C. The supernatant was transferred to a fresh tube and added 0.1 x of 5 M NaCl, mixed gently, then added 1 X of cold isopropanol and finally precipitated overnight at -20°C. The pellet was obtained by centrifuging at 14000 g for 20 min at 4°C. Pellet was washed with 75% ethanol air dried for 10 min. and, resuspended in 5 ml DEPC treated sterile water. Added equal volumes of Phenol:chloroform:isoamylalcohol (25:24:1) to the sample suspension and centrifuged at 14000 g for 15 min at 4°C. The supernatant was collected in a fresh centrifuge tube and added 0.1 X of 5 M NaCl, 1 X of cold isopropanol and precipitated overnight at -20°C. Pellet was obtained by centrifuging at 14000 g for 20 min at 4°C. The pellet was washed with 75% ethanol and resuspended in 500 µl DEPC treated sterile water. To the dissolved pellet, equal volumes of chloroform was added shook vigorously for 2 min and centrifuged at 12000 g for 15 min at 4°C and collected the supernatant. Again added equal volumes of phenol: chloroform (1:1), mixed vigorously for 5 min and centrifuged at 12000 g for 15 min at 4°C and collected the supernatant. Finally the supernatant was transferred to a fresh 1.5 ml sterile Eppendorf tube and add 0.1 V x 5 M NaCl and, 1 X cold isopropanol and precipitated overnight at -20°C. The pellet of RNA was obtained by centrifuging at 12000 g for 20 min at 4°C. After washing in 75% ethanol and air dried for 10 min, the pellet was resuspended in sterile water and the RNA sample obtained was stored at -80°C.

Qualitative Estimation of RNA

The quality of RNA was determined using 1.2% formaldehyde agarose gel electrophoresis. The gel was prepared in DEPC water using 0.36 g agarose, 5.35 ml formaldehyde, 5.9 ml of 5X gel running buffer for 30 ml (Sambrook et al., 2001). Five µl of RNA was mixed with 3 µl of ethidium bromide and 3 µl of 5X gel running buffer and the contents were incubated at 65°C for 3 min in a water bath. The tubes were kept on ice for 5 seconds and 2.5µl gel loading buffer/dye and 2.5µl ethidium bromide (0.1%) were added. The contents were spun briefly and loaded onto the gel. The gel tank was filled with 1X TBE and resolved at 60 V. The RNA was visualized under UV transilluminator.

mRNA isolation

Total RNA obtained from each tissue was used to isolate mRNA using oligotex direct mRNA midi/maxi kit following the procedures described by manufacturer's protocol (Qiagen, CA). The purity of mRNA was determined by the peaks in the image system.

Quantitative estimation of RNA and mRNA

The quantity and the purity of RNA and mRNA were estimated using a nano spectrophotometer (Nanodrop Inc.,). One µl of the RNA was placed in the instrument and the absorbance was read at 260 nm and 280 nm to measure the quantity. The ratio of A260/A280 was calculated to check RNA quality.

Isolation of Differentially Expressed Transcripts to Water Stress

Differentially expressed transcripts due to water stress were identified using Differential Display RT PCR (DDRT PCR) and subtractive hybridization (SH) methods.

Differential Display RT-PCR

Differential Display RT PCR was performed in a 20 µl reaction mixture as per the manufacturer's protocol (GenHunter, TN). The Forward and reverse primers provided by the manufacturer were used.

Reverse transcription of mRNA

First strand synthesis was performed in a total volume of 20 µl. The reaction mixture contained 200 ng total RNA, 2 µl of 2µM one base-anchored primer of H-T11 G 5'-AGCTTTTTTTTTTTG-3' or H-T11 A 5'-AGCTTTTTTTTTTTA-3' or H-T11 C 5'-AGCTTTTTTTTTTTC-3', 1µl of MMLV reverse transcriptase containing 100 units, 1.6 µl of 250 µM dNTP and 2 µl of 5X first strand buffer (250mM Tris pH 8.3, 30 mM $MgCl_2$, 375 mM KCl). The reaction conditions for first strand synthesis are programmed in PCR as 65°C for 5 min, 37°C for 60 min followed by 75°C for 5 min.

Differential Display PCR

The second strand synthesis and PCR amplification was performed in a 20 µl reaction mixture, using 2.0 µl of RT mix from the first strand cDNA. Each reaction mixture contains 2 µl of 10X PCR buffer, 1.6 µl dNTP (25 mM), 2.0 µl of each anchored oligo dT and one of the arbitrary primers H-AP1: 5'-AAGCTTGATTGCC-3'; H-AP2:

5’-AAGCTTCGACTGT-3’; H-AP3: 5’-AAGCTTTGGTCAG-3’; H-AP4: 5’-AAGCTTCTCAACG-3’; H-AP5: 5’-AAGCTTAGTAGGC-3’; H-AP6: 5’-AAGCTTGCACCAT-3’; H-AP7: 5’-AAGCTTAACGAGG-3’; H-AP8: 5’-AAGCTTTTACCGC-3’ and 0.2 µl taq polymerase containing 4 units. The reaction were performed using thermal cycler (MJ Research, Inc., Model PTC-100) programmed to 95°C for 30 sec, followed by annealing at 40°C for 2 min, extension at 72°C for 30 Sec for 40 cycles and then followed by final extension at 72°C for 5 min.

Separation of DDRT PCR Products

The DDRT mixture is denatured with an equal volume of gel loading buffer (95% formamide, 0.1% xylene cyanole FF and 0.1 % bromophenol blue) at 90°C for 2 min. DDRT products were resolved on 6% polyacrylamide and 8 M urea gel by electrophoresis at 60 V. The transcripts in the gel were visualized using silver staining.

Re-amplification of cDNA transcripts

The differential products obtained were isolated from the gel. Selected bands will be cut from the gel and cDNA was eluted by soaking the gel slice in 50 µl TE buffer followed by heating at 100°C for 5 min. The eluted fragments were re-amplified using same set of primers that generated the differential product. The cDNA transcripts obtained from this study were cloned and sequenced.

Cloning of PCR products obtained through DDRT

Cloning of PCR products was carried out using PCR-TRAP cloning system (Gene Hunter Corporation, USA) as per the following procedure.

Ligation

The reamplified PCR products are ligated to PCR-TRAP vector. The reaction mix was prepared in 20 µl with the following reagents: Distilled Water 10 µl, 10 X ligase buffer 2 µl, Insert-ready PCR-TRAP vector (150 ng/µl) 2 µl, PCR product 5 µl, T4 DNA ligase (200 units/ µl) 1 µl. The above ingredients were mixed well and ligated overnight at 16°C.

Transformation

The competent cells provided by Gene Hunterwere thawed in ice for 15 min. The cells were quickly mixed and aliquot 100 µl each in 1.5 ml microfuge tube and 10 µl of ligation mix was added to the tube containing competent cells. The tubes were mixed well and incubated for 45 min. Heat shock was given to the cells for 2 min at 42°C and set the tubes back in ice for 2 min. To this mixture, 400 µl of LB medium (no tetracycline) was added and incubated at 37°C for 1 h. Meanwhile, LB + Tet plates were pre-warmed and 30 µl of X-gal (20 mg/ml) was added to the middle of the plate and let dry and then spread the cells immediately onto the LB Tet plate. The transformed cells were briefly vortex and 200 µl of cells were plated on LB +Tet (20 µg/ml) and incubated the plate upside down overnight at 37°C.

Plasmid isolation

Plasmid inserts were confirmed by colony-PCR method using primers flanking the cloning site of the PCR –TRAP Vector.

Colony lysis

Each colony was picked by clean pipet tip and placed in a microfuge tube containing 50 µl of colony lysis buffer. The tubes were incubated in boiling water for 10 min. Spin the tubes at room temperature, then transferred the supernatant into clean tube. This lysate was used for PCR.

PCR Reaction

For each colony lysate added the following reagents in 20 µl reaction mixture: 10 X PCR buffer 2.0 µl; dNTPs (250 µM) 1.6 µl; Lgh primer (2 µM) 2.0 µl; Rgh primer (2 µM) 2.0 µl; Colony lysate 2.0 µl; Taq DNA Polymerase (200 units/ µl) 0.2 µl. PCR was performed as per the following conditions: 94°C for 30 sec, 52°C for 40 sec and 72°C for1 min and for 30 cycles followed by 5 min extension at 72°C and final incubation at 4°C.

DNA gel electrophoresis

The PCR products (20 µl) were analyzed on 1.5% agarose gel prepared in 1X TBE containing 0.5 µg/ml ethidium bromide. The gel was run in 0.5X TBE. The respective intact (unrestricted) plasmid was also run at 60 V to confirm the restriction and release of the inserts.

Qualitative and Quantitative Estimation of Plasmid DNA

The quality and quantity of plasmid DNA was estimated with a spectrophotometer (Genesys 5). One µl of plasmid DNA was taken in 1 ml of sterile water for quantification. Absorbance at 260 and 280 nm was taken and the DNA purity was assessed by calculating the A260/A280 ratios. Purity of DNA was also assessed on a 1% agarose gel and selected plasmids were sequenced using Lseq and Rseq primers provided by the manufacturer (GeneHunter, TN).

Subtractive hybridization PCR Select

In order to concentrate on low abundant and up regulated genes during water deficit subtractive suppression hybridization was performed to isolate transcripts uniquely expressed in response to water stress. Clonetech PCR-select cDNA subtraction kit was used for obtaining the clones expressed in one population. cDNAs of water stressed leaf samples that had specific (differentially expressed) transcripts were used as tester and cDNAs from control leaf tissue of irrigated plant were used as driver. Tester and driver cDNAs were hybridized and hybrid sequences were then removed. Consequently, remaining unhybridized cDNAs represented genes expressed in the tester, but absent from the driver mRNA (Diatchenko et al., 1996). Two rounds of hybridization and PCR amplification was carried out to normalize and enrich the differentially expressed cDNAs. The procedure is given below as per the manufacturer (Clontech) protocol.

First strand cDNA synthesis

For each tester, driver and the control, poly A+ RNA 2 µg was mixed with 1µl cDNA synthesis primer (10 µM) in a sterile 0.5 ml tube. The contents were mixed and spun briefly. The tubes were incubated at 70ºC in a thermal cycler for 2 min and cooled on ice for 2 min. The tubes were centrifuged briefly. To each reaction tube, added 2µl 5X first strand buffer, 1µl dNTP mix (10 mM each), 1µl AMV reverse transcriptase (20 units / µl) and 1µl sterile water. The tubes were gently vortexed, briefly centrifuged and incubated at 42ºC for 1.5 h in an air incubator. The tubes were then placed on ice to terminate first strand cDNA synthesis and immediately proceeded to second strand cDNA synthesis.

Second Strand cDNA synthesis

To the first strand synthesis reaction (10 µl), added 48.4 µl of sterile water, 16.0 µl 5X second strand buffer, 1.6 µl dNTP mix (10mM), 4.0 µl of 20X second strand enzyme cocktail. The contents were mixed and briefly centrifuged. The tubes were incubated at 16°C in a thermal cycler for 2 h. To the above reaction mixture 2 µl (6 units) of T4 DNA polymerase was added and mixed well. The tubes were incubated at 16°C for 30 min in a thermal cycler. The second strand synthesis was terminated by adding 4µl of 20X EDTA/Glycogen.

cDNA isolatin

One hundred µl of phenol:choroform:isoamyl alcohol (25:24:1) was added to the tubes. The tubes were then thoroughly vortexed and centrifuged at 14,000 rpm for 10

minutes at room temperature. The top aqueous layer was carefully removed and placed in a clean microifuge tube. One hundred µl of choroform:isoamyl alcohol (24:1) was added to the aqueous layer. Forty µl of 4M ammonium acetate and 300 µl of 95% ethanol was added, vortex thoroughly and centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was removed carefully and the pellet was overlaid with 500 µl of 80% ethanol. The tubes were centrifuged at 14,000 rpm for 10 min and the supernatant was removed. The pellet was air dried for 10 min and dissolved in 50 µl of water. Six µl of this was digested with Rsa I for agarose gel electrophoresis to estimate yield and size range of ds cDNA products synthesized.

Rsa I Digestion

This step generates shorter, blunt ended ds cDNA fragments, which are optimal for subtraction and necessary for adaptor ligation. To ds cDNA (43.5 µl), added 5.0 µl of 10XRsa I Restriction Buffer and 1.5 µl Rsa I (10 units/µl). The contents were vortex and centrifuged briefly. The tubes were incubated at 37ºC for 1.5 h. Five µl of the digest was set aside to analyze the efficiency of Rsa I digestion and 20X EDTA/glycogen mix (2.5 ml) was added to terminate the reaction. Later, 50 µl phenol:choroform:isoamyl alcohol (25:24:1) was added and thoroughly vortexed, centrifuged at 14,000 rpm for 10 min at room temperature. The top aqueous layer was carefully removed and placed in a clean microcentrifuge tube. To the aqueous layer, 50 µl of choroform:isoamyl alcohol (24:1) was added and later, 25 µl of 4M ammonium acetate and 187.5 µl of 95 per cent ethanol was added, vortexed thoroughly and centrifuged at 14000 rpm for 20 min at room temperature. The supernatant was removed carefully and the pellet was overlaid with

200 µl of 80% ethanol. The tubes were centrifuged at 14000 rpm for 5 min and the supernatant was removed. The pellet was air dried for 10 min and dissolved in 5.5 µl of water and stored at –20ºC. These 5.5µl samples of Rsa I digested samples served as experimental driver cDNA. In the further step, these samples were ligated with adaptors to create tester cDNA.

Adaptor Ligation

One µl of each Rsa I digested experimental cDNA was diluted with 5 µl of sterile water.

Preparation of adaptor ligated tester cDNA

Ligation master mix was prepared in 10 µl by combining 5 X ligation buffer (2 µl) and T4 DNA ligase (400 units/ µl) in total 5 µl reaction mixture. For the experimental tester cDNA, the reagents presented as below was combined and mixed thoroughly.

Component	Tube 1 Tester 1-1 (µl)	Tube 2 Tester 1-2 (µl)
Diluted tester cDNA	02	02
Adaptor 1 (10µM)	02	-
Adaptor 2 (10µM)	-	02
Master mix	06	06

In a fresh micro centrifuge tube, 2 µl of tester 1-1 and 2 µl of tester 2-1 was mixed, which formed unsubtracted tester control 1-c after ligation was complete. The tubes were centrifuged briefly and incubated at 16ºC overnight. The ligation reaction was stopped by adding 1 µl of EDTA/glycogen mix. The samples were heated at 72ºC for 5 minutes to inactivate the ligase. The tubes were briefly centrifuged. This formed the adaptor ligated tester cDNA. One µl from unsubtracted tester control were taken and diluted into 1 ml of water, which were later used for PCR. The samples were stored at –20ºC.

First Hybridization

For each of the experimental subtraction, the reagents presented below were added to make 4 µl reaction mixture and centrifuged briefly.

Component	Hybridization Sample 1 (µl)	Hybridization Sample 2 (µl)
Rsa I digested driver cDNA	1.5	1.5
Adaptor 1 ligated tester 1-1	1.5	-
Adaptor 2R ligated tester 1-2	-	1.5
4X hybridization buffer	1.0	1.0

The samples were incubated in a thermal cycler at 98º C for 1.5 min. The samples were then incubated at 68º C for 8 hours and then proceed to second hybridization.

Second Hybridization

The reaction mixture containing Driver cDNA (1 µl) was mixed with 4X hybridization buffer (1 µl) and sterile water (2 µl). One µl of this mixture was placed in a microcentrifuge tube and incubated in a thermal cycler at 98ºC for 1.5 min. The tube of freshly denatured driver was removed from the thermal cycler and the following procedure was done to simultaneously mix the driver with hybridization samples 1 and 2. This ensured that the two hybridization samples mixed together only in the presence of freshly denatured driver. The sample interface of the tube containing hybridization sample 2 was gently touched with the pipette tip and the entire sample was drawn into the tip. The entire mixture was transferred to the tube containing hybridization sample 2 and mixed by pipetting up and down. The tube was gently centrifuged and incubated at 68ºC for overnight. Two hundred µl of dilution buffer was added to the tube, mixed and heated in a thermal cycler at 68ºC for 7 min. The tube was stored at –20ºC.

PCR amplification

One µl of each diluted cDNA (i.e., each subtracted sample from step F.6 and the corresponding diluted unsubtracted tester control) was taken into a labeled tube. One µl of the PCR control subtracted cDNA was taken into the tube. Master mix was prepared for all the primary PCR tubes. The reaction mixture contained 10X PCR reaction buffer 2.5 µl, dNTP mix (10mM) 0.5 µl, PCR primer 1 (10 µM) 1.0 µl and, 50X advantage cDNA polymerase mix 0.5 µl. The contents were mixed well and gently vortexed. The master mix (24 µl) was added into each of the reaction tubes and incubated in a thermal cycler at 75ºC for 5 min to extend the adaptors. Thermal cycling was done immediately

at 94°C at 25 sec followed by 27 cycles of 94°C for 10 sec, 66°C for 30 sec and 72°C for 1.5 min. Three µl of each primary PCR mixture was diluted in 27 µl of water. One µl of each diluted primary PCR product was taken for further second PCR. Master mix for the secondary PCR was prepared using the components, 10X PCR reaction buffer 02.5 µl, nested PCR primer 1(10µM) 1.0 µl, nested PCR primer 2 (10µM) 1.0 µl, dNTP mix (10 µM) 0.5 µl and, 50X advantage cDNA polymerase mix 0.5 µl. The contents were mixed well and briefly centrifuged. Twenty four µl of master mix was pipetted out into each reaction tube. Thermal cycling was done for 12 cycles using the parameters of 94°C 10 sec, 68°C 30 sec and, 72°C 1.5 min. The reaction products were stored at –20°C. The PCR mixture obtained was thus enriched for differentially expressed cDNAs.

Cloning of PCR products obtained through Subtractive Hybridization

The PCR mixture enriched with cDNAs obtained from subtractive hybridization was cloned using pGEM cloning vector provided by Promega Inc.

Ligation

Ligation reaction was set up in a 1.5 ml microfuge tube using 2X ligation buffer 5 µl, plasmid vector pGEM T easy vector 1 µl, purified PCR fragment (approximately 0.54 p mol ends) 2 µl, deionized water 1 µl, T4 DNA ligase (5 units) 1 µl. A control ligation reaction was also performed using control PCR fragment provided with the kit. The reaction mix was incubated at 4°C overnight.

Transformation

The high efficiency competent cells (JM 109) were removed and placed on ice bath until just thawed (for 5 min) and gently mixed the cells. The cells were quickly mixed and aliquot 100 µl each in 1.5 ml microfuge tube and 10 µl of ligation mix was added to the tube containing competent cells. The tubes were mixed well and incubated for 45 min. Heat shock was given to the cells for 2 min at 42°C and set the tubes back in ice for 2 min. To this mixture, 400 µl of LB medium (no tetracycline) was added and incubated at 37°C for 1 h. Meanwhile, LB + Tet plates were pre-warmed and 30 µl of X-gal (20 mg/ml) was added to the middle of the plate and let dry and then spread the cells immediately onto the LB Tet plate. The transformed cells were briefly vortexed and 200 µl of cells were plated on LB +Tet (20 µg/ml) and incubated the plate upside down overnight at 37°C.

Plasmid Isolation

Plasmid inserts were confirmed by colony-PCR method using primers flanking the cloning site of the pGEM Vector.

Colony lysis

Each colony was picked by clean pipet tip and placed in a microfuge tube containing 50 µl of colony lysis buffer. The tubes were incubated in boiling water for 10 min. Spin the tubes at room temperature, then transferred the supernatant into clean tube. This lysate was used for PCR. The reagents were mixed for 20 µl of PCR were 10 X PCR buffer 2.0 µl, dNTPs (250 µl) 1.6 µl, Lgh primer 2.0 µl, Rgh primer 2.0 µl, colony

lysate 2.0 µl and Taq DNA polymerase 0.2 µl. The PCR was performed following conditions at 94°C for 30 sec, 52°C for 40 sec and 72°C for 1 min for 30 cycles followed by 5 min extension at 72°C and final incubation at 4°C**.**

DNA gel electrophoresis

The PCR products (20 µl) were analyzed on 1.5% agarose gel prepared in 1X TBE containing 0.5 µg/ml ethidium bromide. The gel was run in 0.5X TBE. The respective unrestricted plasmid was also run at 60 V to confirm the restriction and release of the inserts.

Qualitative and Quantitative Estimation of Plasmid DNA

The quality and quantity of plasmid DNA was estimated in spectrophotometer. One µl of plasmid DNA was taken in 1 ml of sterile water for quantification. Absorbance at 260 and 280 nm was taken and the DNA purity was assessed by calculating the A260/A280 ratios. Purity of DNA was also assessed on a 1% agarose gel and only good quality plasmids were sequenced.

Sequencing and Characterization of Cloned PCR Products

The cDNA sequencing was carried out on CEQ 8800 as follows using DYE Terminator Cycle Sequencing with Quick Start Kit. DNA sequencing reaction was prepared in 20 µl reaction mixture comprised of DNA template 0.5 – 10.0 µl, custom primer (1.6uM) 2.0 µl and DTCS Quick start Master Mix 8.0 µl. For DDRT PCR clones, primers provided by PCR TRAP were used. Polymerase chain reaction was carried out at

90ºC for 20 sec, 50ºC for 20 sec and 60ºC for 4 min for 30 cycles following by holding at 4ºC. Prepared freshly stop solution/glycogen mixture using 3 M sodium acetate (pH 5.2) 2 µl, 10 uM Na_2EDTA (pH 8.0) 2 µl and 20 mg / ml glycogen 1 µl. Five µl of the stop solution/glycogen mixture was added to separate labeled tubes, transferred the sequencing reaction and mixed thoroughly. Added 60 µl cold 95% ethanol/d H_20 from -20ºC freezer and mixed thoroughly, and centrifuged at 14,000 rpm at 4ºC for 15 min. Carefully supernatant was removed with a micropipette and rinsed the pellet with 200 µl 70% ethanol from -20ºC and vacuum dried the tubes for 10 min. The pellet was dissolved in 40 µl of sample loading solution. The samples were transferred to 96 well plates and overlaid with one drop of light mineral oil. The sample plates were loaded into the instrument and started the sequencing program.

Functional annotation of the Isolated Proteins

Sequence similarities of all unique genes / transcripts were annotated on the basis of the existing annotation with sequences to nucleotide sequences in non-redundant databases at Gene bank, European Molecular Biology Laboratory (EMBL), and DNA Data Bank of Japan (DDBJ), Protein data Bank (PDB), National center for Biotechnology Information (NCBI) databases using the FASTA, BLASTN BLASTP and BLASTX Server (Altschul et al., 1990). The sequence data thus obtained was analyzed for their identity using BLASTX (Nucleotide translated-protein) search for its homology with the sequence of a gene already recorded in the database of the NCBI (National Centre for Biological Information). Proteins with BLAST scores above 45 bits and significantly low expected value (E-value) were designated as known function. The lower the E-value

the more significant is the match considering the length of the query. The Expect value (E) is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size. It decreases exponentially as the Score (S) of the match increases.

Section B. PROTEOMICS

Water stress responsive proteins were identified and isolated using high throughput two-dimensional electrophoresis (2-D PAGE). This technique is one of the effective methods for the separation of hundreds of proteins on a gel.

Tissue Preparation

About 2g leaf tissues form control and water stressed plants were homogenized in 20% trichloro acetic acid (TCA) in acetone and washed with acetone, later with ethanol and ethyl acetate (2:1). The powder was air dried and used for protein extraction.

Protein Extraction

Grape leaf powder (25 mg) was homogenized in 750 µl of extraction buffer containing 7.3 M urea, 2M thiourea, 2% CHAPS and 50mM ditheothritol (DTT). The sample tubes were placed in ice during homogenization to avoid degradation. The tubes were centrifuged at 14,000 rpm / 10 min and, the supernatant was transferred to a fresh vial.

Quantification of Proteins

The total protein content of the samples was determined according to method described by Bradford (1976). Bovine Serum Albumin (BSA) was used as the standard to quantify the total protein concentration in the sample. Equal concentration (75µg) of protein was loaded on each isoelectric focusing (IEF) gel tube.

Preparation of Isoelectrofocussing (IEF) and 2-dimensional Sodium dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (2-D SDS-PAGE)

Proteins were separated by two-dimensional gel electrophoresis as described by Basha (1979). The proteins were identified on 2-D electrophoresis, the spots were eluted from the gel and were identified sequences using Liquid Chromatography Mass Spectrometry (LC/MS). In the first dimension, proteins are resolved in according to their isoelectric points (pI) using IEF. Under standard conditions of temperature and urea concentration, the observed focusing points of the great majority of proteins using IEF closely approximate the predicted isoelectric points calculated from the amino acid compositions. In the second dimension, proteins are separated according to their approximate molecular weight using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE). This technique can provide molecular weight approximations (+/- 10%) for most proteins, with some dramatic exceptions. The 2-D PAGE consisted of isoelectric focusing in the first dimension and SDS-PAGE (Lameli, 1970) in the second dimension under denaturing conditions. All 2D and SDS-PAGE gel evaluations were repeated in triplicate. Bromophenol Blue was used as the tracking dye. Preparative gels were stained with colloidal Commassie Brilliant Blue G-250 to visualize

the protein spots. Imaging of the gels was carried out with BIO-RAD gel Documentation System. The proteins were identified and isolated using gel elution technique for further characterization.

Mass spectrometry analysis and Protein Sequence Characterization

To determine the identity and function of the proteins, the spots observed on the 2-D PAGE were eluted, and treated with DTT to break disulphide linkages, alkylated with iodoacetamide and then digested with trypsin. Protein samples were destained and underwent a 14 h tryptic digest at 37ºC. The resultant peptides were extracted in washes of ammonium bicarbonate solution, ACN and 10% formic acid. Extraction solvent was removed under vacuum and the peptides were resuspended in 30 µl of 5% MeOH, 0.5% formic acid. Capillary RP HPLC separation of protein digests (desalted with a PepMap C18 cartridge) was performed using Ultimate Capillary HPLC System (LC Packings, San Francisco, CA). Samples (3 µl) were injected directly onto a PepMap reversed phase C18 column (0.075 x 150 mm) supplied by LC Packing (Dionex). The flow rate after splitting was 320 nl / min. Tandem mass spectrometric analysis was performed online using a hybrid quadrupole time-of-flight instrument (QSTAR XL hybrid LC/MS/MS) equipped with a nanoelectrospray source (Applied Biosystems, Foster City, CA). Tandem mass spectra were acquired using the information Dependent Acquisition mode. The ion spray voltage was 1750 V, the curtain gas was set to 15 (arbitary units) and the declustering potential was 60 V. The raw MS/MS sequence data was BLAST against NCBI non-redundant entries (NIH, Bethesada, MD) using Mascot (Matrix Science version 2.0.01, London UK).

CHAPTER IV

RESULTS AND DISCUSSION

For the first time, the study has been carried out on water deficit stress on molecular and biochemical compositions in Florida Hybrid Bunch grape. The results obtained in this research are described.

Section A. GENOMICS

Experiment 1 Induction of Water Stress to Grape Plants and Measurement of Soil Water Potential

Florida hybrid bunch grape variety 'Suwannee' was used in this study (Figure 4.1). The stem cuttings from the stocks were initiated and planted in five gallon pots. These cuttings were maintained for two years under green house conditions (Figure 4.2). Water stress was induced by withholding irrigation to plants up to 20 days. After 20 days of stress, the plants started showing symptoms of wilting. The experiment was carried out to measure the soil water potential and correlate to the intensity of water stress in the plant. The soil water potential was measured at 5 day interval to determine the water deficit stress. The soil water potential was initially 25 centibars at day one. The control plants maintained the soil water potential between 22 and 24 centibars as they were

regularly irrigated throughout the experiment. Soil water potential was gradually reduced over 20 day period of water stress. Fifth day water stress showed 19.35 while it declined gradually to 9.44 at 20th day of water stress (Table 4.1).

Table 4.1 Soil Water Potential during Progressive Water Stress.

Days Interval	Soil Water Potential	
	Control	Treatment (Water Stress)
5 Day	24.7 ±0.24	19.35 ±0.38
10 Day	23.6 ±0.19	17.15 ±0.98
15 Day	24.5 ±0.28	15.69 ±0.67
20 Day	22.9 ±0.95	9.44 ±0.82

Figure 4.1 Florida Hybrid Bunch Grape 'Suwannee' developed through hybridization of Local Grape species with *Vitis vinifera*

The data indicate the water stress level in the whole plant. The leaf tissue samples were collected in six replications from control and treated plants, frozen in liquid nitrogen immediately and stored at -80º C for genomics and proteomics studies.

Figure 4.2 Two year old Florida Hybrid Bunch Grape 'Suwannee' growing in Greenhouse conditions

Experiment 2: Isolation of RNA and mRNA from Control and Water Stressed Leaf Tissue of Florida Hybrid Bunch grape 'Suwannee'

Total RNA was isolated from both control and stressed leaf tissues at 5, 10, 15 and 20 day stress periods according to the procedure mentioned in chapter II Materials and Methods as described by Lopez-Gomez (1992). The yield of total RNA extracted from control and stressed leaf tissues varied according to the progressive stress periods. The average yield of total RNA extracted from control and treated tissues was 38.8 and 13.6 μg/g of the leaf tissue respectively (Table 4.2). The yield of RNA was reduced with

the increase in stress period in leaf tissue sample (5th day of stress- 17.2 μg/g and 20th day stress- 8.0 μg/g) indicating either the partial degradation of total RNA due to prolonged water deficit stress or the recovery from the water stressed tissue sample is low. The RNA found intact and of high quality without any smears or any sign of degradation (Figure 4.3).

Table 4.2 Total RNA Content in Control and Water Stress leaf tissue of Florida hybrid Bunch Grape 'Suwannee'

Days Interval	RNA (μg/g of leaf tissue)	
	Control	Treatment (Water Stress)
5 Day	42.4	17.2
10 Day	35.0	14.8
15 Day	38.5	14.3
20 Day	39.2	8.0

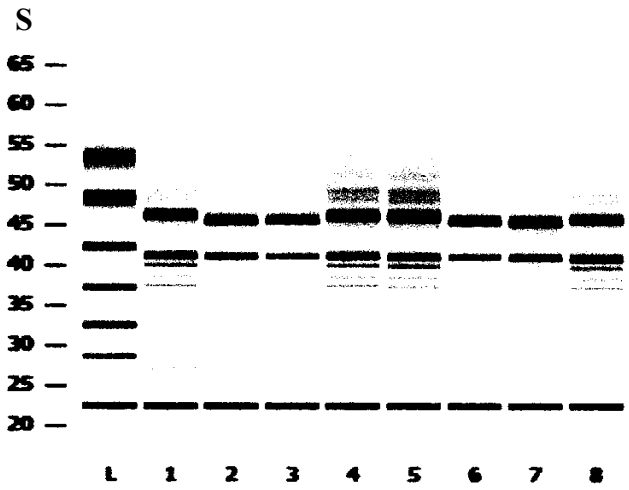

Figure 4.3 Total RNA of both control and stress tissue of Florida Hybrid Grape 'Suwannee'

L: Ladder, Lane 1-4: RNA of Control and Lane 5-8: RNA of Stress. S: Sedimentation.

Total RNA obtained from each tissue was used to isolate mRNA using oligotex direct mRNA midi/maxi kit (catalogue number 72041) following the procedures described by manufacturer's protocol (Qiagen, CA). The purity of mRNA was determined by the peaks in the image system.

Several protocols have been described by various laboratories for the isolation of RNA from plant tissues. Salzman et al. (1999) method yielded RNA but it was found to be inadequate and degraded. This suggests that RNA might have been lost by binding to polysaccharides, polyphenols or other components during extraction. For successful DDRT-PCR and subtractive hybridization it is necessary to use high quality RNA to not to miss any low copy expressed genes. The ability to isolate high quality total RNA and mRNA free of protein, genomic DNA was crucial for molecular analysis such as reverse transcription polymerase chain reaction (RT-PCR). Therefore, an efficient protocol that yields qualitative RNA and mRNA at higher amounts from both control and water

stressed leaf samples is required to obtain the low expressed genes. There are several methods available in the literature. RNA extraction methods of Lopez-Gomez and Gomez-Lim (1992), Salzman et al. (1999), Hu et al. (2002), Iandolino et al. (2004), Thomas and Schiefelbein (2002), Tattersall et al. (2005), commercial kits and Trizol method did not yield sufficient amount of good quality RNA from different grape tissues. In this study, RNA isolation procedure described by Lopez-Gomez and Gomez-Lim (1992) was followed in which RNA extraction was carried out on ice with the buffer pH 7.5 to prevent phenolic oxidation and polysaccharides precipitation. This was followed by precipitating the polysaccharide complex using potassium acetate and overnight precipitation of RNA with LiCl. This method consistently gave good yields of good quality RNA. The procedure described is modified involving addition of 2% insoluble polyvinylpyrrolidone (PVP) which facilitated removal of most of the polyphenols and polysaccharides while pre-warmed extraction buffer helped in inactivating RNase which is high in mature and diseased tissue. Earlier studies indicated that PVP in extraction buffer is incompatible with phenol extraction and binds to nucleic acids (Asif et al. 2000). The insoluble PVP is compatible with the buffer and helps in removing most secondary metabolites. Inclusion of PVP during grinding the tissue helped in recovering higher quantity of RNA (20%) compared to adding PVP in the extraction buffer. Polyvinylpyrrolidone helps in dissociation of complexes of polysaccharides, phenols and other compounds (Ainsworth, 1994), which can be removed later by phenol: chloroform extraction. The RNA yield obtained using pre-warmed extraction buffer was higher (32 to 540 μg per g of fresh sample) compared to the yield obtained with buffer at room temperature (15 to 140 μg per g of fresh sample). Inclusion of an additional re-extraction

step with phenol and chloroform helped remove contaminants and recover high quantity of RNA. Inefficient removal of polysaccharides and polyphenolic compounds results in coprecipitation with RNA, which affects the yield and quality (Logemann et al. 1987). Earlier, several researchers obtained higher yields of qualitative RNA from various tissues of *Satsuma mandarin* and kiwi fruits by using the modified conventional extraction method (Lopez-Gomez and Gomez-Lim, 1992). Hence, LiCl precipitation method was found suitable for isolating good quality and integrated RNA from water stressed and healthy leaf tissues of grape. Total RNA was directly precipitated using cold absolute ethanol/isopropanol instead of LiCl to avoid any water insoluble precipitate and loss of RNA (Liu et al. 1998). There was no significant yield difference when either cold absolute ethanol or isopropanol was used for RNA precipitation. Grape is a woody perennial and contains large amounts of polyphenols and polysaccharides. Because of these compounds isolation of good quality RNA from grapevine tissue is difficult. Most of the published protocols failed to yield sufficient quantity of high quality RNA from various grape tissues suitable for gene expression studies. Our refined protocol with the inclusion of high concentration of PVP, pre-warmed extraction buffer and three extraction steps yielded good quality and quantity RNA, especially from mature and diseased tissue containing high levels of polyphenols and polysaccharides. Intact RNA, high A260/A280 ratio (1.52 to 1.90), high A260/A230 ratio (2.10 to 2.36), higher amount of mRNA recovery (3.4%), consistent cDNA profile through Differential Display RT-PCR, amplification of higher number of subtracted cDNA transcripts through subtractive hybridization and RT-PCR using gene specific primer confirmed the quality of RNA. Hence, this protocol will be useful for isolating high quality RNA suitable for gene

expression studies and also for isolating RNA from plants containing high concentration of polyphenols and polysaccharides.

From the total RNA obtained, sufficiently high quality mRNA was isolated (mRNA is 5% of the total RNA). This was sufficient and proved suitable to carryout subtractive hybridization to isolate specific genes from stressed and control tissues. The samples when stored for prolonged periods of over six months to one year did not yield good quality RNA. The RNA isolated under sterile condition yielded higher quality intact RNA, which yielded sufficiently high quality RNA and to analyze differentially expressed genes in response to water stress.

Experiment 3: Optimization of Primer Combinations of Differential Display RT PCR for High Yield of Differentially Expressed Transcripts

Total RNA form control and stressed leaf samples were used to identify differentially as well as uniquely expressed transcripts to water stress. A total of 24 primer combinations obtained from GenHunter Inc., were used to identify suitable primer pairs to obtain high resolution transcripts using RNA from control and 5^{th} day water stressed leaf tissue. First strand cDNAs were synthesized from control and stress leaf samples (Figure 4.4). Of the 24 primer pairs, 11 primers resolved both up- and down-regulated transcripts. Three primer pairs showed up-regulated transcripts, five primer

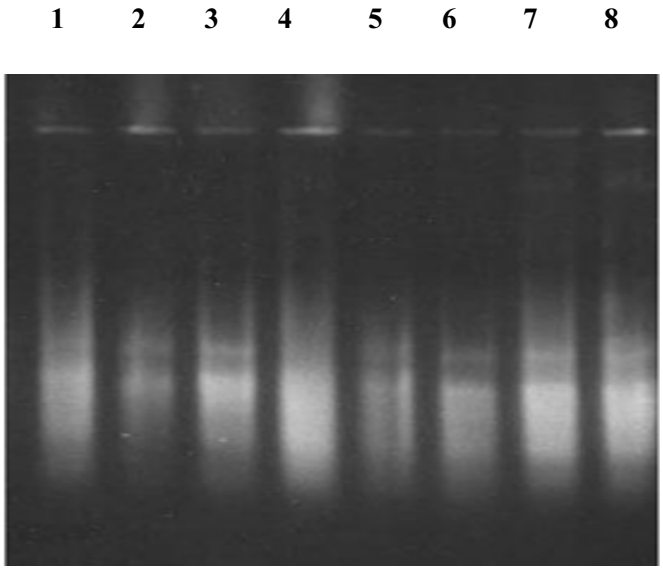

Figure 4.4 cDNA synthesis from RNA of control and stress samples

Lanes: 1 to 4: Control tissue; 5-6: Water stressed sample at 5, 10, 15 and 20 days

Table 4.3 Optimization of Primer Pairs for High Frequency Transcript Profiling to Water Stress

Primer F	Primer R	Up-Regulated	Down-Regulated	New
H T11 G	H AP-1	1	5	2
H T11 G	H AP-2	3	2	3
H T11 G	H AP-3	0	4	1
H T11 G	H AP-4	0	5	1
H T11 G	H AP-5	0	1	0
H T11 G	H AP-7	2	0	2
H T11 C	H AP-1	0	5	1
H T11 C	H AP-2	8	0	0
H T11 C	H AP-3	5	0	4
H T11 C	H AP-4	0	3	8
H T11 C	H AP-8	3	4	0
Total Transcripts Affected		22	29	22

combinations showed down-regulated transcripts. Three primer pairs showed both up- and down- regulated transcripts, while 8 pairs showed induction of new transcripts (Table 4.3).

A total of eleven primer combinations were selected based on the results to study the transcript profile for different stress periods. These primer pairs proved potential for yielding significantly higher number of either up- and down-or newly regulated transcripts to water stress.

Experiment 4: Identification of Differentially Expressed Transcripts to Water Stress in Florida Hybrid Bunch 'Suwannee'

Differential Display RT PCR was performed using total RNA of the control and water stress treated samples at 5, 10, 15 and 20 day periods. First strand cDNA synthesis was performed using selected primer pairs optimized from results obtained in Experiment 3. The second strand synthesis and PCR amplification was performed using corresponding oligo primer and selected arbitary primers. In this experiment, over 50 transcripts were resolved from each primer combination those showing differential expression to water stress treatment. The results showed that expression of cDNA transcripts was greatly affected during the 10 and 15-day stress period. Beyond 15 days of stress, most of the transcripts were suppressed, indicating that over 15 day stress was detrimental to the plant (Figure 4.5). Differentially expressed and unique cDNA transcripts were isolated from the gel and re-amplified using corresponding primer pair/s that generated the PCR products. Maximum number of transcripts was up-regulated at 5^{th} day of stress, while there are no transcripts suppressed or newly synthesized. As the

stress duration increases, there are 4 more transcripts were up regulated and 2 transcripts were down regulated and 4 new were synthesized. During 15th day stress, only one more transcript was up regulated, however, 2 were suppressed and 4 newly synthesized. At 20th day, where the plant almost showed wilting symptoms, 4 transcripts were down regulated (Table 4.4).

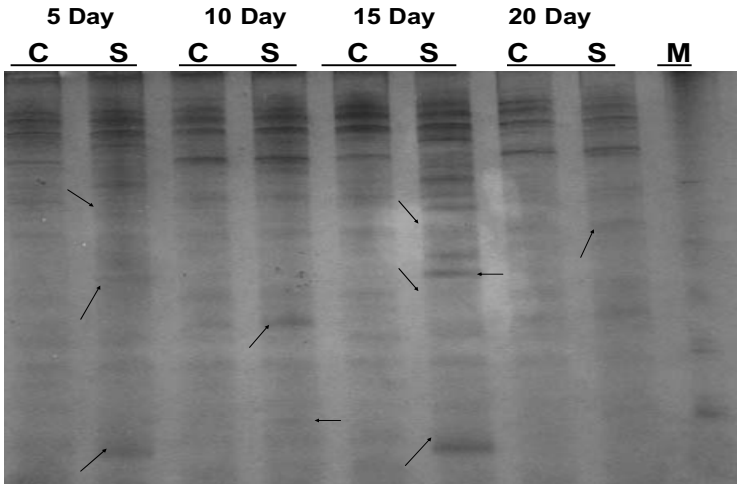

Figure 4.5 Identification of Differentially Expressed Transcripts to Water Stress

Primer:AP1, HT 11G; Direction of Arrows indicate up-down regulation of transcript/s
Note 5 transcripts up regulated, 3 down regulated and 2 newly synthesized.

The data clearly show that, the initial stress level enhanced the expression of transcripts. As the stress prolongs, more number of transcripts were affected up to 15th day stress. Beyond this point, no significant changes were observed because, at 20th day stress, there was decline in the number of transcripts affected so as the total yield of transcripts.

Overall, a total of 14 transcripts were up regulated, nine at 5th day, four at 10th day and one at 15th day of stress periods while 8 transcripts were suppressed (2 each at

10^{th} and 15^{th} day stress and 4 at 20^{th} day stress periods). Eight new transcripts were identified, 4 each at 10^{th} and 15^{th} day of stress periods consequently.

Table 4.4 Differentially Expressed Transcripts During Different Water Stress Periods

Differentially Expressed Transcripts	5 Day	10 Day	15 Day	20 Day	Total
Up Regulated	9	4	1	0	14
Down Regulated	0	2	2	4	8
New	0	4	4	0	8

Cloning and Transformation

Differentially expressed cDNA transcripts were directly cloned into PCR-TRAP vectors (GeneHunter, TN). High frequency of transformation was achieved as indicated by the amplification of recombinant plasmids. These recombinant plasmids of control and water stressed tissues were then transferred to *E. coli* (competent) cells. High yield of recombinant (white) colonies were obtained in DDRT method (Figure 4.5). Selected clones were sequenced and characterized.

Restriction Digestion of recombinant plasmid DNA

In order to confirm the presence of insert in the plasmids, the plasmid DNA was digested with the restriction enzymes as prescribed in the plasmid maps. Different sizes of inserts were observed. This confirmed the presence and quality of plasmids with

inserts, which can be used for PCR amplification with specific primers. Higher molecular weight inserts were obtained, which yielded more or less complete length of genes. Thirty individual plasmids were selected and sequenced in this study.

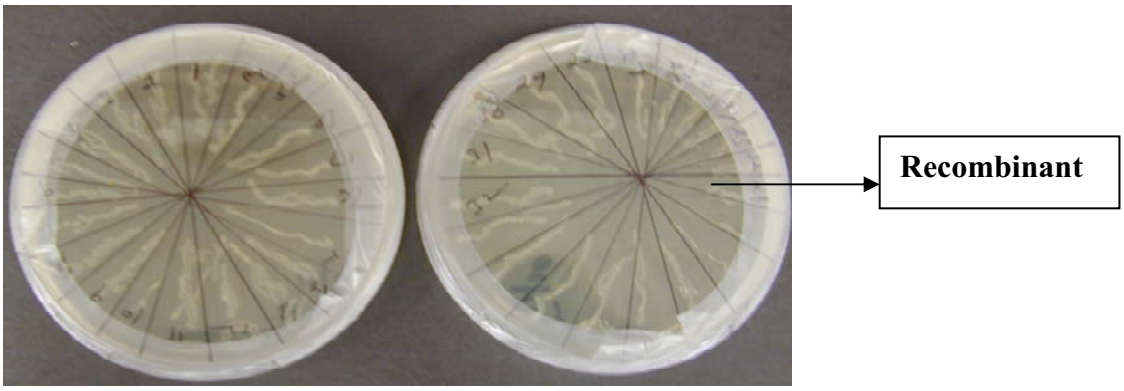

Figure 4.6 Cloning of Differentially Expressed Transcripts through DDRT PCR

Sequencing, Data Analysis and, Characterization of the Transcripts isolated through DDRT

From the raw sequence data obtained the sequence of plasmid and the primer of M13 forward and backward were removed. Then the data having only the sequence of genes were analyzed for their identity using BLASTX search (Basic Local Algorithm Tool X nucleotide translated query vs. protein database) for its homology with the sequence of a gene already recorded in the database of the NCBI (National Center for Biological Information). Out of the 30 characterized genes, most significant genes were annotated and discussed in detail here. Out of selected genes, 3 were up regulated and 4 were down regulated and 3 were newly synthesized genes were analyzed and the function was annotated. The function of these sequences was identified with their expect value

(E-value) and protein homology (Table 4.5). The list of sequences is reported in Appendix A.

Table 4.5 Isolation of differentially expressed genes specific to control and water stressed tissues

Sequence Regulated	Base pairs	Name of Protein	Score (Bits)	E Value
DOWN	430	Sucrose Synthase 1	69	0.2
DOWN	310	Actin	153	1e-41
DOWN	282	AP2 Transcriptional activator	35	10
DOWN	428	Cytochrome b gene	86	6e-17
UP	272	WRK type transcription factor	75	6e-14
UP	410	Putative aquaporin	163	1e-45
UP	717	Protein Kinase	118	2e-26
NEW	431	ABF3	295	9e-60
NEW	367	Isoprene Synthase	28	115
NEW	248	Water-stress inducible protein	31	10

Differential display RT PCR has been proven to be an efficient method to isolate and identify up regulated or down regulate genes to responding to a treatment both in plant and animals (Liang and Pardee, 1992). The approach has been widely used to identify differentially expressed gens to abiotic and biotic stresses in various crop plants (Bauer 1993; Hannappel et al., 1995). Earlier, several reports have shown the potential application of DDRT PCR to identify water stress induced differentially expressed genes in crop plants. DDRT PCR was used to identify genes that are differentially expressed in two-year old birch trees as a response to ozone-induced oxidative stress. One of the

ozone-induced cDNA fragments isolated by DDRT-PCR was used to isolate the corresponding cDNA from an O_3-induced birch cDNA library. Nucleotide sequence analysis suggests that it encodes a mitochondrial phosphate translocator protein, the first one isolated from plants (Kiiskinen et al., 1997). In the present study, the genes were either up-regulated or down regulated or newly synthesized to induced water stress. The most important genes involved in water stress regulation are 1. Sucrose Synthase, 2) Actin, 3) SNF1-Related Protein Kinase, 4) WRKY-type transcription factors, 5) ABF3 and, 6) Isoprene synthase.

Sucrose synthase is an enzyme belongs to family of glycosyltransferases which participates in starch and sucrose metabolism. It is a tetramer with a molecular mass of 320 kD and subunits of 80 kD. It catalyzes glucose and fructose to sucrose. It is an important enzyme which play role in synthesis of nucleotide sugars and saccharides (Zervosen and Elling, 1999). This enzyme is gradually suppressed from 5 day stress period and completely absent by 20^{th} day stress period. The suppression of this enzyme indicate that, the tissue was unable to maintain the synthesis of this protein during water stress regime. Similar observations were found in soybean where leaf water potential of leaves and, sucrose synthase levels reduced on gradual drought stress (Gonzalez et al., 1995). It is suggested that sucrose synthase may play a key role in the regulation of nodule carbon metabolism and, therefore, of nitrogen fixation under drought stress conditions (Gonzalez et al., 1995). Analysis of metabolic enzyme activities and metabolites from well hydrated control and partially dehydrated cotton plants showed that, water stress decreases cotton leaf starch content directly by enhancing α-amylase activity and indirectly by altering sucrose metabolism. Inhibition of sucrose synthetase

causes an sucrose in the cytoplasm and also contributes to excess orthophosphate amassment in the chloroplasts. Orthophosphate inhibits ADP-glucose pyrophosphorylase activity which is responsible for starch synthesis in the chloroplasts (Geigenberger and Stitt 1993). Studies on developmental changes in starch and sucrose content in wheat showed marked reduction in their contents. Sucrose synthase and UDP-glucose pyrophosphorylase (UDP-Gppase), showed higher catalytic activity and more resistance to water stress, as compared with amyloplastic enzymes. Soluble starch synthase was the enzyme most sensitive to water stress in that it responded earlier, and to a greater extent, than the other enzymes. However, under severe dehydration conditions, leading to cessation of growth, the decline in soluble starch synthase activity was less than that for ADP-Gppase. These results suggest that soluble starch synthase is the site of response to water stress by which the rate of grain growth can be affected (Ahmadi and Baker 2001). Sucrose synthase activity was highly correlated with both polypeptide and transcript levels indicating, gene expression is regulated mainly at the mRNA level in the different tissues and organs of developing carrot plants (Veronica et al., 1995).

Our results indicate that actin is gradually down regulated to water stress. Actin is a globular protein found in all eukaryotic cells. It is the monomeric subunit of microfilaments, one of the three major components of the cytoskeleton. Actin was down regulated at 10^{th} day stress and continued to suppress till 20^{th} day stress period. Actin participates in many important cellular functions, including cell division and cytokinesis vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape. It was also reported in root tissue of 5-day old winter wheat seedlings, the water permeability of two transport channels of plasmodesmata

known to inhibit polymerization of cytoskeleton actin filaments, due to water loss in the seedlings. This suggest that in roots of drought-resistant plants, after a moderate water loss, a diffusive water flow through the cytoplasmic symplast increases, while that through the vacuolar symplast decreases. After a high water loss in seedlings, it was noticed a greater increase in water permeability of the cytoplasmic symplast, and a decrease in water permeability of the vacuolar symplast, however, in the roots of low resistant cultivars these changes were poorly expressed. This observation lead to conclusion that, the variety 'Suwannee' might be susceptible to water stress hence the activity of actin is suppressed during water stress (Volobueva et al., 2001). Further it was explained that, under osmotic stress, the critical concentration for G-Ca-ATP actin was reduced for six different osmolytes. These results are interpreted as showing that reducing water activity favored the polymerized state. The nucleotide binding site of the Mg conformation is more closed than the Ca and more closely resembles the closed actin conformation in the polymerized state. These results suggest that the water may come from the cleft of the nucleotide binding site (Fuller and Rand, 1999).

SNF1-Related Protein Kinase was up regulated from 5 day stress period. SNF1-related protein kinase is an osmotic stress-activated protein kinase in *Arabidopsis thaliana* that can significantly impact drought tolerance of *Arabidopsis* plants. Knockout mutants of protein kinase (SRK2C) exhibited drought hypersensitivity in their roots, suggesting that SRK2C is a positive regulator of drought tolerance in *Arabidopsis* roots. It is concluded that, SRK2C is capable of mediating signals initiated during drought stress, resulting in appropriate gene expression. Their research reveals new insights around signal output from osmotic-stress-activated SnRK2 protein kinase as well as

supporting feasibility of manipulating SnRK2 toward improving plant osmotic-stress tolerance (Umezawa et al., 2004).

The role of WRKY-type transcription factors although is still obscure they have multiple roles in the plant defense response and developmental processes. The transcript encoding these transcription factors shown to up regulate at 10^{th} day stress period and continue to express at 20^{th} day stress. Earlier in soybean, 64 *GmWRKY* genes from soybean were identified, and were found to be differentially expressed under abiotic stresses. Nine GmWRKY proteins were tested for their transcription activation in the yeast assay system, and five showed such ability. These results indicate that the three *GmWRKY* genes play differential roles in abiotic stress tolerance, and that *GmWRKY13* may function in both lateral root development and the abiotic stress response (Zhou et al., 2008).

ABF3 is the gene that encodes a transcription factor for the expression of ABA-responsive genes (Oh et al., 2005). In this experiment, this gene is newly expressed to water stress at 10 day and continue to express up to 20 day period of water stress. Abscisic acid (ABA) plays an important role in environmental stress responses of higher plants during vegetative growth *via* regulating the expression of numerous stress-responsive genes. Abscisic acid controls various biochemical, cellular, and developmental aspects of adaptive responses to a variety of common abiotic stresses ultimately leading to physiological changes. ABF3 is an excellent genetic resource for development of crop plants with multiple stress tolerance (Vanjildorj et al., 2005). The mode of gene regulation by ABA appears to be highly conserved among plant species. Transcription factors highly identical to ABFs have also been reported in major crop

species such as rice, wheat, and barley. The high degree of conservation of regulatory elements suggests that ABF3 will function in a wide variety of plant species. For instance, drought tolerance of tobacco is greatly enhanced by ABF3 and similar effects were also observed with other plants.

A new gene identified as isoprene synthase is an enzyme responsible for a hydrocarbon which can significantly affect atmospheric chemistry including reactions leading to tropospheric ozone. This gene was induced at 10 and 15 day water stressed leaf samples continue to express till 20 day stress period. Isoprene emission is remarkably resistant to water stress (Tingey *et al.*, 1981). Water stress found to have a minor effect on isoprene emission (Tingey *et al.*, 1981; Sharkey and Loreto, 1993; Fang *et al.*, 1996; Pegoraro *et al.*, 2004; Funk *et al.*, 2005; Monson *et al.*, 2007). Isoprene synthase activity is quite robust in response to water stress (Brüggemann and Schnitzler, 2002; Brilli *et al.*, 2007). The maintenance of isoprene emission and stimulation by water stress can be interpreted as adaptive in light of the thermo tolerance hypothesis. This is because of water stress is likely to lead to more frequent heat stress as latent heat loss is reduced with reduced water availability. With the new information on temperature and water stress effects on isoprene emission, it is speculated that isoprene emission may help plants cope with stressful conditions (Sharkey and Loreto, 1993).

Experiment 5: Isolation of uniquely expressed genes specific to water stress in control and water stressed tissues using Subtractive Hybridization

Subtractive hybridization is a powerful technique that enables to compare two populations of mRNA. It also enables to obtain clones of genes that are expressed in one

population but not in the other. This single stranded mRNA was reverse transcribed using reverse transcriptase to make it double stranded cDNA. The cDNA of water stressed leaf tissue that contains specific (differentially expressed) transcripts were used as tester and the control leaf tissue cDNA were used as driver and *vice versa.* Tester and driver cDNAs were *Ras* I digested to link the adaptor ligated with suitable adaptors and later were hybridized. The hybrid sequences were then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester, but are absent from the driver mRNA. The PCR reaction was performed with specific primer to enrich differentially expressed cDNAs.

Cloning and Transformation

The differentially expressed cDNAs obtained through selective PCR amplification of both stressed and control leaf tissue mRNA, after subtraction were directly cloned into plasmid pGEM T Easy Vector. Good quality recombinant plasmids were obtained from both the cDNAs of control and water stress tissues through PCR based subtractive hybridization. High number of recombinant colonies were obtained when the plasmids were transferred to *E. coli* (competent) cells, which were selected based on white (recombinant) and blue (non-recombinant) colonies (Figure 4.7 and 4.8) since the vector is lacZ genetically marked. Restriction digestion of the recombinant plasmids was carried out to confirm the cloning and the inserts. The plasmids having inserts were selected for sequencing and characterization (Figure 4.9).

Restriction Digestion of recombinant plasmid DNA

In order to confirm the presence of insert in the plasmids, the plasmid DNA was digested with the restriction enzymes as prescribed in the plasmid maps. Different sizes of inserts were observed, which confirms the presence and quality of plasmids with inserts. Higher molecular weight inserts were obtained, which yielded more or less complete length of genes. Fifty individual plasmids were selected and sequenced in this study.

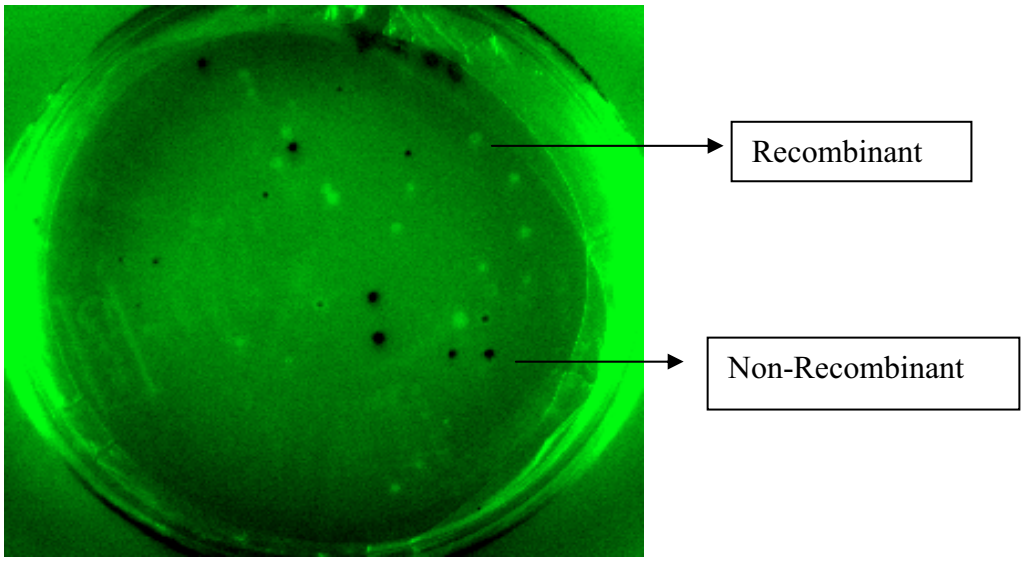

Figure 4.7 Recombinant colonies of E.coli bacteria containing PCR product of Control Tissue from Subtractive Hybridization

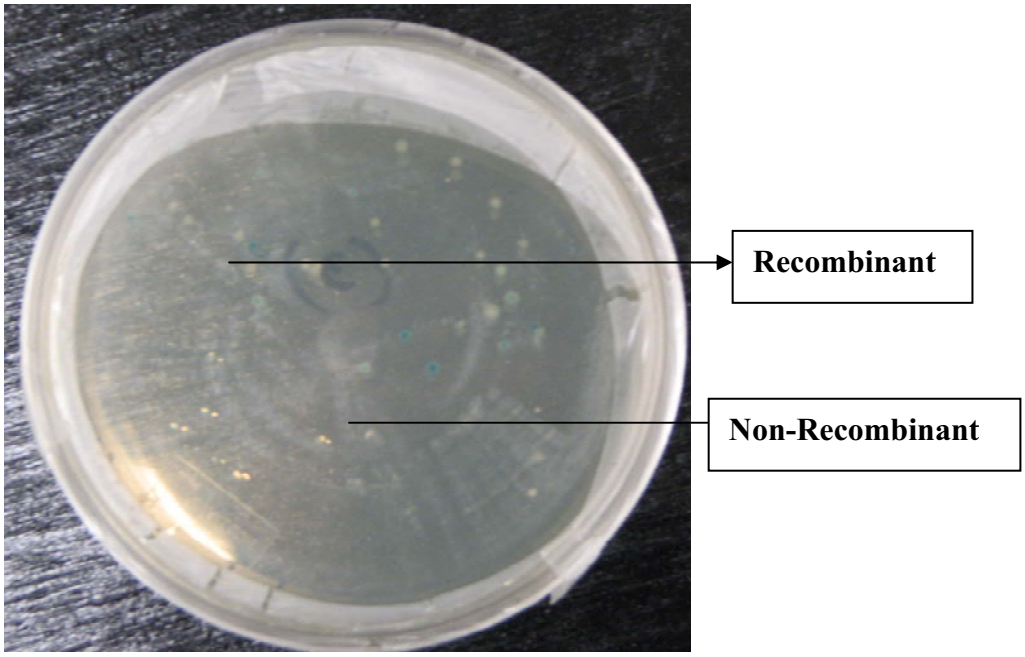

Figure 4.8 Recombinant colonies of *E.coli* bacteria containing PCR product of Water Stressed tissue from Subtractive Hybridization

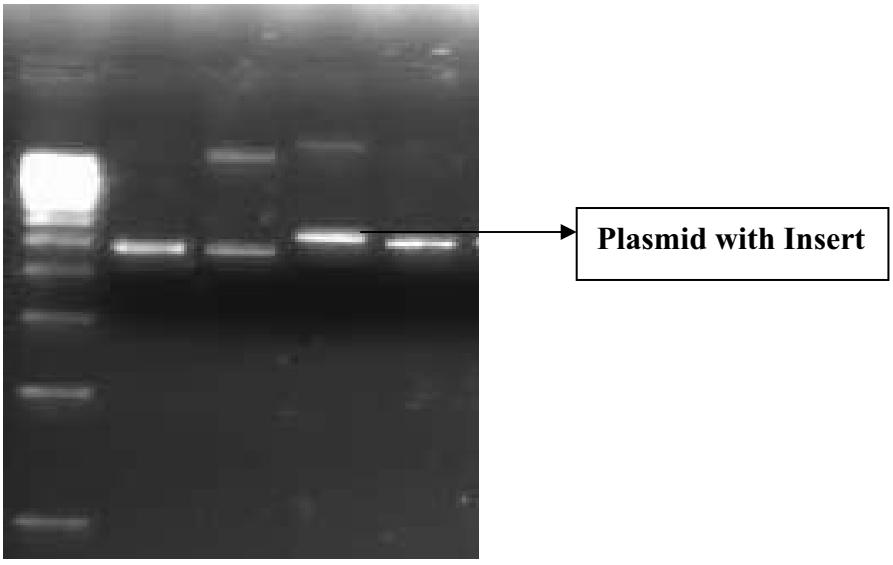

Figure 4.9 Isolation of plasmid showing insert Water stressed tissue from Subtractive Hybridization

Experiment 6: Characterization of the Isolated Transcripts through SH

Sequencing of 45 selected plasmids was carried out on CEQ Beckman Coulter sequencer. From the raw sequence data obtained the sequence of plasmid and the primer of M13 forward and backward were removed. The obtained sequence data was analyzed using BLASTX (Basic Local Alignment Tool - Nucleotide Translated Query vs. Protein Database) search for its homology with the sequence of a gene already recorded in the database of the NCBI (National Center for Biological Information). Out of the 45 characterized genes, 33 from water stress tissue, and 12 from control irrigated tissue obtained through subtractive hybridization. The sequences of different genes are presented in Table 4.6 and 4.7 with their E value (Expected Value) and protein homology. Most significant genes were discussed below.

Subtractive hybridization technique has been successfully used in various crops to isolate developmentally regulated genes and the genes differentially expressed to a certain response. Expression of some of the novel senescence-associated genes (SAGs) such as the responses to age, leaf detachment, ethylene and cytokinin were identified and isolated using this method. A subtractive hybridization approach was used to isolate vernalization-responsive genes from a late-flowering ecotype of *Arabidopsis thaliana*. *EARLI1* is the first *Arabidopsis* gene shown to be stably activated by vernalization. The abundance of its RNA is progressively elevated by vernalization and remains high for at least 20 days at room temperature. The basal level of *EARLI1* RNA is higher in early-flowering ecotypes, but is increased also after vernalization. This gene can be further used to investigate vernalization-specific transcriptional regulation (Willkosz and Schläppi 2000). Suppressive subtractive hybridization was used to create a library

enriched in cadmium-induced cDNAs from cadmium-tolerant *Datura innoxia* to investigate overall molecular responses of a metal tolerant plant. Two differential screening steps were used to screen the cadmium-induced library resulting in 8 putative cadmium-specific cDNAs out of a pool of 94 clones. Reverse transcriptase polymerase chain reaction was used to confirm that 4 of these 8 clones were cadmium-specific, while the other 4 were induced under heat shock or in the no treatment cells in addition to cadmium exposure. One of the 4 cadmium-specific cDNAs had homology to a sulfur transferase-family protein in *Arabidopsis thaliana* (Louie et al., 2003). Sequence analysis of six clones revealed that five clones were related to known proteins including non-specific lipid transfer proteins, early light-induced proteins, ACC oxidase or dehydrins, predicted to be involved in a wide range of physiological processes. The studies demonstrate the potentiality of subtractive hybridization in isolating genes specific to a tissue. Subtractive differential screening was used to isolate ripening-associated cDNAs from a Shiraz grape (*Vitis vinifera L.)* berry cDNA library. A rapid increase in the mRNA levels of a number of cDNAs not present in unripe fruit occurred in grape berries at the onset of ripening. The putative translation products of some of these clones had homologs in other species that are involved in cell wall structure. These included four proline-rich proteins, a small protein that is similar to the non-catalytic, N-terminal domain of some pectin methylesterases, glutamate-rich proteins and the clones encoded putative stress response proteins. These included two thaumatin-like proteins, a metallothionein, a transcription factor, a cytochrome P 450 enzyme, and proteins induced by water, sugar, and/or cold stress in other species. Many of the homologs of the grape cDNAs thought to be involved in cell wall structure or stress-related responses also

accumulate in a developmental manner in other plants. This may indicate that the grape mRNAs accumulate in response to stresses such as the storage of high concentrations of sugars and rapid cell expansion, or they may accumulate as part of the ripening developmental program (Davies and Robinson, 2000).

In this study, we used subtractive hybridization method to isolate differentially expressed genes in response to water stress. We used mRNA recovered from total isolated RNA from control and water stressed leaf tissues. Several of differentially expressed cDNAs were recovered from PCR based subtractive hybridization and were enriched by amplification with specific primers. About 30 genes from water stressed tissue and 20 genes from control leaf tissue were isolated using this technique. This technique enabled to isolate differentially expressed genes in water stressed leaf tissue that are not expressed in control tissue, which would help to address the stress tissue problem. Subtractive hybridization also increases the probability of obtaining differentially expressed rare transcripts.

Genes characterized from control tissue and their functions

Out of 20 sequences identified, the following six selected genes are discussed below as they play critical role in regulating water stress.

Table 4.6 Identification of genes from control tissue obtained through subtractive hybridization

Sequence Number	Base pairs	Protein	Score (Bits)	E score
1	456	Galactinol synthase	185	1e-46
2	271	CER5-like protein	182	7e-46
3	483	Ascorbate peroxidase	31	4.2
4	284	Arginine decarboxylase	97	6e-56
5	403	DHN	29	7.7
6	504	Alcohol dehydrogenase	85	4e-16
7	281	Proline Rip	195	7e-50
8	191	Glutathione transferase7	135	1e-31
9	305	ACC	29	8.9
10	601	Indole 3 acetic b-glycosyl transferase	35	6e-17

The surfaces of plants (principally leaves and stems) exposed to the air are covered in a wax cuticle that prevents them from drying out.

Plant cells have an ABC transporter protein called CER5 that transports hydrophobic wax precursor molecules across the plant cell plasma membrane (Schulz and Frommer, 2004). CER5 gene encodes an ABC transporter localized in the plasma membrane of epidermal cells and it is required for wax export to the cuticle (Pighin et al., 2004). Its synthesis requires extensive export of lipids from epidermal cells to the plant surface. *Arabidopsis cer5* mutants had reduced stem cuticular wax loads and accumulated sheet like inclusions in the cytoplasm of wax-secreting cells. Cuticle, including wax and cutin is the barrier covering plant aerial organs and protecting the

inner tissues. The *Arabidopsis thaliana* ATP-binding cassette (ABC) transporter CER5 has been identified as a wax exporter. It is found that, the expression was light dependent, and the phytohormone ABA up-regulated *AtWBC11* expression. The results support that *AtWBC11* is involved in cuticle development (Luo et al., 2007). In our study, we found this gene only in control leaf tissue and its suppressed to water stress indicating that the plant was not able to synthesize this gene in order to withstand the changes occur during the physiological changes to water stress.

Ascorbate Peroxidase is an antioxidant enzyme which was found significantly affected to water stress in various plants. In our results, this gene was suppressed. On contrary, this antioxidant enzyme, ascorbate peroxidase increased significantly under water stress. Water stress imposed at different stages after anthesis resulted in an increase in lipid peroxidation and a decrease in membrane stability and chlorophyll and carotenoid contents in wheat. It seems that drought tolerance represented by higher membrane stability and chlorophyll and carotenoid contents and lower lipid peroxidation, is related to its higher antioxidant enzyme activity (Sairam and Saxena, 2000). Oxidative injury and antioxidant responses were investigated in two banana genotypes subjected to 40 % PEG-induced water stress. PEG treatment resulted in oxidative injury, as expressed in increased lipid peroxidation and reduced membrane stability index however, greater oxidative injury was detected in banana. Ascorbate peroxidase activity was enhanced under water stress. Higher ascorbate peroxidase and superoxide dismutase activities were associated with greater protection against water stress-induced oxidative injury (Chai et al., 2005).

Arginine decarboxylase is one of the polyamines significantly was affected to drought. The gene encoding arginine decarboxylase is suppressed in stressed sample of FH grape in our study. The study on how polyamines are involved in drought in rice (*Oryza sativa* L.) showed that, the activities of arginine decarboxylase, *S*-adenosyl-L-methionine decarboxylase, and spermidine synthase in the leaves were significantly enhanced by water stress. The results suggest that rice has a large capacity to enhance polyamine biosynthesis in leaves in response to water stress. However, this grape did not show synthesis of this gene to water stress indicating that it has not adapted to water stress. The role of PAs in plant defense to water stress varies with PA forms and stress stages (Yang et al., 2007).

Proline-rich protein is a putative bimodular protein of 126 amino acids with a proline-rich domain and a hydrophobic cysteine-rich domain plus a signal peptide at the N terminal. The PrP gene expression was investigated and demonstrated that it accumulates in leaves and epicotyls of soybean seedlings. The PRP mRNA was also expressed in response to salicylic acid and virus infection. In addition, the PRP gene transcription was regulated by circadian rhythm, salt stress drought stress and plant hormones indicating that the PRP gene might play a role in plant responses to multiple internal and external factors (He et al., 2004). The absence of this gene in stress samples in grape cultivar in this study indicate that, plant is not able to produce certain genes involved in drought tolerance. cDNA clone encoding a proline-, threonine-, and glycine-rich protein was isolated from a wild tomato species (Yu et al., 1996). Northern-blot analysis and in situ hybridization studies revealed that PTGRP is down-regulated by drought stress as also obtained in our results. The level of the mRNA in leaves and stems

of 8-d drought-stressed plants decreased 5- to 10-fold compared with that in regularly watered plants. The mRNA re-accumulated when drought-stressed plants were re-watered. In regularly watered *L. chilense* plants, PTGRP protein was found to be localized in xylem pit membranes and disintegrated primary walls. PTGRP is the first drought-regulated protein that has been precisely localized in the cell wall (Harrak et al., 1999).

Dehydrin has been the model gene for entire transcriptome response for studying the expression pattern to drought. We obtained this gene expression only in control samples. The response of barley to gradual drought over 21 days and low temperature including chilling, freeze–thaw cycles, and deacclimatization over 33 days was investigated (Tommasini et al., 2008). The expression of 13 barley *Dhn* genes mirrored the global clustering of all transcripts, with specific combinations of *Dhn* genes providing an excellent indicator of each stress response.

ACC synthase exists as a multi-gene family whose individual members are differentially regulated, many by various stresses. When plants are subject to a variety of stresses they often exhibit symptoms of exposure to ethylene. Although this relationship usually results from induction of ACC synthase thus raising the concentration of the precursor of ethylene, it is now apparent that there are numerous other ways that stresses produce ethylene-like symptoms. In addition, ACC oxidase, AdoMet synthetase, enzymes in the methionine cycle, and enzymes that conjugate ACC are regulated by stress. In more unusual cases, ethylene production is not increased by stress or may be reduced as it happened in this study. There is evidence for stress effects on perception of ethylene and the potential exists that some steps of the ethylene signal transduction

pathway may be influenced by stress. It is becoming more apparent that a number of stress responses involve interactions with other hormones (Morgan and Drew, 1997). ACC concentrations in loblolly pine needles of both seed sources decreased as water potential began decreasing. Below -1.4 MPa, ACC levels started increasing or remained constant until -2.8 MPa at which time its level increased three-fold. Mean ACC levels in root tissue were slightly higher than the mean levels in the needle tissue roots apparently were more efficient in converting it to ethylene since ethylene production was two to three times higher than needle tissue. The modulation of ethylene synthesis by ACC synthase and ethylene forming enzyme appeared to be influenced by stress level, organ and seed source (Morgan and Drew, 1997).

Genes characterized from stress tissue and their functions

Out of 20 sequences identified, the following six selected genes are discussed below as they play critical role in regulating water stress. We obtained few genes that were also up regulated or newly synthesized in DDRT PCR method. The details are discussed below. The genes found in both methods are 1.SNF1-Related Protein Kinase (Up regulated), Isoprene synthase (found also in DDRT experiment), ABF3 (found also in DDRT experiment) 6) WRKY-type transcription factors (Up regulated).

Table 4.7 Identification of genes from stress tissue obtained through subtractive Hybridization

Sequence Number	Base pairs	Protein	Score (Bits)	E score
1	456	Isoprene synthase	185	1e-46
2	271	Stress enhanced protein	182	7e-46
3	483	Cell Division Protein	31	4.2
4	305	Cytochrome b	29	8.9
5	411	Rab21 gene for water-stress inducible protein	152	152
6	403	Glyoxalase I	29	7.7
7	504	ABF3	85	4e-16
8	281	ASR2 gene	195	7e-50
9	368	Xyloglucan endotransglucosylase	157	2e-36
10	191	AP 2	135	1e-31

The *Asr* gene family exclusively present in plant genomes, is involved in transcriptional regulation. Its members are up-regulated in roots and leaves of water- or salt-stressed plants. The *Asr2* gene (named after abscicic acid, stress, ripening) encodes a putative transcription factor. The gene is expressed in stressed leaf samples. It is reported that, this gene is up-regulated in leaves and roots of tomato plants exposed to water-deficit stress**.** This gene was first cloned and characterized in a cultivar of commercial tomato. There is clear evidence that, this gene had undergone dramatic accelerated rates of amino acid substitutions in tomato lineages living in dry habitats (Frankel, et al., 2003). Additional evidence of adaptive evolution has been reported for *Asr2* in *Solanum chilense* and *S. arcanum*, two species dwelling in habitats with different

precipitation regimes. The extent of nucleotide diversity in *Asr2* differed between species in more than one order of magnitude. In both species we detected evidence of non-neutral evolution, which may be ascribed to different selective regimes, potentially associated to unique climatic features, or, alternatively, to demographic events (Giombin et al., 2008).

The presence of AP2 transcription factor in this study indicate that the water stess is mediated by specific changes in gene expression of phytohormone abscisic acid (ABA) modulates the expression of many genes important to plant growth and development and to stress adaptation (Kizis et al., 2001). In this study, we found that an APETALA2/EREBP-type transcription factor, AtERF7, plays an important role in water stress. AtERF7 interacts with the protein kinase PKS3, which has been shown to be a global regulator of ABA responses. AtERF7 binds to the GCC box and acts as a repressor of gene transcription. *Arabidopsis* overexpressing *AtERF7* show reduced sensitivity of guard cells to ABA and increased transpirational water loss. By contrast, *AtERF7* and *AtSin3* RNA interference lines show increased sensitivity to ABA during germination. Together, our results suggest that AtERF7 plays an important role in ABA responses and may be part of a transcriptional repressor complex and be regulated by PKS3 (Song et al., 2005).

Glyoxalase I (*S*-lactoylglutathione-lyase) is a 56 kDa, heterodimeric protein. It is reported that, a strong biochemical evidence for modulation of glyoxalase I activity by calcium/calmodulin (Ca^{2+}/CaM) is existing. In the presence of Ca^{2+} glyoxalase I showed a significant (2.6-fold) increase in its activity. It also showed a Ca^{2+} dependent mobility shift on denaturing gels. Glyoxalase I was activated by over 7-fold in the presence of

Ca^{2+} (25 μM) and CaM (145 nM) and this stimulation was blocked by the CaM antibodies and a CaM inhibitor, trifluroperazine (150 μM). The stimulation of glyoxalase I activity by CaM was maximum in the presence of Mg^{2+} and Ca^{2+}; however, magnesium alone also showed glyoxalase I activation by CaM (Deswal and Sopory, 1999).

Cytochrome b is a mitochondrial gene induced in response to stress. Although plant cell bioenergetics is strongly affected by abiotic stresses, mitochondrial metabolism under stress is still largely unknown. Interestingly, plant mitochondria may control reactive oxygen species (ROS) generation by means of energy-dissipating systems. Therefore, mitochondria may play a central role in cell adaptation to abiotic stresses, which are known to induce oxidative stress at cellular level. Studies on mitochondria from durum wheat, a species well adapted to drought shown that ATP sensitive plant mitochondria potassium channel these systems are able to dampen mitochondrial ROS production. This was found to occur in mitochondria from both control and hyperosmotic-stressed seedlings. Therefore, the hypothesis of a 'feed-back' mechanism operating under hyperosmotic/oxidative stress conditions was validated. Stress conditions induce an increase in mitochondrial ROS production; ROS activate $PmitoK_{ATP}$ and PUCP that, in turn, dissipate the mitochondrial membrane potential, thus inhibiting further large-scale ROS production. Another important aspect is the chloroplast/cytosol/mitochondrion co-operation in green tissues under stress conditions aimed at modulating cell redox homeostasis. Durum wheat mitochondria may act against chloroplast/cytosol over-reduction: the malate/oxaloacetate antiporter and the rotenone-insensitive external NAD(P)H dehydrogenases allow cytosolic NAD(P)H oxidation;

under stress this may occur without high ROS production due to co-operation with AOX, which is activated by intermediates of the photorespiratory cycle (Pastore et al., 2007).

Section B. PROTEOMICS

A comprehensive proteomic study to understand the biochemical basis of water stress was carried out using high throughput 2-dimensional gel electrophoresis (2-D PAGE).

Experiment 7: Qualitative and quantitative analysis of leaf proteins due to water stress in Florida Hybrid Bunch 'Suwannee'

Protein extraction was carried out following the method described by Basha (1979). The yield of protein content was higher in control tissue when compared to the total protein in water stressed samples. Equal amounts of protein were loaded on 2-dimensional electrophoresis to monitor the changes in protein profile in control and treated samples. A significant difference in the content of protein between control and water stressed leaf tissue was observed. The total protein content was reduced in stressed tissue when compared to control tissue. This is in agreement with the results obtained for RNA contents. We found RNA in higher contents in control than in stressed samples. The average protein content in control tissue was high (3.26mg/g) when compared to water stressed leaf tissue sample (2.75 mg/g) (Table 4.8). This might be due to reduction or degradation of several proteins due to water stress.

Table 4.8 Total Protein content in Control and stress samples of Leaf tissue from Florida Hybrid Bunch 'Suwannee'

Days Interval	Total Protein Content (mg / g. f. wt)	
	Control	Water Stress
5 Day	3.43	3.30
10 Day	3.24	3.0
15 Day	3.50	2.3
20 Day	2.90	2.5

Both qualitative and quantitative differences in proteins were observed in response to water stress. Total proteins were suppressed upon progressive water stress from 5 to 20 day treatment. This was clearly evident from the 2-D PAGE (Figure 4.10). This study indicates that water stress affected the expression of many genes and there by protein synthesis.

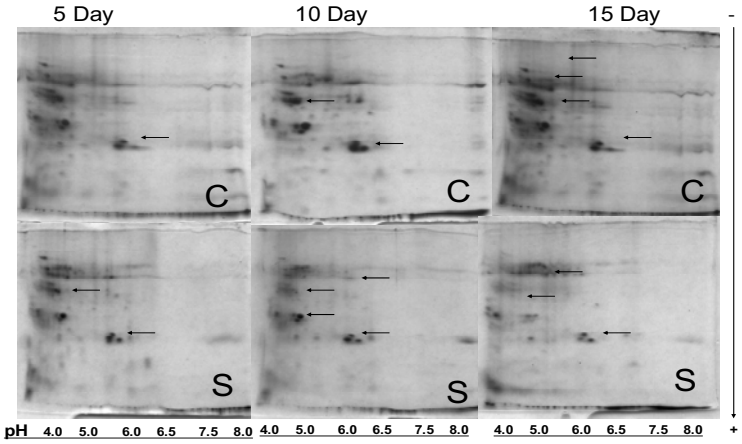

Figure 4.10 Differentially Expressed Leaf Proteins in Response to Water Stress in Florida Hybrid Bunch 'Suwannee'

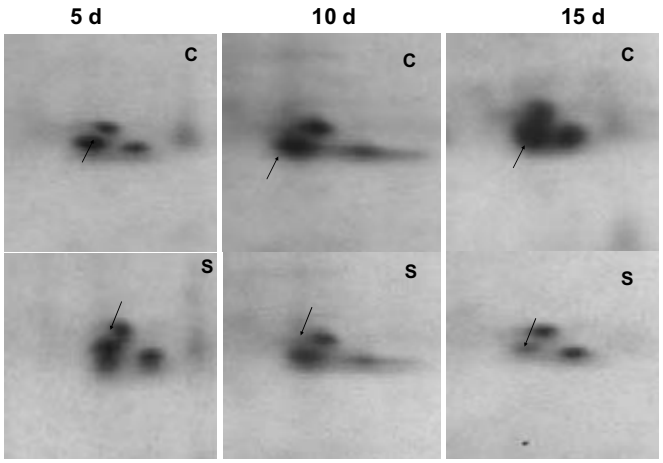

Figure 4.11 Differential expression of protein UV-B repressible Rubisco activase to progressive water stress

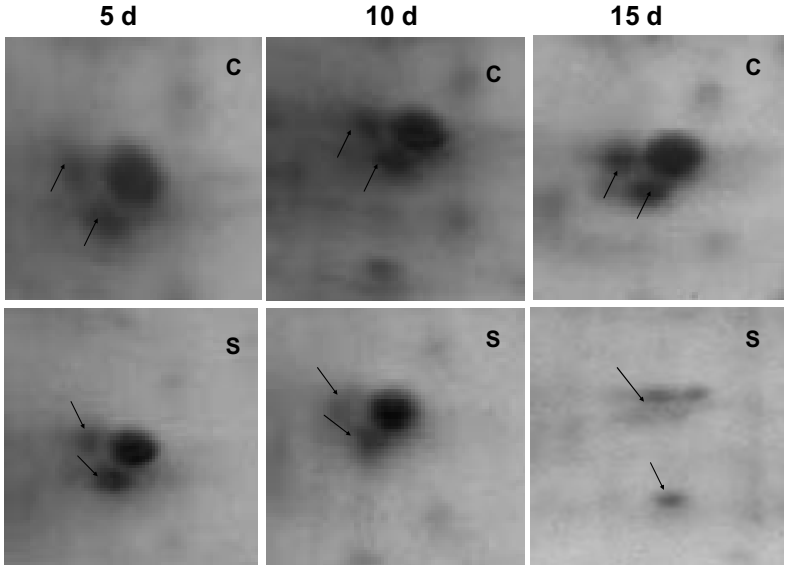

Figure 4.12 Differential expression of protein glyceraldehyde 3-phosphate dehydrogenase and phospho ribulokinase to progressive water stress

Experiment 8: Identification of Differentially Expressed Leaf Proteins to Water Stress in Florida Hybrid Bunch 'Suwannee'

To analyze differentially expressed proteins the gel fragments corresponding to each spot were excised, digested with trypsin and analyzed in LC/MS. Most of the digests yield good quality MS data as shown based on computation of protein probability (Figure 4.13). All protein spots matched protein databases at 95% identification rate. All protein identification reported in Table 4.9 had Mascot scores greater than or equal to two times the accepted significance threshold (95%). Mascot software incorporates a probability based implementation of Mowse algorithm. This helps in finding a protein to the characteristics of the sample, the mass spectrum parameters and the size of the FASTA database searched (Figure 4.14).

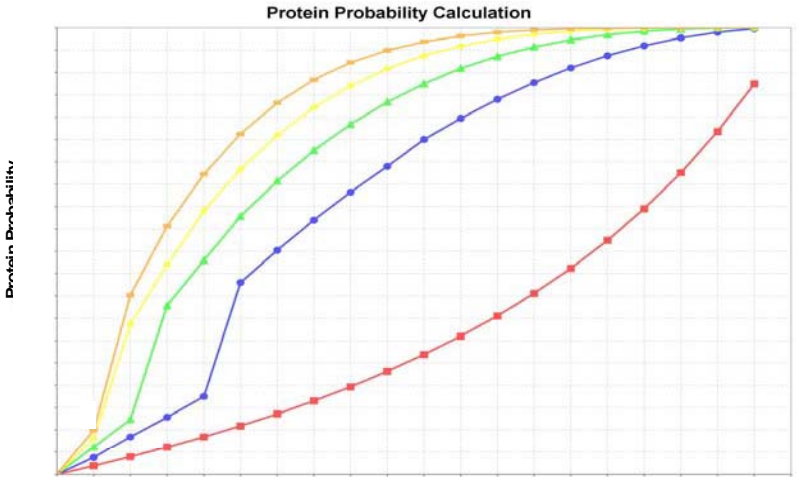

Figure 4.13 Protein Probability Chart obtained in LC/MS Analysis for Rubisco. The curves indicate the highest probability of each peptide sequence

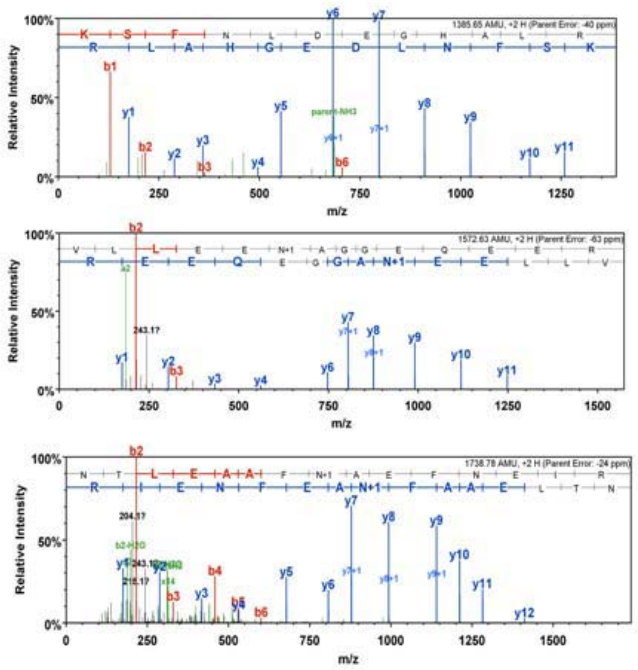

Figure 4.14 Mass Spectrum Analysis of a Peptide of trypsin digested Rubisco protein sample. Peaks of each amino acid of a peptide sequence indicate the probability of the peptide in the sample

Table 4.9 Partial Protein Sequences Differentially Expressed in Leaf tissue of Florida Hybrid Bunch 'Suwannee' obtained by LC/MS Analysis

	Protein Name	Mascot Score	a PM/%	Identified Proteins	[b] Accession Number	Organism matched	SwissProt Acc. No./ c MW kDa
1	LLEYGNMLVQEQENVKR YLSEAALGDANEDAIKR	64.7 49	3/68	Ultraviolet-B-repressible rubisco activase	gi|18476502	*Pisum sativum*	NA/6
2	VAINGFGR AVALVLPTLK	50.7	2/4	Glyceraldehyde-3-phosphate dehydro-genase A subunit	gi|77540210	*Glycine max*	P850113A/43
3	GFYIAPAFMDK MGINPIMMSAGELESGNAGEPAK IGVCTGIFR	60 54.6 64.2	2/18	Rubisco activase precursor	gi|3687676	*Datisca glomerata*	NA
4	LVGNLSWR TPDGGFFTR TDNTCGPEPPLVER	55.3 58.1 51.5	3/6	Violaxanthin de-epoxidase precursor	gi| 1463123	*Nicotiana tabacum*	NA/54.5
5	GYMFTTTAER GEYDESGPSIVHR	67.4 71.6		Actin	gi|32186896	*Gossypium hirsutum*	NA/41.7
6	SFQCELVFAK MGINPIMMSAGELESGNAGEPAK MCCLFINDLDAGAGR EENPRVPIIVTGNDFSTLYAPLIR LVDTFPGQSIDFFGALR LLEYGNMLVMEQENVKR	53.9 85.7 66.7 46.9 46.5 57.8	7/26	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor	gi|266893	*Cucumis sativus*	NA/45.7
7	LTSVFGGAAEPPR GGNPDSNTLISDTTTVICLDDYHSLDR GVTALDPR ANDFDLMYEQVK KPDFDAYIDPQK LDELIYVESHLSNLSTK	74.4 47.1 55.0 55.0 50.0 58.9	6/22	Phosphopentokinase	gi|125578	*Mesembryanthemum crystallinum*	P850112/44.1
8	GLGAGGNPDIGMNAAK AVQAQEGIAALR DAALNAIQSPLLDIGIER	51.3 78.0 81.5	3/10	Cell division protein ftz	gi|115454331	*Oryza sativa* (japonica)	N/A/47.2

a Number of peptides identified via mascot search engine and confirmed by *de novo* sequencing matched/sequence percentage coverage
b Accession Number Using Mascot result in NCBI/Swiss Prot
c Swiss Prot Submission number/Theoretical molecular mass PI

The results obtained form 2-D PAGE and LC/MS analysis revealed that several proteins were suppressed during water stress regime. No protein was found up regulated to water stress treatment. Initial water stress period at 5th day showed little effect on protein suppression. Progressive water stress through 15th day period suppressed most of the major protein spots as shown in Figure 4.10. Beyond 15th day stress, the proteins seem to degrade and no significant protein spots were identified. Major suppressed proteins were sequences and identities were recorded as in Table 4.9. A total of 7 significant proteins that were suppressed to water stress are discussed below. The protein

spots were eluted and identified the homology of the peptide sequences LC/MS (Figures 4.11 and 4.12).

The proteins suppressed to water stress were identified as rubisco, glyceraldehyde 3 phosphate dehydrogenase, ultraviolet B repressive rubisco activase, phosphoribulokinase. Ribulose 1-5 bisphosphate-carboxylase is the largest functional category of proteins involved in carbon assimilation and protein synthesis. The largest functional category of proteins involved in photosynthesis is Rubisco. Rubisco activase, a molecular chaperone, is reported to catalyze Rubisco from an inactive closed conformation to an active open conformation (Salvucci et al., 2006). Our earlier studies in grape indicate that the expression of Rubisco was seen only in the tolerant grape cultivars even upon pathogen infestation, while this protein is down-regulated in infected leaf tissue (Vasathaiah et al., 2008). The expression of Rubisco in tolerant cultivar indicates the ability of plant to overcome water stress and perform normal photosynthesis. Rubisco is the key enzyme which catalyses carbon dioxide fixation and photorespiratory oxidation (Spreitezer and Salvucci, 2002). Glyceraldehyde 3 phosphate dehydrogenase is also an important enzyme of the glycolysis pathway. It catalyzes the synthesis of 1, 3-bisphosphoglycerate, a high energy intermediate used for the synthesis of ATP (Voet and Voet 2004). Ultraviolet B repressive rubisco activase is the enzyme which suppresses the UV B /stress mediated affects on transcription or translation (Casati et al., 2006). Phosphoribulokinase catalyzes ATP dependent phosphorylatio of Ru5P in calvin cycle carbon dioxide fixation and photorespiratory oxidation (Brandes et al., 1996).

CHAPTER V

SUMMARY AND CONCLUSIONS

Grape is one of the largest and most important agricultural commodities in the US agriculture sector, produced for raisins and wine. Both phenolics and flavonoids in wines contribute to the health benefits including reducing the risk of cardiovascular disease, cancer and degenerative diseases associated with aging. Earlier studies showed significant differential expression in genes in response to water deficit conditions among each of the three berry tissues of skin, pulp and seed. Genes with functions in the flavonoid pathway were highly expressed in the skin and the seed, with genes specifically involved in aroma, anthocyanin pathway being over expressed specifically in the skin.

Florida Hybrid Bunch grapes are leading commercial variety in southeast US region for the production of wine. Water stress in this region is greatly affecting the productivity and wine characteristics of these grape cultivars. Very little research is done to study the changes in biochemical and molecular components due to water stress and the correlation with altered wine characteristics and nutraceutical properties in Florida hybrid bunch grapes. These are mainly grown for wine and table grape. These grape are developed from cross between *Vitis* with local varieties and they are PD tolerant and grown in Southeast US region. However, these are susceptible to various biotic and

abiotic stresses such as diseases anthracnose disease, fruit rot, and environmental factors such as water deficit stress.

In the present study we were able to isolate 37 specific genes from both water stressed and control tissues. Since growth and development is affected under environmental stress, regulation of genes involved in RNA and protein metabolism was studied. Identification of potential genes and stress-regulated promoters and proteins will help in defining strategies for developing transgenic plants which could fight the negative impact of environmental stresses on development and yield. The differentially expressed genes determine the tolerance or susceptibility of the cultivar by synthesizing new genes in response to water stress condition. Most were found to be expressed during stress. Most Differential Display RT PCR and Subtractive hybridization showed that, the oxidative stress which caused photosynthetic genes and enzymes suppressed in the tissues due to water stress. High temperature, low transpiration and respiration rates which lead to the stress and affected the activity of many genes and most of the genes expressed were due to oxidative stress, which is evident form the isolation of genes through subtractive hybridization. This condition affects the plant metabolism, translocation of solutes, and eventually berry composition. The study shows the potentiality of DDRT PCR and subtractive hybridization and proteomic approach in identifying and isolating differentially expressed genes and proteins from the water stressed leaf tissue in grape. The data helps in assessing the genes those cause or initiate the stress in grape and help in understanding the physiological processes and metabolic pathways of this stress, there by the problem of stress can be addressed with an appropriate control measures and also helps in developing genetically engineered grape

to enhance the gene synthesis that will improve the tolerance to water stress and thereby improve nutritionally superior Florida Hybrid Bunch Grape genotypes. This research focused on gene expression at the level of transcripts and proteins, and careful profiling of metabolite changes contributing to wine quality. This project will greatly facilitate future gene discovery efforts in grapevines and lead to improvements in both production efficiency and wine quality under adverse growing conditions.

Further studies have to be carried out in order to understand the initiation/cause of water stress, they are metabolites such as amino acids, sugars, proteins and phenolics which are known to affect wine quality will be identified and characterized. Over-expression of several low molecular weight proteins in the leaf during prolonged water stress were observed in hybrid bunch grape. cDNA chip specific for water stress induced genes which will enable identification and study of expression of stress related genes across the genotypes will be prepared. Will isolate genes expressed under water stress, and correlate the expression of these genes with berry development and composition. Identification of metabolic pathways affecting juice and wine quality will be necessary to enhance the enological characteristics of the wine. Such studies provide new insights into the complex interactions between water deficit condition and disease tolerance and changes in nutraceutical properties, and wine characteristics in berry.

Recent advances in genomics have led to improved strategies for engineering stress tolerance in plants. Drought alters gene expression in plants with considerable overlap among these stresses. This research program on Florida Hybrid grape will lead to a better understanding of the genetic mechanisms for drought tolerance in *V. vinifera*. Ultimately this research will improve wine grape production efficiency in drier regions of

the world and a better understanding of the factors that contribute to improved wine quality under abiotic stress conditions.

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APPENDIX A

PARTIAL SEQUENCES OF DIFFERENTIALLY EXPRESSED GENES CHARACTERIZED USING DIFFERENTIAL DISPLAY RT PCR

1. Sucrose synthase1

GCGTCAGGGAGCGCATTGGCGACTCACTCTCTGCCCACCCCAATGAGCTTGTCGCCGTCTTCACCAGGCT
GAAAAACCTTGGAAAGGGTATGCTGCAGCCCCACCAGATCATTGCCGAGTACAACAATGCGATCCCTGAG
GCTGAGCGCGAGAAGCTCAAGGATGGTGCTTTTGAGGATGTCCTGAGGGCAGCTCAGGAGGCGATTGTCA
TCCCCCCATGGGTTGCACTTGCCATCCGCCCTAGGCCTGGTGTCTGGGAGTATGTGAGGGTCAACGTCAG
CCCAACAACAACTTTGTTCTTGAGCTGGACTTTGAGCCATTCAATGCCTCCTTCCCCCGTCCTTCTCTGT
GAGCATGTACCCCTTGCTCAACTTCCTTCGCGCCCACAACTACAAGGGGATGACCATGATGTTGAACGAC
AGAATCCGCAGTCTCAGTGCTCTGCAAGGTGCGCTGAGGAAGGCTGAGGAGCACCTGTCCACCCTACAAG
CGCTAAGCGTGCACAGGAGACTATCCACCTCCTCTTGGACCTCCTGGAGGCCCCAGATCCGTCCACCCTG
AAGCTAATGTCTTGGGTTACCCTGACACCGGAGGCCAGGTTGTCTACATCTTGGATCAAGTGCGCGCTAT
GGAGAACGAAATGCTGCTGAGGATCAAGCAGTGTGGTCTTGACATCACGCCGAAGATCCTTATTGTCACC
GCCATATCCTTCGCGTGCCATTCAGAACAGAAAACGGAATCGTTCGCAAGTGGATCTCGCGATTTGAAGT
CTGGCCGTACCTGGAGACTTACACTGATGACGTGGCGCATGAGATTGCTGGAGAGCTTCAGGCCAATCCT
GACCTGATCATCGGAAACTACAGTGACGGAAACCTTGTTGCGTGTTTGCTCGCCCACAAGATGGGTGTTA
CTCACTGTACCATTGCCCATGCGCTTGAGAAAACTAAGTACCCTAACTCCGACCTCTACTGGAAGAAGTT

2. Putative cyclic nucleotide-dependent protein kinase

GTGAAACAGGGTGGTGAAGGGGACTGCTTCTACGTTGTGGGTAGTGGAGAATTCGAGGTCTTAGCAACTC
AGGATGGAAAGAACGGTGAGGTACCTAGGATCTTGCAGCGTTATACAGCTGAGAAACAATCATCATTCGG
TGAACTCGCCTTAATGCATAACAAGCCGCTTCAGGCTTCTGTACGTGCTGTAGATCACGGAACATTGTGG
GCCTTAAAAAGAGAAGATTTTCGAGGAATTTTGATGTCTGAGTTTTCAAACTTAGCATCCTTGAAGCTTC
TTCGTTCTGTCGATCTTCTTTCCCGTCTTACAATTTTGCAACTAAGCCATGTTGCAGAGTCTCTTTCCGA
AGCTTGCTTTTCTGATGGACAAACAATTGTTACCAAGGACCAAAAACTTCAGGGCCTGTATGTTATCCAG
AAGGGACGCGTAAAAATATCTTTCTGTACAGAGGTGTTGGAGAGTCAAAATGTTTCAAGCCTTACAACTG
GAATCACTAACGAGTATGACAATCTTGAAATCGGAACAGAAGTCTCCATAGAAAAGCATGAAGGAAGTTA
TAAAGCCACTCTTGCAGACCTGGAATGGACAACATGCTTGAGTACAACAGACTGTAGTGAGATTGGGCTC
GTGCATTTGAAAGATAAAGAAAATTTGCTCAGCTTGAAAAGATTTTCAAAGCAAAAGGTGAAAAAGTTAG
GTAAAGAGGCACAAGTATTGAAAGAGCGGAATCTGATGAAGAACGTAATAAAGCCCTCAGCTATTGTTCC
CGAAATCTTGTGTACTTGTGTCGATCAAACATTTGCGGCAATCTTACTGAACACTACTCTTGCCTGTCCT
ATCTCTTCTCTGCTTCACTCTCCGCTTGACGAGTCATCTGTCCGTTTCATTACCGGCTCACTTGTGTCTG
CCATAGAAGACATACACAAGAACGAGATTCTCTTCAGAGGTTCATCCCCCGAGTTACTGATGTTGGATCA
ATCCGGATATCTACAGATTGTAGACTTCAGATTCGCCAAGAAATTGTCCGGGGAACGAACATTTACAATC

3. Actin

CCACGAAACCACATACAACTCCATCATGCAGTGTGATGTTGATATCAGGAAAGACTTGTATGGTAACATTG
TCCTCAGTGGTGGTTCAACTATGTTCCCAGGAATTGCTGACAGAATGGGCAAGGAAATCTCTGCACTGGCC
CCAAGCAGCATGTG

4. Water stress induced protein

ATTAGTGGTTGCTGTGTTACCTGACGTGCCGGCCGATCACCGGAGACAGCTATTGGACCAAGGCTGCGTC
ATCAAGGAGATTCAGCCGGTTTACCCACCGGATAACCAAACTCAGTTTGCTATGGCTTACTACGTCCTCA
ACTACTCTAAACTTCGTATTTGGAAGTTTGTAGAGTACAGCAAGCTGATATACTTAGACGGAGACATACA
AGTGTTTGAGAACATAGATCACTTGTTTGATCTTCCTGACGGCAACTTCTACGCCGTTAAAGACTGTTTC
TGCGAGAAGACTTGGAGCCACACGCCTCAGTACAAGATTGGCTACTGCCAACAGTGTCCGGACAAGGTGA
GATTTCTTGAACATGTACTTCAAAGACATATACAAGCCTATTCCACCAGTTTACAATCTTGTCTTGGCCA

TGCTCTGGAGGCATCCAGAGAACATAGAGCTTAACGAAGCTAAGGTTGTTCATTACTGTGCAGCCGGTGC
TAAGCCTTGGAGGTTCACAGGCCAAGAAGGAAATATGGAGAGGGAAGACATCAAGATGCTTGTAGAGAAA
TGGTGGGACATTTACAACGACGAGTCTCTTGACTACAAAAACTTTAATGTGCATTGCGGACAAAAAGAAG
ATGTTCACAGGAAACCGAAAACCCTTCCACAGTTCTTTACAGATTTGTCTGAAGCTGATGTGCTTCAATG

5. Cytochrome b gene mitochondrial

GTAGAACACGTTATGAGAGATGTTGAAGGGGGCTGGTTGCTCCGTTATATGCATGCTAATGGGGCAAGTA
TGTTTCTCATTGTGGTTCACCTTCATATTTTTCGTGGTCTATATCATGCGAGTTATAGCAGTCCTAGGGA
ATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACAGCTTTTACAGGATACGTACCA
CCTTGGGGTCAGATGAGCTTTTGGGGAGCTACAGTAATTACAAGCTTAGCTAGCGCCATACCTGTAGTAG
GAGATACCATAGTGACTTGGCTTTGGGGTGGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAG
TCTTCATCATTTACTCCCCCTTATTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATAT
GGATCAAATAATCCATTGGGTGTCCATTCAGAGATGGATAAAATTTCTTTTTACCCTTATTTTTATGTAA
AGGATCTAGTAGGTCGGGTAGCTTCTGCTATCTTTTCTTCCATTTGGATTTTTTATGCTCCTAATGTTTT
GGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCTCATATTGTGCCGGAATGGTAT
TTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGACAAATCGGGAGGTGTAGCCGCAATAGCACCAG
TTTTTATATGTCTGTTGGCTTTACCTTTTTTTAAAAGTATGTATGTGCGTAGTTCAAGTTTTCGACCGAT
TCACCAAGGAATATTTTGGTTGCTTTTGGCGGATCGCTTACTACTAGGTTGGATCGGATGTCAACCTGTG

6. 26S Ribosomal RNA gene

TGGATTAACGAGATTCCCACTGTCCCTGTCTACTATCCAGCGAAACCACAGCCAAGGGAACGGGCTTGGC
GGAATCAGCGGGGAAAGAAGACCCTGTTGAGCTTGACTCTAGTCCGACTTTGTGAAATGACTTGAGAGGT
GTAGGATAAGTGGGAGCCGGAAACGGCGAAAGTGAAATACCACTACTTTTAACGTTATTTTACTTATTCC
GTGAATCGGAGGCGGGGATCATCCCCTCCTTTTGGACCCAAGGCGCGCCCAGCGCGCCGATCCGGGCGGA
CGAATACGAACCGTGAAAGCGTGGCCTATCGATCCTTTAGACCTTCGAAATTTGAAGCTAGAGGTGTCAG
AAAAGTTACCACAGGGATAACTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGATCCTTC
GATGTCGGCTCTTCCTATCATTGTGAAGCAGAATTCACCAAGTGTTGGATTGTTCACCCACCAATAGGGA
ACGTGAGCTGGGTTTAGACCGTCGTGAGACAGGTTAGTTTTACCCTACTGATGACAGTGTCGCAATAGTA

7. Putative aquaporin

CTTTTACAGGGCCATCATTTCCGAGTTCGTGGCCACCCTTTTGTTCCTCTACGTTACTGTGTTGACGGTG
ATTGGGTACAAGAACCAGACTGATCCCTACCATCACGGAAATGAATGCAACGGGGTTGGTATTCTTGGCA
TTGCTTGGGCCTTTGGTGGCGTGATCTTCATCCTTGTTTACTGCACCGCCGGCATCTCTGGAGGGCATAT
TAATCCTGCAGTGACGTTCGGGGTATTCCTGGCTAGAAAGGTGTCGCTGGTCCGAGCCGTGATGTACATG
GTGGCCCAGTGCGGGGGAGCCATCTGCGGCTGTGGCCTGGTCAAGCTATTCCAGGATATTTACTACATCA
GGTACGGTGGTGGCGCCAATGAGCTCGCCTCTGGATACAGTATAGGAACGGGGTTGGCTGCTGAGATTAT
TGGCACCTTCGTTCTTGTCTACACCGTCTTCTCTGCTACTGATCCCAAAAGAAATGCAAGAGATTCCCAT
GTACCTGTTTTGGCACCACTCCCCATTGGATTTGCTGTGTTCTTGGTTCACTTAGCCACGATCCCCATCA
CCGGTACCGGAATCAACCCGGCTCGAAGTCTCGGAGCTGCTGTCATCTACAACCAACCCAAAGCCTGGAG
TGACCATTGGGTATTTTGGGCTGGACCCTTCATTGGTGCAGCCATTGCAGCATTCTATCACCAGTTCATA
TTGAGAGCTGGAGCTGTTAAAGCTCTAGGGTCTTTCAAGAGCAGTTCCCACATGTAATGGGGTAATGCAT
TTCTTTCCTTTCCTTTCCCTTTTTTGCCTCGTTTTGTAGTAGTGATGTTGTTGAGATTAATTTATCAACA
ACTCTGCTCTAATAAGCTCCTTTATCAGTTCATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

8. Abscisic acid responsive elements-Binding factor 3

GTACGAAAGCTTGAGTAATGGGGTCTAGATTAAACTTCAAGAGCTTTGTTGATGGTGTGAGTGAGCAGCA
GCCAACGGTGGGGACTAGTCTTCCATTGACTAGGCAGAACTCTGTGTTCTCGTTAACCTTTGATGAGTTT

CAGAACTCATGGGGTGGTGGAATTGGGAAAGATTTTGGGTCTATGAACATGGATGAGCTCTTGAAGAACA
TTTGGACTGCAGAGGAAAGTCATTCAATGATGGGAAACAATACCAGTTACACCAACATCAGCAATGGTAA
TAGTGGAAACACTGTTATTAACGGCGGTGGTAACAACATTGGTGGGTTAGCTGTTGGTGTGGGAGGAGAA
AGTGGTGGTTTTTTCACTGGTGGGAGTTTGCAGAGACAAGGTTCACTTACCTTGCCTCGGACGATTAGTC
GCTGGTGTGGTTAGGGAAGAACCTCAACCGGTGGAGAGTGTAACTAACTTCAATGGCGGATTCTATGGAT
TTGGCAGTAATGGAGGTCTTGGGACAGCTAGTAATGGGTTTGTTGCAAACCAACCTCAAGATTTGTCAGG
AAATGGAGTAGCGGTGAGACAGGATCTGCTGACTGCTCAAACTCAGCCACTACAGATGCAGCAGCCACAG
ATGGTGCAGCAGCCACAGATGGTGCAGCAGCCGCAACAACTGATACAGACGCAGGAGAGGCCTTTTCCCA
AACAGACCACTATAGCATTTTCCAACACTGTTGATGTGGTTAACCGTTCTCAACCTGCAACACAGTGCCA
GGAAGTGAAGCCTTCAATACTTGGAATTCATAACCATCCTATGAACAACAATCTACTGCAAGCTGTCGAT
TTTAAAACAGGAGTAACGGTTGCAGCAGTATCTCCTGGAAGCCAGATGTCACCTGATCTGACTCCAAAGA
GCGCCCTGGATGCATCTTTGTCCCCTGTTCCTTACATGTTTGGGCGAGTGAGAAAAACAGGTGCAGTTCT
GGAGAAAGTGATTGAGAGAAGGCAAAAAAGGATGATAAAGAATAGGGAATCAGCTGCAAGATCCCGCGCT

9. Isoprene synthase

GATGCTGTTACAAAAACTAGCCTTCATGCTACTGCTCTTAGCTTCAGGCTTCTCAGACAGCATGGCTTTG
AGGTCTCTCAAGAAGCGTTCAGCGGATTCAAGGATCAAAATGGCAATTTCTTGAAAAACCTTAAGGAGGA
CATCAAGGCAATACTAAGCCTATATGAAGCTTCATTTCTTGCCTTAGAAGGAGAAAATATCTTGGATGAG
GCCAAGGTGTTTGCAATATCACATCTAAAAGAGCTCAGCGAAGAAAAGATTGGAAAAGACCTGGCCGAAC
AGGTGAATCATGCATTGGAGCTTCCATTGCATCGAAGGACGCAAAGACTAGAAGCTGTTTGGAGCATTGA
AGCATACCGTAAAAAGGAAGATGCAGATCAAGTACTGCTAGAACTTGCTATATTGGACTACAACATGATT
CAATCAGTATACCAAAGAGATCTTCGCGAGACATCAAGGTGGTGGAGGCGTGTGGGTCTTGCAACAAAGT
TCTGAAGGACAAGGGGGAAAACATTCTTCCATACCTAACAAAAGCGTGGGCAGATTTATGCAATGCATTC
CTACAAGAAGCAAAATGGTTGTACAATAAGTCCACACCAACATTTGATGAATATTTCGGAAATGCATGGA
AATCATCCTCAGGGCCTCTTCAACTAGTTTTTGCCTACTTTGCCGTTGTTCAAAACATCAAGAAAGAGGA
AATTGATAACTTACAAAAGTATCATGATATCATCAGTAGGCCTTCCCACATCTTTCGTCTTTGCAACGAC

10. AP2 transcriptional activator (DRF1)

GTAATCTTCCCTGTAATGAATATGCACTCTTGGCGCGGCAAAACCCCAAGGGAGATGCGCTGCCTGTGGC
ATCTATTCTGCGGAAAAAGCGACCTCGGAGATCACGTGATGGGCCTAATTCAGTCTCTGAAACGATCAGG
CGATGGAAAGAAGTGAACCAACAACTGGAGCATGATCCACAGGGTGCAAAGAGGGCGAGGAAGCCACCTG
CAAAGGGTTCAAAGAAGGGCTGTATGCAGGGGAAAGGAGGACCTGAGAATACACAATGTGGATTCCGTGG
TGTAAGGCAACGTACTTGGGGGAAGTGGGTTGCTGAAATTCGGGAGCCAAATCGGGTGAGCAGGCTCTGG
AGGTGTTGTACGTGGTGCTTCAGCATCATGCGAGTCTACTACAACATCCACCAACCACTCAGATGTTGCT
TCTAACTTGCCGCGACAAGCTCAAGCTCTTGAGATTTACTCCCAGCCAGATGTGCTTGAGTCCACCGAAT
CAGTTGTGCTGACTTCTGTTGAGCATTACAGCCATAAAGACAGTGTTCCTGACGCTGGCTCAAGCATTGC
AAGGAGCACATCCGAAGAGGATGTGTTCGAGCCATTGGAGCCTATTTCCAGTTTGCCGGATGGGGAATCT
GACGGTTTTGATATAGAAGAATTATTGAGATTGATGGAAGCCGACCCAATTGAAGTTGAGCCGGTCACTG
GGGGCTCCTGGAATTGTGGAACCAACACTGGCGTGGAAATGGGCCTGCTGGAACCTCTGTACCTGGGTGG

11. Unknown protein

GGTCAAGGGACCAAAAGGTAAAATGGAGCTGGAGCTCAGCCTTAATCGGAGCAGCATCAGCAACAGCAGC
AGCTTCACTCCTAAGCGCCAAGCCAAAAGATCCAACATTCCACCTTATCTCCATAGACCTTACCTCACTG
AAACTAAACCTCCCTGTCCTCGACGCAGAGCTAATGCTTACCGTACACGTCACTAACCCTAACATCGCAG
CCATCCATTACTCCTCCACCAAGATGACAATCCTCTACGATGGCACGGTTCTTGGCTCAGCTGAGGTCAA
GCGCAACACGCGCGGCAGTTCTTTTCTGACGTGGCGAATAGAGAGATGAAACTTGAGGCTAAGCTTACTA
TTGAAGGAGCGGCTAAGGTTTTGTGGTGGGATCATAGTTTTAGGGTTCACGTGGATAGTTTTGTCACCGT

TGATCCTGTTTTTCTTGATGTTATTGGTCAAGAGAATAAGTCTCAGATGGATTTGTTTCTTACTTAAAAA

12. EST

CTACAATCCTCTTCCTAGCAAAGCTTCTTCATCCAATGCTTTTACTACGCTTGCCCTCTTCAAACCCAGA
ACCAAGGCGGTTCCTAAGAAGGCTGCTCCACCGCCGAAGCCAAAGGTTGAAGATGGTATTTTGGTACCTC
CGGTGGCATCGGTTTCACTAAGCAGAATGAGCTCTTCGTGGGACGTGTTGCCATGATTGGATTTGCTGCA
TCGATGTTGGGAGAGGCAATAACAGGAAAAGGAATTCTATCTCAGTTGAACCTAGAGACTGGAATTCCCA
TTTACGAAGCAGAACCACTTCTTCTTTTCTTCATCCTTTTCACCTTGCTCGGAGCCATTGGAGCTTTGGG
TTGTTGGGGAGATTGGCTCACCAGGGGATCGCTTTCTTTCTAATTTGGAGAAATCTATATGGGGAAGGGG
AGCGCTAGCGCGACTCGACCATCGGAACAGGGATTCTCAGCCAGGGAAAACGAGCCACTGGTATCGGTCT
GACGCTCTCTTCTTTTGGGGAAACAGATAGGGAAGCACGGAACCGGAGAATTTCTAGCCGGAAAGGAAGG
AAAGGATGGAAAAGGCATGGAGGGACGAGCAAAAAGCCATAAAGTAAGAAAGGGACAAGGAAAGGCATGG
TCAGGAGGCGCCATGACAGATTTTAGGTGGGGAAGAAGAACCGAGAAGTTGACGCAGTCTAGGGGAAAAG
GCGGTTGATACATCCGCGGACACAGGAGAGACACATAAAGAAAACGGTTGGTGGGAGGTGGGGTAGAGAG

13. EST

ATGGGGCTACAGGCGGTCAAGTATTTGTCAGAGGGAAAGCTGGGGAGCGGTTTGCTGTGAGAAACTCACT
TGCTGAAGCTGTAGTTGAAGGCACTGGAGACCATTGTTGTGAGTACATGACTGGTGGTTGTGTGGTGGTA
CTGGGAAAAGTGGGTAGAAATGTAGCTGCTGGGATGACCGGAGGTTTGGCGTACCTTCTTGATGAGGATG
ACACTTTGATGGCCAAGGTAAATAAAGAAATAGTGAAGGTCCAAAGAGTGACTGCTCCTGTTGGCCAGAT
GCAGCTAAAGAGCCTTATTGAAGCTCATGTTGAAAAAACCGGAAGCTGCAAAGGCGCTGCTATCCTGAAG
GAGTGGGACACGTATCTACCGCTCTTTTGGCAGCTAGTACCACCCAGTGAAGAAGACACCCCCAAAGCTT
GTGCCTCATTCCAGGGAACCAGTGCTGGGCAAGTGACCTCCTTCCCATCTGCATAAGCTAACGATGCTAG
GAATTTTTGACTTAGAATTCGCCACAGATTTATCGGGAAGAGAAATCTTTGGGATCCTTGGATATAAATT
TTTTCCCAAAGAAAAAGGTTATATCCCCCCCAGTGTAATCGCCACCCACAACCTCCCTTTTACAAGCAGA
GGCTCCACCATAGGAAAAGAATTCTAAGGGGATTTTCACAGAAAAGGAAAAACCAATTGCAAGGCTCCCG

14. Water-stress inducible protein

GAAGCATCATAAGCATATGGAGGAAGTGGGTGGACTGGGAACTATGGCCACCGGAGCCTTTGCACTCGTA
ACTCTCTTTACCTTTTTATTCCTTCATTCATTCTCAGATCCACCTTATATGTCTCGACAATGGCGATCTA
ATACTATGTGATCAGTTTTTACTGAAATGGAACACTGTTTGGTCAAATTCTGATTAACTGTTGTTGTCGT
GATTGAAGAGGAGATTGCAGCAGCAGCTGCGGTGGGAGAAGGGGGTTA

15. Putative aquaporin

AGCAGTCTGATCCATGTGGTGGCGTGGGGCTTCTGGGTGTTGCATGGGCCTTTGGTGGGATGATTTTCAT
TCTCGTGTACTGCACTGCCGGAATCTCTGGTGGGCACATCAACCCTGCTGTGACCTTCGGGTTGTTCTTG
GCCAGGAAGGTGTCTCTTATCCGGGCTTTGGCCTACATGGTGGCTCAGTGTTTGGGAGCCATTTGCGGTG
TTGGGTTGGTGAAAGCCTTCATGAAATCCTTCTACAATTCACTTGGTGGTGGTGCCAACTCCGTCGCCGC
CGGCTACAACAAAGGCACAGCTCTTGGTGCTGAAATCATCGGCACTTTCGTGCTCGTGTACACTGTTTTC
TCAGCCACTGACCCCAAGAGAAGCGCCAGAGATTCCCACGTCCCTGTTTTGGCTCCCCTGCCCATTGGGT
TTGCTGTCTTCATGGTCCACCTTGCCACCATCCCCATCACCGGCACCGGCATCAACCCCGCCAGGAGCTT
CGGCGCCGCCGTCATCTACAACAATGAAAAAGTTTGGGACGACCAGTGGATCTTCTGGGTCGGACCATTT
GTGGGAGCACTAGCTGCAGCAGCATACCACCAGTACATACTGAGAGCAGCTGCCATCAAAGCTTTGGGAT
TTGTTTGTTTGTGTGTACCAGAGATGATTATGATGATGATGTTGATTATGAGAGCCCCTCTCTTCTTTTT
TTTGTTTTTTTTTTTTTTCTTTCTTTGTATCTTTTGTTCCATAATTTAATCCTTCTTTGAAAAAAAAAAA
AAAAAAAAAAAAAAAA

16. Putative aquaporin

CAGGGCTCTGATTGCGGAGTTCATTGCCACGCTGCTCTTCCTTTACATTACGGTGCTGACAGTCATCGGC
TACAAGAGCCAGACTGCCGGGGGTGACCCATGCGGTGGTGTTGGCATTCTGGGCATTGCTTGGTCTTTTG
GTGGCATGATCTTTATCCTTGTTTACTGCACTGCCGGCATCTCTGGGGGACACATTAACCCGGCGGTGAC
CTTTGGGCTGTTCCTGGCCCGGAAGGTGTCGCTGATCCGAGCAATATTGTACATGGTGGCTCAGTGTCTT
GGAGCCATTTGTGGTGTGGGTCTCGTCAAAGCCTTCCAATCTGCTTACTATGATCGCTACGGGGGCGGTG
CCAACGAGCTCTCCACCGGCTACAGCAAAGGCACCGGCTTGGGCGCTGAGATCATTGGAACTTTTGTCCT
TGTCTACACCGTCTTCTCTGCAACTGACCCCAAGAGGAGTGCCAGAGACTCCCATGTTCCTGTTCTGGCA
CCTCTTCCAATTGGGTTTGCCGTTTTCATGGTTCACTTGGCCACTATTCCTATCACTGGCACCGGTATCA
ACCCTGCCAGGAGTTTGGGGGCTGCTGTTATCTACAACAATGAGAAGGCCTGGGATGACCAGTGGATCTT
TTGGGTTGGACCCTTCATTGGTGCAGCCATTGCAGCCTTCTACCACCAGTTCATATTGAGAGCTGGAGCT
GTCAAGGCTCTTGGGTCATTCAGGAGCACCGCTCATGTGTGATTTGCAGAGCCATTTTGATACCTTCTTC
CACTGTTATTGGGGGCGAAGAAAAAGAATTTGGAAGGAGGAGAAGTGATTGGAATTGGAAGAATAATTAT
GGAGGTTGTTATTGATATGGATGAGGGCATGAAATTGTAGATACCATCTCCATGGAAATAGGAAGCTTTT
TTTGCTTATGCTTGAACAAAATTCTGTGTTGTGGCTTGTCTTGGAAGTAAAGGGCTTTATTTTGTTTTCC
GAAAAAAAAAAAAAAAAAAAAA

17. Putative aquaporin

TCATTCATGGTCGTTTTGGAGAGCTGGGATTGCCGAGTTCATGGCCACCTTCCTCTTCCTCTACATCACT
GTCTTGACTGTTATGGGTGTTGTCAGGTCCCCCAGCAAGTGTGCCACTGTTGGTATTCAGGGTATTGCTT
GGGCTTTTGGGGGTATGATCTTTGCCCTTGTCTACTGCACTGCTGGTATCTCAGGAGGACACATAAACCC
AGCGGTGACATTTGGCCTGCTCTTGGCCAGAAAGCTGTCTCTAACCCGAGCCGTGTTCTACATGATCATG
CAATGCCTGGGCGCCATCTGTGGCGCAGGTGTGGTGAAGGGGTTCCAGGGACATCAGTATGAGGTGTTAG
GTGGTGGAGCCAATGTTGTGGCTGCTGGCTATTCCAAGGGCGTTGGGCTTGGTGCTGAGATTGTTGGCAC
TTTTGTTCTTGTCTACACTGTTTTCTTTGCAACTGATGCCAAGAGAAATGCCAGAGACTCACATGTCCCT
ATTTTGGCTCCTCTCCCGATTGGGTTTGCAGTGTTCTTGGTTCATCTGGCAACCATCCCCATCACTGGAA
CTGGCATCAACCCTGCTAGGAGTCTGGGGGCCGCCATCGCCTACAACAAAGAGCATGCCTGGGATGACAT
GTGGATTTTCTGGGTTGGACCCTTCATTGGAGCTGCTCTTGCTGCCATGTACCAGCAGATAGTCATCAGA
GCCATTCCATTCAAGAGCAGGGCTTGAGACTTCCATCGCCTTTCTCTGTTGGGCATTTTGTTTCTCTCAT
GAATTCTGTTATCCTTCCCTGCCCCATAGGCAAAAAAAA
AAAAAAAAAAAAAAAAAAAAA

18. Putative aquaporin

ACAAGGACTACAAGGATCTACCACCAGCACCCTTGTTTGAGCCCGGGGAGTTGAAGTCATGGTCTTTTTA
CAGAGCCGGGATTGCTGAGTTCATGGCCACTTTCCTGTTTCTCTATATCACCATTTTGACTGTTATGGGT
GTGAAAAAGTCACCCACCATGTGTGCCAGTGTTGGTATTCAGGGGATTGCTTGGGCTTTTGGTGGTATGA
TCTTTGCCCTTGTCTACTGCACTGCTGGTATCTCAGGAGGACACATCAACCCAGCTGTGACCTTTGGTCT
GCTACTGGCGAGGAAGCTGTCTCTTACCAGGGCAATTTTCTACATCATAATGCAGTGCCTTGGTGCCATC
TGTGGGGCTGGTGTTGTTAAGGGCTTTGAGGGTTCCCAATCCTATGAGGTGTTGGGTGGTGGAGCTAACG
TTGTGAATTCTGGCTACACCAAGGGTGATGGCCTTGGTGCTGAGATTGTTGGCACCTTTGTTCTTGTTTA
CACTGTCTTCTCTGCTACTGATGCCAAGAGAAACGCCAGAGACTCTCACGTCCCTATTTTGGCCCCCCTC
CCCATTGGGTTTGCAGTGTTCTTGGTTCACTTGGCCACCATCCCCATCACAGGAACTGGCATTAACCCAG
CCAGGAGTCTTGGAGCTGCTATCATCTTCAACAGAGAGCATGCATGGGATGACATGTGGATCTTCTGGGT
GGGACCGTTCATTGGAGCTGCTCTTGCTGCCATGTACCAGCAGATAGTCATCAGAGCCATTCCATTCAAG
AGCAGGGCTTGAGACTTCCATCGCCTTTCTCTGTTGTGCATTTTGTTTCTCTCATGTGGATTTGGTGTTT
CTGAAAAAAAAAAAAAAAAAAAAAAAAAAA

19. Putative aquaporin

TGGCGGTTGCTGTTGCCCATGGTTTCGCTCTCTTTGTTGCAGTCGCTATTAGCGCCAACATCTCCGGTGG
CCATGTTAACCCTGCGGTGACCTTCGGGCTGGTTGTTGGTGGTCAGATCACCATTCTCACTGGCATCTTG
TACTGGATTGCCCAGCTTGTTGGCTCCATTCTTGCATGTTTCCTACTCAAACTTGTCACAGGAGGCTTGA
CGACTCCCGTCCATAGTCTTGGAGCTGGGGTTGGAGTCATTGATGCTATTGTCTTCGAGATCGTGATCAC
TTTCGCTCTGGTCTACACCGTCTATGCAACGGCGGTTGACCCGAAGAAGGGCTCACTGGGCATCATTGCA
CCCATTGCCATAGGTCTTGTTGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAATGA
ACCCCGCCCGCTCCTTCGGCCCCGCCGTCGTCAGCGGCGACTTCAAGGACAACTGGATCTACTGGGTGGG
ACCCCTAATTGGAGGTGGCATGGGAGGATCTGTCTATGCAATTATGTACATGGGCTCTGATCATCAACCA
CTAGCGTCCAGCGAATTCTAAGCTGAGTTTTTTTCAGGAACTCAATTGTTTGTTGTTGTCTCAACCCATT
AATGAGGTTGGTGATGTGCAGTAAAAAAAAAAAAAAAAAAAAAAAAAAAA

20. Putative aquaporin

TCACTGACAACGGTTCCACCACTCCCGCTGGCCTCGTTGCCGCCTCTCTGGCTCACGCATTCGCTCTCTT
TGTTGCCGTCTCTGTAGGAGCCAACACCTCCGGTGGGCACGTCAACCCCGCAGTCACCTCCGGTGCCTTC
GTCGGCGGTAACATCACCCTCCTCCGGGGCATCCTATACTGGATTGCTCAGCTTCTTGGGTCCACCGTAG
CTTGCTTGCTACTTAAGTTTTCCACCAACGGCATGACCACCAGCGCCTTCTCTCTGTCTTCAGGAGTGAC
AGTGTGGAACGCGTTTGTTTTCGAGATCGTGATGACCTTCGGGCTGGTGTACACAGTCTACGCCACAGCC
ATTGATCCAAAGAAGGGCAATTTGGGAATTATTGCACCGATTGCCATCGGTTTTATCGTGGGTGCTAACA
TTCTGGCAGGTGGGGCCTTCGACGGTGCCTCAATGAACCCAGCAGTATCATTTGGGCCTGCCTTGGTGAG
CTGGACCTGGACCAACCACTGGGTCTACTGGGCCGGGCCTCTGATCGGAGGAGGGCTTGCTGGACTTGTG
TACGAGGTCTTCTTCATCAGCCACACTCACGAGCAGCTCCCCTCCACAGACTACTGAGCTCATCAATCCA
TGAACAGTTCCGAGGCTTTTGTTTGATGTATTTATTTCTTTACAGGCTTGTTCATGTGTTTATTTCGTGA
GTTTCAGTCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA

21. Putative aquaporin

AGAGTTTTTCTCCATGCTCATCTTTGTTTTTGCTGGGGAAGGCTCGGGCATGGCTTTTAACAAGCTGACG
GATAGTGGGTCGTCAACACCGGCGGGCCTGGTGGCAGCTGCTCTAGCCCATGGCTTCGCTCTGTTCGTGG
CCGTTTCGGTGGGTGCGAACATATCTGGCGGACATGTGAACCCGGCCGTGACGTTTGGAGCCTTTATTGG
CGGACACATAACGTTGTTGAGAGGCATTTTGTATTGGATTGCCCAGCTGCTGGGATCTGTCGTTGCATGC
TTGCTGCTTAAGTTCTCCACCGGTGGATTGGAAACGTCTGCATTCTCCCTATCCTCAGGCGTGTCGGTGT
GGAACGCCCTGGTTTTTGAGATTGTGATGACCTTCGGCCTGGTTTACACAGTGTATGCCACAGCAGTGGA
TCCAAAGAAGGGGAACTTGGGCATTATTGCACCTATTGCAATTGGTTTCATAGTTGGTGCCAACATATTA
GCTGGTGGTGCATTTGATGGTGCTTCCATGAACCCAGCAGTGTCATTTGGGCCTGCTGTTGTTAGCTGGT
CATGGGCCAACCACTGGGTCTACTGGGCCGGGCCTCTCATCGGTGCCGCCATTGCCGCCATCATCTACGA
TCACATCTTCATTGACAGTACACATGAGCAACTGCCCACCACGGATTATTAGGAGCTTGCTATGAATTTC
AAAAAAAAAAAAAAAAAAAAAAAAAAAAA

APPENDIX B

TRANSCRIPTS IDENTIFIED FROM SUBTRACTED LIBRARY

i) Control Leaf Tissue

1. Galactinol synthase

TGGCCGGTAACGGCGACTACGTGAAGGGCGTGGTGGGGCTGGCCAAGGGGCTGAGAAAGGTGAAAACCGC
ATACCCACTTGTGGTGGCGGTACTGCCGGACGTCCCGGCGGAGCACCGCCGCATTCTGGAGGACCAGGGC
CATACAGGTGTTTGGTAACATAGACCATTTGTTTGATTTGGATGATGGGTATTTCTATGCTGTGATGGAC
TGTTTCTGTGAAAAAACGTGGAGCAACTCGCCGCAGTACAAGATTGGGTACTGCCAGCAGTGCCCGGAGA
AGGTGCAGTGCCCGGCGGAGATGGGTCCAGCACCCCCTCTCTACTTCAACGCGGCATGTTTGTGTTTGAG
CCTTGCCTCTCCGTCTATGATGATCTCCTTACCACTCTCAAGATCACAACTCCTACCTCCTTTGCCGAGC
AGGACTATTTGAATATGTTCTTCAGGGACATCTACAAGCCCATTCCACCCACCTACAACCTTGTTTTAGC
CATGCTATGGCGCCACCCAGAGAACATTGACCTTCAAAGAACCAATGTTGTTCACTATTGTGCTGCTGGT
TCAAAACCATGGAGGTATACTGGGAAGGAGGAGAACATGGAGAGGGAGGATATAAAGATGTTGGTGAAGA
AGCTTAGTTTATATAAATAGGAGTTTAATTTTAGGAGTTGGGTTTGGTTTGAAGCCAGGGTGAACATATT
CTTCTTCTTGTAAAGTTTTAAGTCTAGGAGAAGGGTTGTATTGGGAGGATATGTAAGGTTGATGATTTTT
TTTTATTTCCATTTTTGTTGATTGGAATTTGTTGGGTTTATGAATATTGGATGATATGAATGTAACAAAA

2.CER5-like protein

GCTCTTGCAAGGGTTGTACGGCTACGCCGTGCCCGGCAGGATCGTTGCCATCATGGGGCCCTCCGGCTCT
GGCAAGTCCACCCTCCTCGACTCCCTCTCCGGGAGGCTGGCGAGGAATGTGCTCCAGACCGGCAAGGTGC
TGCTCAACGGCAAGAAGAGGCGGCTCGACTTTGGCGCGGTGGCCTACGTGACACAGGAGAACGTTCTGCT
GGGCACGCTGACCGTCCGGGAGACGGTTACCTACTCGGCGCAGCTGCGGCTGCCATCGAGCATGTCAAAG
GCTGAGGTGCGCCGAGTGGTCGACGACACGCTGGACGAGATGGGCCTCCGGGAGTGCGCTGACCGGCCCA
GCGAGGTGTTCGCACTCTTCGACGATCTCTGCCTCCTCTCCAGCGGCGAGAGCGTCTACTTCGGAGACGC
CAAACTTGCACCACAGTTCTTTGCAGAAACCGGATTCCCCTGCCCGAGCCGGAGGAACCCATCCGACCAC
GAGGAGGCAGTGAAGGGCAGCCAAGCGACCTGGTGCAAGCAGCTGCGCACGCTGACCAAGCGCTCCTACA
TCAACATGTACCGAGACTTCGGCTACTACAGGCTGCGCATCATCATCTACGTCCTGATGGCCATCTGCCT
CGGTACCATCTACTACGACGTCGGCAACGGCTACACTGCAATCCAGGCGCGTGCCTCGTGCGGCGGCTTC
GTTTCCGGCTTCATGACGTTCATGTCCATCGGCGGCTTCCCCTCCTTCATCGAGGAGATGAAGGTCTTCT
CCCTCGAGCGGCAGAACGGCCACTACGGCGTCGCCGCCTACATCATATCCAACTTCCTCTCCTCCATGCC
GTTCCTGTTGACCATGTCCTGGGCCAGCGCCTCCATCACATACTGGATGGTCAAGTTCCGGCCAGGATTC

3.Ascorbate peroxidase (APX1) gene

TAGTAATGCGATCACAGTACTTCCATGTTATGTTTTGGTTTGGTGACCGGATTTGGTTTAACTTACTGCC
AGACATAGTTCAACTGTTTACGTTCATAAGCGACTCACACAACTAATGCAATTGTTGTTAGTCTCATGCT
TTAACATCATGGCCAACTAATGAAATGTTTGCTTGACTATTTTAAGACATGAACATGGTGGTGGTGGTTT
TTATTTCTTGGTTGTATGAGAGGTCGATTGGCTTGTGTTGTTGATTATGGGAGTGCAGCACTTCAGATAT
TTTATTCTTGTGGTGCAAGATTTAGCTTATACTAAAGTTGATGTTATTTCTAATGGATGATGATGTAAGT
TATATCAGTTAAAAGTGTATATTTGCACTTGTTCTTACGGTTTATTTTGTACCTGGTGCAGAACAAGTGA
TTCTAAAAGTAAAGGAAGATCATAAACAAAGATTTTATATGCCTACCAGTTACAGGATAAACTATTGAAT
GTGTTATTCTCATGATCTTTCATTTCATTTACTCTTTGTATCAAGATCTGGCACGCTGCATTTGGGAACA
ACGTGCTGGAATAAAGTGTCAATGGTTGGATGACTTAAGAGGAATCAGTTTTGTTCTTATCAAACAGTTA
TCTACCTATAGCTTGCAGGTGTTGTTGCAGTTGAAGTCACTGGAGGCCCCATGATTAATTTTACTCCTGG
CAGAAAGGTTATGTTAATCTATCTTCGAGAGATTGATGTATGTAAAAAAAAAGTACTACCCATCTGCAGC
ATTGGGGAGATGGTCGTGCAATTGCTTCTGTCTATTGCAAGTTGTCTTAAATGAATTAAGAACAGTCTCT
AAAGTAGTCACCTTATGTTTCTTTCAGGATTCAATGATTTCTCCAAAGGAAGGGCGGCTTCCAGATGCTA
AACAAGGTCAACCAAATGTCCAGAAAATCTTAGTTCCTCTAAATCTTCTGGTTAGCGTGTCTAGAAGTTA
4. Abscisic acid responsive elements-Binding factor 3

GTACGAAAGCTTGAGTAATGGGGTCTAGATTAAACTTCAAGAGCTTTGTTGATGGTGTGAGTGAGCAGCA
GCCAACGGTGGGGACTAGTCTTCCATTGACTAGGCAGAACTCTGTGTTCTCGTTAACCTTTGATGAGTTT
CAGAACTCATGGGGTGGTGGAATTGGGAAAGATTTTGGGTCTATGAACATGGATGAGCTCTTGAAGAACA
TTTGGACTGCAGAGGAAAGTCATTCAATGATGGGAAACAATACCAGTTACACCAACATCAGCAATGGTAA
TAGTGGAAACACTGTTATTAACGGCGGTGGTAACAACATTGGTGGGTTAGCTGTTGGTGTGGGAGGAGAA
AGTGGTGGTTTTTTCACTGGTGGGAGTTTGCAGAGACAAGGTTCACTTACCTTGCCTCGGACGATTAGTC
GCTGGTGTGGTTAGGGAAGAACCTCAACCGGTGGAGAGTGTAACTAACTTCAATGGCGGATTCTATGGAT
TTGGCAGTAATGGAGGTCTTGGGACAGCTAGTAATGGGTTTGTTGCAAACCAACCTCAAGATTTGTCAGG
AAATGGAGTAGCGGTGAGACAGGATCTGCTGACTGCTCAAACTCAGCCACTACAGATGCAGCAGCCACAG
ATGGTGCAGCAGCCACAGATGGTGCAGCAGCCGCAACAACTGATACAGACGCAGGAGAGGCCTTTTCCCA
AACAGACCACTATAGCATTTTCCAACACTGTTGATGTGGTTAACCGTTCTCAACCTGCAACACAGTGCCA
GGAAGTGAAGCCTTCAATACTTGGAATTCATAACCATCCTATGAACAACAATCTACTGCAAGCTGTCGAT
TTTAAAACAGGAGTAACGGTTGCAGCAGTATCTCCTGGAAGCCAGATGTCACCTGATCTGACTCCAAAGA
GCGCCCTGGATGCATCTTTGTCCCCTGTTCCTTACATGTTTGGGCGAGTGAGAAAAACAGGTGCAGTTCT
GGAGAAAGTGATTGAGAGAAGGCAAAAAAGGATGATAAAGAATAGGGAATCAGCTGCAAGATCCCGCGCT

5. Arginine decarboxylase

TCGTGAAGAAGGTTTCGGATCCGAAACCAGATTGCGGGCTCGGGTTGCAGCTCCCGCTCATTGTTCGCCT
GCCCGATGTGCTCAAGAACCGGCTGGAGTCGCTCCAGGGGGCGTTCGATCTCGCGATCCAGTCTCACGAC
TACGGGTCCCACTACCAGGGCGTGTTCCCGGTGAAATGCAACCAGGACCGGTTCGTTGTGGAGGACATTG
TCCGGTTCGGCTCGCCGTTCCGGTTCGGATTGGAAGCTGGGTCGAAGCCGGAGCTTCTCTTGGCCATGAG
CTGCTTGTGCAAAGGTAACCCAGAAGCCCTTCTCATCTGCAATGGATTCAAAGACTTCGAGTACATCTCT
CTGGCTCTGTTTGCTCGCAAGCTCGCCTTAAACACAGTGATTGTTCTTGAGCAAGAGGAAGAGCTCGATG
TGGTTATCGATTTGAGCAAGAAGCTAGGTGTTCGACCCGTGATTGGGGCCCGAGCCAAGCTCAAAACCAA
GCATTCGGGTCATTTCGGGTCGACTTCGGGCGAGAAAGGGAAGTTCGGGCTCACTACCACTCAGATTTTA
CGGCAATCAGAGGTGAGTATGAGGCTTGCTTGACATATGCTGATCAGTTGAAACAACGCTGTATTGATCA
GTTCAAAGAAGGGTCTCTGGGCATTGAGCAATTAGCCACTGTTGATGGGCTTTGTGATATGGTTTCGAAA
GCAATCGGGGCATCTGACCCTGTCCGTACGTACCATGTGAATCTCTCGGTTTTTACTTCAATTCCAGACT
TCTGGGGCATTGGGCAGACGTTCCCAATAGTCCCGATTCACCGCCTCGATCAGCGGCCGGCGGTGAGGGG
GATATTGTCGGACTTGACCTGCGACAGTGATGGGAAGATTGACAAGTTCATCGGTGGCGAGTCGAGCCTG

6. Protein kinase

CATAATGGAATATGCTTCTGGCGGTGAACTTTACGAGCGGATTTGCAATGCAGGACGGTTTAGTGAAGAT
GAGGCTCGGTTCTTCTTTCAGCAGCTTCTATCTGGAGTCAGTTATTGTCATTCGATGCAAATTTGCCATC
GTGACCTGAAGCTAGAGAATACATTGTTGGATGGAAGTCCTGCTCCTCGATTAAAAATTTGTGATTTTGG
ATATTCAAAGTCTTCTGTTCTTCATTCACAACCAAAGTCAACTGTTGGTACTCCTGCATACATCGCTCCA
GAGGTACTGCTTCGTCAGGAATATGATGGCAAGATTGCAGATGTATGGTCATGTGGTGTGACCTTATACG
GACGATTTCATGACTGACAATCTTGATCTTGACGATGACATGGATGACTTTGACTCTGAATCTGAAATCG
ACATTGACAGTAGCGGAGAGATAGTTTACGCTCTCTAATAAAAAGCCTTTTTTAACAACCAAAACACTTC
TCTATCTGTTCTAAGACCAGTAGTGTTCTGATCCTCTGGTTTCAAATTCTACCAATTTTTGTATTGTCTC

7. Aldehyde dehydrogenase

GTGGACGCCTACGACGGAAAGACCATCGAGGTGCAAAATCCAGCAACTGGTGAGGTCCTTGCAAACGTGC
CCTGTATGGGCAGCAGAGAAACATCTGATGCTATTGCTTCTGCTCACAGTACATTCTATTCTTGGAGTAA
ACTCACTGCTAGTGAGAGGAGCAAGGCACTAAGAAAATGGTACGATCTAATTATTTCACACAAGGAAGAG
CCTGCACTTCTCATGACACTGGAGCAGGGGAAACCTATGAAAGAAGCCCTTGGTGAGGTCAATTATGGTG
CAAGTTTCATAGAATATTTTGCAGAGGAAGCAAAGCGTATATATGGTGATATCATTCCCCCAACTTTATC
TGATCGCAGATTGTTGGTTCTGAAGCAGCCTGTTGGGGTAGTTGGAGCTATTACACCATGGAATTTTCCA
TTAGCAATGATAACCCGAAAGGTTGGACCAGCTTTGGCCTGTGGCTGCAGTGTTGTTGTCAAGCCATCAG

AAGAGAGTACATCTCAGGGTCCTCTGATTAACGAAGCTGCTGTTCAAAAGGTGGAGAAGTTCATAAATGA
CGCGACTTCAAAGGGAGCAAATGTCATGCTAGGTGGTAAAAGACACAGCCTGGGGATGTCATTTTATGAG
CCAACTGTAGTAGGGAATGTCAGCAATGATATGCTTCTTTTCAGAGAAGAAGTTTTTGGTCCAGTTGCAC
CCCTTATACCATTCAAAACTGAGGAAGAAGCAGTCCATATGGCCAACGATACAAACGCAGGCTTAGCTGC
ATACATATTTACCAAGAGCATACCTCGTTCATGGCGTGTATCTGAATCTCTTGAATACGGCTTAGTTGGG
GTAAACGAGGGAATTATTTCAACAGAGGTGGCACCATTCGGTGGAGTAAAGCAGTCTGGTCTAGGGAGAG
AAGGATCGAAGTACGGCATCGACGAATACTTGGAGCTCAAGTACATCTGCATGGGCAACTTGGGTTGAGT
CCTGAAATCGGCCTCTGGCGAGAAACGCTTGGCAAATGATGAACCGAGAAACCAAATAGGCAGGCGTGCT

8. Xyloglucan endotransglucosylase

AACAGGACAACCTTATATTTTGCAAACAAATGTGTTCACTGGTGGTCAAGGTAACAAAGAACAAAGGATC
TTTCTTTGGTTTGATCCTACAAAAGAGTTCCACAGATACTCCATTCTATGGAATATGTACCAGATTGTGT
TTTTTGTGGACGATGTTCCCATAAGAGTATTCAAGAACAGCAAGGATTTGGGAGTAAAATTTCCATTTGA
TTAATGCTAAGTTTTGTGCTACACAAGGTAAAAGATGGTGGGATCAACCTGAATTTCGTGATCTTGATGC
TGCTCAATGGAGAAGATTAAGGTGGGTTCGTCAAAAATTCACCATTTACAACTATTGCACTGATCGAAAA
CGTTTACCTCAAATACCACCTGAATGTACAAGAGACCGTGACATTTAAATTTTCATATACTCTATTTGGT
CTAATTTATGTTATGTTTTCAAATACCATGTCACATTGTTGTCAAGGATACAAAAGACCATTTCATATCA
TGTGTTTGTTTCTCATTGTATTAGACGTTTTATCGTTTCATTATTTATTTACATTATTGTTCCATCGAGT

9. Glyoxalase I

TATGCAACAAACTATGTTTAGAATTAAAGACCCCAAAGTTAGCCTTGATTTTTATTCTCGCGTTTTGGGC
ATGTCTTTGCTTAAGAGATTGGATTTTCCTGAGATGAAGTTCAGCTTATACTTTATGGGGTATGAGGATA
CAACAGAAGCTCCAAGTAACCCTGTGGATAGAACAGTTTGGACCTTTGCTCAAAAGGCTACAATTGAATT
GACCCATAATTGGGGCACTGAAAGTGATCCAGAGTTCAAAGGGTACCACAATGGCAATTCTGATCCTCGT
GGCTTTGGACACATTGGCATAACTGTTGATGACACCTACAAAGCATGTGAAAGATTTCAGAATCTTGGAG
TCGAGTTTGTTAAGAAACCAGATGATGGGAAAATGAAAGGGATTGCATTTATTAAGGATCCCGATGGTTA
CTGGATTGAACTCTTTGACCGGAAAACAATTGGAAATGTAACAGAAGGTAATGCTTGAGATAAAACCTTC
AGGTAGACACCATGGTATCCAATTCTCTTGTCTTATTTACTTATTCATGTTTAGCGCCCTGTATGTAATT

10. Glutathione transferase 7

GCCGTCTGCGAGTCCCTCCTCATCGTCGAGTACGTCGACGAGGCCTTCGACGGGCCGTCCATCCTGCCGG
CCGACCCCCACGACCGTGCCGTCGCCCGTTTCTGGGCGAACTTCTTGGACACCAAGTTCTCCCAGCCGTT
CTGGCTGGCGTACTGGGCGGAGGGCGAGGCGCAGAAGGCCGTGGTGAAGGAGGCCAAGGAGAACCTGGCG
CTCCTGGAGGCGCAGCTCGGCGGGAAGAGCTTCTTCGGCGGCGACACGCCCGGGTACCTCGACATAGCCG
GTTCCCCGCTCTATGCCAGTGGGCCAGGGACTACAGCTCCAGTGAAGCGCTCAGGCCATGCCTGCCGGAC
AGGGACCGACTCGTTGCCTACTTCACCGAGAACAAGGAGAAGTACAAGACATTTGCCAAGGCAACGTTGC
ATCAGTAGCTGCTAGTTGGGTGCAAACCGCTTCTTTATCTCTGTGTGGAATAATGTATACGTACGTGCTC
AGCTACCGGAGTTGACTGTAGTCAAATTGCTAAAAAAAAAAAAAAAAAAAA

11. PDR20

TCGAGGTGCGGTACGAGAACCTGAATGTGGAAGCGGAGGCGTACGTTGGTAGCAGAGGTTTGCCCACCAT
CCTCAACACCTACGCCAATGTGCTGGAGGGTCTGGCAAACACTCTTCACATAACACCAAATAGGAAGCAG
AAAATATCGATCCTTCACAATGTCAGTGGGATAATCAAGCCTCACAGAATGACCTTGCTTTTGGGTCCTC
CTGGCGCTGGAAAAACCACACTTCTTTTGGCCTTGGCTGGAAATGTCCCTTCAGGTCTAAAGGTATCTGG
ACAAATAACATACAATGGCCATACCATGGATGAATTCGAGCCCCGGAGATCAGCAGCTTATGTTAGTCAA
CATGATCTACATATGGGTGAATTGACGGTTCGTGAGACAGTCAATTTCTCTGCAAAATGTCAAGGAATTG
GCCACCGTTACGATCTGCTAATGGAACTATCAAGGAGAGAAAAGGAAGAAAATATTAAACCAGATCCAGA
ATCAACTGGACTTGACAGCTCCACAACATACAATATTGTGGACTCCATCCGGCAGACCATCCACATTGTT

GGTGGAACAGCAGTCATTGCTTTGTTACAACCTGCACCAGAGACATATGAATTGTTTGATGATATAATCC
TCCTCTCAGACGGTCAGGTCGTCTACAATGGTCCTCGTGAACATGTGCTAGAATTCTTTGAATCAGTTGG
CTTCAAATGTCCTGAGAGAAAAGGCGTAGCAGACTTCTTGCAGGAAGTTACTTCAAGGAAAGATCAGCGG
CAATACTGGATGCATGGTGATGAGACATACCGATATGTTCCTGTTAAGGAGTTTGCAGAGGCATTTCAGT
CTTTCCATGTTGGTCAGGCAATAAGAAGTGAGTTGGCAATCCCATTTGACAAGAGCAGGAGCCACCCTGC
TGCCCTGAAAACATCAAAATATGGTGCTAGCATGAAAGAACTGCTTAAAGCGAACATTGACAGAGAGATA
TTGCTCATGAAAAGGAACTCCTTTGTGTATATATTCAAGGCAACTCAGTTAACACTCATGACATTCATTG
CGATGACTGTCTTTATCCGCACCAATATGCATCATGACTCAATAACAAATGGGGGAATATACATGGGCGC
ACTCTTCTTTGGGATCCTGATGATCATGTTCAACGGATTGGCAGAAGTTGGTCTAACTATTGCAAAACTC
CCTGTTTTCTTCAAGCAAAGGGATCTCCTCTTCTATCCAGCATGGACATACTCCTTACCATCATGGATCA

12. Putative proteinase inhibitor-related protein

TCGACGTCTGCGACGACGCCCACCCCCAGTGCCCCAAGGGCTGCTCGGCCTGCCGCGTGGTGACGCCCAG
CCCTCACAAGACGTTCCGGTGCGCCGACATGAAGAGCACCGTCGATGGCACCTGCGGCGGGCCATGCAAA
AAGCACTGATCCGTTCAGGGCTTCAGTCTCAGACGAGGGGTGCCGCCTAAAATAAAGCTCAGATGAGATG
AGCAGTCATGGCGCCTATGCTTCTCTGTGGGTCGTGCTATACGGCCAACAATATGTATACTGCTATCGTC
GTGTCTTGTCAATGTGTTTCCTCTCTCGAGAAAGGTGGTGGTGCTTGTACTTGTGTTCACCTATCCTCCG
TGAATAAGCACATGTAAAAAAAA

13. Transcription factor

GCGAAGAAGAGAGCTCAAAGGTTAGATCAGAAGGTTGTTCGAAAAGCGTGGAGTCGTCGAAAAAGAAGGG
GAAGAAACAAAGGTATGCGTTTCAAACAAGGAGCCAAGTGGATATTCTTGATGATGGTTATCGATGGAGG
GCAATGTGAAGAAGCAAGTGCAAAGATTAACAGTGGACCAAGAAGTGGTCGTGACAACCTACGAAGGAGT
GCATTCGCATCCCATCGAGAAATCCACCGAAAACTTCGAGCATATTCTCACTCAAATGCAAATCTACTCT
TCTTTCTAGTTAATTCTCTCAAAATCTTTTATACCTTATTTTAGAGCCTATAATTCTTAGAGCTCATGTT
GTAAATTATTTACAAAAATTAGAATGTTACATTTTACGTGTGTTGAAAAGAAAAACCATGCATGATCTGA

14. Isolate Lchce-1 Asr2 gene

CTGAAGAGAAACATCAACACCATCATTTGTTCCACCACAAGAACAGGGAGGATGAGGGTGGAC
CAGTTGATTATGAAAAAGAAGTGAAGCACCACAGCCATCTCGAGAAGATTGGTGAACTTGGTGCTGTTGC
TGCTGGTGCTTTCGCCTTAGTATGTAAATATAATAACATATATTCTATAGTCGATATTTTCTTTTTATAT
AAGTACATCTGATACAAGTCTTCTTTAATTTCTTACGCCGTTTGTGGTTGAATTATTAAATATATAGCAT
GAGAAACACAAGGCAAAGAAGGACCTAGAGAATGCACACAAGCACAAGATAGAGGAAGAGATTGCAGCTG
TTGCTGCAGTTGGTGCAGGTGGATTTGCATTCCATGAACATCATCAGAAAAAAGATGCCAAGAAAGAAAA
AAAGGAAGTTGAGGGTGGACGCCACCACC

15. DRF1

TTGTGGAGCAGAGGAAAGTACCCGGAGTCATGTTCTCGTCAAACCAATAGGAAAAAGCGACCTCGGAGATC
ACGTGATGGGCCTAATTCAGTCTCTGAAACGATCAGGCGATGGAAAGAAGTGAACCAACAACTGGAGCATG
ATCCACAGGGTTCAAAGAGGTCAGCCACCTGCAAAGGGTTCAAAGAAGGGCTGTATGCAGGGGAAAGGAGG
ACCTGAGAATACACAATGTGGATTCCGTGGTGTAAGGCAACGTACTTGGGGGAAGTGGGTTGCTGAAATTC
GGGAGCCAAATCGGGTGAGCAGGCTCTGGTTGGGAACGTTC

16. S-adenosylmethionine decarboxylase uORF and S-adenosylmethionine decarboxylase

GGTGGTGAAGGGGGGGAAATTCGTGAGATCTGTTCCGGATCACGCGTGCGCGCTCGGGAATCGGGGGTTC
CACACATAGCCTCGTCGATTTGAATTTGATGTACTAATGGAGTCTAAGGGTGGCAAGAAGTCTAGCAGTA
GTCGTTCCCTGATGTACGAAGCTCCCCTTGGCTACAGCATCGAGGATCTCCGCCCTGCAGGCGGCATCAA
GAAGTTCTCCGCTGCTTACTCGAACTGCGCGAGGAAGCCATCCTGATAGCTCTCTCGTCAGCCCCATCCT
AGTAGCTTAGAAACCACCTGCTTTTCCATTTTGATCTTTCTAAAATCTCTCTGGCATAGCTGCTTTCCAG
AGCGCCTTGAGATCAGCTTCTCTGAGGCACCTGTCTTCACTGACCCCAATGGAAGGGGACTCCGTGCACT
CTCGCGTGCCCAGATTGACTCTGTTCTCGACCTTGCACGGTGCACCATTGTGTCCAAGCTCTCAAATGAG
GACTTTGACTCTTATGTCTTATCTGAGTCAAGCCTGTTTGTCTACCCATACAAGATGGTGATCAAGACCT
GGTTACACATCGTGTGCTAAGGAGATGACCAAGCTCTCTGGTATCTCGGACATTATCCCAGAGATGGAGA
TCTGTGACTTCGATTTTGAGCCCTGTGGCTACTCCATGAATGCTGTTCATGGCCCTGCTTTCTCAACCAT
TCATGTGACCCCAGAGGACGGCTTCAGCTATGCAAGCTACGAGGTCATGGGCTTCAACCCGGGCTCTCTG

17. Rho-GTPase-activating protein-like (Hsdr4) gene, promoter region

GGGACAATCTTCTCCATAAAAACATCCTTATCTTTTCTCAACAACAACAACAACAACAAAATCTTATCTT
CCCTGGGACTTGTGTTTTTCTTAGTATTCCCTGGGACTTGTTTTTGTAGGCAATTTCCTGGGGCCTTTTT
TTAGACTGAATTTCCTGGGACTTTTTGTTAGACTGAATTTCATGGGACTTGTGTCGGGCTTCTAAGAAAA
TGAGATCCATTCCAGCTCGTTAGGCCTCGTTTGATTAAGGGGGATTGGGGAAGTTTTGAGAGGAAAATTC
CGGGAGGGCCAGAAACCCCCACAGATCCTCGGGGCACCCATTTGGTAGGAGGGGTTTGCTTAGCCCAATC
CCTTCCGTCCCCTTCAATCCCCTCCTATCCATGTGTTTCAAAACCCTCCTCGGGGGACTAGTGGAAAGAA
GGGCTTGTAGTTATCTAAGTGGGGTCCAATGGGATCAATGAAAAAAGTGGGCTGGCCCATTTGTTTCCAA
TGGGAGCCCACTAAAGGAAATTAAGTGGGATGGACCAATTACTCCCACTTAATCCCATCTCCACCTGCAG
CCTGACGGGCCGGGCTCGCGGGGACTCTGCAGCAGGAGGGGCGGCCGGCCATGGCGCTCGCATCGGGCTC
CGGCGGCGACTTCTCCGTGATGGTGATAGGGTCCGACTTCGCGGTGGACGCCGGCGCGGCGCTCCTCACC
CTGCCTATGTGGCTGGCTTCTTTGCTAGTGGTACCATCCCCTGTTGGTCTAGTTTCTTGGTTTGGTAACA

18. 1-aminocyclopropane-1-carboxylate oxidase

GGGTGCGCGAGCAGAGGTTCCTCGAGTTCGCCAGCAAGACGCTCAAGGACGCGGAGGGCGTGAAGGCGGA
AAACCTGGACTGGGAGAGCACCTTCTTCGTCCGCCACCTCCCGGAATCCAACATCGCCGAGATACCGGAC
CTCGACGACGAGTACCGGCGCGTCATGAAGCGGTTCGCCGGCGAGCTGGAGGCGCTTGCGGAGCGGCTGC
TGGACCTGCTGTGCGAGAACCTCGGCCTCGACAAGGGCTACCTCGCGCGGGCGTTCCGCGGGCCCAGCAA
GGGCACCCCGACGTTCGGCACCAAGGTGAGCAGCTACCCGCCGTGCCCGCGCCCGGACCTCGTGAACGGC
CTCCGTGCGCACACCGACGCTGGCGGCATCATCCTGCTGTTCCAGGACGACCGAGTGGGCGGGCTCCAGC
TGCTCAAGGACGGCGAGTGGGTGGATGTGCCGCCCATGCGCCACTCCATCGTGGTCAACCTGGGCGACCA
GCTGGAGGTGATCACCAACGGCAGGTACAAGAGCGTGATGCACCGGGTGGTGGCGCAGCCCGACGGGAAC
AGGATGTCCATCGCGTCCTTTTACAACCCGGGTAGCGACGCGGTCATCTTCCCCGCGCCCGAGCTGGTGA

19. Dessication-related protein

GCTCAGTTCGGCGGTGAGAAATACGGCGGAAGGCACACCGACGAGTACGGAAACCCCATTCAGCAAGGCG
CAGGAGCACACCGCGGAGGAGGCATCATGGGCGGTGGTCAACAAGCCGGCCAGCATGGTACCACCGGCGT
CCTCGGTCATGGAACCGCCGGTCAGCATGGTACCACCGGCGGCGGCCTTGGTCACGGAACCGCCGGTACG
GGCGGTGCCTTGGGTGGCCAGCACCGTCGCTCCGGCAGCTCAAGCAGCTCCTCATCATCTGAAAGCGATG
GAGAAGGTGGTAGGCGAAAGAAGGGGATGAAGGACAAGATGAAGGAGAAGCTTCCCGGCGGCCATGGTAC
TACTACTGATCAGCAGCAATATGGTACGGCAGCAACCCACGGCCAAGCACAGCAGCATGAGAAAAAGGGC
ATCATGGACAAGATCAAAGAGAAGCTTCCCGGCGGCCAGCATTAGAATTAAAACAAATATGAAAAATATG
TAAGACCCACCATAAAATTATTACTACTAAATAATAAGAGGTAAGACCGAGTTATGAGGGGTCTCACACC

20. Plantacyanin

TTGGCTCAGGCTGCCATCTACACTGTTGGAGGTGCTGGTGGTTGGACCTTTAACACTATTGCCTGGCCTA
ATGGGAAAAACTTTAAAGCTGGTGATACACTTGTATTCAACTATAGTCCGGGTGCACACAATGTGGTGGC
AGTGAGCAAAGCAGGGTATGGTAGCTGCAAGACTCCAAGAGGAGCCAAAGTGTATCGGTCAGGAAAAGAT
AAATTGCCATCAATGCTGTTTGATTTTTATTATTATAATAATAATATTGTGTGCTTTTATCTATGTGCTT
TCATCATACCTCTTGTACCTGTTCTAATTTATACACTTCGTGAATCATGTAGAAGTTTTCAATAAATGGA
AAGGTGGGTGTGAATTGTGGTGTGTACCATTTATGTTAGTAAATGTTATTCATCATGGGTGTAGTAAGTT
TACGGCCCAATTGTTTTGAGAGAACGGCATATGACTTGAAAAAAAAAAAA
AAAAAAAA

21. EST from the Forward SSH library

GTAGTGAAGTATCATGTATAGTGCCCTATGTTATAAAGATTGCATGTGTTACTTAAATCTTTCTGTTAGA
TCGTAAGTACGATGCATGCATCATGTTTAATTAACCTGCTTAAGTTACCTTCCCTTTTAAAGTGGTTTGG
GTTAAGTAAGGTTCAATCCTATTCATGCTAACCTTCAGATCCCCCTATCTGTTCTCATCGTCCTAATTCT
GTCTTGTTTTAGGATGGCACGTATGAAGACGACTCCTCGCAAGAAAACGGGTCCGCAAGGAGTGCCGCGT
CATCAGTTGGCCGCCAGAGTTGATGGAGCAAGCAGTAGCCGTAATCCGAACCCGGATTCGGAGTCAGAGG
TTGCAAGGCTTACATCAGAAGTAGAGCGACTGAAGCGCAACATGCGTTTTTGGAAACAGTTCCAGAATGA
CAT

22. EST from the Forward SSH library

GTAGTGAAGTATCATGTATAGTGCCCTATGTTATAAAGATTGCATGTGTTACTTAAATCTTTCTGTTAGA
TCGTAAGTACGATGCATGCATCATGTTTAATTAACCTGCTTAAGTTACCTTCCCTTTTAAAGTGGTTTGG
GTTAAGTAAGGTTCAATCCTATTCATGCTAACCTTCAGATCCCCCTATCTGTTCTCATCGTCCTAATTCT
GTCTTGTTTTAGGATGGCACGTATGAAGACGACTCCTCGCAAGAAAACGGGTCCGCAAGGAGTGCCGCGT
TCCCGGAAAGGAGACAGAGGCCAGGGCTCGTGTTCGTGATTGGACTTTATG

23. Proline-rich protein

CCGGAGCCGGAGCCGAAGCCAAAGCCAAAGCCTCATCCTAAACCCACGCCAAAACCTAAGCCCAAGCCAG
AGCCGGAGCCAAAACCAGTACCTAAGCCTGAGCCTAAACCGGAACCAAAGCCAGAACCAAAACCTGAGCC
TAAGCCTGAACCTAAACCATACCCAGAGCCAAAACCGGAGCCGAAACCAGAGCCAAAACCGGAGCCAAAG
CCAGAACCCAAACCTGAGCCGGAGCCTAAACCTGAGCCTAAGCCCAAAAAGCCAAAACCCGAACCCAAAC
ACCCAAACCCAAACCCAAACCCCACCCCAAACCAA

24. Dehydrin 1b (DHN1b) gene alternatively spliced

CTGGTATGCATGGTGGGGAGCATCAGCAGCAGCCCCACCAACAATCCGGCAGCTCTGTAAGTTGCCATGC
CAAATGCCAAATCGTTATTAATTAATTAGCTTTGATATCTTGGTTTTGTTATTTTGACTGGCTTTGATAC
GCAGTCATCCGAGGACGATGGGCAAGGTGGGAGAAGGAAGAAAGGGATGAAGGAGAAGATAAAAGAGAGG
ATACCGGGCATGGGACGCAAGGACGAGCAGAAGCAAACGAGCGCCACAAGTACGCCGGGGCAGGGGCAGC
AACAGAAAGGAATGATGGAGAAGATCAAGGAGAAGCTGCCTGGGGGCCACTAGGTGTGTCTGGAAATGAA
GGGGACATGGTTTAATCGTTTGTAACTGAAACGCGAGTCTAGGTCCATAGGTCATAGGTGAATGGGTGAT
GCATAAGCGTTTGGTTATGAACCAGACGAGTCTAAATTGAGTTAATAAATTGCTGGACTGCTACTATATG
CAGCCTCGCATCAGCTTTCTGCTTTCTCAATGTAGTCGCTGTATGTCTGTCCTCTTCGTATATTAGTAGC
TGAGTGCTTATTAATGCTACACTTATGTTGTCATTTCTCATAAACTAGATTTTGTTTTGGTGTTACCACC
CCCAATCTGACTTCATGCTTAAGCGCAAGTTTATTGTGCTTTAATTTGATCAAATCTCGATATATTCAGG
TACCCTGTACATGCTCCAATTCAGGTGTTGCAGAAACCACATGTTCACAACGAATAATAAATGTAAATAA

ATGGCTACAGCAAGTTTTAGTATGTATTAGAAGAGAAAAAAAAAAAAAACTGGTGAAAACAGTACACGAA
A

25. Dehydrin 1a (DHN1a) gene alternatively spliced

CACCGACTCCATTGCCTATATAAATCCCAAACCTATTCCCCAACCTTTCATTCATCATCAGACGAAACCA
AGCTTACTGATTGATTCCGATTTCTTGTTGCCGCTTTCATACTCAGTTCCGTTTCAAATTTCATCGATCG
TTTCAACACAATGGCATATCAGCAAGATCCATGCGCCAACCCAACGCATCAGACTGGCAAGACCGGAGGC
CAGACCGACCAGTATGGAAACCCAGTCCATCAAACCGAGGCCATGGGCGCGTATGGTGCGGGAACTGGCA
CTGGTATGCATGGTGGGGAGCATCAGCAGCAGCCCCACCAACAACCCGGCGTGCTTCATCGCTCCGGCAG
CTCTGTAAGTTGCCATGCCAAATGCCAAATCGTTATTAATTAGCTTTGATATCTTGGTTTTGTTATTTTG
ACTGGATTTGATACGCAGTCATCCGAGGACGATGGGCAAGGTGGGAGAAGGAAGAAAGGGATGAAGGAGA
AGATAAAAGAGAGGATACCGGGCATGGGACGCAAGGACGAGCAGAAGCATACGAGCGCCACAAGTACGCC
GGGGCAGGGGCAGCAACAGAAAGGAATGATGGAGAAGATCAAGGAGAAGCTGCCTGGGGGCCACTAGGTG
TGTCTGGAAATGAAGGGGACGTGGTCTAATCGTTTGTAACTGAAACGAGAGTCTAGGTCCATAGGTCATA
GGTGAATGGGTGATGCATAAGCGTTTGGTTATGAACCAGACGAGTCTAAATTGAGTTAATAAATTGCTGG
ACTGCTGCTATATGCAGCCCGGCATCTGCTTTCTGCTTTCTCAATGTAGTCCCTGTATGTCTGTCCTCTT
CGTAT

26. CBF-like transcription factor (Cbf2) gene

TTTCGCACAAACGGAAAACTGGAAAAAAGAAGTTCCGGAAGACGCGACACCCGATATACAGGGGCGTGCG
CCAAAGAAATGAGAACAAATGGGTGAGTGAAGTGCGCGAGCCCAGTAAGAAGTCCAGGATATGGCTAGGC
ACCTTTCCTACTCCGGAAATGGCAGCTAGGGCCCATGATGTGGCTGCCCTAGCGCTCAGAGGCCACTTTG
CTTCCCTCAATTTTCCCGATTCGGCTTGGCGCCTTCCCCGCGCCAGGTCATCCTCCGCAGGAGACGTACA
GTTCGCGGCGATTCAGGCCGCTAAGGCCTTTCAGCAACCTCCATCATCGTCATCTTCTACACCTTTTATA
ATGGATAATATGTCAGCAGGGTCGAGGAAGATACTAGAAACGTCTTCTGTCGTAGACACACCTCAGTTAA
AAAGCCAAAAGAAGGTTGTGGGAGTTTCACCGGTAGATAGTAAGAGCTGGGAGAAAGCCGGAGATGGTTT
CCCGACAGCGTTCGTTGATGAGGAGGCAGTGTTTAATATGCCAGGTTTAATTGACAGCATGGCCGAGGGT
CTGCTTCTTACTCCACCTGCTATGTGTGAAGGCTTCAGTTGGGACGATGCAGTTTCACACATTGACTTGT
CTTTGTGGAATCATGATTTCCTATCTTAAGTTTCTTCTCTTGACATTTCTAGTCAATCAAGTACAGCCCA
GGATGGACTTCAGGT

27. CBF-like transcription factor (Cbf3) gene

CGCCAAAGAAATGGCAATAGATGGGTATGCGAAGTGCGGGATCCCAAAACCAAGTCCAGGATATGGCTTG
GGACTTTTTCCACTCCCGAAATGGCGGCTAGGGCGCATGATGTGGCCGCCCTAGCATTCAGAGGCAATTT
TGCTGCGCTCAATTTCCCCGACTCCGCGTCTCGCCTGCCGCGCGCCAAGTCATCTTCAGCTGGGGACATA
CAGGTGGCGGCCCTTGCGGCTGCCATGGCTTTTCGTCCTGCTGCGCCGTCTTCATCCTCTTCTTATATTT
CACATGTCACTCCCTGTAGCGAGGAATTAGAAACTTCATGCAGTGAAGACTCGCCTCAGTTAGAGAGCCG
AAAAAAGGTTGTGGGAGTTGCATTGGAGGATTCTGAGAGCTCAGAGGGCGCTCCATATGGTTCGAGCACG
GTGTTCATGGATGAGGAGGCATTGTTTAATATGCCAGGTTTGATTAACAGCATGGCAGAAGGTTTGCTCC
CCTAGTGTTCACATT

28. CBF-like transcription factor (Cbf1) gene

ACGACACCCAATATACAGAGGCGTGCGGCAAAGAAATGGGAACAAATGGGTGTGTGAAGTGCGGGAACCC
CTTAAGAAATCCAGGATATGGCTAGGCACCTTTCCCACCCCGAAAATGGCTGCTAGGGCTCATGATGTGG
CTGCCCTAGCGCTTAGAGGCCGCTTTGCTTCCCTCAATTTCCCCGACTCGGCTTGGCGCCTTCCACGGCC
CAAGTCGTCCTCTGCAGAAGACATACAAGTAGCAGCGCTTGAAGCCACCAAGGCTTTCAACCCAACTGCA

CCATCTTCGTCCTCCTTGGCCTCTGCATTGGATAATATGTCAGGAGTTGCAGACTCGAAGAAGGTACTAG
AAACTTCACCAAATGTGGAGTCACCTAAGTTAAAGAGCCAAAGGATGGTTCTGGAAGTTTCTCCGGTGGA
TACTAAGAGGTCAGAGAAGGTTGGAGATGGTTCAACGACAGTGTCCATGGACGAGGAGGCAATGTTCAAT
ATGCAAGGTTTAATTAACAGCATGGCTGAGGGTTTGCTCCTTACTCCACCTGCTATGTGTAAAGGATTCA
GCTGGGATGATGCGACTGATTCCCACATTGACTTGTCTTTGTGGAATGATGATTAGTTTGACATAGGTGC
GAAGAATTAA

29. Germin-like protein 2

AACTCATCATTATAAATACACAACCCCACTTTTTCTCTCTCCCCATTCTAATTTGAGATATCCATCTACT
TCAGGACGTCTGTGTTGCCGATCTCACTTCTGGTGTAAAGGTGAATGGGTTCAGCTGTAAGGACGCGACA
AACATAACTGCAACGGATTTCTTCTTCGACGGCCTGGCGAAACCGGGTCTGACCAACAACTCCATGGGGT
CTCTGGTAACAGGTGCTAATGTGCAGAAGATTCCTGGTCTCAACACCCTTGGCGTCTCTCTCTCCCGCAT
CGACTACGCACCAGGTGGTCTCAACCCACCCCACACTCACCCTCGTGCTACAGAGATGGTGTTCGTGCTT
GAAGGCGAGTTGGATGTGGGGTTCATCACCACCTCCAACACTCTCATTTCCAAGTCCATTAAGAAAGGGG
AGATCTTTGTGTTCCCAAAGGGGTTGGTCCACTTCCAGAAGAACAACGGCGAAGTCCCTGCTGCCGTCAT
ATCTGCTTTTAACAGCCAGTTGCCTGGAACCCAGTCTATTGCGGTGAGCTTGTTTGCTGCCTCACCACCC
GTGCCTAACAATGTGTTGACCAAGGCTTTCCAGGTGGGTACTAAGGAGGTTGAGAAAATCAAGTCCAGAC
CCTTGTCATGTTTACCGCCAAAAAAAAAAAAAAAAAA

30. Germin-like protein 3

CATTTGTGTTGCAATTAGTGACCCCAAGGATGGTGTGTTCGTGAATGGAAAGTTCTGCAAGGATCTAAAG
CTTGCTTCAGCTGATGATTTTTTCTACTATGGGCTCCACATTCCAGGAAACATAACGAACCCAGTTGGCT
CAATGGTCACTCCTGTAAATGTTGAACAAATACCAGGACTTAACACCCTTGGCATATCCATGGTTCGTAT
TGATTATGCACCATACGGTCAAAACCCTCCTCACACACACCCTCGTGCTACTGAGATCCTAGTTGTCTTG
GAGGGAACCCTCTTAGTTGGCTTTGTCACATCCAACAACGAAAACCGCCTCATCAGCAAAGTCCTTTACA
TGCCTTTGCTGGTCTGAGTAGCCAAAATCCAGGTGTTATCACCATAGCAAATGCAGTCTTTGGATCAGAT
CCACCCATCGATCCTGATGTTCTCACCAGGGCCTTCCAACTTGACGAGGATGTGGTCAAGGACCTCCAAT
CCCGCTTCTGGTGGGACAACAACTAAAAGAATATTTAGTTGAGCAAGTGATGAACTATTTGCTTGTTTAA

31. ACC synthase (ACS1) gene

TGGGGCAACGTCCGCCAACGAGACACTCATGTTCTGCCTTGCTAACCCAGGCGAAGCTTTTCTCCTTCCC
ACCCCATACTACCCAGGGTAATTACCTTGCTATTTCTGTTTGCAACTGCTATAAAGCCTTTAAGTTGTTT
ATTGCTTTACGCATGAAATGTAGTTAACTGACTTTTTTTTTGCGATTCTTGCAGATTTGATAGAGGTCTC
AGGTGGCGTACCGGAGTTGAAATTGTACCGATACAGTGTTCTAGTTCGAATGGCTTCCAGATTACTGAAT
CCGCTCTCGAAGAAGCTTACCACCAAGCTCAAAAACGCAGCCTCAAAGTTAAAGGCGTATTGATCACCAA
CCCCTCAAATCCGTTGGGCACCACAACCAGCCGAGATGAGCTCCTCAATCTCCTGGTTAACTTCATTACC
GCAAAAGGAATCCATCTAATTAGCGACGAAATTTATTCCGGTACTGTTTTCGACTCACCAGGCTTCGTGA
GCATCATGGAGGTTCTAATGGACAGGAACTACATGAACACTGAAGTTTGGAAAAGAGTTCACATTGTCTA
AATGCTCATTTCTGGACTTCAAAACACTGGCATTGACTGCCTCAAGAGCAACGCCGGTTTGTTTTGTTGG
GTTGACATGAGACACCTCTTGAGCTCCAACACATTCGAAGCCGAGATGGAACTCTGGAAGAAAATTCTCT

ii) Water Stressed Leaf tissues:

1. Isoprene synthase

GATGCTGTTACAAAAACTAGCCTTCATGCTACTGCTCTTAGCTTCAGGCTTCTCAGACAGCATGGCTTTG
AGGTCTCTCAAGAAGCGTTCAGCGGATTCAAGGATCAAAATGGCAATTTCTTGAAAAACCTTAAGGAGGA

CATCAAGGCAATACTAAGCCTATATGAAGCTTCATTTCTTGCCTTAGAAGGAGAAAATATCTTGGATGAG
GCCAAGGTGTTTGCAATATCACATCTAAAAGAGCTCAGCGAAGAAAAGATTGGAAAAGACCTGGCCGAAC
AGGTGAATCATGCATTGGAGCTTCCATTGCATCGAAGGACGCAAAGACTAGAAGCTGTTTGGAGCATTGA
AGCATACCGTAAAAAGGAAGATGCAGATCAAGTACTGCTAGAACTTGCTATATTGGACTACAACATGATT
CAATCAGTATACCAAAGAGATCTTCGCGAGACATCAAGGTGGTGGAGGCGTGTGGGTCTTGCAACAAAGT
TCTGAAGGACAAGGGGGAAAACATTCTTCCATACCTAACAAAAGCGTGGGCAGATTTATGCAATGCATTC
CTACAAGAAGCAAAATGGTTGTACAATAAGTCCACACCAACATTTGATGAATATTTCGGAAATGCATGGA
AATCATCCTCAGGGCCTCTTCAACTAGTTTTTGCCTACTTTGCCGTTGTTCAAAACATCAAGAAAGAGGA
AATTGATAACTTACAAAAGTATCATGATATCATCAGTAGGCCTTCCCACATCTTTCGTCTTTGCAACGAC

2. Water-stress protein

ACATTACAAAAGAGGGATGTAGTATTTGGAGTTGATCGTAGCGCAAAGGTTTCCTTTGCTGATTCCTACC
CTCAAGTCGATGATGAAATAATGGCACAGGTCAGAACTGTATTTCTTGATGGCCTTCCTCCCTCATGGGA
TGAAGACCGTGTTAAGAAATATCTCAAAAAGTATGGAGCTATTGAGAAAGTCGAACTTGCACGAAACATG
CCAGCTGCCAAGAGAAAGGATTTTGGGTTTGTTACATTTGATACACATGATAATGCTGTTGCATGTACTG
AAGGGATGAGTAACTCTGAGATCGGTGAAGGTGAACACAAGGCAAAAGTAAGGGCTAGACTGTCGAGACC
ATTGCAGAGACCACCTAGAATGAAACATGGATTGAGAGGAAATTTTAGAGTTGGGCATGGTGCCCCGCGA
TTAGCCGCTTACCATCCATCAGGAGTCATCCGTTGAAGCGGCCCATAGATATTAGAGATAGGCGTCCTGC
TATGTCAATGCCAGATAGAGCTAGGCGTTTCCCTCCAGAGAGATCTTATGACAGGAGGCCCCCAGCTCCA
GTTTACCTGAAGAGAAGTCCAAGGAGAGAATACGGGAGGCGTGATGAACTTCCTCCTCCAAGGAGCAGAG
CTACAACTGATTACATTACCAGAGTTCCGGTTGATAGACGTCCCTCTTTGAGGGATGATTATTCACCCCG
GGGATCAGGTTATTCAGACCTCGGTCCTCGTAGTGCTCCCCGTCTTTCTGATAGGCGAGCTTATGCTGAT
GATAGTTATGGGGAGAAGTTTGACCGGCCTTTAGTGGCCTATAGGGAGGGTCGCGGCCGTGATTATGACA
CCATTTCTGGATCAAAGCGTCCGTATGCAGATATGGATGATGTGCCTCGGTACCAAGAAATCGGTGTCCG
TCAGTCTAAGGCACGTTTAGACTACGACATTGGTGGCAGCAGTGCTCGGTATGGAGATACATATAGTGAG
AGGTCTGGACGACCACATGCGGGATATAGTAGCAGCCGATCTATCCCTGTTCATGATTCAGCATATGGCA

3. Universal stress protein (USP) family protein-like

CGGTGGATTTCTCGGAGTGCAGCAAGAAGGCTCTGAACTGGGCGATCGATAACGTGGTTCGAGACGGCGAT
TATCTTATCCTGGTAACCGTCGCTCCCAGTATGAATTACGAGGAAGGCGAGATGCAGCTCTGGGAGACCGT
TGGATCACCGTTGA

4. Cell division protein Os09g0541000

TGGGTAAGTGGTCGCTGTACCGTGCCCTCATCGCGGAGTTCATGGCGACGCTCATCTTCCTCTACGTGAG
CATCGCCACCGTGATCGGGTACAAGAACCAGAGGGCCACCGTCGACGCGTGCACCGGCGTCGGCTACCTC
GGCGTGGCGTGGTCGTTCGGTGCCACCATATTCGTCCTCGTCTACTGCACCGGCGGCGTCTCCGGCGGGC
ACATCAACCCGGCGGTGACGCTGGGCCTCTTCTTCGGGCGGAAGCTGTCGCTCGTCCGCACCGTGCTGTA
CGTCGTGGCGCAGTGCCTCGGCGCCATCGCCGGCGCCGGCATCGTCGGCACGTTCATCCTCGTCTACACC
GTCTTCTCCGCCACCGACCCCAAGCGCACCGCCCGCGACTCCTTCATCCCCGTACTCGTGCCGCTGCCGA
CCGGTGATCGGGGCGTTCTTGGCGGCGGCGTACCACAAGCTGGTGCTGCGCGGCGAGGCCGCCAAGGCGC
TCAGCTCGTTCAGGAGCACCAGCGTGACGGCGTGAGGAAGACGACGATGTTCATTTGATACGAGAGATCG
ATCAGCTGCTTGATTAATTGTTCTTGATTTCGTTGTTCAATTTACAATCTACGACTATGGACGATCATGC
ATATGATATGATCGATGCATGCGTCTGTTGTCAGTTTTATTTGGGACTTTCAGTGTGAGAGTGTGAATGG
TTGTAAAATAAGTGGAGTGCCTGTATGTATCTGTATGCATGCACGGCACGGTACGCGTGTTTATGGATGA

5. Rab21 gene for water-stress inducible protein RAB21

TAACCTATCATTCCCACAATCTAATCCACTTATTTCTCTTCCCATGATCTTATCCTCTCATTTCTCCTCA
CTACTTTTGCATTTGTAGGAAACACAATGACACCGTCGAAGAAAGCTGGTGGAGCACCGTAGCCAGCAAT
CACCAAAACACAGAGGGGAGGAGGTCGGCAGCGGCCATGCGGACGGCGATGAGACAACGCGACGCAAAGA
GGGAGGAGGACGTTGGCGATCATGCTGGTGTTGGCGGAGGAGGTCACTGGCCATGCGAATGACAGCGGGG
CAGCGCAACACAAAAAGGGGGGAGGATGCCGGCGACCACGCTAGTACCATGAAGCAAGATGATGTGAAAG
GGAGGACCGGACGAGGGTTGGACCTCTGCCGCCGACGTGAAGAGCGTGATGTGTAGAAGGAGATGTTAGA
CCAGATGCCGACGCAACTTAGCCCTGCAAGTCACCCGACTGCATATCGCTGCTTGCCCTCGTCCTCATGT
ATGTTAGAATATTCATTCCGTTGAAACAATCTTGGTTAAGGGTTGGAACATTTTTATCTGTTCGGTGAAA
CATCCGTAATATTTTCGTTGAAACAATTTTTATCCGACAGCACCGTCCAACAATTTACACCAATTTGGAC
GTGTGATACATAGCAGTCCCCAAGTGAAACTGACCACCAGTTGAAAGGTATACAAAGTGAACTTATTCAT
CTAAAAGACCGCAGAGATGGGCCGTGGCCGTGGCTGCGAAACGACAGCGTTCAGGCCCATGAGCCATTTA
TTTTTTAAAAAAATATTTCAACAAAAAAGAGAACGGATAAAATCCATCGAAAAAAAAAAACTTTCCTACG
CATCCTCTCCTATCTCCATCCACGGCGAGCACTCATCCAAACCGTCCATCCACGCGCACAGTACACACAC
ATAGTTATCGTCTCTCCCCCCGATGAGTCACCACCCGTGTCTTCGAGAAACGCCTCGCCCGACACCGTAC
GTGCGCCACCGCCGCGCCTGCCGCCTGGACACGTCCGGCTCCTCTCCCGCCGCGCTGGCCACCGTCCACC

6. DNA, cis-acting regulator of water stress specific gene

TTTTGTCACATATACTGCATTGCAACAATTGCCATATATCACTTCTGCCATCCCATTATATAGCAACTCA
AGAATGGATTGATATATCCCTATTACTAATCTAGACATGTTAAGGCTGAGTTGGCAGTCCATCTTCCAAC
CACCACCTTCGTTTCGCGCACATACTTTCAACTACTAATGGTGTGTTTTAAATAGCTTTAAAAAATTATA
TTGATCCATTTTTTTAAAAAAAATAGCTAATACTTAATTAATCACGTGTTAAAAGACCGCTCCGTTTTGC
GTGCCACGGAGGGATAGGTTCACATCCTGCATTACCGAACACAGCCTAAATCTTGTTGTCTAGATTCGTA
GTACTGGATATATTAAATCATGTTCTAAGTTACTATATACTGAGATGAATAGAATAAGTAAAATTAGACC
CACCTTAAGTCTTGATGAAGTTACTACTAGCTGCGTTTGGGAGGACTTCCCAAAAAAAAAAGTATTAGCC
AGAGGCAAGAGCATCCGTATTAACCAGCCTTTTGAGACTTGAGAGTGTGTGTGACTCGATCCAGCGTAGT
TTCAGTTCGTGTGTTGGTGAGTGATTCCAGCCAAGTTTGCGATGGCTTCTCAGCAGGAACGGGCTAGCTA
CCACGCCGGCGAGACCAAGGCCCGCGCCGAGGTGAGGTTTCCCTTCGTGCATCCGCGCGCGCCGACCGCA
CGTACACCACCACGTTTGTCCCATCCGTTGCGCGTGGTTACATTTGTTGTTTGCTTGTTTCGTGCGTTGC
AGGAGAAGACGGGGCGCATGATGGGCACGGCGCAGGAGAAGGCGCGGGAGGCCAAGGACACGGCGTCCGA
CGCCGCGGGGCGCGCGATGGGCAGGGGACACGGCGCCAAGGAGGCGACCAAGGAGAAGGCGTACGAGACC
AAGGACGCGACCAAGGAGAAGGCGTACGAGGCAAAGGACGCGGCCTCCGACGCCACCGGCCGCGCCATGG
ACAAGGGCCGCGCCGCGGGCGCCACGAGGGACAAGGCGTACGATGCCAAGGACAGGGCGGCTGACACGGC
GCAGTCCGCCGCCGACCGCGCCCGCGACGGCGCCGGGCAGACCGGGAGCTACATTGGACAGACCGCCGAG
GCCGCCAAGCAGAAAGCGGCCGGCGCCGCGCAGTACGCCAAGGAGACCGCGATCGCCGGCAAGGACAAGA

7. Arabinogalactan protein

CAACACCCGCCAAACCATCACCGTCCTCGCCATCGACAATGCCGCCATGGCTTCCCTCACCTCCAAGCAC
CTCCCCATTTCCACCCTCAAAAACATCCTCTCCCTCCACGTCCTCCTCGATTACTTCGGCGCTAAAAAAC
TCCACCAAATCACCGACGGCTCTGCCCTTGCTGCCACCATGTACCAAGCCACCGGCTCCGCCCCCGGCAC
TGCCGGTTTTGTCAACATCACCGACCTCAAAGGCGGCAAAGTCGGCTTCGCCGCCACCAACCCCGCCTCT
GATGAAGGCGATTCCGACAGCACACCCTCTCTCAACTCAACCTTCATCAAATCCCTAAAGGAAATCCCTT
ACAACATCTCAGTCATCCAAATCAGCCACATTCTATCCTCACCTACCGCCGAGGCGCCTTCTCCGGCGCC
TTTCCCGACGCACTCGAGGTTTTCACCACCAACACAGAAGGCGGATTAACCGTCTTTTGTCCTTCAGACG
ACGCCTTCAAAGGCTTCCTCCCTAATTTCAAAAACCTAACAAAAGAAGAAAAAAACTCTCTGCTACTCTT
CCATGGAATTCCTGTCTACAACTCCATGGCATTGCTCAAAACCAGCAATGGAGTGATGAACACTCTCGCT
ACCGACGGTAAGAACAAATTCGACTTCACTGTTCAAAACGCAGGTCAGAAAGTGACATTGAAGACGAAGG
CTGTCACCGCGACCATCACCGCTACTCTGTTGGACGAAGATCCAGTTGCGATCTACACCATTGATAAGGT

8. Water-stress protein

ATTGCAGAGACCACCTAGAATGAAACATGGATTGAGAGGAAATTTTAGAGTTGGGCATGGTGCCCCGCGA
GGTGGCCGTTTACCATATTCTCGTGCTCCTCCACCTCGCAGGCCTCCGCCACGTCTTATTCGGCCTGCTG
TTAGCCGCTTACCATCCATCAGGAGTCATCCGTTGAAGCGGCCCATAGATATTAGAGATAGGCGTCCTGC
TATGTCAATGCCAGATAGAGCTAGGCGTTTCCCTCCAGAGAGATCTTATGACAGGAGGCCCCCAGCTCCA
CCATTTCTGGATCAAAGCGTCCGTATGCAGATATGGATGATGTGCCTCGGTACCAAGAAATCGGTGTCCG
TCAGTCTAAGGCACGTTTAGACTACGACATTGGTGGCAGCAGTGCTCGGTATGGAGATACATATAGTGAG
AGGTCTGGACGACCACATGCGGGATATAGTAGCAGCCGATCTATCCCTGTTCATGATTCAGCATATGGCA
GCAGCCGTCATGGAATGAGTTATGGAGGTTCTGCTAGCAGTGCTGATGCTGGTGGTATGTACCCACCGAG

9. Stress-inducible protein

TAGGTGCTGCGTTTATTGGATTGTCCAAGTTTGATGAAGCGGTTGATTCGTATAAGAAAGGATTAGAGAT
TGATCCGAGTAATGAGATGCTTAAATCGGGATTAGCTGATGCTTCGAGATCTAGGGTTTCGTCAAAGTCG
AATCCTTTTGTTGATGCGTTTCAAGGGAAGGAGATGTGGGAGAAGTTGACGGCGGATCCGGGGACTAGGG
TTTATTTGGAGCAGGATGATTTTGTTAAGACGATGAAGGAGATTCAGAGGAACCCTAATAATCTTAATTT
GTATATGAAGGATAAGAGAGTTATGAAGGCTTTAGGGGTTTTGTTGAATGTGAAGTTTGGTGGATCTAGT
GGTGAAGATACTGAGATGAAGGAGGCTGATGAGAGGAAAGAGCCTGAACCGGAGATGGAACCTATGGAGT
TGACGGAGGAGGAGAGGCAGAAGAAGGAGAGAAAGGAGAAGGCTTTGAAGGAGAAAGGGGAAGGAAATGT
ACTGTGACAAGGCTGTTGAAAGAGGCAGAGAACTTCGTTCTGACTTCAAGATGATAGCAAGAGCTCTGAC
TAGAAAAGGATCTGCTCTAGTGAAAATGGCGAGATGCTCGAAAGACTTTGAGCCTGCGATTGAGACTTTC
CAGAAAGCTCTTACAGAGCATCGTAATCCAGATACATTGAAGAAACTGAACGATGCTGAGAAAGTCAAGA
AAGAGCTGGAGCAACAGGAGTACTTTGATCCTACGATAGCCGAGGAGGAGCGAGAGAAAGGTAATGGATT
CTTTAAAGAACAAAAGTATCCAGAGGCAGTGAAGCATTATTCAGAAGCAATCAAAAGAAACCCGAACGAC

10. Stress enhanced protein1

CTCTCGCCTTTTCTCTTCCCAATTCTGGTGCCCTAAAGCTAGCCACAATCACAAACCCAACTTCTACATG
TCGGGTTCATGTTCCGCAACTTGCTGGAATCCGATCCACCTTCGCTTCTGGTTCTCCTCTCTTGCCATTG
AAGTTGAGTATGACCCGTAGAGGAGGAAACAGAGCAGCATCAGTTTCCATAAGAAGTGAGCAAAGTACAG
AAGGAAGCAGTGGTTTGGATATATGGCTTGGTCGTGGCGCCATGGTTGGTTTTGCAGTTGCCATTACTGT
TGAGATTTCCACTGGAAAAGGACTTCTTGAGAATTTTGGAGTAGCAAGTCCATTGCCTACGGTTGCTTTA
CAAGAATCTTGTTTTGTGATGCTGCCGAGGATCATTTTCTTGTATTAAGAATCTTGTATTATATATCACT
GTAACTTCTACATCCATCAGTGGAGAATCTTACATTCTTAGTTTTTTTTGGCAACATACATGATCTATAC

11. Water stress specific subtracted cDNA

CGCTGCCGTGACGGAAGGAAGAGGAGGAGCAAGGAGCACGACGCGATGGGCCACTCCAACGTGTGG
AACTCGCACCCTAAGAACTACGGCCCTGGCTCCCGCGTCTGCCGGGTCTGCGCGAACCCCCAC
TCAGGA

12. Open stomata 1, SNF1-Related protein kinase

AAGGATCTCAAGAAACCCACTTGTGTTGTTGGTTAGATACTTCACGGGTCTCTGAAAACGTCTCTTTCTC
ACAACCATAACTTGATCACCCAATACTCCTTTTCTCATCTTAAAGGCTCAAATTCATCCACGTCACACCG
TTGTTCATTTCCTCTGATGTCTTGGTGTCGTTAGATATTGTCTCCCAAAAAAGAAATCTTCTTGACACAG
AGATTGAAGTCGCAAAGAGACAGAGGAAAGAGGGGGAGAAAATGGATCGACCAGCAGTGAGTGGTCCAAT
GGATTTGCCGATTATGCACGATAGTGATAGGTATGAACTCGTCAAGGATATTGGCTCCGGTAATTTTGGA
GTTGCGAGATTGATGAGAGACAAGCAAAGTAATGAGCTTGTTGCTGTTAAATATATCGAGAGAGTGTTGT
TTTAAAGGCTCTAGGTGTTTCTTTTGTTATGGAACGTGGTATTAATGGTGGGACTTTTTGTATTTGTACA
TGCAGATGTTTGGTCTTGTGGGGTTACTCTGTATGTCATGCTGGTTGGAGCATATCCTTTCGAAGATCCC

GAGGAACCAAAGAATTTCAGGAAAACTATACATAGAATCCTGAATGTTCAGTATGCTATTCCGGATTATG
TTCACATATCTCCTGAATGTCGCCATTTGATCTCCAGAATATTTGTTGCTGACCCTGCAAAGAGGATATC
AATTCCTGAAATAAGGAACCATGAATGGTTTCTAAAGAATCTACCGGCAGATCTAATGAACGATAACACG
ATGACCACTCAGTTTGATGAATCGGATCAACCGGGCCAAAGCATAGAAGAAATTATGCAGATCATTGCAG

13. Water stress specific subtracted cDNA

ATACATGAGAGTAGATAGACAGAGAACATGAAGCAAAACAGAGACACAAGCTGCAGCGGCACACATGGGGC
ACCGGCCCCACCTACCACCACCAGACCGGACGACGCACACTTTATTTAGCCCATGTCACTCGCAGTCCGCA
CCGGGCACTGCTATAGCTACGACACACACACTGTCACACAGAAACCGTCGCGTTCCGGTT

14. Water stress specific subtracted cDNA

AGCCGGGTCCTACTACTACCTCGATGACTAATGAGGAAGTGCAGGTTCTTCTTGGAGAGCCATATGTCGC
ATAATCTTTCCAGAGCGTGGAAAGGAGAAGCTACTTTGAAGCTACCACTGCATCATGGCATGTGACTTGT
TGAATAATTGGTTGCAGGAGTAGAGTGCGCCGGTTATAACTTATAAAGCAGAGCATGCCGTGTTGATACT
GAGTTTTTTGTTGGCTTGACCTATATGAAGAGAAGGATCGGTGTTAAAATGAGATTTGCGAGGGGGAACT
AATTTCTGTTTTTAAGCAATACTGTACTGTCGTTTTGCTATCAAGTATATTTGACCCTCGATTATATAGA
TTACATTGAAGTTTTTGGGCAACAATGTGAAATCTTGTGATAAATCTCTGTGCTTATTGGTTATCTGATT

15. EST from the Forward SSH library

GCGGGAACAAAACGGAAGCTCGATGGCTGACAGGTGGGTGTACCCCCGGCCCACATGTAAAAGACACT
GGATGGGCCCAAAGGAAGAAAGTTTTGTTTCAAGAA

16. EST from the Forward SSH library

ATGCTGCTTCCAAGATCCGTAAGAGAGCTTTCGAGTCAGGTTCATCCCAGTCCAGAGCCCCAGTGGCCAA
CCGTCCGGCTTATCGATCGCCTGCCCCAGGTGCAAGGTTTAGGCCACCGCAAAGGAAGAATCAGAATGCT
CAGCAACCCCAGAGGAACCAGAAACCATTCAAGATAGCATTGCCTCAGGCGAAGATAGGACAAGGTAGTT
CGTCTGGAACTGTGGCTCAAGTGAAGGGACCGTGCTTCAACTGCAACCAACCCGGGCACTTTGCAAAGTT
TTGTCCCTACCCAAAGAAACAGCAGACGCAGTATCAAGCTCGTGCGCATCACACCACCGTGGATGACATC
CCTAAAGGAGAACCGGTAACAGCTGGTATATTTTCTATCAACAATCATCCTGCAGTAGTCTTGTTTGATT

17. AhDSa269 drought stressed subtracted Adapter ligation cDNA

CGCAAGCTTGCTATGAAGCACCCGGATAAGTGGGCCCGGGACCCTTCCACCGCTGGAGAAGCCAAGC
GCCGCTTTCAGCAAATCCAGGAAGCTTACTCAGTGGATCAGTCCAAGAGGTCAATGTACGAT

18. AhDSa262 drought stressed cDNA similar to cyclic nucleotide-and calmodulin-regulated ion channel 9

CGCGCGCGTGTTTTTTATGATAAGAAAGGTTGCGGTGTAGCTTATGCTTAGCACTTTTAGAGATAG
TATTTGTTGAGGGCTGTCTTATTGTCAATTCTTTGGCGATTATAAGATTTGGGTGGTGTGGGGGT
TATTTTTTTGATCAAAGGAAGGGTGAGT

APPENDIX C.

WATER STRESS RESPONSIVE DIFFERENTIALLY EXPRESSED PROTEIN SPOTS SHOWING PEPTIDE MATCHING WITH NCBI DATABASE

The proteins affected in water stress were isolated, and identified the peptide sequences using LC/MS. The identified proteins are listed below (Table 8).

1. LLEYGNMLVQEQENVKR
YLSEAALGDANEDAIKR

gi|18476502 (100%), 5669.7 Da
ultraviolet-B-repressible rubisco activase [Pisum sativum]
3 unique peptides, 3 unique spectra, 3 total spectra, 34/50 amino acids (68% coverage)

P K M T L E K **L L E** **Y G N M L V Q E Q E** **N V K R** V Q L A D K **Y L S E A A L G D A**
N E D A I K R G T F

2.

GLAYDVSDDQQDITR
NFMTLPNIK
SFQCELVFAKMGINPIMMSAGELESGNAGEPAK
MCVLFINDLDAGAGR

gi|10720248 (100%), 48202.4 Da
Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor (RuBisCO activase) (RA), gi|303351
2 unique peptides, 2 unique spectra, 2 total spectra, 72/441 amino acids (16% coverage)

M A A S L S T V G A V N R T L L N L N G S G G G A S G P S S A F F G T S L K K V
I S S R V P N S K L T S G S F K I V A A D K E I E E T Q Q T E G D R W R **G L A Y**
D V S D D Q Q D I T **R** G K G L V D S L F Q A P M D A G T H Y A V I S S H K Y L S
A G L R Q Y N F D N I K D G F Y I A P A F L D K L V V H I A K **N F M T L P N** **I K**
V P L I L G V W G G K G Q G K **S F Q C E** **L V F A K M G I N P** **I M M S A G E L E S**
G N A G E P A K L I R Q R Y R E A S D L I K K G K **M C V L F** **I N D L D A G A G R**
L G G T T Q Y T V N N Q M V N A T L M N I A D N P T N V Q L P G M Y N K E D N A
R V P I I V T G N D F S T L Y A P L I R D G R M E K F Y W A P T R E D R I G V C
K G I F R T D G V P E K D I V E L V D K H P G Q S I D F F G A L R A R V Y D D E
V R K W I S G V G V D S V G K K L V N S K E G P P T F D Q P K M T L D K L L L Y
A S M L V Q E Q E N V K R V Q L A D Q Y L N E A A L G N A N E D A I K S G S F F
K

3.

VAINGFGR
AVALVLPTLK

gi|77540210 (100%), 43221.5 Da
glyceraldehyde-3-phosphate dehydrogenase A subunit [Glycine max]
2 unique peptides, 2 unique spectra, 2 total spectra, 18/403 amino acids (4% coverage)

M A S A T F S V A K P A L Q A N G K G F S E F S G L R S S S G F L P F S R K S S
E D F H S V I A F Q T Y A V G S S G G Y K K G V T E A K L K **V A I N G F G R** I G
R N F L R C W H G R K D S P L D V I A I N D T G G V K Q A S H L L K Y D S I L G
T F D A D V K P V G S N V I S V D G K E I K V V S D R N P A N L P W K D L G I D
L V I E G T G V F V D R E G A G K H I Q A G A K K V L I T A P G K G D I P T Y V
V G V N E Y D Y S P D E P I I S N A S C T T N C L A P F V K V L D Q K F G I I K
G T M T T T H S Y T G D Q R L L D A S H R D L R R A R A A A L N I V P T S T G A
A K **A V A L V L P T** **L K** G K L N G I A L R V P T P N V S V V D L V V Q V S K K T
F A E E V N A A F R E S A D N E L K G I L S V C D E P L V S V D F R C T D V S S
T V D S S L T M V M G D D M V K V I A W Y D N E W G Y S Q R V V D L A D I V A N
K W K

4.

LVGNLSWR
TPDGGFFTR
TDNTCGPEPPLVER

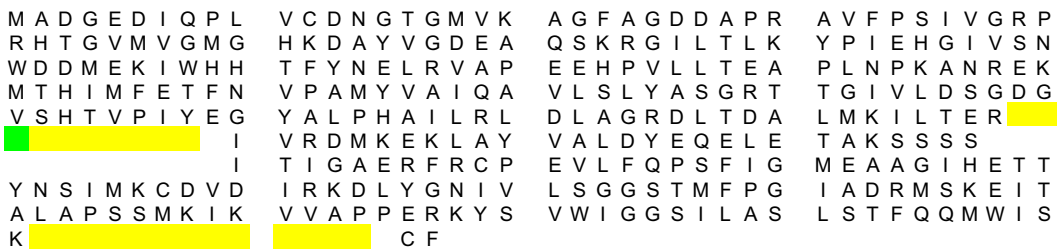
gi|1463123 (100%), 54562.1 Da
violaxanthin de-epoxidase precursor [Nicotiana tabacum]
3 unique peptides, 3 unique spectra, 3 total spectra, 31/478 amino acids (6% coverage)

M A L A P H S N F L A N H E T I K Y Y V G S K L P G H K R F S W G W E D Y F G S
I V V A K I C S S R R I P R Y F R K S P R I C C G L D S R G L Q L F S H G K H N
L S P A H S I N Q N V P K G N S G C K F P K D V A L M V W E K W G Q F A K T A I
V A I F I L S V A S K A D A V D A L K T C T C L L K E C R L E L A K C I S N P A
C A A N V A C L Q T C N N R P D E T E C Q I K C G D L F E N S V V D E F N E C A
V S R K K C V P R K S D V G D F P V P D P S V L V Q K F D M K D F S G K W F I T
R G L N P T F D A F D C Q L H E F H T E E N K **L V G N L S W** **R** I R **T P D G G F F**
T R S A V Q K F V Q D P K Y P G I L Y N H D N E Y L L Y Q D D W Y I L S S K V E
N S P E D Y I F V Y Y K G R N D A W D G Y G G S V L Y T R S A V L P E S I I P E
L Q T A A Q K V G R D F N T F I K **T D N** **T C G P E P P L V E** **R** L E K K V E E G E
R T I I K E V E E I E E E V E K V R D K E V T L F S K L F E G F K E L Q R D E E
N F L R E L S K E E M D V L D G L K M E A T E V E K L F G R A L P I R K L R

5.

GYMFTTTAER
GEYDESGPSIVHR

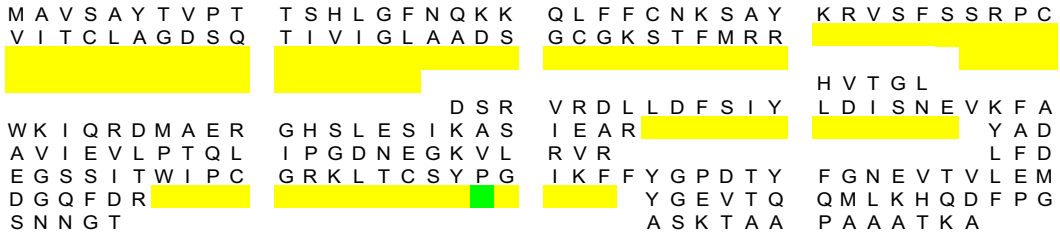
gi|32186896 (100%), 41734.6 Da
actin [Gossypium hirsutum]
2 unique peptides, 2 unique spectra, 2 total spectra, 23/377 amino acids (6% coverage)



6.

LTSVFGGAAEPPRGGNPDSNTLISDTTTVICLDDYHSLDR
GVTALDPRANDFDLMYEQVK
KPDFDAYIDPQK
LDELIYVESHLSNLSTK

gi|125578 (100%), 44115.9 Da
Phosphoribulokinase, chloroplast precursor (Phosphopentokinase) (PRKase) (PRK), gi|167266|gb|AAA33034.1| p
6 unique peptides, 7 unique spectra, 7 total spectra, 89/397 amino acids (22% coverage)



7.

GLGAGGNPDIGMNAAK
AVQAQEGIAALR
DAALNAIQSPLLDIGIER

gi|115454331 (100%), 47242.7 Da
Os03g0646100 [Oryza sativa (japonica cultivar-group)], gi|108710083|gb|ABF97878.1| Cell division protein ftsZ, puta
3 unique peptides, 3 unique spectra, 3 total spectra, 46/452 amino acids (10% coverage)

M M A A P Q L P C C T R L A P P C P G K A A A E A R T L A R S R F R C C A G A A
R P R S F Q K K D S F L D L H P E V T L L R G G D E A A V V A T R K G S P N G S
P L E G L G A P P D H C D Y D G A K I K V V G V G G G G S N A V N R M I E S S M
N G V E F W I V N T D V Q A I R M S P V L P Q N R L Q I G Q E L T R **G L G A G G**
N P D I G M N A A K E S V E S I Q E A L Y G A D M V F V T A G M G G G T G T G G
A P V I A G I A K S M G I L T V G I V T T P F S F E G R R R **A V Q A Q E G I A A**
L R N S V D T L I V I P N D K L L S A V S P N T P V T E A F N L A D D I L R Q G
I R G I S D I I T V P G L V N V D F A D V R A I M Q N A G S S L M G I G T A T G
K S R A R **D A A L N** **A I Q S P L L D I G** **I E R** A T G I V W N I T G G A D M T L F
E V N S A A E I I Y D L V D P N A N L I F G A V I D P S L N G Q V S I T L I A T
G F K R Q D E P E G R T T K G G Q Q T Q G D N G R R P S S A E G S M I E I P E F
L R R R G P S R F P R V

APPENDIX D

PREPARATION OF STOCK SOLUTIONS

Reagents used for isolation of RNA from Leaf tissue

- Homogenization buffer: 4 M Guanidine thiocyanate, 100 mM Tris-HCl, pH 8.0, 25 mM Sodium citrate pH 8.0, 0.5% N-Lauryl sarcosine
- PVP, insoluble
- 5 M NaCl and ß-Mercaptoethanol
- Chloroform : isoamyl alcohol (24:1)
- Tris-saturated phenol (pH 8.0) : chloroform : isoamyl alcohol (25:24:1) (prepared fresh)
- Phenol : Chloroform (1:1)
- Pre-cooled Isopropanol / absolute Ethanol
- 75% Ethanol prepared from DEPC treated water

5x Formaldehyde gel running buffer (500 ml)

Dissolve Sodium acetate 4.1g and MOPS (3-(N-Morpholino) propanesulphonic acid) 10.3g in 400ml sterile DEPC treated water and adjust the pH to 7.0 with 2N NaOH. To this add 5ml of 0.5M EDTA of pH 8.0 and make up the volume to 500ml with sterile DEPC water.

5-Bromo-4-Chloro-Indoly-β-D-Galactoside (X-Gal)

Dissolve 20mg X-gal in 1ml of dimethylformamide. Store the solution at -20°C.

Isopropyl-beta-thiogalactopyranoside (IPTG)

Make a solution of IPTG by dissolving 2g of IPTG in 8ml of distilled water. Adjust the volume of the solution to 10ml with distilled water and sterilize by filtration through a 0.22 micron disposable filter. Dispense the solution into 1ml aliquots and store them at -20°C. It helps in stimulating the production of beta-galactosidase enzyme and helps in expression.

Acrylamide Stock Solution

Acrylamide 3.196 g

DATD 564 mg

H_2O up to 10 ml

Riboflavin Stock Solution:

Riboflavin 0.001g in 25 ml H_2O. Dissolve and filter.

Equilibration Buffer: pH 8.8

50 mM Tris HCl pH 8.8: 16.65 ml

6 M urea: 180.18g

10% Glycerol: 50 ml

2M Thiourea: 76.12 g

2 % SDS: 10 g

10 % Glycerol 50 ml

5 % 2- ME add on the day of use.

DH_2O up to 500 ml adjust pH 6.8 with 1 N HCl or with ½ H_2O/ ½ conc. HCl and add glycerol and refrigerate.

Sample Overlay Buffer:

8 M Urea 4.8 g

0.05% fast green 1 drop

Protein Solubilization Buffer (5mM K_2CO_3)

Upper Chamber Buffer:

NaOH 0.8g in 500 ml H_2O up to 500 ml. Degas prepared fresh.

Lower Chamber Buffer:

H_2SO_4 4.17 ml H_2O 2.5 liter

Lysis Buffer:

9.5 M urea

1% Agarose Gel:

Agarose: 1 g

Bromophenol Blue: 2-4 drops

5% mercaptoethanol

100 ml DH_2O

Vortex it until dissolves and heat it until boils. Add 2-4 drops of Bromophenol Blue. Put it in glass tube of 5 ml each and keep them in refrigerator. Dissolve and add 5 % mercaptoethanol (25 µl) to the glass tube of 5ml just before use.

0.1%(BPB) Bromophenol Blue Solution:

Bromophenol Blue: 2 mg

DH_2O: 20 ml

Ammonium persulfate (APS) for IEF Gels

Dissolve 0.06 g APS (Ammonium persulfate) in 250 µl DH_2O.

IEF Gel Preparation (500 µl for 1 gel)

Urea		273 mg
Acrylamide		78 µl
Ampholines		25µl
48 %	3 - 10	12 µl
33 %	5 -7	8.25µl
19 %	8 – 10.5	4.75 µl
Riboflavin		19 µl
NP-40		10µl
TEMED		.5µl

H_2O up to	500 µl
APS	1.3µl

20% APS solution for 2nd Dimension:

80 mg APS in 400 ml DH2O Vortex it.

Separating (Resolving) Gel Buffer 1.5 M pH 8.8 250 ml

Tris: 45.38 g

SDS (Sodium Dodecyl Sulfate): 1 g

Adjust the pH 8.8 with 1 N HCl or with½ H_2O/ ½ conc. HCl. keep in refrigerator.

Stacking Gel Buffer I M pH 6.8 250 ml

Tris: 30.25 g

SDS: 1 g

Adjust the pH 6.8 with 1 N HCl or with½ H_2O/ ½ conc. HCl. Store in refrigerator.

Running Buffer 1 liter in H_2O

Glycine: 14.4 g

SDS: 1 g

Tris: 3 g

30 % Acrylamide Solution

Acrylamide: 30 gr

N,N-methylene- bis-acryl-amide: 0.8 gr

Add up to 100 ml DH_2O. Dissolve, store in refrigerator.

Resolving Gel (11%)

Acrylamide	3.70 ml
Separating Gel Buffer	2.55 ml
H_2O	3.70 ml

TEMED	4 µl
20% APS	50 µl
Total	10 ml

Stacking Gel	
Acrylamide	0.85 ml
Stacking Gel Buffer	0.65 ml
H_2O	3.45 ml
TEMED	5 µl
20% APS	25 µl
Total	5 ml

Fixative (7/40) Solution: 7% Acetic Acid, 40% Methanol

0.125 % Comassie Blue Staining Solution (1 liter):
Comassie Brilliant Blue R-250: 1.25 g
Acetic Acid: 100 ml
Methanol: 400 ml
DH_2O: 500 ml
Add the 1.25 g Comassie Brilliant blue R-250 to 40% methanol in water and stir it until the dye dissolves. Filter it add acetic acid and store at Room Temperature.

Destaining Solution (6 liters)
DH_2O: 4980 ml
Ethanol: 600 ml
Acetic Acid: 420 ml