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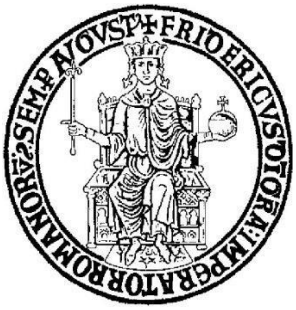

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Comprehensive analysis of stress response and fruit quality mechanisms in tomato: insights into the role of glutathione s-transferases and source-to-sink dynamics

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ABSTRACT

Climate change is dramatically affecting cultivable areas and diminishing natural resources, posing significant challenges to food security worldwide. As a critical horticultural crop and a key model for genetic studies, tomato (*Solanum lycopersicum*) is at the forefront of agricultural research due to its relatively short biological cycle and comprehensive genetic and genomic resources, including a fully and well-sequenced genome.

The primary aim of this doctoral thesis was to develop a comprehensive understanding of stress response mechanisms and fruit quality in tomato, focusing on the role of glutathione S-transferase (gst) genes and the intricacies of source-to-sink dynamics under drought stress.

We started with a thorough bioinformatics analysis of the gst loci in the *S. lycopersicum* according to the latest version of the tomato genome annotation (ITAG 4.1) and for the first time, we have identified and characterized the GST genes in its wild relatives: *Solanum pennellii, Solanum lycopersicoides,* and *Solanum pimpinellifolium*. This analysis included domains, structural features, motifs, phylogenetic relationships, expression patterns, orthologs, and synteny, offering a deep dive into the evolutionary history and functional diversification of the gst gene family. This foundational work highlighted the potential of these genes in environmental stress adaptation and improving fruit quality through a comparative expression analysis, covered different tissues (i.e. leaf, fruit, root) and a wide spectrum of stresses (heat, drought, salt, cold), enabling a detailed elucidation of GST tissue-specific gene expression dynamics in response to abiotic stressors. Furthemore, our study employed polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) to verify the enhanced accuracy of the Glutathione S-transferase (GST) gene family annotations between *Solanum lycopersicum* ITAG 4.1 and ITAG 2.4, underscoring the necessity of ongoing updates and experimental validation for the reliability of genomic data.

The thesis also delved into the functional roles of the *S. lycopersicum* tau Glutathione S-Transferase gene (*Solyc075056420*), which was functionally characterized through its stable over-expression in *Nicotiana tabacum* transgenic plants. The genetic transformation was facilitated using the Agrobacterium tumefaciens strain LBA4404, with the coding sequence regulated by the 35S2 promoter. T2 offspring transgenic plants with different levels of the gst mRNA expression underwent eco-physiological characterization under drought and salinity as well as molecular and metabolic characterization adding insights on the effect of the target genes on the plant response to environmental stresses through the control of the cellular redox potential. The expression of the gst gene appeared to regulate the antioxidant defense system by enhancing the efficiency of some antioxidants while reducing dependence on others. The modulation of the expression of other genes, particularly within the glutathione-ascorbate (GSH-AsA) cycle, seemed to help

optimize the plant's antioxidant response. This may have allowed to manage the accumulation of reactive oxygen species (ROS) more effectively under conditions of both drought and salt stress. A non-redundant reference *S. lycopersicum* core collection was selected from a global collection using genome-wide SNP data. Plants were grown under semi-controlled conditions with full water reintegration (FWR) and halved water reintegration (HWR) and changes in evapotranspiration, CO_2 assimilation, water use efficiency, leaf colorimetric parameters and leaf electrolyte leakage were measured. The most drought-resistant and susceptible phenotypes were selected for a deeper analysis of the plant response to drought in a second experiment. Upon the administration of the differential watering treatments (FWR and HWR), different tissues (leaves, stems, and mature green fruits) were sampled for metabolic and transcriptomic profiling by RNAseq analysis. Numerous differentially expressed genes (DEGs) were identified, indicating significant changes in gene expression that respond to drought stress. Specifically, the Red Setter tomato variety exhibited high shifts in gene expression across all plant tissues between control and stress condition, with 1,018 genes up-regulated and 646 down-regulated in leaves, 328 up-regulated and 835 down-regulated in fruits, and 320 up-regulated and 365 down-regulated in stems. Conversely, the Severianin variety showed a more moderate response, with 288 genes up-regulated and 242 down-regulated in leaves, 578 up-regulated and 545 down-regulated in fruits, and 231 up-regulated and 394 down-regulated in stems. These differentially expressed genes (DEGs) were then analyzed across various pathways to identify genes involved in key biological processes such as photosynthesis, hormone signaling, redox homeostasis, carbohydrate metabolism, solute transport, and cell wall organization. Our study has identified key genes that could better clarify the source-to-sink mechanism in tomato plants, particularly under drought stress. This investigation enhances our understanding of how these plants manage resource allocation and maintain fruit quality in adverse conditions.

Il cambiamento climatico sta colpendo drammaticamente le aree coltivabili e diminuendo le risorse naturali, ponendo sfide significative alla sicurezza alimentare in tutto il mondo. Essendo una coltura orticola fondamentale e un modello chiave per gli studi genetici, il pomodoro (*Solanum lycopersicum*) è in prima linea nella ricerca agricola grazie al suo ciclo biologico relativamente breve e alle risorse genetiche e genomiche complete, compreso un genoma completo e ben sequenziato.

Lo scopo principale di questa tesi di dottorato era quello di sviluppare una comprensione completa dei meccanismi di risposta allo stress e della qualità del frutto nel pomodoro, concentrandosi sul ruolo dei geni glutatione S-transferasi (gst) e sulle complessità delle dinamiche "source-to-sink" in condizioni di stress da siccità.

Abbiamo iniziato con un'analisi bioinformatica approfondita dei loci gst in *S. lycopersicum* secondo l'ultima versione dell'annotazione del genoma del pomodoro (ITAG 4.1) e per la prima volta abbiamo identificato e caratterizzato i geni GST nei suoi parenti selvatici: *Solanum pennellii, Solanum lycopersioides* e *Solanum pimpinellifolium*. Questa analisi ha incluso domini, caratteristiche strutturali, motivi, relazioni filogenetiche, modelli di espressione, ortologi e sintenia, offrendo un'immersione profonda nella storia evolutiva e nella diversificazione funzionale della famiglia dei geni gst. Questo lavoro fondamentale ha evidenziato il potenziale di questi geni nell'adattamento allo stress ambientale e nel miglioramento della qualità del frutto attraverso un'analisi comparativa dell'espressione, coprendo diversi tessuti (foglia, frutto, radice) e un ampio spettro di stress (calore, siccità, sale, freddo), consentendo una delucidazione dettagliata delle dinamiche di espressione genica tessuto-specifiche GST in risposta a fattori di stress abiotici. Inoltre, il nostro studio ha utilizzato la reazione a catena della polimerasi (PCR) e la PCR a trascrizione inversa (RT-PCR) per verificare la maggiore accuratezza delle annotazioni della famiglia di geni glutatione S-transferasi (GST) tra *Solanum lycopersicum* ITAG 4.1 e ITAG 2.4, sottolineando la necessità di aggiornamenti continui e validazione sperimentale per l'affidabilità dei dati genomici

La tesi ha inoltre approfondito i ruoli funzionali del gene glutatione S-transferasi di *S. lycopersicum* tau (*Solyc075056420*), che è stato caratterizzato funzionalmente attraverso la sua sovraespressione stabile nelle piante transgeniche di *Nicotiana tabacum*. La trasformazione genetica è stata facilitata utilizzando il ceppo Agrobacterium tumefaciens LBA4404, con la sequenza codificante regolata dal promotore 35S2. Piante transgeniche figlie di T2 con diversi livelli di espressione dell'mRNA gst sono state sottoposte a caratterizzazione eco-fisiologica in condizioni di siccità e salinità, nonché caratterizzazione molecolare e metabolica, aggiungendo approfondimenti sull'effetto dei geni bersaglio sulla risposta della pianta agli stress ambientali attraverso il controllo dell'attività cellulare del potenziale redox. Sembra che l'espressione del

gene gst regoli il sistema di difesa antiossidante migliorando l'efficienza di alcuni antiossidanti e riducendo la dipendenza da altri. La modulazione dell'espressione di altri geni, in particolare all'interno del ciclo glutatione-ascorbato (GSH-AsA), sembrava contribuire a ottimizzare la risposta antiossidante della pianta. Ciò potrebbe aver consentito di gestire l'accumulo di specie reattive dell'ossigeno (ROS) in modo più efficace in condizioni sia di siccità che di stress salino. Una raccolta principale di riferimento non ridondante di S. lycopersicum è stata selezionata da una raccolta globale utilizzando dati SNP sull'intero genoma. Le piante sono state coltivate in condizioni semi-controllate con reintegrazione completa dell'acqua (FWR) e reintegrazione dell'acqua dimezzata (HWR) e sono stati misurati i cambiamenti nell'evapotraspirazione, nell'assimilazione della CO_2, nell'efficienza nell'uso dell'acqua, nei parametri colorimetrici fogliari e nella perdita di elettroliti fogliari. I fenotipi più resistenti e suscettibili alla siccità sono stati selezionati per un'analisi più approfondita della risposta delle piante alla siccità in un secondo esperimento. Dopo la somministrazione dei trattamenti di irrigazione differenziali (FWR e HWR), diversi tessuti (foglie, steli e frutti verdi maturi) sono stati campionati per il profilo metabolico e trascrittomico mediante analisi RNAseq. Sono stati identificati numerosi geni espressi in modo differenziale (DEG), che indicano cambiamenti significativi nell'espressione genetica che rispondono allo stress da siccità. Nello specifico, la varietà di pomodoro Red Setter ha mostrato elevati cambiamenti nell'espressione genetica in tutti i tessuti vegetali tra condizione controllo e condizione stressante, con 1.018 geni up-regolati e 646 down-regolati nelle foglie, 328 up-regolati e 835 down-regolati nei frutti e 320 up-regolati. regolato e 365 down-regolati negli steli. Al contrario, la varietà Severianin ha mostrato una risposta più moderata, con 288 geni up-regolati e 242 down-regolati nelle foglie, 578 up-regolati e 545 down-regolati nei frutti e 231 up-regolati e 394 down-regolati negli steli. Questi geni espressi in modo differenziale (DEG) sono stati poi analizzati attraverso vari percorsi per identificare i geni coinvolti in processi biologici chiave come la fotosintesi, la segnalazione ormonale, l'omeostasi redox, il metabolismo dei carboidrati, il trasporto dei soluti e l'organizzazione della parete cellulare. Il nostro studio ha identificato geni chiave che potrebbero chiarire meglio il meccanismo "source-to-sink" nelle piante di pomodoro, in particolare in condizioni di stress da siccità. Questa indagine migliora la nostra comprensione di come queste piante gestiscono l'allocazione delle risorse e mantengono la qualità dei frutti in condizioni avverse.

CHAPTER I

GENERAL INTRODUCTION

1.1 Economic and food chain relevance of tomato at global level

The tomato (*Solanum lycopersicum*) belongs to the family of Solanaceae, standing as a cornerstone in global agriculture deeply integrated into human diets. It is a rich source of health-promoting compounds, such as vitamins, carotenoids, and phenolic compounds (Quinet et al., 2019; Conti et al., 2023).

Tomato production has grown significantly in the past few years across all the word. The global production was 186,1 million tons in 2022 according to the FAO statistics (FAOSTAT; https://www.fao.org/faostat). This represents a decrease of 1.7% compared to the 189.3 million metric tons produced in 2020 and an increase of 0.35% compared to the average of the previous three years (2019-2021). In terms of the size of the market of tomato globally, in 2023 China took the lead followed by India, Turkey, USA and Italy (Figure 1).

The global tomato market grew from $165.72 billion in 2022 to $178.65 billion in 2023, with a compound annual growth rate (CAGR) of 7.8%. The market is expected to reach $239.28 billion by 2027 with a CAGR of 7.6%. (Tomatoes Global Market Report, 2023).

However, tomato production faces challenges such as high input costs, pests, diseases, and postharvest losses, which necessitate continuous research and development to address these challenges.

The global economic importance of tomatoes extends beyond their market value. The crop's susceptibility to environmental stresses, poses significant threats to yield and fruit quality. Research focusing on the resilience of tomato cultivars to abiotic stress is crucial for ensuring stable production and mitigating economic losses, particularly considering the ongoing challenges posed by climate change (Conti et al.2023).

Owing to their significant economic value, tomatoes have become a central point of research, serving as a model crop to investigate plant molecular processes related to development, metabolism, and stress response (Quinet et al., 2019). Addressing the challenges in tomato cultivation, including genetic, environmental, and market-related factors, is essential for maintaining its position as a vital component of global food security and economic development.

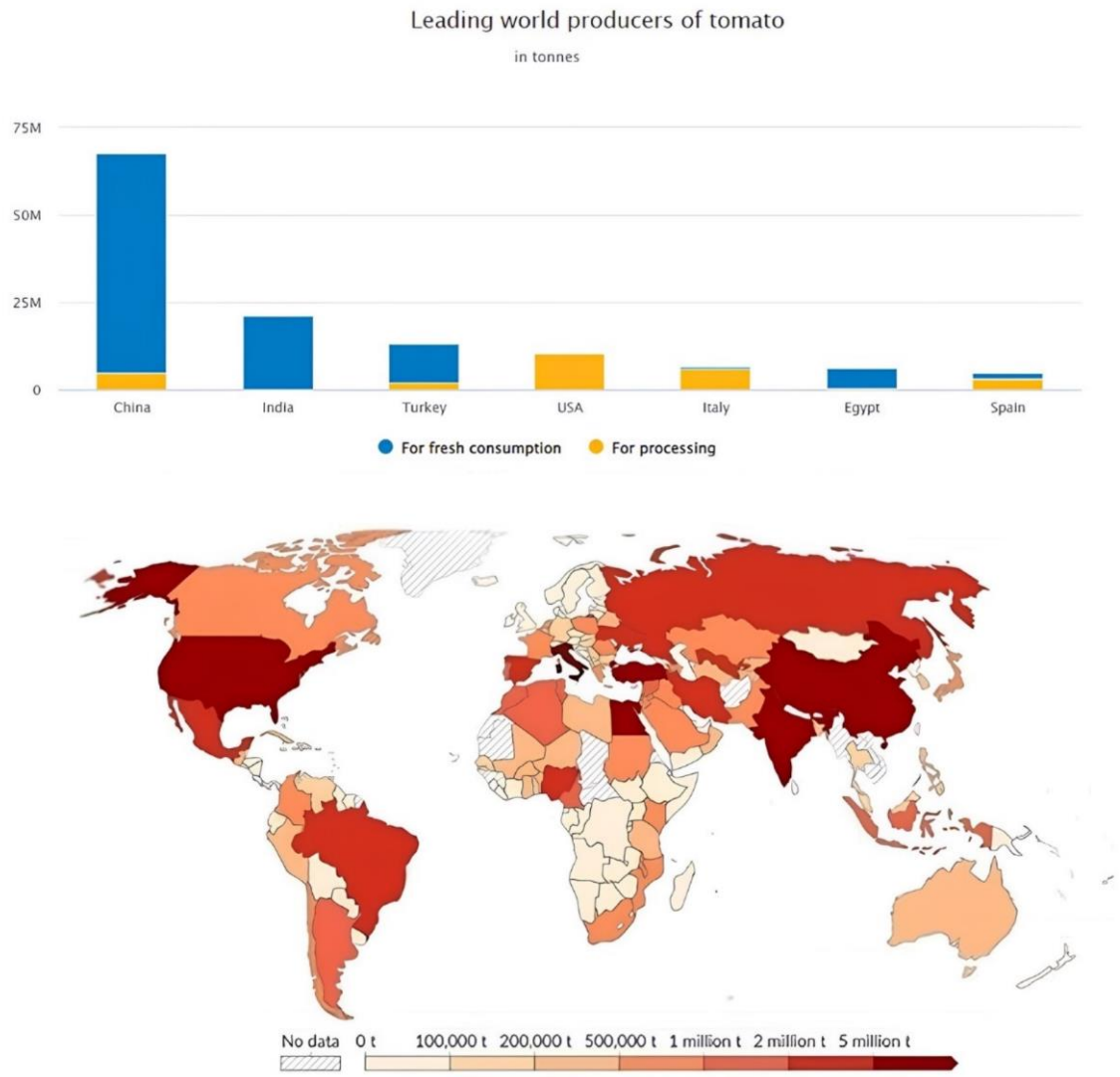

Figure 1. Global tomatoes production. In the upper graph report the ranking of the leading world Countries producing tomatoes. In the lower planisphere illustrates the worldwide estimated values of tomato production in tonnes in 2023 (FAOSTAT, 2023).

1.1.1 Tomato fruit quality and environmental sources of variation

Fruit quality, a main attribute in horticultural production, is determined by a complex interplay of genetic, environmental, and physiological factors. We can divide the tomato quality traits into chemical and physical properties, such as firmness, pH, and total soluble solids, and organoleptic properties, including taste and aroma, and nutritional contents. Tomato fruit is rich in bioactive compounds such as carotenoids, ascorbic acid (AsA) and phenolics, and (Table 1).

The tomato cell wall structure, predominantly constituted of cellulose, hemicellulose, and pectin, emerges as a pivotal determinant in the texture and firmness of the fruit, dynamically evolving across the fruit ripening process. In fact, the ripening process leads to a period of extensive remodeling in the cell wall, mainly governed by the hormone ethylene that orchestrates the expression of various set of genes and the regulation of different enzyme activities. Among others, the ripening process controls the activity of enzymes involved in the synthesis and oxidation of ethylene, inducing cellulase, xyloglucan-endotransglycosylase and other enzymes activating the fruit softening, thus leading to modification of its texture (Karlova et al., 2014; Quinet et al 2019).

The fruit content in Total Soluble Solids (TSS) is another important trait affecting fruit quality. TSS is primarily affected by sugars, organic acids and minerals, and its level affects fruit sweetness and processing yield. Elevated levels of TSS are often associated with enhanced flavor profiles, meeting consumer preferences. During the ripening process, occurring metabolic changes underline an increase in sugar content and a balancing of organic acids, further enhancing the overall fruit quality (Jiang, et al. 2019; Kiferle et al 2022).

Being a rich source of vitamins, minerals, phenolic content, flavonoid groups, dietary fibers, proteins and several antioxidant compounds, tomato helps us effectively in fighting against many types of cancer (Tilahun et al., 2017). In addition, it reduces the risk of developing hypertension and other cardiovascular diseases (Cheng et al., 2017). Tomato is one of the rich sources of natural antioxidants. Therefore, the beneficial properties of tomatoes are mainly attributed to a diverse range of antioxidative, chemo-preventive and anti-proliferative activities of its dietary antioxidants (Çelik et al., 2017). The association between tomato consumption and skin health, in particular the protection against atopic dermatitis, has been reported. This is followed by the impact tomatoes have on the gut microbiome and how this may lead to a reduced risk of liver inflammatory disease and inflammatory bowel diseases. Also, tomatoes improved exercise recovery and decreased muscle damage after physical exertion, modulated immune system, and reduced risk of infertility. Observational and experimental studies highlighting neuroprotection and a role in diabetes-associated oxidative stress have been reported. There are evidence suggesting that lycopene has anticancer properties, anti-angiogenic properties, reduces insulin-

like growth factor (IGF) in blood, and modulates cellular pathways that lead to cancer. Anticancer properties of other components of tomatoes, including its fibre, vitamin C, and phenolic constituent ferulic acid, have also been reported. These compounds are known to decrease the adverse effects of reactive oxygen species (ROS) produced during the normal metabolic reactions such as cellular respiration and photosynthesis, due to environmental stresses or UV irradiation (Foyer, 2018). These ROS cause serious oxidative damage to lipids, DNA, carbohydrates and proteins in our bodies (Yang et al., 2019).

Continued investment in research, including genetic engineering and innovative breeding methodologies will increasingly lead to the development of tomato varieties with enhanced flavor, enriched nutritional profiles, and enhanced resilience to both abiotic and biotic stresses. This dedicated approach is essential for meeting the increasing global demand for high-quality tomatoes, while also boosting their nutritional benefits and fortifying them against environmental challenges and disease.

Table 1. Nutritional value of tomato fruitomato referred to 100g of fresh weight.

Water (g)	94.52
Energy (kcal)	18.00
Protein (g)	0.88
Total lipid (g)	0.20
Carbohydrate, by difference (g)	3.89
Fiber, total dietary (g)	1.2
Sugars, total(g)	2.63
Minerals	
Calcium (mg)	10.00
Iron (mg)	0.27
Magnesium (mg)	11.00
Phosphorus (mg)	24.00
Potassium (mg)	237.00
Sodium (mg)	5.00
Zinc (mg)	0.17
Vitamins	
Vitamin C, total ascorbyc acid (mg)	13.7
Thiamin (mg)	0.04
Riboflavin (mg)	0.02
Niacin (mg)	0.59
Vitamin B-6 (mg)	0.08
Folate, DFE (µg)	15.00
Vitamin A (IU)	833.00
Vitamin E (mg)	0.54
Vitamin K (µg)	7.09

Source: USDA National Nutrient database.

1.1.2 Inheritance of fruit quality traits and their interaction with environmental stresses

The shifting consumer demand for tomatoes that are not only high-yielding but also flavorful and nutritious reflects a broader trend towards healthier and more sustainable food choices. Historically, agricultural breeding practices have often prioritized traits such as yield, shelf-life, and resistance to pests and diseases, sometimes at the expense of taste and nutritional content.

However, as awareness grows regarding the importance of diet in overall health and well-being, consumers are increasingly seeking out foods that not only satisfy hunger but also provide essential nutrients and enjoyable eating experiences. Tomatoes, being a staple ingredient in many cuisines worldwide, have become a focal point for this shift in agricultural breeding practices.

To meet this demand, plant breeders are now leveraging advanced breeding techniques, including traditional breeding methods and cutting-edge biotechnology, to develop tomato varieties with improved flavor profiles and enhanced nutritional content. This may involve selecting for traits such as sweetness, acidity, aroma, texture, and nutrient density.

Moreover, there is a growing emphasis on sustainable agricultural practices, which includes reducing the need for synthetic pesticides and fertilizers. By breeding tomatoes that are naturally resistant to pests and diseases, growers can minimize the use of chemical inputs, thereby improving the environmental sustainability of tomato production.

Most of fruit quality traits have a complex genetic inheritance and have essentially to be addressed by quantitative genetics tools to dissect the underlying genetic diversity. Diallel mating designs are particularly effective for identifying promising lines in breeding programs and for elucidating the genetic basis of desired traits. Fruit quality traits, such as nutritional and sensory properties, are significantly influenced by metabolites (Wang et al., 2015). Quantitative trait loci (QTL), DNA regions associated with a particular quantitative trait, are important because they directly influence traits like taste, nutritional value, and resistance to challenging environmental conditions. Several authors (Schauer et al., 2008; Alseekh et al., 2015) explored how some QTL control specific aspects of tomato metabolism, such as sugar and acid content. Investigation on both cultivated and wild tomato germplasm has revealed metabolic diversity and the potential of wild species for improvement (Schauer et al., 2005; Sauvage et al., 2014). Also, tolerance to combined abiotic stresses of salinity and heat in tomato introgression lines (*Solanum lycopersicum x Solanum pimpinellifolium*) was linked to significant changes in nitrogen metabolism, including increased concentrations of proteins and amino acids in more tolerant plants, and highlighted the importance of nitrogen use efficiency for improving tomato fruit quality under abiotic stress (Lopez et al., 2015).

Hybrid tomato cultivars are predominantly cultivated due to their high productivity, early maturation, uniform fruit quality, and disease resistance (Ingallina et al., 2020). A study reported that tomatoes have the highest number of registered varieties, 89% of which are hybrids (Santamaria and Signore, 2021). This trend highlights the need for further research on metabolites and fruit quality in tomato hybrids.

Heterosis, the superior performance of hybrids over their parental genotypes, is attributed to mechanisms such as dominance complementation, overdominance, and epistasis (Semel et al., 2006; Krieger et al., 2010; Soyk et al., 2020). Various studies have investigated the relationship between genetic distances of parental lines and heterosis in hybrids (Kaushik et al., 2018; Liu et al., 2020), with some using phenotypic, molecular, and genetic data combined with statistical and machine learning techniques to predict heterosis (Andorf et al., 2010; Kumar et al., 2020).

In tomato, heterosis has been noted for traits such as biomass production, yield, fruit quality, and metabolite content (Marchionni Basté et al., 2010; Solieman et al., 2013; Agustina et al., 2021), highlighting its significance in breeding for improved fruit quality.

The mechanisms influencing specific target traits, in any case, remain complex, involving multiple genes, biological processes, and molecular pathways (Li et al., 2019).

Breeding for yield and fruit quality may further increase the efficient use of resources. However, because of the limited possibilities for ambient control, a reduced heritability of important traits may be a major challenge to breeding programs. In-depth knowledge of quantitative genetic parameters and, in particular, genetic variance and heritability should help overcome those challenges. The characteristics of sugar, acidity, and flavours may vary greatly with respect not only to the cultivar but also to the environmental conditions. The solar irradiation experienced during ripening, the harvesting time, and the temperature, water supply, and storage conditions are important parameters that influence internal quality parameters [20, 21]. The heritability for traits related to taste, flavour, and nutritional compounds, i.e., the concentrations of sugars, lycopene, ascorbate and organic acids, ranged from 0.55 to 0.97 (Zörb et al., 2020; Reddy et al., 2020). Negative correlations of sugar concentrations with fruit weight and of organic acid concentrations with fruit weight and yield were frequently observed and suggest trade-offs in breeding for larger fruits and higher yields.

The frequency and length of heat waves and drought events are increasing global water resource depletion, making climate change a growing danger threatening global food security (Hein et al., 2021). Abiotic stressors, including drought, salinity, and temperature extremes, exert a profound influence on the development and quality of tomato fruits. Such stress conditions can induce alterations in fruit size, yield, and metabolite composition, impacting TSS, firmness, and bioactive compound levels. Severe levels of stress can dramatically compromise yield and fruit quality.

Although, moderate stress has been shown to enhance tomato fruit quality, increasing the levels of flavor compounds and beneficial phytonutrients like sugars, organic acids, and antioxidants (Rosales et al., 2011; Ripoll et al., 2016; Egea et al., 2022).

For these reasons, it is essential to plan and produce future crops and crop systems for sustainable agriculture by optimizing productivity and reducing negative environmental effects to guarantee both food and ecosystem security (Tian et al., 2022).

Understanding tomato plants' tolerance mechanisms has been the subject of extensive research efforts, as plant tolerance to abiotic stress is a complex phenomenon (Pineda et al., 2012; Lu et al., 2017). The plant response to environmental stresses leads to morphological alterations, alters plant homeostasis and impacts several physiological processes in plant development (Figure 2). Lower soil water potential due to salinity or reduced soil water availability during drought both result in diminished water uptake by plants, leading to a loss of cell turgor pressure (Cuartero et al., 2006). When it comes to heat stress, daily mean temperatures above 29 °C have a negative impact on tomato fruit yield. While extreme periods of high temperatures are irreversible, and cause significant output losses, the changes caused by under-mild stresses may be reversed (Mesa et al., 2022). Notably, tomato vegetative development is less vulnerable to periodic temperature increases because, even at temperatures as high as 38 °C, structural damage to its photosystem II does not occur (Lu et al., 2017). Oxidative stress, which raises reactive oxygen species (ROS) levels in plants, is a common consequence of several abiotic stimuli. Higher ROS concentrations lead to oxidative damage to nearly all biological components, including proteins, lipids, and nucleic acids, which results in programmed cell death (Kerchev and Van Breusegem, 2022).

Specific stressors elicit distinctive responses in plants. For example, in response to heat stress, plants open their stomata to enhance transpiration and reduce leaf temperature. In contrast, during drought or salt stress, plants close their stomata to save water, thus demonstrating the range of strategies plants employ to cope with various environmental challenges (Rivero et al., 2022).

It has been observed that when tomato plants are subjected to both salinity and heat simultaneously, the impact on their growth is less severe than when they face salinity as a single stressor. This occurs because higher temperatures can lead to increased transpiration, which protects the photosynthesis mechanism. Consequently, there may be a temporary boost in plant growth and CO_2 assimilation rates (Lopez-Delacalle et al., 2020). Depending on the type of abiotic stress, tomato fruits can experience different metabolic changes. Specifically, when heat stress is applied during the later stages of fruit development, both vitamin C and carotenoids are significantly reduced, while the absence instead of water can increase other primary and secondary metabolites (Hernández et al., 2015; Albert et al., 2016).

The strategy of growing agricultural crops under precisely controlled environmental stress has been proven to enhance fruit quality (Fanciullino et al., 2014). This approach can improve tomato quality by increasing levels of sugars, amino acids, and organic acids, enriching the fruit flavor and nutritional value (Quinet et al., 2019). Additionally, plants raised under stress conditions tend to accumulate higher levels of antioxidants through both enzymatic and non-enzymatic systems (Klunklin and Savage, 2017). Since these antioxidants play a crucial role in fighting off oxidative stress such conditions profoundly impact the types and quantities of metabolites found in tomatoes, effectively altering their nutritional profile (Miller et al., 2010).

Applying a moderate level of stress that does not hinder plant development can enhance fruit quality without affecting yield, particularly by increasing antioxidants. For instance, the strategy of controlled salt stress application in greenhouses has been suggested as an effective way to achieve this outcome (Toscano et al., 2019; Meza et al., 2020).

It's evident that these stresses lead to significant changes in how certain genes behave. For successful plant breeding that focuses on fruit quality in tough environments, understanding the function and the expression of these genes, and how they interact with the environment and each other, is key. Several genes that enhance the nutritional quality of tomato fruits under stress have been identified. These genes are involved in building up antioxidants (such as carotenoids and tocopherols), important for the plant's stress response, developing fruit chloroplasts, managing the transition of chloroplasts to chromoplasts in fruits, and responding to light signals which affect fruit quality (Egea et al., 2022).

Figure 2. Environmental factors impacting tomato fruit yield and quality.

1.2 Breeding approaches for enhancing fruit quality stability

The initial stage of plant breeding is the domestication of wild plants, where plant parts or seeds with desired characteristics are preserved for future cropping seasons (Lin et al., 2014). Post-domestication involves creating new, valuable varieties by selecting more desirable traits from the domesticated plants. Historically, plant breeding has focused on selecting visibly desirable traits from cultivated crops. In the modern era, molecular markers have facilitated the selection of desirable traits often linked to one or several quantitative trait loci (QTLs), thereby reducing the time and effort needed for cross-breeding (Foolad and Panthee, 2012).

Linkage drag is a major limitation of traditional crossbreeding methods, as it can introduce undesirable traits from a parental donor along with the desired ones. This challenge has been effectively addressed by genetic engineering techniques, such as transgenesis, which allow for the insertion of only the genes or alleles of interest into an elite plant. However, these products are subject to stringent regulation and require numerous costly and time-consuming trials and biosafety assessments before release into the environment. This is partly due to the requirement of introducing a selection marker, typically derived from non-plant sources, or the integration of foreign DNA into a targeted plant (Bai and Lindhout, 2007).

Another significant plant breeding tool involves generating random mutations in plants using chemical or physical agents, such as gamma rays. These agents induce widespread DNA damage, including double-stranded breaks (DSBs) and nucleotide modifications, resulting in the creation of multiple mutant strains. Backcrossing, although longer, is often necessary to eliminate unwanted mutations and may also fix unforeseen changes in the genomes of the mutant plants (Shelake et al., 2019). The domestication of the cultivated tomato (*Solanum lycopersicum var. lycopersicum*) likely started in the Andean region of Central America, progressing through two intermediate stages with *S. pimpinellifolium* as the first stage's direct ancestor and *S. lycopersicum var. cerasiforme* in the second stage. Fruit size was a key factor in the evolution and selection during domestication (Lin et al., 2014). Other characteristics distinguishing domesticated tomatoes from their wild relatives include growth parameters and fruit traits (Bai and Lindhout, 2007).

According to Bai and Lindhout (2007), the three primary traditional tomato breeding methods—pedigree, hybrid, and backcross breeding—focus on combining various traits for different market and consumption requirements. Due to the long period of selective domestication that reduced genetic diversity among inbred tomato populations (Ranc et al., 2008), cross-pollination among these populations is the simplest and fastest method to achieve genetic variation and subsequent

selection of new varieties with novel traits. The pedigree method records the performance of all progenies across many generations of a hybrid from genetically distinct parents, aiding in the selection of varieties with new traits of interest.

Tomato breeders use backcross breeding to introduce new alleles into cultivated lines and repeatedly backcross progenies with the parental cultivated lines to recover their genetic backgrounds. This approach aims to acquire unique features, such as biotic or abiotic stress tolerance, from wild relatives. These traditional breeding methods are time-consuming and labor-intensive, requiring meticulous monitoring and selection of the best progenies across multiple generations.

Introgression lines (ILs) are a set of lines constructed by using a combination of continuous backcrossing and selfing to replace chromosome fragments of the recipient parent (RP) with chromosome fragments of the donor parent (DP), which can directly transfer the desired traits of interest from wild varieties to domesticated varieties. Some studies demonstrate the usefulness of tomato *S. pennellii* ILs. A first example is the discovery of the Brix 9-2-5 quantitative trait locus (QTL), which is linked to the LIN5 cell wall invertase gene located on chromosome 9. This QTL, uncovered through the study of IL9-2-5, demonstrates the profound impact of ILs in increasing fruit sweetness by elevating °Brix values, (Fridman et al., 2002, 2004). Others QTL related to harvest index and Brix in ripening fruit were investigated using IL2-1 and IL8-3, respectively (Gur et al., 2010; Ikeda et al., 2013). In addition to these studies, the ethylene responsive factor 1 (ERF1) gene, identified in IL7-3, has been highlighted for its significant role in the accumulation of phenolics and ascorbic acid levels in tomato fruits (Di Matteo et al., 2013; Calafiore et al., 2019)

In the genomics era, "marker-assisted selection" (MAS) uses molecular markers to assist in selecting specific genotypes (Collard and Mackill, 2008). Typically, PCR or sequencing is employed to identify the presence of QTLs in hybrid offspring using DNA markers closely linked to the target regions. The ability to sequence an entire plant genome at a low cost has greatly enhanced the efficiency of conventional breeding methods.

MAS has not only improved productivity but also the precision of tomato breeding. By focusing on specific genomic regions associated with desired traits, breeders can minimize the transfer of unwanted traits from wild relatives, thus reducing the occurrence of linkage drag. Precision breeding allows for the development of tomato varieties that maintain desirable characteristics of the cultivated parent, such as flavor, yield, and fruit quality, while also exhibiting enhanced stress tolerance or other targeted traits (Vu et al. 2020).

Genomic Selection (GS) represents an approach that leverages comprehensive genomic information to predict the performance or quality of individuals without directly assessing their

phenotypes. This method has enabled a more rapid and efficient selection process compared to traditional methods. By constructing predictive models from a reference population with known genotypic and phenotypic data, GS allows breeders to estimate the phenotypic potential of individuals based solely on their genetic makeup, significantly accelerating breeding cycles.

A study of underscore the application of GS in tomato breeding, focusing on improving fruit quality, a trait governed by complex polygenic influences and variable heritability (Duangjit et al., 2016). Their findings highlight the optimal use of 122 accessions and 5995 SNPs, pointing out that the density of SNP markers significantly influences prediction accuracy.

A depth analysis into the domestication and evolutionary history of tomato, has revealed a significant selection sweep that has shaped fruit-related traits. The work has identified genetic markers associated with fruit weight and flavonoid content, providing valuable genomic resources for targeted breeding efforts (Yang et al., 2023)

The integration of computer science with genomics is setting the stage for advanced breeding frameworks, especially for crops like tomatoes, aiming to fine-tune improvement processes to meet market and agricultural demands efficiently (Cappetta et al., 2020).

1.3 Biothecnological approaches to improve fruit quality stability

The introduction of innovative plant breeding technologies, commonly referred to as new plant breeding techniques (NPBTs), has significantly revolutionized the field of crop improvement. Among these, cisgenesis and intragenesis stand out as approaches that offer a higher degree of precision and specificity in introducing genetic variations compared to traditional methods.

Cisgenesis involves the transfer of genes between organisms that are conventionally crossable, (the same species), keeping the gene's organization intact. This method offers a more natural and less controversial alternative of the transgenesis for introducing desired traits such as disease resistance or quality improvements (Schouten et al.,2006).

Intragenesis, on the other hand, enables the insertion of recombinant genes derived from the same organism or from closely related organisms, using gene expression vectors to achieve new combinations of genetic traits. This approach is distinguished by its flexibility and the possibility of combining multiple genes in a single step, speeding up the genetic improvement process. One of the first applications of intragenesis was demonstrated by C. M Rommens (2007), who used this technique to develop potato varieties with improved fruit quality and reduced potential for acrylamide formation.

Advancements in gene editing techniques have significantly facilitated the development of new plant varieties with desired traits, effectively overcoming the primary limitations associated with standard genetic modification approaches. Gene editing provides a suite of technologies that allow for precise, targeted modifications without necessarily introducing DNA from other species. This has sparked extensive debates within public opinion, especially regarding whether organisms modified in such subtle ways—sometimes involving the alteration or silencing of a single letter within the genome—should be considered genetically modified organisms (GMOs) This progress has been made possible through the adoption of several strategies that exploit the capabilities of chimeric nucleases. Central to these strategies is a sophisticated system that merges sequence-specific DNA-binding domains with a nonspecific DNA cleavage module. By starting sequence-specific or targeted DNA double-strand breaks (DSBs), this system is intended to allow for precise genomic modifications. This will greatly improve the plant's natural DNA repair mechanisms, which include the error-prone non homologous end joining (NHEJ) and homology-directed repair (HDR) (Gascuel et al., 2017).

Among the forefront NPBTs are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems, with the CRISPR/Cas9 system standing out due to its versatility and efficiency-

The CRISPR/Cas9 system operates based on the adaptive immunity mechanism found in bacteria and archaea, which defends against invading viruses or phages. It employs precisely engineered nucleases to create double-stranded breaks (DSBs) in DNA, which are then repaired through either non-homologous end joining (NHEJ) or homology-directed repair (HDR)/homologous recombination (HR). The NHEJ repair mechanism, recognized for its propensity to introduce errors, can cause insertions, deletions, and nucleotide substitutions. (Tiwary et al., 2023)

The tomato has been a model organism for several pioneering trials. Noteworthy among these are the work of Lor et al. (2014), who successfully knocked out the PROCERA (PRO) gene implicated in gibberellin signaling regulation, and Hilioti et al. (2016), who demonstrated the effectiveness of ZFN by targeting the LEAFY-COTYLEDON1-LIKE4 (L1L4) transcription factor, crucial for plant development.

The CRISPR/Cas9 system, initially discovered as a component of the adaptive immune system in prokaryotes, has emerged as the most promising among NPBTs. Its proven adaptability for precise gene editing across various organisms, including humans, insects, and plants, was well documented (Shalem et al., 2014; Konermann et al., 2015). The system's potential has been further expanded through the development of the "Goldenbraid" technology, which allows for the multi-modular expression of several plant genes within a single construct, thus facilitating the creation of constructs capable of simultaneously modifying multiple targets of interest (Sarrion-Perdigones et al., 2013; Vazquez-Vilar et al., 2016).

This technology has already led to significant achievements in the modification of genes that influence fruit quality and storage potential, underlining the importance of shelf life as a key quality attribute. A notable example of this success is the targeting of the ripening inhibitor (RIN) gene in tomatoes. The RIN gene encodes a MADS-box transcription factor that plays a pivotal role in the regulation of ethylene synthesis and the ripening process of fruits. This strategic intervention demonstrates the technology's capacity to enhance fruit shelf life and quality by precisely modulating the genetic pathways involved in ripening (Ito et al., 2015)."

In *Solanum lycopersicum*, gene targeting has led to significant achievements, such as the precise editing of the pectate lyase (PL) gene, resulting in delayed fruit softening without affecting other ripening-related processes (Uluisik et al., 2016), and the modification of the SlAGAMOUS-LIKE6 (SlAGL6) gene, a MADS-box transcription factor that confers tolerance to heat stress and leads to the development of parthenocarpic fruits (Klap et al., 2016).

Furthermore, generating the CRISPR gene knockout mutants targeting the INVINH1 and VPE5 genes has resulted in a notable enhancement in the concentrations of glucose, fructose, and the overall fruit soluble solids content.

The great potential of NPBTs lies in their ability to precisely modify targets for enhancing nutritional value and reducing anti-nutritional factors. This necessitates further exploration and refinement of gene editing techniques. Integrating CRISPR technology with other DNA-modifying enzymes, such as recombinases, transposases, and enzymes responsible for DNA methylation and acetylation, is expected to open new avenues in gene editing. This suite of tools holds the promise of revolutionizing plant breeding by allowing for the precise and efficient improvement of crop traits. Such progress could align agricultural practices more closely with environmental sustainability and consumer needs (Gascuel et al., 2017).

1.4 Solanum lycopersicum as a model for studying fruit quality traits

The tomato (*Solanum lycopersicum*) occupies a pivotal role in genetic and genomic research, supported by a comprehensive suite of resources that enable a deep understanding of its biological complexities.

At the heart of the tomato's significance is its extensive characterization, which has been well-mapped and annotated. The availability of high-quality genomic sequences lays the groundwork for exploring the genetic factors influencing fruit quality, disease resistance, and other crucial agronomic traits. The First sequencing of the tomato genome by The Tomato Genome Consortium (2012) has been instrumental in establishing the tomato as a key organism in advancing plant research.

Furthermore, the tomato benefits from a rich array of genetic resources, including extensive collections of mutants and introgression lines. Its characterization is further enhanced by detailed genetic maps and molecular markers. These tools are indispensable for navigating the tomato genome, allowing researchers to pinpoint specific genes associated with desirable traits and to track their inheritance through breeding programs.

The integration of advanced bioinformatics tools and specialized databases, such as https://solgenomics.net/, has further solidified the tomato's status as a model organism. Bioinformatics platforms are crucial for analyzing genomic data, gene expression profiles, and protein interactions, offering a comprehensive view of the genetic networks that underpin tomato development and metabolism.

Additionally, the tomato's suitability for genetic transformation protocols has made it an exemplary subject for functional genomics studies. Efficient transformation techniques enable targeted modifications of the tomato genome, providing direct means to assess gene function and to manipulate nutritional and agronomic traits. For all these reasons, genetic improvement of tomatoes has become faster and more efficient.

Besides, the tomato a vital source of important nutrients like lycopene and β-carotene, is considered also the best organism for studying fruit quality traits in the nutritional genetics (Zhu et al., 2018). This purpose not only highlights the tomato's significance in terms of dietary consumption of his essential chemicals, but it also enhances its consideration for exploring the genetic foundations behind fruit nutritional profiles. Because tomatoes and their processed products contain health-promoting phytochemicals like tocopherols and flavonoids, they are considered functional foods (USDA 2022; Figure 3).

A variety of tomato and genetically modified strains with elevated quantities of nutrients including lutein, β-carotene, zeaxanthin, and lycopene have been produced through the process

of domestication and genetic innovation. This development places *Solanum lycopersicum* as a key genetic platform for advancing our understanding significant role of genetic intervention in the amplification of fruit quality attributes (Fraser et al., 2004).

The development of higher anthocyanin levels made possible by the deliberate introduction by Butelli et al. (2008) of transcription factors *Del* and *Rosea1* from *Antirrhinum majus* into the tomato, highlights the tomato's versatility for analyzing the genetic mechanisms controlling phytochemical synthesis and highlights its importance in the field of nutritional genomics as well as its potential to prevent disease (Butelli et al., 2008). The integration of genes like *Aft* and *atv* to increase anthocyanin levels across a range of *S. lycopersicum* strains, as well as the overexpression of genes like AtMYB75 and SlMYB75 in the cultivar MicroTom, demonstrate a variety of genetic strategies for improving fruit pigmentation (Zuluaga et al., 2008; Yan et al., 2020; Jian et al., 2019).

Under conditions of plentiful natural light, the tomato variation Pro35S:BrTT8 displayed a substantial buildup of anthocyanins, a reaction that was not observed in lower light. This behavior points to a complex regulatory mechanism that is influenced by environmental conditions and highlights the role *Solanum lycopersicum* plays in examining gene-environment interactions and how they affect the expression of fruit quality traits (Zhang et al., 2019).

The use of advanced phenotyping and metabolite profiling has been essential in quantitatively assessing how genetic variations influence growth and metabolite profiles, particularly within genome-wide association studies (GWAS) that target tomato fruit quality traits.

Solanum lycopersicum serves as a model for enhancing our understanding of innovative methods for agricultural and nutritional advancements (Liu et al., 2022). Its genetic diversity, coupled with a well characterized genomic structure, positions *Solanum lycopersicum* as a tool for exploring the complex interplay among genetics, environment, and fruit quality.

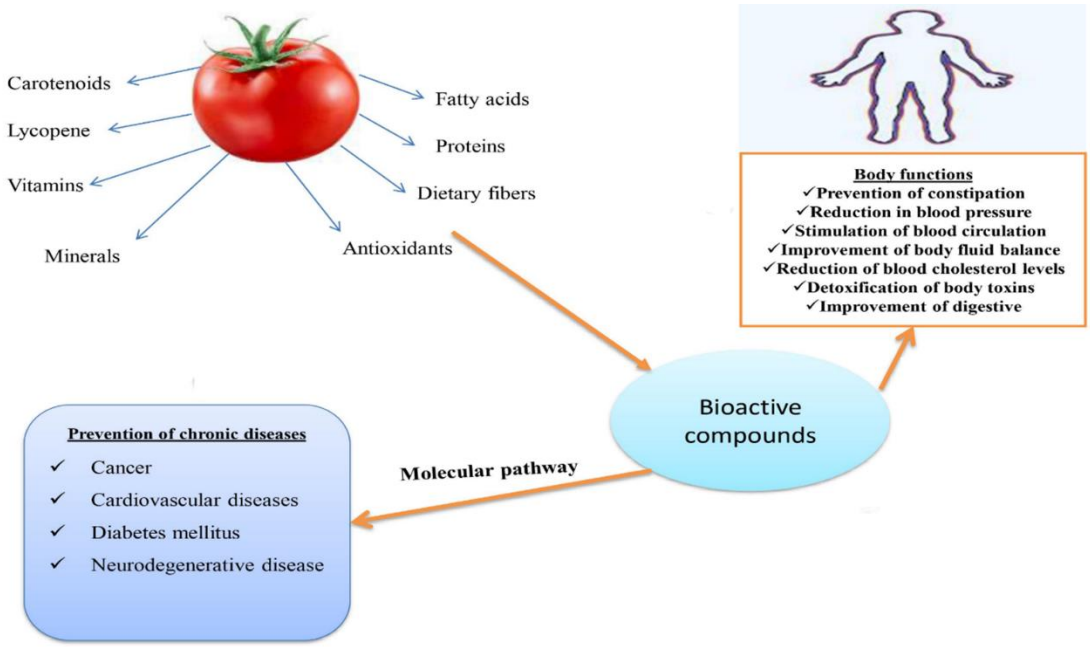

Figure 3. Tomato nutrient composition and its numerous health benefits.

1.5 Aims and thesis structure

The primary aim of this doctoral thesis was to deepen our understanding of stress response mechanisms and fruit quality in tomato. This research specifically focused on the role of glutathione S-transferase (GST) genes and the complex dynamics of source-to-sink relationships under drought stress.

- In chapter II, a bioinformatics analysis of the GST loci in *Solanum lycopersicum* was conducted according to the latest version of the tomato genome annotation (ITAG 4.1). For the first time, GST genes in its wild relatives *Solanum pennellii, Solanum lycopersicoides,* and *Solanum pimpinellifolium* were identified and characterized. The analysis included domains, structural features, motifs, phylogenetic relationships, expression patterns, orthologs, and synteny. A comparative expression analysis was conducted across different tissues (leaf, fruit, root) and a wide spectrum of stresses (heat, drought, salt, cold). The chapter further explored the presence of single nucleotide polymorphisms (SNPs) in GST genes and their correlation with stress response traits.
- In chapter III, the functional role of the *Solyc07g056420* GST gene, a member of the tau class GSTs, was investigated through its overexpression in transgenic *Nicotiana tabacum* plants. The genetic transformation process utilized Agrobacterium tumefaciens strain LBA4404, with gene expression driven by the 35S2 promoter. The T2 offspring transgenic plants underwent eco-physiological, metabolic, and molecular characterization to understand how this GST gene influenced plant responses to drought and salinity stress. Additionally, the study examined how the introduction of this gene affected the expression of other genes within the redox pathway, particularly those involved in the glutathione-ascorbate cycle.
- In chapter IV, the effects of drought stress on source-to-sink dynamics and fruit quality in tomato plants were examined. A core collection of genetically diverse S. lycopersicum specimens was selected using genome-wide SNP data and screened for varying drought responses. Detailed physiological and metabolic profiling of selected phenotypes under controlled drought conditions revealed the regulatory mechanisms influencing photo-assimilation, transport, and allocation of photo-assimilates. Through RNA-seq analysis across different tissues (leaves, stems, fruits), key genes and metabolic pathways were identified that mediate stress-induced changes in source-to-sink dynamics.

CHAPTER II

GLUTATHIONE S-TRANSFERASES: THEIR ROLE IN FRUIT QUALITY AND STRESS RESPONSE

2.1 Introduction

Glutathione S-transferases (GSTs) are phase II metabolic isozymes that catalyze the conjugation of the tripeptide (γ-Glu-Cys-Gly) glutathione (GSH) to a variety of substrates such as endogenous and xenobiotic compounds for detoxification (Hernández et al., 2020) (figure 4). GSH is a key player in the plant response to oxidative stress. In fact, GSH biosynthesis is stimulated when the cell faces stress conditions and develops its defense capability. Ascorbate and NADPH collaborate with GSH in the Foyer-Halliwell–Asada cycle (Potters et al., 2002;) (figure 5) for H_2O_2 detoxification, preserving cells from damages caused by exceeding levels of reactive oxygen species (ROS) (Hernández et al., 2017).

GST proteins typically include two binding domains: the glutathione-binding site (G-site) found at the N-terminal (GST-N) and the substrate-binding site (H-site) found at the C-terminal (Edwards & Dixon, 2005)

Recent studies have made it possible to identify and characterize the superfamily of GST genes in a multitude of plant species. These investigations revealed variations in the number of member genes. For example, *Arabidopsis* includes 53 GST genes (Sappl et al., 2004), *rice* 79 (Jain, Ghanashyam & Bhattacharjee, 2010), *apple* 69 (Jiang et al., 2019), *pepper* 85 (Islam et al., 2019), *pear* 57 (Li et al., 2022) and *fig* 70 (Liu et al., 2023).

The plant GST superfamily can be further divided into 10 classes based on criteria such as gene structure and function, protein sequence similarity, and immunological characteristics: TAU (U), ZETA (Z), PHI (F), dehydroascorbate reductase (DHAR), LAMBDA (L), THETA (T), eukaryotic translation elongation factor 1B γ-subunit (EF1Bγ), microsomal GST (mGST), tetrachlorohydroquinone dehalogenase (TCHQD), and glutathionyl hydroquinone reductase (GHR). TAU and PHI are the most abundant plant GSTs (Islam et al., 2017).

In the tomato (*Solanum lycopersicum*) genome – ITAG annotation 2.4 –, a total of 90 GST genes have been identified: 57 of these genes belong to the TAU class, 7 to LAMBDA, 6 to PHI, 6 to DHAR, 4 to THETA, 3 to EF1Bγ, 2 to ZETA, 2 to GHR, 2 to microsomal, and 1 to TCHQD (Islam et al., 2017).

Environmental stresses such as drought, salinity, and exposure to pollutants can induce oxidative stress, leading to the production of ROS. GSTs mitigate these effects by detoxifying ROS and other toxic metabolites.

Previous studies found TAU-class GSTs in tomato plants, which were able to mitigate oxidative stress-induced cell death in yeast (Kilili et al., 2004). A specific tomato TAU gene (*Solyc07g056480*) showed increased GST activity in response to low temperature-induced oxidative stress (Ding et al., 2022). It was observed that several GST genes were expressed at higher levels in salt-tolerant tomato accessions (Sun et al., 2010). Furthermore, in Arabidopsis plants, overexpression of a TAU tomato gene (LeGSTU2) led to improved tolerance to salt, drought, and osmotic stress, along due to elevated proline levels and enhanced antioxidant capacity (Xu et al., 2015).

In tomato, the expression of GST genes is differentially regulated during fruit maturation, indicating their potential role in the modulation of fruit color, flavor, and nutritional content (Zhou et al., 2022). GSTs are involved also in the metabolism of flavonoids, compounds that contribute to the antioxidant capacity of tomatoes and influence their taste and color (Qi et al., 2022).

Tomato wild relative species like *Solanum pimpinellifolium, Solanum lycopersicoides, and Solanum pennellii* exhibit remarkable tolerance to abiotic stress, flourishing in harsh conditions. This resilience is credited to the altered expression of a wide array of genes, sparking molecular and physiological mechanisms that enable these wild species to adeptly adapt (Loukehaich et al., 2012).

Since the release of the tomato genome into the public domain in 2012, both assembly and gene annotation have been refined and improved.

This work aims to provide a more comprehensive overview of the GST (Glutathione S-Transferase) gene family, emphasizing its significance in abiotic stress tolerance and its implications in fruit quality.

Based on these considerations, in this thesis we performed an update on the identification and characterization of GST genes of *S. lycopersicum* according to the latest version of the tomato genome annotation (ITAG 4.1) and for the first time, we have identified and characterized the GST genes in wild tomato species. Bioinformatic analysis of data from public repositories led to functional insights into the role of GSTs in controlling different biological processes their involvement in the plant response to drought was also investigated.

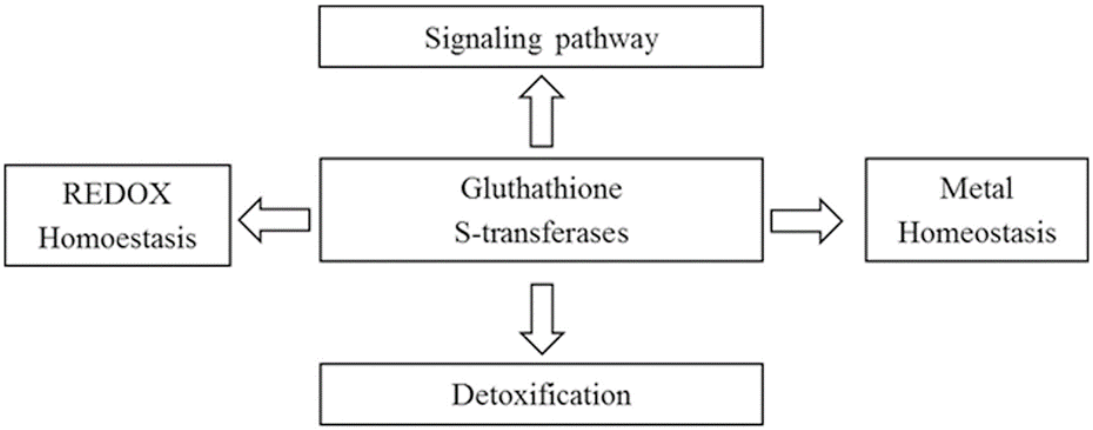

Figure 4. Diagram summarizing biological processes involving GST functions.

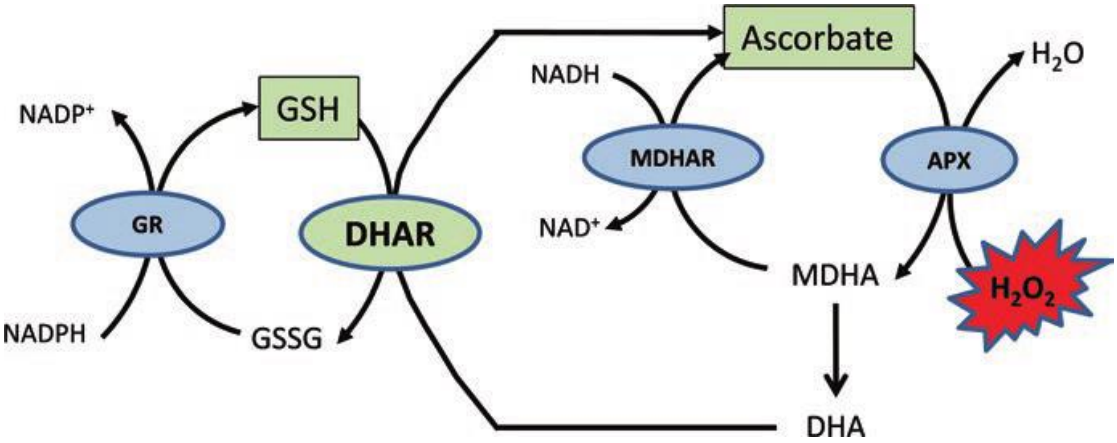

Figure 5. Ascorbate-glutathione (AsA-GSH) cycle in plants. The patway consists of a several redox-coupled reactions whose main function is the scavenging of H_2O_2 and the recycling to the oxidized forms of ascorbate and glutathione.

2.2 Materials and methods

2.2.1 Genome-wide identification of GSTs and PCR validation

FASTA formatted nucleotide and protein sequences GFF3 formatted gene annotation files refer to data provided by Fernandez-Pozo and colleagues (2015) and were retrieved from the Sol Genomics Network (SGN) database (http://www.solgenomics.net). The Arabidopsis GST protein sequences (N. = 53) were downloaded from Araport 11 (https://www.arabidopsis.org/), those of *S. lycopersicum* (N. = 90) were extracted from the multiFASTA file (ITAG 2.40) using the identified genes listed in (Islam et al., 2017). Those sequences were compared to the tomato proteome (ITAG 4.1) using *blastp* with an E-value cutoff ≤ $1e^{-15}$. In addition, the hidden Markov model (HMM) profiles PF02798 (GST-N) and PF00043 (GST-C) were downloaded from the Pfam database (Mistry et al., 2021) and used to search for GST candidates within the tomato proteome (ITAG 4.1) using HmmerWeb version 2.41.2 (Finn et al., 2011) with an E-value threshold ≤ e^{-5}. The protein sequences of *S. pennellii*, *S. pimpinellifolium* and *S. lycopersicoides* were retrieved from the Sol Genomics FTP server. Then, the GST protein sequences of Arabidopsis (N. = 53) and *S. lycopersicum* (N. = 83; ITAG 4.1) were used to search for GST candidates in wild tomato species using *blastp* with an E-value cutoff ≤ $1e^{-15}$. The same HMM profiles were also searched against the proteome of wild tomato species.

Chromosome position and gene structure of all identified GSTs were extracted from GFF3 files downloadable from the Sol Genomics FTP server (https://solgenomics.net/ftp/genomes/).

The exon-intron structures of GST genes were graphically identified by comparing the genomic and coding sequences in the online Gene Structure Display Server (GSDS, http://gsds1.cbi.pku.edu.cn/; Hu et al., 2014). The resulting upstream-downstream exon-intron and intron phase loci were exported from GSDS.

Prediction of subcellular localization of all annotated GSTs was carried out using CELLO v.2.5, a sub-cellular localization predictor tool (http://cello.life.nctu.edu.tw/; Yu et al., 2006).

To verify the enhanced accuracy of the Glutathione S-transferase (GST) gene family annotations provided in the *Solanum lycopersicum* ITAG 4.1 *vs* ITAG 2.4, target sequences were amplified by polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) and Sanger sequenced. DNA and RNA were purified from fully expanded young leaves of the tomato cultivar Heinz 1706. Leaf samples were collected from one-month-old plants grown in plant growth cabinet under a temperature of 20/24 °C, a photoperiod of 18/8h light/dark and a relative humidity of 65%. Leaf samples were homogenized in liquid nitrogen to preserve integrity of nucleic acids. For DNA extraction, the ground tissue was processed using the "Wizard® Magnetic 96 DNA

Plant System" kit by Promega. The RNA was isolated from leaf samples using the InviTrap® Spin Plant RNA Mini Kit from Invitek Molecular. The extracted RNA served as a template for reverse transcription, using the RevertAid First Strand cDNA Synthesis Kit.

Benchling platform (https://benchling.com), was used to design specific primers targeting the GST gene models (genomic and CDS sequences) according to prediction from both ITAG 2.4 and 4.1 releases.

The PCR products were separated via gel electrophoresis and visualized using a GelDoc, providing a confirmation of amplification and so the presence or absence of the targeted GST genes. Furthermore, the amplified products underwent Sanger sequencing and the resulting sequences were aligned against the expected sequences.

2.2.2 Identification of protein domains and motifs

The identification of protein domains was performed with the Conserved Domain Database (CDD) and Resources (Marchler-Bauer et al., 2017; Lu et al., 2020; Wang et al 2023;). GST sequences were searched against the Pfam database using an expected value threshold 0.01.

The MEME online server Version 5.4.1 (http://meme-suite.org/tools/meme; Bailey et al., 2009) was used to identify motifs (ecurring, fixed-length patterns) in GST protein sequences. Parameters were set as follows: motif site distribution = any number of repetitions (anr); maximum motif number = 10; and motif length = 6-200.

Graphical visualization of domains and motifs along the GST sequences was achieved using “biosequence structure illustrator” function within the TBtools software (Chen. et al., 2020).

2.2.3 Phylogenetic analysis

All identified GST protein sequences were used to build a multiple sequence alignment using ClustalW (https://www.clustal.org/; Thompson et al., 2002). The cladogram was made by MEGA XI (http://www.megasoftware.net; Tamura et al., 2021) using the Maximum-likelihood (ML) method and the Jones-Taylor-Thornton (JTT) mode. Node robustness was estimated using the bootstrap method with 500 replications. The cladogram was imported into FigTree (http://tree.bio.ed.ac.uk/software/figtree/) for graphical visualization.

2.2.4 Duplication and Ka/Ks ratio

MCscanX (Wang et al., 2012) was used to identify duplicated GST genes. The 'duplicate_gene_classifier' program implemented in the MCScanX package was used to classify duplicated genes a) whole genome or segmental duplication (WGD), b) tandem duplication (TD), c) proximal duplication (PD), and e) transposed duplication (TRD) pairs. The Ka/Ks calculator in TBtools was used to compute the nonsynonymous (Ka) to synonymous substitution (Ks) rate of gene pairs. Circular plots were formulated with Circos (Krzywinski et al., 2009) to visualize duplications.

2.2.5 Identification of orthologs and analysis of synteny relationships

Orthologs genes among *S. lycopersicum, S. lycopersicoides, S. pimpinellifolium, S. pennellii* and *A. thaliana* were identified using the "OrthoVenn3" tool (https://orthovenn3.bioinfotoolkits.net/; Sun et al., 2023). The OrthoFinder algorithm was run by setting the E-value to $1e^{-2}$) and the inflation value to 1.50.The "one step MCScan X tool" (E-value $\leq 1e^{-10}$; maximum number of blast hits =5) in TBtools was used to explore the synteny relationships of the orthologous GST genes. The latter were represented graphically using the "Dual synteny plot" in TBtools (Chen et al., 2020).

2.2.6 Prediction of protein-protein interaction and identification of transcription factor binding motifs in regulatory regions

Protein-protein interactions between *S. lycoperiscum* GSTs were predicted using STRING (http://string-db.org/ - Franceschini et al., 2013). The minimum interaction score required was set to the medium confidence level (0.4) of possible interactions. The putative proximal regulatory regions of *S. lycopersicum* GSTs (2,000 bp upstream of the translation start site) were extracted from indexed reference chromosome sequences using *samtools faidx* (Denececk 2021 et al., 2021). The transcription factor binding motifs (TFBMs) were identified using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al., 2003) with default settings.

2.2.7 Expression patterns of GST genes

In order to investigate the expression profiles of Glutathione S-transferase (GST) genes in *Solanum lycopersicum*, publicly available RNA-seq data were retrieved from Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) and re-analyzed. The study covered different tissues (i.e. leaf, fruit, root) and a wide spectrum of stresses (heat, drought, salt, cold) enabling a detailed elucidation of GST tissue-specific gene expression dynamics in response to abiotic stressors (Table S1).

In particular, raw RNA-sequencing data in SRA format were downloaded using RSA-toolkit. To convert the datasets in FASTQ format we used the fastq-dump utility. Quality check and trimming of the raw data were performed using Fastp (Chen et al., 2018). The filtered sequences were then aligned to the reference tomato genome utilizing HISAT2 (Kim et al., 2015). Samtools (Li et al., 2009) were used to convert the alignment files into the BAM format. Finally, FeatureCounts (Liao et al., 2013) was used to obtain the number of reads mapped to each target gene. The expression values were then rendered in CPM (Counts Per Million) according to the following formula: CPM = readsMappedToGene * 1/totalNumReads * 10^{-6}. Additionally, we normalized the expression values using the base 2 logarithm to facilitate more meaningful comparisons and interpretations of gene expression levels.

2.2.8 Identification of a tomato non-redundant reference core collection. GST genes and drought stress response traits in different tomato accessions

To investigate the *gst* sequence polymorphisms within the gene pool of the tomato crop, a tomato non-redundant reference core collection was selected within the population of 360 tomato accessions re-sequenced by Lin and colleagues (2014) to comprehensive analyze the tomato evolution across domestication and breeding. Based on 2,340,973 Single Nucleotide Polymorphisms (SNPs) identified, Lin and colleagues (2014) reported Principal Component Analysis (PCA) coordinates. To capture a comprehensive representation of genetic diversity within the tomato species based on their genetic distance, we carried out a filtering process on principal components (PC1 and PC2). The filtration criterion was set to select accessions having coordinate differences on either PC1and PC2 exceeding of the absolute value 0.004. The threshold 0.004 was set to selected accessions with substantial deviation in genetic variation, purging redundancy in the genetic variability (Table S5).

2.2.9 Sequence diversity and GST intra-species pangenome

Variant call format (VCF) files containing polymorphism data for selected accessions of the tomato reference collection were obtained from the AGIS Tomato 360 Resequencing Project hosted and retrieved fromthe Solgenomics website (https://solgenomics.net/ftp/genomes/tomato_360/). In order to identify single nucleotide polymorphisms (SNPs) within the gst genomic sequences, the bedtools (Aaron et al., 2010) and VCFtools (Danecek et al., 2011) were used for SNP calling, and SnpEff (Cingolani et al., 2012) was used for annotating SNPs and predicting their potential effects on the gst genes.

2.2.10 Drought response and trait association to gst sequence diversity

Seeds of the selected accession of the tomato reference collection were obtained from the Rick Tomato Genetics Resource Center of the University of California, Davis. Seeds were sown in seed trays and plantlets were grown for 30 days in a growth chamber at 24 °C, 50% humidity, artificial light with photoperiod of 16/8 h light/dark. Plantlets were transplanted in Ø25cm pots filled with a with a 1:1 mixture of sandy soil and loam and water supply was operated by complete compensation of water lost by evapotranspiration to the field capacity level. When 75% of plants were at the anthesis on the first flower truss, plants underwent two watering treatments with five plants replica per each combination of accession and watering treatment. The two water treatments consisted in the complete restitution of water loss (Full Water Restitution – FWR) and in the restitution of half of the lost water (Half Water Restitution – HWR), respectively (figure 6). Fifteen days later, plants were assessed for leaf CO_2 assimilation rate, transpiration and leaf temperature using a gas exchange analyzer (Licor 6400). Also, leaf color variations were assayed by a portable chromameter (Minolta CR400). Leaf electrolyte leakage (EL) was analyzed using the procedure reported by da Soleimani (2013). Fresh leaves (L1) were immersed for three hours in 0.1 M Mannitol at 20 °C. The electrical conductivity (L1) of the solution was assayed by an electrical conductivity meter (Cond 80 PRO Stirrer, XS). Tubes containing the leaf in mannitol solution underwent heating at 100 °C for 10' and then left to cool and the electrical conductivity assessment was repeated (L2). The EL of each sample was calculated as EL = (L1 / L2) x 100.
The effect of gst SNPs on drought-related phenotypic traits were assessed Mann–Whitney U test using the software IBM® SPSS® Statistics (version 27). SNPs significantly associated with drought-related phenotypic traits have been highlighted through boxplots created with R software (R Core Team, 2021).

Figure 6. Drought impact on plant turgor and color. On the left tomato plant watered by complete restitution of evapotranspirated lost water (FWR). On the right, tomato under drought treatment by compensating half of the evapotranspirated water (HWR) exhibiting acute symptoms. Thirty-five tomato accessions were evaluated, with three control replicates and three drought-stressed replicates for each accession.

2.3 Results

2.4 Discussion

This study provided an in-depth investigation on the roles of glutathione S-transferase genes (gst) in *Solanum lycopersicum* (tomato) and its wild relatives, focusing on their contributions to environmental stress adaptation mechanisms and significance in evolutionary dynamics, development, fruit ripening and quality. In fact, gst genes are reported to control fruit ripening, fruit redox balance and antioxidant activity with possible implications in breeding plants for fruit quality, , including taste, color, and nutritional value (Zhou et al., 2022; Qi et al., 2022).

The diversity and distribution of gst genes in the tomato genome highlighted the evolutionary adaptability of the gst gene family through gene duplication events and diversification. Specifically, the tandem gene duplications, which have been a dominant force driving gst diversification, suggested a mechanism of emergence of new functions possibly related to enhanced stress tolerance and fruit quality (Ugalde et al., 2023; Lan et al., 2016). Lan and colleagues (2016) reported that adjacent genes tend to undergo co-regulation, particularly in the case of tandem duplicates and even after their separation, neighboring gene pairs still exhibit interchromosomal colocalization (Dai et al., 2014). This is consistent with the significance of tandem duplications in enabling adaptive responses to environmental stimuli (Hanada et al., 2008; Zhong et al., 2018).

In *S. lycopersicum*, the whole-genome duplication (WGD) or segmental duplication have been observed among three pairs of genes located on chromosomes 1 and 9, within collinear blocks. These segmental duplications, though relatively rare, play a pivotal evolutionary role by providing additional gene copies that are potential substrates for the evolution of novel functions (Freeling et al., 2009; Koszul et al., 2019).

The subclass diversification within the GST protein class, particularly the prominence of the TAU subclass in *S. lycopersicum*, has been of special interest. This subclass has been closely associated with stress responses that are integral to maintaining cellular homeostasis under stress conditions, which, in turn, can have profound effects on fruit quality by minimizing stress-induced damages that could impair fruit development and ripening processes (Estévez et al., 2020; Cao et al., 2022).

The wide range of predicted subcellular localization among GSTs highlighted their multifunctional roles in various cellular compartments, influencing processes beyond detoxification, such as redox regulation and the modulation of gene expression. These roles have been crucial to directly affect the metabolic pathways responsible for the synthesis of flavor, color, and antioxidant compounds in fruits (Dixon et al., 2002; Rouhier et al., 2008).

Combining the phylogenetic analyses, the Ka/Ks ratios estimation and the analysis of orthology allowed to delve deeper into evolution dynamics of the gst gene family. The presence of positively

selected gene pairs within the gst family suggested they underwent selection pressure that may lead to the development of new functions or the enhancement of existing ones toward an evolutionary adaptation to environmental challenges. Among the genes exhibiting positive selection (Ka/Ks > 1), in *S. lycopersicum* several candidates stand out for their potential adaptive significance. For instance, the gene pairs *Solyc09g011540.2.1-Solyc09g011550.4.1* and *Solyc09g011550.4.1-Solyc09g011590.4.1* not only show positive selection but are also involved in tandem duplication events, suggesting that these genes might have evolved new functions crucial for stress response or other adaptive traits. These genes are located within a cluster on chromosome 9, which is significant for its high density of gst genes and its involvement in adaptive responses. In *S. pennellii*, the gene pair *Sopen11g012580.1-Sopen11g012610.1*; in *S. pimpinellifolium*, the gene pairs *SPIMP02g0057440-SPIMP02g0057450* and *SPIMP06g0202160-SPIMP09g0271810*; and in *S. lycopersicoides*, the gene pairs *Solyd47g050020.1-Solyd07g070010.1* and *Solyd09g055830.1-Solyd09g055840.1*, exhibited positive selection with Ka/Ks ratios greater than 1. These gene pairs highlight the evolutionary adaptation within these wild species, suggesting potential new functions that enhance their ability to withstand environmental stresses. The orthogroup 140 emerged as the largest orthogroup, including almost all gst genes clustered on the chromosome 9 in all examined Solanum genomes except for *S. lycopersicoides*. On the other hand, the orthogroup 4080 was the predominant orthogroup in *S. lycopersicoides*. This suggested that the orthogroup 140 underwent expansion in *S. lycopersicum, S. pennellii,* and *S. pimpinellifolium*, reflecting a likely broadening of gst gene functions, whereas it experienced a contraction in the *S. lycopersicoides* genome. The finding highlighted a likely unique evolutionary path experienced by the species *S. lycopersicoides*.

The conserved synteny among orthologous gst genes across different chromosomes in different species further supports the notion of functional conservation, emphasizing their crucial roles (Catchen et al., 2009). However, non-syntenic genes were detected and arose with a similar pattern in any investigated wild relative compared to the reference *Solanum lycopersicum*. The incomplete gene synteny between chromosomes 9-10 and chromosomes 7-12 suggested that non-synthenic genes have not conserved their ancestral locations across the species during their evolutionary divergence. These genes might have arisen from recent duplicated copies or novel, lineage-specific genes (Bowers et al., 2023).

The protein-protein interaction network analysis revealed significant interactions among GST family members, suggesting their involvement in a wide range of biological pathways. In particular, most GSTs shared interaction with two members of the DHAR sub-class (*Solyc05g054760.5.1 and Solyc11g011250.3.1*). Given that biologically active tau and phi GSTs are dimers and could dissociate in a more reducing environment because of an increase in GSH

intracellular concentration, the activity of several GSTs is controlled by the cellular redox potential (Gallé et al., 2019). In addition, GSTs containing cysteine in the active site (e.g. DHAR) have the ability to form heterodimers with other GSTs, DHAR could act as central regulatory hubs, influencing processes such as development, redox regulation, responses to environmental stresses, signal transduction, and cellular homeostasis (Ding et al., 2020).

The expression profiling of gst genes in different *S. lycopersicum* tissues and in response to different environmental stresses provided a framework for the generic involvement of specific gst genes in the tissue-specific adaptive response to environmental constraints. Several gst genes showed to modulate the abundance of their mRNA in fruit upon abiotic stresses suggesting mediating plant response to stress and fruit ripening and quality. For instance, *Solyc07g056420* exhibited notable up-regulation under various stress conditions, such as drought, cold, heat, and salinity. Similarly, *Solyc01g081250* demonstrated high responsiveness to heat and salinity, highlighting its involvement in stress adaptation mechanisms. The clustering of gst genes on chromosome 7, particularly those responding to heat stress, suggests these genes may have evolved to manage fruit stress responses collectively, making them valuable targets for breeding programs aimed at improving fruit resilience and quality. It could be argued that the modulation of the gst gene expression could be involved in controlling the fruit level of antioxidant beneficial bioactive compounds highlighting their potential for breeding tomato for enhanced fruit nutritional quality (Mailloux et al., 2013; Singh et al., 2022).

The identification in the tomato reference collection of DNA polymorphisms within gst genes highlighted genetic variability to focus on for breeding tolerance to environmental stresses in tomato. Among the identified SNPs, several candidates stand out for their significant roles in drought-responsive traits. For example, the genes *Solyc01g081260* and *Solyc09g074850* show significant associations with CO_2 photoassimilation, indicating their potential role in maintaining photosynthetic efficiency under drought stress. The gene *Solyc10g084400*, associated with both CO_2 photoassimilation and leaf electrolyte leakage, suggests its involvement in multiple stress response pathways. The gene *Solyc04g081740*, associated with changes in WUE, could play a critical role in optimizing water utilization under drought conditions. The SNPs that fall on *Solyc11g011250* are significantly associated with variations in leaf temperature, indicating its role in regulating transpiration and heat dissipation mechanisms. The presence of two SNPs within this gene underscores its importance in contributing to maintaining optimal leaf temperature under drought conditions, which is essential for preventing heat stress and ensuring continued physiological functions. Tolerant tomato phenotypes have the potential to cope with environmental constraints limiting negative effects on yield and fruit quality. However, understanding the biological link between the modulation of fruit quality traits under stress

conditions and the genetic diversity in the gst target genes still remain a challenge. Setting the potential association between fruit quality traits and the genetic diversity in the gst genes, offers a promising perspective to exploit gathered knowledge in developing new biotechnological tools for more efficient breeding approaches (Monroe et al., 2018; Baggs et al., 2020).

In conclusion, this study provided an up-to-date mapping and annotation of the gst gene family members in the Solanum lycopersicum genome and its wild relatives. Our analysis to validate the accuracy of the Glutathione S-transferase (GST) gene family annotations across the ITAG 4.1 and ITAG 2.4 versions of the Solanum lycopersicum highlights the importance of experimental validation to confirm the accuracy and reliability of genomic data.

Results also highlighted several promising candidate genes to target for breeding tomato plant tolerance against abiotic stresses. Possible implications on fruit quality traits have discussed. Our findings added insights on the gst gene family paving the way for the development of advanced breeding approaches for mitigating challenging effects of the global warming.

The complex functional regulation of the gst genes highlighted the need for further proof-of-concept research aimed at testing the hypothesis that targeting gst genes could combine breeding tomato crop stress tolerance and enhancing fruit quality.

2.5 Supplementary

Table S1 List of analyzed Sequence Read Archive files resulting from RNAseq experiments in different tomato tissues under different stresses.

Sequence Read Archive (run IDs)	**Environmental stress**	**Tissue**	**Digital Object Identifier**
SRR11855627 - SRR11855628 - SRR11855629 - SRR11855636 - SRR11855637 - SRR11855638	Drought	Leaf	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151277
SRR11855648 - SRR11855649 - SRR11855650 - SRR11855657 - SRR11855658 - SRR11855659	Heat	Leaf	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151277
SRR26671295 - SRR26671296 - SRR26671297 - SRR26671301 - SRR26671304 - SRR26671305	Cold	Leaf	https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP470214&o=acc_s%3Aa
SRR12172700 - SRR12172702	Drought	Red Ripe Fruit	https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP270909&o=acc_s%3Aa
SRR10998563 - SRR10998564 - SRR10998565 - SRR10998566 - SRR10998569 - SRR10998570	Heat	Red Ripe Fruit	https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP246520&o=acc_s%3Aa
SRR23424152 - SRR23424153 - SRR23424154 - SRR23424158 - SRR23424161 - SRR23424162	Salt	Root	https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP422069&o=acc_s%3Aa

Table S2 List of GST genes identified in S. lycopersicum, S. Pennelli, S. pimpinellifolium and S. lycopersicoides and the predicted sub-cellular localization of the encoded protein.

Locus	Sub-class	Strand	CDS coordinate (5' to 3')	Localization
Solyc01g081250.3.1	TAU	+	73096202-73097558	Cytoplasmic
Solyc01g081260.2.1	TAU	+	73098720-73099603	Mitochondrial
Solyc01g081270.2.1	TAU	+	73101147-73102488	Cytoplasmic
Solyc01g081310.3.1	TAU	+	73126647-73128177	Cytoplasmic
Solyc01g091330.4.1	ZETA	-	77219753-77225928	Chloroplast
Solyc01g099590.5.1	TAU	-	82035338-82041839	Cytoplasmic
Solyc01g102660.4.1	ZETA	+	83678654-83683832	Cytoplasmic
Solyc02g038818.1.1	LAMBDA	+	30724846-30728917	Cytoplasmic
Solyc02g068900.3.1	GHR	+	36794702-36800349	Mitochondrial
Solyc02g081240.1.1	TAU	+	43254627-43255573	Cytoplasmic
Solyc02g081340.3.1	PHI	+	43327302-43328660	Cytoplasmic
Solyc02g081430.3.1	MGST	+	43394666-43396531	PlasmaMembrane
Solyc03g116120.3.1	TAU	-	60050784-60051561	Cytoplasmic
Solyc03g116130.2.1	TAU	-	60054257-60055137	Cytoplasmic
Solyc04g009530.4.1	LAMBDA	-	2979674-2984908	Chloroplast
Solyc04g050560.4.1	THETA	+	46799571-46807100	Extracellular
Solyc04g057890.3.1	TCHQD	-	54330009-54332976	Mitochondrial
Solyc04g064470.2.1	DHAR	-	54949998-54952276	Nuclear
Solyc04g081740.3.1	MGST	+	63640198-63644324	Mitochondrial
Solyc05g006730.5.1	TAU	+	1426366-1428745	Cytoplasmic
Solyc05g006740.5.1	TAU	+	1428803-1430736	Cytoplasmic
Solyc05g006750.3.1	TAU	+	1431319-1432541	Cytoplasmic
Solyc05g013950.1.1	DHAR	-	7490633-7493003	Extracellular
Solyc05g026210.2.1	TAU	-	40253585-40254761	Chloroplast
Solyc05g054760.5.1	DHAR	-	63992443-63996733	Cytoplasmic
Solyc05g161440.1.1	LAMBDA	+	36303901-36304592	Mitochondrial
Solyc06g009020.2.1	PHI	+	2968059-2970275	Cytoplasmic
Solyc06g009040.4.1	PHI	+	2978521-2980532	Cytoplasmic
Solyc06g011280.3.1	ELONGATION FACTOR	-	6296428-6299486	Cytoplasmic
Solyc06g069040.4.1	TAU	-	40474533-40475841	Cytoplasmic
Solyc06g075520.3.1	DHAR	+	44586627-44589509	Extracellular
Solyc06g083770.5.1	GHR	-	46677908-46681572	Chloroplast
Solyc07g021460.3.1	TAU	-	18087719-18089270	Cytoplasmic
Solyc07g056420.4.1	TAU	+	64194793-64197247	Cytoplasmic
Solyc07g056430.3.1	TAU	+	64200651-64202227	Cytoplasmic
Solyc07g056440.3.1	TAU	+	64204521-64206122	Cytoplasmic
Solyc07g056450.3.1	TAU	+	64207993-64209702	Cytoplasmic
Solyc07g056460.3.1	TAU	+	64210129-64211840	Cytoplasmic
Solyc07g056470.3.1	TAU	+	64213290-64215147	Cytoplasmic
Solyc07g056490.5.1	TAU	-	64217497-64219178	Cytoplasmic

Solyc07g056500.4.1	TAU	-	64222292-64223430	Cytoplasmic
Solyc07g056510.3.1	TAU	-	64225651-64227142	Cytoplasmic
Solyc07g161200.1.1	PHI	-	42247656-42257250	Chloroplast
Solyc08g062570.1.1	TAU	+	49587830-49588246	PlasmaMembrane
Solyc08g080900.3.1	THETA	-	62188477-62192508	Cytoplasmic
Solyc08g080910.3.1	THETA	-	62193241-62196947	Cytoplasmic
Solyc09g007150.3.1	LAMBDA	+	793825-797595	Cytoplasmic
Solyc09g011490.3.1	TAU	-	4857023-4858045	Cytoplasmic
Solyc09g011500.3.1	TAU	-	4859658-4861241	Cytoplasmic
Solyc09g011510.2.1	TAU	-	4867501-4868546	Cytoplasmic
Solyc09g011520.3.1	TAU	-	4873881-4874998	Cytoplasmic
Solyc09g011530.3.1	TAU	-	4875847-4876137	Extracellular
Solyc09g011540.2.1	TAU	+	4882513-4883612	Cytoplasmic
Solyc09g011550.4.1	TAU	+	4884282-4885152	Cytoplasmic
Solyc09g011590.4.1	TAU	+	4902850-4913319	Cytoplasmic
Solyc09g011600.3.1	TAU	+	4914321-4915937	Cytoplasmic
Solyc09g011610.4.1	TAU	+	4918483-4920186	Cytoplasmic
Solyc09g011630.3.1	TAU	+	4933589-4935029	Cytoplasmic
Solyc09g011640.5.1.1	TAU	+	4938248-4939836	Cytoplasmic
Solyc09g011650.4.1	TAU	+	4948227-4950749	Cytoplasmic
Solyc09g056180.3.1	DHAR	-	43432642-43436321	Extracellular
Solyc09g063150.3.1	TAU	+	57279355-57281129	Cytoplasmic
Solyc09g074850.4.1	PHI	+	62887115-62889488	Cytoplasmic
Solyc09g091130.5.1	TAU	-	66579858-66581203	Cytoplasmic
Solyc09g091140.4.1	TAU	-	66581836-66583671	Cytoplasmic
Solyc09g160500.1.1	TAU	-	13655384-13656427	PlasmaMembrane
Solyc10g007620.3.1	TAU	-	1799898-1801139	Cytoplasmic
Solyc10g007635.1.1	TAU	-	1804139-1805377	Cytoplasmic
Solyc10g084400.2.1	LAMBDA	-	63112482-63115222	Cytoplasmic
Solyc10g084960.2.1	TAU	+	63467215-63468888	Cytoplasmic
Solyc11g011250.3.1	DHAR	-	4323237-4328911	Mitochondrial
Solyc11g028090.3.1	ELONGATION FACTOR	-	19272853-19273860	Nuclear
Solyc11g028100.2.1	ELONGATION FACTOR	+	19291315-19293950	Cytoplasmic
Solyc11g039930.2.1	DHAR	-	39674171-39674923	Extracellular
Solyc12g011300.2.1	TAU	+	4197079-4198205	Cytoplasmic
Solyc12g011310.2.1	TAU	-	4203131-4204692	Cytoplasmic
Solyc12g011320.2.1	TAU	-	4205535-4207020	Cytoplasmic
Solyc12g044520.2.1	LAMBDA	-	59155196-59159166	Cytoplasmic
Solyc12g044530.3.1	LAMBDA	-	59187654-59191397	Cytoplasmic
Solyc12g056250.2.1	THETA	-	61801797-61804279	Cytoplasmic
Solyc12g062730.3.1	TAU	-	33945067-33946623	Cytoplasmic
Solyc12g094430.1.1	PHI	+	64127267-64129423	Cytoplasmic

Sopen00g007230.1	LAMBDA	+	20245285-20245403	Cytoplasmic
Sopen00g010060.1	TAU	-	30835465-30835974	Cytoplasmic
Sopen01g019540.1	DHAR	+	57153200-57153398	Nuclear
Sopen01g032820.1	TAU	+	90601791-90602281	Cytoplasmic
Sopen01g032830.1	TAU	+	90606571-90607059	Cytoplasmic
Sopen01g032840.1	TAU	+	90610869-90611384	Cytoplasmic
Sopen01g032890.1	TAU	+	90633224-90634014	Cytoplasmic
Sopen01g033680.1	TAU	+	91400746-91401318	Cytoplasmic
Sopen01g037070.1	ZETA	-	95209123-95209361	Chloroplast
Sopen01g043120.1	TAU	-	100259372-100259882	Cytoplasmic
Sopen01g045070.1	ZETA	+	101917323-101917896	Cytoplasmic
Sopen02g012630.1	LAMBDA	+	35532378-35532503	Cytoplasmic
Sopen02g012640.1	LAMBDA	+	35564197-35564322	Cytoplasmic
Sopen02g014600.1	DHAR	+	38539606-38539877	Nuclear
Sopen02g018000.1	GHR	+	42042845-42043500	Mitochondrial
Sopen02g025880.1	TAU	+	48974807-48975223	Cytoplasmic
Sopen02g025970.1	PHI	+	49048463-49049124	Cytoplasmic
Sopen02g026070.1	MGST	+	49138100-49138465	PlasmaMembrane
Sopen03g035090.1	TAU	-	70109871-70110283	Cytoplasmic
Sopen03g035100.1	TAU	-	70113276-70113643	Cytoplasmic
Sopen04g004690.2	LAMBDA	-	3130629-3130925	Chloroplast
Sopen04g025740.1	TCHQD	-	66714099-66714236	Nuclear
Sopen04g035370.1	MGST	+	76317520-76318027	Chloroplast
Sopen05g002680.1	TAU	+	1499533-1500181	Chloroplast
Sopen05g002690.1	TAU	+	1501383-1502002	Cytoplasmic
Sopen05g033230.1	DHAR	-	76603136-76603460	Cytoplasmic
Sopen06g003820.1	PHI	+	3275014-3275864	Cytoplasmic
Sopen06g003840.1	PHI	+	3292568-3293118	Cytoplasmic
Sopen06g005470.1	ELONGATION FACTOR	-	7266386-7266490	Cytoplasmic
Sopen06g026330.1	TAU	-	53400796-53401167	Cytoplasmic
Sopen06g035180.1	GHR	-	60103408-60104196	Mitochondrial
Sopen07g010540.1	TAU	-	21297967-21298240	Cytoplasmic
Sopen07g029790.1	TAU	+	75375005-75375682	Cytoplasmic
Sopen07g029800.1	TAU	+	75380263-75380848	Cytoplasmic
Sopen07g029810.1	TAU	+	75384166-75384747	Cytoplasmic
Sopen07g029820.1	TAU	+	75386610-75386960	Extracellular
Sopen07g029830.1	TAU	+	75390039-75390622	Cytoplasmic
Sopen07g029840.1	TAU	+	75392487-75393046	Cytoplasmic
Sopen07g029850.1	TAU	-	75394892-75394935	Cytoplasmic
Sopen07g029860.1	TAU	-	75398273-75398645	Cytoplasmic
Sopen07g029870.1	TAU	-	75408608-75408955	Cytoplasmic
Sopen08g029200.2	THETA	-	68630987-68631134	Cytoplasmic
Sopen08g029220.1	THETA	-	68635521-68635694	Cytoplasmic

Sopen09g002020.1	LAMBDA	+	809776-810053	Cytoplasmic
Sopen09g006240.1	TAU	-	5104696-5105047	Cytoplasmic
Sopen09g006250.1	TAU	-	5107829-5108257	Cytoplasmic
Sopen09g006280.1	TAU	-	5115617-5115987	Cytoplasmic
Sopen09g006290.1	TAU	-	5122834-5123207	Cytoplasmic
Sopen09g006300.1	TAU	-	5125079-5125307	Cytoplasmic
Sopen09g006310.1	TAU	+	5142263-5142864	Cytoplasmic
Sopen09g006320.1	TAU	+	5143953-5144538	Cytoplasmic
Sopen09g006330.2	TAU	+	5147939-5148539	Cytoplasmic
Sopen09g006340.1	TAU	+	5153838-5154394	Cytoplasmic
Sopen09g006350.1	TAU	+	5156606-5157151	Cytoplasmic
Sopen09g006360.1	TAU	+	5161566-5162177	Cytoplasmic
Sopen09g006370.1	TAU	+	5166888-5167291	Cytoplasmic
Sopen09g006390.1	TAU	+	5203211-5203751	Cytoplasmic
Sopen09g006400.1	TAU	+	5207537-5208066	Cytoplasmic
Sopen09g006410.1	TAU	+	5211218-5211719	Cytoplasmic
Sopen09g006420.1	TAU	+	5222169-5222645	Cytoplasmic
Sopen09g006440.1	TAU	+	5240025-5240586	Cytoplasmic
Sopen09g006450.1	TAU	+	5244753-5245188	Cytoplasmic
Sopen09g006460.1	TAU	+	5249783-5250401	Cytoplasmic
Sopen09g010950.1	TAU	-	18143312-18143680	Cytoplasmic
Sopen09g025320.1	TAU	+	71115099-71115539	Chloroplast
Sopen09g034110.1	TAU	-	82110521-82110709	Cytoplasmic
Sopen09g034120.1	TAU	-	82112652-82112999	Cytoplasmic
Sopen10g003550.1	TAU	-	2057065-2057454	Cytoplasmic
Sopen10g017090.1	DHAR	-	45576165-45576193	Cytoplasmic
Sopen10g033840.1	LAMBDA	-	80912912-80913074	Cytoplasmic
Sopen10g034420.1	TAU	+	81268977-81269576	Cytoplasmic
Sopen11g006030.1	DHAR	-	4351456-4351713	Mitochondrial
Sopen11g012580.1	ELONGATION FACTOR	-	17938254-17938419	Extracellular
Sopen11g012610.1	ELONGATION FACTOR	+	17961286-17961878	Cytoplasmic
Sopen12g006630.1	TAU	+	5179399-5180007	Cytoplasmic
Sopen12g006640.1	TAU	-	5181948-5182401	Cytoplasmic
Sopen12g006650.1	TAU	-	5184779-5185251	Cytoplasmic
Sopen12g017180.1	TAU	+	42700600-42701051	Cytoplasmic
Sopen12g022810.1	LAMBDA	-	59044403-59044472	Cytoplasmic
Sopen12g022820.1	LAMBDA	-	59069074-59069142	Nuclear
Sopen12g022830.1	LAMBDA	-	59095379-59095482	Nuclear
Sopen12g022850.2	LAMBDA	-	59141134-59141272	Cytoplasmic
Sopen12g026980.1	TAU	+	70858032-70858615	Chloroplast
Sopen12g028770.1	THETA	-	77971539-77971739	Cytoplasmic
Sopen12g031310.1	PHI	+	80442625-80443444	Cytoplasmic
Sopen12g032870.1	TAU	-	81623448-81623639	Cytoplasmic

SPIMP01g0027300	TAU	+	77770330-77776615	Cytoplasmic
SPIMP01g0027350	TAU	+	77800703-77802070	Cytoplasmic
SPIMP01g0028230	TAU	+	78553082-78554587	Cytoplasmic
SPIMP01g0032370	ZETA	-	81860152-81866424	PlasmaMembrane
SPIMP01g0040230	ZETA	+	88320593-88328184	Cytoplasmic
SPIMP02g0057440	LAMBDA	+	32318046-32322259	Extracellular
SPIMP02g0057450	LAMBDA	+	32333134-32336477	Cytoplasmic
SPIMP02g0057460	LAMBDA	+	32348272-32352511	Cytoplasmic
SPIMP02g0063120	GHR	+	38642502-38648149	Mitochondrial
SPIMP02g0070910	TAU	+	45320604-45321549	Cytoplasmic
SPIMP02g0071010	PHI	+	45406679-45408460	Cytoplasmic
SPIMP02g0071100	MGST	+	45474738-45476497	PlasmaMembrane
SPIMP03g0112390	TAU	-	60598179-60605001	Cytoplasmic
SPIMP04g0123180	LAMBDA	-	2969571-2975046	Chloroplast
SPIMP04g0134940	THETA	+	49078048-49090706	Mitochondrial
SPIMP04g0139100	TCHQD	-	56778029-56781126	Mitochondrial
SPIMP04g0139770	DHAR	-	57433120-57435883	PlasmaMembrane
SPIMP04g0149470	MGST	+	66153377-66157531	Mitochondrial
SPIMP05g0152360	TAU	+	1529908-1533774	Cytoplasmic
SPIMP05g0152370	TAU	+	1534493-1535634	Cytoplasmic
SPIMP05g0152380	TAU	+	1536686-1537966	Cytoplasmic
SPIMP05g0158960	DHAR	-	7825140-7826566	Extracellular
SPIMP05g0165190	LAMBDA	+	37363078-37364634	Cytoplasmic
SPIMP05g0165920	TAU	-	41388598-41392938	Cytoplasmic
SPIMP05g0175480	DHAR	-	65588575-65592882	Cytoplasmic
SPIMP06g0180280	PHI	+	3108742-3110750	Cytoplasmic
SPIMP06g0180290	PHI	+	3119089-3120990	Cytoplasmic
SPIMP06g0181480	ELONGATION FACTOR	-	6632282-6635252	Cytoplasmic
SPIMP06g0196060	TAU	-	42673282-42674635	Cytoplasmic
SPIMP06g0202160	DHAR	+	46795926-46799271	Extracellular
SPIMP06g0205110	GHR	-	48954552-48957455	Chloroplast
SPIMP07g0214860	TAU	-	18555721-18557157	PlasmaMembrane
SPIMP07g0223260	TAU	-	58987699-58993441	Cytoplasmic
SPIMP07g0228470	TAU	+	63706491-63728306	Cytoplasmic
SPIMP07g0228480	TAU	-	63728552-63730202	Cytoplasmic
SPIMP07g0228490	TAU	-	63733371-63738134	Cytoplasmic
SPIMP08g0245530	TAU	+	52069035-52069830	Cytoplasmic
SPIMP08g0256390	THETA	-	64903822-64909146	Cytoplasmic
SPIMP09g0259900	LAMBDA	+	803247-806992	Cytoplasmic
SPIMP09g0264560	TAU	-	4953630-4954680	Cytoplasmic
SPIMP09g0264570	TAU	-	4956068-4957630	Cytoplasmic
SPIMP09g0264580	TAU	-	4979745-4980787	Cytoplasmic

SPIMP09g0264600	TAU	-	4986206-4987183	Cytoplasmic
SPIMP09g0264620	TAU	+	4994958-4997630	Cytoplasmic
SPIMP09g0264630	TAU	+	4998992-5000496	Cytoplasmic
SPIMP09g0264640	TAU	+	5015172-5018485	Cytoplasmic
SPIMP09g0264650	TAU	+	5030461-5038302	Cytoplasmic
SPIMP09g0264660	TAU	+	5039383-5040651	Cytoplasmic
SPIMP09g0264670	TAU	+	5052502-5068574	Cytoplasmic
SPIMP09g0271810	DHAR	-	45519825-45524521	Nuclear
SPIMP09g0275660	TAU	+	60029124-60030882	Cytoplasmic
SPIMP09g0279760	PHI	+	65499068-65507759	Nuclear
SPIMP09g0284150	TAU	-	69336809-69340471	Cytoplasmic
SPIMP10g0289360	TAU	-	1904645-1910286	Cytoplasmic
SPIMP10g0311710	LAMBDA	-	66348088-66350872	Cytoplasmic
SPIMP10g0312240	TAU	+	66701923-66703633	Cytoplasmic
SPIMP11g0319170	DHAR	-	4292630-4298306	Mitochondrial
SPIMP11g0326230	ELONGATION FACTOR	-	20898171-20900048	Extracellular
SPIMP11g0326240	ELONGATION FACTOR	+	20915231-20918605	Cytoplasmic
SPIMP11g0330110	DHAR	-	41087283-41090544	Nuclear
SPIMP12g0343590	TAU	+	4134058-4135437	Cytoplasmic
SPIMP12g0343600	TAU	-	4140070-4141669	Cytoplasmic
SPIMP12g0343610	TAU	-	4142321-4144015	Cytoplasmic
SPIMP12g0349580	TAU	+	30752056-30755535	Chloroplast
SPIMP12g0350360	TAU	-	36608721-36610377	Cytoplasmic
SPIMP12g0356650	LAMBDA	-	62463219-62467390	Cytoplasmic
SPIMP12g0356670	LAMBDA	-	62477992-62490959	Mitochondrial
SPIMP12g0358510	THETA	-	65337772-65340259	Cytoplasmic
SPIMP12g0361200	PHI	+	67684863-67687374	Cytoplasmic
SPIMP12g0362770	TAU	-	68820170-68821470	PlasmaMembrane
Contig2482g050040.1	TAU	-	10468-11429	Chloroplast
Contig2619g050030.1	ZETA	-	15387-19002	Cytoplasmic
Contig2655g050020.1	PHI	-	12551-14289	Cytoplasmic
Contig2756g050030.1	TAU	-	3284-4167	Cytoplasmic
Contig2756g050040.1	TAU	-	6137-18769	Cytoplasmic
Contig300g050040.1	MGST	-	23650-27453	Chloroplast
Contig47g050010.1	TAU	-	907-2368	Chloroplast
Contig47g050020.1	TAU	-	18589-20074	Cytoplasmic
Contig764g050020.1	PHI	-	4370-10991	Cytoplasmic
Solyd01g073300.1	TAU	-	114211136-114211914	Cytoplasmic
Solyd01g073350.1	TAU	-	114235865-114239627	Cytoplasmic
Solyd01g073360.1	TAU	-	114241245-114242096	Cytoplasmic
Solyd01g075750.1	ZETA	-	117042492-117048236	Chloroplast
Solyd01g081570.1	TAU	-	122402167-122403128	Chloroplast

Solyd01g083460.1	ZETA	+	124267232-124272022	Cytoplasmic
Solyd02g061070.1	GHR	+	63040385-63044979	Mitochondrial
Solyd02g069830.1	TAU	+	71837872-71838819	Cytoplasmic
Solyd02g069940.1	PHI	+	71910562-71912420	Cytoplasmic
Solyd02g070030.1	MGST	+	71986147-71987471	PlasmaMembrane
Solyd03g077580.1	TAU	-	79202336-79203089	Cytoplasmic
Solyd03g077590.1	TAU	-	79205878-79206737	Cytoplasmic
Solyd03g077650.1	TAU	-	79296180-79296774	Cytoplasmic
Solyd03g077660.1	TAU	-	79300796-79301655	Cytoplasmic
Solyd04g054440.1	LAMBDA	-	4414848-4420192	Chloroplast
Solyd04g067590.1	TCHQD	-	80632646-80633886	Nuclear
Solyd04g078720.1	MGST	+	93891258-93895171	Chloroplast
Solyd05g051790.1	TAU	+	1964002-1970793	Cytoplasmic
Solyd05g060840.1	LAMBDA	+	20741633-20742118	Cytoplasmic
Solyd05g062200.1	TAU	-	31040419-31044502	Cytoplasmic
Solyd05g062890.1	LAMBDA	+	44755653-44758589	PlasmaMembrane
Solyd05g072160.1	DHAR	-	98096700-98100821	Cytoplasmic
Solyd06g050520.1	PHI	-	457432-464062	Cytoplasmic
Solyd06g055340.1	ELONGATION FACTOR	-	22890232-22893055	Cytoplasmic
Solyd06g070700.1	TAU	-	82561734-82562810	Cytoplasmic
Solyd06g079070.1	GHR	-	89283864-89286438	Chloroplast
Solyd07g070010.1	TAU	+	92915279-92933044	Cytoplasmic
Solyd07g070030.1	TAU	+	92946998-92951995	Cytoplasmic
Solyd07g070040.1	TAU	-	92952527-92963264	Cytoplasmic
Solyd08g072760.1	THETA	-	85927115-85936583	Cytoplasmic
Solyd09g051050.1	LAMBDA	+	858299-876605	Cytoplasmic
Solyd09g055740.1	TAU	-	5891887-5896065	Cytoplasmic
Solyd09g055750.1	TAU	-	5896095-5908469	Cytoplasmic
Solyd09g055770.1	TAU	+	5911638-5952812	Cytoplasmic
Solyd09g055780.1	TAU	+	5956535-5957068	Cytoplasmic
Solyd09g055790.1	TAU	+	5968723-5971062	Cytoplasmic
Solyd09g055800.1	TAU	+	5971076-5983355	Cytoplasmic
Solyd09g055820.1	TAU	+	6033990-6034499	Cytoplasmic
Solyd09g055830.1	TAU	+	6039018-6042148	Cytoplasmic
Solyd09g055840.1	TAU	+	6045495-6047914	Cytoplasmic
Solyd09g058120.1	TAU	+	14130054-14130909	Cytoplasmic
Solyd09g063650.1	TAU	+	94299400-94300565	Chloroplast
Solyd09g067750.1	PHI	+	103030781-103032987	Cytoplasmic
Solyd09g072440.1	TAU	-	108389171-108390140	Chloroplast
Solyd09g072450.1	TAU	-	108391234-108392857	Cytoplasmic
Solyd10g052610.1	TAU	-	2129221-2134679	Cytoplasmic
Solyd10g066470.1	TAU	+	86154359-86159584	Cytoplasmic
Solyd10g066480.1	TAU	+	86184399-86185806	Cytoplasmic

Solyd10g066490.1	TAU	+	86188978-86189610	Cytoplasmic
Solyd10g068950.1	LAMBDA	+	88639132-88641834	Cytoplasmic
Solyd11g055460.1	DHAR	-	5292890-5293696	Mitochondrial
Solyd11g060890.1	ELONGATION FACTOR	-	22946891-22949106	Cytoplasmic
Solyd12g055600.1	TAU	+	5411943-5413355	Cytoplasmic
Solyd12g055610.1	TAU	-	5420001-5424979	Cytoplasmic
Solyd12g055760.1	TAU	+	5552233-5557759	Cytoplasmic
Solyd12g061120.1	THETA	-	49569435-49570590	Nuclear
Solyd12g061250.1	TAU	-	51916745-51920708	Chloroplast
Solyd12g062060.1	TAU	+	67837142-67838749	Cytoplasmic
Solyd12g063310.1	TAU	-	78319250-78320135	Cytoplasmic
Solyd12g065710.1	LAMBDA	-	88336720-88340620	Mitochondrial
Solyd12g067730.1	THETA	-	92576727-92579354	Cytoplasmic
Solyd12g067750.1	THETA	-	92585183-92587686	Cytoplasmic
Solyd12g070740.1	PHI	+	95921648-95923610	Cytoplasmic
Solyd12g072450.1	TAU	-	97335521-97337323	PlasmaMembrane

Table S3. Duplicated gene pairs, non-synonymous and synonymous substitution statisticsand orthogroups of gst genes in the tomato and its wild relative genomes.

Gene name	Parental	Duplication mode	Ka	Ks	Ka/Ks	orthogroup
Solyc01g081250.3.1	*Solyc01g081260.2.1*	Tandem	0,168	0,409	0,411	3103
Solyc01g081260.2.1	*Solyc09g011500.3.1*	WGD or segmental	0,328	NaN	NaN	3103
Solyc01g081270.2.1	*Solyc01g081310.3.1*	Tandem	0,421	3	0,148	3103
Solyc01g081310.3.1	*Solyc09g011630.3.1*	WGD or segmental	0,415	1,858	0,224	13144
Solyc01g091330.4.1	*Solyc01g102660.4.1*	Proximal	0,304	1,952	0,156	17788
Solyc01g099590.5.1	*Solyc09g063150.3.1*	WGD or segmental	0,824	1,938	0,425	17577
Solyc01g102660.4.1	*Solyc01g091330.4.1*	Proximal	0,304	1,952	0,156	3443
Solyc02g038818.1.1	*Solyc09g007150.3.1*	Dispersed	0,094	0,327	0,288	604
Solyc02g068900.3.1	*Solyc06g083770.5.1*	Dispersed	0,648	NaN	NaN	6067
Solyc02g081240.1.1	*Solyc12g011300.2.1*	Dispersed	0,420	1,429	0,294	18111
Solyc02g081340.3.1	*Solyc09g074850.4.1*	Dispersed	0,558	NaN	NaN	18111
Solyc02g081430.3.1	*single*	Singleton	-	-	-	7067
Solyc03g116120.3.1	*Solyc03g116130.2.1*	Tandem	0,313	1,514	0,207	3419
Solyc03g116130.2.1	*Solyc03g116120.3.1*	Tandem	0,313	1,514	0,207	3419
Solyc04g009530.4.1	*Solyc02g038818.1.1*	Dispersed	0,296	1,496	0,198	15844
Solyc04g050560.4.1	*Solyc12g056250.2.1*	Dispersed	0,336	0,811	0,414	26629
Solyc04g057890.3.1	*single*	Singleton	-	-	-	7143
Solyc04g064470.2.1	*Solyc06g075520.3.1*	Dispersed	0,182	0,302	0,602	1399
Solyc04g081740.3.1	*single*	Singleton	-	-	-	11163
Solyc05g006730.5.1	*Solyc05g006740.5.1*	Tandem	0,161	0,569	0,283	5332
Solyc05g006740.5.1	*Solyc05g006750.3.1*	Tandem	0,354	3,1	0,114	5332
Solyc05g006750.3.1	*Solyc05g006740.5.1*	Tandem	0,354	3,1	0,114	8873
Solyc05g013950.1.1	*Solyc05g054760.5.1*	Proximal	0,207	0,478	0,432	1399
Solyc05g026210.2.1	*Solyc05g006750.3.1*	Proximal	0,307	1,151	0,267	22195
Solyc05g054760.5.1	*Solyc05g013950.1.1*	Proximal	0,207	0,478	0,432	6837
Solyc05g161440.1.1	*Solyc09g007150.3.1*	Dispersed	0,256	0,638	0,401	23062
Solyc06g009020.2.1	*Solyc06g009040.4.1*	Tandem	0,085	0,251	0,34	1000
Solyc06g009040.4.1	*Solyc06g009020.2.1*	Tandem	0,085	0,251	0,34	1000
Solyc06g011280.3.1	*Solyc02g081340.3.1*	Dispersed	0,804	1,918	0,419	4174
Solyc06g069040.4.1	*Solyc10g084960.2.1*	Dispersed	0,459	NaN	NaN	19081
Solyc06g075520.3.1	*Solyc09g056180.3.1*	Dispersed	0,165	0,195	0,849	1399
Solyc06g083770.5.1	*Solyc02g068900.3.1*	Dispersed	0,648	NaN	NaN	14901

Solyc07g021460.3.1	*Solyc08g062570.1.1*	Dispersed	0,260	0,457	0,569	4081
Solyc07g056420.4.1	*Solyc07g056430.3.1*	Tandem	0,083	0,259	0,322	226
Solyc07g056430.3.1	*Solyc07g056440.3.1*	Tandem	0,043	0,153	0,281	226
Solyc07g056440.3.1	*Solyc07g056450.3.1*	Tandem	0,067	0,314	0,214	226
Solyc07g056450.3.1	*Solyc07g056460.3.1*	Tandem	0,095	0,445	0,213	226
Solyc07g056460.3.1	*Solyc07g056470.3.1*	Tandem	0,044	0,150	0,29	226
Solyc07g056470.3.1	*Solyc07g056490.5.1*	Tandem	0,197	2,128	0,093	226
Solyc07g056490.5.1	*Solyc07g056500.4.1*	Tandem	0,101	0,409	0,248	226
Solyc07g056500.4.1	*Solyc07g056490.5.1*	Tandem	0,101	0,409	0,248	226
Solyc07g056510.3.1	*Solyc07g056500.4.1*	Proximal	0,262	1,481	0,177	26998
Solyc07g161200.1.1	*Solyc09g074850.4.1*	Dispersed	0,125	0,128	0,98	1000
Solyc08g062570.1.1	*Solyc12g062730.3.1*	Dispersed	0,191	0,338	0,564	4081
Solyc08g080900.3.1	*Solyc08g080910.3.1*	Tandem	0,140	0,268	0,52	8614
Solyc08g080910.3.1	*Solyc08g080900.3.1*	Tandem	0,140	0,268	0,52	8614
Solyc09g007150.3.1	*Solyc10g084400.2.1*	Dispersed	0,161	0,83	0,194	604
Solyc09g011490.3.1	*Solyc09g011500.3.1*	Tandem	0,101	0,403	0,25	140
Solyc09g011500.3.1	*Solyc01g081260.2.1*	WGD or segmental	0,107	0,426	0,251	140
Solyc09g011510.2.1	*Solyc09g011520.3.1*	Tandem	0,157	0,331	0,473	140
Solyc09g011520.3.1	*Solyc09g011530.3.1*	Tandem	0,181	0,414	0,436	140
Solyc09g011530.3.1	*Solyc09g011520.3.1*	Tandem	0,181	0,414	0,436	23323
Solyc09g011540.2.1	*Solyc09g011550.4.1*	Tandem	2,717	1,376	1,975	140
Solyc09g011550.4.1	*Solyc09g011590.4.1*	Tandem	2,588	1,516	1,707	140
Solyc09g011590.4.1	*Solyc09g011600.3.1*	Tandem	0,134	0,349	0,3840	140
Solyc09g011600.3.1	*Solyc09g011610.4.1*	Tandem	1,694	NaN	NaN	140
Solyc09g011610.4.1	*Solyc09g011600.3.1*	Tandem	1,694	NaN	NaN	140
Solyc09g011630.3.1	*Solyc01g081310.3.1*	WGD or segmental	0,166	0,516	0,321	22437
Solyc09g011640.5.1.1	*Solyc09g011630.3.1*	Tandem	0,166	0,516	0,321	140
Solyc09g011650.4.1	*Solyc09g011640.5.1.1*	Proximal	0,407	1,298	0,314	4080
Solyc09g056180.3.1	*Solyc11g039930.2.1*	Dispersed	0,163	0,175	0,935	1399
Solyc09g063150.3.1	*Solyc01g099590.5.1*	WGD or segmental	0,376	1,210	0,311	19681
Solyc09g074850.4.1	*Solyc12g094430.1.1*	Dispersed	0,499	NaN	NaN	1000
Solyc09g091130.5.1	*Solyc09g091140.4.1*	Tandem	0,323	0,661	0,488	6602
Solyc09g091140.4.1	*Solyc09g091130.5.1*	Tandem	0,323	0,661	0,488	6602
Solyc09g160500.1.1	*Solyc09g011530.3.1*	Dispersed	0,368	0,881	0,418	23323
Solyc10g007620.3.1	*Solyc10g007635.1.1*	Dispersed	0,272	1,342	0,203	19372

Solyc10g007635.1.1	*Solyc12g011300.2.1*	Dispersed	0,224	NaN	NaN	226
Solyc10g084400.2.1	*Solyc02g038818.1.1*	Dispersed	0,180	0,830	0,217	10844
Solyc10g084960.2.1	*Solyc06g069040.4.1*	Dispersed	0,459	NaN	NaN	140
Solyc11g011250.3.1	*Solyc06g075520.3.1*	Dispersed	0,507	NaN	NaN	14678
Solyc11g028090.3.1	*Solyc11g028100.2.1*	Tandem	0,205	0,311	0,658	23493
Solyc11g028100.2.1	*Solyc11g028090.3.1*	Tandem	0,205	0,311	0,658	4174
Solyc11g039930.2.1	*Solyc04g064470.2.1*	Dispersed	0,066	0,121	0,547	1399
Solyc12g011300.2.1	*Solyc10g007635.1.1*	Dispersed	0,224	NaN	NaN	20192
Solyc12g011310.2.1	*Solyc12g011320.2.1*	Tandem	0,359	3,019	0,119	226
Solyc12g011320.2.1	*Solyc12g011310.2.1*	Tandem	0,359	3,019	0,119	23537
Solyc12g044520.2.1	*Solyc12g044530.3.1*	Tandem	0,021	0,082	0,255	604
Solyc12g044530.3.1	*Solyc12g044520.2.1*	Tandem	0,021	0,082	0,255	604
Solyc12g056250.2.1	*Solyc04g050560.4.1*	Dispersed	0,336	0,811	0,414	11157
Solyc12g062730.3.1	*Solyc07g021460.3.1*	Dispersed	0,100	0,137	0,727	4081
Solyc12g094430.1.1	*Solyc02g081340.3.1*	Dispersed	0,533	NaN	NaN	20297
Solyc12g097080.2.1	*Solyc06g069040.4.1*	Dispersed	0,468	NaN	NaN	20312
Sopen00g007230.1	*Sopen09g002020.1*	Dispersed	0,016	0,086	0,19	604
Sopen00g010060.1	*Sopen07g010540.1*	Dispersed	0,061	0,16	0,384	4081
Sopen01g019540.1	*Sopen10g017090.1*	Dispersed	0,263	0,34	0,773	1399
Sopen01g032820.1	*Sopen01g032830.1*	Tandem	0,011	0,081	0,142	3103
Sopen01g032830.1	*Sopen01g032840.1*	Tandem	0,042	0,184	0,229	3103
Sopen01g032840.1	*Sopen01g032890.1*	Tandem	0,427	4,281	0,100	3103
Sopen01g032890.1	*Sopen01g032840.1*	Tandem	0,427	4,281	0,100	13144
Sopen01g033680.1	*Sopen10g034420.1*	Dispersed	0,366	1,390	0,263	13509
Sopen01g037070.1	*Sopen01g045070.1*	Proximal	0,309	1,805	0,171	17788
Sopen01g043120.1	*Sopen01g032840.1*	Proximal	0,512	2,188	0,234	17577
Sopen01g045070.1	*Sopen01g037070.1*	Proximal	0,309	1,805	0,171	3443
Sopen02g012630.1	*Sopen02g012640.1*	Tandem	0,019	0,042	0,45	604
Sopen02g012640.1	*Sopen02g012630.1*	Tandem	0,019	0,042	0,45	604
Sopen02g014600.1	*Sopen01g019540.1*	Dispersed	0,268	0,309	0,867	1399
Sopen02g018000.1	*Sopen02g026070.1*	Dispersed	1	NaN	NaN	6067
Sopen02g025880.1	*Sopen12g006650.1*	Dispersed	0,491	1,583	0,31	18111
Sopen02g025970.1	*Sopen12g031310.1*	Dispersed	0,52	NaN	NaN	7448
Sopen02g026070.1	*single*	Singleton	-	-	-	7066
Sopen03g035090.1	*Sopen03g035100.1*	Tandem	0,31	1,313	0,236	3419
Sopen03g035100.1	*Sopen03g035090.1*	Tandem	0,31	1,313	0,236	3419

Sopen04g004690.2	*Sopen10g033840.1*	Dispersed	0,268	1,744	0,154	15844
Sopen04g025740.1	*Sopen04g035370.1*	Dispersed	1	NaN	NaN	7143
Sopen04g035370.1	*single*	Singleton	-	-	-	11163
Sopen05g002680.1	*Sopen05g002690.1*	Tandem	0,278	0,796	0,349	5332
Sopen05g002690.1	*Sopen05g002680.1*	Tandem	0,278	0,796	0,349	8873
Sopen05g033230.1	*Sopen02g014600.1*	Dispersed	0,055	0,224	0,247	6837
Sopen06g003820.1	*Sopen06g003840.1*	Tandem	0,083	0,226	0,368	1000
Sopen06g003840.1	*Sopen06g003820.1*	Tandem	0,083	0,226	0,368	1000
Sopen06g005470.1	*Sopen11g012580.1*	Dispersed	0,241	0,422	0,57	4174
Sopen06g026330.1	*Sopen01g043120.1*	Dispersed	0,442	2,275	0,194	19081
Sopen06g035180.1	*Sopen02g018000.1*	Dispersed	0,619	NaN	NaN	14901
Sopen07g010540.1	*Sopen00g010060.1*	Dispersed	0,061	0,16	0,384	4081
Sopen07g029790.1	*Sopen07g029800.1*	Tandem	0,077	0,216	0,358	226
Sopen07g029800.1	*Sopen07g029810.1*	Tandem	0,065	0,163	0,402	226
Sopen07g029810.1	*Sopen07g029820.1*	Tandem	0,32	0,78	0,411	226
Sopen07g029820.1	*Sopen07g029830.1*	Tandem	0,203	0,359	0,566	226
Sopen07g029830.1	*Sopen07g029840.1*	Tandem	0,084	0,374	0,224	226
Sopen07g029840.1	*Sopen07g029850.1*	Tandem	0,201	1,314	0,153	226
Sopen07g029850.1	*Sopen07g029860.1*	Tandem	0,115	0,336	0,341	226
Sopen07g029860.1	*Sopen07g029850.1*	Tandem	0,115	0,336	0,341	226
Sopen07g029870.1	*Sopen07g029860.1*	Proximal	0,264	1,474	0,179	26998
Sopen08g029200.2	*Sopen08g029220.1*	Tandem	0,137	0,286	0,478	8614
Sopen08g029220.1	*Sopen08g029200.2*	Tandem	0,137	0,286	0,478	8614
Sopen09g002020.1	*Sopen00g007230.1*	Dispersed	0,016	0,086	0,19	604
Sopen09g006240.1	*Sopen09g006250.1*	Tandem	0,081	0,459	0,176	140
Sopen09g006250.1	*Sopen09g006280.1*	Tandem	-	-	-	140
Sopen09g006280.1	*Sopen09g006290.1*	Tandem	-	-	-	140
Sopen09g006290.1	*Sopen09g006300.1*	Tandem	-	-	-	140
Sopen09g006300.1	*Sopen09g006290.1*	Tandem	-	-	-	140
Sopen09g006310.1	*Sopen09g006320.1*	Tandem	0,247	0,848	0,291	140
Sopen09g006320.1	*Sopen09g006330.2*	Tandem	0,125	0,604	0,207	140
Sopen09g006330.2	*Sopen09g006320.1*	Tandem	0,125	0,604	0,207	140
Sopen09g006340.1	*Sopen09g006350.1*	Tandem	0,105	0,362	0,289	140
Sopen09g006350.1	*Sopen09g006360.1*	Tandem	0,119	0,36	0,331	140
Sopen09g006360.1	*Sopen09g006370.1*	Tandem	0,126	0,345	0,364	140
Sopen09g006370.1	*Sopen09g006390.1*	Tandem	0,132	0,324	0,408	140

Sopen09g006390.1	*Sopen09g006370.1*	Tandem	0,132	0,324	0,408	140
Sopen09g006400.1	*Sopen09g006410.1*	Tandem	0,138	0,443	0,311	140
Sopen09g006410.1	*Sopen09g006420.1*	Tandem	0,165	0,431	0,383	140
Sopen09g006420.1	*Sopen09g006410.1*	Tandem	0,165	0,431	0,383	23323
Sopen09g006440.1	*Sopen09g006450.1*	Tandem	0,188	0,492	0,383	22437
Sopen09g006450.1	*Sopen09g006440.1*	Tandem	0,188	0,492	0,383	140
Sopen09g006460.1	*Sopen079010540.1*	Dispersed	-	-	-	4080
Sopen09g010950.1	*Sopen09g006300.1*	Dispersed	0,131	0,422	0,311	140
Sopen09g025320.1	*Sopen09g034110.1*	Tandem	0,42	1,476	0,285	19681
Sopen09g034110.1	*Sopen09g034120.1*	Tandem	0,396	0,859	0,461	6602
Sopen09g034120.1	*Sopen09g034110.1*	Tandem	0,396	0,859	0,461	6602
Sopen10g003550.1	*Sopen12g006630.1*	Dispersed	0,257	NaN	NaN	226
Sopen10g017090.1	*Sopen01g019540.1*	Dispersed	0,263	0,34	0,773	1399
Sopen10g033840.1	*Sopen04g004690.2*	Dispersed	0,268	1,744	0,154	10844
Sopen10g034420.1	*Sopen09g006310.1*	Dispersed	0,212	1,124	0,189	140
Sopen11g006030.1	*Sopen05g033230.1*	Dispersed	0,335	NaN	NaN	14678
Sopen11g012580.1	*Sopen11g012610.1*	Tandem	0,233	0,23	1,014	23493
Sopen11g012610.1	*Sopen11g012580.1*	Tandem	0,233	0,23	1,014	4174
Sopen12g006630.1	*Sopen07g029840.1*	Dispersed	0,225	1,474	0,153	20192
Sopen12g006640.1	*Sopen12g006650.1*	Tandem	0,355	1,924	0,184	226
Sopen12g006650.1	*Sopen12g006640.1*	Tandem	0,355	1,924	0,184	23537
Sopen12g017180.1	*Sopen09g006360.1*	Dispersed	0,057	0,158	0,361	140
Sopen12g022810.1	*Sopen12g022820.1*	Tandem	0,016	0,079	0,206	604
Sopen12g022820.1	*Sopen12g022830.1*	Tandem	0,125	0,203	0,615	604
Sopen12g022830.1	*Sopen12g022820.1*	Tandem	0,125	0,203	0,615	604
Sopen12g022850.2	*Sopen12g022830.1*	Proximal	0,375	1,034	0,363	604
Sopen12g026980.1	*Sopen05g002680.1*	Dispersed	0,236	0,759	0,311	5332
Sopen12g028770.1	*Sopen12g031310.1*	Tandem	1,464	NaN	NaN	11157
Sopen12g031310.1	*Sopen12g028770.1*	Tandem	1,464	NaN	NaN	20297
Sopen12g032870.1	*Sopen09g034110.1*	Dispersed	0,395	0,902	0,437	20312
SPIMP01g0027300	*SPIMP01g0027350*	Tandem	0,437	2,672	0,164	3103
SPIMP01g0027350	*SPIMP01g0027300*	Tandem	0,437	2,672	0,164	13144
SPIMP01g0028230	*SPIMP10g0312240*	Dispersed	0,361	0,979	0,368	13509
SPIMP01g0032370	*SPIMP01g0040230*	Tandem	0,385	2,495	0,154	17788
SPIMP01g0040230	*SPIMP01g0032370*	Tandem	0,385	2,495	0,154	3443
SPIMP02g0057440	*SPIMP02g0057450*	Tandem	0,218	0,182	1,199	604

SPIMP02g0057450	*SPIMP02g0057460*	Tandem	0,082	0,124	0,659	604
SPIMP02g0057460	*SPIMP02g0057450*	Tandem	0,082	0,124	0,659	604
SPIMP02g0063120	*SPIMP06g0205110*	Dispersed	0,648	NaN	NaN	6067
SPIMP02g0070910	*SPIMP12g0343610*	Dispersed	0,529	1,807	0,293	18111
SPIMP02g0071010	*SPIMP12g0361200*	Dispersed	0,528	NaN	NaN	7448
SPIMP02g0071100	*single*	Singleton	-	-	-	7066
SPIMP03g0112390	*SPIMP08g0245530*	Dispersed	0,204	0,97	0,21	3419
SPIMP04g0123180	*SPIMP10g0311710*	Dispersed	0,27	1,757	0,154	15844
SPIMP04g0134940	*SPIMP12g0358510*	Dispersed	0,282	0,845	0,334	26629
SPIMP04g0139100	*single*	Singleton	-	-	-	7143
SPIMP04g0139770	*SPIMP11g0330110*	Dispersed	0,064	0,077	0,828	1399
SPIMP04g0149470	*single*	Singleton	-	-	-	11163
SPIMP05g0152360	*SPIMP05g0152370*	Tandem	0,383	2,824	0,136	5332
SPIMP05g0152370	*SPIMP05g0152380*	Tandem	0,042	0,062	0,684	8873
SPIMP05g0152380	*SPIMP05g0152370*	Tandem	0,042	0,062	0,684	8873
SPIMP05g0158960	*SPIMP05g0175480*	Proximal	0,24	0,561	0,428	1399
SPIMP05g0165190	*SPIMP12g0356650*	Dispersed	0,286	0,467	0,613	23062
SPIMP05g0165920	*SPIMP05g0152380*	Proximal	0,168	0,23	0,728	22195
SPIMP05g0175480	*SPIMP04g0139770*	Proximal	0,166	0,627	0,266	6837
SPIMP06g0180280	*SPIMP06g0180290*	Tandem	0,084	0,256	0,329	1000
SPIMP06g0180290	*SPIMP06g0180280*	Tandem	0,084	0,256	0,329	1000
SPIMP06g0181480	*SPIMP11g0319170*	Dispersed	0,943	2,765	0,341	4174
SPIMP06g0196060	*SPIMP01g0027350*	Dispersed	0,454	3,544	0,128	19081
SPIMP06g0202160	*SPIMP09g0271810*	Dispersed	0,108	0,054	2,002	1399
SPIMP06g0205110	*SPIMP02g0063120*	Dispersed	0,648	NaN	NaN	14901
SPIMP07g0214860	*SPIMP12g0350360*	Dispersed	0,027	0,053	0,511	4081
SPIMP07g0223260	*SPIMP0790228480*	Proximal	0,387	1,267	0,306	226
SPIMP07g0228470	*SPIMP07g0228480*	Tandem	0,218	1,154	0,189	25867
SPIMP07g0228480	*SPIMP07g0228490*	Tandem	0,101	0,422	0,24	226
SPIMP07g0228490	*SPIMP07g0228480*	Tandem	0,101	0,422	0,24	226
SPIMP08g0245530	*SPIMP07g0214860*	Dispersed	0,124	0,276	0,449	4081
SPIMP08g0256390	*SPIMP12g0358510*	Dispersed	0,269	0,598	0,45	8614
SPIMP09g0259900	*SPIMP10g0311710*	Dispersed	0,148	0,819	0,181	604
SPIMP09g0264560	*SPIMP09g0264570*	Tandem	0,104	0,407	0,256	140
SPIMP09g0264570	*SPIMP09g0264580*	Tandem	0,125	0,406	0,309	140
SPIMP09g0264580	*SPIMP09g0264600*	Tandem	0,117	0,249	0,47	140

SPIMP09g0264600	*SPIMP09g0264620*	Tandem	0,168	0,536	0,314	140
SPIMP09g0264620	*SPIMP09g0264630*	Tandem	0,117	0,551	0,212	140
SPIMP09g0264630	*SPIMP09g0264640*	Tandem	0,164	0,579	0,282	140
SPIMP09g0264640	*SPIMP09g0264630*	Tandem	0,164	0,579	0,282	140
SPIMP09g0264650	*SPIMP09g0264660*	Tandem	0,146	0,436	0,336	140
SPIMP09g0264660	*SPIMP09g0264650*	Tandem	0,146	0,436	0,336	140
SPIMP09g0264670	*SPIMP09g0264660*	Proximal	0,26	0,711	0,365	4080
SPIMP09g0271810	*SPIMP06g0202160*	Dispersed	0,108	0,054	2,002	1399
SPIMP09g0275660	*SPIMP09g0284150*	Proximal	0,378	1,152	0,328	19681
SPIMP09g0279760	*SPIMP06g0180280*	Dispersed	0,287	2,061	0,139	11880
SPIMP09g0284150	*SPIMP09g0275660*	Proximal	0,378	1,152	0,328	6602
SPIMP10g0289360	*SPIMP07g0228470*	Dispersed	0,282	1,557	0,181	19372
SPIMP10g0311710	*SPIMP04g0123180*	Dispersed	0,27	1,757	0,154	10844
SPIMP10g0312240	*SPIMP09g0264670*	Dispersed	0,3	1,124	0,267	140
SPIMP11g0319170	*SPIMP11g0326240*	Proximal	0,937	2,252	0,416	14678
SPIMP11g0326230	*SPIMP11g0326240*	Tandem	0,285	0,395	0,721	23493
SPIMP11g0326240	*SPIMP11g0326230*	Tandem	0,285	0,395	0,721	4174
SPIMP11g0330110	*SPIMP11g0326240*	Proximal	1,028	NaN	NaN	1399
SPIMP12g0343590	*SPIMP12g0343600*	Tandem	0,275	1,124	0,244	20192
SPIMP12g0343600	*SPIMP12g0343610*	Tandem	0,361	2,333	0,155	226
SPIMP12g0343610	*SPIMP12g0343600*	Tandem	0,361	2,333	0,155	23537
SPIMP12g0349580	*SPIMP05g0152360*	Dispersed	0,128	0,326	0,393	5332
SPIMP12g0350360	*SPIMP07g0214860*	Dispersed	0,027	0,053	0,511	4081
SPIMP12g0356650	*SPIMP12g0356670*	Tandem	0,133	0,144	0,919	604
SPIMP12g0356670	*SPIMP12g0356650*	Tandem	0,133	0,144	0,919	604
SPIMP12g0358510	*SPIMP04g0134940*	Dispersed	0,282	0,845	0,334	11157
SPIMP12g0361200	*SPIMP09g0279760*	Dispersed	0,515	NaN	NaN	20297
SPIMP12g0362770	*SPIMP09g0284150*	Dispersed	0,379	1,154	0,328	20312
Contig47g050010.1	*Solyd01g073350.1*	Proximal	0,49	3,271	0,15	-
Contig2482g050040.1	*Solyd01g081570.1*	Proximal	0,004	0	NaN	17577
Contig2619g050030.1	*Solyd01g083460.1*	Dispersed	0,003	0	NaN	3443
Contig2655g050020.1	*Solyd02g069940.1*	Proximal	0,011	0,042	0,266	7448
Contig2756g050030.1	*Solyd01g073350.1*	Proximal	0	0	NaN	3103
Contig2756g050040.1	*Solyd01g073360.1*	Proximal	0,032	0,064	0,49	3103
Contig300g050040.1	*Solyd04g078720.1*	Dispersed	0,001	0,032	0,043	11163
Contig47g050020.1	*Solyd07g070010.1*	Proximal	0,033	0,029	1,147	226

Contig764g050020.1	*Solyd06g050520.1*	Proximal	0	0	NaN	1000
Solyd01g073300.1	*Solyd01g073350.1*	Tandem	0,41	3,001	0,136	13144
Solyd01g073350.1	*Solyd01g073360.1*	Tandem	0,056	0,16	0,35	3103
Solyd01g073360.1	*Solyd01g073350.1*	Tandem	0,056	0,16	0,35	3103
Solyd01g075750.1	*Solyd01g083460.1*	Proximal	0,336	1,969	0,171	3103
Solyd01g081570.1	*Contig2482g050040.1*	Proximal	0,004	0	NaN	17577
Solyd01g083460.1	*Contig2619g050030.1*	Proximal	0,003	0	NaN	3443
Solyd02g061070.1	*Solyd06g079070.1*	Dispersed	0,64	NaN	NaN	6067
Solyd02g069830.1	*Solyd12g055600.1*	Dispersed	0,422	1,338	0,315	18111
Solyd02g069940.1	*Contig2655g050020.1*	Dispersed	0,011	0,042	0,266	7448
Solyd02g070030.1	*single*	Singleton	-	-	-	7066
Solyd03g077580.1	*Solyd03g077590.1*	Tandem	0,3	1,542	0,194	3419
Solyd03g077590.1	*Solyd03g077650.1*	Tandem	0,272	1,269	0,214	3419
Solyd03g077650.1	*Solyd03g077660.1*	Tandem	0,273	1,351	0,202	3419
Solyd03g077660.1	*Solyd03g077650.1*	Tandem	0,273	1,351	0,202	3419
Solyd04g054440.1	*Solyd10g068950.1*	Dispersed	0,268	2,192	0,122	15844
Solyd04g067590.1	*single*	Singleton	-	-	-	7143
Solyd04g078720.1	*Solyd04g067590.1*	Dispersed	1,068	NaN	NaN	11163
Solyd05g051790.1	*Solyd05g062200.1*	Proximal	0,297	0,933	0,319	8873
Solyd05g060840.1	*Solyd10g068950.1*	Dispersed	0,026	0,084	0,306	10844
Solyd05g062200.1	*Solyd05g051790.1*	Proximal	0,297	0,933	0,319	22195
Solyd05g062890.1	*Solyd12g065710.1*	Dispersed	-	-	-	-
Solyd05g072160.1	*Solyd11g055460.1*	Dispersed	0,323	NaN	NaN	6837
Solyd06g050520.1	*Contig764g050020.1*	Dispersed	0	0	NaN	1000
Solyd06g055340.1	*Solyd11g060890.1*	Dispersed	0,026	0,186	0,137	4174
Solyd06g070700.1	*Solyd03g077650.1*	Dispersed	0,463	3,250	0,143	19081
Solyd06g079070.1	*Solyd02g061070.1*	Dispersed	0,64	NaN	NaN	14901
Solyd07g070010.1	*Solyd07g070030.1*	Tandem	0,094	0,391	0,241	25867
Solyd07g070030.1	*Solyd07g070040.1*	Tandem	0,205	1,493	0,137	19372
Solyd07g070040.1	*Solyd07g070030.1*	Tandem	0,205	1,493	0,137	226
Solyd08g072760.1	*Solyd12g061120.1*	Dispersed	0,009	0,011	0,816	8614
Solyd09g051050.1	*Solyd12g065710.1*	Dispersed	-	-	-	11867
Solyd09g055740.1	*Solyd09g055750.1*	Tandem	0,135	0,525	0,256	140
Solyd09g055750.1	*Solyd09g055770.1*	Tandem	0,152	0,463	0,329	4080
Solyd09g055770.1	*Solyd09g055750.1*	Tandem	0,152	0,463	0,329	4080
Solyd09g055780.1	*Solyd09g055800.1*	Proximal	0,171	0,148	1,151	4080

Solyd09g055790.1	*Solyd09g055800.1*	Tandem	0,195	0,687	0,283	22437
Solyd09g055800.1	*Solyd09g055820.1*	Tandem	0,074	0,185	0,403	4080
Solyd09g055820.1	*Solyd09g055830.1*	Tandem	0,039	0,046	0,837	4080
Solyd09g055830.1	*Solyd09g055840.1*	Tandem	0,038	0,028	1,349	4080
Solyd09g055840.1	*Solyd09g055830.1*	Tandem	0,038	0,028	1,349	4080
Solyd09g058120.1	*Solyd09g055740.1*	Proximal	0,154	0,543	0,283	140
Solyd09g063650.1	*Solyd09g072440.1*	Proximal	0,384	1	0,384	19681
Solyd09g067750.1	*Contig764g050020.1*	Dispersed	0,242	2,198	0,11	1000
Solyd09g072440.1	*Solyd09g072450.1*	Tandem	0,328	0,672	0,488	6602
Solyd09g072450.1	*Solyd09g072440.1*	Tandem	0,328	0,672	0,488	6602
Solyd10g052610.1	*Solyd07g070040.1*	Dispersed	0,301	1,872	0,161	19372
Solyd10g066470.1	*Solyd10g066480.1*	Tandem	0,044	0,074	0,599	140
Solyd10g066480.1	*Solyd10g066490.1*	Tandem	0,037	0,067	0,547	140
Solyd10g066490.1	*Solyd10g066480.1*	Tandem	0,037	0,067	0,547	140
Solyd10g068950.1	*Solyd05g060840.1*	Dispersed	0,026	0,084	0,306	14678
Solyd11g055460.1	*Solyd05g072160.1*	Dispersed	0,323	NaN	NaN	14678
Solyd11g060890.1	*Solyd06g055340.1*	Dispersed	0,026	0,186	0,137	4174
Solyd12g055600.1	*Solyd12g055610.1*	Proximal	0,304	1,080	0,282	20192
Solyd12g055610.1	*Solyd12g055760.1*	Tandem	0,004	0,022	0,196	226
Solyd12g055760.1	*Solyd12g055610.1*	Tandem	0,004	0,022	0,196	226
Solyd12g061120.1	*Solyd08g072760.1*	Proximal	0,009	0,011	0,816	-
Solyd12g061250.1	*Solyd05g051790.1*	Dispersed	0,117	0,222	0,527	5332
Solyd12g062060.1	*Solyd03g077580.1*	Dispersed	0,265	0,911	0,291	4081
Solyd12g063310.1	*Solyd09g055750.1*	Dispersed	0,169	0,533	0,318	140
Solyd12g065710.1	*Solyd05g062890.1*	Dispersed	-	-	-	604
Solyd12g067730.1	*Solyd12g067750.1*	Tandem	0,018	0,039	0,478	11157
Solyd12g067750.1	*Solyd12g067730.1*	Tandem	0,018	0,039	0,478	11157
Solyd12g070740.1	*Solyd09g067750.1*	Dispersed	0,507 79	NaN	NaN	20297
Solyd12g072450.1	*Solyd09g072450.1*	Dispersed	0,333	0,893	0,373	20312

Table S4. Occurrence of stress-related cis-regulatory elements within the 2 kbp upstream the start codon of S. lycopersicum gst genes. **ABRE**: cis-acting element involved in the abscisic acid responsiveness. **AuxRR-core:** cis-acting regulatory element involved in auxin responsiveness. **DRE:** cis-acting element involved in dehydration: low-temp and salt stresses. **ERE:** ethylene-responsive element. **GARE-motif**: gibberellin-responsive element. **LTR:** cis-acting element involved in low-temperature responsiveness. **MBS:** MYB binding site involved in drought-inducibility**. TC-rich repeats:** cis-acting element involved in defense and stress responsiveness. **TCA-element**: cis-acting element involved in salicylic acid responsiveness. **TGACG-motif:** cis-acting regulatory element involved in the MeJA-responsiveness. **W box**: wounding and pathogen response.

LOCUS	ABRE	AuxRR-core	DRE	ERE	GARE-motif	LTR	MBS	TC-rich repeats	TCA-element	TGACG-motif	W box
Solyc01g081250	5			1		1	1	1		1	1
Solyc01g081260				1		1	1		2		
Solyc01g081270		3						1			2
Solyc01g081310	1						2	3			2
Solyc01g091330	2			2			1		1		
Solyc01g099590	4			1				1			
Solyc01g102660	4			6		2			1		
Solyc02g038818	3	1		3					1	1	3
Solyc02g068900	4			3			2	1	1	2	3
Solyc02g081240	1						1			1	1
Solyc02g081340	7			2	1	1	2	2		1	1
Solyc02g081430	1			3				1	1		1

Solyc03g116120	3		2		1		1			
Solyc03g116130	1	1		1	1	1		1		
Solyc04g009530		4						2		
Solyc04g050560	1		1		3		1	1		
Solyc04g057890	1	2					1	3		
Solyc04g064470	2		1	1	1			4	1	
Solyc04g081740	2							2		
Solyc05g006730	3	4	1		1		1	2		
Solyc05g006740	2				1			1	1	
Solyc05g006750	4	2		1			1	3		
Solyc05g013950		1						1	1	
Solyc05g026210	1	1			1		1		1	
Solyc05g054760		2				1				
Solyc05g161440	3	1	1	1					1	
Solyc06g009020	1	4					1	1		
Solyc06g009040	2	1				1		3	1	
Solyc06g011280	4	11					1	3		

Solyc06g069040	1			4							2
Solyc06g075520				3			1				
Solyc06g083770	3	1		1		3		1		3	1
Solyc07g021460	8	1				1	1	1	1	1	1
Solyc07g056420						1		1	1		
Solyc07g056430	5			3	1					3	
Solyc07g056440				2	1	1		1			
Solyc07g056450	4	1		1					2	2	
Solyc07g056460	5	1		4	1		1	1			5
Solyc07g056470	3			2				1		1	
Solyc07g056490	5			2	1			1			
Solyc07g056500	3	1		6			1	1			1
Solyc07g056510	5				1		3	1	1	1	1
Solyc07g161200	1	1					2			1	
Solyc08g062570			1			1	1			1	
Solyc08g080900	1			2	1		1		1		2
Solyc08g080910										2	1

Solyc09g007150	2	4						2	1
Solyc09g011490	1				1				2
Solyc09g011500	2	2					1	2	
Solyc09g011510	2	2			2		1	2	1
Solyc09g011520	2	4		1	1		1		1
Solyc09g011530		3							
Solyc09g011540	3	4	1					2	2
Solyc09g011550	2	1			1				
Solyc09g011590		6				1		2	3
Solyc09g011600	1	4		2				1	
Solyc09g011610	1	2		1					
Solyc09g011630	1		1	3			1	1	2
Solyc09g011640	2	1					1	1	1
Solyc09g011650		1	1		1		1		
Solyc09g056180				3		2		1	2
Solyc09g063150	2	5	1	1			3	3	
Solyc09g074850	1	1				1	1	1	

Solyc09g091130	2		2				1		3	
Solyc09g091140	1	1	2				3		1	
Solyc09g160500	5		1		1	1			1	2
Solyc10g007620	1					2	3	1	4	
Solyc10g007635			2	1		1				
Solyc10g084400	2	1	1		1	2	1	1	1	
Solyc10g084960	3		3					2	3	
Solyc11g011250	2		7	1		3	1			
Solyc11g028090	2	1				2				
Solyc11g028100	1		1			1		1	1	
Solyc11g039930	1		2	1				1	2	1
Solyc12g011300	1		2		1				3	1
Solyc12g011310	1		4			1				1
Solyc12g011320	2				1			1		1
Solyc12g044520	3	1	2	1	1			2		3
Solyc12g044530	2	1	2		1			1		3
Solyc12g056250	2		2		1				2	2

Solyc12g062730	15	1	1		1	2	1
Solyc12g094430	6	1	2		1		
Solyc12g097080	3		4	1	1		1

Table S5. Summary of SNPs' calling in the S. lycopersicum reference collection of 35 accessions and predicted phenotypic effect.

Accession individual code	**Name**	**Number of SNPs**	**SNPs with HIGH impact effect**	**SNPs with MODERATE impact effect**	**SNPs with LOW impact effect**
TS-122	Rutgers	9		4	
TS-127	Hacienda Calera	259		44	35
TS-130	Severianin	13	1	3	1
TS-133	Peto95-43	71	2	8	15
TS-135	Hacienda Rosario	43		7	3
TS-139	Red Setter	37	1	7	3
TS-143	Florida 7547	10		4	1
TS-151	T-5	117	2	19	18
TS-170	Cuor di bue	42	1	6	7
TS-171	UC-82	61	1	8	10
TS-185	B-L-35	6		1	1
TS-186	Rowpac	101	2	12	8
TS-190	italian cann	84	2	9	15
TS-195	Ace	5		1	
TS-204	Florida 7060	27		6	4
TS-206	Prince Borghese	5		1	
TS-211	NC 84173	47		6	7
TS-212	Flora-Dade	29	1	7	5
TS-224	Guayaquil	107		7	10
TS-237	Platense	17		5	1
TS-242	Ayacucho	54		13	4
TS-249	Merida	33		7	3
TS-253	Heinz 1706-BG	2			1
TS-256	TS-256	71	1	13	5
TS-263	Rio Grande	18	2	1	3
TS-264	King Humbert #1	29		6	3

TS-277	Hunt100	37	1	5	5
TS-278	Early Santa Clara	40		4	2
TS-3	M-82	90	2	10	14
TS-4	Hawaii 7998	108		12	7
TS-48	Mexico City	86		15	13
TS-49	Earliana	28	1	3	1
TS-68	Chiclayo	356	3	42	53
TS-69	Huachinango	31		7	3
TS-7	Micro-Tom	106	3	13	20

CHAPTER III

FUNCTIONAL CHARACTERIZATION OF A GLUTATHIONE S-TRANSFERASE GENE UNDER SALT AND DROUGHT STRESS

3.1 Introduction

Abiotic stresses, such as drought, salinity, and exposure to heavy metals, pose significant challenges to global agriculture, directly affecting plant physiology, crop yield and quality. Genetic engineering emerges as a promising frontier for enhancing plant tolerance to abiotic stresses, a strategy aimed at contributing to more resilient agricultural systems to limit yield losses and quality deterioration often generated by severe environmental stresses. However, understanding gene functions and its temporal-spatial pattern of expression, its interaction with other genes and outcomes of its regulation on the phenotype is crucial to design effective breeding-oriented biotechnological strategies. Therefore, the functional characterization of a gene includes the investigation of transcription, translation, mechanisms of post-transcriptional and post-translational regulation, biochemical regulation of biosynthetic fluxes, the response of the gene to forces challenging homeostasis. The efficiency of such studies in understanding gene functions is dramatically enhanced by the availability of genetic and genomic resources of the target species, including a reliable genome sequence, a deeply curated annotation, databases and bioinformatic tools for data mining, and cheap sequencing technologies allowing for the detailed analysis of plant genomes and dissection of the genetic basis of agronomic traits.

Glutathione S-Transferases (GSTs) are a class of proteins mainly involved in the detoxification and oxidative stress response encoded by a complex gene family exhibiting gene duplication, divergent evolution, and functional specialization (Islam et al., 2014). The characterization of *gst* genes in model plants like tobacco and Arabidopsis has provided insights into their potential to enhance stress tolerance and the accumulation of antioxidant bioactive compounds. In fact, the overexpression of a *Pyrus pyrifolia gst* in transgenic tobacco lines (Liu et al., 2013) enhanced plant tolerance to a range of abiotic stresses including drought, salinity, and cadmium exposure. This was accomplished by normal plant growth under stress conditions and reduction in superoxide anion concentration, suggesting effective detoxification of Reactive Oxygen Species (ROS). Mutation of the *Arabidopsis thaliana AtGSTU17* gene (Chen et al., 2012) increased

tolerance to drought and salinity through the accumulation of GSH (Glutathione) and ABA (Abscisic Acid). Tobacco plants overexpressing the *Glycine max GmGSTU4* gene exhibited increased salt tolerance accomplished by the production of protective metabolites, such as proline and trehalose (Kissoudis et al., 2015). *LeGSTU2*, a GST gene from *Lycopersicon esculentum* was over-expressed in Arabidopsis leading to increased tolerance to osmotic and salt stresses. Proline, malondialdehyde, and antioxidative enzyme activity changes were correlated with the increased tolerance of transgenic plants, arguing the potential of the target gene for enhancing tomato tolerance and fruit quality (Xu et al., 2015).

Previous research carried out in our laboratory on a tomato introgression line, correlated the up-regulation of the *Solyc07g056420* gene encoding a tau Glutathione S-Transferase (GST) with the fruit hyper-accumulation of phenolics compounds (Di Matteo A. et al., 2013). In Chapter II, bioinformatics analysis revealed that the *Solyc07g056420* gene consisted of two exons and one intron and presented both GST_C_Tau and GST_N_Tau domains. This gene was characterized by a tandem duplication and was subcellularly localized in the cytoplasm. Additionally, three stress-related cis-regulatory elements were found in its promoters. Expression pattern analysis in *S. lycopersicum* showed that *Solyc07g056420* exhibited high expression in leaves in response to drought, cold, and heat stress, as well as in roots in response to salinity.

The main objective of our research was to functionally characterize the *Solyc07g056420* gene to gain the proof-of-concept on the role of the target gene in controlling the antioxidant synthesis and accumulation and the redox state of plant tissues. A more general goal of the study was to elucidate a possible effect of a modulation in the plant redox potential might affect plant tolerance to environmental stresses. Toward this goal, the target *S. lycopersicum* gst gene was over-expressed in transgenic *Nicotiana tabacum* plants and the effect of drought and salt stresses was assessed by eco-physiological, metabolic, and molecular investigations. Particular emphasis was given to the redox balance between ROS and antioxidant compounds.

3.2 Materials and methods

3.2.1 Engineering expression vectors and plant transformation

The *Solyc07g056420* CDS sequence was identified in the Solgenomics database (https://solgenomics.net) and used to design the cloning strategy. In particular, the cloning of the target sequence between the promoter (p35S) and the terminator (t35S) of the CaMV 35S protein in the cloning vector and the subsequent sub-cloning of the expression cassette into the destination vector (the binary expression vector) were carried out using the Gateway® system (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the Gateway® Technology User Manual (https://tools.thermofisher.com/content/sfs/manuals/gatewayman.pdf). The Gateway® technology is based on the bacteriophage λ site-specific recombination system and promotes the insertion of a nucleotide fragment into a vector by recombination. The Recombination is conservative (there is no loss or addition of nucleotides), site-specific and exploits specific sequences (att sites), having approximately 15 complementary bp, present both in the virus genome and on the bacterial chromosome. Lambda recombination uses two att sites: attB, on the regions flanking the gene of interest, and attP, on the bacteriophage chromosome. After recombination two further sites are obtained: attL and attR which are involved in the second phase of the process in order to obtain the binary vector.

Lambda recombination is catalyzed by a mixture of enzymes that bind to specific sequences (att sites), recognize target sites, cleave them, and then covalently bind DNA molecules. Recombination occurs following a double exchange of strands and the formation of bonds of DNA molecules in a new arrangement. The proteins involved in the recombination reaction differ depending on whether the lambda bacteriophage uses the lytic or lysogenic pathway. The lysogenic pathway is catalyzed by an Integrase (Int) of the bacteriophage λ and by Integration Host Factor (IHF) proteins of E. coli (contained in the BP Clonase™ enzyme mixture) while the lytic pathway is catalyzed by the Integrase (Int) proteins and Bacteriophage λ excisionase (Xis) and E. coli Integration Host Factor (IHF) protein (contained in the LR Clonase™ enzyme blend). Gateway® technology uses the lambda recombination system to facilitate the transfer of heterologous DNA sequences (bounded by modified att sites) between vectors (Hartley et al., 2000). Two recombination reactions form the basis of Gateway® technology. The BP Reaction that facilitates the recombination of an attB substrate (attB-PCR product or linearized attB expression clone) with an attP substrate (donor vector) to create an input clone containing attL. This reaction is catalyzed by the BP Clonase™ enzyme blend. The LR reaction, however,

facilitates the recombination of an attL substrate (input clone) with an attR substrate (destination vector) to create an expression clone containing attB. This reaction is catalyzed by the LR Clonase™ enzyme blend. The wild-type λ att recombination sites have been modified to improve the efficiency and specificity of the Gateway® BP and LR recombination reactions. In particular, mutations were made to the central regions of the att sites to eliminate the stop codon and to ensure the specificity of the recombination reactions and to maintain orientation and reading phase. Mutations were introduced into the short (5 bp) regions flanking the central 15 bp regions of attB sites to minimize the formation of single-stranded secondary structures of attB plasmids (e.g., ssDNA or mRNA phagemids).A portion of 43 bp of the attR site was removed to make the in vitro attL x attR reaction irreversible and more efficient (Bushman et al., 1985). To allow recombinant cloning and efficient selection of entry or expression clones, most Gateway® vectors contains two att sites flanking a cassette containing: a) the ccdB gene for negative selection (present in donor, target and supercoiled entry vectors); b) the gene for chloramphenicol resistance (CmR) for counter selection (present in the donor and in the destination vectors). After a BP or LR recombination reaction, this cassette is replaced by the gene of interest. The presence of the ccdB gene allows negative selection of donor and destination vectors (and some entry vectors) in E. coli after recombination and transformation. The CcdB protein interferes with E. coli DNA gyrase (Bernard and Couturier, 1992), thereby inhibiting the growth of most E. coli strains (e.g. DH5α™,TOP10). When recombination occurs (between a destination vector and an input clone or between a donor vector and an attB-PCR product), the ccdB gene is replaced by the gene of interest. Cells containing vectors with the ccdB gene that did not take part in the recombination reaction or byproducts bearing the ccdB gene cannot grow. This allows for high-efficiency recovery of desired clones.

To PCR amplify the target sequence, an appropriate primer pair was used. The sequence of the forward primer included a) four guanine residues (G) at the 5′ end followed (b) by the 25 bp attB1 site, (c) by 18-25 bp of specific sequences to the target and d) two additional nucleotides to maintain the correct reading frame with the attB1 region (these two nucleotides cannot be AA, AG or GA, because these additions create a translation termination codon). The sequence of the reverse primer included, a) four guanine (G) residues were inserted at the 5′ end followed by b) the 25 bp attB2 site, c) 18-25 bp sequence specific to the CDS of interest, and d) an additional nucleotide to maintain the correct reading frame with the attB2 region. The reverse primer was designed taking into account the removal of the stop codon. Primer sequences were as follow:

Solyc07g056420.2_att_Fw

GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGCTAATGATGAGGTGATTCTG

Solyc07g056420.2_att_Rv

GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATTCAAGTCCAAGCTTTTCCCTA

To PCR amplify the target CDS, 20 ng of cDNA from leaves of *Solanum lycopersicum* var. Red Setter were used as a template in a reaction of 100 µL containing 10 µL of 10x Pfx Reaction Buffer, 2 µL of 450 mM MgSO, 16 µL of 1.25 mM dNTPs, 5 µL of 1 µM forward primer , 5µL of 1 µM reverse primer and 0.5µL of 2.5 U μL^{-1} Platinum® Pfx DNA Polymerase (Thermo Fisher Scientific Catalog no. 11708-013). The reactions was incubated in a Veriti Thermal Cycler (Life Technologies) using 30 cycles and a Ta of 58 °C.

To purify the attB-PCR products, 150 µl of TE pH 8.0 were added to 50 µl amplification reaction containing the attB-PCR product. 100 µl of PEG 8000 30% and 30 mM $MgCl^2$ were added to the reaction. After mixing thoroughly by vortexing, the sample was immediately centrifuged at 10,000 x g for 15 minutes at room temperature. The supernatant was removed, and the pellet was dissolved in 50 µl of TE pH 8.0. The quality and quantity of the recovered attB-PCR product was verified on an agarose gel according to manufacturer instructions.

The attB-PCR product was subsequently cloned into the donor vector (pDONR/ZEO) using the BPclonase enzyme complex. To prepare the BP reaction, 50 ng of attB-PCR product (100 fmol) were added to 300 ng of pDONR/ZEO, 4 µl of 5X BP Clonase™ Reaction and TE Buffer (pH 8.0) to16 µl in a 1.5 ml tube. After removal from the -80°C freezer, the tube containing the BP Clonase enzyme complex was vortexed twice and then 4 µL were added to the reaction. The reaction was incubated at 25°C for 14 hours and then 2 µl of Proteinase K were added to the reaction and a further 10 min incubation at 37°C was carried out before proceeding with the transformation of electrocompetent *E. coli* cells.

To produce the expression vector pK2GW7,0:Solyc07g056420_CDS, a selected clone pDONR/Zeo:Solyc07g056420_CDS was recombined with the destination vector pK2GW7,0, an 11,135 bp binary vector harboring between LB and RB the gene NptII for kanamycin resistance, the ccdB sequence flanked attR1 and attR2 sites preceded and followed by the 35S2 promoter and the 35S terminator, respectively. LR recombination reaction was set adding 300 ng of the input clone pDONR/Zeo:Solyc07g056420_CDS, 300 ng of the destination vector pK2GW7,0, 4 µl of the 5X LR Clonase™ Reaction Buffer and TE Buffer (pH 8.0) to16 µl. Then 4 µl of LR Clonase™ Enzyme Mix was added and the reaction was briefly vortexed twice. The reaction was incubated at 25°C overnight before adding 2 µl of a Proteinase K solution and incubating for 10

min at 37°C. 1 µl of LR recombination reaction was used for the transformation of One Shot® TOP10 electrocompetent cells.

The expression vector pK2GW7,0:Solyc07g056420_CDS was transformed in *Agrobacterium tumefaciens* strain LBA4404 and a transformed colony was used to co-cultivate tobacco (*Nicotiana tabacum* L. cv. Samsun-NN) leaf explants derived from in vitro cultured plants using the procedure detailed by Chiaiese et al., (2012).

3.2.2 Development of transgenic T2 lines

T_0 plants were selected by PCR amplification of the $35S^2$ promoter sequence and then propagated on selective media and grown *in vivo* in a controlled environment within a growth chamber at 24 °C, 65 % humidity, photoperiod 16/8 h light/dark until they reached the flowering stage. Flowering plants were individually isolated with paper bags to prevent any potential cross-pollination. The dehydrated capsules were collected subsequently to harvest seeds for the T1 generation. The seeds obtained from self-fertilization of T1 tobacco plants were sterilized with 70% ethanol, followed by 2.5% sodium hypochlorite with Tween 20, agitated for 20 minutes. To remove any residual hypochlorite and ethanol, three washes with autoclaved water were performed under sterile conditions. The seeds were then placed in Petri dishes on selective growth medium (P0C + Kanamycin 50). Control plates were prepared by sowing Samsung NN wild-type seed on POC without selection agents. The plates were incubated in a growth chamber at a temperature of 24°C ± 1, with a light intensity of 100 $\mu E*m^{-2}/s$ and a 16/8 h light/dark photoperiod. T_1 progenies segregating 3:1 were selected for further analysis. Approximately 40 days after sowing, 86 seedlings were transferred to a 91-hole polystyrene seedtray *in vivo*. Seedtray was covered with a transparent plastic film to prevent excessive moisture loss and filled with a peat-based substrate. Desalted water was used for irrigation. T_1 plants were selected by PCR amplification of the $35S^2$ promoter sequence. Sixty days after sowing, 14 WT seedlings and 72 T_1 seedlings were transplanted *in vivo* in pots filled with a sterilized substrate made of a 1:3 soil to peat mixture.

T2 seeds were collected from isolated T1 selected plants and screened on selective media. T2 progenies lacking segregation were selected for further characterization. T2 plants were selected by PCR amplification of the $35S^2$ promoter sequence.

3.2.3 Drought and salt stress application

Three months-old T2 tobacco plants were used. To investigate the plant response to drought, wild-type plants and transgenic lines underwent two watering treatments. The first one consisted in the complete reintegration of evapo-transpired water (FWR) estimated by loss of weight. The second treatment consisted in the reintegration of half of the water loss (HWR). Six plant replications for each combination of genotype x treatment were used. Fourteen days later the first differentiating watering, plants underwent further eco-physiological, chemical, and molecular characterizations. To assess plant response to salinity, wild-type plants and transgenic lines underwent two watering treatments. The first one consisted in the supplementation of plant with water whereas the second one consisted in supplementing plants with 200 mM NaCl (EC 43 mS/cm). The specific concentration of 200 mM NaCl was chosen based on its ability to induce a measurable stress response without causing immediate plant death, thus allowing the assessment of stress tolerance mechanisms. This concentration is commonly used in tobacco plant stress studies to simulate high salinity conditions (Agarwa et al., 2016; Ahmad et al., 2020). After 14 days plants were sampled for further analysis.

3.2.4 Molecular characterization

Total DNA was extracted from 100 mg of leaf sample powdered in liquid nitrogen using mortar and pestle. The material was extracted using the "Wizard® Magnetic 96 DNA Plant System" Kit (Promega), following the manufacturer's guidelines. The concentration and purity of the extracted DNA sample were assessed using a NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific). To confirm the transgenic event of the T2 plants, PCR amplification targeting the NptII marker gene was performed. To examine the effects of environmental stress on plant molecular responses, we investigated transcriptional changes within the glutathione-ascorbate (GSH-AsA) cycle in T2 transgenic plants. In particular, the quantification of target mRNAs was performed using the TaqMan qPCR technology. For each target gene sequence and the endogenous control, a primer pairs and a probe were designed using the PrimerQuest™ Tool available at https://eu.idtdna.com/ (Table S6).

TaqMan qPCR reactions were set using the PrimeTime® Gene Expression Master Mix following the manufacturer instructions. The qPCR analysis was run using a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). The gene expression data were normalized using the

housekeeping gene *tobacco actin* as endogenous control. For each tested sample three technical replicas were used. Results were expressed as Fold Change (FC), namely logarithm in base 2 of RQ. Data are being validated with statistical analysis by IBM® SPSS® Statistics software through Student's t-test with 1000 bootstrap ($p < 0.05$).

3.2.5 Phenotypic and metabolic characterization

A Li-6400 gas exchange system (Li-Cor) was used to assess leaves for CO_2 assimilation rate, stomatal conductivity and transpiration rate, water use efficiency and leaf temperature. Leaf colorimetric analysis was performed with a portable chromameter (Minolta CR400). The Relative Water Content (RWC) is the amount of water present in a leaf at a given moment, compared to the maximum quantity that it can absorb. The RWC was calculated by sampling a fully expanded leaf from the plant, weighing its fresh weight (FW) and immersing it in deionized water for 12h at 4°C in the dark. At the end of the incubation, the turgid weight (TW) was determined by weighing the leaf from which the excess water was removed by blotting paper. The leaf was dried in an oven at 65°C until constant weight and the dry weight (DW) recorded. The RWC was calculated according to the formula:

$$\text{RWC} = \frac{(\text{FW}-\text{DW})}{(\text{TW}-\text{DW})} \text{ x } 100.$$

The metabolic characterization was based on an exploration of biochemical parameters that involved the physiological state and stress response mechanisms of the plants under investigation. Dry matter content, alongside an array of compounds such as flavonoids, phenolics, hydrogen peroxide, photosynthetic pigments, anthocyanins, and antioxidant compounds were measured. We have employed three technical replicates for each biological replicate.

The dry matter content was assessed with a Moisture Analyzer MJ33 (Mettler Toledo).

Leaf flavonoids content was measured according to the procedure reported by Zhishen J. et al., (1999) with some modifications. 0.2 g of frozen sample powder were extracted with 2 ml of an 80% methanol/water solution and incubated at 60°C for 5 minutes. After incubation, the samples were vortexed, cooled on ice, centrifuged, and the supernatant was analyzed for flavonoid content. The absorbance was measured at 405 nm, allowing us to express the total flavonoid content in milligrams of quercetin equivalents per 100 g of fresh weight (FW).

Leaf total phenolics were quantified according to content followed, utilizing a procedure described by Mandal et al., (2013) with some modifications. This included homogenizing 0.1 g of frozen grounded sample with 2 ml of 60% methanol, followed by a 5-minute incubation at

60°C and centrifugation. The supernatant was then treated with Folin-Ciocalteu's reagent and sodium carbonate. Absorbance was assessed at 760 nm the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per 100 g of FW.

The leaf level of hydrogen peroxide was assessed according to the procedure reported by Loretoand Velikova (2001).

The extraction and quantification of photosynthetic pigments were carried out according to Hartmut et al. (1983), using 100% acetone. Anthocyanins were assessed using a protocol by Giusti and Wrolstad (2001).

The leaf antioxidant capacity was evaluated with the ABTS methodology according to Kerchev et al. (2008) and results were expressed as Trolox equivalents per 100 g of FW.

3.3 Results

3.4 Discussion

Abiotic stresses can impact dramatically on plant growth, yield, and quality and climate change is leading to increased exposure to environmental constraints because of warmer temperatures, more frequent episodes of extreme heat, increased drought conditions, and desertification, and in some regions, more frequent and extreme storms and flooding. Whitin this scenario, developing plant varieties tolerant to such conditions has become critical for mitigating global warming effects by enhancing agro-system resilience and food security. The research works here discussed deal with the functional characterization of the tomato gene *Solyc07g056420* encoding for a TAU GST, by heterologous expression in tobacco to investigate its involvement in plant response to salinity and drought.

Salinity and drought are major current issues causing significant economic losses and concerns in areas facing a continuous decrease in freshwater reserves and an increase in saline and sodic soils used for agriculture (Saxena et al., 2019).

Plants respond to different stressors by deploying an elaborate array of molecular defenses to lessen detrimental effects on growth, development, and yield. Central to their defense strategy is the control of reactive oxygen species (ROS), which can even severely damage cellular components if exciding the cellular capability for the redox balance homeostasis (Huang et al., 2019).

To mitigate ROS-induced damages and control ROS-mediate oxidative burst, plants have evolved a sophisticated antioxidant system, including enzymatic and non-enzymatic defenses. Enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (POX), actively convert ROS into less harmful molecules. SOD, for example, transforms superoxide radicals into hydrogen peroxide, subsequently reduced to water and oxygen by CAT and POX (Rajput et al., 2021).

To complement the activity of antioxidant enzymes in counteracting ROS, non-enzymatic antioxidants, including ascorbate, tocopherol, glutathione (GSH), flavonoids, and carotenoids, directly scavenge ROS or enhance the activity of enzymatic processes (Jomova et al., 2023). A critical process of the antioxidant system the glutathione-ascorbate cycle, also referred to as Foyer-Halliwell-Asada pathway, essential to detoxify exciding ROS. As part of this process, ascorbate peroxidase reduces hydrogen peroxide to water (Hasanuzzama et al., 2019; Pandey et al., 2015) using ascorbate (As) as donor the oxidize to monodehydroascorbate, spontaneously disproportionating to ascorbate and dehydroascorbate. Monodehydroascorbate can also be enzymatically reduced back to ascorbate by the monodehydroascorbate reductase (MDHAR) by using electrons from NADH as well as dehydroascorbate can be reduced to ascorbate by the

dehydroascorbate reductase (DHAR) that use electrons from glutathione (GSH). This process contributes to limit the dehydroascorbate degradation, keep the redox balance and enhance the total as pool. Furthermore, glutathione disulfide (GSSG), that is the oxidized form of GSH, is reduced back to GSH by glutathione reductase (GR) using NADH electrons. Furthermore, the balance between GSH/GSSG is critical for controlling plant response to stress and is mainly regulated by fine tuning biosynthetic enzyme activities such as glutathione reductase and) and GSH-related enzyme such as glutathione peroxidase and glutathione S-transferases (GSTs) (Hasanuzzama et al., 2019).

GSTs are key players within the antioxidant defense. Among other functions, GST are involved in GSH reduction, as recycling, ROS peroxidation, and xenobiotic detoxification by condensation of GSH to xenobiotics, thereby enhancing their solubility and removal. This agrees with several research papers involving GSTs in plant stress response and tolerance and emphasize the evolutional significance of many GST genes in promoting plant adaptation to challenging environments and their potential in plant breeding (Hasanuzzaman et al., 2017).

Our results highlighted the role of the tomato *gst* gene Solyc07g056420 in regulating the tobacco response to abiotic stresses. However, the effects of the transgene expression showed to be dependent on the specific stressor. In particular, the expression of the *gst* gene modified the plant response to salt stress by inducing a more severe reduction of the plant height and a greater increase in the root biomass, reduced the impact on the leaf RWC without affecting photo-assimilation and stomatal conductance, leaf colorimetric parameters and chlorophyll contents. Conversely, under drought response, the *gst* expression enhanced the aboveground biomass loss and the reduction in leaf RWC, maintained the higher leaf colorimetric a/b ratio and increased the leaf chlorophyll a and b levels, maintained higher levels of photo-assimilation and stomatal conductivity and reduced leaf temperature.

The stress-dependent action of the *Solyc07g056420* gene is particularly evident when comparing transgenic plants to wild-type plants in terms of photo-assimilation and stomatal conductance. Under salt stress, we observed a reduction in both parameters, but there was no significant difference between the transgenic plants and the wild-type plants. Conversely, under drought stress, transgenic plants exhibited a smaller percentage loss in photo-assimilation and stomatal conductance compared to wild-type plants across all levels of transgene expression. Improved stomatal conductance and photo-assimilation allow the plants to maintain higher rates of gas exchange, which is essential for photosynthetic activity. This helps prevent excessive leaf temperatures and supports metabolic processes that are crucial under water-limited conditions. The *Solyc07g056420* gene appears to help transgenic plants manage their internal water use more

effectively under drought stress, allowing them to maintain critical physiological processes that support growth and stress tolerance, compared to plants under salt stress.

The increase in root biomass under salt stress is presumably consistent with the attempt of the plant to maintain an efficient root activity and enhance efficiency in the exclusion of ions present in the circulating solution, diverting energy from the above-ground parts (Balasubramaniam et al., 2023). This response is amplified by the expression of the gst gene. These results suggest that the *gst* gene interfere with the growth process and these effects could be mediated by signaling pathways that are only partially affected by drought stress. Also, compensatory mechanisms could be involved in mitigating the impact of the transgene on growth under drought conditions. Additionally, the decrease in biomass observed in plants with intermediate levels of transgene expression (1<FC<2) under drought stress could point to a critical vulnerability induced by that expression level. This finding suggests that the modification might disrupt a delicate balance in the plant's stress response or resource allocation (Abbas et al., 2023).

However, most of phenotypes expressed by transgenic plants under salt and drought stress are largely differentiated from wild-type plants in T2 offsprings with lower levels of transgene expression, while the effects of the transgene expression resulted almost completely nullified in transgenic plants with the highest expression. These results could indicate interference of GST activity with growth processes at higher expression levels. Moreover, at higher transgene expression levels, compensatory phenomena may occur whereby the overexpression of the transgene could be accompanied by the downregulation of other endogenous GST genes. Therefore, in an attempt by the biological system to re-establish homeostasis, the effect of transgene expression could diminish to the point of being unquantifiable in the highest expression categories (El-Brolosy et al., 2017).

The different pattern of leaf chromatic alterations observed in response to stress in transgenic plants likely associated with differences in the accumulation of certain metabolites (Guihua et al., 2022). Transgenic plants showed wider increase in chlorophyll a and b than wild-type plants upon drought stress. Consistently, a higher reduction in the leaf b* component than wild types would be expected. Nevertheless, transgenic plants showed higher levels of the leaf b* component than wild type, indicating a shift towards yellow, and their variation upon drought was lower. Other chemical components have likely compensated the increase in leaf chlorophyll, keeping high the expression of the leaf b* component. Also, the higher increase in the a/b ratio observed in transgenic plants expressing the *gst* gene at 1<FC<2 under drought stress suggest higher accumulation of red pigments or decrease of yellow pigments, possibly as a protective response to stress.

Consistently with the observed trends in leaf chlorophyll a and b, the expression of the *gst* gene did not impact the reduction in photo-assimilation and stomatal conductivity upon salt stress. However, the collapse of the latter two photosynthetic-related functions under drought stress was strongly mitigated by the *gst* transgene expression and this was also in agreement with the lower leaf temperature observed in transgenic plants under drought.

The higher leaf RWC observed in transgenic plants under salinity is likely a consequence of the increased root growth and the enhanced capacity of the root system to reintegrate leaf water loss precenting reduction in the photo-assimilation rate and stomatal conductivity. Conversely, the reduced leaf RWC in transgenic plants under drought might result from the increased photo-assimilation rate and stomatal conductivity and the inability of the plant to compensate for the increased water requirement through an appropriate hydraulic modification of the soil-plant system. The presumed rise of the threshold in the hydraulic differential for the stomatal closure (Bharath et al., 2021) might result in compromising the plant ability to cope with drought.

Our results suggested the involvement of the *gst* transgene in the regulation of the redox balance leading to changes in the readiness of the plant to respond to stress. Transgenic plants showed lower levels of hydrogen peroxide and anthocyanins and responded to salt stress with lower increases in leaf ascorbate and antioxidant capacity in the protein fraction and higher increase in the antioxidant capacity in the lipophilic and hydrophilic fractions. The reduced accumulation of anthocyanins in leaves of transgenic plants under salt stress suggests that the *gst* gene is involved in controlling the oxidative stress response (Dabravolski et al., 2023; Landi et al., 2015), possibly through the modulation of lipophilic and hydrophilic antioxidants (Martínez-Lorente et al., 2024). The reduced level of ROS consequent to an increased redox potential might explain the lack of activation of the stomatal closure.

Conversely, transgenic plants responded to drought stress with higher increase in hydrogen peroxide and total phenolics and lower increases in anthocyanins. Also, transgenic plants maintained higher levels of flavonoids. The higher increase in hydrogen peroxide suggests that transgenic plants under drought experienced higher oxidative stress (Leonora et al., 2016). This is consistent with the observed wider increase in phenolics that are often associated with oxidative stress and modulation of the antioxidant defense system (Singhal et al., 2015; Kumar et al., 2023). In previous research, the hyper-accumulation of phenolic compounds was observed in the fruits of a tomato introgression line overexpressing *Solyc07g056420* (Di Matteo A. et al., 2013). However, in this study, we evaluated the leaves and observed differential effects depending on the type of stress. The effects on the plant response were mediated by differently controlling oxidative stress depending on the stressor. Specifically, under salt stress, there was a reduction in phenolic compounds and modulation of antioxidants, suggesting an adaptive response aimed at

maintaining cellular stability rather than immediate oxidative defense. Conversely, under drought stress, there was an increase in phenolic content and flavonoid levels, indicating an active engagement of the antioxidant defense system to counteract higher oxidative stress conditions induced by water deficit.

To summarize, the *gst* expression in the heterologous tobacco system has been involved in the plant response to abiotic stress. Effects on the plant response were mediated by controlling differently the oxidative stress depending on the stressor. In order to investigate molecular mechanisms and genes involved in controlling the cellular redox balance (Schieber et al. 2014), tobacco genes involved in the Foyer-Halliwell-Asada pathway underwent expression profiling. This cycle plays a crucial role in detoxifying reactive oxygen species (ROS) and maintaining cellular redox balance (Hasanuzzaman et al., 2019).

Transgenic *gst* plants showed lower expression of three ascorbate peroxidase genes out of four that was compensated upon salt stress. Similarly, a glutathione peroxidase gene out of two was downregulated in transgenic plants both under unstressed and salt stressed conditions. Ascorbate peroxidase and glutathione peroxidase are involved in removing hydrogen peroxide and release oxidative stress. This was consistent with the observed down-regulation of monodehydroascorbate reductase and dehydroascorbate reductase genes that are involved in the recycling of the oxidized forms of ascorbate. The up-regulation of two glutathione reductase genes suggested an increased demand for reduced glutathione possibly depending on the over-expression of the gst transgene. Overall, our model suggests that the expression of the *gst* gene induced lower levels of oxidative stress accomplished by high expression rate of glutathione reductase genes and reducing sensitivity to the salinity stress.

Conversely, the over-expression of the *gst* gene in transgenic tobacco plants resulted in the down-regulation of four ascorbate peroxidase genes, one glutathione peroxidase gene, one dehydroascorbate reductase gene and one glutathione reductase gene. These findings suggest that the *gst* gene reduce the cellular redox potential upon drought response consistently leading to increased levels of ROS and early stomata closure.

Overall, the tomato *gst* gene is involved in fine-tuning the plant's response to environmental stress through the control of the antioxidant system. Our hypothesis is that *gst* gene provides a regulatory hub that modulates various genes in the GSH-AsA cycle (Foyer et al., 2011) contributing to the adaptive plasticity for a tailored response to the specific stresses.

3.5 Supplementay

Table S6. List of primers and labeled probes for quantitative PCR analysis using TaqMan technology.

Name	Annotation	Sequence
Actine_Fw	Actine	5’- AGGCTGTCCTTTCCTTGTATG -3’
Actin_Rv	Actine	5’- CAAAGCATGACCCTCGTAGAT -3’
Actin_Pb	Actine	5’ – FAM-TGTGTTGGATTCTGGTGATGGTGTGA –ZEN-IBFQ-3’
Solyc07g056420_Fw	GST	5’- CGAACAAGAGGCAGGTAAGAA -3’
PbSolyc07g056420	GST	5'-6-FAM-AAAGTGTTGGAGGGAGCACTTGGA-3IABkFQ-3'
Solyc07g056420_Rv	GST	5’- CAGCAGTAGAACCCAATGAGAG-3’
Nitab_050_Fw	GPX	5'-GGCCTGGAAATATTGGCATTT-3'
Nitab_050_Probe	GPX	5'-6-FAM-AATTTGGCGAGGAAGAACCAGGGA-3IABkFQ-3'
Nitab_050_Rv	GPX	5'-TAGGGAACTCTGACTTGAAACG-3'
Nitab_060_Fw	DHAR	5'-TCCCTACGTGAAGTCGTACA-3'
Nitab_060_Probe	DHAR	5'-6-FAM-AATCATTCATCAACACGCGGGCG-3IABkFQ-3'
Nitab_060_Rv	DHAR	5'-CAACCCTCAATGACATCCTCTT -3'
Solyc02g083620_Fw	APX	5'-CAAAGGACGAAGAGGCTTTCT-3'
Solyc02g083620_Probe	APX	5'-6-FAM-TGCAGAGTCACACAAGAAGCTCTCAG-3IABkFQ-3'
Solyc02g083620_Rv	APX	5'-TTGAAGCAAGATGAAGGTGGT-3'
Solyc02g086710_Fw	MDHAR	5'-TGTAATTGGTGGTGGCTACAT-3'
Solyc02g086710_Probe	MDHAR	5'-6-FAM-CACCAAAGAAGCAGCACATTCCATTCC-3IABkFQ-3'
Solyc02g086710_Rv	MDHAR	5'-GAGTAAATAGGCGGGCCATAC-3'
Solyc06g005150_Fw	APX	5'-TCTTTGACAACTCATACTTTACGGA-3'
Solyc06g005150_Probe	APX	5'-6-FAM-CTGCTTTCCGCCCCCTTGTTG-3IABkFQ-3'
Solyc06g005150_Rv	APX	5'-TCTTCATCCGCAGCATATTTCT-3'
Solyc08g080940_Fw	GPX	5'-GTGAATGGTGATAATGCTGCTC-3'
Solyc08g080940_Probe	GPX	5'-6-FAM-TGAAATCAAGCAAAGGTGGGTTCTTTGG-3IABkFQ-3'
Solyc08g080940_Rv	GPX	5'-AGGAATTTGGAGAAGTTCCACT-3'
Solyc09g007270_Fw	APX	5'-TGAGTGGAGAGAAAGAAGGTCT-3'
Solyc09g007270_Probe	APX	5'-6-FAM-CTCCAGCTACCATCAGACAAAGCACT-3IABkFQ-3'

Solyc09g007270_Rv	APX	5'-GGCGGAAGACTGGATCTTCTA-3'
Solyc09g009390_Fw	MDHAR	5'-GGAGAAGGGTGGAATTAAGACAG-3'
Solyc09g009390_Probe	MDHAR	5'-6-FAM-TATGCTGTGGGTGATGTTGCCACT-3IABkFQ-3'
Solyc09g009390_Rv	MDHAR	5'-TCAGCAGATTTGCGAGAATGA-3'
Solyc09g065900_Fw	GR	5'-AACTCTACTCGGCTAAGAACATAC-3'
Solyc09g065900_Probe	GR	5'-6-FAM-CGCCCATTTATCCCAGACATTCCTGG-3IABkFQ-3'
Solyc09g065900_Rv	GR	5'-GCAAATCAAGGGCAGCATC-3'
Solyc09g091840_Fw	GR	5'-ACTGGATGGCACCAAGATAAG-3'
Solyc09g091840_Probe	GR	5'-6-FAM-ACTGGTAGTAGGGCTCATCGTCCA-3IABkFQ-3'
Solyc09g091840_Rv	GR	5'-CAAGCTCAAGGCTTCATCTGA-3'
Solyc11g018550_Fw	APX	5'-AGGAAATTGTTGCACTTTCTGG-3'
Solyc11g018550_Probe	APX	5'-6-FAM-AGATCCAGACCAGAGCGTAGTGGT-3IABkFQ-3'
Solyc11g018550_Rv	APX	5'-CAGTCCATGATTGTCCTCCAG-

CHAPTER IV

INVESTIGATING SINK-TO-SOURCE STRENGTH IN TOMATO TO IDENTIFY GENES CONTROLLING THE INTERACTION BETWEEN STRESS RESPONSE AND FRUIT QUALITY TRAITS IN TOMATO

4.1 Introduction

Tomato (*Solanum lycopersicum* L.) is an important source of carbohydrates and antioxidants, among others (Zhao et al., 2019; Quinet et al., 2019; Gerszberg et al., 2015; Zhu et al., 2018). Tomato is a worldwide important crop and a model plant for studying fleshy fruit ripening and development, and fruit quality (The Tomato Genome Consortium, 2012; Sun et al., 2020; Zsögön et al., 2017). Fruit quality components include external and internal features. The external quality factors are fruit size, color, and texture which are easily visually appreciated (Quinet et al., 2019). Internal traits include the level of sugar and vitamins, bioactive compounds (anthocyanin, lycopene, and malate) and flavor (Wang et al., 2019; Razzaq et al., 2019; Wang et al., 2019). Intensive domestication and breeding practices have enhanced tomato yield, fruit quality, color, mechanical resistance to harvesting, shelf life, pest and disease resistance (Kazachkova et al., 2021), often overlooked flavor and decreased genetic variability. Sugars, along with phytohormones, control the growth, development, and metabolism of organs to determine sugar dynamics in tomato fruits (Beckles et al., 2012). These metabolites strongly affect fruit quality trais and are strictly controlled by source-to-sink balance that, in turn, is mainly regulated by genetic variability and environmental factors.

Understanding the dynamic regulation of physiological mechanisms controlling the source-to-sink balance across plant development and its response to challenging environments is crucial to foster knowledge-informed plant breeding approaches for yield and fruit quality. Crop yield and fruit quality traits results from complex dynamically-regulated relationship between photo-assimilation, transport and photo-assimilate allocation processes and all are shaped by genotypic variability, environmental factors and agricultural management (Yan et al., 2022; Fukushima et al., 2018).

In plants, the translocation of photo-assimilates mainly occurs from sources, such as leaves, to sinks, such as fruits, and is fundamental for growth and development. Sucrose is the major translocated photo-assimilate in tomato across a pressure gradient maintained by the osmotic potential between phloem loading/unloading sites (Beckles et al., 2012). Sucrose transported from the symplast are mainly targeted to fruit cells (Beckles et al., 2012). However, sucrose may also reach out fruit being hydrolyzed in the apoplast by a cell wall invertase, and the resultant fructose and glucose molecules are, then, imported via plasma membrane hexose transporters. Additionally, the ability of tomato fruits to unload sucrose is crucially affected by changes in the activity of sucrose synthase (SuSy), which cleaves sucrose within the fruit (D'Aoust et al., 1999). Overall, SuSy and invertase activities are major determinants of fruit sink strength (Li et al., 2012; Datir and Ghosh, 2020) as they form the sucrose gradient used to transport sucrose from phloem under a concentration gradient (Clepet et al., 2021; Qu et al., 2018). Although photosynthesis in fruit is generally not essential, the relationship between phloem loading of sucrose in source tissues and its unloading in sink tissues through central carbon metabolism is closely linked to the quality of tomato fruit (Kahlau and Bock, 2008; Lytovchenko et al., 2011).

Invertases operate the transmembrane sucrose hydrolysis to glucose and fructose and occurs in isoforms with varying biochemical characteristics and subcellular localizations (Sun et al., 2020). They are mainly involved in regulating sucrose transport to distinct tissues and have critical roles in carbohydrate partitioning, plant response to abiotic and biotic stresses unassisted or in conjunction with phytohormones and other important developmental processes (Li et al., 2012). Based on their biochemical properties and subcellular localization (Dinar, 1981; Koch, 2004; Rojo et al., 2003), invertases are classified as acid/vacuolar, neutral/cytoplasmic, and extracellular invertases (Nguyen-Quoc and Foyer, 2001; Qu et al., 2018).

Sugar unloading is a developmental regulated process. It occurs in early fruit developmental stages primarily by the symplast to gradually decline at further stages (Quinet et al., 2019). At these early stages, only a small amount of sucrose is unloaded by apoplastic invertase (Beckles et al., 2012; Beauvoit et al., 2014), whereas vacuolar acid invertase and neutral invertase are activated by gibberellic acid shortly after anthesis. However, the tomato QTL Brix9-2-5 was associated to a deletion in the third exon of the LIN5 gene (Draffehn et al., 2010; Baxter et al., 2005) and found to regulate yield and fruit quality traits such as size, soluble solids content and glucose and fructose concentrations.

This transport is heavily influenced by environmental changes, including water stress, inducing plants to tightly regulate the synthesis of photo-assimilates and their distribution (Lemoine et al., 2013; Osorio et al., 2014). Under water stress, tomato plants exhibit altered carbon allocation in their leaves, which can significantly impact sucrose metabolism (Micallef et al., 1995). In the

early stages of water deficit, there is a decrease in water uptake by the roots, leading to altered water movement within the plant, reduced cellular turgor pressure, and consequently stomatal closure (Tombesi et al., 2015; Wang et al., 2018). Simultaneously, endogenous levels of the hormone abscisic acid (ABA) increase, further signaling stomatal closure. In this way, plants lose less water through the leaves but also obtain less carbon for photosynthesis (Pastenes et al., 2005). In the long term, the reduction in CO_2 available for assimilation leads to a slowdown in the Calvin cycle (Dias & Brüggemann 2010; Jedmowski et al., 2014), excessive reduction of photosystem II, subsequent production of reactive oxygen species (ROS) causing cellular damage (Gururani et al., 2015), consequently, a reduced power of the source to sink strength (Rodrigues et al., 2019).

As a consequence of variations in carbon source-sink relationships depending on environmental challenges, metabolic signals mediate regulative gene expression mechanisms that modulate sucrose and its cleavage products (Datir and Ghosh, 2020). High invertase activities are reported to increase sucrose imports into young tomato fruits, sugar signaling regulates high sucrose import pathways and cell wall (Li et al., 2012), different invertases show different response to varied stresses and activities in different organs (Qu et al., 2018), invertase regulation at transcriptional and post-transcriptional levels is influenced by hormones, oxygen supply, pathogens, protein inhibitors, and sugars (Long et al., 2002; Zeng et al., 1999; Link et al., 2004; Trouverie et al., 2004).

The major goal of this study was the identification of genes for enhancing tomato fruit quality. In this light, a comparative investigation on the relationship between drought-affected source-to-sink strength and fruit quality in tomato drought-sensitive and drought-tolerant genetic backgrounds was carried out. A worldwide reference tomato collection was selected and screened for differential drought responses focused on leaf photosynthesis-related traits. Contrasting phenotypes were selected to confirm their physiological and metabolic response to drought. A transcriptomic profiling was carried out by RNA-seq approach across leaf, stems and fruits in contrasting phenotypes responding to drought stress to elucidate regulative mechanisms controlling photo-assimilation and translocation underlying fruit quality traits.

This study enabled the identification of key genes and metabolic processes mediating stress-induced alterations of source-to-sink strength fruit quality traits. Prospectively, several candidate genes have the potential to be exploited for breeding tomato fruit quality in challenging environments for more sustainable and resilient agro-systems.

4.2 Materials and methods

4.2.1 Experiment design, drought stress application and first screening

A non-redundant *S. lycopersicum* reference core collection of 35 tomato accessions was selected from a larger collection of worldwide accessions (Lin et al., 2014). Their genetic diversity highlighted 2,340,973 Single Nucleotide Polymorphisms (SNPs) and was reported by Lin and colleagues (2014) according to Principal Component Analysis (PCA) coordinates. To capture a comprehensive representation of genetic diversity and remove redundancy in allelic variation, accessions having differences on their principal component coordinates (PC1 and PC2) exceeding the absolute value of 0.004 were selected. The threshold 0.004 was set to have a member for most of identified component intervals and yielded 35 accessions.

Seeds were obtained from the "C.M. Rick Tomato Genetics Resource Center (TGRC)" tomato genebank of the University of California Davis. The study also involved an additional 11 tomato local varieties, kindly provided by the "Campania Germplasm Bank" and one variety (Seccagno) from "Comunità Montana Alta Irpinia". To enrich the screening, 5 cultivars with the trait of interest, "heat tolerance", as identified by the TGRC (Saladette, Malintka 101, Hotset, NC HS-1, and H5), were included., along with another 23 cultivars available from our Department of Agriculture at the University of Naples Federico II (UNINA) (Table S7).

The collection was screened for drought tolerance at the fruit set stage of the first flower truss (figure 46). The study was set up according to a completely randomized design, with two water treatments. Seeds were sown in seed trays and plantlets were grown for 30 days in a growth chamber at 24 °C, 50% humidity, artificial light with photoperiod of 16/8 h light/dark. Plantlets were transplanted in Ø25cm pots filled with a 1:1 mixture of sandy soil and loam and water supply was operated by complete compensation of water lost by evapotranspiration to the field capacity level. When 75% of plants were at the anthesis on the first flower truss, plants underwent two watering treatments with five plants replica per each combination of accession and watering treatment. The two water treatments consisted in the complete restitution of water loss (Full Water Restitution – FWR) and in the restitution of half of the lost water (Half Water Restitution – HWR), respectively. Fifteen days later, plants were assessed for gas exchange rates and colorimetric variations in leaves upon drought. A gas exchange analyzer (Licor LI-6400) was used to measure photoassimilation rate (µmol CO_2 m^{-2} s^{-1}), transpiration (mmol H_2O m^{-2} s^{-1}), stomatal conductance (mol H_2O m^{-2} s^{-1}), WUE (Water Use Efficiency), and leaf temperature (°C). The colorimetric leaf response was assessed using a portable CR-400 colorimeter (Konica Minolta) that returned the trichromatic coordinates L*, a*, and b* (figure 47). Leaf membrane stability was assessed by

Electrolyte Leakage (EL). Specifically, leaf samples were incubated for three hours in 0.1 M mannitol at 20°C and the electrical conductivity (L1) was measured using a Cond 80 PRO conductometer (XS Instruments). Samples were incubated at 100°C for 10 minutes and the electrical conductivity was assessed again (L2) after samples cool at room temperature. The EL of each sample was calculated as loss of electrolyte from the fresh tissue out of the full potential electrolyte release according to the formula EL = (L1/L2) * 100 (Hnilickova et al., 2019).
Most contrasting phenotypes (most drought-sensitive and drought-tolerant) were selected for further characterization.

Figure 46. Greenhouse screening of the reference tomato collection for drought response. Seventy-five tomato accessions were evaluated, with six biological replicates used for each treatment: three as controls and three subjected to drought stress.

Figure 47. Gas exchange and colorimetric analysis measurements were performed with the Licor LI-6400 (on the left and on the right) and the Konica Minolta CR-400 portable colorimeter (in the middle picture), respectively.

4.2.2 Second drought stress experiment: phenotypic characterization

According to a two-step design, the most tolerant (UC-82 and Red Setter) and susceptible tomato accessions (Saladette, Florida 7060, Severianin) were selected for further phenotypic characterization. Seeds were sown in seed trays and plantlets were grown for 30 days in a growth chamber at 24 °C, 50% humidity, artificial light with photoperiod of 16/8 h light/dark. Plantlets were transplanted in lysimeters having a diameter of Ø125cm filled with a 1:1 mixture of sandy soil and loam. Plants were supplied with water to compensate completely the water lost by evapotranspiration to the field capacity level. When 75% of plants showed fruit set on the first flower truss, plants underwent two differentiating watering treatments according to a completely randomized design with three plants replica per each combination of genotype x watering treatment. The two water treatments consisted in supplying water to keep the soil water potential between 10 and 20 kPa in the control treatment and between 100 and 150 kPa in the drought treatment (Figure 48). Differentiating watering treatments were applied for four weeks (Figure 49). The leaves were analyzed at different time points (once for week) for leaf CO_2 assimilation rate, transpiration, leaf temperature using a LI-6400 gas exchange analyzer (Licor; Figure 50). Fruits were sampled (Figure 51) at various ripening stages — specifically Mature Green (MG), Breaker (BR), Turning (TU), Light Red (LR), and Red Ripe (RR) — and processed to assess the soluble solids content (SSC). Fruits were individually split into parts by longitudinal cut and the pulp was isolated by eliminating the seeds, the liquid and the locular gel and the columella tissue. The remaining pericarp was frozen in liquid nitrogen and grounded to a fine powder using a stainless steel blender (Waring).

Figure 48. Installation of tensiometric probes in the root exploration zone and recording of the soil water potential by a portable data logger.

Figure 49. Drought stress experiment in lysimeters under plastic tunnel served by a drip irrigation system equipped with volumetric counter of water supplied volume at Portici (NA). The tomato accessions tested included two drought-resistant varieties (UC-82 and Red Setter) and three drought-susceptible varieties (Severianin, Florida 7060, and Saladette). Six biological replicates were used for each genotype: three as controls and three subjected to drought stress.

Figure *50*. The LI-6400 gas exchange analyzer used in the experiments.

Figure 51. Tomato fruits sampled at different ripening stages.

4.2.3 RNA extraction

Total RNA was extracted and purified from powdered tomato tissues stored at -80 °C according to Griffiths et al. (1999) with some modifications. An amount of 4 g of frozen powder was added to 12 ml of Extraction Buffer (6% w/v 4-aminosalicylic acid, 1% w/v 1,5-naphthalenedisulphonic acid, 50mM Tris-HCl pH 8.3, 5% w/v Phenol Solution (100 g phenol crystals, 14 ml m-cresol, 0.1 g 8-hydroxy-quinoline, 30 ml DEPC-treated water)) and an equal volume of phenol/chloroform solution (500 g Phenol crystals, 0.5 g 8-hydroxy-quinoline, 500 ml chloroform, 20 ml iso-amyl alcohol, 200 ml 100mM Tris-HCl pH 8.0) in 30 ml Oakridge tubes. Tubes were vigorously shaken and then centrifuged at 10,000 rpm (15 min at RT). The supernatant was recovered, added to 15 ml of phenol/chloroform solution in a clean Oakridge tube, vigorously shaken and the mix centrifuged at 10,000 rpm (15 min at RT). Hence, a volume of 11 ml of the aqueous phase was transferred to a clean Oakridge tube and the nucleic acid was precipitated for 1 h at -20°C with the addition of 27.5 ml of ice-cold pure ethanol and 1.1 ml of 3 M sodium acetate (pH 6.0). RNA was next pelleted by centrifuging at 10,000 rpm (15 min, 4 °C) and the pellet washed in 70% ethanol. Upon dissolving the pellets in 2 ml of DEPC-treated water by vortexing the tubes, 2 ml of 2X CTAB Extraction Buffer (1.4 M NaCl, 2% w/v CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM EDTA pH 8.0) and 4 ml of CTAB Precipitation Buffer (1% CTAB w/v, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) were added and the mixture centrifuged at 12,000 rpm (30 min at RT). The resulting pellet was re-suspended in 400 ml of 1.4 M NaCl and precipitated again at -20 °C (for 1 h to over-night) by the addition of 1 ml of ice-cold pure ethanol. The samples were then centrifuged at max speed (10 min, 4 °C), the pellets washed in 500 ml of 70% ethanol, re-dissolved in 400 ml of DEPC-treated water, and incubated at 50 °C for 5 min. The RNA was next extracted twice by adding a volume of 400 ml of phenol/chloroform solution. Thereafter, the nucleic acid was precipitated in 0.1 volume of 3 M sodium acetate (pH 6.0) and 3 volumes of ice-cold 100% ethanol at -20 °C (for 1 h to over-night). The samples were washed with 500 ml of 70% ethanol. The pellet was re-dissolved in 180 µl of DEPC-treated water and the remaining DNA was eliminated by treating the specimen with 1 µl of RQ1 DNase (Promega) following the addition of 20 µl of RQ1 DNase Reaction Buffer. The mixture was incubated at 37 °C for 30 min. The RNA was next extracted twice by adding a volume of 300 ml of phenol/chloroform solution. The nucleic acid was precipitated in 0.1 volume of 3 M sodium acetate (pH 6.0) and 3 volumes of ice-cold 100% ethanol at -20 °C (for 1 h to over-night). The samples were washed with 500 ml of 70% ethanol. The pellet was re-dissolved in 50 µl of DEPC-treated water and incubated at 50 °C for 5 min. The quality of the RNA was assessed using a Bio

Rad SmartSpec 3000 and integrity was confirmed using the AGILENT 2100 Nano Bioanalyzer on a RNA 6000 nano chip.

4.2.4 Transcriptomic profiling by RNAseq analysis

An RNAseq analysis was performed on leaves, fruits, and stems from Red Setter and Severianin tomato plants, under unstressed and drought-stressed conditions.

RNA sequencing was provided in outsourcing by Sequentia Biotech SL (C/ del Dr. Trueta, 179, Sant Martí, Barcelona). Sequencing of 36 poly A-enriched RNA libraries at 40M PE 150bp was run on an Illumina platform. Raw sequencing data underwent initial quality assessment and preprocessing using fastp, which facilitated the trimming of adapters and removal of low-quality sequences (Chen et al., 2018).

The reads were aligned to the *Solanum lycopersicum* reference genome version SL4.0 using HISAT2 (Kim et al., 2015). Post-alignment, Samtools (Li et al., 2009) was run to convert SAM files to BAM format, streamlining the data for gene quantification. Gene-level quantification was achieved using featureCounts (Liao et al., 2013), which mapped the reads to genes.

To address differences in library size and composition, the expression data were normalized using the Trimmed Mean of M-values (TMM) method. The Principal Component Analysis (PCA) and the linear regression R^2 were carried out in R software (R Core Team, 2021) to identify and remove non-homogeneous biological replicates. Specifically, replicates with an R^2 below 0.95 were excluded to provide a consistent dataset for future analysis.

The R platform (R Core Team, 2021) was also used to run DESeq2 and EdgeR to selectively filter out low-abundance genes and perform the differential expression analysis. Changes in the mRNA expression were calculated as log2 of the expression level in the actual condition compared to the reference condition, namely fold change (FC). Differentially expressed genes (DEGs) were selected to have an adjusted p-value (padj) at < 0.05 and a FC in absolute value higher than 0.5.

4.2.5 Functional Annotation with GO, mapping on KEGG pathways and MapMan visualization.

To provide DEGs with functional annotation, the PANTHER classification system was used for Gene Ontology (GO) annotation (Thomas et al., 2022). The PANTHER tool (version 15.0, http://pantherdb.org) facilitates the assignment of DEGs to GO terms across three categories Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF).
The KEGG Automatic Annotation Server (KAAS; Moriya et al., 2007) was used for mapping DEGs on KEGG (Kyoto Encyclopedia of Genes and Genomes; Kanehisa et al. 2010) pathways.The KEGG Automatic Annotation Server (KAAS, accessible at https://www.genome.jp/kaas-bin/kaas_main) provided functional annotation based on bidirectional BLAST comparisons against the KEGG GENES database. KAAS results include KO assignments that were used to map genes to their corresponding KEGG pathways, highlighting the metabolic pathways involved. Both GO and KEGG analyses were run using an adjusted p-value lower than 0.05. To assist data mining and understand possible implications of DEGs into regulatory mechanisms and metabolic DEGs were submitted to the Mercator4 tool (version 2.0, http://mapman.gabipd.org/web/guest/app/mercator) for automated functional annotation (Schwacke et al., 2019). Mercator provided an assignment of DEGs to MapMan bins by analyzing protein sequences against multiple databases, including TAIR, KEGG, and SwissProt. Finally, the software MapMan version 3.6.0RC1 (Usadel et al., 2009) was used for pathway visualization. This analysis enabled to focus on most implicated pathways such as photosynthesis (Table S8), redox homeostasis (Table S9), carbohydrate metabolism (Table S10), response of specific phytohormones (Table S11), cell wall organization (Table S12), and solute (Table S13).

4.3 Results

4.4 Discussion

As part of their plasticity, plants adapt the allocation of resources from source to sink in response to environmental conditions. The strength or efficiency of resource allocation between source is dynamically regulated across growth and development and is adjusted depending on different environmental factors such as light intensity, temperature, water availability, nutrient levels, and response to pest attack or pathogen infection. Plants experiencing water scarcity respond by adjusting photosynthetic activity and carbohydrate metabolism, thus affecting the transfer of nutrients from source to sink. Drought can severely affect crop yield and quality of produce, particularly when the latter is a biological fruit such as tomato. Several studies proved that drought stress can hinder phloem transport mechanisms, thereby reducing the delivery of nutrients to developing sinks (Sevanto, 2018). Furthermore, drought conditions can prompt a metabolic shift in plants from growth-oriented processes to survival-oriented ones, influencing the allocation of resources among roots, stems, and leaves to optimize water usage and maintain homeostasis (Yang et al., 2021). The identification of physiological mechanisms and regulative processes possibly impairing drought response and reduction in source-to-sink strength could enhance the efficiency of new knowledge-based breeding strategies to develop new tolerant crop systems for resilient agro-ecosystems.

As part of our study, an *S. lycopersicum* tomato reference collection of worldwide genotypes was developed based on SSR data and screened for drought tolerance. Contrasting phenotypes (i.e. Severianin, Saladette, Florida 6070, Red Setter and UC82) were selected based on their divergent physiological stability in the leaf's response to water scarcity. A second experiment was carried out to further characterize differential strategies to cope with water scarcity in the selected genotypes and validate their contrasting drought tolerance. Among others, Red Setter performed with moderate drop in leaf photo-assimilation rate, stomatal conductance, and yield x SSC (product of yield and fruit soluble solid content) under water scarcity and was identified as a more drought-tolerant genotype. By contrast, leaf gas-exchange parameters and yield x SSC performances were largely compromised under drought conditions in Severian that was identified as more drought-sensitive counterpart of Red Setter.

Leaf chlorophyll content provides indications on the photosynthetic rate and plant growth. The decrease in chlorophyll content under drought stress conditions typically suggests oxidative stress and could be the result of chlorophyll photooxidation and/or degradation. In addition, carotenoids in higher plants have many roles, including the regulation of light harvesting, ROS-scavenging, and preventing oxidative damage, thereby increasing plant stress tolerance. The decline in the

abundance of photosynthetic pigments could have a negative impact on the effective photosynthetic performance and crop yield.

Screening the reference collection for gas exchange rates showed a decline in CO_2 photo-assimilation and transpiration rates across most of accessions under drought conditions. However, the genotype UC82 exhibited an exceptional increase in CO_2 assimilation, suggesting an effective physiological mechanism to cope with water deficit. Other genotypes such as M82 and Red Setter performed good levels of drought tolerance, with lower decrease in transpiration rate under water scarcity. Typically, increases in leaf stomatal conductance would correspond to increased photosynthesis rates. This is because higher CO_2 concentration inside the leaf would enable more carbon fixation into photosynthetic sugars. Most of screened tomato genotypes performed according to the expected pattern, showing that as stomatal conductance decreases, photosynthesis rates also drop. However, a few genotypes maintained photo-assimilation rate at higher levels despite reduced stomatal conductance suggesting more effective strategies to tolerate drought. This could involve more efficient internal CO_2 recycling within the leaf mesophyll or differences in leaf anatomy or biochemistry that allow plants to better utilize the available resources. UC82 stood out for higher levels of Water Use Efficiency (WUE). Also, UC82 and Red Setter tomato plants maintained lower increases in leaf temperatures upon drought, suggesting that their strategies to increase WUE might also mitigate heat stress.

To delve deeper into gene expression mechanisms controlling the plant response to drought, a systemic biology approach for comparative transcriptomic profiling of leaves, stems, and fruits from Red Setter and Severianin under contrasting levels of soil water potential was undertaken.

Plant response to drought involves a complex regulatory network of multilayered and multigene interactions (Kuromori et al., 2022). Transcriptomic and proteomic approaches are promising in deciphering the mechanisms of plant stress tolerance and have been successfully applied in the study of many plant species (Parihar et al., 2019; Zhuang et al., 2014;).

To uncover the molecular underpinnings that control the assimilation, translocation and allocation of photosynthates in response to drought and outcomes yield and fruit quality, we focus on transcriptomic within photosynthesis, redox potential, carbohydrate metabolism, phytohormone responses, cell wall organization, and solute transport.

Among other processes, photosynthesis under drought stress is critically influenced by the capacity of the plant to manage and adapt to water scarcity. This physiological process is central to plant growth and survival, as it directly affects the source-to-sink dynamics that determine the distribution and allocation of assimilated carbon and other nutrients throughout the plant. Water scarcity affects the basic structural organization, which inhibits carbon assimilation and damages the photosynthetic apparatus (Golldack et al., 2011; Ali and Ashraf, 2011). Under mild to

moderate drought conditions, stomatal limitations are primarily responsible for reduced photosynthesis. As drought severity increases, non-stomatal factors become the predominant cause of reduced photosynthetic activity. This shift from stomatal to non-stomatal limitations involves changes in internal CO_2 concentration and the regulation of photosynthetic biochemical activities, ultimately leading to a decrease in photosynthetic efficiency and increased oxidative stress (Wang et al., 2018).

Our results highlighted specific transcriptomic response to drought in Red Setter and Severianin. In particular, leaves of the more tolerant Red Setter genotypes expressed upregulation of a cytochrome *b6/f* complex gene, suggesting its crucial role in maintaining electron transport between photosystem II (PSII) and photosystem I (PSI) under stress conditions. The cytochrome b6/f complex is pivotal for cyclic electron transfer and serves as a redox-sensing hub, which is essential for regulating light harvesting and gene expression in photosynthesis. The enhancement of this complex could potentially increase the photosynthetic capacity and efficiency, making it a strategic target for improving stress tolerance (Malone et al. 2021).

Additionally, Red Setter leaf and mature green fruits exhibited up-regulation of two *PsbO* genes within the PSB complexes. PSB complexes are important for maintaining the structural integrity and operational efficiency of PSII during photosynthesis. Also known as the oxygen-evolving complex protein 33 (*OEC33*), *PsbO* plays a critical role in the protection and repair mechanisms of PSII, which are vital under stress conditions. Up-regulation of *PsbO* genes suggests an enhanced capability to manage and repair PSII, ensuring sustained oxygen evolution and photosynthetic performance. This adaptive response can help the plant maintain its photosynthetic activity and survive better under adverse environmental conditions such as drought stress (Sasi et al., 2018; Gururani et al., 2012). These photosynthesis-related genes might be good candidates to target for breeding strategies to enhance tomato drought tolerance (Rodrigues et al., 2019).

In plants, drought stress can lead to excessive production of reactive oxygen species (ROS), which could damage lipids, proteins, DNA, and carbohydrates (Miller et al., 2008). Part of the tolerance response evolved by plants consists of enhancing the antioxidant defense mechanisms to effectively eliminate excess of ROS (Gill et al., 2010).

Several antioxidant genes including three ascorbate peroxidase, two glutathione S-transferases, and three thioredoxins were upregulated in Red Setter leaves, indicating that these enzymes are relevant to the response to drought stress. GSTs can catalyze the binding of glutathione nucleophile molecules to various foreign electrophilic substances, contributing to detoxification. Overexpression of the *GsGST* gene engineered from wild soy has enhanced the drought and salt tolerance in transgenic tobacco (Ji et al., 2010), while *LeGSTU2* engineered from tomato actively improved the salt and drought tolerance of *A. thaliana* (Xu et al., 2015). Ascorbate peroxidase

(*APX*) is an important antioxidant enzyme involved in the metabolism of active oxygen in plants and is the key enzyme for the elimination of H_2O_2 in chloroplasts. Previous studies have shown that *APX* activity in rice seedlings exposed to PEG was significantly higher than that in control plants (Sharma et al., 2005). Thioredoxins (*TRX*) modulate metabolic responses under stress conditions. Following similar reductions in relative water content (~50%), Trx mutants subjected to two drought cycles showed significantly higher maximum quantum efficiency (Fv/Fm) and increased drought tolerance. Trx mutant plants showed a quicker recovery after two drought cycles, as observed by greater accumulation of secondary metabolites and greater stomatal conductance (Fonseca-Pereira et al., 2018). Key genes of the ascorbate pathway were upregulated in Red Setter and Severianin fruits. Severainin fruits highlighted the upregulation of a VTC1 homologous gene. The *VTC1* gene, also known as GDP-D-mannose pyrophosphorylase, is crucial in the biosynthesis of ascorbic acid (vitamin C) in plants. *VTC1* catalyzes the conversion of mannose-1-phosphate to GDP-D-mannose, one of the key steps in the biosynthetic pathway leading to the production of ascorbic acid. Ascorbate is an important antioxidant in plants, protecting cells from oxidative damage caused by ROS, and plays a crucial role in the response to environmental stress, including water, salt, and temperature stresses. Ascorbate not only serves as a cofactor for several vital enzymatic reactions but is also involved in the regulation of growth, cell division, and differentiation. Moreover, it modulates plant responses to hormones and participates in regulating physiological processes such as photosynthesis and respiration (Wang et al., 2013; Akram et al., 2017). Manipulation of *VTC1* expression and, consequently, ascorbate pool size, is a strategy explored to improve plant tolerance to environmental stresses and to increase their nutritional content (Conklin et al., 1997, 1999; Badejo et al., 2007). In SV fruit, a strong up-regulation of a 1-Cys peroxiredoxin was detected. Peroxiredoxins are antioxidant enzymes that catalyze the reduction of alkyl hydroperoxides to alcohols and hydrogen peroxide to water. 1-Cys Prxs function to relieve mild oxidative stresses and as molecular chaperones under severe conditions during seed germination and plant development. Overoxidation controls the switch in function of 1-Cys-Prxs from peroxidases to molecular chaperones (Kim et al., 2011). The NADPH oxidase (Rboh) is a key enzyme in the process of generating ROS in plants, which are essential signaling molecules in many physiological processes, including stress response (Kaur et al., 2016) The upregulation of NADPH oxidase (*Rboh*) in the stem of Red Setter, unlike the downregulation observed in leaves and fruits, could indicate a complex stress response strategy that varies among different plant tissues. This phenomenon might suggest that while the leaves and fruits attempt to minimize damage from reactive oxygen species (ROS) by limiting their production, the stem might actively use an increase in ROS to mediate cellular signaling and adaptive responses to stress. In this way, ROS in the stem could not only serve to strengthen the

plant's support structures but also act as signals to activate defense mechanisms in other tissues, thus orchestrating a coordinated and systemic response to drought stress. This is consistent with the upregulation of the UPBEAT transcription factor, which is involved in ROS homeostasis. Managing oxidative stress is crucial as ROS levels often spike under drought conditions, potentially damaging cellular structures (Zhu et al., 2010).

Interestingly, the gene *Solyc07g056420*, classified as a tau glutathione S-transferase, was characterized in Chapter III where its overexpression in transgenic tobacco enhanced antioxidant capacity and modulated the redox pathway, particularly the glutathione-ascorbate cycle. This gene exhibited significant downregulation in the stems of Red Setter under drought conditions. The observed downregulation suggests a potential reduction in redox buffering capacity, which could be a strategic response to manage oxidative stress differently across plant tissues. This could imply that in stems, the plant might rely on alternative antioxidant mechanisms or prioritize other physiological processes over redox balance to cope with water deficit. Further investigation is required to elucidate the precise role of *Solyc07g056420* in drought response, but its differential regulation highlights its potential as a candidate gene for developing drought-tolerant varieties with improved fruit quality traits.

Drought limits the assimilation of carbohydrates by affecting photosynthesis and alters the source-to-sink transport. The reduction in carbohydrate production at the source leads to a decreased supply to the sinks. This scarcity of resources can force plants to prioritize which sinks receive the limited available sugars, often favoring essential survival functions such as root development and seed or fruit production. Moreover, plants may increase carbohydrate storage mechanisms or alter carbohydrate metabolism pathways to adapt to the stress, which includes increased conversion of sugars into osmolytes that help in maintaining cell turgor and osmotic balance.

Plant response to drought includes changes in the expression of genes involved in carbohydrate metabolism such as sucrose synthase, which plays a pivotal role in sucrose breakdown and reallocation. Similarly, invertases regulate the hydrolysis of sucrose and several transport proteins that modulate the efficiency of sugar transport from source to sink tissues. These genetic adjustments are crucial for plants to effectively manage reduced photosynthetic output and altered carbohydrate distribution (Winter and Huber, 2000). Results underlined that genes involved in sucrose and starch metabolism were centrally implicated in the plant's response to water scarcity. Red Setter leaves reduced the expression of genes involved in invertase activities, such as cell wall acid invertase and alkaline sucrose-specific invertase likely to reduce the export of sucrose. Also, up-regulation of sucrose synthase genes indicates a prioritization of sucrose synthesis and accumulation, likely for maintaining osmotic balance and providing sugars for respiration under reduced photosynthesis (Chaves et al., 2003; Du et al., 2020; Mohammadkhani & Heidari, 2008).

Consistently, down-regulation of genes involved in starch biosynthesis (e.g., ADP-glucose pyrophosphorylase) could indicate a reallocation of carbon from starch to more immediate uses such as sucrose synthesis or other stress response metabolites (Halford et al., 2010; Mohammadkhani & Heidari, 2008). Upregulation of myo-inositol oxygenase in Red Setter leaves points towards a cellular reinforcement strategy by modifying cell wall composition. The increased transcription of a starch granule initiation factor and of a phosphoglucan phosphatase which are involved in starch granule synthesis and starch mobilization, respectively, suggest a nuanced regulation of carbon allocation within the stressed plant (Roitsch & Gonzalez, 2004). Severianin leaves also underwent down-regulation of cell wall acid invertase. Moreover, the significant up-regulation of sucrose synthase along with beta amylase genes suggests active sucrose mobilization and an accelerated production of glucose and consequently, galactose, which might be part of a strategy to ensure adequate energy and metabolite flow under drought conditions (Du et al., 2020). Red Setter stems displayed down-regulation of genes involved in sucrose biosynthesis, while the increased expression of pyruvate decarboxylase and alcohol dehydrogenase suggests increased glycolytic oxidation. This might reveal an attempt to maximize energy production under restricted photosynthetic activity (Mathan et al., 2020).

The source-to-sink strength in plants responding to drought is crucially modulated phytohormones. ABA is critical for recruiting stress-related mechanisms and orchestrating adaptation of the plant physiology to drought conditions. ABA levels were exponentially elevated in Arabidopsis, wheat, rice, tomato, soy, maize, and sesame under drought conditions (Zhang et al., 2006; Dossa et al., 2019). ABA-mediated stomatal closure reduces water loss by decreasing transpiration rate. Additionally, ABA progressively increases hydraulic conductivity and stimulates root cell elongation, allowing plants to recover from water-limited conditions (Daszkowska-Golec et al., 2016). Several studies have demonstrated the fundamental role of ABA in regulating the expression of genes, proteins, and enzymatic activities involved in plant cell dehydration (Chen et al., 2020; Yu et al., 2020).

Ethylene regulates critical plant growth and development processes, as well as the responses to abiotic stresses. This hormone is known for its ability to mediate fruit ripening, leaf senescence, and responses to stress conditions such as drought and salinity. Under drought conditions, ethylene modulates stomatal closure, contributing to reduced water loss through transpiration, a vital mechanism for plant survival in arid environments (Fatma et al., 2022). Recent research has demonstrated that ethylene interacts with other hormonal pathways, such as abscisic acid (ABA), to coordinate a comprehensive response to water stress (Salazar et al., 2015). Our results highlighted a different pattern of regulation of genes related to hormonal response to drought in the sensitive Severianin and in the more tolerant Red Setter plants. Red Setter leaves showed

upregulation of ABA synthesis and perception genes, suggesting an intensification of the ABA response to enhance water stress tolerance through mechanisms such as stomatal closure and ABA accumulation (Li et al., 2017). Previous studies have also shown that ABA could improve the plant's ability to eliminate ROS by activating antioxidant enzymes (Zhou et al., 2019). Red Setter leaves also exhibited reduced expression of ethylene biosynthetic genes and increased expression of genes involved in the sensing pathway. This might indicate an adaptation to moderate growth and promote senescence in response to stress (Iqbal et al., 2017). By contrast, Severianin leaves down-regulated ABA receptor genes, suggesting a reduced perception of ABA and a decreased ability to respond to water stress (Zhu et al., 2002). Red Setter fruits expressed downregulation of genes involved in the synthesis of ABA and ethylene, in Severianin fruits showed upregulation of genes controlling the synthesis of both phytohormones (NCED and *ACC* oxidase). This could indicate a different pattern of fruit development and ripening response to stress, with likely impact on fruit quality traits (Ebrahimi et al., 2022).

The primary cell wall is highly hydrated (60% to 80% water) (Frary et a., 2015). Drought affects the water potential of the cell, and the cell wall counterbalances physical stress brought about by variations in turgor pressure by inducing changes in the structure and composition of the cell wall components. The plant cell wall can be mechanically more flexible or rigid to control tissue growth (Wu et al., 2000; Moore et al., 2008). However, plants use multiple strategies to counterbalance drought stress through morphological and physiological changes, acting through various signaling cascades that lead to osmotic adjustments (Ezquer et al., 2020). Additionally, the modified mechanical properties and composition of the cell wall affect the internal balance of metabolic processes (such as carbon source/sink balances). Both Red Setter and Severianin exhibit transcriptional variations in cellulose synthase and cellulose modification genes, which is consistent with the findings that cellulose microfibrils are crucial in maintaining plant biomass and structural integrity under stress (Rao et al., 2017). Cellulose synthase genes are activated through complex signaling pathways, including brassinosteroids which impact *CesA* gene expression (Xie et al., 2011). This suggests a strategic enhancement of structural biomass to counteract the physical stresses induced by drought, where turgor pressure and cellular integrity could be compromised. The marked upregulation of expansin genes observed on Red Setter and Severianin leaves could reflect the role of expansins in facilitating cell wall loosening, allowing for cell expansion and adaptive reconfiguration under drought stress (Marowa et al., 2016). This mirrors observations in other studies where overexpression of expansin genes led to enhanced drought tolerance in Arabidopsis and other species (Liu et al., 2019), highlighting their role in modulating cell wall plasticity to maintain growth and development under water-limited conditions. Several pectin-related genes have shown changes in the expression upon drought,

including those involved in biosynthesis and degradation, indicating active remodeling of the cell wall which is essential for maintaining water content and cell integrity under drought (An et al., 2008). The modulation of pectin structure can facilitate the formation of hydrogel capsules that protect cells from damage, as noted in more drought-tolerant wheat cultivars (Leucci et al., 2008). The activity in genes related to xyloglucan and hemicellulose modification supports the role of these components in adjusting cell wall extensibility and strength (Campbell et al., 1999). Such modifications are critical in ensuring the cell wall's mechanical adaptability to changing environmental conditions, allowing for continued growth and stress mitigation. Upregulation in genes involved in cutin and suberin biosynthesis occurring particularly in Severianin, suggests an enhancement of the plant's external barriers to increase drought response (Yang et al., 2019). This is agreement with the hypothesis that cell wall functions also as protective physical barriers to minimize water loss and maximize resistance to external biotic and abiotic stresses. Strong upregulation of laccase genes involved in lignin polymerization in Red Setter points to an increased lignin content, which can fortify the cell wall and contribute to overall stress resilience. Our study highlighted the importance of sugar transporters and *ABCG* transporters, with a particular focus on the regulatory mechanisms underlying source-to-sink strength.

Drought stress significantly impacts plant metabolism, particularly influencing the accumulation and distribution of sugars, which reset the source-sink relationships and affect carbon partitioning. This adjustment is crucial as plants strive to maintain cellular processes under environmental stress. Notably, disruptions in sugar transport impact how sugars are distributed between source and sink organs, which is vital for managing energy demands during stress (Lemoine et al., 2013; Williams et al., 2000).

The alteration of carbon assimilation and sugar distribution is a responsive mechanism to drought stress, where the source-sink dynamics are heavily regulated by diverse transporters. A decline in source strength (carbon assimilation and export) due to stress impacts sink strength (sugar import and use), underlining the interdependence within the plant (Muller et al., 2011).

Soluble sugar content tends to increase during drought, aiding in maintaining osmotic homeostasis and scavenging reactive oxygen species (ROS) (Kaur et al., 2021).

ATP-binding cassette (*ABC*) transporters are primary membrane-associated transporters that form a large and ubiquitous family playing important roles in plant physiological development (Theodoulou et al., 2015), including organ growth and development, nutrient acquisition, response to abiotic and biotic stress, disease resistance, and plant-environment interactions (Aryal et al., 2015; Do et al., 2018). Compared to other eukaryotes, plants possess more abundant *ABC* transporters, particularly the *ABCG* subgroup (Dhara et al., 2021).

ABCG transporters are involved in different processes in many plant species, including pathogen response, formation of diffusion barriers, abiotic stress response, and transport of lipids and phytohormones (Jarzyniak et al., 2020). Several *ABCGs* have been identified as ABA transporters required for the long-distance translocation of ABA (Zhang et al., 2021; Kuromori et al., 2010; Kang et al., 2015).

In Red Setter leaves and fruits, we observed a downregulation of several genes for *ABCG* transporters, which could indicate a reduction in the activity of exporting ABA and other lipophilic compounds, affecting membrane permeability and hormonal response signaling (Do et al., 2018). Also, several sugar transporters were downregulated within Red Setter leaves, stems and fruit and in Severianin fruits possibly reflecting stress response strategies to limit the removal of energetic resources and increase sugar conservation in leaves, a potentially advantageous mechanism under drought conditions. This could be associated with a decrease in sugar export from the leaves, which ultimately results in growth inhibition as the plant adopts a strategy to withstand water stress conditions (Xue et al., 2016). Furthermore, this downregulation could be strategic, as it helps to retain sugars within the vacuoles to maintain osmotic potential and turgidity under stress conditions (Eom et al., 2011; Leach et al., 2017).

The Figure 83 represents a transcriptomic model illustrating the changes occurring in the drought-tolerant Red Setter variety. This model highlighs how Red Setter maintains higher photosynthetic efficiency under stress conditions leading to higher source-to-sink strength and increased fruit antioxidant activity.

A crucial aspect of this mechanism is the upregulation of glutathione S-transferases (*GSTs*), particularly Phi (*Solyc12g094430*) and Lambda (*Solyc10g084400*). This upregulation promotes the activation of 2-Cys peroxiredoxin (*2-CysPrx*), underscoring their pivotal role in maintaining redox homeostasis.

2-CysPrx, with thioredoxins (*TRXs*), are integral to the electron transport chain (ETC) during photosynthesis. Among the various *TRXs*, *TRXm* plays a significant role in modulating the photosynthetic apparatus. *2-CysPrx* and the *TRXm*, which are upregulated in Red Setter, interact with key components of the ETC, ensuring the proper flow of electrons and maintaining photosynthetic efficiency under stress conditions.

Another key component in this model is the NADPH-dependent chloroplast thioredoxin reductase (*NTRC*), that enhances the function of the NDH complex. This complex facilitates the transport of protons into the thylakoid lumen, generating a proton gradient that drives ATP synthase activity. The resultant increase in ATP production is essential for energy-demanding processes, particularly under drought stress. Moreover, *NTRC* stimulates the SOQ1 factor, inhibiting non-photochemical

quenching (NPQ) and thus preventing the dissipation of excess energy as heat, which further enhances photosynthetic efficiency (Nikkanen and Rintamäki, 2019).

Furthermore, NTRC enhances the Calvin-Benson-Bassham (CBB) cycle, promoting carbon fixation. This enhancement leads to increased production of photoassimilates, which is vital for maintaining growth and productivity under drought conditions (Nikkanen and Rintamäki, 2019).

The upregulation of Lambda and Phi *GSTs* leads to the downregulation of the other Tau *GSTs*. This redirection ensures that GSH is concentrated on essential enzymes involved in photosynthesis and carbon fixation, particularly in the Calvin cycle. This enhances photosynthetic efficiency and carbon assimilation. Additionally, the increased GSH demand triggers higher activity of ascorbate peroxidase, glutathione peroxidase, and glutathione reductase to manage excess reactive oxygen species (ROS), ensuring balanced oxidative stress management and sustained photoassimilation.

Moving on to the stem, we observed that increased photoassimilate production results in the overexpression of sucrose synthase (*SUS*). This enzyme is crucial as it catalyzes the conversion of sucrose and *UDP* (uridine diphosphate) into *UDP*-glucose and fructose. The activity of sucrose synthase is vital for regulating the levels of sucrose within the plant, facilitating its breakdown and mobilization for energy and carbon supply.

Under drought conditions, the overexpression of sucrose synthase in Red Setter ensures that photoassimilates, primarily carbohydrates, are efficiently converted and mobilized. This process predominantly involves the breakdown of sucrose into simpler sugars that are easily transported through the phloem to sink tissues, such as growing roots, fruits, and seeds. The high concentration of sucrose in the symplast creates a gradient that facilitates this efficient transport.

Moreover, there is a down-regulation of the auxin transporter in Red Setter. Auxin is a plant hormone that regulates various aspects of growth and development, including the allocation of carbohydrates. The reduced expression of the auxin transporter limits the movement of carbohydrates towards the apices. As a result, Red Setter exhibits reduced vegetative growth under stress conditions compared to other plants. This strategic allocation of resources ensures that energy is conserved and directed towards essential processes, enhancing the plant's overall stress tolerance and efficiency in utilizing available resources.

This shift in resource allocation is particularly beneficial under drought conditions, where the plant needs to optimize its energy use. By prioritizing the transport of sucrose to critical sink tissues and limiting unnecessary vegetative growth, Red Setter maintains better growth and productivity even under adverse conditions

In the fruits of Red Setter, the upregulation of cell wall invertases plays a pivotal role in enhancing sucrose unloading, a process crucial for fruit development. Cell wall invertases hydrolyze sucrose

into glucose and fructose, facilitating their uptake and utilization by fruit tissues. This efficient conversion and transport of sugars are essential for the growth and ripening of fruits, ensuring that developing fruits receive an adequate supply of energy and carbon.

Moreover, there is an increased expression of enzymes involved in glycolysis and glucuronate interconversion. These enzymes support metabolic flexibility by allowing the plant to efficiently convert glucose into pyruvate and other intermediates, which can be used for energy production and biosynthetic processes. This flexibility is particularly important under stress conditions, as it helps the plant manage its energy resources more effectively.

Additionally, genes involved in cell wall biosynthesis and reorganization are upregulated in the fruits. These genes encode for components such as hemicellulose and pectins, which are critical for maintaining cell wall structure and function. Upregulation of these genes ensures that the cell walls remain robust and flexible, maintaining cell turgor and structural integrity. This is crucial for sustaining fruit growth and development, particularly under drought conditions, where maintaining water balance and structural stability is essential. Our results add new insights into strategies that plants evolved to manage resource allocation and maintain fruit quality in adverse conditions. The exploration of transcriptional mechanisms within specific pathways allowed the identification of several candidate genes to be targets for enhancing fruit quality in more efficient crops by using natural water resources. In particular, our results allow for designing new knowledge-informed breeding strategies based on conventional or biotechnology-assisted approaches for developing new crops for more resilient agro-ecosystems.

Differentially expressed genes are still to be validated by an independent technology and TaqMan qRT-PCR experiments are currently in progress for selected transcripts. This will support the accuracy of our differential expression findings.

This study highlights the potential of integrating genetic, transcriptomic, and eco-physiological data to innovate and improve plant varieties, thereby establishing a solid groundwork for future advancements in plant science and agricultural biotechnology.

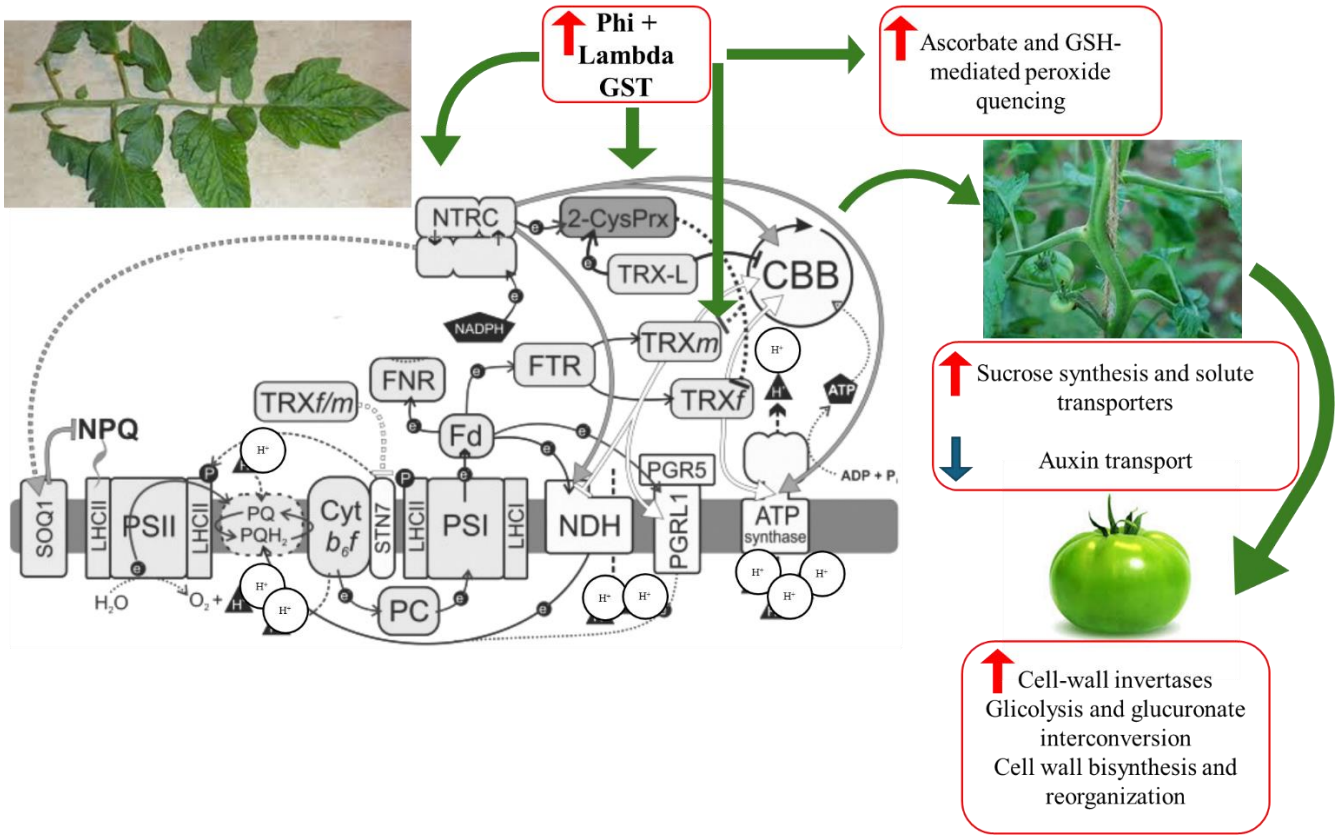

Figure 83. Regulative model illustrating mechanisms involved in the tolerant response to drought. Upregulation of a lambda and a phi gst genesin leaves, downregulate GST genes, reduce competition for GSH and increase the expression of other GST family member like glutathione peroxidase, ascorbate peroxidase and dehydroascorbate reductase that contributes ROS detoxification. NTRC activates NDH complex, ATP synthase, and CBB cycle, which helps to balance the redox poise between light and carbon fixation reactions. NTRC may also mediate the SOQ1-dependent down-regulation of NPQ to prevent heat dissipation under low light. Fd-TRX system keeps the redox-regulated photosynthetic enzymes active under moderate and higher light intensities. In relation to NTRC, TRXm has an antagonist role in the regulation of the electron flow in the thylakoid membrane. 2-CysPRXs are involved in the oxidation of chloroplast TRX systems. Dotted arrows represent hypothetical or potentially indirect effect on photosynthetic proteins. This is accomplished by the increased expression of a sucrose synthase gene in petioles and by the upregulation of genes involved in sucrose download and cell wall biosynthesis and remodeling in fruits inducing higher increase in antioxidant activity.

4.5 Supplementary

Table S7, List of names, individual codes, and sources of seed accessions used for drought stress screening. The seeds were obtained from the C.M. Rick Tomato Genetics Resource Center (TGRC), University of Naples Federico II (UNINA), the Campania Germplasm Bank, and the Comunità Montana Alta Irpinia.

Accession name	Accession individual code	Seed source
Ace	LA0516	TGRC
Arancione determinato	cod-29	UNINA
Ayacucho	LA0134C	TGRC
B-L-35	LA4347	TGRC
Canestrino	cod-99	UNINA
Cannellino flegreo	cod-F	Campania Germplasm Bank
Cento scocche	cod-S	Campania Germplasm Bank
Chiclayo	LA0395	TGRC
Costoluto genovese	cod-53	UNINA
Costoluto genovese	cod-100	UNINA
Cuor di bue	TS-170	TGRC
Earliana	LA3238	TGRC
Early Santa Clara	LA0517	TGRC
Fiaschello Battipagliese	cod-127	UNINA
Fiaschetto di Torre Guaceto	cod-87	UNINA
Flora-Dade	LA3242	TGRC
Florida 7060	LA3840	TGRC
Florida 7547	LA4025	TGRC
Giallo determinato (da serbo)	cod-34	UNINA
Guardiolo	cod-G	Campania Germplasm Bank
Guayaquil	LA0410	TGRC
H5	LA2375	TGRC
Hacienda Calera	LA0113	TGRC
Hacienda Rosario	LA0466	TGRC
Hawaii 7998	LA3856	TGRC
Heinz 1706-BG	LA4345	TGRC
Hotset	LA3320	TGRC
Huachinango	LA1459	TGRC
Hunt100	LA3144	TGRC
ISCI - BATTIPAGLIA - Sel. PC 09	cod-43	UNINA
ISCI - BATTIPAGLIA - Sel. PC 14	cod-48	UNINA
italian canner	TS-190	TGRC
King Humbert #1	LA0025	TGRC
Ladino di Panocchia	cod-102	UNINA
M-82	LA3475	TGRC
Malintka 101	LA3120	TGRC
Merida	LA1462	TGRC

Mexico City	LA0146	TGRC
Micro-Tom	LA3911	TGRC
Nasone del Cavallino - Sel. To 1-13	cod-117	UNINA
NC 84173	LA4354	TGRC
NC HS-1	LA3847	TGRC
pantano romanesco	cod-17E	UNINA
Pera di Albenga sel. Riviera	cod-77	UNINA
Peto95-43	LA3528	TGRC
Piennolo Pollena	cod-PP	Campania Germplasm Bank
Piennolo rosso	cod-PR	Campania Germplasm Bank
Piennolo vesuviano	cod-PV	Campania Germplasm Bank
Pisanello	cod-81	UNINA
Pizzutello "La Piana"	cod-114	UNINA
Platense	LA3243	TGRC
Pomodoro Costoluto Rosso di Rotonda	cod-68	UNINA
Pomodoro di Belmonte - PBL02	cod-71	UNINA
Pomodoro di Sorrento - PS01	cod-54	UNINA
Pomodorino giallo	cod-PG	Campania Germplasm Bank
Pomodoro giallo Blocky-pepper	cod-94	UNINA
Pomodoro Pera d'Abruzzo - PPA02	cod-66	UNINA
Pomodorino Reginella	cod-R	Campania Germplasm Bank
Prince Borghese	LA0089	TGRC
Red Setter	TS-139	TGRC
Rio Grande	LA3343	TGRC
Rosa cilentano biloculare	cod-86	UNINA
Rowpac	LA3214	TGRC
Rutgers	LA1090	TGRC
Saladette	LA2662	TGRC
San marzano	LA3008	TGRC
San Marzano 20 SMEC	cod-20	Campania Germplasm Bank
San Marzano 24 SMEC	cod-24	Campania Germplasm Bank
scatolone di Bolsena	cod-28E	UNINA
Seccagno	cod-X	Comunità montana Alta Irpinia
Severianin	LA2413	TGRC
T-5	LA2399	TGRC
TS-256	LA2260	TGRC
UC-82	LA1706	TGRC
Vesuviano	cod-V	Campania Germplasm Bank

Table S8. Fold Change in response to drought of differentially expressed genes (DEGs) mapped to the photosynthesis pathway across leaf, stem, and fruit of two tomato varieties, Red Setter and Severianin. The fold change values illustrate the difference in gene expression between control and drought-stressed treatments.

Photosintesys patwhay											
	Leaf				**Stem**				**Fruit**			
Red setter	**Log2FC**	**Severianin**	**Log2FC**	**Red setter**	**Log2FC**	**Severianin**	**Log2FC**	**Red Setter**	**Log2FC**	**Severianin**	**Log2FC**
Solyc09g014520	1.39697738	Solyc09g011080	1.015634	solyc04g071930	1.3929567	solyc00g500063	1.375024	solyc01g028810	0.49908832	solyc07g017950	4.155767
Solyc06g063370	1.26988792			solyc01g097910	-1.1071578			solyc07g042250	0.93081653	solyc11g069790	1.16572
Solyc01g105030	1.22170578			solyc07g066310	-0.9024711			solyc01g088610	0.6186434	solyc12g044250	2.149252
Solyc01g105050	1.70096679			solyc02g085950	-1.7805635			solyc09g011080	0.95956725	solyc10g007290	1.07127
Solyc02g065400	1.16529143			solyc02g085940	-2.2808628						
Solyc02g090030	1.12537976			solyc03g034220	-1.2904968						
Solyc07g044860	1.06639556			solyc11g065740	-1.2560605						
Solyc02g079950	1.49505883			solyc05g013150	-0.8386008						
Solyc07g066310	0.82444621			solyc10g086580	-1.7773205						
Solyc01g109040	1.23304855			solyc03g111610	-1.2748855						
				solyc11g009020	-1.9358732						

Table S9. Fold Change in response to drought of differentially expressed genes (DEGs) mapped to the carbohydrate metabolism pathway across leaf, stem, and fruit of two tomato varieties, Red Setter and Severianin. The fold change values illustrate the difference in gene expression between control and drought-stressed treatments.

Carbohydrate metabolism patwhay											
	Leaf				**Stem**				**Fruit**			
Red setter	**Log2FC**	**Severianin**	**Log2FC**	**Red setter**	**Log2FC**	**Severianin**	**Log2FC**	**Red Setter**	**Log2FC**	**Severianin**	**Log2FC**
Solyc10g083290	-2.37418731	Solyc10g083290	-1.923916	solyc07g065900	-2.2805164	solyc02g066950	0.8056779	solyc09g010080	2.1662166	solyc03g083910	-4.666631
Solyc04g081440	-0.84398122	Solyc09g098590	1.4172947	solyc04g071340	-2.073151			solyc10g083290	-2.8722475	solyc01g109790	2.1369274
Solyc07g042520	4.380875205	Solyc06g062430	1.2565958	solyc10g083290	-1.5629971			solyc12g009300	0.8622901	solyc03g096730	0.8939395
Solyc09g098590	2.036982011	Solyc08g007130	-1.582668	solyc12g009300	1.8960259						
Solyc12g008650	-3.64302564	Solyc08g077530	-1.074681	solyc10g076510	2.1089787						
Solyc09g008260	0.744768503	Solyc02g086530	2.0045291	solyc06g059740	1.3494873						
Solyc11g007830	0.707077416			solyc01g110360	-1.5510992						
Solyc12g062250	0.959201706			solyc02g084440	-0.7979903						
Solyc03g112500	8.639326943			solyc10g054870	-2.2886965						

Table S10. Fold Change in response to drought of differentially expressed genes (DEGs) mapped to the REDOX homeostasis pathway across leaf, stem, and fruit of two tomato varieties, Red Setter and Severianin. The fold change values illustrate the difference in gene expression between control and drought-stressed treatments.

REDOX homeostasis patwhay											
	Leaf			Stem				Fruit			
Red setter	Log2FC	Severianin	Log2FC	Red setter	Log2FC	Severianin	Log2FC	Red Setter	Log2FC	Severianin	Log2FC
Solyc12g094430	0.634380613	Solyc06g048410	0.9929172	solyc02g082760	-1.6552984	solyc08g014000	1.238011	solyc06g048410	1.0375196	solyc01g081310	-1.050634
Solyc10g084400	1.319851715	Solyc12g096320	1.1511878	solyc01g099620	3.270662			solyc01g081310	-1.0557123	solyc09g011560	-2.148959
Solyc07g042440	1.412244302	Solyc05g006750	-1.258222					solyc01g086680	-5.215668	solyc09g011590	-4.519159
Solyc01g079820	0.543240615	Solyc09g011490	-0.995175					solyc06g069040	-3.260402	solyc09g011600	-0.810771
Solyc06g005150	0.920463949	Solyc09g011560	-0.915368					solyc09g011520	-6.8520346	solyc03g096040	7.629849
Solyc06g005160	0.878752878	Solyc12g019740	1.3396415					solyc09g011590	-4.7954903	solyc05g009950	0.8451088
Solyc09g007270	2.898744155	Solyc09g007270	1.809184					solyc09g011600	-1.2744874	solyc03g096730	0.8939395
								solyc09g011630	-7.154637	solyc04g054690	-1.834436
								solyc10g084960	-2.5155933		
								solyc10g084400	-1.629195		
								solyc08g008670	1.0109557		
								solyc03g117980	-1.1380407		
								solyc01g102700	-1.2036474		
								solyc01g099210	-1.9625357		

Table S11. Fold Change in response to drought of differentially expressed genes (DEGs) mapped to the phytohormone response pathway across leaf, stem, and fruit of two tomato varieties, Red Setter and Severianin. The fold change values illustrate the difference in gene expression between control and drought-stressed treatments.

Phytohormone response patwhay											
Leaf				**Stem**				**Fruit**			
Red setter	**Log2FC**	**Severianin**	**Log2FC**	**Red setter**	**Log2FC**	**Severianin**	**Log2FC**	**Red Setter**	**Log2FC**	**Severianin**	**Log2FC**
solyc04g071960	1.9600974	solyc06g050500	-2.548388	solyc06g061180	-0.7450229	solyc10g076410	-1.7251595	solyc07g056570	-1.1671394	solyc08g016720	2.7983344
solyc01g095700	0.8273628	solyc10g076410	-2.242628	solyc11g013310	1.2889004	solyc10g085310	-1.3115215	solyc06g068940	-2.216391	solyc06g050500	-1.152261
solyc01g108210	2.431741	solyc10g085310	-1.928714	solyc12g095750	-1.7994398	solyc02g091520	-0.93821466	solyc04g007500	-0.85000515	solyc09g015380	-3.287383
solyc08g061560	1.0500922	solyc07g040990	0.9517903	solyc12g008900	-6.694672	solyc12g040800	-1.4977818	solyc04g074080	0.91728896	solyc12g040800	-0.896936
solyc07g049530	-1.4512855	solyc02g093890	3.262083	solyc02g036350	-1.7595645	solyc11g006040	1.9424592	solyc07g049530	-1.914199	solyc04g015750	0.7471243
solyc09g089610	1.6496093	solyc09g089610	2.7884324	solyc03g119910	-2.0530396	solyc12g017530	-1.8380923	solyc12g005940	-2.091899	solyc04g074080	1.2905835
solyc11g006180	1.2086937	solyc11g006180	1.6636764					solyc09g089610	0.89840996	solyc06g066020	2.925645
solyc01g096810	-0.6811493	solyc02g036350	-1.24332					solyc10g079690	1.7551019	solyc06g084070	6.296524
		solyc07g049550	-1.623017					solyc03g122340	-1.151142	solyc07g008020	4.737675
										solyc09g083280	2.6010494
										solyc06g074070	0.872758
										solyc01g068410	4.0810165
										solyc06g059730	6.9266787
										solyc12g006690	1.6466628
										solyc01g095080	-2.201263
										solyc02g081190	-0.821113
										solyc07g026650	3.2529933
										solyc09g089610	2.9306774
										solyc10g079690	6.6646457
										solyc12g017530	3.3379483
										solyc12g096570	4.1660523
										solyc01g006540	-7.488668

Table S12. Fold Change in response to drought of differentially expressed genes (DEGs) mapped to the Cell Wall organization pathway across leaf, stem, and fruit of two tomato varieties, Red Setter and Severianin. The fold change values illustrate the difference in gene expression between control and drought-stressed treatments.

Cell wall organization patwhay												
	Leaf			Stem				Fruit			
Red setter	Log2FC	Severianin	Log2FC	Red setter	Log2FC	Severianin	Log2FC	Red Setter	Log2FC	Severianin	Log2FC
solyc03g070440	3.9405594	solyc01g102580	-0.689531	solyc09g075350	2.788773	solyc08g076320	0.7125731	solyc02g072240	2.395461	solyc04g071650	1.5346209
solyc12g055970	1.6635088	solyc12g098810	0.9142343	solyc12g008530	2.295382	solyc07g052320	0.83877516	solyc04g071650	1.0345522	solyc09g072820	3.2445881
solyc12g088240	2.1916757	solyc12g088240	1.0893954	solyc12g089250	4.9480505	solyc01g091050	2.2905781	solyc09g072820	2.4285245	solyc05g005080	0.712181
solyc03g115830	1.868779	solyc03g031840	0.8145925	solyc01g104950	1.7009355	solyc06g069380	1.5396098	solyc04g078990	7.570816	solyc03g070440	4.638271
solyc09g008320	2.0401363	solyc01g112000	-1.317076	solyc06g062580	2.2917788	solyc01g006350	0.8882191	solyc11g005760	5.6063156	solyc09g010210	2.123921
solyc02g065530	1.9076277	solyc08g077900	5.0038104	solyc05g005170	4.1715927	solyc01g006370	1.5339134	solyc09g007420	7.65733	solyc11g040340	3.112881
solyc04g078990	7.2863636	solyc03g065250	0.9044318	solyc05g007830	1.5267239	solyc01g073750	0.8461523	solyc06g068770	1.4132482	solyc04g076650	7.001537
solyc06g034370	-2.4381347	solyc03g117800	0.8965041	solyc06g005560	3.1402807	solyc03g111570	0.8791268	solyc11g011300	3.7117345	solyc11g011300	2.8903592
solyc11g019910	1.5845472			solyc06g051800	1.7070621	solyc07g053980	0.8870302	solyc10g077080	6.501824	solyc04g072850	1.8101221
solyc06g069380	0.8451344			solyc03g083900	1.7502656	solyc07g056260	0.97066575	solyc02g093230	-1.9529905	solyc05g052390	5.4759474
solyc10g005200	1.3324097			solyc08g079090	1.8849553	solyc08g076320	0.7125731	solyc02g093250	-1.6547658	solyc09g010995	7.0999856
solyc09g007660	3.4762032			solyc12g057140	1.1419019	solyc07g052320	0.83877516	solyc06g068440	-1.41823	solyc12g009490	4.130244
solyc11g069250	4.345093			solyc01g105010	1.7100198	solyc01g102350	1.2173827	solyc09g014240	3.699445	solyc05g018510	1.487857
solyc05g014000	4.5647883			solyc02g092790	0.9003050	solyc04g005430	1.1744556	solyc10g085090	7.439222	solyc06g065670	1.4373672
solyc06g049050	2.50924			solyc04g063210	1.222326			solyc06g074390	-1.3901525	solyc08g083190	2.1326065
solyc10g076830	8.437427			solyc03g116910	-1.5218334			solyc12g100270	-5.101333	solyc08g081220	3.8468077
solyc10g085090	3.9993012			solyc06g074390	-1.3556595			solyc03g117800	-0.8892841	solyc05g055400	2.02921
solyc05g052340	3.8245857			solyc03g117800	-1.1864648			solyc02g080330	-5.800581	solyc11g007540	4.002657
solyc05g052400	6.5085645			solyc10g080840	-0.9917351						
solyc09g014240	3.4792395			solyc03g019760	-1.0633129						
solyc06g076330	4.0999146										

Table S13. Fold Change in response to drought of differentially expressed genes (DEGs) mapped to the solute transport pathway across leaf, stem, and fruit of two tomato varieties, Red Setter and Severianin. The fold change values illustrate the difference in gene expression between control and drought-stressed treatments.

Solute transport patwhay											
Leaf				Stem				Fruit			
Red setter	Log2FC	Severianin	Log2FC	Red setter	Log2FC	Severianin	Log2FC	Red Setter	Log2FC	Severianin	Log2FC
solyc05g014390	-2.215087	solyc07g020790	0.7068127	solyc08g076720	-1.2979252	solyc07g065320	1.3393153	solyc01g096190	-1.6436698	solyc05g018510	1.487857
solyc04g015970	-0.83486366	solyc01g098490	1.9675934	solyc12g044820	0.9950079	solyc09g064440	0.91318536	solyc02g064680	-1.2438676	solyc05g056470	8.066169
solyc06g070960	-2.186643			solyc01g105450	-1.4171934	solyc06g066600	1.2609895	solyc04g077870	-0.68540657	solyc06g065670	1.4373672
solyc01g105450	-1.4905679			solyc03g007690	2.7636325			solyc07g017780	-0.7202476	solyc03g032040	-0.978225
solyc08g067610	-1.4197665			solyc03g019760	-1.0633129			solyc05g010120	-0.9255295	solyc05g018230	-1.068696
solyc09g091660	-1.213025			solyc05g055330	-0.943782			solyc06g062780	-0.9066708	solyc02g062860	-6.900272
solyc09g091670	-2.5713751			solyc03g093400	-0.8184083			solyc05g053600	-2.45548	solyc01g068410	4.0810165
solyc09g074230	-1.0355896			solyc01g109460	-0.8801879			solyc05g053610	-3.2228458	solyc06g059730	6.9266787
solyc09g075820	-1.1443093			solyc11g017010	-0.9160845			solyc08g067610	-3.7030177		
solyc12g010690	-0.8728924							solyc09g091670	-4.8592167		
								solyc08g079790	0.88213843		
								solyc12g089180	-0.75232226		
								solyc02g079220	-2.4158335		
								solyc09g075820	-1.6020678		

CHAPTER V

CONCLUSIONS

Tomato (*Solanum lycopersicum*) stands as a globally significant horticultural crop not only for its extensive use in various cuisines around the world but also for its nutritional value, offering vital vitamins, minerals, and antioxidants. It also serves as a key model organism in plant genetic studies due to its well-documented genomic resources and relatively short growth cycle. However, the cultivation of tomatoes is increasingly challenged by climate change, which brings about more frequent and severe drought conditions, potentially compromising both yield and fruit quality.
My PhD, carried out at UNINA (University of Naples Federico II) in collaboration with the Mountain Community and North Carolina State University, focused on an in-depth analysis of stress response mechanisms and fruit quality in tomato, particularly examining the gene family of glutathione S-transferase (gst) genes and the complex source-to-sink dynamics under drought conditions.
For this purpose, three main works were carried out: 1) Comprehensive bioinformatics characterization of the glutathione S-transferase (GST) loci in *S. lycopersicum* and its wild relatives, *Solanum pennellii, Solanum lycopersioides,* and *Solanum pimpinellifolium*; 2) Functional characterization of the tau Glutathione S-Transferase (*Solyc075056420*) in response to drought and salinity stresses; 3) Selection and detailed evaluation of a core collection of *S. lycopersicum* under different irrigation regimes to assess drought resilience and investigate source-to-sink dynamics through eco-physiological and transcriptomic analyses in selected tomato accessions with contrasting phenotypes.

The main results are summarized in the following points:

- An exhaustive bioinformatics analysis of the glutathione S-transferase (gst) genes in the *Solanum lycopersicum* was carried out according to the latest ITAG 4.1 genome annotation. This detailed survey was expanded to include the genomes of wild tomato relatives *Solanum pennellii, Solanum lycopersioides*, and *Solanum pimpinellifolium*, uncovering a rich diversity in gene domains, structural motifs, and phylogenetic connections. Our investigation utilized polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) to verify and compare the annotations of the Glutathione S-transferase (GST) genes in *Solanum lycopersicum* between ITAG 4.1 and ITAG 2.4. This approach underscores

the importance of continuous updates and experimental validations in ensuring the accuracy and reliability of genomic annotation.

- An exploration of publicly available RNA-seq data from *Solanum lycopersicum* was carried out. This analysis successfully identified specific gst genes (*Solyc06g009020, Solyc06g011280, Solyc01g102660, Solyc05g054760, Solyc07g056420, Solyc07g056490, Solyc10g084400, Solyc05g056490 and Solyc11g028100*) that exhibit enhanced expression to various stress conditions in distinct tissues such as leaves, fruits, and roots. These genes have emerged as candidates for further investigation due to their potential role in the plant's response to environmental stimuli and fruit ripening.

- Analysis of SNP data within the gst gene coding sequences (CDS) was carried out, identifying a total of 2179 SNPs. Notably, 15 SNPs were significantly associated with the phenotypic traits related to drought tolerance in tomatoes.

- The heterologous expression of the tau glutathione S-transferase (gst) gene from *Solanum lycopersicum* in *Nicotiana tabacum* was successfully facilitated using *Agrobacterium tumefaciens* strain LBA4404, with the coding sequence under the control of the 35S2 promoter. This genetic transformation enabled the detailed eco-physiological and molecular characterization of T2 progeny under environmental stress conditions, providing valuable insights into the gene function.

- Transgenic tobacco expressing the tau glutathione S-transferase (gst) gene from *Solanum lycopersicum* exhibited enhanced tolerance to salt and drought stresses. Under salt stress, these plants adjusted their antioxidant system, notably enhancing glutathione reductase activity while reducing ascorbate peroxidase and monodehydroascorbate reductase expression. This adjustment aids in managing oxidative stress induced by salinity. Similarly, upon drought conditions, gst expression improved RWC and increased flavonoid levels, enhancing antioxidant capacity.

- A non-redundant reference *S. lycopersicum* core collection of worldwide accessions was selected from a global collection using genome-wide SNP data. Two water stress experiments were conducted to identify tomato varieties more sensitive and more tolerant to drought. Detailed eco-physiological assessments were taken, including evapotranspiration, CO_2 assimilation, water use efficiency, leaf colorimetric parameters, and electrolyte

leakage. These analyses identified more tolerant varieties such as "Red Setter" and "UC82" and the more sensitive ones such as "Severianin", "Florida 7060" and "Saladette".

- RNA-seq analyses were performed on leaves, stems, and fruits to compare gene expression between the control condition and the drought stress treatment in Red Setter and Severianin. In the leaf tissues of the Red Setter genotype, significant changes in the expression were observed for 948 genes up-regulated and 582 down-regulated. In the fruits, 231 genes were up-regulated and 621 down-regulated, while in the stems, 312 genes were up-regulated and 354 down-regulated. For the Severianin genotype, the leaf tissue showed 178 genes both up-regulated and down-regulated, the fruits responded with 481 up-regulated and 331 down-regulated genes, and the stems displayed 223 up-regulated and 383 down-regulated genes.

- Examination of DEGs for critical pathways involved in plant response to drought highlighted several key genes. These genes play roles in vital processes such as photosynthesis, carbohydrate metabolism, redox homeostasis, phytohormonal responses, cell wall organization, and solute transport. Understanding these adaptations, which facilitate the efficient allocation of resources from source tissues (like leaves) to sink tissues (such as fruits), is crucial for designing innovative strategies for breeding. This knowledge sheds light on how plants maintain fruit quality and optimize resource use under stress and candidate key genes as potential targets for genetic engineering. Currently, breeding programs are in progress in order to develop new tomato genotypes with enhanced plant tolerance to environmental challenges and higher quality under adverse conditions.

The findings from this PhD research open several cues for future investigation and potential applications in tomato breeding and agricultural practices. The functional characterization of the glutathione S-transferase (gst) gene family in *Solanum lycopersicum* and its wild relatives has alloed to established important insights for future genetic studies and breeding programs. Several gst genes might operate significant roles in stress responses and are candidate genes for targeted breeding programs aimed at enhancing drought tolerance and fruit quality in tomatoes.

Genetic engineering and breeding programs can leverage the identified candidate genes, particularly those involved in redox homeostasis and stress response. However, furthere researches to validate their roles in stress tolerance and fruit quality are needed.

Additionally, GWAS approaches could investigate superior gst alleles and marker-assisted selection could be exploited to piramidize superior gst alleles to develop new improuved cultivars. The most up-to-date genomic selection approaches could help in selecting superior gst allele combination for more tolerant genotypes. Also, combining bioinformatics, structural genomics and functional genomics might lead to a deep elucidation of the gst contribution to tolerance. For example, CRISPR/Cas9 and other gene-editing technologies could be utilized to further investigate gene functions and their interactions within the broader network of stress response and fruit development pathways.

The development of drought-tolerant tomato varieties has significant implications for sustainable agricultural systems. These varieties can potentially reduce water usage, lower reliance on chemical inputs, and improve the overall sustainability of tomato production systems.

In conclusion, this research significantly advances our understanding of the genetic and molecular mechanisms underlying stress tolerance and fruit quality in tomatoes. The integration of bioinformatics, transcriptomics, and eco-physiological analyses provides a comprehensive framework for future studies and practical applications. The outcomes of this PhD project have the potential to drive innovation in tomato breeding, contributing to more resilient and sustainable agricultural systems in the face of global climate challenges. Further proof-of-concept research is needed to validate these findings and explore their practical applications in breeding programs. Future studies should focus on the functional validation of candidate genes in tomato and other crops, as well as the development of biotechnological tools for more efficient and targeted breeding approaches. The continued integration of multi-omics data will be crucial in advancing our understanding of plant stress responses and developing crops that can thrive in challenging environmental conditions.

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