Molecular approaches to characterize and improve abiotic stress tolerance in broccoli and other crops

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Acknowledgements

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Abstract/Resumen/Resum

Abstract

Climate change has increased the exposure of crops to stresses like drought and salinity and, thus, it has a negative impact on plant performance and crop yield in a world with an increasing population. This threatens food security. There is an interest in developing new varieties and cultivars of crops with enhanced tolerance to abiotic stresses. With this aim, we developed an approach in crops with high economic and nutritional values, such as broccoli and melon, to identify physiological and biochemical traits that could be limiting factors for abiotic stress and, thus are likely biotechnological targets for improving abiotic stress tolerance or may be useful for predicting tolerance of uncharacterized varieties.

In the present thesis, we used physiology and metabolomics to identify as distinctive traits for drought stress tolerance in broccoli, net photosynthesis, water use efficiency, stomatal conductance, abscisic acid, metabolites related to sulfur metabolism and other molecules such as urea, quinic acid and gluconic acid lactone. On the other hand, in melon, we found water potential and several amino acids as serine, phenylalanine, glycine, isoleucine, asparagine and tryptophan to be differential traits among tolerant and sensitive cultivars under drought stress. Regarding salt stress tolerance, we identified Na^+/K^+ ratios as a distinctive trait for both broccoli and melon whereas in broccoli, but not melon, transpiration, and stomatal conductance were useful physiological traits. Concerning biochemical traits, hormones, such as abscisic acid, jasmonic acid and indole-3-acetic acid, metabolites of the Krebs cycle and total content of the related glutathione, cysteine and methionine and proline proved to be indicative of saline stress responses in broccoli. However, in melon, we found only proline, phenylalanine and histidine as a distinctive trait of salt stress tolerance. We also used metabolomic tools to identify that γ-Aminobutyric acid correlates with a better taste in broccoli.

Given the importance of sulfur metabolism in stress tolerance, as well as its link with the nutritional properties of broccoli, we also carried out the characterization of the serine O-acetyltransferase enzymes in broccoli. Among the eight isoforms found in this species, our data suggest that the most promising biotechnological targets for enhancing stress tolerance are the BoSAT1d and the BoSAT3 paralogues.

Resumen

El cambio climático ha aumentado la exposición de los cultivos a estreses como la sequía o la salinidad y, por tanto, tiene un impacto negativo en el rendimiento de las plantas y los cultivos en un mundo con una población en aumento en el que la seguridad alimentaria está amenazada. Existe un interés en desarrollar variedades y cultivares con mayor tolerancia a estreses abióticos. Con esta finalidad desarrollamos un abordaje, en cultivos con un alto valor económico y nutricional como brócoli y melón, para identificar rasgos fisiológicos y bioquímicos posiblemente limitantes de estrés abiótico y, por tanto, podrían ser dianas biotecnológicas para mejorar la tolerancia a estrés abiótico o que pueden ser útiles para predecir la tolerancia de variedades no caracterizadas previamente.

En la presente tesis, empleamos fisiología y metabolómica para identificar como rasgos distintivos de tolerancia a estrés por sequía en brócoli, la fotosíntesis neta, la eficiencia en el uso del agua, la conductancia estomática, el ácido abscísico, metabolitos relacionados con el metabolismo del azufre y otras moléculas como la urea, el ácido quínico y el ácido glucónico lactona. Por otra parte, en melón encontramos el potencial hídrico y varios aminoácidos como serina, fenilalanina, glicina, isoleucina, asparagina y triptófano como rasgos distintivos entre cultivares tolerantes o sensibles bajo estrés por sequía. Al respecto del estrés salino, identificamos el ratio Na^+/K^+ como rasgo distintivo tanto para brócoli como para melón mientras que sólo en brócoli, la transpiración y la conductancia estomática como rasgos fisiológicos útiles y, en cuanto a rasgos bioquímicos, hormonas como el ácido abscísico, el ácido jasmónico y el ácido indol-3-acético así como metabolitos del ciclo de Krebs y el contenido total de los relacionados glutatión, cisteína y metionina y prolina, se mostraron como indicadores de respuestas a estrés salino en brócoli. Sin embargo, en melón, encontramos prolina, fenilalanina e histidina como rasgo distintivo de tolerancia a estrés salino. También utilizamos herramientas metabolómicas para identificar que el ácido γ-aminobutírico correlaciona con un mejor sabor del brócoli.

Además, dada la importancia del metabolismo del azufre y la tolerancia al estrés, así como su relación con las propiedades nutricionales del brócoli, llevamos a cabo la caracterización de las enzimas serina O-acetiltransferasas de brócoli. La evidencia que

encontramos sugiere que las dianas biotecnológicas más prometedoras para mejorar la tolerancia al estrés son los parálogos BoSAT1d y BoSAT3.

Resum

El canvi climàtic ha augmentat l'exposició dels cultius a estressos com la sequera o la salinitat i, per tant, té un impacte negatiu en el rendiment de les plantes i els cultius en un món amb una població en augment on la seguretat alimentària està amenaçada . Hi ha un interès a desenvolupar varietats i cultivars amb més tolerància a estressos abiòtics. A aquest efecte desenvolupem un abordatge, en cultius amb un alt valor econòmic i nutricional com ara bròquil i meló, per identificar trets fisiològics i bioquímics possiblement limitants d'estrès abiòtic i, per tant, podrien ser dianes biotecnològiques per millorar la tolerància a estrès abiòtic o que poden ser útils per predir la tolerància de varietats no prèviament caracteritzades.

En aquesta tesi, utilitzem fisiologia i metabolòmica per identificar com a trets distintius de tolerància a estrès per sequera en bròquil, la fotosíntesi neta, l'eficiència en l'ús de l'aigua, la conductància estomàtica, l'àcid abscísic, metabòlits relacionats amb el metabolisme del sofre i altres molècules com la urea, l'àcid quínic i l'àcid glucònic lactona. D'altra banda, en meló trobem el potencial hídric i diversos aminoàcids com a serina, fenilalanina, glicina, isoleucina, asparagina i triptòfan com a trets distintius entre cultivars tolerants o sensibles sota estrès per sequera. Pel que fa a l'estrès salí, identifiquem la ràtio Na+/K+ com a tret distintiu tant per a bròquil com per a meló mentre que només en bròquil, la transpiració i la conductància estomàtica com a trets fisiològics útils i, quant a trets bioquímics, hormones com l'àcid abscísic , l'àcid jasmònic i l'àcid indol-3-acètic així com metabòlits del cicle de Krebs i el contingut total dels relacionats glutatió, cisteïna i metionina i prolina, es van mostrar com a indicadors de respostes a estrès salí en bròquil. Tot i això, en meló, trobem prolina, fenilalanina i histidina com a tret distintiu de tolerància a estrès salí. També utilitzem eines metabolòmiques per identificar que l'àcid γ-aminobutíric correlaciona amb un millor sabor del bròquil.

A més, atesa la importància del metabolisme del sofre i la tolerància a l'estrès, així com la seva relació amb les propietats nutricionals del bròquil, duem a terme la caracterització dels enzims serina O-acetiltransferases de bròquil. La seva evidència suggereix que les dianes biotecnològiques més prometedores per millorar la tolerància a l'estrès són els paràlegs BoSAT1d i BoSAT3.

Abbreviations

Abbreviations

Ψ_w: Water potential

A: Photosynthetic rate/adenine

ABA: Abscisic acid

ABRE: Abscisic acid-responsive element

ANOVA: Analysis of variance

APS: Adenosine 5'-phosphosulfate

AREB: ABA-Responsive Element Binding Protein

AVP1: *Arabidopsis thaliana* vacuolar H^+-pyrophosphatase

Bo: *Brassica oleracea*

BSA: Bovine serum albumin

CDPK: Calcium-dependent Protein kinase

C_i: Sub-stomatal concentration of CO_2

CIPK: CBL-interacting protein kinases

Co-A: Coenzyme A

Ct: Cycle threshold

Cv: Cultivar

DAPI: 4'6-diamidino-2-phenylindole

DHJA: Dehydrojasmonic acid

DMSO: Dimethyl sulfoxide

DRE: Dehydrtion responsive element

E: Transpiration

eV: Electronvolt

F: Quantum yield

Fv/Fm: Variable fluorescence/Maximum fluorescence

GABA: γ-aminobutyric acid

GFP: Green fluorescent protein

GMO: Genetically modified organism

G_s: Stomatal conductance

GSH: Reduced glutathione

H^+: Proton

His/H: Histidine

HNO_3: Nitric acid

HKT1: High-Affinity K^+ transporter 1

HSP: Heat shock protein

IAA: Indole-3-acetic acid

JA: Jasmonic acid

LEA: Late embryogenesis abundant

Leu/L: Leucine

LMWS: Low molecular weight sulfhydryls

MAPK: Mitogen-activated protein kinase

MES: 2-(N-morpholino)ethanosulfonic acid

MoClo: Molecular cloning

MRT: Multiple Range Test

MSTFA: N-methyl-N-[trimethylsilyl]trifluoroacetamide

MYA: Million years ago

NADPH: Nicotinamide adenine dinucleotide phosphate

NHX1: Na^+/H^+ antiporter 1

KAT1: K^+ channel in *Arabidopsis thaliana* 1.

OAS: O-acetylserine

OASTL: O-acetylserine-(thio)liase

OD: Optical density

PAPS: 3′-phosphoadenosine-5′-phosphosulfate

PCA: Principal Component Analysis

PSII: Photosystem II

P-value: Probability value

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

RD22BP1: Responsive to Dehydration 22 Binding Protein 1

RIN: RNA integration number

SA: Salicylic acid

SAT: Serine O-acetyltransferase

SE: Standard error

ROS: Reactive oxygen species

SD: Synthetic defined medium

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNF1: Sucrose non-fermenting

SnRK2: Sucrose non-fermenting 1-related protein kinase 2

SOS1: Salt-overlay-sensitive 1

SOS3. Salt-overlay-sensitive 3

TBP1: TATA-box-binding protein 1

TCA: Trichloroacetic acid

TLeaf: Leaf temperature

TOF: Time-of-Flight Mass Spectrometry

TRPV1: Transient Receptor Potential Vanilloid 1

UBQ2: Ubiquitin2

UPLC: Ultra-Performance Liquid Chromatography

WUE: Water use efficiency

WUEinst: Instantaneous water use efficiency

WUEintr: Intrinsic water use efficiency

General introduction

General introduction

1. Stress in plants

Stress in plants could be defined as 'external conditions that adversely affect growth, development and productivity' (Buchanan *et al*., 2000), since plants are impacted by both abiotic and biotic environmental factors across various levels. Beyond affecting plant physiology and triggering resistance responses, these factors can also induce changes in the dynamics of the genome (Molinier *et al*., 2006).

However, the concept of stress itself is relative since a certain environmental situation may be stressful for some species and non-stressful for others. Plants are sessile organisms and, thus, they are not able to escape from stressful conditions, so along millions of years of evolution, they have acquired and developed mechanisms that allow them to live in diverse environments to the point that plants have colonized almost every environment on Earth independently of the altitude or latitude (Dentant, 2018).

Plant stress can be categorized into two main types: abiotic stress and biotic stress. Abiotic stress is caused by environmental factors and can include either physical (such as cold, drought, heat) or chemical stressors (like salt or toxins). Biotic stress, in contrast, arises from exposure to living organisms or viruses (Gull *et al*, 2019).

Plants are under constant assault by biotic agents, such as herbivores, other plants, insects, bacteria, fungi, viruses, viroids or nematodes, which produce not only an ecological, but also an economic impact (Pimentel *et al*., 2001).

The majority of the pathogens invade previously damaged tissues. Once inside the plant, they use several strategies to feed upon them. For example, necrotrophy, which consists of killing the host cell and decomposing the plant tissue (van Kan, 2006), and, biotrophy, in which the infected host plant cells remain alive for several days. However, if there is an initial cycle of biotrophy combined with a final phase of necrotrophy, the pathogen is considered hemibiotrophic (Perfect & Green, 2001).

Any deviation from optimal external conditions, such as an excess or deficit in the chemical or physical environment is considered abiotic stress. Depending on the causal agents, this stress can be classified in various types as drought, salinity, heat, cold, frost,

waterlogging, environmental pollution, deficiency in mineral elements or mechanical stress (caused by wind or compact soil). Among these types of abiotic stresses, drought-caused water stress is the most prevalent in limiting crop productivity (Bray, 2000).

Drought can be defined through a meteorological, agricultural, hydrological or socioeconomic viewpoint. From an agricultural and physiological perspective, drought occurs when the available water for plants in the soil is decreased due to low soil moisture at a certain time. Drought-caused water stress occurs when the transpiration rate from leaf surfaces is higher than the water uptake by roots (Salehi-Lisar *et al*., 2016).

Water deficiency leads to harmful alterations in cellular components. The proper functioning of proteins and biomembranes, which depends on their biologically active conformation, relies on maintaining an intact hydration shell. Severe drought stress can lead to an impairment of amino acid synthesis, protein metabolism, the dark phase of photosynthesis or respiration, and can ultimately disrupt the osmotic equilibrium within the cell (Koyro *et al*, 2012).

Drought stress symptoms appear as a reduction of leaf area and stem length, reduced growth rate and loss of turgor or reduced leaf water potential, as well as by altering the reproductive development of plant and enhancing the abscission of leaves and fruits, leading to the reduction of crop yield (Farooq *et al*., 2009).

Salt stress is the adverse effect of excess minerals such as Na^+ and/or Cl^- (Munns, 2005). It is considered to be one of the most serious limiting factors for crop growth and production, because salinization affects more than 6% of the Earth's land area and approximately 20% of the world's irrigated land (Saeedipour, 2011). Salt stress influences various crucial processes, including germination, growth, photosynthetic pigments and photosynthesis, water relations, nutrient balance, oxidative stress and, thus, yield (Parihar *et al*., 2015).

The impact of salinity on plant growth takes place in two distinct phases. Initially, there is a rapid osmotic phase that hampers the growth of young leaves. This is followed by a

slower ionic phase that accelerates the senescence of mature leaves, among other effects (Parvaiz, & Satyawati, 2008).

Salt stress diminishes water potential and disrupts ion homeostasis, leading to toxicity, since, on one hand, changes in the ionic environment result in alterations of the membrane potential, leading to the damage of biomembranes and, on the other hand, proteins experience negative impacts on their hydration and charge, promoting their precipitation and reducing their activity (Castillo *et al*., 2007). This altered water status initially reduces growth and restricts plant productivity. Ultimately, these detrimental effects manifest themselves at the whole-plant level, resulting in decreased productivity or, in the most severe cases, plant death.

1.2. The general response of plants to stress

Plant stress responses develop through a dynamic process comprising several phases. First of all, an alarm phase takes place, which is concomitant with the activation of response mechanisms. Secondly, there is a resistance phase, which takes place if the cellular metabolism of the plant has adapted to the new conditions allowing the plant to reach a new optimal physiological state in which the plant achieves the maximum degree of resistance. In subsequent steps, there is an exhaustion phase that occurs when stress persists excessively or becomes severe, which can lead to the death of the plant or, if the stress disappears, a final recovery phase, which leads to the establishment of a new homeostasis (Kosová *et al*., 2015).

Plants undergo various morphological, physiological, metabolic and molecular changes in response to environmental challenges, but early sensory components in plants are not well understood yet (Stephan *et al*, 2016). However, the perception of either biotic or abiotic stress triggers a rapid but transient increase in intracellular Ca^{2+} that seems to be associated to calcium channels, pumps and transporters (Knight *et al*, 1997).

When faced with abiotic stress, plants activate mechanisms to reduce water loss through stomatal closure, impacting their photosynthetic capacity by decreasing CO_2 absorption and fixation. Photosystem II (PSII) is particularly sensitive to CO_2 limitations, and the induction of photoinhibition reduces its efficiency, enhancing reactive oxygen species

(ROS) production (Noctor *et al*., 2014). Consequently, metabolic adjustments take place in order to manage water loss and oxidative stress. To integrate environmental stimuli and modulate physiological responses, plants synthesize hormones, such as ABA, that often engage in crosstalk, which, at a molecular level, involves interactions with various transcription factors of stress-responsive genes (Arbona *et al*., 2017). Also, protein turnover and its degree of phosphorylation participate in the regulation of the stress response (Damaris & Yang, 2021).

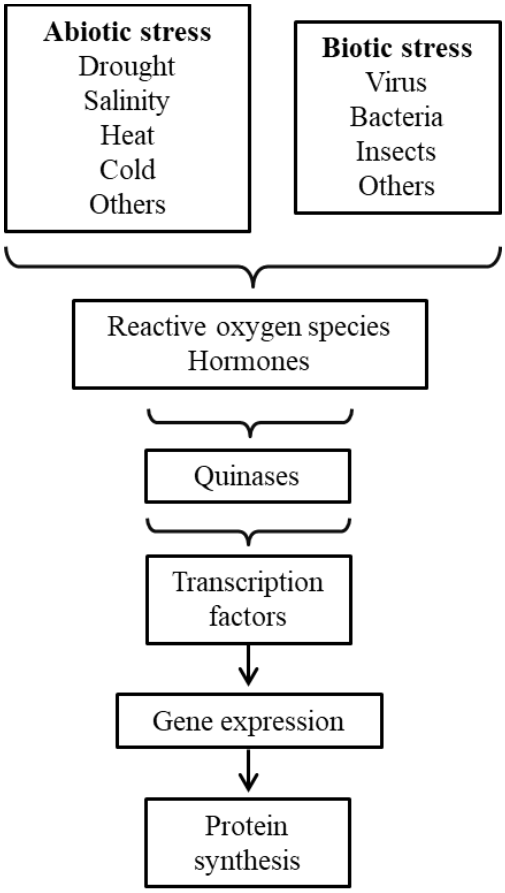

Figure 1. Mechanisms of stress signalling, with particular focus on points of convergence between abiotic and biotic stress (adapted from Azcón-Bieto & Talón, 2008).

1.2.1 Physiological, biochemical and molecular responses to drought stress

In order to respond to drought stress, plants have mechanisms of stress avoidance or stress tolerance. Some species avoid drought stress by conserving water through, for instance, stomatal closure or by alterations in the root/shoot relation. They may also exposure to drought by culminating their life cycle within a window of favourable moisture conditions and producing dormant seeds prior to the arrival of dry seasons. On the other hand, once the water potential is has diminished, plants tolerate the stress carrying out an osmotic adjustment through the accumulation of compatible solutes (Ahanger *et al*., 2014).

Stomatal closure is one of the first responses to water stress. The decrease in stomatal conductance that takes place correlates with less CO_2 assimilation and, thus, the decline in net photosynthesis, whose recovery depends on the capacity to adjust the stomatal and mesophyll conductance (Kumar *et al*., 2018). In many species, drought stress also causes a decline in chlorophyll content (Anjum *et al*., 2011), as well as a reduction of root hydraulic conductivity (Aroca *et al*., 2012).

Regarding molecular responses, regulation can occur at a transcriptional, post-transcriptional and post-translational level, although most studies focus on transcriptional processes (Luo *et al*., 2012). Plants detect when their roots experience water scarcity and communicate this signal to the aerial part by promoting the synthesis of ABA, via the activation of several genes of its biosynthetic pathway. In addition to inducing stomatal closure, ABA also regulates the expression of genes responsible for drought stress tolerance through two types of transcriptions factors: ABRE-binding proteins and DRE-binding proteins. The latter class of transcription factors are named after a 9 bp conserved sequence known as the dehydration responsive element (DRE) (Takahashi *et al*., 2018) and they are activated by SNF1-related kinases. These genes regulate additional transcription factors and signalling-related proteins, as well as LEA (late embryogenesis abundant) proteins (Yoshida *et al.,* 2014), which are extremely hydrophilic proteins whose intracellular accumulation stabilizes other proteins and membranes during drought stress, especially in the presence of sugars like trehalose

(Hand *et al*., 2011). Nevertheless, it should be mentioned that there are several ABA-independent pathways involved in the response to drought stress (Shinozaki *et al*., 2007).

Protein kinases from the calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) families, as well as calcineurin B-like protein-interacting kinases (CIPK), play significant roles in enhancing drought tolerance (Kumar *et al*., 2018). Among one of the ABA-activated protein kinases, SnRK2, phosphorylates the potassium channel KAT1, inhibiting its activity to prevent stomatal opening via K^+ influx (Sato *et al*., 2009).

Another type of proteins that are induced under drought stress are heat shock proteins (HSPs). This class of molecules exhibit robust cytoprotective properties, contributing to cellular stability by preserving proteins in their proper conformations, inhibiting the aggregation of misfolded proteins, facilitating the refolding of denatured proteins, and eliminating detrimental polypeptides that emerge under stressful circumstances (Grigorova *et al*., 2011). Also, there are heat shock factors (HSFs) that bind to promoter regions of HSPs (Pelham, H. R. B., 1982).

As mentioned, one response to drought stress is stomatal closure to reduce transpiration. However, as a consequence of stomatal closure there is a reduction in the CO_2/O_2 ratio within the chloroplast due to the restriction of atmospheric CO_2 diffusion into the plant. Under these conditions, the photo-reduction of O_2 by photosystem I is favoured. As a result of this, ROS, such as superoxide radical, hydrogen peroxide, hydroxyl radical or singlet oxygen are formed. These species cause oxidative damage in the cell, so plants have acquired antioxidant defence mechanisms, which can be enzymatic or non-enzymatic (Hernandez *et al*., 2012). Moreover, the amplification of ROS during stress serves as a warning signal, likely participating both upstream and downstream of ABA-dependent signalling pathways during drought stress (Cruz De Carvalho, 2008).

The Halliwell-Asada pathway is responsible for inactivating the superoxide anion and hydrogen peroxide: the enzyme superoxide dismutase converts the superoxide anion into hydrogen peroxide, which is then degraded by the enzyme ascorbate peroxidase through the reduction of ascorbate to monodehydroascorbate radical. Subsequently, the

enzyme monodehydroascorbate reductase regenerates ascorbate, with reduced glutathione (GSH) serving as the reducing power source used to regenerate ascorbate catalysed by the dihydroascorbate reductase enzyme. The cycle concludes with the action of the glutathione reductase enzyme, which transforms oxidized glutathione into the reduced form by oxidizing NADPH (Avashti *et al*., 2018). On the other hand, ROS attack membrane lipids producing peroxides. In this case, α-tocopherol detoxifies the radicals formed by transforming into α-tocopheroxyl radical and, with the action of ascorbate, α-tocopherol is regenerated. Subsequently, monodehydroascorbate reductase transforms the formed monodehydroascorbate radical into ascorbate (Szarka *et al.*, 2012).

In addition, carotenoids have the important role of preventing the formation of singlet oxygen by the quenching of chlorophyll *a* fluorescence and releasing it as heat (Ramel *et al*., 2012). Thus, the reversible interconversion of the carotenoids violaxanthin and zeaxanthin, the xanthophyll cycle, is a promising target for genetic engineering to enhance stress tolerance. In Arabidopsis, the overexpression of the *ChVDE gene*, that encondes a violaxanthin de-epoxidase led to enhanced photosynthetic, respiration and transpiration rates under drought stress in comparison with the wild type (Sun *et al*., 2019).

In order to maintain turgor pressure and facilitate water absorption under stress, plants try to maintain the internal water potential significantly lower than that of the soil, which is typically achieved by elevating the concentration of cell solutes by its absorption from the soil or by synthesizing compatible solutes in greater quantities (Ahanger *et al*., 2014). The most frequent compatible solutes are proline and glycine-betaine. However, sugars, such as glucose or fructose or amino acids, such as glutamine, are also relevant in their role as compatible osmolytes (Ramakrishna & Singh Gill, 2018).

1.2.2. Physiological, biochemical and molecular responses to salt stress

During the initial osmotic phase, the impact of salinity on water balance becomes pivotal, prompting stomatal closure and impeding leaf expansion. In the subsequent ionic phase, the ion-dependent salinity response emerges, spanning a more extended

period, which implies the accumulation of ions in the shoot, reaching toxic levels and leading to premature leaf aging (Negrão *et al*., 2017).

Under moderate levels of soil salinity, the root has the ability to prevent the absorption of potentially harmful ions and, also, plants avoid injury from salts by either excluding ions from their leaves or compartmentalizing them within vacuoles (Wang *et al*., 2019). When salts are excluded from the leaves, plants utilize organic substances to decrease the osmotic potential of the cytoplasm and vacuole, thereby reducing the leaf water potential. Among these components, there are the aforementioned proline and glycine-betaine (Gupta & Huang, 2014).

The regulation of the ionic homeostasis in salt stress conditions is linked to the rapid increase in intracellular Ca^2, that activates the SOS3 protein of the SOS (salt-overlay-sensitive) pathway, which, in turn, activates the SOS2. The SOS2 protein kinase phosphorylates the SOS1 protein, a Na^+/ H^+ exchanger in the cell membrane. Also, the SOS3-SOS2 complex also inhibits the Na^+ cell membrane-transporter HKT1 and activates NHX1, a Na^+/H^+ antiporter of the tonoplast (Ji *et* al., 2013).

Also, the Ca^{2+} peak activated by salt stress, along with its associated phosphorylation events, appear to promote ABA biosynthesis. Indeed, upon ABA accumulation, this molecule can also upregulate the expression of its biosynthetic genes through the calcium-signaling pathway and can, as well, activate its catabolic enzymes to degrade it. During salt stress, increased ABA signalling could also enhance the up-regulation of MAPKs related to salt tolerance in certain species (Ryu & Cho, 2015). Thus, ABA-regulated Ca^{2+}-related kinases and SnRKs have the potential to adjust salt stress tolerance in some species by influencing gene expression and directly phosphorylating ABA and stress-related transcription factors (Tuteja, 2007). Among other transcription factors as MYC/MYB or RD22BP1, the ABA-dependent salinity stress signalling pathway activates basic leucine zipper transcription factors known as AREB. These factors bind to the ABRE element to induce the stress-responsive gene *RD29A*. Also, the downstream genes of these AREB transcription factors in response to stress include numerous stress-responsive genes. Among them, there are genes that encode LEA class proteins, so that all of them contain ABRE sequences in their promoters (Nakashima, & Yamaguchi-Shinozaki, 2013).

Also, in some species, ABA induces the synthesis of the protein osmotin (Singh *et al*., 1989), which regulates cellular osmolarity through metabolic and structural adaptations or by sequestering solutes into compartments. Transgenic plants overexpressing osmotin exhibited increased leaf expansion, higher chlorophyll levels and relative water content. Therefore, this protein has a role in safeguarding chlorophyll and photosynthetic machinery. It also induces the activation of downstream genes involved in upregulating biosynthetic pathways that lead to the accumulation of proline. (Hakim *et al*., 2018).

Upon salt stress, salicylic acid biosynthesis and concentration are typically significantly increased although it is usually linked to plant biotic stress responses. It is, in fact, documented to engage in significant cross talk with other plant growth regulators such as ABA and it is also linked with proline (Saraf *et al*., 2018). It facilitates ion exclusion and/or compartmentalization, aids in osmotic adjustments, reduces lipid peroxidation, stimulates the synthesis of stress-induced protein kinases and regulates the oxidative system to counteract lethal oxidative events (Singh & Gautam, 2013).

The antioxidant system, comprising both enzymatic and non-enzymatic components, plays a crucial role in neutralizing ROS induced by salinity stress. Salinity tolerance is positively associated with the activity of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, ascorbate peroxidase and glutathione reductase, as well as with the accumulation of non-enzymatic antioxidant compounds, like ascorbate (Sarker & Oba, 2020).

Also, helicase proteins have a relevant function on enhancing or maintaining photosynthesis and antioxidant mechanisms to promote salinity tolerance in plants (Gupta & Huang, 2014). Indeed, the overexpression of the helicase gene *PDH45* conferred tolerance to salt stress in rice improving the photosynthetic and the antioxidant machinery (Gill *et al*., 2013). What's more, when overexpressing in Arabidopsis a DEAD-box RNA helicase gene, *AtRH17*, the enhancement of salt-stress tolerance takes place through a different pathway than the well-known ABA-dependent and ABA-indepent pathways (Nguyen, *et* al., 2018).

2. Climate change and stress in agriculture

Climate change poses a worldwide threat to global food and nutritional security. It has led to an increase in temperature of a rate of over 0.2 ºC per year over the 2013-2022 period. These changes have been attributed to anthropogenic causes (Forster *et al.*, 2023) and they have been associated with an alteration in precipitation patterns (Leisner, 2020), increasing the exposure of crops to drought. Also, in many cases, the water and, therefore, the soil, gets salinized due to excessive irrigation and the increased water demand, which lowers the phreatic level. Taken together, it is clear that climate change has reduced crop yield (Raza *et al*., 2019). Although different regions have different vulnerabilities, the extreme temperatures and low rainfall are the most limiting factors of crop productivity in Southern Europe (Olesen *et. al.*, 2011). Despite this negative impact, it is also crucial to consider that in contrast to salinity and drought, elevated atmospheric CO_2 concentration has opposing effects on plants, given that it enhances photosynthesis and decreases stomatal resistance in C3 plants, thereby increasing water use efficiency. This improvement also reduces photorespiration (Urban, 2003). Also, regarding biotic stress, climate change is predicted to affect the impact of diseases on crops by altering pathogen development, survival rates and host susceptibility (Elad & Pertot, 2014).

This negative impact on plant performance and crop yield is likely to be aggravated in the near future because continued greenhouse gas emissions will intensify the exposure of crops to biotic and abiotic stresses (Dahal *et. al.*, 2019). Therefore, in addition to generating stress-tolerant crop varieties, a number of technological approaches have been developed to counteract the impact of climate change, such as rainwater harvesting, micro-irrigation, crop diversification, raised-bed planting, precision nutrient application, leaf colour charts, crop residue management, zero tillage or crop residue management (Malhi *et al*, 2021).

In addition, the population of the world is increasing, mainly in arid or semiarid areas (Anderson *et al.*, 2020). A probable scenario is that by 2050 world population will reach a number that threatens its nourishment (Bailey-Serres *et al.*, 2019). As a consequence, one of the major challenges of the 21st century is to reduce food security risk and

increase agricultural yield, goals that will become more difficult due to the growing population and global warming (Wollemberg *et al.*, 2016).

The importance of addressing issues related to stress in crops becomes even more evident when considering their economic value. Between 2000 and 2021, global primary crop production increased by 54%, reaching 9.5 billion tonnes, which means an increase of 3.3 billion tonnes since 2000. Cereals, comprising slightly less than one-third of the total production, emerged as the leading crop group in 2021, followed by sugar crops (22%), vegetables and oil crops (12% each). Fruits, along with roots and tubers, each contributed 9-10% to the total production. The value of primary crop production increased at a slightly faster rate compared to the quantities produced, increasing by 57% from USD 1.8 trillion in 2000 to USD 2.8 trillion in 2021 (Figure 2). However, in Europe, this value increased only a 19% and it was the only region in the world in which there was not an increase in the value of primary crop production (FAO, 2023**).**

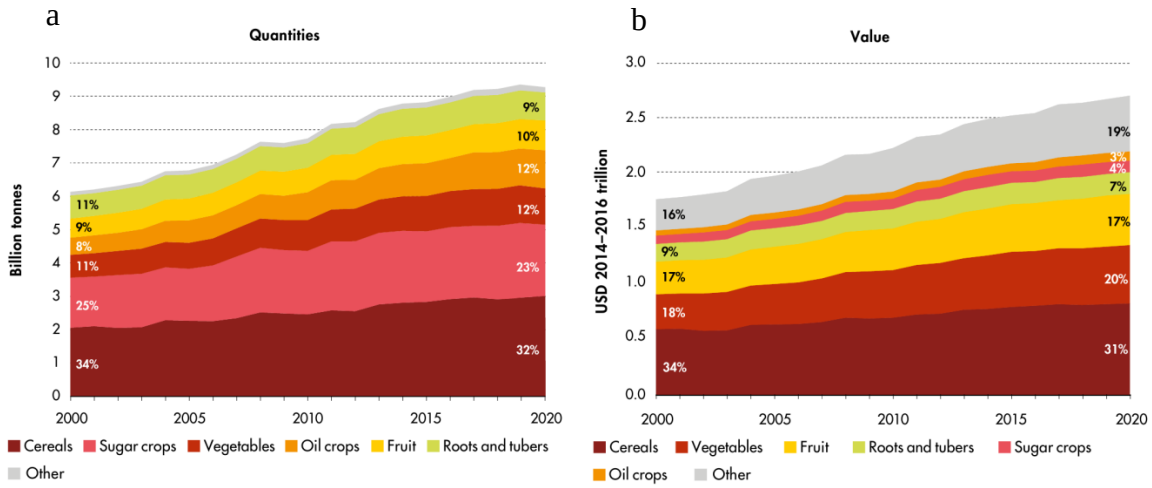

Figure 2. World production of crops in billions of tonnes (a) and its economic value in trillions of USD (b) (FAO, 2023).

3. Strategies for improving stress tolerance to abiotic stress

Numerous genes linked to plant responses to abiotic stresses have been biotechnologically used to produce stress-tolerant plants. One of these approaches is the

overexpression of genes encoding enzymes for the biosynthesis of protective metabolites, thereby demonstrating the potential of transgenic strategies (Marco *et al*, 2015). In contrast, traditional breeding techniques, like mutagenesis and crossing with wild relative species, have been widely used to obtain more productive varieties, but, when aiming to develop materials resilient or tolerant to stress conditions, this approach becomes challenging and often yields limited success (Whelan & Lema, 2015).

Another strategy relies on screening physiological traits for drought and salt tolerance (Forster *et al*., 2000). Contrasting the physiological reactions between tolerant and sensitive varieties or cultivars can serve as a valuable method for anticipating the response of uncharacterized ones prior to their cultivation. Although there are general physiological responses to stress, such as stomatal closure, they can depend on the species. These studies also enable us to determine the physiological characteristics that are regulated in response to salt and drought stress across various populations (Taïbi *et al*., 2017).

Transcriptomics, proteomics and metabolomics, have emerged in recent years to comprehensively explore the biology of organisms. Metabolomics stands out as the most versatile among all 'omics' technologies, as it is very useful for explaining phenotypes, since primary metabolites (sugars or amino acids) and secondary metabolites (antioxidants) reflect the integration of gene expression, protein interactions and various regulatory processes. Therefore, the presence, absence and relative accumulation of specific metabolites along with gene and/or protein expression data, offer precise markers for identifying tolerant crop varieties in breeding programs (Arbona *et al*., 2013).

3. 1. Metabolomics and taste

Metabolomics has gained significant attention for its relationship with sensory evaluation. The chemical properties of food, including flavour (taste and smell) and texture, have garnered increasing interest due to their impact on the sensory experience of food. These types of analyses provide a comprehensive overview of the chemical composition underlying sensory properties (Shu *et al*., 2023). For instance, peptides like aspartame or certain amino acids, such as alanine and leucine, enhance the sweet taste

(Yasuura *et* al, 2014), whereas sour taste is the result of hydrogen ions found in acidic solutions, encompassing organic acids, inorganic acids and acid salts (Fritz *et al*., 2021). Biotechnological approaches involving metabolites and taste have been carried out in crops like tomato, which shows better taste caused by the accumulation of volatile terpenoid compounds through the introduction of a geraniol synthase (Klee, 2010).

Sometimes, taste alone can condition consumer preferences, thereby influencing the market demand (Florkowski, 2014). Also, added sugars and fats are considerably cheaper compared to recommended 'healthy' diets that are focused around lean meats, whole grains, fresh vegetables and fruits. Energy-dense foods, apart from the appeal of good taste and affordability, usually have large portion sizes, all of this likely contributing significantly to overeating and weight gain. Economic disparities in access to healthier food options, contribute to much higher rates of obesity, as well (Drewnowski & Darmon, 2005).

4. Broccoli

Broccoli (*Brassica oleracea* var. *italica* L., n=9), is a crop that belongs to the Brassicaceae family, that includes numerous economically important crops used for food, oilseed or condiments. The diverse wild germoplasm of this family offers an interesting gene pool for breeding, with many wild crucifers serving as valuable model species for research, like *Arabidopsis thaliana* (Warwick, 2011).

Research indicates that broccoli may reduce the risk of cancer due to its rich array of bioactive compounds. These include vitamins C and E, quercetin, kaempferol glycosides, as well as various glucosinolates, such as glucobrassicin and glucoraphanin. Notably, sulphoraphane, derived from glucoraphanin through hydrolysis, appears to be a key bioactive component responsible for much of the anticancer properties of broccoli (Jeffery & Araya 2009).

Over the past decade, broccoli consumption has increased significantly, driven by growing awareness of its abundance in polyphenols, flavonoids, vitamins, minerals and fiber, coupled with its low caloric content. Spain is the fourth largest producer of broccoli world-wide, being China the largest producer followed by India and the United

States (Nagraj *et al*., 2020). In the world in 2022, more than 26 million tonnes of broccoli and cauliflower were produced (FAO, 2023).

5. Melon

Melon (*Cucumis melo* L.) is a diploid eudicot plant species (2n = 24), valued for its unique biological characteristics and economic significance. It is classified within the Cucurbitaceae family (Garcia-Mas *et al*., 2012), that also includes many commercially important crops as cucumbers, pumpkins and watermelons. Melon is the most diverse species of the *Cucumis* genus, presenting considerable diversity in the characteristics of the fruit, such as size, shape, colour, texture, taste and composition (Stepansky *et al.*, 1999). The vegetative growth and reproductive phases of melon plants require significant water uptake, making them susceptible to the detrimental effects of drought stress. These effects have become more prevalent due to climate change and the phenomenon of global warming (Sharma *et al*, 2014).

Melons are not a significant source of proteins, but they are a good source of fiber, minerals, pro-vitamin A and vitamin C, as well as antioxidants like carotenoids and, also, phenolic compounds as flavonoids and alkaloids (Manchali *et al*., 2021). In addition, melon is categorized as a low-sugar fruit due to its sugar and carbohydrate contents. Therefore, its consumption contributes to a healthy diet and, consequently, a high economic value (Lester, G., 1997). Indeed, in 2022, more than 28 million tonnes of cantaloupes and other melons were produced (FAO, 2023). China is the world's first producer with 14,2 million tons in 2022, with Spain ranking 11th with over half a million tons of melons produced (Ritchie *et al*., 2023).

6. Sulfur assimilation in a stress tolerance framework

Sulfur is very abundant, and it is an essential component of the amino acids cysteine and methionine (Giordano & Raven, 2014). The thiol group of cysteine is responsible not only for the antioxidant activity and therefore stress resistance role of glutathione, but it also forms disulfide bridges that determine protein tertiary structure. In addition, cysteine is the precursor of several vitamins, cofactors, glucosinolates and methionine (Romero, *et al.,* 2014). Glucosinolates are molecules whose content is particularly high in broccoli. As mentioned above, these metabolites have been reported to have cancer-

prevention capabilities (Holst *et al.*, 2004) and they contribute to broccoli flavour to some extent as well (Baik *et al.*, 2003).

Sulfur enters plant cells through sulfate transporters. Before it can undergo reduction, it is activated through adenylation to form adenosine 5'-phosphosulfate (APS) by the ATP sulfurylase. Subsequently, APS is reduced to sulfite by a specific reductase, which in turn is reduced to sulfide (Figure 3, Kopriva *et al*., 2009).

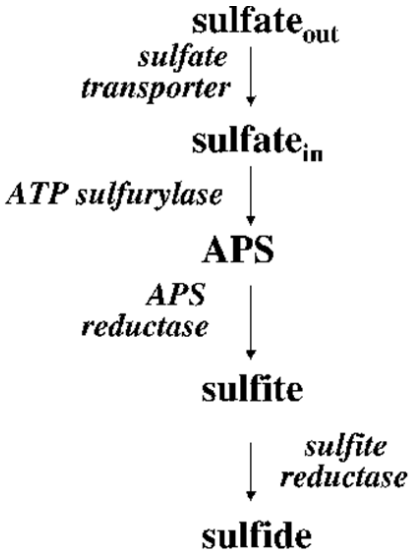

Figure 3. Initial steps of the sulfur assimilation pathway in plants (adapted from Kopriva *et al*., 2009).

This reduced sulfide then participates in the last step of sulfate assimilation, cysteine biosynthesis (Figure 4). As will be discussed in chapter V, the biosynthesis of cysteine has two enzymatic steps. The first step is catalysed by the serine O-acetyltransferase (SAT), which converts L-serine and acetyl-CoA to O-acetylserine. The second and last step is catalysed by the O-acetylserine-(thio)liase (OASTL), in which O-acetylserine is sulfydrylated, resulting in cysteine. O-acetylserine synthesis, catalysed by SAT, is the rate-limiting step, since in the cases studied to date, the OASTL activity is higher than the SAT activity (Figure 5, Heeg *et al*., 2008). The activity of SAT is tightly regulated, including feedback inhibition by cysteine (Olsen, L. R., 2004).

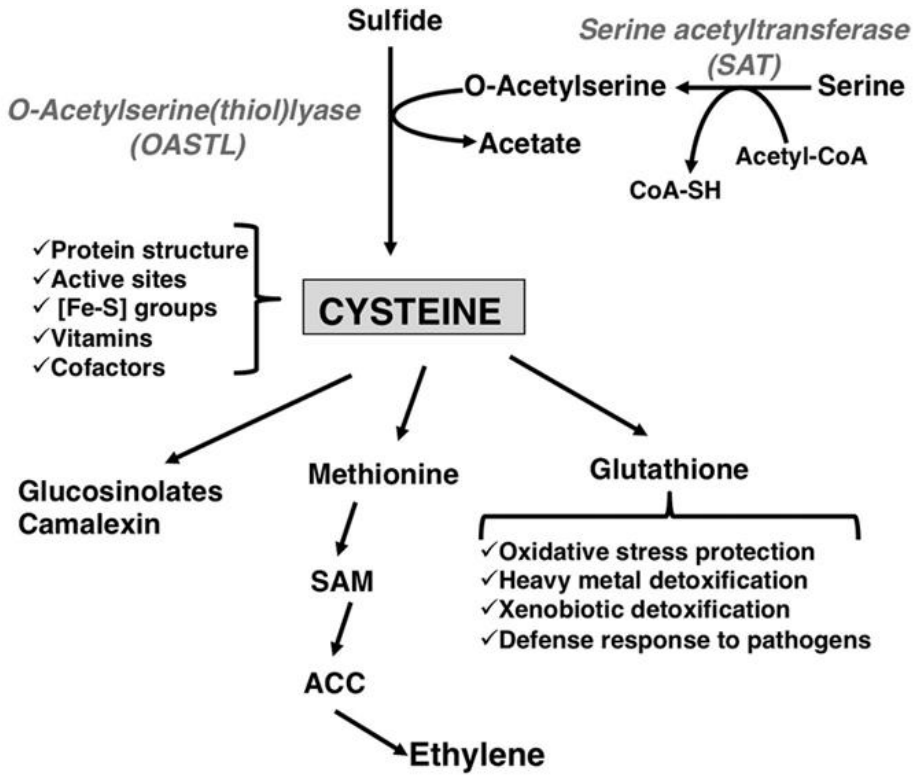

Figure 4. Enzymatic pathways in cysteine biosynthesis and an overview of the functions of cysteine in plant metabolism. (adapted from Romero *et al.*, 2014).

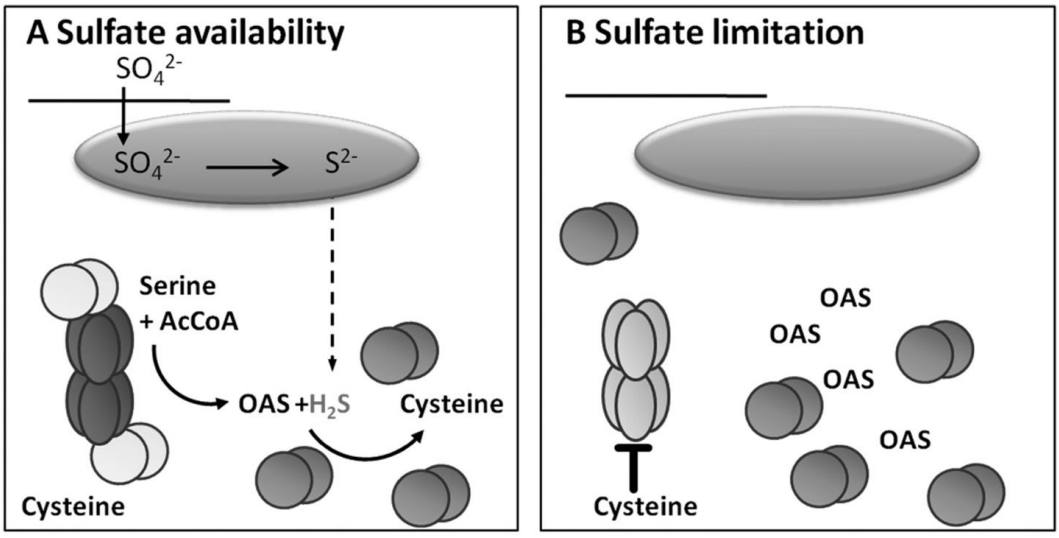

Figure 5. Model for regulatory function of the cysteine synthesis complex under sulfate availability or limitation. Dark ellipses correspond to active SATs and light ellipses correspond to inactive SATs. Dark circles correspond to active OASTLs and light circles correspond to inactive OASTLs (Wirtz *et al.*, 2012).

It has been previously shown that the manipulation of initial steps of the sulfur assimilation pathway can lead to abiotic stress tolerance. For example, the overexpression of ATP sulfurylase in *Brassica juncea* was shown to increase the levels of total thiols and sulfur levels, leading to tolerance to abiotic stressors, such as toxic metals and metalloids (Wangeline *et al*., 2004). Also, in transgenic potato plants expressing a bacterial serine acetyltransferase, an increase of cysteine and glutathione in leaves was observed (Harms *et al.,* 2001). Finally, transgenic tobacco plants overexpressing a serine acetyltransferase increased levels of cysteine and glutathione and showed a marked resistance to oxidative stress (Blaszcyk *et al*., 2003). These examples clearly indicate that characterization of enzymes involved in the sulfur assimilation pathway can lead to the identification of biotechnological targets for stress tolerance in different plant species. Therefore, taking into account the nutritional properties of broccoli and the global context that threatens food security, tackling the sulfur assimilation route on this species in order to obtain stress-resistant varieties has a major interest.

In the present thesis, we aimed to identify distinctive biochemical and physiological traits for abiotic stress tolerance in two species with agronomic interest, melon and broccoli. The underlying idea is to identify factors that are determinant for stress tolerance that can be used to predict the potential tolerance of uncharacterized varieties and could be developed as possible biotechnological targets in the future. Also, considering that broccoli lacks widespread popularity among consumers (Scott & Downey, 2011), we performed an analysis taking advantage of metabolomic tools to select a group of molecules that align with the sensory assessment of various cultivars, suggesting that they could serve as promising targets for broccoli breeding strategies aimed at enhancing taste and, therefore, fostering the purchase of a considered healthy product with the subsequent economic impact. In addition, based on our studies, we also decided to characterize a specific family of broccoli genes as possible biotechnological targets. We chose the serine acetyltransferase enzymes that play a key role in the cysteine synthesis pathway, because of its significant implications in abiotic stress tolerance.

Objectives

Objectives

The main objective of this thesis is the identification of potential biotechnological targets for enhancing abiotic stress tolerance in broccoli and in melon as well as to improve broccoli taste.

1. Identification of physiological and biochemical distinctive traits for drought and salt stress tolerance in broccoli (*Brassica oleracea* L. var. *Italica* Plenck).

2. Identification of physiological and biochemical distinctive traits for drought and salt stress tolerance in melon (*Cucumis melo* L.).

3. Identification of metabolites influencing broccoli taste.

4. Genome-wide and functional characterization of the serine acetyltransferase enzymes in broccoli.

Chapter I

Chapter I

The physiological and molecular characterization of the differential response of broccoli (*Brassica oleracea* var. *Italica*) cultivars reveal limiting factors for broccoli tolerance to drought stress

Sergio Chevilly, Laura Dolz-Edo, José M. López-Nicolás, Luna Morcillo, Alberto Vilagrosa, Lynne Yenush, and José M. Mulet. (2021)
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Abstract

Broccoli is a cruciferous crop rich in health-promoting metabolites. Due to several factors, including anthropogenic global warming, aridity is increasing in many cultivation areas. There is a great demand to characterize the drought response of broccoli and use this knowledge to develop new cultivars able to maintain yield under water constraints. The aim of this study is to characterize the drought response at the physiological and molecular level of different broccoli (*Brassica oleracea* L. var. *Italica* Plenck) cultivars, previously characterized as drought-sensitive or drought-tolerant. This approach aims to identify different traits which can constitute limiting factors for drought stress tolerance in broccoli. For this purpose, we have compared several physiological parameters and the complete profile of amino acids, primary metabolites, hormones and ions of drought tolerant and drought sensitive cultivars under stress and control conditions. We have found that drought-tolerant cultivars presented higher values of methionine, abscisic acid and lower amounts of urea, quinic acid and the gluconic acid lactone. Interestingly, we have also found that drought treatment increases the levels of most essential amino acids in leaves and in florets. Our results have established physiological and molecular traits useful as distinctive markers to predict drought tolerance in broccoli, or which could be reliably used for breeding new cultivars adapted to water scarcity. We have also found that a drought treatment increases the content of essential amino acids in broccoli.

Key words: Broccoli, Drought stress, Plant hormones, Essential amino acids, Primary metabolites.

Introduction

The human population is currently above 7.000 million and is expected to increase to 9.000 million in 2050. To provide a robust food supply for this growing population, agricultural productivity must increase concomitantly. In the current context of anthropogenic global warming, increasing temperatures and CO_2 concentrations in the atmosphere, the precipitation regimes are changing, increasing aridity and limiting the amount of water available for agriculture (Mahajan *et al*., 2005). Drought affects millions of people per year and is considered one of the main causes of famines. Breeding has improved the effectiveness of some crops under drought stress (Sinclair *et al*., 2011). But there is still a need to develop novel cultivars of crops able to grow in arid lands, which will allow for the extension of cultivable lands or increase the productivity of established agricultural soils and thus, increase food production and diminish the water footprint. This is especially urgent in drought-prone areas devoted to the production of horticultural crops, such as the Mediterranean area, California, Florida or South Africa, among many others. Predicted climate change is expected to exacerbate the negative impact of extreme drought events (McDowell *et al*., 2011), moreover drought is one of the main factors driving deforestation in developing countries (Zaveri *et al*., 2020).

There is considerable knowledge regarding the physiology, biochemistry and molecular genetics of plants under drought stress, but turning this knowledge into new cultivars of crops able to maintain yield under drought stress or adapted to arid environments has proven to be very difficult (Ashraf *et al*., 2010). Genetic engineering has shown limited success thus far. Although there are many descriptions in the literature of drought tolerant crops by means of genetic engineering (Hu *et al*., 2014), there are only two cultivars in the market whose trait conferred by the transgene is drought tolerance: the Droughtgard® maize (Wang *et al*¸2015) and very recently the soybean expressing the HB4 transcription factor from sunflower (Ribichich *et al*., 2020). The problem is that in most cases the strategy is a bottom-up approach starting at the molecular level, and thus the selection of the transgene is based on evidence provided by results in other plants or data base mining of results of gene expression during abiotic stress. This approach may fail as it does not detect the limiting factor(s) or because significant tolerance in the field is mediated by many genes with additive effects. To avoid this problem, several

experimental designs have proven to be effective, such as screening for genes in heterologous systems (Serrano *et al*., 2003; Locascio *et al*., 2019). However, to take advantage of this knowledge, new GMO crops must be developed, which is still a problem to market in many countries and requires a long and expensive process of regulation prior to approval.

Here we present an alternative top-down strategy: to use field experience to identify tolerance markers at the molecular level (Sinclair *et al*., 2011). Broccoli (*Brassica oleracea* L. var. *Italica* Plenck) is a major horticultural crop, cultivated in temperate areas. In 2018, the world production of broccoli and cauliflower was about 25 million tones, with China and India as the main producers worldwide, accounting for 70% of the total production. USA is the main producer in the Americas, and Spain is the top producer in Europe (data available in FAO sums up broccoli and cauliflower) (FOASTAT, 2020). Broccoli is also a source of dietary health-promoting molecules (Mukherjee *et al*., 2008; Keck *et al*., 2003; Aghdam *et al*., 2020). Most of these molecules are resistant to standard cooking techniques (Wu*et al*., 2018), therefore broccoli is recommended in most diets. Maintaining the yield of this highly nutritional crop and diminishing the environmental impact on its production is the objective of most breeders. To attain this objective, it is necessary to increase its tolerance to drought stress or at least, decrease the water footprint (Hoekstra *et al*., 2012) of broccoli production.

The comparison of the physiological and molecular responses among drought-tolerant and drought-sensitive populations to identify differential traits has proven to be a useful strategy to characterize the abiotic response of specific crops (Taïbi *et al*., 2016; Souana *et al*., 2020) or even forest species (Taïbi *et al*., 2017; Taïbi *et al*., 2018). To apply this strategy to broccoli, we have identified drought-tolerant and drought-sensitive pre-commercial cultivars of this crop based on field and greenhouse experiments. Then, we have characterized the physiological and molecular responses of these different broccoli cultivars under controlled drought stress, aiming at identifying distinctive traits for drought tolerance. Studies at the molecular level require the use of controlled greenhouse conditions, given that, in the field, there are many variables (such as the presence of pathogens, different light exposure, wound stress caused by strong wind, rain or insect attack or mechanical stimulation) that can differentially affect plants and

therefore generate excess variability in the results. The data generated in the current study may be useful to predict if broccoli cultivars that have not been tested in field trials will be suitable for planting in drought-prone areas, and to breed for novel cultivars with increased amounts of metabolites or physiological traits that are limiting under drought stress. Therefore, this approach constitutes a top-down-top strategy, because the final objective is to transfer the knowledge acquired in the laboratory to the field, by determining which cultivars are going to be more resistant to drought stress based on the physiological, biochemical or metabolomic profile.

Materials and Methods

Plant Material and treatments

This study was performed using four broccoli pre-commercial cultivars, provided by SAKATA Iberica Seeds SLU (Valencia, Spain).. Cultivars were pre-selected among 12 pre-commercial varieties based on their survival and fitness under drought conditions. We confirmed the reproducibility of the results employing controlled drought-stress greenhouse experiments (Supplemental Figure 1). Plants were grown following common procedures reported in the literature for this species. Greenhouse conditions were as follows: 16 h light/8 h dark (200 μmol m^{-2} s^{-1} of light intensity), at 24 ± 2ºC and 70 ± 5% relative humidity, in pots containing a 1:2 vermiculite:soil mixture arranged in a complete random block design with six blocks where the different seed sources were randomized within the block. Plants were watered to full capacity every 2 days with complete Hoagland's nutrient solution (Hoagland *et al*., 1950) containing all essential macro and micro-nutrients as described (Saporta *et al*., 2019). After 5 weeks of growth, healthy plants of similar size from each cultivar, accounting for 5 replicates per cultivar and treatment, were randomly assigned to control and drought treatment. Control plants were irrigated every 2 days, whereas drought conditions were applied by withholding water until total weight (plant and container) was reduced to 60% of their initial weight. To obtain the florets, plants were grown for three months under greenhouse conditions (16 h light/8 h dark (200 μmol m^{-2} s^{-1} of light intensity), at 24 ± 2ºC and 70 ± 5% relative humidity in individual plant pots (25 cm diameter, 25 cm height). Once florets were developed drought treatment was applied through withholding water until seedling weight (plant and container) was reduced to 60% of their initial weight, while control plants were kept with normal watering.

Physiological measurements

Measurements were performed in the third youngest leaf according to previous studies (Taïbi *et al*., 2017). The water potential (Ψw, -MPa) was measured with a Schölander pressure pump (model PMS-1000, PMS Instruments, Corvallis, OR, USA) in five plants of each cultivar and treatment. A CIRAS-3 portable photosynthesis system (PP Systems, Amesbury MA, USA) was used for gas exchange determinations. The

conditions were saturating light (1500 μmol of photons m^{-2} s^{-1}), with a temperature of 25ºC, controlled ambient CO_2 (390 μmol mol^{-1} CO_2) and a relative humidity of approximately 55%. The instantaneous determination of net CO_2 assimilation -photosynthesis- (A, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol m^{-2} s^{-1}), transpiration (E, mmol H_2O^{-2} s^{-1}) and instantaneous water use efficiency (WUE, μmol CO_2 $mmol^{-1}$ H_2O), which is defined by the relationship between photosynthesis and stomatal conductance, were determined in the same leaves in five replicates for each cultivar.

Amino acid analysis

Glutathione (GSH) and free amino acids were extracted from 0.1 grams of lyophilized leaves according to the method described previously (Mulet *et al*., 2004). In brief, plant material was pooled and homogenized using mortar and pestle. Each pooled sample (0.10 g of dry weight) was heated 12 min. at 95C in 2% isocitrate buffer (pH 2 with HCl) (Qu *et al*., 2001). One to ten dilutions of these extractions were injected in a Beckman Gold amino acid automatic analyzer. The analysis was carried out following the protocol provided by the manufacturer, using a sodium citrate system and ninhydrin for detection as described (Gisbert *et al*., 2020).

Ion content determination

Ions were determined as described previously (Rios *et al*., 2012). Briefly, samples of the third youngest leaf from the indicated plants (about 1 g) were lyophilized for 3 days. Dry weight was determined, and ions were extracted by a 30 min. incubation in 1 ml of 0.1M HNO_3 at room temperature. Then samples were centrifuged, and supernatant was diluted with 4 ml of milliQ water and filtered (0.22 μM). Sodium and potassium were measured in a plasma emission spectrophotometer (Shimadzu), as described (Durgbanshi *et al*., 2005).

Hormone determinations

Abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and indoleacetic acid (IAA) were quantified as described previously (Roessner *et al*., 2000). Freeze-dried

lyophilized tissue from the third youngest leave (50 mg) was extracted with 2 ml of water after spiking with $[^2H_6]$-ABA, $[^2H_3]$-PA, dehydrojasmonic acid (DHJA), and $[^{13}C]$-SA applying mechanical stress with a ball mill (MillMix20, Domel, Zelezniki, Slovenia). Extracts were centrifuged (4000g, 10 min, 4ºC), supernatants were collected and the pH was adjusted to 3 with acetic acid. The extract was partitioned twice against diethyl ether. The upper layer was collected and evaporated (Speed Vac, Jouan, Saint HerblainCedex, France). The dry residue was resuspended in 10% MeOH, sonicated, filtered (0.22 µM, Albet S.A., Barcelona, Spain), and injected into an UPLC system (Acquity SDS, Waters Corp., Milford, MA). Analytes were separated using a reversed-phase C18 column (Gravity, 1.8 µm, 50 × 2.1 mm, Macherey- Nagel, Düren, Germany) using a 300 µl min^{-1} linear gradient of ultrapure H_2O (A) and MeOH (B) (both supplemented with 0.01% acetic acid). The gradient was: (0-2 min) 90:10 (A:B), (2-6 min) 10:90 (A:B) and (6-7 min) 90:10 (A:B). For quantification we used a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. Quanfitation of hormones was achieved based on a standard curve. Three biological replicates per cultivar and treatment were analyzed for each sampling time and each sample was measured twice.

Metabolomic analysis

The third youngest leave was collected and lyophilized and then homogenized with a mechanical tissue disruptor in the presence of liquid nitrogen before obtaining 10 milligrams of sample powder for each replicate. Four biological replicates of each cultivar and treatment were analyzed using a method modified from (Freeman *et al*., 2004). Powder was extracted in 1.4 ml 100% methanol and 60 µl of an internal standard (0.2 mg ribitol in 1 ml of water). The mixture was heated for 15 min. at 70ºC and centrifuged (10 minutes (min.); 20.000 g). The supernatant was transferred to a glass vial, then 750 µl of chloromethane and 1.5 ml of water were added. The mixture was vortexed for 15 seconds and centrifuged for 15 min. at 20.000 g. 150 µl aliquots of the methanol/water supernatant were dried by evaporation for 6–16 h.

For derivatization, dry residues were dissolved in 40 µl of 20 mg/ml methoxyamine hydrochloride in pyridine and incubated for 90 min. at 37ºC, followed by addition of 70

µl MSTFA (N-methyl-N-[trimethylsilyl]trifluoroacetamide) and 6 µl of a retention time standard mixture (3.7% [w/v] mix of fatty acid methyl esters ranging from 8 to 24C) and further incubation for 30 min. at 37ºC.

Sample volumes of 2 µl were injected in split and splitless mode to increase the metabolite detection range in a 6890 N gas chromatograph (Agilent Technologies Inc. Santa Clara, CA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI). Gas chromatography was performed on a BPX35 (30 m × 0.32 mm × 0.25 µm) column (SGE Analytical Science Pty Ltd., Australia) with helium as the carrier gas at a constant flow of 2 ml/min. The liner was set at 230ºC. The oven program was 85ºC for 2 min, 8ºC/min. ramp until 360ºC. Mass spectra were collected at 6.25 spectra s^{-1} in the m/z range 70–800 and ionization energy of 70 eV. Chromatograms and mass spectra were evaluated using the CHROMATOF program (LECO, St. Joseph, MI).

Statistical Analysis

Analysis of variance was carried out to determine significant differences between means at a $p < 0.05$ level. Homogeneous groups were separated using the Duncan multiple range test (MRT) test. In all cases, data were examined for normality and homogeneity of variances and assessed for any violations of assumptions. The data analysis for this project was generated using SPSS software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: USA).

Results

Physiological measurements

The first aim of our study was to test the physiological response of the plants (visual fitness upon irrigation withdrawal, fresh weight and drought weight ratio under control and stress conditions and ratio of stress/control conditions) and to check the stress conditions to validate the experimental design (Supplemental Figure 1). To check the effect of the selected drought stress conditions, we determined several functional parameters. Under drought stress, water potential decreased significantly (about 4-fold), indicating that the plants were indeed experiencing drought stress. The same response was observed for the other parameters, such as maximal PSII efficiency (Fv/Fm) andtranspiration (E). For photosynthetic rates (A), WUE and stomatal conductance (gs) the drought tolerance cultivars exhibited a significant improvement when compared to the drought sensitive (Figure 1).

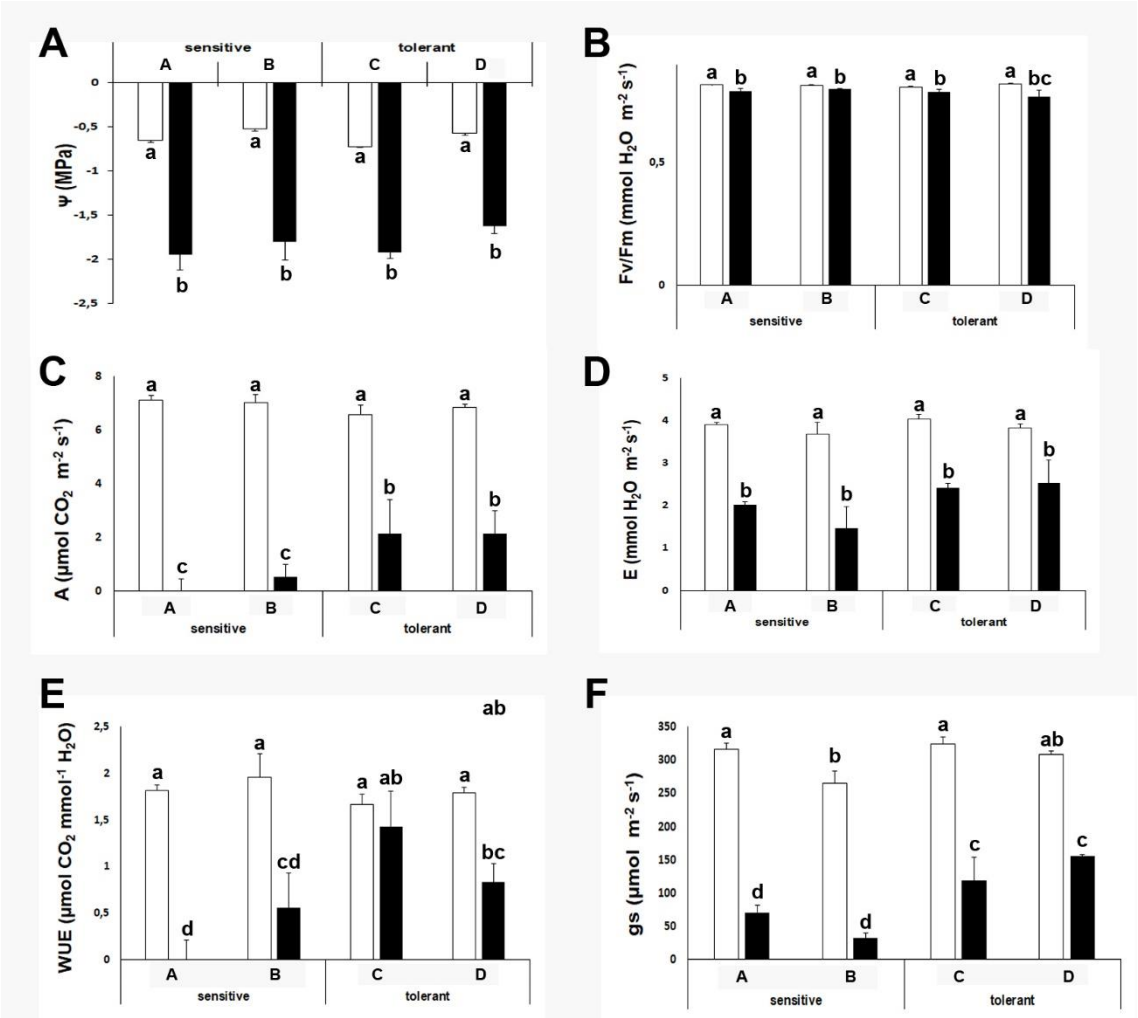


Figure 1. Physiological measurements. Water potential (Ψw) (A); Maximal efficiency of PSII (Fv/Fm) (B); Net photosynthesis (A) (C); transpiration (E) (D); instantaneous water use efficiency (WUE) (E) and stomatal conductance (gs) (F), of drought-sensitive and drought-tolerant cultivars under watered (white bars) and drought-stress (black bars) treatments. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 5$). Scale bars are mean + Statistical Error (SE).

Glutathione and free amino acids

Drought stress induces oxidation, and under these conditions, the biosynthesis of sulfur containing amino acids may become limiting due the requirement of cysteine for the biosynthesis of glutathione (GSH) (Hayat *et al*., 2012). In our study, GSH accumulated to higher levels in drought-tolerant plants, independently of the stress (Figure 2A). The levels of methionine (Met) were higher in drought-tolerant plants after stress (Figure 2B), while the levels of serine (Ser), which is required for cysteine and methionine biosynthesis, showed a similar pattern to GSH (Figure 2C). We did not find a distinctive pattern for cysteine (Figure 2D).

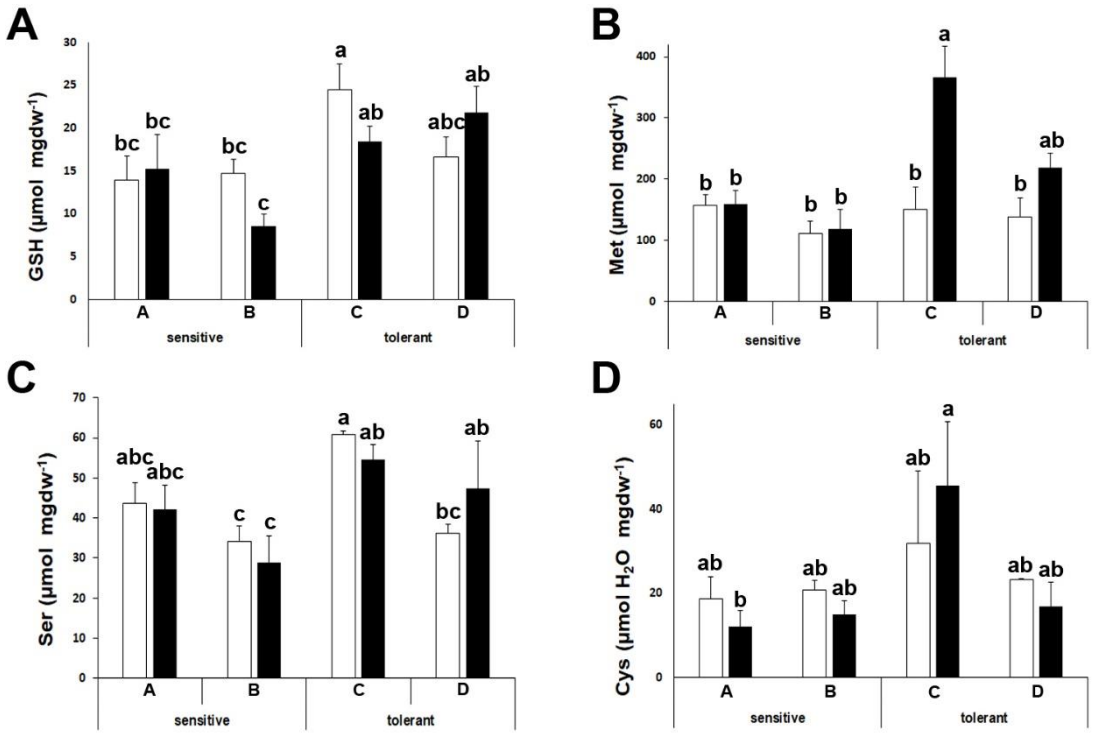


Figure 2. Glutathione, sulfur containing amino acids and serine determination. Glutathione (GSH) (A); methionine (Met) (B); serine (Ser) (C) and cysteine (Cys) (D) content of drought-sensitive and drought-tolerant cultivars under watered (white bars) and drought stressed (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean + SE.

We further investigated the levels of free amino acids. Some amino acids can act as precursors for osmolytes, act as osmolytes themselves or even could have previously undescribed functions in stress tolerance. For instance, proline (Pro) and glycine (Gly) are related to osmotic adjustment and can act as osmolytes or, in the case of proline, even as an antioxidant (Locascio *et al*., 2019). As expected, proline accumulated under drought stress, although the magnitude of this change did not correlate with the tolerance to drought stress (Figure 3A). Glycine levels showed a slight and non-significative decrease upon stress and levels were similar for sensitive and tolerant cultivars (Figure 3B).

We examined the patterns of the others amino acids looking for differences between sensitive and tolerant cultivars. The alanine (Ala) content decreased in stressed plants, but tolerant cultivars maintained a higher level (Figure 3C). Leucine (Leu), also a hydrophobic amino acid, showed the opposite pattern, as levels increased after stress,

but the content was lower for tolerant cultivars independently of the treatment (Figure 3D). Aspartic acid (Asp) levels changed divergently among sensitive (decrease) and tolerant (increase or maintain) cultivars (Figure 3E). A similar effect was found for glutamine (Gln), which increased upon stress in sensitive cultivars, while tolerant cultivars presented higher levels that were maintained in stressed plants (Figure 3F).

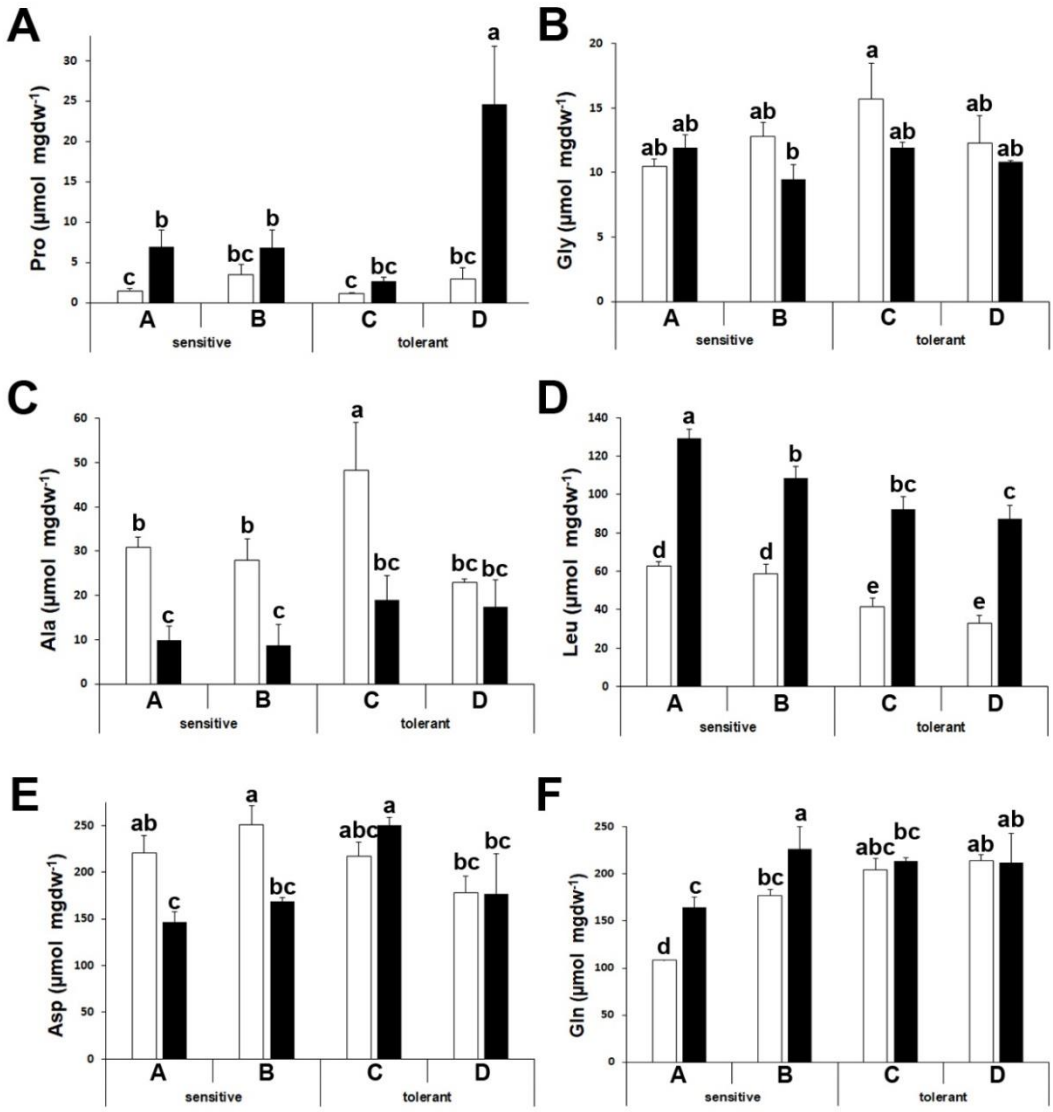


Figure 3. Amino acids which can act as osmolytes or with differential patterns between stress-sensitive and stress-tolerant cultivars. Proline (Pro) (A); glycine (Gly) (B); alanine (Ala) (C); leucine (Leu) (D); aspartic acid (Asp) (E) and glutamine (Gln) (F) content of drought-sensitive and drought-tolerant cultivars under watered (white bars) and drought stressed (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean + SE.

For some other amino acids, we did not observe differences among sensitive and tolerant cultivars, but there is no description available in the literature regarding the behavior of the pools of the free proteinic amino acid under drought stress in broccoli. Arginine (Arg), lysine (Lys), histidine (His), phenylalanine (Phe) and isoleucine (Ile) showed increased levels after stress (Figure 4 A-E), whereas valine levels where stable and unaffected by stress (Figure 4F). Threonine (Thr) and glutamic acid (Glu) levels decreased after stress (Figure 4 G and H).

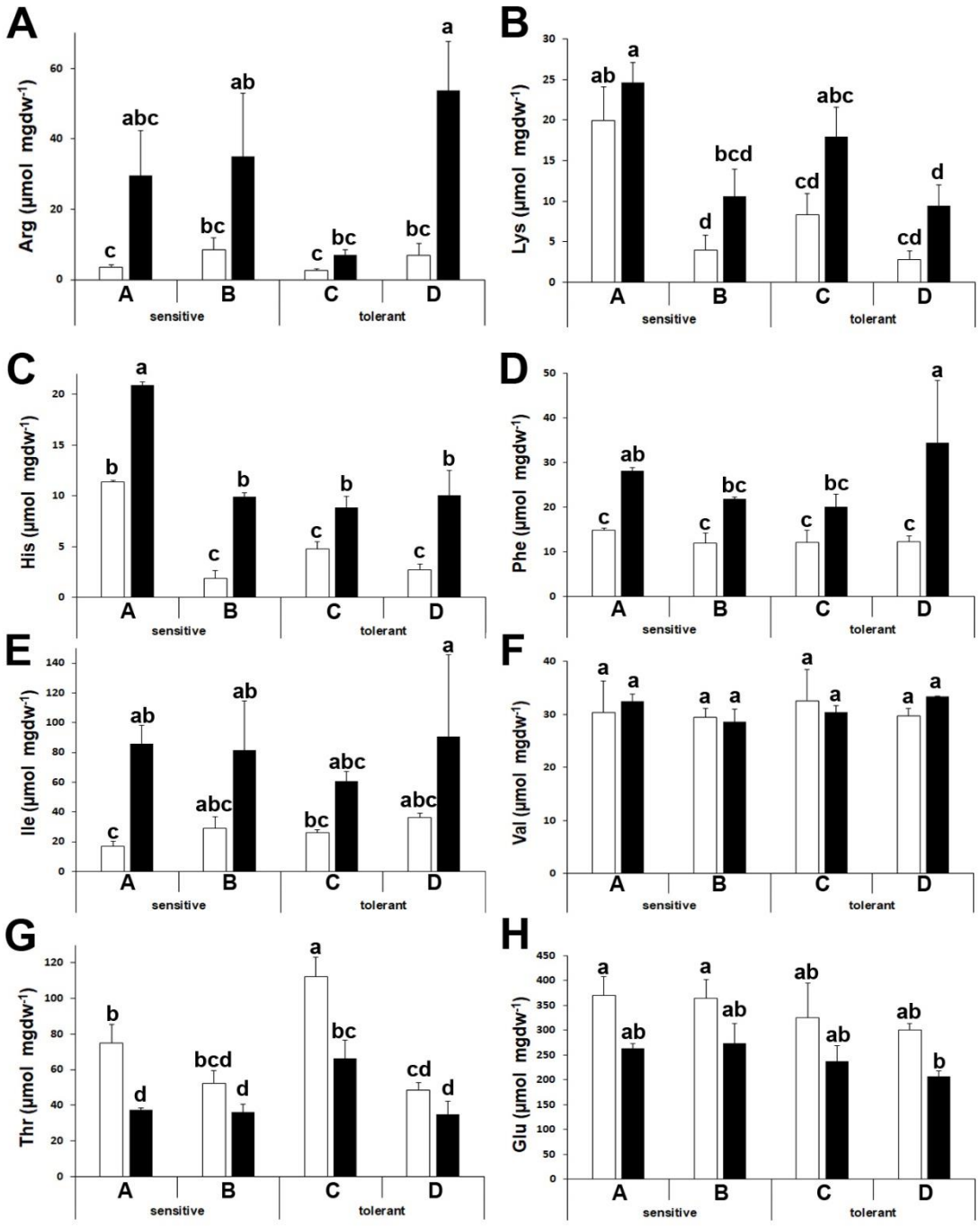


Figure 4. Amino acids with similar patterns between stress-sensitive and stress-tolerant cultivars. Arginine (Arg) (A); lysine (Lys) (B); histidine (His) (C); phenylalanine (Phe) (D); isoleucine (Ile) (E); valine (Val) (F); threonine (Thr) (G) and glutamic acid (Glu) (H) content of drought-sensitive and drought-tolerant seed sources under watered (white bars) and drought stressed (black bars) treatments. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean + SE.

Levels of essential amino acids in florets

Analyzing the results from amino acid determinations (Figures 3 and 4), we noticed that drought increases the levels of amino acids essential for the human diet. However, these levels were measured in leaves from 5-week-old plants, rather than the edible part of broccoli (florets). This opens the possibility that a drought treatment could increase the content of essential amino acids in broccoli and thus increase its nutritional value. To test this hypothesis, we cultivated a different cultivar until the development of the bud, we applied the drought stress and determined the content of essential amino acids in florets. We used a different cultivar to confirm that we were observing a general pattern for broccoli independent of the cultivar (Figure 5A). We could observe an increase in all essential amino acids, except for Met. Increases ranged from 1.2-fold to 10-fold (Figure 5B).

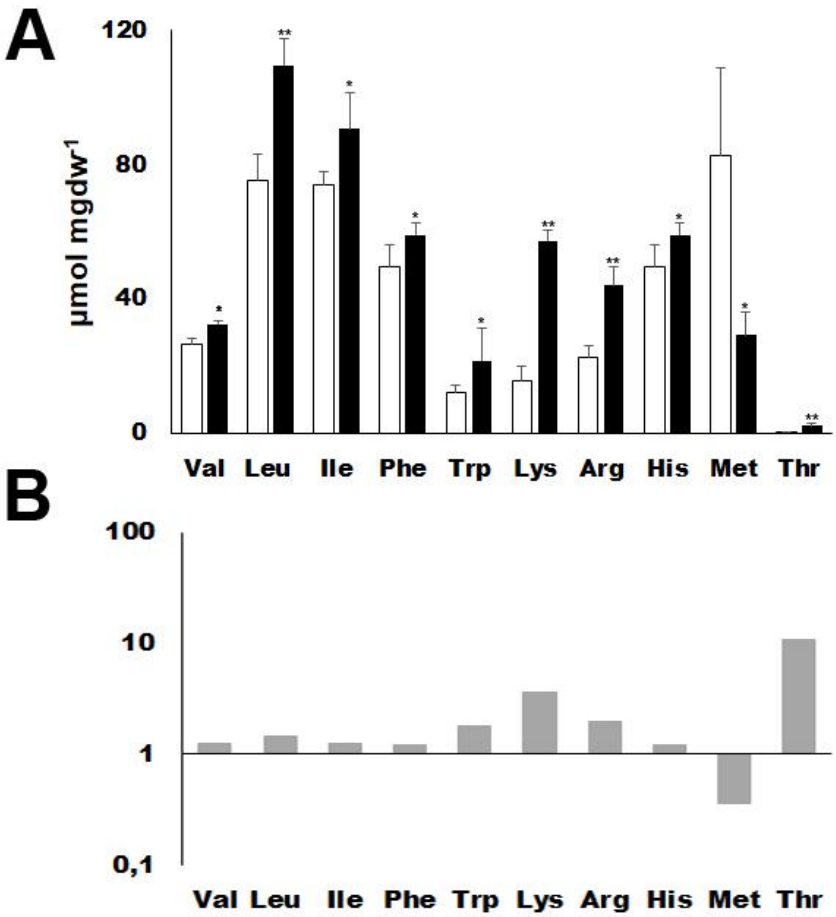


Figure 5. Determination of essential amino acids in florets. Content of the indicated amino acids in florets under watered (white bars) and drought-stressed (black bars) treatments (A) and ratio of the stress/control content (B). Asterisks indicate the p values (* = $p < 0.01$ and ** = $p<0.001$), as determined by t-student's test (n = 3). Scale bars are the mean + SE.

Sodium and potassium content

Plant cells must maintain a stable ionic environment under a range of external conditions. Potassium is the major ion in the internal medium of plants; the concentration in the cell cytoplasm must be stable and about 150 mM, independently of the external concentration. At the same time, sodium, an abundant cation in most soils, must be maintained outside the cytoplasm due to its toxicity. In addition, potassium fluxes are determinant to basic cellular processes involved in the drought stress response, such as stomatal aperture (Voss *et al.*, 2013). Potassium also can act as an osmolyte under stress conditions. We investigated whether the potassium content in leaves could be a distinctive feature for drought tolerance in broccoli. We determined sodium and potassium content in leaves under control and stress conditions. We

observed only minor differences among cultivars and treatments for both cations, suggesting that potassium homeostasis is not a limiting factor for drought tolerance in the cultivars analyzed (Figure 6).

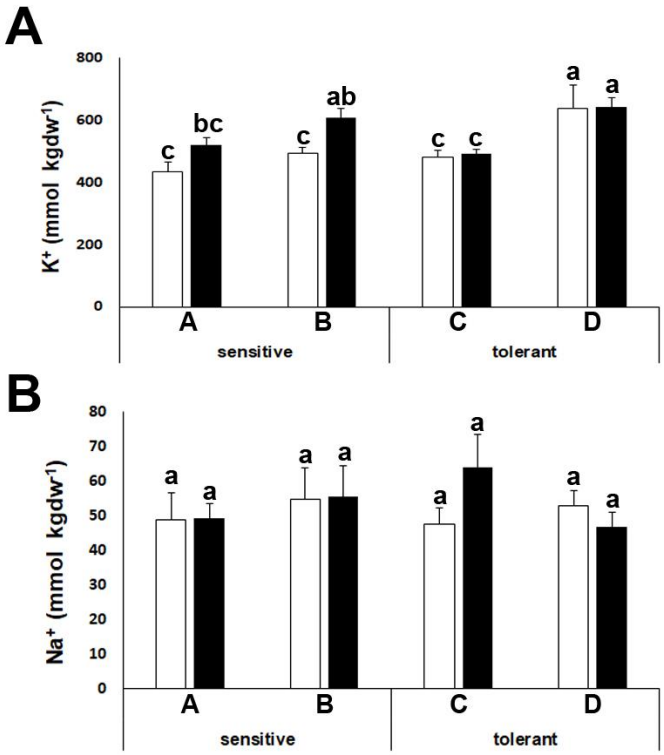


Figure 6. Ion content determination. Potassium (K^+) (A) and sodium (Na^+) (B) content of drought-sensitive and drought-tolerant cultivars under watered (white bars) or drought-stressed (black bars) treatments. Data with different letters differ significantly ($p<0.05$), as determined by Duncan's MRT test (n=8). Scale bars are mean + SE.

Hormone determinations

Hormones play a major role in stress responses as they are responsible for transducing the signal to the whole plant. Abscisic acid (ABA) is the main hormone responsible for the abiotic stress response, and, as expected, its levels increased upon stress (Figure 7A). The relative increase was higher in drought-tolerant cultivars (Figure 7A). Auxin (IAA) and jasmonic acid (JA) levels decreased upon stress, but we did not observe a distinctive pattern (Figure 7 B and C). Salicylic acid (SA) has been described as being able to increase the tolerance to abiotic stress in horticultural crops when applied exogenously (Souana *et al*., 2020). We found that levels of SA were stable upon stress. Levels diverged about 2- to 3-fold between cultivars, but again, we did not

observe a distinctive pattern between drought-tolerant and drought-sensitive cultivars (Figure 7D).

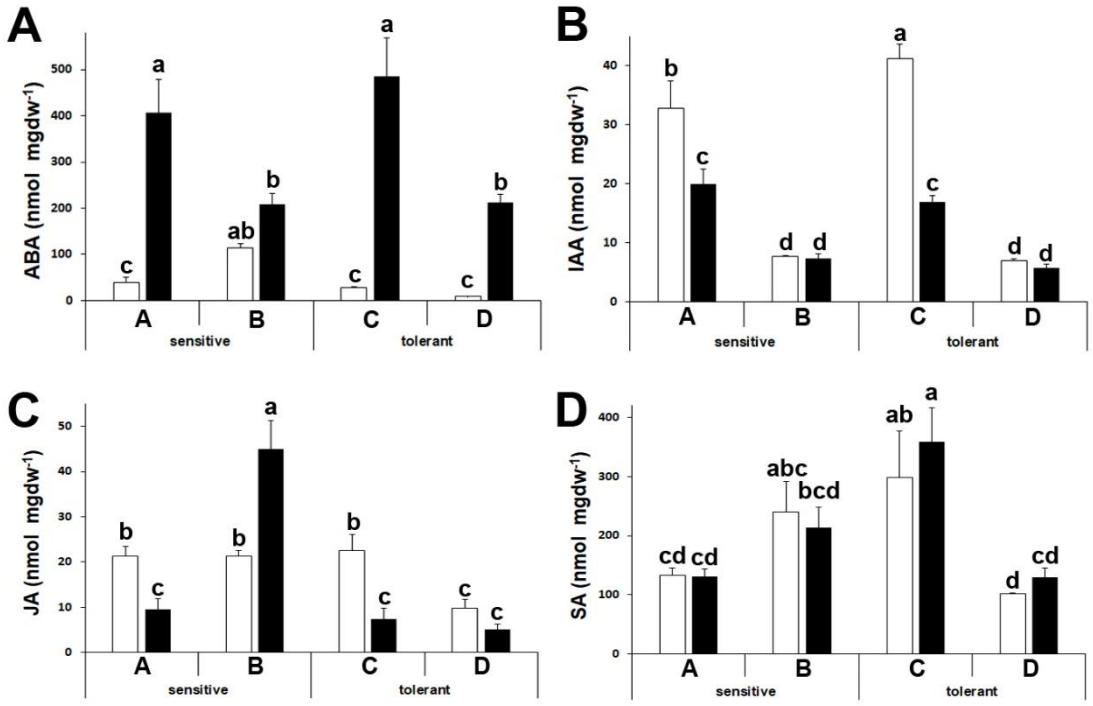


Figure 7. Hormone levels. Abscisic acid (ABA) (A); indolacetic acid (IAA) (B); jasmonic acid (JA) (C) and salicylic acid (SA) (D) content of drought-sensitive and drought-tolerant cultivars under watered (white bars) and drought-stressed (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 6$). Scale bars are mean + SE.

Primary metabolite analysis

Finally, we investigated whether we could identify distinctive traits among the cultivars by analyzing primary metabolites. We did not observe any distinctive pattern when we analyzed sugars or intermediates of the Krebs cycle (data not shown), but we found that urea and quinic acid levels increased upon stress in drought-sensitive cultivars, while decreasing in the drought-tolerant ones (Figure 8 A and B). Also, the levels of the lactone of gluconic acid were higher for drought tolerant cultivars under control conditions (Figure 8C).

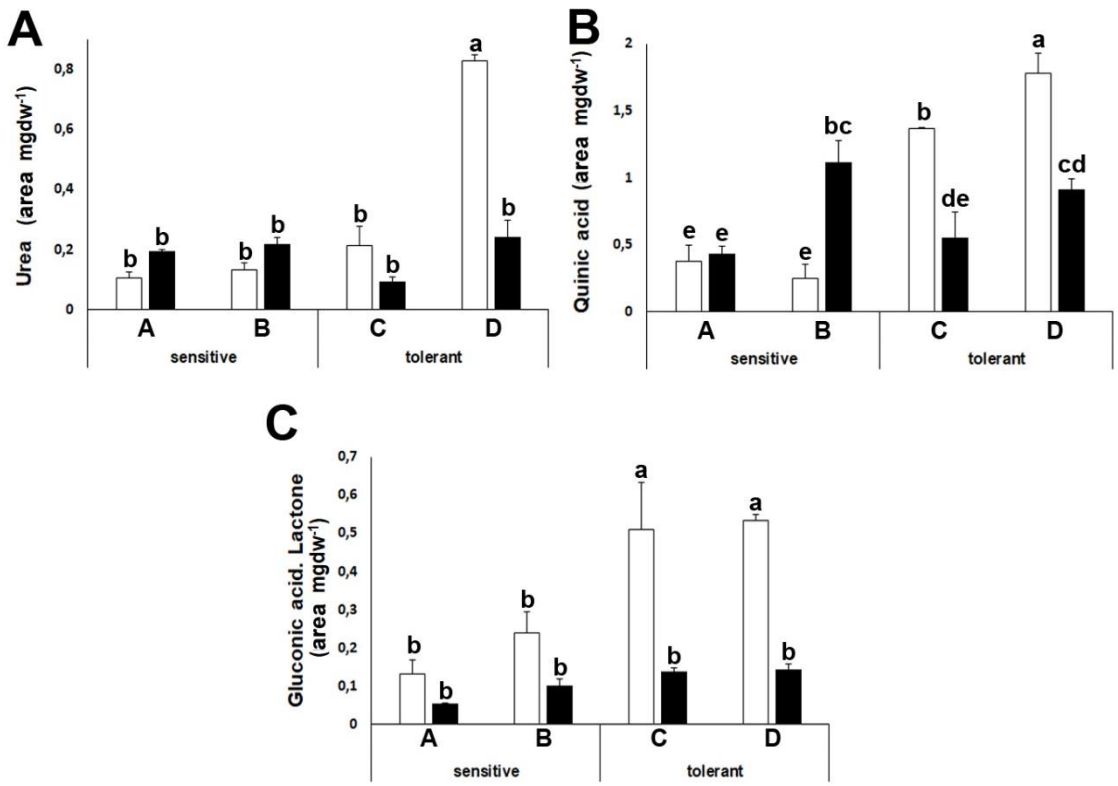


Figure 8. Levels of primary metabolites with differential patterns among cultivars. Urea (A); quinic acid (B) and gluconic acid lactone (C) of drought-sensitive and drought-tolerant cultivars under watered (white bars) or drought-stressed (black bars) conditions. The units are the area of the peak per mg of sample. Data with different letter differ significantly ($p<0.05$), as determined by Duncan's MRT test (n=4). Scale bars are the mean + SE.

Discussion

We designed this study to find limiting factors for drought stress tolerance in broccoli using a molecular and physiological approach. The identified differential traits could be useful for breeding new cultivars of broccoli with less water requirement. The main findings are summarized in supplemental figure 2.

Throughout this study, the well-established marker of drought stress, water potential (Ψw), and the known drought stress response molecules, Pro and ABA, exhibited the expected differences with respect to control plants. These results validate our experimental design and confirm that the plants in the greenhouse were affected by drought stress. Interestingly, from these three parameters, we only found a differential response between tolerant and sensitive cultivars for ABA, pointing out that the amount of this hormone may be limiting under drought stress conditions. Potassium can also act as an osmolyte, but we have shown here that it is not the limiting factor for drought stress tolerance, as we did not find significant differences among tolerant and sensitive cultivars, nor a significant increase upon drought stress. A known strategy of plants during drought stress is to downregulate the energy status to avoid oxidation (Taïbi *et al*., 2017; Li *et al*., 2017; Golldack *et al*., 2014). We have observed this downregulation in broccoli (Figure 1B). At the physiological level gs, A and WUE differ significatively among cultivars, indicating that they could be used as markers for stress tolerance.

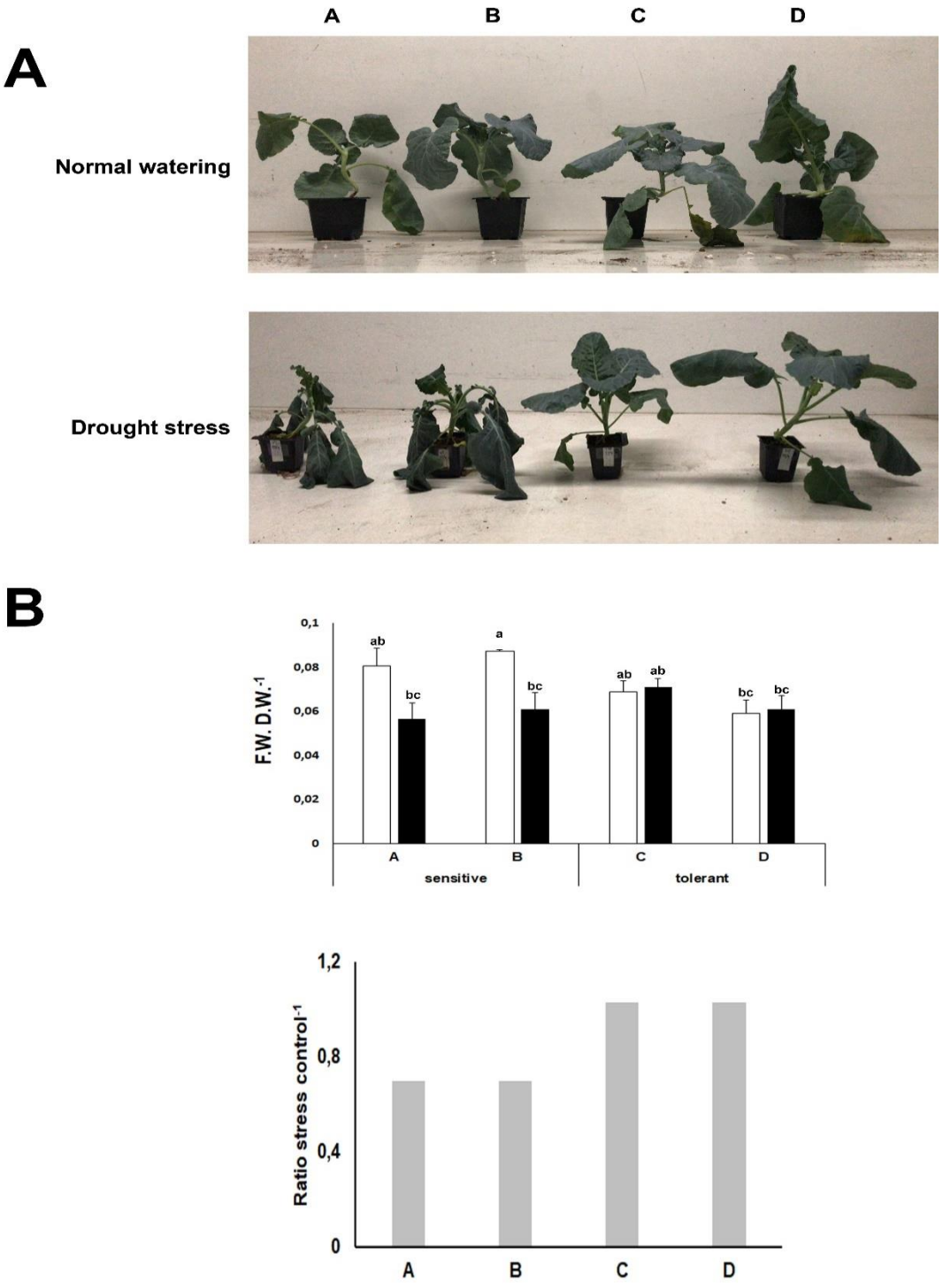
It is known that one of the main problems caused by drought is oxidative damage (López-Martín *et al*., 2008) The biosynthesis of cysteine from serine, and specifically the activity of the serine O-acetyltransferase (Hayat *et al*., 2004; Labudda *et al*., 2014) is a known limiting step for abiotic stress tolerance. Several reports indicate that GSH is considered to be the most important thiol involved in the prevention of oxidative damage in plants (Xu *et al*., 2019; Harun *et al*., 2020). In addition, broccoli is rich in sulfur-containing molecules, such as glucosinolates. It has been suggested that the levels of GSH (required for the biosynthesis of glucosinolates) and Met (one of the main precursors) are important to maintain the biosynthesis of pivotal molecules for the defense against herbivores in broccoli (Wang *et al*., 2019). We could see that the levels of GSH, Met and Ser in leaves were higher for drought-tolerant plants, indicating that in broccoli, sulfur metabolism is also limiting for drought stress tolerance, and that the

antioxidant response involving glutathione or sulfur containing proteins is a limiting factor for tolerance (Figure 2).

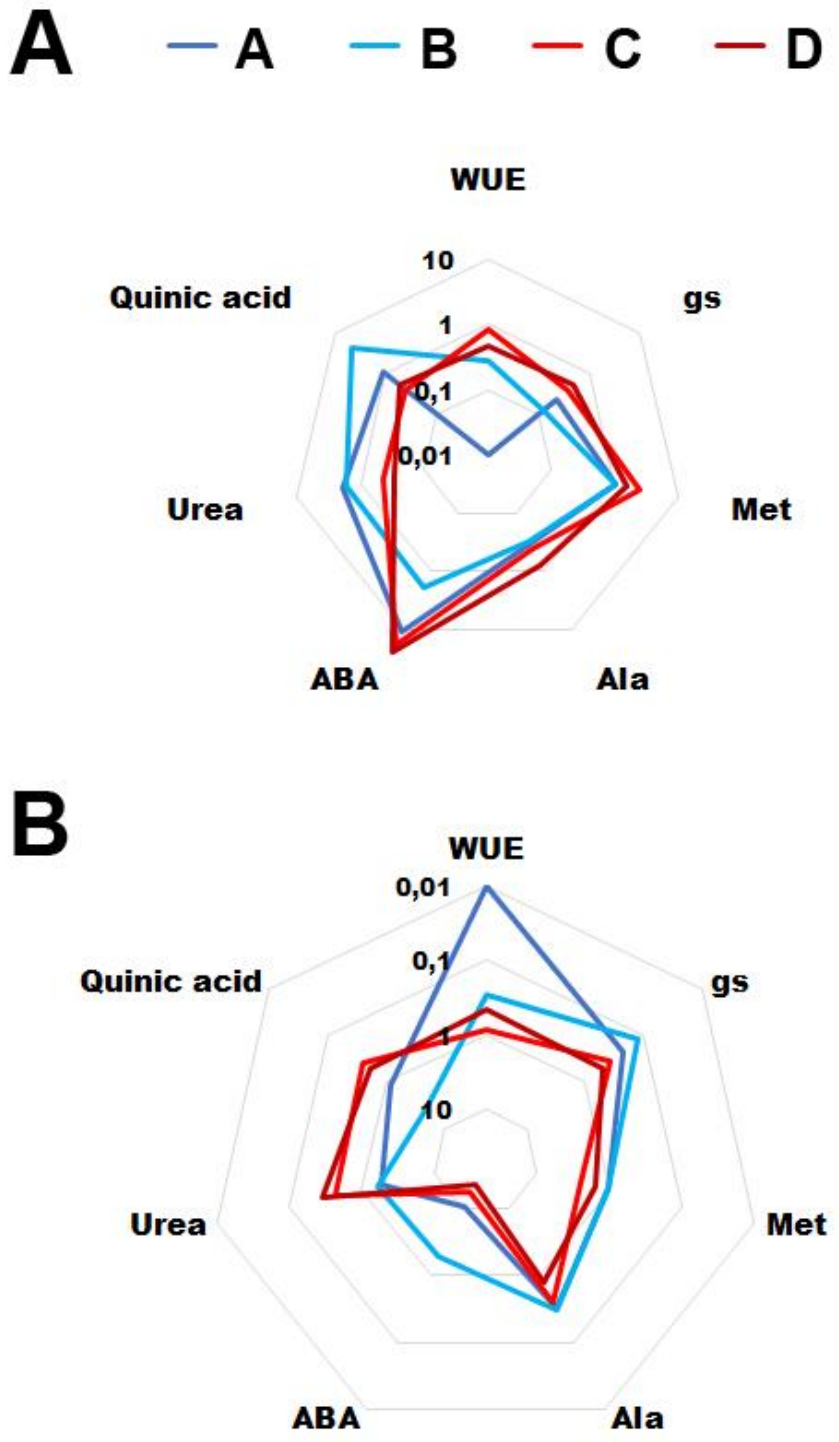
We have mentioned before that the content in ABA is a limiting factor for drought tolerance. Hormone responses are as expected for broccoli under abiotic stress (Witte *et al*., 2011), but the levels of IAA, JA or SA are not a distinctive trait in our cultivars, as hormonal levels did not correlate with tolerance (Figure 7). Also, we have found that urea and quinic acid decreased in drought-tolerant cultivars (Figure 8). In our experimental conditions, all plants had the same level of nitrogen fertilization, so observed changes do not represent changes in nitrogen uptake from the soil, but in nitrogen metabolism. The main source of urea is the degradation of arginine (Winter *et al*., 2011). We have found that arginine accumulates upon drought stress (Figure 4A), so the observed decrease in urea could be explained by an inhibition of the arginase activity, the enzyme that converts arginine into urea. In addition to urea, Arg is the immediate precursor of several molecules related to stress responses, such as nitric oxide (NO), ornithine and agmatine (Aghdam *et al*., 2019; Tapiero *et al*., 2002) and is also the precursor of creatine, polyamines, and glutamate (Hasanuzzaman *et al*., 2018). External application of Arg has also been shown to alleviate drought stress in some crops (Teixeira *et al*., 2013). Therefore, Arg might play a crucial role in stress recovery and a decrease in its turnover to urea may be a marker for drought stress tolerance. We also found a distinctive pattern for quinic acid and the gluconic acid lactone, although its biochemical interpretation is not obvious. We can summarize these results stating that we have found that high levels of Met and ABA, together with low levels of urea, quinic acid and gluconic acid lactone constitute a signature for drought tolerance in broccoli.

The strategy that we have used has another interesting advantage. It is known that stress can increase the organoleptical (Craig *et al*., 2004) or health-promoting properties (Zaicovski *et al*., 2008) of several crops. So, studying the chemical profile of broccoli during drought stress could be a useful tool, not only for maintaining yield under adverse conditions, but to describe conditions in which its nutritional content may increase. Water stress has been shown to delay post-harvest yellowing in broccoli florets (Li *et al*., 2014). Here, we show that drought increases the content in all essential amino acids (except Met), in the edible part of broccoli (Figure 5). There are previous

descriptions in the literature of treatments that can be applied to broccoli and could enhance its nutritional content or delay its senescence (Xu *et al*., 2012). Although broccoli is not considered a rich protein source, we have observed that drought treatment enhances its nutritional content. It is interesting to note that Met increases in leaves but decreases in florets. As mentioned before, Met is the precursor of glucosinolates and other molecules related to stress defense, such as polyamines. It is likely that the biosynthesis of these molecules under stress is more determinant in leaves. Taken all together, we have identified several limiting factors for broccoli tolerance to drought stress which could define novel targets for breeding programs or for the biotechnological improvement of broccoli aiming at creating novel cultivars adapted to drought-prone areas.



Supplemental figure 1. Representative plants of each cultivar under normal watering (upper panel) or after 6 days of drought stress (lower panel) (A); The third leave of each plant was collected, and fresh weight and dry weight was determined of drought-sensitive and drought-tolerant cultivars under watered (white bars) and drought-stress (black bars) treatments (upper panel) the ratio between stress and control conditions (lower panel) (B). Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 5$). Scale bars are mean + Statistical Error (SE).



Supplemental figure 2. Summary of the main findings of this study. Radial diagrams of the ratio between stress/control content (A) and control/stress content (B). Values are represented in a decimal logarithmic scale.

Chapter II

Chapter II

Identification of distinctive physiological and molecular responses to salt stress among tolerant and sensitive cultivars of broccoli (*Brassica oleracea* var. *Italica*)

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Abstract

Salt stress is one of the main constraints determining crop productivity, and therefore one of the main limitations for food production. The aim of this study was to characterize the salt stress response at the physiological and molecular level of different Broccoli (*Brassica oleracea* L. var. *Italica* Plenck) cultivars that were previously characterized in field and greenhouse trials as salt sensitive or salt tolerant. This study aimed to identify functional and molecular traits capable of predicting the ability of uncharacterized lines to cope with salt stress. For this purpose, this study measured different physiological parameters, hormones and metabolites under control and salt stress conditions. This study found significant differences among cultivars for stomatal conductance, transpiration, methionine, proline, threonine, abscisic acid, jasmonic acid and indolacetic acid. Salt tolerant cultivars were shown to accumulate less sodium and potassium in leaves and have a lower sodium to potassium ratio under salt stress. Analysis of primary metabolites indicated that salt tolerant cultivars have higher levels of several intermediates of the Krebs cycle and the substrates of some anaplerotic reactions. We have found that the energetic status of the plant, the sodium extrusion and the proline content are the limiting factors for broccoli tolerance to salt stress. Our results establish physiological and molecular traits useful as distinctive markers to predict salt tolerance in Broccoli or to design novel biotechnological or breeding strategies for improving broccoli tolerance to salt stress.

Key words: salt stress, broccoli, molecular markers, metabolomics, crop improvement, Krebs Cycle, Amino acids, Anaplerotic reactions.

Introduction

Nearly the 70% of earth's surface is covered by salty water and about 10% of terrestrial habitats are affected by salt. In addition, anthropogenic global warming is altering the weather patterns and thus threatening agricultural production (Bisbis *et al*., 2018; Van Passel *et al*., 2017). Climate change is predicted to have two impacts that will worsen the salinization of land: the direct inundation of coastal areas by seawaters and the increased aridity (IPCC, 2017). With rising temperatures, the pressure on aquifers will increase and therefore also the chances of sea water infiltration due to the decrease in the phreatic level. In addition, repeated cycles of irrigation and evaporation combined with high levels of fertilization induce the accumulation of salts and thus soil salinization. Coping with salt is one of the major problems of agriculture and the presumed scenario will be much worse in the near future (Tripathi *et al*., 2016).

Halophytes (i.e. plants able to complete their life cycle under saline conditions that would prevent growth and/or reproduction in most species) are rare (Flowers *et al*., 2008). Less than 2% of flowering plants are halophytes (Flowers *et al*., 2015), but this trait has emerged in at least 100 different angiosperm families (Santos *et al*., 2016). From the evolutionary point of view, it seems that salt tolerance may be a macroevolutionary self-destructive trait, gained often but frequently lost by reversal or extinction (Bromham *et al*., 2020). From the horticultural point of view, breeding for salt tolerant crops has proven to be very difficult. The use of biotechnological crops and new breeding techniques is also very limited. In the literature, there is information on only two successful field trials of crops transformed with a gene able to increase salt tolerance (Kotula *et al*., 2020): barley expressing the *Arabidopsis thaliana* vacuolar H^+-pyrophosphatase (*AVP1*) (Schilling *et al*., 2014) and wheat expressing the vacuolar Na^+/H^+ antiporter gene *AtNHX1* from *Arabidopsis thaliana* (Xue *et al*., 2004).

The presence of salt in the irrigation water, mainly sodium chloride, causes two problems during plant development. First, in the soil, outside the plant, the salt is able to retain the water, preventing its absorption by the roots, thus making an effect similar to drought. On the other hand, when sodium cations enter the plant, they induce toxicity, as sodium displaces potassium cations, which are the main ions inside plant cells. This displacement interferes with different biochemical and physiological processes and can

lead to cell death. Plants have developed complicated mechanisms to maintain ion homeostasis, mainly aimed at keeping the potassium concentration within the cell high, and the sodium concentration low (Serrano *et al*., 1999).

The genus *Brassica* includes some important species of agronomic interest, including broccoli (*Brassica oleracea* L. var. *italica*, Plenck), a crop cultivated in temperate climates. Broccoli production has gained importance in recent years for its considerable nutritional value provided by its richness in bioactive compounds such as vitamins C and E, quercetin or kaempferol glycosides (Jeffery *et al*., 2009), as well as by the presence of glucosinolates, a group of about 120 molecules derived from amino acids (Fahey *et al*., 2001) with a ß-D-thioglucose moiety (Zinoviadou *et al*., 2017). In plants, glucosinolates have been shown to play a role in the defense against biotic stress (Hanschen *et al*., 2014), but in humans it has been proven that, apart from contributing to the characteristic flavor of broccoli, some compounds produced upon hydrolysis of glucosinolates, such as sulforaphane, may reduce the risk of lung, breast, gastric, prostate or kidney cancer (Gupta *et al*., 2014; Li *et al*., 2010).

Broccoli is considered a crop moderately tolerant to salt stress. Its tolerance is higher than other common vegetables such as lettuce, onion, maize or carrot (Shannon *et al*., 1998). Salt stress in broccoli causes a two-phase growth decrease (Munns *et al*., 2002). The first phase of the growth decrease is the consequence of salt surrounding the roots and the second phase results from the internal injury due to salt accumulation in leaves (Kawasaki *et al*., 2001). In general, the *Brassicaceae* family can tolerate salt stress by osmolyte accumulation, Na^+ exclusion and a relatively high K^+ retention ability (Shahzad *et al*., 2021). Prior studies have also determined the changes in several physiological parameters upon salt stress, for instance a study comparing three commercial cultivars determined that leaf water potential only changes in the long term, and that the changes in the transpiration rate and stomatal conductance depend on the cultivar (Muries *et al*., 2013). To our knowledge there are no studies in the literature comparing the hormone levels or the amino acid and metabolite contents of different cultivars with different levels of stress tolerance under control and salt stress conditions. In the specific case of broccoli, the characterization of a cultivar as tolerant to salinity has always been made *a posteriori*, on the basis of the empirical evidence obtained in the field or the experience of farmers who have used certain cultivars. Many times,

cultivars are used without having any information *a priori* on whether they are adapted to salty soils, with the consequent drop in production and loss of income for the farmer. It is also interesting to generate new cultivars able to withstand salt stress, as it has been demonstrated that salt stress enhances the nutritional quality of broccoli (Lopez_Berenguer *et al*., 2009). In addition, broccoli has considerable potential because of its susceptibility to biotechnological modifications, affording the possibility to design strategies to improve its resistance to various stresses (Metz *et al*., 1995). Although it has been an active field of investigation, there is no commercial cultivar of broccoli generated using biotechnological tools in the market (Kumar & Srivastava, 2016).

Previous studies carried out in our laboratory on non-model species, such as *Pinus halepensis* (Taïbi *et al*., 2018), *Phaseolus vulgaris* (Taïbi *et al*., 2016) or *Vicia faba* (Souna a *et al*., 2020), have demonstrated the usefulness of physiological or molecular markers to characterize abiotic stress tolerant cultivars. In the present paper, there is a characterization, using different physiological and chemical strategies, of two salt tolerant and two salt sensitive cultivars with the goal of identifying distinctive traits at the physiological or molecular level. Thus, the aim of our study was to determine if this study could observe differences in any physiological or molecular parameter among different cultivars and/or treatments. The identification of these traits will allow us to predict whether uncharacterized field cultivars are likely to be salt tolerant or salt sensitive and this information will also help to identify the limiting factors for broccoli salt tolerance. This knowledge will facilitate the breeding of new salt tolerance cultivars and will supply farmers with new cultivars with enhanced production in the context of climate change and help to provide consumers with broccoli with enhanced nutritional content (López-Berenguer *et al*., 2009).

Materials and methods

Plant material and experimental conditions

Seeds of salt tolerant or salt sensitive cultivars were provided by SAKATA Iberica Seeds SLU (Valencia, Spain). The selection was based on field performance and confirmed by greenhouse tests under controlled salt stress. All are pre-commercial hybrid lines which are being developed by the company and are not available in the market yet.

The experimental design included two main factors: 1) stress level (control plants /salinity-stressed plants) and 2) cultivar (two previously characterized as salt sensitive and two as salt tolerant). For different experiments seeds were germinated in a Petri dish with filter paper. After five days, they were transferred to a substrate (50% kekkila peat, 25% perlite, 25% vermiculite) in TEKU cultivation trays, series PL 2838/24 with wells of 5.5 x 6 cm and volume of 149 mL. Greenhouse conditions were as follows: 16 h light/8 h dark (200 μmol m^{-2} s^{-1} of light intensity), at 24 ± 2ºC and 70 ± 5% relative humidity. The experimental design consisted of an aleatory placement where each block was composed by 4 pots per tray and one plant per pot. Each experiment consisted in 3 individuals × 4 cultivars x 2 treatments (24 total plants) and was replicated 3 to 5 times. Plants were watered with Hoagland solution. After 5 weeks irrigation was kept (control plants) or salinity stress conditions were applied by watering with Hoagland solution plus 220 mM NaCl. Samples were taken or measures were performed after six days of stress treatment (Sanoubar *et al*., 2016). The same experimental design was used for determining the amino acid, hormone, ion and metabolite content. Samples were taken from the third youngest leave. In all cases, the number of samples per experiment (n) refers to biological replicates from different plants.

Physiological measurements

Plants were grown under greenhouse conditions and stress was applied as described above. Physiological measurements were performed as described in Taibi *et al*., (2017) in the same leaves that were used for the rest of the determinations (i.e. third youngest leaf). The water potential (Ψw, -MPa) was measured with a Schölander pressure pump

(model PMS-1000, PMS Instruments, Corvallis, OR, USA). For gas exchange measurements this study used a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury MA, USA) under the following conditions: saturating light (1500 μmol of photons m^{-2} s^{-1}), temperature of 25ºC, ambient CO_2 390 μmol mol^{-1} CO_2 and relative humidity of approximately 55%. The instantaneous determination of net CO_2 assimilation -photosynthesis- (A, μmol CO_2 $m^{-2}s^{-1}$), transpiration (E, mmol $H_2O^{-2}s^{-1}$), stomatal conductance (gs, mol $m^{-2}s^{-1}$) and instantaneous water use efficiency (WUE, μmol CO_2 $mmol^{-1}H_2O$) were determined in the same leaves in four replicates for each cultivar and condition.

Amino acid analysis

100 mg of lyophilized leaf was grounded with a mortar and pestle in the presence of liquid nitrogen. The resulting powder was homogenized for 30 seconds with 2 mL of 2% citrate buffer pH 2 (Mulet *et al*., 2004), boiled at 95ºC for 12 minutes and centrifuged for five minutes at 13000 *g*. The supernatant was filtered through a 0,22-micrometer pore-size non-sterile filter. 1/10 dilutions of these extracts were injected into an automatic Beckman Gold amino acid analyzer. The analysis was carried out according to the protocol supplied by the manufacturer, using a system of ninhydrin and sodium citrate for detection.

Hormone quantification

Plant hormones were quantified according to the method described in Durgbanshi *et al*., 2005). In brief, lyophilized tissue from the third youngest leaf (50 mg) was extracted in 2 mL of water after spiking with [$^2H_6$]-ABA, [$^2H_3$]-PA, dehydrojasmonic acid (DHJA), and [^{13}C]-SA using a ball mill (MillMix20, Domel, Zelezniki, Slovenija). After the mechanical treatment, samples were centrifuged (4000 *g*, 10 min, 4°C) and the supernatants were collected. The pH was adjusted to 3 using acetic acid. Then extract was extracted using diethyl ether. This process was repeated twice. The upper layer was recovered and evaporated (Speed Vac, Jouan, Saint Herblain, France). To resuspend the dry residue, we used 10% MeOH aided by gentle sonication. Then we passed filtered the solution (0,22μM Albet S.A., Barcelona, Spain), and injected it into a UPLC system (Acquity SDS, Waters Corp., Milford, MA) and analyzed as described (Durgbanshi *et*

al., 2005). Three biological replicates per cultivar and treatment were analyzed for each sampling time.

Ion content determination

Ions were determined as described previously (Gisbert *et al*., 2020). Briefly, samples of the third youngest leaf from 1-month-old plants (about 1 g) were dried at 70°C for 4 days. Dry weight was determined, and ions were extracted by a 30 min incubation in 1 mL of 0.1M HNO_3 at room temperature. Then samples were centrifuged, and supernatant was diluted with 4 mL of milliQ water and filtered (0.22 µM pore-size). Sodium and potassium were measured in a plasma emission spectrophotometer (Shimadzu), as described (Rios *et al*., 2012).

Primary metabolite analysis

Primary metabolite analysis was performed at the Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) Metabolomics Platform by a method modified from that described by Roessner *et al*., 2000 (Roessner *et al*., 2000). 10 mg of leaves per sample were homogenized with liquid nitrogen and extracted in 1400 µL 100% methanol and 60 µL internal standard (0.2 mg ribitol in 1 mL of water). The mixture was extracted for 15 min at 70ºC, then the extract was centrifuged for 10 minutes at 20,000 g. Supernatant was transferred to a glass vial and we added 750 µl of chloroform and 1,5 mL of milliQ water. After mixing and centrifuging at 20,000 g aliquots (0.15 mL) were taken and dried. Samples were analyzed as described in Roessner *et al*., 2000).

Statistical design and analysis

The main treatment effects were analyzed by using the general linear model (ANOVA) considering three fixed factors: treatment (control and salinity), sensitivity degree to salinity (sensitive and tolerant) and cultivar (with four levels) as a factor nested within sensitivity degree. These analyses were performed with the SPSS v.25.0 statistical package (IBM SPSS Statistics for Windows, Armonk, NY, USA; IBM Corp.). The means were considered to be significantly different at $p < 0.05$ after Duncan's new

multiple range test (MRT). In all cases, we used one factor analysis of Variance (ANOVA) and all groups were analyzed independently. For Figure 9B data in each line and treatment were normalized against the value of cultivar 1.

Results

Physiological measurements

As expected, this study found a negative effect of salinity on water potential (Ψw) in both salt-tolerant and salt-sensitive cultivars. Values under salt stress significantly decreased by 2.5- to 3-fold compared to control values, indicating that plants were indeed stressed (Figure 1A). The salinity treatment also had a negative effect on stomatal conductance (gs) and transpiration (E) (Figure 1B and C). For both variables, values were lower for salt-sensitive cultivars, while results were about two-fold higher in salt-tolerant cultivars under stress. In fact, the results of these variables in salt-tolerant cultivars under salinity were similar to values under control conditions (Figure 1B and C). This study found a clear positive effect on the water use efficiency (WUE) of salt-sensitive plants under stress (i.e., cultivar 1), while salt-tolerant cultivars retained similar values under stress and control conditions, indicating that were dealing better with the stress (Figure 1D). This study also observed a negative effect of salt stress on photosynthesis (A) and on substomatic CO_2 concentrations, although the intensity of changes was lower than for gs and E. Regarding cultivars, the differences among salt tolerant and salt sensitive cultivars were not significant for photosynthesis (Figure 1E), but results showed higher substomatical CO_2 concentrations for salt-tolerant cultivars, and again, similar to values obtained for non-stressed plants (Figure 1F).

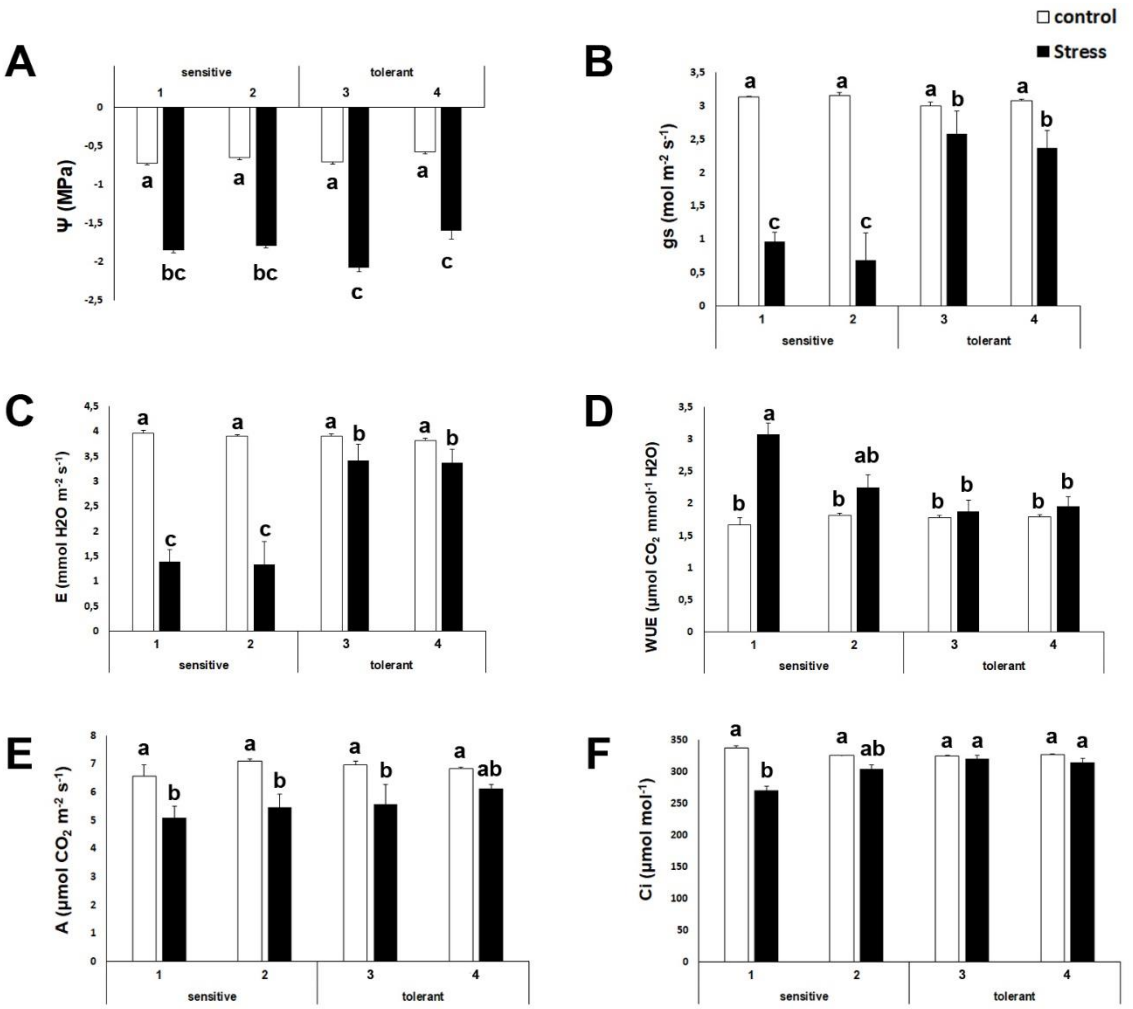


Figure 1. Physiological measurements. Water potential (Ψw) (A); stomatal conductance (gs) (B); transpiration (E) (C); instantaneous water use efficiency (WUE) (D); Net photosynthesis (A) (E) and CO_2 substomatical concentration (Ci) (F) of salt-sensitive and salt-tolerant cultivars under control (white bars) and stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 5$). Scale bars are the mean + Statistical Error (SE).

Free amino acids

Once confirmed the tolerance and sensitivity of the selected cultivars at the physiological level, this study further investigated the level of essential metabolites. There is no description available in the literature regarding the behavior of the free amino acid pools under salt stress in *Brassica oleracea* var. *Italica*, so this study investigated the complete free amino acid profile in our plants under the studied conditions.

It has been shown that in response to abiotic stress the biosynthesis of sulfur containing amino acids may become limiting, specifically due to the requirement of cysteine for the biosynthesis of glutathione (GSH), which is required to cope with the oxidative stress induced by abiotic stress (Mulet *et al*., 2004; Freeman *et al*., 2004). GSH is also required for the biosynthesis of glucosinolates (Harun *et al*., 2020). The total content of GSH and cysteine (Cys) was less in salt tolerant cultivars, under stress and control conditions (Figure 2 A and B). Methionine (Met) is a precursor of aliphatic glucosinolates (Liu *et al*., 2020). The salt sensitive cultivars showed increased amounts of Met under salt stress, but the level was stable for the tolerant cultivars (Figure 2C). This study did not find a distinctive pattern for serine (Ser) levels (Figure 2D).

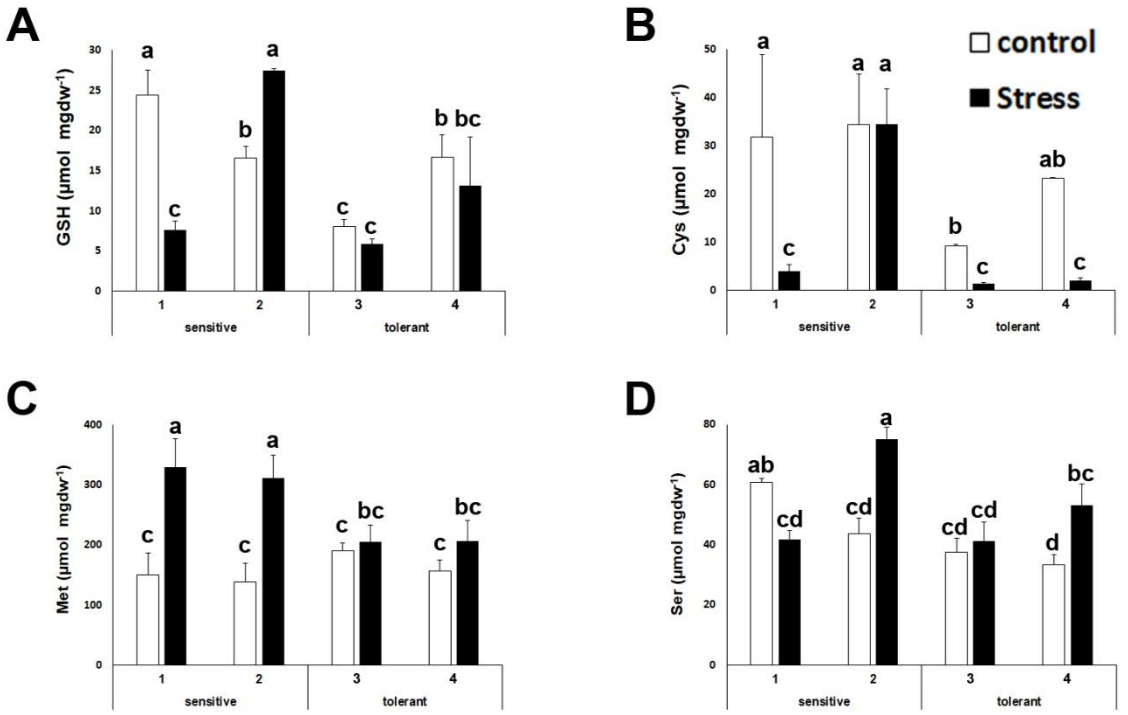


Figure 2. Glutathione, sulfur containing amino acids and serine determination. Glutathione (GSH) (A), cysteine (Cys) (B), methionine (Met) (C) and serine (Ser) (D) levels of salt-sensitive and salt-tolerant cultivars under control (white bars) and stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean + SE.

Some amino acids can act as precursors for osmolytes or act as osmolytes themselves. Proline (Pro) is related to osmotic adjustment and has been described to accumulate in some *Brassicaceae* plants under drought stress (Podda *et al*., 2019). This study also observed that proline accumulated upon salt stress. Interestingly, this accumulation was

higher in salt tolerant cultivars (Figure 3A). On the other hand, histidine (His), asparagine (Asn), threonine (Thr) and lysine (Lys) levels in stressed and control conditions were lower for salt tolerant cultivars (Figure 3 B-E).

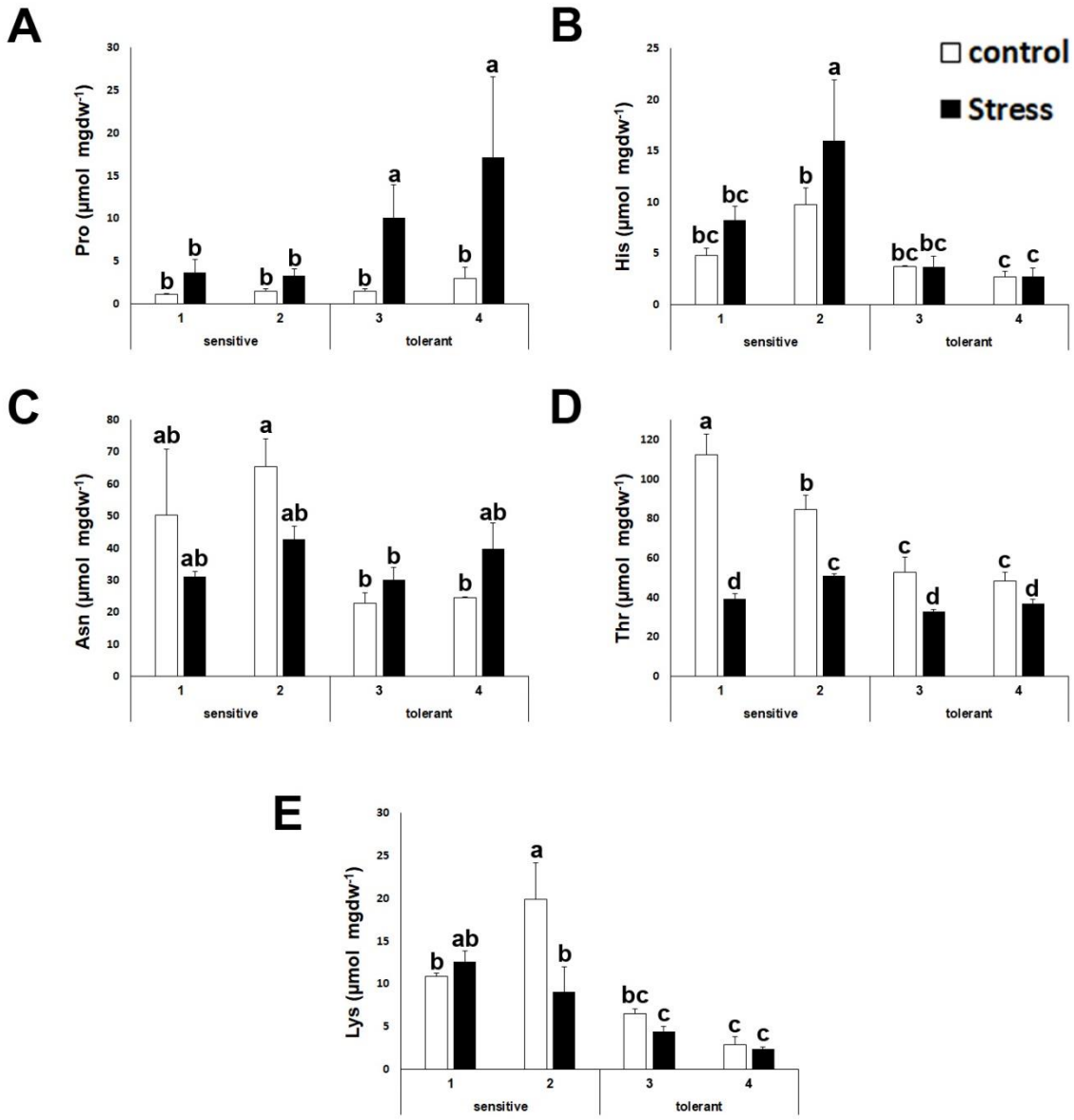


Figure 3. Amino acids with differential accumulation patterns between stress sensitive and stress tolerant cultivars. Proline (Pro) (A); histidine (His) (B); asparagine (Asn) (C); threonine (Thr) (D) and lysine (Lys) (E) levels of salt-sensitive and salt-tolerant cultivars under control (white bars) and stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean + SE.

For other amino acids, this study did not observe differential patterns between salt-tolerant and salt-sensitive cultivars, but this study found a distinctive stress response.

Alanine (Ala) decreased its content about 3- to 5-fold (Figure 4A). The phenylalanine (Phe) and valine (Val) content stayed stable (Figure 4 B and C), while arginine (Arg) increased from 2- to 5-fold and isoleucine (Ile) increased between 50% to 3-fold (Figure 4 D and E).

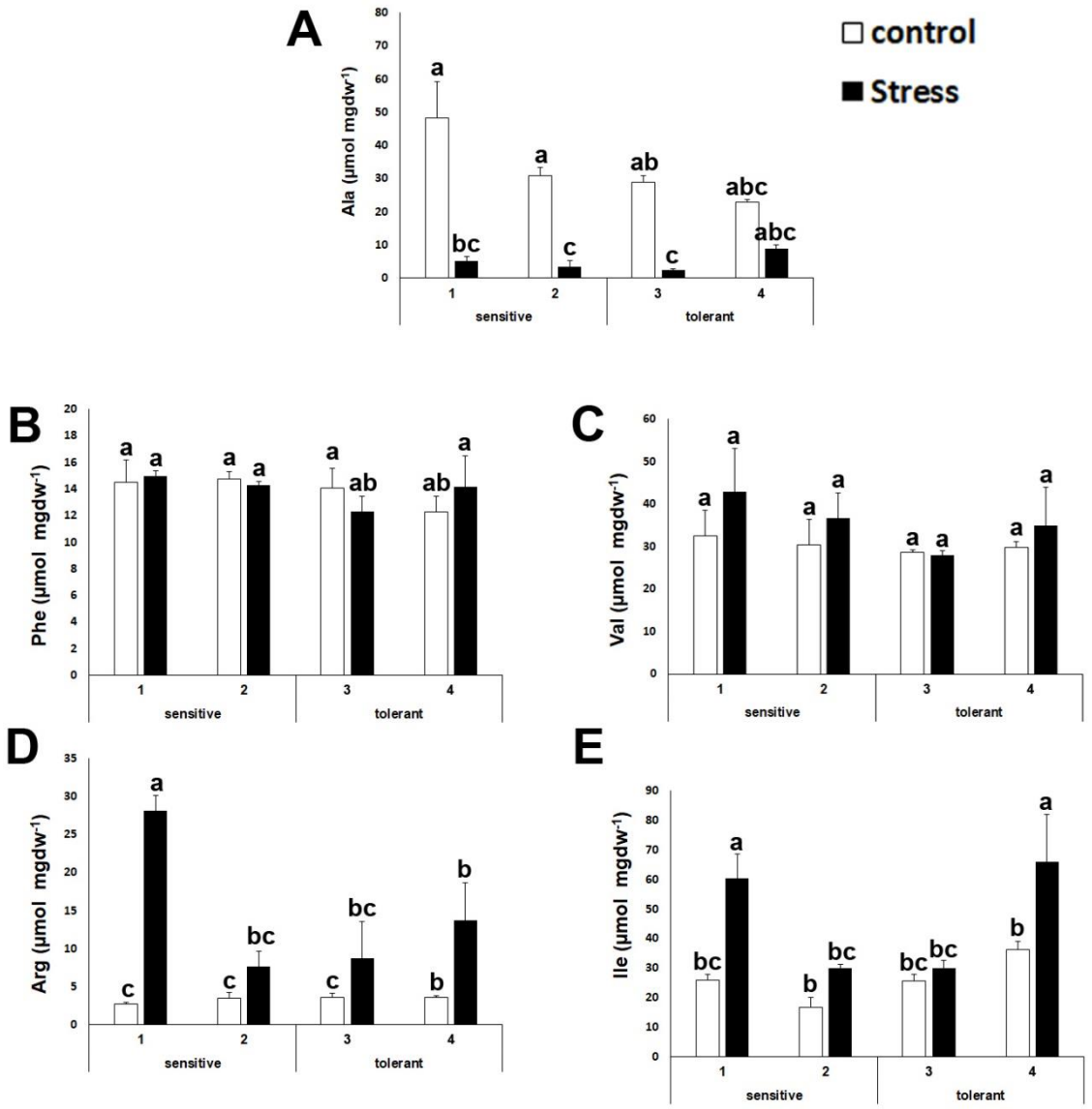


Figure 4. Amino acids with similar accumulation patterns between stress sensitive and stress tolerant cultivars. Alanine (Ala) (A); phenylalanine (Phe) (B); valine (Val) (C); Arginine (Arg) (D) and isoleucine (Ile) (E) levels of salt-sensitive and salt-tolerant cultivars under control (white bars) and stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean + SE.

Hormone measurements

Plant hormones mediate salt stress responses and thus regulate plant growth adaptation (Yu *et al*., 2020). This study investigated the differences in the hormone levels among cultivars under control and salt stress conditions. As expected, abscisic acid levels increased upon salt stress. Interestingly, salt tolerant cultivars showed decreased basal levels under control conditions, but exhibited a higher level upon salt stress, so the increase was 2- to 4-fold higher in salt tolerant cultivars (Figure 5A). Jasmonic acid levels decreased in all cases upon salt stress, but the levels were lower under basal conditions for salt tolerant cultivars and the decrease was about 3-fold less (Figure 5B). Indoleacetic and salicylic acid levels were also lower for salt tolerant cultivars (Figure 5 C and D).

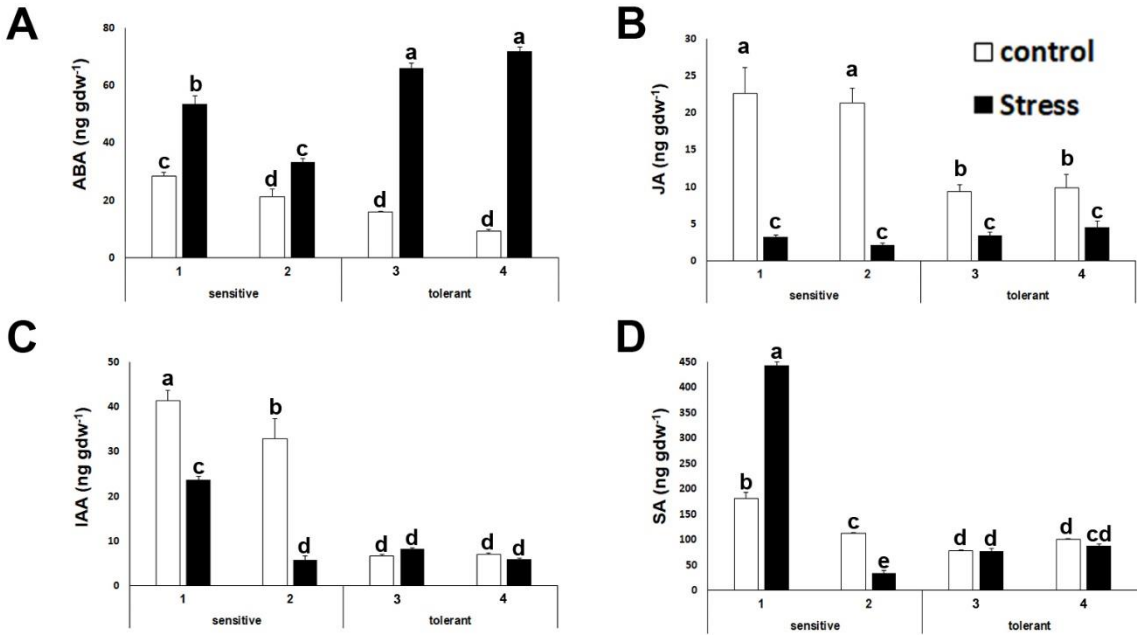


Figure 5. Hormone content. Abscisic acid (ABA) (A); jasmonic acid (JA) (B); indoleacetic acid (IAA) (C) and salicylic acid (SA) (D) content of salt-sensitive and salt-tolerant cultivars under control (white bars) and stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 6$). Scale bars are mean + SE.

Sodium and potassium determination

Sodium and potassium accumulation in leaves of control and stressed plants was determined. This study found that leaves from stress tolerant plants accumulate less sodium than leaves of sensitive plants (Figure 6A), and less potassium (Figure 6B), but

the Na^+/K^+ ratio is significantly lower for salt tolerant cultivars (Figure 6C), indicating that the loss of potassium is less than the uptake of sodium.

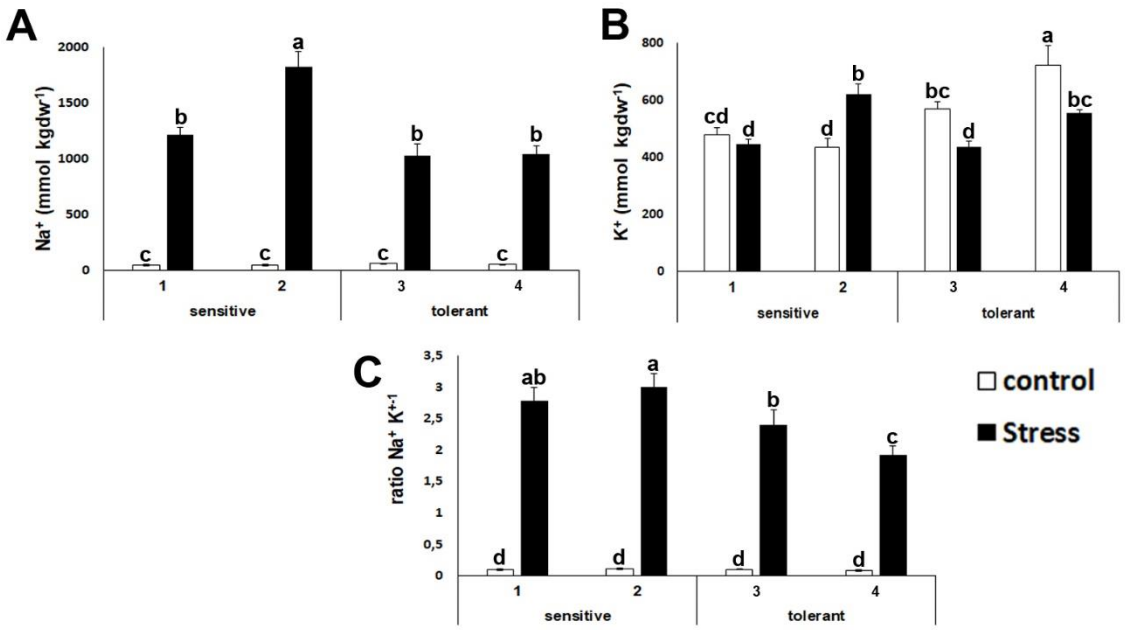


Figure 6. Ion content determination. Sodium content (Na^+) (A), potassium content (K^+) (B) and ratio Na^+/K^+ (C) content of salt-sensitive and salt-tolerant cultivars under control (white bars) or stressed (black bars) treatments. Data with different letter differ significantly ($p<0,05$), as determined by Duncan's MRT test (n=8). Scale bars are mean + SE.

Primary metabolite analysis

This study found that intermediates of the Krebs cycle were present in higher amounts in salt tolerant plants under control or stressed conditions. This study could confirm this for citric, succinic, malic and fumaric acids (Figure 7 A-D). This study also observed this pattern for aspartic and glutamic acid (Figure 7 E-F). Both amino acids are the substrates of two anaplerotic reactions which feed the Krebs cycle, as aspartic acid is the precursor of oxalacetate and glutamate is both the product of the reaction of aspartic acid with α-ketoglutarate and the precursor of α-ketoglutarate.

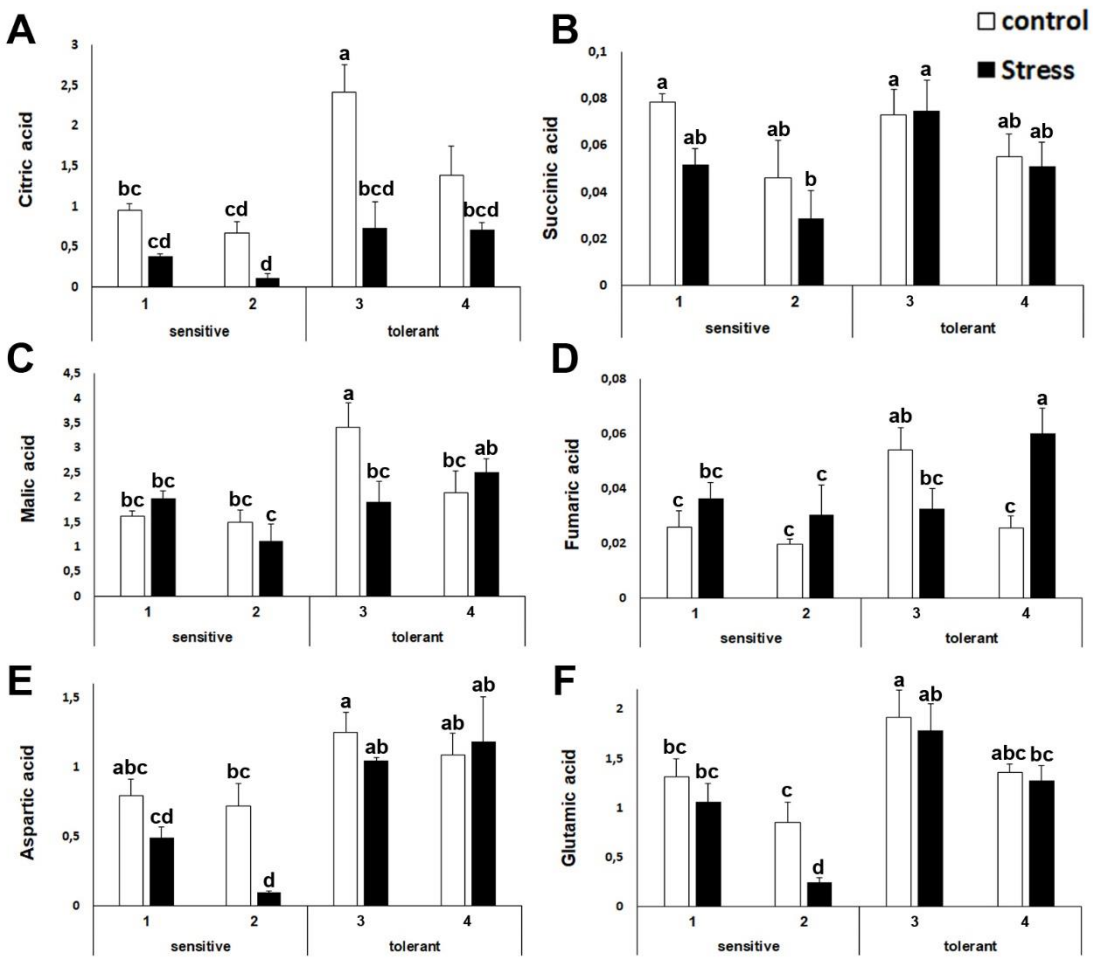


Figure 7. Content of primary metabolites related to the Krebs cycle. Citric acid (A); succinic acid (B); malic acid (C); fumaric acid (D); aspartic acid (E) and glutamic acid (F) levels of salt-sensitive and salt-tolerant cultivars under control (white bars) or stressed (black bars) treatments. The units are the area of the peak per mg of sample. Data with different letter differ significantly ($p<0,05$), as determined by Duncan's MRT test (n=4). Scale bars are mean + SE.

This study also found that for some metabolites there was an effect due to salt stress, but no differential trend among sensitive and tolerant cultivars was observed. This study determined that upon salt stress the levels of myoinositol, hydroxyproline, γ-amino butyric acid (GABA) and galactinol increased 2- to 4-fold in leaves from salt stressed plants (Figure 8 A-D). On the other hand, gluconic acid and the lactone of gluconic acid decreased upon salt stress (Figure 8 E-F).

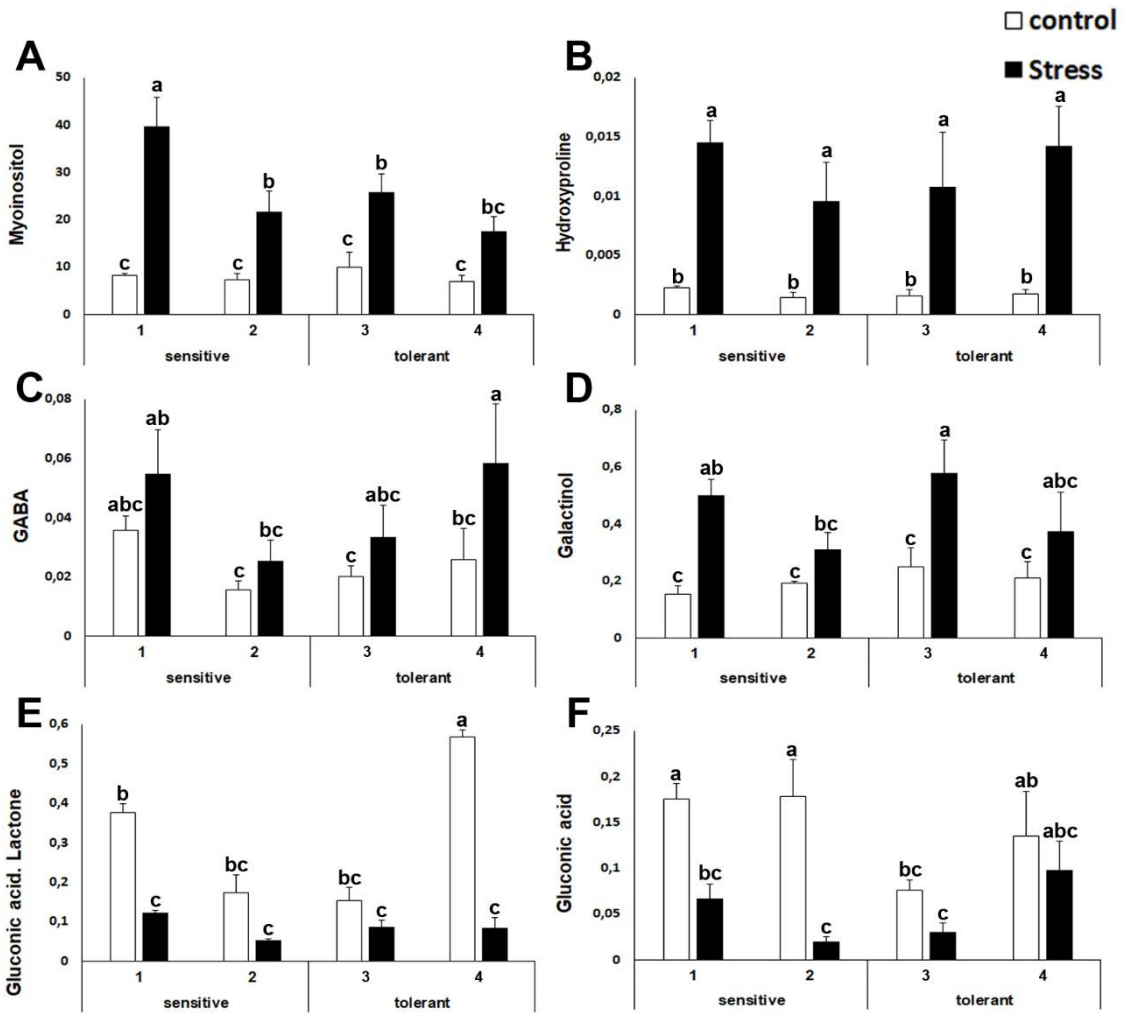
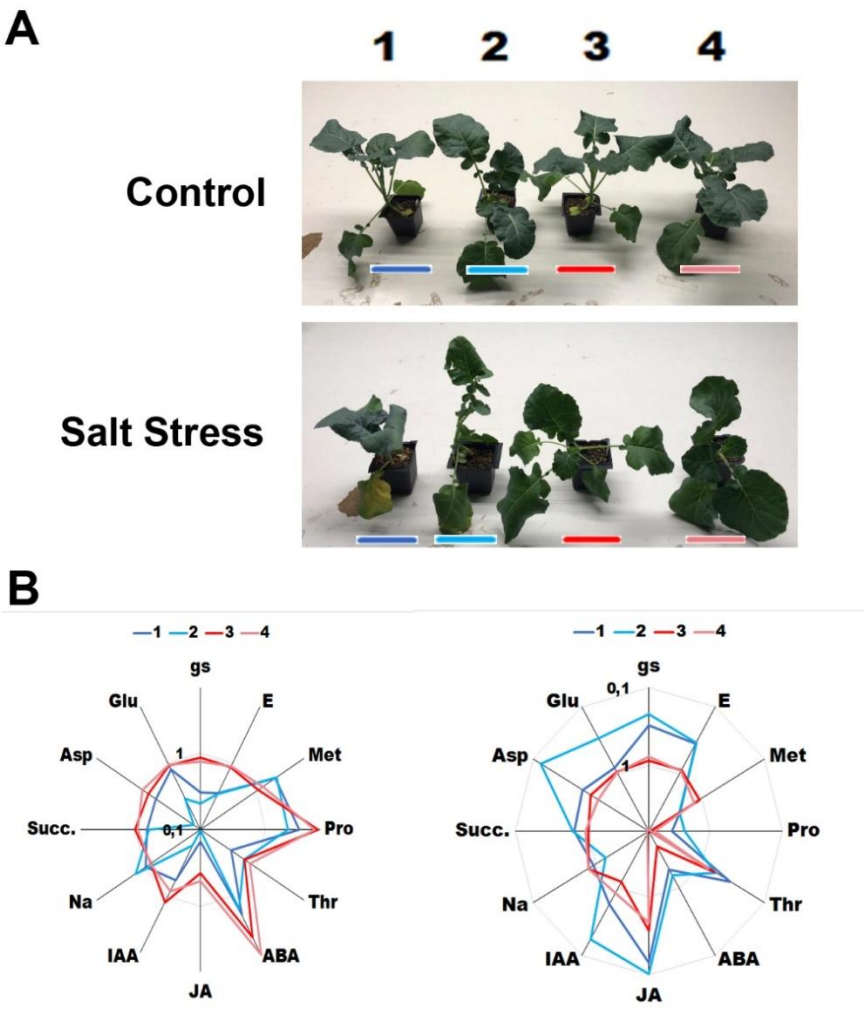


Figure 8. Content of primary metabolites altered by salt stress. Myoinositol (A); hydroxyproline (B); γ-aminobutyric acid (GABA) (C); galactinol (D); gluconic acid lactone (E) and gluconic acid (F) content of salt-sensitive and salt-tolerant cultivars under control (white bars) or stressed (black bars) treatments. The units are the area of the peak per mg of sample. Data with different letter differ significantly ($p<0,05$), as determined by Duncan's MRT test (n=4). Scale bars are mean + SE.

Discussion

This study has compared the effect of salt stress at the molecular and physiological level in two salt tolerant and two salt sensitive broccoli cultivars in order to identify distinctive traits among these cultivars that could predict the performance of uncharacterized cultivars under salt stress conditions and constitute limiting factors for salt stress tolerance. The main findings are summarized in figure 9.


A
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Cys
Met
Ser
Pro
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Phe
Val
Arg
Ile
ABA
JA
IAA
SA
K
Na
K/Na
Citric acid
Succinic acid
Malic acid
Fumaric acid
Aspartic acid
Glutamic acid
Myoinositol
Hydroxyproline
GABA
Galactinol
Gluconic acid Lactone
Gluconic acid

Figure 9. Summary of the main findings of this study. Representative plants of each cultivar under normal watering (upper panel) or after 6 days of salt stress (lower panel) (A). Radial diagrams of the ratio between stress/control content (left) or control/stress content (right). The tolerant lines are shown in shades of blue and the sensitive lines in shades of red. The values are represented in a decimal logarithmic scale (B). Heat map of all the results obtained in the present study. Green indicates higher normalized values, yellow average values while red indicates lower values (C).

A statistical analysis of all the physiological results indicated that the significant differences were linked to the stress sensitivity/tolerance of the cultivar, and not to individual cultivars (Table 1), thus validating our experimental design.

Table 1. Summary statistics of the general linear model and p-values (F) for the effect of treatments (control and salinity), degree of sensitivity to salinity (sensitive and tolerant), cultivars within sensitivities and the interaction between treatments and degree of sensitivity on broccoli plant performance. The numbers in bold indicate significant effects ($p<0.05$) and italics denote marginally significant effects ($p<0.1$).

Variable	Treatment (T) F(p)	Sensitivity (S) F(p)	Cultivar (V) F(p)	TxS F(p)
WP	266.43 (<0.001)	0.10 (0.751)	4.74 (0.358)	1.13 (0.358)
gs	35.61 (<0.001)	9.45 (0.006)	0.06 (0.945)	4.51 (0.015)
E	42.08 (<0.001)	17.91 (0.001)	0.04 (0.961)	6.80 (0.003)
WUE	7.00 (0.015)	*3.61 (0.070)*	0.61 (0.550)	*0.43 (0.092)*
A	6.72 (0.017)	2.66 (0.118)	0.88 (0.430)	1.07 (0.383)
Ci	13.70 (0.001)	*3.20 (0.086)*	0.64 (0.535)	4.22 (0.016)

As mentioned before all cultivars, both salt-sensitive and salt-tolerant were stressed by salinity, according to water potential measurements. However, salt tolerant cultivars were able to better withstand the stress, since values under stressed conditions were very similar to values obtained under control conditions for most of the parameters measured. In all samples tested, the water potential (Ψw) exhibited significant reductions in salt-stressed broccoli (Figure 1A). The validity of the experimental design was also confirmed by the increase in ABA (Figure 5A) and Pro (Figure 3A), which are

standard indicators that the plant is responding to abiotic stress. The effect on stomatal conductance, transpiration and substomatal CO_2 concentration was minor in the salt tolerant plants, probably reflecting that these cultivars were less affected by salt stress. Photosynthetic rates were less affected by salt stress; however, some tolerant cultivars had the same rates under control and salt stress conditions and no changes in transpiration were observed, in contrast to that observed in the salt sensitive plants (Figure 1).

Sulfur metabolism is pivotal in broccoli physiology. The synthesis of cysteine from serine and the subsequent biosynthesis of GSH is a key aspect of antioxidant defense. The serine acetyl transferase enzyme has been described as the main limiting factor for abiotic stress tolerance in several plants (Mulet *et al*., 2004; Freeman *et al*., 2004). Some reports confirm that GSH is the most important thiol involved in the prevention of oxidative damage in plants (Labudda *et al*., 2014; Pyngrope *et al*., 2013). In broccoli, salt stress induces an antioxidant response, which involves the enzymes involved in the regeneration of GSH (Shah *et al*., 2021). In addition, sulfur and GSH are required for the biosynthesis of glucosinolates. This study observed that GSH, Cys and Met accumulation was lower in salt tolerant cultivars (Figure 2). It has been proposed that salt-tolerant species have higher glutathione content and higher redox states in comparison with salt-sensitive species (Shalata *et al*., 2001; Khan *et al*., 2008; Chaparzadeh *et al*., 2004). Nevertheless, in the case of broccoli, it has been shown that glutathione increases after 24 hours of stress and then decreases, and that the main accumulation is observed in roots (Hernandez *et al*., 2010). Therefore, the GSH and sulfur amino acid content in leaves may not be determinant for salt tolerance, and the observed low amounts may indicate that they are being used for the biosynthesis of other molecules (i.e glucosinolates) or are being accumulated in other tissues of the plant. Among others, Met is a substrate for the synthesis of various polyamines with important roles in stress tolerance (Groppa *et al*., 2008; Alcázar *et al*., 2010). Therefore, it is likely that the lower level observed in salt tolerant plants indicates that Met is being recruited for the biosynthesis of molecules related to the stress response. Proline, in addition to its role as an amino acid, is an important osmolyte (Verbruggen *et al*., 2008). As expected, the proline content increased after salt stress (Zaghdoud *et al*., 2012). In fact, broccoli is one of the vegetables capable of accumulating very high levels of proline (Bandurska *et al*., 2013). This study found a distinctive pattern, as salt tolerant

plants presented higher accumulation of proline, both under stress and control conditions, indicating that increased proline accumulation correlates with the salt tolerance of the plant (Figure 3A).

The hormone levels constitute a distinctive factor for salt tolerance or sensitivity in broccoli (Figure 5). Interestingly the ratio of the levels of ABA, JA and IAA from stress and control conditions were higher in salt tolerant cultivars, unveiling another distinctive trait among sensitive and tolerant cultivars (Figure 9 A and B). Salicylic acid levels were lower for tolerant plants. This result is surprising since in some crops it has been shown that external application of salicylic acid is able to alleviate the effects of salt stress (Souana *et al*., 2020).

Potassium is the major cation determining the intracellular ionic environment in plants (Serrano *et al*., 1999; Rodríguez-Navarro *et al*., 2000). Our results indicate that salt tolerant cultivars accumulate less sodium in leaves, thus indicating that the role of the sodium extrusion systems is more limiting than sodium accumulation in the vacuole. There is another interesting outcome. The presence of salt in the soil has an osmotic effect, due to the ability of the ions to retain water. If tolerant cultivars have less sodium and less potassium, in principle, they must have less osmotic potential inside the cell. That means that tolerant cultivars should compensate this loss of ions with osmolytes. This study has found that tolerant cultivars accumulate more proline (Figure 3A). The current model for salt tolerance mechanism in brassica states that this family has a multiplicity of mechanisms, and among them, osmolyte accumulation, sodium extrusion and potassium retention (Shahzad *et al*., 2021). Our experimental design further develops this model as it allows for the identification of the limiting factors for salt tolerance at least in early stages of development (5-6 weeks), as our experiments target to unveil the differences among sensitive and tolerant varieties. Taken together, the ability to extrude sodium, and to compensate for the loss of potassium with osmolytes (such as proline) may be a signature of tolerant cultivars, pointing to proline biosynthesis and sodium extrusion from roots or the cytosol (and not vacuolar accumulation) as the limiting factors for tolerance at the whole plant level, while the ability to accumulate potassium does not appear to be a distinctive factor. Therefore, this study has further defined the current model for salt stress tolerance in broccoli. In addition, this study has discovered that the energetic metabolism, at several steps, is

also a distinctive trait. This is in agreement with the fact that the salt stress response is energetically costly, and thus explains why it is a self-destructive trait, gained often by selection, but frequently lost by reversal or extinction when the selection agent (salt stress) disappears (Bromham *et al*., 2020). Another valuable outcome of this study is that it has described that the levels of several amino acids and metabolites change upon stress, although their levels are not limiting for stress tolerance (Figure 4 and Figure 8). This contrasts to what is known for other crops like tomato, where the changes in the levels of amino acids are a distinctive trait for salt tolerance (Santa-Cruz *et al*., 1999).

As mentioned before, from the bioenergetic point of view, the stress response is expensive. The biosynthesis of osmolytes and the maintenance of ion homeostasis requires large amounts of energy that must be diverted from other physiological processes, mostly related to plant development. This explains why under stress, plants slow or completely arrest their developmental program and yield decreases, which leads to important agricultural losses. Our metabolomic analysis pointed out that salt tolerant cultivars of broccoli have higher amounts of intermediates of the Krebs cycle, and of two substrates of anaplerotic reactions. The Krebs cycle is the main catabolic process for carbohydrates produced in the Calvin cycle. This study has shown before that physiological parameters related to gas exchange were also distinctive for salt tolerant plants and this holds true for broccoli as well. In addition, the anabolism and catabolism of carbohydrates, and thus, the ability to produce energy, is a main determinant for salt tolerance in broccoli, together with the increased osmolyte biosynthesis and sodium extrusion.

This study has used a Greenhouse-based approach to determine differential traits at the physiological and molecular levels between salt-tolerant and salt-sensitive cultivars of broccoli at the initial stages of plant development (5-6 weeks). Our results indicate that the most distinctive trait for salt tolerance in broccoli is related to the ability to maintain photosynthesis and carbohydrate catabolism under salt stress, the levels of proline, the hormone content upon stress, and the ability to extrude sodium (Figure 9).
Taken together, this study proposes that the analysis of proline, hormone levels (i.e. ABA) or Krebs cycle intermediates (i.e. succinic acid) under salt stress in leaves of 5- to 6-week- old plants could be a fast and reliable method to screen for broccoli cultivars tolerant to salt stress. Our findings also may constitute the basis to develop

biotechnological strategies, such as assisted or precision breeding techniques, to generate novel salt tolerant cultivars.

Chapter III

Chapter III

Distinctive traits for drought and salt stress tolerance in melon (*Cucumis melo* L.)

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Abstract

Melon (*Cucumis melo* L.) is a crop with important agronomic interest worldwide. Because of the increase of drought and salinity in many cultivation areas as a result of anthropogenic global warming, the obtention of varieties tolerant to these conditions is a major objective for agronomical improvement. The identification of the limiting factors for stress tolerance could help to define the objectives and the traits which could be improved by classical breeding or other techniques. With this objective, we have characterized, at the physiological and biochemical levels, two different cultivars (sensitive or tolerant) of two different melon varieties (Galia and Piel de Sapo) under controlled drought or salt stress. We have performed physiological measurements, a complete amino acid profile and we have determined the sodium, potassium and hormone content. This has allowed us to determine that the distinctive general trait for salt tolerance in melon are the levels of phenylalanine, histidine, proline and the Na^+/K^+ ratio, while the distinctive traits for drought tolerance are the hydric potential, isoleucine, glycine, phenylalanine, tryptophan, serine and asparagine. These could be useful markers for breeding strategies or to predict which varieties are likely perform better under drought or salt stress. Our study has also allowed us to identify which metabolites and physiological traits are differentially regulated upon salt and drought stress between different varieties.

Keywords: Melon, *Cucumis melo*, salt stress, drought stress, amino acids, plant hormones, ion content.

Introduction

Melon (*Cucumis melo L.*) is a major crop with great agronomic and economic interest, considered a gourmet food in several markets and cultures. One of the main problems for melon farming is that its cultivation demands a lot of water (Cabello *et al*., 2009). In the current context of anthropogenic global warming and the subsequent climate change, aridity is increasing in traditional cultivation areas, and thus, melon culture is subjected to increasing abiotic stress, which compromises the yield. Specifically, drought stress is increasing, and salt stress is directly related to this water scarcity, given that excessive irrigation increases the salt deposition in the soil and diminishes the phreatic level, thus enabling the infiltration of sea water. It is estimated that 20% of all arable land and almost half of the land with water availability are affected by salts, significantly reducing yield below the genetic potential of most crops (Botella *et al*., 2007; Chandna *et al*., 2014). As a result of salinization, crop yields are declining while arable land is being irreversibly lost (Nawaz *et al*., 2010). High salinity levels also increase soil pH. In addition, saline stress leads to deterioration of soil structure and prevents the air-water balance, essential for biological processes occurring in the roots (Galvan-Ampudia *et al*., 2013). Saline soils reduce the biomass production of crops affecting important biochemical and physiological processes in the plant (Serrano *et al*., 1999).

We have generated considerable knowledge at the biochemical level and physiological level regarding how abiotic stress affects basic physiological processes, the cellular function and even the biochemical targets, but there are still large gaps in our knowledge about the limiting factors for stress responses. More specifically, we are lacking knowledge regarding which traits could be improved by breeding or genetic engineering that would have a major impact on plant growth and development under stress conditions. This explains the low success in breeding novel crops that are adapted to saline soils or are able to maintain yield under drought stress conditions (Ashraf *et al*., 2009). Proof of this scarcity of results is that there are only two GMO cultivars on the market whose trait is drought tolerance: the Droughtgard maize from BASF and the HB4 soy from Agroceres (Wang *et al*., 2015; Ribichich *et al*., 2020). To date, there are no marketed biotechnological crops with enhanced yield under saline conditions.

Several strategies have been developed to identify the limiting factors for stress tolerance. Evaluating the physiological and biochemical response of stress tolerant and stress sensitive plants is a well-established strategy to discover differential traits for abiotic stress tolerance (Taibi *et al*., 2017; Taibi *et al*., 2018; Chevilly *et al*., 2021). All these analyses have been performed testing different cultivars from the same variety and a single stress. We have further developed this concept by evaluating, in the same analysis, different stresses and different cultivars of two different varieties to find limiting factors which are not particular to a specific variety or stress. In this report, we have applied this strategy to a pivotal horticultural crop for the economy in the Mediterranean area. There are several reports evaluating Galia melon performance under salt stress in field conditions (Akrami & Arzami, 2018; Akrami *et al*., 2019), but so far, there are no studies evaluating the plant response at the initial stages of development under controlled conditions. This work has been designed to determine the differences at the physiological and biochemical levels between different melon genotypes under two different abiotic stresses. These varieties had previously been characterized as sensitive or tolerant to abiotic stress. We have subjected these varieties to controlled drought or salinity stress, and have monitored different physiological or biochemical parameters, in order to find changes that are relevant among varieties or treatments. This will allow us to identify the limiting factors in abiotic stress tolerance and will help to define novel breeding strategies.

Materials and methods

Plant material

The four pre-commercial varieties of melon *(Cucumis melo* L.) seeds used were provided by Enza Zaden and referred to as Cv. 1, Cv. 2, Cv. 3 and Cv. 4. Cv. 1 is a Galia melon (*Cucumis melo* Cv. *reticulatus*) tolerant to abiotic stress, Cv. 2 is a Galia type melon sensitive to abiotic stress; Cv. 3 melon is a Piel de Sapo (a.k.a. Santa Claus Melon; *Cucumis melo* Cv. *inodorus*) tolerant to abiotic stress and Cv. 4 is a Piel de Sapo sensitive to abiotic stress.

Experimental design

For different experiments, 20 seeds of each variety were germinated in a Petri dish with moist sterile Whatmann filter paper. After five days, seedlings were transferred to a substrate (50% kekkila peat, 25% perlite, 25% vermiculite) in individual plant pots 12 cm diameter x 8 cm height. The experimental design consisted of an aleatory placement where each block was composed by 4 pots per tray and one plant per pot. Each experiment consisted in 5 individuals × 4 varieties x 3 treatments (60 total plants). Plants were watered with Hoagland solution. After 3 weeks, when plants reached the four-leaf phase, irrigation was maintained (control plants), limited (drought stress) or watered with Hoagland solution plus 220 mM NaCl (salt stress). Samples were taken or measurements were performed after six days of stress treatment (salt stress) or when the total weight (plant and container) was reduced to 60% of their initial weight (drought stress), at about 9 days. In all cases, the number of samples per experiment (n) refers to biological replicates from different plants (between 3 to 5). All samples for each treatment were collected at the same time. Plants were grown in a phytotron at 25 ± 2^{o}C, humidity of 50-60% and a photoperiod of 16h light/8h darkness (200 μmol m^{-2} s^{-1} of light intensity). All experiments were replicated to check the reproducibility of the results.

Physiological measurements

The water potential (Ψ_w, MPa) was measured with a Schölander pressure pump (model PMS-1000, PMS Instruments, Corvallis, OR, United States). Stomatal conductance (g_s,

mmol H_2O $m^{-2}s^{-1}$), the sub-stomatal concentration of CO_2 (C_i), photosynthetic rate (A, µmol CO_2 $m^{-2}s^{-1}$), transpiration (E, mmol H_2O $m^{-2}s^{-1}$), water use efficiency (WUE, µmol CO_2 $mmol^{-1}H_2O$) and leaf temperature through infrared Thermometry (Tleaf, °C), were determined with a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury MA). The measurements were recorded under saturating light conditions (1500 µmol quanta m^{-2} s^{-1}), with a temperature of 25°C, and ambient CO_2 concentration of 400 mol $^{-1}$ CO_2 and a relative humidity of approximately 55%. Chlorophyll fluorescence indices (i.e., Fv/Fm and Quantum yield) were measured with a portable pulse-amplitude modulated chlorophyll fluorometer (PAM-2100, Heinz Walz, Effeltrich, Germany). These measurements of the photosystem II efficiency were performed once the plants were adapted to darkness for thirty minutes, on the same leaves where stomatal conductance and photosynthesis were determined. All measures were performed on the third youngest full-developed leaf of each plant, analyzing a total of five plants per variety.

Amino acid analysis

One gram of the third youngest leaf was taken, lyophilized and ground with a mortar and pestle in the presence of liquid nitrogen. The resulting powder was homogenized for 30 seconds with 2 mL of 2% citrate buffer pH 2 (Mulet *et al*., 2004) and centrifuged for five minutes at 13000 *g*. The supernatant was filtered through a 25-micrometer pore-size non-sterile filter. 1/10 dilutions of these extracts were injected into an automatic Beckman Gold amino acid analyzer. The analysis was carried out according to the protocol supplied by the manufacturer, using a system of ninhydrin and sodium citrate for detection. Measurements were normalized to dry weight.

Hormone quantification

Plant hormones were determined following the method of Durgbanshi (Durgbanshi *et al*., 2005). Briefly, lyophilized samples were ground to powder in the presence of liquid nitrogen. 200 mg per replicate were purified with solid phase extraction columns (SPE; reverse phase and ion exchange), using internal deuterated standards. The analysis was carried out using UPLC-mass spectrometry (Acquity SDS, Waters Corp., Milford, MA). Measurements were normalized to dry weight.

Ion content determination

Ions were determined as described (Gisbert *et al*., 2020). Briefly, samples of the third youngest leaf from 1-month-old plants (about 1 g) were dried at 70 °C for 4 days. Dry weight was determined, and ions were extracted by a 30 min incubation in 1 mL of 0.1M HNO_3 at room temperature. Then samples were centrifuged, and the supernatant was diluted with 4 mL of milliQ water and filtered (22 µM). Sodium and potassium were measured in a plasma emission spectrophotometer (Shimadzu), as described (Rios *et al*., 2012). Measurements were normalized to dry weight.

Statistical analysis

The ANOVA was performed by using the SPSS software v.25.0 statistical package (IBM SPSS Statistics for Windows, Armonk, NY, USA; IBM Corp.). The means were considered to be significantly different at $p < 0.05$ after Duncan's new multiple range test (MRT) (Duncan, 1955).

Results

Physiological determinations

Several responses of plants to abiotic stress occur at the physiological level. We investigated whether we could identify differential responses among varieties or cultivars. As expected, the water potential increased upon stress between 1,12 and 1,25 for salt stress and 1,3 and 2,67 for drought stress (expressed as -MPa; Fig. 1A), thus validating our experimental design. The tolerant cultivars presented higher values. The salinity treatment had a negative effect on stomatal conductance (gs), while the drought stress had a more modest effect on this parameter, observing minor differences when compared to the corresponding control (Fig. 1B). A similar pattern was found with transpiration (E) and photosynthesis (A), which was stable upon drought stress, but decreased upon salt stress. Interestingly A also decreased upon drought stress in the tolerant Galia cultivar (Fig. 1C and 1D). Maximum efficiency of photosystem II (determined as Fv/Fm) and quantum yield presented minor, but in some cases significant changes (Fig. E and F). Water Use efficiency, intrinsic and instantaneous, decreased under drought stress and increased upon salt stress in Galia plants, but was stable in Piel de Sapo (Cv. 3; Fig. 1G and 1H).

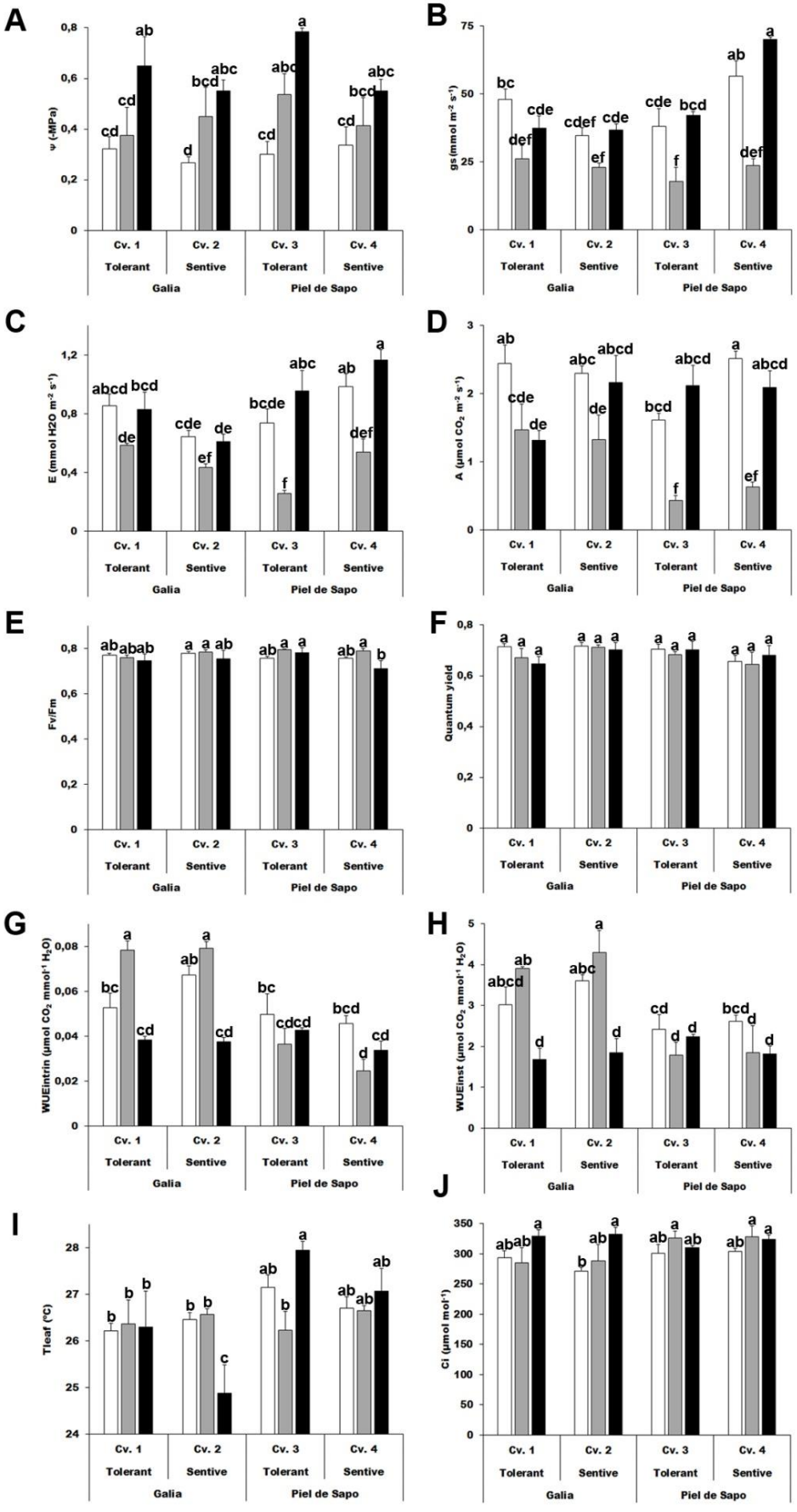
A
ψ (-MPa)
B
gs (mmol m-2 s-1)
C
E (mmol H2O m-2 s-1)
D
A (µmol CO2 m-2 s-1)
E
Fv/Fm
F
Quantum yield
G
WUEintrin (µmol CO2 mmol-1 H2O)
H
WUEinst (µmol CO2 mmol-1 H2O)
I
Tleaf (°C)
J
Ci (µmol mol-1)
Cv. 1
Tolerant
Cv. 2
Sentive
Cv. 3
Tolerant
Cv. 4
Sentive
Galia
Piel de Sapo

Figure 1. Physiological measurements. Water potential (Ψw) (A); stomatal conductance (gs) (B); transpiration (E) (C); Net photosynthesis (A) (D); Quantum efficiency of photosystem II (Fv/Fm) (E); Quantum yield (F); intrinsic water use efficiency (WUEintr) (G); instantaneous water use efficiency (WUEinst) (H); Leaf temperature (Tleaf) (I) and sub-stomatal CO_2 concentration (Ci) (F) of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3) or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (grey bars) and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 5$). Scale bars are the mean ± standard error (SE). Experiment was replicated with similar results.

We also determined the leaf temperature and found a differential response among varieties. In Galia, the leaf temperature decreased in the tolerant variety upon drought stress about 4%, and in the Piel de Sapo the leaf temperature increased in the tolerant variety about 0,3% (Fig. 1I). We observed minor effects on the sub-stomatical CO_2 concentration (Ci) (Fig. 1J).

Amino acid measurements

Once we had studied the response of the selected varieties and cultivars at the physiological level, we further investigated the level of amino acids. First, we focused on the hydrophobic amino acids (Fig. 2). In most cases, there was no distinctive pattern. However, for leucine (Leu), the content increased under salt stress (between 40-115%), and to a minor extent, under drought stress (between 0 to 67%) (Fig. 2B). Glycine (Gly) can act as an osmolyte and is a precursor of antioxidant molecules, such as the tripeptide glutathione. Its content under stress conditions correlated with tolerance to stress, but only for the Piel the Sapo variety (Fig. 2E). Similarly, phenylalanine (Phe) levels under drought stress correlated with sensitivity (Fig. 2F). Finally, we observed a 7-fold increase in Tryptophan (Trp) content in Piel de Sapo sensitive cultivar under drought stress conditions (Fig. 2G).

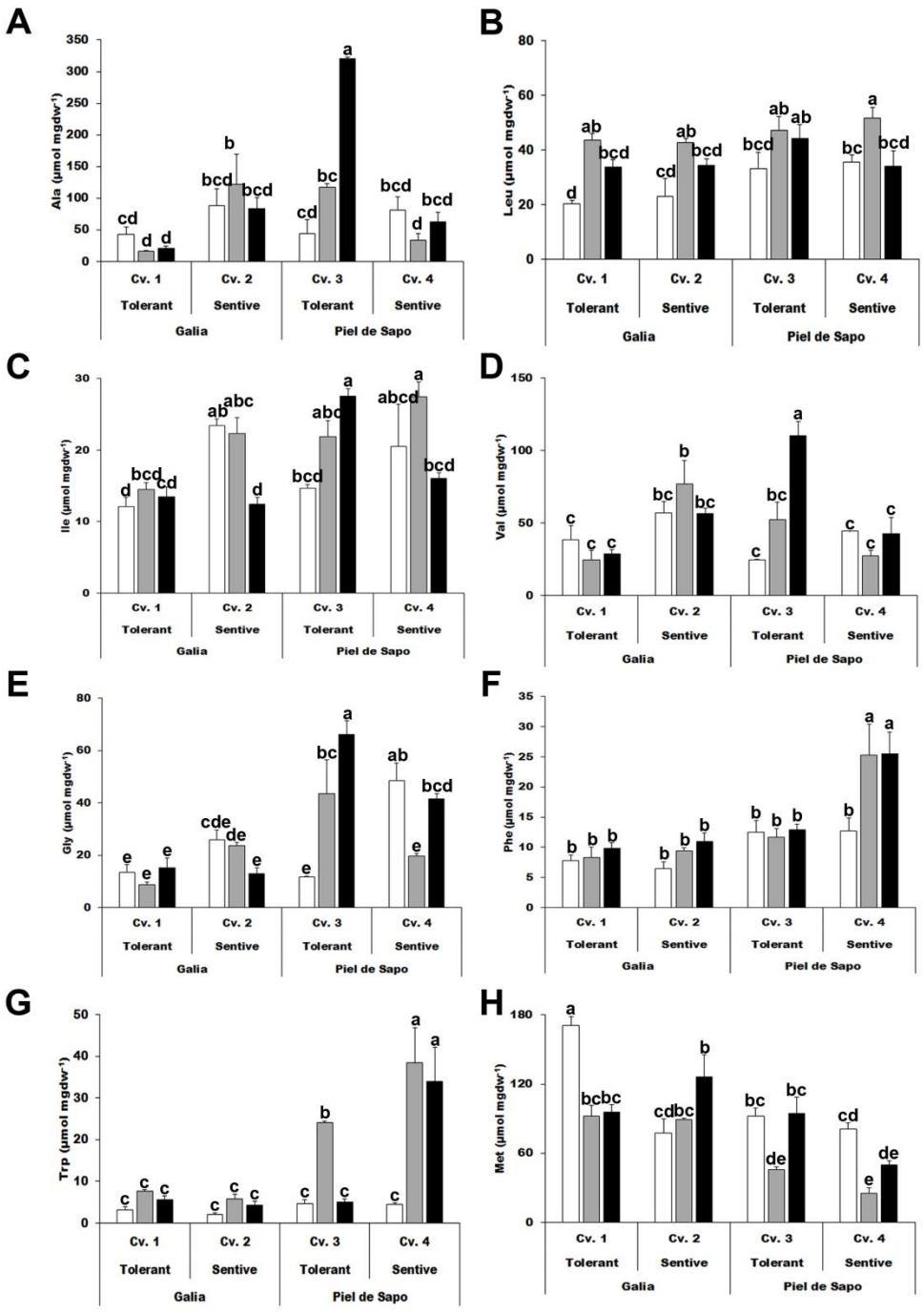

Figure 2. Hydrophobic amino acids. Alanine (Ala) (A); leucine (Leu) (B); isoleucine (Ile) (C); valine (Val) (D); glycine (Gly); phenylalanine (Phe); tryptophan (Trp) and methionine (Met) of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3) or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (grey bars) and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test (n = 3). Scale bars are mean ± SE. Experiment was replicated with similar results.

We further investigated the polar amino acids. Serine (Ser) levels increased, between 2,3- to 4-fold, under salt stress (Fig. 3A) and similar results were obtained for asparagine (Asn). For other amino acids, such as threonine (Thr), cysteine (Cys), proline (Pro) or glutamine (Gln), we did not find a distinctive pattern (Fig. 3).

We also studied the charged amino acids and found that an increase in the levels of lysine (Lys) correlate with salt tolerance, but for drought tolerance only in the case of the Galia cultivar (Fig. 4A). Also, an approximate 4-fold increase of histidine (His) content was observed under drought stress conditions for Galia cultivars (Fig. 4C). Aspartic acid (Asp) levels behaved in disparate manners: in Galia they increased under salt stress in the sensitive cultivar (Cv. 2), while in Piel de Sapo, they increased in the tolerant cultivar (Cv. 3; Fig. 4D). Glutamic acid (Glu) levels increased under salt and drought stress with respect to the control only in Piel de Sapo (Fig. 4E).

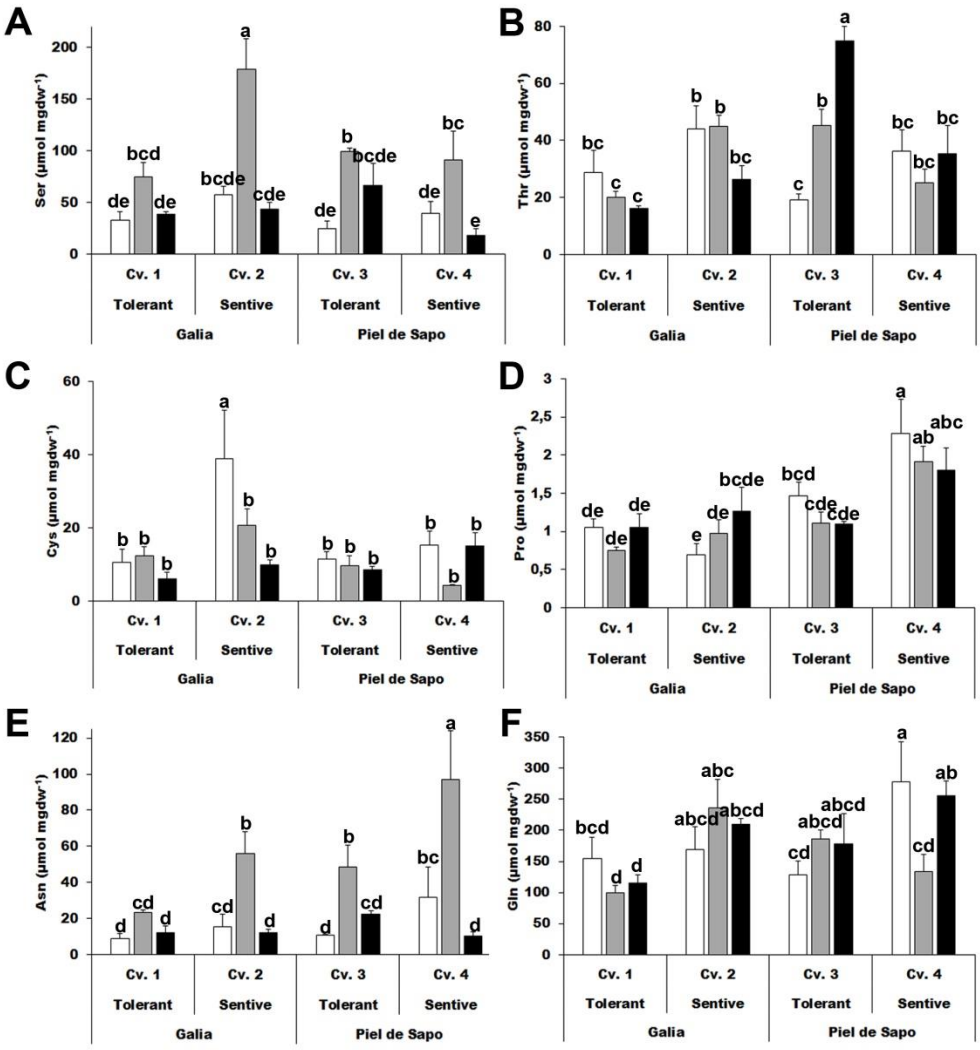

Figure 3. Polar amino acids. Serine (Ser) (A); threonine (Thr) (B); cysteine (Cys) (C); Proline (Pro) (D); asparagine (Asn) (E) and glutamine (Gln) (F) of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3) or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (grey bars) and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test (n = 3). Scale bars are mean ± SE. Experiment was replicated with similar results.

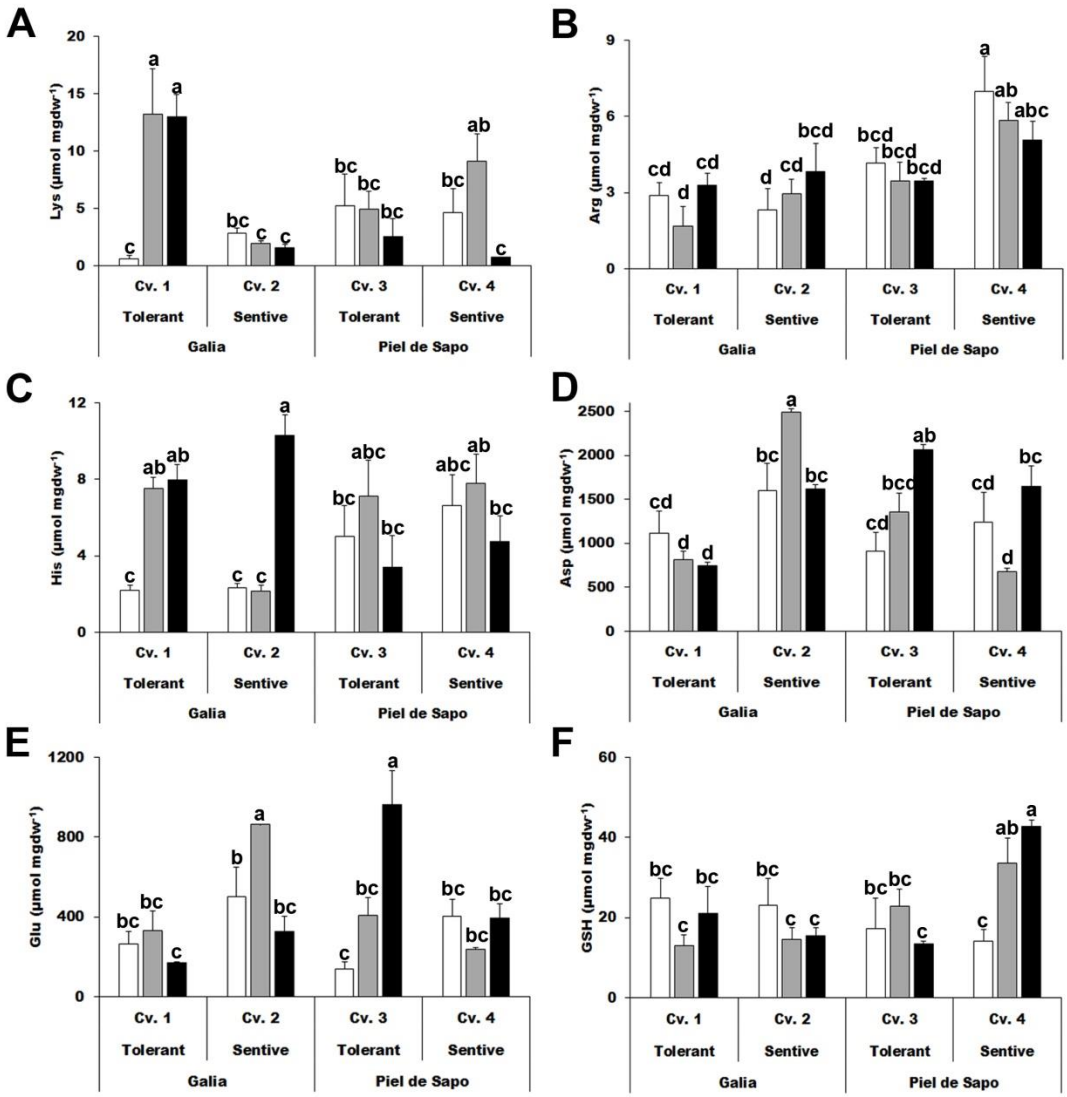

Figure 4. Charged amino acids. Lysine (Lys) (A); arginine (Arg) (B); Histidine (His) (C); aspartic acid (Asp) (D); glutamic acid (Glu) (E) and glutathione (GSH) (F) content of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3) or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (grey bars) and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test (n = 3). Scale bars are mean ± SE. Experiment was replicated with similar results.

Sodium and potassium content

We determined the ion content of the investigated varieties and cultivars under control and stress conditions. As expected, the potassium content decreased under salt stress conditions (between 5% to 40%), as sodium competes with potassium. Under drought stress, the potassium content also decreased about 10%. Potassium has been described to act as an osmolyte, but according to our results, that is not its main

role in the investigated plants (Fig. 5A). Sodium levels behaved differently depending on the variety. Sodium levels were higher in the tolerant cultivar in Galia plants, while the levels were lower in tolerant cultivars in Piel de Sapo plants (Fig. 4B). In all cases, the Na^+/K^+ ratio was higher for tolerant cultivars (Fig. 4C).

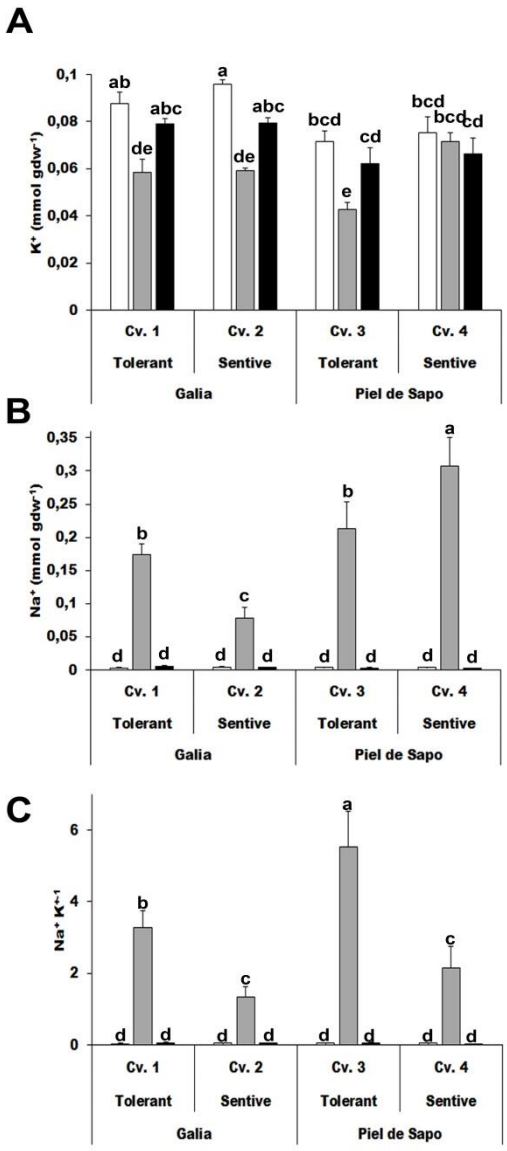

Figure 5. Ion content determination. Potassium content (K^+) (A), sodium content (Na^+) (B) and the Na^+/K^+ ratio (C) of the tolerant Galia genotype (Cv. 1), sensitive Galia genotype (Cv. 2), tolerant Piel de Sapo genotype (Cv. 3) or sensitive Piel de Sapo genotype (Cv. 4) under control (white bars), salt stress (grey bars) and drought stress (black bars) conditions. Data with different letter differ significantly ($p<0,05$), as determined by Duncan's MRT test (n=5). Scale bars are mean ± SE. Experiment was replicated with similar results.

Hormone determination

One of the most determinant aspects of stress tolerance is the hormonal response. Hormones, such as abscisic acid (ABA) or salicylic acid (SA), are directly involved in the response to abiotic stress, while other hormones, such as indolacetic acid (IAA) or jasmonic acid (JA) are mainly related to growth, but indirectly may affect the response to abiotic stress. We determined the levels of different hormones under control and stress conditions. IAA levels increased 17-fold under salt stress in the tolerant cultivar of the Piel de Sapo variety (Fig. 6A). Levels of JA decreased upon stress in the tolerant Galia cultivar (70% for salt stress and 43% in drought stress) and increased (1,91-fold for salt stress and 3,53-fold drought stress) in the sensitive cultivar (Fig. 6B). As expected, ABA levels increased upon stress, but the increase was more pronounced in Piel de Sapo plants under salt stress (between 8-11-fold) (Fig. 6C). SA levels also increased upon stress but, again, only in Piel de Sapo plants (Fig. 6D).

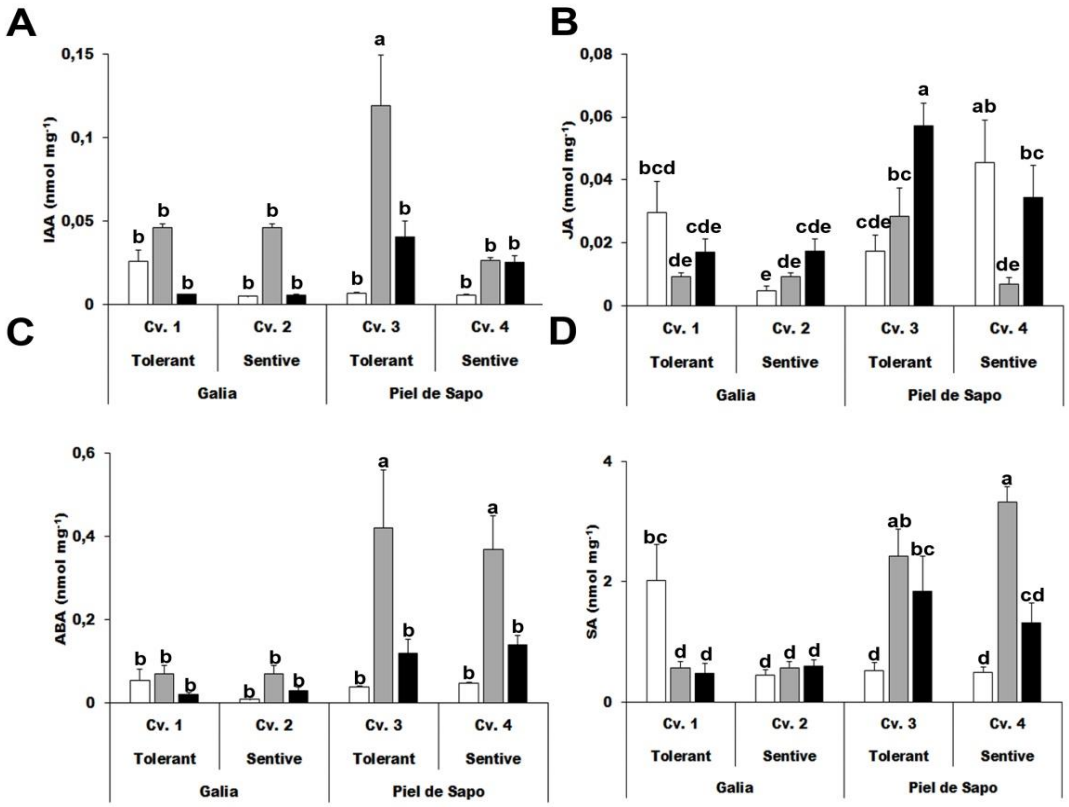

Figure 6. Hormone levels. Indolacetic acid (IAA) (A); jasmonic acid (JA) (B); abscisic acid (ABA) (C) and salicylic acid (SA) (D) content of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3) or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (grey bars) and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test (n = 5). Scale bars are mean ± SE. Experiment was replicated with similar results.

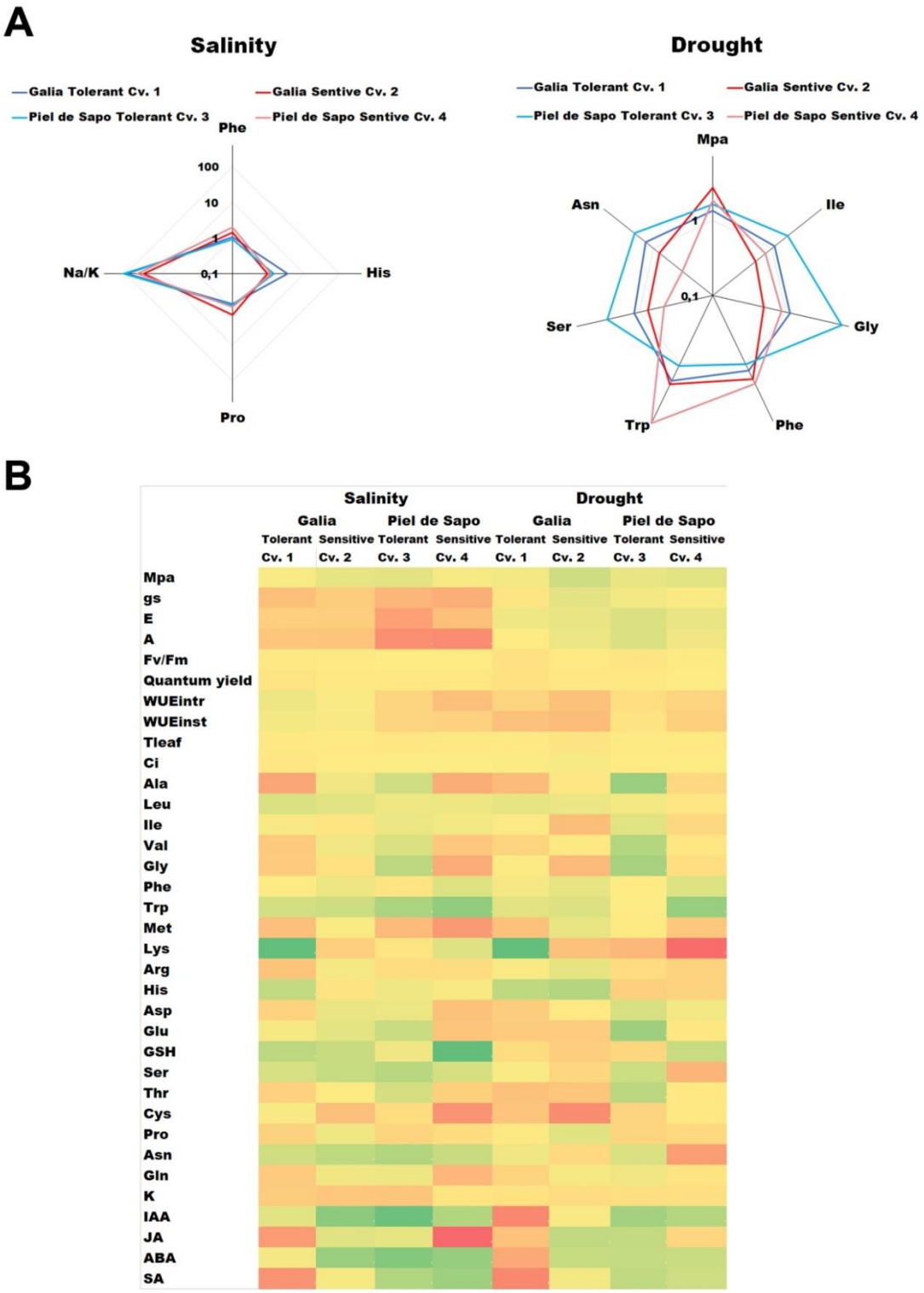

Figure 7. Summary of the main findings of this study. (A) Radial diagrams of the ratio between stress/control levels under salt stress (left) or stress/control levels under drought stress (right). The tolerant cultivars are shown in shades of blue and the sensitive cultivars in shades of red. The values are represented in a decimal logarithmic scale. (B) Heat map of all the results obtained in the present study. Green indicates higher stress/control values, yellow average stress/control values while red indicates lower stress/control values in a decimal logarithmic scale.

Discussion

The main objective of this study is to compare physiological and biochemical responses of two cultivars of two different varieties, to both salt and drought stress, in order to find common patterns among different varieties. We included stress tolerant and sensitive cultivars as well to gain further insight into differential responses within varieties. Through the relativization of data (i.e., the ratio of the value under stress with respect to the value under control conditions) we have found that, irrespectively of the variety, tolerance to salt stress correlates with higher ratios (stress/control) for His (3.4 and 1.42 for tolerant, vs, 0.92 and 1.18 for sensitive) and Na^+/K^+ (11.,37 and 91.3 for tolerant vs. 31.4 and 47.6 for sensitive) and lower Phe (0.93 and 1.06 for tolerant vs. 1.45 and 2.0 for sensitive) and Pro ratios (0.71 and 0.75 for tolerant vs. 1.41 and 0.83 for sensitive). In the case of drought stress, tolerance correlates with increased ratios of Ile (1.87 and 1.1 for tolerant vs. 0.78 and 0.5 for sensitive), Gly (5.68 and 1.12 for tolerant vs. 0.85 and 0.5 for sensitive), Ser (2.72 and 1.17 for tolerant vs. 0.75 and 0.46 for sensitive) and Asn (2.12 and 1.38 for tolerant vs. 0.79 and 0.32 for sensitive), and decreased hydric potential and decreased ratios of Phe (1.26 and 1.03 for tolerant vs 2.0 and 1.69 for sensitive) and Trp (1.08 and 1.79 for tolerant vs. 2.0 and 7.61 for sensitive) (Fig. 7A and supplemental table 1). All the results are summarized in the form of a heat map in figure 7B. The numerical data of the ratios of the Stress/control for all values are presented in Supplemental table 1.

One interesting aspect of our results is that, among varieties, physiological parameters are not a distinctive trait for abiotic stress tolerance. Several previous studies have determined the effect of stress on melon physiology. In a recent study on muskmelon genotypes under drought stress, the net photosynthetic rate, stomatal conductance (Gs) and the transpiration (E) rate decreased (Ansari *et al*., 2019). Other reports described similar decreases in stomatal conductance in genotypes different from the ones that we have used in this study (Kusvuran, 2012; Wang *et al*., 2016). There are reports indicating that during drought stress in melon, there was a significant increase in water use efficiency in drought tolerant genotypes (Akhoundnejad % Dasgan, 2019). In our case, under drought conditions, we did not observe any significant differences with the control values, that is, efficiency is maintained, although

in this report plants were grown in field conditions and measures were taken in older plants.

We have also calculated the differential traits between Galia and Piel de Sapo irrespectively of their stress tolerance (Supplemental Figure 1). In this case, the Piel de Sapo variety showed higher Stress/control ratio for E, A, WUEintr and WUEinst under salt stress (Fig. 8). In agreement with our results, it has been previously described that under salt stress in muskmelon there is a significant increase in WUEintr and WUEinst with respect to control conditions (Ansari *et al*., 2018). Our data under saline stress conditions showed no changes for Galia plants, but we confirmed the increase in our conditions for Piel de Sapo cultivars. The fact that most of the differences observed in the physiological traits are variety dependent and not stress dependent may be explained by the differences in the leaf morphology.

There is no description available in the literature regarding the behavior of the free amino acid pools under salt and drought stress in *Cucumis melo* comparing stress and different varieties, so here we have investigated the complete free amino acid profile in our plants under the studied conditions. Salt tolerance correlated with higher levels of His in tolerant plants. This has been related to tolerance against heavy metals as it can chelate them, but its role in salt stress tolerance has not been described. Proline it is known to act as and osmolyte, but we have found that tolerant plants have less stress/control ratio than the sensitive ones, pointing out that the increases observed upon salt stress are not determinant for salt stress tolerance. In the case of drought stress, Ile, Gly, Ser and Asn were higher in tolerant varieties. Glycine can act as an osmolyte and also is a component of the tripeptide glutathione. Serine is also a precursor of cysteine and other stress-related molecules. On the other hand, high stress/control ratios of Phe correlated with sensitivity to stress, and it is the only molecule that decreased under conditions of both drought and salt stress. Phe is a precursor of several molecules, among them lignin, a pivotal molecule for cell wall biosynthesis. The accumulation of Phe in sensitive cultivars, irrespectively of the varieties, may be a symptom that basic plant processes like cell wall biosynthesis are more affected by stress than in tolerant cultivars.

Potassium is the major ion in the cytoplasm and thus is largely responsible for the intracellular ionic environment. Sodium is toxic for melon plants and must be extruded from the cell or accumulated in the vacuole. Regarding ion accumulation, our results suggest that the limiting factor for stress response is the ability to accumulate sodium (Serrano *et al*., 1999; Rodríguez-Navarro, 2000). Under salt stress, plants can extrude sodium from the root, or take it up, transport it to the aerial part and accumulate it in the vacuoles (Arzani & Ashraf, 2016). The higher Na^+ and Na^+/K^+ ratio of a salt-tolerant variety in our study may be explained by the vacuolar accumulation of sodium in the leaf tissues. Similar results regarding the Na^+/K^+ ratio and water use efficiency were observed in a field trial with *Cucumis melo cv. Huanghemi* (Tedeschi *et al*., 2017) so the trend is the same, even when we compare field/greenhouse conditions and early development/late develompment. Here we demonstrate that the ability to accumulate sodium and maintain a high Na^+/K^+ ratio is a distinctive trait for tolerant cultivars. We did not find any distinctive pattern with the potassium levels under drought stress. Therefore, the role of potassium as an osmolyte is not a limiting factor for drought tolerance in these melon cultivars.

Melon is a climacteric fruit, so its hormonal levels are subjected to drastic changes (Dunlap *et al*., 1996). There are several descriptions in the literature of the hormonal levels in cucurbit plants under abiotic stress. For instance, exogenous application of SA increases drought tolerance in muskmelon (Korkmaz *et al*., 2007), similar to what is observed in other cultivated plants (Souana *et al*., 2020). In addition, SA and JA levels increase upon spermidine addition and increase tolerance to salt stress (Radhakrishnan & Lee, 2013). Also, JA levels tend to increase in cucumber plants subjected to drought stress (Llanes *et al*., 2016). It has also been described that under mild or moderate water stress, IAA content tend to increase (Huang *et al*., 2018). ABA is the main player in the abiotic stress response in plants, and it has also been described to increase upon abiotic stress in melon (Sun *et al*., 2013). When we studied the phytohormone levels, we did not find any common pattern among the sensitive or tolerant varieties and cultivars studied, although the levels of SA and IAA were higher in Piel de Sapo cultivars (between 3- and 7-fold for SA and 4- to 17-fold for IAA).

Taken together, we have performed a complete study of two different melon varieties comparing sensitive and tolerant cultivars and applied statistical tools to the results to

find common patterns in salt or drought stress responses that could be useful to predict the behavior of uncharacterized varieties and cultivars and to design novel classical or biotechnological breeding strategies. Varieties or cultivars with increased His content and/or the ability to accumulate sodium (likely in the vacuoles) may display improved tolerance to salt stress, while novel varieties with enhanced levels of Ile, Gly, Ser and Asn could show better performance under drought stress conditions. High levels of Phe seem correlate with diminished tolerance to abiotic stress. Thus, our results have provided a useful framework for future studies which will examine the ability of these parameters to predict stress tolerance performance in additional melon varieties and cultivars.

Chapter IV

Chapter IV

Identification of distinctive primary metabolites influencing broccoli (*Brassica oleracea*, var. *Italica*) taste

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Abstract

Broccoli (*Brassica oleracea* L. var. *Italica* Plenck) is a cruciferous crop considered a good source of micronutrients. Better taste is a main objective for breeding, as consumers are demanding novel cultivars suited for a healthy diet, but more palatable. This study aimed to identify primary metabolites related to cultivars with better taste, according to a consumers panel. For this purpose, we performed a complete primary metabolomic profile of 20 different broccoli cultivars grown in the field and contrasted the obtained data with the results of a consumer panel which evaluated the taste of the same raw buds. A statistical analysis was conducted to find primary metabolites correlating with better score in the taste panels. According to our results, sugar content is not a distinctive factor for taste in broccoli. Accumulation of the amino acids leucine, lysine and alanine, together with Myo-inositol negatively affected taste, while a high content of γ-aminobutyric acid (GABA) is a distinctive trait for cultivars scoring high in the consumers panels. A Principal Component Analysis (PCA) allowed us to define three different groups according to the metabolomic profile of the 20 broccoli cultivars studied. Our results suggest molecular traits that could be useful as distinctive markers to predict better taste in broccoli or to design novel biotechnological or classical breeding strategies for improving broccoli.

Keywords: Broccoli; *Brassica oleracea*, taste, consumers, metabolites, GABA

Introduction

Broccoli (*Brassica oleracea* var. *Italica*) is a plant that belongs to the *brassicaceae* family. During the last decades, its importance has increased. In 1980, the global production of broccoli and cauliflower was 5.94 million metric tons, while in 2019 the production increased to 26.9 million metric tons (FAOSTAT, 2020), with China and India as the main producers. This increase in production has come as a consequence of increasing consumer demand. One of the reasons explaining this dramatic increase is that broccoli is considered to be part of a healthy diet, as it provides valuable molecules and micronutrients. Broccoli is rich in vitamins C and E, quercetin or kaempferol glycosides (Jeffery *et al*., 2009). Broccoli is also rich in glucosinolates, a group of about 120 molecules derived from amino acids that have a β-D-glucopyranose residue linked through a sulfur atom to a (Z)-N-hydroximinosulfate ester, plus a variable R group (Fahey *et al*., 2001). These molecules confer the characteristic pungent flavor of broccoli. Moreover, some products derived from glucosinolate hydrolisis, such as sulforaphane, may reduce the risk of lung, breast, gastric, prostate or kidney cancer (Gupta *et al*., 2014; Li *et al*., 2010).

Broccoli is not very popular among some consumers, especially children (Tauriello *et al*., 2021). Some adult consumers also dislike broccoli, and this could be explained by genetic variations related to the capsaicin receptor TRPV1, which may render some populations very sensitive to components present in broccoli, thus explaining the aversion (Everaerts *et al*., 2011). Studies on organoleptic and chemical properties of broccoli buds have been carried out to meet consumer demands and to promote the consumption of healthy foods. Different broccoli cultivars diverge in external and internal sensory attributes. Among the factors that influence consumer preferences there are visual aspects (color), taste (bitter and sweet), and flavor aspects. Flavor has been defined as a mingled but unitary experience which includes sensations of taste, smell, and pressure, and often cutaneous sensations such as warmth, color, or mild pain. Flavor depends on different parameters, including aromatic volatiles, and especially, the sugar/acid ratio (Kramer *et al*.,1959; Barret *et al*., 2010). In broccoli, flavor has been described as green/grassy, spicy, broccoli-like, cabbage-like, cauliflower-like, kohlrabi-like, leek-like and mouth-feel pungent (Schnhof *et al*., 2004).

Important differences have been described in terms of taste and flavor among broccoli cultivars. The molecular mechanism underlying these large differences may be explained, at least in part, by changes in the metabolic profile. To confirm this hypothesis, we analyzed the buds of 20 different broccoli cultivars grown in field. In parallel, the organoleptic characteristics of the buds were evaluated by a consumer panel. Our analysis has identified a small group of molecules which correlate with the qualification of different cultivars in the sensory panel that may constitute good targets for future strategies of breeding broccoli for better taste.

Materials and Methods

Plant Material and Treatments

This study was performed using twenty different broccoli cultivars (single-crossed hybrids) provided by SAKATA Iberica Seeds SLU (Valencia, Spain). All the cultivars used in this study are precommercial, not available in the market and codified by a single number (Figure 1). Plants were grown in field conditions following common procedures reported in the literature for this species (Farnham *et al*., 2011). Specifically, to avoid variability due to different environmental or cultivation conditions we used 40 plants per plot and 2 repetitions of each plot. All cultivars were cultivated in the same conditions and location. The sowing date was November of 2020 and the planting date December 2020. As plots approached maturity, crop was evaluated every 2-5 days so that samples were collected for the tasting study when they reached optimal commercial state. Harvesting and evaluation dates were: 09-Mar, 16-Mar, 23-Mar, 29-Mar, and 06-Apr depending on the cultivar. Three heads of each cultivar were sampled at random. Florets were cut from the stem and tasted raw. Aroma and flavor, characteristics were given a subjective score from 1 to 5, with 5 = highest quality and 1= lowest quality. Then samples were frozen in liquid nitrogen for the metabolomic analysis to assure that metabolic content of the analyzed buds corresponded to those whose taste has been evaluated.

Metabolite analysis

Frozen buds were lyophilized and then ground with a mechanical tissue disruptor in the presence of liquid nitrogen. 10 mg of sample powder were used for each replicate. The method used in this report was previously described in Roessner *et al*., (2000). We used 4 biological replicates. Extraction was performed using methanol and chloromethane and finally dried by evaporation.

Derivatization and injection was performed as described in Roessner *et al*., (2000). We collected the mass spectra at 6.25 spectra s–1 in the *m/z* range 70–800. Ionization energy of 70 eV was used. For the evaluation of the mass spectra we used the CHROMATOF program (LECO, St. Joseph, MI).

Statistical Analysis

Correlation analysis and graphics for this project were generated using excel software (Microsoft, 2022). PCA was carried out using the Singular Value Decomposition algorithm implemented by the sckit learn library, after standarizing the data using the sckit learn standard scaler (Pedregosa *et al*., 2011).

Results

Determination of the metabolite content

Samples from the 20 cultivars included in the study were analyzed for their metabolic content (Figure 1). The histograms for each metabolite in each cultivar, with its error bars can be found in supplemental data 1.

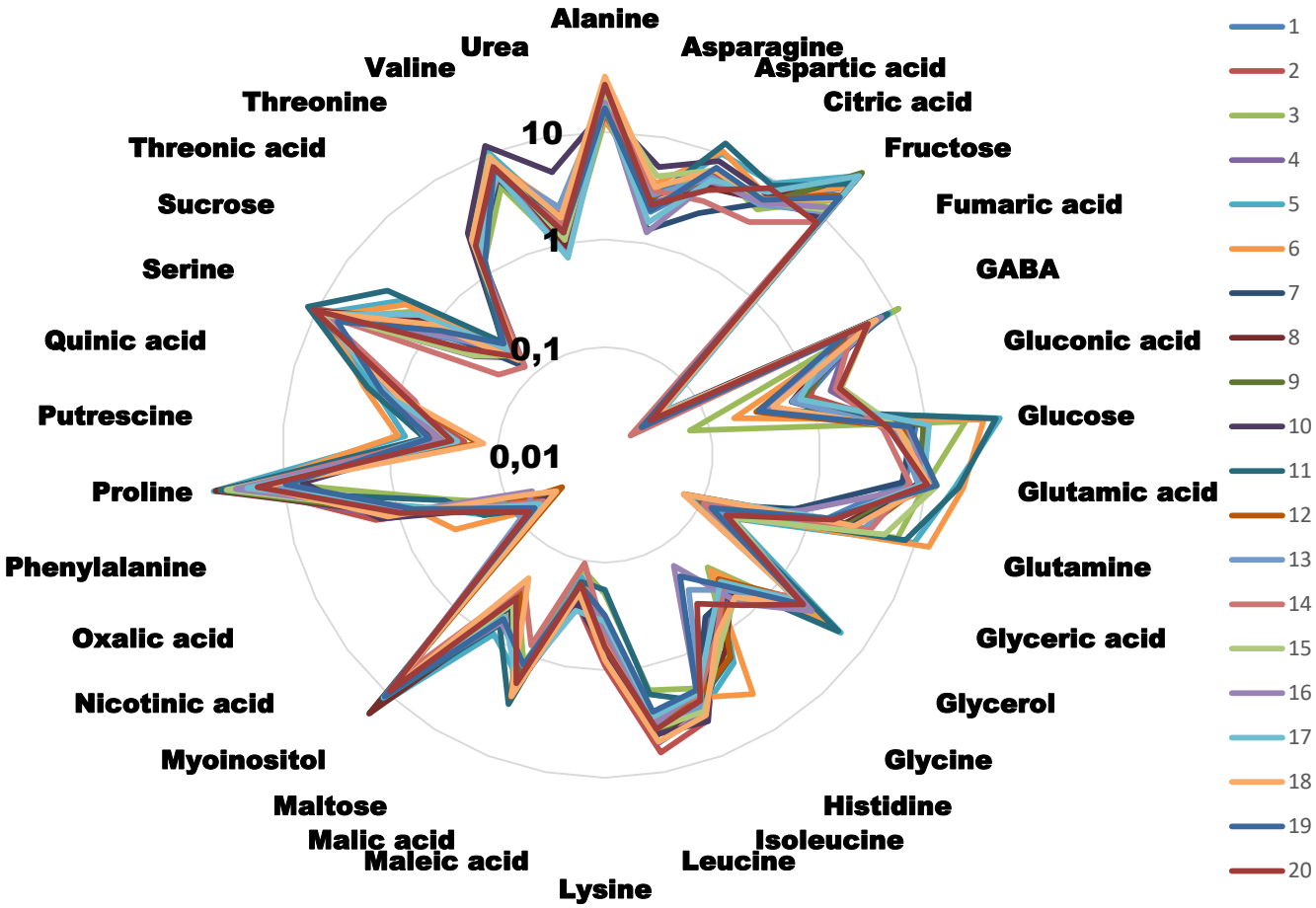

Figure 1. Radial diagram of the metabolite amounts of the different cultivars. Values are the area of the peak per mg of sample represented in a decimal logarithmic scale. Measures are the average of 4 independent biological replicates. Different colors represent different cultivars. For clarity error bars have not been represented, but in most of the cases they represent less than 5% of the total value.

All plants were cultured and harvested under similar conditions. Some metabolite levels were very stable among the different cultivars, while other presented large variations. We evaluated this natural variability by calculating the standard deviation for the different levels obtained for each metabolite in each cultivar (Figure 2). 79,5% of the metabolites analyzed presented standard deviations lower than 2, indicating that most of the primary metabolites analyzed were stable among varieties. On the other hand, we found the major variability in the levels of glucose, proline and fructose (>9).

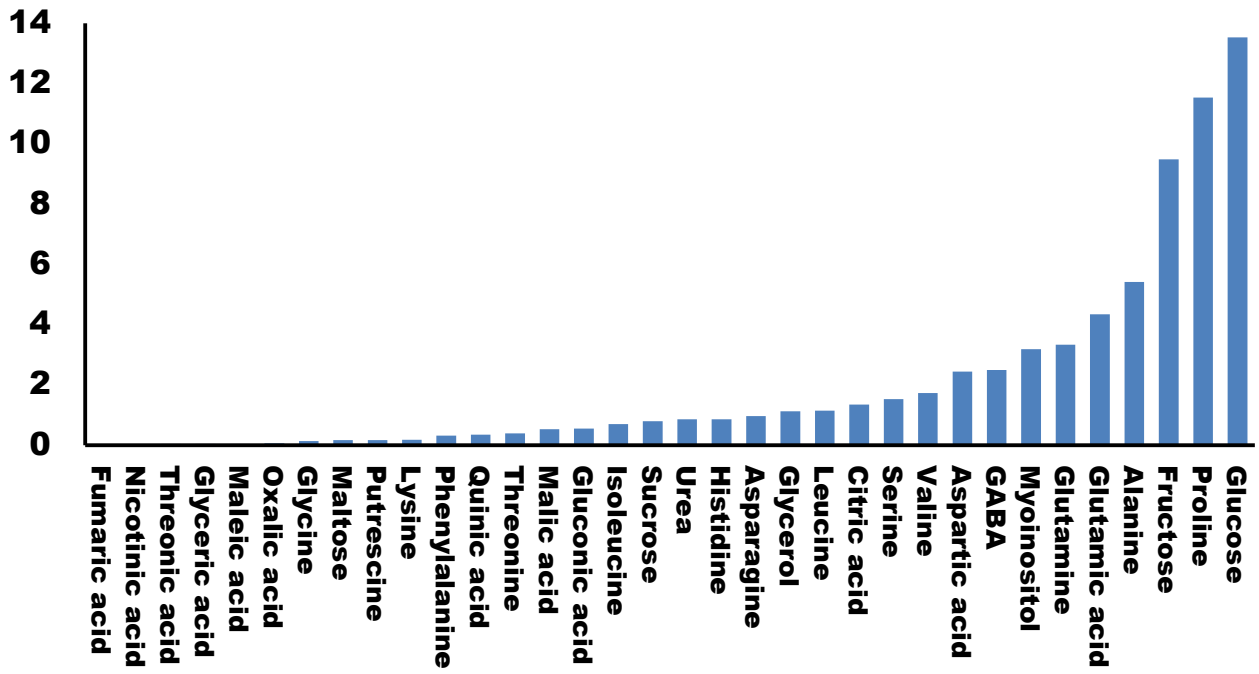

Figure 2. Variability of primary metabolites in different broccoli cultivars. Graphic representation of the standard deviation calculated for the values (area of the peak per mg of sample) of each metabolite in different cultivars.

Correlations between metabolites and tasting score

Raw buds from the 20 cultivars were evaluated by a panel for their taste properties and assigned a qualification which ranged from 1 to 5 (1 being the lowest and 5 the highest). We performed a regression analysis of the qualification for each cultivar with the amount of each metabolite. As expected, we found very low values of R^2 for most of the

molecules analyzed. Specifically, 81% of the metabolites presented an R^2<0,1 suggesting that no correlation exists between the metabolite levels and the taste of the buds. Only 5 metabolites presented an R^2 higher than 0.15. Among them, there were 2 hydrophobic amino acids (alanine and leucine), one charged amino acid (lysine), one non-proteinogenic amino acid (GABA) and the cyclic polyalcohol myo-inositol (Figure 3). All the calculated regressions can be found in supplementary file 2.

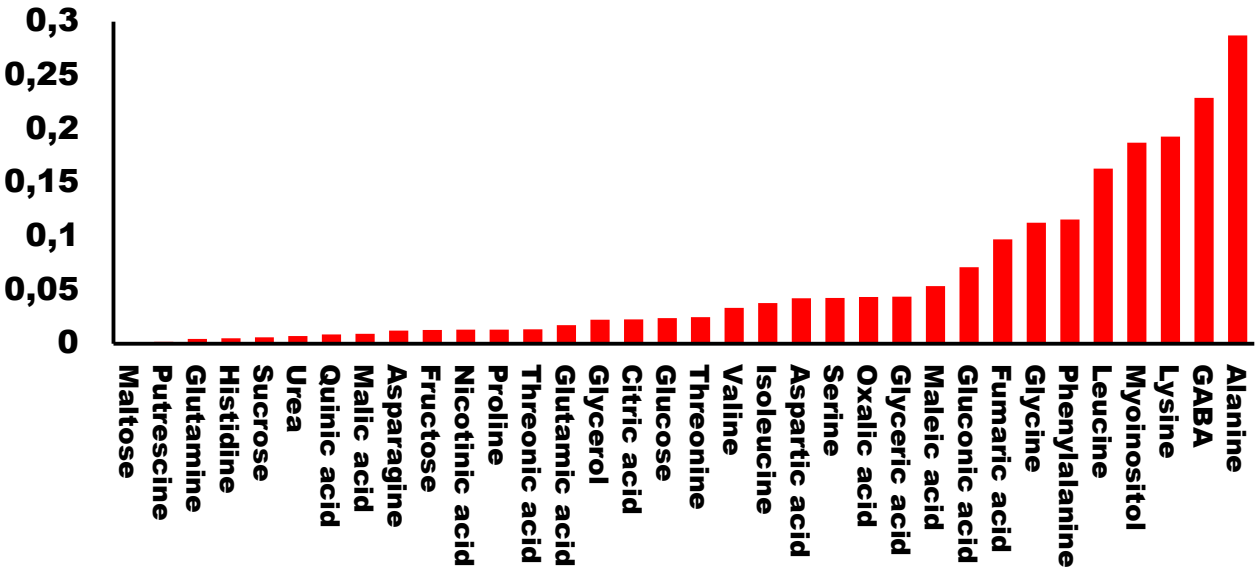

Figure 3. Correlation coefficient (R^2) between each analyzed metabolite and the tasting score.

We analyzed the correlations with a score higher than 0.15. GABA presented a regression slope higher than 0, which indicates a positive correlation between GABA content and taste. For the remaining four, the regression slope was <0, indicating that a higher amount of metabolite correlated with a lower score in the taste panel (Figure 4).

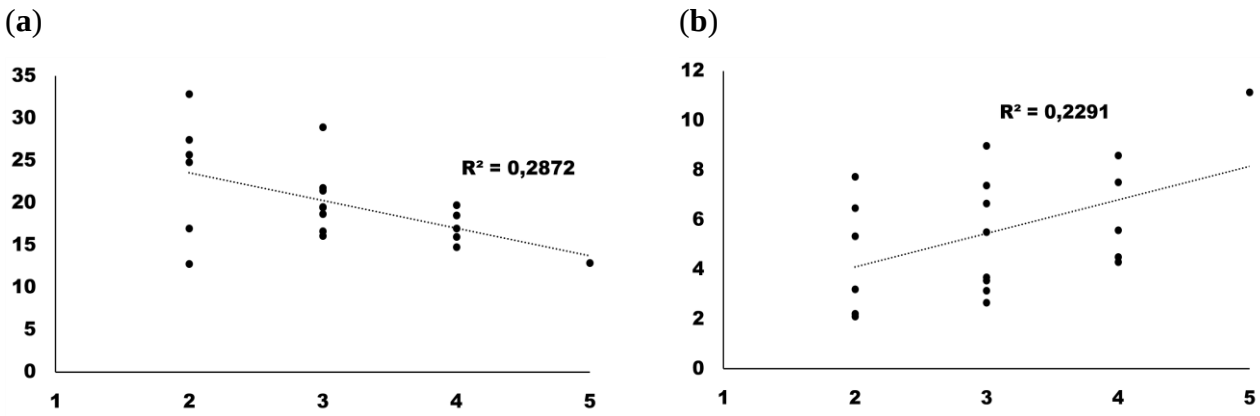

Figure 4. Regression analysis for the two metabolites with the highest correlation: (a) Alanine; (b) GABA.

Principal component analysis (PCA) of the data

We further analyzed our metabolomic data to determine whether the analyzed cultivars could be grouped according to their metabolomic profiles. For this, we performed a principal component analysis (Figure 5).

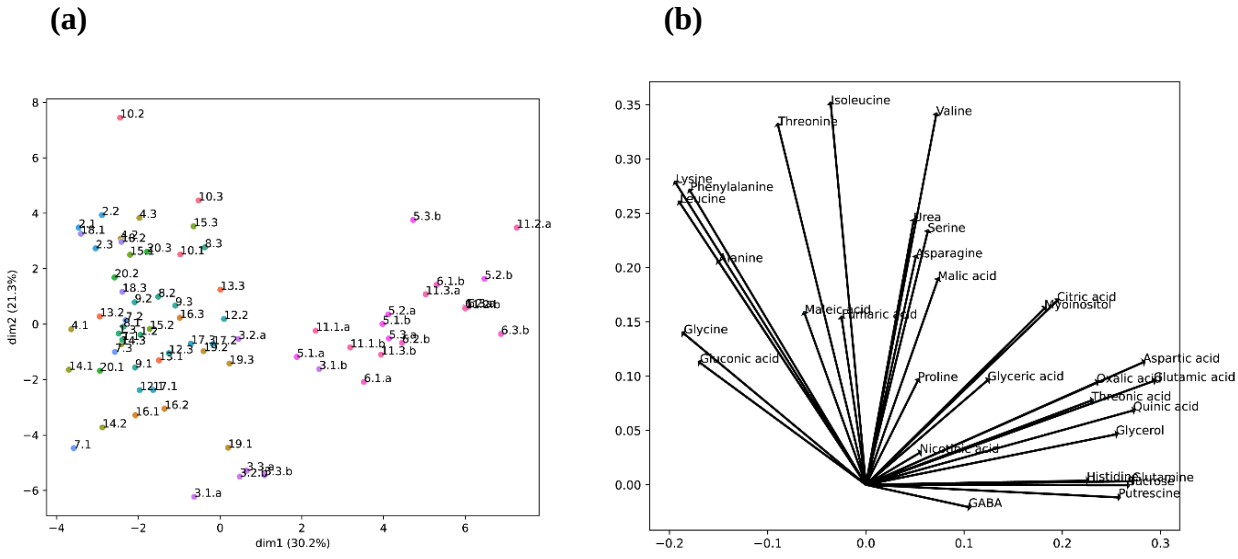

Figure 5. PCA results: (a) PCA projections of the cultivars in the first two principal components. The percentage shown in the axis labels are the percentage of the variance by each principal component; The first number represents a different cultivar, the second number a biological replicate and the letter a technical replicate. (b) Composition of the first two principal components.

The PCA created two principal components, two axes that define a new space, calculated from a linear correlation of the original variables, the metabolite levels. The contribution of each metabolite to the new axes, the two new components, is shown in Figure 5b and the location of the samples in these new axes is shown in Figure 5a. In this projection the cultivars were segregated in three main groups. One group was formed by cultivar 3, the other group by cultivars 5, 6 and 11, while the remaining cultivars constituted a different group (Figure 5a). We observed that the metabolites negatively correlating with taste (lysine, alanine and leucine) grouped together. In fact, phenylalanine, which also presented a high degree of correlation (>0,1) appeared in the same group. Importantly, GABA, the metabolite correlating with good taste, appeared isolated and in the opposite sector of the PCA (Figure 5b).

Discussion

We have previously studied the primary metabolome of broccoli and its relation to abiotic stress. We have found that under salt stress the most limiting factors are the citric acid cycle, as cultivars with higher a content of these components were more tolerant to salt stress (Chevilly *et al*., 2021). We performed a similar study under drought stress and found that drought-tolerant cultivars had lower amounts of urea, quinic acid, and the gluconic acid lactone. Interestingly drought-stressed broccoli accumulated more essential amino acids. (Chevilly *et al*., 2021). Having observed that this kind of approach can be used to find the biochemical basis of macroscopic processes, we wanted to use an adaptation of this methodology to investigate the metabolites involved in broccoli taste or flavor.

The pungent taste associated with broccoli has been related to the presence of glucosinolates, a complex family of molecules derived from some amino acids. A recent study showed that glucosinolates are important in the bitter taste perception of Brussel sprouts, given that this concentration is higher than in broccoli (Wieczorek *et al*., 2022). The influence of glucosinolates in final taste has been observed in other reports, althpugh the same reports found a low variability among cultivars (Hansen *et al*., 1997).

We wanted to determine which primary metabolites could be related to broccoli taste. For this we compared the metabolomes of 20 different cultivars, grown under field conditions with the taste qualifications assigned by a consumer panel. We used field conditions in order to make the experiment as similar as possible to the conditions in which the standard consumer is going to find the broccoli in the supermarket. Even though we used buds of broccoli cultivated in field and compared 20 different cultivars, most of the metabolites were stable and variability was very low. Among the metabolites with higher variability, we found sugars, such as glucose of fructose, and the imino acid, proline. These three molecules could present considerable differences among cultivars due to variations in the genetic backgrounds. However, since they are also related to drought stress response (Xiong *et al*., 2002), we cannot discard that these changes could be due to environmental factors, although we have previously shown that none of these metabolites are limiting for the drought stress response (Chevilly *et al*., 2021).

Surprisingly, we did not find any correlation among those cultivars scoring high in the taste panel and their sugar content. We identified five molecules that presented a high correlation (>0.15) with taste, either negative or positive. Interestingly, three amino acids (lysine, alanine and leucine) negatively correlated with good taste. Alanine is considered to have a sweet taste, similar to saccharine (Schiffman *et al*., 1975), although is able to interact with glutamate receptors (Bryant *et al*., 1991; Eddy *et al*., 2017), and so could be influencing the taste perception. Leucine is known to have a bitter, strong and unpleasant odor (Mukai *et al*., 2007). The case for lysine is similar, as its presence has also been related to bad taste (Watanabe *et al*., 2017). Alanine and leucine are present in high quantities compared to other metabolites, thus reinforcing the idea that their presence is responsible for the low taste scores. Lysine levelss, on the other hand, are lower than the other two amino acids and so it's effect on taste perception may not be as important (figure 1).

The other metabolite that negatively correlated with broccoli taste was myo-inositol. This molecule has a sweet taste (Alarcon *et al*., 2017), and has been related to good taste in some snacks (Gambús *et al*., 2012) and sweetness in wine (Hufnagel *et al*., 2008). Myo-inositol is also found in higher concentrations in naturally-ripened kiwi, as compared to exogenous ethylene-induced ripened kiwi (Lim *et al*., 2017). Surprisingly, in our study myo-inositol correlated with bad taste. Other sugars, such as fructose and glucose, were present in relatively high amounts (Figure 1) and were very variable among cultivars we analyzed (Figure 2). However, we did not find any correlation with taste (Figure 3). This suggests that a positive evaluation of broccoli taste is not related to sweetness and that there are likely to be other molecules competing with or inhibiting the sweet taste or having an antagonistic effect. This is in agreement with a recent report (Wieczorek *et al*., 2022) in which it was shown that sweetness plays significant role in the perception of some Brassica vegetables, but not broccoli.

The only metabolite which presented a positive correlation with taste was GABA, a non-proteic amino acid. This metabolite has been associated with the development of sweet-acidic taste in pineapple (Gao *et al*., 2022). It was also shown to correlate with better taste after glycine-betaine treatment in peach (Jia *et al*., 2022). Interestingly GABA has been described as a health-promoting functional compound (Gramazio *et al*., 2020). The only gene-edited crop currently commercialized is a tomato cultivar with a

higher content of GABA (Nonaka *et al*., 2017). This crop is available in the Japanese market (Ezura *et al*., 2022), but there is no published information on its taste compared to non-edited tomato with less GABA content.

In conclusion, by comparing the metabolomic profile of twenty different broccoli cultivars, we have found that amino acids have a pivotal role in determining taste perception. Leucine, alanine and lysine correlate with worse taste, while GABA correlates with better taste. We failed to find a correlation between abundant sugars and positive taste evaluation. In fact, we observed a negative correlation for myo-inositol, suggesting that the sweet taste is not dominant for broccoli. These results can determine future prospects for classical breeding of new cultivars or biotechnological improvement aiming at less content in leucine, lysine, myo-inositol and alanine and higher content in GABA. This later trait could also further enhance the health-promoting properties of broccoli.

Chapter V

Chapter V

Genome-wide and functional characterization of the serine acetyltransferase enzymes in broccoli (*Brassica oleracea* var. *italica*) identifies two targets for biotechnological improvement.

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Article submitted to *Plant physiology and biochemistry*. I was highly involved in the experimental work and in the elaboration of the manuscript

Abstract

Serine acetyltransferase (SAT; EC 2.3.1.30) is the rate limiting step in cysteine (Cys) biosynthesis. In broccoli (*Brassica oleracea* var. *Italica*), this pathway is particularly important due to its high content in glucosinolates, which are a family of sulfur-containing defensive metabolites that have health-promoting properties when incorporated to the diet. In the current study, we have performed bioinformatic, molecular and biochemical characterization of the SAT isoforms encoded in the broccoli genome. We identified eight SAT enzymes in the broccoli genome, belonging to the three described subgroups according to the intron/exon structure. By characterizing their promoters and studying their expression levels in response to abiotic stress in a tissue-specific manner, we found that *BoSAT1d* was the isoform most responsive to abiotic stress, while the isoform *BoSAT3* had a high expression which was unaffected by stress. Enzymatic activity was very similar for all the studied proteins, except for *BoSAT2*, which presented a much lower activity. When assessing subcellular localization, we found that broccoli enzymes localize to the cytoplasm, cytoplasm and nucleus or chloroplast, but, in contrast to other plants, we did not find any mitochondrial-localized isoform. Taken together, our data support a model in which the chloroplastic isoform produces cysteine at a fixed rate, while cytoplasmic biosynthesis is the main source of cysteine for stress response molecules such as glutathione or glucosinolates. Additionally, our results indicate that *BoSAT1d* and *BoSAT3* may be promising targets for improving sulfate assimilation in broccoli and thus increase the stress resistance and the nutritional benefits.

Keywords: Serine acetyltransferase, Broccoli, Salt stress, Drought stress, Sulfur metabolism, Cysteine.

Introduction

Anthropogenic global warming and the subsequent increment in temperature and alteration in precipitation patterns (reviewed by Leisner, 2020), having enormous impacts in ecosystems (Taibi *et al*., 2014) and are increasingly exposing crops to drought. In parallel, the increasing water demand combined with excessive irrigation are inducing soil salinity, either directly or via the decrease of the phreatic level which facilitates sea water inclusion in aquifers. Coping with the negative effects of drought and salinity is one of the major challenges for agriculture (Campbell *et al*., 2016), which will require increasing food production yields to feed the growing world population.

One of the strategies to provide a healthy diet for a growing population is to increase vegetable intake (Demaio & Rockström, 2015). There is growing interest in obtaining varieties of vegetables rich in health-promoting molecules such as antioxidants that can also be cultivated under adverse environments. Broccoli (*Brassica oleracea* var. *italica*) is a plant of *Brassicacea* family with a remarkable nutritional interest given its low-calorie and high fiber content (Cheng, *et al.*, 2014) and enrichment of glucosinolates, a diverse group of secondary metabolites involved in plant defense against herbivores (Mithen, 2001) widely distributed in plants and specifically in the Brassicacea family (Fahey *et al.*, 2001). Glucosinolates are responsible for the pungent flavor of broccoli (Schonhof *et al.*, 2004) and present extensive beneficial effects when incorporated into the diet. They have been reported to prevent cancer (Holst, *et al.*, 2004; Keum *et al*., 2004), tumor growth and *Helicobacter pylori*-induced gastritis (Lund *et al.*, 2003; Fahey *et al.*, 2002; Thornalley, 2002) and to have anti-inflammatory effects (Sim *et al.*, 2023). From the agronomical point of view, broccoli is cultivated in temperate climates. China and India are the most important global producers, while Spain is the main producer and exporter in Europe. Broccoli production is increasingly threatened by drought and salinity (Zhou *et al*., 2019), and thus, there is growing interest in understanding broccoli stress response mechanisms which can contribute to the development of novel stress-tolerant varieties/cultivars.

Sulfur assimilation is one of the limiting steps for abiotic stress tolerance. Sulfur containing molecules, such as the reductant tripeptide glutathione, play a crucial role in the plant defense system against stress-induced oxidation (Elskens & Penickx, 1997). Cysteine (Cys) synthesis is the last step of sulfate assimilation (Bogdanova & Hell,

1997) and plays a major role in an essential macronutrient. Cys biosynthesis is a two-step process. First, serine-O-acetyltransferase (SAT; EC 2.3.1.30) catalyzes the formation of O-acetyl serine from acetyl-CoA and L-serine. Second, O-acetyl serine is subsequently sulfhydrylated by O-acetylserine (thiol) lyase (OASTL), resulting in Cys and CoA. In plants, OASTL cellular activity is in large excess compared to SAT activity (Saito, 2000; Heeg *et al.*, 2008). O-acetyl serine is the limiting factor for Cys synthesis (Wirtz & Hell, 2003) and therefore subjected to tight regulation. SAT enzymes function in a Cysteine Synthase (CS) complex composed by the reversible association of a SAT hexamer and two OASTL dimers in a Cys synthase complex (Wirtz *et al.*, 2004). In this complex, OASTLs remain inactive and function as regulatory subunits that stimulate SAT activity by counteracting the Cys-feedback inhibition (Wirtz *et al.*, 2010). Several studies have shown that the O-acetyl serine is limiting for abiotic stress responses and that overexpression of this enzyme enhances both glutathione accumulation and stress tolerance (Freeman *et al.*, 2004; Mulet *et al.*, 2004) and stress tolerant varieties of pine (Taibi *et al*., 2017), broccoli (Chevilly *et al.*, 2021) and poplar (Shi *et al*., 2022) correlate with higher accumulations of sulfur containing molecules.

Plant genomes encode several SAT isoforms that present specific expression patterns, differential subcellular localizations and distinct catalytic properties. In the plant model *Arabidopsis thaliana*, there are five functional SAT enzymes that can be classified into three groups (I-III) according to their phylogenetic origin classification and intron-exon structure (Watanabe *et al*., 2008) Group I includes one member, SAT5, a cytosolic enzyme encoded in two exons, which is sensitive to Cys inhibition; Group II contains two members, AtSAT1 and AtSAT3, the most abundant enzymes, insensitive to Cys inhibition and encoded in a single exon; and Group III includes AtSAT2 and AtSAT4, which are cytosolic enzymes with a lower substrate affinity than the other isoforms (Noji *et al*., 1998; Kawashima *et al*., 2005).

Characterizing the enzymes involved in cysteine biosynthesis in broccoli is a strategy to identify candidates to enhance stress tolerance, either by biotechnological or breeding approaches (Freeman *et al*., 2004). In addition, given that the glucosinolates are sulfur containing molecules with extremely interesting nutritional benefits, improving sulfate assimilation in broccoli can not only improve agronomical yield under adverse

conditions, but also add nutritional content and health-promoting properties. In this study, we have characterized the SAT gene family from broccoli. By applying a combination of bioinformatics, molecular biology and biochemical approaches, we define the role of broccoli SATs under normal and stress conditions and identify the most suitable candidates for the genetic improvement of this crop.

Materials and methods

Bioinformatics Analysis

The gene sequences of the *Arabidopsis thaliana SAT (AtSAT)* were obtained from TAIR and their orthologues in *Brassica oleracea* broccoli genome (BOL) (Parkin *et al.* 2014) were mined with BLASTP. Phylogenetic analysis was performed using the maximum likelihood method with MEGA-X software (Kumar *et al.,* 2018). The duplication pattern of SAT genes was obtained with MCscanX and the Dual Synteny Plotter from TBtools (Chen *et al*., 2023) using TAIR10 and BOL genome annotations sequences obtained from Ensembl Plants (Yates et al., 2022). Gene structure was analyzed using the GSDS 2.0 software (Hu *et al.,* 2015). We performed the promoter *cis*-element analysis with NewPLACE software (Higo *et al*., 1999). Protein properties were predicted with ExPASy server tools (Gasteiger *et al*., 2005) and conserved motifs were analyzed using ESPript 3.0 software (Robert *et al.,* 2014). 3D structural prediction was obtained with AlphaFold (Varadi *et al*., 2022).

Plant materials and growth conditions

Seeds of *B. oleracea* var. *italica* cv. Naxos were obtained from SAKATA Iberica Seeds SLU (Valencia, Spain). Seeds were sown in pots containing a 1:2 vermiculite/soil mixture and grown in the greenhouse under long day conditions (16 h light/8 h dark, 200 μmol m^{-2} s^{-1} of light intensity) at 24 ± 2 °C and 70 ± 5% relative humidity. Plants were watered to full capacity every 2 days with complete Hoagland's nutrient solution containing all essential macronutrients and micronutrients (Hoagland & Arnon, 1950). Root and leaf tissue was collected from plants grown for 5 weeks, whereas bud tissue was obtained from 5-month-old plants. Salinity-treated samples were obtained from plants additionally watered for 6 days with Hoagland's nutrient solution supplemented with 220 mM NaCl. Drought was applied by withholding water until the total weight (plant and container) was reduced to 60% of their initial weight and leaves lost turgor.

Gene expression analysis

Total RNA from the different tissues and treatments were extracted using NucleoSpin® RNA Plant kit (Macherey-Nagel; Düren, Germany) following manufacturer's

instructions. RNA integrity was assessed by loading samples in agarose gels and Bioanalyzer (RIN>6). Synthesis of cDNA was performed using PrimeScript RT reagent kit (Perfect Real Time) from Takara (Kusatsu, Japan). qRT-PCR was carried out in a QuantStudio™ 3 - 96-Well 0.1 mL Block (Thermo Fischer; Waltham (Ma), USA) using 5x PyroTaq EvaGreen qPCR Mix Plus (ROX) from Molecular Bioline (Cmb) (Cultek; Madrid, Spain) and 10 µM of each primer. Primers used for qPCR are listed in Supplementary Table S1. They were designed in non-conserved regions and spanning adjacent exons (when possible). Primer specificity was checked by sequencing the PCR amplicons. The obtained Ct data were corrected for the primer efficiencies experimentally determined (see Supplementary Table S1) using the online tool Relative Quantification (Thermo Fischer Cloud; Waltham (Ma), USA). Relative expression was calculated by the ΔCq method as $[1+(\text{Efficiency}/100)]^{-\Delta Cq}$ (Taylor et al., 2019). *BoUBQ2* and *BoTBP1* were used as internal controls (Brulle et al., 2014) after validating that their expression remained constant under salinity and drought treatments. We noticed that the expression of *BoUBQ2* and *BoTBP1* was tissue-dependent and therefore it was not possible to compare gene expression among different tissues**.** Normalized relative expression is calculated as the Relative expression normalized to the untreated controls. Data represented are averages ± SD of three biological replicates (different plants).

Plasmid construction

All plasmids generated in this work are listed in Supplementary table S2. Oligonucleotides used for plasmid generation can be found in Supplementary table S3.
For heterologous expression in yeast, *SAT* genes from *B. oleracea* were cloned using the modular cloning system (MoClo) (Lee *et al*., 2015). In brief, we amplified the ORFs from broccoli cDNA in batches introducing synonymous point mutations to remove *BsmBI* and *BsaI* cleavage sites. These amplicons were introduced in the MoClo entry vector pYTK001 using *BsmBI*-assembly and further assembled using *BsaI* into transcriptional units that contained the yeast *PGK1* constitutive promoter, the *SAT* CDS fused to a Venus Ct-tag through a flexible Gly-Ser linker and the yeast *ENO1* terminator in a backbone with the *URA3* gene as the selection marker.
For *N. benthamiana* experiments, GoldenBraid plasmids expressing the *BoSATs* were generated. In brief, the *BoSAT* CDS were amplified from the MoClo vectors with

specific oligos to adapt the flanking sequences to the of the GoldenBraid 4.0 grammar. The amplicons were then introduced into the entry vector pUPD2 using the *BsmBI* restriction enzyme target sites. Multipartite assembly through restriction-ligation reactions with the *BsaI* enzyme were performed to assemble transcriptional units containing the *BoSAT* fused to a GFP Ct-tag, the 35S promoter and the tNOS terminator in a α1 KAN^R destination vector.

Yeast growth and complementation assay

The osmosensitive *Saccharomyces cerevisiae* strain JM164 (W303-1A, mat a/α, *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 GAL+, gpd1::TRP1*) (Gisbert et al., 2020) was transformed with an empty plasmid or the vector to express each of the *BoSATs*. Transformants were selected, grown, and maintained in Synthetic Dextrose (SD) –URA medium (0,7% Yeast Nitrogen Base, 2% dextrose, 50 mM succinic acid pH 5.5, 60 μg/mL adenine, 30 μg/mL histidine, 100 μg/mL leucine), unless indicated otherwise.
For complementation assays, serial 10-fold dilutions of yeast cultures were spotted onto YPDA plates (1 % Yeast extract, 2% peptone, 2% dextrose, plus 1% adenine) alone or supplemented with 1.7 M sorbitol or with 0.8 M NaCl to assay osmotic or salinity tolerance. Plates were grown at 28ºC for four days (Pérez-Valle et al., 2010).

Determination of low molecular weight sulfhydryls (LMWS)

Exponentially growing yeast cultures (50 mL) of the strain JM164 (OD_{600} of 0,4-0,8) grown on YPDA were pelleted and resuspended with 300 μL sodium phosphate buffer (100 mM; pH 8). Cells were lysed by vortexing in the presence of glass beads with three pulses of 30 sec. Then, 175 μL of sodium phosphate buffer were added. After a 5 min centrifugation, 25 μL of the supernatant were transferred to a new tube and 20 μL of Tri Cloro acetic Acid (TCA; 50% w/v) and 25 μL NaOH 2 M were added. Finally, 25 μL of Ellman reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] 8 mg/mL were added in order to measure absorbance at 412 nm with an extinction coefficient of 13,6 $mM^{-1}cm^{-1}$and referred to a Cys standard calibration curve.

Determination of enzymatic activity

The enzymatic activity of each of the broccoli SATs were determined as described in (Cherest and Surdin-Kerjan, 1992). Briefly, cells in 100 mL of culture in SD+AHL with OD=1 were pelleted and resuspended in 400 µL phosphate buffer 10 mM, pH 7.5 with 10% glycerol. Then, 400 µL of glass beads were added and were put under agitation in a 7ºC chamber, three cycles of 1 min of agitation with incubations of 1 min on ice in between shaking. The samples were then centrifuged and 100 µL of supernatant were taken to a final volume of 1 mL with 10 mM L-serine and 0.15 mM acetyl-CoA. Samples were then incubated 30 minutes at 30ºC. Then, Ellman reagent was added to a final concentration of 0.75 mM and the absorbance was measured at 412 nm. Protein extracts were loaded into a 8% polyacrylamide gel so that it was carried out a sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and, after the transference to a nitrocellulose membrane, it was incubated with a primary antibody (α-GFP Rabbit polyclonal PABG1, Chromotek: Planegg; Germany) in a final dilution of 1:10000 in blocking buffer and, then, with a secondary antibody (Anti-Rabbit IgG, peroxidase-linked NA934, Cytiva Life sciences; Marlborough; Ma; USA) in a final dilution of 1:10000 in the blocking buffer to detect the SAT enzymes fused Venus (Nagai *et al*., 2002). The quantification of the intensity of the bands was obtained with the ImageJ software (Schneider *et al*., 2012).

Subcellular localization in Saccharomyces cerevisiae

Exponentially growing yeast cells of the strain JM164 were centrifuged, and pellets were stained with a mitochondrial marker (MitoTracker®Red; Thermo Fisher scientific; Waltham; Ma; USA) or nuclear dye (DAPI). For mitochondrial staining, pelleted cells were incubated during 30 minutes at 30ºC with MitoTracker 500 mM (dissolved in DMSO) following manufacturer's instructions. For nuclear staining, DAPI (4′,6-diamidino-2-phenylindole), cells were fixed with 1mL of ethanol and stained for 5 minutes with DAPI at a final concentration of 0,1 µg/mL. After the staining, cells were washed and resuspended in the appropriate SD media preheated at 30ºC and images were taken in ZEISS LSD 780 AxioObserver.Z1 confocal microscope (Jena, Germany). The excitation/emission filters used were 410-492/405 for DAPI, 588-652/561 for MitoTracker and 494-543/488 for GFP.

Subcellular localization in Nicotiana benthamiana

Agrobacterium tumefaciens strain C58 was co-transformed with the plasmid containing the GFP fusion proteins as well as the P19 plasmid (Addgene #68214), to inhibit silencing. *Nicotiana benthamiana* epidermal cells were infiltrated with the transformed *A. tumefaciens* strains. After 48h, subcellular localization was assessed by fluorescence confocal microscopy using *Nicotiana* leaves or protoplasts.

For the protoplast purification, infiltrated leaves were cut into 0.5 mm strips and digested for 5 hours in the dark using the enzymatic solution -1.5% cellulase RS, 0.75% macerozyme, 0.5 M mannitol, 1 mM $CaCl_2$, 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.7, 0.1% Bovine serum albumin (BSA). Images were obtained with a ZEISS LSD 780 AxioObserver Z1 confocal microscope (Jena, Germany), with the excitation/emission filters 638-703/488 for chlorophyll auto-fluorescence and 494-543/488 for GFP. Following the visualization, protein extracts were obtained from the infiltrated leaves and western blot assay was performed following the procedures detailed in the ‘determination of enzymatic activity’ section.

Results

Identification of SAT enzymes in broccoli

We used the amino acid sequences of *A. thaliana* SATs (AtSATs) to mine in the *Brassica oleracea* genome database (Liu et al., 2014) in the search of SAT orthologs. We identified 8 SAT genes in broccoli and named them according to their similarity to the Arabidopsis enzymes (Table 1). To study the evolutionary relationships, we constructed a phylogenetic tree with MEGA-X software with the maximum likelihood method using the protein sequences of both broccoli and *Arabidopsis thaliana* SAT enzymes. In addition, to confirm the relationships found with the tree, we analyzed the gene structure with GSDS 2.0 software, paying special attention to the number of exons of BoSAT enzymes, which indicates the corresponding SAT enzyme group (Figure 1). We identified four homologues of *AtSAT1* in broccoli, *BoSAT1a*, *BoSAT1b*, *BoSAT1c* and *BoSAT1d*, and one of *AtSAT3*, *BoSAT3*. All these contain one exon and belong to group II. *AtSAT5* showed two homologues, *BoSAT5a* and *BoSAT5b*, both two containing two exons and clustering in SAT group I. The paralogues *AtSAT2* and *AtSAT4* presented only one homologue, *BoSAT2*, sharing the nine-exon structure typical of group III.

Arabidopsis thaliana and the *Brassica* species diverged 43.2 MYA ago (Lysak *et al*, 2005). About 15,9 MYA (Liu et al., 2014), the *Brassica* genus underwent a whole genome triplication event. We confirmed the phylogenetic relationships found in our bioinformatics analysis by studying the synteny between the chromosome location of the SATs in Arabidopsis and *Brassica oleracea*. The identified BoSAT enzymes were distributed along five of the nine *Brassica oleracea* chromosomes. The syntenic analysis confirmed the phylogenetic relationship among *AtSAT2* and *BoSAT2*, *AtSAT3* and *BoSAT3*, and, lastly, *AtSAT5*, *BoSAT5a* and *BoSAT5b* (Figure 1b). *AtSAT1* has 4 different orthologues in the *Brassica oleracea* genome. The synteny analysis confirmed the common phylogenetic origin with AtSAT1 of *BoSAT1a* and *BoSAT1c* located in chromosome 6, and *BoSAT1b* located in chromosome 3, while *BoSAT1d* was the only paralogue with no syntenic relationship with *AtSAT1*, suggesting that a recent duplication from *BoSAT1c* that occurred after the divergence with the Arabidopsis tribe.

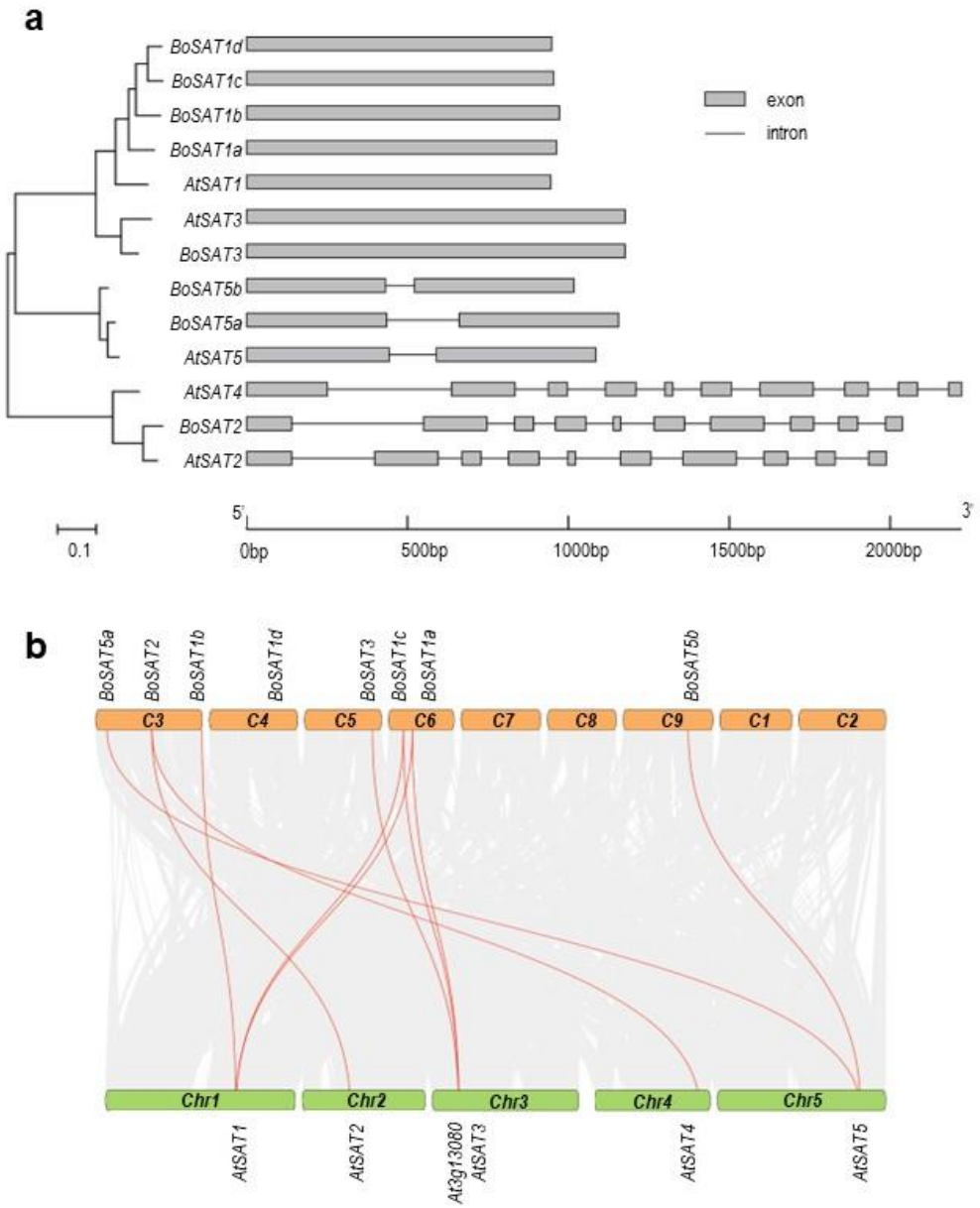


Figure 1. Phylogenetic tree, gene structure and syntenic relationship between *Arabidopsis thaliana* and SAT enzymes and their homologues in broccoli (*Brassica oleracea* var. *italica*). (a) The phylogenetic tree was built with the maximum likelihood method using the protein sequences and the exon-intron structures were represented following their phylogenetic relationships. Grey boxes and black lines indicate exons and introns, respectively. (b) Synteny analysis of SAT genes and their chromosomal distribution. Gray lines in the background indicate the collinear blocks within *B. oleracea* (orange) and *A. thaliana* genome (green) and red lines highlight the syntenic SAT gene pairs.

We also performed a bioinformatics analysis on the amino acid sequence. Whereas the length of BoSAT-coding genes ranged from 1120 (*BoSAT1a*) to 2394 (*BoSAT2*) base pairs, the length of BoSAT enzymes ranged from 309 (*BoSAT5a*) to 391 (*BoSAT3*)

residues. The isoelectric point ranged from 5,72 (*BoSAT2*) to 8,85 (*BoSAT3*). The molecular weight ranged from 32,54 kD (*BoSAT5a*) to 42,78 kD (*BoSAT3*) (Table 2). Comparing the amino acid sequences of SAT genes, we found a high similarity among *AtSAT* and *BoSAT* (Figure 2). All BoSATs except *BoSAT2*, shared the C-terminal Ile essential for the docking with the OASTL and the formation of the CSC complex, (Yi et al., 2013). Like its Arabidopsis ortholog *AtSAT2*, *BoSAT2* did not present a neutral amino acid in this position, but rather a negatively charged aspartic acid residue, followed by a C-terminal extension, known to act as a negative regulatory domain (Liu *et al*., 2022).

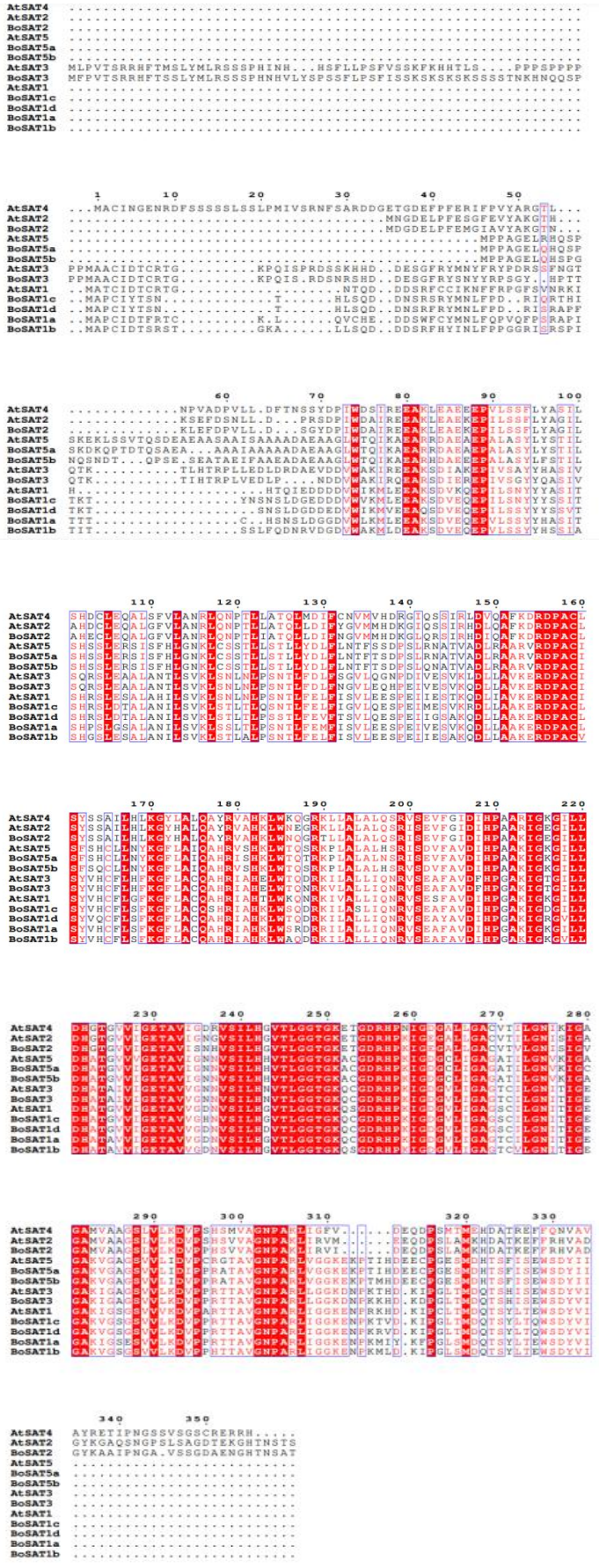

AtSAT4
AtSAT2
BoSAT2
AtSAT5
BoSAT5a
BoSAT5b
AtSAT3
BoSAT3
AtSAT1
BoSAT1c
BoSAT1d
BoSAT1a
BoSAT1b

Figure 2. Alignment of AtSAT and BoSAT protein sequences. The sequences were aligned using the ESPript 3.0 software. Red highlighting indicates identical residues. Regions with over 70% similarity are framed in blue boxes, while residues in black font indicate less than 70% similarity. A black triangle indicates the Ct Ile required for CSC formation.

Next, we used the BoSATs' amino acid sequences to perform a 3D structural prediction with AlphaFold software (Figure 3). All BoSAT sequences led to coherent structures compatible with SAT function containing a N-terminal α–helical domain and a C-terminal ß–helix domain, like other known bacterial and plant SATs (Yi *et al*., 2013).

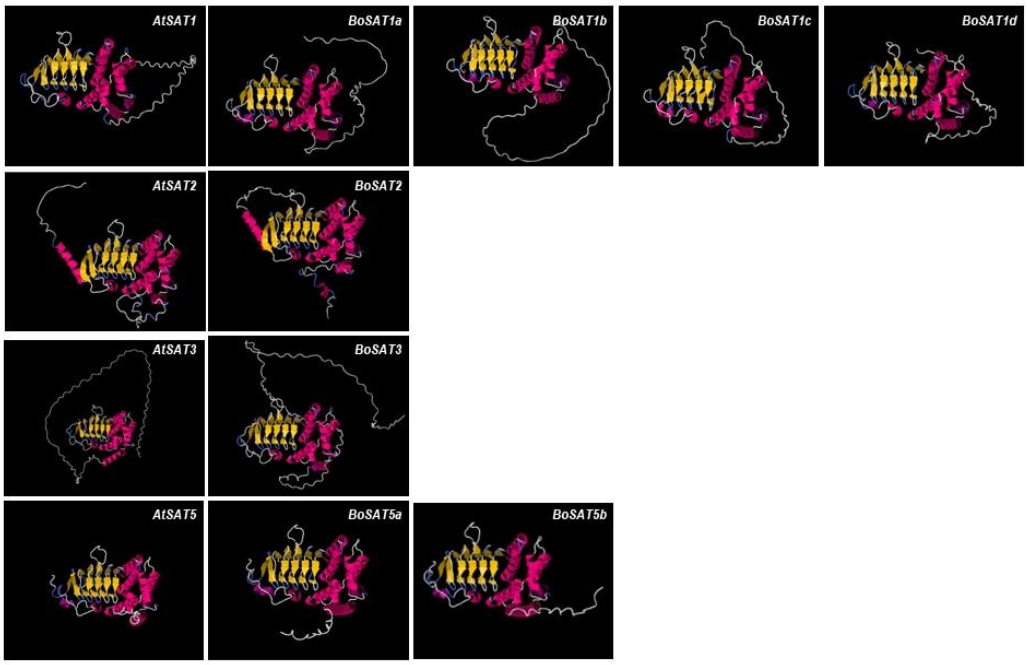


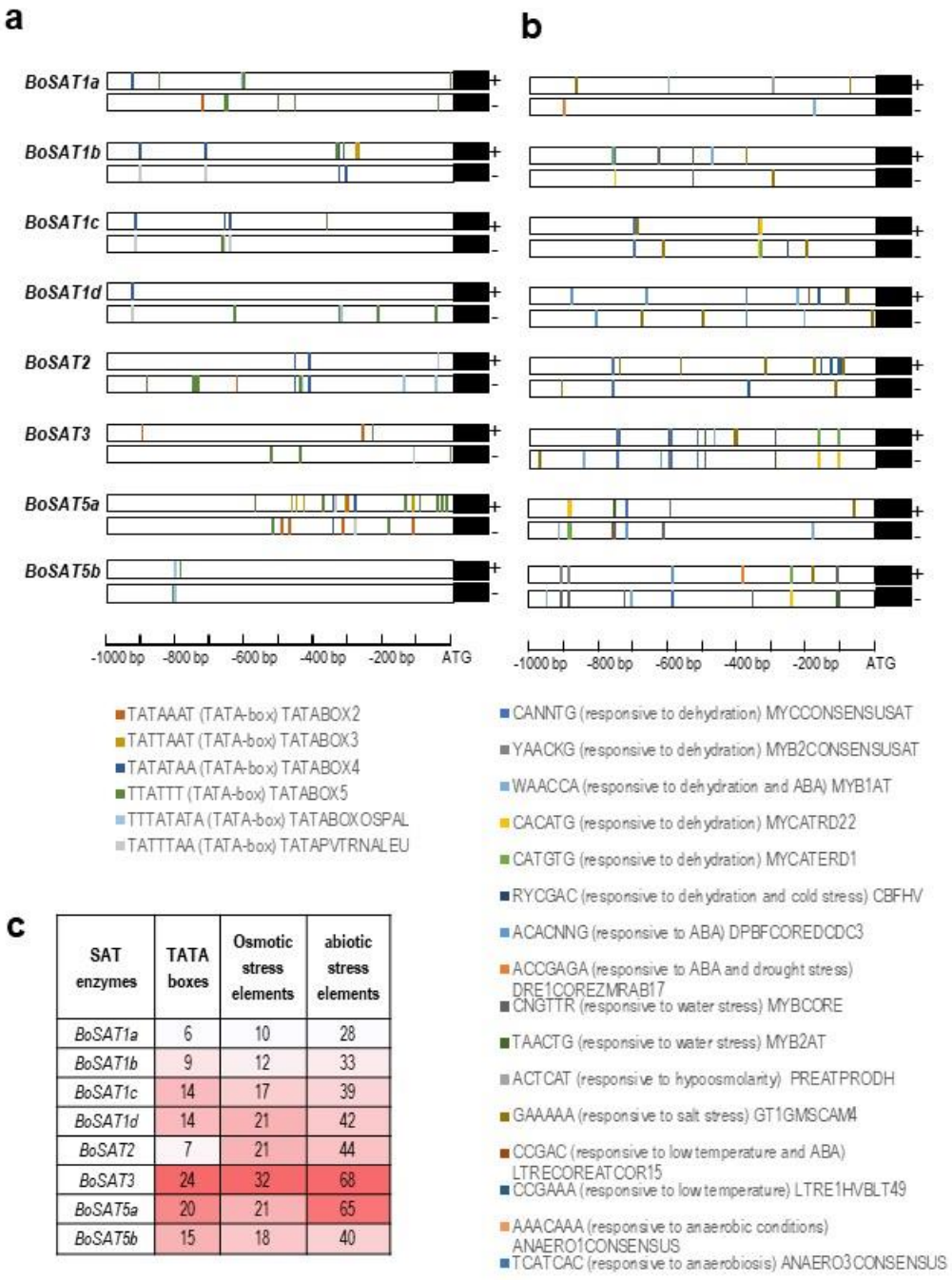
Figure 3. Comparative 3D structures between the SATs present in Arabidopsis and their orthologues in broccoli. The structures are displayed in the same orientation to highlight differences among them. Colors are assigned according to the secondary structure of the region: alpha helices are shown in pink and beta-sheets in yellow.

Analysis of predicted cis regulatory elements in the promoters of BoSAT *genes*

To understand the potential role of SAT broccoli genes, we performed an analysis of their promoters. We considered the promoter region as the 1kb preceding the start codon of *BoSAT* and performed a search of cis stress elements using the NewPLACE software (Higo *et al*., 1999). We first studied the presence of TATA boxes (Figure 4a). All *BoSAT* presented canonical TATA boxes, suggesting that they could be potentially

expressed in broccoli (Savinkova *et al*., 2023). Because TATA box number usually positively correlates with an increased gene expression (Zhang et al., 2017), we compared the number of predicted TATA boxes among the paralogues (Figure 4c). *BoSAT3* and *BoSAT5a* promoters contained the most TATA boxes (24 and 20, respectively), followed by *BoSAT5b*, *BoSAT1c* and *BoSAT1d*, which contained 14-15. *BoSAT1a*, *BoSAT1b* and *BoSAT2* contained less than 10 TATA boxes. Most of the identified TATA boxes were located more than 100 bp upstream the start codon. These variations may explain differences in promoter activity among the genes.

SAT genes have been previously linked to osmotic and oxidative stress tolerance (Ahmad *et al*., 2016). Therefore, we were interested in investigating the presence of abiotic stress-responsive elements in the BoSAT promoters (Figure 4b). We identified many elements involved in osmotic stress (Figure 5b-c), including the dehydration stress elements **CANNTG**, **YAACKG**, WAACCA, CACATG, and CATGTG; the ABA-responsive elements ACACNNG, ACCGAGA and RYCGAC; the water-stress-responsive elements CNGTTR and TAACTG; the hypo-osmolarity-responsive element ACTCAT and the salt responsive element GAAAAA. Additionally, we found the low temperature-responsible elements CCGAC and CCGAAA and the anaerobiosis elements AAACAAA and TCATCAC. Taken together, the *BoSAT* promoter regions contain several *cis* regulatory elements related to osmotic stress, low temperature and anaerobiosis, suggesting a role of this gene family in the response of broccoli to different types of abiotic stress.



c

SAT enzymes	TATA boxes	Osmotic stress elements	abiotic stress elements
BoSAT1a	6	10	28
BoSAT1b	9	12	33
BoSAT1c	14	17	39
BoSAT1d	14	21	42
BoSAT2	7	21	44
BoSAT3	24	32	68
BoSAT5a	20	21	65
BoSAT5b	15	18	40

Figure 4. Analysis of the promoter TATA-boxes and cis-elements of BoSATs. The 1kb DNA sequence upstream of the ATG starting codon of BoSATs were analyzed using the online analysis tool PLACE software (Higo *et al*., 1999) in order to identify the putative TATA-boxes (a) and putative abiotic stress response elements (b). (c) Total amount of putative TATA-boxes osmotic stress or abiotic stress elements. Cells are colored in shades or red according to the relative number of elements.

Tissue-specific expression of SAT *genes in* Brassica oleracea

To study the tissue-specific expression patterns of *BoSATs*, we performed RT-qPCR of these genes in broccoli leaves, roots and buds using the housekeeping genes *UBQ2* and *TBP1* as controls (Brulle *et al*., 2014) (Figure 5). *BoSAT3* was expressed at very high levels in leaves and roots (Figure 5a). Interestingly, this gene was apparently expressed an order of magnitude above the rest of *BoSAT* genes in both roots and leaves, which, correlated with the high number of TATA boxes in its promoter. This supports the idea that *BoSAT3* could be the main SAT isoform in broccoli. *SAT1b* and *SAT5a* were highly expressed in both leaves and roots as well. *BoSAT2* showed moderate expression in roots and low expression in leaves. *BoSAT5b* was expressed in roots, with low expression in buds and no detectable expression in leaves, indicating that *BoSAT5b* may be a root specific BoSAT isoform. Overall *BoSAT* expression was higher in leaves and roots compared to buds, suggesting that SAT activity may not be as relevant in this tissue. Only *BoSAT1a* was expressed more strongly in buds than in other tissues. The expression levels of *BoSAT1c* and *BoSAT1d* were very low in all tissues, indicating that these genes make a limited contribution to the pool of SAT activity in broccoli.

Expression of BoSAT *genes under osmotic stress*

Given the prominence of osmotic stress elements in *BoSAT* promoters (Figure 5), we aimed to define the transcriptional response of *BoSAT* genes under drought and salinity. To uncover possible tissue-specific differences, we performed this study in leaves, roots and buds. In leaves (Figure 5b), *BoSAT1b* was specifically induced by salinity, while *BoSAT1a*, *BoSAT1c* and *BoSAT1d* were induced under both salinity and drought. Remarkably, *BoSAT1d* was induced over a 100-fold under drought and nearly 10-fold under salinity, indicating that this gene may have a significant role in the abiotic stress response of broccoli. The expression of *BoSAT2*, *BoSAT3* and *BoSAT5a* remained

unchanged under either drought or salinity. We did not detect any *BoSAT5b* expression in leaves, even under stress.

In roots (Figure 5c), we only detected substantial inductions for two genes, *BoSAT1c* and *BoSAT1d*. *BoSAT1c* was induced specifically under drought. *BoSAT1d* expression responded to both stressors, with a remarkable 10-fold induction under drought. Again, these strong inductions of *BoSAT1d* point towards an important role of this isoform in the abiotic stress response in broccoli.

In buds (Figure 5d), *BoSAT* expression remained unaffected under osmotic stress. This may reflect that roots and leaves respond to abiotic stress, and the reproductive tissues are less affected by these treatments. Only minor inductions (2-3x fold) were observed in the case of *SAT1a*, *SAT1b* and *SAT3* under drought and *SAT1d* under both salinity and drought, yet the variability among the replicates was high and therefore the observed differences were not significant.

Altogether, our data show that *BoSAT* genes are differentially regulated in response to drought or salt stress and uncovers *BoSAT1d* as a gene highly responsive to abiotic stress, with strong and robust inductions particularly under drought.

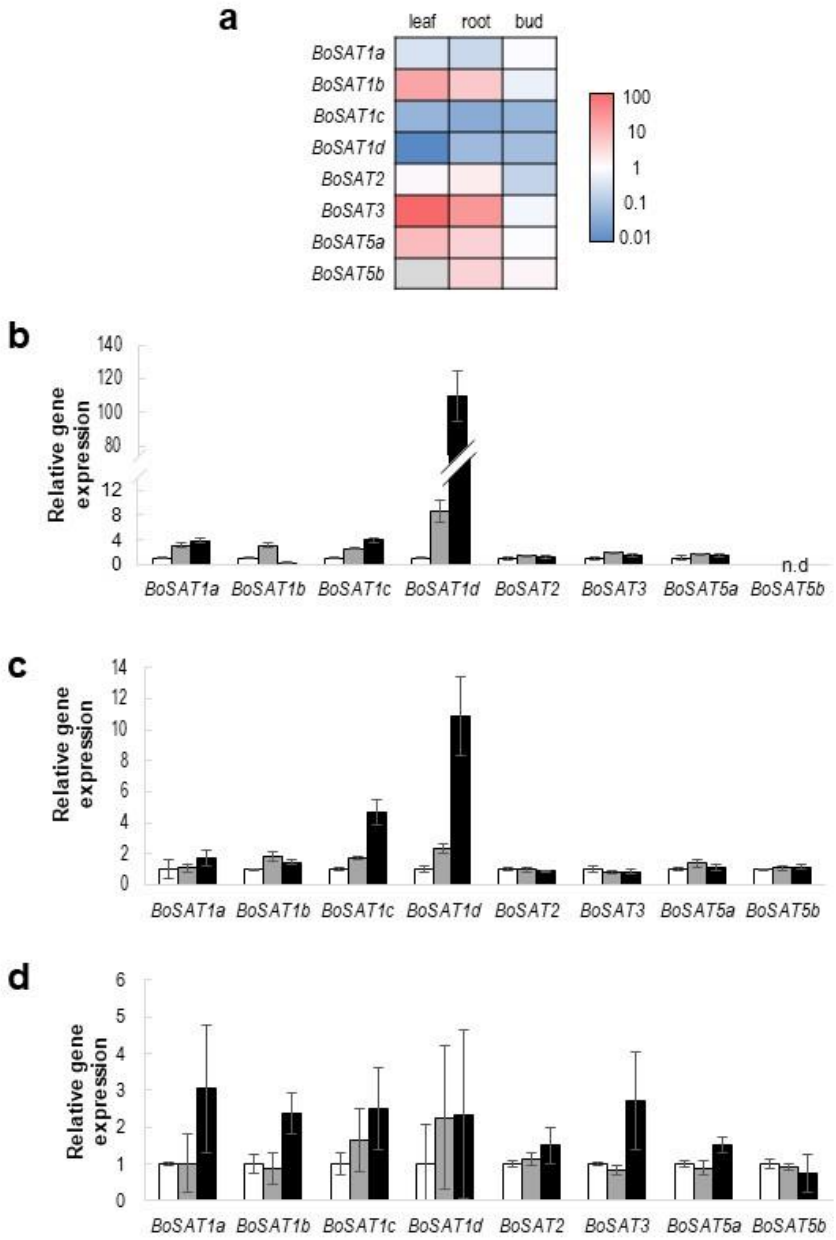


Figure 5. Expression pattern of BoSATs in different tissues under control conditions and water stress. (a) Expression pattern of *BoSATs* in different tissues under control conditions. Total RNA was obtained from different broccoli plant tissues as detailed in materials and methods. Gene expression levels are normalized to the expression level of the reference genes *BoUBQ2* and *BoTBP1*. (b-d) Total RNA was obtained from leaves (b), roots (c) or bud (d) from plants exposed to control (white), salinity (grey) or drought (black) for 6 days. Gene expression levels are normalizaed to the expression level of the reference genes *BoUBQ2* and *BoTBP1* and relative to untreated control conditions. Data are averages ± SEM of three biological repeats.

BoSAT enzyme activity characterization in yeast

To perform a molecular characterization of BoSATs, we cloned the most representative BoSAT isoforms from each group: *BoSAT1b*, *BoSAT1d*, *SoSAT2*, *BoSAT3* and *BoSAT5a*. We discarded from the analysis *BoSAT1a* and *BoSAT1c* due to their high conservation with their paralogous genes and *BoSAT5b* for the same reason and its low level of expression in leaves. As the plant material we were using was not the same that was used for the *Brassica oleracea* genome database (Liu *et al*., 2014), we sequenced the indicated genes and compared their sequence with the sequence in the database. We found the following changes for *BoSAT1b*: A61G (Asn21>Asp), C310A (Leu104>Ile) for *BoSAT2* and T732C, C735T, C777T, T789C, G813A, C843T, G855T, C939G; T219A for *BoSAT3*.

To validate that the BoSAT orthologs identified were functional *in vivo*, we performed a functional study by heterologous expression in yeast, as plant SAT overexpression in the yeast osmosensitive mutant *gpd1Δ* has been shown to confer tolerance to osmotic stress. As a positive control, we used the *Beta vulgaris SAT* used in our previous study (Mulet et al., 2004). All the *BoSAT* genes conferred tolerance to salt and osmotic stress (sorbitol) when overexpressed in the *gpd1Δ* mutant, with some differences (Figure 6a). *BoSAT1d, BoSAT3 and BoSAT5a* achieved the strongest growth recovery under osmotic stress, while the strongest growth complementation in the presence of salt was achieved by *BoSAT1b* and *BoSAT1d* expression. These data suggest that *BoSAT1d* may be more determinant under abiotic stress.

Since the growth complementation could be due to indirect effects, we also measured activity of the heterologously expressed enzymes by determining the content of low molecular weight sulfhydryls (LMWS) under control conditions and osmotic stress (sorbitol, 1.7M). Expression of BoSATs did not change LMWS accumulation under control conditions (Figure 6b). Interestingly, under osmotic stress, *BoSAT* expression significantly increased the level of LMWS probably because of the accumulation of glutathione, whose production is limited by cysteine biosynthesis (Liedschulte et al., 2010). When comparing *BoSATs*, and in line with the stronger yeast complementation, *BoSAT1d* expression leads to a higher LMWS accumulation.

We also measured enzymatic activity directly from raw yeast extracts. Yeast lacks endogenous SAT activity, which allows for the direct determination of the activity of the heterologous BoSAT isoforms (Mulet *et al*., 2004). To determine specific enzymatic activity, we expressed Venus-tagged BoSATs and normalized the activity relative to the amount of enzyme, after validating that the tagging did not affect enzyme functionality (Supplementary figure S1). BoSAT1b, BoSAT1d, BoSAT3 and BoSAT5a presented a stronger SAT activity in crude extracts (Supplementary Figure S2a). When looking at the levels of each protein, we observed large differences among the BoSATs, yet all of them were expressed under a constitutive promoter (Supplementary figure S2b). This observation points towards post-traductional regulation of BoSAT amounts, at the level of mRNA/protein stability and/or translation efficiency. After normalization by the relative amount of protein (Figure 6c), we observed that BoSAT1b, BoSAT1d, BoSAT3 *and* BoSAT5a presented a similarly high activity, while BoSAT2 *was* 4-5 times less active, probably due to its problems for docking with the yeast OAS-TL because the lack of a terminal neutral amino acid.

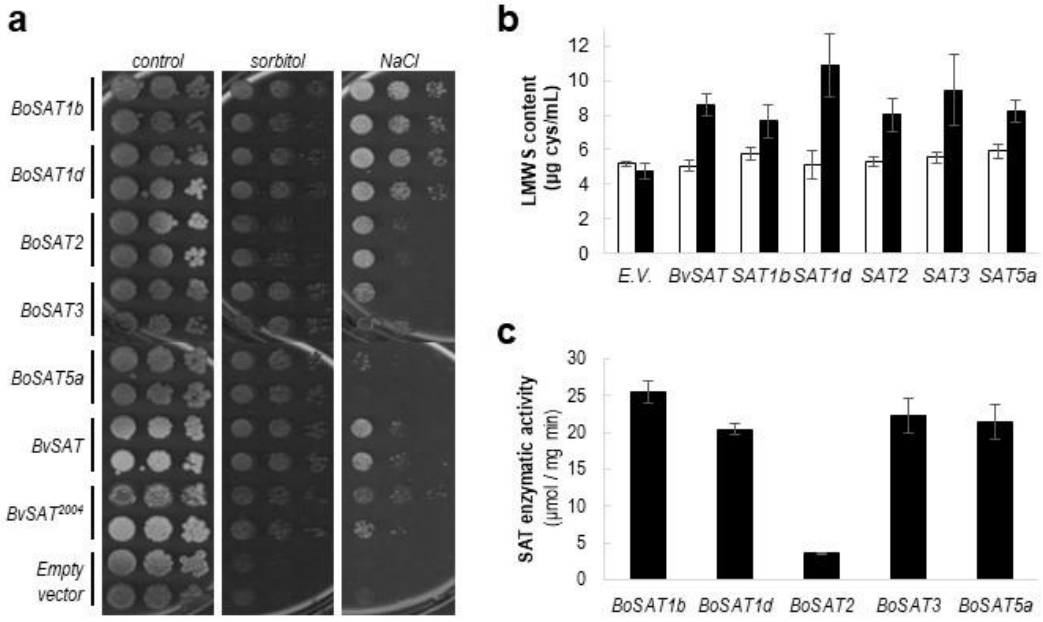


Figure 6. BoSAT activity *in vivo*. The indicated BoSATs were over-expressed in the yeast strain JM164. (a) 10-fold dilutions of yeast cultures were spotted onto YPDA plates supplemented with sorbitol or NaCl, as indicated. BvSAT^{2004} corresponds to the vector used in Mulet *et al*., 2004, while BvSAT was the same gene subcloned into the plasmid used for the BoSATs cloned in this work. (b) Low molecular weight sulfhydryl (LMWS) content expressed as cysteine equivalents measured from crude extracts under control conditions (empty bars) or osmotic stress (filled bars). Osmotic stress was applied by incubation of yeast cultures on YPDA supplemented with 1,7M sorbitol for 90 minutes. (c) BoSAT enzymatic activity normalized vs the amount of SAT protein. Raw values used for the quantification of enzymatic activity and the normalization of BoSAT protein levels can be found in Supplementary figure S2. Dat shown (b-c) are averages ± SEM of at least two biological repeats.

Subcellular localization of BoSATs

SAT enzymes localize to different compartments which may entail different functional specialization (Saito, 2000). As an initial approximation to study BoSAT subcellular localization, we analyzed the localization of the selected isoforms expressed in yeast (Figure 7, Supplemental figure 3). BoSAT1b, BoSAT1d, BoSAT2 and BoSAT5a showed a uniform cytosolic and nuclear localization while BoSAT3 was found in foci that co-localized with the mitochondrial stain MitoTracker®Red suggesting this enzyme could be mitochondrial, similar to its *A. thaliana* ortholog AtSAT3.

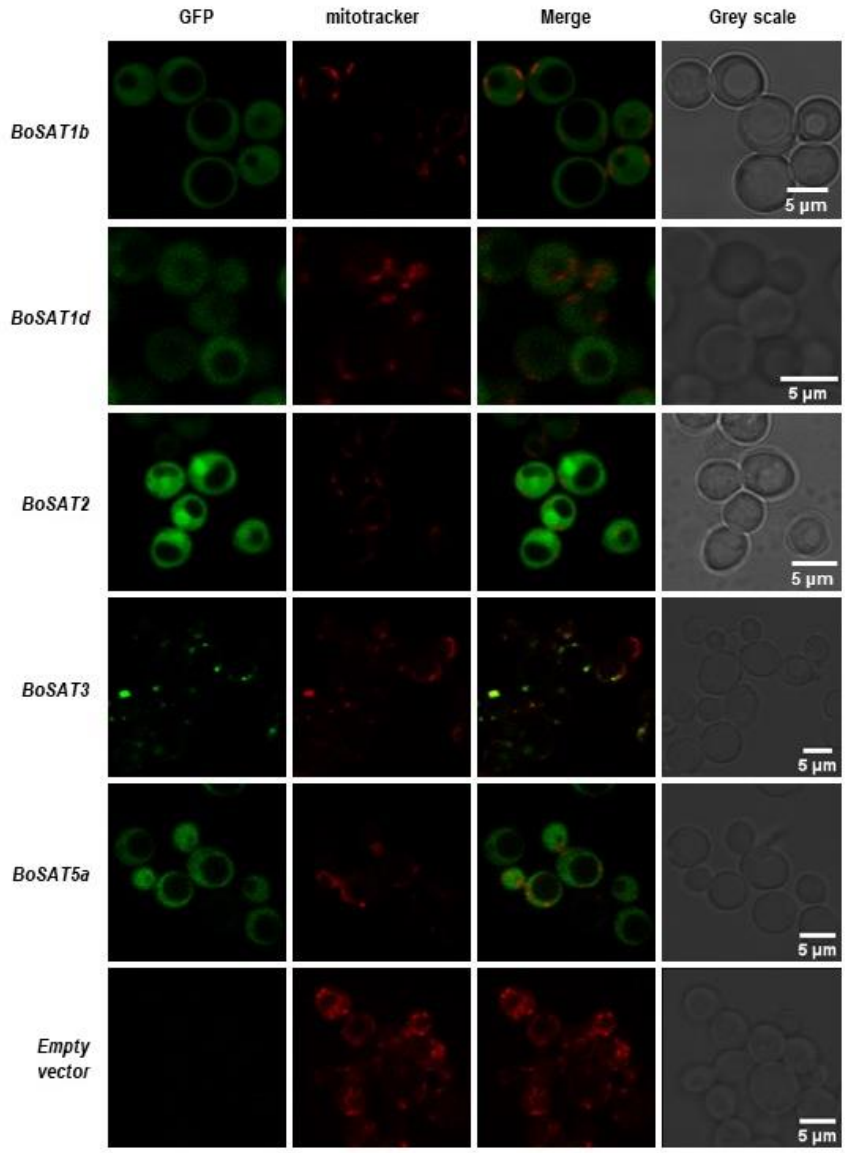


Figure 7. Subcellular localization of BoSATs in yeast. The indicated BoSAT5s tagged with GFP were over-expressed in yeast and images were taken under fluorescence microscopy after a mitochondrial staining (mitotracker).

To validate these preliminary findings in a plant model, we transiently expressed the selected BoSATs in *Nicotiana benthamiana* leaves and study localization in protoplasts obtained from the transfected epidermal cells (Figure 8). BoSAT1b and BoSAT1d were localized in the cytosol as the GFP signal in leaves expressing these isoforms filled the whole cytoplasm. BoSAT2 and BoSAT5a were cytoplasmic but also accumulated in the nucleus. As in yeast, BoSAT3 formed foci, but they co-localized with the chlorophyll autofluorescence signal, indicating chloroplastic localization. The same was observed in full leaves obtained from *N. benthamiana* (Supplementary figure S4). To ensure the

detected GFP signal corresponded to the GFP-tagged BoSATs, we performed western blotting from the infiltrated leaves, which confirmed the GFP signal detected corresponded to the tagged enzymes (expected size of the 60-70kDa) and discarded the accumulation of free GFP (27kDa; Supplementary figure S4).

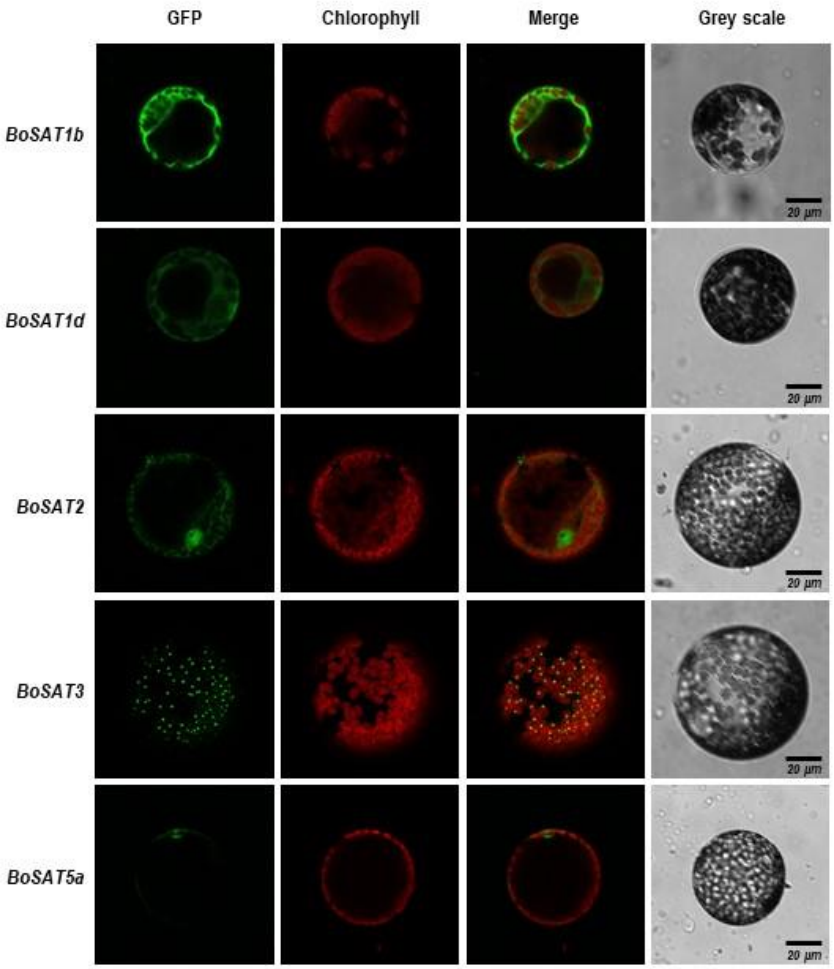


Figure 8. Subcellular localization of BoSATs in *N. benthamiana* protoplasts. Protoplasts were obtained from *N. benthamiana* leaves 2 days after agroinfiltration with plasmids containing BoSATs tagged with GFP. Chlorophyll autofluorescence was used to assess chloroplast localization.

Discussion

The study of Cys biosynthesis in broccoli is a pivotal question, not only for increasing our knowledge of a basic biochemical pathway, but also for applied science. Broccoli is grown in drought-prone areas, and most of the antioxidant response, essential for stress adaptation, depends on sulfur containing molecules, such as glutathione. In addition, glucosinolates have an essential role in broccoli metabolism as they are the main defense molecules against herbivores or pathogens and are health-promoting molecules due to their anticarcinogenic activity. A complete description of the enzymes participating in the Cys biosynthesis in broccoli will help future developments and may provide novel and healthier broccoli cultivars that are more resistant to abiotic stress.

We have found that SAT genes in broccoli are grouped in three groups according to their genomic structure. This structure is widely conserved, not only in the model plant *Arabidopsis thaliana* (Watanabe *et al*, 2008), but in plants from other species, such as *Lycopersicum esculentum* (tomato) (Liu *et al*., 2022). The main difference is that, in Arabidopsis, group 3 (with 9 exons) has two members (*AtSAT2* and *AtSAT4*) while in broccoli only one orthologue appears closely related to AtSAT2.

The expression analysis indicated that the chloroplastic *BoSAT3* isoform is the most highly expressed, while *BoSAT1d* is the one with the strongest response to salt and drought stress, both in roots and leaves (Figure 6b-c). In leaves, the level of *BoSAT1d* expression was about 5-fold higher under salt stress and 10-fold under drought stress. The basal expression agreed with the number of TATA boxes found in the promoter region (Figure 5, 6). The other *BoSAT1* genes also respond to abiotic stress, but to a lesser extent. This suggests that Cys biosynthesis is essential in the abiotic stress response and at the whole plant level. We did not detect any regulation by abiotic stress for *BoSAT2, BoSAT3* nor *BoSAT5*. Also, in the edible part of broccoli, the buds, the levels of expression were lower and the changes upon stress were not significative (Figure 6d).

The regulation of Cys biosynthesis at least partially depends on the subcellular localization of the SAT enzymes (Haas *et al*., 2008). We have found that proteins

encoded by the same genome structure (1 exon) have different localizations. The BoSAT1 paralogues are localized in the cytoplasm, while BoSAT3 is localized associated to the chloroplast (Figure 9). In Arabidopsis and grapevine, SAT orthologues with one exon (Group II) are localized to the mitochondria (Watanabe *et al*., 2008; Tavares *et al*., 2015), while in tomato this orthologue has been shown to be localized in the chloroplast (Liu *et al*., 2022), which is in agreement with what we observe for BoSAT3 in broccoli. The member of the subgroup 1 (two exons) studied here, BoSAT5a, presented a dual nuclear and cytoplasmic localization. In Arabidopsis and grapevine, members of subgroup I are only localized in the cytosol (Watanabe *et al*., 2008; Tavares *et al*., 2015) and in tomato they are mitochondrial (Liu *et al*., 2022). The only member of the group III (10 exons) found in the broccoli genome was localized also in the nucleus and cytoplasm. The tomato orthologue is cytoplasmic (Liu *et al*., 2022), and this is also the case in Arabidopsis (Watanabe *et al*., 2008) and grapevine (Tavares *et al*., 2015).

The most striking difference is that none of the Broccoli SATs localize to the mitochondria. Although *BoSAT3* localizes to the mitochondria when expressed in yeast (Figure 8), in plants, it is located in the chloroplast (Figure 9), so the regulation in broccoli is different to other plants, as all the plants investigated up to now, have at least one mitochondrial isoform. It has been described that the mitochondrial biosynthesis is the rate limiting step (Wirtz, *et al*., 2012) and that this is consistent with the fact that serine production in the mitochondria is the major source of this amino acid in photosynthetic tissues (Voll *et al*., 2006) and alteration in this distribution affects sulfur metabolism (Rosa-Téllez, *et al*., 2024). Also, a nuclear localization of SAT enzyme was not shown before. When we determined the enzymatic activity, we found that the activity of the BoSAT1a, 1b, 3 and 5a were similar while activity of BoSAT2 was about 5-fold lower (Figure 7c). This is also different to what has been observed in other crops such as *Pisum sativum*, where the major enzymatic activity was found in the mitochondria (Droux, 2003). If we consider both the specific enzymatic activity and the expression levels (Figure 6, 7c), it becomes clear that in broccoli Cys biosynthesis depends primarily on group II SAT enzymes, as the most expressed isoform are the chloroplastic *BoSAT3* followed by the cytoplasmic *BoSAT1b*. The expression of *BoSAT3* and *BoSAT1b* are not affected by stress.

In plants, sulfur containing molecules are pivotal for plant stress responses (Youssefian *et al*., 2001; Domínguez-Solís *et al*., 2004; Na and Salt 2011) and Cys is the first organic sulfur containing molecule formed by plant anabolism from inorganic sulfate, essential for the subsequent formation of molecules such as glutathione and phytochelatins (Romero *et al*., 2014). As mentioned before, the most striking difference that we have found compared to other plants where SATs have been characterized is that there are no mitochondrial isoforms. Moreover, the chloroplastic isoform is not regulated by stress. For instance, in tomato, the main stress response is chloroplastic (Liu *et al*., 2022), but in broccoli it seems to be cytoplasmic (Figure 6, 9). *Brassicacea* are rich in glucosinolates (Raiola *et al*., 2017). Over 120 GLS have been identified in *Brassicaceae* (Van Etten & tookey 2018). There are three major groups: aliphatic, derived mainly from methionine, but also from valine, leucine or isoleucine; aromatic, derived from phenylalanine or tyrosine; and indole, derived from tryptophan (Liu et al., 2020). The major glucosinolate in broccoli is 1-S-[(1E)-5-(methylsulfinyl)-N-(sulfonatooxy)pentanimidoyl]-1-thio-β-D-glucopyranose, commonly named glucoraphanin, which is derived from di-homomethionine, (a methionine chain elongated twice) (Agerbick & Olsen 2012). Cys is a precursor for methionine biosynthesis, which occurs in the chloroplast. The first step of glucoraphanin biosynthesis (the formation of di-homomethionine) also occurs in the chloroplast, while the other steps are carried out in the cytosol, including the incorporation of sulfate groups by the universal sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and glutathione (Sikorska-Zimmy and Beneduce, 2021). Methionine is required for many metabolic processes in addition to protein biosynthesis, such as polyamine or auxin biosynthesis. A tentative model would be that BoSAT3 is providing cysteine for methionine biosynthesis in the chloroplast at a standard rate, and BoSAT1b and BoSAT5a would provide cysteine in the cytoplasm, also at a standard rate under normal growth conditions, while BoSAT1d produces cysteine in a stress-responsive manner, mainly for the biosynthesis of glutathione, which can be used directly for stress responses or for the biosynthesis of glucoraphanin (Wirtz *et al*., 2012).

It has been reported that the carboxy-terminus is essential for the interaction of SATs with the next enzyme of the pathway, OASTL (Tavares *et al*., 2015). We have found that all the paralogues in broccoli share the essential Isoleucine in the C-terminus except BoSAT2 (Figure 3). Taken together (expression, activity and amino acid sequence), our

data suggest that the main point of regulation is *BoSAT1d* expression as the enzymatic activity is similar to the other isoforms, and the conservation at the carboxy-terminus does not indicate a different regulation at the level of complex formation. The low activity of BoSAT2 may be explained by its lack of a terminal Ile, which would difficult its docking to the OASTL. The physiological role of the observed nuclear localization for several isoforms remains to be elucidated. To our knowledge this is the first description of a SAT enzyme in the nucleus.

In conclusion, future biotechnological developments aimed to improve broccoli tolerance to abiotic stress or increase the content in health-promoting molecules should focus on increasing the expression of *BoSAT1d* or *BoSAT3,* as both are the most relevant serine O-acetyltransferase genes present in the broccoli genome or even trying to force BoSAT3 expression to the mitochondria to create and extra-pool of Cys.

Table 1. SAT members in *Brassica oleracea*. Broccoli has eight homologues of the SAT enzymes whereas in Arabidopsis there are five SAT enzymes.

Arabidopsis enzyme	Arabidopsis ORF	Broccoli locus	Broccoli protein ID	Broccoli enzyme	Exons	Broccoli chromosome location	Start (bp)	End (bp)
AtSAT1	*At1g55920*	*LOC106298495 Bo6g051550*	XP013590081	BoSAT1a	1	6	14456011	14457130
		LOC106328405 Bo3g185740	XP013622294	BoSAT1b	1	3	64884624	64885789
		LOC106296968 Bo6g035890	XP013588686	BoSAT1c	1	6	8917743	8919104
		LOC106337552 Bo4g040020	XP013632114	BoSAT1d	1	4	9167035	9168283
AtSAT2	*At2g17640*	*LOC106329084 Bo3g093600*	XP013623123	BoSAT2	10	3	34152622	34155015
AtSAT3	*At3g13110*	*LOC106295080 Bo5g132920*	XP013586311	BoSAT3	1	5	41361785	41363313
AtSAT4	*At4g35640*	-	-	-	-	-	-	-
AtSAT5	*At5g56760*	*LOC106328077*	XP013621888	BoSAT5a	2	3	6809432	6810720

		Bo3g019690						
		LOC106316954 *Bo9g126280*	XP013610273	BoSAT5b	2	9	39710026	39711216

Table 2. Characterization of BoSATs, indicating the *Arabidopsis thaliana* orthologue in the first column.

Arabidopsis enzyme	Broccoli enzyme	Genomics (bp)	CDS	Protein (aa)	pI	MW (kDA)
AtSAT1	BoSAT1a	1120	963	320	5,99	34,47
	BoSAT1b	1166	972	323	5,99	34,73
	BoSAT1c	1362	954	317	6,12	34,51
	BoSAT1d	1249	948	315	5,87	34,46
AtSAT2	BoSAT2	2394	969	322	5,81	34,53
AtSAT3	BoSAT3	1529	1176	391	8,85	42,78
AtSAT4	-	-	-	-	-	
AtSAT5	BoSAT5a	1289	930	309	6,66	32,54
	BoSAT5b	1191	927	308	5,84	32,51

Supplementary table S1. qPCR primers used in this work. The qPCR efficiency obtained experimentally for each pair of primers is also indicated.

Gene	**Primer name (ID)**	**Sequence**	**qPCR Efficiency**
BoSAT1a	SAT1a Fw2 (418)	CAGTCATGTTGTCTCGGTCAG	85%
	SAT1a Rv2 (419)	GAGAGGAGGGAATGGTTGTTATG	
BoSAT1b	SAT1b Fw3 (422)	TCTCCACACAACACAACCATTCC	93%
	SAT1b Rv3 (423)	AAAGAAGGGCTTTTCCGGTGC	
BoSAT1c	BoSAT1-cFWD (331)	CTGGACGGTGAAGATGATG	101%
	BoSAT1-cRVS (332)	GCGACGTAATGGAAGAGTAG	
BoSAT1d	BoSAT1-dFWD (333)	CTTCCCTGATCGGATTTCAC	110%
	BoSAT1-dRVS (334)	CGTCGGATTGAGCTTCTTC	
BoSAT2	SAT2 Fw2 (406)	CATGGAACAGGAGTGGTCATAG	103%
	SAT2-Rv2 (407)	TCCCGGTTCCTCCTAAAGT	
BoSAT3	SAT3 Fw2 (408)	AACACGCTCTTCGATCTCTTC	91%
	SAT3 Rv2 (409)	CGCGGGATCTCTCTCTTTAAC	
BoSAT5a	BoSAT5-aFWD (339)	CTTCTCCCATTGCCTTCTC	99%
	BoSAT5-aRVS (340)	GCGAACACTTCGGAGATAC	
BoSAT5b	BoSAT5-bFWD (341)	TACGTGATCCGGCTTGTA	71%
	BoSAT5-bRVS (342)	GGAGACTCGTGAGTGAAGA	
BoUBQ2	BolC.UBQ2.a-FWD (319)	CATCGAGCCGTCTTTAATGG	104%
	BolC.UBQ2.a-RVS (320)	GTGGCCACACTTCTTCTTC	
BoTBP1	BolC.TBP1.a-FWD (321)	GCTTTGCAGGCTCGTAAT	120%
	BolC.TBP1.a-RVS (322)	CTCTTGGCTCCAGTACAAAC	

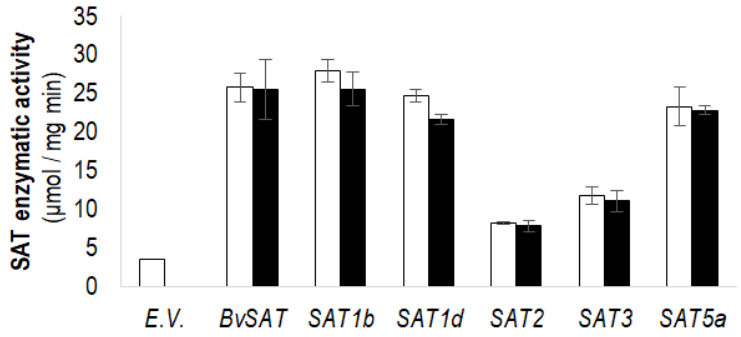
Supplementary table S2. Plasmids used in this work.

Cloning system and application	**Plasmid name**	***E. coli* Selection marker**
MoClo vectors for heterologous over-expression in *Saccharomyces cerevisiae*	pPGK1-BoSAT1b-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT1b-Venus-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT1d-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT1d-Venus-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT2-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT2-Venus-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT3-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT3-Venus-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT5a-tENO1 (CEN, URA3)	Kan
	pPGK1-BoSAT5a-Venus-tENO1 (CEN, URA3)	Kan
Golden braid vectors for localization studies in *Nicotiana benthamiana*	35S-BoSAT1b-tNOS	Kan
	35S-BoSAT1b-GFP-tNOS	Kan
	35S-BoSAT1d-tNOS	Kan
	35S-BoSAT1b-GFP-tNOS	Kan
	35S-BoSAT2-tNOS	Kan
	35S-BoSAT1b-GFP-tNOS	Kan
	35S-BoSAT3-tNOS	Kan
	35S-BoSAT1b-GFP-tNOS	Kan
	35S-BoSAT5a-tNOS	Kan
	35S-BoSAT1b-GFP-tNOS	Kan

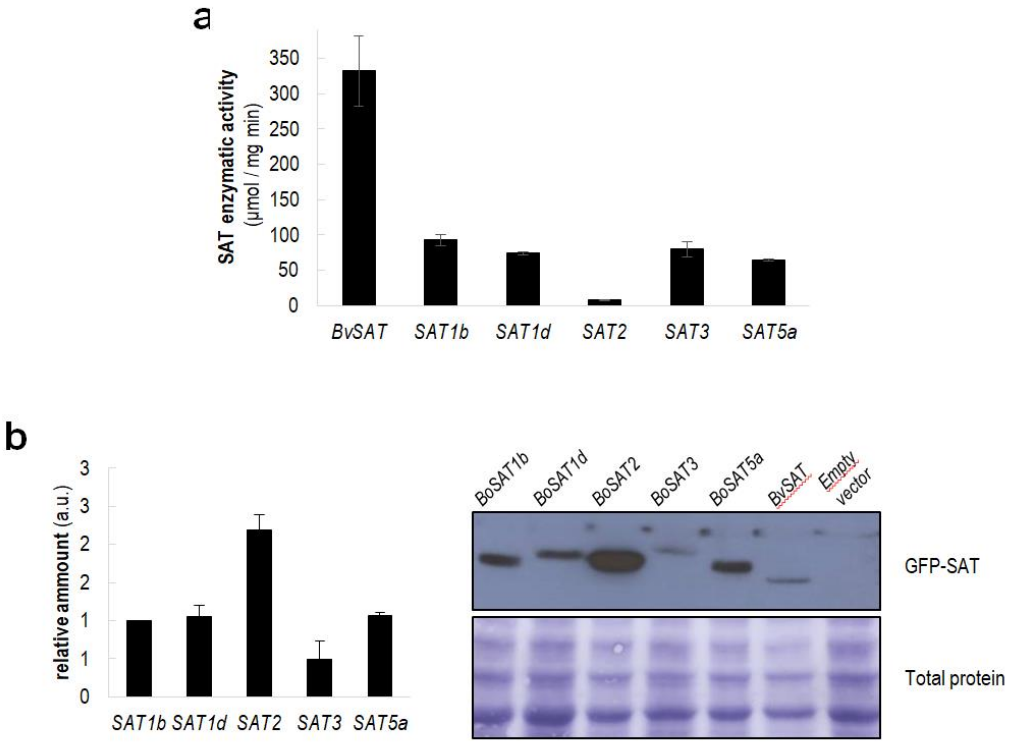
Supplementary table S3. Oligonucleotides used to generate the plasmids of this work**.**

Primers used for the MoClo system (yeast heterologous over-expression)		
Gene	**Name (primer ID)**	**Sequence**
BoSAT1b	BoSAT1b-P3-Fw (432)	GCATCGTCTCATCGGTCTCAT**ATG**GCACCCTGCATCGACACTTC
	BoSAT1b-P3-Rv (433)	ATGCCGTCTCAGGTCTCAGGATCCGATGACATAATCAGACCACTCGGT
	BoSAT1b-PCR1-Rv (435)	GCGCCGTCTCGGGATCTCTTTCCTTTGCTGC
	BoSAT1b-PCR2-Fw (436)	GCGCCGTCTCGATCCAGCTTGCGTAAGCTACG
BoSAT1d	BoSAT1d-P3-Fw (437)	GCATCGTCTCATCGGTCTCATATGGCACCCTGCATCTACAC
	BoSAT1d-P3-Rv (438)	ATGCCGTCTCAGGTCTCAGGAT**CC**GATAACATAATCAGACCATTGGGTCAAATAC
	BoSAT1d-PCR2-Fw (440)	GCGCCGTCTCGATCCAGCTTGCTTAAGCTACG
	BoSAT1d-PCR2-Rv (441)	GCGCCGTCTCGCAGTCTCTCCGATCACCACC
	BoSAT1d-PCR3-Fw (442)	GCGCCGTCTCGACTGCGGTGGTCGGAGACAACG
BoSAT2	BoSAT2-P3-Fw (443)	GCATCGTCTCATCGGTCTCATATGGACGGCGACGAGCTCCC
	BoSAT2-P3-Rv (444)	ATGCCGTCTCAGGTCTCAGGATCCTGTTGCGCTGTTGGTGTGTC
	BoSAT2-PCR1-Rv (446)	GCGCCGTCTCGGAAGACGGTTGGCTAGGACG
	BoSAT2-PCR2-Fw (447)	GCGCCGTCTCGCTTCAAAACCCAACCTTGTTGGC
	BoSAT2-PCR2-Rv (448)	GCGCCGTCTCGCAGTCTCACCTATGACCACT
	BoSAT2-PCR3-Fw (449)	GCGCCGTCTCGACTGCTGTGATAAGCAACCAT
	BoSAT2-PCR3-Rv (450)	GCGCCGTCTCGGGGACGGGTCTTGCTCATCA
	BoSAT2-PCR4-Fw (451)	GCGCCGTCTCGTCCCTGGCAATGAAACATGAT
		BoSAT3
	BoSAT3-P3-Fw (452)	GCATCGTCTCATCGGTCTCATATGTTCCCGGTCACAAGTCG
	BoSAT3-P3-Rv (453)	ATGCCGTCTCAGGTCTCAGGATCCAATAACATAATCCGACCACTCGGA
	BoSAT3-PCR1-Rv (455)	GCGCCGTCTCGAAACGATTGGCTCTCTCTCG
	BoSAT3-PCR2-Fw (456)	GCGCCGTCTCGGTTTCCGGCTACTACCAGGCCTCGATCGTTTCGCAACGAT

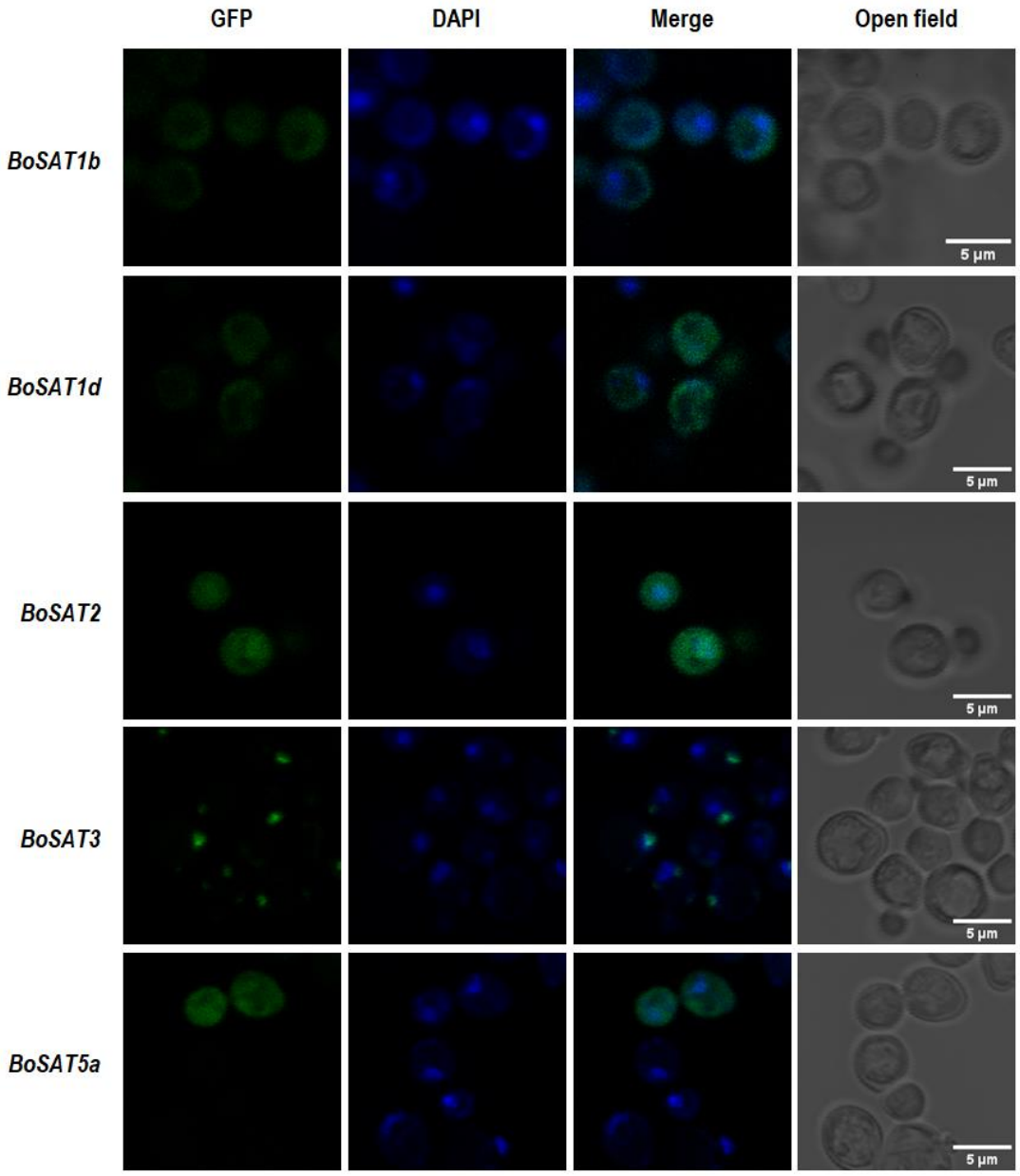
	BoSAT3-PCR2-Rv (457)	GCGCCGTCTCGCAGTCTCCCCGATCACAATC
	BoSAT3-PCR3-Fw (458)	GCGCCGTCTCGACTGCGGTGGTGGGGAACAA
BoSAT5a	BoSAT5a-P3-Fw (459)	GCATCGTCTCATCGGTCTCATATGCCGCCGGCCGGAGAACT
	BoSAT5a-P3-Rv (460)	ATGCCGTCTCAGGTCTCAGGATCCAATGATGTAGTCTGACCATTCCGAG
	BoSAT5a-PCR1-Rv (462)	GCGCCGTCTCGGGGACGAGTGAGAAAGAATCGTC
	BoSAT5a-PCR2-Fw (463)	GCGCCGTCTCGTCCCTCGAGCGATCCATCTC
Primers used for the Golden braid system (localization studies in *N. benthamiana*)		
Gene	**Name (primer ID)**	**Sequence**
BoSAT1b	BoSAT1b-Fwd	GCGCCGTCTCGCTCGAATGGCACCCTGCATCGACAC
	BoSAT1b-Rvs	GCGCCGTCTCGCTCACGAACCGACATAATCAGACCACTCGG
BoSAT1d	BoSAT1d-Fwd	GCGCCGTCTCGCTCGAATGGCACCCTGCATCTACAC
	BoSAT1d-Rvs	GCGCCGTCTCGCTCACGAACCAACATAATCAGACCATTGGGTC
BoSAT2	BoSAT2-Fwd	GCGCCGTCTCGCTCGAATGGACGGCGACGAGCTCCC
	BoSAT2-Rvs	GCGCCGTCTCGCTCACGAACCTGCGCTGTTGGTGTGTCCGT
BoSAT3	BoSAT3-Fwd	GCGCCGTCTCGCTCGAATGTTCCCGGTCACAAGTCG
	BoSAT3-Rvs	GCGCCGTCTCGCTCACGAACCAACATAATCCGACCACTCGG
BoSAT5a	BoSAT5a-Fwd	GCGCCGTCTCGCTCGAATGCCGCCGGCCGGAGAACT
	BoSAT5a-Rvs	GCGCCGTCTCGCTCACGAACCGATGTAGTCTGACCATTCCG



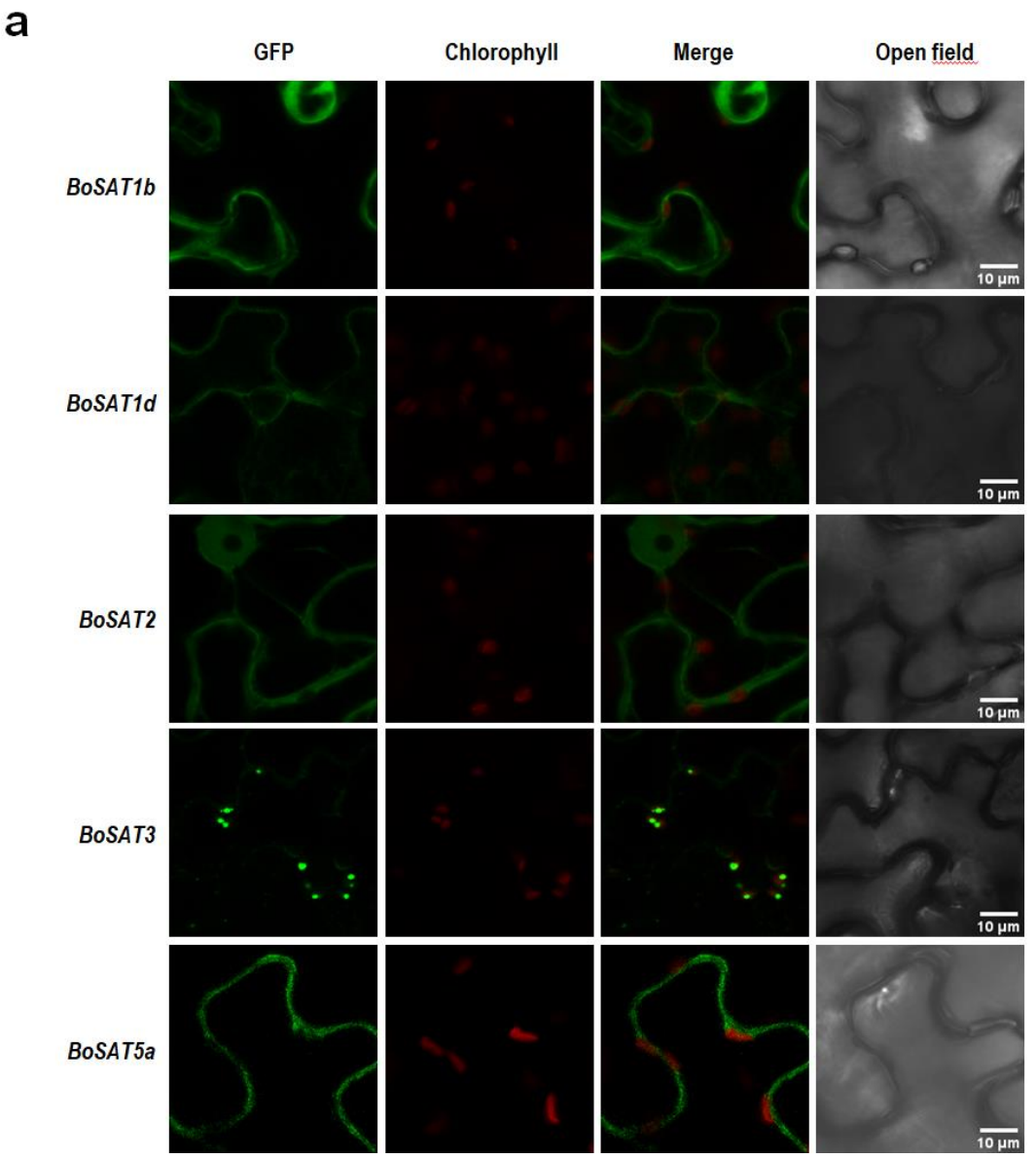
Supplementary figure S1. Effect of Venus tagging on BoSAT activity. The indicated BoSATs, either tagged (filled bars) or untagged (empty bars) were over-expressed in the yeast strain JM164 and enzymatic activity was measured as µmol of substrate converted per mg of protein per minute. Data shown are averages ± SEM of three biological determinations of at least two biological replicates.

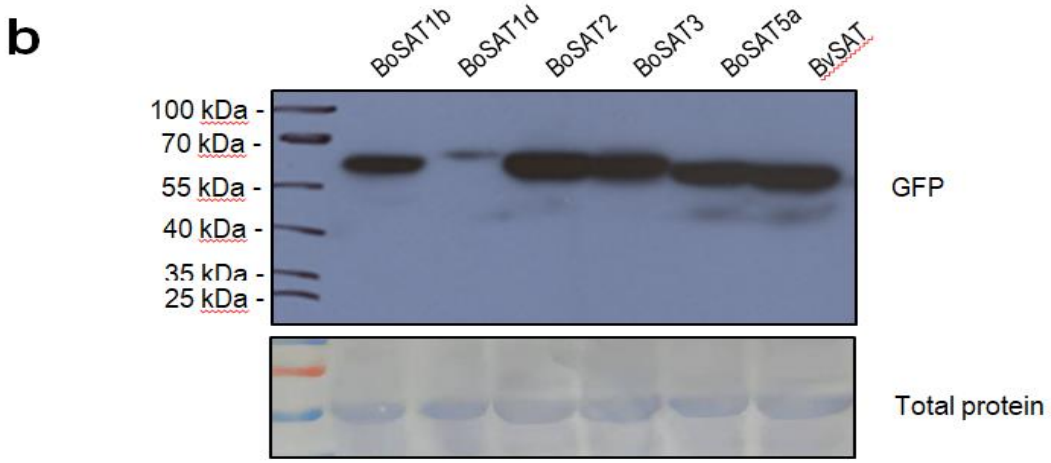


Supplementary figure S2. BoSAT raw enzymatic activity and expression levels upon over-expression in yeast. (a) SAT enzymatic activity measured as µmol of substrate converted per mg of protein per minute was determined as indicated in materials and methods. (b) Relative expression levels of BoSAT upon over-expression in yeast (right panel) and a representative western blot (left panel). Data shown are averages ± SEM of at least two biological replicates.



Supplementary figure S3. Subcellular localization in yeast. The indicated BoSATs tagged with GFP were over-expressed in yeast and images were taken under fluorescence microscopy after a nuclear staining (DAPI).


a
GFP
Chlorophyll
Merge
Open field
BoSAT1b
BoSAT1d
BoSAT2
BoSAT3
BoSAT5a
10 µm


b
BoSAT1b
BoSAT1d
BoSAT2
BoSAT3
BoSAT5a
BySAT
100 kDa -
70 kDa -
55 kDa -
40 kDa -
35 kDa -
25 kDa -
GFP
Total protein

Supplementary figure S4. Subcellular localization in *N. benthamiana*. *N. benthamiana* leaves were imaged 2 days after agroinfiltration with plasmids containing *BoSATs* tagged with GFP. (a) Microscopy images are shown. Chlorophyll autofluorescence was used to assess chloroplast localization. (b) Protein extracts obtained from the agroinfiltrated leaves were resolved by SDS-PAGE and GFP was detected by western blotting.

General discussion

General discussion

The overexpression of certain enzymes, or the identification of biochemical or physiological markers for abiotic stress tolerance have been proved to be useful for improving the overall yield of crops in a world with an increasingly growing population and increasing areas exposed to abiotic stress.

As previously mentioned, searching metabolomic datasets for biomarkers could be useful for future breeding programs aimed at cultivating stress tolerant varieties (Choudhury *et al*., 2021). However, the metabolomic response and tolerance traits can be different among species, or even varieties or cultivars. For example, under salt stress, *Oriza sativa* exhibited an increase in phenylalanine, threonine and valine whereas, *Thellungiella salsuginea* experienced a decrease in threonine and valine (Anzano *et al*., 2022). Also, barley was observed to exhibit salt tolerance by maintaining growth even under high salinity conditions in contrast with the case of other glycophytes, such as citrus, where the capability to minimize chloride ion uptake into the aerial parts is considered a marker of tolerance (Arbona *et al*., 2013).

Also, physiological traits can be markers for stress tolerance (Bayuelo-Jiménez *et al*., 2012). For instance, in *Phaseolus* species, the stomatal conductance is reduced in salt-sensitive species, and, in sugar cane, drought-tolerant varieties had better water status than the non-tolerant ones (Gomathi *et al*., 2020).

1. Molecular and physiological distinctive traits for drought stress tolerance in broccoli and melon

We found in melon certain factors associated with drought tolerance: higher stress/control ratios of isoleucine, glycine, serine and asparagine. Also, decreased water potential and decreased phenylalanine and tryptophan stress/control ratios were observed in tolerant varieties.

However, in the case of broccoli, we found that, under stress, higher net photosynthesis, water use efficiency and stomatal conductance as physiological parameters are related to drought stress tolerance, as well as the ABA stress/control ratio and the levels of serine, methionine (under stress) and glutathione, suggesting that sulfur metabolism is also a limiting factor for drought stress tolerance in broccoli. Similar results were found in *Pinus halepensis* (Taïbi *et al*., 2017). However, urea, quinic acid and gluconic acid lactone had lower stress/control ratios in tolerant varieties. Also, in tolerant varieties, the levels of alanine under stress are higher and the total content of leucine, lower. Despite not being a marker for stress tolerance, almost all essential amino acids accumulated upon drought stress in leaves and in the edible part of broccoli.

2. Molecular and physiological distinctive traits for salt stress tolerance in broccoli and melon

In melon, we found that Na^+/K^+ ratios were higher in tolerant varieties as well as in the case of histidine, whose stress/control ratio also correlated with salt tolerance. However, the stress/control ratios of phenylalanine and proline were lower in tolerant varieties.

In broccoli, transpiration and stomatal conductance were higher in tolerant varieties under stress and the hormones ABA, JA and the auxin IAA showed a higher stress/control ratio. Also, the total content of glutathione, methionine (under stress) and cysteine, that are tightly linked, as shown in Chapter V, correlated with sensitive varieties, as, also, in the case of histidine, asparagine, lysine and threonine. We observe the opposite in the case of metabolites related to the Krebs cycle, such as citric acid, fumaric acid, aspartic acid and glutamic acid, as their amounts increase in tolerant varieties. Proline was not only induced under salt stress, but also its accumulation was higher in tolerant varieties. We also found that tolerant varieties accumulate less sodium and potassium in leaves under stress and have a lower Na^+/K^+ ratio, opposite to what we found in melon. Therefore, in broccoli, salt stress tolerance seems to be associated with the extrusion of sodium to other parts of the plant, whereas in melon, it seems to be linked to the accumulation of sodium in the vacuoles.

Altogether, the majority of the traits differ among the species (Table 1) and, also, we observed that the mechanisms of stress response present substantial differences between the two species.

In this work, we present several biochemical and physiological traits that are distinctive among varieties of melon or broccoli that are tolerant and sensitive to drought and salt stress and they may be limiting factors for abiotic stress tolerance. Thus, they will be evaluated to determine their usefulness for predicting tolerance of previously uncharacterized varieties or to improve already existing varieties.

Table 1. Potential physiological and biochemical markers for stress tolerance to drought and salt stress in broccoli and melon. The potential markers involve changes in the stress/control ratio or the levels of a given molecule, as mentioned in the corresponding paragraph.

		Broccoli	Melon
Potential markers for drought tolerance	Physiological	Net photosynthesis, WUE Stomatal conductance	Water potential
	Biochemical	Serine, ABA Methionine Glutathione, urea Quinic acid Gluconic acid lactone, Alanine, leucine	Serine Phenylalanine Glycine, isoleucine Asparagine Tryptophan
Potential markers for salinity tolerance	Physiological	Transpiration Stomatal conductance Na^+/K^+ ratios	Na^+/K^+ ratios
	Biochemical	ABA, JA, IAA Glutathione, cysteine Methionine, citric acid Fumaric acid Aspartic acid Glutamic acid, proline Asparagine, histidine Threonine, lysine	Histidine Phenylalanine Proline

3. Metabolomics and taste in broccoli

A significant amount of studies regarding broccoli taste have been focused in glucosinolates, given their health-promoting characteristics. However, they have been proven to be related to a bitter or pungent taste (Chiu *et* al., 2019). Phenolic compounds are also associated with broccoli bitterness (Zabaras *et al*., 2012).

Broccoli is a crop with high economic value but, due to its taste, it is not very popular. A metabolomic approach was harnessed to identify that GABA is a metabolite that correlates positively with the good taste of broccoli, whereas lysine, alanine, leucine, and myo-inositol correlated with a bad taste. GABA has health benefits and it has been proved to be associated with better taste in pineapple (Gao *et al*., 2022) and peach (Jia *et al*., 2022). Therefore, enhancing the accumulation of GABA is an interesting biotechnological approach. The limiting step of the GABA biosynthesis and, thus, the most promising target, is the decarboxylation of glutamate to produce GABA and CO_2 in the cytosol by the glutamate decarboxylase enzyme (Fait *et al*., 2007). Also, tackling the biosynthetic pathways of the four molecules found to correlate with bad taste could be a useful strategy.

Also, when cooked, there are changes in the taste and the aroma of broccoli florets and stems. Indeed, during the roasting process, several sulfur-containing compounds were generated. Up to the 15-minute mark of roasting, the majority of volatiles increased over time or were newly formed, whereas most-sulfur containing compounds are no longer detected after 20 minutes of roasting, so this may need to be assessed when considering the impact of the manipulation of taste-related metabolites (Hong *et al*., 2023).

4. Characterization of the serine O-acetyltransferases enzymes in broccoli

Cysteine biosynthesis is the last step of sulfate assimilation in plants. It is a pathway of biotechnological interest not only because its role as a precursor of glucosinolates, glutathione or other important molecules, but also because among the enzymes

involved, we can find promising biotechnological targets for improvement of tolerance to abiotic stress, especially in species with agronomic interest.

Serine O-acetyltransferase catalyses the rate limiting step for cysteine synthesis and it comprises a different number of isoforms depending on the species. The *Arabidopsis thaliana* genome encodes five SAT enzymes, SAT1, SAT2, SAT3, SAT4 and SAT5. SAT2, SAT4 and SAT5 are cytosolic enzymes, with SAT2 and SAT4 exhibiting low expression levels. In contrast, SAT1 is expressed in plastids, while SAT3 localizes to the mitochondria (Kawashima *et al*., 2005). However, there are four SATs in *Vitis vinifera* and in tomato (Tavares *et al*,. 2015; Liu *et al*., 2022). In broccoli, we found eight SATs: BoSAT1a, BoSAT1b, BoSAT1c, BoSAT1d, BoSAT2, BoSAT3, BoSAT5a and BoSAT5b. We observed that all cloned enzymes were functional and conferred tolerance to salt and osmotic stress in yeast and that, except for the chloroplastic BoSAT3, the enzymes participate in cysteine production in the cytoplasm, and also in the nucleus in the case of BoSAT2 and BoSAT5a. Consequently, we suggest that the most promising candidates for biotechnological targeting to enhance stress tolerance in broccoli are BoSAT1d and BoSAT3, since BoSAT1d was the most induced upon stress and BoSAT3 exhibited the highest expression levels and the enzymatic activity is similarly high in both cases. The activity of SAT2 was the lowest probably due to the absence of the isoleucine that is essential for the formation of the complex with the OASTL (Tavares *et al*., 2015).

So far, no species without a mitochondrial SAT enzyme have been described. Recent unpublished results in our laboratory show that in *Beta vulgaris* there are only two SATs and neither of them localize to the mitochondria. Indeed, although it was thought that the location of both SAT and OASTL enzymes in three compartments (chloroplasts, mitochondria and cytosol), was due to the impermeability of endomembranes to cysteine because of the reactivity of the thiol group (Lunn *et al.*, 1990), it has been proven that cysteine is exchangeable between these compartments, where cysteine synthesis is regulated specifically (Heeg *et al.,* 2008 & Watanabe *et al.*, 2008).

As explained in Chapter II, sulfur metabolism seems to be limiting for drought stress tolerance in broccoli. Accordingly, the overexpression of a SAT enzyme is a promising approach to enhance tolerance to drought stress in this species. Regarding melon, the

SAT family has not been characterized yet, but a database search shows that there are four of these enzymes (https://www.ncbi.nlm.nih.gov/, [consulted 2024 May]). However, in this species, we did not see any pattern of accumulation of sulfur-containing molecules upon drought and salinity stresses. Therefore, it seems unlikely that sulfur metabolism has a pivotal role in melon, in contrast to broccoli. A biotechnological approach overexpressing enzymes involved in biosynthetic pathways of molecules that we observed to accumulate in tolerant varieties of melon like glycine could be also considered. For instance, the overexpression of a serine hydroxymethyltransferase-3, that converts serine to glycine, confers tolerance to salt stress in Arabidopsis (Mishra *et al*., 2019). This approach may be successful in melon as well.

Despite the fact that the serine O-acetyltransferase is the rate-limiting step for cysteine synthesis, the overexpression of OASTL in plants increases the number of thiols and the resistance to abiotic stress in tobacco (Sirko *et al.*, 2004). OASTL proteins are β-substituted alanine synthases and belong to the superfamily of pyridoxalphosphate-containing enzymes (Hatzfield *et al.*, 2000). Among the three functional OASTL found in *Arabidopsis thaliana*, OASTLA, OASTLB and OASTLC are encoded by, respectively, *OASTLA* (*At4g14880*), *OASTLB* (*At2g43750*) and *OASTLC* (*At3g59760*) genes. OASTLA is cytosolic, whereas OASTLB is in plastids and OASTLC is mitochondrial. In broccoli, OASTL enzymes have not been characterized yet so, in future research, broccoli OASTL isoforms could be pursued as biotechnological tools to improve abiotic stress resistance properties. We could expect these enzymes to be in the same location as SATs since they form a physical complex. As mentioned in Chapter V, the activity of SAT and OASTL enzymes is regulated by the formation of this complex. More specifically, SAT enzymes are only active when in complex, whereas OASTL is only active when it is in its free form. Sulfide favours the formation of the SAT-OASTL complex and O-acetylserine, induces its dissociation (Sirko *et al.*, 2004). This pathway is also regulated by feedback inhibition by cysteine, which involves competition between serine and cysteine for binding in the active site of some of the SAT enzymes and a cysteine-triggered conformational alteration in the C-terminal segment of it (Olsen, L. R., 2004).

There are several protocols for generating transgenic broccoli plants (Metz *et al*., 1995; Ravanfar & Aziz, 2015; Henzi *et al*., 2000; Chen *et al*., 2001) and transgenic melon plants (Bezirganoglu *et al.*, 2014*;* Vallés & Lasa, 1994; Akasaka-Kennedy *et al*., 2004; Choi *et al*., 2012), so there are effective protocols that can be used in order to pursue future approaches to evaluate the usefulness of the biotechnological targets proposed in this work.

Conclusions

Conclusions

1. In broccoli, by testing tolerant and sensitive varieties, we identified the following drought stress tolerance distinctive traits: under stress, increased net photosynthesis, water use efficiency and stomatal conductance. Also, higher stress/control ratio of ABA, higher levels of alanine (under stress), serine, methionine (under stress) and glutathione as well as lower stress/control ratio of urea, quinic acid, gluconic acid lactone and lower levels of leucine. For salt tolerance, we identified the following distinctive traits: increased transpiration and stomatal conductance under stress, higher stress/control ratio of ABA, JA, IAA and higher levels of fumaric acid, aspartic acid, glutamic acid and proline as well as and lower total content of glutathione, cysteine, methionine (under stress), histidine, asparagine, threonine and lysine as well as a lower Na^+/K^+ ratio.

2. The traits for drought stress tolerance identification in melon were: higher stress/control ratios of isoleucine, glycine, serine and asparagine and lower ones of phenylalanine and tryptophan as well as decreased water potential. Regarding salt stress tolerance in melon, we identified in tolerant varieties higher Na^+/K^+ ratios and stress/control ratio of histidine and lower stress/control ratios of phenylalanine and proline.

3. We identified that GABA correlates with good taste, while lysine, alanine, leucine and myo-inositol were associated with bad taste in broccoli.

4. We identified eight *SAT* genes in broccoli and we found expression of all of them in leaf, root and bud with the exception of *BoSAT5b* in roots. *BoSAT1d* is the paralogue most induced under stress, especially upon drought stress, whereas *BoSAT3* is the one with constitutively higher expression. We cloned five genes encoding the most relevant enzymes and all of them conferred tolerance to salt and osmotic stress in yeast. Their enzymatic activity was similarly high in all cases, except in *BoSAT2*. We conclude that *BoSAT1d* and *BoSAT3* are likely to be the most promising biotechnological targets for enhancing stress tolerance.

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