UNIVERSITÀ DEGLI STUDI DI PADOVA

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in BIOTECHNOLOGY FOR FOOD SCIENCE

Investigating Drought Stress Response and Memory in Sunflower (*Helianthus annuus*) and tomato (*Solanum lycopersicum*)

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Abstract

Drought stress is a major limiting factor in plant growth and productivity; this challenge is compounded by the rapid growth of the global population and the increasing food demands, particularly dealing with important economic crops such as sunflower (*Helianthus annuus*) and tomato (*Solanum lycopersicum*). In this study, we examine the response and memory of sunflower and tomato plants subjected to drought stress and subsequent recovery, drought stress was applied through withholding water in the first stage of plant growth, followed by a rewatering period. Morphological and physiological alterations such as leaf wilting, stomatal conductance and transpiration parameters were evaluated to assess stress conditions and time points for sample collection. Moreover, to better understand plant chromatin regulation and transcriptomic under stress, ChIP protocol and RNA analysis were also evaluated. The results of both morphological and physiological analysis showed minimal alteration in sunflower genotypes under drought stress, while tomato plants exhibited more severe effects, reaching quasi-complete stomatal closure and a decrease in all measured parameters. However, both plants demonstrated fast recovery upon rewatering. Chromatin extraction, sonication and purification for immunoprecipitation, as well as RNA extraction, were performed successfully on leaf tissue samples. The PCR amplification of specific drought stress genes in sunflower plants was also achieved.

This study provides valuable information on the differential drought responses of sunflower and tomato plants using integrated morphological, physiological and molecular techniques. The developed ChIP protocol can proceed with the application of ChiP seq along with RNA seq enabling the investigation of drought effects on plants, their response and memory providing new hints on drought tolerance crop improvement.

Keywords: Drought Stress; Sunflower; Tomato; stress response and memory; ChIP Immunoprecipitation; Transcriptomics.

1. INTRODUCTION

1.1. European project CROPINNO

The sunflower research has been held under the EU-funded CROPINNO project in order to enhance the scientific innovation capacity of IFVCNS (The Institute of Field and Vegetable Crops), which is Serbia's national agricultural research institute.

CROPINNO Twinning Horizon Europe project has established a collaboration network between IFVCNS and four international research institutions: CSIC-IAS (Cordoba, Spain), UNIPD (Padova, Italy), FZJ (Jülich, Germany) and UROS (Rostock, Germany). The main objective is to develop innovative solutions and promote resilient and sustainable agricultural production, especially in the early stages of crop cultivation. These solutions aim to cultivate climate-smart crops, as well as diverse and nutritious crops for healthier and more sustainable options for consumers. Additionally, this collaboration seeks to enhance the ability of crops to respond to challenges such as climate change and biotic/abiotic stress. To accomplish these goals, the utilization of bioinformatic tools in conjunction with various omics technologies (such as genomics, transcriptomics, proteomics, metabolomics, and epigenomics) will facilitate the identification of target genes and markers associated with complex traits in *Helianthus annuus.*

1.2. Sunflower (Helianthus annuus)

A member of the family *Asteraceae* consisting of over 23,000 species and presenting an impressive range of diversity, this includes tall trees that can reach heights of 30 meters, as well as small herbs that barely reach 1 centimeter high. This family exhibits various combinations of male and female reproductive structure parts. In the case of the common sunflower *Helianthus,* the central florets are hermaphroditic, while the head is radiated. Sunflowers are characterized by their variation in terms of gender, which promotes increased outcrossing and genetic recombination (Bohm & Stuessy, 2013).

The *Helianthus* genus consists of 51 species, with 14 being annual and 37 being perennial, the chromosome number is n=17, within it there are diploid species, such are *H. annuus* L., tetraploid, and hexaploidy (6n=102). Sunflower is a nutritional food source, producing oil rich in mono- and polyunsaturated fats and other phytochemicals such as antioxidants, proteins and vitamin B that meet consumer demand and health (Adeleke & Babalola, 2020). It has a long history of use in ethnomedicine and pharmacologic studies, *Helianthus annuus* contains various bioactive

compounds as phenol and terpene compounds that contribute to its anti-inflammatory, antioxidant, and antimicrobial properties (Bashir et al., 2015).

The cultivation of sunflowers has spread throughout the world mainly because sunflowers are an important oil seed plant and are considered a source of high-quality edible oils, as well as their ability to adapt to different climatic and soil conditions (Adeleke & Babalol, 2020). Interspecific hybridization plays a crucial role in sunflower breeding, particularly when the cultivated form has reached limited variability, it becomes necessary to look for desirable genes from the wild type. The major goals in sunflower breeding remain higher seed and oil yield with improved quality, in addition to developing resistance to different stresses and parasites such as broomrape, this was done through weed control using transgenic herbicide resistance genes like imidazolinone IMI, which have been transferred from wild types using backcross breeding (Kaya et al., 2013).

Sunflower is a native crop in North America, it was initially domesticated by the Indians before being introduced to Europe. However Recent archaeological evidence indicated that sunflower was domesticated first in the central USA and this was supported by analysis using RFLP haplotype, (SSR) diversity and QTL. The first breeding efforts for higher seed and oil-yielding varieties took place in Russia. Currently, sunflower cultivation covers approximately 23 million hectares worldwide, with a production of around 30 million metric tons (Kaya et al., 2011), over half of the global sunflower production and cultivation area is concentrated in the Black Sea region (Kaya et al., 2008).

1.3. Tomato (Solanum lycopersicum)

Tomatoes (*Solanum lycopersicum*) are considered model plants for fruit development and plant protection in the family *Solanaceae*, this family comprises between 1000 and 2000 species with large morphological variability and ecological adaptability (Aoki et al., 2010). The tomato chromosomes are composed of pericentric heterochromatin and distal euchromatin, with a high concentration of repeats within and around centromeres, in chromomeres and at telomeres. Euchromatin genes and transcripts exhibit significantly higher densities of recombination, on the other hand, chloroplast insertions and conserved microRNA (miRNA) genes are distributed more evenly throughout the genome (The Tomato Genome Consortium, 2012).

Tomatoes are of huge importance as a food staple worldwide, it is the second largest horticulture in terms of both production and consumption. Tomatoes are rich in vitamins, pro-vitamins, minerals and secondary metabolites, its cultivation spans various regions, ranging from temperate to tropical

and subtropical areas (Gutierrez & Elisabeth, 2018). *S. lycopersicum* undergoes self-pollination and it has the ability to grow under diverse environmental conditions, including air quality, temperatures, and climates (Waheed et al., 2020).

However, to ensure the successful growth of *Solanum lycopersicum L*, adequate water supply, and optimal temperature play a crucial role, this is particularly important because many commercially grown tomato varieties are sensitive to drought and high temperatures, especially during the reproductive stage (Petrović et al., 2021).

1.4. Drought stress

The global population is facing rapid growth and it is expected to reach approximately six billion by the end of 2050. However, the productivity of food is declining as a result of different abiotic stresses. Consequently, mitigating these losses has become a significant concern for all nations to meet the increasing food demands. One of the abiotic stresses that contributes to this issue is drought, also referred to as water deficit stress (Mahajan & Tuteja, 2005). Drought, an inevitable and limiting factor in crop production, poses a major challenge in the context of climate change. It prevails across various environments and is projected to increase in the future, disregarding geographical boundaries and lacking explicit warnings (Hussain et al., 2018; Seleiman et al., 2021).

This major abiotic stress has a negative impact on plant morphology, physiology, biochemistry, and molecular attributes. As a result plant biomass, quality, and energy are reduced, as well as grain quality and yields. These reactions' severity varies depending on the species, in extreme cases prolonged drought stress can lead to plant mortality. This environmental stress is mainly caused by temperature changes due to global warming, changes in light intensity and insufficient or low rainfall (Pivovaroff et al., 2016; Seleiman et al., 2021).

The severity of drought is unpredictable due to various factors such as the occurrence and distribution of rainfall, evaporative demands and the moisture-storing capacity of soils (Masroor et al., 2022). The decrease in water supply and/or the increase in evaporative demand, results in a deficit of soil water, this means that the amount of water available in the soil is lower than the reference condition requirements of plants. In addition, water plays an important role in soil degradation and nutrient movements, simultaneously affecting plant nutrition. Water stress has a significant impact on the functioning and survival of organisms from individual cells to the entire ecosystem (Volaire, 2018).

When plants are affected by drought stress, the photosynthesis rate decreases, the main factors contributing to this decline can be attributable to the closure of the stomatal resulting from a reduction in CO_2 levels, or non-stomatal reason by a decrease in photosynthesis activity in the mesophyll tissue, or both combinations. The specific effects of these two factors (stomatal, non-stomatal, or both) vary depending on experimental treatment, plant species, cultivar, age, and developmental stage (Bhargava & Sawant, 2013; Liang et al., 2020).

The effects of drought stress on plants are observed by different changes in the morphological structure of their leaves, stems, and roots. In general, plant height decreases, leaves wilt and decrease their areas, as well as leaves fall which leads to the leaf number change. Furthermore, the thickness and tissue density of the leaves increase. Root elongations and root-shoot ratio dynamics are also affected (Yang et al., 2021). Moreover, under conditions of water deficit, fresh and dry weights are significantly reduced (Maqsood et al., 2022). In addition to the outer appearance, the internal structure of the plant also undergoes modifications. The thickness of the cuticles increases, and even the coverage of wax and osmophilicity also increases (Chen et al., 2020). Furthermore, research shows that crop growth under water shortage is reduced due to inhibition of cell elongation and expansion as well as mitosis disruption (Khatun et al., 2021).

Crop productivity is also significantly affected, various stages during reproductive development are especially sensitive to environmental stresses, which may lead to complete sterility and severe yield losses (Begcy & Dresselhaus, 2018). The disruptive effect of drought primarily stems from its interference with the ionic and osmotic equilibrium of the cell (Mahajan & Tuteja, 2005).

Mozgova et al. (2019), regrouped the various changes resulting from drought in crops into morphological changes such as germination, plant height and plant biomass reduction. Physiological changes like water content, photosynthetic activity, decrease of pigment content, and membrane integrity. Biochemical changes such as the accumulation of osmoprotectants as proline, sugars, and antioxidants. Furthermore, genetically by inducing molecular change such as altering the expression of stress-related genes.

1.5. Plant response to drought stress

As previously mentioned, stress causes structural and/or metabolic dysfunctions, including damage to structure, protein functions, and enzyme activities. However, plant replies depend on the duration and the severity of the stress, as well as their genetic traits. Plants can be prone to eustress or distress, where eustress occurs during low stress doses and triggers reactions such as the synthesis

of metabolites and stress tolerance. Meanwhile, exposure to a high and constant dose of stress can lead to physiological instability, disruption of homeostasis, and possibly death of the plant, however, distress can also trigger protective responses to mitigate stress damage (Bandurska, 2022; Rico Chávez et al., 2022). To better distinguish these two responses, eustress is a form of low-level, adaptive, and positive form of stress, conversely distress is a form of high-level, non-adaptive, and negative stress, which can lead to new metabolic balances under new circumstances (Sonmez et al., 2023).

Due to the plant's sessile nature, notable mechanisms of response have been observed, including enhancing water absorption and conduction, minimizing water loss, conserving growth stages, adopting an upright and rolling posture to minimize radiation exposure and subsequent water loss as well as rapid closing stomata to support water conservation (small and dense stomata; Li et al. 2021). Moreover, stomata closing during drought stress might initiate the production of reactive oxygen species (ROS), by initiating the production of superoxide and singlet oxygen (Li et al., 2023). Plants produce ROS as the first layer for defense but the increased level negatively affects photosynthesis, as well as impairs the assembly and repair of photosystem II (PSII), and affects chloroplast development (Qi et al., 2018).

Bandurska, (2022) provides an explanation of the concept of adaptation and acclimation (hardening) response in order to cope with stress conditions. Adaptation involves developmental, morphological, and physiological traits that help the growth under adverse conditions, it refers to evolutionary adjustments that are genetically determined and hereditary constitutive. Acclimatation on the other hand comprises structural, physiological, and biochemical alterations that enable the survival of an individual plant in new environmental conditions.

Adaptation or acclimation leads to plant development by various strategies to cope with stress. Those strategies involve a series of morphological, physiological, and biochemical adaptations, based on the high diversity of species, these strategies can be categorized into escape, avoidance, and tolerance mechanisms **(Fig 1)** (Abobatta, 2019; Li et al., 2021).

Escape strategy: refers to a classical adaptive mechanism used by plants to bypass the negative effects of drought on their survival, this mechanism involves accelerating the reproductive phase of the plant, enables the completion of the entire life cycle before the drought event. This strategy is commonly observed in perennial species and some annual grasses, those plants stop the vegetative phase and prioritize seed production using rapid phenological development and

developmental plasticity (Shavrukov et al., 2017). Drought-escaping plants typically do not undergo special morphological, physiological, or biochemical modifications. Instead, they exhibit plant plasticity by varying the duration of the transition from vegetative to reproductive stages, showing fast phenological development, such as early flowering and maturation. The escape is characterized by reduced stomatal conductivity, low transpiration, and increased photosynthetic carbon gain. Although escape allows survival, it may lead to decreased crop yield (Vassileva et al., 2023).

Avoidance strategy can be classified as water savers or water spenders, this strategy aims to retain enough water, first by minimizing water loss and maintaining a high internal water potential in the cell and preventing tissue damage throughout physiological adaptation, it includes reducing transpiration/evapotranspiration and photosynthesis rates. Plants with an avoidance strategy generally have smaller leaf areas with lower stomatal conductance and decreased leaf numbers, and secondly by optimizing water uptake through morphological adjustments by adopting changes in root morphology as elongation. The other adjustments include maintaining low metabolic rates, leaf wilting and increased wax deposition on the leaf surface. The avoidance strategy can be associated with limited productivity (Zia et al., 2021; Bandurska, 2022; Trovato et al., 2023).

Tolerance strategy encompasses resisting low internal water content while sustaining growth during drought, this implies surviving despot experiencing water deficit. This strategy is achieved by enhancing cell membrane stability through osmotic adjustment and cellular elasticity, which ultimately leads to maintaining the tissue turgor. Additional, strategies include also complex regulation of gene expression, protein turnover, and DNA/protein repair. It is worth noting that this strategy could be linked to high productivity (Basu et al., 2016; Pamungkas et al., 2022).

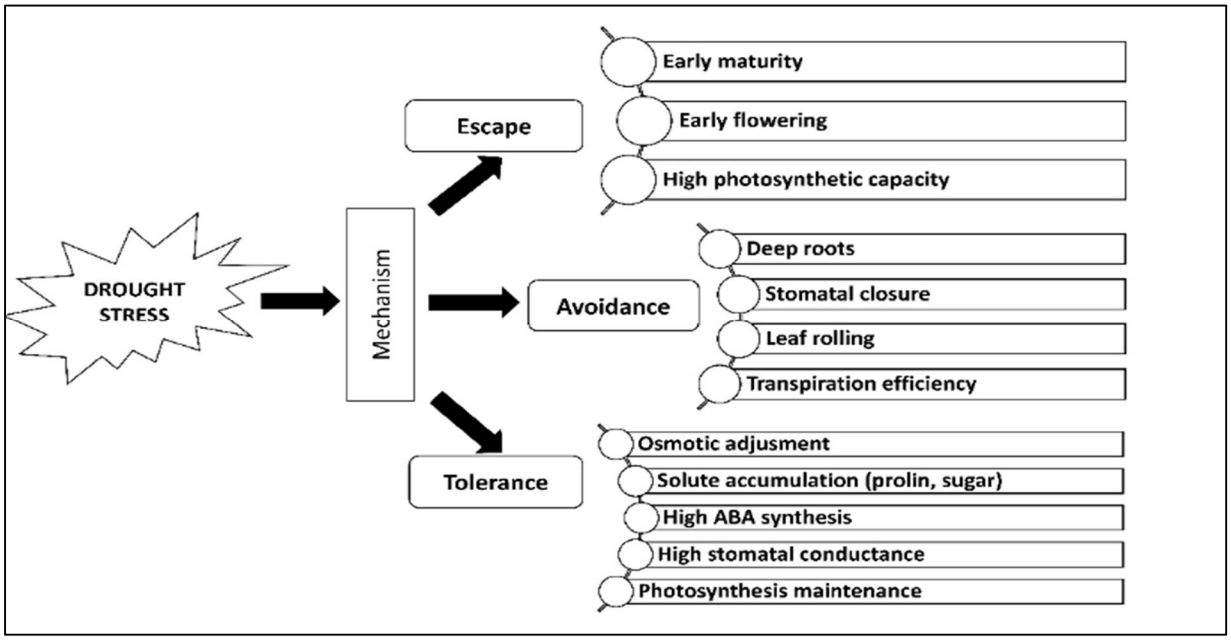

Figure 1: Crops mechanism in drought stress (Pamungkas et al., 2022).

In response to drought stress, it has been noticed that plants modify their root structure, their hormone balance, and redox status, as well as regulate the leaf wax thickness, stomatal development and their activities (Liu et al., 2023). Drought triggers distinct pathways for stress signaling that lead to synthesizing proteins that prevent cell damage, these pathways notably involve hormones such as abscisic acid (ABA), jasmonic acid (JA), and ethylene (Mahmood et al., 2019; Tiwari et al., 2017). In addition to the previous hormones, salicylic acid, auxin, gibberellin, and brassinosteroids are also involved in diminishing drought stress through various mechanisms, including promoting stomatal closure, enhancing root branching, formation and activating defense responses (Iqbal et al., 2022).

Abscisic acid (ABA) is an important signal molecule in plants, which effectively addresses drought conditions and promotes its overall growth and development (Ng et al., 2014). ABA initiates a variety of physiological processes, including stomata closure, root system modulation, soil microbial community organization, transcription and post-transcription gene expression activation, and metabolic adjustments. In particular, the ABA-dependent protein AREB1 plays an important role in regulating plant response to drought by stimulating the expression of downstream stress-related target genes (de Melo et al., 2020; Muhammad Aslam et al., 2022). The roots are the first to detect a decrease in water supply, thus communicating the leaves through the production of Abscisic Acid (ABA), this signal then triggers the closure of the stomata. The limited water

availability to cells due to the poor flow of water from roots to leaves caused the loss of cell turgor leading to an inhibition in growth (Bhargava & Sawant, 2013).

Research by Shamloo-Dashtpagerdi et al. (2015) showed that water shortages lead to changes in regulation proteins such as transcription factors and posttranscriptional factors, kinase, phosphatase, and signal molecules. In addition, Bandurska (2022) points out that severe and long-term droughts lead to the production of various protective proteins, such as LEA proteins, dehydrins, and chaperones. Additionally, compatible compounds like proline, glycine-betaine, proline-betaine, trehalose, raffinose, mannitol, and pinitol are involved in enzyme and membrane protection.

Sunflower, one of the important crops globally, is commonly cultivated in rain-fed areas. It has emerged as a major player in the oilseed production in the world, ranking fourth with an 8% share. However, the production of seeds and oil is greatly affected by drought. Despite being generally acknowledged as a drought-resistant crop due to its capacity to adapt and endure water scarcity, certain sunflower varieties are susceptible to drought. Therefore, the initial step in plant breeding involves the screening of tolerant accessions from the existing germplasm. This process is crucial to identifying sunflower varieties that can withstand and thrive underwater restrictions (Razzaq et al., 2017; Hussain et al., 2018; Ahmad et al., 2022). A recent study by Keipp et al. (2020) has shown that facing drought stress during the grain filling in the growth phase caused a decline in sunflower oil yield, this reduction was due to the decrease in seed weight caused by the cell extension reduction.

Despite being a crop with deep roots, sunflower is highly susceptible to water stress, leading to significant reductions in yield. Furthermore, plant organs are particularly affected. To shield against drought, sunflower plants undergo various morphological and physiological changes. (Pekcan et al., 2016). The phenotypic responses indicate significant differences between early and late drought treatments, in more detail, sunflowers that experienced early drought show diminished overall growth but demonstrated a high capacity for water acquisition after the drought stress phase, resulting in increased aboveground biomass and leaf area with more noticed shift in phenotypic correlations. On the other hand, sunflowers exposed to late drought were small compared to the previous plants but exhibited efficient water use (Janzen et al., 2023).

Drought stress poses challenges for plants therefore making it a crucial concern when it is exposed to economically important crops such as tomatoes. Tomatoes (*Solanum lycopersicum* L.) have an

enormous economic value in all regions of the world, especially in the Mediterranean areas: During drought stress, tomatoes change at both genetical and proteomic levels, in particular, through genes coding osmotins, dehydrins, and kinases. While at the physiological level, tomatoes adjust photosynthesis, modulate the ABA level, and alter the sugar metabolism (Conti et al., 2023).

1.6. Omics technique and drought stress

1.6.1. Transcriptomic, Proteomic and Genomic

Recently, the use of high-throughput sequencing has introduced powerful and effective research tools that can improve our understanding of the molecular mechanisms under stress in plants. This has greatly helped identify potential genes associated with various abiotic stress tolerances and has greatly assisted in the identification of potential genes associated with various abiotic stress tolerances, as well as the discovery of numerous molecular markers, functional genes, and regulatory genes through genome sequencing. Those emerging technologies such as transcriptome analysis, digital gene expression, small RNA deep sequencing, proteomics, and metabolomics are expected to wrap new ways of studying stress resistance in vegetable crops (Zhuang et al., 2014; Roychowdhury et al., 2023).

Drought differs from other stressors due to the complex nature of how crops respond to it and also to the limited mechanism knowledge compared to other abiotic stressors. To gain a thorough understanding of the fundamental biological and cellular processes activated by crop plants during periods of stress, the combination of various omics technologies such as transcriptomics, metabolomics, and proteomics offers a holistic comprehension of cellular dynamics in the presence of this water scarcity (Zargar et al., 2022).

Researchers have utilized molecular tools such as genome-wide identification and comparative proteomic analysis in recent years to study how plants adapt their gene regulatory networks to cope with water scarcity. These advancements in omics and transgenic strategies have provided opportunities to underline the effects on crop growth and yield. Many transcriptomics and proteomics studies have been conducted on various crops such as rice, corn, wheat, barley, and chickpea under water pressure conditions, examining the mechanisms of drought tolerance (Singh et al., 2022). For example, Wu et al. (2022) identified 14 candidate genes that could play a crucial role in the drought response of sunflowers by integrating genome-wide association studies and RNA sequencing. In addition, Liang et al. (2017) revealed 71 genes involved in the response of sunflowers to abiotic stimuli through transcriptomic analysis of leaves and roots, of these genes, nine are likely to be associated with water-related responses.

In a study conducted in 2023 by Shen et al., they discovered three genes: bHLH025, NAC53, and SINAT3, which may play an important role in increasing sunflower drought resistance, these results were obtained by combining physiological measurements with transcriptomic analysis. Other drought-resistant genes that were identified are involved in the synthesis and signaling of ABA and take an important role in minimalizing the impact of drought stress on sunflowers (*Helianthus annuus* L.). This study also showed that drought leads to the accumulation of reactive oxygen species (ROS), thereby increasing the activity of antioxidant enzymes and osmolyte content. A separate proteomics study provided other information, the proteomics investigation of Ghaffari et al. (2017) revealed that drought stress caused alterations in 18 proteins in sensitive lines and 24 proteins in tolerant lines.

The study of tomatoes under drought stress was also often carried out using omics techniques. For example, in 2023 in the study of Liu et al., transcription and post-transcription changes in tomato plants exposed to drought stress were conducted and it was discovered that the expression of 3765 genes and 294 proteins had changed significantly.

Similarly, after subjecting two tomato genotypes (M82 and IL2-5) to drought deficit, a high-throughput sequencing of miRNA profiles unfolds significant changes in the expression of 32 conserved and 68 novel miRNAs. These miRNAs are known to play a vital function in stress response. Furthermore, many target genes associated with stress resistance have been identified (Liu et al., 2018). In another study by Rai et al. (2021), the proteomic reaction of tomato leaves to drought stress was analyzed by two-dimensional gel electrophoresis, the results indicate that the alteration in protein levels after drought stress was mainly related to defense responses, oxidative stress, detoxification, protein synthesis, energy metabolism, and carbon metabolism.

It is important to mention that drought-tolerance genes and proteins exhibit varying functions across different plant species, tissues, varieties, and stress levels (Liu et al., 2023).

Gaining an understanding of the phenotypic traits that enhance plant performance under stressful conditions and comprehending the morphological, physiological, genetic, and molecular mechanisms along with how plants perceive and transmit signals to activate responses for drought tolerance is essential and can contribute significantly to the development of drought-resistant genotypes for cultivation and sustainable agriculture (Mishra & Singh, 2010 ; Saremirad & Mostafavi, 2020).

1.6.2. Chromatin

The epigenetic level is an additional level of gene expression regulation, where modifications can take place either at the chromatin or mRNA (Bhargava & Sawant, 2013). Epigenetics is the study of heritable alterations in gene function that do not involve modifications in the DNA sequence. These alterations in cellular and physiological phenotypic traits can arise from external or environmental influences, or they can be inherent in the regular developmental process (Duan et al., 2018). In eukaryotes, chromosomes have been categorized into two distinct types according to their degree of compaction: heterochromatin, which represents the most condensed structure, and euchromatin the less compact form with the 'beads on a string' shape (Morrison & Thakur, 2021). The genetic information is organized into nucleosomes, which are the fundamental components blocks of euchromatin, a nucleosome is composed of about 147 base pairs of DNA which are wrapped 1.7 times around two sets of four core histone proteins (H2A, H2B, H3, and H4**) (Fig 2a)**, the 14 contact points between the histone and DNA contribute to chromatin compactness and stability (Dobrovolskaia & Arya, 2012).

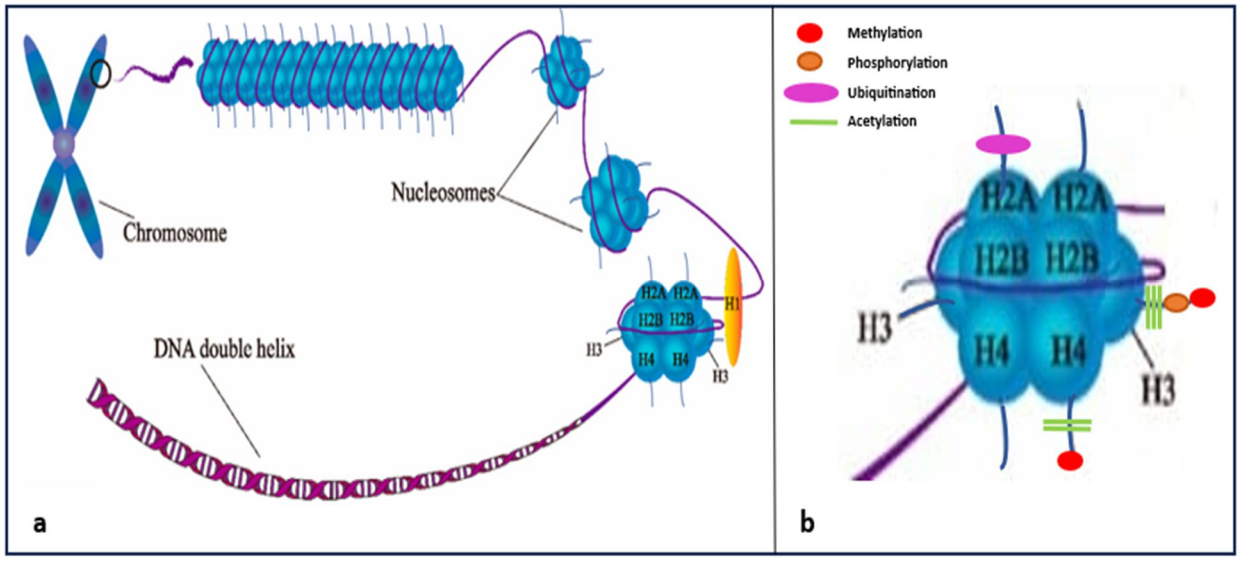

Figure 2: a. Model of chromatin b. Schematic example of histone modifications (Li et al., 2021) modified.

The structure of chromatin is subjected to dynamic regulation through multiple epigenetic mechanisms, including DNA methylation, histone posttranslational modifications (PTMs), ATP-dependent chromatin remodeling, placement of histone variants, and regulation with noncoding RNAs (Liu et al., 2010). Histone modifications in plants have been shown to impact a wide range of biological processes, these include cell growth, light and temperature response, flowering, hormone response, and circadian regulation (Moreno-Pérez et al., 2021).

Histone post-translational modifications (PTMs) incorporate various histone residues modifications, such as acetylation and deacetylation, methylation, phosphorylation and others. Histone methylation is being extensively studied, histone methylation takes place at lysine and arginine residues, involving different methyl groups such as (mono-, di-, and tri-) methylation. Another extensively researched histone modification is histone acetylation, which is believed to induce a more relaxed chromatin state by unraveling, leading to transcription activation facilitation. On the contrary, histone deacetylation returns the open chromatin structure into a closed tangled one, thus hindering transcription (Halder et al., 2022; Xie & Duan, 2023).

The majority of histone modification sites **(Fig 2b)** are located on the N-terminal tail of histones, however, they can occur also at nucleosome core regions. These modifications act as histone code to regulate gene expression and chromatin structure (Jenuwein & Allis, 2001). Many histone modifications take place at specific lysine residues on the N-terminal tails of histones, and play a role in regulating chromatin activity in different manners. For example, histone H3 lysine 9 acetylation (H3K9ac) and histone H3 lysine 4 tri-methylation (H3K4me3) are mainly enriched on active promoters (Kouzarides, 2007; Halder et al., 2022). Moreover, phosphorylation, ubiquitination, sumoylation, glycosylation, and ADP-ribosylation are also involved. These histone modifications are recognized through enzymes known as readers, and specific enzyme complexes referred to as writers and erasers catalyze and remove these histone marks, respectively (Ramirez-Prado et al., 2018; Kang et al., 2022).

In *Arabidopsis thaliana*, the eukaryotic marks H3K9ac and H3K4me3 are responsible for the chromatin dynamic. When this plant experiences a water deficit, the level of H3K4me3 is altered significantly causing the upregulating or downregulating of related genes, similarly the case of barley plants, it has been noticed an increase or decrease in H3K4me3 and H3K4me2 modifications (Halder et al., 2022). Moreover, the trimethylation of histone H3 lysine 27 (H3K27me3) is associated with a repressive chromatin state and gene silencing (Gan et al., 2015).

DNA methylation is an inheritable epigenetic modification that entails the transfer of a methyl group to the C-5 position of the cytosine ring of DNA through DNA methyltransferases (DNMTs). Within plants, cytosines can be methylated in either symmetrical (CG or CHG) or asymmetrical (CHH, where H represents A, T, or C) contexts (Jin et al., 2011).

Lastly in both plant and animal species, chromatin modulators, such as siRNAs and miRNAs, hold a significant role. These modulators directly affect chromatin and trigger RNA-dependent DNA methylation (RdDM) (Begcy & Dresselhaus, 2018).

Chromatin dynamics, which are governed by epigenetic mechanisms, have a crucial function in the regulation of gene expression that enables plants to respond to stress . These dynamics encompass alterations in chromatin structure, modifications, and remodeling, which ultimately affect the accessibility of DNA for transcriptional machinery (Probst & Mittelsten Scheid, 2015). Drought stress modifies histone and initiates the activation of gene responses, the plants exposed to drought stress exhibited increased levels of H39ac (Wang et al., 2021).

Histone modifications such as H3K4 trimethylation, H3K9 acetylation, H3 Ser-10 phosphorylation, H3 phosphoacetylation, and H4 acetylation were detected in conjunction with the activation of stress-responsive genes (Chinnusamy et al., 2009). Histone modification H3K4me3 was elevated during drought stress with more enrichment under severe drought, this led to the activated expression of genes triggered by drought. Upon rewatering and recovery phase, H3K4me3 was eliminated from the upregulated genes, demonstrating its correlation with drought gene responses (Shi et al., 2024).

The stability and reversibility of chromatin alterations are closely linked to the dynamic nature of epigenetic networks. These networks play an important role in maintaining epigenetic memory, including somatic memory and intergenerational/transgenerational memory (Kakoulidou et al., 2021). Environmental pressures may induce somatic and transgenerational memory in plants, resulting in changes in transcriptional stress-responsive genes. These changes can lead to sustained (Type 1) or temporary (Type 2) activation or repression of genes, as well as the addition or removal of specific chromatin modifications (which may trigger molecular memory formation). After the recovery from the initial stress, the chromatin becomes stable and the Memory persists, if the plant encounters a second stress, it triggers the response of these memory genes. Which can be faster, stronger, and more sensitive (Mozgova et al., 2019). These epigenetic mechanisms are supreme in stress memory formation and have the potential to transmit it to future generations of stress-exposed plants (Ramirez-Prado et al., 2018).

The stress memory can be regulated also by DNA modification and microRNAs (miRNA) (Mozgova et al., 2019). Small non-coding RNAs (miRNAs and siRNAs) can initiate DNA methylation and histone modifications, when plants experience drought these modifications enable

the plants to retain a memory, this memory is achieved through the up-and down-regulation of small RNAs, which in turn leads to specific gene expression changes. Ultimately, this mechanism enhances the plants' resistance to future stress events (Bandurska, 2022).

1.7. Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is one of the most effective techniques for investigating the interactions between epigenetic factors or specific proteins bound to genomic DNA regions (e.g., transcription factors with target genes). Using ChIP allows the recognition of specific histones with posttranslational modifications at targeted genomic regions. In addition, ChIP enables the determination of interaction with candidate target genes. This versatile method also allows for comparing chromatin states between normal and stress conditions, making it a valuable tool in studying chromatin dynamics (Begcy & Dresselhaus, 2019).

The major steps for ChIP are as follows: crosslinking where formaldehyde is used to covalently link proteins and DNA; followed by fragmentation, where the DNA-protein complexes are sheared either mechanically with sonication or by enzymatic digestion. Finally immunoprecipitation is then performed to enrich the soluble cross-linked DNA-protein complexes using specific antibodies. Then a reverse cross-linking by an acid or elevated temperature, and after that DNA purification. Finally, the retrieved DNA is analyzed by PCR amplification or qPCR with gene-specific primers, southern blotting, DNA-ChIP, or ChIP seq **(Fig 3)** (Gade & Kalvakolanu, 2012; Begcy & Dresselhaus, 2019). One of the frequent cross-linking agents used is formaldehyde because of its convenient reversibility and ability to create close bonds (Das et al., 2004).

The two main types of ChIP techniques are X-ChIP and N-ChIP, The X-ChIP technique involves using formaldehyde-fixed chromatin and then fragmentation through sonication, while the N-ChIP method uses unfixed native chromatin that is digested by nuclease enzymes. Each approach has its own advantages (Turner, 2001). The ChIP method has gained considerable strength due to its specific resolution of nucleotides, the absence of noise, no limits on genome coverage, and the versatility of application for various types of tissue (Nagaki et al., 2015).

Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) has proved to be a reliable method for analyzing the pattern of histone modification and is widely used for analyzing DNA–protein interactions throughout the genome. This technique has been successfully applied to various plant species such as Arabidopsis, rice, maize, and *Populus trichocarpa* (Li et al.,2021; Hino et al., 2023).

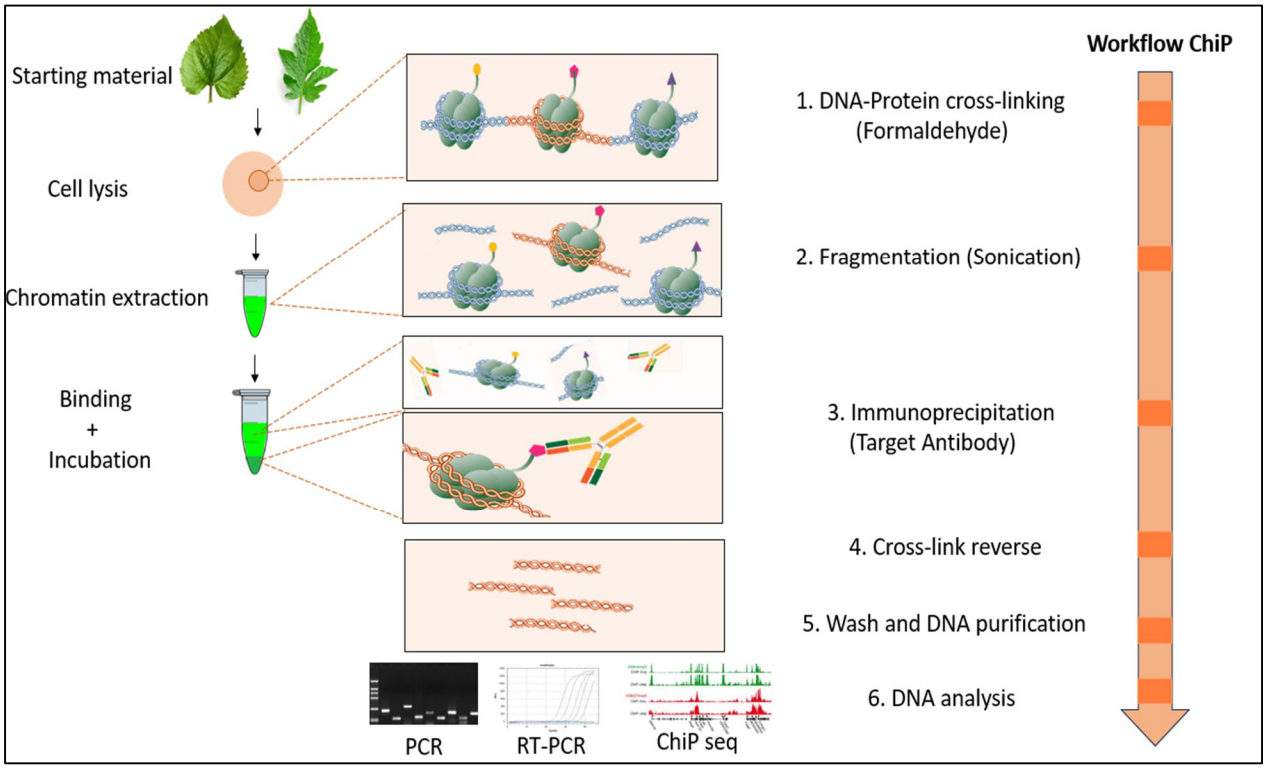

Figure 3: Workflow of the ChIP protocol.

2. AIM OF THE STUDY

Drought stress is one of the limiting factors for crop growth and productivity, therefore we aim in this study to investigate the responses and memory effects caused by this abiotic stress in two economically important crops sunflower and tomato, we focus in this research on three levels:

- Morphological and Physiological responses: by applying mild drought stress to early-stage plants followed by a recovery period with an examination of drought impact on leaf wilting, stomatal conductance, transpiration, chlorophyll content, and anthocyanin index.
- Chromatin level change: Investigate the transition of chromatin status during drought stress and recovery by developing an efficient chromatin immunoprecipitation (ChIP) protocol to study specific histone modification associated with drought response genes.
- RNA extraction for studying transcriptomic responses.

3. MATERIALS AND METHODS

3.1. Plant materials and stress protocol

A collection of 30 sunflower inbred lines were tested under *in vitro* conditions using PEG 6000 in Serbia to obtain a smaller panel of genotypes:

- Ha-26-PR and AB-OR-8 (drought susceptible lines)

- IMI-AB-12-PR and DF-AB-2 (drought tolerant lines)

AB-OR-8 and Ha-26-PR genotypes were used in the drought stress protocol.

Tomato inbred lines used for drought stress and chromatin extraction are Red Setter and M82.

Sunflower leaves used for chromatin extraction and immunoprecipitation were obtained from a mix of genotypes:

First replicate

- IMI.AB.12 PR.MSO control Jan 2. 21/02/2023
- IMI.AB.12 PR.MSO control Jan 3. 21/02/2023
- DF.AB.2 Primary leaves Jan 2. 21/02/2023
- DF.AB.2 Primary leaves Jan 3. 21/02/2023

Second replicate

- Df.AB2. MSO-0 Primary leaves Jan 4. 21/02/2023
- IMI.AB.12 PR.MSO control Jan 1. 21/02/2023
- IMI.AB.12 PR.MSO control Jan 4. 21/02/2023
- IMI.AB.12 PR. Primary leaves. 21/02/2023
- MS.O.Glass 1. PR. 17/11/2023
- MS.O.Glass 3. PR. 17/11/2023

To apply drought stress **(Fig 4)**, the plants were grown in pots in a greenhouse at the Experimental Farm L. Toniolo of the University of Padova, Agripolis campus, Italy. One seed was sown per pot during the summer growing season under controlled conditions, with 30 seeds per genotype for sunflower and 120 seeds per genotype for tomato. At the two leaves stage, plants were divided into control and stressed plants, control plants with 100% water availability, and stressed plants with 70% water availability. Water availability was determined by measuring the weight of the pots every two days, and then adding the required amount of water, a dehydration protocol was applied at three time points**:**

1. T0 (before stress): Well-watered
2. T1 (drought stress): Water reduced
3. T2 (recovery): Rewatering

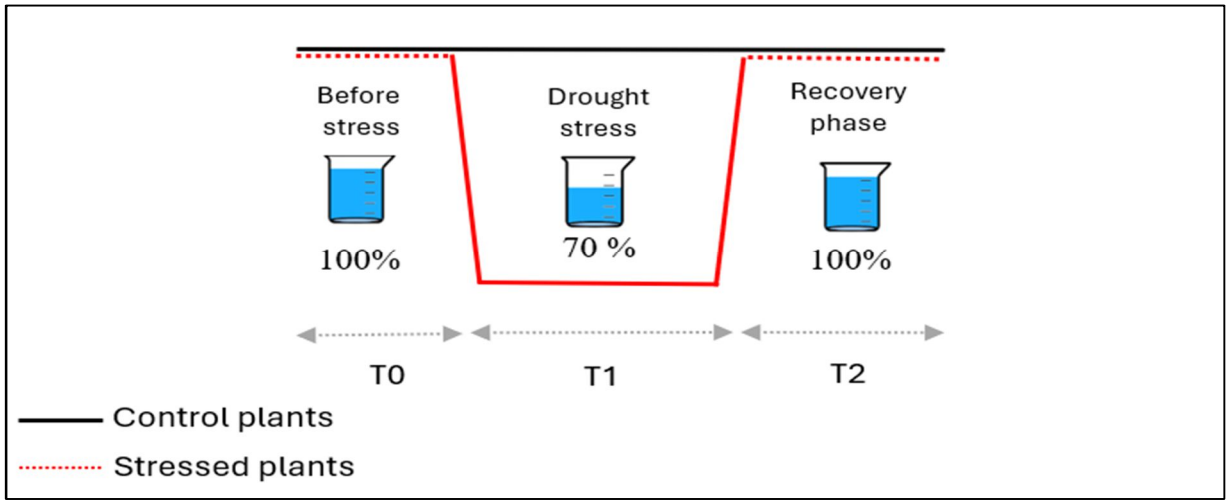

Figure 4: Drought stress protocol for sunflower and tomato plants.

The stress conditions and time points for the collection of plant materials were chosen based on the morphological appearance as leaf wilting and physiological parameters; the stomatal conductance (gsw, mol m^{-2} s^{-1}), transpiration (E apparent, mmol m^{-2} s^{-1}), chlorophyll content (Chl, µg/cm²), and anthocyanin index (Anth).

These parameters were taken and recorded with LI-Cor 600 and Dualex instruments in the morning between 9.30 a.m. to 11 a.m., two or three leaf readings per plant were collected, and additional repetitions were carried out as necessary.

3.1.1. Tissue collection

At each time point, upper leaves (two leaves) were collected from control plants and either stressed plants (at T1) or recovered plants (at T2), the number of independent plants used for each genotype and treatment are summarized in **Table 1.**

Table 1: Number of plants for each genotype and treatment used for leaf sampling.

Genotypes	Control plants			Stressed plants T1	Recovery Plants T2
	T0	T1	T2		
AB-OR-8	/	3	3	10	9
Ha-26-PR	/	3	2	4	5
Red setter	5	5	5	24 (two biological replicates)	24 (two biological replicates)
M82	5	5	5	30 (two biological replicates)	30 (two biological replicates)

After sampling, leaves were immediately placed in separate tubes previously labeled, flash-frozen in liquid nitrogen, and then stored at -80°C for subsequent experiments.

3.1.2. Reagent setup

All buffers used were prepared using autoclaved stock solutions on the day of use and kept on ice until needed. Phenylmethyl sulfonyl fluoride (PMSF, Sigma), Na-butyrate, and protease inhibitor cocktail (PI, Sigma) should be added into the solutions just before use.

Reagents for chromatin extraction

Nuclear Isolation Buffer NIB

10 mM HEPES pH 7.6, 1 M sucrose, 5 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 0.6% Triton X100, 0.5 mM PMSF.

Lysis Buffer

50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 10 mM Na-butyrate.

Reagents for chromatin immunoprecipitation

Low Salt Buffer

20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS.

High Salt Buffer

20 mM Tris–HCl pH 8.0, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS.

LNDET Buffer

20 mM Tris–HCl pH 8.0, 250 mM LiCl, 1% Nonidet P-40, 1% deoxycholate.

TE Buffer

10 mM TRIS pH 8.0, 1 mM EDTA.

Reagents for PCR amplification

10× PCR Buffer, 50m mM $MgCl_2$, Biostab PCR optimiser, 24 mM dNTPs, 10 µM forward Primer, 10 µM reverse primer, Taq DNA polymerase.

3.2. Chromatin extraction and immunoprecipitation assay

The assay described was obtained as described in Canton et al. (2022) experiment with the application of modifications.

3.2.1. Chromatin extraction and fixation procedure

Leaf material was finely powdered with liquid nitrogen and transferred into a 50 ml tube, adding a cold NIB in a ratio of 1g of tissue in 25 ml of NIB. Chromatin was fixed by adding 1% formaldehyde, incubated for 15' at room temperature (RT), and the reaction was blocked for 5' with 3.4 ml of 1M Glycine. Subsequently, the lysate was filtered through a single layer of Miracloth (Millipore) into a new pre-chilled tube and centrifuged at 5000×g for 15' at 4°C followed by

supernatant discard, the white pellet was then washed thrice with 3 ml of cold NIB without adding Triton X-100 and PMSF, then centrifugation at 5000×g for 15' at 4°C with supernatant discard in each wash, ultimately, the pellet was suspended in 500 µl of Lysis Buffer (containing 10 mM Na-butyrate and protease inhibitor cocktail) and stored at -80 °C.

3.2.1.1. Reverse crosslinking to check chromatin extraction/fixation efficiency

A fraction of the extracted chromatin step was sub-divided into a control de-crossed (+DC, reverse crosslinking will be performed) and a control not de-crossed (-DC, no reverse crosslinking). The +DC was reverse cross-linked with 0.2 M NaCl for 16 h at 65°C. An equal volume of FIAC-phenol/chloroform/ isoamyl alcohol (25:24:1) was added, vortexed then centrifugated at 14,000×g for 10' at RT, the aqueous phase was recovered then the FIAC step was repeated. Samples were precipitated with 1/125 volumes of Glycogen and 2.5 volumes of absolute cold ethanol at -80°C for at least 3h, followed by centrifugation at 14,000 ×g for 30' at 4°C, pellet collection, 70% cold ethanol washing, and second centrifugation at 14,000 ×g for 10' at 4°, after that, the supernatant was discarded and the pellet was dried at -20 °C. The extract was resuspended in 10 µl of double distilled water and treated with 1mg/ml of RNase A at 37°C for 30', the efficiency was finely checked by running a gel electrophoresis 1%.

3.2.2. Chromatin Sonication

Sunflower chromatin was sheared using the Quick Start M220 focused ultrasonicator at 13% amplitude with separately 3, 6, and 9 cycles, each cycle is [15s On / 15s Off] ×2, after the On round the tube is immediately placed in ice for the Off round.

For tomato chromatin, Diagenode's Bioruptor Plus sonicator was used at a maximum power of 1000 mA with an automatically control temperature at 4°C, the extract was fragmented for 12, 20, and 30 cycles separately, where each cycle is 45s On/15s Off.

3.2.3. Reverse crosslinking to check chromatin sonication efficiency

Fragmented chromatin was centrifuged at 16000×g for 10' at 4°C. A fraction of the aliquote (+DC) was reverse crosslinked with 0.2 M NaCl for 16 h at 65°C, two cycles of FIAC were held as previously mentioned and incubated at -80 °C for 3h, the precipitation was carried out with 1/125 volumes of Glycogen and 2.5 volumes of absolute cold ethanol. the aliquote was centrifuged for 30' at full speed at 4°C. The pellet was washed with 70% cold ethanol and centrifuged for 5' at 4 °C, lately dried at -20 °C. The sample was resuspended in TE-Pk Buffer and additionally treated with 1 mg/ml RNAse A at 37°C for 30', following proteinase K treatment by adding 10 µg/µl of

Proteinase K and for 1 h at 42°C. Subsequently, one cycle of FIAC was held. The recovered supernatant was purified with QIAquick PCR purification kit (QIAGEN). The concentration and quality check were determined by measuring OD260/230 and OD260/280 ratio on a NanoDrop 2000c spectrophotometer (Thermo Scientific) for tomato and quantified by Qubit 2.0 Fluorometer for sunflower, the size of fragments was checked by gel electrophoresis 1.2% by loading 200 ng of sonicated chromatin, a fraction will be use used as an input sample (IP) in the following PCR evaluation.

3.2.4. Chromatin Immunoprecipitation

The chromatin immunoprecipitation was carried out over three days only with sunflower plant leaves. Day 1 involved Dynabeads proteins G preparation, pre-clearing of the chromatin sample, antibody preparation (H3K4me3), and the immunoprecipitation step, day 2 consisted of a wash and DNA elution, and lastly day 3 involved DNA precipitation and purification, the detailed procedure is found in **Annex 1.**

Summarily, for immunoprecipitation, 7 µg of chromatin was used for each technical replicate and 4 µg antibody precipitation reaction H3K4me3 (Active Motif, Cat. 39159). The histone-DNA complexes were immunoprecipitated overnight at 4°C adding H3K4me3 antibody, Chromatin without the addition of any antibody (No Ab sample) was used as a negative control, Dynabeads protein G (Invitrogen) was used for pre-clarification incubation at 5 °C for 4-5h and the antibody at 5 °C for 2h. The beads linked to histone-DNA immunoprecipitated complexes were sequentially washed, once with Low Salt Buffer, High Salt Buffer, LNDET, and twice with TE (Tris-EDTA) Buffer. The chromatin was also eluted twice with 0.1M $NaHCO_3$ and 1% SDS and the cross-linking was reversed as previously described. DNA samples were precipitated with 1/125 Glycogen (Sigma) and 2.5 volumes of absolute ethanol at -80°C for 3 h. DNA was resuspended in TE Buffer after the pellet collection, later pallet wash with 70% ethanol and dryness at -20°C, DNA was extracted through a single FIAC step and then treated with RNAse A at 37°C for 30' with and a following treatment with proteinase K for 1 h at 42°C was held. DNA was purified with QIAquick PCR purification Kit, and the concentration check was determined on a NanoDrop 2000c spectrophotometer (Thermo Scientific). The ChIpped DNA was finely stored at -20 °C for further experiments.

3.3. Ribonucleic acid (RNA) extraction and reverse transcription

The leaves used in RNA extraction were obtained from AB-OR-8 genotype ($CTRL_1$, $CTRL_2$) on previous plant growth experiment, and M82 genotype (CTRL.T0, CTRL.T1, CTRL.T2, ST_1.T1, ST_2.T1, ST_1.T2, ST_2.T2).

Total RNA was extracted from 50-70 mg of frozen and grinded leaves using RNeasy Plant Mini kit (Qiagen), RNA concentration and quality was determined by measuring OD260/230 and OD260/280 ratio on a NanoDrop 2000c spectrophotometer (Thermo Scientific), while the integrity of extracted RNA was determined by agarose gel 1.2% by loading 400 ng of RNA.

cDNA synthesis was performed with the SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions only for the sunflower genotype.

3.4. PCR Validation

PCR amplification was applied to evaluate the efficacy of primers in amplifying target drought marker genes, previously identified in *in vitro* studies in sunflower plants. The DNA used for this experiment is the input of purified DNA after the chromatin sonication. This step is performed before proceeding with the RNA seq and qPCR experiments, PCR was performed to test a total of 10 primers designed at the 5' **(Table 2).**

The PCR reaction consisted of 5' at 96°C in stage one, followed by 35 cycles of 95°C for 1', 58°C for 30s, 72°C for 40s followed by 72°C for 12', after the PCR reaction 100 ng of DNA was loaded per lane in a volume of 10 µl on a 1% gel electrophoresis.

Table 2: Sequences of primers used for the PCR amplification in sunflower Input (IP).

Primer name	Sequence
FATA_5'_Fwd	GTAATGGCGGTGAAGGTTGATGAGC
FATA_5'_Rev	AACTCCTGCAACAGATTCGCAATCG
FATB_5'_Fwd	AACCTCACTCTGGAGCCAAGACATC
FATB_5'_Rev	TCGGTTTTGAAGCCATGCGTGATAC
HaDhn1_5'_Fwd	ACTACGAGAGCCCAATTCACTCCAC
HaDhn1_5'_Rev	TAACCCCGTGGAACCATAATCACCC
Nup155_5'_Fwd	TCAATGCCCCGAATACAGTGGTGAA
Nup155_5'_Rev	ACGGTTGTAGTGAAACCTCTGCGTA
HaNAC76_5'_Fwd	TGTTTGCAAGAAAGAGCCTTGGGAC
HaNAC76_5'_Rev	CCCCTATTCGTCTTGATCGTCCGAT
HaNAC84_5'_Fwd	GGTTACCGACCCGATGACCCAATTA
HaNAC84_5'_Rev	ACTCTTTCTCCCCAAACATCGCCTT
GAPB_5'_Fwd	GCTGTGTCTGCTCAATTTACTCCCA
GAPB_5'_Rev	CCACCACTATCATTGACCACCACCA
Cu/Zn SOD_5'_Fwd	GGTTGCGGTTCTTAGCAGCAGTGAG
Cu/Zn SOD_5'_Rev	TAGTGAGGACCAGTTGACATGCACC
Fructokinase 2_5'_Fwd	ATCTGCTCTCTGTTTCGGTTCGGTT
Fructokinase 2_5'_Rev	ACACTTGCCAGCACTTTTCCACTTC
CDPK2_5'_Fwd	TCACTCTCCGCTTCGTGTTCTGATT
CDPK2_5'_Rev	CGGCTTCGAAATCCGTTCCAAATCA

3.5. Statistical analysis

All the graphs, calculations, and statistical analyses were performed using GraphPad Prism software version 8.0.2 for Windows 2019, The comparison of means between different groups of numerical variables was performed using one-way ANOVA, P value less than 0.05 ($p < 0.05$) was considered as statistically significant, while The standard deviation was measured by Microsoft Excel.

4. RESULTS

4.1. Plant Development in Response to Stress

The two genotypes of sunflower plants showed signs of stress through morphological changes after 12 days of dehydration treatment, this appeared in slight leaf wilting and small parts yellowing **(Fig 5)**. However, all physiological analyses indicated a non-significant difference between control and stressed sunflower plants with minimal presence of stress.

After 12 days of drought stress, as the graphs in Figures 5 and 6 show, the stomatal conductance and the transpiration rates of the stressed plant of genotype HA has decreased, respectively 34.74%, and

27.33% lower than the control plant. In contrast, the rates of the genotype AB showed a similar rate for the two parameters. Likewise, the chlorophyll and anthocyanin contents showed almost equal rates for the two genotypes in all sunflower plants.

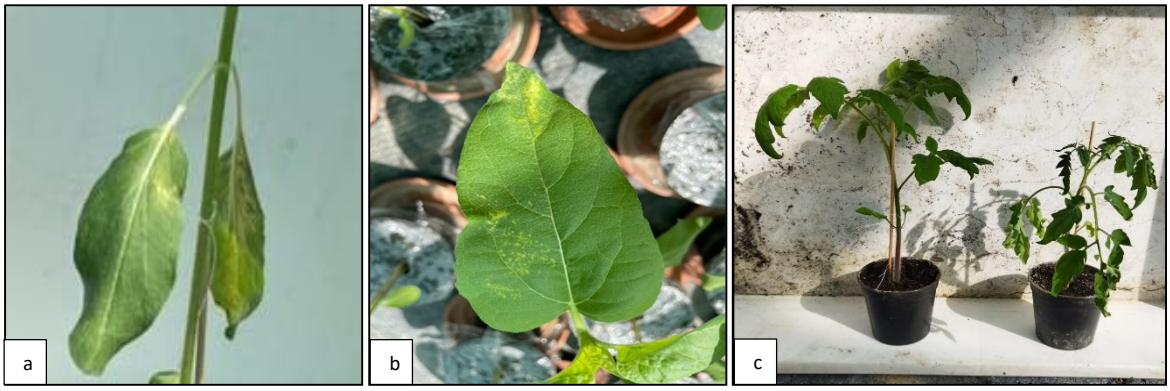

Figure 5: Morphological changes during drought stress treatment a. leaf wilt in sunflower, b. yellowing parts in sunflower leaves and c. difference between controls and stressed tomato plants (control on the left and stressed on the right).

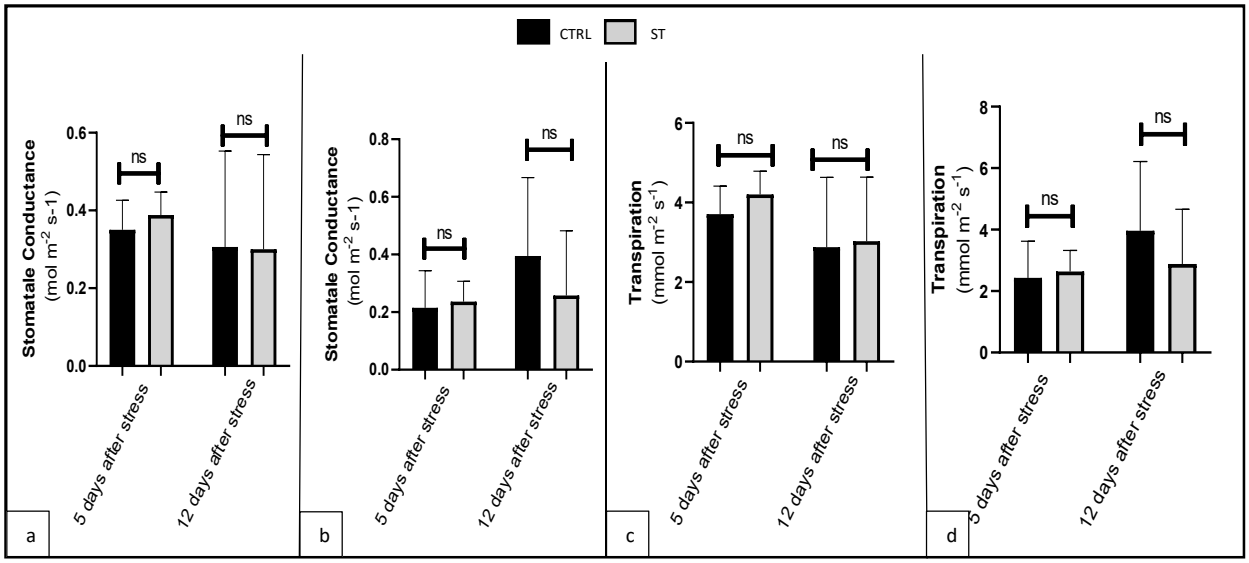

Figure 6: Stomatal conductance (gsw) and transpiration (E_apparent) rates of the leaves after 5 and 12 days of drought stress in two genotypes of sunflower (a. gsw AB, b. gsw HA, c. E_apparent AB, d. E_apparent HA). All values are the mean of 25 AB plants and 14 Ha plants. An ANOVA statistic test $P< 0.05$ was performed and no significant differences were indicated as "ns".

The morphological observation during the restoration of water showed that a recovery period of 5 days was enough for the regrowth of plants and led to the quick restoration of leaf turgor, while the only significant difference detected was an increase of anthocyanin content in AB controls and stressed plants at T2 compared to T1 time points **(Fig 7c)**, the analysis of other recorded parameters showed no significant difference between the two time points and within the same time points in controls and stressed plants.

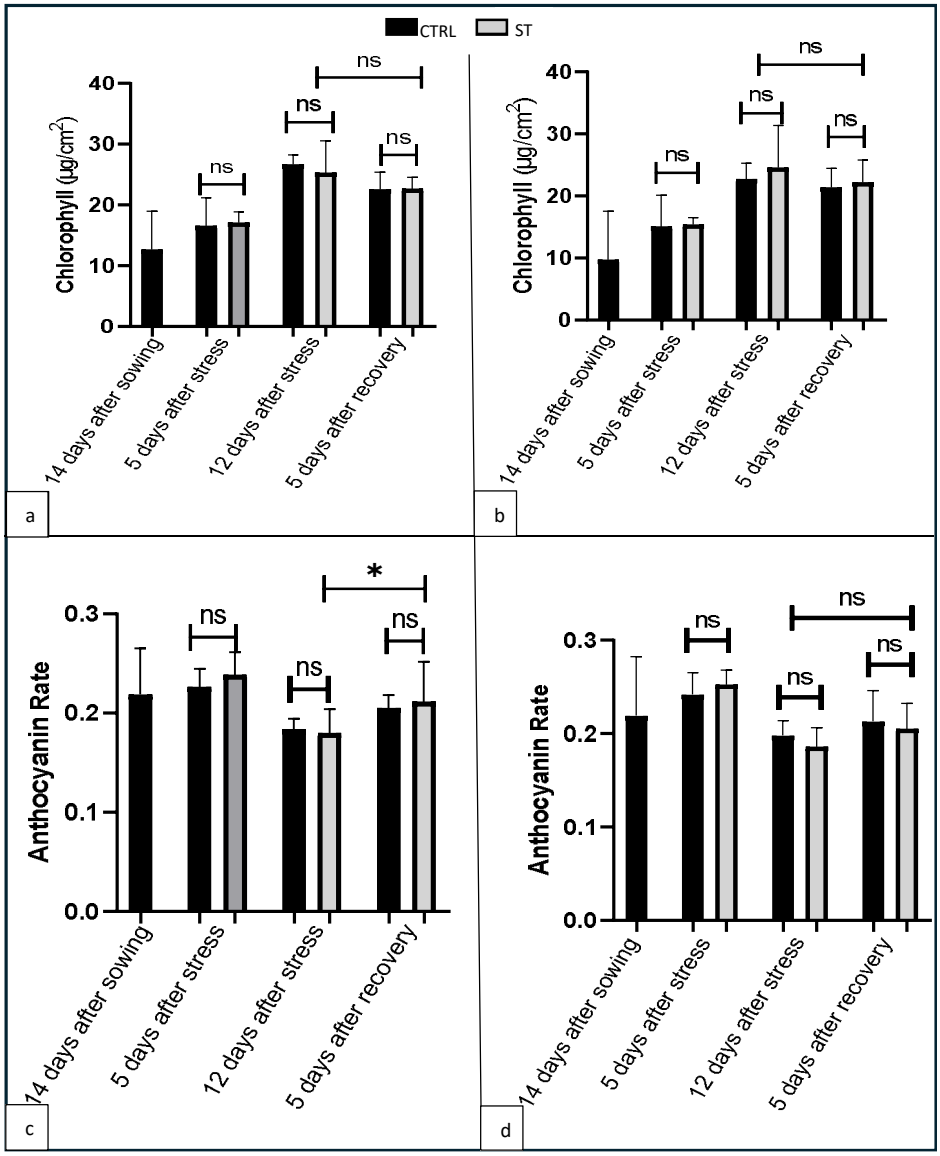

Figure 7: Chlorophyll (Chl) and anthocyanin (Anth) contents during the dehydration time points, T0: after 14 days of seed sowing, T1: 5 and 12 days of drought stress, T1: 5 days of recovery in two genotype sunflower leaves, a. Chl AB, b. Chl Ha, c. Anth AB and d. Anth Ha. All values are the mean of 25 AB plants and 14 Ha plants. An ANOVA statistic test $P< 0.05$ was performed with significant difference treatments indicated as *, while "ns" refers to no significant difference.

On the contrary, time course analysis revealed that tomato plants exhibited both morphological as leaf wilting and a decrease in plant height **(Fig 5c)**, as well as physiological indications of perceived stress after 13 days of dehydration, where gradually all physiological parameters decreased significantly (Brown-Forsythe and Welch's ANOVA test with $P < 0.05$), reaching a quasi-complete stomatal closure, and a decline in the mean transpiration rate of the stressed plants by 83.94% (1st R) and 75.37% (2nd R) for M82 plants, 93.30% (1st R) and 84.88% (2nd R) for Red Setter plants concerning the corresponding control plant **(Fig 8).**

The values also revealed that the anthocyanin content decreased significantly in both lines, on the other hand, as shown in Figure 9 a and b, the chlorophyll content showed a trend to increase under drought conditions with an increase of the mean by 25.21% (1st R) and 28.60% (2nd R) in M82 leaves, 37.60% (1st R) and 29.04% (2ndR) in Red Setter leaves compared to controls plants.

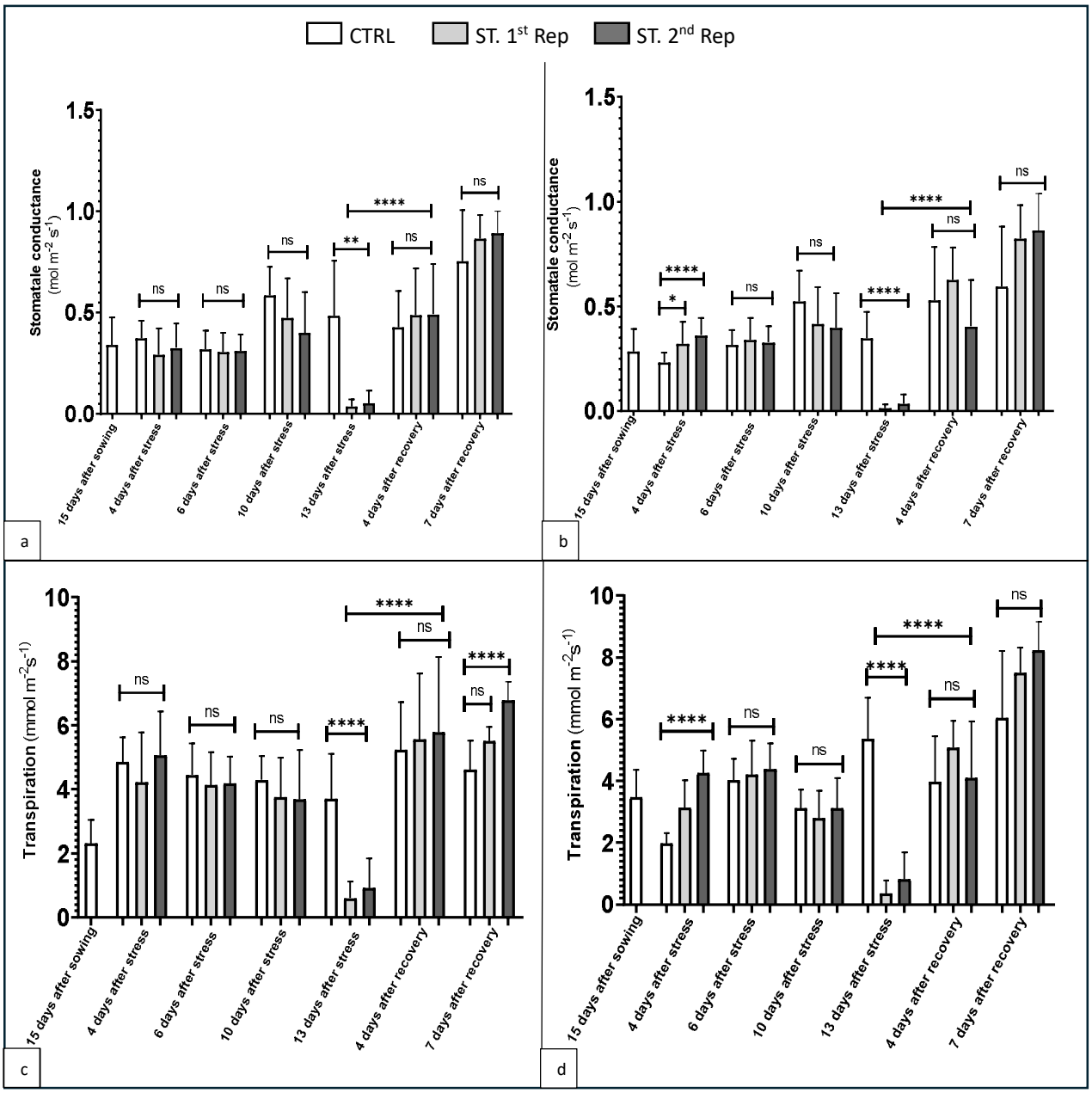

Figure 8: Stomatal conductance (gsw) and transpiration (E_apparent) rates during the dehydration time points, T0: after 15 days of seed sowing, T1: 4, 6, 10, and 13 days of drought stress, T2: 4 days of recovery in two genotype tomato leaves, a. gsw M82, b. gws Red Setter, c. E_Apparent M82 and d. E_Apparent Red Setter. All values are the mean of two biological replicates, with 63 plants for M82 and 75 plants for the Red Setter. An ANOVA statistic test $P< 0.05$ was performed with significant difference treatments indicated as *****, while "ns" refers to no significant difference.

Similarly to sunflower plants and after drought stress, a 4 day recovery period was required for tomato plants, where morphological observations revealed that plant regrowth and leaf turgidity were regained. Furthermore, the physiological measurements at 4 days after recovery in both tomato genotypes showed a significant increase in all parameters (except a decrease in chlorophyll content) compared to the last T1 time point (13 days after stress) and values close to the control plants at the same T2 time point.

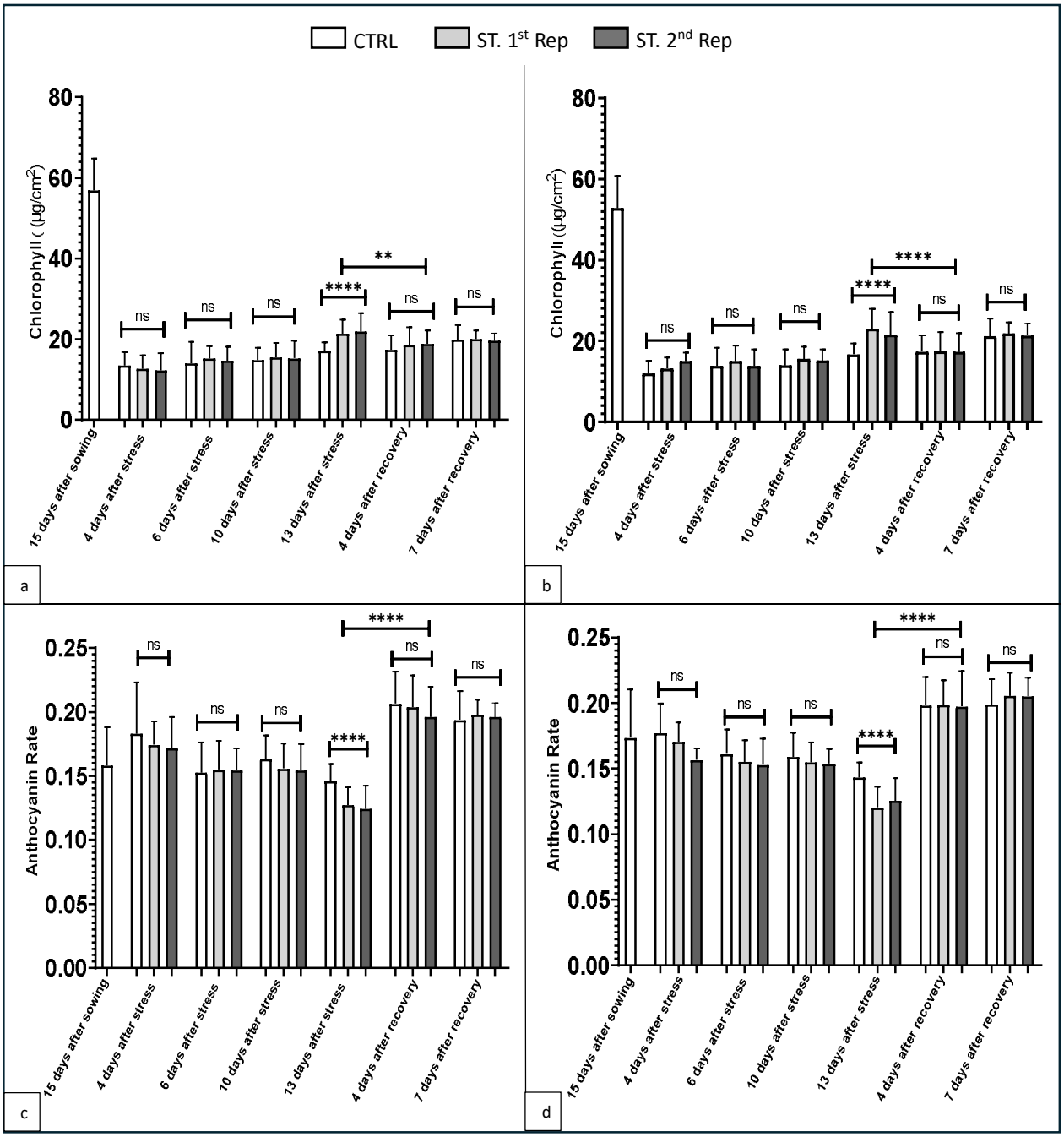

Figure 9: Chlorophyll (Chl) and anthocyanin (Anth) content during the dehydration time points, T0: after 15 days of seed sowing, T1: 4, 6, 10, and 13 days of drought stress, T2: 4 days of recovery in two genotype tomato leaves, a. Chl M82, b. Chl Red Setter, c. Anth M82 and d. Anth Red Setter. All values

are the mean of two biological replicates, with 63 plants for M82 and 75 plants for Red Setter. An ANOVA statistic test $P < 0.05$ was performed with significant difference treatments indicated as *, while "ns" refers to no significant difference.

4.2. Chromatin extraction and fixation

In both sunflower and tomato **(Fig 10)**, the +DC (reverse crosslinked) lane on agarose gel shows a clear, high molecular weight band, that could confirm the successful chromatin extraction and the effectiveness of reverse crosslink, whereas the -DC (not reversed crosslinked) lane that is used as negative controls did not show any visible band**,** indicating the requirement of reverse crosslinking procedure for the isolation of DNA from cross-linked samples, the distinct bands indicate that the extracted chromatin maintained its structural integrity.

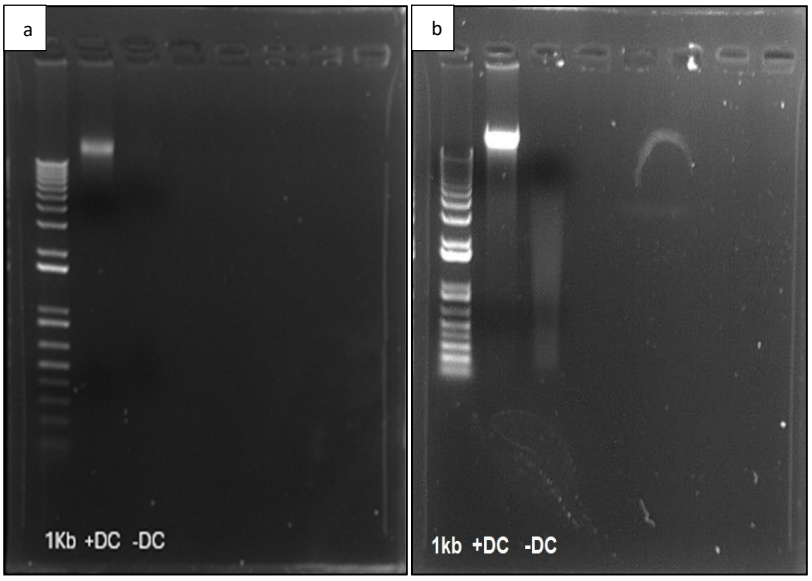

Figure 10: Fixation efficiency of chromatin extracted from leaves tissues from a. sunflower and b.tomato, crosslinked with 1% formaldehyde, subsequently decrosslinked +DC or not decrosslinked -DC, DNA was isolated using phenol/chloroform extraction. Finely checked by running gel electrophoresis 1% with a 1kb Invitrogen ladder.

4.3. Chromatin sonication

Chromatin fragmentation is a crucial step in ChIP protocol, we tested different numbers of cycles to determine the optimum number of cycles to achieve the required sheared DNA fragment range, as performing more cycles resulted in greater fragmentation of chromatin as can be seen in Figure 11.

- For sunflower samples, 9 cycles showed the most compact smear ranging from approximately 500 bp to 2000 bp, with an intense portion around 700 bp to 1000 bp which could be suitable for real-time PCR.

- For tomato samples, in the 20x lane, a smear appears approximately between 200 and 1500 bp with a compact band of small molecular weight roughly between 200 bp to 500 bp likely in the optimal range for both real-time PCR and ChIP-seq.

A second fragmentation replicate was performed in chromatin from tomato leaf chromatin, under the same conditions using the Diagenode's Bioruptor Plus sonicator with only 20 cycles (45 s ON / 15 sec off) on high power mode, the efficiency of fragmentation was confirmed by loading 200 ng in the first and second lanes and 400 ng in the third and fourth lanes of an agarose gel 1.2% **(Fig 12)**.

The smear patterns of the four replicates are approximately between 200 bp to 2000 bp, at the bottom of each smear range, a narrow distinct band falls between approximately 200 bp to 400 bp was observed, the more the amounts of chromatin uploaded the more the bands are clear and intense, in particular in R3 and R4.

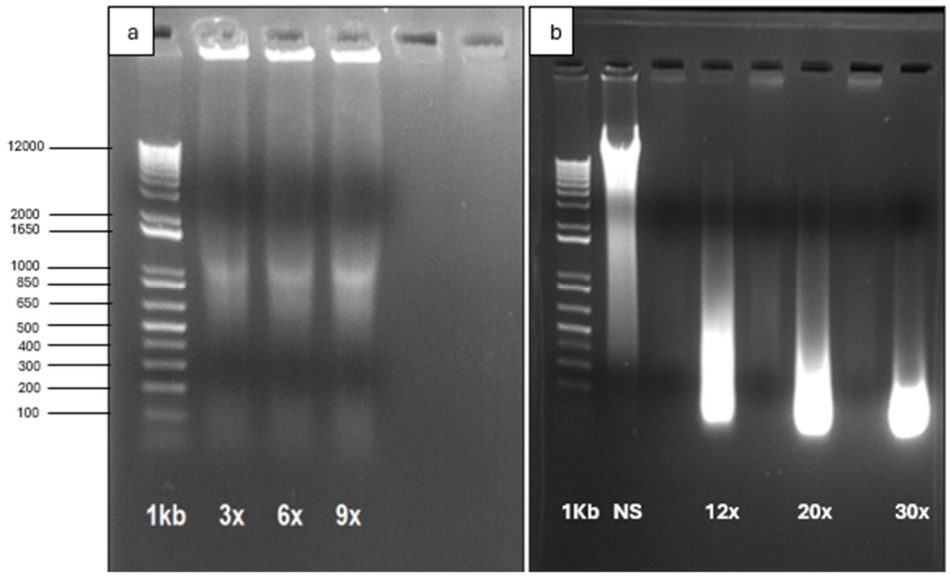

Figure 11: Chromatin physical shearing analyses followed by a reverse crosslinking phase, DNA is isolated using phenol/chloroform extraction, loading 200 ng on 1.2% agarose gel with a 1kb Invitrogen ladder. a. Sunflower chromatin shearing check after the application of 13% amplitude with [15s On / 15s Off]×2 shearing cycles 3, 6, and 9 separate cycles. b. Tomato chromatin shearing check after the application of maximum power with 45s On/15s Off shearing cycle 12, 20, 30 separate cycles.

After chromatin sonication and DNA purification, the Qubit quantification results for sunflower input (IP) were as follows: the first replicate had a concentration of 65 ng/µl and the second replicate had a concentration of 34.2 ng/µl; for tomato samples the concentration and quality were

measured using a nanodrop giving a concentration of 82.1 ng/µl withs Ab260/280 ratios of 1.74 and Ab260/230 ratios of 1.88.

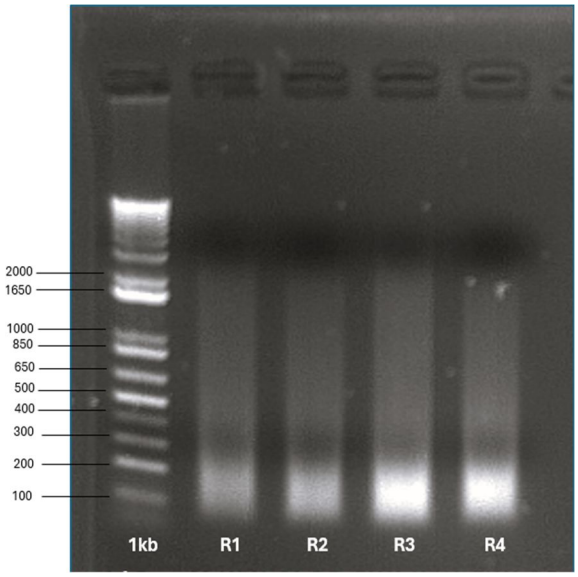

Figure 12: Tomato chromatin shearing check after the application of maximum power for 20 cycles with 45s On/15s Off, followed by a reverse crosslinking phase, a DNA isolated using phenol/chloroform extraction followed by loading 200 ng in R1, R2, and 400 ng in R3, R4 on 1.5% agarose gel with a 1kb Invitrogen ladder.

These results indicate that the required quantity and quality of chromatin are sufficient to proceed to the next step, which is chromatin immunoprecipitation.

4.4. Chromatin Immunoprecipitation

The concentration values of the immunoprecipitated sunflower chromatin after the DNA purifications, checked at the Nanodrop, are:

- [+Ab] = 2.4 ng/uL
- [-Ab] = 3.2 ng/uL

Further analysis should be performed to check the immunoprecipitation success using a single genotype of sunflower plants per sample.

4.5. Ribonucleic acid (RNA) extraction

4.5.1. RNA isolated from sunflower leaves

The RNA concentration of both replicates extracted from sunflower leaves is relatively high, while the purity in the A260 / 280 ratios is higher than 2.00 as shown in Table 3, however, the purity in the A260 / 230 ratios of both replicates is slightly less than 2.00, which could indicate the presence of contamination by peptides, carbohydrates, or phenols. However, Biskup et al. (2020) mentioned

that many studies reported the possibility of performing downstream applications as RT-PCR even with low 260/230 ratios.

Table 3: NanoDrop assessment of RNA quantity and purity extracted from sunflower leaf samples.

Sample	Symbol	Concentration ng/µL (NanoDrop)	Purity - A260/280 (NanoDrop)	Purity - A260/230 (NanoDrop)
$CTRL_1$	R1	515.8	2.09	1.29
$CTRL_2$	R2	1297.9	2.16	1.65

4.5.2. RNA isolated from tomato leaves

As Table 4 reveals, extraction efficiently produced high-quality RNA from all extracted tomato leaves including controlled and stressed samples and at all time points T0, T1, and T2. The purity at A260/A280 and A260/A230 ratios were higher than 2.00, implying the absence of contamination.

Table 4: NanoDrop evaluation of RNA quantity and purity extracted from tomato leaf samples.

Sample	Symbol	Concentration ng/uL (NanoDrop)	Purity - A260/280 (NanoDrop)	Purity - A260/230 (NanoDrop)
CTRL.T0	C1	1283.9	2.15	2.32
CTRL.T1	C2	2276.9	2.1	2.34
CTRL.T2	C3	1485.5	2.15	2.29
SR_1.T1	C4	1578.8	2.14	2.36
SR_2.T1	C5	1465.2	2.15	2.37
SR_1.T2	C6	1212.2	2.15	2.42
SR_2.T2	C7	1089.2	2.15	2.31

The RNA visualised on agarose gels for both plants **(Fig 13)** indicated that the extracted RNA is of high quality and integrity with clear ribosomal bands for 28 S, 18 S, 16 S, and 5 S, the bands between the 16 S and the 5 S are the 23 S rRNA fragments. No high-molecular weight gDNA can be observed because we performed optional on-column DNase digestion to eliminate genomic DNA contamination.

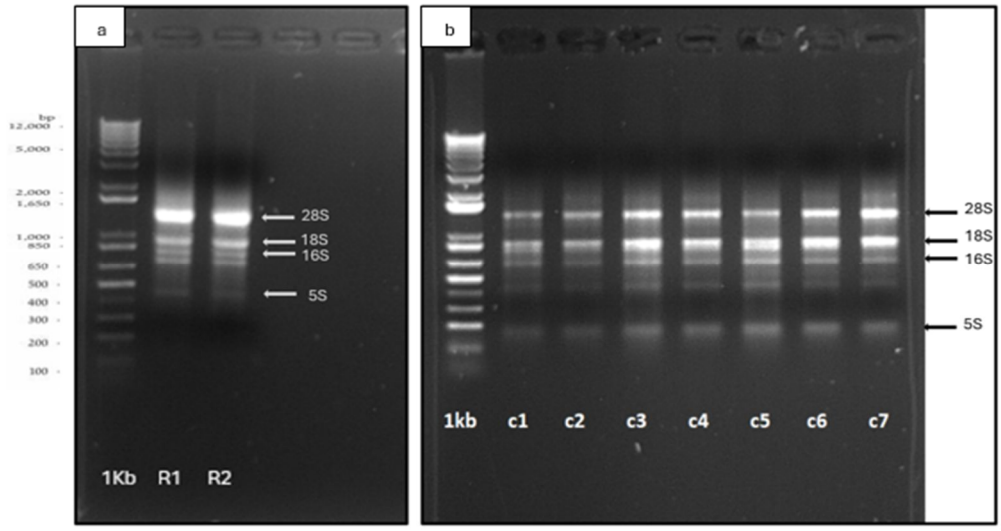

Figure 13: Totale RNA extracted from leaf tissue from a. sunflower control plants (R1 and R2).b. tomato controlled and stressed plant at T0.T1 and T2 (c1, c2,,c3, c4, c5, c6, c7) RNA was isolated by the RNeasy Plant Mini kit (Qiagen) then 400 ng was loaded in each lane of 1.2% agarose gel with 1kb Invitrogen ladder.

The integrity and concentration of RNA extracted from both plants could be used in future reverse transcription-qPCR and RNA sequencing experiments.

4.6. PCR Validation

After PCR amplification, it was found that a total of 8 primer pairs were able to amplify the target sequences of sunflower input (IP) (the purified DNA after chromatin sonication), these primer pairs are FATB, FATA, HaDhn **(Fig 14a)**, Nup155, HaNAC76, HaNAC84, GABP and CDPK2 (Figure 10 b). Bands of 300 bp were observed in all of these primer pairs except for the GABP primer pairs band, which was around 500 bp. However, Cu/Zn SOD and Fructokinase 2 primer pairs were unable to amplify *Helianthus annuus* DNA successfully.

Note: in the first PCR experiment **(Fig 14a)**, the GABP primer pairs did not amplify the PCR template, while the CDPK2 primer pairs exhibited two bands in the positive control and a band in the negative control, Consequently, these two reactions were repeated in a second PCR to obtain more reliable results **(Fig 14b)**.

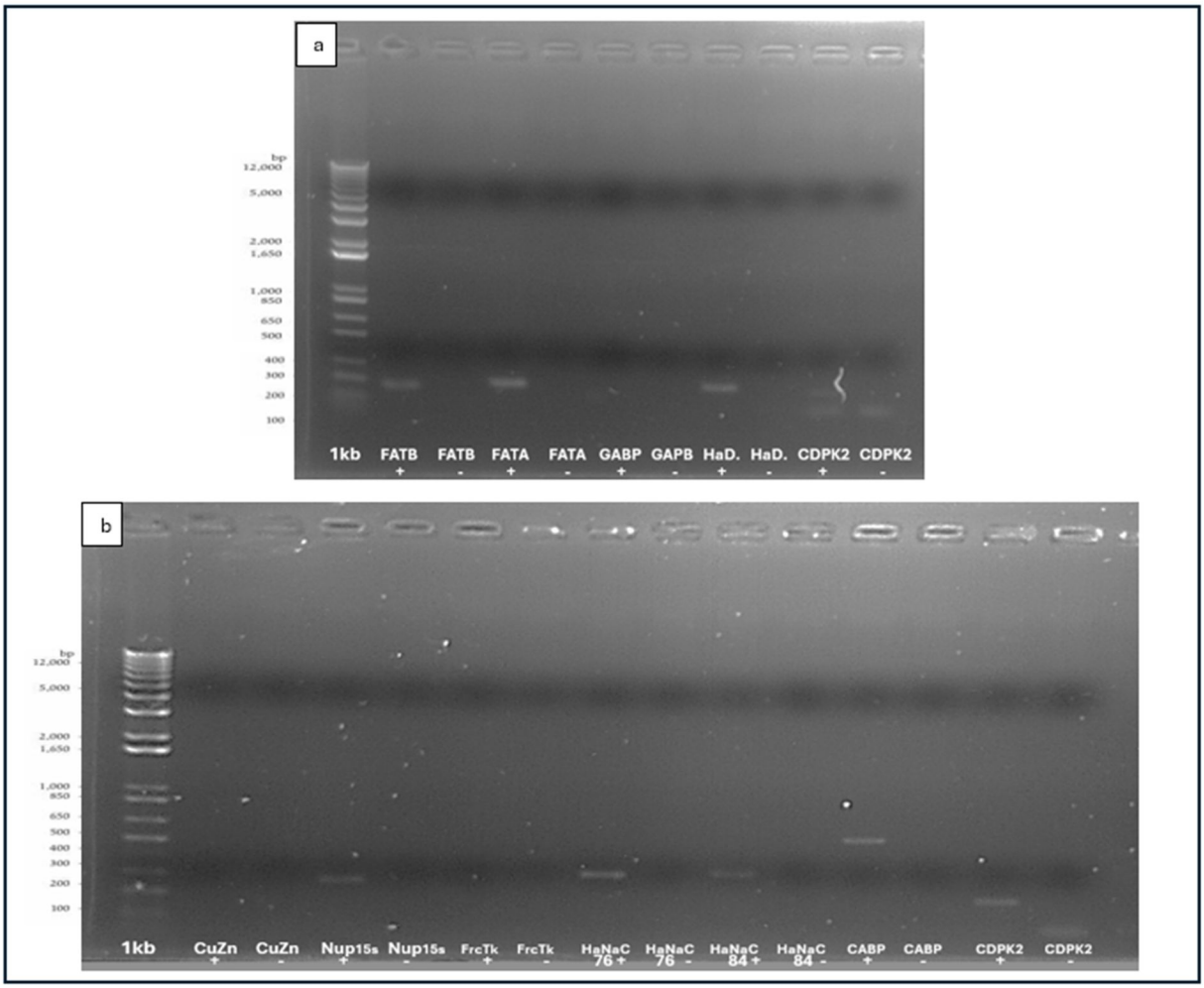

Figure 14: Agarose gel electrophoresis (1%) of PCR amplicons obtained with specific primers for target drought genes to amplify 100 ng of the input DNA (IP) of *Helianthus annuus* leaves. Loading of 1kb Invitrogen ladder in the first lane; the genes tested are: a). FATB, FATA, GABP, HaD. and CDPK2, b) CuZN, Nup15s, FrcTK, HaNaC76, HaNaC 84, CABP and CDPK2, respectively. Positive and negative control lanes were included for each primer.

5. DISCUSSION

In the beginning, all sowed seeds were irrigated well to get good germination. A mild and prolonged drought stress was imposed from the two-leaf stage of the seedling, after the full leaf expansion. As mentioned in several studies, such as Backhaus et al. (2014) and Forestan et al. (2019), repeated mild drought periods increase the tolerance of plants toward severe drought stress. Furthermore a mild drought stress could function in genes stress memory, such as transcriptional and epigenetic memory, in which stress-induced chromatin changes that affect transcription. However, it is also reported that severe drought stress causes stronger enrichment/changes in the histone marks than mild drought stress in model plants *Arabidopsis thaliana* (Ost et al., 2023).

According to Bandurska (2022), the level of dehydration depends on stress severity and duration, as well as adaptive responses to drought stress and the water absorption ability from deep soil layers. Nezami et al. (2008), reported after applying a treatment of 60 % and 30% of field capacity (FC) on sunflower plants, drought stress caused a decline in the plant's height, plants dry matter, stem diameter, head size, seed number, and weight, remarkably the stress severity at 60 % of FC had a higher negative impact on the plant than the 30 % of FC. Similar to our experience, Hussain et al. (2018) and Seleiman et al. (2021), mentioned that sunflower plants are considered moderately drought-tolerant crops, but the decline in soil moisture can lead to leaf wilting, as well as leaf yellowing. In addition, stressed sunflower plants in our trials exhibit partial stomatal closure, Debaeke et al. (2021) reinforce that sunflower plants could adapt to another physiological process by stomatal closure under water scarcity to increase the resistance to gaseous loss.

To prevent soil evaporation, the tops of pots were sealed with a plastic sheet, only leaving one hole for the plant steam. As our finding in sunflower transpiration rates, Bona et al. (2000) reported that while transpiration in progressively increasing water stress was almost similar to that of control plants until the point of very low water availability was reached at about 30% of water availability, thus confirming the great adaptability of sunflower plants to water stress. In contrast, chlorophyll content according to Ahmad et al. (2021), was decreased under drought conditions in all tested sunflower cultivars, except one.

Our experiments point out a correlation between the species and the duration of their response to drought stress. Indeed, even with the application of equal drought stress severity tomato plants showed a higher drought stress impact than the sunflower plants at almost equal drought stress duration. These results could be explained by Laxa et al. (2019) observation, which states that the time needed by plants to reach and respond to stress may vary significantly between species.

From comparing the results, it is clear that species responded differently to drought stress. However, the genotypes of each species responded similarly, yet an interesting comparison between tomato (S. lycopersicum cv. M82) and its wild relative (S. pennellii) in Lupo and Moshelion (2024) experiment, indicates that domesticated tomato plants had a higher transpiration rate and higher stomatal conductance under drought conditions when compared to the wild tomato, these results highlight that wild tomato had a higher sensitivity to soil water content depletion (showed in stronger stomatal closure).

Our results show a complete stomatal conductance close, in tomato plants after 15 days of drought stress, these results are in line with those obtained by Tamburino (2017), the stomatal conductance rates decreased continuously during water withholding, reaching a value of 2% of that controls plants in tomato (*Solanum lycopersicum* L.), followed by complete recovery after 6 days of the rewatering period showing an increase in stomatal conductance levels.

Jangid and Dwivedi (2016), also mentioned a reduction in stomatal conductivity and transpiration rates under drought stress: This decline is linked to an increase in ABA concentration, while the chlorophyll content has decreased contrary to our experiment. However, several studies have reported an increase in chlorophyll content under drought stress: for example tomato plants after one week of dehydration treatment and cereal grasses (Rosa et al., 2023). Furthermore, experiments have shown that chlorophyll content varies among tomato genotypes during drought stress: Therefore it can be used as indicative of oxidative damage, proving that tomato genotypes that show a less decrease in chlorophyll content under stress are likely to have more protective mechanisms function against chlorophyll degradation (Napar et al., 2022).

As showed in the results section, there was a notable decline in anthocyanin levels in both genotypes of tomato, suggesting that both genotypes could be susceptible to drought stress. This hypothesis could be reinforced by Napar et al. (2022): they suggest that anthocyanin accumulation can be considered a marker of stress tolerance in tomato plants, as it is significantly increased in drought stress plants compared to controlled plants with an upregulation of stress related genes; the increased level of anthocyanin is strongly depended on tomato genotypes. Moreover, previous studies on *Arabidopsis*, tobacco, and tomato have found that transgenic plants with higher anthocyanin content have higher drought resistance than wild-type plants, evidencing that low anthocyanin accumulation is correlated with drought sensitivity plants (Li and Ahammed, 2023).

One of the important aspects we encounter in the ChIP protocol by starting with a soft tissue with an H3K4me3 antibody that has been validated for ChIP analysis in other studies, this procedure ties well with Schmidt et al. (2019), wherein the nature (cell or tissue) and the amount of starting material as well as the antibody used (available or new) guide the success of ChIP experiment.

Vacuum infiltration has been introduced to ensure the penetration of the DNA–protein cross-linking solution into plant cells (Saleh, 2008), on top of that several studies, start the ChIP protocols by extracting nuclei then using β-mercaptoethanol like Ranawaka et al. (2020), or isolating protoplast from leaves in Lee et al. (2017) experiment. In our experiment, we avoid vacuuming, isolation of

clear nuclei, and adding β-mercaptoethanol, to maintain the structure of chromatin and shorten the procedures.

As prior referred the main difference between X-ChIP and N-ChIP is the reverse crosslinking, the choice between these two techniques depends on the aim of the study: X-ChIP is used for studying all types of proteins making it more sensitive, besides the possibility of using less amount of cells and antibody. It is important to use the right percentage in the fixation step as it reduces protein rearrangement, in spit of the case of excess it can lead to difficulties in DNA fragmentation (Das et al., 2004).

Kaufmann et al. (2010) and Zhu et al. (2014) recommended the use of formaldehyde for reverse crosslinking, first to preserve chromatin structure during the isolation and secondly for its ability to capture weaker and transient protein-DNA interactions. They also mention that chromatin sonication is highly dependent on the instruments used, like Diagenode which has been successfully used in plant ChIP experiments and may reduce the average fragment size and, consequently, enhance positional resolution as we found in the sonicated chromatin from tomato plants.

Chromatin processing, including chromatin washing and fragmentation, is always considered to be a key step in obtaining a good result for all ChIP experiments. Different ChIP protocols have performed their sonication conditions differently. Usually, the fragment size ranges from 200–2000 bp, however, the sonication conditions depend on the tissue, chromatin sample volume, and sample concentration (Haque et al 2018). For ChIP application the size of the fragments is crucial, the length between 250 and 750 bp is considered good depending on the intended use, either gene target or the whole genome sequencing. Performing sonication instead of enzymatic MNase-mediated fragmentation is preferred since formaldehyde crosslinking could restrict the enzyme function (Canton et al., 2022). Overall our findings are in accordance with the findings reported by Ricardi et al. (2014), on sonicated chromatin from tomato leaves with a size of fragments of 400 bp.

A good ChIP protocol may also be defined as one applicable to a broad variety of plant tissues, yielding high quality and acceptable amount of end DNA, and limited false signals upon amplification. The duration of the protocol and cost efficiency are considered additional features that make a protocol attractive to researchers (Saleh, 2008).

After the application of fixation/extraction of chromatin, immunoprecipitation analysis was performed to identify genes in which H3K4me is enriched. This modification is associated with active chromatin and enriched around TSSs "transcriptional start sites" (He et al. 2011).

Successful trials in other plants such as *Arabidopsis thaliana* indicate that histone modifications are related to the activation/inactivation of drought-inducible genes during the stress/recovery process and suggest that H3K4me3 functions as an epigenetic marker of stress memory (Kim et al., 2012). Similar studies were performed in barely on two histone modifications H3K4me3 and H3K9ac: they found 129 genes enriched in H3K4me3 and 2008 genes in H3K9ac in response to drought (Ost et al 2023). Other ChIP immunoprecipitation followed by ChIP seq was performed on stressed tomato leaves to identify the genomic loci that interact with the ASR1 protein *in vivo*, which is known to play a role in the plant adaptation to drought stress (Ricardi et al., 2014).

Asp (2018), highlighted that in the last 15 years, researchers tended to merge chromatin immunoprecipitation (ChIP) with quantitative PCR (qPCR) for quick and easy analysis of anything chromatin-related as long the antibody is available. This corroborates the possibility of coupling these tools for studying plant responses under drought stress.

The conjugation of the previous techniques with transcriptomic has been also recommended to investigate the relationship between histone modification enrichment and the transcriptional activation of drought-inducible genes, as it is performed in the experiment of Song (2019), where RT-qPCR analyses showed that H3K9ac was positively associated with gene expression of those drought-responsive genes in *Brachypodium distachyon*.

The genes used for PCR amplification were used previously in other experiences and were correlated to drought stress, such as putative oleoyl-[acyl-carrier-protein] hydrolase (FATB), which plays an important role in drought-induced wax biosynthesis (Sanjari et al., 2021) and putative NAC domain-containing protein 5 (HaNAC76), which might exert a positive regulatory role in drought response in sunflower (Li et al., 2021).

6. CONCLUSION AND FUTURE PERSPECTIVES

This study established protocols to induce drought stress and analyze chromatin modification and transcriptomics in sunflower and tomato plants. An efficient chromatin immunoprecipitation (ChIP) protocol on crosslinked chromatin (X-Chip) from soft tissues (leaves) of *Helianthus annuus* has been developed for studying epigenetic signatures of stress adaptation in response to mild drought stress followed by a recovery period: at present for tomato plants (*Solanum lycopersicum)*

the first steps of immunoprecipitation protocol has been applied successfully as chromatin extraction and sonication, the following steps will be performed shortly.

A successful RNA extraction and purification for both plant species were conducted, coupled with positive PCR amplifications of target drought marker genes for sunflower. These procedures create a foundation for future ChIP-seq for analyzing genome-wide distributions and/or specific distribution at target loci of single-post-transcriptional histone modifications combined with RNA-Seq data, to identify epigenetic and gene expression signatures associated with drought tolerance and susceptible lines.

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Annex 2

This protocol was imported from Canton et al. (2022) with minor modifications.

Chromatin immunoprecipitation by dynabeads™ protein G

Day 1

1. Dynabeads Protein G preparation

Before proceeding with the preparation of Dynabeads ™ Protein G aliquots (ThermoFisher Scientific,10004D), it is necessary to evaluate the number of biological samples and antibodies that will be employed including the negative control that is the sample without antibodies. The following steps are referred to as a single aliquot.

1.1 Vortex the Dynabeads™ Protein G and pipette 100 µl in an empty 1.5 ml tube.
1.2 Centrifuge at **1000×*g*** for 2 min at RT. Note: Before using the magnetic separator, we suggest the use of a centrifuge to collect at the bottom the beads to facilitate the following separation with a magnetic rack).
1.3 Use a magnetic separator rack (ThermoFisher Scientific, 12321D) to remove the supernatant and add 1 ml of Lysis Buffer to the beads.
1.4 Incubate the tubes at RT on a rotating incubator with gentle rotation for 5 min.
1.5 Centrifuge 700×*g* for 2 min at RT.
1.6 Repeat the wash steps (1.3–1.5) three more times.
1.7 Add 100 µl of Lysis Buffer. Mix well by pipetting.
1.8 Sub-divide the Dynabeads™ Protein G into two aliquots (50 µl each) and store them on ice.
1.9 add 10 µl per 1ml of inhibitor cocktail (PMSF and protease inhibitor sigma)
1.10 add 10 µl per 1ml of Sodium butyrate 1M

2. Pre-clearing of chromatin sample

2.1 Centrifuge one of the tubes containing 50 µl of Dynabeads™ Protein G at 700×*g* for 2 min at RT.
2.2 Use a magnetic separator rack to remove the supernatant.
2.3 Transfer the chromatin sample previously extracted and sheared, into the tube containing Dynabeads TM Protein G pellet. Note: 7 µg of chromatin
2.4 Incubate the tubes at 4–10 °C on a rotating incubator with gentle rotation for 4–5 h.

3. Antibodies preparation

3.1 Prepare 250 µl of Lysis Buffer containing 4 µg of antibody against H3K4me3 (Active Motif, 39159). Preserve on ice until use.
3.2 Centrifuge at 700×*g* for 2 min at 4 °C the second tube containing 50 µl of Dynabeads™ Protein G previously prepared in step 1.8.
3.3 Remove the supernatant using a magnetic separator rack and add the 250 µl of Lysis Buffer + Antibody prepared in step 3.1.
3.4 Incubate the tubes at 4–10 °C on a rotating incubator with gentle rotation for 2 h.
3.5 Centrifuge at 700×*g* for 2 min at 4 °C.
3.6 Use a magnetic separator rack to remove the supernatant and add the 500 µl of Lysis Buffer.
3.7 Incubate the tubes at RT on a rotating incubator with gentle rotation for 5 min.
3.8 Centrifuge at 700×*g* for 2 min at 4 °C.
3.9 Use a magnetic separator rack to remove the supernatant and add the 500 µl of Lysis Buffer + BSA 5mg/ml (First Wash).
3.10 Incubate the tubes at RT on a rotating incubator with gentle rotation for 5 min.
3.11 Centrifuge at 700×*g* for 2 min at 4 °C.
3.12 Repeat steps 3.9–3.11 twice.
3.13 Use a magnetic separator rack to remove the supernatant and add the 500 µl of Lysis Buffer containing BSA 5 mg/ml. Incubate the tubes at 4–10 °C on a rotating incubator with gentle rotation for 2 h.

4. Immunoprecipitation

4.1 Centrifuge the Dynabeads Protein G derived from step 3.13 at 700×*g* for 2 min at 4 °C.
4.2 Use a magnetic separator rack to remove and discard the supernatant. Place the tubes containing the pellet on ice.
4.3 Centrifuge the tubes containing the chromatin + Dynabeads™ Protein G (from pre-clearing steps) at 700×*g* for 2 min at 4 °C.
4.4 Use a magnetic separator rack to recover the supernatant and throw away the Dynabeads™

Protein G pellet. Transfer the pre-cleared supernatant into the tubes containing the pellet derived from step 4.2.
4.5 Incubate the tubes O/N at 4–10 °C on a rotating incubator with gentle rotation.

Day 2

1. Washes and DNA elution

1.1 Centrifuge at 700*g* for 2 min at 4 °C.
1.2 Use a magnetic separator rack to remove the supernatant and add the 500 µl of Low Salt Buffer.
1.3 Incubate the tubes at RT on a rotating incubator with gentle rotation for 5 min.
1.4 Centrifuge at 700×*g* for 2 min at 4 °C.
1.5 Use a magnetic separator rack to remove the supernatant and add the 500 µl of High Salt Buffer.
1.6 Incubate the tubes at RT on a rotating incubator with gentle rotation for 5 min.
1.7 Centrifuge at 700×*g* for 2 min at 4 °C.
1.8 Use a magnetic separator rack to remove the supernatant and add the 500 µl of LNDET Buffer.
1.9 Incubate the tubes at RT on a rotating incubator with gentle rotation for 5 min.
1.20 Centrifuge at 700×*g* for 2 min at 4 °C.
1.21 Use a magnetic separator rack to remove the supernatant and add the 500 µl of TE Buffer.
1.22 Repeat the wash with TE buffer twice.
1.23 Centrifuge at 700×*g* for 2 min at 4 °C.
1.24 Use a magnetic separator rack to remove the supernatant and add the 260 µl of NaHCO3
0.1 M and 1% SDS. Vortex and incubate the tubes at 65 °C on a rotating incubator with gentle rotation for 30 min. Vortex every 15 min.
1.25 Centrifuge at 700×*g* for 2 min at RT.
1.26 Use a magnetic separator rack to recover the supernatant and transfer it to a new 1.5 ml tube.
1.27 Repeat the elution by adding another 260 µl of $NaHCO_3$ 0.1M and 1% SDS. Vortex and incubate the tubes at 65 °C on a rotating incubator with gentle rotation for 30 min. Vortex every 15 min.
1.28 Centrifuge at 700×*g* for 2 min at RT.
1.29 Use a magnetic separator rack to recover the supernatant and combine it with the first elution, to get a final volume of 500 µl.
1.30 Add a final concentration of 0.2 M NaCl and incubate for 16 h at 65 °C.

Day 3

1. DNA precipitation and purification

1.1 Spin down and add 1/125 volumes of glycogen and 2.5 volumes of EtOH 100%. Mix by inverting the tubes and incubating at **− 80 °C** for 2 h.
1.2 Centrifuge the samples at 14,000×*g* for 15 min at 4 °C. Discard the supernatant.
1.3 Wash the pellet with 1 ml of EtOH 70%. Vortex to detach the pellet.
1.4 Centrifuge the samples at 14,000×*g* for 15 min at 4 °C. Discard the supernatant.
1.5 Dry well the pellet at **-20 °C.**
1.6 Suspend the pellet by adding 300 µl of TE buffer for proteinase K. Add 30 µl of RNase A 10 mg/ml and incubate for 30 min at 37 °C.
1.7 Add 2 µl of Proteinase K (10 µg/µl) and incubate for 1 h at 42 °C.
1.8 Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), vortex, and centrifuge
at 14,000×*g* for 5 min at RT.
1.9 Recover the supernatant in a new 2 ml tube.
1.10 Follow the protocol for Purification by QIAquick PCR purification kit (QIAGEN) with manufactory instructions.
1.11 Elute in 80 µl of Elution Buffer (supplied by Kit).
1.12 One microliter of this ChIPed DNA and an appropriate dilution of input (from 1:60 to
1:100) can be used for qPCR control ("pulldown efficiency evaluation of immunoprecipitation") before performing analyses.
1.13 Store the ChIPed DNA at – 20 °C.

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Chromatin features and epigenetic-mediated mechanisms in *Helianthus annuus L.* in the climate change scenario

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UNIVERSITÀ DEGLI STUDI DI PADOVA

5.09

INTRODUCTION

Agriculture is one of the "victims" of climate change and the consequences, including water deficiency, significantly impact production and yields of crops. CROPINNO is a Twinning Horizon Europe project that focuses on initial stages of the crop improvement, production and implementation of modern breeding tools for creation and introduction into production of climate-resilient commercial varieties. To achieve these objectives, integrative approaches that combine omics technologies (genomics, transcriptomics, proteomics and metabolomics) by using bioinformatic tools will facilitate the identification of target genes and markers for complex traits in *Helianthus annuus L.* and facilitate crop breeding for adaptation to the changing environment.

MATERIALS & METHODS

STEP 1: 30 sunflower inbred lines were tested in *in vitro* conditions using PEG 6000 to obtain a smaller panel of genotypes:

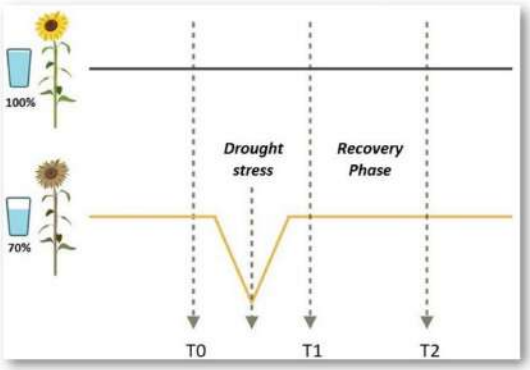
- drought susceptible (**Ha-26-PR**, **AB-OR-8**)
- drought tolerant (**IMI-AB-12-PR**, **DF-AB-2**)

STEP 2: To assess if there is a correlation between the *in vitro and* field drought stress response, some of the previously selected line were grown in pots in a greenhouse during summer and a dehydration protocol was applied (**Fig. 1**). Stress conditions and time points for tissue collection were chosen based on:

- **CO_2 assimilation**
- **stomatal conductance**
- **efficiency of photosystem II**

STEP 3: Chromatin and transcriptome analysis of drought stressed plant leaves are in progress. In Chromatin Immunoprecipitation (ChIP) experiments we are analyzing:

- the genome-wide distribution of two histone modifications (**H3K4me3**, **H3K27me3**)
- the genome-wide distribution of the histone-variant **H2A.Z**



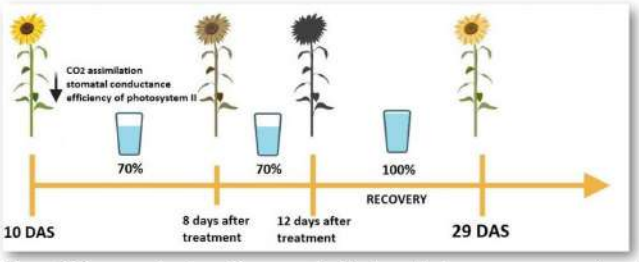


Figure 1. Common treatment frame applied in drought stress-memory experiments and time course frame of the dehydration applied protocol. The analysis of the leaf transcrptome and epigenome after a recovery phase will allow us to identify epigenetic stress-memory marks and stress-memory genes.

RESULTS

About 20 drought marker genes were selected in *in vitro* experiments and are begin tested to assess if there is a correlation in marker expression in the same tolerant and susceptible lines between *in vitro* and *in vivo* conditions, before proceeding with RNA-Seq experiments:

FATA / FATB	Oleoyl-[acyl-carrier-protein] **hydrolase**
HaNAC23 / HaNAC24	**Transcription factor** NAM family
caffeoyl-CoA 3-O-methyltransferase	Caffeoyl-CoA O-**methyltransferase**

An efficient **chromatin immunoprecipitation (ChIP) protocol** for studying epigenetic signatures of stress adaptation in response to extended drought and recovery in *Helianthus annuus L.* has been optimized (**Fig. 2**):

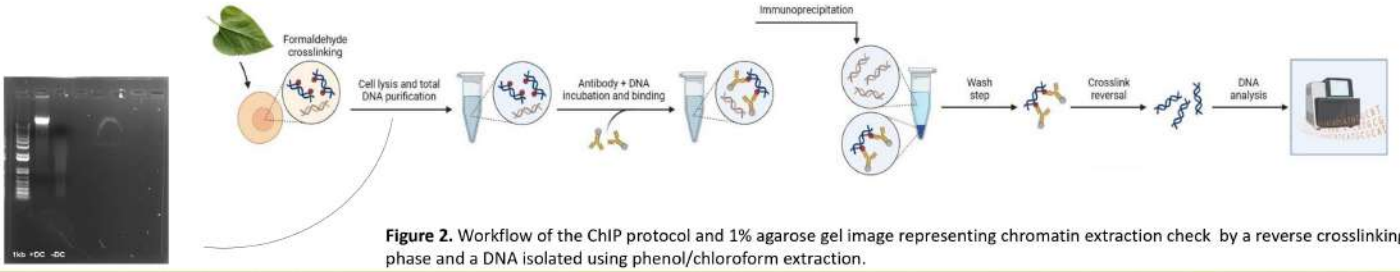


Figure 2. Workflow of the ChIP protocol and 1% agarose gel image representing chromatin extraction check by a reverse crosslinking phase and a DNA isolated using phenol/chloroform extraction.

CONCLUSIONS AND PERSPECTIVES

CROPINNO aims to the implementation of multi-omics tools for increase climate resilience of sunflower. Integration of RNA-Seq and ChIP-Seq data, will unveil possible direct correlations between stress-induced genes, transcriptional variation and histone modification levels and allow the identification of a robust list of epigenetic targets that affect different stress-responsive pathways. Expression (e)QTLs analysis in segregating population will enable the identification of *cis* and *trans*-factors in sunflower genome that regulate the expression levels of drought response key genes.

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