GENERATION OF CITRUS PLANTS WITH HIGHER TOLERANCE TO WATER AND SALINE STRESS

Jesús Lucina Romero Romero

2018

Pontificia Universidad Católica de Chile Facultad de Agronomía e Ingeniería Forestal

Generation of Citrus Plants with Higher Tolerance to Water and Saline Stress

Jesús Lucina Romero Romero

Thesis
Para optar al grado de

Doctor
Ciencias de la Agricultura

Santiago, Chile, June 2018

Thesis presented as part of the requirements for the degree of

Doctor in Ciencias de la Agricultura, approved by the

Thesis Committee

_____

Dr. Patricio Arce Johnson, Advisor

_____

Dr. Pilar Gil

_____

Dr. Michael Geoffrey Handford

_____

Dr. Basilio Alejandro Carrasco Gálvez

Santiago, June 2018

I dedicate this thesis to ...

My Heavenly Father, who is always my hope and my strength, my God in whom I trust; To my children, who will always be the engine of my life. To my parents, for all the love and unconditional support. To my brothers for encouraging me during all these years and to my heart, for keeping me in difficult times and sharing this life with me.

This work was supported by the doctoral grant CONICYT (63130094), and the COTEBAL (1865) of the CIIDIR-IPN Unidad Sinaloa

Acknowledgements

I would like to thank in the first place Dr. Patricio Arce Johnson, my teacher guide and thesis tutor, who during all these years has always given me his trust, friendship, support and encouraged to take new professional and academic challenges.

I thank the members of my committee, Professors Pilar Hil, Michael Geoffrey Handford, Basilio Alejandro Carrasco Gálvez and especially my advisor Patricio Arce for receiving me in his laboratory.

We would also like to thank Dr. Claudio Inostroza-Blancheteau of the Universidad Católica de Temuco and Dr. Marjorie Reyes-Díaz, of the Universidad de la Frontera, for their excellent disposition and collaboration in this work.

I also thank all members of the "Laboratory of Molecular Biology and Plant Biotechnology", especially A Valeria Borjas and Sandy Rojas, for their work on *in vitro* cultivation activities, greenhouse plant management and friendship. To Consuelo Medina, for her willingness to answer questions in the laboratory and finally to Maria Elena Santos Cervantes, Erika Camacho Beltrán, Francisca Parada and Denisse Laborie for their friendship and support in the happy and difficult moments of these years.

CONTENTS

CHAPTER 1.

General Introduction…….8

CHAPTER 2.

Increased drought and salinity tolerance in *Citrus aurantifolia* (Mexican lemon) plants overexpressing Arabidopsis *CBF3* gene…….....46

CHAPTER 3.

Stomata regulation by tissue-specific expression of the *Citrus sinensis* MYB61 transcription factor improves water-use efficiency in Arabidopsis……81

CHAPTER 4.

Model and Conclusions…….……...……...…….117

CHAPTER 1

GENERAL INTRODUCTION

Citrus genus: Taxonomy and current state

Citrus trees are one of the most important persistent leaf fruit crops in tropical, subtropical, and select temperate regions (Kahn et al., 2001; Zhang, 2014) and are cultivated in more than one hundred countries. In regards to taxonomy, the Citrus genus belongs to the Rutaceas family, which includes 162 species (Tanaka, 1977). While there are various taxonomic descriptions of the genus, the taxonomy described by Swingle and Reece (1967) is most accepted:

Family: *Rutaceae*

Subfamily: *Aurantoideae*

Division: *Embriophyta Siphonogama*

Subdivision: *Angiospermae*

Class: *Dicotyledonea*

Subclass: *Rosidae*

Superorder: *Rutanae*

Order: *Rutales*

The true citrus trees are comprised of six genera belonging to the subtribe Citrinae (Clymenia, Eremocitrus, Microcitrus, Poncirus, Fortunella and Citrus (Swingle and Reece, 1967). In 1976, Barrett and Rodas suggested that within the subgenus Citrus there were only three true "basic" species: lemon (*C. medica L.),* mandarins (*C. reticulata Blanco*), and grapefruit (*C. maxima L. Osbeck*). Subsequently, Scora (1988) pointed to *C. halimi* as another true species within Citrus subgenus. Currently the subgenus Eucitrus is the most widely cultivated Citrus.

Citrus plants are usually medium-sized trees with a height of about 6 meters, although some species can reach 15 meters. Most species have a single trunk comprised of very hard wood. Depending on the species, the treetop can be slender or very wide. Flowers range from 2-4 cm in diameter and are aromatic, individual, and often perfect including both pistil and functional stamen. The fruit is a fleshy, hesperidium, indehiscent berry that varies in size, color, shape and juice; the diameter of the fruits can vary from 4 cm (lemon) to more than 25 cm (grapefruit) (Figure 1). Seeds are whitish to greenish, flattened and angular, and usually polyembryonic, meaning they have multiple embryos that can germinate. Most of the species are diploid (2n = 8 chromosomes) and have a relatively small genome (382 Mpb).

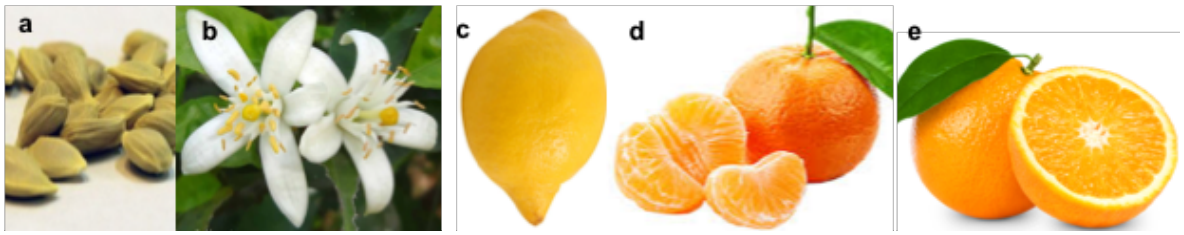


Figure 1. Citrus. a) Seeds, b) Flower, c) Lemon fruit Eureka, d) Mandarins fruit, e) Orange fruit

The Citrus genus is native to the tropical and subtropical regions of India and southern China, reaching northern Australia and New Caledonia. In China, the cultivation of oranges and grapefruit goes back to 2400 years B.C. (Manner et al., 2006). Currently, the cultivation of these trees is widely distributed in the tropical and

subtropical regions between the parallels 44º N and 41º S (Agustí, 2000). This genus has more than 145 species and is the most important genus in this family. The seven most important species within the citrus include *C. sinensis* (sweet oranges), *C. reticulata* (mandarin), *C. clementina* (clementines), *C. lemon* (lemons), *C. paradisi* (grapefruit), *C. grandis* (grapefruit), *C. medica* (citrons) and *C. aurantium* (bitter orange).

Recent studies using genomic, phylogenetic, and biogeographical strategies indicate that citrus plants originated in the southeast Himalayas in a region that includes eastern Assam, northern Myanmar, and western Yunnan (Wu et al., 2018). The ancestry of 46 citrus accessions was recently determined using 588,583 single nucleotide polymorphisms (SNPs) of an ancestral origin derived from three species, *C. medica*, *C. maxima* and *C. reticulate* (Wu et al., 2018). This analysis revealed that grapefruit was present in 24 of the 28 mandarins evaluated and suggested classification of three mandarin types: Mandarins as a pure species (type 1), mandarins mixed with the grapefruit haplotypes P1 or P2 (type 2), and mandarins and sweet oranges generated due to additional introgressions of grapefruit with mandarins of type 2 (type 3). Subsequent crossings between sweet oranges and mandarins or type 3 mandarins generated the mandarins marketed today (Figure 2).

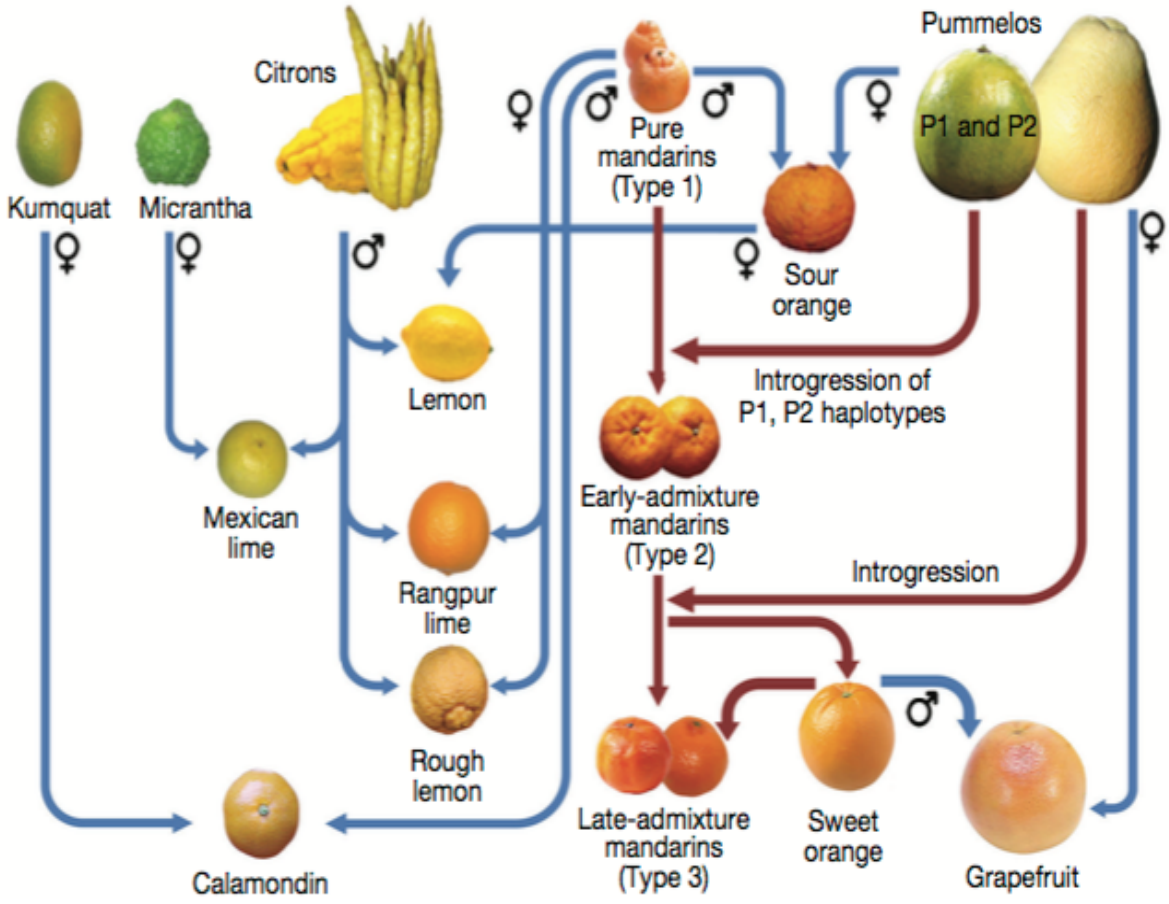


Figure 2. Genealogy of the main citrus species.

The fruits of these trees provide numerous nutrients such as vitamins, fiber, calcium, potassium, and folic acid, which yield beneficial health outcomes and reduce health risks (Jia et al., 2017). Globally, citrus production totals 130 million tons and has grown 16% over the last five years (FAO, 2016). World citrus production is distributed primarily in five countries: China, Brazil, India, United States, and Mexico, which alone produces two thirds of the global supply (Liu et al., 2012; FAO, 2016). Currently, Chile ranks 36th in world citrus production and 11th in

the export of this fruit (FAO, 2016). The main citrus fruits grown in Chile are sweet orange (*Citrus sinensis (L.) Osbeck*), clementines and tangerines (*Citrus clementina L.* and *Citrus reticulata Blanco*), and lemons (*Citrus lemon (L.) Burm.*) (ASOEX, 2011). This sector has experienced greater than 300% the last 10 years (Figure 3), with production increasing from 75,000 tons in 2005 to 250,000 tons in 2017 (ODEPA-CIREN 2015) (Figure 3).

Figure 3. Evolution of Chilean citrus exportation between 2005 and 2017.

The main citrus producing regions in Chile are located in the central northern zone of the country, including region III (Atacama, 23.1%), region V (Valparaíso,

27.6%), region XIII (Metropolitan, 28.9%), and region VI (Libertador Bernardo O'Higgins, 19,4%) (ODEPA, 2012). The Atacama region is the earliest citrus ripening zone in the country, where the color and aroma (flavor) of orange, lemon, and tangerine fruits are most intense (Gompertz, 1999; Arentsen 2002). However, due to limited water availability in this valley, the area of citrus cultivation is very small (162 hectares), equivalent to 0.9% of the national area (Gompertz, 1999).

Effect of water deficiency on plants

The earth's volume of water, an essential resource for all living things, reaches 1,400 million km^3. However, 97.5% of water is held by oceans and seas and salt water, while only 2.5% corresponds to fresh water. Further, only 0.003% of water is potentially available to humans (45,000 km^3) and only 9,000 to 14,000 km^3 are available for capture and use to support of economic activities. Of the total freshwater withdrawals (3,830 km^3/year), the highest proportion (70%) is allocated for agricultural applications, 30% is destined for industrial purposes, and 10% is used domestically (FAO, 2007; Molden et al., 2007; Ruane et al., 2008; Faures, 2012).

Drought is one of the most important meteorological phenomena facing the world today due to its long-term impact on water resources, agricultural production, and economic activity (Wang et al., 2014). The percentage of the planet affected by drought has more than doubled in the last 40 years, especially in arid and semi-arid regions, affecting more people in the world than any other natural phenomenon (Cook et al., 2018). Climatic projections made by Wallace (2000) indicate that the

number of people living in water scarce areas could reach 67% by the year 2050. Work by Schewe et al. (2014) indicates that a 2°C increase in the current global temperature could translate to more than 20% of the world's population living in conditions of absolute water scarcity (Figure 4A). This scenario seems inevitable at the end of the 21st century (Schiermeier, 2014; Zandalinas et al., 2018). Likewise, precipitation is expected to be increasingly erratic in the coming years, generating higher average annual rainfall in higher latitudes and the equatorial Pacific, while precipitation in many regions of medium latitude and dry subtropical regions will decrease (Figure 4B).

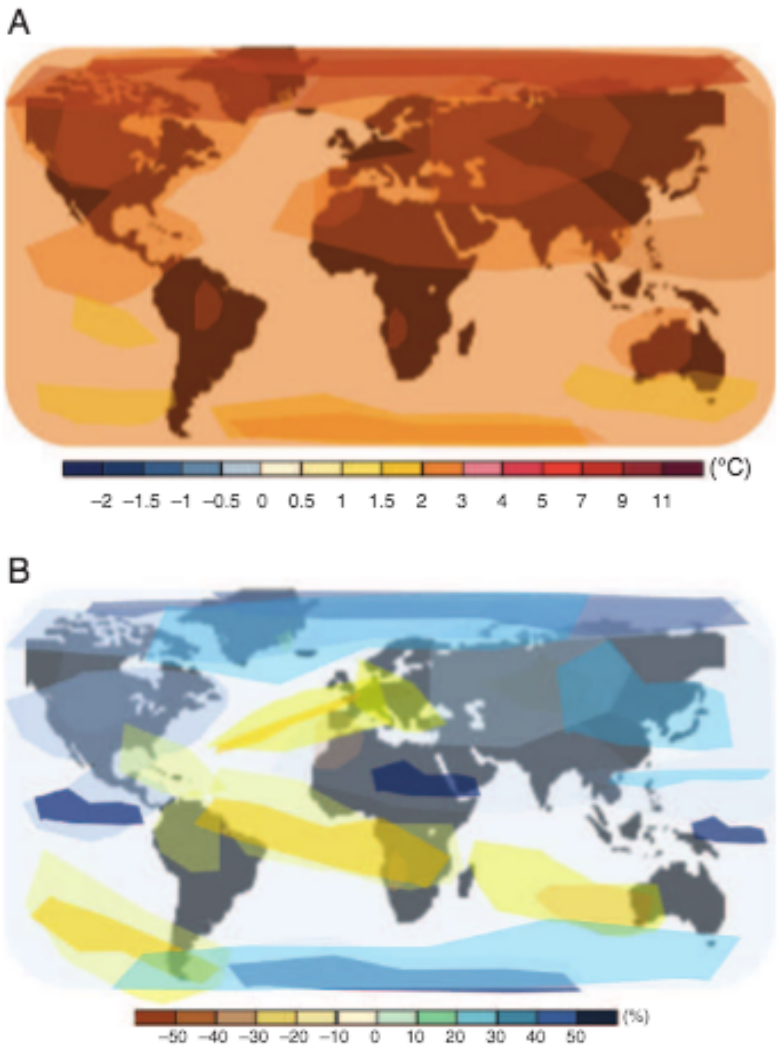


Figure 4. Prediction of changes in average surface temperature (A) and rainfall (B) by the year 2100. The color scale indicates the decrease or increase in Celsius degrees (A) and the percent change in precipitation (Zandalinas et al., 2018).

Considering the projections described above, a water crisis is expected in the future, not only due to the lack of water but also due to the misuse of available water resources (Molden, 2007). Today, climate change is exacerbating drought events in various parts of the world, including the United States, East Africa, Australia, Brazil,

China and Spain. In these countries, drought periods have increased in frequency, intensity, and duration (Van Loon et al., 2016). Chile is no exception: since 2010 the region between Coquimbo and Araucanía has experienced a water deficit of 30%. Due to its territorial extension and persistence, this event has been named *megasequía*. Consequently, aridification is expected in the central and southern zone of Chile during the present century, increasing the occurrence and intensity of droughts similar to the *megasequía* (CR2, 2015).

In addition, agricultural areas requiring irrigation have grown worldwide, with a 100% increase between the year 1960 (140 million hectares) and 2000 (280 million hectares) (Tilman et al., 2002; Pretty, 2008). The considerable growth in agricultural production is associated with increased yields due to higher pesticide and fertilizer use (mostly nitrogen and phosphorus) as well as improved access to water for irrigation. Between 1965 and 1985 average yields increased by 56% worldwide but slowed to 20% growth by 2005 (Foley et al., 2011), indicating that technological advances have not been sufficient to ensure constant growth over time. At the same time, agricultural expansion has irreversibly damaged the environment through intense water use, conversion of natural lands to agricultural ecosystems, and contamination of various terrestrial, aquatic, and subterranean aquatic habitats with excessive nutrients (phosphorus and nitrogen) and pesticides.

Plants require large quantities of water for proper development. Water accounts for 80-95% of the fresh weight of plants Taiz and Zeiger, 2010). However, plants only conserve 3% of the total volume of water absorbed, which is used for photosynthesis or other metabolic processes (Taiz and Zeiger, 2010). As example,

citrus plants have a particularly high water demand, requiring 100 liters of water/tree/day during the sixth year of growth (Shalhevet and Levy, 1990).

Given the importance of water in the development of plants, water deficiency significantly affects cellular functions, generating severe negative impacts on development and reproduction. Fundamental processes such as photosynthesis are affected by water shortage events. Direct effects of water deficiency relate to decreased diffusion of CO_2 through stomata and mesophyll and alterations in photosynthetic metabolism (reducing the content, activity, and regeneration of enzymes involved in the Calvin cycle). Indirect effects include secondary oxidative stress, which can damage the photosynthetic apparatus (Munns, 2011; Dodd and Ryan, 2016). The ability of certain plants to tolerate or overcome water stress is granted by particular physiological and cellular mechanisms, some of which are related to completing their life cycle during the wet season when water is readily available or maximizing water acquisition by generating a large root system to extract water at greater depths and storing water inside the cell as it happens in the plants of CAM metabolism (Dodd and Ryan, 2016) or to induce the abscission of the foliar area.

Decreased leaf area is associated with decreased water loss through transpiration, the process by which plants lose about 95% of water during the day (Taiz and Zeiger, 2010). Osmotic adjustment is another response generated in plant tissue during a water stress event. This response involves increasing the concentration of solutes (osmolytes) such as sugars (mannitol, sorbitol and trehalose), proline, and glycine betaine in the interior of the cell in order to reduce

water potential, allow the influx of water into the cells, and maintain turgor (Taiz and Zeiger, 2010; Dodd and Ryan, 2016). In the same way, water stress induces increased disposition of waxes on the leaf surface with the aim of reducing water loss from the epidermis. In addition, water stress induces changes in the expression of genes related to the biosynthesis of osmolytes, proteases, LEA proteins (Late embryogenesis abundant), aquaporins (transmembrane proteins that form channels to facilitate transport of water), and the abscisic acid (ABA) response, a phytohormone broadly related to stomatal closure in plants (Taiz and Zeiger, 2010; Dodd and Ryan, 2016). ABA is perceived by the PYR/PYL/RCAR receptors (Pyrabactin resistance/pyr1-like/regulatory component of aba receptor) in the guard cells, activating a signaling cascade that culminates in stomatal closure. In the absence of ABA, Protein phosphatase 2C (PP2Cs) inhibits protein kinases that act as positive regulators of stomatal closure by dephosphorylation. ABA receptors interact with PP2Cs, but their affinity is low in the absence of ABA. In contrast, when ABA binds to its receptors, the interaction with PP2Cs strengthens, inhibiting phosphatase activity and allowing the activation of protein kinases such as OST1 (Open stomata 1) and CPKs (calcium-dependent protein kinases), which activate anionic channels by phosphorylation. This mechanism generates anion efflux, depolarization of the plasma membrane, and consequent activation of K^+ efflux channels. The efflux of anions, K^+, and water decreases the turgor of the guard cells and prompts stomatal closure (Figure 4) (Kollist et al., 2014). Located in the leaf epidermis, stomata are comprised of a pair of specialized cells named guard cells and allow gaseous exchange between the plant and the atmosphere. Plants open stomata to assimilate CO_2 through photosynthesis, but doing so renders the plant

susceptible to water loss through transpiration. Accordingly, appropriate regulation of stomatal opening and closing is vital for proper plant growth (Schroeder et al., 2001a; Taiz and Zeiger, 2010).

Rerspiration is a physical process that depends on both the difference in concentration of water vapor between the spaces of the leaf and the outside as well as the resistance to diffusion (r). Resistance may be classified into two types: stomatal resistance of the leaf (rs), associated with diffusion through the stomata; and resistance of the stationary layer of the leaf (rb), related to the stationary layer of air near the surface of the leaf through which water vapor must diffuse in order to reach the turbulent air of the atmosphere (Taiz and Zeiger, 2010).

Several environmental factors such as light, high humidity, and low CO_2 concentration induce stomatal opening by generating hyperpolarization of the plasma membrane by an influx of ions (K^+, Cl^-, $NO3^-$) and through production of malate (Schroeder et al., 2001a; Kim et al., 2010; Araujo et al., 2011) In contrast, darkness (in C3 and C4 plants), high internal CO_2 concentration, drought, and high ozone concentration induce stomatal closure through depolarization of the plasma membrane, which induces efflux of the same ions and conversion of malate to starch, resulting in decreased turgor of the guard cells and stomatal closing (Schroeder et al., 2001a; Schroeder et al., 2001b; Kim et al., 2010; Kollist et al., 2014).

Stomatal opening is mainly induced by blue light, which is perceived by photoreceptors associated with the plasma membrane called phototropins (PHOT1 and PHOT2). These photoreceptors have a serine/threonine (Ser/Thr) kinase

domain and are autophosphorylated and activated upon stimulation by blue light. PHOTs are thought to be inactivated by dephosphorylation by type 2A phosphatase proteins (PP2As). Upon activation, PHOTs interact with 14-3-3 regulatory proteins and phosphorylate a protein kinase called BLUS1 (blue light signaling 1). The signaling cascade downstream of BLUS1 is unknown. However, it is known that blue light signaling culminates in activation of the H^+-ATPase pump of the plasma membrane by phosphorylation, which pumps protons (H^+) out of the cell, resulting in hyperpolarization of the plasma membrane and activation of K^+ influx rectifier channels. This produces an influx of K^+ and water, which increases the turgor of the cells, opening the pore of the stoma and activating H^+-ATPases. Blue light inhibits anionic channels of the plasma membrane which contributes to hyperpolarization (Chen et al., 2012; Kollist et al., 2014) (Figure 5). Other photoreceptors such as cryptochromes (CRYs) and phytochromes (phys) also participate in the regulation of the stomatal opening, but the signaling pathways mediated by these photoreceptors have not been characterized in detail.

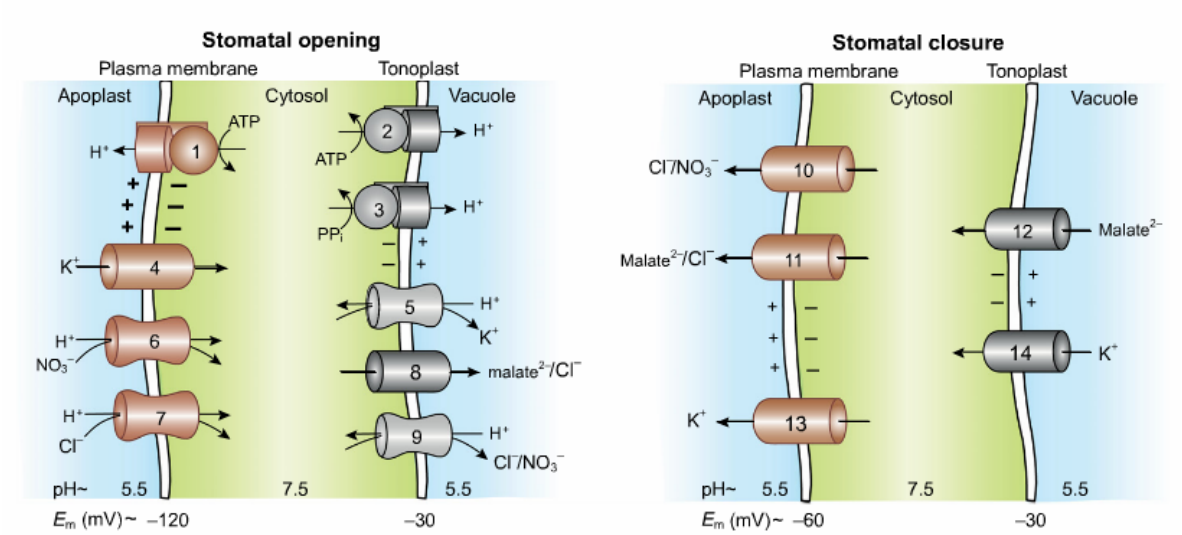


Figure 5: Diagram of the movement of ions in the opening or closing of stomata. The transporters located in the plasma membrane of the guard cells and those located in the tonoplast or vacuolar membrane are represented. The arrows indicate the direction of movement of the ions for both the opening (left) and the closing (right). The change in the potential of the membranes for each event is also represented in the lower part of the figure (Adapted to Kollist et al., 2014).

Effect of salinity on plants

Saline stress is another important condition that affects normal plant development due to the reduction of soil water potential. Approximately, 830 million hectares are affected by salinity (> 6% of the total surface of the planet). According to FAO (2008) for the year 2050 is projected 50% of agricultural land salt. Salinity is a soil condition, defined by a high concentration of soluble salts. Soils with electrical conductivity (CE)> 4 dSm-1 are considered saline (40 mM NaCl) (Qadir et al., 2014). Most fruit trees and agricultural crops are sensitive to salinity, which is reflected in a decrease in their productivity. The accumulation of salt in the soil not

only affects normal plant functions, but also degrades the structure of the soil, resulting in decreased porosity and permeability. The salts that contribute most to salinity are Na^{+}, Ca^{+2}, Mg^{+2}, Cl^{-} and SO_4^{-2}. Salinity can be measured based on electrical conductivity or osmotic potential (Negrão et al., 2017).

Plants can be classified according to their salinity resistance. Very tolerant plants, such as halophytes, have managed to adapt naturally to saline conditions. Examples are the species belonging to the *Chenopodiaceae* family and the *Prosopis* genus. However, the plants most sensitive to salinity belong to the group of glycophytes such as onions, corn, lettuce and citrus. In the particular case of citrus, it has been established that the yield remains unchanged until the electrical conductivity of the soil saturation extract (CEe) reaches 1.4-1.5 dS m^{-1}. From this threshold, for each increment of 1 dS m^{-1} of CEe the yield is reduced between 13 and 13.5% (Maas and Grattan, 1999).

Saline and water stress are directly related because higher concentrations of soluble salts decrease water potential. Therefore, most plants respond to stress (water and salt) with the same physiological and molecular mechanisms because both cases share the same limiting factor, water. However, plants that face saline stress must additionally withstand high concentrations of ions such as Na^{+}, Cl^{-}, and CO_3^{-2}.

The main effects caused by saline stress inside the cell include ionic imbalance and osmotic stress (Bartels and Sunkar, 2005), which result in oxidative damage. Drastic changes in ion concentration and homeostasis lead to molecular

damage that decrease growth rats or even death of the plant. Normally, cells respond to decreased water content by shrinking and relaxing their cells walls, which decreases turgor while increasing the concentration of solutes. This is the first biophysical effect of water stress; therefore, activities that depend on turgor, such as leaf expansion and root elongation, are inevitably affected (Figure 6).

Salts dissolved in water generate lower osmotic potential, which translates into lower water potential. The solutes in a higher concentration in a solution produce an effect similar to that of water deficit because absorption of water is limited. Saline stress also has a specific effect related to the ions themselves.

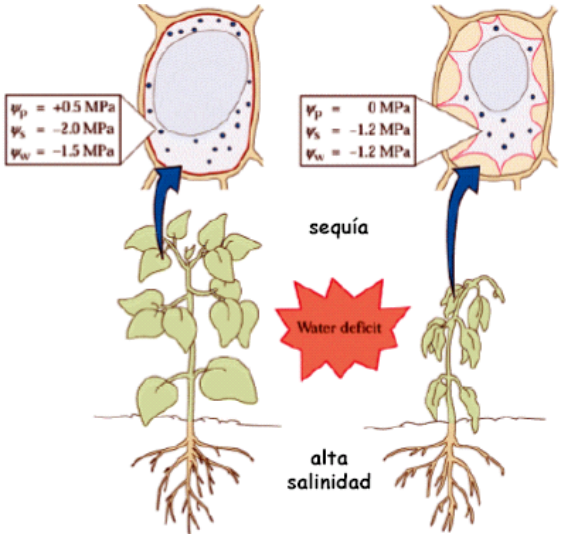


Figure 6. **Water potential changes in the cell by water deficit.** (Taiz and Zeiger, 2010)

Salinity also produces oxidative damage, generated by the accumulation of reactive oxygen species (ROS). ROS are partially reduced forms of atmospheric oxygen (O_2) that result from the excitation of O_2 to form singlet oxygen ($^1O_2$) or the

transfer of one, two, or three electrons to O_2 to form superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), or the hydroxyl radical (OH^-), respectively. While many sources of ROS are present in plant cells, chloroplasts and mitochondria represent the most significant sources of ROS as a product of photosynthesis and respiration. The negative effect caused by excess ROS is known as oxidative stress. The main effects of oxidative stress are alterations at the level of the plasma membrane, interruption of enzymatic activity, changes in the availability of nutrients, and damage in the photosynthetic apparatus. Plants have developed various mechanisms of detoxification and protection to repair the damage caused by ROS, such as the production of enzymes and "antioxidant" metabolites, which allow them to survive in oxidizing conditions. The antioxidant system is responsible for reacting with these agents to achieve detoxification. The ROS detoxifying systems works through two mechanisms, enzymatic and non-enzymatic. The enzymatic systems that inactivate ROS are SOD (superoxide dismutase), APX (ascorbate peroxidase), GPX (glutathione peroxidase), and CAT (catalase) (Wang et al., 2003). SOD is the first enzyme that acts to eliminate ROS, catalyzing dismutation of the superoxide ion (O_2^-) to H_2O_2. This ROS is then reduced to water by the peroxidases APX, GPX, or CAT. The peroxidases APX and GPX require regeneration of their reducing agents, Asc (ascorbate) and GSH (reduced glutathione), respectively, which are mediated by $NAD(P)H^+$.

Non-enzymatic detoxification systems are composed of cellular redox buffers such as ascorbate and glutathione. GSH is oxidized by ROS to GSSG, while Asc is oxidized to MDAsc (monodehydroascorbate) and to DHAsc (dehydroascorbate).

The oxidized products GSSG and MDA-DHA must be reduced by enzymes to GSH and ASA. A high ratio of Asc/MDAsc + DHAsc and GSH/GSSG is essential for the inactivation of ROS (Figure 7).

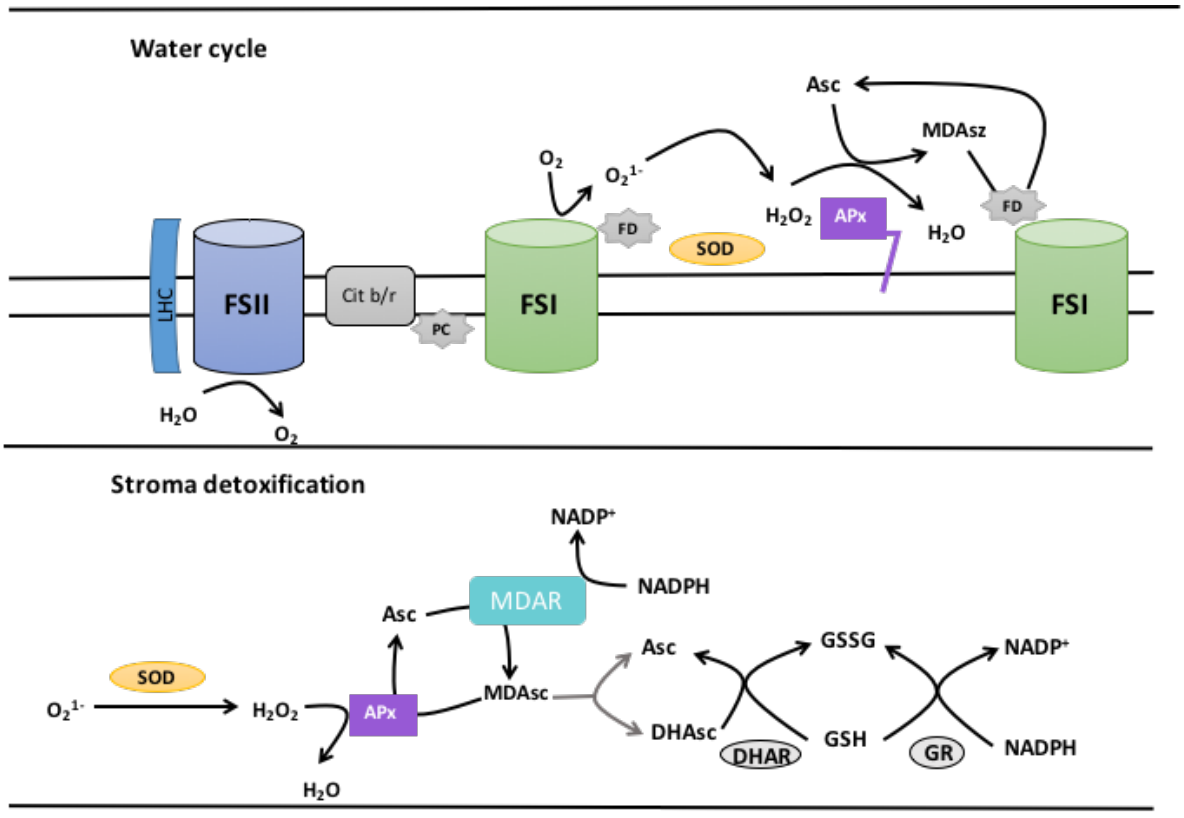


Figure 7. Above: The superoxide generated at the level of Photosystem I (FSI) is eliminated in the water cycle by the concerted activity of SOD and APx. Below: the peroxide and superoxide that escape detoxification are reduced in the stroma by a pathway involving SOD, APx, monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). Asc, ascorbate; MDAsc, monodehydroascorbate; GSSG, oxidized glutathione; GSH, reduced glutathione. Adapted from Taiz and Zeiger, 2010.

Physiological and molecular response of citrus to abiotic stress: Room for genetic improvement.

Biotechnological strategies have been proposed to increase water-use efficiency (WUE), which describes the accumulation of biomass (A) and water consumed (E) (Lambers et al., 2008). The overall aim of these strategies is to reduce the rate of transpiration of the plant without significantly reducing the assimilation of CO_2, ultimately generating highly productive plants with lower water requirements (Cominelli et al., 2010; Cominelli and Tonelli, 2010). Another strategy to increase WUE is to decrease the number of stomata on the leaf surface of plants (Hughes et al., 2017). Laporte et al. (2002) showed that expression of the NADP-dependent malic enzyme (ME, which converts malate and $NADP^+$ to pyruvate, CO_2 and NADPH) in tobacco decreased stomatal conductance (*gs*) and increased the mass of fresh tissue per unit of water consumed without affecting growth. Similarly, overexpression of the *HvEPF1* gene in barley generated transgenic lines with stomatal density and significantly lower *gs*, without decreasing grain yield. These lines showed improved WUE and greater tolerance to drought (Hughes et al., 2017).

Considering food demand projections, more sustainable agricultural practices will be necessary in order to maintain high crop yield while simultaneously minimizing environmental impacts. Implementing sustainable agricultural practices will require biotechnological tools for the generation of monocultures with greater WUE, innovative methods to supply nitrogen and phosphorus, and the integral management of pests to reduce dependence on pesticides (Tilman, 2002; Pretty, 2008). Plants grown under natural conditions are regularly subjected to different

combinations of abiotic stress. Citrus trees are among the most water demanding woody fruit species with water accounting for 85% of their fresh weight versus 50% for other plants. Decreased productivity due to water and/or salinity stress are common in locations where citrus are cultivated (Pérez-Pérez et al., 2009; Álvarez et al., 2015a).

Under current citriculture practices, all commercial varieties are grafted onto rootstock, which provides distinct advantages for the growth and fruit production of the plant (Agustí, 2003). The use of rootstocks allows for control of the vigor, productivity, and quality of the fruit in a given environment and confers greater resistance to various abiotic stressors such as low or high temperatures, salinity, and water stress (Pinochet, 1990; Khan and Khender, 2007; Vélez et al., 2012), though differences arise between different rootstocks. For example, the orange cultivar 'Lane late' exhibits a more efficient biomass/water production ratio when grafted onto the "Carrizo" rootstock than the "Cleopatra" rootstock. Controlled deficit irrigation (CDR) techniques represent another development in the citrus varieties marketed today. This technique provides water savings, lower energy consumption, lower production costs, greater efficiency in the use of water resources, and reduction of pollution risks due to deep percolation (Vélez et al., 2012). Implementation of this technique during filling and ripening of the fruit improves quality by generating a higher content of soluble solids and acidic compounds (Peng and Rave 1998). In the case of lemon and limes, CDR is used in summer to induce flowering out of season. In the orange "Lane beats" reduction of the water supply

generates a delay in the harvest without diminishing production (Pérez-Pérez et al., 2009).

Several authors have demonstrated that the response of citrus plants faced with water deficit events or a combination of abiotic stressors (i.e. severe drought and high temperatures) depends on several factors such as phenology, physiological status, irrigation water quality, genotype, and degree and intensity of stress (Núñez-Vázquez et al., 2017). Citrus plant adaptations to abiotic stressors are varied, but the most relevant are restriction of vegetative growth, reduction of stomatal conductance (*gs*) to avoid water loss by transpiration, decrease in the assimilation of CO_2 and yield, and abscission of leaves and fruit (Pérez-Pérez et al., 2009; Álvarez et al., 2015a). Other cases have reported decreased hydraulic conductivity of the root, generation of waxy layers that occlude the stomata and decrease the water loss, and changes in the angle of inclination and curl of leaves to reduce the intercepted surface and radiation to reduce transpiration (Núñez-Vázquez et al., 2017; Agustí, 2003; Vélez et al., 2012). Consequently, photosynthesis can be directly affected by limitation in CO_2 diffusion though the mesophyll and the stomata, alterations in the photosynthetic metabolism, and modifications in the activity and regeneration of enzymes involved in the Calvin cycle. Moreover, these abiotic stress defense mechanisms may indirectly affect photosynthesis through oxidative stress, which can damage the photosynthetic apparatus (Dodd and Ryan, 2016).

Metabolic and osmoregulation adaptations that involve the accumulation of solutes compatible with normal cellular function have also been reported in response

to water deficiency (Barry et al., 2004; Navarro et al., 2010; Zandalinas et al., 2018). Previous studies have reported an increase in the content of phytohormones such as ABA, jasmonic acid (JA), salicylic acid (SA) (Wang et al., 2012; Wang et al., 2015), and ethylene (Vélez et al., 2012; Zandalinas et al., 2018) or a rapid decrease in gibberellin content (Mahouachi et al., 2005) in response to this abiotic stress. It is known that SA induces the genes *ICS* and *PR1* among others and maintains the integrity of the cell membrane in plants in addition to protecting Photosystem II (PSII) (Clarke et al., 2009; Coqueiro et al., 2015).

The hormone ABA triggers stomatal closure by inducing genes that code for transcription factors (TFs) such as MYB44 and MYB15 (Jung et al., 2008; Cominelli et al., 2010). The induction of numerous genes which may be involved in signaling and transcriptional control has been reported in response to various types of abiotic stress. Examples include MyC, Map kinases and SOS kinases (Shinozaki and Yamaguchi-Shinozaki, 1997), phospholipases (Zhai et al., 2012), and transcription factors including HSF, CBF/DREB, and ABF/ABRE (Zhu, 2002; Von Koskull-Döring et al., 2007; Kang et al., 2002; Álvarez et al., 2015b). Moreover, genes related to membrane stability or protein protection (i.e. heat-shock proteins), chaperones, LEA proteins (Thomashow, 1999; Bray, 1997; Bohnert and Sheveleva, 1998; Ingram and Bartels, 1996; Vierling, 1991), osmoprotectors and free radical scavengers (Bohnert and Sheveleva, 1998), and genes linked to ion transporters and aquaporins (Serrano et al., 1999; Tyerman et al., 1999; Blumwald et al., 2000) have also been implicated in response to abiotic stress.

In Carrizo citrange, mutation of the gene *P5CS* (Δ1-pyrroline-5-carboxylate synthetase) has been shown to affect proline synthesis, generating plants tolerant to water deficiency (Molinari et al., 2004). In other citrus species, overexpression of the yeast gene *HAL2* in *Carrizo citrange* and the rough lemon (*Citrus jambhiri* Lush) was shown to confer higher tolerance to saline stress (Cervera et al., 2000; Ali et al., 2012), while similar findings were observed for overexpression of betaine aldehyde dehydrogenase from *Atriplex hortensis* (AhBADH) in trifoliate orange (Fu et al., 2011); for the *BjGlyI* and *PgGlyII* genes that code for glyoxylases in *Carrizo citrange* (Álvarez et al., 2015a); and for arabidopsis CBF3 in *Citrus macrophylla* (Álvarez et al., 2015b) in regards to saline stress tolerance.

Genetic improvement in Citrus

New varieties of citrus have been developed through conventional improvement over the last several centuries. However, there are only few examples of successful varieties and rootstocks (Khan and Khender, 2007). Characteristics such as sterility of the ovule and pollen, mechanisms of self-incompatibility, polyembryony, high heterozygosity, and long periods of juvenility have complicated genetic improvement of this species through conventional crosses (Gmitter et al., 1992). Therefore, biotechnological tools have emerged as a promising complement to genetic improvement in citrus. One such biotechnological tool is *in vitro* culture. Several regeneration methods have been developed based on the culture of apical buds, nodal sections, epicotyls, and freshly germinated seeds (Bordón et al., 2000). The development of transgenic, intragenic, or cisgenic citrus plants is currently an interesting option, because these technologies offer several distinct advantages

such as increasing the agronomic value of cultivated plants, allowing use of tissues and plant cells in bioreactors for the production of proteins and metabolites of economic importance, and generating basic knowledge related to the action of genes during development or other biological processes in plants (Dale, 1995).

The success of biotechnological tools in agriculture is reflected by the significant increase in cultivation of transgenic crop plants of great agroeconomic importance such as soybean, cotton, rice, corn, and canola (Birch, 1997; Mohammad et al., 2017) across the world.

The genetic transformation of citrus plants began more than two decades ago. In 1992, Moore reported for the first time the integration of transgenes in Carrizo citrange with Agrobacterium, using segments of hypocotyls as explants. Most recent protocols for the transformation of various citrus species such as Carrizo citrange (Cervera et al., 1998), sour orange (Ghorbel et al., 2000), Carrizo macrophylla, *Citrus lemon* (lemon) and Cleopatra mandarin (Ghorbel et al., 2001), have been generated using internodal segments of juvenile greenhouse plants as explants. To date, various citrus rootstocks have been transformed with the aim of shortening juvenility by expressing floral induction genes such as *Leafy* and *Apetala1* (Peña et al., 2001), gene stacking analysis, or gene pyramiding (Cervera et al., 2009).

Strategies employed to increase abiotic stress tolerance in citrus plants include mutation of the P5CS gene in Carrizo citrange (Molinari, 2004); transformation of trifoliate orange with the gene coding for betaine aldehyde dehydrogenase from *Atriplex hortensis* (AhBADH) (Fu, 2011); expression of the

yeast HAL2 gene in Carrizo citrange (Cervera et al., 2000) and rough lemon (*Citrus jambhiri* Lush) (Cervera et al., 2000; Ali, 2012); expression of *AtHXK* (gene that encodes the enzyme hexokinase which phosphorylates glucose and fructose) in cell stores, which decreased stomatal conductance and transpiration without negatively effecting photosynthesis rate while increasing the WUE (Lugassi et al., 2015); expression of arabidopsis glyoxylases in roots of Carrizo citrange for salt stress tolerance (Álvarez et al., 2015a); and expression of Arabidopsis CBF3/DREB1A in *Citrus macrophylla* in response to salt stress (Álvarez et al., 2015b).

All the reports mentioned above focused on rootstocks, which are naturally more robust and stress tolerant than citrus varieties. In field conditions, the grafted aerial portion provides the leaves and conductive tissue, the main organ of the plant responsible water loss. Expanding the characterization of citrus varieties in response to hydric and saline stress is particularly timely when considering the 2ºC increase in average soil temperature that is expected in the next 50 years (Schewe et al., 2014). Optimizing the use and availability of water in water intensive plants such of citrus is of utmost importance. The goal of this thesis was to generate transgenic or intragenic varieties of *Citrus aurantifolia* (lemon) and *Citrus sinensis* (orange valence) expressing the transcription factors CBF3 of *A. thaliana* and MYB61 of *C. sinensis*, respectively, and to evaluate at the physiological and molecular level the response of these lines to water deficit and saline stress.

This thesis was implemented in consideration of the following context:

- Water deficit and saline stress are growing problems in Chile and globally.
- Citrus trees are highly sensitive to stress

- Citriculture is projected to have great economic potential for the country.

- Methodologies for the genetic transformation of citrus have been developed and could be applied to increase water deficit and saline stress tolerance in this species.

This background allowed us to propose the following **hypothesis**:

Constitutive or temporal expression of the transcription factors *CBF3* or *MYB61* in commercial varieties of citrus confers greater tolerance to water deficit and saline stress.

To answer this hypothesis, the following general and specific goals were proposed:

Main Goal

To generate citrus and arabidopsis plants that overexpress the transcription factors *AtCBF3* and *CsMYB61* with the aim of conferring improved tolerance to water and saline stress

Specifics goals:

1. Identify, clone, and sequence the *Citrus sinensis MYB61* homologous gene and the promoter of the *Citrus sinensis MYB15* gene.

2. Analyze at the molecular level the integration and expression of the *AtCBF3* and *CsMYB61* genes in *A. thaliana* and Citrus varieties.

3. Phenotypically characterize transgenic lines of Citrus and Arabidopsis plants expressing *AtCBF3* and *CsMYB61* and evaluate their tolerance to water and saline stress under controlled conditions.

The results of these goals are presented in the form of scientific manuscripts submitted or accepted for publication. Specific goal 1 is presented in chapter 3 with the article "Stomata regulation by tissue-specific expression of the *Citrus sinensis* MYB61 transcription factor improves water-use efficiency in Arabidopsis". This article was accepted to the Journal Plant Physiology and Biochemistry. Specific goals 2 and 3 are presented in chapters 2 and 3, respectively, with the publication “Increased drought and salinity tolerance in *Citrus aurantifolia* (Mexican lemon) plants overexpressing Arabidopsis CBF3 gene”submitted to the Journal Molecular Breeding.

References

Agustí M. (2003). Citricultura. Segunda edición. Ed. Mundiprensa (España). 423p.

Agustí, M. (2000). Citricultura. Ediciones Mundi Prensa, pp. 416.

Ali S, Mannan A, El Oirdi M, Waheed A, Mirza B. (2012). Agrobacterium-mediated transformation of rough lemon (*Citrus jambhiri* Lush) with yeast HAL2 gene. BMC Research Notes 5: 285-292.

Álvarez-Gerding Ximena, Cortés-Bullemore Rowena, Medina Consuelo, Romero-Romero Jesus L., Inostroza-Bacheteau Claudio, Aque Felipe and Arce-Johnson Patricio (2015a). Improved Salinity Tolerance in *Carrizo Citrange* Rootstock through Overexpression of Glyoxalase System Genes. BioMed Research International.

Álvarez-Gerding Ximena, Espinoza Carmen, Inostroza-Bacheteau Claudio and Arce-Johnson Patricio. (2015b). Molecular and physiological changes in response to salt stress in *Citrus macrophylla* W plants overexpressing Arabidopsis CBF3/DREB1A. Plant Physiology and Biochemistry 92 (2015) 71-80.

Araujo W., Fernie A. and Nunes-Nesi A. (2011). Control of stomatal aperture: a renaissance of the old guard. Plant Signal Behav 6(9): 1305-1311.

Arentsen, JI. Caracterización climática y de calidad de fruta en naranjas Navel (*Citrus sinensis* L. (Osbeck)) cv. Newhall en nueve zonas citrícolas de Chile. (2002). Proyecto de Título. Facultad de Agronomía e Ingeniería Forestal. Pontificia Universidad Católica de Chile, Santiago, 31 pp.

ASOEX (2011). Estadísticas, Comité de Cítricos de Chile, http://www.comitedecitricos.cl/es/estadisticas/exportaciones/evolucion.

Barrett H. and Rhodes A., (1976). A numerical taxonomic study of affinity relationships in cultivated Citrus and its close relatives. Syst. Bot. 1: 105-136.

Barry G., Castle W., Davies F. (2004). Rootstocks and plant water relations affect sugar accumulation of citrus fruit via osmotic adjustment. Journal of the American Society for Horticultural Science; 129: 881-889.

Bartels D. & Sunkar, R. (2005). Drought ans salt tolerance in plants. Critical Reviews in Plant Sciences, 24: 23-58.

Birch R. (1997). Plant transformation: problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol. 48, 297-326.

Blumwald E., Aharon G., Apse M. (2000). Sodium transport in plant cells. Biochimica et Biophysica Acta 1465: 140-151.

Bohnert H., Sheveleva E. (1998). Plant stress adaptations-making metabolism move. Curr Opin Plant Biol 1: 267-274.

Bordón Y., Guardiola J. L., García-Luis A. (2000). Genotype affects the morphogenic responsein vitro of epicotyl segments of Citrus rootstocks. Annals of Botany 86: 159-166.

Bray E. (1997). Plant responses to water deficit. Trends Plant Sci 2: 48-54.

Cervera M., Navarro L., Peña. (2009). Gene stacking in 1-year-cycling APETALA1 citrus plants for a rapid evaluation of transgenic traits in reproductive tissues. Journal of Biotechnology 140: 278-282.

Cervera M., Ortega C., Navarro A., Navarro L., Peña L. (2000). Generation of transgenic citrus plants with the tolerance-to-salinity gene HAL2 from yeast. Journal of Horticultural Science and Biotechnology 75: 26-30.

Cervera M., Pina J., Juarez J., Navarro L., Pena L. (1998). Agrobacterium-mediated transformation of citrange: factors affecting transformation and regeneration. Plant Cell Rep 18: 271-278.

Clarke S., Cristescu S., Miersch O., Harren F., Wasternack C., Mur L. (2009) Jasmonates act with salicylic acid to confer basal thermotolerance in Arabidopsis thaliana. New Phytol 182:175– 187.

Cominelli E. and Tonelli C. (2010). Transgenic crops coping with water scarcity. N Biotechnol 27(5): 473-477.

Cominelli E., Galbiati M. and Tonelli C. (2010). Transcription factors controlling stomatal movements and drought tolerance. Transcription 1(1): 41-45.

Cook Benjamin I., Smerdon Jason E., Seager Richard, Coats Sloan. (2018). Global Warming and Drought in the 21st Century.

Coqueiro D., de Souza A., Takita M., Rodrigues C., Kishi L., Machado M. (2015). Transcriptional profile of sweet orange in response to chitosan and salicylic acid. BMC Genomics 16:1-14.

CR2 (2015). La megasequía 2010-2015: Una lección para el futuro, Centro de Investigación del Clima y la Resiliencia (CR2): 1-27.

Chen C., Xiao Y., Li X. y Ni M. (2012). Light-regulated stomatal aperture in Arabidopsis. Molecular Plant 5(3): 566-572.

Dale P. (1995). Regulation and field trialling of transgenic crops. Trends in Biotechnology. 13, 398-403.

Dodd I.C. and Ryan A. (2016). Whole-plant physiological responses to water-deficit stress. eLS. John Wiley & Sons.

FAO. (2008). FAO Land and Plant Nutrition Management Service. http://www.fao.org/ ag/agl/agll/spush.

FAO (2016). Citrus fruit fresh and processed statistical.

FAO (2007) "Water at a Glance."

Faures J. (2012). Coping With Water Scarcity: An Action Framework For Agriculture And Food Security.

Foley J., Ramankutty N., Brauman K., Cassidy E., Gerber J., Johnston M., Mueller N., O'Connell C., Ray D., West P., Balzer C., Bennett E., Carpenter S., Hill J., Monfreda C., Polasky S., Rockstrom J., Sheehan J., Siebert S., Tilman D. and Zaks D. P. (2011). Solutions for a cultivated planet. Nature 478 (7369): 337-342.

Fu X., Khan E., Hu S., Fan Q., Liu J. (2011). Overexpression of the betaine aldehyde dehydrogenase gene from *Atriplex hortensis* enhances salt tolerance in the transgenic trifoliate orange (Poncirus trifoliata L. Raf.). Environ Exp Bot 74: 106-113.

Ghorbel R., Dominguez A., Navarro L., Pena L. (2000). High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of citrus tristeza virus. Tree Physiol 20: 1183-1189.

Ghorbel R., La-Malfa S., López M., Petit A., Navarro L., Peña L. (2001). Additional copies of virG from pTiBo542 provide a super-transformation ability to Agrobacterium tumefaciens in citrus. Physiol Mol Plant Pathol 58: 103-11.

Gmitter F., Grosser J., Moore G. (1992). Citrus. In Biotechnology of perennial crops (ed. F. Hammerschlag and R. L. Litz), pp. 335-369. Wallingford, Oxon, U.K: CAB Intl.

Gompertz, M. (1999). Caracterización climática de nueve zonas citrícolas de Chile. Tesis, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Santiago.48 pp.

Hughes J., Hepworth C., Dutton C., Dunn J., Hunt L., Stephens J., Waugh R., Cameron D., Gray J. (2017). Reducing stomatal density in barley improves drought tolerance without impacting on yield. Plant Physiol 174: 776–787.

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

Jia Hongge, Zhang Yunzeng, Orbovi'c Vladimir, Xu Jin, White Frank F., Jones Jeffrey B. and Wang Nian. (2017). Genome editing of the disease susceptibility gene CsLOB1 in citrus confers resistance to citrus canker.. Plant Biotechnology Journal, pp. 817–823.

Jung C, Seo J, Han S, Koo Y, Kim C, Song S, Nahm B, Choi Y, Cheong J. (2008). Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. Plant Physiol 146: 623-635

Kahn, T., Krueger R., Gumpf D., Roose M., Arpaia M., Batkin T., Bash J., Bier O., Clegg M., Cockerham S., Coggins Jr C., Durling D., Elliott G., Mauk P., McGuire P., Orman C., Qualset C., Roberts P., Soost R., Turco J., Van S., Zucker B. (2001). Citrus genetic resources in California: Analysis and recommendations for long-term conservation. Report No. 22. University of California Division of Agriculture and Natural Resources, Genetic Resources Conservation Program, Davis, CA, USA.

Kang J., Choi H., Im M., Soo Y. (2002). Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. The Plant Cell 14: 343-357.

Khan IA, Khender WJ. (2007). Citrus Breeding. In IA Khan, ed, Citrus Genetics, Breeding and Biotechnology. CAB International, UK, pp 1-8.

Kim T., Bohmer M., Hu H., Nishimura N. and Schroeder J. (2010). Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca^{2+} signaling. Annu Rev Plant Biol 61: 561-591.

Kollist H., Nuhkat M. and Roelfsema M. (2014). Closing gaps: linking elements that control stomatal movement. The New phytologist 203(1): 44-62.

Lambers, H., Chapin F. and Pons T. (2008). Plant physiological ecology. New York, Springer.

Laporte, M., B. Shen and Tarczynski M. (2002). Engineering for drought avoidance: expression of maize NADP-malic enzyme in tobacco results in altered stomatal function. Journal of experimental botany 53(369): 699-705.

Liu Y., Heying E., Tanumihardjo S. (2012). History, global distribution, and nutritional importance of citrus fruits. Comprehensive Reviews in Food Science and Food Safety 11: 530-545.

Lugassi N., Kelly G., . Fidel L, Yaniv Y., Attia Z., Levi A., Alchanatis, Moshelion V., Raveh E., Carmi N. and Granot D. (2015). Expression of Arabidopsis Hexokinase in Citrus Guard Cells Controls Stomatal Aperture and Reduces Transpiration. Frontiers in plant science 6: 1114.

Mahouachi J., Gómez-Cadenas, Primo-Millo E., Talon M., (2005). Antagonistic Changes between Abscisic Acid and Gibberellins in Citrus Fruits Subjected to a Series of Different Water Conditions *Jour- nal of Plant Growth Regulation*,; 24:179-187.

Manner Harley I., Buker Richard S., Easton Smith V., Ward D. and Elevitch Craig R. (2006). Citrus (citrus) and Fortunella (kumquat). Species Profiles for Pacific Island Agroforestry. www.traditionaltree.org.

Maas EV, Grattan SR. (1999). Crops yields as affected by salinity. Agricultural drainage. Agron. Monog. 38, Ed Skaggs, R.W.; van Schilfgaarde, J. ASA-CSSA-SSSA, Madison, Wis., pp 55-109.

Mohammad Abass Ahanger, Nudrat Aisha Akram, Muhammad Ashraf, Mohammed Nasser Alyemeni, Leonard Wijayaand Parvaiz Ahmad. (2017). Plant responses to environmental stresses-from gene to biotechnology. AoB PLANTS 9: plx025; doi:10.1093/aobpla/plx025.

Molden D. (2007). International Water Management Institute. Comprehensive Assessment of Water Management in Agriculture (Program) Water for food, water for life: a comprehensive assessment of water management in agriculture. London; Sterling, VA, Earthscan.

Molinari H., Marur C., Bespalhok Filho J., Kobayashi A., Pileggi M., Leite Junior R., Pereira L., Vieira L. (2004). Osmotic adjustment in transgenic citrus rootstock *Carrizo citrange* (*Citrus sinensis* Osb. x *Poncirus trifoliate* L. Raf.) overproducing proline. Plant Science 167: 1375-1381.

Moore G., Jacono C., Neidigh J., Lawrence S., Cline K. (1992). Agrobacterium-mediated transformation of Citrus stem segments and regeneration of transgenic plants. Plant Cell Rep 11: 238-242.

Munns, R. (2011). Plant adaptations to salt and water stress: differences and commonalities. Advances in Botanical Research 57: 1-32.

Navarro J., Pérez-Pérez Romero J., Botía P. (2010). Analysis of the changes in quality in mandarin fruit, produced by deficit irrigation treatments. Food Chemistry; 119:1591-159.

Negrão S., Schmöckel S. and Tester M. (2017). Evaluating physiological responses of plants to salinity stress. Annals of Botany 119: 1–11.

ODEPA (2012) Estadísticas de frutales.

ODEPA-CIREN (2015) Catastro frutícola, Principales resultados, Región de Coquimbo.

Peng YH, Rave E. (1998). Effect of differing irrigation regimes on fruit quality, yield, fruit size and net CO_2 assimilation of Mihowase Satsuma *The Journal of Horticultural Science* Biotech; 73: 229- 234.

Peña L, Martín-Trillo M, Juárez J, Pina JA, Navarro L, Martínez-Zapater JM. (2001). Constitutive expression of Arabidopsis LEAFY or APETALA1 genes in citrus reduces their generation time. Nat Biotechnol 19: 263-267.

Pérez-Pérez J., Robles J., Botía P. (2009). Influence of deficit irrigation in phase III of fruit growth on fruit quality in 'lane late' sweet orange. Agricultural Water Management, 96: 969-974.

Pinochet H. (1990). Antecedentes para la elección de portainjertos en cítricos. Aconex 27: 9-16.

Pretty J. (2008). Agricultural sustainability: concepts, principles and evidence. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 363(1491): 447-465.

Qadir M., Quillerou E., Nangia V. Murtaza G., Singh M., Thomas R., Drechsel P., Noble A. (2014). Economics of salt-induced land degradation and restoration. Natural Resources Forum 38: 282–295.

Ruane J., Sonnino A., Steduto P. and Deane C. (2008). Coping with Water Scarcity: What Role for Biotechnologies?.

Scora R., (1988). Biochemistry, taxonomy and evolution of modern cultivated Citrus. Proc. Int. Soc. Citricult. VI. Congr. vol. 1. Margraf Publishers, Weikersheim, Germany, pp.277-289.

Schewe J., Heinke J., Gerten D., Haddeland I., Arnell N., Clark D., Dankers R., Eisner S., Fekete B., Colon-Gonzalez F., Gosling S., Kim H., Liu X., Masaki Y., Portmann F., Satoh Y., Stacke T., Tang Q., Wada Y., Wisser D., Albrecht T., Frieler K., Piontek F., Warszawski L. and Kabat P. (2014). Multimodel assessment of water scarcity under climate change. Proceedings of the National Academy of Sciences of the United States of America 111(9): 3245-3250.

Schiermeier Q. (2014). Water risk as world warms. Nature 505(7481): 10-11.

Schroeder J., Allen G., Hugouvieux V., Kwak J. and Waner D. (2001a). Guard Cell Signal Transduction. Annu Rev Plant Physiol Plant Mol Biol 52: 627-658.

Schroeder J., Kwak J. and Allen G. (2001b). Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature 410(6826): 327-330.

Serrano R., Mulet J., Rios G., Marquez J., Larrinoa I., Leube M., Mendizabal I., Pascual-Ahuir A., Proft M., Ros R., Montesinos C. (1999). A glimpse of the mechanisms of ion homeostasis during salt stress. Journal of Experimental Botany 50: 1023.

Shalhevet J., Levy Y. (1990). Citrus trees In: Stewart BA, Nielsen, DR (eds) Irrigation of Agricultural Crops A S A Madison, Wis Mon no 30 p 951-986.

Shinozaki Kazuo and Yamaguchi-Shinozaki Kazuko. (1997). Gene Expression and Signal Transduction in Water-Stress Response. Plant Physiol. (1997) 115: 327-334.

Swingle W. And Recee. (1967). The botany of citrus and its wild relatives. In: The Citrus Industry, vol. 1. University of California Press, Berkeley, CA, USA, pp. 389-390.

Taiz L. and Zeiger E. (2010). Plant Physiology. Sinauer Associates, Inc., Publishers Sunderland, Massachusetts 792 p.

Tanaka T., (1977). Fundamental discussion of Citrus classification. Stud. Citrol. 14: 1-6.

Tilman D., Cassman K., Matson P., Naylor R. and Polasky S. (2002). Agricultural sustainability and intensive production practices. Nature 418(6898): 671-677.

Thomashow M. (1999). Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50: 571-599.

Thomashow M., Gilmour S., Stockinger E., Jaglo-Ottosen K., Zarka D. (2001). Role of the Arabidopsis CBF transcriptional activators in cold acclimation. Physiol Plant 112:171–175 .

Tyerman S., Bohnert H., Maurel C., Steudle E., Smith J. (1999). Plant aquaporins: their molecular biology, biophysics and significance for plant water relations. Journal of Experimental Botany 50: 1055-1071.

Van Loon A., Gleeson T., Clark J., Van Dijk A., Stahl K., Hannaford J., Di Baldassarre G., Teuling A., Tallaksen L., Uijlenhoet R., Hannah D., Sheffield J., Svoboda M., Verbeiren B., Wagener T., Rangecroft S., Wanders N. and Van Lanen H. (2016). Drought in the Anthropocene. Nature Geosci 9(2): 89-91.

Vélez Javier E., Álvarez-Herrera Javier G., Alvarado-Sanabria Oscar H. (2012). El Estrés Hídrico en Cítricos (*Citrus* spp.): Una revisión Water stress on citric fruit (*Citrus* spp.): a review. ORINOQUIA - Universidad de los Llanos - Villavicencio, Meta. Colombia Vol. 16 - No 2.

Vierling E. (1991). The roles of heat-shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42: 579-620.

Von Koskull-Döring P., Scharf K., Nover L. (2007). The diversity of plant heat stress transcription factors. Trends Plant Sci 12: 452-45.

Wallace J. (2000). Increasing agricultural water use efficiency to meet future food production. Agriculture, Ecosystems and Environment 82:105–119.

Wang C., Liu Yang, Li Si-Shen, Han Guan-Zhu. (2015). Insights in to the Origin and Evolution of the Plant Hormone Signaling Machinery. DOI: https://doi.org/10.1104/pp.114.247403.

Wang L., Zhao Y., Reiter R., He C., Liu G., Lei Q., Zuo B., Zheng X., Li Q., Kong J. (2014). Changes in melatonin levels in transgenic Micro-Tom tomato overexpressing ovine AANAT and ovine HIOMT genes. J Pineal Res. 56:134-42.

Wang Z., Bai M., Oh E., Zhu J. (2012). Brassinosteroid signaling network and regulation of photomorphogenesis. Annu Rev Genet 46: 701–724.

Wang W., Vinocur B. and Altman, A. (2003). Plant responde to drought, salinity and extreme temperatures: towards genetics engineering for stress tolerance. Planta, 218: 1 - 14.

Wu Guohong A., Terol J., Ibañez V., López-García A., Pérez-Román E., Borredá C., Domingo C., Tadeo Francisco R., Carbonell-Caballero J., Roberto A., Curk F., Du D., Ollitrault P., Roose Mikeal L., Dopazo J., Gmitter Frederick G. Jr, Rokhsar Daniel S. and Talon M. (2018). Genomics of the origin and evolution of *Citrus.* Nature doi:10.1038/nature25447.

Zandalinas S., Balfagón D., Arbona V., Gómez-Cadenas A. (2018). Regulation of citrus responses to the combined action of drought and high temperatures depends on the severity of water deprivation. Physiol Plant. 2018 Apr. 162(4):427-438. doi: 10.1111/ppl.12643. Epub 2017 Nov 9.

Zhai S., Gao Q., Xue H., Sui Z., Yue G., Yang A., Zhang J. (2012). Overexpression of the phosphatidylinositol synthase gene from *Zea mays* in tobacco plants alters the membrane lipids composition and improves drought stress tolerance. Planta 235: 69-84.

Zhang J. (2014). Lasiodiploidia theobromae in Citrus Fruit (Diploidia Stem-End Rot). Postharvest Decay. Pag. 309-335. doi.org/10.1016/B978-0-12-411552-1.00010-7. pp. 361-396.

Zhu J. (2002). Salt and drought stress signal transduction in plants. Ann Rev Plant Biol 53: 247-273.

CHAPTER 2

INCREASED DROUGHT AND SALINITY TOLERANCE IN *CITRUS AURANTIFOLIA* (MEXICAN LEMON) PLANTS OVEREXPRESSING ARABIDOPSIS CBF3 GENE

Jesús L. Romero-Romero, Claudio Inostroza-Blancheteau, Marjorie Reyes-Díaz, Juan Pablo Matte, Felipe Aquea, Pilar M. Gil, Carmen Espinoza and Patricio Arce-Johnson

Laboratorio de Biología Molecular y Biotecnología Vegetal, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

Increased drought and salinity tolerance in *Citrus aurantifolia* (Mexican lemon) plants overexpressing Arabidopsis CBF3 gene

Jesús L. Romero-Romero[1,2,3], Claudio Inostroza-Blancheteau[4], Marjorie Reyes-Díaz[5,6], Juan Pablo Matte[1], Felipe Aquea[7,8], Pilar M. Gil[2], Carmen Espinoza[1] and Patricio Arce-Johnson[1]*

[1]Facultad de Ciencias Biológicas, Departamento de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile, Santiago, Chile.

[2]Facultad de Agronomía e Ingeniería Forestal, Pontifica Universidad Católica de Chile, Santiago, Chile.

[3]Instituto Politécnico Nacional, CIIDIR, Unidad Sinaloa, Departamento de Biotecnología Agrícola, San Joachín, Guasave, Sinaloa, México

[4]Núcleo de Investigación en Producción Alimentaria, Facultad de Recursos Naturales, Departamento de Ciencias Agropecuarias y Acuícolas, Universidad Católica de Temuco, Temuco, Chile.

[5]Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Temuco, Chile.

[6]Center of Plant, Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus (BIOREN-UFRO), Universidad de La Frontera, Temuco, Chile.

[7]Laboratorio de Bioingeniería, Facultad de Ingeniería y Ciencias, Universidad Adolfo Ibáñez, Santiago, Chile

[8]Center for Applied Ecology and Sustainability (CAPES), Santiago, Chile.

*Corresponding author: Patricio Arce-Johnson. E-mail: parce@bio.puc.cl

Abstract

Citrus are a globally important fruit crop. Abiotic stressors such as drought and salinity adversely affect citrus plant growth, production, and survival. With the aim of improving drought tolerance in citrus plants, we constructed transgenic lines of *Citrus aurantifolia* overexpressing the *Arabidopsis* transcription factor *CBF3.* All transgenic lines grew comparably to wild type plants. Molecular and quantitative real-time analyses showed high expression of *CBF3* in three selected transgenic lines. During a 15 days treatment of water deficit by cessation of irrigation, the transgenic lines showed lower stomatal conductance and transpiration paired with photosynthesis greater than or equal to WT plants, which translated into more efficient water use. The genes *SIP1* and *GOLS* showed similar or greater expression in one of the transgenic lines with respect to control plants. Moreover, transgenic lines were more tolerant to saline stress and presented a greener phenotype with increased chlorophyll content in leaf discs compared to WT plants, indicating lower electrical conductivity in solution. Additionally, all transgenic lines exhibited significantly less accumulation of reactive oxygen species than WT plants. Together, these results demonstrate the potential for heterologous expression of the *CBF3* gene to mediate tolerance to hydric and saline stress in citrus plants.

Keywords: Drought tolerance; Abiotic stress; Transgenics Citrus; CBF3 transcription factor

1. Introduction

Citrus fruits are the most important persistent leaf fruit in tropical and subtropical regions of the world and are cultivated in more than one hundred countries (Zhang et al. 2014). These fruits provide various nutrients such as vitamins, fiber, calcium, potassium, and folic acid which yield important health benefits (Jia et al. 2017). Citrus face biotic and abiotic challenges including water deficiency and high salinity, which affect development and production and limit the distribution of this species. The physiological response of citrus to drought events includes decreased leaf area, increased root length, and limited photosynthetic rate (Amin et al. 2016). Effects on photosynthesis may be direct: reduced CO_2 diffusion through the stomata and mesophyll paired with alterations in photosynthetic metabolism and activity of enzymes involved in the Calvin cycle; or indirect: damage to the photosynthetic apparatus caused by oxidative stress (Dodd and Ryan 2016). Biochemical and morphological strategies that protect cells from damage and dehydration have also been described (Dodd and Ryan 2016). One of these mechanisms is osmotic adjustment, the process by which the concentration of solutes increases in the cell in order to decrease the water potential and maintain the turgor of the cell (Taiz and Zeiger 2010; Dodd and Ryan 2016).

Several studies have shown that drought tolerance is related to regulation of gene expression, accumulation of metabolites, or modification of key proteins under a reduced water potential (Amin et al. 2016). Moreover, water stress induces the synthesis of abscisic acid (ABA), which triggers stomatal closure by inducing the expression of transcription factors such as MYB44 and MYB15 (Jung et al. 2008;

Cominelli et al. 2010). In several plants, overexpression of *CBF3* regulates the expression of genes sensitive to stress from drought, cold, and salinity (Yamaguchi-Shinozaki and Shinozaki 1994).

Citrus trees are characterized by high water demand and acute sensitivity to salinity. Several studies have reported increased stress tolerance in transgenic lines of citrus species, including the expression of betaine aldehyde dehydrogenase from *Atriplex hortensis* in trifoliate orange (Fu et al. 2011), the expression of yeast HAL2 in *Carrizo citrange* (Cervera et al. 2000; Ali et al. 2012), the expression of the glyoxylases genes *BjGlyI* and *PgGlyII* in *Carrizo citrange* (Álvarez-Gerding et al. 2015a), and the expression of *CBF3* from Arabidopsis in *Citrus macrophylla* (Álvarez-Gerding et al. 2015b). These studies, however, focused on rootstocks and not on commercial varieties. In the field, commercial varieties are grafted onto rootstock, with the commercial varieties providing the conductive tissue in the leaves. Importantly, this tissue is the main organ of the plant responsible for water loss through the stomata. Expanding the characterization of citrus varieties in response to hydric and saline stress is particularly timely when considering the 2ºC increase in average soil temperature that is expected in the next 50 years (Schewe et al. 2014).

In this work, we report the physiological and molecular response of *Citrus aurantifolia* (mexican lemon) overexpressing the *Arabidopsis thaliana* transcription factor CBF3, resulting in transgenic lines with increased drought and salinity tolerance in greenhouse conditions. This work demonstrates the potential for

heterologous expression of CBF3 to mediate tolerance to hydric and saline stress in a commercial variety of citrus.

2. Materials and methods

2.1 Plant material.

Citrus aurantifolia seeds were soaked in a fungicide solution of 1% Captan (1 g/L) in a shaker at 30ºC for 12h. Seeds were then surface sterilized with a solution of 1% NaClO for 10 min and rinsed four times with sterile distilled water to remove the seed coat. Seeds were germinated in MT medium with 0.7% agar (w/v). To prevent contamination, 2 ml/L Plant Preservative Mixture (PPM, Plant Cell Technology, Washington, USA) was added without sucrose. Groups of fifteen seeds were then sown and placed in a dark germination chamber at 29ºC ± 2ºC for 3-4 weeks to promote the formation of etiolated epicotyls.

2.2 *Citrus aurantifolia* transformation and plant regeneration.

The previously described pMDC84-CBF3 constructs (Alvarez-Gerding et al. 2015a) were used to generate stable transgenic *C. aurantifolia* lines. The strain EHA105 *of A. tumefaciens* carrying pMDC84-CBF3 was used to perform genetic transformation. Epicotyl segments were transformed as previously described by Cervera et al. (1998). Subsequently, epicotyls were placed in selection medium with hygromycin at a concentration of 4 mg/L. After three weeks in darkness, explants were transferred to a growth chamber at 23ºC± 2ºC with a 16 h light photoperiod and sub-cultured every two weeks in medium supplemented with hygromycin for selection of transgenic shoots. After three weeks of subculture, shoots were observed at the ends of the explants and individualized in root induction medium (MT medium supplemented with naphthalene acetic acid (NAA) 1 mgL^{-1}) when they reached 2-3

cm. Finally, the rooted shoots were acclimatized in hermetic transparent pots containing 50% perlite and 50% peat moss in a greenhouse and individualized when they reached an approximate size of 15 cm. The clonal propagation of the lines was performed after 20 weeks of growth by selecting axillary buds and submerging them in NAA 25 mg L^{-1} followed by placement in pots with substrates for the drought experiments.

2.3. Molecular characterization of transformed plants

The lines rooted in hermetic transparent pots with resistance to hygromycin were screened by PCR analysis with the primers 35SPROM 5′-GACGTAAGGGATGACGCACAAT-3′ and CBF300-Rev 5′-ATAACTCCATAACGATACGTC- 3′ to yield an expected amplification product of 675 bp using genomic DNA isolated from citrus leaves from WT and transgenic lines as template (Alvarez-Gerding et al. 2015a).

2.4. Quantitative Gene Expression

The quantification of *CBF3* expression in transgenic lines was evaluated by qRT-PCR. RNA extraction and cDNA synthesis were performed as described previously (Cañon et al. 2013). Primers qCBF3-Fwd 5′-CTAATATGGCAGAAGGGATGC-3′ and qCBF3-Rev 5′- AACTCCATAACGATACGTCGTC-3′ were used to analyze the expression of *CBF3* with an expected amplification product of 99 bp. In the same way, the expression of the following four genes related to the abiotic stress response in citrus were evaluated by qRT-PCR. These genes are Cold-Response (COR15-

Fwd: 5′–TTGGGGTGTCTACTCAAGGTG-3′ and COR15-Rev: 5′–TTCACATTGACAAAAGGCAAA-3′), P5CS1 (P5CS1-Fwd: 5′–ACGATGTGCGTGCTGCTA-3′ and P5CS1-Rev: 5′–GCACCAAGTCCAAATCGTG-3′), raffinose synthase (SIP1-Fwd: 5′–GGAGTGAAGGGAAGTGGTGA-3′ and SIP1-Rev: 5′ ATAGCAACCATGTGGCCTTC -3′), and galactinol synthase (GOLS-Fwd: GCGAGAAAACATGGAGCAA and GOLS-Rev: GAGATGCTGGGCTCAAACA; the expected amplification products for these genes were 118, 176, 110 and 145pb respectively. Tubuline was used as a housekeeping gene to normalize gene expression. Stratagene model Mx3000P and the SensimixTM SYBR®-Green kit were used for all qRT-PCR analysis.

Relative transcript abundance was calculated considering the efficiency of the PCR reaction measuring the fluorescence of SYBR Green Master Reagent Kit (Agilent Technologies, Santa Clara, USA) for less than 40 cycles with three independent biological replicates and two technical replicates.

$$\Delta Ct_{Eff} = \frac{Eff_{Reference}{}^{Ct_{Reference}}}{Eff_{Target}{}^{Ct_{Target}}}$$

$$\Delta\Delta Ct_{Eff} = \frac{\left(\frac{Eff_{Reference}{}^{Ct_{Reference}}}{Eff_{Target}{}^{Ct_{Target}}}\right)_{Transgenic}}{\left(\frac{Eff_{Reference}{}^{Ct_{Reference}}}{Eff_{Target}{}^{Ct_{Target}}}\right)_{Wt}}$$

Relative transcript abundance was calculated using the GED method (Gene Expression's Ct Difference, ΔCt and ΔΔCt methods). Negative controls (RT- and no-

template) were incorporated in all qPCR assays. Melting curves were analyzed on each real time RT-PCR reaction to corroborate single PCR product.

2.5. Stomatal density

Stomatal density was measured using the abaxial side of mature leaves from 3-4 months olds plants. Briefly, impressions were prepared from both sides of the central vein by the application of a dental silicon layer. Once the silicon had dried, impressions were covered with transparent nail polish. This film was then carefully placed on microscope slides and covered with coverslips. Stomata number per 0.25 mm^2 surface was recorded using a digital camera attached to a binocular microscope (SMZ800, Nikon Corp., Tokyo, Japan).

2.6 Stomatal conductance

Stomatal conductance (gs) was determined in fully expanded leaves using a steady state porometer (Leaf Porometer, Decagon Divices, Pullman, WA, USA) by measuring vapor concentration at two different points in the diffusion path. Four leaves per plant and per treatment were analyzed. Measurements were taken over a 30s period and reported in mmol m^{-2} s^{-1} according to the manufacturer. All physiological parameters were recorded between 09:00 and 11:00 a.m. with a temperature of 20°C and approximately 80% relative humidity in greenhouse.

2.7. Analysis of transgenic plants in drought conditions

To evaluate the drought tolerance, five-month-old transgenic lines of mexican lemon

and untransformed wildtype (WT) plants were transferred to 2L pots containing substrate mix of peat and perlite (1:1). Drought conditions were simulated in six-month-old plants by withholding irrigation for 5, 10, or 15 days. The control treatment (0 d) included well-watered plants, which received 80% field capacity irrigation. Plant survival rates were recorded one week after rewatering. To compare the water loss rates of WT and *CBF3* transgenic lines, the relative water content was determined at 0, 10, and 15 days of treatment by genotype. A foliole was taken from each plant and its fresh weight (FP) was recorded using an analytical balance. The leaves were then left floating in a petri dish with distilled water for 4 hours at 22°C in a growth chamber and subsequently re-weighed on the analytical balance to obtain their weight in turgor (PT). Finally, the leaves were completely dehydrated in an oven at 80 °C for 48 hours, after which their dry weight (PS) was obtained. The RWC was calculated according to the following equation:

$$RWC(\%) = \left[\frac{PF - PS}{PT - PS}\right] \cdot 100$$

Gas exchange was also evaluated at the beginning and at the end of the water stress treatment (days 0, 10, or 15) through the use of the photosynthesis system (IRGA LI-6400XT of LI-COR, Lincoln, Nebraska, United States). Gas exchange parameters were determined in vivo in fully expanded leaves. Five measurements were made per plant with 5 plants per genotype. A PAR light source of 100 μmol $m^{-2}s^{-1}$ was used. Temperature (20ºC±2ºC), relative humidity (70-80%), and CO_2 concentration were controlled in the growth chamber. External CO_2 was applied to obtain a concentration of 400 μmol mol^{-1} (ppm), with a flow of 300 ml min^{-1}. Net assimilation

rate of carbon (A, μmol CO_2 $m^{-2}s^{-1}$), stomatal conductance to water vapor (stomatic conductance, mol H_2O $m^{-2}s^{-1}$), transpiration rate (E, mmol H_2O $m^{-2}s^{-1}$), and instantaneous water use efficiency (WUE, calculated as A/E, mmol CO_2 / mol H_2O) were measured. Additionally, a fully-expanded fourth leaf was harvested following 0, 10, or 15 days of drought exposure, frozen immediately in liquid N_2, and stored at 80°C. At each harvest, three replicates of each mexican lemon line were taken with five plants in each experimental group. The expression of different genes was evalulated using qRT-PCR.

2.8 Leaf senescence and chlorophyll content

Fully expanded leaves were extracted from transgenic lines and WT plants of the same age three months after the transfer to substrate and washed briefly with deionized water. Three 1 cm diameter leaf discs obtained from three different plants of each line were suspended in 3 ml of sodium chloride (NaCl) in a concentration of 100 mM (> 30 $mScm^{-1}$) or in sterile deionized water as the control treatment for 24 hours (Singla-Pareek et al. 2008). Chlorophyll content was determined with dimethyl sulfoxide (DMSO) following the technique described by Hiscox and Israelstam (1979). This analysis was performed with the three leaf discs used in the foliar senescence analysis described above. Following placement in 100 mM NaCl (area each 1 cm^2 disc, 25 mg of total fresh weight) for 24 hours, leaf discs were incubated in 7 ml of DMSO for 30 min at 65^oC in a water bath. Subsequently, 1 ml of extract was transferred to a disposable polyethylene cuvette and absorbance was measured at 645 and 663 nm in a spectrophotometer previously calibrated with pure DMSO. Total chlorophyll content was calculated and the result was expressed as

mg/ml/gFW using the follow equation and converted to mg/cm^2 per leaf area:

$$Chl(A+B) = 0.0202 \cdot A_{645} + 0.00802 \cdot A_{663}$$

2.9 Histochemical detection of H_2O_2 and electrical conductivity

Discs from full leaves of transgenic lines and WT plants of comparable age and size were suspended in 7 ml of 100 mM NaCl for 24 hours to stimulate oxidative burst, resulting in the generation of reactive oxygen species and liberation of solutes to the environment upon deterioration of the cell wall. Sterile deionized water was used as an experimental control. To determine the concentration of H_2O_2, leaf discs were transferred to an aqueous solution containing 1 mgml^{-1} 3,3'-diaminobenzidine (DAB) -HCl (Sigma-Aldrich Co. St. Louise, MO, USA) with pH 3.8 and incubated for 6 hours in the dark at room temperature (Eherenfel et al. 2005; Thordal-Christensen et al. 1997). Chlorophyll was subsequently removed from the foliar samples using 80% ethanol at 60^{o}C (Carvalho et al. 2008). The images were captured using a digital camera attached to a Binocular Magnifier SMZ800 (Nikon Corp., Tokyo, Japan). Electrical conductivity (µmho) of the suspension generated from treatment of the leaf disks with 100 mM NaCl was determined using the HM Digital COM-100 EC/TDS/TEMP meter (HM Digital, Redondo Beach CA, USA) previously calibrated with sterile distilled water.

2.10 Statistical analyses

Data were analyzed using a single factor ANOVA to determine a significant difference from at least one pair of samples, while the Student's t-test ($p \leq 0.05$) was

used for pairwise comparison of samples. The F-test for two samples was conducted to test for equal variance. If the test was accepted, a t-test for two samples assuming equal variance was used; otherwise a t-test for two samples assuming unequal variance was used.

3. Results

We sought to clone and overexpress Arabidopsis transcription factor *CBF3* in the mexican lemon to improve drought and salinity tolerance in a commercial variety of citrus. Transgenic lines were selected for physiological evaluation based on expression level of CBF3. Three transgenic lines showed significant differences in *CBF3* expression compared to WT plants (Figure 1). No phenotypic differences were observed between the transgenic lines and WT plants. Specifically, none of the *CBF3* transgenic lines exhibited significant differences in stomatal density (Figure 2a) or stomatal conductance under normal growth conditions (Figure 2b). To assess drought tolerance in the transgenic citrus lines, irrigation was withheld for 15 days. Five six-month-old plants of each transgenic line were grown in 1 L pots under greenhouse conditions. Relative water content (RWC) was evaluated over time. Two of the three transgenic lines presented a higher RWC compared to WT plants at 10 days. This difference persisted through day 15, with the exception of one transgenic line which showed significantly lower RWC than WT plants (6.65% versus 15%, Table 1).

Stomatal conductance was measured before the treatment and at days 10 and 15 post-treatment. Stomatal conductance was significantly lower in the three transgenic lines compared to WT plants at 10 and 15 days (Figure 3a). In addition, the transgenic lines presented similar transpiration rates than the WT plant at day 10 post-treatment. At day 15, however, all the transgenic lines exhibited lower transpiration rates than WT plants (Figure 3b). Likewise, the rate of photosynthetic assimilation of carbon was similar in transgenic lines at day 10 and 15 compared to

WT plants, except for one of these plants that showed significantly lower rates at both times compared to WT plants (Figure 3c). Finally, water use efficiency was found to be significantly higher in all three transgenic lines compared to WT plants at day 15 post-treatment.

In order to understand the physiological and molecular mechanisms mitigating increased drought tolerance, the expression of the marker genes *P5CS1, COR15, GOLS,* and *SIP1* were evaluated (Figure 4). These genes have been reported as targets of *CBF3* in various species. Differences in the level of expression were found in the expression of all four target genes evaluated in transgenic with respect to WT plants. All the marker genes are upregulated in the LM14 line, while the other lines shown different pattern, even some of them are downregulated in the transgenic lines LM2. (Figure 4).

Overexpression of *CBF3* is known to confer stress tolerance in other plants. In order to evaluate if *CBF3* overexpression in mexican lemon similarly improves salinity tolerance, an in vitro assay was designed and implemented. Several 1 cm discs were extracted from fully expanded leaves of each transgenic plant and treated with 100mM NaCl for 24 hours. At the end of the treatment, total chlorophyll and production of reactive oxygen species (ROS) were quantified (Figure 5 and 6). Lower foliar senescence was observed in the three transgenic lines compared to the WT plants (Figure 5a), which correlated with a higher chlorophyll content in the leaf discs (Figure 6a). Likewise, transgenic lines yielded lower concentrations of ROS (Figure 5b), which correlated with lower electrical conductivity (Figure 6b). Taken together,

these results demonstrate that overexpression of *CBF3* in the mexican lemon confers tolerance to drought and salinity stress.

4. Discussion

Drought and salinity are two of the most critical abiotic conditions that limit the productivity of fruit trees, especially in species with high water demand such as the citrus. Productive citrus plants consume an estimated 100 liters of water/day/tree in the sixth year of growth, placing them among the most water demanding fruit trees (Shalhevet 1990). The physiological and molecular response of citrus plants to drought conditions (Balfagón et al. 2018) and salinity (Gueta-Dahan 1997; Avsian-Kretchmer et al. 2004; Lopez-Climent et al. 2008) has been reported. However, all previous work on citrus plants has focused exclusively on rootstocks (Cervera et al. 2000; Molinari et al. 2004; Fu et al. 2011; Ali et al. 2012; Alvarez-Gerding et al. 2015a; Álvarez-Gerding et al. 2015b; Balfagón et al. 2018). In this work, a commercial variety of lemon (*Citrus aurantifolia*) was genetically modified with the aim of generating a drought and salinity tolerant plant through overexpression of the Arabidopsis transcription factor *CBF3*. Three transgenic lines of mexican lemon were selected for physiological and molecular analysis, with all of them showing significant differences in *CBF3* expression compared to WT plants (Figure 1). Under normal irrigation conditions, transgenic plants were similar to WT plants in regards to the number of stomas and stomatal conductance (Figure 2). These three lines were further evaluated for drought and salinity tolerance. Drought tolerance was established using a 15-day irrigation cessation test according to the protocol described for Arabidopsis (Rizhsky et al. 2004), tomato (Govind et al. 2013) and rice (Xu et al. 2011).

Transgenic plants showed higher RWC after drought treatment compared to WT plants. RWC is a parameter routinely used to determine the water status of plants

following water stress experiments (Lenka et al. 2018). Previous reports characterize a RWC value of 6% as slight stress (Hsiao 1973) similar to that generated in line LM2. In contrast, WT plants at day 15 presented higher stress which may be explained by reduced their capacity to retain water. In this same drought treatment, stomatal conductance and transpiration were lower in transgenic plants at day 10 and especially at day 15 compared to WT plants (Figure 3a and 3b). Our results are similar to those reported in tomato (Govind et al. 2013) and rice (Xu et al. 2011), in which the expression of *CBF3* or its homologous gene conferred increased tolerance to abiotic stressors.

Although *CBF3* has been described as a transcription factor that acts through an ABA-independent pathway (Thomashow et al. 2001), the low stomatal conductance observed under drought treatment suggests indirect participation of *CBF3* in the regulation of stomatal closure. Recently, numerous genes that participate in the regulation of the opening and closing of stomata have been described in Arabidopsis (Takahashi et al. 2013; He et al. 2017), demonstrating a complex molecular mechanism of control. Many of the genes that control stomatal closure belong to large families such as NAC, AP2/ERF, MYB, bZIP, MYC, WRKY, and Cys2His2 zinc-finger (Nakashima et al. 2012; Mizoi et al. 2012; He et al. 2017), some of which may be target genes of *CBF3*.

In general, the transgenic lines yielded similar physiological responses characterized by decreased net photosynthesis, stomatal conductance, and transpiration paired with increased water use efficiency (Figure 3; Table 1). Under stress conditions that restrict CO_2 fixation, the rate of reducing power production is

greater than the rate of its use by the Calvin cycle. This mechanism protects against excess reducing power, an important strategy under water and salinity stress conditions (Chaves et al. 2009), suggesting that mexican lemon overexpressing CBF3 shown physiological performance improving their yield under drought.

Genes related to abiotic stress were significantly induced in transgenic lines (Figure 4). Several studies have reported that increased expression of the genes *COR15A* and *LEA4/5* is related to the drought response in citrus (Sanchez-Ballesta et al. 2004; Gimeno et al. 2009). Moreover, expression of *P5CS1* and *GOLS*, genes associated with compatible osmolytes, has been shown to increase in high salinity conditions (Alvarez-Gerding et al. 2015a). *SIP1* and *GOLS* act on biosynthesis of raffinose, one the most important osmolytes that accumulates in plants subjected to drought and salinity conditions (Silva-Ortega et al. 2008).

While expression of *SIP 1and GOLS* was higher in transgenic lines LM14, suggesting that *CBF3* may induce the concentration of raffinose under abiotic stress. Experiments performed by Molinari et al. (2004) demonstrated significant accumulation of proline, another osmolyte, in the leaves of the citrus rootstock *Carrizo citrange* under abiotic stress.

We additionally evaluated oxidative damage induced by drought and salinity using a leaf disc incubation experiment in transgenic and WT lines. Sodium chloride 100 mM deteriorated WT lines, with the discs exhibiting discoloration characteristic of reduced chlorophyll a+b concentration (Figure 5 and 6). This finding is consistent with results obtained in transgenic tobacco plants under high drought and salt

treatments overexpressing GmDREB3, in which visual loss of chlorophyll in leaf discs and decreased chlorophyll concentration, were observed (Chen et al., 2009). These results confirm a protective relationship between overexpression of *CBF3* and drought and salinity tolerance in leaves. In the absence of *CBF3*, these abiotic conditions induce ionic and osmotic stress, generating ROS such as H_2O_2 that yield oxidative damage (Singla-Pareek et al., 2008; Alvarez-Viveros et al., 2013). In our study, we evaluated the induction of H_2O_2 during a 24 hour incubation with 100 mM of sodium chloride, and demonstrated that WT discs exhibited dark brown coloration compared to translucent light brown transgenic lines (Figure 5 and 6). This evidence suggests that *CBF3* may confer protective effects in regards to ROS generation in plants subject to drought and salinity stress (Kasugga et al., 1999).

In conclusion, our work indicates that overexpression of the transcription factor CBF3 in mexican lemon, a commercial citrus variety, confers increased drought and salinity tolerance, a finding which may support the cultivation of commercial citrus in extreme environments.

Authors and contributors

JLRR conceived the study and designed the experiments with PAJ. JLRR, CIB, MRD, CE and JPM performed the experiments and analyzed the data. JLRR, CIB, FA, PG and PAJ drafted the paper. All authors contributed to the revision of the manuscript and approved the final version.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgment

The authors thank Valeria M. Borjas Mendez for technical assistance. Jesús L. Romero-Romero was supported by PhD fellowship CONACYT (SC392465), COTEBAL: 1865 and CONICYT Doctoral Scholarship for foreigners (63130094). This work was supported by FONDECYT 1150220 to PAJ, CAPES FB-002-2014 to FA and CONICYT PAI 82140040 to JPM. We thank Alyssa Grube for assistance in language support.

Reference

Ali S, Mannan A, El Oirdi M, Waheed A, Mirza B (2012) Agrobacterium-mediated transformation of rough lime (Citrus jambhiri Lush) with yeast HAL2 gene. BMC Research Notes 5: 285-292.

Alvarez-Gerding X, Cortés-Bullemore R, Medina C, Romero-Romero JL, Inostroza-Bacheteau C, Aquea F, Arce-Johnson P (2015a) Improved Salinity Tolerance in *Carrizo Citrange* Rootstock through Overexpression of Glyoxalase System Genes. BioMed Research International 2015:827951

Alvarez-Gerding X, Espinoza C, Inostroza-Bacheteau C and Arce-Johnson P (2015b) Molecular and physiological changes in response to salt stress in *Citrus macrophylla* W plants overexpressing Arabidopsis CBF3/DREB1A. Plant Physiology and Biochemistry 92:71-80.

Álvarez-Viveros M, Inostroza-Blancheteau C, Timmermann T, González M, Arce-Johnson P (2013) Overexpression of *GlyI* and *GlyII* genes in transgenic tomato (*Solanum lycopersicum* Mill.) plants confers salt tolerance by decreasing oxidative stress. Molecular Biology Reports 40: 3281-3290.

Amin F, Davood B (2016) Effect of Drought Stress and its Mechanism in Plants. International Journal of Life Sciences 10: 1-6.

Avsian-Kretchmer O, Gueta-Dahan Y, Lev-Yadun S, Gollop R, Ben-Hayyim G (2004) The Salt-Stress Signal Transduction Pathway That Activates the *gpx1* Promoter Is Mediated by Intracellular H_2O_2, Different from the Pathway Induced by Extracellular H_2O_2. Plant Physiology 135:1685-1696.

Balfagón Damián, Zandalinas Sara I., Baliño Pablo, Muriach María, Gómez-Cadenas Aurelio (2018). Involvement of ascorbate peroxidase and heat shock proteins on citrus tolerance to combined conditions of drought and high temperatures. Plant Physioloy and Biochemistry 127, 194-199.

Cañon P, Aquea F, Rodríguez-Hoces de la Guardia A, Arce-Johnson P (2013) Functional characterization of *Citrus macrophylla BOR1* as a boron transporter. Physiol Plant. 149:329-339

Carvalho L, Santos S, Velile B, Amancio S (2008) *Solanum lycopersicon* Mill. and *Nicotiana benthamiana* L. under high light show distinct responses to anti-oxidative stress. J Plant Physiol 165:1300-1312.

Cervera M, Ortega C, Navarro A, Navarro L, Peña L (2000) Generation of transgenic citrus plants with the tolerance-to-salinity gene HAL2 from yeast. Journal of Horticultural Science and Biotechnology 75: 26-30.

Cervera M, Pina J, Juarez J, Navarro L, Peña L (1998) Agrobacterium-mediated transformation of citrange: factors affecting transformation and regeneration. Plant Cell Rep 18: 271-278.

Chen M, Xu Z, Xia L, Li L, Cheng X, Dong J, Wang Q, Ma Y (2009) Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, *GmDREB3* in soybean (*Glycine max L.*). J. Exp. Bot. 60: 121-135.

Cominelli E, Galbiati M, Tonelli C (2010) Transcription factors controlling stomatal movements and drought tolerance. Transcription 1: 41-45.

Dodd I, Ryan A (2016) Whole‐Plant Physiological Responses to Water‐Deficit Stress. In: eLS. John Wiley & Sons Ltd, Chichester. http://www.els.net [doi: 10.1002/9780470015902.a0001298.pub3]

Eherenfeld N, Cañon P, Stange C, Medina C, Arce-Johnson P (2005) Tobamovirus coat protein CPCG induces an HR-like response in sensitive tobacco plants. Mol Cells 19:418–427.

Fu X, Khan E, Hu S, Fan Q, Liu J (2011) Overexpression of the betaine aldehyde dehydrogenase gene from Atriplex hortensis enhances salt tolerance in the transgenic trifoliate orange (Poncirus trifoliata L. Raf.). Environ Exp Bot 74: 106-113.

Gimeno V, Syvertsen J, Nieves M, Simón I, Martínez V, García-Sánchez F (2009). Additional nitrogen fertilization affects salt tolerance of lime trees on different rootstocks. Scientia Horticulturae 121: 298-305.

Govind K, Neha P, Sushma R, Sanjeev K, Major S (2013) Expression of *rd29A::AtDREB1A/CBF3* in tomato alleviates drought-induced oxidative stress by regulating key enzymatic and non-enzymatic antioxidants. Plant Physiology and Biochemistry 69, 90-100.

Gueta-Dahan Y, Yaniv Z, Zilinskas B, Ben-Hayyim G (1997) Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. Planta 203: 460–469.

Hiscox J, Israelstam G (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332–1334.

Hsiao TC (1973) Plant Responses To Water Stress. Annual Review of Plant Physiology and Plant Molecular Biology 24: 519-570.

Jia H, Zhang Y, Xu J, White F, Jones J Wang N. (2017). Genome editing of the disease susceptibility gene CsLOB1 in citrus confers resistance to citrus canker. Plant Biotechnology Journal 15:817–823.

Jung C, Seo J, Han S, Koo Y, Kim C, Song S, Nahm B, Choi Y, Cheong J. (2008). Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. Plant Physiol 146: 623-635.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology 17: 287-291.

Yamaguchi-Shinozaki K, Shinozaki K. (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. The Plant Cell 6: 251-264.

Lenka S, Muthusamy S, Chinnusamy V, Bansal Kailash C. (2018). Ectopic expression of rice *PYL3* enhances cold and drought tolerance in *Arabidopsis thaliana.* Molecular Biotechnology 60:350–361.

López-Climent M, Arbona V, Pérez-Clemente R, Gómez-Cadenas A. (2008). Relationship between salt tolerance and photosynthetic machinery performance in citrus. Environ Exp Bot 62: 176-184.

Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF family transcription factors in plant abiotic stress responses. Biochimica et Biophysica Acta 1819: 86–96.

Molinari H, Marur C, Bespalhok Filho J, Kobayashi A, Pileggi M, Leite J, Pereira L, Vieira L (2004) Osmotic adjustment in transgenic citrus rootstock Carrizo citrange (*Citrus sinensis* Osb. x *Poncirus trifoliate* L. Raf.) overproducing proline. Plant Science 167: 1375-1381.

Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozak K (2012) NAC transcription factors in plant abiotic stress responses. Biochimica et Biophysica Acta 1819: 97–103.

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R (2004) When defense pathways collide. The response of arabidopsis to a combination of drought and heat stress. Plant Physiology 134: 1683–1696.

Sanchez-Ballesta M, Rodrigo M, Lafuente MT, Granell A, Zacarias L (2004) Dehydrin from Citrus, which confers in vitro dehydration and freezing protection activity, is constitutive and highly expressed in the flavedo of fruit but responsive to cold and water stress in leaves. J Agric Food Chem 52: 1950-1957.

Shalhevet J, Levy Y (1990) Citrus trees In: Stewart BA, Nielsen, DR (eds) Irrigation of Agricultural Crops A S A Madison, Wis Mon no 30 p 951-986.

Schewe J, Heinke J, Gerten D, Haddeland I, Arnell N, Clark D, Dankers R, Eisner S, Fekete B, Colon-Gonzalez F, Gosling S, Kim H, Liu X, Masaki Y, Portmann F, Satoh Y, Stacke T, Tang Q, Wada Y, Wisser D, Albrecht T, Frieler K, Piontek F, Warszawski L, Kabat P (2014) Multimodel assessment of water scarcity under climate change. Proceedings of the National Academy of Sciences of the United States of America 111: 3245-3250.

Silva-Ortega C Ochoa-Alfaro A., Reyes-Agüero J, Aguado-Santa Cruz G, Jiménez-Bremont J (2008) Salt stress increases the expression of *p5cs* gene and induces proline accumulation in cactus pear. Plant Physiology and Biochemistry 46:82-92.

Singla-Pareek S, Yadav S, Pareek A, Reddy M, Sopory S (2008) Enhancing salt tolerance in a crop plant by overexpression of glyoxalase II. Transgenic Research 17:171-80.

Taiz L, Zeiger E (2010) Plant Physiology. Sinauer Associates, Inc., Publishers Sunderland, Massachusetts 792 p.

Thordal-Christensen H, Zhang Z, Wei Y, Collinge D (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 11:1187–1194.

Xu M, Li L, Fan Y, Wan J, Wang L (2011) *ZmCBF3* overexpression improves tolerance to abiotic stress in transgenic rice (Oryza sativa) without yield penalti. Plant Cell Rep. 30:1949–1957.

Zhang J (2014) *Lasiodiploidia theobromae* in Citrus fruit (Diploidia Stem-End Rot). Postharvest Decay 309-335.

Figure Legends

Figure 1. **Molecular analysis of the *C. aurantifolia* lines transformed with CBF3.** Expression analysis of CBF3 by qRT-PCR ΔCt method in lines LM2, LM7, LM14, and WT. Bars represent mean of three plants ± SD.

Figure 2. Analysis of stomatal density and stomatal conductance in lines of mexican lemon overexpressing CBF3 a) Stomatal density of leaves in transgenic *C. aurantifolia* lines transformed with CBF3 (LM2, LM7 and LM14) and untransformed plants (WT) under control conditions. b) Stomatal conductance of *C. aurantifolia WT* and transgenic lines LM2, LM7 and LM14. Bars represent means of five plants ± SD. The Tukey test was used to determine significant differences ($p \leq 0.05$) (n=4).

Figure 3. Evaluation of ecophysiological parameters of transgenic lines of *C. aurantifolia* subjected to irrigation cessation. Gas exchange parameters were evaluated in plants subjected to water stress at the beginning (day 0) and end (day 15) of treatment using a system of photosynthesis (IRGA). (a) Stomatal conductance, (b) Net photosynthesis, (c) Transpiration, (d) Water use efficiency. The average ± standard deviation of 5 plants per genotype (n = 5) are shown. Asterisks indicate significant differences with respect to the WT plant on the same day (Two-way ANDEVA and Bonferroni test; * P≤0,05 and ** P≤0,01).

Figure 4. Relative expression analysis of putative CBF3 target genes in response to drought. Transcript abundance was normalized using the housekeeping gene Tubulin. WT and transgenic *C. aurantifolia* LM2, LM7 and LM14 lines were subjected to 15 days of irrigation cessation. The genes *COR47* (cold-regulated), *P5CS* (D1-pyrroline-5-carboxylate synthase), *GOLS* (galactinol synthase), and *SIP1* (raffinose synthase) were analyzed using ΔΔCt method.

Figure 5. Phenotypic analysis of disc leaves of transgenic plants under salinity stress (a). Chlorophyll content (B). Histochemical detection of reactive oxygen species (H_2O_2) in WT and transgenic plants subjected to saline treatment (100 mM

NaCl) for 24 hours.

Figure 6. Biochemical evaluation of tolerance to salinity in mexican lemon overexpressing CBF3. (A) Content of chlorophyll a+b (mg cm^{-2}) and (B) electrical conductivity in transgenic lines and WT plants under saline treatment. (*) indicates significant differences between the lines ($p \leq 0.05$).

Figure 1.

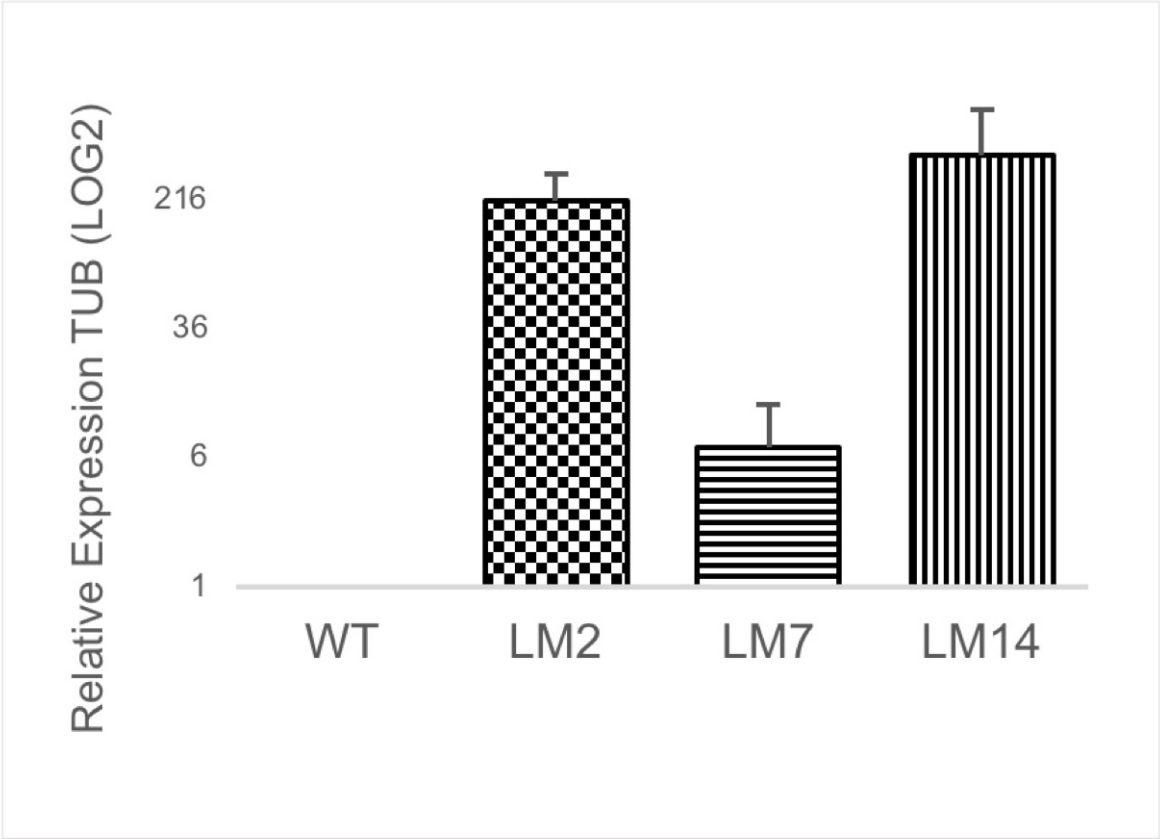


Figure 2.

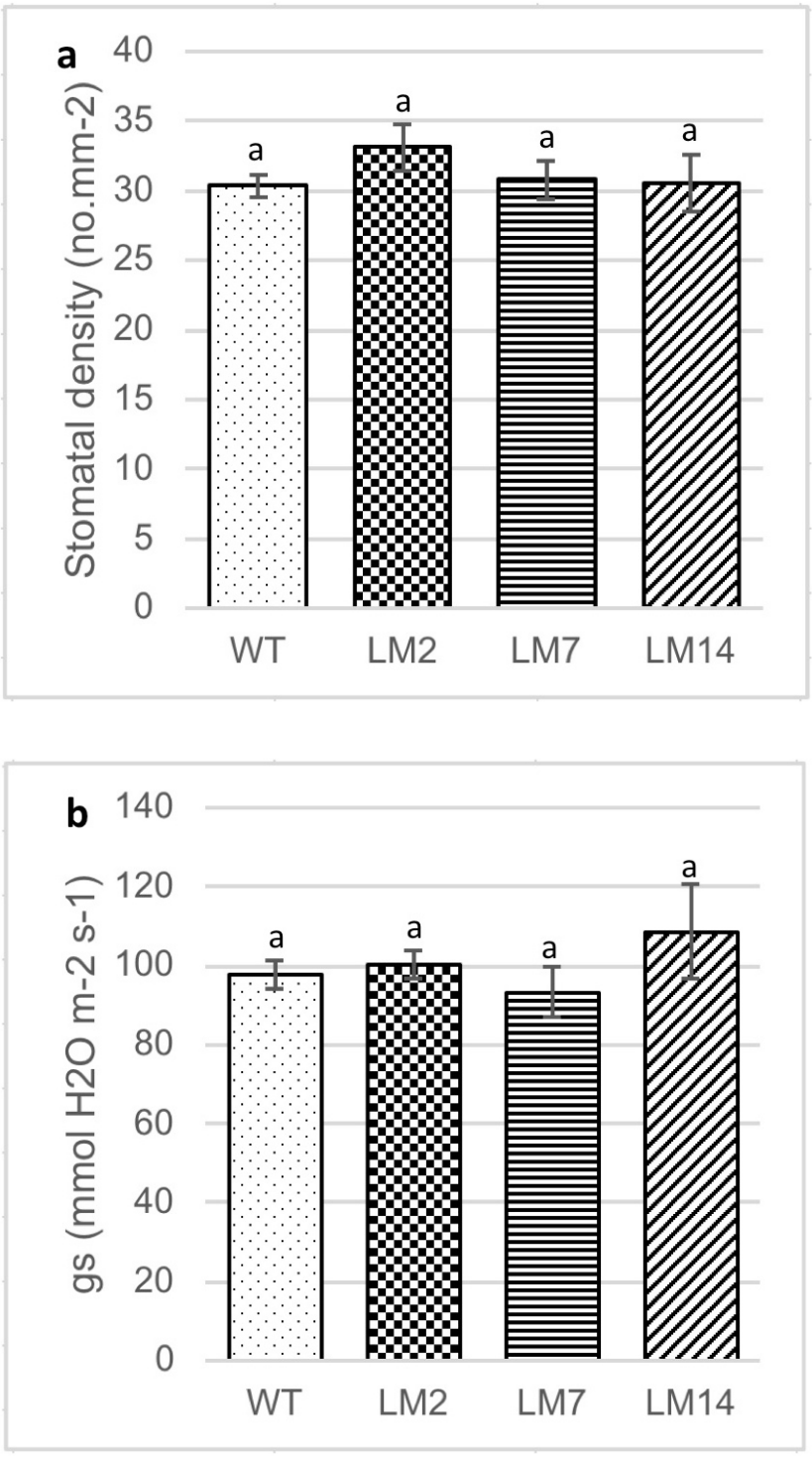

a
Stomatal density (no.mm-2)
40
35
30
25
20
15
10
5
0
a
a
a
a
WT
LM2
LM7
LM14
b
gs (mmol H2O m-2 s-1)
140
120
100
80
60
40
20
0
a
a
a
a
WT
LM2
LM7
LM14

Figure 3.

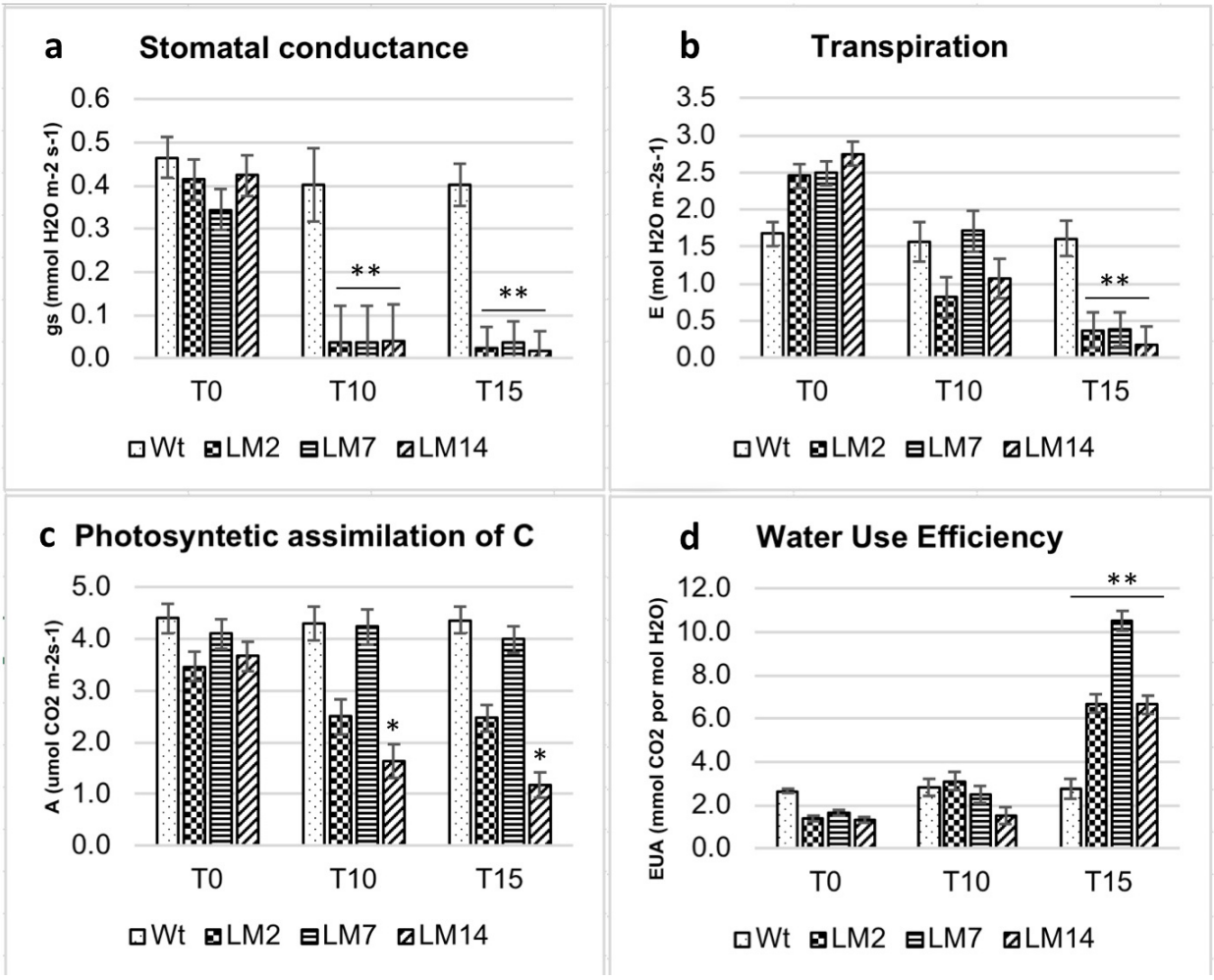

a
Stomatal conductance
gs (mmol H2O m-2 s-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
**
T0
T10
T15
Wt
LM2
LM7
LM14
b
Transpiration
E (mol H2O m-2s-1)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
T0
T10
T15
Wt
LM2
LM7
LM14
c
Photosyntetic assimilation of C
A (umol CO2 m-2s-1)
5.0
4.0
3.0
2.0
1.0
0.0
*
*
T0
T10
T15
Wt
LM2
LM7
LM14
d
Water Use Efficiency
EUA (mmol CO2 por mol H2O)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
**
T0
T10
T15
Wt
LM2
LM7
LM14

Figure 4.

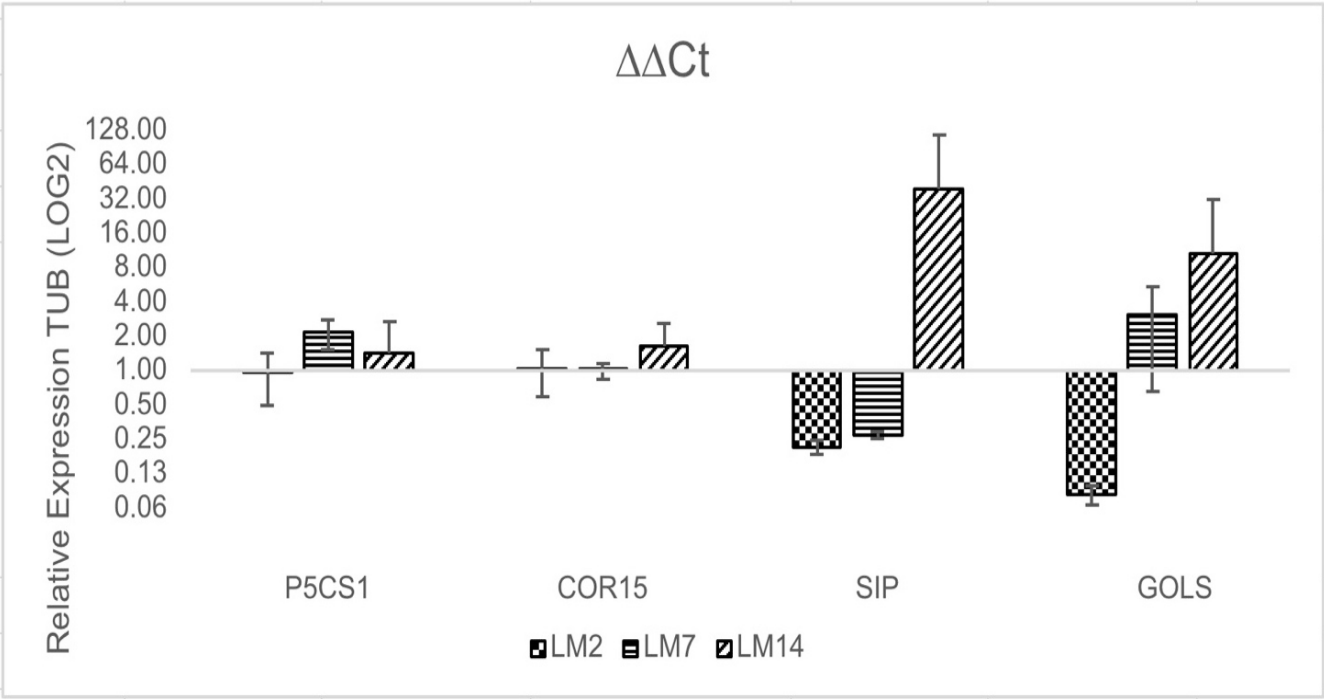

ΔΔCt
Relative Expression TUB (LOG2)
128.00
64.00
32.00
16.00
8.00
4.00
2.00
1.00
0.50
0.25
0.13
0.06
P5CS1
COR15
SIP
GOLS
LM2
LM7
LM14

Figure 5.

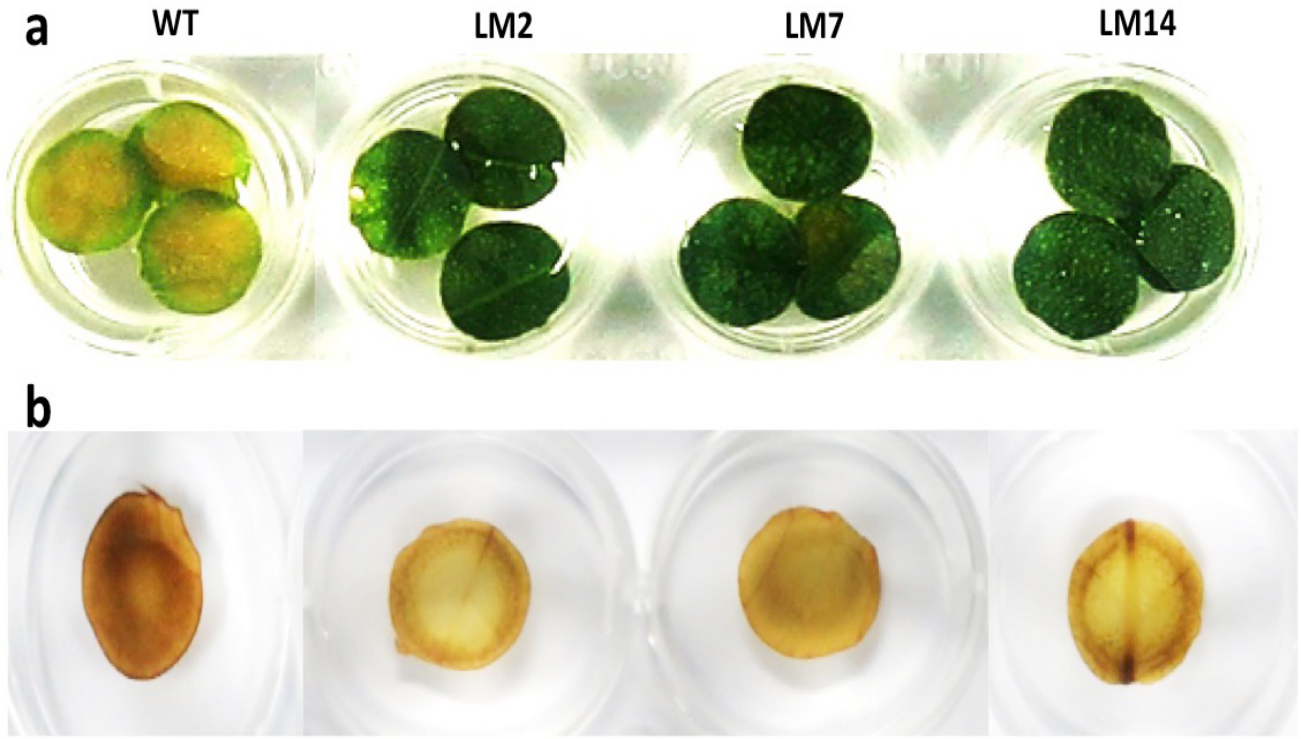


Figure 6.

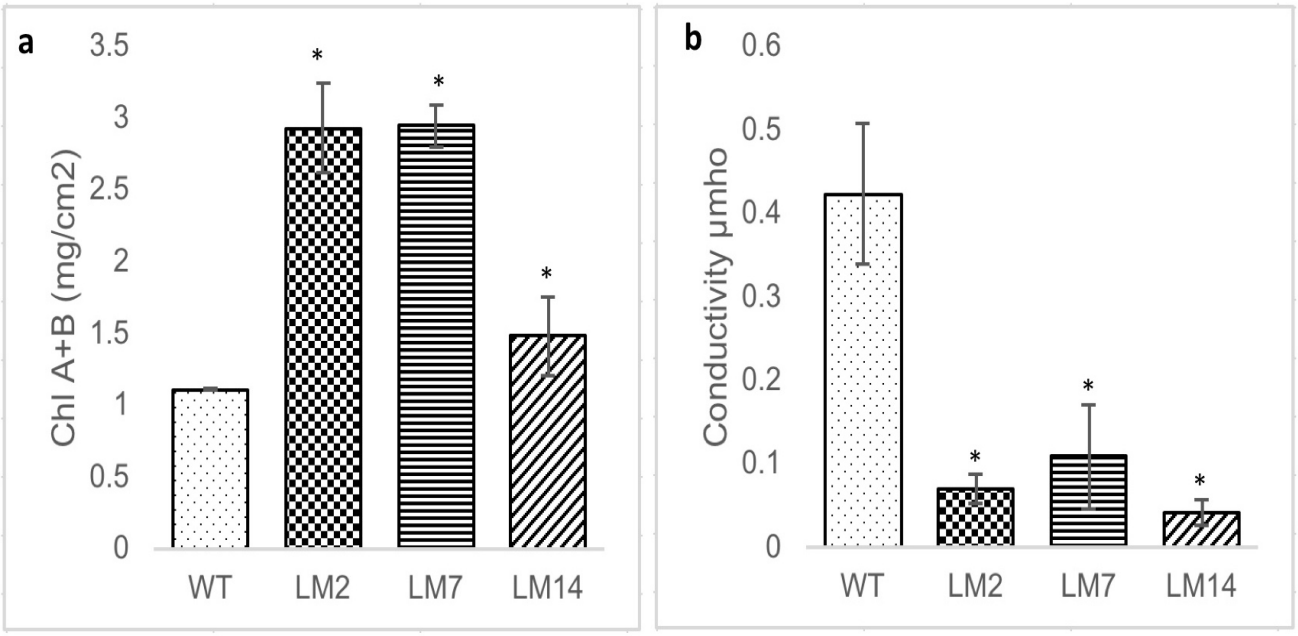

a
Chl A+B (mg/cm2)
3.5
3
2.5
2
1.5
1
0.5
0
WT
LM2
LM7
LM14
*
b
Conductivity µmho
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
LM2
LM7
LM14
*

Table 1. Relative water content (RWC) in lines of mexican lemon overexpressing CBF3 and control plants under water deficit. RWC was analyzed at 0, 10, and 15 days of irrigation cessation.

Average RWC (%)

Line	day 0	Day 10	Decrease	Day 15	Decrease
WT	98,18	89,62	9,56	83,15	15,03
LM2	98,45	94,20	***4,25**	91,80	***6,65**
LM7	98,49	92,63	5,86	90,37	8,12
LM14	97,99	94,32	***3,67**	88,44	9,55

* Indicates significant differences with respect to WT ($p<0,05$)

CHAPTER 3

STOMATA REGULATION BY TISSUE-SPECIFIC EXPRESSION OF THE *CITRUS SINENSIS* MYB61 TRANSCRIPTION FACTOR IMPROVES WATER-USE EFFICIENCY IN ARABIDOPSIS

Jesús L. Romero-Romero, Claudio Inostroza-Blancheteau, Daniela Orellana, Felipe Aquea, Marjorie Reyes-Díaz, Pilar M. Gil, Juan Pablo Matte and Patricio Arce-Johnson

Laboratorio de Biología Molecular y Biotecnología Vegetal, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

Stomata regulation by tissue-specific expression of the *Citrus sinensis* MYB61 transcription factor improves water-use efficiency in Arabidopsis

Jesús L. Romero-Romero[1,2,3], Claudio Inostroza-Blancheteau[4], Daniela Orellana[1], Felipe Aquea[5,6], Marjorie Reyes-Díaz[7,8], Pilar M. Gil[2], Juan Pablo Matte[1] and Patricio Arce-Johnson[1]*

[1]Facultad de Ciencias Biológicas, Departamento de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile, Santiago, Chile.

[2]Facultad de Agronomía e Ingeniería Forestal, Pontifica Universidad Católica de Chile, Santiago, Chile.

[3]Instituto Politécnico Nacional, CIIDIR, Unidad Sinaloa, Departamento de Biotecnología Agrícola, San Joachín, Guasave, Sinaloa, Mexico

[4]Núcleo de Investigación en Producción Alimentaria, Facultad de Recursos Naturales, Departamento de Ciencias Agropecuarias y Acuícolas, , Universidad Católica de Temuco, Temuco, Chile.

[5]Laboratorio de Bioingeniería, Facultad de Ingeniería y Ciencias, Universidad Adolfo Ibáñez, Santiago, Chile

[6]Center for Applied Ecology and Sustainability (CAPES), Santiago, Chile.

[7]Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Temuco, Chile.

[8]Center of Plant, Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus (BIOREN-UFRO), Universidad de La Frontera, Temuco, Chile.

[*]Corresponding author: Patricio Arce-Johnson

E-mail: parce@bio.puc.cl

Keywords**:** Drought tolerance; Abiotic stress; Water use efficiency; Arabidopsis; cisgenic; MYB61 transcription factor; Citrus.

Abstract

Water-use efficiency (WUE) is a quantitative measurement of biomass produced per volume of water transpired by a plant. WUE is an important physiological trait for drought response to mitigate the water deficiency. In this work, a cisgenic construction from *Citrus sinensis* was developed and its function in the improvement of WUE was evaluated in Arabidopsis. Sequences of the *CsMYB61* coding region, a transcription factor implicated in the closure of stomata, together with a putative stomata-specific promoter from *CsMYB15*, were identified and cloned. The protein encoded in the *CsMYB61* locus harbors domains and motifs characteristic of MYB61 proteins. In addition, a 1.2 kb promoter region of the gene *CsMYB15 (pCsMYB15)* containing regulatory elements for expression in guard cells and in response to Abscisic Acid (ABA) and light was isolated. In Arabidopsis, *pCsMYB15* directs the expression of the reporter gene *GUS* in stomata in the presence of light. In addition, transgenic lines expressing the *CsMYB61* coding region under transcriptional control of *pCsMYB15* have a normal phenotype under *in vitro* and greenhouse conditions. These transgenic lines exhibited a smaller opening of the stomata pore, lower stomatal conductance and respiration rate, enhanced sensitivity to exogenous ABA, and high drought stress tolerance. Our results indicate that stomata-specific expression of *CsMYB61* enhances water use efficiency under drought conditions in Arabidospis.

1. Introduction

Regulation of the opening and closing of stomata is a key physiological function for the proper development and survival of plants (Taiz and Zeiger, 2002). Water use efficiency (WUE), which relates biomass generation to water use (Tardieu et al., 2018), is a common metric of plant performance under drought stress. Advances in biotechnological tools have generated plants that consume less water and perform superiorly under water deficit conditions. The use of such engineered plants promises agricultural benefits through reduced water demand and offers the potential for establishment of crops in less exploited arid zones (Cominelli et al. 2010a; Cominelli et al. 2010b). Citruses are one of the most important fruit crops in tropical and subtropical regions, but citrus producing areas are frequently subject to water deficiency, affecting both vegetative and reproductive processes (Vu et al., 1988). Considering that water scarcity will likely increase as a product of global climate change, drought tolerance is a desirable characteristic for citrus sustainability and/or expansion. Among plant strategies for drought stress tolerance, higher regulation of stomata could be a good physiological strategy (Blatt et al., 2017)

Transcription factors that participate in the control of stomatal movements have been identified and characterized. The MYB transcription factors represent one of the largest families in plants and share the MYB DNA binding domain. In Arabidopsis, four transcription factors of the R2R3-MYB family that are involved in the modulation of stomatal movements have been characterized: AtMYB15, AtMYB44, AtMYB61, and AtMYB60 (Cominelli et al. 2005; Liang et al., 2005; Jung et al., 2008; Ding et al., 2009; Galbiati et al., 2011). The first three play a role in

stomatal closure, while MYB60 participates in stomatal opening. Previous studies in our laboratory contributed to characterization of the MYB60 gene in *Vitis vinifera* where its participation in stomatal opening was demonstrated (Galbiati et al., 2011). In contrast, AtMYB61 is expressed specifically in the guard cells where it acts as a transcriptional regulator of stomatal closure, having a role in the regulation of stoma pore size through an ABA-independent pathway and in the light-to-dark transition (Liang et al., 2005). Thermography analysis revealed that *35S:MYB61* plants were approximately 0.5°C warmer than WT plants, whereas *myb61* mutant plants were approximately 0.5°C cooler than WT plants. This suggests that MYB61 alone is necessary and sufficient for the partial closure of stomata (Liang et al., 2005).

Transcription factors that participate in the regulation of stomatal opening and closing in the citrus genus have not yet been described. Identifying citrus MYB61 homologs is relevant to developing genetic tools for WUE improvement in these water intensive fruit crops. In this work, the coding sequence of MYB61 and 1.2 kb of the putative promoter of the MYB15 – a light- and ABA- inducible gene – from *Citrus sinensis* was identified and cloned. A cisgenic construction using both sequences was developed and its role in WUE improvement and drought tolerance was evaluated in Arabidopsis plants.

2. Materials and methods

2.1 Plant material

Arabidopsis thaliana (Col-0) were grown under long day conditions (16 h light /8 h dark cycle and 70-80 µmol m^{-2} s^{-1}) on Petri dishes containing 0.5X MS salts, 1% w/v sucrose and 0.7% agar. Fourteen day-old Arabidopsis plants were transferred to pots with sterile substrate (peat:vermiculite 2:1) and irrigated regularly with tap water supplemented with B5 mineral nutrients.

2.2 Cloning of genomic sequences of pCsMYB15 and CsMYB61

The genomic sequences of pCsMYB15 and CsMYB61 were identified using the *Citrus sinensis* Annotation Project (Huazhong Agricultural University) and Phytozome (http://www.phytozome.net/). The promoter region (1242 pb) of *MYB15* from *C. sinensis* was amplified by high fidelity genomic PCR using the specific primers (Table S1). The resulting fragment was inserted by recombination in the vector pKGWFS7.0 that contains the GUS reporter gene. For preparing *MYB61* overexpression lines, *MYB61* cDNA from *C. sinensis* was amplified by RT-PCR with the specific primers (Table S1). In the same way, the terminator of MYB61 from *C. sinesis* (1000 bp fragment) was amplified by high fidelity genomic PCR using the specific primers (Table S1). The resulting fragments were cloned into the plasmid vector pMF1. The correct assembly of the different sequences (promoter MYB15, CDS MYB61, and terminator MYB61) was confirmed by sequencing of DNA. The two constructions (pKGWF7:pCsMYB15: GUS and pMF1:pCsMYB15: CsMYB61) were used to transform Arabidopsis using the floral dip method (Clough and Bent,

1998). Homozygous transgenic lines were selected in the T3 generation and used in subsequent experiments.

2.3 Quantitative RT-PCR analyses

RNA was extracted as described by (Cañon et al., 2013). The real-time PCR analysis was performed using SYBR Green Master Reagent Kit (Agilent Technologies, Santa Clara, USA). The CLATHRIN gene was used as internal control. The PCR reaction used 40 cycles with three biological replicates. The primers qCsMYB61-Fwd and qCsMYB61-Rev (Table S1) were used to analyze the expression of *CsMYB61*.

2.4 Histochemical staining analysis

The expression of pCs*MYB15* in Arabidopsis vegetative organs was investigated by analyzing GUS staining signals in four independent promoter::GUS reporter lines in light and dark conditions, as described by (Aquea et al., 2010)

2.5 Measurement of density and stomatal aperture

The abaxial side of mature leaves was used to prepare an impression with the application of a dental silicon layer (2 leaves per plant, 3 plants per line, n=3). Once the silicon had dried, the impressions were covered with transparent nail polish. This film was then carefully detached from the leaf, placed on microscope slides and covered with coverslips. Stomata number per 0.001 mm^2 surface per plant was recorded (3 fields per print, 18 fields per line), using a NIKON Eclipse 80ie optic microscope and the stomatal number per mm^2 was calculated. The stomatal aperture was measure placing the detached leaves (from five-week-old plants) in the

stomata opening solution (50 mmol/L KCl, 10 mol/L $CaCl_2$, and 10 mmol/L MES, pH 6.15) for 6 h (three hours in darkness and three hours in a lighted growth chamber [PAR 70-80 µmol m^{-2} s^{-1}] with 95% humidity at 22°C) as described by (Pei et al., 1997). Subsequently, leaves were placed on a slide using water and were observed and photographed in an optical microscope coupled to a digital camera using a total increase of 400X.

2.6 Analysis of pCsMYB15:CsMYB61 plants with ABA and drought stress condition

The *pCsMYB15:CsMYB61* plants were evaluated under ABA and drought treatment using five-week-old plants. Transgenic lines and WT plants were sprayed with 10 µmol/L of ABA, and leaves were harvested after 3 h of incubation and fixed in 2.5% glutaraldehyde (v/v) in sodium cacodylate 0.268M pH 7.2 at 4°C for 3 h. Leaves were subsequently rinsed with sodium cacodylate 0,268M pH 7,2 and dehydrated with increasing concentrations of acetone (50, 70, 95 and 100%, 1 h each immersion). Leaves then underwent a critical point drying and were mounted on an aluminum support for application of a gold bath using the variam/vacuum division equipment (vacuum evaporator PS 100E). The observation was made in 400X and 3000X magnifications in the SEM LEO 1420VP Scanning Electron Microscope. Control samples were collected just before the treatments. A one-way ANOVA and Tukey test with $p \leq 0.05$ were used to compare means. In order to assess potential drought tolerance of *pCsMYB15:CsMYB61* plants, six-week-old WT and transgenic plants were grown in mixed soil (peat and vermiculite 1:1) deprived of water for two weeks and then watered once. The rates of plant survival were recorded one week after

watering. To compare the water loss rates of WT and Cs*MYB61* transgenic lines, the relative water content was determined at 0, 5, 7 and 11 days of treatment by genotype using WT plants as control. For this, leaves were taken from each plant and their fresh weight (FW) was obtained using an analytical balance. Then, the leaves were left in a petri dish floating in distilled water for 4 h at 22°C in a growth chamber, and then reweighed on the analytical balance to obtain their weight in turgor (WT). Finally, the leaves were completely dehydrated in an oven at 60-70°C for at least 48 h, after which their dry weight (DW) was recorded. The RWC was calculated according to the following equation:

$$RWC\ (\%) = [(FW-DW) / (WT-DW)] \times 100$$

2.7 Physiological parameters

Physiological parameters was determined *in vivo* on attached leaves from the second to fourth node of shoots using a portable CO_2 infrared gas analyzer (Licor LR6400, LI-COR Bioscience, Inc., Lincoln, Nebraska, USA) equipped with a cuvette which controlled the light source (300 µmol $m^{-2}s^{-1}$), temperature (20ºC), humidity and CO_2. External CO_2 from air was applied to obtain a reference concentration of 400 ppm with a flow rate of 300 ml min^{-1} and 80% external relative humidity. Net photosynthesis (µmol CO^2 $m^{-2}s^{-1}$), stomatal conductance (gsmol H_2O $m^{-2}s^{-1}$), transpiration rate (E, mmol H_2O $m^{-2}s^{-1}$), and instantaneous water use efficiency (EUA, calculated as A/E, mmol CO_2/mol H_2O) were also determined.

3. Results

a. Tissue-specific expression of putative CsMYB15 promoter

Genetic regulation of stomatal movement depends mainly on an efficient gene expression control system. Previous reports have shown that the promoter of MYB15 is active not only in vegetative and reproductive organs but also in the guard cells of stomata in Arabidopsis during the day (Ding et al., 2009). With the aim to drive specific expression of a transcriptional regulator of stomatal closure, a putative promoter (1.2 kb upstream of the annotated ATG start codon) of MYB15 from *Citrus sinensis* was amplified and cloned (pCsMYB15). The sequence obtained was analyzed in silico and showed at least 10 binding motifs for Dof zinc finger transcription factors ((T/A)AAAG), which play a critical role in guard-cell expression of KST1 promoter activity in potato plants (Plesch et al., 2001). In addition, other elements related to ABA- and light responses are present in pCsMYB15 (Table S2; Figure S1). To monitor the specific activity of this genomic sequence, pCsMYB15 was amplified by PCR and cloned into a GUS reporter vector and transformed into Arabidopsis plants by floral dip. Eight transgenic lines were selected and GUS activity was evaluated during plant development in the presence or absence of light. Figure 1 illustrates that GUS activity is observed in stoma but only in presence of light. The activity of this promoter was not observed in darkness, suggesting that the light-responsive elements identified in the promoter sequence are functional. GUS activity was also observed in roots (data not shown).

3.2 Citrus sinensis MYB61 homologous gene

Selection of a candidate citrus homologue of the AtMYB61 transcription factor was based upon an in silico search in the *Citrus* genome database followed by amplification of the gene by PCR, cloning, and sequencing. A schematic representation of the gene sequence is shown in Figure 2a. The translated amino acid sequence showed 57.2% and 50.3% similarity with AtMYB61 and AaMYB1, respectively (Figure 2a). A phylogenetic tree of MYB61 from different plants species was created, revealing thus homology with trees species (Figure 2b). The exon–intron structure reveals that CsMYB61 has 3 exons and 2 introns. This genetic structure is also present in AtMYB61 and the size of the exons is almost identical in the 2 genes analyzed (Figure 3a). To determine whether the putative transcription factor homologue could control stomata closing during the day, the pCsMYB15 sequence was assembled into a transformation vector upstream of the putative CsMYB61 gene generating the cisgenic construction pMF1-CsMYB61 (Figure S2). Additionally, a stop codon was cloned 1000 bp downstream of CsMYB61 to serve as a transcriptional terminator. Arabidopsis plants were transformed with construction and numerous lines resistant to kanamycin were selected. Nine lines yielded a PCR amplification product of the expected size of approximately 1230 bp corresponding to CsMYB61 (data not shown). The expression level of CsMYB61 was analyzed by qRT-PCR and four of them showed detectable levels, with line L5 demonstrating the highest expression (Figure 3b).

3.3 Functional evaluation of *pCsMYB15:CsMYB61* in Arabidopsis

The ability of these lines to regulate stomata opening and closing under basal conditions was assessed using the print of their stomata in enamel. No significant differences between the WT lines and the nine transgenic lines with CsMYB61 gene were observed with respect to stomatal density (Figure S3). However, the transgenic lines L1, L2, L5 and L6 showed significantly lower opening of their stomata compared to the WT plant (Figure 4A). In these lines stomatal opening ranged from 1 to 1.7 μm compared to the WT plant in which stomata were opened 2.8 μm on average. The stomatal-to-opening ratio with respect to the length of the stomata is significantly lower in the same lines (Figure 4B). To evaluate ABA sensitivity, the selected transgenic plants were grown in optimal conditions under an irrigation regime and treated with foliar application of 10 μm of ABA. Leaves were collected, fixed, and analyzed with a scanning electron microscope 4 hours after treatment. Distilled water was used as control. Both the WT line and the transgenic lines showed lower stomatal opening in response to ABA compared to the control condition. However, stomatal closure was more pronounced in transgenic lines, especially in lines L2 and L6 (Figure 4C). Quantification of this phenotype showed significantly lower opening than the control plant (0.013 μm), ranging from values of 0.004 μm for lines L2 and L6 to 0.007 μm for line L1 (Figure 4D). In addition, the selected transgenic lines showed a decrease in their relative water content (RWC) with respect to controls after 11 days of irrigation cessation (table 1).

In order to evaluate if transgenic plants showed higher WUE, a stop watering assay was performed. Selected lines were grown under optimum conditions of light

and soil moisture (maintained at field capacity) for 35 days, after which transgenic and WT plants (five replicas per line) were subjected to water withholding for 11 days. Net photosynthesis (A_N), stomatal conductance (gs), and transpiration rate (E) were evaluated and the WUE was determined. Stomatal conductance (Figure 5B) and transpiration rate (Figure 5C) were significantly lower in the transgenic line 2 compared to WT. Net photosynthesis showed higher values in transgenic plants after 11 days of irrigation cessation compared to the control plant (Figure 5D). In respect to WUE, the transgenic lines 2 and 5 showed a greater value after 11 days of water withholding (Figure 5E). Rehydration followed this treatment, and the phenotype was recorded after 5 days. The WT plants failed to recover, whereas the transgenic lines expressing CsMYB61 returned to a nearly normal phenotype (Figure 5A). Taken together, our results show that regulation of stomatal opening through the tissue-specific expression of transcription factor CsMYB61 in Arabidopsis significantly increases WUE in water deficient conditions.

4. Discussion

Harnessing transcription factors, which regulate the expression of the stress-responsive genes, represents a powerful strategy to modulate drought resistance and prevent plant productivity loss. In the present study, the expression of *CsMYB61* under transcriptional control of *pCsMYB15* in Arabidopsis significantly increased WUE under water limited conditions. The higher *pCsMYB15:CsMYB61* expression lines exhibited significantly lower opening of their stomata compared to the WT plant and this response is sensitive to ABA application (Figure 4). In addition, these plants showed greater photosynthetic assimilation paired with lower stomatal conductance and transpiration rate (Figure 5), suggesting that this genetic construct has the potential to improve yield under water-limited conditions. The 35S promoter is frequently used to evaluate the function of a gene; however, unusual phenotypes are observed under normal conditions in some cases (Hsieh et al., 2002). In this work, we constructed a stoma-specific *CsMYB61*-expressing vector and generated *pCsMYB15:CsMYB61* transgenic Arabidopsis plants. This promoter was chosen due to its tissue specificity and its activity in light. Analysis of GUS activity under transcriptional control of the CsMYB15 promoter revealed the reporter gene to be expressed predominantly in stomata in presence of light (Figure 1). This pattern of expression agrees with the literature, where AtMYB15 participates in the regulation of stomatal closure (Cominelli et al., 2010b; Ding et al., 2009). CsMYB61 is the *Citrus sinensis* AtMYB61 (At1g09540) homologous gene, which potentially controls resource acquisition and allocation in plant. AtMYB61 expression was both sufficient and necessary to bring about reductions in stomatal aperture with consequent

effects on gas exchange (Liang et al., 2005). The results obtained in this work are consistent with our hypothesis that specific expression of a gene that participates in the closing of stomata normally during the night will contribute to a lower stomatal opening when expressed during the day. Accordingly, our results are consistent with those reported for the constitutive over-expression of AtMYB61 in Arabidopsis in which the opening of the stomata pore was partially reduced (Liang et al., 2005) and of AaMYB1, an orthologous gene to AtMYB61 in *A. annua*, in which stomata were generated with 45% less pore opening compared to the WT plant (Matías-Hernández et al., 2017). In our experiments, the transgenic plants showed increased sensitivity to ABA in comparison to non-transformed plants (Figure 4). As ABA mediates plant response to drought stress, this results suggests that expression of CsMYB61 under transcriptional control of the promoter CsMYB15 should be increased in water limited conditions and induce enhanced stomatal closing, as was observed. In order to evaluate tolerance to drought conditions, a water withholding experiment was performed. The transgenic lines showed less water loss compared to control plants, with a decrease of RWC ranging from 10% to 39% in comparison to WT plants, which RWC decreased by more than 55% (Table 1). This results agrees with the hypothesis that plants with smaller stomatal pores have lower water loss by transpiration, as has already been demonstrated in Arabidopsis for similar conditions (Cominelli et al., 2005; Liang et al., 2005; Galbiati et al., 2011). Similar result happen when barley plants with significantly reduced stomatal density show an improve in drought tolerance without impacting on yield (Hughes et al., 2017)

Plants with smaller stomatal pore openings and decreased transpiration exhibit decreased photosynthesis. In C3 plants such as Arabidopsis, the influx of CO_2 from the atmosphere to the leaf is less than the efflux of water from the leaf into the atmosphere. This is partly due to differences in concentration and the size of CO_2 and water molecules as well as the fact that fixed CO_2 must cross the plasma membrane, the cytoplasm of mesophyll cells, and the chloroplast envelope. However, there are several reports showing that a decrease in stomatal conductance could increase WUE without affecting photosynthesis. Expression of the maize malic enzyme in tobacco plants (Laporte et al., 2002) mutations of the transcription factor GTL1 in Arabidopsis (Yoo et al., 2010) and expression of *AtHXK1* in guard cells in citrus fruits (Lugassi et al., 2015) are examples of how lower stomatal conductance produces an increase in WUE. Therefore, our strategy to increase WUE by decreasing stomatal conductance without significantly affecting photosynthesis is possible as supported by our results and examples in the literature.

In conclusion, our observations regarding transgenic Arabidopsis expressing *CsMYB61* under the *CsMYB15* promoter showed that stoma-specific expression improved WUE. Manipulation of CsMYB61 in citrus could have promising applications in stress management and plant performance. Further experiments are now needed to characterize the effects of *pCsMYB15:CsMYB61* in citrus and evaluate its stomatal regulatory mechanism in a fruit tree.

Authors and contributors

JLRR conceived the study and designed the experiments with PAJ. JLRR, CIB, DO, MRD and JPM performed the experiments and analyzed the data. JLRR, FA, PG and PAJ drafted the paper. All authors contributed to the revision of the manuscript and approved the final version.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgment

The authors thank Valeria M. Borjas Mendez for technical assistance. Jesús L. Romero-Romero was supported by PhD fellowship CONACYT (SC392465), COTEBAL: 1865 and CONICYT Doctoral Scholarship for foreigners (63130094). This work was supported by FONDECYT 1150220 to PAJ, CAPES FB-002-2014 to FA and CONICYT PAI 82140040 to JPM. We thank Alyssa Grube for assistance in language support.

References

Aquea, F., Johnston, A., Cañon, P., Grossniklaus, U., Arce-Johnson, P., 2010. TRAUCO, a Trithorax-group gene homologue, is required for early embryogenesis in Arabidopsis thaliana. Journal Experimental Botany 61, 1215-1224

Blatt, M., Brodribb, T., Torii, K., 2017. Small pores with a big impact. Plant Physiology 174, 467-469

Cañon, P., Aquea, F., Rodríguez-Hoces de la Guardia, A., Arce-Johnson, P., 2013. Functional characterization of Citrus macrophylla BOR1 as a boron transporter. Physiologia Plantarum 149, 329-339.

Clough, S., Bent, A., 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant journal 16, 735-743.

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

Cominelli, E., Galbiati, M. and Tonelli, C., 2010a. Transcription factors controlling stomatal movements and drought tolerance. Transcription 1, 41-45.

Cominelli, E. and Tonelli, C., 2010b. Transgenic crops coping with water scarcity. New Biotechnol 27, 473-477.

Ding, Z., Li, S., An, X., Liu, X., Qin, H. Wang, D., 2009. Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in Arabidopsis thaliana. Journal of genetics and genomics 36, 17-29.

Galbiati, M., Matus, J., Francia, P., Rusconi,F., Canon, P., Medina, C., Conti, L., Cominelli, E., Tonell, C., Arce-Johnson, P., 2011. The grapevine guard cell-related VvMYB60 transcription factor is involved in the regulation of stomatal activity and is differentially expressed in response to ABA and osmotic stress. BMC plant biology 11, 142.

Hsieh, T. Lee, J., Charng, Y., Chan, M., 2002. Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. Plant Physiology 130, 618-626.

Hughes, J., Hepworth, C., Dutton, C., Dunn, J., Hunt, L., Stephens, J., Waugh, R., Cameron, D., Gray, J., 2017. Reducing Stomatal Density in Barley Improves Drought Tolerance without Impacting on Yield. Plant Physiology 174, 776-787

Jung, C.,. Seo, J.,. Han, S., Koo, Y.,Kim, C., Song, S., Nahm, B., Choi, Y., Cheong, J., 2008. Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. Plant Physiology 146, 623-635.

Krens, F., Schaart, J., van der Burgh, A., Tinnenbroek-Capel, I., Groenwold, R., Kodde, L., Broggini, G., Gessler, C., Schouten, H., 2015. Cisgenic apple trees; development, characterization, and performance. Frontiers Plant Science 6, 286.

Laporte, M.,, Shen, B.,. Tarczynski,M., 2002. Engineering for drought avoidance: expression of maize NADP-malic enzyme in tobacco results in altered stomatal function. Journal Experimental Botany 53, 699-705.

Liang, Y., Dubos, C., Dodd, I., Holroyd, G.,Hetherington, A.,Campbell, M., 2005. AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in Arabidopsis thaliana. Current biology 15, 1201-1206.

Lugassi, N., Kelly, G., Fidel,L., Yaniv, Y., Attia,Z., Levi,A., Alchanatis,V.,. Moshelion, M., Raveh,E., Carmi, N., Granot, D., 2015. Expression of Arabidopsis Hexokinase in Citrus Guard Cells Controls Stomatal Aperture and Reduces Transpiration. Frontiers Plant Science 6, 1114.

Matías-Hernández, L., Jia, W., Yang, K., Tang, K., Brodelius P., Pelaz, S., 2017. AaMYB1 and its orthologue AtMYB61 affect terpene metabolism and trichome development in Artemisia annua and Arabidopsis thaliana. Plant Journal 90, 520-534

Pei, Z., Kuchitsu, K., Ward, J., Schwarz, M., Schroeder, J., 1997. Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and abi1 and abi2 mutants. Plant Cell 9, 409-423.

Plesch, G., Ehrhardt, T., Mueller-Roeber, B., 2001. Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression. Plant Journal 28, 455-464.

Taiz, L., Zeiger, E., 2002. Plant physiology. Sunderland, Mass., Sinauer Associates, Publishers.

Tardieu, F., Simonneau, T., Muller B., 2018. The physiological basis of drought tolerance in crops plants: A scenario-dependent probabilistic approach. Annual Review of Plant Biology 69

Vu, J., Yelenosky, G., 1988. Water deficit and associated changes in some photosynthetic parameters in leaves of valencia orange (Citrus sinesis L. Osbeck). Plant Physiology 88, 375-378.

Yoo, C., Pence, H., Jin, J., Miura,K., Gosney,M., Hasegawa P., Mickelbart, M., 2010. The Arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. Plant Cell 22, 4128-4141.

Legend of figures

Figure 1. **Histochemical assays of the expression pattern of GUS under the control of the *CsMYB15* promoter in p*CsMYB15*–GUS transgenic Arabidopsis.** A non-transgenic WT plant was used as a control. Both plants were grown in darkness and light during 24 h before the

histochemical assay.

Figura 2. Sequence and phylogenetic analysis of CsMYB61. A) Alignment of the putative CsMYB61 protein with its homologous proteins from Arabidopsis and Artemisia annua**.** The R2 and R3 domains are marked in green and yellow respectively. The signature of subgroup 13 is marked in blue. Identical amino acids are marked with an asterick. B) Phylogenetic analysis of CsMYB61 and other MYB61 proteins from different species. The amino acid sequences were subjected to Clustal W using the neighbor-joining method in MEGA 7.1. At, Arabidopsis thaliana; Cs, Citrus sinensis; Zm, Zea mays; Aa, Artemisia annua; Ad, Arachis duranensis; Cp, Cucurbita pepo; Pa, Prunus avium; Cc, Citrus clementina; Me, Manihot esculenta; Qs, Quercus suber

Figure 3. A schematic representation of the exon-intron structure of the genomic sequence of AtMYB61 and CsMYB61 and expression in Arabidopsis transgenic lines. **A)** The exons and introns are represented with boxes and black lines, respectively. Numbers above exons refer to their size in nucleotides. The distribution of the R2 and R3 repeats of the DNA binding domain MYB is highlighted in dark gray and light gray, respectively. **B)** The transcript abundance was

normalized to the lowest expression for this gene. Bars represent means of three plants ± standard error.

Figure 4. Stomatal opening analysis in A. thaliana transgenic lines expressing the CsMYB61 transcription factor under water limited conditions. Stomatal response to ABA applications in A. thaliana lines overexpressing CsMYB61. a) Stomatal opening (pore width) determined through optical microscopy complemented with a representative image of the appearance of the stomata in these conditions in WT plants and those overexpressing CsMYB61. b) ratio between the stomatal opening and the length of the stoma for the transgenic lines overexpressing CsMYB61. Asterisks show significant differences with respect to WT. Statistical differences were observed after performing a one-way ANDEVA and Bonfferoni test that compared the lines with respect to WT. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. a) Visualization of the stomata by scanning electron microscopy (SEM) in response to exogenous application of ABA in transgenic lines and WT (3000 X magnification, bar = 30 µm). 100 µm of ABA was used. b) Stomatal opening of WT and transgenic lines of A. thaliana overexpressing CsMYB61. The bars represent the mean ± SD (n=90 stomata per plant) ≤ 0.05.

Figure 5. Physiological parameters of *A. thaliana* transgenic lines expressing the CsMYB61 transcription factor under drought stress treatments at the beginning (day 0) and end (day 11). a) Phenotype of transgenic lines after 11 days of treatment (b) Stomatal conductance, (c) CO_2 assimilation, (d) Transpiration, (e) Instant water use efficiency. The averages ± SD of 5 plants per genotype (n = 5) are shown. Asterisks indicate significant differences with respect to WT in the same days

(one-way ANOVA and Bonferroni test; * P <0.05 and ** P <0.01).

Figura S1. Promoter analysis of CsMYB15. The analysis was performed by PLACE, A Database of Plant Cis-acting Regulatory DNA Elements. Binding sites are represented by the symbols described in the figure

Figura S2. **Schematic representation of cisgenic construction.** The pMF1 plasmid was used as was described by Krens et al., 2015

Figura S3. Stomatal density of Arabidopis WT and transgenic line expressing CsMYB61.

Figure 1.

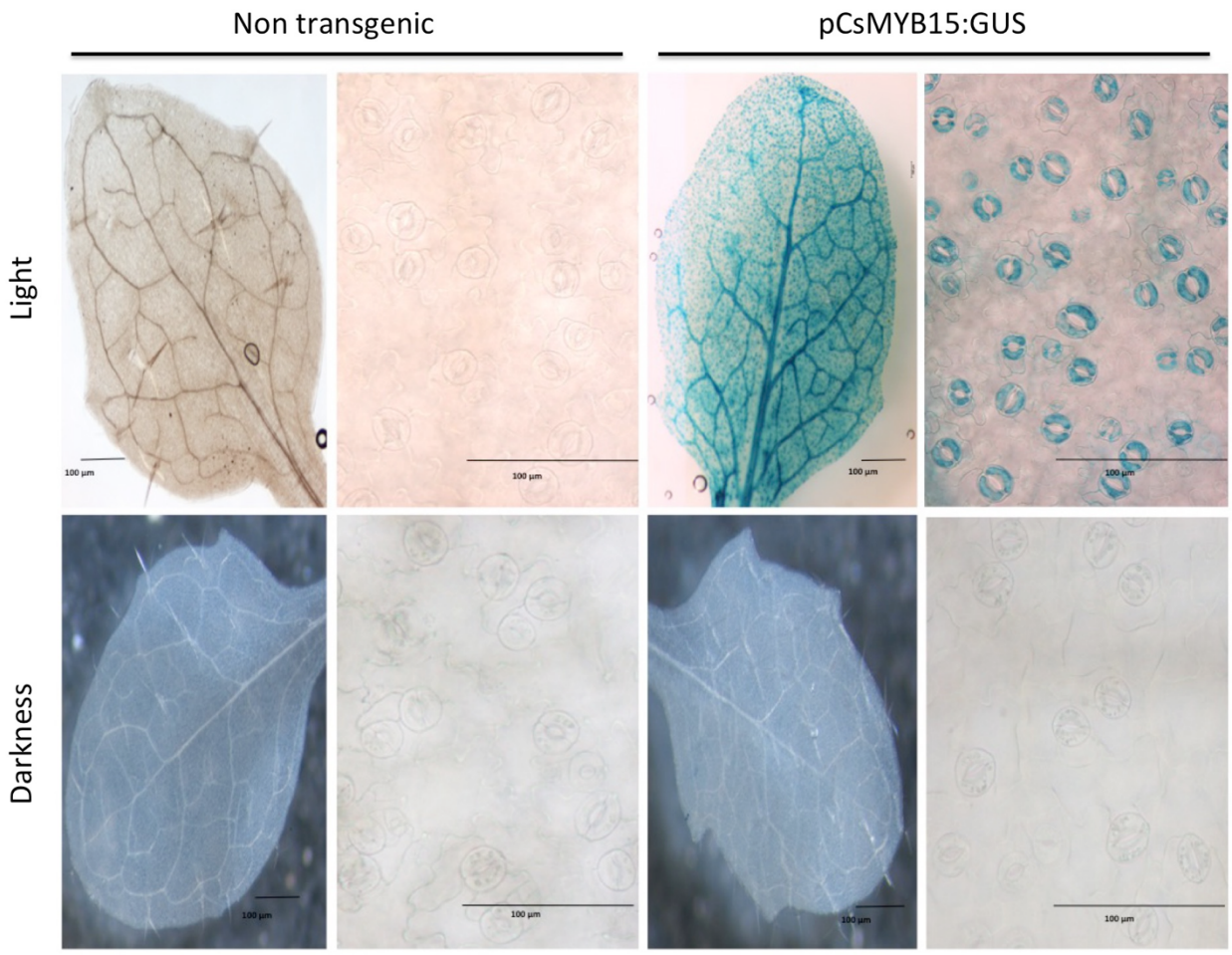
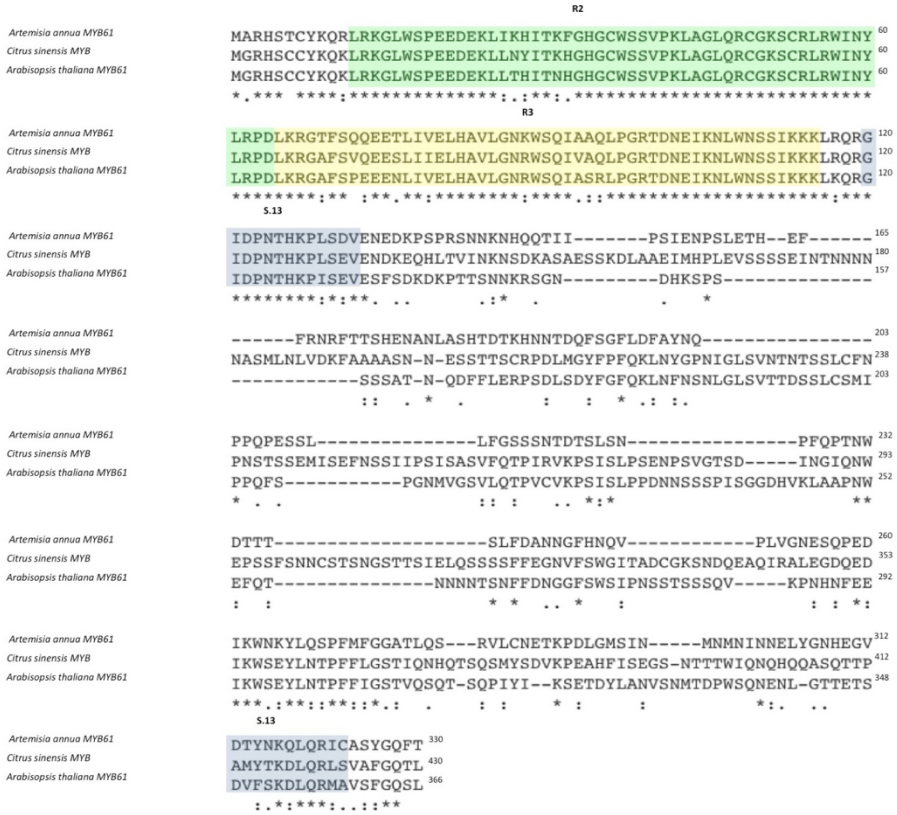


Figure 2.

A


R2
R3
S.13
S.13
Artemisia annua MYB61
Citrus sinensis MYB
Arabisopsis thaliana MYB61

B

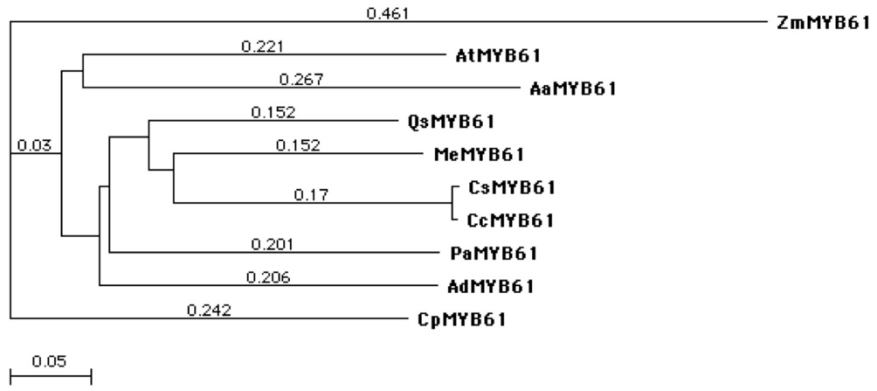

0.461
ZmMYB61
0.221
AtMYB61
0.267
AaMYB61
0.152
QsMYB61
0.03
0.152
MeMYB61
0.17
CsMYB61
CcMYB61
0.201
PaMYB61
0.206
AdMYB61
0.242
CpMYB61
0.05

Figure 3.

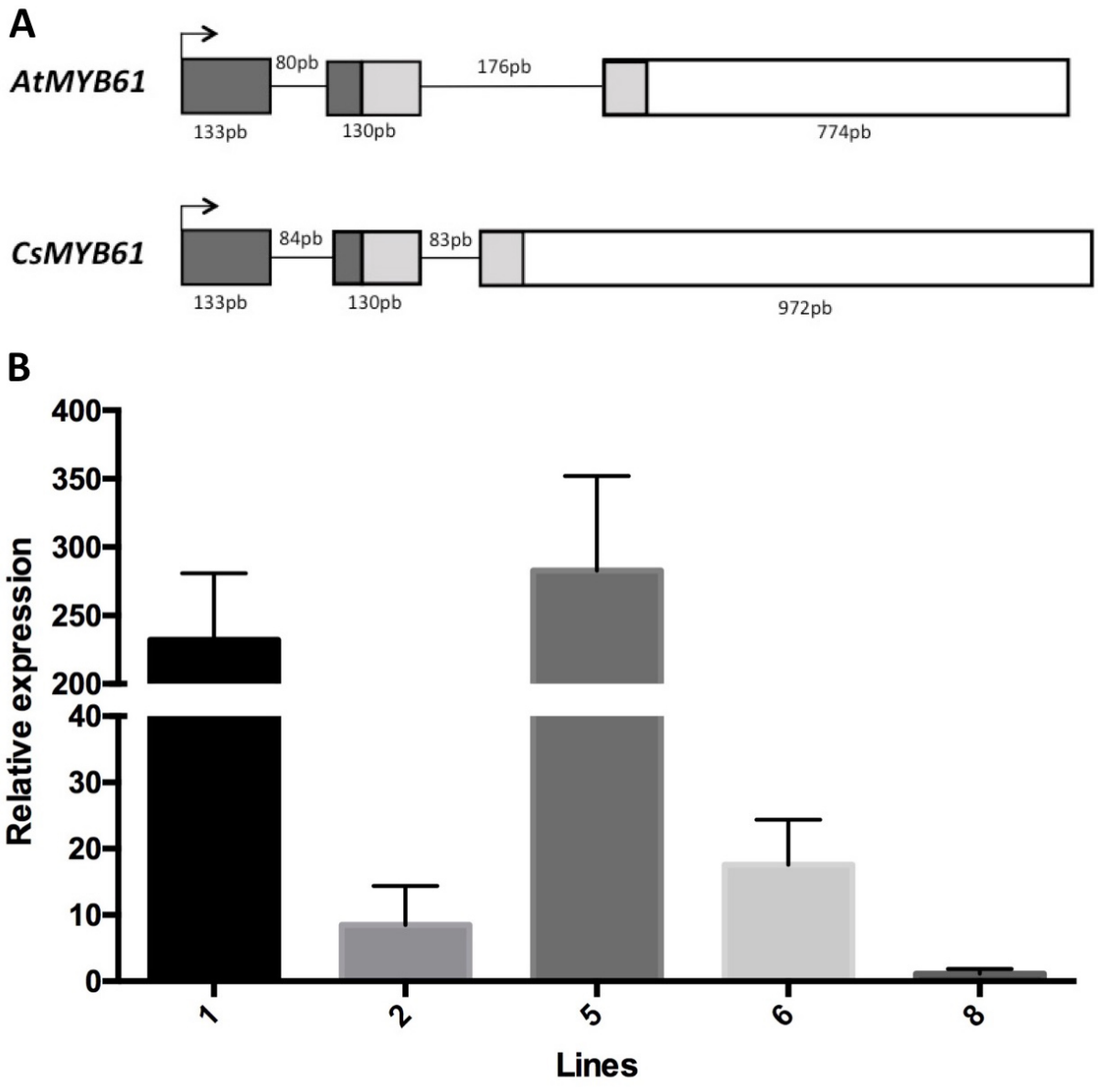

A
AtMYB61
80pb
176pb
133pb
130pb
774pb
CsMYB61
84pb
83pb
133pb
130pb
972pb
B
Relative expression
400
350
300
250
200
40
30
20
10
0
1
2
5
6
8
Lines

Figure 4.

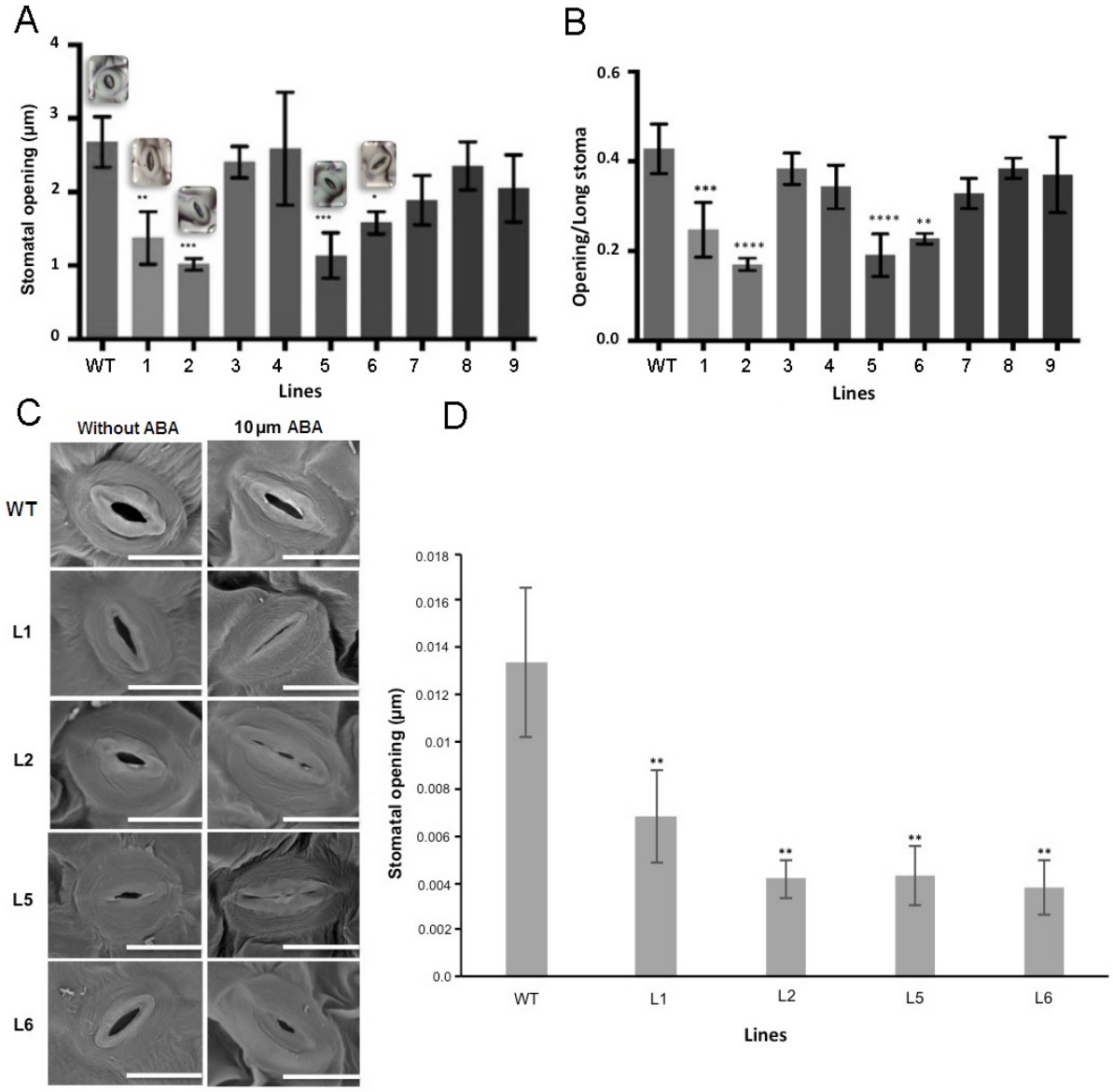

A
Stomatal opening (µm)
Lines
WT 1 2 3 4 5 6 7 8 9
B
Opening/Long stoma
Lines
C
Without ABA
10 µm ABA
WT
L1
L2
L5
L6
D
Stomatal opening (µm)
WT L1 L2 L5 L6
Lines

Figure 5.

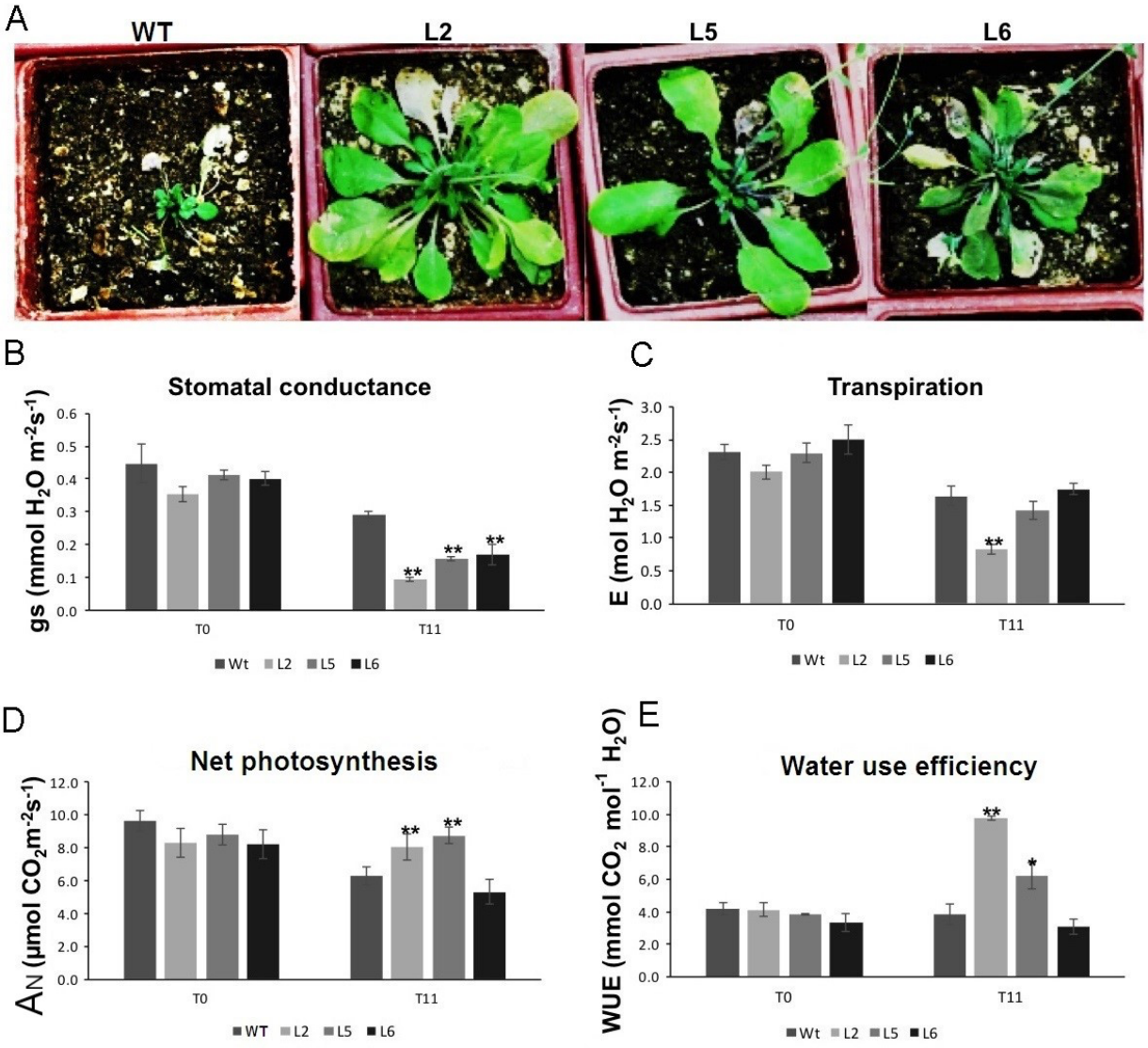

A
WT
L2
L5
L6
B
Stomatal conductance
gs (mmol H2O m-2s-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
T0
T11
Wt L2 L5 L6
C
Transpiration
E (mol H2O m-2s-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
T0
T11
Wt L2 L5 L6
D
Net photosynthesis
AN (µmol CO2m-2s-1)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
T0
T11
WT L2 L5 L6
E
Water use efficiency
WUE (mmol CO2 mol-1 H2O)
12.0
10.0
8.0
6.0
4,0
2.0
0.0
T0
T11
Wt L2 L5 L6

Figure S1.

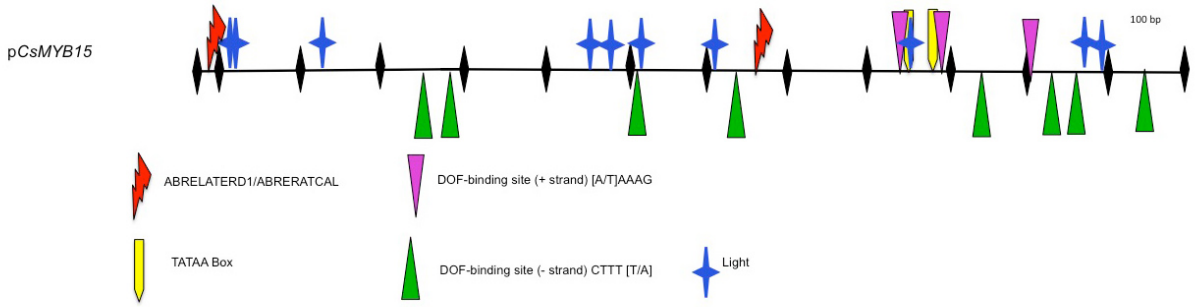

pCsMYB15
100 bp
ABRELATERD1/ABRERATCAL
DOF-binding site (+ strand) [A/T]AAAG
TATAA Box
DOF-binding site (- strand) CTTT [T/A]
Light

Figure S2.

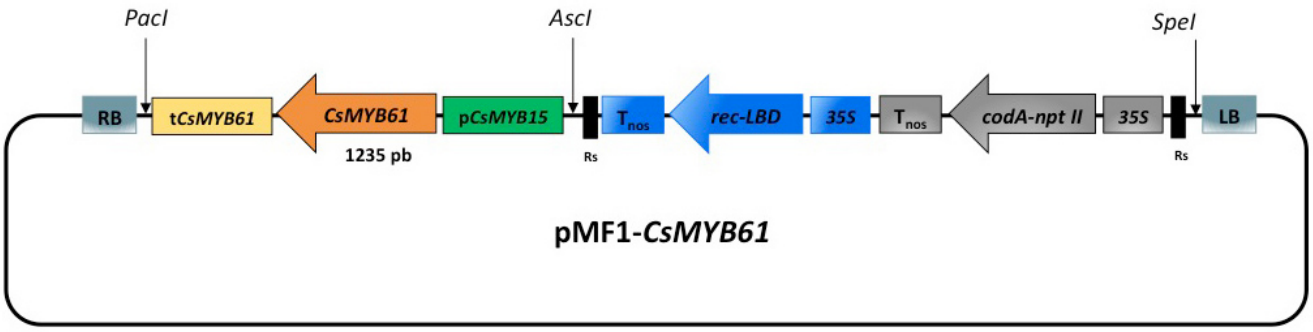

PacI
AscI
SpeI
RB
tCsMYB61
CsMYB61
1235 pb
pCsMYB15
Rs
T nos
rec-LBD
35S
T nos
codA-npt II
35S
Rs
LB
pMF1-CsMYB61

Figure S3.

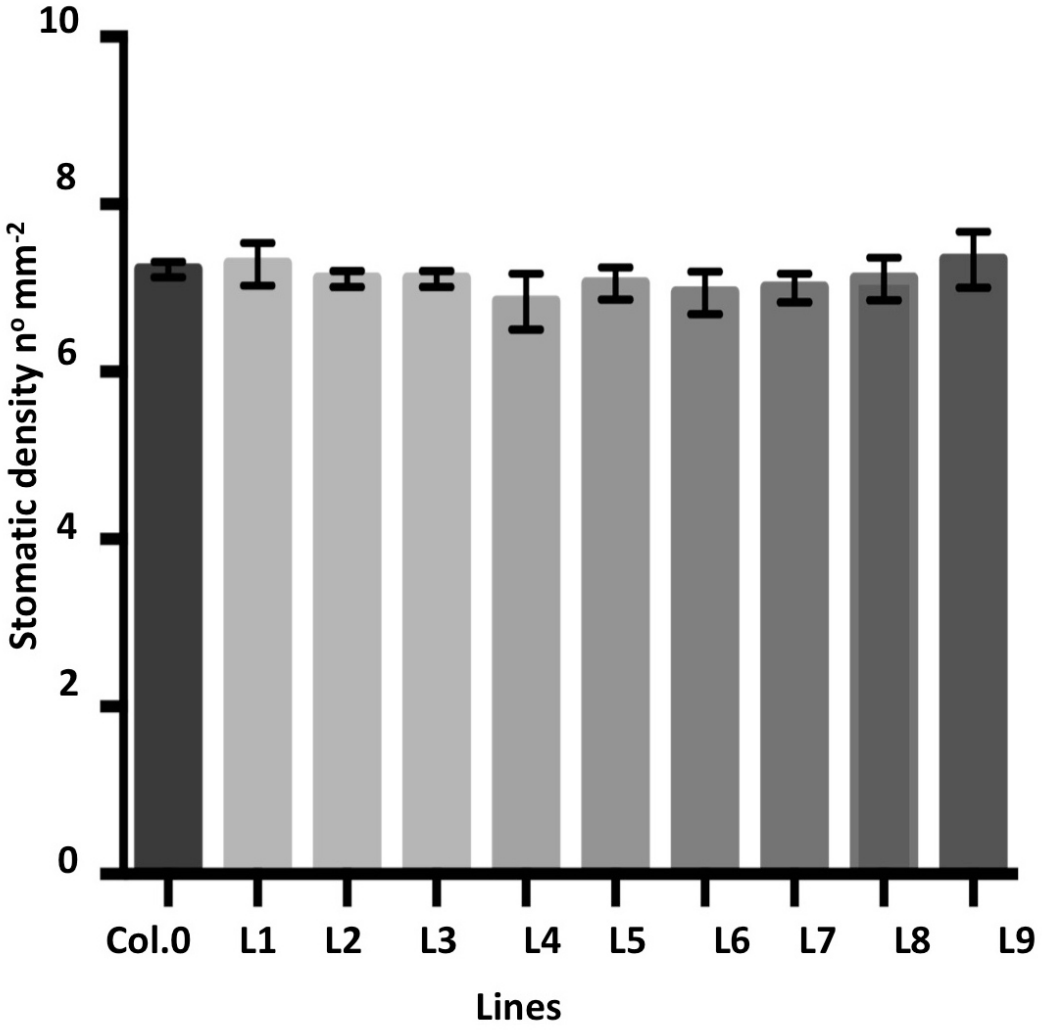

10
8
6
4
2
0
Stomatic density n° mm-2
Col.0
L1
L2
L3
L4
L5
L6
L7
L8
L9
Lines

Table 1. Relative water content (RWC) in transgenic lines with transcription factor *CsMYB61* and wild type plants (WT) under drought stress. Relative water content in the lines subjected to drought stress treatment at 0, 5, 7 and 11d of cessation of irrigation until the water content was invariant.

Line	RWC average (%)		
	Day 0	Day 11	Decrease
WT	95.44	42.62	55.34
L1	95.58	58.13	39.19
L2	93.87	84.36	10.14*
L5	94.29	75.03	20.43*
L6	86.99	52.84	39.26

* Asterisks indicate significant differences with respect to WT plants on the same day. Five plants per line and WT were used

Table S1. **Gene-specific primers used for gene expression analysis in Arabidopsis.**

Gene	Primers	Sequence (5'→3')	Reference
pCsMYB15	Forward	CACCCATTTAATGGACTAATGAACTCCT	This work
	Reverse	C CTTGTTAATTTCTTTCAAACTAGT	
CsMYB61 (cds)	Forward	GTTTAAACATGGGGAGGCATTCTTG	This work
	Reverse	GGTACCCTAAAGGGTTTGTCCAAAAG	
CsMYB61 (terminator)	Forward	GGTACCCTTGTAGAATCAAAAGGAAGA	This work
	Reverse	TTAATTAACTCTTATCCTTGCAATCAATC	
qCsMYB61	Forward	GCCGCTTTCTGAGGTTGA	This work
	Reverse	AGAAGCAGCTGCAGCAAAC	

Table S2. **Putative cis-acting elements of the promoter of CsMYB15**

Cis-element	Position	Sequence	Funtion
ABRELATERD1	1186 (+) 1022 (-)	ACGTG (+) CACGT (-)	ABA-response element
ARE	884 (+)	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction
BOX 4	1086 (+)	ATTAAT	Part of a conserved DNA module involved in light responsiveness
BOX II	1184 (+)	ACGTGGC	Light-responsive element
Sp1	1142 (+)	CC[G/A]CCC	Light-responsive element
Motivo-CATT	87 (+)	GCATTC	Light-responsive element
GATA-BOX	349 (+) 590 (+) 689 (+) 754 (+)	GATA	Light-responsive element
G-BOX	1021 (+)	CACGAC	*cis*-acting regulatory element involved in light responsiveness
Motivo-TCT	361 (+)	TCTTAC	Part of a light-responsive element
Motivo-GT1	717 (+)	GGTTAAT	Light-responsive element
DOF	746 (+) 787 (+) 914 (+) 150 (-) 174 (-) 399 (-) 528 (-) 831 (-) 841 (-) 930 (-)	AAAAG (+) CTTTT (-)	guard-cell expression element

CHAPTER 4

MODEL AND CONCLUSIONS

In this thesis work it was demonstrated that the manipulation of transcription factors type CBF3 and MYB61 allow the plant to increase the tolerance to water and saline stress and increase its efficiency in the use of water (WUE). The specific stoma expression of the transcription factor CsMYB61 low in transcriptional control of the pCsMYB15 promoter whose expression is day and mediated by ABA allowed the partial closure of the stoma. This affected stomatal conductance (gs), trasnpiracion (E) without affecting photosynthesis (A) which resulted in an increase in WUE.

The following model proposes a possible control of the stomata regulation mediated by the transcription factors of the MYB family. It is shown that an important stimulus of stomatal regulation is light that induces the FT MYB60 wich induce the opening of stoma. On the other hand, darkness (absence of light) stimulates CsMYB61 closing the stoma. Additionally, water stress, another important stimulus of stomatal regulation, induces the synthesis of the ABA hormone, which stimulates MYB44 and MYB15 closing the stoma.

The construction developed in this thesis (pCsMYB15 :: CsMYB61) allowed for the first time to demonstrate that under light conditions the stoma could be partially closed under conditions of water stress and thereby improve the WUE in the plants.

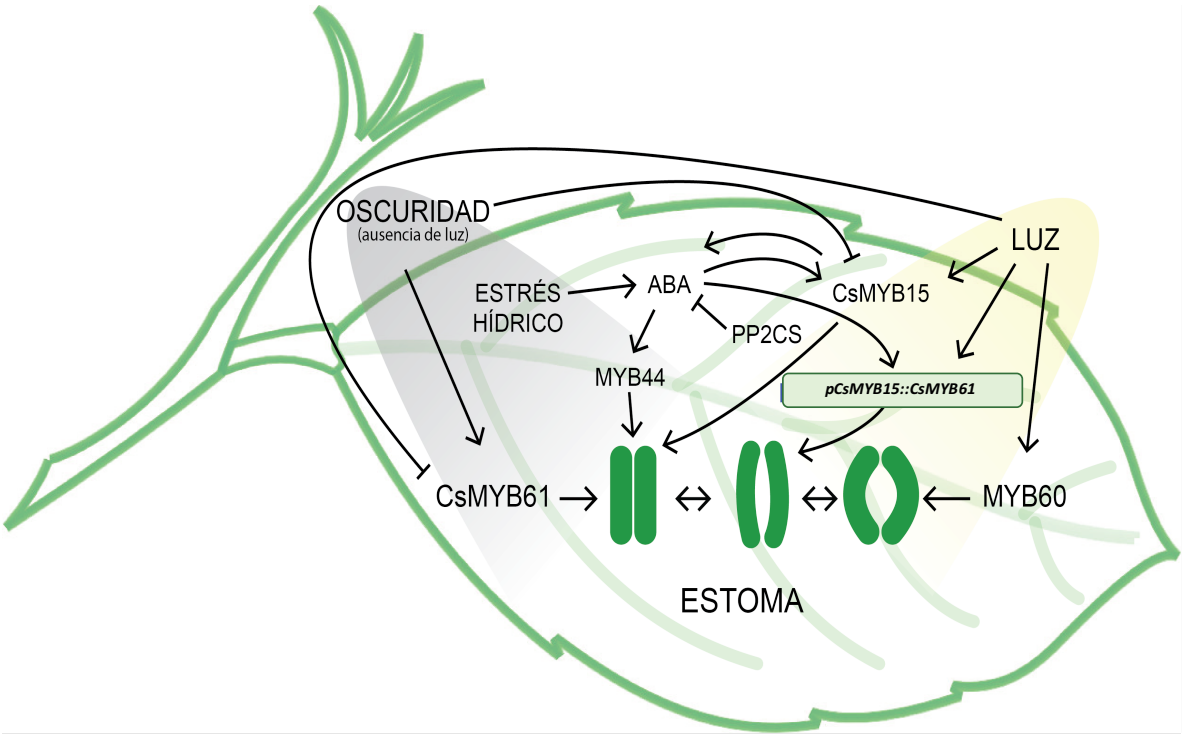


Figure 1. Model of the regulation of stomatal opening in plants by the family of the transcription factor MYBs.

Las conculsiones especificas que resultan de esta tesis son las siguientes:

It is possible increase WUE and the tolerance to salinity in the model plant *Arabidopsis thaliana* and in plants of agricultural interest such as *Citrus aurantifolia* (lemon) through expression of the transcription factor CBF3.

Stomatal specific expression of CsMYB61 in guard cells by CsMYB15 promoter in arabidopsis plants, reduces stomatal opening, conductance, and transpiration without affecting negatively photosynthesis.

It is possible to increase WUE in citrus and arabidopsis plants by manipulating gene expression associated with stomatal opening and closing.

The resolution of the main goal of the thesis through the set of results obtained allowed to demonstrate the hypothesis.