UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Scienze Agrarie, Alimentari, Forestali e Ambientali

Settore Scientifico Disciplinare AGR/03

INVESTIGATION ON THE GENETIC BASIS OF ENVIRONMENTAL STRESS IN FRUIT TREE CROPS

IL DOTTORE

JUBINA BENNY

IL COORDINATORE

CH.MO PROF. VINCENZO BAGARELLO

IL TUTOR

CH.MO PROF. TIZIANO CARUSO

CO TUTOR

CH.MO PROF. ANNALISA MARCHESE

CICLO XXXIII

2021

Table of Contents

ABSTRACT

Plant stress can be divided into two major categories: abiotic stress and biotic stress. Abiotic stress happens when plants are exposed to the environment either physically or chemically. There is an emergency in developing crop varieties that are tolerant to abiotic stresses to ensure food security and safety in the coming years. Multiple abiotic stress like drought, heat, frost at flowering and nutrient deficiency can cause an erratic fruiting behavior or following extreme events, the death of the plants. Plants require an optimal level of nutrients and essential minerals for their growth and development that are mainly acquired from soil by their roots. Nutrient deficiency is an environmental stress that can seriously affect fruit production and quality. In the past decades, agriculturalists relied only on the traditional methods to identify the stresses. In this postgenomic era, called the "*omic*" era, transcriptional and translational research on model plants has provided many valuable information on many horticultural species.

In the present dissertation, the objective of the first study was to identify, and map key genes involved in drought response on leaves across different crops. The study is the first to provide RNA-Seq data analysis related to transcriptomic responses towards drought across different fruit tree crops. The second study was conducted to identify essential genes involved in general plant abiotic stress conditions and those involved in specific and unique in different abiotic stresses. A pipeline composed of pathway and gene set enrichment analysis, protein-protein interaction networks, and gene visualization tools were employed. The next study aims to identify genes that serve as potential targets to develop cultivars with enhanced drought and salinity resistance and/or tolerance across different fruit tree crops in a biotechnological sustainable way. An "omic" experimental plan was developed to investigate and understand a physiological stress presumably due to nutritional deficiencies causing premature flower bud abscission in pistachio that leads to alternate bearing behavior. The aim of this analysis was to provide insights into the transcript changes between inflorescence buds and fruits in bearing and non-bearing shoots to identify the molecular mechanism causing premature inflorescence bud abscission, which is linked to alternate bearing in the Italian pistachio cultivar Bianca.

Key molecular physiological conclusions were generated based on the identification of conserved gene sets, pathways, and gene networks involved in abiotic stress resistance/tolerance. The experiment provides a valid approach to ask additional questions with respect to how plants respond to stress. Identifying key information in transcriptomic data is very important, especially when the "omic" study deals with plant responses to stresses in field conditions where a high number of variables and disturbing factors may affect the analysis. The proper understanding of plant stress response mechanisms under specific stresses can draw a better view for improving worldwide food production.

1. CHAPTER 1

1.1. General Introduction

Plants are exposed to many different environmental stresses, which are also referred to as some external conditions that adversely affect development, growth or reduces and limit plants' productivity. These stresses will trigger a plant response by altering gene expression, cellular metabolism, crop yields, changes in growth rates, etc. A sudden change in the environmental condition reflects on plant stress. Several metabolic dysfunctions exhibit on plants due to it is exposing to some stresses. If the stress is mild or short term as the effect is temporary, the plants can recover from such injuries, while if the stresses are severe, it leads to the plant death. Such types of plants can be considered as stress susceptible plants. However, plants can escape from stress to an extent. The exposure to stress leads to acclimation to that specific stress in a time-dependent manner in stress-tolerant plant species. Plant stress can be divided into two major categories: abiotic stress and biotic stress.

Biotic stress is imposed on plants by viruses, bacteria, fungi, nematodes, insects, arachnids, and weeds. The agents causing biotic stress to deprive their host of its nutrients can lead to plant death. Dure to the pre- and postharvest losses to the farmers, biotic stresses become a significant stress condition. However, plants can counteract biotic stresses by some sophisticated strategies despite lacking the adaptive immune system. Such sophisticated defense mechanisms that can act against the stress are controlled by the genetic code stored inside the plant. There are several genes in the plant genome against these stresses (resitence genes). It is the climatic condition in which the crop lives decide what type of biotic stress may be imposed on crop plants and the crop species' ability to resist that stress. Since insects reduce leaf area by chewing and virus infections reduce photosynthesis rate per leaf area, many biotic stresses affect photosynthesis.

Abiotic stress happens because plants are exposed to the environment either physically or chemically. Abiotic stress is imposed on plants by non-living factors such as temperature, sunlight, salinity, floods, cold, and drought. Abiotic stresses such as excessive watering (waterlogging), extreme temperatures (cold, frost, and heat), drought (water stress), salinity, and mineral toxicity negatively impact the growth, development, yield, and seed quality of crop and other plants. In the future, it is predicted that freshwater scarcity will increase, and ultimately the severity of the abiotic stresses will intensify. Therefore, there is an emergency in developing crop varieties that are tolerant to abiotic stresses to ensure food security and safety in the coming years.

1.2. Abiotic stresses

Global losses in agriculture caused by pests are estimated at 25-40% for the major crops, representing a value of over €500 billion worldwide. Moreover, crops usually attain only about 50% of their potential yield because of abiotic stresses (drought, heat, cold, water logging, high salinity, and toxic compounds). Plants have evolved complex mechanisms to perceive external signals and translate these into an optimal adaptive response to maximize the chance for survival, under combinations of various biotic and abiotic stress conditions. So far plant responses to stress situations have been extensively studied at molecular level, including their response under multi-stress conditions. In plants, the change in the gene expression pattern due to the stress response can affect the productivity and growth rate (Alcazar et al., 2006) and can also cause erratic bearing behaviour. Therefore, it is necessary to identify the genes responsible for abiotic stresses to understand the stress response mechanism. The first line of defense of a plant against abiotic stress is in its roots. If the soil holding the plant is healthy and biologically diverse, the chances of a plant surviving stressful conditions will be high. At the same time, there will be many disruptions like change at the osmotic level inside the plant cell's cytoplasm during its fight against abiotic stress, and changes at hormonal level (Shinozaki et al., 2003).

Some of the major abiotic stresses are explained below:

- **Drought**

Due to the continuous increase in temperature and CO_2 levels, drastic climatic changes are happening worldwide. One of the primary reasons for drought stress is the uneven distribution of rainfall. Due to the severe drought conditions, the soil water available to the plants is decreased, which causes the premature death of the plants. The first response of the plant, which is subjected to drought stress, is the plant growth arrest. The shoots' growth will be reduced during the drought conditions, which indeed reduce their metabolic demands. The plants will synthesize the protective compounds under drought by mobilizing metabolites needed for the osmotic adjustment (Riemann et al., 2015).

- **Salinity**

The crop yield and production are affected globally due to the soil affected by salinity. Salt stress reduces the growth of crops and production in many ways. Two immediate effects imposed on crop plants by salt stress are osmotic stress and ion toxicity. More salt will limit plants' ability to take up water and minerals like K^+ and Ca^{2+} from the soil. Thus, the osmotic pressure under salinity stress in the soil solution exceeds the plant cells' osmotic pressure. These direct effects of salinity stress cause some secondary effects like assimilating production, reduced cell expansion, membrane function, and decreased cytosolic metabolism (Riemann et al., 2015).

- **Cold**

One of the major abiotic stresses that decrease the productivity of agricultural crops is cold stress. It affects the quality and post-harvest life of the crops. In order to prevent themselves from stress, plants will modify their molecular mechanism to adjust to the stress environment. The chilling and freezing conditions are harmful to the plant. To adapt to such a situation, plants acquire tolerance against such lethal cold weather by a process called acclimation.

However, there are many different crops that are still incompetent in cold acclimation. The stress will affect the cellular and molecular function of the plant. The cold stress induces many different signal transduction pathways such as protein kinase, Abscisic acid (ABA), Ca^{2+}, protein phosphate, and ROS (Ramu etal., 2016).

- **Heat**

The rise in temperature across the globe has become an important factor that affects the growth of plants, along with their productivity. The percentage of photosynthetic efficiency, crop yield, and seed germination decline when plants encounter heat stress. At the same time, during the heat stress, it was also observed that the anther is dysplastic, and the tapetal cells also tend to lose their functionality. The combination of salt and heat stress results in physiologically conflicting responses that are also observed during drought and heat stress. Heat stress results in increased respiration and will therefore require more water uptake by the plant to attain turgor pressure. This leads to more salt uptake and will therefore increase salt stress even further (Mittler e al., 2015). Although with each individual stress plants are shown to have the ability to survive, the survival rate of plants that encounter a combination of salt and heat stress is dramatically decreased (Qin et al., 2011).

- **Toxin**

The use of chemical fertilizers, sewage water irrigation, and growing industrialization has been a reason for the presence of toxic metals in soil. This, in turn, leads to harmful effects to the soil-plant ecosystem (Ashraf et al., 2007).

- **Nutritional Stress**

Plants require an optimal level of nutrients and essential minerals for their growth and development that are mainly acquired from soil by their roots. Nutrient deficiency is an environmental stress that can seriously affect fruit production and quality. An excess has a negative effect on soil biology while scarcity has a negative impact on growth and development. In addition, nutrient deficiency can disturb the plant's antioxidant system, as nutrients are needed for antioxidant biosynthesis. Under nutrient deficiency conditions, some secondary metabolite compounds, like phenolic compound, are produced in plants (Peleg et al., 2011).

Multiple abiotic stress like drought, heat, late frost at flowering and nutrient deficiency can cause an erratic fruiting behavior in plants. Fruit trees exhibit many irregularities in yield, including failure to produce fruit despite luxurious growth. A common abnormality observed in fruit trees across the world is alternate bearing. Alternate bearing is an important characteristic of many tree crops, such as avocados, oranges, apples, olive, almond, pistachio, pecan.
It is suggested that three mechanisms are apparently involved in the maintenance of the alternate bearing condition in fruit tree species (Shalom et al., 2012):

(a) Flowering site limitations
(b) Hormonal control
(c) Nutritional control

It is proved that after an "ON" year, in which most of the tree energy is directed towards the growth and development of the fruits, the energy reserves are significantly reduced. There are multiple visible causes, decreased shoot growth and flower buds or imperfect flowers, but all

occur in association with a heavy crop, suggesting a carbohydrate resource limitation to shoot growth, or flower initiation, induction or differentiation (Khezri et al., 2020). The figure 1.1 showed possible hypothesis associated with alternate bearing.

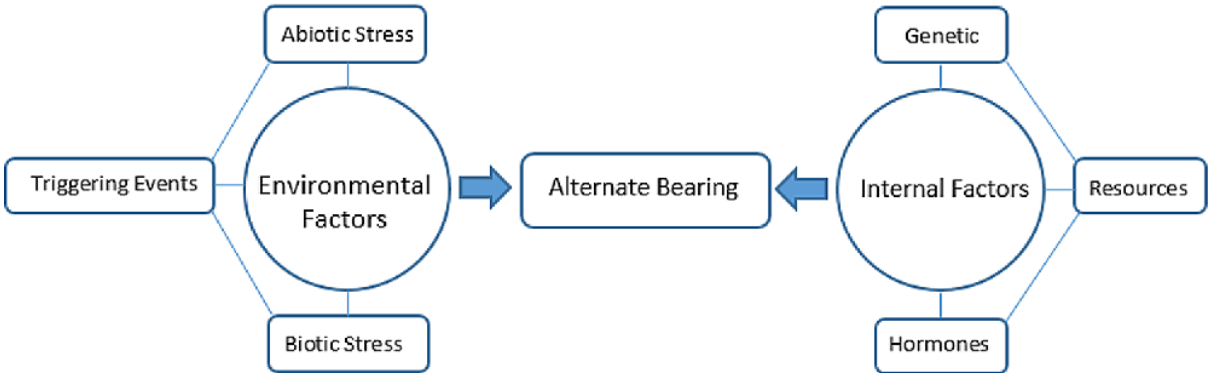


Figure 1.1: Hypotheses associated with alternate bearing intensity (Khezri et al., 2020)

1.3. Pre- and Post-genomic era

The earth temperature will increase by 3-5°C in the coming years. Uneven rainfall and an increase in temperature lead to environmental imbalance. The activities such as inappropriate irrigation, usage of excessive fertilizers, and exploitation of metal resources can lead to salt stress. Under these circumstances, plants will probably encounter more frequently, concurrently, both drought and salinity stresses. Therefore, it is necessary to develop stress-tolerant cultivars to secure food security. Molecular work is to be done at the genetic level to develop plants' mechanisms to prevent them from different types of stress conditions.
In the past decades, agriculturalists relied only on the traditional methods to identify the stresses. The abiotic stress suppresses seed germination vegetative growth, leaf area, and root biomass . This, indeed, will decrease in average fruit weight or the number of fruits produced by a plant. In salinity stress, fruit enlargement is suppressed during the cell expansion phase because of water uptake into the fruit, which is the motive for cellular expansion. During the pre-genomic era, researchers studied only the physiological changes such as stomal closure, water intake percentage, root weight, leaf area, photosynthesis, carbohydrate influx, etc. But recent studies showed that extremely complex traits like stress should be studied at genomic levels to gain insight into key molecular and genetic features behind the stress.

- **Bioinformatics tools and platform**

Plants have evolved mechanisms to perceive these environmental challenges, transmit the stress signals within cells as well as between cells and tissues, and make appropriate adjustments in their growth and development to survive and reproduce. In recent years, significant progress has been made on many fronts of the stress signaling research,

particularly in understanding the downstream signaling events that culminate at the activation of stress- and nutrient limitation-responsive genes, cellular ion homeostasis, and growth adjustment. However, the revelation of the early events of stress signaling, particularly the identification of primary stress sensors, still lags behind.
Modern biotechnology tools, such as tissue culture and genetic engineering, offer an alternative to conventional breeding to generate new cultivars with enhanced agronomic and nutritional characteristics (Dai et al., 2015). In recent years, sequence-specific genome editing technologies were found to be useful tools for crop improvement and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (Cas9) (Cheng et al., 2013) is the newest and most widely used genome editing technology for the study of the function of genes and for the development of mutant lines with enhanced tolerance to biotic and abiotic stresses, herbicide resistance or improved yield. In the last decades, transgenic crops have been developed and genetic modification has been performed to confer resistance against abiotic stresses like drought, salinity, cold etc. (Meng et al., 2018; Lynch et al., 2018). The CRISPR/Cas9-mediated genome editing technology has opened a new opportunity for rapid development of disease resistant crop varieties by either stacking of disease resistant (R) gene(s) or disruption/deletion of susceptibility genes (Evans et al., 2011).

Due to the recent progress in next-generation sequencing (NGS) technologies, the emergent postgenomic era has enabled advances not only for model plants and major cereal crops but also for horticultural crops, which comprise a great diversity of species. In this postgenomic era, transcriptional and translational research on model plants has provided many valuable information on many horticultural species. The physiological basis for these stress responses is in integrating many transduced events into a comprehensive signaling pathway network. A central place in this transduction network is occupied by the plant hormones. These hormones help in coordinating cellular processes such as cell division, elongation, and differentiation, which are the fundamental basis for higher plant development and related character expressions.
Next-Generation Sequencing (NGS) methods have been widely adopted over Sanger sequencing, referred to as "first-generation" sequencing due to their dropping costs and ability to sequence DNA at an unprecedented speed. The huge amounts of data generated by NGS have extended the understanding of structural and functional genomics through the concepts of "omics," providing new insight into the workings and meaning of genetic conservation and diversity of living things. NGS technologies can be applied for multiple applications such as

- Sequencing the Whole-Exome (WES) to identify the genetic variants
- Whole transcriptome sequencing (RNA-seq), which helps to understand the expression of transcripts
- Targeted (TS) or candidate gene sequencing to sequence only the genomic regions of interest to identify variants.
- Methylation Sequencing (MeS) or Bisulfite Sequencing to investigate epigenetic modification.

In plant research, NGS technologies have become crucial tools for the assembly of crop reference genomes, transcriptome sequencing for the study of gene expression, whole-genome molecular marker development, and identification of markers in known-function genes. Plants respond to abiotic stresses via dynamic and complex reactions that accompany

molecular, cellular, and physiological changes in plant tissues. To understand the responses of plants to abiotic stresses, diverse crop breeding approaches have been applied from traditional breeding methods to variable -omics methods, such as next-generation sequencing (NGS).

RNA sequencing (RNA-Seq) uses the capabilities of high-throughput sequencing methods to provide higher coverage and greater resolution of the dynamic nature of the transcriptome. Thereby elucidate different physiological and pathological conditions. This technology consists of converting RNA molecules to a library of cDNA fragments with adaptors. These fragments are sequenced, and the resulting reads are either aligned to a reference genome (if available) or assembled de novo, followed by transcript quantification.

The first step in transcriptome sequencing is the isolation of RNA from a biological sample. To ensure a successful RNA-Seq experiment, the RNA should be of sufficient quality to produce a library for sequencing (Deokar et al. 2011). Following RNA isolation, the next step in transcriptome sequencing is the creation of an RNA-Seq library, which can vary by the selection of RNA species and between Next Generation Sequencing (NGS) platforms. The construction of sequencing libraries principally involves isolating the desired RNA molecules, reverse-transcribing the RNA to cDNA, fragmenting, or amplifying randomly primed cDNA molecules, and ligating sequencing adaptors. Many protocols focus on enriching mRNA molecules before library construction by selecting polyadenylated (poly A) RNAs. In this approach, the 3′ poly-A tail of mRNA molecules is targeted using poly-T oligos covalently attached to a given substrate (e.g., magnetic beads). In RNA-Seq preparation methods, RNA is converted into cDNA because most sequencing technologies require DNA libraries. Most cDNA synthesis protocols create libraries that were uniformly derived from each cDNA strand, thus representing the parent mRNA strand and its complement. The overview of RNA-Seq library preparation is given in **Figure 1.1**.

Many reads that can be generated per sequencing run (e.g., a single lane of an Illumina HiSeq 2500 generates up to 750 million paired end reads) permits the analysis of increasingly complex samples. The introduction of unique 6-bp indices, also known as “barcodes,” to each RNA-Seq library enables the pooling and sequencing of multiple samples in the same sequencing reaction. The barcodes identify which sample the read originated from. Most high-throughput sequencing platforms use a sequencing-by-synthesis method to sequence tens of millions of sequence clusters in parallel. In recent years, the sequencing industry has been dominated by Illumina, which applies an ensemble-based (i.e., sequencing many identical copies of a DNA molecule) sequencing-by-synthesis approach.

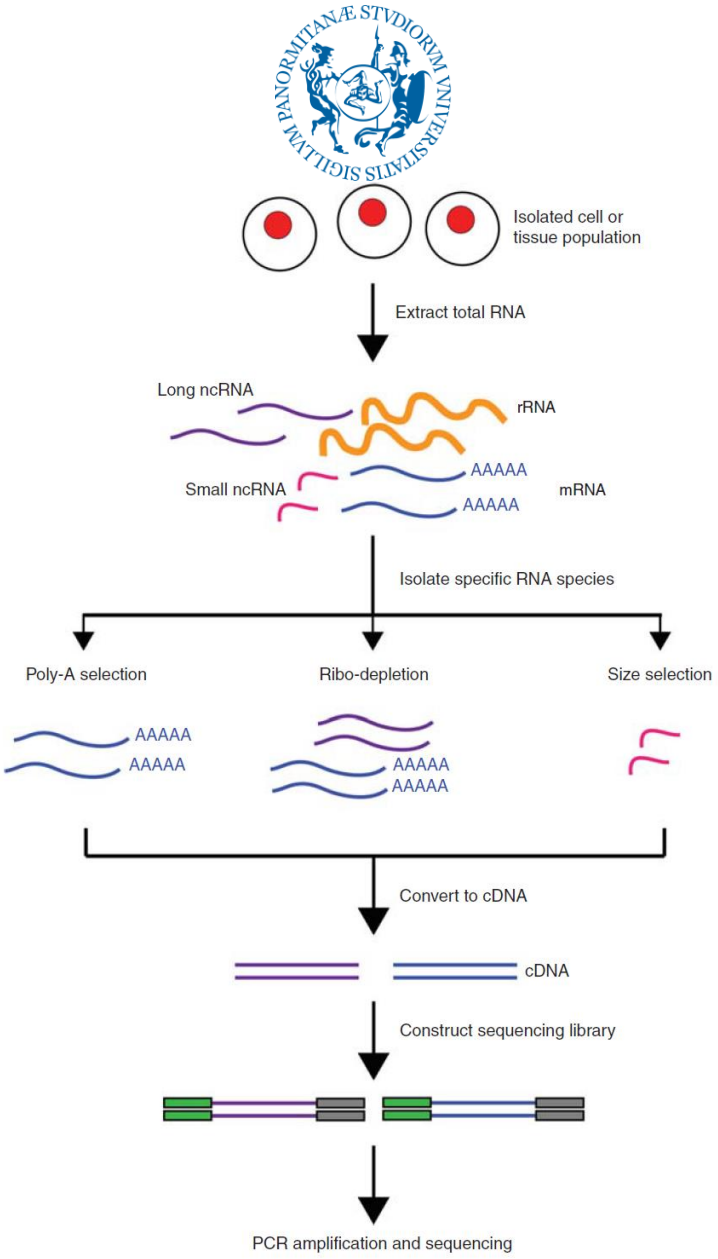


Figure 1.2: Overview of RNA-Seq library preparation (Kukurba et al. 2015)

Global gene expression profiling using RNA-Seq technologies has been widely used to study biological and cellular responses to plants' oxidative stress responses. Since the number of such transcriptome studies is growing, it is very significant to have a comprehensive analysis by integrating multiple studies to identify robust gene expression signatures that would be subtle in individual studies. Such studies are known as meta-analysis.
Systematic literature review and meta-analysis are increasingly popular in agricultural sciences. The meta-analysis technique has been applied in numerous fields, for example, psychology, law, management, education, medicine, and even policy formulation. Across various fields, meta-analysis has been used to examine the following:

- strength of the relationship between two variables
- effectiveness of treatments or interventions
- accuracy of theories
- validity of measuring instruments

- validity of procedures and
- presence of moderation effects

Meta-analysis facilitates derogating or decimating potential biases associated with individual studies and improving statistical power to detect subtle but biologically meaningful variations through increased sample sizes (Balan et al., 2018).

- **Plant stress perception, signaling and responses**

Since the development of NGS, the transcriptome has been widely studied to gain insights into the molecular mechanisms by which plant species adapt to their environment. Currently, transcriptome data analyses of plants are performed in various organisms under diverse conditions, including exposure to abiotic stresses. The first layer of protection against abiotic stress is the construction of structural barriers such as waxy cuticles, pigments, trichomes and antimicrobial metabolites to prevent or attenuate invasion and stress induction. If the first layer of protection is breached or it is not sufficient, plant immune system takes action by recognizing non-self-molecules and signals from stressed or injured cells, and respond to that by activating an effective counter response (Hoang et al., 2017; Imran et al., 2018). Potential threats can be perceived by the plant via both extracellular and intracellular receptors that bind to substrates. Although the receptor substrates for abiotic stresses remain unknown, these stress signals are known to be perceived by different receptors including CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE 1 (CRLK1), RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and CYSTEINE-RICH REPEAT RECEPTOR-LIKE KINASE 5 (CRK5), which are involved in cold stress and drought tolerance and abscisic acid (ABA) signaling, respectively (Kawasaki et al., 2005; Mao et al., 2011).

Abiotic and biotic stress perception subsequently leads to the phosphorylation and activation of receptor kinases resulting in a rapid calcium (Ca2+) influx and phosphorylation of receptor-like cytoplasmic kinases (RLCKs) and calcium-dependent protein kinases (CPKs) that recruit and phosphorylate respiratory burst oxidase homolog D (RbohD; Orcheski et al., 2016; Najafi et al., 2018). Activation of RbohD results in the production of extracellular reactive oxygen species (ROS) that depolarizes plant cells within minutes after elicitor application (Pandey et al., 2013). Both Ca2+ and ROS were shown to act as second messengers and spread throughout the plant, activating plant stress signaling.

Besides rapid second messengers induced signaling, receptor activation leads to downstream mitogen activated kinase (MAPK) signaling that activates transcription factors (TFs) involved in stress signaling and regulation (Mittler et al., 2011). These include TFs from different families such as, ABA-responsive element-binding proteins (AREBs), WRKYs, APETALA 2 (AP2)/ethylene-responsive element-binding factors, myeloblastosis (MYB) TFs, myelocytomatosis (MYC) TFs, basic domain-leucine zipper (bZIP) TFs (e.g., TGA binding TFs), and zinc finger proteins (ZFPs; Cao et al., 2017; Li et al., 2017). These transcription factors are known to regulate different stress-driven signaling pathways including the production of phytohormones that amplify stress signals.

Depending on the nature of the stress, plants make use of phytohormone-driven signaling pathways to amplify stress signaling, including the production and accumulation of ABA,

salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) Phytohormonal accumulation was shown to be linked to early plant stress signaling involving ROS production, showing the importance of general and early stress responses in stress specific phytohormonal regulation. The figure 1.3 showed a simplified working model of a signaling network of plant responses to abiotic stress.

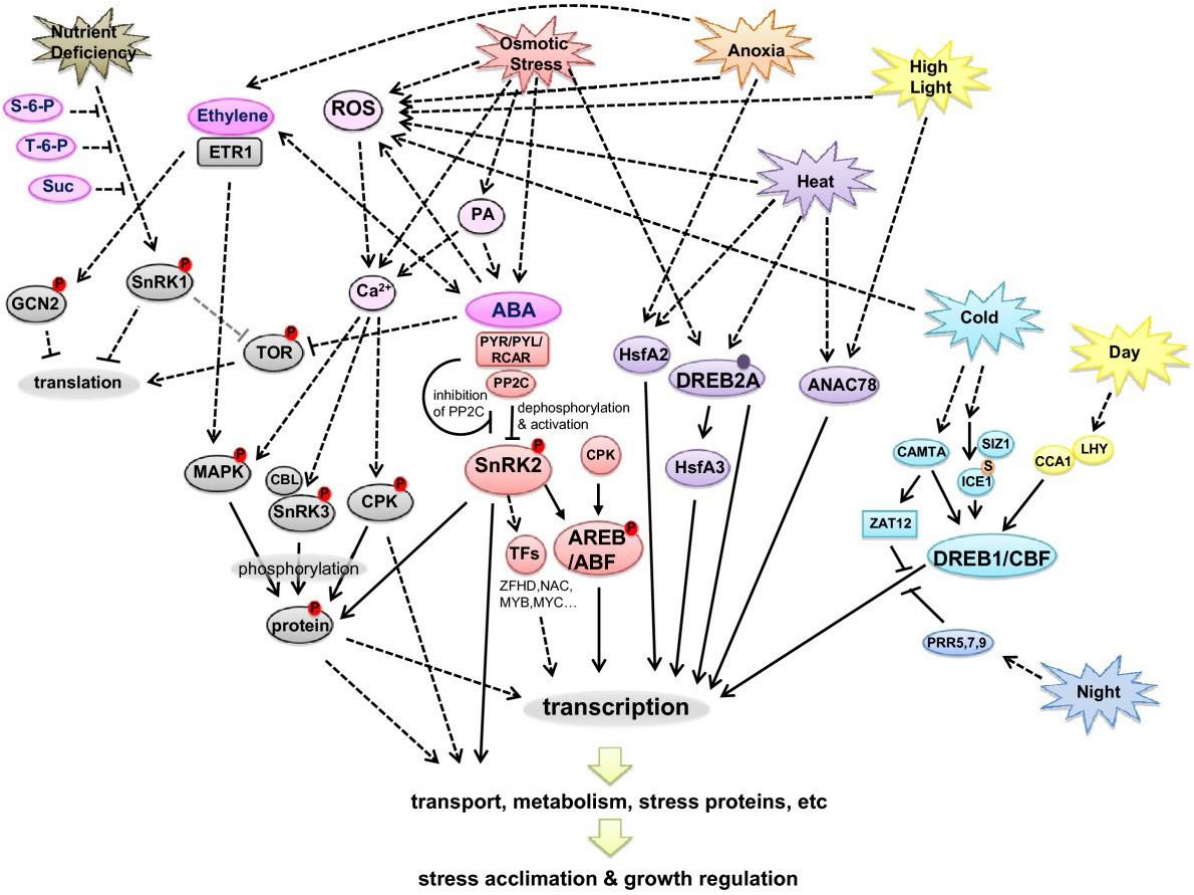


Figure 1.3: A simplified working model of a signaling network of plant responses to abiotic stress (Khadka et al., 2019)

1.4. Research Objectives

The main objective of my Ph.D. projects was to perform a comprehensive study of the genetic basis of environmental and physiological stress in fruit tree crops through the application of meta-analytical technique and RNA-Seq analysis. The study helped in obtaining the gene expression data pertaining to abiotic stress in fruit tree crops to detect the strongly associated genes, pathways, and gene set categories. Identifying key information in transcriptomic data is very important, especially when the "omic" study deals with plant responses to stresses in field conditions where a high number of variables and disturbing factors may affect the analysis. A wide range of stress due to drought, salinity, and heat adversely affects plant growth and productivity worldwide. There were individual

transcriptome studies based on individual stress on different crops, which lacks the significance of identifying the potential genes, which are vulnerable to any abiotic stress. The proper understanding of plant stress response mechanisms under specific stresses can draw a better view for improving worldwide food production.

In this first study, the objective was to identify, and map key genes involved in drought response on leaves across different crops. In this work, I have conducted a meta-analysis of 9 RNA-Seq studies conducted in 7 crops to deliver conserved and reliable genomic information exploitable by breeding to enhance drought resistance in crops. I analyzed (in a most comprehensive manner as possible) RNA-Seq data in crops (herbaceous, tree fruit crops, model plant) under drought using the same bioinformatics pipeline to deliver functional genomics knowledge that will guide molecular breeding to enhance drought tolerance and resistance in crops. Data were dissected using an integrated approach of pathway- and gene-set enrichment analysis, Mapman visualization tool, gene ontology analysis, and inferred protein-protein interaction network. In summary, taken together with all the findings, I propose a model of plant response to drought; and I anticipate that these insights will assist in the development of genetic resistance and implementing strategies to enhance resistance. My study is the first to provide RNA-Seq data analysis related to transcriptomic responses towards drought across different crops.

The second study was conducted to identify essential genes involved in general plant abiotic stress conditions and those involved in specific and unique in different abiotic stresses. Bioinformatics analysis of previously published RNA-Seq studies on leaves was performed by carefully selecting published studies related to four abiotic stress factors: drought, salinity, cold, and heat. To achieve my aim, I considered the following steps which helped me to increase the specificity of the study, which were a) download row data from the literature for analysis b) use single bioinformatics pipeline for data analysis, c) use reference genome downloaded from a single source (phytozome), and d) remove the genes which play a role in tree and fruit development. So, my focus was to conduct raw data analysis by developing a bioinformatics pipeline using the reference genome from a single source. A pipeline composed of pathway and gene set enrichment analysis, protein-protein interaction networks, and gene visualization tools were employed. The study provided insights into molecular regulatory systems controlling resistance/tolerance/susceptibility to four significant abiotic stresses in plants.

The next study aims to identify genes that serve as potential targets to develop cultivars with enhanced drought and salinity resistance and/or tolerance across different fruit tree crops in a biotechnological sustainable way. In that study, I conducted a meta-analysis by selecting six RNA-Seq studies with similar experimental design (timing and intensity of stresses) conducted in five fruit tree crops in order to deliver conserved and reliable genomic information for enhancing drought and salinity crop resistance/tolerance. I analyzed, in the most comprehensive manner possible, RNA-Seq data in fruit tree crops under drought and salinity using the same bioinformatics pipeline used in my previous analysis. The most important players among the huge amount of data generated by every single RNA-Seq study were identified and mapped on the chromosomes to develop next-generation markers (i.e., based on epigenetic mechanisms). Key molecular physiological conclusions were generated based on the identification of conserved gene sets, pathways, and gene networks involved in

abiotic stress resistance/tolerance. The experiment provides a valid approach to ask additional questions with respect to how plants respond to stress.

From these experiments, I came up with a bioinformatic approach that can serve as a common pipeline to answer many major abiotic stress issues faced by fruit crops. One such issue that was least studied was alternate bearing in pistachio tree. An experimental plan was developed to discuss and understand the molecular mechanism causing premature flower bud abscission that leads to alternate bearing in pistachio. This study can be considered as the first study reporting and documenting the gene expression profiling associated with inflorescence bud abscission. The aim of this analysis was to provide insights into the transcript changes between inflorescence buds in bearing and non-bearing shoots in order to identify the molecular mechanism causing premature inflorescence bud abscission, which is linked to alternate bearing in the Italian pistachio cultivar Bianca. The results demonstrated the nutritional theory and the involvement of a complex network of hormonal signals and cross talk in the inflorescence bud drops of fruiting shoots. These findings have important implications for the horticultural management of this fruit species.

The final study was done on fruit samples from Italian pistachio cultivar Bianca to identify the molecular mechanism causing premature inflorescence bud abscission and therefore to complete the previous analysis. In this study, RNA seq analysis was carried out in fruits of “ON” and “OFF” shoots of the cultivar Bianca, for two consecutive years to investigate the presence of inhibitory signals or genes relate to hormone biosynthesis directly or indirectly linked to the premature fall of the inflorescence buds, considered the main cause of alternate bearing behavior of Pistachio tree. From my findings, it is evident that one of the leading causes of premature inflorescence bud abscission is the shortage of nutrients. Hormone applications may mitigate the phenomenon; however, accurate management of resources like carbohydrates and mineral elements directly or indirectly linked to the mechanism can modulate the rate of alternating production. At the same time, the finding of putative biomarkers, in the future, may lead to a reduction of the inflorescence buds and the possibility to balance the alternate bearing phenomenon.

2. CHAPTER 2

Experiment 1: Identification of key genes and its chromosome regions linked to drought responses in leaves across different crops through meta-analysis of RNA-Seq data

DOI: https://doi.org/10.1186/s12870-019-1794-y

2.1. Introduction

Drought is one of the most severe and increasing environmental factors affecting agricultural production. The water uptake, plants adaptation and long-term evolution of plant species are affected by drought stress (Lynch et al., 2013). Plant requires a substantial change in the metabolism, which includes regulation of transcription and gene expression and extensive transcriptome reprogramming to withstand with adverse environmental stress conditions like drought (Janiak et al., 2018). Therefore, transcriptomic studies offer great insight into the mechanisms of plant stress responses. Among the small plant molecules, hormones play an important role in the modulation of the complex plant physiological and molecular responses to drought. Abscisic acid is the key hormone modulating water loss and cellular growth maintenance (Peleg et al., 2011). However, this is only one among the many key players in the complex molecular networks underlying crop responses to environmental stresses. The outcome of the responses is regulated by complex crosstalk where small molecules (such as hormones) play a specific role of inhibition/induction of key proteins in stress signal reception, transmission, and responses such as kinases, phosphatases, and transcription factors, defensive responsive genes (Krasensky et al., 2012). Some key transcription factor (TF) families such as MYB, WRKY, and bZIPs have been involved differently depending on the type of stress. Some TFs have been object of genetic engineering to improve stress tolerance in model and crop plants (Qin et al., 2011). Transcriptomic studies are essential in gaining insight into the crop responses to drought by identifying specific genes involved in plant responses to water stresses highlighting each crop's peculiarities and identifying which genes are the base of diverse drought tolerance and resistance mechanism. Since data of each study are typically related to only one season, this may lead to reduced reliability of the conclusions driven by each study. Indeed, it is essential to find a pipeline to compare data across species in order to strengthen the meaning of every single study, validating published works across species and reducing the environmental variability that affects their reliability. This kind of works named meta-analysis is lacking in crops, especially at the transcriptomic level. Therefore, it is highly desirable to put more efforts in developing extensive studies to systematically understand drought-stress-related mechanisms in crops, which will accelerate the development of new crop varieties with improved stress resistance to increase agricultural sustainability and food supply for a highly growing world population.

RNA-Sequencing (RNA-Seq) is a rapid technique for genome-wide gene expression analysis (Martinelli et al., 2013). With the emergence of this technique, the high-throughput transcriptomic technologies have been revolutionized. This technique can be considered an efficient way to identify genes and gene families encoding proteins involved in different metabolic pathways related to the study's object. Next-generation sequencing methods have

enabled to understand the gene expression data in both quantitative and qualitative manner (Zhao et al., 2018) and can be used for obtaining sequences on a large scale with high sequencing depth. It is crucial to gain insight into the complex networks of crop environmental stress responses by elucidating the molecular basis of drought-stress transduction pathways and drought tolerance mechanisms to assist in developing drought-tolerant crops. Omic approaches have been used to validate RNA-Seq data related to environmental stress responses. However, transcriptomic studies present some drawbacks represented by the following: 1) high presence of false-positive results that requires validation with other platforms, 2) data are generally affected by environmental, experimental, developmental and genetic conditions, 3) experiments are typically not repeated and conducted in only one season, 4) data are highly affected by the environment, especially when performed in field conditions, 5) few replicates are usually performed due to the high costs of these analysis and the scarce integration between transcriptomic and other omic platforms. Meta-analysis improves the reproducibility of RNA-Seq studies because: 1) it filters the most meaningful information linked with the object of study, 2) eliminates data affected by environmental variability, 3) reduces false-positive results, 4) increase the number of virtual replicates, 5) integrates multiple datasets. Meta-analysis studies should be combined with statistical modeling for each sample to unveil intrinsic mechanisms (Zhai et al., 2017).

2.2. Aim of the research

In the present work, a meta-analysis was conducted with 9 RNA-Seq studies conducted in 7 crops to deliver conserved and reliable genomic information exploitable by breeding to enhance drought resistance in crops. The RNA-Seq data were analyzed (in a most comprehensive manner as possible) in crops (herbaceous, tree fruit crops, model plant) under drought using the same bioinformatics pipeline to deliver functional genomics knowledge that will guide molecular breeding to enhance drought tolerance and resistance in crops. The aim was to shed light into drought response mechanisms conserved across crops instead of identifying specific responses in each crop. The purpose was mainly to answer the two following unresolved questions:

1. Which genes and molecular mechanisms are conserved across species and can be considered strictly modulating drought responses in plants?

2. How leaf development affects crop molecular responses to drought and which genes are playing a key role in drought resistance at different developmental stages?

2.3. Materials and Methods

- Search strategy for selection of RNA-Seq studies

Articles published dealing in response to drought in both tree fruit crop and herbaceous species were collected. These studies were identified from Scopus and PubMed if they respect the following criteria as follows: (i) consist of RNA-seq analysis, (ii) included at least one of the following terms in title and abstract: drought, leaf, stress, abiotic stress, water

stress, (iii) studies provided raw data submitted in public databases. These criteria resulted in a selection of 9 articles comprising of total 42 samples (Table 2.1).

Table 2.1: Articles, crops, number of samples, tissue and sample description (control vs treatment) included in the analysis

Articles	Crops	No. of sample	Tissue	Sample Description		Duration of stress
				Control	Treated	
Clauw et al. (2015)	*Arabidopsis thaliana*	6	Seedling leaves	Control1 (ERR754071) Control2 (ERR754083) Control3 (ERR754090)	Treated1 (ERR754061) Treated1 (ERR754065) Treated3 (ERR754082)	The third seedling leaves were harvested at 10 DAS (Days after stress)
Song et al. (2016)	*Zea mays* cv. B73 (Study1)	2	Mature leaves	Control (SRR4054956)	Treated (SRR4048280)	Leaves were collected after 15 DAS
Corso et al. (2015)	*Vitis vinifera* cv. M4	4	Young leaves	Control1 (SAMN02393571) Control2 (SAMN02393572)	Treated1 (SAMN02393596) Treated2 (SAMN02393595)	Leaves were collected after 10 DAS
Li et al. (2017)	*Zea Mays* cv. B73 (Study 2)	4	Mature leaves	Control 1 (SRR3984708) Control 2 (SRR3984749)	Drought1 (SRR3984782) Drought2 (SRR3984791)	Plant were grown without watering until their third leaves were fully expanded
Pieczynski et al. (2018)	*Solanum tuberosum* cv. Gwiazda	10	Mature leaves	Gwiazda_D01 (SRR5448182) Gwiazda_D02 (SRR5448183) Gwiazda_D03 (SRR5448184)	Gwiazda_D6_1 (SRR5448185) Gwiazda_D6_2 (SRR5448186) Gwiazda_D6_3 (SRR5448187) Gwiazda_D10_1 (SRR5448188) Gwiazda_D10_2 (SRR5448189, SRR5448190) Gwiazda_D10_3 (SRR5448191)	Leaves were collected after 6 DAS and 10 DAS
Orcheski et al. (2016)	*Malus X domestic*	4	Seedling leaves	WR1 (SRR3160181) WR2 (SRR3160208)	PR1 (SRR3160081) PR2 (SRR3160180)	Seedling leaves were harvested after 14 days
Liu et al. (2017)	*Solanum lycopersicum*	4	Seedling leaves	SCK (SRR5282480)	SD (SRR5282481)	The third seedling leaves were

Articles	Crops	No. of sample	Tissue	Sample Description		Duration of stress
				Control	Treated	
	cv. M82			TCK (SRR5282476)	TD (SRR5282478)	harvested at 10 DAS (Days after stress)
Salman et al. (2016)	*Vitis vinifera* cv. Summer Black	2	Mature leaves	Control (SRR3466603)	Treated (SRR3466604)	Mature leaves were collected with the interval of 5 days from 0 to 20 days
Liu et al. (2015)	*Triticum aestivum* cv. TAM107	6	Mature leaves	Control1 (SRR1542404) Control2 (SRR1542405)	Treated1 (SRR1542406) Treated2 (SRR1542407) Treated3 (SRR1542408) Treated 4 (SRR1542409)	Leaves were collected at 6 h after stress

If different time point series were present in the same study, a single time point was selected (10 DAS or whichever is nearer). This selection was done since most of the analyzed studies were performed at 10 DAS. The selected studies were grouped based on leaf developmental stage (seedling leaves, young and mature leaves). Raw data were downloaded and analyzed through a pipeline generated for the meta-analysis. The complete pipeline used for this study is provided in Figure 2.1.

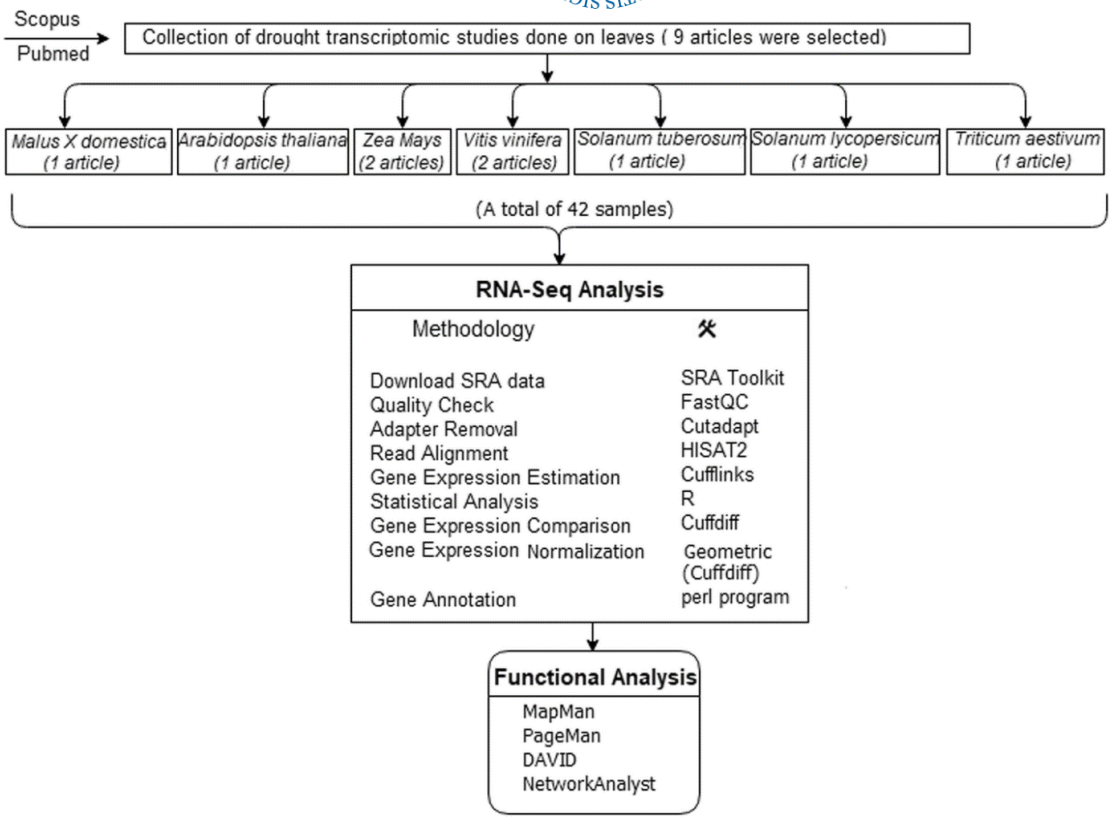


Figure 2.1: Workflow of the meta-analysis of the 9 transcriptomic studies related with drought stress in leaf tissue. Functional and statistical data analysis were indicated.

- Read alignment, gene differential expression and annotation

For all the 9 articles, the latest available version of the corresponding crop genome and its annotation file were downloaded from Phytozome (https://phytozome.jgi.doe.gov). The raw data files were downloaded from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) and EMBL ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) according to the accession number given in the article and converted to FASTQ format using SRA toolkit version 2.3.5. Raw data underwent pre-processing by trimming low-quality bases followed by adaptor sequence removal to obtain high-quality clean reads using cutadapt version 1.8.1. The pre-processed high-quality reads were mapped to the corresponding genome with HISAT2 version 2.1.0 (Kim et al., 2015) using the default parameters. The resulted output of HISAT2 was then used for the identification of differentially expressed genes using Cuffdiff tool in Cufflinks version 2.2.1 pipeline with default parameters. Up- and down-regulated genes with p-value < 0.05 were considered for downstream functional analysis. The DEGs selected were annotated using corresponding crop genome mapping file downloaded from the Phytozome. Custom made in-house Perl script was used for the selection of genes and mapping.

- Statistical and cluster analysis

The DEGs corresponding to each study separately analyzed, with p-value < 0.05, were then taken for the statistical analysis. Using p.adjust function of R, all the statistical tests were corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate. This

approach can make the FDR at the desired level of α (in this study 0.05) by adjusting the P-values. R software was used for the statistical analysis. Differences among the selected studies were adjusted using the sample normalization. In order to remove systematic variation between different species, the normalization procedure served as a crucial pre-processing step to adjust for the different sample sequencing depths and other confounding technical effects. I used the geometric normalization method where FPKMs and fragment counts are scaled via the median of the geometric means of fragment counts across all libraries, as described in (Anders et al., 2010). The dendrogram was generated for identifying the clustering patterns of the considered studies. The grouping of the clusters for dendrogram was done using the Euclidean distance measure.

- Gene set enrichment analysis

I mapped the entire differentially regulated gene IDs of each plant species to Arabidopsis thaliana and found out the corresponding best hit TAIR ID using the annotation file downloaded from Phytozome. I used MapMan (Thimm et al., 2004) with the Arabidopsis thaliana mapping file (http://mapman.gabipd.org/) to map and visualize the metabolic overview, hormone regulation, secondary metabolism, transcription factors, and protein targeting. Firstly, I visualized the drought-regulated genes in common in at least 6 of 9 studies. Secondly, I visualized the drought-regulated genes in common between the three studies in seedlings and finally in common between the five in mature leaves. The PageMan analysis, plugin of MapMan, was used to visualize differences among metabolic pathways using Wilcoxon tests, no correction, and an over-representation analysis (ORA) cutoff value of 3. All the homologous TAIR IDs of the 9 studies were searched against the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (Huang et al., 2009) Web server (https://david.ncifcrf.gov/). The gene ontology information related to the biological process was extracted from the DAVID result.

- Gene mapping in crop chromosomes

The drought-regulated genes involved in abiotic stress, hormone metabolism and transcription factors were selected for the chromosome mapping. I found out the corresponding chromosome number, start and end of the drought-regulated gene IDs from the annotation file downloaded from Phytozome (https://phytozome.jgi.doe.gov) using custom made Perl script. These genes were then mapped towards the chromosome according to their chromosome number, start and end points.

- Protein-protein interaction network

NetworkAnalyst (Xia et al., 2014), a web-based tool for network-based visual analytics of protein-protein interaction networks, was used. The list of homologous TAIR IDs from 6 of 9 studies were uploaded and mapped against the STRING interactome database with default parameters (confident score cutoff = 900 and with experimental evidence) provided in NetworkAnalyst. The networks between drought-regulated genes in seedlings and in mature leaves corresponding to the list of the visualized genes in MapMan were also obtained. To study the key connectives and to simplify the large network, I selected “Minimum Network”.

2.4. Results

Based on the search criteria described in Methods, found 22 RNA-Seq were studies: 7 were performed in roots, 12 in leaves, 3 in fruits. Among leaf studies, 3 of them have no raw data available. Indeed, the analysis was performed using 9 studies (Clauw et al., 2015, Song et al.,

2017, Corso et al., 2015, Li et al., 2017, Pieczynski et al., 2017, Orcheski et al., 2016, Liu et al., 2017): 5 dealing with mature leaves, 1 in young leaves and the other 3 in seedlings. The 9 studies comprise of 2 fruit tree crops and 5 herbaceous ones. The selected species belong to C3 and C4 plants. Photosynthesis is the process that plants use to turn light, carbon dioxide, and water into sugars that fuel plant growth, using the primary photosynthetic enzyme Rubisco. C3 plants do not have the anatomic structure nor the abundance of PEP carboxylase to avoid photorespiration like C4 plants. The articles and crops selected for the study, number of up- and down-regulated genes were listed in Table 2.2.

Table 2.2: Table 1 The number of up-regulated and down-regulated genes in response to drought for each study. Number of up- and down-regulated genes in common in at least 6, 7, 8, 9 of 9 studies

Article	Crop	Sample Information		
		Total	Up	Down
Clauw et al. (2015)	*Arabidopsis thaliana*	17,230	8184	9046
Song et al. (2016)	*Zea mays* cv. B73 (Study1)	11,693	5611	6082
Corso et al. (2015)	*Vitis vinifera* cv. M4	11,114	6154	4960
Li et al. (2017)	*Zea mays* cv. B73 (Study 2)	10,601	5225	5376
Pieczynski et al. (2018)	*Solanum tuberosum* cv. Gwiazda	10,843	6409	4434
Orcheski et al. (2016)	*Malus X domestica*	16,700	8545	8155
Liu et al. (2017)	*Solanum lycopersicum* cv. M82	9746	5164	4582
Haider et al. (2017)	*Vitis vinifera* cv. Summer Black	9420	2866	6554
Liu et al. (2015)	*Triticum aestivum* cv. TAM107	11,556	5830	5726
Commonly regulated in 9 of 9 articles		0	0	0
Commonly regulated in strictly 8 of 9 articles		12	5	7
Commonly regulated in strictly 7 of 9 articles		15	11	4
Commonly regulated in strictly 6 of 9 articles		351	147	204

The analysis resulted in the identification of a total of 108,903 genes in which 53,988 were up-regulated and 54,915 were down-regulated. For each of the analysis, the total number of genes range from 9420 to 17,230. The number of genes up-regulated was in a range of 2866 to 8184 and down-regulated genes were span from 4582 to 9046. The two *Vitis vinifera* studies form a cluster showing an overall transcriptomic similarity towards the analysis to drought (Figure 2.2).

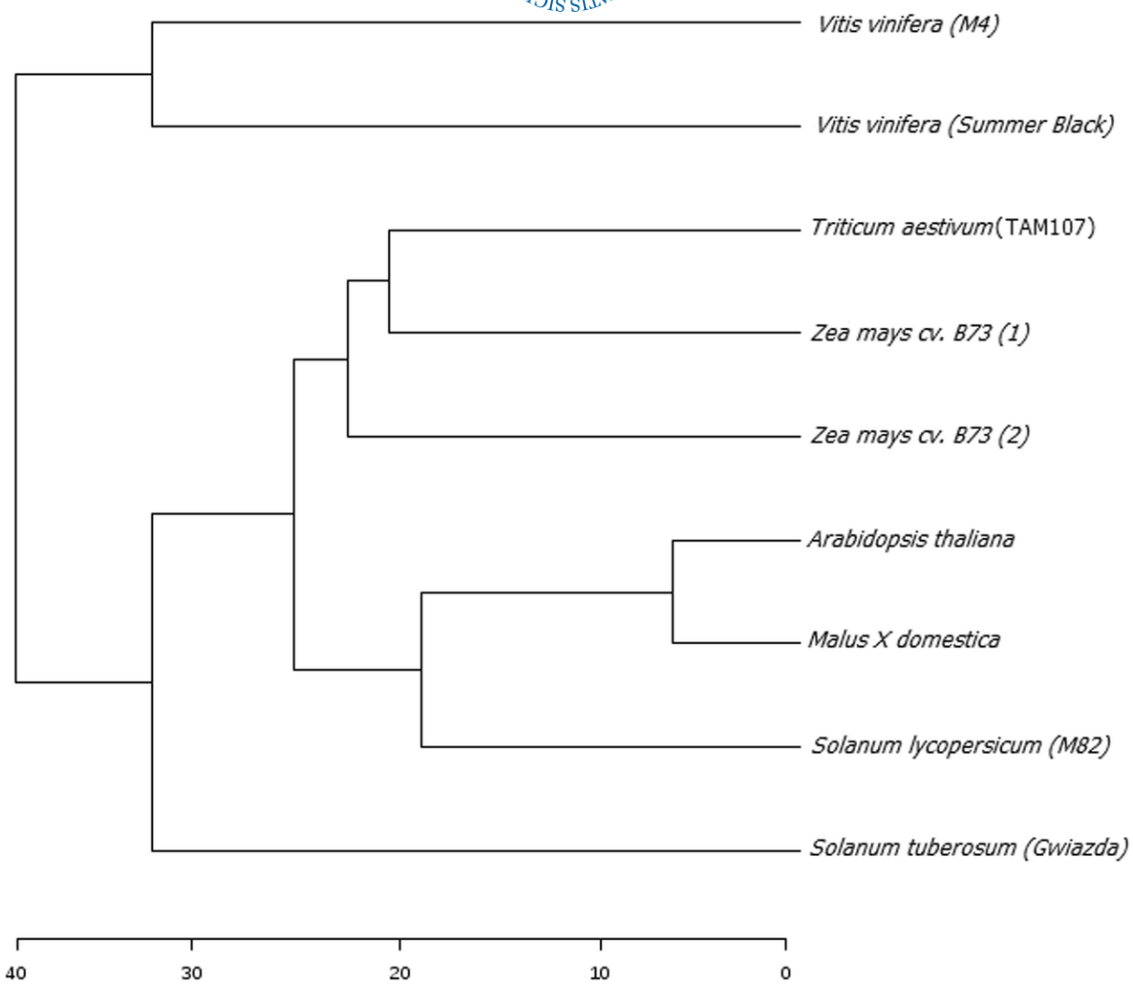


Figure 2.2: Dendrogram showing the hierarchical relationship among the RNA-seq studies selected for the meta-analysis. Resulted log2FC values of the analysis were used for generating the tree. Plant species used for the analysis (9 studies) were indicated

Although the transcriptomic responses in the two maize studies were very similar, the closeness of one maize study to wheat study was higher than between the two maize studies. The similarity in drought responses among apple, Arabidopsis thaliana and tomato were related since they dealt with drought responses in seedling leaves.

- Gene set and pathway enrichment analysis

DAVID software was used to identify the common biological processes affected by drought at transcriptomic level considering the drought-regulated genes in at least 6 of 9 studies. Metabolic pathways divided in up- or down-regulated by drought along with GO ID, its GO term, count, p-values and Benjamini values were shown (Table 2.3).

Table 2.3: Significantly regulated biological processes (FDR < 0.05) which are commonly regulated in at least 6 of 9 transcriptomic studies

GO_ID	GO_TERM	Count	P-value	Benjamini test
DOWN-REGULATED				
GO:0006633	fatty acid biosynthetic process	3	7.02E-250	5.80E-247
GO:0015031	protein transport	6	2.93E-129	1.21E-126
GO:0016192	vesicle-mediated transport	4	1.08E-117	2.96E-115
GO:0046777	protein autophosphorylation	5	3.73E-117	7.70E-115
GO:0006468	protein phosphorylation	13	4.54E-32	7.49E-30
GO:0006839	mitochondrial transport	3	8.95E-26	1.23E-23
GO:0006096	glycolytic process	3	1.83E-23	2.16E-21
GO:0006412	Translation	9	7.14E-23	7.38E-21
GO:0000398	mRNA splicing, via spliceosome	3	3.95E-21	3.62E-19
GO:0071555	cell wall organization	3	3.95E-21	3.62E-19
GO:0006349	regulation of gene expression by genetic imprinting	3	4.28E-21	3.54E-19
GO:0010025	wax biosynthetic process	3	5.84E-21	4.39E-19
GO:0009611	response to wounding	5	4.47E-19	3.08E-17
GO:0006855	drug transmembrane transport	3	1.07E-17	6.77E-16
GO:0009409	response to cold	6	7.87E-15	4.65E-13
GO:0009553	embryo sac development	3	2.10E-12	1.15E-10
GO:0048364	root development	4	2.84E-12	1.47E-10
GO:0006629	lipid metabolic process	4	5.96E-11	2.90E-09
GO:0009058	biosynthetic process	3	3.39E-09	1.56E-07
GO:0009416	response to light stimulus	4	3.48E-08	1.51E-06
GO:0016310	Phosphorylation	4	4.45E-08	1.84E-06
GO:0009826	unidimensional cell growth	3	5.57E-08	2.19E-06
GO:0009723	response to ethylene	3	7.02E-08	2.64E-06
GO:0009751	response to salicylic acid	3	7.02E-08	2.64E-06
GO:0006508	Proteolysis	6	1.36E-07	4.89E-06
GO:0009555	pollen development	3	2.58E-07	8.86E-06
GO:0006886	intracellular protein transport	3	3.09E-07	1.02E-05

GO_ID	GO_TERM	Count	P-value	Benjamini test
GO:0032259	Methylation	3	3.09E-07	1.02E-05
GO:0055085	transmembrane transport	3	3.09E-07	1.02E-05
GO:0045893	positive regulation of transcription, DNA-templated	3	4.61E-07	1.47E-05
GO:0051301	cell division	3	7.84E-07	2.40E-05
GO:0006511	ubiquitin-dependent protein catabolic process	3	7.84E-07	2.40E-05
GO:0006952	defense response	6	9.34E-07	2.76E-05
GO:0006457	protein folding	3	2.42E-06	6.90E-05
GO:0006979	response to oxidative stress	3	2.42E-06	6.90E-05
GO:0008152	metabolic process	3	3.55E-06	9.78E-05
GO:0007275	multicellular organism development	3	4.73E-06	1.26E-04
GO:0016567	protein ubiquitination	3	5.79E-06	1.49E-04
UP-REGULATED				
GO:0006351	transcription, DNA-templated	14	7.89E-06	1.98E-04
GO:0006355	regulation of transcription, DNA-templated	15	8.21E-06	1.99E-04
GO:0055114	oxidation-reduction process	6	1.23E-05	2.90E-04
GO:0006970	response to osmotic stress	5	3.37E-05	7.74E-04
GO:0009737	response to abscisic acid	8	3.64E-05	8.11E-04
GO:0009845	seed germination	4	3.82E-05	8.29E-04
GO:0006396	RNA processing	4	3.84E-05	8.13E-04
GO:0042542	response to hydrogen peroxide	3	5.36E-05	0.001106
GO:0009636	response to toxic substance	3	5.78E-05	0.001163
GO:0009408	response to heat	4	5.78E-05	0.001163
GO:0009624	response to nematode	3	6.12E-05	0.001203
GO:0009414	response to water deprivation	5	6.12E-05	0.001203
GO:0009734	auxin-activated signaling pathway	4	6.53E-05	0.001253
GO:0009738	abscisic acid-activated signaling pathway	4	7.53E-05	0.001412
GO:0009908	flower development	4	1.07E-04	0.001968

GO_ID	GO_TERM	Count	P-value	Benjamini test
GO:0006470	protein dephosphorylation	3	1.29E-04	0.002319
GO:0006810	Transport	5	1.29E-04	0.002319
GO:0009651	response to salt stress	6	1.36E-04	0.00238
GO:0015979	Photosynthesis	3	1.36E-04	0.00238
GO:0009733	response to auxin	4	1.38E-04	0.002364
GO:0007165	signal transduction	5	1.51E-04	0.00255
GO:0009873	ethylene-activated signaling pathway	3	1.90E-04	0.003131
GO:0009735	response to cytokinin	3	2.35E-04	0.003805
GO:0035556	intracellular signal transduction	3	2.61E-04	0.004137
GO:0042742	defense response to bacterium	3	2.61E-04	0.004137
GO:0046686	response to cadmium ion	3	3.34E-04	0.005191
GO:0005975	carbohydrate metabolic process	3	3.35E-04	0.005117
GO:0009793	embryo development ending in seed dormancy	3	3.35E-04	0.005117

No GO-terms related to the biological process were commonly drought-regulated in at least 7 of 9 studies. Among at least 6 of 9 articles, 38 GO-terms were down-regulated while 28 were up-regulated. Among them, it is worthy to mention some of the biological pathways that are known to be repressed by the drought stress such as wax biosynthesis and cell wall organization, fatty acid biosynthesis, protein phosphorylation. On the opposite, the study identified some GO-terms that were up-regulated in response to water stress such as response to osmotic stress, response to abscisic acid, response to water deprivation, abscisic-activated signalling pathway, response to salt stress, response to hydrogen peroxide.

- Abiotic stress responses

Genes mapped in the abiotic stress-related GO-terms identified by DAVID are shown in Figure 2.3.

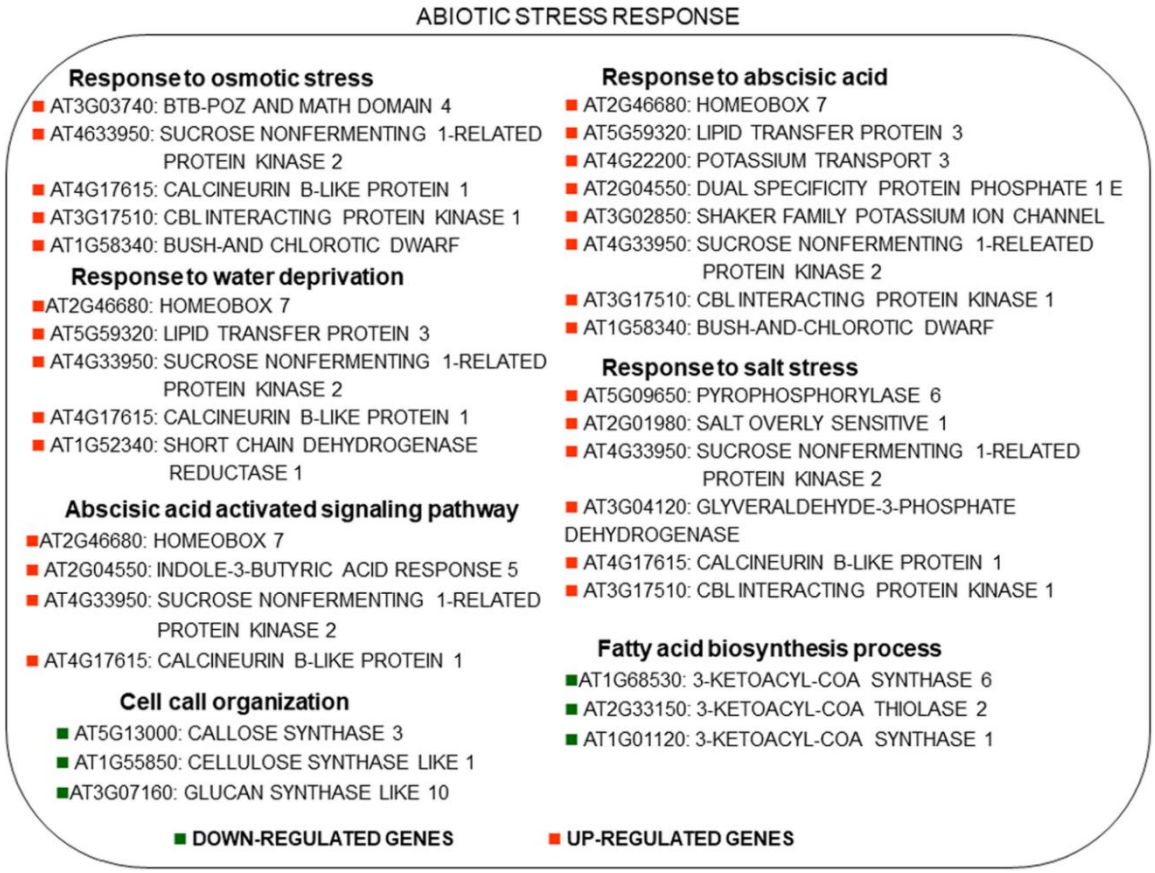


Figure 2.3: Drought-regulated genes involved in abiotic stress-related categories which are commonly regulated in at least 6 of 9 studies were indicated. Genes were identified as Arabidopsis thaliana orthologs of each gene of the analyzed plant species. Red indicated up-regulation and green indicated the down-regulation in response to drought

Among the drought up-regulated genes involved in osmotic and salt stress, it is worth to mention the sucrose-related protein kinase and the CBL interacting protein kinase, the salt overly sensitive 1, and pyrophosphorylase 6. In the category of "response to water deprivation", there was up-regulation of homeobox 7, lipid transfer protein 3, open stomata 1, calcineurin B-like protein. Four genes were up-regulated by drought and involved in "abscisic acid-activated signalling" while 8 of the drought up-regulated genes were involved in "response to abscisic acid". Drought repressed three genes involved in fatty acid biosynthesis such as 3-ketoacyl-coa synthase 1 and 6 and 3-ketoacyl-coa thiolase 2.

- Secondary metabolism, cellular responses, signalling

MapMan web-tool was used to identify transcriptomic effects of drought in key selected categories such as secondary metabolism, cellular responses and signalling (Figure 2.4).

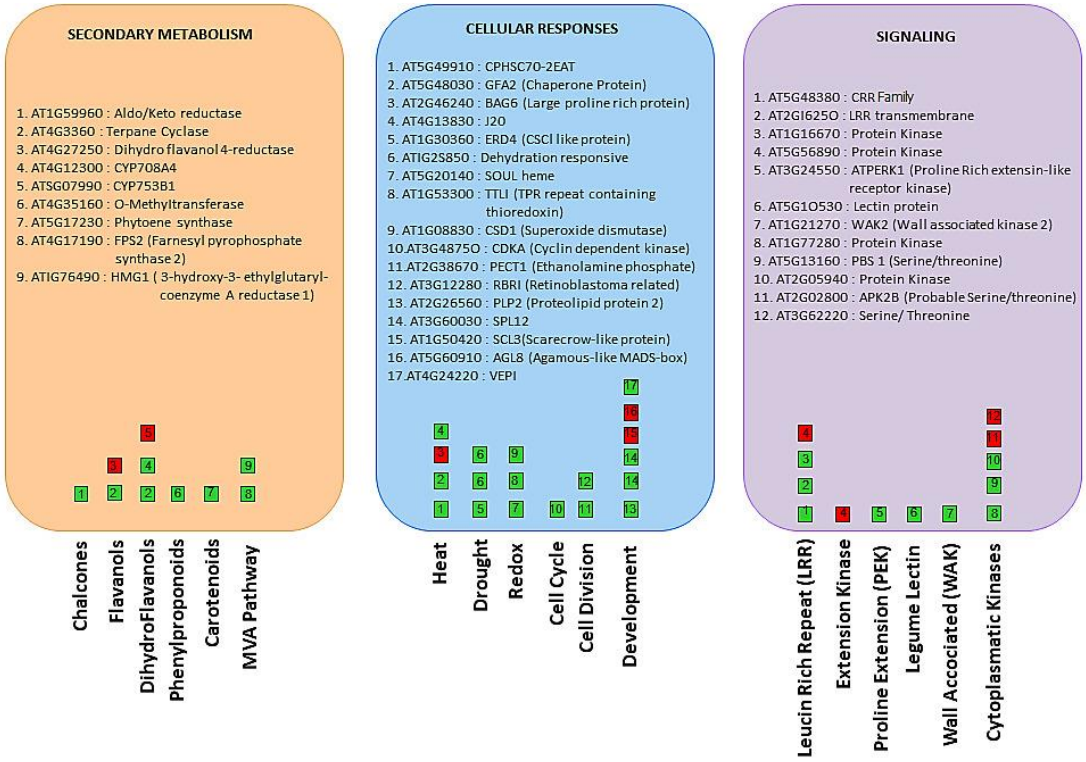


Figure 2.4: MapMan overview showing transcriptomic effects of drought in key categories selected such as secondary metabolism, cellular responses and signaling. Genes were identified as Arabidopsis orthologs of each genes of the analyzed plant species. Red means up-regulated and green means down-regulated.

Among the secondary metabolism, the drought-repressed genes were involved in terpene pathways such as terpene cyclase, phytoene synthase, farnesyl pyrophosphate synthase 2. Cellular response genes were mostly inhibited by drought. MADS transcription factors like AGL8 (agamous-like MADS-box) and SCL3 (scarecrow-like protein) were enhanced. Relating to signalling mechanisms, genes encoding for 2 leucin rich repeat genes, 3 protein kinases, a proline-rich extension like receptor kinase, a lectin protein were repressed. On the other hand, a protein kinase (AT5G56890), and two serine/threonine kinases were up-regulated.

- Transcription factors and hormones

Among the drought-up regulated transcription factors, it is worthy to mention the induction of AL1 (alfin-like), UGKYAH (trihelix), WRKY20, zinc ion binding, two homeobox genes (one CDF2 and an SDG26 (SET domain)). Among the repressed ones, there were two bHLH

members, a MYB factor (TKI1), two ABA-related TF (ABI3VP1), and ARR2 (cytokinin-related) (Figure 2.5).

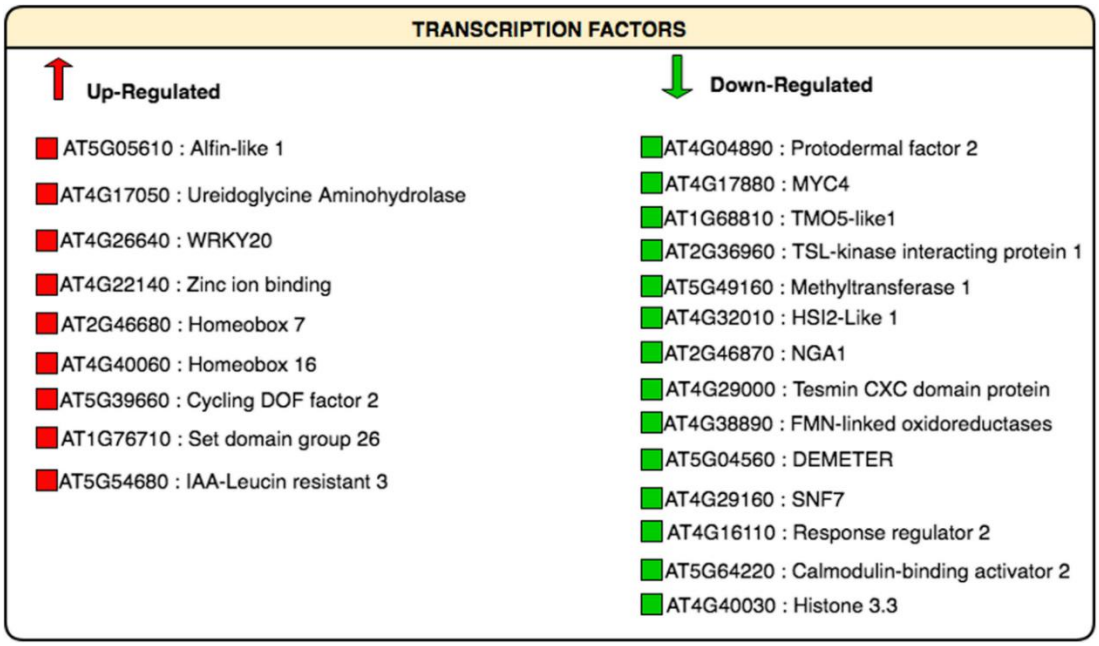


Figure 2.5: Drought-regulated genes involved in transcription factors which are commonly regulated in at least 6 of 9 studies. Genes were identified as Arabidopsis thaliana orthologs of each gene of the analyzed plant species. Red indicated up-regulation and green indicated the down-regulation in response to drought

Figure 2.6 summarized the drought-regulated genes involved in hormone-related categories. Ethylene and salicylic acid pathways were repressed by drought whereas auxin, abscisic acid, cytokinin, ethylene pathways were mostly up-regulated. Water deprivation down-regulated three genes responsive to ethylene and three responsive to salicylic acid (such as glutathione-s-transferase 2) while it up-regulated several genes responsive to auxin, abscisic acid, cytokinin and ethylene activated signalling pathway. Among the auxin-responsive genes it is worth to mention the enhancement of indole-3-butyric acid response 5 and the phytochrome associated protein 2. Relating to abscisic acid there was an up-regulation in homeobox 7, lipid transfer protein 3, shaker potassium ion channel, SNF1, potassium transport 3. The cytokinin responsive gene, heat shock protein 93, and three ethylene-related genes, ERF1, SKP1 and DREB were enhanced.

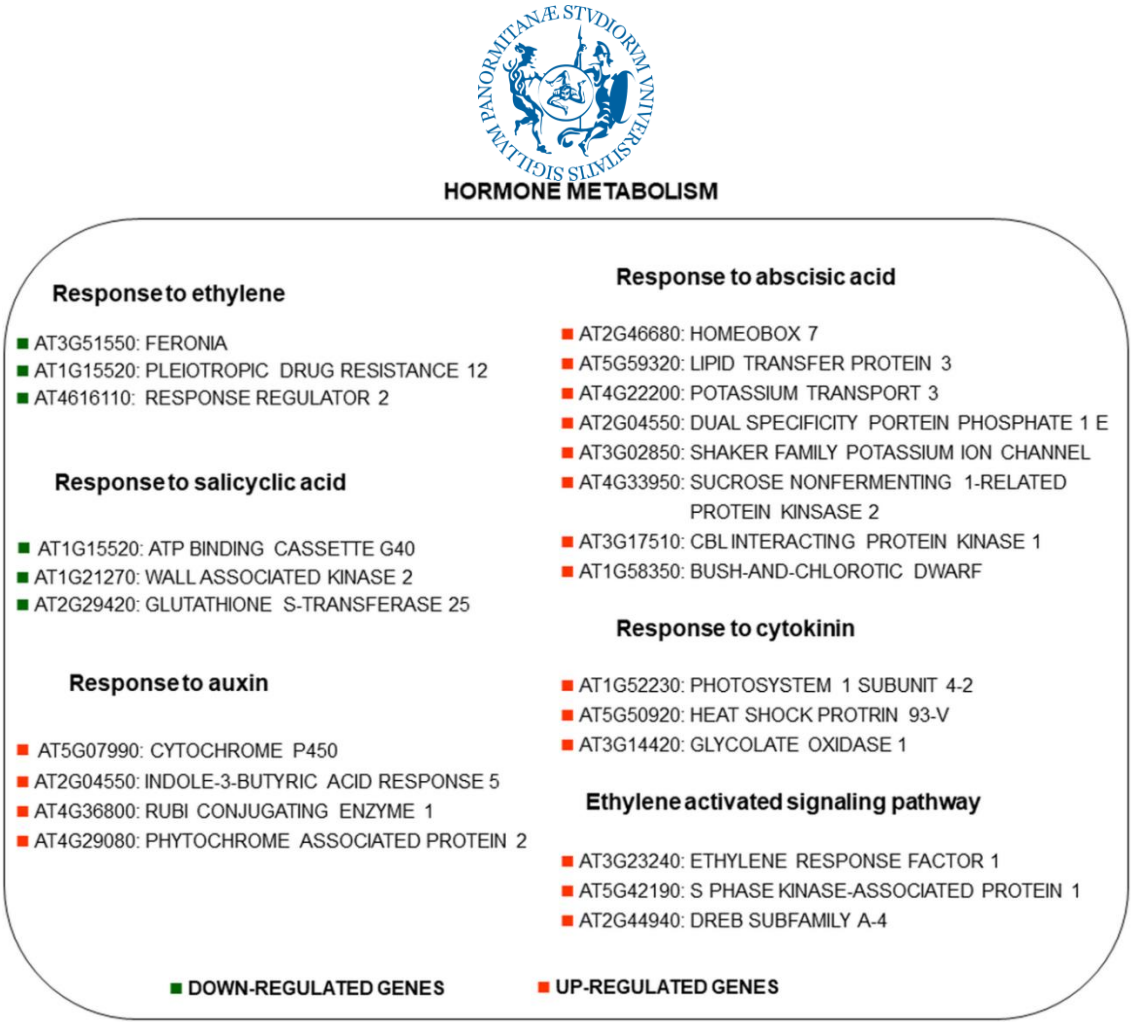


Figure 2.6: Drought-regulated genes involved in hormone-related categories which are commonly regulated in at least 6 of 9 studies were shown. Genes were identified as Arabidopsis orthologs of each gene of the analyzed plant species. Red indicated up-regulation and green indicated the down-regulation in response to drought.

- Protein-protein network analysis

The protein-protein interaction (PPI) network analysis comprises of 351 drought-related genes commonly regulated in at least 6 of 9 studies. Minimum default settings were used to reduce the number of interacting proteins and the complexity of the networks (Figure 2.7).

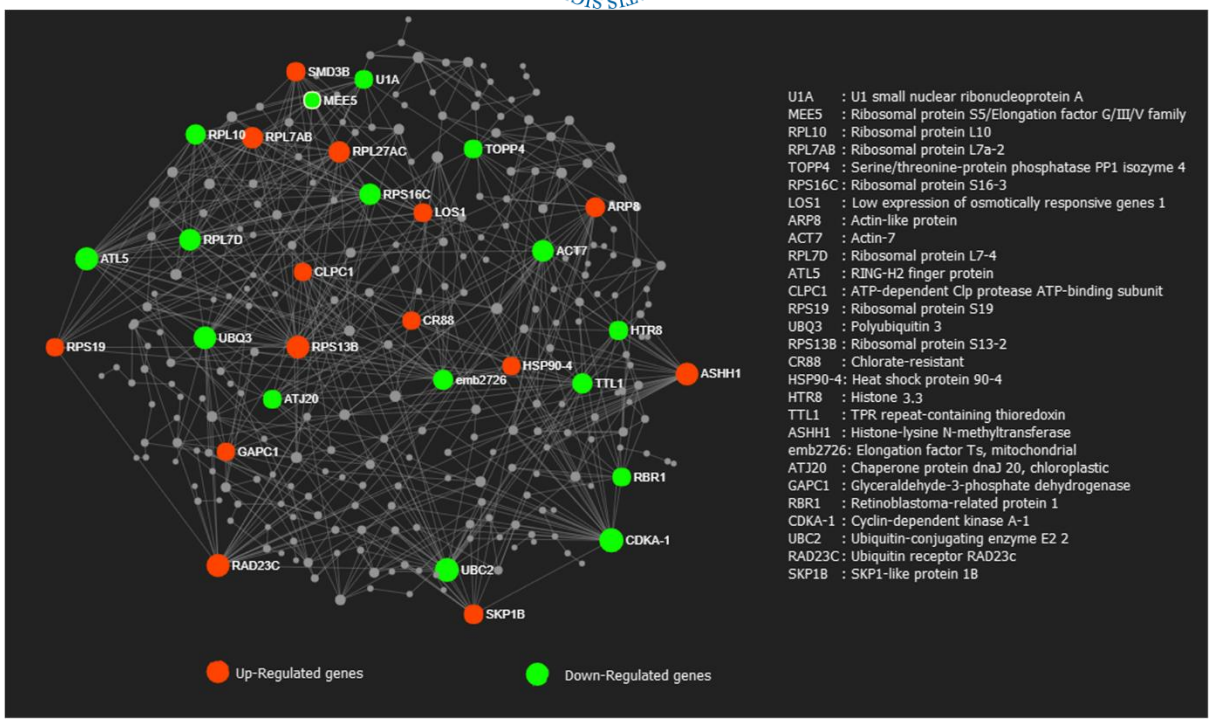


Figure 2.7: Protein-protein interaction network analysis predicted for genes commonly regulated in 6 of 9 studies based on Arabidopsis knowledgebase. Proteins encoded by genes having high degree of betweeness are shown in red color (up-regulated) and green color (down-regulated)

Some key genes with a high number of interactions (> 20) were highlighted. Among the up-regulated hub (highly interacting) proteins it is worthy to notice some key proteins that may play a key role in drought response such as LOS1 (Low expression of osmotically responsive genes 1), HSP90–4 (heat shock protein 90–4), SKP1B (SKP1-like protein 1B), CR88 (chlorate resistant). Interestingly, drought down-regulated highly interactive proteins such as ATL5 (ring H2 finger protein), UBQ3 (polyubiquitin 3), TTL1 (TP repeat-containing thioredoxin), ATJ20, (chaperone protein DNAJ 20), CDKA-1 (Cyclin Dependent Kinase A-1). PPI network analysis was performed for drought-regulated in common between the three seedling studies and between the five studies on mature leaves. In seedling, two major hub proteins WDR5A (histone methylase component) and ASHH1 (histone lysine N methyltransferase) were up-regulated. In mature leaves, an LRR receptor-like serine/threonine protein kinase was repressed while CDKF-1 (cyclin dependent kinase F-1) was up-regulated.

- Chromosome mapping of key drought-regulated genes in crops

Key genes encoding genes related to abiotic stress responses, transcription factors, hormone metabolism (obtained from DAVID software) were mapped in the respective chromosomes of the 7 crops. There was a total of 55 genes. Interestingly, I observed that in some species there was not a homogeneous distribution of these genes across chromosomes since some chromosomes contained a higher number of them. While in apple, potato, and tomato there was a similar distribution of these genes in the chromosomes, whereas, in *Zea mays, Triticum aestivum* and *Arabidopsis thaliana* there was a higher presence of these genes in some of the

chromosomes. In maize, a total of 29 abiotic stress-related genes were mapped to chromosome 1 implying that the chromosome 1 regions should contain more genes involved in drought resistance than the other chromosome regions. In *Arabidopsis thaliana*, 17 genes were mapped to chromosome 4. In *Triticum aestivum* chromosome 2 (2A + 2B + 2D) and 5 (5A + 5B + 5D genome) mapped respectively 15 and 12 genes. This work allowed to identify which chromosome might contain more genes involved in drought resistance and will guide the identification of new molecular markers linked with drought resistance.

- Drought-regulated transcriptomic responses at different leaf stage

Attention was paid on the drought-responsive genes at different leaf developmental stages. Comparing the three studies dealing with drought transcriptomic responses in seedlings (tomato, *Arabidopsis thaliana* and apple), 934 commonly drought-regulated genes were identified (Figure 2.8).

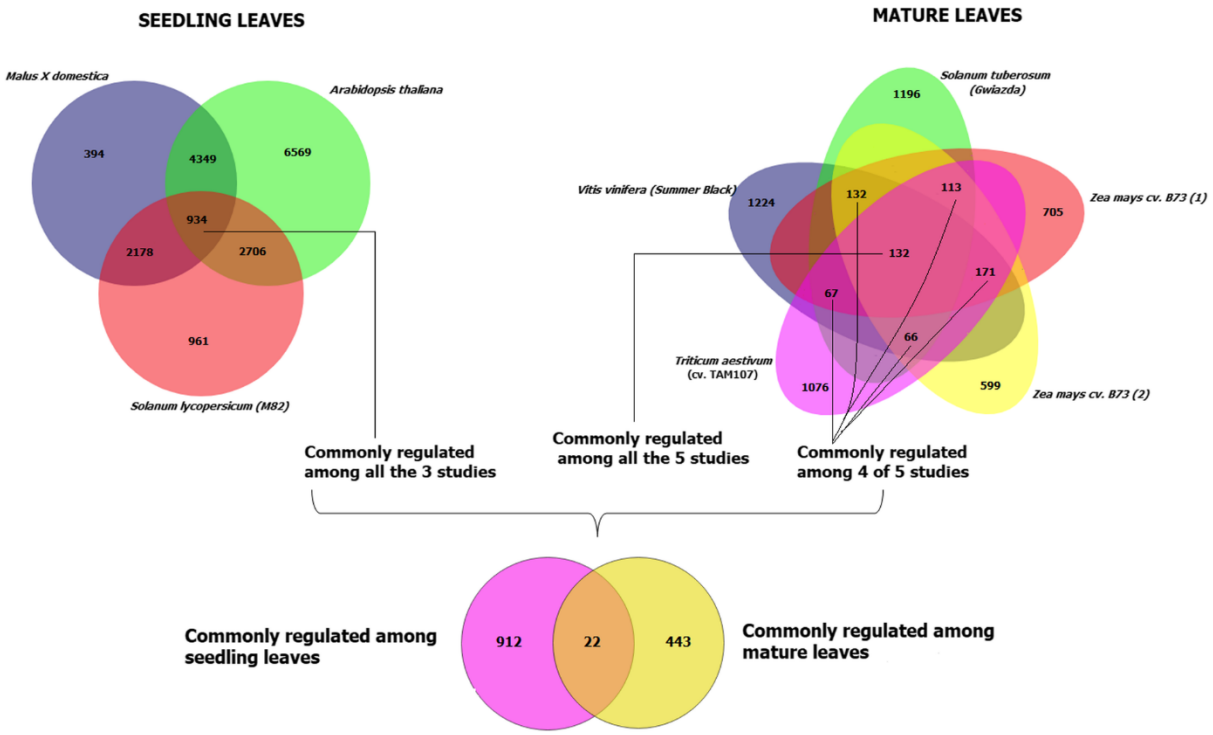


Figure 2.8: Comparison between transcriptomic responses to drought during leaf development (in seedlings and mature leaves). Venn-diagram showing the number of commonly regulated and unique genes responsive to drought in the three seedling studies and in the five studies dealing with mature leaves.

On the other hand, 465 genes were commonly drought-regulated in at least 4 of 5 studies in mature leaf tissues. Finally, comparing the two lists of drought-regulated genes, 912 genes were specifically drought-regulated in seedlings, 443 in mature leaves and 22 in common between the two types of leaves. These results demonstrated that transcriptome reprogramming in response to drought depends on different leaf developmental stage.

- Drought-responsive genes in seedlings

Among the genes that were regulated by drought in seedlings, I paid attention to those belonging to key categories playing an important role in drought response modulation such as hormones, transcription factors and abiotic defence responses. Relating to hormones, two ethylene-related genes (ERF4 and ethylene-responsive element binding protein (ESE3, AT5G25190)) were up-regulated in all the three seedling studies. In addition, there were other 5 up-regulated genes involved in auxin (TIR1, auxin-responsive protein (AT4G38840)), abscisic acid (HVA22), gibberellin (KAO2, GA4). On the other hand, there were 8 down-regulated hormone-related genes: oxidoreductase B2 and AIR9 (auxin-related), AREB3 (abscisic acid), BAK1 (brassinosteroid-related), CKX7(cytokinins), gibberellin-20-oxidase2 and gibberellin-2-beta-dioxygenase (gibberellins). Three genes involved thioredoxin pathways were also up-regulated: APRL5, PDIL5–1, ATY1. Unexpectedly there were also some heat stress-related genes repressed such as (HSP17.8, ARL1, GFA2, HSP98.7). Regarding with transcription factors, there were some key categories that were commonly up-regulated among crops such as MYBs (MYB3, MYB94, MYB1), bHLH, and homeobox. Relating to the WRKY family, two genes were up-regulated (WRKY53 and WRKY20) while one gene was repressed (WRKY22). Interestingly, the SET-domain family was mainly up-regulated.

- Drought-responsive genes in mature leaves

Genes involved in the same categories that are commonly drought-regulated in at least 4 of 5 studies (cellular responses, hormones, transcription factors) were studied in detail. In total 4 hormone-related genes were drought-repressed such as an auxin-responsive (RRT4; O-fucosyltransferase family protein), two ABA-related genes (NCED4, HVA22A) and one salicylic acid-related (UDP-glucosyltransferase). ERF1, a key player in jasmonic acid-ethylene crosstalk was up-regulated by drought in mature leaves. Unexpectedly I observed that most of the genes encoding transcription factors were repressed by drought including YABBY5, ARR2, BLH6, TRFL2, three zinc finger proteins and other 5 genes. Alfin-like 1 was the only up-regulated transcription factor. Relating to another primary metabolism, it is worth notice that two genes involved in phospholipid biosynthesis were repressed (phosphatidylserine synthase and galactolipid galactosyltransferase).

2.5. Discussion

- Common drought responses across plant species in all kind of leaves

This study enabled the identification of drought-regulated genes conserved across species, addressing the first question of the aim of the analysis. Twenty-seven genes were up- or down-regulated in response to drought in at least 7 of 9 studies. Some of them required particular attention considering that they have been previously linked to drought responses in single studies. They were ERF1 (involved in ethylene signalling), WRKY20 and Alfin-like 1, zinc finger ocre domain protein 1 (transcription factors), serine carboxypeptidase 27 and protein kinase 2B (involved in signalling). The involvement of these genes in drought responses is discussed below.

Among the drought-regulated 351 genes in at least 6 of 9 studies, attention was paid on the hormone, transcription factor and stress defence categories. Among those genes related to osmotic stress, the up-regulation of sucrose nonfermenting1–related protein kinase2 (SNF1-related protein kinase; also named as SnRK2) was conserved across species. This member

belongs to a family of genes that have been previously associated with osmotic stresses (Boudsocq et al., 2005). Kobayashi et al., 2004 showed that these members are induced by osmotic stress and that three of them are activated through an ABA-dependent manner. Its role is extremely important in guard cells where it is playing a key role as a central hub to mediate ABA signalling (Yoshida et al., 2005). Taken together, the meta-analysis confirmed that this gene should be an important player in sensing water deprivation in leaf tissues in different crops. This gene should be considered as a target for crop genetic engineering for the development of molecular markers associated with drought-resistance in crops.

The work identified two genes involved in abiotic stress signalling: a calcineurin B-like (CBL) calcium sensor protein and a CBL interacting protein kinase 1. Calcineurin B-like proteins (CBLs) represent a unique family of plant calcium sensors that relay signals by interacting with a family of protein kinases, designated as CBL-interacting protein kinases (CIPKs). A previous study indicated that CIPK23 play this important role in water stress response by interaction with the calcium sensors CBL1 and CBL9 that synergistically regulates CIPK23. As suggested by (Cheong et al., 2007), the different combination of CIPK and CBL members should be responsible for cell-specific signalling responses (osmotic stress or potassium uptake) in different organs (leaves or roots). Based on these findings, it is possible to speculate that the simultaneous induction of calcineurin B-like calcium sensor protein (AT4G17615) and CBL interacting protein kinase 1 (AT3G17510) across 6 of 9 studies implies that the two proteins should play a major role in the activation of rapid drought sensing. This hypothesis implies that these two genes might be also considered as good targets for molecular breeding to enhance abiotic stress resistance.

Relating to GO term “response to water deprivation”, three additional genes were up-regulated such as homeobox 7, lipid transfer protein 3 (LTP3), short dehydrogenase reductase 1 (ABA2). Homeobox 7 belongs to Homeodomain-leucine zipper (HD-Zip) family proteins which are transcription factors related to environmental stress responses in plants. A member of homeobox family has been shown to confer resistance to drought in *Helianthus annuus* (sunflower) through over-expression (Dezar et al., 2005). LTP3 is known to bind to lipids and its over-expression enhanced drought tolerance through the action of MYB96 that directly binds to its promoter (Guo et al., 2013). ABA2 is a NAD- or NADP-dependent oxidoreductases involved in ABA biosynthesis. This gene is responsive to ABA exogenous treatment (Zhou et al., 2014). The analysis showed that a SOS3 like calcium binding protein was commonly induced by drought in 6 of 9 crops. This gene encodes a member of the calcineurin B-like calcium sensor gene family and mediates salt tolerance by regulating ion homeostasis in Arabidopsis. I also observed an up-regulation of salt over sensitive 1 (SOS1) that is a key player of the Salt-Overly-Sensitive (SOS) pathway, essential for maintaining a normal ion ratio in the cytoplasm in salt conditions (Huang et al., 2012). Salinity is biphasic stress composed by an initial change of osmotic conditions followed by a subsequent stage of ionic modifications. Indeed, SOS1 plays an important role in the second phase of salinity stress. Transgenic over-expression of this gene has shown to induce drought tolerance in *Arabidopsis thaliana* demonstrating that improved resistance to salt stress can be obtained by limiting Na^+ accumulation in plants (Shi et al., 2003). Being drought mainly osmotic stress, the induction of this gene implies a possible role of this gene in the response to osmotic changes too. This might be explained by the fact that water deprivation has the consequence to increase the levels of soil ion concentrations which indirectly causing salt stress. The meta-analysis highlighted the repression of fatty acid biosynthesis in response to drought in leaves. It is known that water deficit inhibits fatty acid desaturation and drought

resistance has been linked with a reduction of fatty acid metabolism in cotton resulting in greater stability of the membrane system (Pham-Thi et al., 1985).
Relating to hormones, I found several drought-regulated genes in common between 6 of 9 studies. Three genes were involved in ABA biosynthesis and signalling, two genes in auxin response, two genes involved in ethylene-related pathways. ABA2 Arabidopsis thaliana mutants showed a reduced drought tolerance in comparison to wild type implying that the up-regulation of this gene should be a benefit for drought resistance. The meta-analysis showed another unexpected result: ABA3 was repressed in response to water stress in 6 of 9 crops. This gene is a basic leucine zipper (bZIP)-type ABRE-binding protein that was shown to be up-regulated by drought in vegetative tissues (Uno et al., 2000). Although at first glance, the results on both AREB2 and HVA22 seem to be in contrast with published findings, the repression of this gene in response to drought might be due to differences in the analyzed time points and drought intensity between studies.
Relating to ethylene biosynthesis, I found that ACS12 was constitutively repressed by drought. It is generally accepted that ethylene is involved in mediating plant responses to abiotic stress. ACS cereal mutants showed to have delayed leaf senescence in drought conditions. Mutant leaves continue to be photosynthetically active under water stress implying that leaf function is maintained (Young et al., 2004). These findings showed that ethylene may serve to determine the onset of natural senescence and regulate drought-induced senescence. Based on these findings, I may speculate that the repression of ACS12 in leaves should be beneficial to inhibit ethylene biosynthesis and consequently improve drought resistance. ERF1 is known to be involved in plant disease resistance (Singh et al., 2002) but its role in abiotic stress responses is less clear. ERF proteins are characterized by an ERF DNA binding domain. These transcription factors bind to multiple cis elements such as DRE/CRT and CE1 elements, involved in stress responses (Zhang et al., 2009). In *Arabidopsis thaliana*, the expression of ERF1 enhanced tolerance to drought. hypothesized that ERF1 was linked with enhanced drought resistance in rice through the induction of ABA2. Since I found that both ABA2 and ERF1 were induced in 6 of 9 crops, the findings confirm this hypothesis rendering these two genes potential targets for enhancing resistance to drought. Among the conserved drought up-regulated transcription factors, it is worth to mention WRKY20, a member of WRKYs. This finding agrees with published works that demonstrated an increased drought tolerance due to the over-expression of WRKY20 in *Arabidopsis thaliana* (Luo et al., 2013).

- Drought-regulated genes at different leaf developmental stages

I answered the second question by identifying the drought-regulated genes commonly expressed among the three transcriptomic studies dealing with seedling responses and among the five studies performed on mature leaves. The findings highlighted that drought has very different transcriptomic effects on leaves depending on their developmental stage. Indeed, the identification of expression QTLs for drought resistance in leaves should clearly take in high consideration which developmental stage is considered. Among the 22 drought-regulated genes commonly expressed seedling and mature leaves it is worth noticing a transcription factor (alfin-like 1) and a heat shock protein (HSC70–7). Several members of Alfin-like TFs were up-regulated in response to different abiotic stresses in *Brassica oleracea*. The role Alfin-like TFs in enhancing salt stress and drought resistance is well-known when it is over-expressed in roots (Winicov et al., 2000). Alfin-like 1 is a transcription co-activator (Lee et

al., 2009) that contains a typical PHD finger binding promoter element of PRP2, a salt inducible gene. This study meta-analysis lets hypothesize that the role of this transcription factor might have a similar function in leaves.

- Drought-regulated genes in seedlings

The up-regulation of two ethylene signalling genes in seedlings (ERF4 and one EREBP (ESE3)) implies that ethylene might have a promoting effect in drought response at early leaf development. These findings agree with previously published works that showed that the over-expression of ERF4 promoted adaptation to salt stress and drought (Seo et al., 2010). This gene is a transcriptional repressor that suppressing a repressor of defence response genes positively regulates shoot growth and water-stress tolerance in rice during early growth stages (Joo et al., 2013). ESE3 belongs to a family of ethylene response factor (ERF) genes that are involved in enhancing salt tolerance. Results of the work confirmed that the up-regulation of ethylene signalling should play a key role in drought resistance. HVA22 is an ABA-responsive gene regulated by environmental stresses. The up-regulation of HVA22 has been shown to be tissue-specific and in response to drought in barley. The analysis confirmed this evidence showing an opposite trend of expression between seedlings and mature leaves. The results challenged the hypothesis that this gene should enhance drought resistance in mature leaves.

MYB is a large family of transcription factors well-known to be involved in drought. The transgenic over-expression of MYB1 enhanced drought resistance (Dai et al., 2007). MYB94 activates cuticular wax biosynthesis in *Arabidopsis thaliana* and might be important in drought response (Lee et al., 2017). The analysis confirmed the role of these two MYB factors in seedling response to drought implying that they should be considered potential targets for enhancing drought resistance. The induction of MYB factors in drought is reported in the selected articles (Liu et al., 2017). I also found that WRKY53 was up-regulated in seedlings in response to drought confirming previous findings that showed WRKY53 drives the inhibition of stomatal closure by reducing H_2O_2 content facilitating stomatal opening by promoting starch degradation and consequently inhibiting drought tolerance. The induction of WRKY20 in response to drought should allow a positive effect on drought tolerance in crops since the over-expression of this gene improved plant yields in soybean and enhanced drought tolerance in alfalfa (Ning et al., 2017). The protein-protein interaction analysis showed that WDR5A was up-regulated in all three seedling studies in response to drought. This confirmed the important role of this protein in drought responses. WDR5A is a regulating nitric oxide accumulation and NOS-like activity in guard cells to modulate stomatal closure for adaptive plant response to drought (Liu et al., 2016). In seedlings, this gene should drive the closure of the stomata and the survival of leaf cells under water deprivation.

- Drought-regulated genes in mature leaves

In mature leaves, this meta-analysis showed that ERF1 was up-regulated. The same result can be seen in (Song et al., 2017). The role of this gene in drought response has been previously discussed. Considering the mature leaf datasets all together, the role of ERF1 in modulating the expression of antioxidant and detoxifying proteins that protect cell components in leaf mature tissues is highlighted. ERF1 should work as a ‘regulatory gene’ under different stress

conditions, changing the expression of 'functional genes' acting as detoxification and osmotic adjustment enzymes or proteins to protect cells from damage.
BAG6 is a Calmodulin (CaM)-binding transcription activators (CAMTA), which translates calcium signatures into different biochemical, and molecular pathways (Evans et al., 2011)and acts as a multi-functional protein that regulates apoptotic-like processes involved in different abiotic stresses.
From this analysis it is evident that this gene was up-regulated in mature leaves across the different crops. Indeed, it is possible to speculate that this gene should be involved in signalling mechanisms in response to drought stress. Calcium (Ca^{2+}) works as a secondary messenger in plants, and it is involved in different responses to different environmental stresses (Pandey et al., 2013). These transcription factors modulate many functional genes involved in stress tolerance in plants including drought and regulate the expression of ERFs (Janiak et al., 2015). Based on these findings it is possible to hypothesize that the two genes BAG6 and ERF1 might be linked in a common signalling response to drought in crops in mature leaves. Interestingly the analysis showed that HSP70 was repressed by drought. The heat shock protein 70s (Hsp70s) and heat shock factors (Hsfs) play key roles in protecting plant cells or tissues from various abiotic stresses (Li et al., 2017). It was observed that heat shock proteins play as activators or repressors, suggesting that these proteins might be modulated by both the activation and the repression mechanisms under stress condition (Wen et al., 2017). Indeed, the effect of the repression of HSP70 in mature leaves under drought must be further investigated. Cyclin dependent kinases (CDKs) are signalling proteins induced by stresses such as drought (Campo et al., 2014). Since this gene was a highly interacting protein in drought-related gene networks, it can be speculated that the induction of CDKF1 in mature leaves should play an important role in the promotion of drought resistance in crops.

2.6. Conclusion

Taken together all these findings, I proposed a model of plant response to drought shown in Figure 2.9.

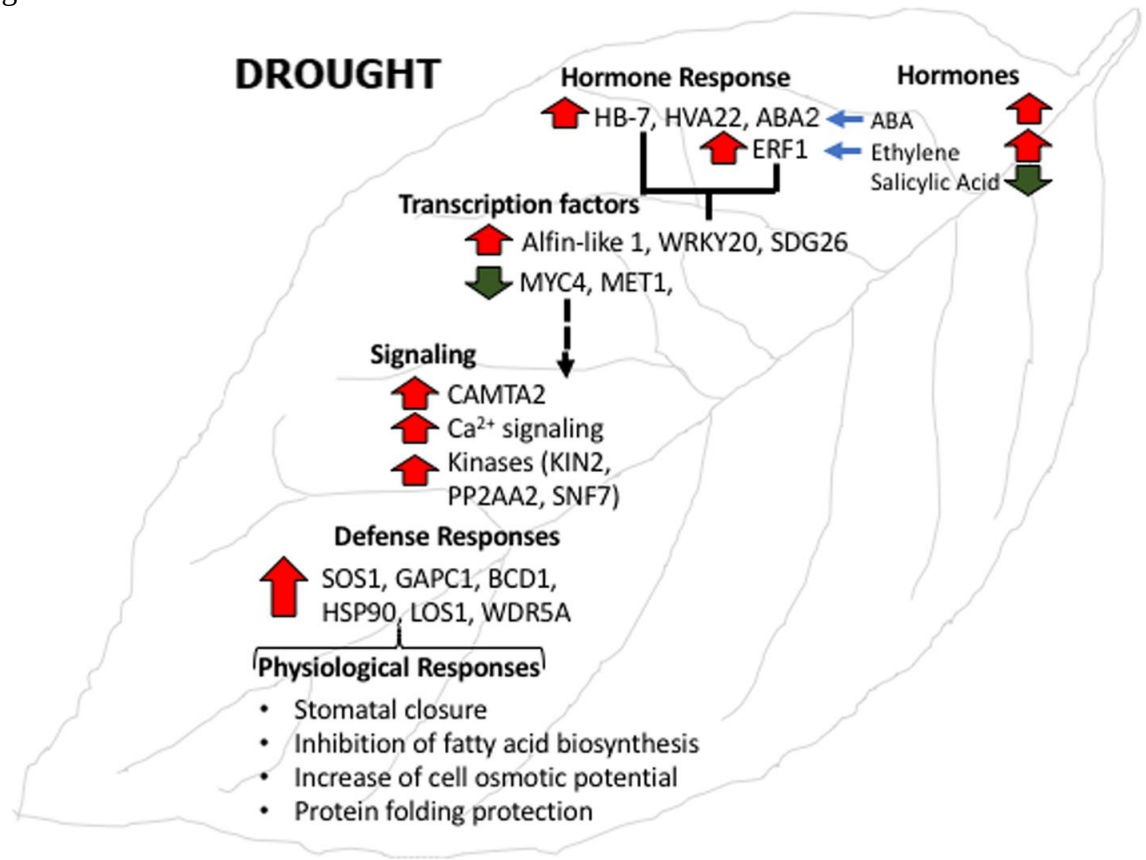


Figure 2.9: A model of transcriptional modulation of plant responses to drought in leaves. Important genes identified by the meta-analysis belonging to key functional categories and their consequent involvement in physiological responses were indicated

The first plant response should be the induction of the biosynthesis of key hormones such as ABA and ethylene driving the activation of key signalling proteins (ERF1, ABA2 and HB7). These proteins should promote the fine-tuned transcriptional modulation through the cross-talk of a complex network of transcription factors (Alfin-like 1, WRKY20, SDG26). The up-regulation of key proteins in the signal transduction (CAMTA2, KIN2, and SNF7) should provoke the induction of proteins involved in physiological defensive responses represented by stomatal closure, inhibition of fatty acid biosynthesis, an increase of osmotic potential and protection of protein folding. Molecular breeding for drought resistance should focus on these genes.

3. CHAPTER 3

Experiment 2: Identification of conserved genes linked to different abiotic stresses in leaves among different plant species.

DOI: https://doi.org/10.1071/FP20028

3.1. Introduction

Various environmental stresses such as drought, salt, cold and heat will affect the development, productivity, and quality of plants (Najafi et al. 2018). Due to the global climate change, some stress factors (e.g. heat, drought, and salinity) are becoming more prevalent and therefore the impact of these abiotic environmental stresses is becoming more significant. The simultaneous occurrence of several abiotic stress factors is particularly lethal to crops, and as a response, plants have evolved complex molecular networks to cope with and survive such environmental stresses (Najafi et al. 2018). Due to the rapid progresses of the next-generation sequencing technologies, the number of transcriptomic studies has increased exponentially in ISI/Scopus databases in the last 10 years. Hence, numerous transcriptomic studies dealing with abiotic environmental stress responses have been conducted in a few plant species including *Arabidopsis thaliana*. (Ding et al. 2013; Imran et al. 2018), *Malus domestica Borkh*. (Wu et al. 2015; Yang et al. 2017) and *Vitis vinifera* L. (Rienth et al. 2014; Zenoni et al. 2016; Benny et al. 2019a). Although each of these studies allows insights into the genes, pathways and functional gene categories implicated in specific combinations of stress and plant species, obtained data are characterised by low reliability because of high external and uncontrolled environmental variability. Transcriptomic studies are usually conducted in a specific environment, using a single time of sampling, and usually with a low number of biological replicates, so results are often highly speculative. The power of these studies is reduced by the evidence that the potential key players in abiotic stress resistance/tolerance are regulated by a high number of physiological, developmental, and environmental conditions. Thus, conclusions about the key transcriptomic mechanisms behind plant abiotic stress responses in plants are generally unreliable, and evidence to support hypotheses is weak. In an environmental context that becomes increasingly hostile and complex, a meta-analysis could provide a valuable tool since it aims to compare different transcriptomic studies concerning the same research purposes, identifying common molecular features and strengthen the power of next-generation sequencing (NGS) approaches reducing environmental variability and virtually increasing biological replicates. A meta-analysis could aid in a better understanding of the mechanisms underlying the problems of environmental stress that can compromise crop productivity and food security. Further, a meta-analysis could compare differentially regulated genes and affected pathways among different studies using the same bioinformatic methods (Rawat et al. 2015). In addition, a comparison of the molecular mechanisms related to different stress conditions would allow validation of potential candidate genes involved in specific and exclusive plant abiotic stress responses. Such information is crucial to shed light on the molecular regulatory networks related to abiotic stress responses in plants and to deliver stronger scientific evidence that could be used for next-generation crop breeding programs. This approach has been already used to identify key conserved genes involved in both biotic and abiotic stresses (Balan et al. 2017, 2018; Benny et al. 2019b).

3.2. Aim of the Research

The present study was conducted to identify key major genes involved in general plant abiotic stress conditions and those involved in specific and unique pattern of different abiotic stresses factors. I performed a bioinformatics analysis of previously published RNA-Seq studies on leaves through a careful selection of published studies related to four abiotic stress factors: drought, salinity, cold and heat. Finally, a meta-analysis could also provide information about an early alert for plant physiological status under stress and aid the development of more sustainable management strategies.

3.3. Materials and Methods

- Search strategy to identify published studies for bioinformatic analysis

The published RNA-Seq studies related to abiotic stress responses in leaf tissues were searched using Scopus and PubMed with the combination of keywords 'transcriptomics' 'leaf' and 'abiotic stress' that were published in or before June 2018. I found 11 articles related to my purpose of meta-analysis of abiotic stress responses in plant leaves using next generation sequencing approaches and with availability of raw data in public databases. Among these, I selected eight articles with publically available raw data (Xu et al. 2014; Corso et al. 2015; Forestan et al. 2016; Haider et al. 2017; Li et al. 2017a; Liu et al. 2017; Orcheski and Brown 2017; Shumayla et al. 2017). From these considered studies, one manuscript was related with salinity (Forestan et al. 2016), four other works related to drought (Corso et al. 2015; Haider et al. 2017; Liu et al. 2017; Orcheski and Brown 2017), one paper related to cold (Xu et al. 2014), one related to heat (Shumayla et al. 2017) and the last one was related to salinity, heat and cold (Li et al. 2017a). So, in total, I gathered four articles related to drought, two works related to salinity, two studies dealing with cold and two related to heat. The raw files (SRA format) of the eight articles dealing with abiotic stress responses in leaves were downloaded from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra). In total, 68 samples were analysed. I downloaded the raw data of all the 'abiotic stress' selected studies and performed RNA-Seq analysis using a single analysis pipeline in Figure 3.1 to obtain the differentially expressed genes (DEGs).

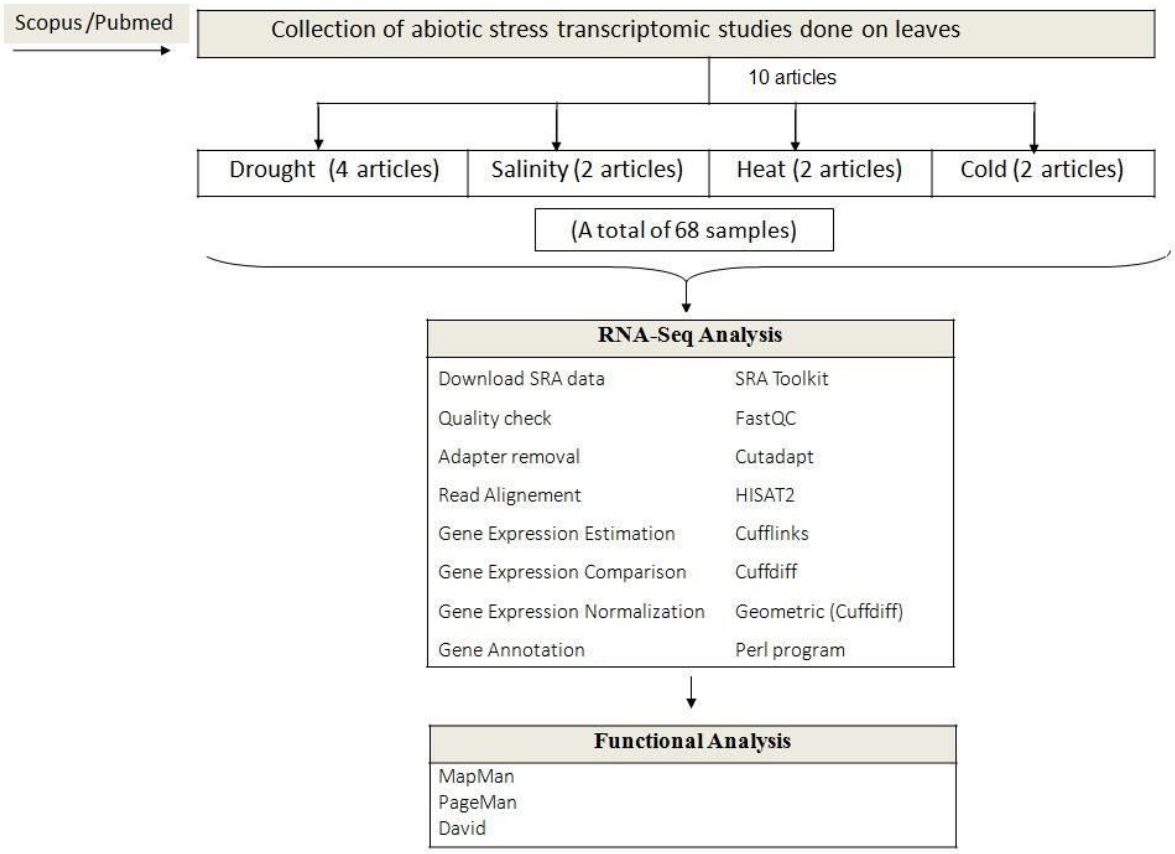


Figure 3.1. Workflow of the meta-analysis of the 10 transcriptomic studies related with abiotic stress in leaf tissue. Functional data analyses are indicated.

- Read alignment, gene differential expression and annotation

For all the articles selected, the latest available version of the corresponding crop genome and its annotation file were downloaded from Phytozome (https://phytozome.jgi.doe.gov). The raw data files were downloaded from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) and EMBL ArrayExpress (https://www.ebi.ac.uk/arrayexpress) according to the accession number given in the article and converted to FASTQ format using SRA toolkit ver. 2.3.5. Raw data underwent pre-processing by trimming low quality bases followed by adaptor sequence removal to obtain high-quality clean reads using cutadapt version 1.8.1. The pre-processed high-quality reads (Phred-score > 30) were mapped to the corresponding genome with HISAT2 ver. 2.1.0 using '-novel-splicesite' option along with the default parameters. The resulted output of HISAT2 was then used for the identification of differentially expressed genes using Cuffdiff tool in Cufflinks version 2.2.1 pipeline with default parameters. Only up- and downregulated genes obtained with fold change cut-off (log2 FC > 0.5 or log2 FC < _0.5) and P-value < 0.05 were considered for the meta-analysis. The DEGs selected were annotated using corresponding crop genome mapping file downloaded from the Phytozome. Each of the Arabidopsis IDs were then selected and searched for identifying the corresponding orthologs using PANTHER and the functional similarities were identified using the UniProtKb and ensembl plants compara. DEGs were subjected to functional and

enrichment analyses after identification of the corresponding orthologous genes in *Arabidopsis thaliana* (L.) Heynh. Since each study involving plants different from *Arabidopsis*, the entire list of gene IDs corresponding to DEGs was mapped to *A. thaliana*, and the best corresponding TAIR (The Arabidopsis Information Resource) IDs were found by using the annotation file downloaded from Phytozome. During mapping to Arabidopsis orthologs, cases of 'many-to-one' mapping were solved by calculating an average expression value (log2 fold change).

- Gene set enrichment analysis

I used MapMan (Thimm et al. 2004) with the *A. thaliana* mapping file to map and visualise the hormone regulation, secondary metabolism, and transcription factors The unique genes present in each of the stress separately were visualised. The PageMan (Usadel et al. 2006) analysis, plugin of MapMan, was used to visualise differences among metabolic pathways using Wilcoxon tests, no correction, and an over-representation analysis (ORA) cut-off value of 3. All the homologous TAIR IDs of the studies were searched against the Database for Annotation, Visualisation, and Integrated Discovery (DAVID) ver. 6.8 (Huang et al. 2009). The gene ontology information related to biological process was extracted from the DAVID result.

- Statistical analysis

The DEGs corresponding to each study were analysed separately when they had a P-value < 0.05 and log2 FC >0.5 or log2FC<_0.5. All statistical tests were corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate using p.adjust function of R. By adjusting the P-values, this approach can make the FDR at the desired level of a (in the present study = 0.05). Differences among the selected studies were adjusted using the sample normalisation. To remove systematic variation between different species, the normalisation procedure served as a crucial pre-processing step to adjust for the different sample sequencing depths and other confounding technical effects. The geometric normalisation method was used where FPKMs and fragment counts are scaled via the median of the geometric means of fragment counts across all libraries.

- Protein–protein interaction network

NetworkAnalyst, a web-based tool for network-based visual analytics of protein–protein interaction networks, was used (https://www.networkanalyst.ca). The list of unique homologous TAIR IDs for each gene uniquely modulated by each abiotic stress were uploaded and mapped against the STRING interactome database with default parameters (confident score cut-off = 900 and with experimental evidence) provided in NetworkAnalyst. The networks between drought-regulated genes in seedlings and in mature leaves corresponding to the list of the visualised genes in MapMan were also obtained. To study the key connectives and to simplify the large network, I selected 'minimum network'.

3.4. Results

The articles and crops selected for the study, number of up- and downregulated genes are listed in Table 3.1.

Table 3.1: Transcriptomic studies dealing with abiotic stress responses used for meta-analysis. Number of up-regulated and down-regulated genes were indicated for each study

Authors	Crop	Stress	No. of sample	Sample description		Sample Information		
				Control	Treated	Total	up	down
Li P et al., 2017	Maize	Salinity	4	Control1 (SRR3984708) Control2 (SRR3984749)	Salinity1 (SRR3984762) Salinity2 (SRR3984771)	264	264	0
Forestan el al., 2016	Maize	Salinity	4	Control1 (GSM1826055) Control2 (GSM1826071)	Treated1 (GSM1826057) Treated2 (GSM1826073)	424	424	0
Li P et al., 2017	Maize	Heat	4	Control1 (SRR3984708) Control2 (SRR3984749)	Heat1 (SRR3984794) Heat2 (SRR3984795)	228	228	0
Shumayl a et al., 2017	Wheat	Heat	4	Control 1 (SRR1542404) Control 2 (SRR1542405)	Treated1 (SRR1542412) Treated 2 (SRR1542413)	154	0	154
Li P et al., 2017	Maize	Cold	4	Control1 (SRR3984708) Control2 (SRR3984749)	Cold1 (SRR3984802) Cold2 (SRR3984815)	219	219	0
Xu et al., 2014	Vitis	Cold	2	Control (SRR922004)	Treated (SRR922126)	179	179	0
Haider et al., 2017	Vitis	Drought	2	Control (SRR3466603)	Treated (SRR3466604)	174	174	0
Orcheski and Brown, 2017	Malus	Drought	4	Control 1 (SRR3160181) Control 2 (SRS1283365)	Treated1 (SRR3160081) Treated2 (SRR3160180)	432	178	254
Liu et al., 2017	Tomato	Drought	4	SCK (SRR5282480) TCK (SRR5282476)	SD (SRR5282481) TD (SRR5282478)	159	88	71
Corso et al., 2015	Vitis	Drought	4	Control1 SAMN02393571 Control2 SAMN02393572	Treated1 SAMN0239359 Treated2 SAMN0239359 5	2889	2141	748

- Hormone-related pathways

Drought stress enhanced expression of some brassinosteroids like 3-oxo-5-a-steroid 4-dehydrogenase, STEROL 1 and DWARF 5 and have opposite effects on the expression of cycloartenol synthase 1 and brassinosteroid insensitive 1 (Figure 3.2).

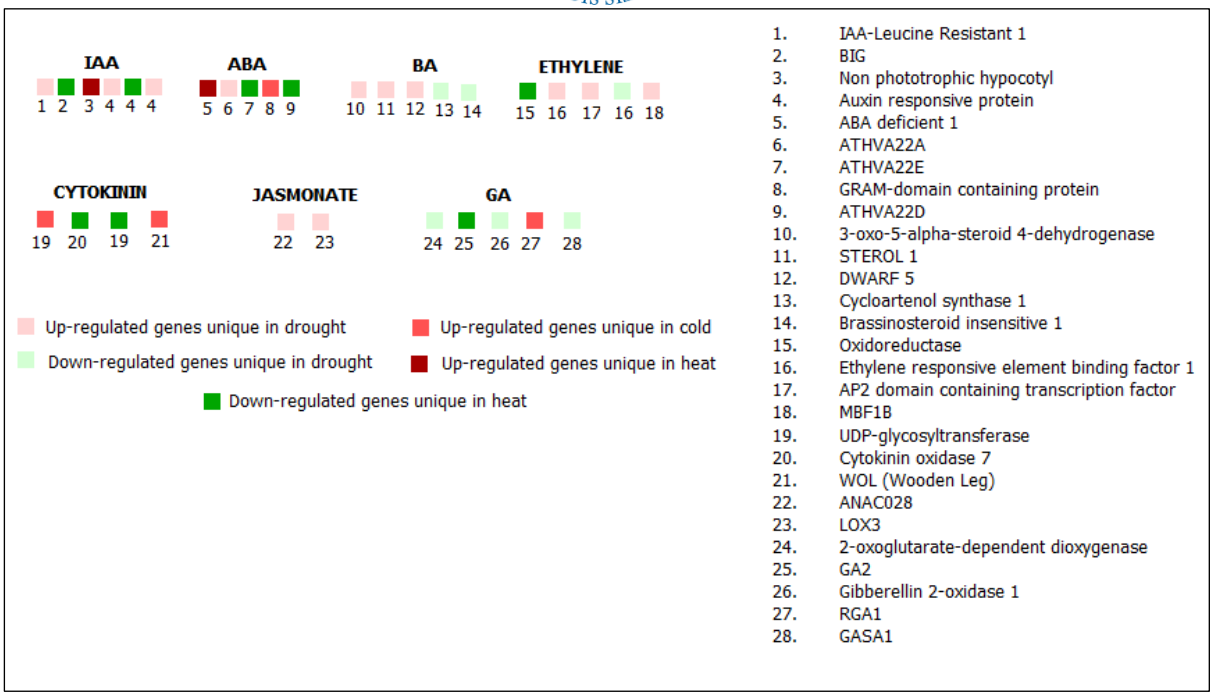


Figure 3.2. Abiotic stress-regulated genes involved in hormone-related categories that are uniquely regulated in the studies are shown. Genes were identified as Arabidopsis orthologs of each gene of the analysed plant species. Red indicates upregulation and green indicates downregulation in response to stress.

Two key jasmonate genes were upregulated by drought stress. All the gibberellin related genes (GASA1, gibberellins 2-oxidase 1 and 2-oxoglutarate-dependent dioxygenase) were downregulated by the effect of drought. Several genes involved in ethylene biosynthesis and signalling were enhanced by drought stress such as MBF1B, AP2andERF1. Heat stress downregulated one ethylene related gene – oxidoreductase. Cold stress upregulated ABA (GRAM domain containing protein), cytokinin (UDP-glycosyltransferase and WOL) and gibberellin (RGA1). Heat stress enhanced the expression of a gene involved in IAA (non-phototrophic hypocotyl) response.

- Secondary metabolism

Secondary metabolism was significantly modulated by the expression of genes involved in the different analysed stress studies (Figure 3.3).

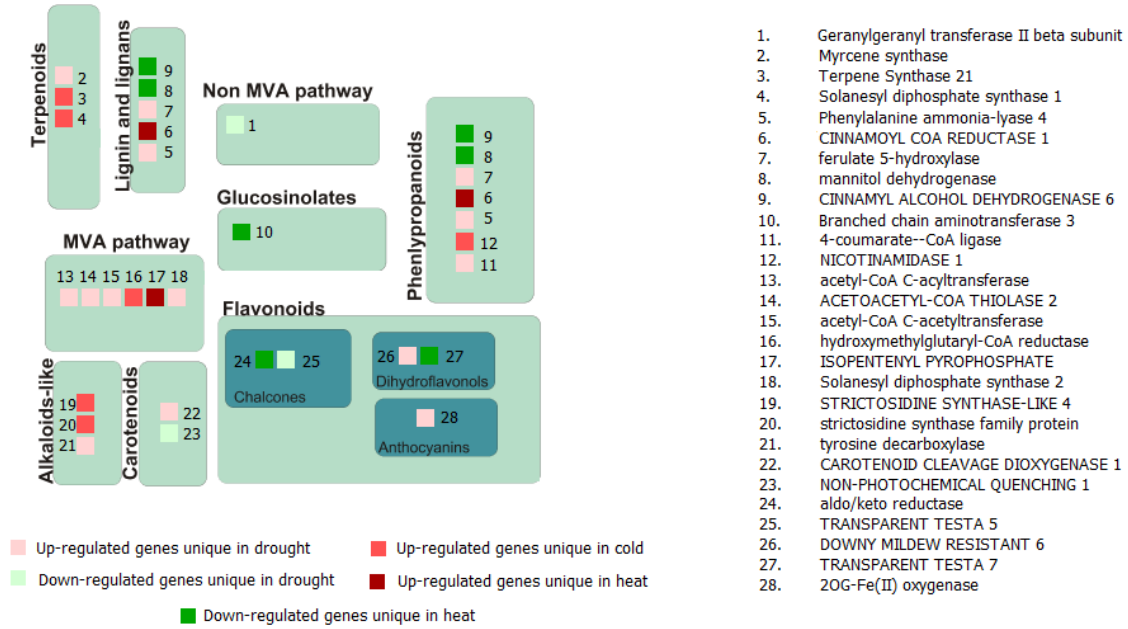


Figure 3.3: Abiotic stress-regulated genes involved in secondary metabolism categories which are uniquely regulated in the studies were shown. Genes were identified as Arabidopsis orthologs of each gene of the analyzed plant species. Red indicated up-regulation and green indicated the down-regulation in response to stress

I noted that of the different stress related categories that come under mevalonic acid (MVA) pathway, terpenoids and alkaloids were upregulated. Heat stress repressed most of the flavonoids (aldo-keto reductase, TRANSPARENT TESTA7), lignin and lignans (mannitol dehydrogenase and CINNAMYL ALCOHOL DEHYDROGENASE 6), phenylpropanoids and glucosinolates (branched chain aminotransferase 3) but upregulated the CINNAMYL COA REDUCTasE 1 and ISOPENTENYL PYROPHOSPHATE. The expression of cold stress-related genes involved in terpenoid (terpene synthase 21 and solanesyl diphosphate synthase 1) and alkaloids (STRICTOSIDINE SYNTHASE).

- Transcription factors

I used MapMan software to demonstrate the effect of abiotic stress in transcription factors and to identify the crucial and specific genes response in each type of abiotic stresses, Transcription factors were drastically affected by three of four analysed abiotic stresses (drought, cold, heat stresses; Figure 3.4).

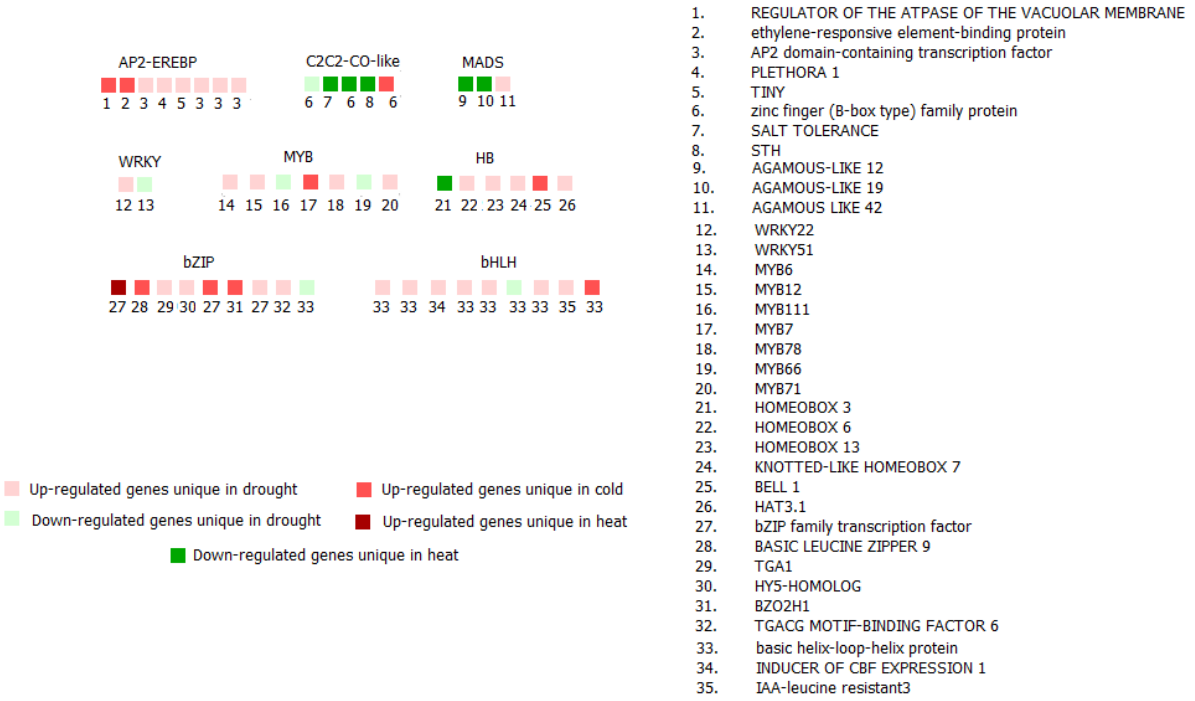


Figure 3.4: Abiotic stress-regulated genes involved in transcription factors categories which are uniquely regulated in the studies were shown. Genes were identified as Arabidopsis orthologs of each gene of the analyzed plant species. Red indicated up-regulation and green indicated the down-regulation in response to stress

Drought stress induced key genes encoding AP2-EREBPs such as AP2 domain containing transcription factor, PLT1 and TINY, one MADS box transcription factors (AGL42), a WRKY factor (WRKY22), four MYB factors (MYB6, MYB12, MYB78, MYB71), four homoebox genes (HB6, HB13, KNAT7, HAT3.1), four bZIP members (TGA1, HY5-Homologue, bZIP TFs, TGA6), three bHLH TFs (Bhlh protein, ICE1, ILL3). Among the downregulated genes in response to water deprivation there were two MYB factors (MYB111 and MYB66), one C2C2-CO-like member (B-box zinc finger), one WRKY gene (WRKY51), one bZIP transcription factor and one bHLH member. Cold stress enhanced two AP2-EREBPs genes (ACA4, ERFs), three bZIP members. I also found that another three well known drought-regulated transcription factors (MYB7, BELL1 and 1 bHLH member) were enhanced. Heat stress specifically induced one bZIP TF. Other genes were repressed in response to heat, such as three C2C2-CO-LIKE (B-box zinc finger, Salt Tolerance, STH), two MADS box (AGAMOUS-LIKE 12, AGAMOUS-LIKE 19) and one homeobox (HOMEOBOX3).

- Gene set enrichment analysis

Gene enrichment analysis was conducted using PageMan to identify any relation between the expression and function of differentially expressed genes in different abiotic stress conditions (Figure 3.5).

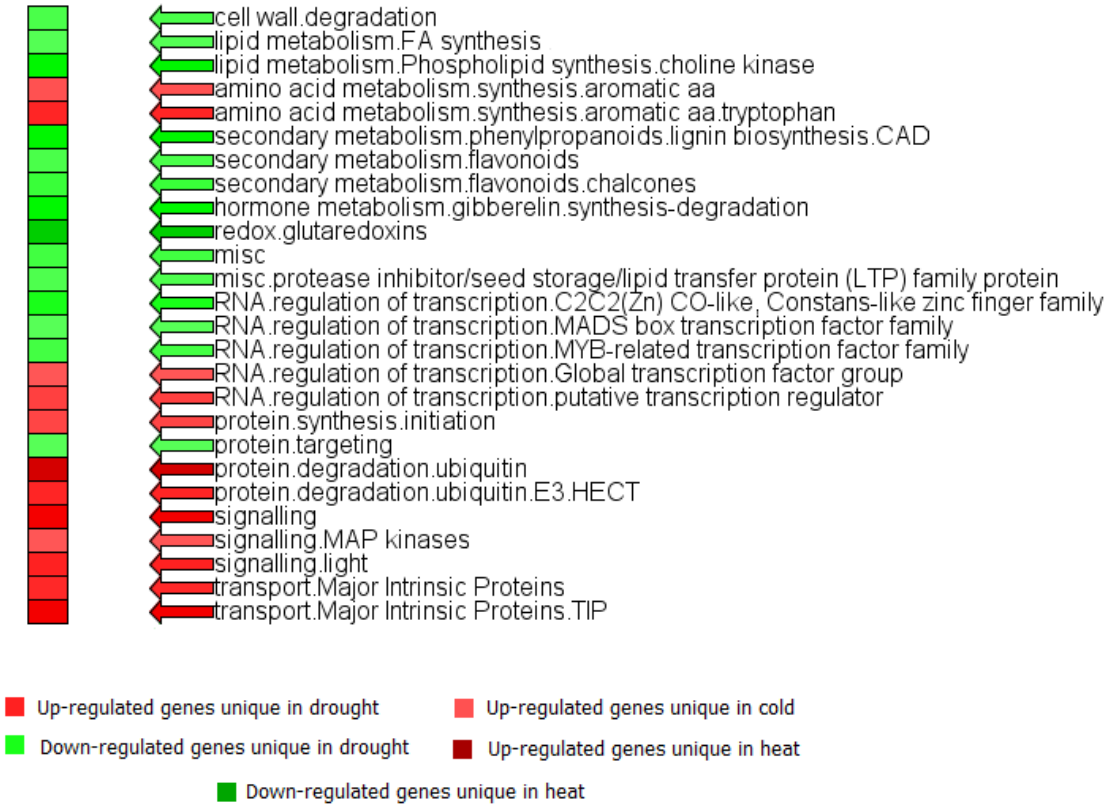


Figure 3.5: The PageMan analysis was used to visualize differences among metabolic pathways using Wilcoxon tests, no correction, and an over-representation analysis (ORA) cutoff value of 3.

Analysis showed that drought stress downregulated several genes categories such as those implicated in cell wall degradation, lipid metabolism (fatty acid synthesis, phospholipid choline kinase synthesis), secondary metabolism (phenylpropanoids, flavonoid, flavonoid chalcones), hormone metabolism gibberellin (synthesis and degradation), RNA processing (miscRNA), lipid transfer protein (LTP), RNA regulation of transcription C2C2(Zn) co-like constans-like zinc finger family genes, and genes involved in RNA regulation of transcription MADS-box transcription factor family. Other genes involved in protein targeting process were also downregulated by drought stress. In contrast, aromatic amino acid metabolism genes, protein degradation ubiquitin ligases HECT genes, genes involved in signalling, in light signalling and key genes encoding major intrinsic proteins such as those encoding tonoplast (TIPs), where upregulated by drought. Heat stress enhanced specifically key genes implicated in ubiquitin-mediated protein degradation and repressed glutaredoxin genes. Cold stress upregulated key genes implicated in RNA regulation, and involved in initiation of protein synthesis, and encoding MAP kinases signalling pathways.

- Biological process enrichment analysis

DAVID software was used to identify the gene ontologies (biological process, cellular component, molecular function) that were significantly affected by the three types of abiotic stresses. Metabolic pathways were divided into those up- or downregulated by drought, and gene-onthology (GO) ID, GO terms, count and P-values are shown in Table 3.2.

Table 3.2: Significantly regulated biological processes (FDR < 0.05) which are uniquely regulated transcriptomic studies

GO_ID	Description	Count	P-Value	Expression
Unique genes in Drought				
GO:0015992	proton transport	3	0.032473	Down
GO:0045087	innate immune response	9	0.00227	Up
GO:0015979	photosynthesis	13	0.003343	Up
GO:0006096	glycolytic process	8	0.011903	Up
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	11	0.015973	Up
GO:0008652	cellular amino acid biosynthetic process	7	0.033405	Up
GO:0009768	photosynthesis, light harvesting in photosystem I	4	0.040003	Up
Unique genes in Cold				
GO:0006874	cellular calcium ion homeostasis	3	0.03036	Up
GO:0006816	calcium ion transport	3	0.0322	Up
Unique genes in Heat				
GO:0008152	metabolic process	10	1.39E-05	Down
GO:0009813	flavonoid biosynthetic process	5	5.08E-03	Down
GO:0052696	flavonoid glucuronidation	4	0.016338	Down
GO:0009992	cellular water homeostasis	3	0.010365	Up
GO:0006457	protein folding	5	0.03212	Up

I identified GO terms that were upregulated by drought such as innate immune response, photosynthesis, photosynthesis, light harvesting in PSI, glycolytic process, transmembrane receptor protein tyrosine kinase signalling pathway and cellular amino acid biosynthetic process. In contrast, proton transport was downregulated in response to water stress. Heat stress downregulated GO terms related to metabolic process, flavonoid biosynthetic process and flavonoid glucuronidation, but upregulated those related to cellular water homeostasis and protein folding. Cold stress enhanced cellular calcium ion homeostasis and calcium ion transport.

- Protein–protein network analysis

The protein–protein interaction (PPI) network analysis was comprised of unique genes from each of the abiotic stress selected for the study. Minimum default settings were used to reduce the number of interacting proteins and the complexity of the networks (Figure 3.6).

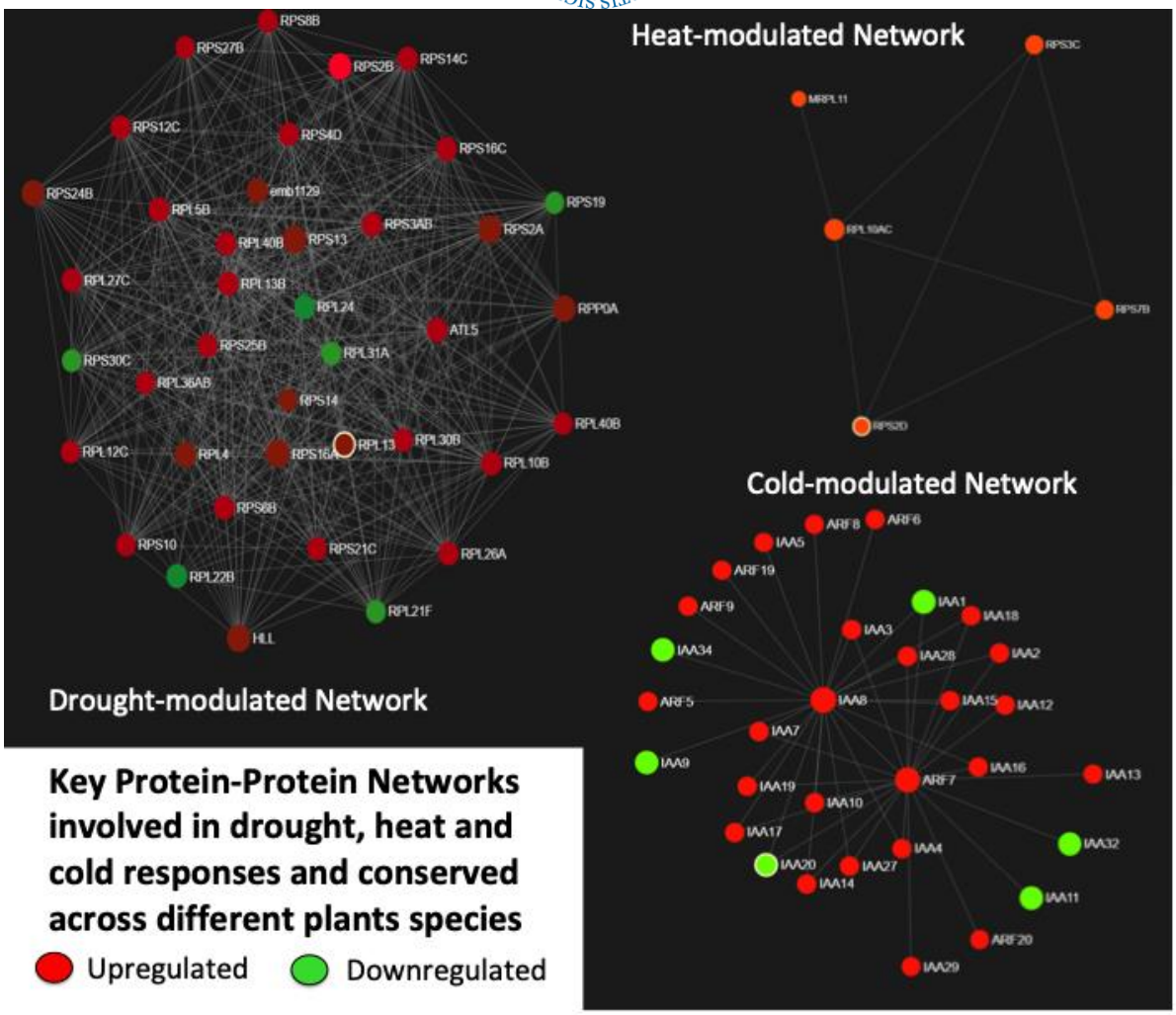


Figure 3.6: Protein-protein network analysis is shown using STRING software among uniquely differentially regulated genes in response to drought, cold and heat. Key highly interactive proteins were indicated. Red means upregulated and green means downregulated by each stress.

Among the upregulated hub (highly interacting) proteins I noted some key proteins that may play a key role in drought response such as HLL, RPS12C, RPS4D and RPP0A. Further, drought downregulated highly interactive proteins such as RPS30C, RPL24 and RPS19. PPI network analysis was performed for unique genes in cold stress, and showed a downregulation in IAA34, IAA9 and IAA20 and an upregulation in ARF5 and IAA3. Among the genes uniquely modulated by heat, I could find only upregulation in MRPL11, RPS3C, RPL10AC, RPS7B and RPS2D.

3.5. Discussion

The present study was conducted in order to shed light onto the abiotic stress response mechanisms in plants, and to identify specific responses to each abiotic stress condition. It was hoped that the identification of commonalities between similar independent studies would help us discover the most associated genes to the subject of the study and allow us to

focus on the functional analysis only on those common findings (Benny et al. 2019a). Available RNA-Seq datasets related to abiotic stress responses in leaf tissues were used in order to deliver functional genomic information linked with exclusive molecular responses to specific types of abiotic stresses. The selection of papers included in the meta analysis was based on four points. First, the type of tissue subjected to the transcriptomic analysis: I selected studies related to leaves but excluded studies concerned with other tissue types. Second, the availability of raw data (many of the published papers that I selected; the raw data were unavailable). Third, the type of stress: I focused on the most significant abiotic stresses affecting crops today, these being drought, salt, heat and cold stress. Finally, the read alignment of the reference genome had to be of sufficient quality (i.e. have a high percentage of read annotation, mapping and homology with correspondent Arabidopsis orthologue). These selection criteria resulted in 10 transcriptomic studies dealing with the chosen abiotic stress factors among a number of species. Similar meta-analyse of transcriptomic data have been conducted in single plant species including as *A. thaliana* (Rest et al. 2016), rice (Muthuramalingam et al. 2017) and sunflower (Ramu et al. 2016a).

- Transcription factors

Regarding transcription factors, the study revealed that drought stress significantly upregulated three APETALA2/ethylene-responsive element binding protein (AP2/EREBP) transcription factors (AP2 domain containing transcription factor, PLT1 and TINY), whereas two of them (ACA4, ERFs) were induced by cold stress. AP2/EREBP family of transcription factors are well known to be involved in various environmental stresses responses including biotic and abiotic stresses such as pathogen infection, drought salinity and temperature (Dietz et al. 2010; Liu and Zhang 2017; Balan et al. 2018). Sun et al. (2008) reported that the expression of TINY, a DREB-like factor was induced by drought stress in Arabidopsis, and suggested that TINY plays a role in the crosstalk between biotic and abiotic stress-responsive gene expressions by connecting the DRE- and ERE-mediated signalling pathways (Sun et al. 2008). Previous studies have reported that overexpression of ERF family genes increases tolerance to a wide range of abiotic stresses in different plant species such as *Arabidopsis*, rice, tomato and tobacco (Park et al. 2001; Aharoni et al. 2004; Guo et al. 2004; Zhang and Huang 2010; Schmidt et al. 2013). These observations are in agreement with the data. I found that one MADS box transcription factors (AGL42) was induced by drought, whereas two of them (AGAMOUS-LIKE 12, AGAMOUSLIKE 19) were repressed in response to heat. MADS-box genes are known to be key players in many developmental processes in plants such the flower development and floral induction (Causier et al. 2002), as well as crucial regulators in response to abiotic stresses (Gupta et al. 2012). However, MADS-box genes are also important molecular regulators of plant responses to low temperature, photoperiod and plant hormones such as cytokinin, ethylene and gibberellins (Lozano et al. 1998; Ando et al. 2001; Duan et al. 2006; Lee et al. 2008; Li et al. 2016). In addition, Jia et al. (2018) suggested a probable involvement of LcMADS1, LcMADS2, LcMADS3, LcMADS7 and LcMADS9 genes in abiotic stress responses in sheep grass (Jia et al. 2018). Indeed, LcMADS1 and LcMADS2 genes were significantly upregulated by cold stress, LcMADS3 gene was upregulated in response to mannitol and ABA and LcMADS9 was induced by salt stress (Jia et al. 2018). In Oryza sativa, OsMADS26, an AGL12-class gene, have been reported to be involved in drought tolerance (Khong et al. 2015). These data partially agree with the meta-analysis, which showed that AGL42, AGAMOUS-LIKE 12, AGAMOUS-LIKE 19 may be

crucial regulators involved in abiotic stress responses. The meta-analysis revealed that WRKY22 and WRKY51 are drought-regulated genes conserved across different plant species. WRKY family is well known to play important roles in abiotic stress responses, including, salinity (Niu et al. 2012; Liang et al. 2017), drought (Luo et al. 2013; Sun et al. 2015; Li et al. 2017b) oxidative stress (Yan et al. 2014), nutrient stress (Chen et al. 2009; Su et al. 2015; Dai et al. 2016), heat (Cai et al. 2015; He et al. 2016) and pathogen infection (Liu and Bai 2005; Maoet al. 2011; Chen et al. 2013; Dey et al. 2014). Sanchita et al. (2014) reported that WRKY51 was found to have differential expression under abiotic stresses whereas WRKY22 and WRKY51 were enhanced by drought and cold stresses in Chiifu Brassica rapa. In addition, it was reported that WRKY51 enhanced the lateral root formation in response to abiotic stresses or nutrition in wheat (Hu et al. 2018). MYB family is a well-known TF category that plays a major role in organ development, regulation of primary and secondary metabolism, flavonoid biosynthesis and response to various biotic and abiotic stresses (Jin and Martin 1999; Li et al. 2015; Qi et al. 2015). However, the large number of members of this family makes identification of orthologs commonly regulated by each abiotic stress across plant species very difficult. The results showed that four MYB factors (MYB6, MYB12, MYB78 and MYB71) were enhanced in response to water deprivation whereas two (MYB111 and MYB66) were repressed. In contrast, cold stress enhanced MYB7. Many studies reported the induction of MYB factors under drought conditions (Clauw et al. 2015; Li et al. 2017a; Benny et al. 2019a). Thus, the overexpression of MYB1 enhanced drought resistance in transgenic Arabidopsis plants (Dai et al. 2007), and it was also reported that both MYB96 (Lee et al. 2014, 2016) and MYB94 (Lee et al. 2016) activated cuticular wax biosynthesis in A. thaliana. Additionally, Lee et al. (2014, 2016) suggested that these genes might play important role in drought stress responses in plants via activating this adaptive mechanism. I found that flavonoid pathways were modulated by each abiotic stress. In this regard, MYB12 has shown to increase flavanol biosynthesis and accumulation, which contributes to reduced water loss and enhanced drought tolerance in Arabidopsis (Nakabayashi et al. 2014). Further, two MYBS in the meta-analysis (MYB12 and MYB111) were found to be regulated by different light spectra in the turnip B. rapa, suggesting roles in light stress responses in addition to drought (Wang et al. 2012). Moreover, the MYB7 played a key role in the ABA-mediated regulation of salt and osmotic stress via ABA insensitive 5 (ABI5). In fact, MYB7 repressed ABI5 expression during seed germination, positively influenced the content of anthocyanins (which are crucial pigments in the abiotic stress responses), and positively regulated the lateral root growth under salinity (Kim et al. 2015; Skubacz et al. 2016). Ruan et al. (2018) reported that MeMYB111 transcription factor was responsive to ABA, drought, and cold stresses in cassava leaves, and suggested that this gene might has a role in ABA signalling during abiotic stress responses. Four HOMEOBOX genes (HB6, HB13, KNAT7 and HAT3.1) were enhanced under drought, conditions, whereas HOMEOBOX3 was repressed in response to heat.

The results suggest that homeobox genes HB6, HB13, KNAT7 and HAT3.1 may be involved in drought responses, whereas HOMEOBOX3 TF may be involved in heat responses. In rice, it was found that OsHOX22/OsHOX24 homeobox proteins might be considered as negative regulators in abiotic stress responses (Bhattacharjee et al. 2016). KNAT7 is a component of a transcription network regulating secondary cell wall biosynthesis, whose function remains unclear, although in Arabidopsis, KNAT7 is considered as a negative regulator of secondary wall biosynthesis (Li et al. 2012). HAT3.1 was one of the first transcription factors discovered with the typical PHD finger domain in plants (Schindler et al. 1993). Later, 45

others of this type of TFs were identified in Arabidopsis, 44 in rice and 67 in maize (Wang et al. 2015). These large families play diverse roles during plant growth and development. Arabidopsis PHD-domain ALFIN1-like proteins were shown to promote seed germination (Molitor et al. 2014), and in Arabidopsis, they are involved in regulating flowering by modifying the SOC1/FT chromatin conformation (López-Gonzalez et al. 2014). It has been also suggested that MS1 – another member of this family – plays a key role in pollen development (Fernández Gómez and Wilson 2014). However, the literature on these TFs is huge, so the role of this type of TF in environmental stress responses is yet to be fully elucidated. Wei et al. (2009) reported that the expression of six GmPHDs was induced by drought stress in soybean. Among them, GmPHD4/5 expression was enhanced under cold stress conditions, whereas GmPHD2/5 was identified to regulate salt stress responses (Wei et al. 2009; Wu et al. 2011). In maize, 15 of 67 ZmPHDs were revealed to respond to abiotic stresses, such as drought and salinity (Wang et al. 2015). Moreover, nine PtPHDs were differentially expressed under drought, salt and cold stresses (Wu et al. 2016). Sun et al. (2017) reported differential expression of OsPHDs gene under environmental stresses in rice, including ABA (abscisic acid), water deficit, cold and high cold (Sun et al. 2017). OsPHD1/7/8/13/33 were differentially expressed under drought and cold stresses, OsPHD5/17 were downregulated under water deficit and cold stresses whereas OsPHD3/44/28 showed differential expression under Cd and ABA stresses (Sun et al. 2017).
I found that four bZIP members (TGA1, HY5-Homologue, TGA6 and an uncharacterized bZIP TF) were induced by drought, three of them (bZIP 9, bZIP TFs, BZO2H1) were enhanced by cold stress and one was enhanced by heat. These results suggest a possible role of this TF family in abiotic stress responses. Xu et al. (2016) reported that the overexpression of bZIP TF (ZIP110) improved salt tolerance in soybean suggesting its role as a positive regulator involved in salt stress tolerance. In addition, functional analysis of GmbZIP110 have found in regulating many downstream target genes by binding to the ACGT motif in A. thaliana (Cao et al. 2017). In potato (Solanum tuberosum), the overexpression of a hot pepper bZIP like transcription factor CaBZ1 in transgenic plants improved drought stress tolerance (Moon et al. 2015). Hence, in transgenic alfalfa (Medicago sativa), the overexpression of ABF3 using sweet potato oxidative stress-inducible promoter SWPA2 improved growth under water deprivation (Wang et al. 2016). I found that three genes encoding bHLH TFs (an uncharacterized bHLH protein, ICE1 and ILL3) were enhanced under drought whereas one was downregulated. A previous study identified TGA1 and TGA4 transcription factors as putative regulatory factors that mediate nitrate responses in Arabidopsis roots (Alvarez et al. 2014). A recent study highlighted that bHLH genes are related to biotic and abiotic stress tolerance in wheat (Wang et al. 2019). For hormone-related pathways, the results show that drought stress specifically enhanced genes involved in ethylene-related pathways (biosynthesis, signalling, response), which is known to play an important role in activating plant responses to water deprivation and flooding (Lee et al. 2009; Ramu et al. 2016b). Ethylene is responsible in activating signals affiliated with the synthesis of many transcription factors that controls the gene activation/ repression during stress such as the ethylene response factor ERF1 (Bastola et al. 1998; Young et al. 2004; Seo et al. 2010). In A. thaliana, ERF1 has been shown to be induced by both salinity and drought stresses (Cheng et al. 2013); however, the present study showed that the ERF1 is induced in leaves only by drought stress. Similar results were found previously, confirming that ERF1 is upregulated in mature leaves of different crops under drought conditions (Song et al. 2017; Benny et al. 2019a). Brassinosteroids represent another class of plant growth regulator (Adam and

Marquardt 1986) for which related genes are differentially regulated in response to drought. Studies have reported that brassinosteroids (BRs) mediate abiotic stresses such as salinity, heat and drought (Clouse and Sasse 1998). The results show that some brassinosteroids such as 3-oxo-5-a-steroid 4-dehydrogenase, STEROL 1 and DWARF 5 were upregulated, whereas two (cycloartenol synthase 1 and brassinosteroid insensitive 1) were repressed. There results agree with previous findings showing the induction of 3-oxo-5-a-steroid 4-dehydrogenase in rice roots under drought conditions (Muthurajan et al. 2018).

Gibberellins are one of the most important groups of phytohormone in plants for the modulation of growth and development (Bari and Jones 2009). According to the study, a number of gibberellin-related genes show opposite trends of expression. For example, GASA1, gibberellins 2-oxidase 1 and 2-oxoglutarate-dependent dioxygenase were downregulated by the effect of drought, but gibberellin (RGA1) was upregulated by cold. Similar results have been found by Zhu (2016), which suggests a putative interaction between RGA1 and a cold stress sensor required for chilling tolerance (Ma et al. 2015; Zhu 2016). Moreover, GASA1 seems to play an important role under both abiotic (puffing) and biotic (Huanglongbing) stresses in citrus peel tissues (Martinelli et al. 2015). The results indicate that the drought stress alters GA metabolism and signalling: GA signalling is reduced in response to cold, salt, and osmotic stresses (Colebrook et al. 2014). Drought and high salinity increased the expression of three breadfruit GA2-oxidase genes (AaGA2ox1, AaGA2ox2 and AaGA2ox4) and their possible involvement in abiotic stress response resistance was discussed by Zhou and Underhill (2016). The 2- oxoglutarate-dependent dioxygenase (2OGD) superfamily represents the second largest enzyme family in the plant genome whose members are involved in various oxygenation/hydroxylation reactions. In addition, 2-oxoglutarate-dependent dioxygenase gene may be involved in chilling stress responses in tomato by regulating JA accumulation and the expression of genes related with JA biosynthetic and signalling under chilling stress (Hu et al. 2019).

The phytohormone ABA is known as important factor in plant growth and development in response to various environmental conditions including drought stress (Riemann et al. 2015). The main function is the control of stomata opening and closure to reduce water loss via transpiration (Wilkinson and Davies 2010; Mittler and Blumwald 2015). The induction of ABA synthesis presents one of the fastest phytohormonal responses to abiotic stresses, thereby triggering ABA-inducible gene expression (Yamaguchi- Shinozaki and Shinozaki 2006). Shinozaki and Yamaguchi- Shinozaki. (2007) also showed that in plants, high salinity or drought stress causes ABA accumulation and obvious changes in genes expression. Nevertheless, I showed that cold stress upregulated a GRAM domain containing protein involved in ABA-related pathways. The GRAM domain has ~70 amino acids, a length usually found in glucosyl transferases and other membrane-associated proteins (Doerks et al. 2000). Generally, this domain seems to be involved in membrane-associated processes such as intracellular protein- or lipid-binding signalling pathways (Doerks et al. 2000).

In relation to heat stress, the meta-analysis highlighted an enhanced expression of a genes involved in IAA (non-phototrophic hypocotyl) response. The phytohormone auxin, IAA, plays an important role in a plant's responses to abiotic stresses (Bari and Jones 2009). IAA also plays a crucial part in the adaptation of plants to salinity (Iqbal et al. 2014), and participates in increasing the growth of root and shoot of plants under heavy metal or salinity stresses (Sheng and Xia 2006; Egamberdieva 2009). Salinity has been reported to reduce IAA levels in plants such as maize. In fact, auxin tends to enhance the transcription of several genes called primary auxin response genes, which have been characterised and identified in

many plant species such as soybean, rice and Arabidopsis (Javid et al. 2011). Thus, auxins present a powerful constituent in the defence responses via many gene regulations and crosstalk mediation (Bari and Jones 2009; Ghanashyam and Jain 2009). PPI networks highlighted the role played by auxin-related genes in cold responses and particularly ARF7 and IAA8, which were shown to be at a core position of network composed by several auxin-related genes. The phytohormones Jasmonate (JAs) represent signalling molecules that regulate plant growth and orchestrate systemically and locally the responses to many abiotic stress factors (Turner et al. 2002; Pauwels et al. 2009). Many studies have shown that JA levels are increased after drought and salt stresses exposure (Creelman and Mullet 1995; Wang et al. 2001). Further, experiments conducted on rice leaves and roots exposed to drought and salinity showed an increased content of JAs, and induced JA biosynthesis genes (Moons et al. 1997; Tani et al. 2008). Another study reported that the content of Jasmonate is enhanced by sorbitol treatment to a degree enough to initiate JA-responsive gene expression (Kramell et al. 2000). The present study corroborates these previous results and showed the enhancement of two key genes involved in Jasmonate genes that were also upregulated by drought stress. Moreover, under drought stress, endogenous JA content increased in maize root cells (Xin et al. 1997). Nevertheless, the main function of JA in drought stress remains unclear and controversial, since in some studies JA has been reported to improve the tolerance whereas it caused a reduction in growth in other works. This may be explained by the fact that the responses to drought conditions depend generally on the type and tissue of plant studied, the duration and intensity of the stress applied, and the dosage of JA applied (Lee et al. 1996; Kim et al. 2009).

- Primary and Secondary Metabolism

It was reported that the overexpression of selenenyl diphosphate synthase 1 (SPS1) enhanced tolerance to photo-oxidative stress in Arabidopsis plants which was related to their increased capacities for plastoquinone-9 biosynthesis (Ksas et al., 2015). The work also revealed that drought stress repressed fatty acid biosynthesis in leaves. Another primary metabolism pathway that was repressed by drought stress in leaves was phospholipid biosynthesis. Benny et al. (2019) reported that two genes involved in phospholipid biosynthesis (phosphatidylserine synthase and galactolipid galactosyltransferase) were repressed under drought and reported that water deficit inhibits fatty acid desaturation. Another previous study associated with drought resistance in cotton also produced a reduction of fatty acid metabolism which results in greater stability of the membrane system (Anh et al., 1985).
The results showed an up-regulation of different categories of genes involved in secondary metabolism and selectively regulated by drought, salinity, heat and cold. In fact, these stress related genes come under MVA pathway that is known to be responsible for terpenoid biosynthesis which comprise a series of metabolites with peculiar protection roles to biotic attacks (Tholl, 2015). In addition, as chemical signals, several volatile sesquiterpenes are implied in activating plant defence mechanisms to respond to biotic stresses. Terpenoids are widespread in plants and should have played an important role in plant evolution as response to different biotic and abiotic aspects (Balan et al., 2017). Flavonoids are also crucial in defense against environmental stress such abiotic and biotic stresses. Nakabayashi et al. (2014) showed that flavonoids played an important role as a mitigator of oxidative and drought stress in Arabidopsis.

These molecules are the most abundant hydrophilic antioxidants in fruits and own significant biological activities in humans playing an important role in the prevention of human disease and maintaining of good health (Peluso et al., 2018). Flavonoids and anthocyanins are found in many fruits and vegetables and most of them are coloured compounds, especially red fruits, grape skins, pomegranate, loquat, blueberries, red cabbages (Rop et al., 2010; Gentile et al., 2016; Mannino et al., 2019; Passafiume et al., 2019). The results confirmed that drought stress clearly enhanced flavonoids while heat stress repressed most of these genes (i.e. aldo/keto reductase, TRANSPARENT TESTA70). It was reported in previous studies that aldo-keto reductase activity and gene expression increased with osmotic and salt stress and abscisic acid (ABA), which plays a key role in abiotic stress responses in rice, wild oat, barley, and *Xerophyta viscosa* (Li and Foley, 1995; Mundree et al., 2000; Roncarati et al., 1995). AKR gene expression increased in Bromegrass under low temperature exposure and ABA treatment suggesting a role of AKR enzymes in cold stress tolerance (Lee and Chen, 1993). AKR gene expression also increased with various other abiotic stress factors, including heat, drought, heavy metals, and UV-B in digitalis and alfalfa (Gavidia et al., 2002 ; Hegedüs et al., 2004; Hideg et al., 2003; Oberschall et al., 2000). An over-induction of aldo–keto reductase was previously linked to oxidative and heat stress tolerance in rice (Turóczy et al. 2011). By the same way, the expression of phenylpropanoid genes was induced by drought and cold but repressed by heat. The protective roles that are played by phenylpropanoid in plants against both biotic and abiotic stresses, are well-known (Liu et al., 2015). This beneficial activity is due to the inhibition of the formation of reactive oxygen species (ROS) as reported by Commisso et al. (2016). In this context, carotenoids are antioxidant molecules that protect plants from photooxidative processes, performing an effective scavenging action against ROS. Carotenoids are natural pigments with polyisoprene structure known to play important roles in plants as antioxidants and constitute photosynthetic organelles present in all the superior plants, mosses, ferns and algae resulting attractants for pollinators and seed dispersers for plants (Cazzonelli, 2011; Khoo et al., 2011). Generally, these molecules are involved in photosynthesis and photoprotection. Carotenoids are not synthesized by humans and animals, so the diet consisting of fruits and vegetables only provide the greatest contribution from exogenous carotenoids (Massenti et al., 2015; Perrone et al., 2016). They can work in different ways to improve health or to slow down a pathological state, thus counteracting oxidative stress (Perrone et al., 2014, 2016). They have a fundamental role in counteracting oxidative stress in humans and animals. In clinical and research settings, carotenoids in the blood or tissues can be detected after dietary intake (Perrone et al., 2016; Peluso et al., 2018). Therefore, plasma carotenoids or skin carotenoids may be a suitable indicator of total antioxidant status (Massenti et al., 2015; Perrone et al., 2016). Regarding with their specific role in plants, a recent function for carotenoids has recently emerged and relates to the response of plants to environmental stresses. ROS can oxidize carotenoids and produce reactive electrophilic species (RES), characterized by a carbonyl function adjacent to a double bond that is able to react with nucleophilic atoms (such as S and N) common to many biological molecules such as thiols (Farmer and Mueller, 2013). Consequently, thiol modification by these electrophilic lipids (RES) could activate transcription factors, thus inducing gene responses (Levonen et al., 2004) RES or oxidation of beta carotenoids derivate are potential signal molecules the concentration of which increases in plants exposed to environmental stress (such as heat stress). For example, oxidized carotenoid molecules exogenously have been shown to influence the transcription of genes involved in cell survival and stress responses (Loeffler et al., 2005). The dominant gene

families encode glutathione-S-transferases (GSTs), UDP-glucosyl transferases, cytochrome P450 and transporters. The genes down-regulated by the RES were involved in cell walls, cell division and auxin signaling. Furthermore, carotenoid oxidation molecules are bioactive compounds. For example, products derived from the enzymatic oxidation of carotenoids possess important signaling functions in plants. The abscisic acid hormone is an example of a molecule derived from the enzymatic oxidation of neoxanthin (Nambara and Marion-Poll, 2005) involved in the responses of plants to the environment stress and to pathogens, and also plays a role in seed germination, in the early development of the embryo and in stomatal regulation. My research group has identified the carotenoid cleavage dioxygenase 1 (CCD1) that was up regulated in drought stress. The gene that encoding the enzyme was involved in apocarotenoid biogenesis molecule of 20 or 27 atoms of C that originated from the oxidation of beta carotene. Photosynthesis can be inhibited by high concentrations of carotenoid RES (Shao et al., 2011). At this regard, my results confirm that the NON-PHYTOCHEMICAL QUENCHING gene was down regulated by drought stress.

I observed that genes involved in lignin and lignans (mannitol dehydrogenase and CINNAMYL ALCOHOL DEHYDROGENASE 6), phenylpropanoids and glycosylates were mostly repressed by heat stress. This evidence is well-documented in literature (Moura et al., 2010). Commisso et al. (2016) reported that the levels of most metabolites *declined* sharply after *heat stress as result of* cell death and subsequent metabolite degradation due to protein denaturation and aggregation, affect the RNA stability, membrane fluidity and integrity (Wahid et al., 2007). On contrast, two metabolite CINNAMYL COA REDUCTASE 1 (CCR1) and ISOPENTENYL PYROPHOSPHATE were increased after heat stress (Wahid et al., 2007). On the other hand, cold stress enhanced genes involved in terpenoid (terpene synthase 21 and selenenyl diphosphate synthase 1) and in alkaloids (STRICTOSIDINE SYNTHASE). Previous works highlighted the increase of CCR under different stress conditions such as wound or pathogen infection (Lauvergeat et al., 2001; Kawasaki et al., 2006). Nevertheless, the precise molecular role of lignin biosynthesis genes in abiotic stress remains unclear. Hence, several hypotheses have been proposed. The most accepted one is that lignin related enzymes such as CCR are associated with the drought and salt stress tolerance mechanisms (Chazen and Neumann, 1994; Kawasaki et al., 2001; Kim et al., 2006; Lee et al., 2007). Taken together all these findings could drive the conclusion that CCR1 is closely associated to heat stress responses. Concerning the terpene synthase, Lee G et al. (2014) showed that rice terpene synthase 20 (OsTPS20) plays a major role in producing terpene volatiles during the abiotic stress (Lee G et al. 2014). Another recent study identified three new terpene synthase genes in Santalum spp. demonstrating that TPS1 play important roles in chemical defense and in protection against light and temperature stress (Zhang et al., 2019).

3.6. Conclusion

Although this meta-analysis cannot provide definitive information that can be quickly transferred in molecular tools for crop breeding, I have provided more insights into molecular regulatory networks controlling resistance/tolerance/susceptibility to 4 major abiotic stresses in plants. Next step will be their mapping in each crop chromosomes thanks to the ongoing projects of re-sequencing using the exponential progresses of next-generation sequencing technologies. This essential work will speed up the delivery of molecular markers for sustainable agronomic approaches for a future agriculture that will face the highly threatening

effects of a rapid climate change. Essential insights in the hormonal crosstalk modulating simultaneous abiotic stress responses were provided: up-regulation of jasmonate-related genes was linked to drought, while gibberellin repression was down-regulation by drought and heat. Cold stress induced genes involved in ABA, cytokinin and gibberellins. Relating to transcription factors, I found that different categories are involved in specific responses to abiotic stresses: AP2-EREBP, MADS, WRKY22, MYB, homeobox genes members were linked to drought stress while cold stress was associated to induction of MYB7 and BELL 1. Heat repressed C2C2-CO-LIKE, MADS and HOMEOBOX3. Last important findings of my meta-analysis were:

1) induction of ubiquitin-mediated protein degradation by heat
2) up-regulation of MAP Kinases by cold stress.

4. CHAPTER 4

Experiment 3: Gaining insight into exclusive and common transcriptomic features linked to drought and salinity responses across fruit tree crops

DOI: https://doi.org/10.3390/plants9091059

4.1. Introduction

Drought and salinity are considered two major environmental factors affecting plant productivity and plant distribution. Therefore, it is necessary to understand plant tolerance toward drought and salinity, forming a major research topic. Drought stress represents a critical issue at reproductive stages for crop production because it impairs key physiological processes involved in yield and its components such as bud development, flowering, and fruit ripening. There are significant differences within the same species in response to drought stress, especially at the root level (Lynch et al., 2018). Drought-resistant cultivars are those that more efficiently modulate carbohydrate partitioning toward seed filling, contrasting drought stress during the pod-filling stage. It was shown that a more efficient modulation of sucrose transport favors an efficient carbon mobilization toward seeds (Cuellar-Ortiz et al., 2008). Drought stress also reduces water uptake and affects the rapid and long-term adaptation mechanisms of plant species to climate change. Identifying the molecular mechanisms and key genes involved in drought and salinity resistance is essential for efficient next-generation molecular breeding. Plants can perceive abiotic stresses and elicit appropriate responses with altered metabolism, growth, and development. These regulatory circuits include stress sensors, signaling pathways comprising a network of protein–protein reactions, transcription factors, and hormones, and finally the output proteins or metabolites (Benny et al., 2019). Plants are sessile organisms. Indeed, water and salt stress occur frequently, and, since plants cannot move, they developed strategies to adjust themselves with these challenges either via adaption mechanisms or via specific growth habits to avoid stress conditions. These plant cryptic way of resisting to harsh environmental stresses are modulated by a complex regulatory network that is only barely elucidated. Differential stress tolerance could be attributed to differences in plant reactivity in terms of stress perception, signal transduction and appropriate gene expression programs, or other novel metabolic pathways that are restricted to tolerant plants (Zhu et al., 2001). Exposure to drought or salt stress triggers many common reactions in plants. Both stresses cause cellular dehydration, which causes osmotic stress and water movement from the cytoplasm into the extracellular space. The stresses induce reactive oxygen species and radical ions, which in turn show a negative effect on the cellular structures and metabolism. Even though the early responses toward salinity and drought are the same, the responses toward ionic components are different. Decrease of photosynthesis or hormonal crosstalk regulation such as increased levels of abscisic acid (ABA) is a common physiological feature of both stresses. High intracellular concentrations of sodium and chloride ions are specific issues of salinity stress (Flowers et al., 2015). Over the last decade, thousands of genes involved in drought stress responses were identified, which can be included in two groups (Meng et al., 2018). One

group of genes directly protects the plants against drought stress by regulating water transport (aquaporin) (Alexandersson et al., 2010) or by protecting the integrity of cellular membrane and macromolecules. The second group of genes (receptor proteins, protein kinases, protein phosphatases, and transcription factors) regulates signal perception, signal transduction, and amplification (Shinozaki et al., 2003). Many key genes for salinity tolerance relating to oxidation–reduction processes, ion transport and chloride channels, hormone-related genes, like ethylene perception-related and ABA, as well as many transcription factors, were discovered. Fruit trees must exist under adverse environmental conditions over years and, therefore, require not only drought/salinity adaptiveness but also flexibility toward the metabolism of hormones, transcription factors (TF), etc. to adjust with changing conditions. For example, some key transcription factor (TF) families such as MYB, WRKY, basic leucine zipper (bZIPs) were found to be involved in a different manner depending on the type of stress (Hoang et al., 2017). Drought and salinity tolerance in fruit trees is usually achieved via biochemical modification of the cellular metabolism. (Chen et al., 2014). Transcriptomic studies are important in identifying specific genes involved in water and salinity stresses in different species. These types of analysis help in recognizing which genes are the basis of diverse abiotic tolerance and resistance mechanisms. However, transcriptomic approaches have several drawbacks. Most transcriptomic studies are generally related to only one season, which may lead to low reliability of conclusions of these studies. RNA-Seq data are affected by high environmental variability, often presenting false-positive results. Therefore, it is necessary to adapt bioinformatic pipelines to enhance the comparison of data obtained across different species in order to strengthen the meaning of every single study and validate the published works reducing the environmental variability (Benny et al., 2019). Meta-analysis is a statistical technique for combining the findings from independent studies. It is used to determine the effectiveness of a treatment or to study a factor affecting a process combining data from randomized similar studies. Meta-analysis provides a precise estimation of treatment's effect giving weight to the size of the different studies included in the analysis. The current study is focused on fruit tree responses toward drought and salinity, as well as major genes which can be utilized by genetic engineering for the development of tolerant species. Thus, a meta-analysis of all the transcriptomic studies can play a vital role in selecting the most frequent and most significant differentially expressed genes (DEGs) among the complete list of differentially regulated genes.

4.2. Aim of the Research

In the present work, I conducted a meta-analysis by selecting six RNA-Seq studies with similar experimental design (timing and intensity of stresses) conducted in five fruit tree crops in order to deliver conserved and reliable genomic information for enhancing drought and salinity crop resistance/tolerance. I analyzed, in the most comprehensive manner possible, RNA-Seq data in fruit tree crops under drought and salinity using the same bioinformatics pipeline used in the previously published meta-analysis. The most important players among the huge amount of data generated by every single RNA-Seq study were identified and mapped on the chromosomes to develop next-generation markers (i.e., based on epigenetic mechanisms). Key molecular physiological conclusions were generated based on the identification of conserved gene sets, pathways, and gene networks involved in abiotic stress resistance/tolerance. This study provides a valid approach to ask additional questions with respect to how plants respond to stress.

4.3. Materials and Methods

- Search Strategy for Selection of RNA-Seq Studies

For the analysis, the most relevant articles on drought and salinity stress response in fruit crops, together with one herbaceous species, were taken into consideration. These studies, identified from Scopus and PubMed, were considered suitable when abiding by the following three criteria: (i) presenting RNA-Seq sequencing methodology; (ii) mentioning at least one of the following terms in title and abstract: drought, salinity, root, stress, and abiotic stress; (iii) the presence of publicly accessible raw data. These criteria were met in six articles on a total of 26 samples (Table 4.1).

Table 4.1. Articles, crops, number of samples, stress, and sample description (control vs. treatment) included in the analysis.

Article	Stress	Species	No. of samples	Control	Treated	Duration of stress
Khadka et al. 2019	Drought	*Vitis riparia* Michx	6	SRR6494883 (Control1)	SRR6494880 (Treated 1)	Roots were harvested at 14 days after stress (DAS)
				SRR6494884 (Control2)	SRR6494881 (Treated 2)	
				SRR6494885 (Control3)	SRR6494882 (Treated 3)	
Feng et al. 2017	Drought	*Prunus mahaleb* L.	6	SRR5112808 (Control1)	SRR5112805 (Treated1)	Roots were harvested at 15 DAS
				SRR5112809 (Control2)	SRR5112806 (Treated2)	
				SRR5112810 (Control3)	SRR5112807 (Treated3)	
Ksouri et al. 2016	Drought	*Prunus persica*	6	SAMEA3861653 (Control 1)	SAMEA3861656 (Treated1)	Roots were harvested at 16 DAS
				SAMEA3861654 (Control2)	SAMEA3861657 (Treated2)	
				SAMEA3861655 (Control3)	SAMEA3861658 (Treated3)	
				SRR6770841 (Control 2)	SRR6770840 (Treated 2)	
Bazakos et al. 2015	Salinity	*Olea europaea* L. cv. Kalamon	2	SRR891235 (Control1)	SRR886308 (Treated1)	Roots were harvested at 90 DAS
Yaish et al. 2017	Salinity	*Phoenix dactylifera* L. cv.	2	SRR4034943 (Control1)	SRR4034944 (Treated1)	Roots were harvested at

		Khalas				45 DAS
Radwan et al. 2015	Salinity	*Phoenix dactylifera* L. cv. Deglet Beida	4	SRR2027988 (Control 1)	SRR2029376 (Treated 1)	Roots were harvested at 60 DAS
				SRR2029378 (Control 2)	SRR2029381 (Treated 2)	

The selected studies were grouped based on stress: three articles were focused on drought and three articles were focused on salinity. For the functional analysis, the following groups were considered:

(A) Commonly regulated genes among three articles in drought.

(B) Commonly regulated genes among three articles in salinity.

(C) Commonly regulated genes among both (A) and (B).

The entire workflow of the study is given in Figure 4.1.

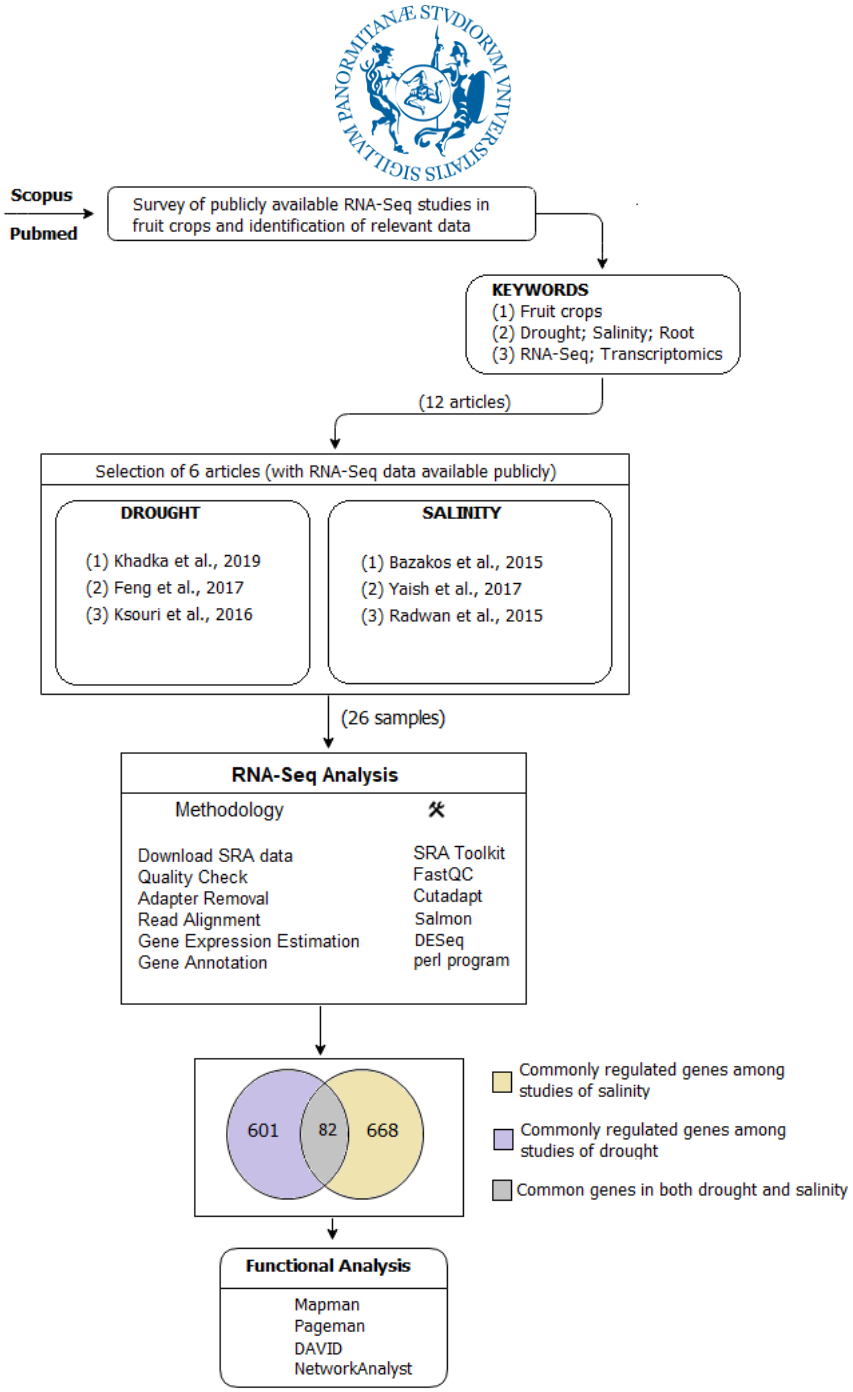


Figure 4.1. Workflow of the meta-analysis of the six transcriptomic studies related to drought and salinity stress in root tissue. Functional and statistical data analysis are indicated.

- Read Alignment, Gene Differential Expression, and Annotation

For each of the six articles, the relative crop genome and the annotation file were downloaded from Phytozome (https://phytozome.jgi.doe.gov) and the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). According to the accession number provided in the selected articles, raw data were downloaded from the NCBI sequence read archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) and European Molecular Biology Laboratory (EMBL) ArrayExpress (https://www.ebi.ac.uk/arrayexpress/). The raw data were then converted to FASTQ format using SRA toolkit version 2.3.5. I trimmed low-

quality bases and adapter sequences from the raw data using Cutadapt version 1.8.1 to obtain high-quality clean reads. These reads were aligned to the corresponding genome using Salmon version 0.14.0 with default parameters. To aggregate the transcript-level quantification to the gene level for gene-level differential expression analysis, I used the R package called tximport. The quantification results of salmon were then given to DESeq2 for the differential expression analysis. Up- and downregulated genes with p-value < 0.05, log2FC ≤ -2, and log2FC ≥ 2 were considered for downstream functional analysis. The statistical tests were corrected using the Benjamini–Hochberg false discovery rate (FDR) procedure with the help of the p.adjust function of R. The annotation of DEGs selected was performed using the related crop genome mapping files retrieved from the Phytozome database. For the selection of genes and their genome, along with chromosome mapping, a custom-made in-house Perl script was employed.

- Statistical and Cluster Analysis

The DEGs corresponding to each independently studied research work, having a p-value < 0.05, were then analyzed by undertaking appropriate statistical tests, corrected for multiple comparisons using the p.adjust function of R and FDR (Benjamini et al., 1995). By adjusting the p-values, the false discovery rate (FDR) was selected to a desired level of $\alpha = 0.01$. Sample normalization was adopted in order to avoid systematic variation among the studies selected for the meta-analysis. The normalization served as a crucial and rigorous pre-processing step to adjust the sequencing depths and technical effects. Geometric normalization was used, whereby fragments per kilobase of transcript per million (FPKM) and fragment counts were scaled via the median of the geometric means of fragment counts across all libraries. R software was used for all statistical analyses. The dendrogram was constructed using Euclidean distance measure for identifying the clustering patterns of the examined drought and salinity studies.

- Gene Set and Pathway Enrichment Analysis

All the DEGs from each study were taken and aligned to the Arabidopsis thaliana reference genome for obtaining the best hit “The Arabidopsis Information Resource” (TAIR) ID. MapMan (Thimm et al., 2004) (http://mapman.gabipd.org/) was used for mapping and the visualization of key metabolic pathways such as secondary metabolism, hormone regulation, transcription factors, and protein targeting using the Arabidopsis thaliana mapping file. The drought-regulated genes common in three of three studies were visualized first; then, the common salinity-regulated genes among the three studies in the salinity group and, at last, the common genes between drought and salinity stresses were visualized. Differences among metabolic pathways were visualized by the PageMan (Usadel et al., 2006) analysis, a plugin of MapMan, by means of the Wilcoxon test algorithm, without any correction, using an over-representation analysis (ORA) cut-off value of 3. The TAIR IDs produced from the analysis of the each group were searched against the DAVID (Huang et al., 2009) version 6.8 Web server (https://david.ncifcrf.gov/). The information related to biological process, cellular component, and molecular function were retrieved from the GO result.

- Mapping of Genes to Corresponding Chromosomes

The chromosome mapping was done by selecting the commonly regulated abiotic stress-related genes involved in both drought and salinity. With the help of a custom-made Perl script, I fetched chromosome number along with the start and end of the commonly regulated

gene IDs, and then I located the chromosome number, with start and endpoints of each species accordingly.

- Protein–Protein Interaction Network

NetworkAnalyst (Xia et al., 2004), a web-based tool for network-based visual analytics for gene expression profiling, meta-analysis, protein–protein interaction network analysis, and visual exploration, was used for individual data annotation and analysis. The list of homologous TAIR IDs from three groups was uploaded separately and mapped against the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) interactome database with default parameters (confident score cut off = 900 and with experimental evidence) provided in NetworkAnalyst. To study the key connectives and to simplify the large network, I selected the "Minimum Network" setting provided by STRING. Networks were modified indicating if genes were up- or downregulated in response to each stress.

- Validation Analysis

I implemented a leave-one-out cross-validation (LOOCV) methodology for validating the expression value of the 82 common (hub) genes. The dataset was split into two: a training set and a test set for the validation. I discarded one sample from the main dataset for testing and selected the others for training.

4.4. Results

- Transcriptomic Responses to Drought and Salinity

Twelve RNA-Seq studies in public databases matching the chosen selection criteria were found. Six of them had no raw data available thus were excluded. The analysis was performed using six studies: three dealing with drought and the other three dealing with salinity.

The articles, plant species, and the number of up- and downregulated genes for each article are listed in Table 4.2. The analysis resulted in the identification of a total of 36,909 genes, of which 18,404 were upregulated and 18,505 were downregulated. Taking the stress-related genes toward salinity response, 51.55% of the genes were upregulated and 53.01% were downregulated. When considering the drought-related genes, 50.46% of the total number of stress-related genes were upregulated and 49.53% were downregulated.

Table 4.2. The number of upregulated and downregulated genes in response to drought/salinity for each study; those commonly regulated in drought, salinity, and both drought and salinity are given.

Articles	**Crops**	**Sample Information**		
		Total	**Down**	**Up**
DROUGHT				
Khadka et al. 2019	*Vitis riparia* Michx	5,021	2,950	2,071
Feng et al. 2017	*Prunus mahaleb* L.	6,959	3,056	3,903
Ksouri et al. 2016	*Prunus persica*	5,856	2,829	3,027
SALINITY				

Bazakos et al. 2015	*Olea europaea* L. cv. Kalamon	6,060	2,982	3,911
Yaish et al. 2017	*Phoenix dactylifera* L. cv. Khalas	5,504	3,585	1,919
Radwan et al. 2015	*Phoenix dactylifera* cv. Deglet Beida	6,676	3,103	3,573
Commonly Regulated in drought		683	349	334
Commonly Regulated in salinity		750	390	360
Commonly Regulated among both drought and salinity		39	16	23
Common genes among drought and salinity		82		

The first comparison was performed using the three studies in salinity to find common genes regulated among them. In total, 750 genes were common, implying their conserved role in response toward salinity. A second comparison was done comparing the three works related to drought. In total, 683 genes were common in all the three drought studies. A third comparison was done on the 683 drought-related genes and 750 salinity-related genes to find genes common among both salinity and drought. This latter comparison highlighted 82 differentially regulated genes involved in drought and salinity. There were 39 genes that showed the same trend of expression: 23 were all upregulated and 16 were all downregulated. I also paid special attention to these 39 genes (Table 4.3) in the downstream functional analysis.

Table 4.3. Comparison highlighting 39 genes with the same trend of expression among drought and salinity (23 were all upregulated and 16 were all downregulated). The gene identifier (ID), expression type, description, functional term, and category are given.

Gene ID	**Expression type**	**Description**	**Functional Term**
AT5G11700	Down	Ephrin type-B receptor Ephrin type-B receptor	Vacuole
AT5G56270	Down	Probable WRKY transcription factor 2	DNA-binding transcription factor activity
AT1G15060	Down	Alpha/beta hydrolase family protein	hydrolase activity
AT1G04910	Down	O-fructosyl transferase 1	carbohydrate metabolic process
AT5G24090	Down	Acidic endo chitinase	chitin catabolic process
AT1G67180	Down	Zinc finger (C3HC4-type RING finger) protein	Cell cycle
AT3G61790	Down	E3 ubiquitin-protein ligase SINAT3	ubiquitin-dependent protein catabolic process
AT4G02570	Down	CUL1 AT4G02570 protein	auxin-activated signaling pathway

AT2G42520	Down	DEAD-box ATP-dependent RNA helicase 37	nucleic acid binding
AT5G47650	Down	Nudix hydrolase 2	metal ion binding
AT5G25930	Down	Leucine-rich repeat receptor-like protein kinase	protein phosphorylation
AT4G32010	Down	B3 domain transcription repressor VAL2	regulation of transcription, DNA-templated
AT3G19840	Down	Pre-mRNA-processing protein 40C	mRNA processing
AT2G27900	Down	RABA5d	endocytic recycling
AT5G14720	Down	Protein kinase superfamily protein	phosphorylation
AT4G32850	Down	Polynucleotide adenylyl transferase 4	nucleotidyltransferase activity
AT4G03500	Up	Ankyrin repeat family protein	Membrane
AT1G15520	Up	PLEIOTROPIC DRUG RESISTANCE 12	abscisic acid transport
AT1G02520	Up	ATP-BINDING CASSETTE B11	transmembrane transport
AT3G06880	Up	Transducin/WD40 repeat-like superfamily protein	response to stress
AT5G64813	Up	LIGHT INSENSITIVE PERIOD1	Cytoplasm
AT4G31210	Up	DNA topoisomerase, type IA	metal ion binding
AT5G07990	Up	CYTOCHROME P450 75B1	oxidation-reduction process
AT5G52450	Up	Protein DETOXIFICATION 16	response to nematode
AT2G36690	Up	Germination insensitive to ABA mutant 2, GIM2	oxidation-reduction process
AT5G11040	Up	VAN4, VASCULAR NETWORK DEFECTIVE 4	cytokinesis by cell plate formation
AT5G23150	Up	ENHANCER OF AG-4 2, HUA2	regulation of transcription by RNA polymerase II
AT3G14270	Up	FAB1B, Forms aploid & binucleate cells 1B	phosphatidylinositol phosphorylation
AT2G03810	Up	18S pre-ribosomal assembly gar2-like protein	regulation of asymmetric cell division
AT4G26270	Up	PFK3, Phosphofructokinase 3	fructose 6-phosphate metabolic process
AT5G58003	Up	C-terminal domain Phosphatase-like 4	dephosphorylation of RNA polymerase II

			C-terminal domain
AT4G35160	Up	N-acetyl serotonin O-methyl transferase	Methylation
AT2G45550	Up	CYTOCHROME P450	oxidation-reduction process
AT2G19130	Up	S-locus lectin protein kinase family protein	phosphorylation
AT4G02590	Up	UNE12, Unfertilized EMBRYO SAC 12	regulation of defense response
AT3G48190	Up	Serine/threonine-protein kinase (ATM)	DNA damage checkpoint
AT5G54310	Up	AGD5, ARF-GAP domain 5	activation of GTPase activity
AT2G26330	Up	Quantitative Resistance to Plectosphaerella 1	receptor serine/threonine kinase binding
AT2G27920	Up	SCPL51, Serine Carboxypeptidase-Like 51	Proteolysis

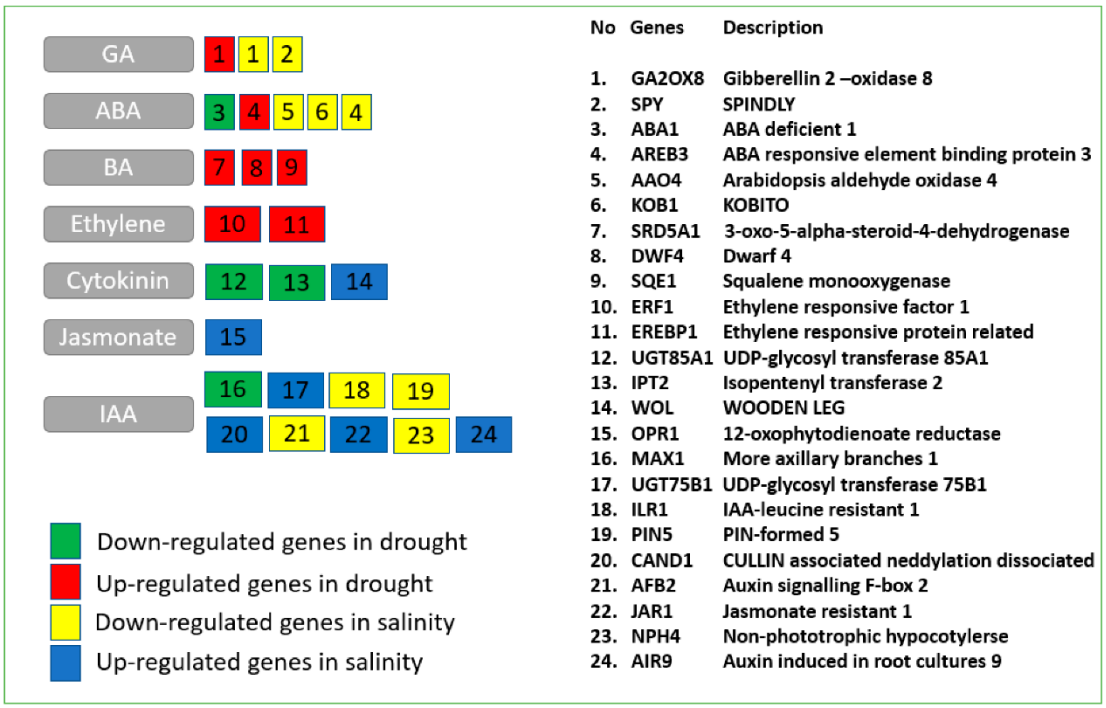
- Gene Set and Pathway Enrichment Analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) software was used to annotate the functionalities of genes corresponding to drought and salinity at the transcriptomic level taking the list of drought-regulated genes (common among three studies) and salinity-regulated genes (common among three studies). Among the drought studies, two GO terms were downregulated while 13 were upregulated. It is worth mentioning some of the biological pathways that are well known to be enhanced by drought stress such as response to abscisic acid, response to jasmonic acid, defense response, protein phosphorylation, and heterochromatin maintenance. On the contrary, some GO terms that were downregulated in response to water stress were the following: response to carotenoid biosynthetic process and embryo development ending in seed dormancy. While considering the salinity responses, 14 GO terms were downregulated and 14 were upregulated. GO terms such as regulation of jasmonic acid-mediated signaling pathway, response to cadmium ion, ubiquitin-dependent protein catabolic process, cellular heat acclimation, and regulation of stomatal movement showed an enhancement toward the salinity stress, whereas leaf senescence, response to cytokinin, auxin metabolic process, late nucleophagy, transmembrane receptor protein tyrosine kinase signaling pathway, and micro autophagy of nucleus were repressed. When comparing the GO terms corresponding to each stress, no GO terms related to the biological processes were commonly downregulated among drought and salinity. On the other hand, pathways encoding regulation of defense response, transmembrane transport, and metal ion binding were enhanced toward both drought and salinity responses.

- Transcriptomic Responses Related to Hormone Metabolism

I focused my attention to the hormonal-related genes considering the key role played by hormonal crosstalk in the modulation of abiotic stress responses in plants. When focusing on drought stress studies, water deprivation downregulated two genes responsive to cytokinin

(uridine diphosphate (UDP)-glycosyltransferase (UGT85A1) and isopentenyl transferase 2 (IPT2)), one responsive to abscisic acid (ABA) (ABA deficient 1 (ABA1)), and one indole acetic acid (IAA) gene (more axillary branches 1 (MAX1)). On the other hand, it upregulated several genes responsive to abscisic acid, gibberellin, brassinosteroids (BRs), and ethylene-activated signaling pathway. Among the brassinosteroid-related genes, it is worth mentioning the enhancement of 3-oxo-5-alpha-steroid 4-dehydrogenase (SRD5A1), DWARF 4 (DWF4), and squalene monooxygenase (SQE1) (Figure 4.2)



No	Genes	Description
1.	GA2OX8	Gibberellin 2 –oxidase 8
2.	SPY	SPINDLY
3.	ABA1	ABA deficient 1
4.	AREB3	ABA responsive element binding protein 3
5.	AAO4	Arabidopsis aldehyde oxidase 4
6.	KOB1	KOBITO
7.	SRD5A1	3-oxo-5-alpha-steroid-4-dehydrogenase
8.	DWF4	Dwarf 4
9.	SQE1	Squalene monooxygenase
10.	ERF1	Ethylene responsive factor 1
11.	EREBP1	Ethylene responsive protein related
12.	UGT85A1	UDP-glycosyl transferase 85A1
13.	IPT2	Isopentenyl transferase 2
14.	WOL	WOODEN LEG
15.	OPR1	12-oxophytodienoate reductase
16.	MAX1	More axillary branches 1
17.	UGT75B1	UDP-glycosyl transferase 75B1
18.	ILR1	IAA-leucine resistant 1
19.	PIN5	PIN-formed 5
20.	CAND1	CULLIN associated neddylation dissociated
21.	AFB2	Auxin signalling F-box 2
22.	JAR1	Jasmonate resistant 1
23.	NPH4	Non-phototrophic hypocotylerse
24.	AIR9	Auxin induced in root cultures 9

Figure 4.2. Drought- and salinity-regulated genes involved in hormone-related categories commonly regulated in the studies are shown. Genes were identified as Arabidopsis orthologs of each gene of the analyzed plant species. Red and green indicate the up- and down-regulated genes in drought, whereas blue and yellow indicate the up- and down-regulated genes in salinity.

Related to salinity stress, there was an upregulation in 12-oxophytodienoate reductase (OPR1), wooden leg (WOL), UDP-glucosyltransferase 75B1 (UGT75B1), cullin-associated and neddylation-dissociated (CAND1), jasmonate resistant 1 (JAR1), and auxin induced in root cultures 9 (AIR9). At the same time, genes encoding for auxin, gibberellins (gibberellin 2 oxidase 8 (GA2OX8)), and abscisic acid (aldehyde oxidase 4 (AO4), KOBITO 1 (KOB1), ABA-responsive element binding protein 3 (AREB3)) were downregulated.

- Transcription Factors (TFs)

TFs are special proteins that control the transcription of genes, and many of them are, therefore, expressed in a genotype-, tissue-, and stress-specific manner. A total of 45 major TFs were differentially expressed, with 16 downregulated in both drought and salinity conditions and 23 commonly upregulated. The most expressed TF families were WRKY, basic helix–loop–helix (bHLH), MYB, trihelix-factor, APETALA2/ethylene-responsive element binding protein (AP2-EREBP), homeobox (HB), and GATA. Among downregulated

genes, it is worth mentioning ephrin type-B receptor, WRKY2, O-fructosyltransferase 1, and a zinc finger (C3HC4-type really interesting new gene (RING) finger) family protein. Among the upregulated genes, it is worth noting the expression of serine carboxypeptidase-like 51, adenosine diphosphate (ADP)-ribosylation factors-GTPase-activating proteins (ARF-GAP) domain 5, serine/threonine-protein kinase, ataxia telangiectasia mutated (ATM), and transducin/WD40 repeat-like superfamily protein, due to their involvement in plant response to abiotic stresses and ABA-dependent plant development.

- Stress-Related Genes Involved in Both Drought and Salinity

Genes mapped to the abiotic stress (drought/salinity)-related categories were identified using MapMan, and they are shown in Figure 4.3. Among the common drought upregulated genes, it is worth mentioning the DNAJ-like 20 (J20), DNAJ heat shock N-terminal domain-containing protein, dehydration 22 (RD22) (nutrient reservoir), and 4-phosphopantetheine adenylyl transferase (ATCOAD). In the category of salt stress-related genes, I observed an upregulation of DNAJ heat shock N-terminal domain-containing protein and a downregulation of Luminal binding protein 2 (BIP2), chloroplast heat-shock protein 70-2, heat-shock cognate protein 70-1 (HSC70-1), dehydration responsive protein, and RD22.

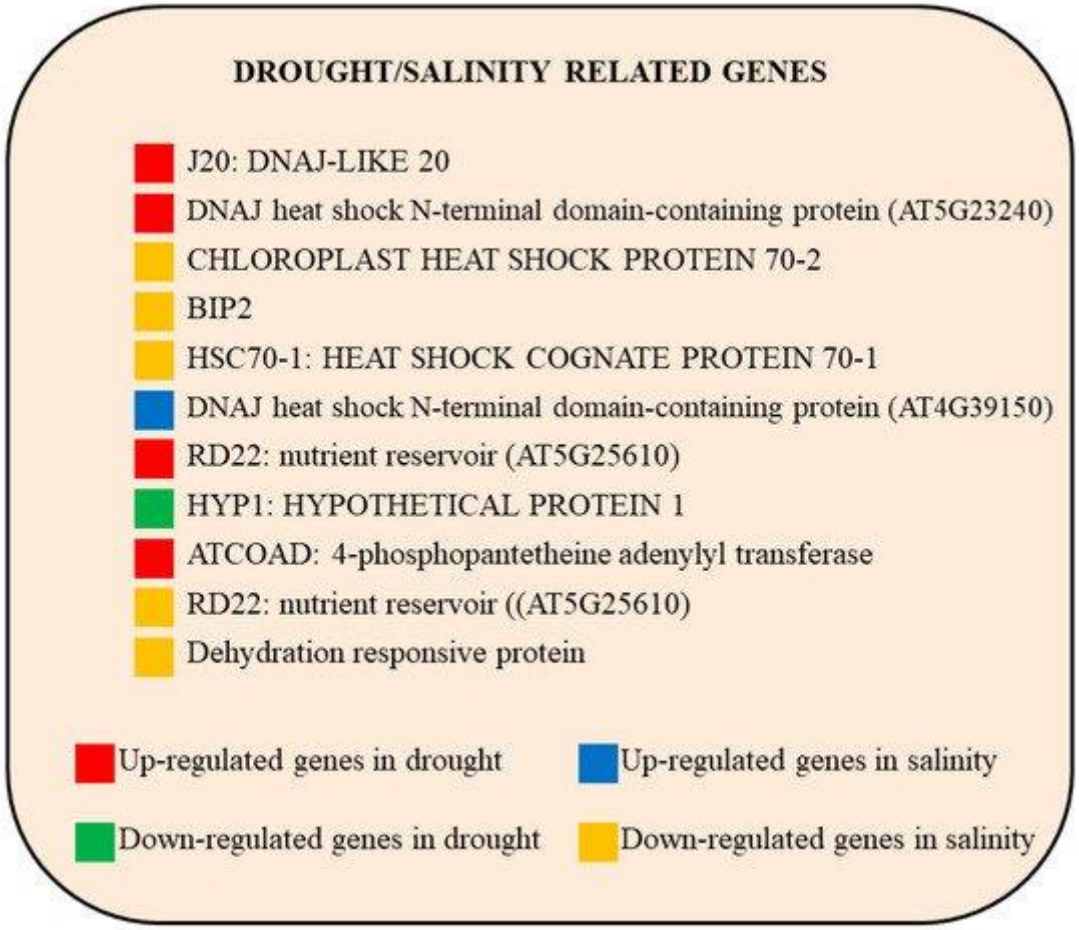


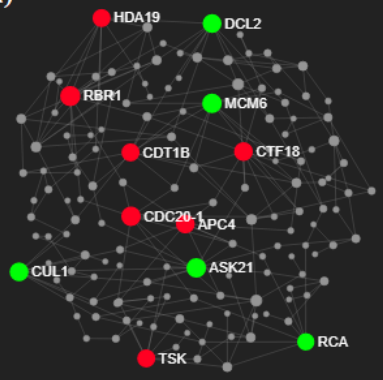
Figure 4.3.Drought/salinity-regulated genes involved in abiotic stress-related categories commonly regulated in all eight studies are indicated. Genes were identified as Arabidopsis thaliana orthologs of each gene of the analyzed plant species. Red indicates up-regulation and

green indicates down-regulation in response to drought stress, whereas blue and yellow indicate up- and down-regulated genes in salinity.

- Protein–Protein Interaction Network Analysis in Response to Abiotic Stresses

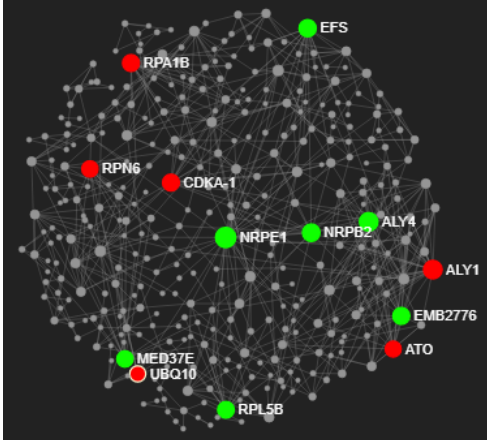
The protein–protein interaction (PPI) network analysis comprised three networks based on the minimum default settings used to reduce the number of interacting proteins and the complexity of the networks (Figure 4.4). Some key genes with a high number of interactions (>20) were highlighted.

(a)



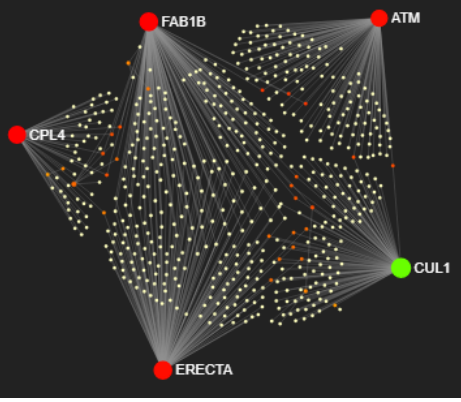
Gene ID	Description
HDA19	Histone deacetylase 19
DCL2	dicer-like 2
RBR1	Retinoblastoma-related protein 1
MCM6	MINICHROMOSOME MAINTENANCE 6
CDT1B	cyclin-dependent protein serine/threonine kinase
CTF18	Chromosome transmission fidelity protein 18
CDC20-1	cell cycle-regulated E3 ubiquitin-protein ligase
APC4	Anaphase-promoting complex subunit 4
ASK21	SKP1-like protein 21
CUL1	Cullin-1
RCA	RUBISCO ACTIVASE
TSK	TONSOKU

(b)



Gene ID	Description
EFS	CAROTENOID CHLOROPLAST REGULATORY1
RPA1B	REPLICATION PROTEIN A 1B
RPN6	NON-ATPASE SUBUNIT 9
CDKA-1	Cyclin-dependent kinase A-1
NRPE1	DEFECTIVE IN MERISTEM SILENCING 5
NRPB2	EMBRYO DEFECTIVE 1989
ALY4	DNA-BINDING DOMAIN OF ZN-FINGER PARP 1
ALY1	ALWAYS EARLY 1
EMB2776	E3 ubiquitin ligase complex
ATO	ATROPOS
MED37E	Probable mediator of RNA polymerase II
UBQ10	Polyubiquitin 10
RPL5B	OLIGOCELLULA 7

(c)



Gene ID	Description
FAB1B	FORMS APLOID AND BINUCLEATE CELLS 1B
ATM	Serine/threonine-protein kinase
CPL4	C-TERMINAL DOMAIN PHOSPHATASE-LIKE 4
ERECTA	QUANTITATIVE RESISTANCE TO PLECTOSPHAERELLA 1
CUL1	Cullin-1

Figure 4.4. Protein–protein interaction network analysis predicted for genes commonly regulated in (a) three of three drought studies, and (b) three of three salinity studies; (c) genes commonly regulated in six of six studies of both drought and salinity based on Arabidopsis knowledgebase. Proteins encoded by genes having a high degree of betweenness are shown in red (up-regulated) and green (down-regulated).

Interestingly, drought downregulated highly interactive proteins such as rubisco activase (RCA), S-phase kinase-associated protein 1 (SKP1)-like protein 21 (ASK21), and dicer-like 2 (DCL2), while it upregulated proteins such as histone deacetylase 19 (HDA19), cyclin-dependent protein serine/threonine kinase (CDT1B), retinoblastoma-related protein 1 (RBR1), and cell cycle-regulated E3 ubiquitin-protein ligase (CDC20-1) (Figure 4.4a). PPI network analysis was performed for salinity-regulated genes showing an enhancement of three major hub proteins, polyubiquitin 10 (UBQ10), atropos (ATO), and cyclin-dependent kinase A-1 (CDKA-1), along with a repression of embryo defective 1989 (NRPB2), DNA-binding domain of Zn-finger poly ADP ribose polymerase (PARP) 1 (ALY4), and E3 ubiquitin ligase complex (EMB2776) (Figure 4.4b). Among the upregulated hub (highly interacting) proteins that were present in both drought and salinity categories, it is worth noting that some proteins may play a key role in abiotic response such as forms aploid and binucleate cells 1B (FAB1B), ATM, quantitative resistance to plectosphaerella 1 (ERECTA), and one downregulated protein, cullin-1 (CUL1) (Figure 4.4c).

- Genes Involved in General Dehydration Stresses

I paid special attention to the 39 (23 were upregulated and 16 were downregulated) genes showing a similar expression pattern in both drought- and salinity-related studies (Table 4.3). It is worth mentioning that both salinity and drought stress downregulated WRKY transcription factor 2, CUL1, nudix hydrolase 2, O-fructosyltransferase 1, and E3 ubiquitin-protein ligase, whereas commonly upregulated genes were phosphofructokinase 3 (PFK3), cytochrome P450 75B1, N-acetyl serotonin O-methyltransferase, ATM, and serine carboxypeptidase-like 51 (SCPL51)

These 39 key genes were mapped onto the respective chromosomes of the crops. There was no homogeneous distribution of these genes across the genome observed in some of the species. The presence of a higher number of commonly regulated genes was identified in some chromosomes. In grape and olive, a homogeneous distribution of the genes in the chromosomes could be found. However, in peach, most of the genes were present in one chromosome. A total of 13 abiotic stress-related genes being mapped to chromosome 1 of peach might imply the importance of the involvement of chromosome 1 in drought and salinity resistance compared to other chromosome regions. This evidence should be taken in careful consideration by molecular breeders. This work helped in the identification of significant regions in the chromosome that contain numerous genes involved in drought and salinity. This can help guide the linking of new molecular markers capable of drought/salinity resistance.

- Leave-One-Out Cross-Validation (LOOCV) of Meta-Analysis

I employed the LOOCV approach in order to validate the 82 hub genes identified from the study. This method can predict the difference between control and treated samples. I could identify a predictive accuracy of 95.03% with an area under curve (AUC) value of

0.934 for the expression levels of these genes. These results validate the meta-analysis approach to finding the hub genes responsible for the stress response.

4.5. Discussion

Roots are the first organs to be exposed to water deficiency and salt stress, and they are the first tissue to sense drought and salinity conditions. Signaling cascades transfer chemical signals toward shoots to initiate molecular responses that lead to the biochemical and morphological changes, allowing plants to be protected against water loss and salinity and to tolerate stress conditions (Kwasniewski et al., 2015). Here, I present an overview of signaling network and gene expression regulation pathways that are actively induced in roots of fruit crops under drought and salinity stress, as these stresses are the most limiting factors of crop yield, especially in smallholder systems (Polania et al., 2016). Although it is possible to identify a good percentage of the genetic variability due to additive genetics (major genes/alleles), probably around 20–50%, these major genes are yet to be identified. This is mainly because functional genomic studies (especially RNA-seq) present data with high variability and often contrasting evidence due to the diverse experimental conditions of studies that often escape the control of researchers. For example, in the study, I considered two studies from the same species (*Phoenix dactylifera*) but of different cultivar under salinity stress. The stress duration was different for both the studies. While comparing the DEGs from both studies, a total of 5504 genes were identified from Yaish et al., 2017, while 6676 DEGs were identified from Radwan et al., 2015. This shows that the differences in the number and type of genes modulated by stress by different cultivars are due to genotypic variance, environmental differences, different time points and stress intensity, sampling time, different growth parameters, and ways of cultivation. This is why it is important to perform meta-analyses of previously published data instead of investing more economic resources in new studies. The aim is to identify strongly associated gene loci with both drought and salt stress in order to deliver reliable molecular markers to be used in a molecular marker-assisted selection, aimed at creating new cultivars with resistance/tolerance to these strictly connected abiotic stresses. It is worth mentioning that the ongoing climate change occurring worldwide is probably affecting these two abiotic stresses more than others. The aim is to create cultivars that are beneficially responsive to multiple stresses to face the multiple harsh conditions.

- The Role of Hormones in Drought and Salinity Responses

The meta-analysis highlighted unexpectedly the role of hormones in complex gene regulatory networks of plant responses to abiotic stresses. Indeed, I found three genes involved in BR-related pathways that were all upregulated in response to drought (SRD5A1, DWF4, and SQE1). BRs are polyhydroxylated steroidal hormones involved in many plant physiological processes such as hypocotyl elongation, root modulation, stomata regulation, gametophyte growth and development, and germination (Rozhon et al., 2019). However, recently, their role in plant adaptation to drought was shown (Fàbregas et al., 2018). Plants with reduced biosynthesis of BRs are typically dwarfed and show dark green, curled leaves, small petioles, reduced hypocotyls and internodes, delayed flowering, and less fertility. On the other hand, plants with enhanced BRs show higher height and longer hypocotyls (Nie et al., 2017). Indeed, I speculated that the upregulation of brassinosteroids in response to drought would allow reducing the detrimental effects on key physiological processes in plants that are involved in seed production and, consequently, in crop yield. The induction of BR genes in

tolerant/resistant genotypes should be taken under consideration in future validation approaches using transgenics and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) technologies.

The basipetal transport of auxin was inhibited in plants under water stress, provoking losses of cotyledonary petioles and early leaf loss (Davenport et al., 1997). In addition, auxin transport inhibitors and drought had a synergistic action on leaf loss. Osmotic stress provoked a significant enhancement in the basipetal transport of auxins, implying a link between drought responses and polar auxin transport in plants. Contrasting evidence was observed for IAA-related genes in response to salinity; while IAA-resistant leucine 1, PIN-FORMED 5 (PIN5), AFB2, and Non-phototrophic hypocotyls (NPH4) were commonly repressed among the analyzed studies, UGT75B1, CAND1, JAR1, AIR9 were upregulated. These data partially agree with previous findings showing an involvement of AIR9 and JAR1 in drought responses in barley through the action of miR2406 (Fard et al., 2017). UGT75B1 is an auxin-related gene that controls cellular ABA content and activity through glycosylation. UGT75B1 is induced by osmotic stress, salinity, and ABA (Chen et al., 2020). Overexpression of UGT75B1 in *Arabidopsis thaliana* provokes higher seed germination rates, larger stomatal aperture, and seedling greening in response to salt, drought, and osmotic stresses (Dalal et al., 2018). It is known that auxin plays an important role in plant growth and development. Its spatial distribution among plant tissues is modulated by polar localization of PIN-formed (PIN) auxin efflux carrier transporters, which constitute a large family. The overexpression of PIN3 was shown to promote drought resistance (Tognetti et al., 2010). Several publications showed that auxin signaling plays a vital role in stress responses in plants (Benny et al., 2020), while fewer studies focused on the auxin transport response under difficult environments. It is important to note that MYB, WRKY, and AP2-EREBP were highly repressed, suggesting their role in the abiotic stress response and plant growth processes (Ksouri et al., 2016).

The role of ethylene in drought resistance is well known (Khadka et al., 2019; Amirbakhtiar et al., 2019). The transgenic induction of ethylene response factor 1 (ERF1) in wheat enhanced resistance to salt and drought stress, inducing an increase in chlorophyll content, as well as superoxide dismutase and peroxidase activity (Xing et al., 2016). These effects were probably mediated by the modulation of expression levels of some stress responsive genes. ERF1 belongs to the large family of AP2/ERF genes involved in response to drought and salt stresses. However, the role of AP2/ERF genes is contrasting, since some AP2/ERF genes have negative effects such as AP23 (Zhuang et al., 2010). Comparative analyses between susceptible and tolerant genotypes also confirmed a role of ERF1 in drought conditions (Deokar et al., 2011). The meta-analysis shows an induction of ERF1 in drought conditions, agreeing with the previous evidence.

- Key Genes and Chromosome Regions in Abiotic Stress Tolerance/Resistance

The transgenic over-expression of some WRKY members was shown to promote drought tolerance in *Arabidopsis thaliana* such as WRKY20 (Feng et al., 2017). Here, I identified a commonly upregulated WRKY that could be a future target for promoting drought tolerance using a transgenic approach (WRKY6). In addition, I found an upregulation of a transducin/WD40 repeat-like superfamily protein in response to both abiotic stresses, and this is confirmed by previous findings that showed a role of this gene in modulating ABI5 stability and abscisic acid responses in drought conditions (Wan et al., 2019). I found that

three DNAJs were commonly induced by drought, and the role of these heat-shock proteins in drought tolerance was confirmed previously. In fact, overexpression of a J-domain protein increased drought tolerance in transgenic Arabidopsis (Xia et al., 2014). In addition, over-expression of Arabidopsis DnaJ (Hsp40) induced NaCl-stress tolerance (Zhao et al., 2010). Looking at the mapping of the 39 common genes with the same trend of expression (up in both, down in both), it is possible to see that some crops showed an inhomogeneous distribution of these genes among the different chromosomes of the analyzed crop species. I found that chromosomes contained a different density of abiotic stress-related genes in peach, while, in grape and olive, their distribution seemed to be similar. In peach, 13 abiotic stress-related genes were mapped to chromosome 1. These findings highlight the need to focus on these chromosomes to develop molecular markers associated with drought and salinity resistance in these crops. Indeed, the meta-analysis showed that the mapping of the identified genes will help in understanding which genomic regions are linked to abiotic stress resistance, helping the development of sustainable breeding strategies based on next-generation molecular markers.

- A Hypothetical Transductional Signal in Response to Osmotic Stresses

The discovery of common features between the two types of osmotic stress in the transductional signal at the transcript level will allow the identification of reliable target genes that play a key role in drought/salinity tolerance/resistance. The role of 39 common genes in gene regulatory networks in response to general osmotic stress is shown in Figure 4.5. Five genes, involved in hormone signaling, were up-regulated: ATB11 and ATP-dependent permease (PDR12) (ABA), germination insensitive to ABA mutant 2 (GIM2) (gibberellins), acetyl serotonin O-methyltransferase (ASMT) (salicylic acid), and SPCL51 (brassinosteroids). There is previous evidence that these genes are involved in drought or salinity tolerance/resistance (Khan et al., 2019). PDR12 is a PDR-type ABC transporter that mediates cellular uptake of abscisic acid, and mutant experiments demonstrated that this gene facilitates stomata closure and enhances drought tolerance (Kang et al., 2010). Plants over-expressing a member of the same ABC transporter family showed increased resistance to drought and salt stress (Kim et al., 2004). GIM2 enhanced GA biosynthesis while inhibiting ABA biosynthesis. GIM2 mutant seeds showed an ABA-insensitive phenotype during the germination and post-germination stage (Xiong et al., 2018). A serine carboxypeptidase was shown to regulate BRI1 involved in brassinosteroid signaling (Li et al., 2001), while another member of the same family is involved in brassinosteroid-mediated responses to both biotic and abiotic stresses (Sakamoto et al., 2008). Related to signal transduction of drought/salinity stress, the following kinases were commonly modulated among drought- and salinity-related studies: S-locus lectin protein kinase, serine, LRR receptor-like protein kinase (AT5G25930), and protein kinase (AT5G14720). Among transcription factors, it is worth mentioning ankyrin repeat family protein (upregulated), WRKY2 (downregulated), and C2HC4 RING finger (AT5G14720) (downregulated). Ankyrin repeat family members were also shown to be modulated by drought, and a RING zinc finger ankyrin protein was isolated and characterized from drought-tolerant *Artemisia desertorum* (Yang et al., 2008). Interestingly, I found that WRKY2 was repressed by drought and salinity, and this was unexpected since these genes were shown to be induced by NaCl and mannitol stress. This gene is a nuclear-localized transcription factor, and its role in osmotic stress needs to be clarified (Jiang et al., 2009). Nevertheless, studies showed that the expression of WRKY2 gene in *Poncirus trifoliata* was suppressed by 27−50% upon exposure to prolonged drought stress (Banerjee et

al., 2015). Moreover, the expression of this gene increased initially when both cold-tolerant Poncirus and cold-sensitive *Citrus maxima* (pummelo) were exposed to cold stress. However, the gene expression subsided in both cold-tolerant Poncirus and cold-sensitive pummelo after exposure to 1 h and one day of cold stress, respectively (Şahin-Çevik et al., 2012). The reason behind the repression of WRKY2 in the analysis could be the duration of the stress (14–90 DAS) selected for the study. Among the defense response genes, I identified WD40D, which, in wheat, functions as a positive regulator of salt stress and osmotic stress responses. This evidence was demonstrated by the downregulation of TaWD40D through virus-induced gene silencing, which provoked a decrease in relative water content and reduced growth compared to non-silenced lines. Indeed, it was already hypothesized that this gene might be used for the genetic improvement of stress tolerance in crop plants (Kong et al., 2014). In addition, a fructosyl transferase (FUT) was linked with an increased tolerance to osmotic stress in *Pyropia tenera* (Wi et al., 2017), and the data confirmed this evidence. The upregulation of protein detoxification 16 may be explained by the well-known fact that the upregulation of detoxification processes generally drives enhanced resistance to abiotic stresses, linked to increased radical ions and highly reactive oxygen species.

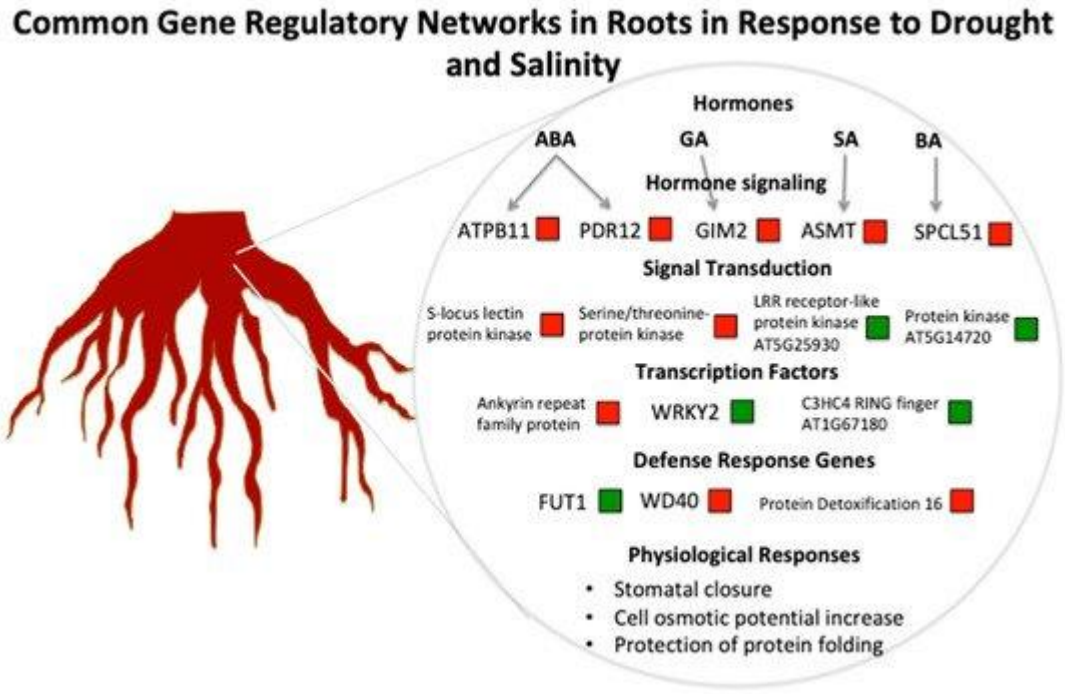


Figure 4.5. Main gene regulatory networks in common between responses to drought and salinity. Key genes involved in hormonal signaling, transduction signal, transcription regulation, and defense responses identified by the meta-analysis are indicated together with physiological effects. Upregulated genes are shown in red, while downregulated genes are shown in green.

4.6. Conclusion

In conclusion, I believed that the information provided by this work may be useful in developing molecular markers linked to these 39 genes or at least a subset of them (Figure 4.5); moreover, this study can facilitate targeting them with innovative biotechnological tools (transgenesis, genome editing) to create genotypes with enhanced resistance to drought/salinity stress resistance in crops. Studies confirmed that the abiotic stress-related genes identified in this study can be selected as molecular markers usable for the improvement of these complex quantitative traits (Cimò et al., 2017). This meta-analysis identified genes serving as potential targets for molecular breeding activities to develop cultivars with enhanced drought and salinity resistance and tolerance across different crops in a biotechnologically sustainable way.

5. CHAPTER 5

Experiment 4: Transcriptome analysis of *Pistacia vera* inflorescence buds in bearing and non-bearing shoots reveals the molecular mechanism causing premature flower bud abscission

DOI: https://doi.org/10.3390/genes11080851

5.1. Introduction

Pistachio (*P. vera* L.) originates in the arid areas of central Asia, in the areas of the Caspian Sea (Iran) and the territories between Afghanistan and Kyrgyzstan (Caruso et al., 1995). The pistachio is a wind-pollinated deciduous, dioecious tree which presents cyclic variation of fruiting, usually of two years, in which heavy production occurs during the "ON" year and less/no production in the following "OFF" year. The mechanism regulating the alternate bearing phenomenon in pistachio is unique (Khezri et al., 2020). In a mature pistachio tree, all the main vegetative and reproductive phases are carried out by the plant in a short period, between the mid of March and the end of May. The growth pattern of the current season's shoot is exclusively dominant, and it extends from the vegetative terminal bud of the previous season's shoot. Under each of the compound leaf on the current season's growth, there is a single axillary bud. Most of these axillary buds differentiate into inflorescence primordia; therefore, flowering and fruit production occurs on 1-year-old wood (Crane et al., 1987; Ferguson et al., 2016). Thus, unlike other alternate bearing crop species, pistachio produces floral buds on current-year shoot but, in the "ON" year, inflorescence buds start to detach starting from the basal end of the current-year shoot and then towards the apical end (Khezri et al., 2020). Meanwhile, on the contrary, there is bud retention in low crop load ("OFF") years. Bud abscission is considered the visible mechanism underlining the alternate bearing (Khezri et al., 2020). In order to simplify this phenomenon by outlining a time-line, lower buds start to abscise or drop at the end of June and continues in July and August, determining the heavy reduction in production in the next year, thus resulting in an "OFF" year. The figure below shows the growth pattern of the shoot during the "ON" and "OFF" season (Figure 5.1).

Figure 5.1. (A): Fruit clusters on one-year-old wood and lateral inflorescence buds on current year's bearing shoot of *P. vera* L. indicated by yellow circles (June "ON"); (B): one-year-old wood and lateral inflorescence buds on current year's not-bearing shoot (July "OFF"); (C): Red circles show the sites of inflorescence bud abscission in current year's bearing shoot (July "ON").

The physiological mechanism, which triggers the inflorescence buds drop linked to the alternate bearing behavior in pistachio, is not completely clear and two hypotheses are

considered, one of which involves nutritional factors and the other involves hormonal factors. On the basis of the nutritional hypothesis, the competition of the growing embryos with the new inflorescence buds for the use of metabolites, carbohydrates and nitrogen can be the main cause of inflorescence bud dropping (Crane et al., 1972; Sparks et al., 1974). The hormonal hypothesis suggests that some growth regulators are directly involved in bud abscission. However, subsequent studies conducted on the levels of abscisic acid (ABA) in fruits and inflorescence buds did not show any relationship between the levels of this hormone and the bud drop (Takeda et al., 1980).

The nutritional theory suggests that the inflorescence bud drop occurs in coincidence with the period of embryo growth and is more intense when the crop on one-year-old shoot is heavy, since the embryo represents the strongest "sink" (Spann et al., 2008). This temporal coincidence of bud drops, and nut development suggests a competition between the developing embryo and inflorescence buds for the available resources. The lack of competitive ability of inflorescence buds compared to fruit in attracting the photosynthates produced by the leaves was demonstrated by tracking the translocation of the radioactive carbon isotope, C14 (Takeda et al., 1980). It also confirmed that in branches that are subjected to annular decortications, also known as shoot girdling (removal of a bark ring from the base of the current year's shoot to separate it from the fruitescences), it is possible to reduce the inflorescence bud drop by 70% (Vemmos et al., 2012). Similar results emerged from a study on the accumulation of nitrogen, phosphorus and potassium on the various organs of the branch, showing that the inflorescence buds of non-bearing branches accumulated significantly greater quantities of macro elements, compared to the inflorescence buds of the bearing branches (Baninasab et al., 2007).

Various studies have also highlighted the direct correlation between the fruit load and the intensity of the drop of the inflorescence buds (Nzima et al., 1997). Studies proved that the presence of the infructescence decrease the growth of the leaves and of the shoot axis (Stevenson et al., 2000), and that plants deprived of the fruits for the next years accumulate more carbohydrates and thus they express a greater potential for growth compared to those left in the normal year production cycle (Marra et al., 1998). Many studies showed significant changes in starch content and the difference in translocation of starch in tissues of "ON" and "OFF" trees (Marra et al., 2018).

Moreover, in pistachio, within the canopies of "OFF" trees, it is possible to find some "ON" shoots and within canopies of "ON" trees, there always some "OFF" shoots; therefore, the theory of shoot or branch autonomy should be considered (Takeda et al., 1980). Shoot autonomy in fruit trees depends on resource (carbon, water, nutrients and hormone distribution) availability. These results underline the importance of reserve substances which, although stored in the permanent organs of the plant during the "OFF" year, are not enough for the full expression of the vegetative growth potential and the fructification. Interesting results have emerged from the study of the influence of polyamines (putrescine, spermine and spermidine) on the inflorescence bud drops (Gündeşli et al., 2019). In general, the level of polyamines is negatively correlated with inflorescence bud drops.

Genetic mechanisms involved in alternate bearing have been recently studied by transcriptomic analyses in some fruit crops, such as apple (Guitton et al., 2016), citrus (Shalom et al., 2012) and olive (Yanik et al., 2013), where the alternate bearing is explained

as the lack of flower bud initiation and their morphological differentiation, unlike in pistachio. In citrus, the fruit load critically affects bud fate before that flower induction occurs and an alternate bearing signal may be generated in the fruit or in another organ that perceives the flowering initiation and the change of key metabolic pathways (Shalom et al., 2012). It has been demonstrated in many fruit crops that "ON" and "OFF" crop status is associated with changes in the expression of flowering control genes (Guitton et al., 2016; Yanik et al., 2013). Genes regulating trehalose and flavonoid metabolism and genes homologous to Squamosa promoter binding-like (SPL) were found induced in "OFF" buds of citrus (Shalom et al., 2012).

In apple, microarray analysis showed that flower induction genes were differentially regulated between "ON" and "OFF" inflorescence buds and critical changes occur in expression of genes involved in oxidative stress, cell wall biogenesis, carbohydrate biosynthesis and lipid metabolism (Guitton et al., 2016). In olive, a cDNA library experiment performed on different developmental stages of leaves and fruits in "ON" and "OFF" trees showed that P450 monooxygenase and two dehydrins were more expressed in leaves of "ON" trees than in leaves of "OFF" trees (Guitton et al., 2016). Furthermore, in "ON" olive trees, a UDP-glucose epimerase, an acyl-CoA binding protein, a triose phosphate isomerase and a putative nuclear core anchor protein were more expressed in fruits. In "ON" and "OFF" olive trees, differences in miRNA-targeted genes were also found involved in main hormone signal-transduction pathways and carbohydrate metabolism which can be potentially associated in alternate bearing processes (Yanik et al., 2018). Preliminary transcriptional analysis in pistachio showed that in inflorescence buds of "ON" bearing shoots, photosynthesis related genes were down-regulated and some terpenoids related genes were up-regulated (Martinelli et al., 2018).

5.2. Aim of the Research

The aim of this analysis is to provide insights into the transcript changes between inflorescence buds in bearing and non-bearing shoots in order to identify the molecular mechanism causing premature inflorescence bud abscission, which is linked to alternate bearing in the Italian pistachio cultivar Bianca.

5.3. Materials and Methods

- Plant Material, RNA Extraction, Processing and Sequencing

The transcriptomic analysis was conducted taking the tissue samples from one mature P. vera (L.) tree of the cultivar "Bianca", grown inland of Sicily (37°30′ Lat. N), in 27th of June 2018 and in 22nd of July 2019. The inflorescence buds from bearing ("ON") and non-bearing ("OFF") branches were analyzed. Bearing branches showed from 40 to 50 fruits; non-bearing had no fruits. I collected 4–6 inflorescence buds each from three branches (considered as three biological replicates) of the same tree during the "ON" and "OFF" status of June and July which constitute a total of 12 samples. All bud samples were immediately frozen in liquid nitrogen after collection and stored at −80 °C. The samples were grounded in liquid nitrogen and total RNA extraction was performed with the SpectrumTM Plant Total RNA Kit (Sigma-Aldrich, Milan, Italy) employing 100 mg of frozen tissue. RNA quality and RNA

Integrity Number (RIN) were checked by using the Bioanalyzer. Libraries were obtained using the TruSeq RNA-Seq sample prep kit from Illumina (Illumina, Inc., San Diego, CA, USA). The 12 samples were loaded into one lane of an Illumina flow cell, and clusters were created by Illumina Bot. The clusters were sequenced using the service provided by BMR Genomics (Padua, Italy) at ultra-high throughput on the Illumina HiSeq 2000 (Illumina Inc.) to obtain single reads per sample, each 75 bp long.

- De Novo Assembly, Evaluation and Annotation

The quality of the raw sequences generated from transcriptome sequencing was assessed with FastQC (version 1.16) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). With respect to the FastQC report, the low-quality bases (Q-score < 30) were removed using custom made Perl script and the adaptor sequences were removed using cutadapt (version 2.0). The filtered reads were then aligned against Silva database (https://www.arb-silva.de/) using bowtie (Langmead et al., 2009) (version 2.3.4.1) in order to remove rRNA reads and to obtain clean reads. The total pre-processed reads from all 12 samples were then de novo assembled using Trinity (Grabherr et al., 2011) (version 2.8.4) using default parameters. The transcripts from Trinity assembly were further clustered using CD-Hit-EST (Li et al., 2006) (version 4.6.8), using a clustering threshold of 98% identity to reduce redundancy. The assembly statistics were obtained using the transrate (Smith-Unna et al., 2016) (http://hibberdlab.com/transrate/) program. The assembly was evaluated with BUSCO (Simão et al., 2015)(version 3.0.2), a tool that assesses genome completeness based on the presence of single-copy orthologs, using the green plant dataset (viridiplantae_odb10). The complete workflow of the Pistachio de novo transcriptome assembly and annotation were summarized in Figure 5.2.

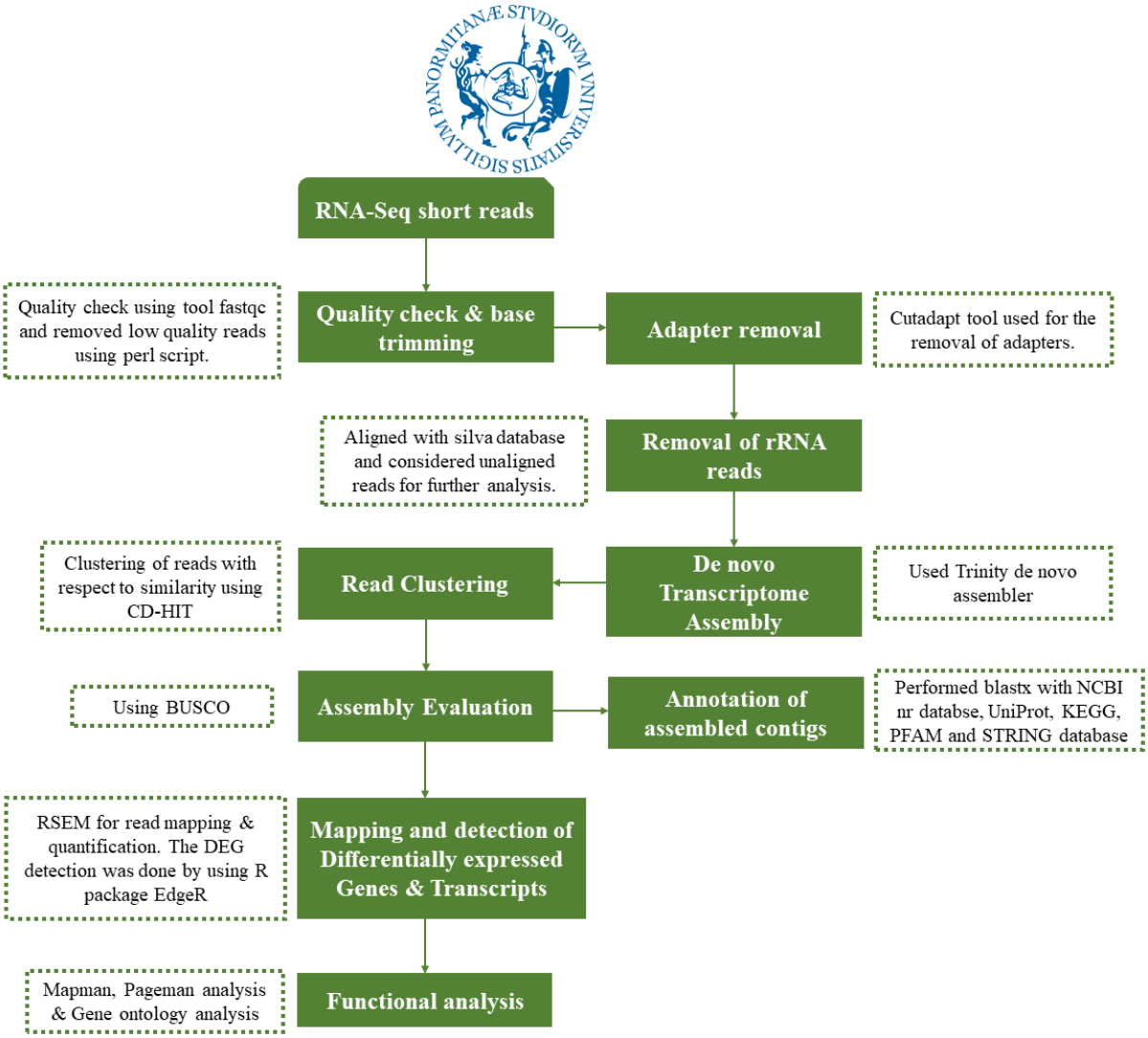


Figure 5.2: Workflow of the de-novo analysis of the transcriptomic studies related with inflorescence bud abscission in bud tissue.

The obtained contigs were annotated using BLASTx program (http://www.ncbi.nlm.nih.gov/BLAST/) with an E-value threshold of 1 × 10−5 to NCBI nr database (https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins), UniProt protein database (https://www.uniprot.org), InterPro database (https://www.ebi.ac.uk/interpro/), KEGG database (http://www.genome.jp/kegg), PFAM database (https://pfam.xfam.org) and STRING database (https://string-db.org). I considered only the contigs corresponds to 'Viridiplantae' and the unannotated contigs for the final transcriptome assembly. RNA-Seq data were deposited in NCBI's sequence read archive (SRA) under accession number PRJNA623387.

- Differentially Expressed Genes (DEG) between Stages

To estimate the expression levels of the Trinity reconstructed transcripts, I used RSEM (Li et al., 2011). RSEM is a package used to estimate the gene and isoform expression levels from RNA sequence data. The expected count matrix derived from RSEM is given as the input for edgeR (Robinson et al., 2009). The comparison selected for the study is given in Table 5.1.

Table 5.1. The number of total genes, up-regulated and down-regulated genes in inflorescence buds in current year non-fruiting shoot "OFF" and in inflorescence buds in fruiting shoots "ON".

Comparison (inflorescence bud)	Differentially expressed genes	Up-regulated	Down-regulated
July “OFF” vs. July “ON”	1,087	247	840
June “OFF” vs. July “OFF”	2,299	976	1,323
June “ON” vs. July “OFF”	2,450	591	1,859
June “OFF” vs. July “ON”	2,768	820	1,948
June “ON” vs. July “ON”	3,882	712	3,170
June “OFF” vs. June “ON”	1,844	1,409	435

Genes represented with an adjusted p-value (FDR) lower than 0.01 and at least a two-fold change were only considered as significantly differentially expressed in the pairwise comparison of the samples. In addition, the functional-enrichment analysis was performed to identify which gene ontology (GO) terms and metabolic pathways that were significantly enriched in differentially expressed genes (DEGs).

- Gene Enrichment and Functional Analysis

The final contigs were aligned against TAIR10 (https://www.arabidopsis.org) protein sequence using blastx program, in order to get the corresponding TAIR Id. The blastx result files were parsed and generated a Pistachio mapping file for Mapman containing the five categories (a) Nearly identical: Score ≥ 1000 and e-value = 0 (b) Highly similar: Score ≥ 1000 and e-value ≠ 0 OR (Score ≥ 500 & Score < 1000) and e-value = 0 (c) Moderately similar: (Score ≥ 200 & Score < 1000) and e-value ! = 0 (d) Weakly similar: (Score ≥ 100 & score < 200) (e) Very weakly similar: (Score < 100) based on the blastx score and e-value. I used MapMan (http://mapman.gabipd.org/) (Thimm et al., 2004) with the Pistachio mapping file to map the gene IDs and visualize the metabolic overview, hormone regulation, CHO metabolism, secondary metabolism and transcription factors using two generated files: (1) related to “ON” and “OFF” stages of bud, (2) related to two time-point (June and July).

The PageMan analysis plugin of MapMan was used to visualize differences among metabolic pathways using Wilcoxon tests, no correction, and an over-representation analysis (ORA) cutoff value of 3. I considered all the differentially expressed genes present that are related to the comparison of “ON” and “OFF”, June and July for the PageMan analysis. The TAIR IDs were searched against the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (Huang et al., 2007) Web server (https://david.ncifcrf.gov/). The gene ontology information related to the biological process was extracted from the DAVID result.

5.4. Results

- De Novo Transcriptome Assembly and Annotation

To examine the inflorescence bud abscission phenomenon of *P. vera* relate to alternate bearing, inflorescence buds from three separate shoots of the same tree were collected and sequenced from bearing and non-bearing shoots. The picking dates of the material coincide with a period of the initial competition between fruit and inflorescence buds, not causing inflorescence bud drop, and a period of strong completion, causing the drop of inflorescence buds.

The sequencing of the data of June produced 199 million raw reads (60 Gb of data), whereas July produced 196 million reads (59 Gb of data) as a single-end. The high-quality single-end reads with an average quality score of 38 were selected for the transcriptome assembly after trimming off the low-quality bases and adapters from the June and July data sets. The total pre-processed reads were then de novo assembled using Trinity and transcripts from Trinity assembly were further clustered using CD-Hit-EST.

I used RSEM for the quantification of the genes. The count matrix generated I then taken as the input by edgeR. The downstream analysis resulted in the identification of a total of 14,330 genes in which 4755 were up-regulated and 9575 were down-regulated. For each of the analysis, the total number of genes ranged from 1087 to 3882. The number of genes up-regulated was in a range of 247 to 1409 and down-regulated genes were spans from 435 to 3170. Subsequently, the assembled transcripts were annotated by BLASTX against a non-redundant (NR) protein database, PFAM, KEGG, Uniprot/Swissprot, InterPro and STRING databases. It is likely that the cv. Bianca faces a limitation of resources around the third week of June, when the first sampling of the plant material was made and, that in a month, it reaches its maximum peak, corresponding to the second sampling period when the drop of inflorescence buds started. June “OFF” vs. July “ON” corresponds to the most divergent scenarios. The Venn diagram shows the overlap of the genes for “ON” and “OFF” seasons of June and July (Figure 5.3).

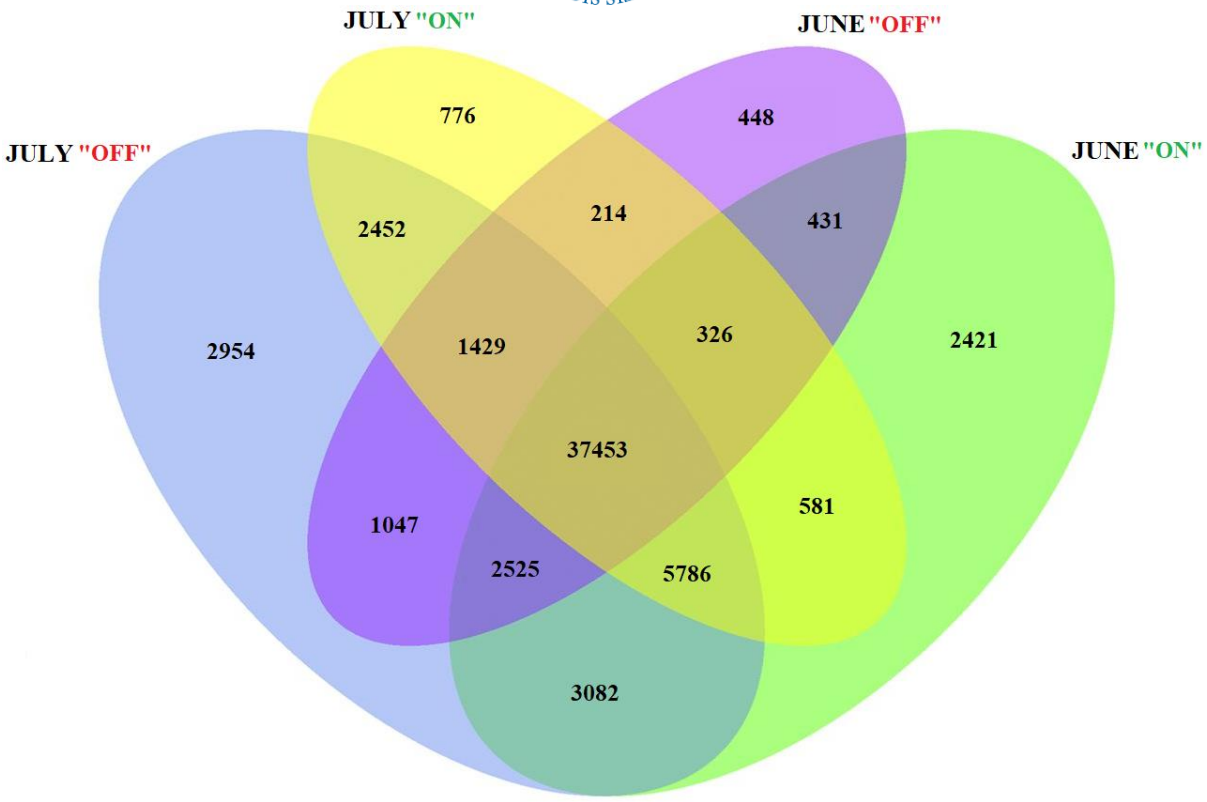


Figure 5.3: The Venn-diagram shows the overlap of the genes for “ON” and “OFF” inflorescence buds of June and July.

The figure shows 37,453 genes were common among all the seasons which might have a role in the developmental process rather than the alternate bearing. The main comparison is focused on “ON” and “OFF” period of June to investigate thoroughly different pathways and processes of the bud abscission. This comparison can also avoid factors like physiological and developmental changes that might occur in the bud during the two different time points (June and July). To add strength to the conclusion, a comparative study on the effect of crop load during the “OFF” and “ON” period of July is also investigated.

- Effect of Crop Load on Photosynthesis During in June “OFF” vs. June “ON” Inflorescence Buds

The changes in gene expression in “ON” inflorescence buds do not reflect an enhancement of photosynthetic activity when compared to “OFF” year inflorescence bud from non-fruiting shoots). Most of the genes involved in photosynthesis were up-regulated during the “OFF” year. Both photosystem II PSII polypeptide subunits, MAF1, a global repressor of RNA polymerase III (Pol III) and PDE335 (Pigment defective 335) showed an up-regulation during June “OFF”. In contrast, a gene calling for CRR3 (chloro-respiratory reduction 3) was repressed during the “OFF” period. The genes encoding for cytochrome (UGT76D1), ATP synthase (PDE332) and cyclic electron flow (PGR5-LIKE A) were enhanced during the June “OFF” period.

- Effect of Crop Load on Starch Metabolism in June "OFF" vs. June "ON" Inflorescence Buds

Crop load had much greater effects in reducing starch contents and limiting the starch accumulation. Therefore, the study on the relationship between crop load and starch metabolism helps in assessing the functional distribution of starch in "ON" and "OFF" flower buds. The genes encoded for sucrose phosphate synthase (SPS1F), less adhesive pollen 5 and starch synthase 4 were enhanced in the tissue of inflorescence buds of the "OFF" current year shoots (Figure 5.4).

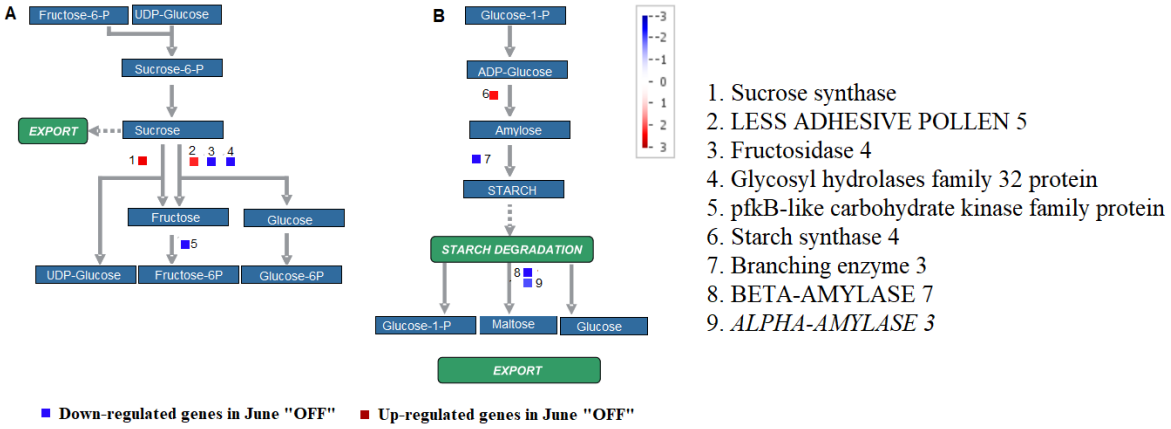


Figure 5.4. Figure shows the Mapman pathways in sucrose-starch metabolism. Figure highlights differentially expressed genes between inflorescence buds in the non-bearing shoot (June "OFF") and inflorescence buds in bearing shoot (June "ON") in sucrose degradation (A) and starch synthesis (B) pathways. Individual genes were represented by small squares. The color scale indicates the log2 FC value. Red represents up-regulation and blue represents down-regulation in June "OFF" relative to June "ON".

These enzymes are involved in the formation of carbohydrate reserves (Kozlowski et al., 1997). The genes encoded ALPHA-AMYLASE 3, BETA-AMYLASE 7, fructosidase 4, glycosyl hydrolases family 32 and pfkB like carbohydrate kinase, which was involved in carbohydrate degradation was repressed.

- Effect of Crop Load Status on Polyamine and Transcription Factors in Inflorescence Buds June "OFF" vs. June "ON" Shoots

This section of study was conducted to examine the role of free polyamines in the inflorescence bud abscission. The "OFF" inflorescence buds exhibited significantly higher polyamine (PA) and spermidine (Spd) enhancement than the "ON" ones, during most of the period. In "OFF" inflorescence buds, the genes encoding for thermospermine synthases (ACL5), probable polyamine transporter, Polyamine oxidase 1 isoform 1 and spermidine synthase (speE) were enhanced. On the contrary, the expression of S-adenosyl-L-methionine-dependent methyltransferases was repressed.

In June "OFF" inflorescence buds, three bZIP (bZIP61, leucine zipper transcription factor 16 and G-box binding factor 3), ARF7 (Auxin response factor 7), WRKY19, zinc ion binding and four homeobox genes (Enhanced drought tolerance 1, BEL1-like homeodomain 3, IFL1

and HB-8) were down-regulated, while one histone (ULI3) gene, two alfin-like members, two MYB factor (MYB103 and MYB14), three WRKY (WRKY31, WRKY72, and WRKY53), all the histone related factors and AS2 were up-regulated. In June "ON" buds, the study reported the enhancement of MYB factors (MYB60, MYB3 and MYB106), WRKY factors (WRKY19 and WRKY49) and Histone acetyltransferases.

- Gene Set and Pathway Enrichment Analysis During in June "OFF" vs. June "ON" Inflorescence Buds

DAVID software was used to identify the biological processes, cellular components and molecular functions affected by crop load at transcriptomic level considering the differentially expressed genes in the June "OFF" and June "ON". While comparing, June "OFF" buds and June "ON" buds, 53 GO terms were down-regulated, whereas 31 were up-regulated. The biological pathways that are known to be repressed during "OFF" response to salicylic acid, chloroplast envelope, circadian rhythm, response to auxin, ion transmembrane transport, apoplast and proteolysis were found in my analysis. In contrast, I identified some GO-terms that were up-regulated in response to alternate bearing, such as nutrients ion transport, ABA catabolic process, gibberellin catabolic process, amino acid transmembrane transport and carbohydrate metabolic process.

- Effect of Crop Load on Hormone Metabolism in June "OFF" vs. June "ON" Inflorescence Buds

The objective of the current section was to study the role of hormone in inflorescence bud abscission. The genes involved in hormone-related categories are summarized in Figure 5.5.

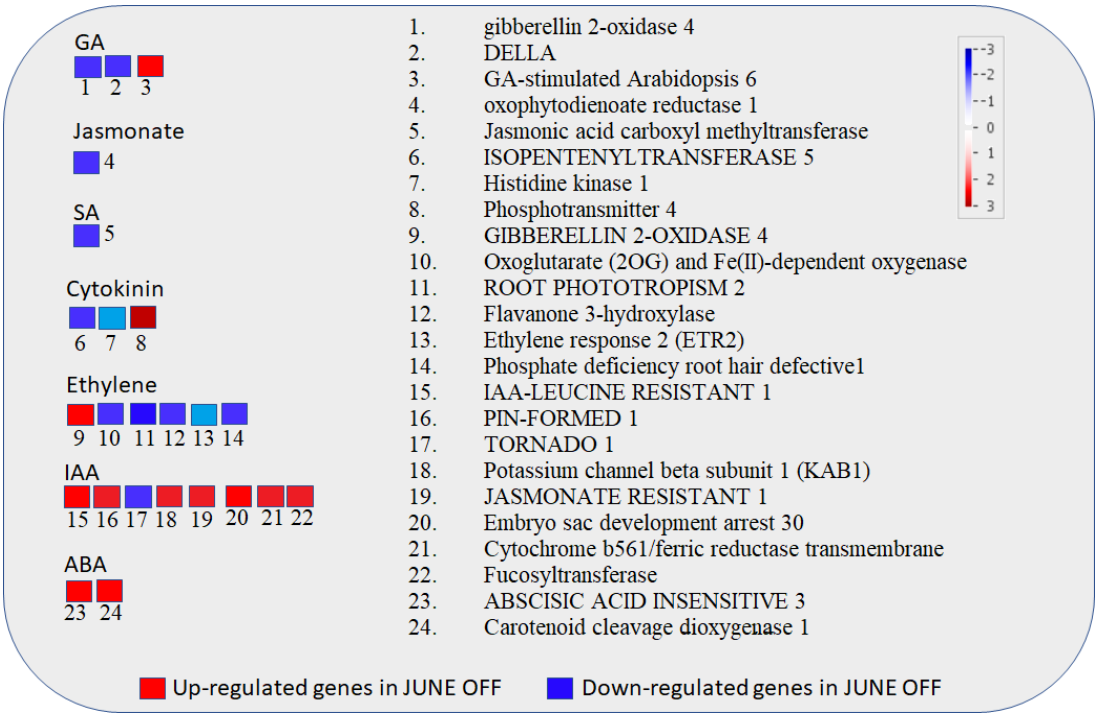


Figure 5.5: Figure shows hormone metabolism in Pistachio among the June "OFF" vs. June "ON" inflorescence bud comparison. The color scale indicates the log2 FC value. Red represents up-regulation and blue represents down-regulation in June "OFF" buds relative to June "ON" buds.

Repression of ethylene and gibberellin pathways were identified in inflorescence buds of "OFF", whereas ABA and IAA pathways were mostly up-regulated. In June "OFF" inflorescence buds, five genes responsive to ethylene, two genes responsive to gibberellin and two genes responsive to cytokinin were down-regulated (Figure 5.5). Relating to auxin-responsive genes, down-regulation of PIN formed 1 and up-regulation of TOR, Potassium channel beta subunit 1 (KAB1) and Jasmonate resistant 1 were observed. Relating to ABA there was an up-regulation in abscisic acid insensitive 3, lipid transfer protein 3, shaker potassium ion channel, SNF1, potassium transport 3, phosphotransmitter 4 and Carotenoid cleavage dioxygenase 1. Several genes involved in ethylene biosynthesis and signaling such as Oxoglutarate (2OG) and Fe (II)-dependent oxygenase, Root phototropism 2, Flavanone 3-hydroxylase, Ethylene response 2 and phosphate deficiency root hair defective 1 were repressed during in "OFF" buds.

- Effect of Crop Load on Ubiquitin and Autophagy Dependent Degradation in June "OFF" vs. June "ON" Inflorescence Buds

The results on the effect of crop load on ubiquitin and autophagy-dependent degradation could be a tool for understanding the premature inflorescence bud abscission presumably associated to the alternate bearing mechanism of P. vera. The genes in inflorescence buds from "ON" and "OFF" shoots, that were involved in ubiquitin and autophagy-dependent categories were summarized in Figure 5.6.

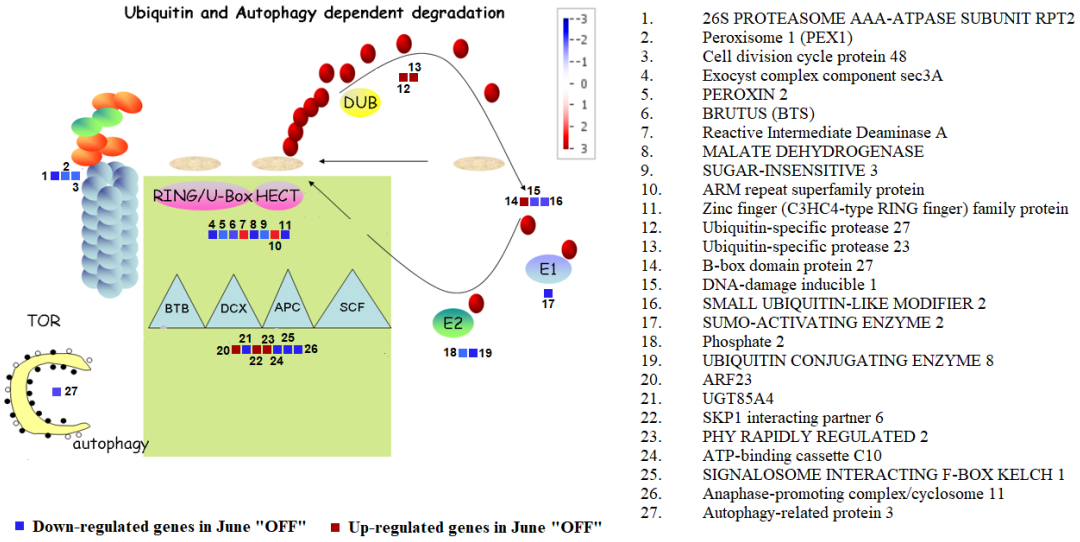


Figure 5.6. Figure shows Ubiquitin and Autophagy dependent degradation in Pistachio among the June "OFF" vs. June "ON" inflorescence bud comparison. The color scale indicates the log2 FC value. Red squares represent up-regulation and blue squares represent down-regulation in June "OFF" relative to June "ON".

It is worthy to mention that most of the genes were repressed in "OFF" inflorescence buds. During this season, genes responsive to autophagy (ATG8C: Autophagy related protein 3) and genes responsive to ubiquitin proteasome (PAG1: Proteasome Alpha Subunit G1 and Cytochrome P450) were down-regulated. While discussing the E3 RING/U-BOX genes, it is worthy to mention the down-regulationof PEROXIN 2, BRUTUS (BTS), Malate dehydrogenase, Sugar-insensitive 3 and MAPK in OFF buds. Relating to the up-regulated

genes, I noticed genes such as reactive intermediate deaminase A, ARM repeat superfamily protein, B-box domain protein 27, ARF23, SKP1 interacting partner 6 and PHY rapidly regulated 2 (Figure 5.6).

- Effect of Crop Load on Carbohydrate Metabolism and Mobilization in June "OFF" vs. June "ON" Inflorescence Buds

The objective of the current section was to verify the role of CHO reserves and mobilization as a cause or effect of the drop of inflorescence buds in P. vera. The relationship among the carbohydrate metabolism and mobilization pathway in Pistachio and the inflorescence buds from non-bearing shoots (June "OFF") and bearing shoots, June "ON" is indicated in Figure 5.7.

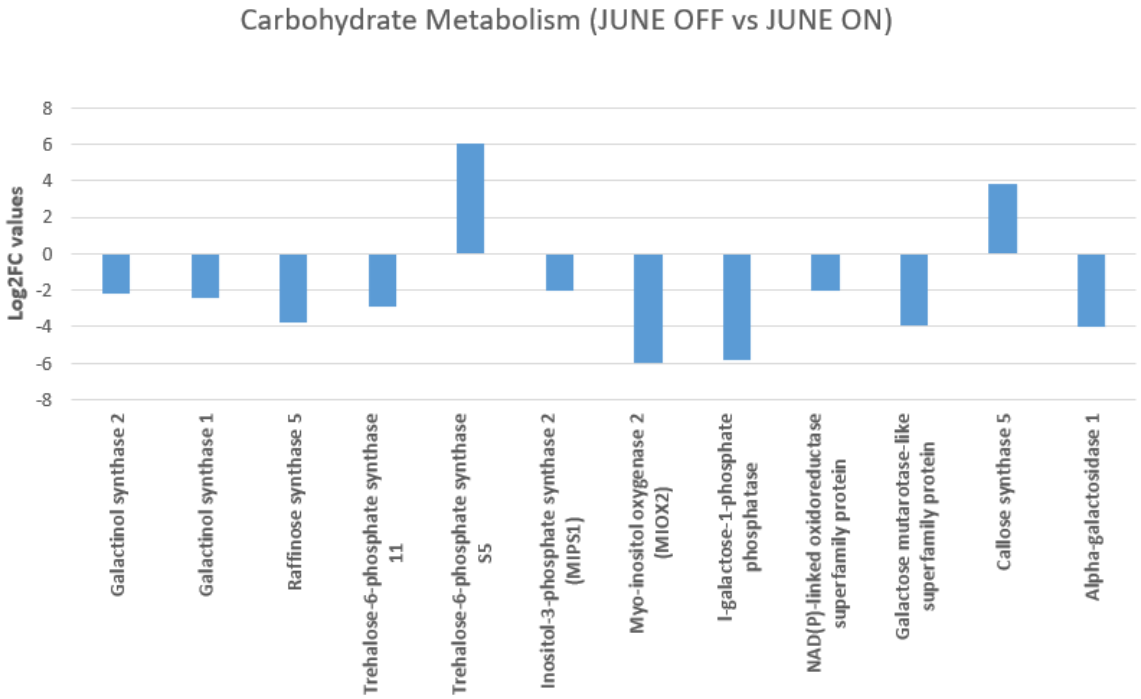


Figure 5.7. Figure shows carbohydrate metabolism and mobilization pathway in Pistachio among the June "OFF" vs June "ON" inflorescence bud comparison. The red circle represents the value of log2 fold change. The line indicates the effects on carbohydrate levels driven by differential expression of different CHO metabolism genes.

The study showed that the pistachio inflorescence bud of non-fruiting shoots "OFF" required low amounts of carbohydrates due to the lack of fruits at the time and thus accumulated some starch. Similarly, I also found that the inflorescence buds of bearing and non-bearing pistachio shoots differed in their carbohydrate storage and mobilization patterns, suggesting that the in-season carbon mobilization might influence the flower bud abscission directly or indirectly linked to the alternate bearing. Raffinose synthase gene (Raffinose synthase 5 (RS5), two galactinol synthase genes (Galactinol synthase 1 and Galactinol synthase 2) and MIOX2 showed repression in the "OFF" buds, whereas sugar alcohols, such as callose synthase and trehalose-6-phosphate synthase 11, showed an up-regulation (Figure 5.7).

- Comparison between “ON” and “OFF” Inflorescence Buds of “JUNE” and “JULY”

A comparative study of differently regulated genes among the “ON” and “OFF” inflorescence buds collected in “June” and “July” summarizes that, during the “OFF” season of July, there is a gradual reduction of raffinose synthase 1 and MIOX2 as I identified in June “OFF”. The enhancement of hormones like ABA and, at the same time, reduction of gibberellin and ethylene indicates that July “OFF” is gradually showing the same pattern as that of June “OFF”. SnRK1 and TOR down-regulated both the cases; therefore, no programmed cell death (PCD) and autophagy occurs during July “OFF” and makes plant stable for the next upcoming “ON” season. During the comparison of the inflorescence buds in fruiting shoots “ON” of June and July, I could find that almost all the genes during July “ON” participate in a similar way as that of “ON” June. This comparison proves that gene expression profiling associated with “ON” season of June and July and “OFF” season of June and July are similar proving the importance of these genes in the flower bud abscission and alternate bearing.

- Effects of Crop Load in July “OFF” vs. July “ON” Inflorescence Buds

An enhancement ABA was identified in inflorescence buds of July “OFF”, whereas ethylene and gibberellin pathways were mostly down-regulated. In July “OFF” inflorescence buds, three genes responsive to ethylene (ETR2, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, Flavanone 3-hydroxylase) one gene responsive to gibberellin (gibberellin 2-oxidase 4) and one gene responsive to cytokinin (Isopentenyl transferase 5) were down-regulated. Relating to auxin-responsive genes, down-regulation of PIN formed 1 and auxin F-box protein 5 and the up-regulation of TOR, Potassium channel beta subunit 1 (KAB1) and Glycoside Hydrolase Family 3 were observed. Relating to ABA there was an up-regulation in SNF1, Abscisic acid insensitive 3 and Carotenoid cleavage dioxygenase 1.

The comparative study of the July “OFF” vs July “ON” produced similar results to the results of June “OFF” vs June “ON”. The genes encoded for sucrose phosphate synthase (SPS1F) and starch synthase 4 were enhanced in the inflorescence buds of the July “OFF”. The genes encoded ALPHA-AMYLASE 3, BETA-AMYLASE 7 and fructosidase 4 were repressed. Most of the genes involved in photosynthesis were up-regulated during the July “OFF” year, similar to the results of June “OFF”. The photosystem II PSII polypeptide subunits and PDE335 (Pigment defective 335) showed an up-regulation, whereas gene calling for CRR3 (chloro-respiratory reduction 3) was repressed during July “OFF”. The genes encoding for cytochrome (UGT76D1), ATP synthase (PDE332) and cyclic electron flow (PGR5-LIKE A) were enhanced during the “OFF” period.

5.5. Discussion

The growth of the endocarp of the cultivar Bianca is from the first week of May to the end of June, while the growth of the embryo is from the first week of July to the end of August. In “ON” trees, most of the inflorescence bud’s abscission starts at the end of June and continues in July and August. None of the works to date could confirm the involvement of flowering promoter and repressor genes in regulating inflorescence bud’s abscission in pistachio. This study provides insights into the transcript changes between inflorescence buds in bearing and non-bearing shoots in order to identify the molecular mechanism causing premature

inflorescence bud abscission, which is linked to the alternate bearing in the Italian pistachio cultivar “Bianca”.

The relationships between the flower bud drops linked to alternate bearing and the carbohydrate storage have been mentioned in several studies (Marra et al., 2009). It generally seems evident that in pistachio trees, nutrients are stored during the “OFF” year and that they are used for reproductive growth in the following year (Weinbaum et al., 1994). There are significant changes in starch content and different translocation of starch in the tissues of “ON” and “OFF” trees (Marino et al., 2018) and it has been suggested that the mobilization of stored carbohydrates may cause inflorescence bud abscission in pistachio. The role of individual sugars in the process of inflorescence bud abscission has not yet been investigated.

In the study, genes encoding for sucrose phosphate synthase (SPS1F), degradation sucrose invertase (A/N-InvE), starch synthase 4, callose synthase and trehalose-6-phosphate synthase 11 were enhanced in the June “OFF” inflorescence buds, whereas BETA-AMYLASE 7, ALPHA-AMYLASE 3, branching enzyme 3, fructosidase 4, glycosyl hydrolases family 32, and pfkB like carbohydrate kinase were repressed. This supports the nutritional theory demonstrating that nutrients are stored during the “OFF” year to be used for reproductive growth the following year in pistachio trees. Interestingly, in rice and sugar, hormone signals regulated ALPHA-AMYLASE 3 enzyme expression, which catalyzed starch degradation (Lu et al., 1998). In particular, sugar starvation promoted the expression of ALPHA-AMYLASE 3 that resulted in the up-regulation in pistachio “ON” inflorescence buds.

Raffinose synthase gene (Raffinose synthase 5 (RS5), two galactinol synthase genes (Galactinol synthase 1 and Galactinol synthase 2) and MIOX2 showed enhancement in the study in inflorescence buds of bearing shoots (“ON”). The raffinose family of oligosaccharides has a wide range of predicted functions and are currently emerging as crucial molecules during stress response in plants (Zuther et al., 2012), because of their membrane-stabilizing, antioxidant and, perhaps, predictable signaling functions (Valluru et al., 2011). They participate in several cellular functions, such as transport and storage of sugars (Sengupta et al., 2015), signaling molecule following pathogen attack and wounding (Couée et al., 2006), signal transduction (Xue et al., 2007), membrane trafficking (Thole et al., 2008) and mRNA export (Okada et al., 2009). Recent transcriptional profiling data in Arabidopsis thaliana showed up-regulation of the Myo-inositol oxygenase (MIOX) genes under limited energy or nutrient conditions shows consistent with my results indicating an up-regulation in “ON” buds. MIOX2 plays a prominent role in the oxidation of inositol for the needs of the plant in different tissues and it is involved in the biosynthesis of nucleotide sugar precursors for cell-wall matrix polysaccharides (Kanter et al., 2005).

Interestingly, trehalose-6-phosphate (T6P), that was up-regulated in “OFF” inflorescence buds, seem to play a central role in sugar metabolism regulation in plants (Ponnu et al., 2011). It has been proposed that T6P is transported by an unknown mechanism into plastids, where it induces starch synthesis via thioredoxin-mediated activation of AGPase, and that there is a regulatory loop which involves T6P, SnRK1 (a gene that represses plant growth, inhibited by T6P) and bZIP11 that control sucrose availability and utilization. In source leaves, T6P fine-tunes sucrose levels by adjusting sucrose synthesis, while it regulates Sucrose consumption in sink organs, probably acting via multiple mechanisms, including inhibition of the SnRK1 gene (Figueroa et al., 2016). T6P regulates growth in relation to

sucrose supply by adjusting biosynthetic reactions and through regulating hormone signaling like auxin either directly or indirectly (Paul et al., 2010).

In the pistachio tree, it has been demonstrated that the accumulation of nitrogen, phosphorus and potassium is greater in inflorescence buds of non-bearing branches, compared to the ones of the bearing branches (Baninasab et al., 2007), and that the nutrient contents of the trees and annual nutrient consumption are influenced by the alternate bearing. The results showed an enhancement of nitrogen permease regulator of amino acid transport activity 3 and carbon-nitrogen hydrolase, which is supporting the fact that the concentration of nitrogen (N) was higher in the inflorescence buds, leaves, and fruits of non-fruiting branches (OFF) than in the analogous "ON" structures. Competition between flower buds and developing nuts for N might play an important role.

The study showed up-regulation of potassium ion channel, magnesium dechelatase (SGR), magnesium-chelatase subunit (ChlH), CSC1-like protein (Calcium-dependent channel) and calcium permeable stress-gated cation channel (TMEM63) during in "OFF" inflorescence buds. Some studies found that N, K, Ca and Mg content were affected by crop load in olive leaves, showing lower values following the "ON" year (Fernández-Escobar et al., 1999). However, the information on the effects of fruiting on nutrient concentrations of different organs of pistachio trees relative to bud abscission is limited. In the "OFF" inflorescence buds of June, I found genes encoding for sugar phosphates accumulation including substrates of the Calvin cycle, glycolysis, and the pentose phosphate pathway. Sugar phosphates transformed into sucrose and transport to fruit. This can reduce the sugar phosphates in the source tissues of trees with strong sink tissue such as fruit. Whereas, in the inflorescence buds of "OFF" shoots, the absences of fruits lead to the accumulation of sugar phosphates and starch. Studies showed that the expression of some of the genes and proteins involved in the Calvin cycle is up-regulated in "OFF" trees (Yanik et al., 2013).

Studies on the stomatal transpiration rates in another alternating species, such as the olive tree, have not shown any variation between plants in "ON" and "OFF" (Proietti et al., 2013), contrary to what occurs in species like orange or strawberry (Syvertsen et al., 2003). Studies on photosynthesis and production of photosassimilate in pistachio have shown a decline in "ON" trees during mid-July, which could be due to early senescence and the fall of the leaves (Marino et al., 2018). A similar decline in photosynthesis due to leaf aging has been reported for apple trees (Butler et al., 1981) and olive trees (Proietti et al., 2013). In the present study, the up-regulation of both the PSII polypeptide subunits of the photosystem II, MAF1, a global RNA polymerase III (Pol III) and PDE335 (defective Pigment 335) repressor that can be found in the "OFF" buds of June indicate that pistachios have the ability to maintain relatively high photosynthetic rates.

In plants, nutrient limitation due to sink competition leading to sugar starvation is perceived as nutritional stress and generate changes in the redox status promote the synthesis of free radicals which can cause transient oxidative stress due to an increase of ROS generation (Morkunas et al., 2012), that can be neutralized by some adaptive mechanisms which can protect the cells from oxidative damage. The cells subjected to sugar starvation at the beginning try to adapt to this deficiency through a gradual metabolic reorganization that implies the substitution of carbohydrate metabolism by protein and lipid metabolism and that change may cause autophagy (Rose et al., 2006). Variations in sugar levels induce changes in

ROS production and sugar starvation can cause the activation of ROS production, as indicated by transcriptome profiling analysis, where sucrose starvation results in activation of oxidative stress genes, such as catalase (Contento et al., 2004).

It has been found that plant processes, such as cell division, morphogenesis and stress responses, were affected by the involvement of polyamines (PAs)-putrescine (Put), spermidine (Spd), spermine (Spm), cadaverine (Cad) and thermospermine (t-Spm) (Alcázar et al., 2018). The free polyamines could have an important physiological function in the development of flower bud abscission, which causes alternate bearing in pistachio trees. A significant decrease in polyamines (Pas; Put and Spd) in shoots and leaves of "ON" trees during the heavy bud, abscission period was reported while an increase detected during the same period in "OFF" trees, indicating an association between flower bud abscission and the level of PAs in pistachio. In Satsuma mandarin, during the "ON" season, polyamines were accumulated in the stem which can suppress flowering and cause fruit bearing (Nishikawa et al., 2012). It is possible that a decrease in N concentrations in plant tissues may cause a decrease in polyamines, as they can represent nitrogenous sources or as signal molecules that regulate the fruitlet abscission process in grapevine (Aziz et al., 2003). Many studies have highlighted that abscission or ethylene biosynthesis can be delayed with low levels of S-adenosylmethionine (SAM). During this phase, PAs and ethylene compete and PAs can become dominant. The low concentrations of PAs can trigger the senescence and cause abscission (Gomez-Jimenez et al., 2010).

In the study, polyamines related genes exhibited significantly higher enhancement in the inflorescence buds of non-fruiting branches (OFF) than the "ON" fruiting ones, in accord with the recent study of Gündeşlí et al., 2019. The genes encoding for thermospermine synthases (ACL5), probable polyamine transporter, Polyamine oxidase 1 isoform 1 and spermidine synthase (speE) were enhanced during the "OFF" seasons. These references, along with the results, support the fact that polyamines could play a crucial role in the inflorescence bud abscission of pistachio. A high level of polyamines is known to act as antisenescence agents and counteract the activity of abscisic acid and ethylene (Khezri et al., 2010. The competition between polyamines and ethylene pathways for S-adenosil methionine (AdoMet) or the inhibition of ACC syntase or ethylene forming enzyme (EFE) by polyamines can result in a mechanism that can modulate physiological events, including senescence and flower bud abscission.

The role of hormonal factors involved in inflorescence bud abscission was studied by many authors in pistachio leading to contrasting results (Vemmos et al., 1994); however, only recently lower levels of auxin in most of the organs of "ON" pistachio trees during kernel development have been directly implicated in bud abscission (Gündeşli et al., 2010). Exogenous application of auxins prevented inflorescence bud abscission in pistachio (Pontikis et al., 1990). In another study conducted in citrus the auxin amount is in a positive relationship with abscission by causing a delay of abscission, resulting in improvement in fruit quality and yield. In the research, auxin was down-regulated in "ON" buds. The study shows that auxin conjugates play an important role in IAA metabolism, temporary storage reserves and inflorescence bud abscission.

In the auxin-responsive gene category, differentially expressed in the present study, it is worth to mention the down regulation of TOR that I found in June "OFF" inflorescence buds

and up-regulated in June “ON”. The regulation of autophagy by TOR and SnRK1 or SNF1-related kinase is conserved in plants (Ahn et al., 2011). In Arabidopsis, AuTophaGy-related1 (ATG1) kinase complex and ATG13 together generate a complex which can regulates autophagy, nitrogen deprivation and short-term carbon starvation. Furthermore, this ATG1-ATG13 complex are sensitive to the nutrient level mediated by TOR (Marshall et al., 2018). https://dev.biologists.org/content/145/13/dev160887—ref-115 SnRK1 complex is activated by energy deprivation, abiotic stresses and starvation but suppressed by glucose in Arabidopsis (Baena-González et al., 2007). SnRK1 and TOR can target phosphorylation substrates to sense energy and nutrient levels and coordinate transcriptome, metabolism, cell growth and development (Wurzinger et al., 2018). Interestingly I found the down regulation of SnRK1 or SNF1-related kinase in June “OFF” inflorescence buds and an enhancement in June “ON”. TOR signaling plays an important role in stem and progenitor cell function and regulation that modulate proliferation and maintenance, cell-cell interactions and sink-source organ communication.

There are several studies reporting the involvement of ABA biosynthesis or ethylene perception critical for sugar signaling (Arenas-Huertero et al., 2000). The ethylene signal is transmitted via a pathway that includes a transcriptional cascade, and EIN3 has been identified as a critical component within this cascade (Guo et al., 2004). The regulation of EIN3 by ethylene and sugar indicates the cross talk between the two signaling pathways. Remarkably, I have found that the transcription of ethylene is also down-regulated by glucose in June “OFF” inflorescence buds, whereas ABA encoding genes like carotenoid cleavage dioxygenase 1 and abscisic acid insensitive 3 were up-regulated during June “OFF” inflorescence buds.

Interestingly, in barley, the antagonism between ABA and GA has been demonstrated to be an essential factor controlling the metabolism in aleurone cells and the PCD. GA induces the production of hydrogen peroxide (H2O2) and α-amylases in aleurone cells which lead to hydrolyse stored starch (Ishibashi et al., 2012). Thus, the high level of GA expression that I found in June “ON” inflorescence buds can be an indication of the shortage of sugar and a signal for inducing starch degradation to supply the carbohydrate need. In studies on abiotic stress, responses showed the involvement of polyamines in PCD through the production of hydrogen peroxide (H2O2) and Nitrogen oxide (NO) (Takács et al., 2016). Abiotic stress conditions induce an excess of spermidine into the apoplast, where it is catabolized by the enzyme PA oxidase, producing reactive oxygen species (ROS) such as H2O2 and/or other nitrogenous molecules (N) through different cascades (Moschou et al., 2012). H2O2 accumulation can cause the induction of PCD or stress tolerance, depending on the levels of intracellular Pas (Corpas et al., 2019). PCD is strictly regulated by the ratio of PA anabolism to catabolism, while ROS generation/accumulation has a crucial role in cell fate decision (Moschou et al., 2008).

PA catabolism by amine oxidases, copper-containing amine oxidases (CuAOs), flavin-containing PA oxidases (PAOs) and the parallel production of H2O2 can result in two different scenarios. High H2O2 levels lead to programmed cell death (PCD) (Yang et al., 2018), while low H2O2 level is efficiently scavenged by enzymatic/nonenzymatic antioxidant factors that help plants to survive abiotic stress, using different defense mechanisms (Pitino et al., 2017). In the present study, ROS related genes such as peroxisome 1 (PEX1), isocitrate dehydrogenase, COPPER/ZINC SUPEROXIDE DISMUTASE,

FLAVANONE 3-HYDROXYLASE, Peroxidase, HXXXD-type acyl-transferase family protein as well as many stress-related genes (Disease resistance protein, Glycosyl hydrolase, Cysteine-rich secretory proteins, Serine/threonine-protein kinase MAPK/ERK KINASE 4, VASCULAR ASSOCIATED DEATH1, Leucine-rich repeat protein kinase, Riboflavin synthase-like superfamily protein and Pentatricopeptide repeat) were found down-regulated in June "OFF" inflorescence buds vs June "ON" inflorescence buds. It is well known in both animals and plants that peroxisome PEX genes are induced by the universal stress signal, H2O2 (López-Huertas et al., 1999).

Most of the genes involved in ubiquitin and autophagy dependent categories were repressed during the June "OFF" inflorescence buds, (ATG8C: Autophagy related protein 3, ubiquitin proteasome, PAG1: Proteasome Alpha Subunit G1, Cytochrome P450, E3 RING/U-BOX genes, PEROXIN 2, BRUTUS (BTS), Malate dehydrogenase, Sugar-insensitive 3 and MAPK genes). It has been found that BTS may act as an E3 ligase, which catalyzes the final step in the protein ubiquitination via the 26S proteasome (Matthiadis et al., 2016). During June "OFF" inflorescence buds, I noticed the up-regulation of reactive intermediate deaminase A, ARM repeat superfamily protein members of the U-Box E3 Ubiquitin Ligase Family, B-box domain protein 27, ARF23, SKP1 interacting partner 6 and PHY rapidly regulated 2.

In the study, three bZIP transcription factors were found to be down-regulated during the June "OFF" inflorescence buds. bZIP61 harbor various stress-related cis-elements, indicating this bZIP related gene may involve in response to multiple abiotic stresses. In rice, OsbZIP genes, like OsbZIP16, act as positive regulators of drought and osmotic stress (Agarwal et al., 2019). The bZIP61 and bZIP16 were found up-regulated in June "ON" bud. The Auxin response factors were found to be down-regulated during the June "OFF" buds, while ARF7 was found up-regulated in June "ON" bud. In A. thaliana two related auxin response factors, ARF7 and ARF19 act as transcriptional activators of early auxin response genes during lateral root formation. Three WRKY (WRKY31, WRKY72 and WRKY53) transcription factors having a key role in response to many different environmental stresses were up-regulated in June "OFF" inflorescence buds. AP2/EREBP (APETALA2/ethylene-responsive element-binding protein) transcription factors were found up-regulated in June "ON" inflorescence buds. Interestingly, in rice, OsAP2/EREBP plays an important role in the crosstalk of signaling pathways of different kinds of stresses (Sharoni et al., 2010).

The evidence of transcriptomics results allowed the elaboration of a model that supports the nutritional theory and elucidates for the first time the role of hormones, polyamines and ROS in inflorescence buds abscission likely associated to the alternate bearing behavior of the pistachio. I speculated that when the level of sugar is not critical, as indicated by the down-regulation of genes involved in starch demolition (ALPHA-AMYLASE 3 and ALPHA-AMYLASE 7) and up-regulation of starch synthase 4, like in June "OFF" inflorescence buds, SnRK1 complex is suppressed by sugars or by trehalose-6-phosphate (T6P), considered a fine-tunes of sucrose levels, which is up-regulated and the transcription of ethylene and GA are down-regulated, as well as many stress relate genes and ubiquitin and autophagy-dependent genes. Auxin related genes, on the contrary, are up-regulated, indicating a possible accumulation of this hormone, inducing cell growth and perhaps the down-regulation of TOR. The oxidization of polyamines, such as Spd, occurs in the apoplast at a slow rate, with moderate production of H2O2, which activates the ROS-dependent protective pathway that does not trigger PCD or autophagy (Xiong et al., 2013) (Figure 5.8).

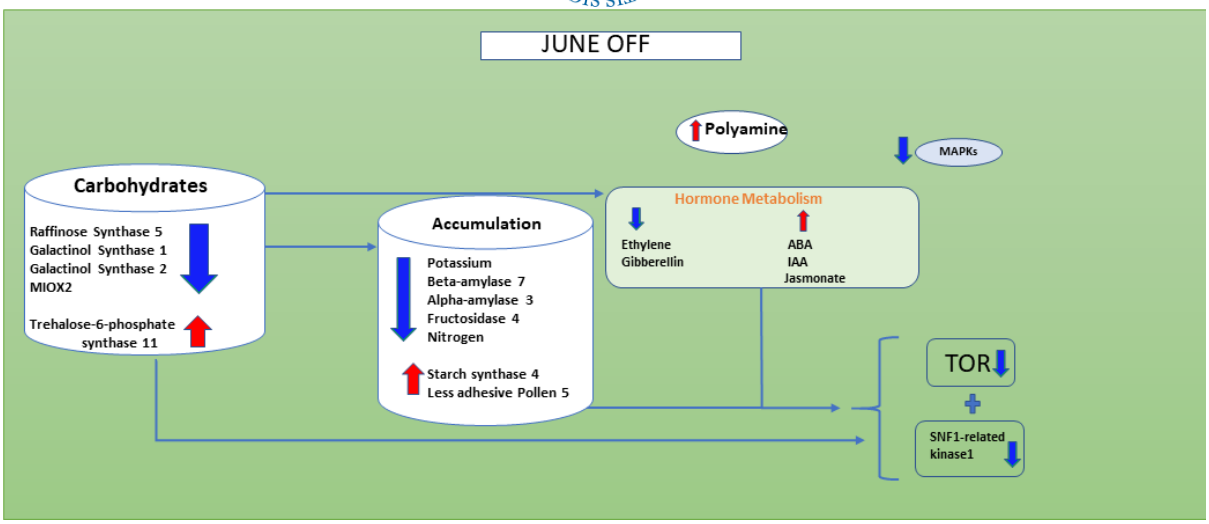


Figure 5.8. A figure showing the down-regulated genes in the inflorescence buds of non-fruiting branches of June "OFF" season. Red shows the up-regulated genes and blue shows the down-regulated genes. In this situation the inflorescence buds do not occur.

In June "ON" inflorescence buds when the degradation of starch occurs as indicated by the up-regulation of ALPHA-AMYLASE 3, T6P is down-regulated and SNF1-related kinase 1 and TOR are activated. TOR signaling networks seem involved in cell-cell interactions, sink-source organ communication and autophagy. In the study, Raffinose synthase gene 5 (RS5), galactinol synthase genes (Galactinol synthase 1 and Galactinol synthase 2) and MIOX2 showed enhancement in "ON" inflorescence buds. In June "ON" inflorescence buds, spermidine oxidation occurs faster with the high production of H2O2 inducing PCD pathway and PA are down-regulated. Interestingly, genes of the GA pathway, up-regulated in June "ON", may also increase the production of hydrogen peroxide (H2O2), which can represent a signal for inducing starch degradation to supply the carbohydrate need. Furthermore, in conjunction with low PA expression, down-regulation of auxin was also found resulting altogether in flower bud abscission (Figure 5.8). It is very interesting to note the enhancement of some transcription factors in July "ON", which presumably increase programmed cell death and autophagy by promoting a more substantial abscission of inflorescence buds since the depletion of nutrients is greater due to the intense growth of embryos.

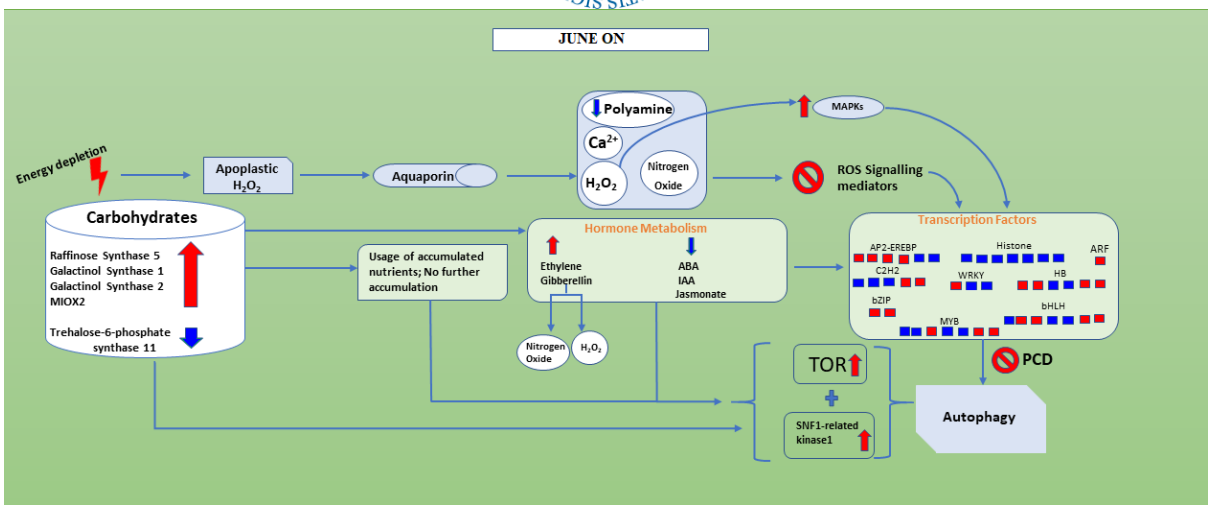


Figure 5.9. A figure showing the hypothetical molecular mechanism behind inflorescence buds abscission in fruiting branches of June "ON" season. Red and blue show the up-regulated and down-regulated genes in inflorescence buds of the June "ON" season, respectively.

This work concludes that, in the "OFF" inflorescence buds of June, the genes corresponding to carbohydrate show reduction compared to June "ON" inflorescence bud. Furthermore, there is a higher amount of accumulation of starch (BETA-AMYLASE 7, ALPHA-AMYLASE 3 and Fructosidase 4), nitrogen and potassium in June "OFF" compared to June "ON". The hormones such as ethylene and gibberellin are showing down-regulation and ABA, IAA and Jasmonate are showing up-regulation when compared with June "ON" inflorescence buds; I can conclude that these hormones play an important in the production of Nitrogen oxide, Polyamine and H2O2, which eventually target cell death and autophagy during the June "ON" period. As predicted, there is no such signaling taking place for H2O2 and ROS and polyamines show change towards its enhancement in June "OFF". Therefore, during June "OFF" inflorescence buds, no PCD and autophagy occur (Figure 5.8) and makes plant stable for the next upcoming "ON" season (Figure 5.9). Interestingly it seems that in pistachio exogenous application of PA can reduce many physiological disorders and inflorescence bud abscission (Kamiab et al., 2015), and preliminary experiments are currently being carried out in the cultivar Bianca to detect the dose and the timing of treatment.

5.6. Conclusion

These results highlighted how the lack of resources (carbohydrates and mineral elements) in *P. vera* can be the main cause triggering a cascade of events involving hormones and ROS which end, through autophagy phenomena, with the abscission of inflorescence buds, directly or indirectly linked to the mechanism of alternating production. This study provided further support to the theory of shoot autonomy in pistachio with regards to flower bud abscission and identified key genes and hormones associated with inflorescence bud abscission, the knowledge of which could also lead, in future, to a reduction of the inflorescence buds drop, through the development of biomarkers, and the possibility to modulate the alternate bearing.

Experiment 5: De-novo discovery and analysis of the *Pistacia vera* (L.) fruits enable the identification of genes and hormones linked to inflorescence bud abscission.

5.7. Introduction

The development of genomic and transcriptomic studies has contributed to a better understanding of the molecular and physiological processes involved in the bud abscission phenomenon. The recent transcriptomic experiment on inflorescence buds of 'ON' and 'OFF' trees of the cultivar Bianca described in the experiment 4 showed that the lack of resources (primarily carbohydrates) was the leading cause of inflorescence bud abscission. The study showed that the SnRK1 gene complex, the auxin-mediated TOR gene, ROS, genes responsive to ubiquitin and autophagy and genes involved in the biosynthetic pathways of auxins and polyamines, leads to the premature inflorescence bud abscission of the loaded "ON" branches (Benny et al. 2020).

This study completes the experiment 4 on the transcriptomic of inflorescence buds of “ON” and “OFF” shoots of the cultivar Bianca and gives further insight into the nutritional factors and hormonal factors involved.

5.8. Aim of the Research

In the present study, RNA seq analysis was carried out in fruits of “ON” and “OFF” shoots of the cultivar Bianca, to investigate the presence of inhibitory signals or genes relate to hormone biosynthesis directly or indirectly linked to the premature fall of the inflorescence buds, considered the main cause of alternate bearing behaviour of Pistachio tree.

5.9. Materials and Methods

- Plant Material, RNA extraction, processing, and sequencing

The transcriptomic analysis was conducted taking the tissue samples from 1 mature *Pistacia vera* (L.) tree of the cultivar Bianca, grown inland of Sicily (37° 30’ Lat. N) in 27th of June and in 22nd of July 2019. The fruit tissue taken from bearing (“ON”) and non-bearing (“OFF”) branches were analysed. Bearing branches showed from 40 to 50 fruits; non-bearing had very few fruits from three to eight fruits. Four to six fruits were collected each from three branches (considered as three biological replicates) of the same tree during the “ON” and “OFF” status of June and July which constitute a total of 12 samples. All samples were immediately frozen in liquid nitrogen after collection and stored at -80 ° C. The samples were grounded in liquid nitrogen and total RNA extraction was performed with the Spectrum Plant Total RNA Kit (Sigma-Aldrich) employing 100 mg of frozen tissue. RNA quality and RNA Integrity Number (RIN) were checked by using the Bioanalyzer. Libraries were obtained using the TruSeq RNA-Seq sample prep kit from Illumina (Illumina, Inc., CA, USA). The 12 samples were loaded into one lane of an Illumina flow cell, and clusters were created by Illumina Bot. The clusters were sequenced using the service provided by BMR Genomics (Padua, Italy) at ultra-high throughput on the Illumina HiSeq 2000 (Illumina Inc.) to obtain single reads per sample, each 75bp long. The denovo assembly, evaluation and annotation have been done using the similar method of that experiment 4.

- Differentially Expressed Genes (DEG) between Stages

To estimate the expression levels of the Trinity reconstructed transcripts, I used RSEM. RSEM is a package used to estimate the gene and isoform expression levels from RNA sequence data. The expected count matrix derived from RSEM is given as the input for edgeR. The comparison selected for the study is given in Table 6.1.

Table 6.1. The number of total genes, up-regulated and down-regulated genes in fruits in current year non-bearing shoot "OFF" and in fruits in bearing shoots "ON".

Comparison	Differentially expressed genes	Up-regulated	Down-regulated
June "OFF" vs. June "ON"	1,536	702	834
July "OFF" vs. July "ON"	950	482	468

Genes represented with an adjusted P-value (FDR) lower than 0.01 and at least a two-fold change were only considered as significantly differentially expressed in the pairwise comparison of the samples. In addition, the functional-enrichment analysis was performed to identify which GO terms and metabolic pathways that were significantly enriched in DEGs. The gene enrichment and functional analysis have been done using the similar methods used in experiment 4.

5.10. Results

- De novo transcriptome assembly and annotation

. To examine the inflorescence bud abscission phenomenon of *P. vera* relate to alternate bearing, fruit tissue from three separate shoots of the same tree were collected and sequenced from bearing and non-bearing shoots. The picking dates of the material coincide with a period of the initial competition between fruit and inflorescence buds, not causing inflorescence bud drop and a period of strong completion causing the drop of inflorescence buds. The sequencing of the data of June produced 227 million raw reads whereas July produced 234 million reads as a single-end. The high-quality single-end reads with an average quality score of 38 were selected for the transcriptome assembly after trimming off the low-quality bases and adapters from the June and July data sets. The total pre-processed reads were then de novo assembled using Trinity and transcripts from Trinity assembly were further clustered using CD-Hit-EST. The assembly was evaluated with BUSCO to assess the transcriptome assembly by measuring the completeness of the transcriptome based on evolutionary present universal single-copy orthologs. The number of up- and down-regulated genes along with the total number of genes obtained in each sample comparison were listed in Table 6.1.

I used RSEM for the quantification of the genes. The count matrix generated I then taken as the input by edgeR. The downstream analysis resulted in the identification of a total of 34,409 genes in which 16,701 were up-regulated and 17,708 were down-regulated. For each of the analysis, the total number of genes range from 905 to 11,250. The number of genes up-regulated was in a range of 482 to 5,102 and down-regulated genes were spans from 468 to 6,148. Subsequently, the assembled transcripts were annotated by BLASTX against a non-

redundant (NR) protein database, PFAM, KEGG, Uniprot/Swissprot, InterPro and STRING databases. It is likely that the cv. Bianca faces a limitation of resources around the third week of June, when the first sampling of the plant material was made and, that in a month, it reaches its maximum peak, corresponding to the second sampling period when the drop of inflorescence buds started. June "OFF" vs July "ON" corresponds to the most divergent scenarios. The main comparison is focused on "ON" and "OFF" period of June to investigate thoroughly different pathways and processes of the bud abscission. This comparison can also avoid factors like physiological and developmental changes that might occur in the bud during the two different time points (June and July).

- Effect of crop load on photosynthesis in fruits during June "OFF" vs June "ON"

While comparing to the "OFF" year fruits from non-bearing shoots, the changes in gene expression in "ON" fruits do reflect an enhancement of photosynthetic activity. Most of the genes involved in photosynthesis were down-regulated during the "OFF" year. The photosystem II PSII polypeptide subunit and photosystem II LHC-II subunits (CHLOROPHYLL PROTEIN 24, LIGHT-HARVESTING CHLOROPHYLL B-BINDING 2 and light harvesting complex gene 1) were down-regulated during June "OFF". In contrast, a gene calling for photorespiration, D-isomer specific 2-hydroxyacid dehydrogenase and ATP synthase (PIGMENT DEFECTIVE 332) were enhanced during the "OFF" period. The genes encoding for calvin cycle ribulose bisphosphate carboxylase and transketolase 2 were repressed during the June "OFF" period.

- Effect of crop load on starch metabolism in fruits during June "OFF" vs June "ON"

The study on the relationship between crop load and starch metabolism help in assessing the functional distribution of starch in "ON" and "OFF" fruits. The genes encoded for sucrose transporter 4, sucrose synthase 3 and heteroglycan glucosidase 1 were enhanced in the fruit tissue of the "OFF" current year shoots. The genes encoded for ALPHA-AMYLASE 3, BETA-AMYLASE 8 and fructosidase 4, which was involved in starch degradation was repressed (Figure 6.1).

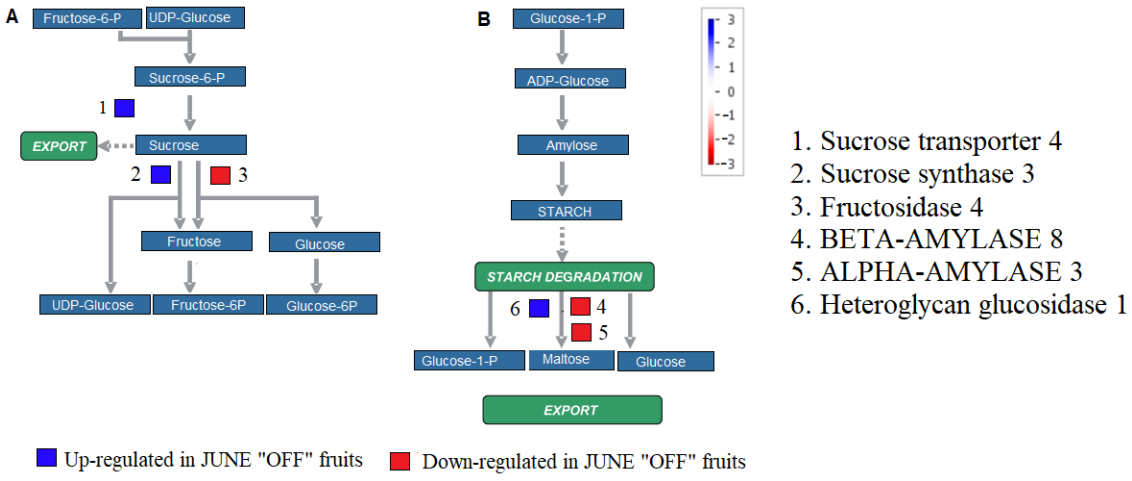


Figure 6.1. Figure shows the Mapman pathways in sucrose-starch metabolism. Figure highlights differentially expressed genes between fruits in the non-bearing shoot (June "OFF") and bearing shoot (June "ON") in sucrose degradation (A) and starch synthesis (B) pathways. Individual genes were represented by small squares. The color scale indicates the log2 FC value. Red represents down-regulation and blue represents up-regulation in June "OFF" relative to June "ON".

- Effect of crop load status on polyamine and transcription factors in fruits during June "OFF" vs June "ON" shoots

The "OFF" fruits exhibited significantly higher polyamine (PA) and spermidine (Spd) enhancement than the "ON" year fruits. In "OFF" fruits, the genes encoding for thermospermine synthases (ACL5), probable polyamine transporter, and spermidine synthase 1 (speE) were enhanced. On the contrary, the expression of S-adenosyl methionine carrier 2 was repressed.
In June "OFF" fruits, all the genes related to bZIP (bZIP69, leucine zipper transcription factor 18, bZIP61, bZIP65 and trichome birefringence-like 41), WRKY13, and two homeobox genes (Enhanced drought tolerance 1 and Homeodomain GLABROUS 8) were down-regulated. While five WRKY factor (WRKY40, WRKY75, ABA-overly sensitive 1, mitogen activated protein kinase and UDP-glycosyltransferase) and two Aux/IAA related genes (argonaute 5 and indole-3-acetic acid inducible 30) were up-regulated (Figure 6.2). In June "ON" fruits, the study reported the enhancement of C2H2 factors (zinc finger protein 7, transparent testa 1, leafy cotyledon 1 and L-glutamine D-fructose-6-phosphate) and MADS factors like AGAMOUS-like 104 and floral homeotic protein apetala 1.

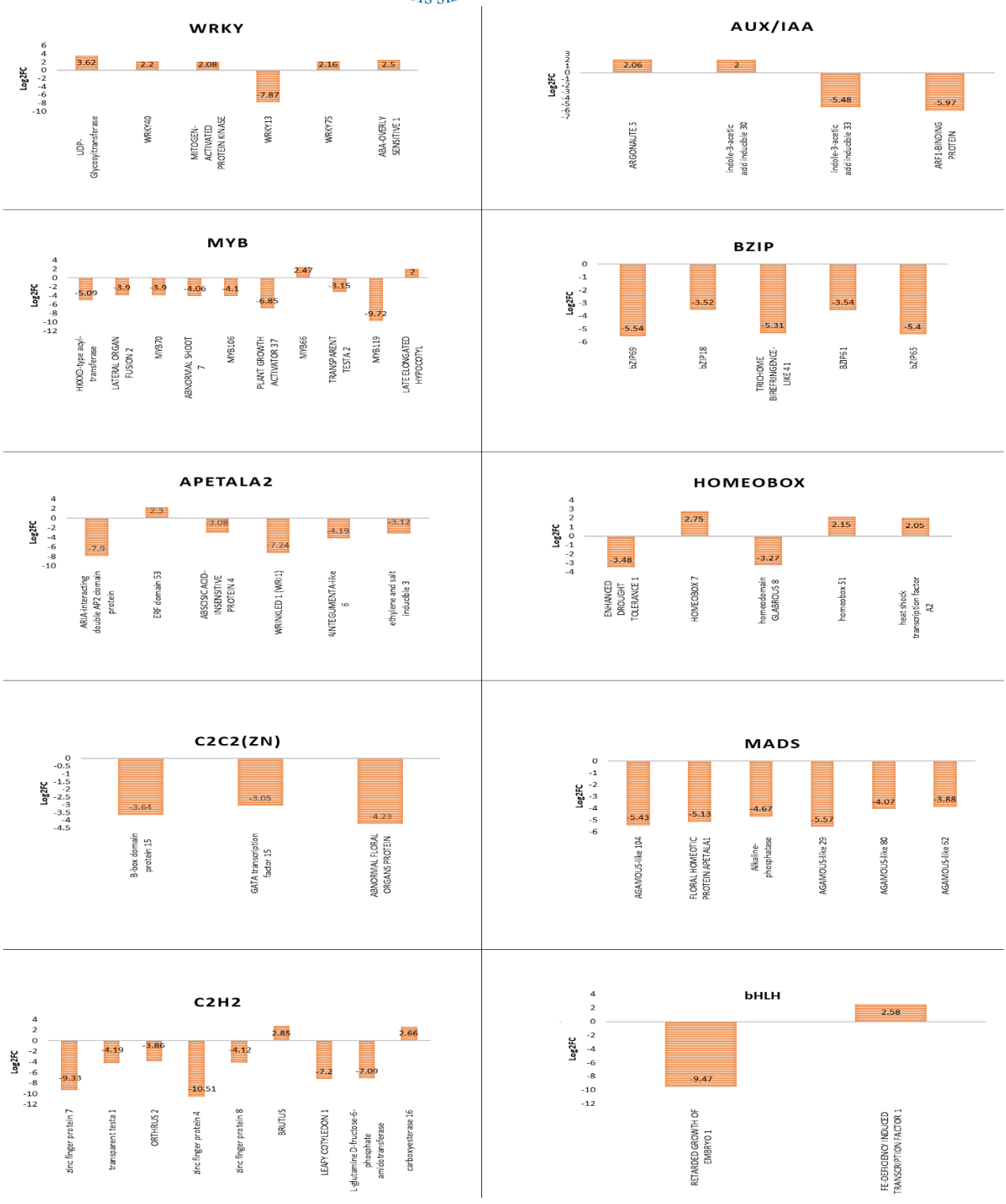


Figure 6.2. Figure shows transcription factors among the June "OFF" vs. June "ON" fruit comparison. The y-axis indicates the log2 FC value. The bar represents the differentially expressed genes in June "OFF" fruits relative to June "ON" fruits.

- Effect of crop load on hormone metabolism in June “OFF” vs June “ON” fruits

The objective of the current section was to study about the role of hormone in fruits tissue that may lead to bud abscission. The genes involved in hormone-related categories were summarized in Figure 6.3.

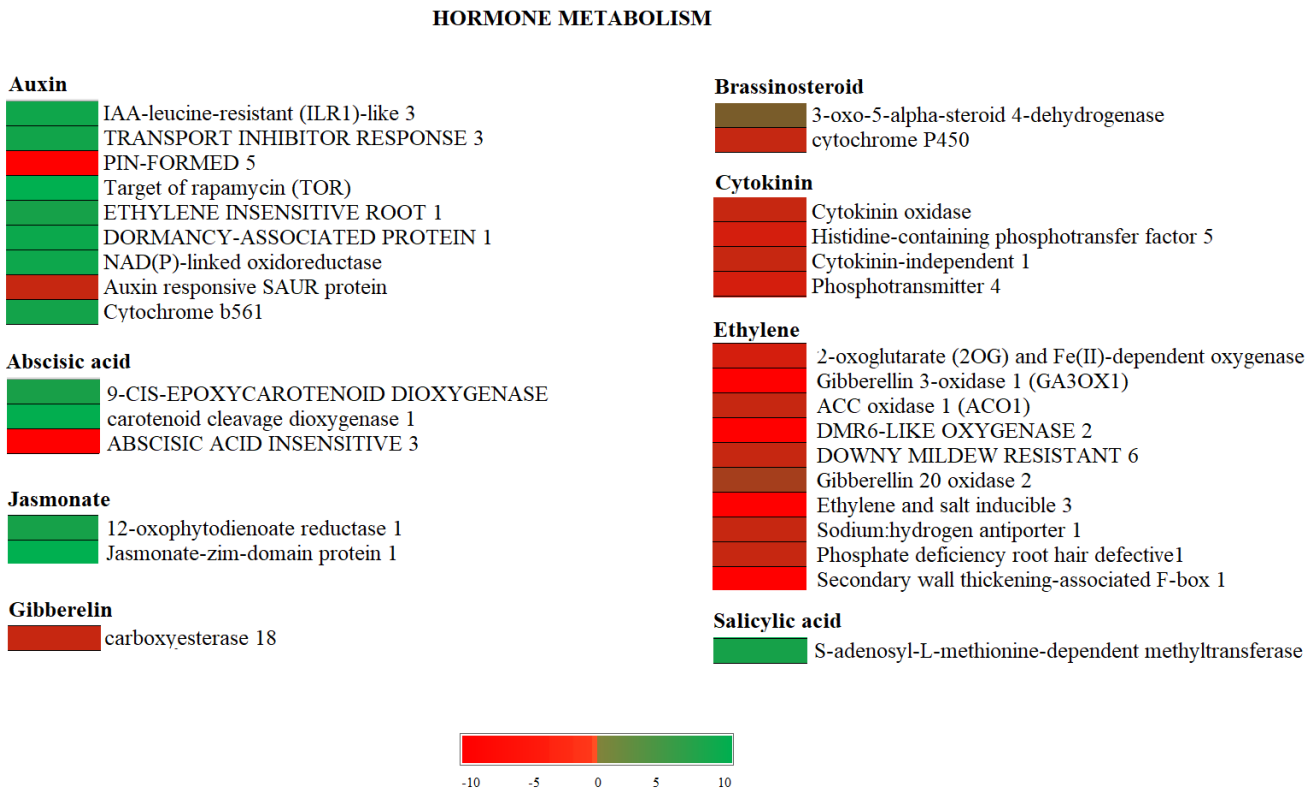


Figure 6.3. Figure shows hormone metabolism in Pistachio among the June “OFF” vs. June “ON” fruits comparison. The colour scale indicates the log2 FC value. Red represents down-regulation and green represents up-regulation in June “OFF” fruits relative to June “ON” fruits.

Repression of ethylene, gibberellin and cytokinin pathways were identified in June “OFF” fruits whereas ABA, IAA and Jasmonate pathways were mostly up-regulated. In June “OFF” fruits, all the genes responsive to ethylene, gibberellin, brassinosteroid and cytokinin were down-regulated. Relating to auxin-responsive genes, down-regulation of PIN formed 1 and SAUR protein and the up-regulation of target of rapamycin (TOR), Ethylene insensitive root 1, and cytochrome B561 were observed. Relating to ABA, there was a down-regulation in abscisic acid insensitive 3, and up-regulation in Carotenoid cleavage dioxygenase 1 and 9-cis-epoxycarotenoid dioxygenase. Several genes involved in ethylene biosynthesis and signaling such as 2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase, Gibberellin 3-oxidase 1 (GA3OX1), ACC oxidase 1 (ACO1), DOWNY mildew resistant 6 and phosphate

deficiency root hair defective 1 were repressed during in “OFF” fruits. I also observed an up-regulation in S-adenosyl-L-methionine-dependent methyltransferase related to Salicylic acid (Figure 6.3).

- Effect of crop load on carbohydrate metabolism and mobilization in June “OFF” vs June “ON" fruits

The relationship among the carbohydrate metabolism and mobilization pathway in Pistachio fruit from non-bearing shoots (June “OFF”) and bearing shoots (June “ON”) was indicated in Figure 6.4.

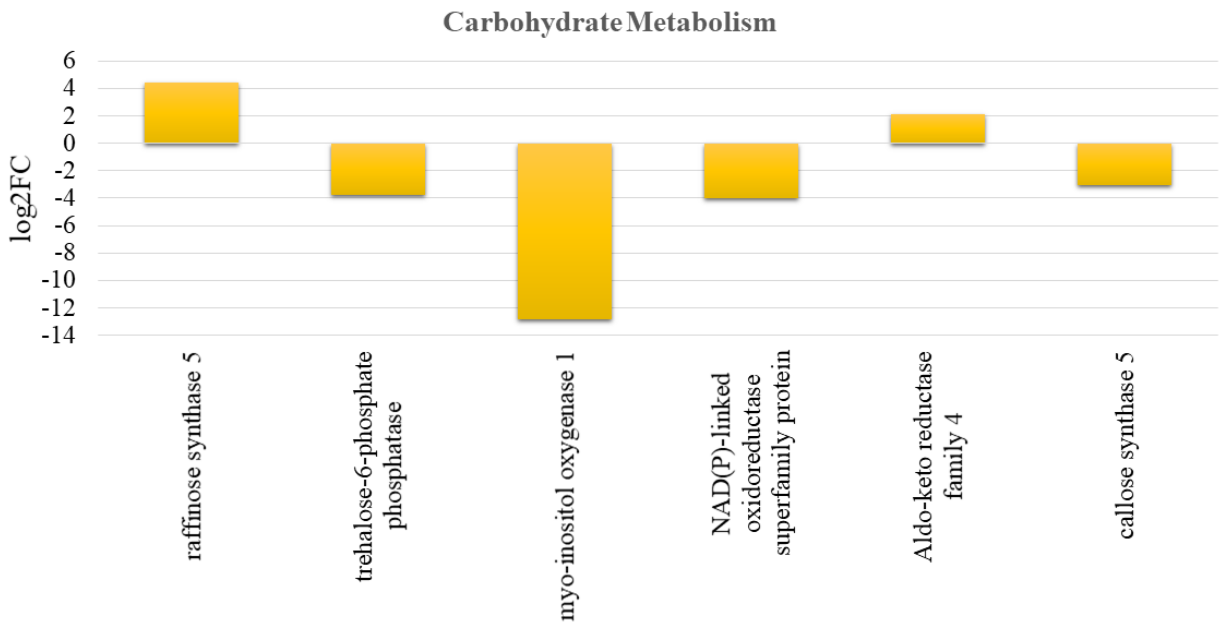


Figure 6.4. Figure shows carbohydrate metabolism and mobilization pathway in Pistachio among the June “OFF” vs June “ON” fruit comparison. The y-axis represents the value of log2 fold change. The bar indicates the effects on carbohydrate levels driven by differential expression of different CHO metabolism genes.

The study showed that the pistachio fruit of non-bearing shoots (“OFF”) required low amounts of carbohydrates due to the lack of fruits at the time and thus accumulated some starch. Similarly, I found genes encoding for carbohydrate storage in the fruits of bearing and non-bearing pistachio shoots is repressed and suggest that the in-season carbohydrate metabolism might influence the flower bud abscission directly or indirectly linked to the alternate bearing. Raffinose synthase gene (Raffinose synthase 5 (RS5), and aldo-keto reductase family 4 were enhanced during June “OFF”. Whereas sugar alcohols such as callose synthase 5, trehalose-6-phosphate synthase, NAD(P)-linked oxidoreductase protein and MIOX2 were showed repression in the “OFF” fruits (Figure 6.4).

- Comparison between “ON” and “OFF” fruits of “JUNE” and “JULY”

During the comparison of the fruits “ON” of June and July, almost all the genes during July “ON” participate in a similar way as that of “ON” June. This comparison proves that gene expression profiling associated with “ON” season of June and July and “OFF” season of June and July are similar proving the importance of these genes in the flower bud abscission and alternate bearing.

- Effects of Crop Load in July “OFF” vs. July “ON” fruits

An enhancement ABA and salicylic acid was identified in fruits of July “OFF”, whereas ethylene and gibberellin pathways were downregulated. In July “OFF” fruits, seven genes related to ethylene (2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, Gibberellin 3-oxidase 1, ACC oxidase 1, DMR6-like oxygenase 2, downy mildew resistant 6, ethylene and salt inducible 3 and sodium:hydrogen antiporter 1), one gene responsive to gibberellin (gibberellin 20 oxidase 2) and three genes responsive to cytokinin (histidine-containing phosphotransfer factor 5, cytokinin-independent 1 and phosphotransmitter 4) were down-regulated (Figure S3). Relating to auxin-responsive genes, down-regulation of PIN formed 5 and auxin responsive SAUR protein and the up-regulation of TOR, Ethylene insensitive root 1, sugar transporter protein 12, NAD(P)-linked oxidoreductase and cytochrome b561 were observed. Relating to ABA there was a down-regulation of Abscisic acid insensitive 3 and up-regulation of Carotenoid cleavage dioxygenase 1 and 9-cis-epoxycarotenoid dioxygenase.

The comparative study on starch metabolism of the July “OFF” vs July “ON” produced Similar results to the results of June “OFF” vs June “ON”. The genes encoded for sucrose synthase 1, sucrose synthase 3 and heteroglycan glucosidase 1 were enhanced in the fruit tissue of the “OFF” July shoots. The genes encoded for ALPHA-AMYLASE 3, BETA-AMYLASE 8 and fructosidase 4, which was involved in starch degradation was repressed during July “OFF”.

In July “OFF” fruits, all the genes related to bZIP (leucine zipper transcription factor 18, bZIP61, bZIP65 and trichome birefringence-like 41), WRKY13, and two homeobox genes (Enhanced drought tolerance 1 and Homeodomain GLABROUS 8) were down-regulated. While four WRKY factor (WRKY75, ABA-overly sensitive 1, mitogen activated protein kinase and UDP-glycosyltransferase) and two Aux/IAA related genes (argonaute 5 and indole-3-acetic acid inducible 30) were up-regulated. In July “ON” fruits, the study reported the enhancement of APETALA2 (ethylene and salt inducible 3, ARIA-interacting double AP2 domain protein, WRINKLED 1) and MADS factors like AGAMOUS-like 104 and floral homeotic protein apetala 1.

5.11. Discussion

Previous studies on the role of carbohydrate in inflorescence bud abscission showed that the fruit is dominant in competing for photosynthates compared to inflorescence buds in pistachio. The study also concluded that inflorescence bud abscission pistachio occurs due to the deficiency of carbohydrates transferring from the adjacent leaves (Crane and Nelson (1971, 1972; Marino et al. 2018). The recent transcriptomic experiment on inflorescence buds of 'ON' and 'OFF' trees of the cultivar Bianca by Benny et al. (2020) described in the experiment 4 proved that the lack of resources (primarily carbohydrates) was the leading

cause of inflorescence bud abscission. This report is consistent with the result I achieved. The genes such as Aldo-keto reductase and raffinose synthase (RS5) showed an up-regulation in the present study in 'ON' fruits. RS's are crucial molecules during stress response (S Sengupta, 2015). They are involved in several cellular functions, such as transport and storage of sugars (I Couée, 2006), signaling molecule following pathogen attack and wounding, signal transduction, membrane trafficking (H Xue, 2007), and mRNA export (M Okada, 2009).

In many fruit crops, such as apple (Guitton B, Kelner JJ et al., 2016), citrus (Shalom L, Samuels S et al., 2012), olive (Yanik H, Turktas M et al.,2013), the alternate bearing inhibits flower bud initiation and their morphological differentiation during a heavy crop load. Therefore, the research has usually been focused on genes involved in floral initiation. However, the pistachio tree shows a peculiar alternate bearing behavior, as it differentiates inflorescence buds every year, that abscise in "on year" with massive crop load. In the previous study (Benny et al. 2020), there were no genes related to floral initiation, while several genes responsive to hormone showed a direct or indirect link to the premature flower bud abscission (Benny et al. 2020).

In this study interestingly, in fruits, genes typically involved in floral initiation showed a different expression. In July "ON" fruits, it is evident of an enhancement of APETALA2 (ethylene and salt inducible) WRINKLED 1 and MADS factors like AGAMOUS-like 104 and floral homeotic protein APETALA 1. APETALA2 (AP2) encodes a member of the AP2/EREBP (ethylene-responsive element-binding protein) class of transcription factors involved in floral organ identity and specification, ovule, and seed development (Ohto et al. 2005). AP2 may also function during vegetative growth since it is also expressed at the mRNA level in both stem and leaf. WRINKLED1 (WRI1, At3g54320) is an APETALA2/ethylene-responsive element binding protein (AP2/EREBP) transcription factors regulating the expression of genes involved in carbon allocation into oil or triacylglycerol (TAG) in plants (Cernac and Benning, 2004; Snell et al. 2019).

In many species, it has been proved that WRI1 can orchestrate the regulation of many genes involved in shuffling carbon from starch and sucrose into fatty acid synthesis during the glycolysis process (Maeo et al. 2009Snell et al. 2019). Recently, it was discovered that SUCROSE-NON-FERMENTING-1-RELATED PROTEIN KINASE-1 (SnRK1) could affect the turnover rate of WRI1 by phosphorylating its tandem AP2-domains by controlling the proteasomal degradation (Zhai et al., 2017). In Arabidopsis, it has been demonstrated that trehalose 6-phosphate interacts with subunits of the SnRK1-complex, leading to reduced phosphorylation of WRI1, which is stabilized and therefore, Trehalose 6-phosphate positively regulates fatty acid biosynthesis (Zhai et al., 2018).

Many plants accumulate triacylglycerol (TAG), starch, and storage proteins in their seeds as energy reserves for the seedlings (Athenstaedt and Daum, 2006). In developing Arabidopsis seeds, carbon is initially stored as starch and afterward degraded and remobilized into TAG biosynthesis. The sequestration of carbon to energy-dense storage molecules, like starch and TAG, during seed filling implies a shift of the carbon flow from source tissues into newly established sink tissue and an allocation of carbon within the sink into the synthesis of specific storage molecules. (Ruuska et al., 2002). In this work, it is evident that "ON" fruits act as the strongest sink and here it occurs the carbon allocation into oil.

MADS-box proteins function as a significant regulator for many plant-developmental processes, including flower senescence, flowering time, floral organ specification, gametophyte, embryo, and seed development (Smaczniak et al., 2012). MADS-domain

protein interactions, either with members of the other proteins or with same family, could explain their specificity and ability to orchestrate different developmental programs that respond to internal and external signals such as hormones.

In the study, the upregulation of MADS-box genes in the "ON" season fruits imply their involvement in fruit development. It is now known that the MADS-box gene, Agamous, is directly involved in the activation of the jasmonic acid (JA) biosynthesis gene (Ito et al., 2007). JA shows synergistic and/or antagonistic effects with abscisic acid (ABA), ethylene (ET), salicylic acid (SA), and other plant hormones in environmental stress resistance response, and it can inhibit O3-induced programmed cell death (Raza et al. 2020) while its role in controlling age related senescence is still under investigation (Jibran et al., 2013). In the study it is evident that there exists a negative correlation among JA and ET, however the role of JA is not clear.

In apple, it has been reported that hormone-related genes like GA biosynthesis and degradation are more likely to be responsible for alternate bearing than flowering genes (Guitton et al. 2012). However, the bud abscission in pistachio does not appear to be linked to gibberellin in developing fruits and buds (Lin et al. 1984), as well as abscisic acid levels (Takeda and Crane 1980). It has been reported that bud abscission in pistachio does not appear to be linked to gibberellin in developing fruits and buds (Lin et al. 1984). Likewise, Abscisic acid (ABA) and gibberellin are the hormones that regulate a wide range of plant processes (Reid 1985; Zhang and Zhang 2009). However, it was found that bud abscission in pistachio is not attributed to abscisic acid levels in developing buds and fruits (Takeda and Crane 1980). I can see a consistent result in the study when comparing results from both inflorescence bud and fruit. All the genes calling for abscisic acid and gibberellin were down-regulated in both inflorescence bud and fruit.

The study showed an up-regulation of auxin-related genes during the "off" season and a drastic down-regulation in “ON” fruits consistent with the down-regulation previously observed in “ON” inflorescence buds (Benny et al. 2020) which strongly supports the hormonal involvement in the alternate bearing. Interestingly auxin is implicated in a substantial cross-talk with TOR signaling pathway, that resulted up-regulated in off fruits, auxin-mediated activation of TOR leads to the translation of specific messages (Schepetilnikov et al., 2013), can reduce stress-mediated autophagy (Pu et al., 2017), and it is involved in meristem activation (Li et al., 2017). It is also evident that auxin exert inhibitory effects on cytokinin pathway and signaling mechanisms, as it has been reported in different studies (Gündeşli et al. 2020).

Cytokinin in ON fruit shows a strong up-regulation and subsequent increase of the sink strength in competition with inflorescence buds in ON shoots which were found having down-regulated cytokinin (Benny et al. 2020). This can be assumed like a sign of competition happening between pistachio fruits and inflorescence buds during the “ON” and “OFF” season. Sink competition due to lack of nutrients can induce oxidative stress and ROS accumulation leading to autophagy in inflorescence buds (Benny et al. 2020).

Interestingly ET responsive genes, including transcription factors regulating the expression of genes involved in the allocation of Carbon into oil are up-regulated in “ON” fruits, confirming their sink strength. Nevertheless, it has been found that endogenous free polyamines, especially spermine and spermidine, have a regulatory role and negative correlation with the inflorescence bud abscission (Roussos et al. 2004), and exogenous application of free polyamines significantly decreased the flower bud abscission (Baninasab and Rahemi 2008). The study showed significant results that prove the negative correlation

between polyamine and bud abscission in pistachio trees. The competition between polyamines and ethylene pathways for S-adenosyl methionine can result in a mechanism that can modulate physiological events, including senescence and inflorescence bud abscission.
Stevenson and Shackel (1998) suggested that selecting a cultivar with low alternate bearing behavior should be physiologically achievable only if the alternate bearing trait is not caused by limited carbohydrate availability. The previous study on inflorescence bud proved that Raffinose synthase gene (Raffinose synthase 5 (RS5), and MIOX2 showed repression in the "OFF" buds, but sugar alcohols, such as callose synthase and trehalose-6-phosphate synthase 11, showed an up-regulation. Whereas these gene showed exact opposite expression pattern in "OFF" fruits proved that a competition between fruits and inflorescence buds for carbohydrate occurs which in turn proved its importance in alternate bearing.

5.12. Conclusion

From my findings, it is evident that main leading causes of premature inflorescence bud abscission is the shortage of nutrients. This study substantially confirmed what emerged in the transcriptomic study conducted on inflorescence buds of Pistachio. It is evident that the lack of nutrients triggers a competition between sinks, and the fruit is the strongest sink. the lack of carbohydrates influences the genes that control the availability, the allocation, and the partition of carbohydrates among sinks. Sugars and hormones signal nutritional stress and trigger a cascade that leads to the abscission of weak sinks such as inflorescence buds in over-loaded "ON" shoots.

GENERAL CONCLUSION

My analysis has provided definitive information about molecular regulatory networks controlling resistance/tolerance/susceptibility towards major abiotic stresses in plants. This can be quickly transferred in molecular tools for crop breeding. The plant responses towards drought stress should be through the induction of the biosynthesis of key hormones such as ABA and ethylene driving the activation of key signalling proteins (ERF1, ABA2 and HB7). These proteins should promote the fine-tuned transcriptional modulation through the crosstalk of a complex network of transcription factors. Relating to transcription factors, I found that different categories are involved in specific responses to abiotic stresses: AP2-EREBP, MADS, WRKY22, MYB, homeobox genes members were linked to drought stress while cold stress was associated to induction of MYB7 and BELL 1. Heat repressed C2C2-CO-LIKE, MADS and HOMEOBOX3. Last important findings of my meta-analysis were:

- induction of ubiquitin-mediated protein degradation by heat
- up-regulation of MAP Kinases by cold stress

The up-regulation of key proteins in the signal transduction should provoke the induction of proteins involved in physiological defensive responses represented by stomatal closure, inhibition of fatty acid biosynthesis, an increase of osmotic potential and protection of protein folding.

The work on the transcriptome analysis of the *Pistacia vera* inflorescence buds in bearing and non-bearing shoots reveals the molecular mechanism causing premature flower bud abscission. This work concludes that, in the “OFF” inflorescence buds of June, the genes corresponding to carbohydrate show reduction compared to June “ON” inflorescence bud. Furthermore, there is a higher amount of accumulation of starch (BETA-AMYLASE 7, ALPHA-AMYLASE 3 and Fructosidase 4), nitrogen and potassium in June “OFF” compared to June “ON”. The hormones such as ethylene and gibberellin are showing down-regulation and ABA, IAA and Jasmonate are showing up-regulation when compared with June “ON” inflorescence buds; I can conclude that these hormones play an important role in the production of Nitrogen oxide, Polyamine and H2O2, which eventually target cell death and autophagy during the June “ON” period.

The final study on the Pistacia vera fruits of “ON” and “OFF” shoots of the cultivar Bianca complete the experiment 4 on buds and gives further insight into the nutritional factors and hormonal factors involved. From my findings, it is evident that main leading causes of premature inflorescence bud abscission is the shortage of nutrients. Hormone applications may mitigate the phenomenon; however, accurate management of resources like carbohydrates and mineral elements directly or indirectly linked to the mechanism can modulate the rate of alternating production. At the same time, the finding of putative biomarkers, in the future, may lead to a reduction of the inflorescence buds and the possibility to balance the alternate bearing phenomenon.

Acknowledgement

I praise God, the Almighty for providing me with this opportunity and granting me the capability to proceed successfully. I am very grateful to the many people who have been such great inspiration, support and help to me during this project, and my graduate career.

First and foremost, I am extremely grateful to my doctoral supervisor Prof. Tiziano Caruso and co-supervisor Dr Annalisa Marchese, for their invaluable support, scientific supervision, hard work, constant guidance, cooperation, patience, advice, and motivation. Professor Tiziano Caruso has been a vital part to all my doctoral successes, and a great role model and inspiration for all students in academia. I am also very grateful to Dr. Annalisa Marchese for helping me enormously for the improvisation and final formatting of this dissertation.

I would like to thank Prof. Francesco Paolo Marra and Prof. Federico Martinelli for continuing to support me beyond my PhD project and entrusting me with instructing their knowledge in alternative bearing mechanism in Pistachio.

Thank you to my PhD coordinator, Professor Vincenzo Bagarello and Carmelo Priolo for supported me throughout the doctoral process.

Thank you to Professor Wendy Harwood for her support and words of encouragement and for giving me the possibility to work in her laboratory at the Norwich Research Park, Norwich.

Thank you to my lab colleagues Bipin, Silvia, Giovanna, Emilio, Rafael, and Sebastiano for their support.

And last but certainly not least, my family, especially my husband who has supported me throughout my academic. Without their tremendous understanding and encouragement in the past few years, it would be impossible for me to complete my study.

REFERENCES

1. Adam, G., & Marquardt, V. (1986). Brassinosteroids. *Phytochemistry*, *25*(8), 1787–1799. https://doi.org/10.1016/s0031-9422(00)81151-6
2. Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., van Arkel, G., & Pereira, A. (2004). The SHINE Clade of AP2 Domain Transcription Factors Activates Wax Biosynthesis, Alters Cuticle Properties, and Confers Drought Tolerance when Overexpressed in Arabidopsis. *The Plant Cell*, *16*(9), 2463–2480. https://doi.org/10.1105/tpc.104.022897
3. Alcazar, R., Marco, F., Cuevas, J., Patron, M., Fernanado, A., & Carrasco, P. (2006). Involvement of polyamines in plant response to a biotic stress. *Biotechnology Letters*, *28*, 1867. https://doi.org/10.1071/bl19047
4. Alexandersson, E., Danielson, J. Å. H., Råde, J., Moparthi, V. K., Fontes, M., Kjellbom, P., & Johanson, U. (2010). Transcriptional regulation of aquaporins in accessions of Arabidopsis in response to drought stress. *The Plant Journal*, *61*(4), 650–660. https://doi.org/10.1111/j.1365-313x.2009.04087.x
5. Alvarez, J. M., Riveras, E., Vidal, E. A., Gras, D. E., Contreras-López, O., Tamayo, K. P., Aceituno, F., Gómez, I., Ruffel, S., Lejay, L., Jordana, X., & Gutiérrez, R. A. (2014). Systems approach identifies TGA1 and TGA4 transcription factors as important regulatory components of the nitrate response of *Arabidopsis thaliana* roots. *The Plant Journal*, *80*(1), 1–13. https://doi.org/10.1111/tpj.12618
6. Amirbakhtiar, N., Ismaili, A., Ghaffari, M. R., Nazarian Firouzabadi, F., & Shobbar, Z.-S. (2019). Transcriptome response of roots to salt stress in a salinity-tolerant bread wheat cultivar. *PLOS ONE*, *14*(3), e0213305. https://doi.org/10.1371/journal.pone.0213305
7. Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Nature Precedings*, 4282. https://doi.org/10.1038/npre.2010.4282.2
8. Ando, S., Sato, Y., Kamachi, S. ’., & Sakai, S. (2001). Isolation of a MADS-box gene (ERAF17) and correlation of its expression with the induction of formation of female flowers by ethylene in cucumber plants (*Cucumis sativus* L.). *Planta*, *213*(6), 943–952. https://doi.org/10.1007/s004250100571
9. Anh, T. P. T., Borrel-Flood, C., Vieira da Silva, J., Marie Justin, A., & Mazliak, P. (1985a). Effects of water stress on lipid metabolism in cotton leaves. *Phytochemistry*, *24*(4), 723–727. https://doi.org/10.1016/s0031-9422(00)84884-0
10. Ashraf, M., & Foolad, M. R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, *59*(2), 206–216. https://doi.org/10.1016/j.envexpbot.2005.12.006
11. Balan, B., Caruso, T., & Martinelli, F. (2017). Gaining Insight into Exclusive and Common Transcriptomic Features Linked with Biotic Stress Responses in Malus. *Frontiers in Plant Science*, *8*, 1569. https://doi.org/10.3389/fpls.2017.01569

12. Balan, B., Marra, F. P., Caruso, T., & Martinelli, F. (2018). Transcriptomic responses to biotic stresses in *Malus x domestica*: a meta-analysis study. *Scientific Reports*, *8*(1), 19348. https://doi.org/10.1038/s41598-018-19348-4
13. Banerjee, A., & Roychoudhury, A. (2015). WRKY Proteins: Signaling and Regulation of Expression during Abiotic Stress Responses. *The Scientific World Journal*, *2015*, 1–17. https://doi.org/10.1155/2015/807560
14. Bari, R., & Jones, J. D. G. (2008). Role of plant hormones in plant defence responses. *Plant Molecular Biology*, *69*(4), 473–488. https://doi.org/10.1007/s11103-008-9435-0
15. Bastola, D. R., & Pethe. (1998). A novel zinc-finger protein in alfalfa roots that binds to promoter elements in the salt-inducible MsPRP2 gene. *Plant Molecular Biology*, *38*, 1123. https://doi.org/10.1093/nar/pmb734
16. Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x
17. Benny, J., Marra, F. P., Giovino, A., Balan, B., Caruso, T., Martinelli, F., & Marchese, A. (2020). Transcriptome Analysis of *Pistacia vera* Inflorescence Buds in Bearing and Non-Bearing Shoots Reveals the Molecular Mechanism Causing Premature Flower Bud Abscission. *Genes*, *11*(8), 851. https://doi.org/10.3390/genes11080851
18. Benny, J., Perrone, A., Marra, F. P., Pisciotta, A., Caruso, T., & Martinelli, F. (2019a). Deciphering transcriptional regulation mechanisms underlining fruit development and ripening in Vitis vinifera. *Journal of Berry Research*, *9*(4), 641–664. https://doi.org/10.3233/jbr-190418
19. Benny, J., Pisciotta, A., Caruso, T., & Martinelli, F. (2019a). Identification of key genes and its chromosome regions linked to drought responses in leaves across different crops through meta-analysis of RNA-Seq data. *BMC Plant Biology*, *19*(1), 1794. https://doi.org/10.1186/s12870-019-1794-y
20. Bhattacharjee, A., Khurana, J. P., & Jain, M. (2016). Characterization of Rice Homeobox Genes, OsHOX22 and OsHOX24, and Over-expression of OsHOX24 in Transgenic Arabidopsis Suggest Their Role in Abiotic Stress Response. *Frontiers in Plant Science*, *7*, 627. https://doi.org/10.3389/fpls.2016.00627
21. Boudsocq, M., & Laurière, C. (2005). Osmotic Signaling in Plants. Multiple Pathways Mediated by Emerging Kinase Families. *Plant Physiology*, *138*(3), 1185–1194. https://doi.org/10.1104/pp.105.061275
22. Cai, H., Yang, S., Yan, Y., Xiao, Z., Cheng, J., Wu, J., Qiu, A., Lai, Y., Mou, S., Guan, D., Huang, R., & He, S. (2015). CaWRKY6 transcriptionally activates CaWRKY40, regulates *Ralstonia solanacearum* resistance, and confers high-temperature and high-humidity tolerance in pepper. *Journal of Experimental Botany*, *66*(11), 3163–3174. https://doi.org/10.1093/jxb/erv125

23. Campo, S., Baldrich, P., Messeguer, J., Lalanne, E., Coca, M., & San Segundo, B. (2014). Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation. *Plant Physiology*, *165*(2), 688–704. https://doi.org/10.1104/pp.113.230268
24. Cao, L., Yu, Y., Ding, X., Zhu, D., Yang, F., Liu, B., Sun, X., Duan, X., Yin, K., & Zhu, Y. (2017). The Glycine soja NAC transcription factor GsNAC019 mediates the regulation of plant alkaline tolerance and ABA sensitivity. *Plant Molecular Biology*, *95*(3), 253–268. https://doi.org/10.1007/s11103-017-0643-3
25. Causier, B. (2002). PLANT BIOLOGY: MADS-Box Genes Reach Maturity. *Science*, *296*(5566), 275–276. https://doi.org/10.1126/science.1071401
26. Cazzonelli, C. I. (2011). Carotenoids in nature: insights from plants and beyond. *Functional Plant Biology*, *38*(11), 833. https://doi.org/10.1071/fp11192
27. Chazen, O., & Neumann, P. M. (1994). Hydraulic Signals from the Roots and Rapid Cell-Wall Hardening in Growing Maize (*Zea mays* L.) Leaves Are Primary Responses to Polyethylene Glycol-Induced Water Deficits. *Plant Physiology*, *104*(4), 1385–1392. https://doi.org/10.1104/pp.104.4.1385
28. Chen, L., Zhang, L., Li, D., Wang, F., & Yu, D. (2013). WRKY8 transcription factor functions in the TMV-cg defense response by mediating both abscisic acid and ethylene signaling in Arabidopsis. *Proceedings of the National Academy of Sciences*, *110*(21), E1963–E1971. https://doi.org/10.1073/pnas.1221347110
29. Chen, T.-T., Liu, F.-F., Xiao, D.-W., Jiang, X.-Y., Li, P., Zhao, S.-M., Hou, B.-, & Li, Y.-. (2020). The Arabidopsis UDP-glycosyltransferase75B1, conjugates abscisic acid and affects plant response to abiotic stresses. *Plant Molecular Biology*, *102*(4–5), 389–401. https://doi.org/10.1007/s11103-019-00953-4
30. Chen, X., Lin, S., Liu, Q., Huang, J., Zhang, W., Lin, J., Wang, Y., Ke, Y., & He, H. (2014). Expression and interaction of small heat shock proteins (sHsps) in rice in response to heat stress. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1844*(4), 818–828. https://doi.org/10.1016/j.bbapap.2014.02.010
31. Chen, Y.-F., Li, L.-Q., Xu, Q., Kong, Y.-H., Wang, H., & Wu, W.-H. (2009). The WRKY6 Transcription Factor Modulates PHOSPHATE1 Expression in Response to Low Pi Stress in Arabidopsis. *The Plant Cell*, *21*(11), 3554–3566. https://doi.org/10.1105/tpc.108.064980
32. Cheng, M.-C., Liao, P.-M., Kuo, W.-W., & Lin, T.-P. (2013). The Arabidopsis ETHYLENE RESPONSE FACTOR1 Regulates Abiotic Stress-Responsive Gene Expression by Binding to Different cis-Acting Elements in Response to Different Stress Signals. *PLANT PHYSIOLOGY*, *162*(3), 1566–1582. https://doi.org/10.1104/pp.113.221911
33. Cheong, Y. H., Pandey, G. K., Grant, J. J., Batistic, O., Li, L., Kim, B.-G., Lee, S.-C., Kudla, J., & Luan, S. (2007). Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in

Arabidopsis. *The Plant Journal*, *52*(2), 223–239. https://doi.org/10.1111/j.1365-313x.2007.03236.x

34. Cimò, G., Marchese, A., & Germanà, M. A. (2016). Microspore embryogenesis induced through in vitro anther culture of almond (*Prunus dulcis* Mill.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *128*(1), 85–95. https://doi.org/10.1007/s11240-016-1086-2
35. Clauw, P., Coppens, F., De Beuf, K., Dhondt, S., Van Daele, T., Maleux, K., Storme, V., Clement, L., Gonzalez, N., & Inzé, D. (2015a). Leaf Responses to Mild Drought Stress in Natural Variants of Arabidopsis. *Plant Physiology*, *167*(3), 800–816. https://doi.org/10.1104/pp.114.254284
36. Clouse, S. D., & Sasse, J. M. (1998). BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development. *Annual Review of Plant Physiology and Plant Molecular Biology*, *49*(1), 427–451. https://doi.org/10.1146/annurev.arplant.49.1.427
37. Colebrook, E. H., Thomas, S. G., Phillips, A. L., & Hedden, P. (2013). The role of gibberellin signalling in plant responses to abiotic stress. *Journal of Experimental Biology*, *217*(1), 67–75. https://doi.org/10.1242/jeb.089938
38. Commisso, M., Toffali, K., Strazzer, P., Stocchero, M., Ceoldo, S., Baldan, B., Levi, M., & Guzzo, F. (2016). Impact of Phenylpropanoid Compounds on Heat Stress Tolerance in Carrot Cell Cultures. *Frontiers in Plant Science*, *7*, 1439. https://doi.org/10.3389/fpls.2016.01439
39. Corso, M., Vannozzi, A., Maza, E., Vitulo, N., Meggio, F., Pitacco, A., Telatin, A., D'Angelo, M., Feltrin, E., Negri, A. S., Prinsi, B., Valle, G., Ramina, A., Bouzayen, M., Bonghi, C., & Lucchin, M. (2015a). Comprehensive transcript profiling of two grapevine rootstock genotypes contrasting in drought susceptibility links the phenylpropanoid pathway to enhanced tolerance. *Journal of Experimental Botany*, *66*(19), 5739–5752. https://doi.org/10.1093/jxb/erv274
40. Creelman, R. A., & Mullet, J. E. (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences*, *92*(10), 4114–4119. https://doi.org/10.1073/pnas.92.10.4114
41. Cuellar-Ortiz, Soniam, De La Paz Arrieta-Montiel, Maria, Acosta-Gallegos, Jorge, & Covarrubias, Alenjandraa (2008). Relationship between carbohydrate partitioning and drought resistance in common bean. *Plant, Cell & Environment*, *31*(10), 1399–1409. https://doi.org/10.1111/j.1365-3040.2008.01853.x
42. Dai, X., Wang, Y., & Zhang, W.-H. (2015). OsWRKY74, a WRKY transcription factor, modulates tolerance to phosphate starvation in rice. *Journal of Experimental Botany*, *67*(3), 947–960. https://doi.org/10.1093/jxb/erv515
43. Dai, X., Xu, Y., Ma, Q., Xu, W., Wang, T., Xue, Y., & Chong, K. (2007a). Overexpression of an R1R2R3 MYB Gene, OsMYB3R-2, Increases Tolerance to Freezing, Drought, and Salt Stress in Transgenic Arabidopsis. *Plant Physiology*, *143*(4), 1739–1751. https://doi.org/10.1104/pp.106.094532

44. Dai, X., Xu, Y., Ma, Q., Xu, W., Wang, T., Xue, Y., & Chong, K. (2007b). Overexpression of an R1R2R3 MYB Gene, OsMYB3R-2, Increases Tolerance to Freezing, Drought, and Salt Stress in Transgenic Arabidopsis. *Plant Physiology*, *143*(4), 1739–1751. https://doi.org/10.1104/pp.106.094532
45. Dalal, M., Sahu, S., Tiwari, S., Rao, A. R., & Gaikwad, K. (2018). Transcriptome analysis reveals interplay between hormones, ROS metabolism and cell wall biosynthesis for drought-induced root growth in wheat. *Plant Physiology and Biochemistry*, *130*, 482–492. https://doi.org/10.1016/j.plaphy.2018.07.035
46. Davenport, R. J., Reid, R. J., & Smith, F. A. (1997). Sodium-calcium interactions in two wheat species differing in salinity tolerance. *Physiologia Plantarum*, *99*(2), 323–327. https://doi.org/10.1111/j.1399-3054.1997.tb05419.x
47. Deokar, A. A., Kondawar, V., Jain, P. K., Karuppayil, S. M., Raju, N. L., Vadez, V., Varshney, R. K., & Srinivasan, R. (2011). Comparative analysis of expressed sequence tags (ESTs) between drought-tolerant and -susceptible genotypes of chickpea under terminal drought stress. *BMC Plant Biology*, *11*(1), 70. https://doi.org/10.1186/1471-2229-11-70
48. Dey, S., Wenig, M., Langen, G., Sharma, S., Kugler, K. G., Knappe, C., Hause, B., Bichlmeier, M., Babaeizad, V., Imani, J., Janzik, I., Stempfl, T., Hückelhoven, R., Kogel, K.-H., Mayer, K. F. X., & Vlot, A. C. (2014). Bacteria-Triggered Systemic Immunity in Barley Is Associated with WRKY and ETHYLENE RESPONSIVE FACTORs But Not with Salicylic Acid. *Plant Physiology*, *166*(4), 2133–2151. https://doi.org/10.1104/pp.114.249276
49. Dezar, C. A., Fedrigo, G. V., & Chan, R. L. (2005). The promoter of the sunflower HD-Zip protein gene Hahb4 directs tissue-specific expression and is inducible by water stress, high salt concentrations and ABA. *Plant Science*, *169*(2), 447–456. https://doi.org/10.1016/j.plantsci.2005.04.008
50. Dietz, K.-J., Vogel, M. O., & Viehhauser, A. (2010). AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signalling. *Protoplasma*, *245*(1–4), 3–14. https://doi.org/10.1007/s00709-010-0142-8
51. Ding, Y., Liu, N., Virlouvet, L., Riethoven, J.-J., Fromm, M., & Avramova, Z. (2013). Four distinct types of dehydration stress memory genes in *Arabidopsis thaliana. BMC Plant Biology*, *13*(1), 229. https://doi.org/10.1186/1471-2229-13-229
52. Doerks, T., Strauss, M., Brendel, M., & Bork, P. (2000). GRAM, a novel domain in glucosyltransferases, myotubularins and other putative membrane-associated proteins. *Trends in Biochemical Sciences*, *25*(10), 483–485. https://doi.org/10.1016/s0968-0004(00)01664-9
53. Duan, K., Li, L., Hu, P., Xu, S.-P., Xu, Z.-H., & Xue, H.-W. (2006). A brassinolide-suppressed rice MADS-box transcription factor, OsMDP1, has a negative regulatory role in BR signaling. *The Plant Journal*, *47*(4), 519–531. https://doi.org/10.1111/j.1365-313x.2006.02804.x

54. Egamberdieva, D. (2009). Alleviation of salt stress by plant growth regulators and IAA producing bacteria in wheat. *Acta Physiologiae Plantarum*, *31*(4), 861–864. https://doi.org/10.1007/s11738-009-0297-0
55. Evans, T. I. A., Hell, J. W., & Shea, M. A. (2011). Thermodynamic linkage between calmodulin domains binding calcium and contiguous sites in the C-terminal tail of CaV1.2. *Biophysical Chemistry*, *159*(1), 172–187. https://doi.org/10.1016/j.bpc.2011.06.007
56. Fàbregas, N., Lozano-Elena, F., Blasco-Escámez, D., Tohge, T., Martínez-Andújar, C., Albacete, A., Osorio, S., Bustamante, M., Riechmann, J. L., Nomura, T., Yokota, T., Conesa, A., Alfocea, F. P., Fernie, A. R., & Caño-Delgado, A. I. (2018). Overexpression of the vascular brassinosteroid receptor BRL3 confers drought resistance without penalizing plant growth. *Nature Communications*, *9*(1), 200. https://doi.org/10.1038/s41467-018-06861-3
57. Fard, E. M., Bakhshi, B., Keshavarznia, R., Nikpay, N., Shahbazi, M., & Salekdeh, G. H. (2017). Drought responsive microRNAs in two barley cultivars differing in their level of sensitivity to drought stress. *Plant Physiology and Biochemistry*, *118*, 121–129. https://doi.org/10.1016/j.plaphy.2017.06.007
58. Farmer, E. E., & Mueller, M. J. (2013). ROS-Mediated Lipid Peroxidation and RES-Activated Signaling. *Annual Review of Plant Biology*, *64*(1), 429–450. https://doi.org/10.1146/annurev-arplant-050312-120132
59. Feng, Y., Liang, C., Li, B., Wan, T., Liu, T., & Cai, Y. (2017). Differential expression profiles and pathways of genes in drought resistant tree species *Prunus mahaleb* roots and leaves in response to drought stress. *Scientia Horticulturae*, *226*, 75–84. https://doi.org/10.1016/j.scienta.2017.07.057
60. Fernández Gómez, J., & Wilson, Z. A. (2014). A barley PHD finger transcription factor that confers male sterility by affecting tapetal development. *Plant Biotechnology Journal*, *12*(6), 765–777. https://doi.org/10.1111/pbi.12181
61. Flowers, T. J., & Colmer, T. D. (2015). Plant salt tolerance: adaptations in halophytes. *Annals of Botany*, *115*(3), 327–331. https://doi.org/10.1093/aob/mcu267
62. Fogacci, F., Cicero, A. F. G., Derosa, G., Rizzo, M., Veronesi, M., & Borghi, C. (2017). Effect of pistachio on brachial artery diameter and flow-mediated dilatation: A systematic review and meta-analysis of randomized, controlled-feeding clinical studies. *Critical Reviews in Food Science and Nutrition*, *59*(2), 328–335. https://doi.org/10.1080/10408398.2017.1368007
63. Forestan, C., Aiese Cigliano, R., Farinati, S., Lunardon, A., Sanseverino, W., & Varotto, S. (2016). Stress-induced and epigenetic-mediated maize transcriptome regulation study by means of transcriptome reannotation and differential expression analysis. *Scientific Reports*, *6*(1), 30446. https://doi.org/10.1038/srep30446
64. FW, Y., & Feng, X. Q. (2015). Abscisic acid biosynthesis and catabolism and their regulation roles in fruit ripening. *Phyton*, *84*(2), 444–453. https://doi.org/10.32604/phyton.2015.84.444

65. Gavidia, I., Pérez-Bermúdez, P., & Seitz, H. U. (2002). Cloning and expression of two novel aldo-keto reductases from *Digitalis purpurea* leaves. *European Journal of Biochemistry*, *269*(12), 2842–2850. https://doi.org/10.1046/j.1432-1033.2002.02931.x
66. Gentile, C., Reig, C., Corona, O., Todaro, A., Mazzaglia, A., Perrone, A., Gianguzzi, G., Agusti, M., & Farina, V. (2016). Pomological Traits, Sensory Profile and Nutraceutical Properties of Nine Cultivars of Loquat (*Eriobotrya japonica* Lindl.) Fruits Grown in Mediterranean Area. *Plant Foods for Human Nutrition*, *71*(3), 330–338. https://doi.org/10.1007/s11130-016-0564-3
67. Ghanashyam, C., & Jain, M. (2009). Role of auxin-responsive genes in biotic stress responses. *Plant Signaling & Behavior*, *4*(9), 846–848. https://doi.org/10.4161/psb.4.9.9376
68. Guo, L., Yang, H., Zhang, X., & Yang, S. (2013). Lipid transfer protein 3 as a target of MYB96 mediates freezing and drought stress in Arabidopsis. *Journal of Experimental Botany*, *64*(6), 1755–1767. https://doi.org/10.1093/jxb/ert040
69. Guo, Z.-J., Chen, X.-J., Wu, X.-L., Ling, J.-Q., & Xu, P. (2004). Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. *Plant Molecular Biology*, *55*(4), 607–618. https://doi.org/10.1007/s11103-004-1521-3
70. Gupta, S. K., Rai, A. K., Kanwar, S. S., Chand, D., Singh, N. K., & Sharma, T. R. (2011a). The single functional blast resistance gene Pi54 activates a complex defence mechanism in rice. *Journal of Experimental Botany*, *63*(2), 757–772. https://doi.org/10.1093/jxb/err297
71. Haider, M. S., Zhang, C., Kurjogi, M. M., Pervaiz, T., Zheng, T., Zhang, C., Lide, C., Shangguan, L., & Fang, J. (2017a). Insights into grapevine defense response against drought as revealed by biochemical, physiological and RNA-Seq analysis. *Scientific Reports*, *7*(1), 13464. https://doi.org/10.1038/s41598-017-13464-3
72. He, G.-H., Xu, J.-Y., Wang, Y.-X., Liu, J.-M., Li, P.-S., Chen, M., Ma, Y.-Z., & Xu, Z.-S. (2016). Drought-responsive WRKY transcription factor genes TaWRKY1 and TaWRKY33 from wheat confer drought and/or heat resistance in Arabidopsis. *BMC Plant Biology*, *16*(1), 806. https://doi.org/10.1186/s12870-016-0806-4
73. Hegedüs, A., Erdei, S., Janda, T., Tóth, E., Horváth, G., & Dudits, D. (2004). Transgenic tobacco plants overproducing alfalfa aldose/aldehyde reductase show higher tolerance to low temperature and cadmium stress. *Plant Science*, *166*(5), 1329–1333. https://doi.org/10.1016/j.plantsci.2004.01.013
74. HIDEG, É., NAGY, T., OBERSCHALL, A., DUDITS, D., & VASS, I. (2003). Detoxification function of aldose/aldehyde reductase during drought and ultraviolet-B (280-320 nm) stresses. *Plant, Cell & Environment*, *26*(4), 513–522. https://doi.org/10.1046/j.1365-3040.2003.00982.x
75. Hoang, X. L. T., Nhi, D. N. H., Thu, N. B. A., Thao, N. P., & Tran, L.-S. P. (2017). Transcription Factors and Their Roles in Signal Transduction in Plants under Abiotic

Stresses. *Current Genomics*, *18*(6), 600. https://doi.org/10.2174/1389202918666170227150057

76. Hu, T., Wang, Y., Wang, Q., Dang, N., Wang, L., Liu, C., Zhu, J., & Zhan, X. (2019). The tomato 2-oxoglutarate-dependent dioxygenase gene SlF3HL is critical for chilling stress tolerance. *Horticulture Research*, *6*(1), 127. https://doi.org/10.1038/s41438-019-0127-5
77. Hu, Z., Wang, R., Zheng, M., Liu, X., Meng, F., Wu, H., Yao, Y., Xin, M., Peng, H., Ni, Z., & Sun, Q. (2018). TaWRKY51 promotes lateral root formation through negative regulation of ethylene biosynthesis in wheat (*Triticum aestivum* L.). *The Plant Journal*, *96*(2), 372–388. https://doi.org/10.1111/tpj.14038
78. Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2008a). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*(1), 44–57. https://doi.org/10.1038/nprot.2008.211
79. Huang, X.-S., Liu, J.-H., & Chen, X.-J. (2010). Overexpression of PtrABF gene, a bZIP transcription factor isolated from *Poncirus trifoliata*, enhances dehydration and drought tolerance in tobacco via scavenging ROS and modulating expression of stress-responsive genes. *BMC Plant Biology*, *10*(1), 230. https://doi.org/10.1186/1471-2229-10-230
80. Huang, Z., Long, X., Wang, L., Kang, J., Zhang, Z., Zed, R., & Liu, Z. (2012). Growth, photosynthesis and H+-ATPase activity in two Jerusalem artichoke varieties under NaCl-induced stress. *Process Biochemistry*, *47*(4), 591–596. https://doi.org/10.1016/j.procbio.2011.12.016
81. Imran, Q. M., Hussain, A., Lee, S.-U., Mun, B.-G., Falak, N., Loake, G. J., & Yun, B.-W. (2018). Transcriptome profile of NO-induced Arabidopsis transcription factor genes suggests their putative regulatory role in multiple biological processes. *Scientific Reports*, *8*(1), 18850. https://doi.org/10.1038/s41598-017-18850-5
82. Iqbal, N., Umar, S., Khan, N. A., & Khan, M. I. R. (2014). A new perspective of phytohormones in salinity tolerance: Regulation of proline metabolism. *Environmental and Experimental Botany*, *100*, 34–42. https://doi.org/10.1016/j.envexpbot.2013.12.006
83. Janiak, A., Kwaśniewski, M. ł., & Szarejko, I. (2015a). Gene expression regulation in roots under drought. *Journal of Experimental Botany*, *67*(4), 1003–1014. https://doi.org/10.1093/jxb/erv512
84. Janiak, A., Kwasniewski, M., Sowa, M., Gajek, K., Żmuda, K., Kościelniak, J., & Szarejko, I. (2018). No Time to Waste: Transcriptome Study Reveals that Drought Tolerance in Barley May Be Attributed to Stressed-Like Expression Patterns that Exist before the Occurrence of Stress. *Frontiers in Plant Science*, *8*, 2212. https://doi.org/10.3389/fpls.2017.02212
85. Javid, M. G., & Sorooshzadeh, A. (2001). The role of phytohormones in alleviating salt stress in crop plants. *Australian Journal of Crop Science*, *5*(1), 726. https://doi.org/10.1186/1999-3110-55-38

86. Jia, J., Zhao, P., Cheng, L., Yuan, G., Yang, W., Liu, S., Chen, S., Qi, D., Liu, G., & Li, X. (2018). MADS-box family genes in sheepgrass and their involvement in abiotic stress responses. *BMC Plant Biology*, *18*(1), 1259. https://doi.org/10.1186/s12870-018-1259-8
87. Jiang, W., & Yu, D. (2009). Arabidopsis WRKY2 transcription factor mediates seed germination and postgermination arrest of development by abscisic acid. *BMC Plant Biology*, *9*(1), 96. https://doi.org/10.1186/1471-2229-9-96
88. Joo, J., Lee, Y. H., Kim, Y.-K., Nahm, B. H., & Song, S. I. (2013). Abiotic stress responsive rice ASR1 and ASR3 exhibit different tissue-dependent sugar and hormone-sensitivities. *Molecules and Cells*, *35*(5), 421–435. https://doi.org/10.1007/s10059-013-0036-7
89. Kang, J., Hwang, J.-U., Lee, M., Kim, Y.-Y., Assmann, S. M., Martinoia, E., & Lee, Y. (2010). PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. *Proceedings of the National Academy of Sciences*, *107*(5), 2355–2360. https://doi.org/10.1073/pnas.0909222107
90. Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., & Bohnert, H. J. (2001). Gene Expression Profiles during the Initial Phase of Salt Stress in Rice. *The Plant Cell*, *13*(4), 889. https://doi.org/10.2307/3871347
91. Kawasaki, T., Koita, H., Nakatsubo, T., Hasegawa, K., Wakabayashi, K., Takahashi, H., Umemura, K., Umezawa, T., & Shimamoto, K. (2005). Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. *Proceedings of the National Academy of Sciences*, *103*(1), 230–235. https://doi.org/10.1073/pnas.0509875103
92. Khadka, V. S., Vaughn, K., Xie, J., Swaminathan, P., Ma, Q., Cramer, G. R., & Fennell, A. Y. (2019). Transcriptomic response is more sensitive to water deficit in shoots than roots of *Vitis riparia* (Michx.). *BMC Plant Biology*, *19*(1), 19. https://doi.org/10.1186/s12870-019-1664-7
93. Khan, S., Anwar, S., Yu, S., Sun, M., Yang, Z., & Gao, Z.-. (2019). Development of Drought-Tolerant Transgenic Wheat: Achievements and Limitations. *International Journal of Molecular Sciences*, *20*(13), 3350. https://doi.org/10.3390/ijms20133350
94. Khong, G. N., Pati, P. K., Richaud, F., Parizot, B., Bidzinski, P., Mai, C. D., Bès, M., Bourrié, I., Meynard, D., Beeckman, T., Selvaraj, M. G., Manabu, I., Genga, A., BRUGIDOU, C., Do, V. N., Guiderdoni, E., Morel, J.-B., & Gantet, P. (2015). OsMADS26 negatively regulates resistance to pathogens and drought tolerance in rice. *Plant Physiology*, pp.01192.2015. https://doi.org/10.1104/pp.15.01192
95. Khoo, H.-E., Prasad, K. N., Kong, K.-W., Jiang, Y., & Ismail, A. (2011). Carotenoids and Their Isomers: Color Pigments in Fruits and Vegetables. *Molecules*, *16*(2), 1710–1738. https://doi.org/10.3390/molecules16021710

96. Kim, D., Langmead, B., & Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nature Methods*, *12*(4), 357–360. https://doi.org/10.1038/nmeth.3317
97. Kim, E. H., Kim, Y. S., Park, S.-H., Koo, Y. J., Choi, Y. D., Chung, Y.-Y., Lee, I.-J., & Kim, J.-K. (2009). Methyl Jasmonate Reduces Grain Yield by Mediating Stress Signals to Alter Spikelet Development in Rice. *Plant Physiology*, *149*(4), 1751–1760. https://doi.org/10.1104/pp.108.134684
98. Kim, J. K., Bamba, T., Harada, K., Fukusaki, E., & Kobayashi, A. (2006). Time-course metabolic profiling in Arabidopsis thaliana cell cultures after salt stress treatment*. *Journal of Experimental Botany*, *58*(3), 415–424. https://doi.org/10.1093/jxb/erl216
99. Kim, J., Hyun, W., Nguyen, H., Jeong, C., Xiong, L., Hong, S., Lee, H. (2014). AtMyb7, a subgroup 4 R2R3 Myb, negatively regulates ABA-induced inhibition of seed germination by blocking the expression of the bZIP transcription factor ABI5. *Plant, Cell & Environment*, *38*(3), 559–571. https://doi.org/10.1111/pce.12415
100. Kim, S., Kang, J.-, Cho, D.-I., Park, J. H., & Kim, S. Y. (2004). ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *The Plant Journal*, *40*(1), 75–87. https://doi.org/10.1111/j.1365-313x.2004.02192.x
101. Kobayashi, Y., Yamamoto, S., Minami, H., Kagaya, Y., & Hattori, T. (2004). Differential Activation of the Rice Sucrose Nonfermenting1–Related Protein Kinase2 Family by Hyperosmotic Stress and Abscisic Acid. *The Plant Cell*, *16*(5), 1163–1177. https://doi.org/10.1105/tpc.019943
102. Kong, D., Li, M., Dong, Z., Ji, H., & Li, X. (2014). Identification of TaWD40D, a wheat WD40 repeat-containing protein that is associated with plant tolerance to abiotic stresses. *Plant Cell Reports*, *34*(3), 395–410. https://doi.org/10.1007/s00299-014-1717-1
103. Kramell, R., Miersch, O., Atzorn, R., Parthier, B., & Wasternack, C. (2000). Octadecanoid-Derived Alteration of Gene Expression and the "Oxylipin Signature" in Stressed Barley Leaves. Implications for Different Signaling Pathways. *Plant Physiology*, *123*(1), 177–188. https://doi.org/10.1104/pp.123.1.177
104. Krasensky, J., & Jonak, C. (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany*, *63*(4), 1593–1608. https://doi.org/10.1093/jxb/err460
105. Ksas, B., Becuwe, N., Chevalier, A., & Havaux, M. (2015). Plant tolerance to excess light energy and photooxidative damage relies on plastoquinone biosynthesis. *Scientific Reports*, *5*(1), 10919. https://doi.org/10.1038/srep10919
106. Ksouri, N., Jiménez, S., Wells, C. E., Contreras-Moreira, B., & Gogorcena, Y. (2016). Transcriptional Responses in Root and Leaf of *Prunus persica* under Drought Stress Using RNA Sequencing. *Frontiers in Plant Science*, *7*, 7. https://doi.org/10.3389/fpls.2016.01715

107. Kukurba, K. R., & Montgomery, S. B. (2015). RNA Sequencing and Analysis. *Cold Spring Harbor Protocols*, *2015*(11), pdb.top084970. https://doi.org/10.1101/pdb.top084970
108. Kwasniewski, M., Daszkowska-Golec, A., Janiak, A., Chwialkowska, K., Nowakowska, U., Sablok, G., & Szarejko, I. (2015). Transcriptome analysis reveals the role of the root hairs as environmental sensors to maintain plant functions under water-deficiency conditions. *Journal of Experimental Botany*, *67*(4), 1079–1094. https://doi.org/10.1093/jxb/erv498
109. Lauvergeat, V., Lacomme, C., Lacombe, E., Lasserre, E., Roby, D., & Grima-Pettenati, J. (2001). Two cinnamoyl-CoA reductase (CCR) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry*, *57*(7), 1187–1195. https://doi.org/10.1016/s0031-9422(01)00053-x
110. Lee, B.-R., Kim, K.-Y., Jung, W.-J., Avice, J.-C., Ourry, A., & Kim, T.-H. (2007). Peroxidases and lignification in relation to the intensity of water-deficit stress in white clover (*Trifolium repens* L.). *Journal of Experimental Botany*, *58*(6), 1271–1279. https://doi.org/10.1093/jxb/erl280
111. Lee, G. W., Lee, S., Chung, M.-S., Jeong, Y. S., & Chung, B. Y. (2014). Rice terpene synthase 20 (OsTPS20) plays an important role in producing terpene volatiles in response to abiotic stresses. *Protoplasma*, *252*(4), 997–1007. https://doi.org/10.1007/s00709-014-0735-8
112. Lee, S. B., Kim, H., Kim, R. J., & Suh, M. C. (2014a). Overexpression of Arabidopsis MYB96 confers drought resistance in *Camelina sativa* via cuticular wax accumulation. *Plant Cell Reports*, *33*(9), 1535–1546. https://doi.org/10.1007/s00299-014-1636-1
113. Lee, S. B., Kim, H. U., & Suh, M. C. (2016). MYB94 and MYB96 Additively Activate Cuticular Wax Biosynthesis in Arabidopsis. *Plant and Cell Physiology*, *57*(11), 2300–2311. https://doi.org/10.1093/pcp/pcw147
114. Lee, S., Choi, S. C., & An, G. (2008). Rice SVP-group MADS-box proteins, OsMADS22 and OsMADS55, are negative regulators of brassinosteroid responses. *The Plant Journal*, *54*(1), 93–105. https://doi.org/10.1111/j.1365-313x.2008.03406.x
115. Lee, S. P., & Chen, T. H. H. (1993). Molecular Cloning of Abscisic Acid-Responsive mRNAs Expressed during the Induction of Freezing Tolerance in Bromegrass (*Bromus inermis* Leyss) Suspension Culture. *Plant Physiology*, *101*(3), 1089–1096. https://doi.org/10.1104/pp.101.3.1089
116. LEE, T.-M., LUR, H.-S., LIN, Y.-H., & CHU, C. (1996). Physiological and biochemical changes related to methyl jasmonate-induced chilling tolerance of rice (*Oryza sativa* L.) seedlings. *Plant, Cell and Environment*, *19*(1), 65–74. https://doi.org/10.1111/j.1365-3040.1996.tb00227.x
117. Lee, W. Y., Lee, D., Chung, W.-I., & Kwon, C. S. (2009a). Arabidopsis ING and Alfin1-like protein families localize to the nucleus and bind to H3K4me3/2 via

plant homeodomain fingers. *The Plant Journal*, *58*(3), 511–524. https://doi.org/10.1111/j.1365-313x.2009.03795.x

118. Levonen A.L., Landar, A., Ramachandran, A., Ceaser, E.K., Dickinson, D. A., Zanoni, G., Morrow, J. D., & Darley-Usmar, V.M. (2004). Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochemical Journal*, *378*(2), 373–382. https://doi.org/10.1042/bj20031049

119. Li, B., & Foley, M. E. (1995). Cloning and characterization of differentially expressed genes in imbibed dormant and after ripened *Avena fatua* embryos. *Plant Molecular Biology*, *29*(4), 823–831. https://doi.org/10.1007/bf00041171

120. Li, C., Ng, C. K.-Y., & Fan, L.-M. (2015). MYB transcription factors, active players in abiotic stress signaling. *Environmental and Experimental Botany*, *114*, 80–91. https://doi.org/10.1016/j.envexpbot.2014.06.014

121. Li, E., Bhargava, A., Qiang, W., Friedmann, M. C., Forneris, N., Savidge, R. A., Johnson, L. A., Mansfield, S. D., Ellis, B. E., & Douglas, C. J. (2012). The Class II KNOX gene KNAT7 negatively regulates secondary wall formation in Arabidopsis and is functionally conserved in Populus. *New Phytologist*, *194*(1), 102–115. https://doi.org/10.1111/j.1469-8137.2011.04016.x

122. Li, F., Li, M., Wang, P., Cox, K. L., Duan, L., Dever, J. K., Shan, L., Li, Z., & He, P. (2017). Regulation of cotton (*Gossypium hirsutum*) drought responses by mitogen-activated protein (MAP) kinase cascade-mediated phosphorylation of GhWRKY59. *New Phytologist*, *215*(4), 1462–1475. https://doi.org/10.1111/nph.14680

123. Li, J., Nam, K. H., Vafeados, D., & Chory, J. (2001). BIN2, a New Brassinosteroid-Insensitive Locus in Arabidopsis. *Plant Physiology*, *127*(1), 14–22. https://doi.org/10.1104/pp.127.1.14

124. Li, P., Cao, W., Fang, H., Xu, S., Yin, S., Zhang, Y., Lin, D., Wang, J., Chen, Y., Xu, C., & Yang, Z. (2017a). Transcriptomic Profiling of the Maize (*Zea mays* L.) Leaf Response to Abiotic Stresses at the Seedling Stage. *Frontiers in Plant Science*, *8*, 290. https://doi.org/10.3389/fpls.2017.00290

125. Li, Q., Huo, Q., Wang, J., Zhao, J., Sun, K., & He, C. (2016). Expression of B-class MADS-box genes in response to variations in photoperiod is associated with chasmogamous and cleistogamous flower development in *Viola philippica*. *BMC Plant Biology*, *16*(1), 832. https://doi.org/10.1186/s12870-016-0832-2

126. Liang, Q.-, Wu, Y.-, Wang, K., Bai, Z.-, Liu, Q.-, Pan, Y.-, Zhang, L., & Jiang, B.-. (2017). *Chrysanthemum* WRKY gene DgWRKY5 enhances tolerance to salt stress in transgenic chrysanthemum. *Scientific Reports*, *7*(1), 5170. https://doi.org/10.1038/s41598-017-05170-x

127. Liu, C., & Zhang, T. (2017). Expansion and stress responses of the AP2/EREBP superfamily in cotton. *BMC Genomics*, *18*(1), 3517. https://doi.org/10.1186/s12864-017-3517-9

128. Liu, J., Osbourn, A., & Ma, P. (2015). MYB Transcription Factors as Regulators of Phenylpropanoid Metabolism in Plants. *Molecular Plant*, *8*(5), 689–708. https://doi.org/10.1016/j.molp.2015.03.012
129. Liu, M., Yu, H., Zhao, G., Huang, Q., Lu, Y., & Ouyang, B. (2017a). Profiling of drought-responsive microRNA and mRNA in tomato using high-throughput sequencing. *BMC Genomics*, *18*(1), 3869. https://doi.org/10.1186/s12864-017-3869-1
130. Liu, W. -. C., Li, Y. -. H., Yuan, H. -. M., Zhang, B. -. L., Zhai, S., & Lu, Y. -. T. (2016). WD40-REPEAT 5a functions in drought stress tolerance by regulating nitric oxide accumulation in Arabidopsis. *Plant, Cell & Environment*, *40*(4), 543–552. https://doi.org/10.1111/pce.12723
131. Liu, X. Q., Bai, X. Q., Qian, Q., Wang, X. J., Chen, M. S., & Chu, C. C. (2005). OsWRKY03, a rice transcriptional activator that functions in defense signaling pathway upstream of OsNPR1. *Cell Research*, *15*(8), 593–603. https://doi.org/10.1038/sj.cr.7290329
132. Liu, Z., Xin, M., Qin, J., Peng, H., Ni, Z., Yao, Y., & Sun, Q. (2015). Temporal transcriptome profiling reveals expression partitioning of homeologous genes contributing to heat and drought acclimation in wheat (*Triticum aestivum* L.). *BMC Plant Biology*, *15*(1), 511. https://doi.org/10.1186/s12870-015-0511-8
133. Loeffler, C., Berger, S., Guy, A., Durand, T., Bringmann, G., Dreyer, M., von Rad, U., Durner, J., & Mueller, M. J. (2004). B1-Phytoprostanes Trigger Plant Defense and Detoxification Responses. *Plant Physiology*, *137*(1), 328–340. https://doi.org/10.1104/pp.104.051714
134. López-González, L., Mouriz, A., Narro-Diego, L., Bustos, R., Martínez-Zapater, J. M., Jarillo, J. A., & Piñeiro, M. (2014). Chromatin-Dependent Repression of the Arabidopsis Floral Integrator Genes Involves Plant Specific PHD-Containing Proteins. *The Plant Cell*, *26*(10), 3922–3938. https://doi.org/10.1105/tpc.114.130781
135. Lozano, R., Angosto, T., Gómez, P., Payán, C., Capel, J., Huijser, P., Salinas, J., & Martínez-Zapater, J. M. (1998). Tomato Flower Abnormalities Induced by Low Temperatures Are Associated with Changes of Expression of MADS-Box Genes. *Plant Physiology*, *117*(1), 91–100. https://doi.org/10.1104/pp.117.1.91
136. Luo, X., Bai, X., Sun, X., Zhu, D., Liu, B., Ji, W., Cai, H., Cao, L., Wu, J., Hu, M., Liu, X., Tang, L., & Zhu, Y. (2013a). Expression of wild soybean WRKY20 in Arabidopsis enhances drought tolerance and regulates ABA signalling. *Journal of Experimental Botany*, *64*(8), 2155–2169. https://doi.org/10.1093/jxb/ert073
137. Lynch, J. P. (2013). Steep, cheap and deep: an ideotype to optimize water and N acquisition by maize root systems. *Annals of Botany*, *112*(2), 347–357. https://doi.org/10.1093/aob/mcs293
138. Lynch, J. P. (2018). Rightsizing root phenotypes for drought resistance. *Journal of Experimental Botany*, *69*(13), 3279–3292. https://doi.org/10.1093/jxb/ery048

139. Ma, Y., Dai, X., Xu, Y., Luo, W., Zheng, X., Zeng, D., Pan, Y., Lin, X., Liu, H., Zhang, D., Xiao, J., Guo, X., Xu, S., Niu, Y., Jin, J., Zhang, H., Xu, X., Li, L., Wang, W., … Chong, K. (2015). COLD1 Confers Chilling Tolerance in Rice. *Cell*, *160*(6), 1209–1221. https://doi.org/10.1016/j.cell.2015.01.046

140. Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., & Zhang, S. (2011). Phosphorylation of a WRKY Transcription Factor by Two Pathogen-Responsive MAPKs Drives Phytoalexin Biosynthesis in Arabidopsis. *The Plant Cell*, *23*(4), 1639–1653. https://doi.org/10.1105/tpc.111.084996

141. Martinelli, F., Ibanez, A. M., Reagan, R. L., Davino, S., & Dandekar, A. M. (2015). Stress responses in citrus peel: Comparative analysis of host responses to Huanglongbing disease and puffing disorder. *Scientia Horticulturae*, *192*, 409–420. https://doi.org/10.1016/j.scienta.2015.06.037

142. Martinelli, F., Reagan, R. L., Uratsu, S. L., Phu, M. L., Albrecht, U., Zhao, W., Davis, C. E., Bowman, K. D., & Dandekar, A. M. (2013). Gene Regulatory Networks Elucidating Huanglongbing Disease Mechanisms. *PLoS ONE*, *8*(9), e74256. https://doi.org/10.1371/journal.pone.0074256

143. Meng, H.-L., Zhang, W., Zhang, G.-H., Wang, J.-J., Meng, Z.-G., Long, G.-Q., & Yang, S.-C. (2018). Unigene-based RNA-seq provides insights on drought stress responses in *Marsdenia tenacissima*. *PLOS ONE*, *13*(11), e0202848. https://doi.org/10.1371/journal.pone.0202848

144. Mittler, R., & Blumwald, E. (2015). The Roles of ROS and ABA in Systemic Acquired Acclimation. *The Plant Cell*, *27*(1), 64–70. https://doi.org/10.1105/tpc.114.133090

145. Molitor, A. M., Bu, Z., Yu, Y., & Shen, W.-H. (2014). Arabidopsis AL PHD-PRC1 Complexes Promote Seed Germination through H3K4me3-to-H3K27me3 Chromatin State Switch in Repression of Seed Developmental Genes. *PLoS Genetics*, *10*(1), e1004091. https://doi.org/10.1371/journal.pgen.1004091

146. Moon, S.-J., Han, S.-Y., Kim, D.-Y., Yoon, I. S., Shin, D., Byun, M.-O., Kwon, H.-B., & Kim, B.-G. (2015). Ectopic expression of a hot pepper bZIP-like transcription factor in potato enhances drought tolerance without decreasing tuber yield. *Plant Molecular Biology*, *89*(4–5), 421–431. https://doi.org/10.1007/s11103-015-0378-y

147. Moons, A., Prinsen, E., Bauw, G., & Van Montagu, M. (1997). Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. *The Plant Cell*, *9*(12), 2243–2259. https://doi.org/10.1105/tpc.9.12.2243

148. Moura, J. C. M. S., Bonine, C. A. V., de Oliveira Fernandes Viana, J., Dornelas, M. C., & Mazzafera, P. (2010). Abiotic and Biotic Stresses and Changes in the Lignin Content and Composition in Plants. *Journal of Integrative Plant Biology*, *52*(4), 360–376. https://doi.org/10.1111/j.1744-7909.2010.00892.x

149. Mundree, S. G., Whittaker, A., Thomson, J. A., & Farrant, J. M. (2000). An aldose reductase homolog from the resurrection plant *Xerophyta viscosa* Baker. *Planta*, *211*(5), 693–700. https://doi.org/10.1007/s004250000331
150. Muthurajan, R., Rahman, H., Manoharan, M., Ramanathan, V., & Nallathambi, J. (2018). Drought responsive transcriptome profiling in roots of contrasting rice genotypes. *Indian Journal of Plant Physiology*, *23*(3), 393–407. https://doi.org/10.1007/s40502-018-0381-9
151. Muthuramalingam, P., Krishnan, S. R., Pothiraj, R., & Ramesh, M. (2017). Global Transcriptome Analysis of Combined Abiotic Stress Signaling Genes Unravels Key Players in *Oryza sativa* L.: An In-silico Approach. *Frontiers in Plant Science*, *8*, 300. https://doi.org/10.3389/fpls.2017.00759
152. Najafi, S., Sorkheh, K., & Nasernakhaei, F. (2018). Characterization of the APETALA2/Ethylene-responsive factor (AP2/ERF) transcription factor family in sunflower. *Scientific Reports*, *8*(1), 11576. https://doi.org/10.1038/s41598-018-29526-z
153. Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., Matsuda, F., Kojima, M., Sakakibara, H., Shinozaki, K., Michael, A. J., Tohge, T., Yamazaki, M., & Saito, K. (2013). Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *The Plant Journal*, *77*(3), 367–379. https://doi.org/10.1111/tpj.12388
154. Nie, S., Huang, S., Wang, S., Cheng, D., Liu, J., Lv, S., Li, Q., & Wang, X. (2017). Enhancing Brassinosteroid Signaling via Overexpression of Tomato (*Solanum lycopersicum*) SlBRI1 Improves Major Agronomic Traits. *Frontiers in Plant Science*, *8*, 8. https://doi.org/10.3389/fpls.2017.01386
155. Ning, W., Zhai, H., Yu, J., Liang, S., Yang, X., Xing, X., Huo, J., Pang, T., Yang, Y., & Bai, X. (2017). Overexpression of Glycine soja WRKY20 enhances drought tolerance and improves plant yields under drought stress in transgenic soybean. *Molecular Breeding*, *37*(2), 614. https://doi.org/10.1007/s11032-016-0614-4
156. Niu, C., Wei, W., Zhou, Q., Tian, A., Hao, Y., Zhang, W., Ma, B., Lin, Q., Zhang, Z., Zhang, J., & Chen, S. (2012). Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants. *Plant, Cell & Environment*, *35*(6), 1156–1170. https://doi.org/10.1111/j.1365-3040.2012.02480.x
157. Oberschall, A., Deák, M., Török, K., Sass, L., Vass, I., Kovács, I., Fehér, A., Dudits, D., & Horváth, G. V. (2008). A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses. *The Plant Journal*, *24*(4), 437–446. https://doi.org/10.1111/j.1365-313x.2000.00885.x
158. Orcheski, B., & Brown, S. (2016a). High-throughput sequencing reveals that pale green lethal disorder in apple (*Malus*) stimulates stress responses and affects senescence. *Tree Genetics & Genomes*, *13*(1), 1097. https://doi.org/10.1007/s11295-016-1097-5

159. Pandey, N., Ranjan, A., Pant, P., Tripathi, R. K., Ateek, F., Pandey, H. P., Patre, U. V., & Sawant, S. V. (2013). CAMTA 1 regulates drought responses in *Arabidopsis thaliana*. *BMC Genomics*, *14*(1), 216. https://doi.org/10.1186/1471-2164-14-216

160. Park, J. M., Park, C.-J., Lee, S.-B., Ham, B.-K., Shin, R., & Paek, K.-H. (2001). Overexpression of the Tobacco Tsi1 Gene Encoding an EREBP/AP2-Type Transcription Factor Enhances Resistance against Pathogen Attack and Osmotic Stress in Tobacco. *The Plant Cell*, *13*(5), 1035. https://doi.org/10.2307/3871362

161. Passafiume, R., Perrone, A., Sortino, G., Gianguzzi, G., Saletta, F., Gentile, C., & Farina, V. (2019). Chemical–physical characteristics, polyphenolic content and total antioxidant activity of three Italian-grown pomegranate cultivars. *NFS Journal*, *16*, 9–14. https://doi.org/10.1016/j.nfs.2019.06.001

162. Pauwels, L., Inzé, D., & Goossens, A. (2009). Jasmonate-inducible gene: what does it mean? *Trends in Plant Science*, *14*(2), 87–91. https://doi.org/10.1016/j.tplants.2008.11.005

163. Peleg, Z., & Blumwald, E. (2011). Hormone balance and abiotic stress tolerance in crop plants. *Current Opinion in Plant Biology*, *14*(3), 290–295. https://doi.org/10.1016/j.pbi.2011.02.001

164. Pieczynski, M., Wyrzykowska, A., Milanowska, K., Boguszewska-Mankowska, D., Zagdanska, B., Karlowski, W., Jarmolowski, A., & Szweykowska-Kulinska, Z. (2017). Genomewide identification of genes involved in the potato response to drought indicates functional evolutionary conservation with Arabidopsis plants. *Plant Biotechnology Journal*, *16*(2), 603–614. https://doi.org/10.1111/pbi.12800

165. Polania, J. A., Poschenrieder, C., Beebe, S., & Rao, I. M. (2016). Effective Use of Water and Increased Dry Matter Partitioned to Grain Contribute to Yield of Common Bean Improved for Drought Resistance. *Frontiers in Plant Science*, *7*, 600. https://doi.org/10.3389/fpls.2016.00660

166. Qi, L., Yang, J., Yuan, Y., Huang, L., & Chen, P. (2015). Overexpression of two R2R3-MYB genes from *Scutellaria baicalensis* induces phenylpropanoid accumulation and enhances oxidative stress resistance in transgenic tobacco. *Plant Physiology and Biochemistry*, *94*, 235–243. https://doi.org/10.1016/j.plaphy.2015.06.007

167. Qin, F., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2011). Achievements and Challenges in Understanding Plant Abiotic Stress Responses and Tolerance. *Plant and Cell Physiology*, *52*(9), 1569–1582. https://doi.org/10.1093/pcp/pcr106

168. Radwan, O., Arro, J., Keller, C., & Korban, S. S. (2015). RNA-Seq Transcriptome Analysis in Date Palm Suggests Multi-Dimensional Responses to Salinity Stress. *Tropical Plant Biology*, *8*(3–4), 74–86. https://doi.org/10.1007/s12042-015-9155-y

169. Ramu, V. S., Paramanantham, A., Ramegowda, V., Mohan-Raju, B., Udayakumar, M., & Senthil-Kumar, M. (2016). Transcriptome Analysis of Sunflower Genotypes with Contrasting Oxidative Stress Tolerance Reveals Individual- and Combined- Biotic and Abiotic Stress Tolerance Mechanisms. *PLOS ONE*, *11*(6), e0157522. https://doi.org/10.1371/journal.pone.0157522
170. Ramu, V. S., Swetha, T. N., Sheela, S. H., Babitha, C. K., Rohini, S., Reddy, M. K., Tuteja, N., Reddy, C. P., Prasad, T. G., & Udayakumar, M. (2015). Simultaneous expression of regulatory genes associated with specific drought-adaptive traits improves drought adaptation in peanut. *Plant Biotechnology Journal*, *14*(3), 1008–1020. https://doi.org/10.1111/pbi.12461
171. Rawat, N., Kiran, S. P., Du, D., Gmitter, F. G., & Deng, Z. (2015). Comprehensive meta-analysis, co-expression, and miRNA nested network analysis identifies gene candidates in citrus against Huanglongbing disease. *BMC Plant Biology*, *15*(1), 568. https://doi.org/10.1186/s12870-015-0568-4
172. Rest, J. S., Wilkins, O., Yuan, W., Purugganan, M. D., & Gurevitch, J. (2016). Meta-analysis and meta-regression of transcriptomic responses to water stress in Arabidopsis. *The Plant Journal*, *85*(4), 548–560. https://doi.org/10.1111/tpj.13124
173. Riemann, M., Dhakarey, R., Hazman, M., Miro, B., Kohli, A., & Nick, P. (2015). Exploring Jasmonates in the Hormonal Network of Drought and Salinity Responses. *Frontiers in Plant Science*, *6*, 1077. https://doi.org/10.3389/fpls.2015.01077
174. Rienth, M., Torregrosa, L., Luchaire, N., Chatbanyong, R., Lecourieux, D., Kelly, M. T., & Romieu, C. (2014). Day and night heat stress trigger different transcriptomic responses in green and ripening grapevine (*Vitis vinifera*) fruit. *BMC Plant Biology*, *14*(1), 108. https://doi.org/10.1186/1471-2229-14-108
175. Roncarati, R., Salamini, F., & Bartels, D. (1995). An aldose reductase homologous gene from barley: regulation and function. *The Plant Journal*, *7*(5), 809–822. https://doi.org/10.1046/j.1365-313x.1995.07050809.x
176. Rop, O., Reznicek, V., Valsikova, M., Jurikova, T., Mlcek, J., & Kramarova, D. (2010). Antioxidant Properties of European Cranberrybush Fruit (*Viburnum opulus* var. *edule*). *Molecules*, *15*(6), 4467–4477. https://doi.org/10.3390/molecules15064467
177. Rozhon, W., Akter, S., Fernandez, A., & Poppenberger, B. (2019). Inhibitors of Brassinosteroid Biosynthesis and Signal Transduction. *Molecules*, *24*(23), 4372. https://doi.org/10.3390/molecules24234372
178. Ruan, M.-B., Yang, Y.-L., Li, K.-M., Guo, X., Wang, B., Yu, X.-L., & Peng, M. (2018). Identification and characterization of drought-responsive CC-type glutaredoxins from cassava cultivars reveals their involvement in ABA signalling. *BMC Plant Biology*, *18*(1), 1528. https://doi.org/10.1186/s12870-018-1528-6
179. Sah, S. K., Reddy, K. R., & Li, J. (2016). Abscisic Acid and Abiotic Stress Tolerance in Crop Plants. *Frontiers in Plant Science*, *7*, 571. https://doi.org/10.3389/fpls.2016.00571

180. SAIJYO, R., & URITANI, I. (1971). Biosynthesis of Terpenoids in Tea Plant. *Agricultural and Biological Chemistry*, *35*(13), 2132–2134. https://doi.org/10.1271/bbb1961.35.2132
181. Sakamoto, H., Matsuda, O., & Iba, K. (2008). ITN1, a novel gene encoding an ankyrin-repeat protein that affects the ABA-mediated production of reactive oxygen species and is involved in salt-stress tolerance in *Arabidopsis thaliana*. *The Plant Journal*, *56*(3), 411–422. https://doi.org/10.1111/j.1365-313x.2008.03614.x
182. Sanchita, Dhawan, S. S., & Sharma, A. (2013). Analysis of differentially expressed genes in abiotic stress response and their role in signal transduction pathways. *Protoplasma*, *251*(1), 81–91. https://doi.org/10.1007/s00709-013-0528-5
183. Schindler, U., Beckmann, H., & Cashmore, A. R. (1993). HAT3.1, a novel Arabidopsis homeodomain protein containing a conserved cysteine-rich region. *The Plant Journal*, *4*(1), 137–150. https://doi.org/10.1046/j.1365-313x.1993.04010137.x
184. Schmidt, R., Mieulet, D., Hubberten, H.-M., Obata, T., Hoefgen, R., Fernie, A. R., Fisahn, J., San Segundo, B., Guiderdoni, E., Schippers, J. H. M., & Mueller-Roeber, B. (2013). SALT-RESPONSIVE ERF1 Regulates Reactive Oxygen Species-Dependent Signaling during the Initial Response to Salt Stress in Rice. *The Plant Cell*, *25*(6), 2115–2131. https://doi.org/10.1105/tpc.113.113068
185. Seo, Y. J., Park, J.-B., Cho, Y.-J., Jung, C., Seo, H. S., Park, S.-K., Nahm, B. H., & Song, J. T. (2010a). Overexpression of the ethylene-responsive factor gene BrERF4 from *Brassica rapa* increases tolerance to salt and drought in Arabidopsis plants. *Molecules and Cells*, *30*(3), 271–277. https://doi.org/10.1007/s10059-010-0114-z
186. Shao, J., Xu, Y., Wang, Z., Jiang, Y., Yu, G., Peng, X., & Li, R. (2011). Elucidating the toxicity targets of β-ionone on photosynthetic system of *Microcystis aeruginosa* NIES-843 (*Cyanobacteria*). *Aquatic Toxicology*, *104*(1–2), 48–55. https://doi.org/10.1016/j.aquatox.2011.03.014
187. Sheng, X.-F., & Xia, J.-J. (2006). Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. *Chemosphere*, *64*(6), 1036–1042. https://doi.org/10.1016/j.chemosphere.2006.01.051
188. Shi, H., Lee, B.-, Wu, S.-J., & Zhu, J.-K. (2002). Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nature Biotechnology*, *21*(1), 81–85. https://doi.org/10.1038/nbt766
189. Shinozaki, K., & Yamaguchi-Shinozaki, K. (2006). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*, *58*(2), 221–227. https://doi.org/10.1093/jxb/erl164
190. Shinozaki, K., Yamaguchi-Shinozaki, K., & Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology*, *6*(5), 410–417. https://doi.org/10.1016/s1369-5266(03)00092-x
191. Shumayla, Sharma, S., Taneja, M., Tyagi, S., Singh, K., & Upadhyay, S. K. (2017). Survey of High Throughput RNA-Seq Data Reveals Potential Roles for

lncRNAs during Development and Stress Response in Bread Wheat. *Frontiers in Plant Science*, *8*, 1019. https://doi.org/10.3389/fpls.2017.01019

192. Singh, K. (2002). Transcription factors in plant defense and stress responses. *Current Opinion in Plant Biology*, *5*(5), 430–436. https://doi.org/10.1016/s1369-5266(02)00289-3

193. Skubacz, A., Daszkowska-Golec, A., & Szarejko, I. (2016). The Role and Regulation of ABI5 (ABA-Insensitive 5) in Plant Development, Abiotic Stress Responses and Phytohormone Crosstalk. *Frontiers in Plant Science*, *7*, 1884. https://doi.org/10.3389/fpls.2016.01884

194. Song, K., Kim, H. C., Shin, S., Kim, K.-H., Moon, J.-C., Kim, J. Y., & Lee, B.-M. (2017a). Transcriptome Analysis of Flowering Time Genes under Drought Stress in Maize Leaves. *Frontiers in Plant Science*, *8*, 267. https://doi.org/10.3389/fpls.2017.00267

195. Su, T., Xu, Q., Zhang, F.-C., Chen, Y., Li, L.-Q., Wu, W.-H., & Chen, Y.-F. (2015). WRKY42 Modulates Phosphate Homeostasis through Regulating Phosphate Translocation and Acquisition in Arabidopsis. *Plant Physiology*, *167*(4), 1579–1591. https://doi.org/10.1104/pp.114.253799

196. Sun, J., Hu, W., Zhou, R., Wang, L., Wang, X., Wang, Q., Feng, Z., Li, Y., Qiu, D., He, G., & Yang, G. (2014). The *Brachypodium distachyon* BdWRKY36 gene confers tolerance to drought stress in transgenic tobacco plants. *Plant Cell Reports*, *34*(1), 23–35. https://doi.org/10.1007/s00299-014-1684-6

197. Sun, M., Jia, B., Yang, J., Cui, N., Zhu, Y., & Sun, X. (2017). Genome-Wide Identification of the PHD-Finger Family Genes and Their Responses to Environmental Stresses in Oryza sativa L. *International Journal of Molecular Sciences*, *18*(9), 2005. https://doi.org/10.3390/ijms18092005

198. Sun, S., Yu, J.-P., Chen, F., Zhao, T.-J., Fang, X.-H., Li, Y.-Q., & Sui, S.-F. (2007). TINY, a Dehydration-responsive Element (DRE)-binding Protein-like Transcription Factor Connecting the DRE- and Ethylene-responsive Element-mediated Signaling Pathways inArabidopsis. *Journal of Biological Chemistry*, *283*(10), 6261–6271. https://doi.org/10.1074/jbc.m706800200

199. Tani, T., Sobajima, H., Okada, K., Chujo, T., Arimura, S.-, Tsutsumi, N., Nishimura, M., Seto, H., Nojiri, H., & Yamane, H. (2007). Identification of the OsOPR7 gene encoding 12-oxophytodienoate reductase involved in the biosynthesis of jasmonic acid in rice. *Planta*, *227*(3), 517–526. https://doi.org/10.1007/s00425-007-0635-7

200. Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J., Müller, L. A., Rhee, S. Y., & Stitt, M. (2004a). Mapman: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal*, *37*(6), 914–939. https://doi.org/10.1111/j.1365-313x.2004.02016.x

201. Tognetti, V. B., Van Aken, O., Morreel, K., Vandenbroucke, K., van de Cotte, B., De Clercq, I., Chiwocha, S., Fenske, R., Prinsen, E., Boerjan, W., Genty, B., Stubbs, K. A., Inzé, D., & Van Breusegem, F. (2010). Perturbation of Indole-3-Butyric Acid Homeostasis by the UDP-Glucosyltransferase UGT74E2 Modulates Arabidopsis Architecture and Water Stress Tolerance. *The Plant Cell*, *22*(8), 2660–2679. https://doi.org/10.1105/tpc.109.071316

202. Turner, J. G., Ellis, C., & Devoto, A. (2002). The Jasmonate Signal Pathway. *The Plant Cell*, *14*(suppl 1), S153–S164. https://doi.org/10.1105/tpc.000679

203. Turóczy, Z., Kis, P., Török, K., Cserháti, M., Lendvai, Á., Dudits, D., & Horváth, G. V. (2011). Overproduction of a rice aldo–keto reductase increases oxidative and heat stress tolerance by malondialdehyde and methylglyoxal detoxification. *Plant Molecular Biology*, *75*(4–5), 399–412. https://doi.org/10.1007/s11103-011-9735-7

204. Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2000). Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Sciences*, *97*(21), 11632–11637. https://doi.org/10.1073/pnas.190309197

205. Usadel, B., Nagel, A., Steinhauser, D., Gibon, Y., Bläsing, O. E., Redestig, H., Sreenivasulu, N., Krall, L., Hannah, M. A., Poree, F., Fernie, A. R., & Stitt, M. (2006). PageMan: An interactive ontology tool to generate, display, and annotate overview graphs for profiling experiments. *BMC Bioinformatics*, *7*(1), 535. https://doi.org/10.1186/1471-2105-7-535

206. Van Zandt, P. A., Tobler, M. A., Mouton, E., Hasenstein, K. H., & Mopper, S. (2003). Positive and negative consequences of salinity stress for the growth and reproduction of the clonal plant, *Iris hexagona*. *Journal of Ecology*, *91*(5), 837–846. https://doi.org/10.1046/j.1365-2745.2003.00806.x

207. WAHID, A., GELANI, S., ASHRAF, M., & FOOLAD, M. (2007). Heat tolerance in plants: An overview. *Environmental and Experimental Botany*, *61*(3), 199–223. https://doi.org/10.1016/j.envexpbot.2007.05.011

208. Wan, X., Zou, L.-H., Zheng, B.-Q., & Wang, Y. (2019). Circadian Regulation of Alternative Splicing of Drought-Associated CIPK Genes in *Dendrobium catenatum* (Orchidaceae). *International Journal of Molecular Sciences*, *20*(3), 688. https://doi.org/10.3390/ijms20030688

209. Wang, L., Xiang, L., Hong, J., Xie, Z., & Li, B. (2019). Genome-wide analysis of bHLH transcription factor family reveals their involvement in biotic and abiotic stress responses in wheat (*Triticum aestivum* L.). *3 Biotech*, *9*(6), 1742. https://doi.org/10.1007/s13205-019-1742-4

210. Wang, Y., Wang, Q., Zhao, Y., Han, G., & Zhu, S. (2015). Systematic analysis of maize class III peroxidase gene family reveals a conserved subfamily involved in

abiotic stress response. *Gene*, *566*(1), 95–108. https://doi.org/10.1016/j.gene.2015.04.041

211. Wang, Y., Zhou, B., Sun, M., Li, Y., & Kawabata, S. (2012). UV-A Light Induces Anthocyanin Biosynthesis in a Manner Distinct from Synergistic Blue + UV-B Light and UV-A/Blue Light Responses in Different Parts of the Hypocotyls in Turnip Seedlings. *Plant and Cell Physiology*, *53*(8), 1470–1480. https://doi.org/10.1093/pcp/pcs088
212. Wang, Z., Su, G., Li, M., Ke, Q., Kim, S. Y., Li, H., Huang, J., Xu, B., Deng, X.-P., & Kwak, S.-S. (2016). Overexpressing Arabidopsis ABF3 increases tolerance to multiple abiotic stresses and reduces leaf size in alfalfa. *Plant Physiology and Biochemistry*, *109*, 199–208. https://doi.org/10.1016/j.plaphy.2016.09.020
213. Wei, W., Huang, J., Hao, Y.-J., Zou, H.-F., Wang, H.-W., Zhao, J.-Y., Liu, X.-Y., Zhang, W.-K., Ma, B., Zhang, J.-S., & Chen, S.-Y. (2009). Soybean GmPHD-Type Transcription Regulators Improve Stress Tolerance in Transgenic Arabidopsis Plants. *PLoS ONE*, *4*(9), e7209. https://doi.org/10.1371/journal.pone.0007209
214. Wen, F., Wu, X., Li, T., Jia, M., Liu, X., Li, P., Zhou, X., Ji, X., & Yue, X. (2017). Genome-wide survey of heat shock factors and heat shock protein 70s and their regulatory network under abiotic stresses in *Brachypodium distachyon*. *PLOS ONE*, *12*(7), e0180352. https://doi.org/10.1371/journal.pone.0180352
215. Wi, J., Jung, H. S., Im, S., Yang, S., Park, E.-J., Hwang, M. S., Jeong, W.-J., & Choi, D.-W. (2017). A nuclear fucosyltransferase-like protein, PtFUT, from marine red alga *Pyropia tenera* (Rhodophyta) confers osmotic stress tolerance. *Journal of Applied Phycology*, *30*(1), 717–727. https://doi.org/10.1007/s10811-017-1241-0
216. Wilkinson, S., & Davies, W. J. (2010). Drought, ozone, ABA, and ethylene: new insights from cell to plant to community. *Plant, Cell & Environment*, *33*(4), 510–525. https://doi.org/10.1111/j.1365-3040.2009.02052.x
217. Winicov, I. (2000). Alfin1 transcription factor overexpression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. *Planta*, *210*(3), 416–422. https://doi.org/10.1007/pl00008150
218. Wu, S., Wu, M., Dong, Q., Jiang, H., Cai, R., & Xiang, Y. (2016). Genome-wide identification, classification, and expression analysis of the PHD-finger protein family in *Populus trichocarpa*. *Gene*, *575*(1), 75–89. https://doi.org/10.1016/j.gene.2015.08.042
219. Wu, T., Pi, E.-X., Tsai, S.-N., Lam, H.-M., Sun, S.-M., Kwan, Y., & Ngai, S.-M. (2011). GmPHD5 acts as an important regulator for crosstalk between histone H3K4 di-methylation and H3K14 acetylation in response to salinity stress in soybean. *BMC Plant Biology*, *11*(1), 178. https://doi.org/10.1186/1471-2229-11-178
220. Wu, T., Wang, Y., Zheng, Y., Fei, Z., Dandekar, A. M., Xu, K., Han, Z., & Cheng, L. (2015). Suppressing Sorbitol Synthesis Substantially Alters the Global Expression Profile of Stress Response Genes in Apple (*Malus domestica)* Leaves. *Plant and Cell Physiology*, *56*(9), 1748–1761. https://doi.org/10.1093/pcp/pcv092

221. Xia, J., Benner, M. J., & Hancock, R. E. W. (2014). NetworkAnalyst - integrative approaches for protein–protein interaction network analysis and visual exploration. *Nucleic Acids Research*, *42*(W1), W167–W174. https://doi.org/10.1093/nar/gku443

222. Xia, J., Kong, D., Xue, S., Tian, W., Li, N., Bao, F., Hu, Y., Du, J., Wang, Y., Pan, X., Wang, L., Zhang, X., Niu, G., Feng, X., Li, L., & He, Y. (2014). Nitric oxide negatively regulates AKT1-mediated potassium uptake through modulating vitamin B6 homeostasis in Arabidopsis. *Proceedings of the National Academy of Sciences*, *111*(45), 16196–16201. https://doi.org/10.1073/pnas.1417473111

223. Xin, Z.-, Zhou, X., & Pilet, P.-E. (1997). Level changes of jasmonic, abscisic, and indole-3yl-acetic acids in maize under desiccation stress. *Journal of Plant Physiology*, *151*(1), 120–124. https://doi.org/10.1016/s0176-1617(97)80047-8

224. Xing, L., Zhao, Y., Gao, J., Xiang, C., & Zhu, J.-K. (2016). The ABA receptor PYL9 together with PYL8 plays an important role in regulating lateral root growth. *Scientific Reports*, *6*(1), 2016. https://doi.org/10.1038/srep27177

225. Xiong, W., Ye, T., Yao, X., Liu, X., Ma, S., Chen, X., Chen, M.-L., Feng, Y.-Q., & Wu, Y. (2018). The dioxygenase GIM2 functions in seed germination by altering gibberellin production in Arabidopsis. *Journal of Integrative Plant Biology*, *60*(4), 276–291. https://doi.org/10.1111/jipb.12619

226. Xu, W., Li, R., Zhang, N., Ma, F., Jiao, Y., & Wang, Z. (2014). Transcriptome profiling of *Vitis amurensis*, an extremely cold-tolerant Chinese wild Vitis species, reveals candidate genes and events that potentially connected to cold stress. *Plant Molecular Biology*, *86*(4–5), 527–541. https://doi.org/10.1007/s11103-014-0245-2

227. Xu, Z., Ali, Z., Xu, L., He, X., Huang, Y., Yi, J., Shao, H., Ma, H., & Zhang, D. (2016). The nuclear protein GmbZIP110 has transcription activation activity and plays important roles in the response to salinity stress in soybean. *Scientific Reports*, *6*(1), 366. https://doi.org/10.1038/srep20366

228. Yaish, M. W., Patankar, H. V., Assaha, D. V. M., Zheng, Y., Al-Yahyai, R., & Sunkar, R. (2017). Genome-wide expression profiling in leaves and roots of date palm (Phoenix dactylifera L.) exposed to salinity. *BMC Genomics*, *18*(1), 600. https://doi.org/10.1186/s12864-017-3633-6

229. Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology*, *57*(1), 781–803. https://doi.org/10.1146/annurev.arplant.57.032905.105444

230. Yan, H., Jia, H., Chen, X., Hao, L., An, H., & Guo, X. (2014). The Cotton WRKY Transcription Factor GhWRKY17 Functions in Drought and Salt Stress in Transgenic Nicotiana benthamiana Through ABA Signaling and the Modulation of Reactive Oxygen Species Production. *Plant and Cell Physiology*, *55*(12), 2060–2076. https://doi.org/10.1093/pcp/pcu133

231. Yang, M., Zhang, Y., Zhang, H., Wang, H., Wei, T., Che, S., Zhang, L., Hu, B., Long, H., Song, W., Yu, W., & Yan, G. (2017). Identification of MsHsp20 Gene Family in *Malus sieversii* and Functional Characterization of MsHsp16.9 in Heat Tolerance. *Frontiers in Plant Science*, *8*, 1761. https://doi.org/10.3389/fpls.2017.01761

232. Yang, X., Sun, C., Hu, Y., & Lin, Z. (2008). Molecular cloning and characterization of a gene encoding RING zinc finger ankyrin protein from drought-tolerant *Artemisia desertorum*. *Journal of Biosciences*, *33*(1), 103–112. https://doi.org/10.1007/s12038-008-0026-7

233. Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., & Shinozaki, K. (2005). The Regulatory Domain of SRK2E/OST1/SnRK2.6 Interacts with ABI1 and Integrates Abscisic Acid (ABA) and Osmotic Stress Signals Controlling Stomatal Closure in Arabidopsis. *Journal of Biological Chemistry*, *281*(8), 5310–5318. https://doi.org/10.1074/jbc.m509820200

234. Young, T. E., Meeley, R. B., & Gallie, D. R. (2004). ACC synthase expression regulates leaf performance and drought tolerance in maize. *The Plant Journal*, *40*(5), 813–825. https://doi.org/10.1111/j.1365-313x.2004.02255.x

235. Zhai, P., Yang, L., Guo, X., Wang, Z., Guo, J., Wang, X., & Zhu, H. (2017). MetaComp: comprehensive analysis software for comparative meta-omics including comparative metagenomics. *BMC Bioinformatics*, *18*(1), 1849. https://doi.org/10.1186/s12859-017-1849-8

236. ZHANG, M., LIU, W., & BI, Y.-P. (2009). Dehydration-responsive element-binding (DREB) transcription factor in plants and its role during abiotic stresses. *Hereditas (Beijing)*, *31*(3), 236–244. https://doi.org/10.3724/sp.j.1005.2009.00236

237. Zhang, X., Niu, M., Teixeira da Silva, J. A., Zhang, Y., Yuan, Y., Jia, Y., Xiao, Y., Li, Y., Fang, L., Zeng, S., & Ma, G. (2019). Identification and functional characterization of three new terpene synthase genes involved in chemical defense and abiotic stresses in *Santalum album*. *BMC Plant Biology*, *19*(1), 1720. https://doi.org/10.1186/s12870-019-1720-3

238. Zhang, Z., & Huang, R. (2010). Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor TERF2/LeERF2 is modulated by ethylene biosynthesis. *Plant Molecular Biology*, *73*(3), 241–249. https://doi.org/10.1007/s11103-010-9609-4

239. Zhao, S., Li, C.-I., Guo, Y., Sheng, Q., & Shyr, Y. (2018). RnaSeqSampleSize: real data based sample size estimation for RNA sequencing. *BMC Bioinformatics*, *19*(1), 2191. https://doi.org/10.1186/s12859-018-2191-5

240. Zhao, Z., Zhang, W., Yan, J., Zhang, J., Liu, Z. L. X., & Yi, Y. (2010). Over-expression of Arabidopsis DnaJ (Hsp40) contributes to NaCl-stress tolerance. *African Journal of Biotechnology*, *9*(7), 972–978. https://doi.org/10.5897/ajb09.1450

241. Zhou, J., Wang, J., Li, X., Xia, X.-J., Zhou, Y.-H., Shi, K., Chen, Z., & Yu, J.-Q. (2014). H2O2 mediates the crosstalk of brassinosteroid and abscisic acid in tomato

responses to heat and oxidative stresses. *Journal of Experimental Botany*, *65*(15), 4371–4383. https://doi.org/10.1093/jxb/eru217

242. Zhou, Y., & Underhill, S. J. R. (2015). Breadfruit (*Artocarpus altilis*) gibberellin 20-oxidase genes: sequence variants, stem elongation and abiotic stress response. *Tree Genetics & Genomes*, *11*(4), 909. https://doi.org/10.1007/s11295-015-0909-3

243. Zhu, J. K. (2001). Plant salt tolerance. *Trends Plant Science*, *39*(6), 66–71. https://doi.org/10.1007/bf00014816

244. Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology*, *53*, 247. https://doi.org/10.3390/6040049

245. Zhu, J.-K. (2016). Abiotic Stress Signaling and Responses in Plants. *Cell*, *167*(2), 313–324. https://doi.org/10.1016/j.cell.2016.08.029

246. Zhuang, J., Chen, J.-M., Yao, Q.-H., Xiong, F., Sun, C.-C., Zhou, X.-R., Zhang, J., & Xiong, A.-S. (2010). Discovery and expression profile analysis of AP2/ERF family genes from *Triticum aestivum*. *Molecular Biology Reports*, *38*(2), 745–753. https://doi.org/10.1007/s11033-010-0162-7