THALITA MASSARO MALHEIROS FERREIRA

DECIFRANDO A TOLERÂNCIA À SALINIDADE EM PLANTAS: ANÁLISE DE PROMOTORES, GENES CANDIDATOS E O SISTEMA DE DEFESA ANTIOXIDANTE

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biotecnologia Vegetal, para a obtenção do título de Doutora.

Prof. Dr. Manoel Teixeira Souza Júnior
Orientador

Prof. Dr. Jaire Alves Ferreira Filho
Coorientador

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DECODING SALINITY TOLERANCE IN PLANTS: PROMOTER ANALYSIS, CANDIDATE GENES, AND THE ANTIOXIDANT DEFENSE SYSTEM

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biotecnologia Vegetal, para a obtenção do título de Doutora.

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RESUMO GERAL

A salinidade do solo se configura como um obstáculo crescente para a agricultura mundial, afetando mais de 100 países em todos os continentes. Esse estresse abiótico impacta negativamente as plantações, reduzindo a produtividade e ameaçando a segurança alimentar. Diante desse cenário, torna-se crucial a busca por soluções eficazes para garantir a sustentabilidade da produção agrícola. Nesse contexto, a transcritômica surge como uma ferramenta poderosa para investigar as complexas respostas moleculares das plantas à salinidade. A transcritômica, através da técnica de RNA-Seq, também conhecida como sequenciamento shotgun de transcritoma completo, permite a caracterização detalhada do transcritoma em uma célula, tecido ou organismo em um determinado momento. Essa tecnologia possibilita a identificação da maioria dos genes expressos em um estágio específico, a diferenciação de diferentes isoformas e a quantificação dos níveis de expressão dos transcritos. Para validar os resultados do RNA-Seq, outras técnicas como a PCR quantitativa em tempo real (qRT-PCR) podem ser utilizadas. Este estudo explora a complexa resposta do sistema de defesa antioxidante em duas espécies tolerantes à salinidade: beldroega (*Portulaca oleracea* L.) e gliricidia (*Gliricidia sepium* (Jacq.) Kunth) aclimatadas ao estresse salino extremo. O estudo se concentrou em um grupo pré-selecionado de genes que codificam enzimas antioxidantes chave e moléculas redox, reconhecidas por seu papel crucial no sistema de defesa antioxidante da planta. Esses genes foram investigados por meio de qRT-PCR, análise filogenômica e caracterização estrutural e funcional adicional. Os resultados para beldroega revelaram uma regulação positiva significativa de quatro enzimas antioxidantes e duas moléculas redox especificamente nas folhas estressadas pelo sal, não nas raízes. Esse achado sugere que a regulação diferencial do sistema antioxidante nas folhas contribui para a tolerância da beldroega à salinidade. Os resultados para gliricidia destacam os mecanismos regulatórios que governam as enzimas antioxidantes e as moléculas redox em plantas adaptadas a um estresse salino muito alto, evidenciando um papel predominante do sistema radicular nesta resposta. Além disso, este estudo buscou selecionar, anotar e validar genes induzíveis por estresse - e seus promotores - expressos diferencialmente nas folhas de dendezeiro (*Elaeis guineensis* Jacq.) sob estresse salino. Os resultados para o dendezeiro demonstraram que a análise do transcritoma levou à seleção de 14 genes que passaram por anotação estrutural e funcional, além de terem sua expressão validada pela técnica qRT-PCR. Estes estudos representam uma aplicação pioneira de RNA-Seq e qRT-PCR para desvendar a contribuição do sistema de defesa antioxidante para a notável resiliência da beldroega e gliricídia ao estresse

salino elevado. O estudo com dendê ajudou a validar o processo de seleção precisa de genes responsivos ao estresse salino com perfil de expressão específico e predefinido e sua sequência promotora. Estes resultados representam um passo importante na identificação de genes-chave para o desenvolvimento de dendê tolerante à salinidade.

Palavras-chave: Beldrogea; gliricídia; dendê; salinidade; estresse abiótico; transcritômica; resistência; RNA-Seq; and qRT-PCR.

GENERAL ABSTRACT

Soil salinity poses an increasing obstacle to global agriculture, affecting over 100 countries across all continents. This abiotic stress negatively impacts crops, reducing productivity and threatening food security. In this scenario, the search for effective solutions to ensure the sustainability of agricultural production becomes crucial. In this context, transcriptomics emerges as a powerful tool to investigate the complex molecular responses of plants to salinity. Transcriptomics, through the RNA-Seq technique, also known as whole transcriptome shotgun sequencing, allows for the detailed characterization of the transcriptome in a cell, tissue, or organism at a specific time. This technology enables the identification of most genes expressed at a specific stage, the differentiation of different isoforms, and the quantification of transcript expression levels. To validate RNA-Seq results, other techniques such as quantitative real-time PCR (qRT-PCR) can be employed. This study explores the complex response of the antioxidant defense system in two salt-tolerant species: purslane (*Portulaca oleracea* L.) and gliricidia (*Gliricidia sepium* (Jacq.) Kunth) acclimated to extreme salt stress. The study focused on a pre-selected group of genes encoding key antioxidant enzymes and redox molecules, recognized for their crucial role in the plant's antioxidant defense system. These genes were investigated using qRT-PCR, phylogenetic analysis, and additional structural and functional characterization. Purslane results revealed significant upregulation of four antioxidant enzymes and two redox molecules specifically in salt-stressed leaves, not in roots. This finding suggests that differential regulation of the antioxidant system in leaves contributes to purslane's salt tolerance. Gliricidia results highlight the regulatory mechanisms governing antioxidant enzymes and redox molecules in plants adapted to very high salt stress, evidencing a predominant role of the root system in this response. This finding paves the way for future research on the crucial role of roots in the salt tolerance of this species. Oil palm results demonstrated that transcriptome analysis led to the selection of 14 genes that underwent structural and functional annotation, in addition to having their expression validated by qRT-PCR. These studies represent a pioneering application of RNA-Seq and qRT-PCR to unravel the contribution of the antioxidant defense system to the remarkable resilience of purslane and gliricidia to high salt stress. The oil palm study helped validate the process of accurate selection of salt stress-responsive genes with a specific and predefined expression profile and their promoter sequence. These results represent an important step towards identifying key genes for the development of salt-tolerant oil palm.

Keywords: Purslane; gliricidia; oil palm; salinity; abiotic stress; transcriptome; resistance; RNA-Seq; and qRT-PCR.

INDICADORES DE IMPACTO

A crescente salinidade do solo representa um desafio global com profundos impactos sociais, tecnológicos, econômicos e culturais. Mais de 100 países enfrentam essa questão, que ameaça a segurança alimentar e a produtividade agrícola. A busca por soluções sustentáveis é crucial, e nesse cenário, a transcritômica surge como uma ferramenta tecnológica promissora. A redução da produtividade agrícola devido à salinidade do solo tem um impacto direto na segurança alimentar. Em comunidades que dependem fortemente da agricultura, a perda de colheitas pode levar à escassez de alimentos e ao aumento dos preços, afetando desproporcionalmente as populações mais vulneráveis. Isso pode gerar deslocamento de pessoas de áreas rurais, aumento da pobreza e instabilidade social. Economicamente, a diminuição da produção agrícola resulta em perdas financeiras significativas para agricultores e países, impactando o Produto Interno Bruto (PIB) e a balança comercial de nações exportadoras de commodities agrícolas. Além disso, os custos associados à recuperação de solos salinos ou ao desenvolvimento de novas terras agrícolas são elevados, exercendo pressão sobre os orçamentos governamentais e os recursos disponíveis para outras áreas essenciais. A necessidade de combater a salinidade impulsiona o avanço tecnológico na agricultura. A transcritômica, com a técnica de RNA-Seq, exemplifica essa evolução. Essa tecnologia permite investigar as complexas respostas moleculares das plantas ao estresse salino, identificando genes expressos e seus níveis de expressão. Estudos como os realizados com beldroega, gliricídia e dendezeiro demonstram a aplicação pioneira do RNA-Seq e qRT-PCR para desvendar os mecanismos de defesa antioxidante das plantas. O conhecimento gerado por essas pesquisas pode levar ao desenvolvimento de cultivares mais tolerantes à salinidade através de técnicas de melhoramento genético e biotecnologia, como a edição de genes. Isso representa um salto tecnológico na busca por soluções sustentáveis para a produção de alimentos em ambientes desafiadores. A salinidade do solo também pode ter impactos culturais, especialmente em regiões onde a agricultura é intrínseca à identidade e ao modo de vida das comunidades. A perda de terras férteis pode significar a perda de práticas agrícolas tradicionais, conhecimentos ancestrais e laços com a terra. Isso pode levar a uma desestruturação social e cultural, afetando a resiliência das comunidades e a transmissão de saberes entre gerações. A necessidade de adaptar as práticas agrícolas e o desenvolvimento de novas tecnologias pode, por outro lado, fomentar a inovação e o aprendizado, gerando novas abordagens para a relação humana com o meio ambiente e a produção de alimentos. Em suma, a salinidade do solo é um problema multifacetado que exige soluções integradas. A pesquisa em transcritômica oferece

uma esperança tecnológica, mas os desafios sociais, econômicos e culturais persistem, demandando uma abordagem holística para garantir um futuro agrícola sustentável. Em suma, a salinidade do solo é um desafio complexo que exige soluções integradas. A tecnologia, como a transcritômica, oferece esperança, mas é preciso uma abordagem holística para garantir um futuro agrícola sustentável.

IMPACT INDICATORS

Rising soil salinity represents a global challenge with profound social, technological, economic, and cultural impacts. More than 100 countries face this issue, which threatens food security and agricultural productivity. The search for sustainable solutions is crucial, and in this scenario, transcriptomics emerges as a promising technological tool. Reduced agricultural productivity due to soil salinity has a direct impact on food security. In communities that depend heavily on agriculture, crop failures can lead to food shortages and rising prices, disproportionately affecting the most vulnerable populations. This can lead to displacement of people from rural areas, increased poverty, and social instability. Economically, reduced agricultural production results in significant financial losses for farmers and countries, impacting the Gross Domestic Product (GDP) and trade balance of agricultural commodity-exporting nations. Furthermore, the costs associated with restoring saline soils or developing new agricultural land are high, putting pressure on government budgets and resources available for other essential areas. The need to combat salinity drives technological advancement in agriculture. Transcriptomics, using RNA-Seq, exemplifies this evolution. This technology allows us to investigate the complex molecular responses of plants to saline stress, identifying expressed genes and their expression levels. Studies such as those conducted with purslane, gliricidia, and oil palm demonstrate the pioneering application of RNA-Seq and qRT-PCR to unravel plants' antioxidant defense mechanisms. The knowledge generated by this research can lead to the development of more salinity-tolerant cultivars through genetic improvement and biotechnology techniques, such as gene editing. This represents a technological leap in the search for sustainable solutions for food production in challenging environments. Soil salinity can also have cultural impacts, especially in regions where agriculture is intrinsic to communities' identity and way of life. The loss of fertile land can mean the loss of traditional agricultural practices, ancestral knowledge, and ties to the land. This can lead to social and cultural disruption, affecting community resilience and the transmission of knowledge between generations. The need to adapt agricultural practices and develop new technologies can, on the other hand, foster innovation and learning, generating new approaches to human relationships with the environment and food production. In short, soil salinity is a multifaceted problem that requires integrated solutions. Transcriptomics research offers technological hope, but social, economic, and cultural challenges persist, demanding a holistic approach to ensure a sustainable agricultural future. In short, soil salinity is a complex challenge that requires integrated solutions. Technology, such as transcriptomics, offers hope, but a holistic approach is needed to ensure a sustainable agricultural future.

SUMMARY

FIRST PART

1 GENERAL INTRODUCTION

Population growth and climate change place increasing pressure on the biomass production system, affecting the supply of food, fiber, feed and bioenergy on regional, national and global scales (Negacz *et al*., 2022). This complex situation requires comprehensive solutions that involve the scientific community, political analysts, policymakers, and other stakeholders (Dodson *et al*., 2020). About a fifth of the world's agricultural land, equivalent to a vast and significant area, faces the challenges posed by saline and sodic soils. This problem, which affects more than 100 countries, represents a crucial obstacle to global food security. Estimates indicate that soil salinity and sodicity cause annual losses of US$27.3 billion, resulting in reduced agricultural productivity and putting the livelihoods of millions of people at risk (Negacz *et al*., 2022; Zaman *et al*., 2018).

Salinity is caused by excess Na^+ and Cl^- ions found in soil solution and water. The three effects observed in plants due to salinity are: (i) reduction in water potential, (ii) ionic imbalance or disturbances in homeostasis and (iii) ionic toxicity. Exposure of plants to high salinity can lead to death or reduced productivity, compromising fundamental processes such as growth, photosynthesis, protein synthesis, energy metabolism and lipid metabolism (Herbette *et al*., 2011). The production and accumulation of reactive oxygen species (ROS) in plants, caused by various abiotic stresses, including salinity, result in the severe destruction of organelles and cellular functions. This causes peroxidation of membranes, damage to the cell membrane, degradation of biological macromolecules and, ultimately, cell death. The ability of plants to eliminate the toxic effects of ROS appears to be the main determinant of their tolerance to different stresses. Antioxidants are the first line of defense against damage caused by free radicals and are essential for optimal plant cell health (Rajput *et al*., 2021).

The diversity of adaptive mechanisms that minimize the effects of saline stress is more efficient in halophyte species than in glycophytes (Sharma *et al*., 2012). Salinity tolerance mechanisms in plants can be divided into three categories: (1) physiological, related to growth, water relations, ion homeostasis, photosynthesis, yield and senescence; (2) biochemical, involving antioxidant compounds, compatible solutes and hormonal response; and (3) molecular, referring to gene regulation (Rajput *et al*., 2021).

Tolerance strategies in stressed plants include a series of physiobiochemical mechanisms. Among the enzymatic components are superoxide dismutase (SOD), catalase

(CAT), peroxidases (POX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferases (GST), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Non-enzymatic components include ascorbic acid (AA), glutathione (GSH), phenolic compounds, alkaloids, flavonoids, carotenoids, free amino acids, and α-tocopherols (Zhou *et al*., 2018).

The transcriptome represents the small percentage of the genetic code that is transcribed into RNA molecules. By analyzing the transcriptome, we can gain valuable insights into the essential biological processes that define cell functionality in different contexts. Transcriptome analysis in plants and other organisms can help identify genes with desirable characteristics, such as resistance to diseases or stresses, for the development of more resilient varieties (Rani; Sharma, 2017). While RNA-Seq is a powerful tool, it is important to validate your results through other techniques, such as quantitative real-time PCR (qRT-PCR). qRT-PCR confirms gene expression detected by RNA-Seq, providing an additional level of reliability to the data. The combination of RNA-Seq and qRT-PCR offers a comprehensive view of gene expression, allowing quantification and validation of genes of interest with high accuracy (Liu *et al*., 2016). *Portulaca oleracea*, popularly known as purslane, is a succulent herbaceous plant classified as one of the most important invasive plants in the world. Due to its high nutritional value and wide range of pharmacological effects, including anti-inflammatory, antibacterial, antioxidant and antiulcerogenic properties, purslane is one of the medicinal species listed by the World Health Organization (Zhou *et al*., 2015). Purslane has high adaptability to extreme soil conditions, being able to grow in environments under water stress, salinity and poor nutrients. Therefore, it has been considered a potential model plant to study resistance to abiotic stresses (Montoya-García *et al*., 2022).

Economically, *Gliricidia sepium* stands out for improving water infiltration, increasing soil water retention capacity, reducing soil erosion and restoring and improving soil quality, resulting in greater crop yields (Diouf *et al.,* 2017). Furthermore, it is known for its ability to adapt to diverse soil types, including eroded acidic, sandy, heavy clayey, calcareous and alkaline soils (Rahman *et al*., 2019). The salinity tolerance limits of gliricidia and its responses to salt stress are not yet well understood (Rahman *et al*., 2019). Studies by Rahman and colleagues have postulated that proline, which has shown increased accumulation under salt stress, may help gliricidia plants adjust to water deficit conditions. Proline participates in metabolic signaling and is known to be metabolized by its own family of stress-responsive enzymes (Phang *et al*., 2010).

Oil Palm is an essential source for multiple industries, the African oil palm (*Elaeis guineensis* Jacq.), popularly known as oil palm, is a species native to Africa and champion in productivity among oilseeds (Corley; Tinker, 2015). With an average productivity of 2.94 tons per hectare, oil palm surpasses other traditional oilseeds such as sunflower (0.74 t/ha), rapeseed (0.72 t/ha) and soybeans (0.46 t /ha) (Ritchie; Soopner; Roser, 2021). This high productivity makes it essential for food security in several regions of the world (Adade, 2022). Palm oils have diverse applications, serving different sectors, such as the food industry (68%); in industrial applications (27%), and as biofuels (5%).

This study represents a pioneering application of RNA-Seq and qRT PCR to unravel the contribution of the antioxidant defense system (genes encoding antioxidant enzymes and redox molecules) to the remarkable resilience of purslane and the adaptation phenotype of gliricidia to high salt stress. In relation to the study in oil palm, we sought to select a set of profile-specific salt stress-inducible genes, and their promoters, differentially expressed in leaves under salt stress.

SECOND PART

Artigo 1 - Book chapter - Redigido conforme norma do periódico científico - Versão publicada	
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ARTIGO 1 - Genetic Engineering of Purslane (*Portulaca Oleracea* L.)[1]

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Abstract

Portulaca oleracea L., popularly known as purslane, is an herbaceous succulent plant classified as one of the most important invasive weeds in the world. Due to its high nutritional level and wide range of pharmacological effects, involving anti-inflammatory, antibacterial, antioxidant, and antiulcerogenic, purslane is one of the medicinal species listed by the World Health Organization. In addition, purslane produces several phytochemicals, including flavonoids, alkaloids, and terpenoids, which confer different pharmacological activities and make the plant highly attractive for use in the most diverse industries. It has high adaptability to extreme soil conditions, able to grow and spread in environments under drought stress, salinity, and poor nutrients; and has been presented as a potential model plant to study resistance to abiotic stresses. Among other purslane traits of interest to the agriculture sector, is worth to mention phytoremediation and allelopathy, thus being a sustainable alter- native in organic agriculture. Here, we report a bibliometric analysis of purslane *in vitro* tissue culture and genetic modification/editing and discuss opportunities and limitations to exploit the biotechnological potential of purslane as a source of valuable bio-molecules for many different industries.
Keywords: purslane, medicinal plant, multipurpose species, genetic transformation, tissue culture, biolistic, agrobacterium, abiotic stresses

1. Introduction

Portulaca oleracea L. (**Figure 1**), the most well-known species of the *Portulaca* genus, is commonly known as purslane, or common purslane, according to Ref. [1]. This genus belongs to the family Portulacaceae, order Caryophyllales, superorder Caryophyllanae, class Magnoliopsida, subdivision Spermatophytina, division Tracheophyta, superdivision Embryophyta, infrakingdom Streptophyta, and sub- kingdom Viridiplantae [2, 3].

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Figure 1.
Purslane (Portulaca oleracea L.).

Purslane is classified as a multipurpose plant species [4]. Plants cultivated for the purpose of providing more than one significant contribution to the production Medicinal Plants – Chemical, Biochemical, and Pharmacological Approaches and/or service functions of a land use system are defined as multipurpose plants. They are classified according to the attributes of the plant species and the functional role of it in the technology under consideration, be it linked to the agricultural, pharmaceutical, chemical, or other economic sector.

2. Socioeconomic importance of purslane

2.1 A medicinal plant recommended by the World Health Organization

According to the World Health Organization (WHO), purslane is one of the most used medicinal plants. Known as a "Global Panacea"—a remedy supposed to heal all sicknesses, it is used extensively in folk medicine due to its wide array of health effects [5, 6]. The ethnobotanical importance of purslane led to various studies confirming its pharmacological properties. Those studies support its use as an antibacterial [7], anti-inflammatory, antioxidant [8], neuro- and hepatoprotective [6], antidiabetic [9], and antiulcerogenic agent [10], among other applications. In addition, it is reportedly a highly nutritious plant, being among the top terrestrial sources of essential fatty acids, tocopherol, ascorbic acid, glutathione, and other components, which suggests its nutraceutical potential [11, 12]. These valuable chemical constituents result from purslane's diverse set of chemical pathways.

All organisms have an integrated network of chemical reactions meticulously mediated and regulated by enzymes. It encompasses primary and secondary metabolic pathways synthesizing various organic compounds [13]. More specifically, primary metabolism involves generating components required for growth and development. Its products often serve as intermediates for the production of specialized chemicals that comprise the secondary metabolism, which plays a crucial part in a plant's interaction with the environment [14]. Processes that result in primary metabolites are highly conserved, while those of secondary metabolites are lineage- specific and continuously influenced by abiotic and biotic factors. That results in the formation or suppression of bioactive compounds that confer specific properties to the plant, intending to promote its survival and protection [15, 16].

Following a simple classification, secondary metabolites are divided into three main groups: phenolics, nitrogen-containing compounds, and terpenoids. Each is obtained through different biosynthetic pathways, resulting in chemicals with distinctive structures that confer

valuable properties. These are building blocks to the manufacturing of high-added-value products focusing on health, nutrition, or agriculture, and thus are of enormous importance within the scientific and industrial framework [16, 17]. Purslane has a rich and unique content of these bioactive compounds that, individually or synergistically, provides beneficial effects and explains its extensive use in folk medicine [6, 12]. The following paragraphs will give insight into some highly important secondary metabolite groups and their known activities.

Flavonoids, which comprise the phenolic group, are among the main active ingredients from purslane—with kaempferol, luteolin, apigenin, myricetin, and quercetin as its major components. In addition, novel structures, namely portulacanones and oleracones, were first isolated from this plant. Studies have shown the anticancer [18, 19], anticholinesterase [20], anti-inflammatory, and antioxidant effects of these flavonoids [21, 22]. Furthermore, families belonging to Portulacaceae produce betalains, known as nitrogen-containing plant pigments with limited occurrence in nature [23, 24]. This subgroup is natural colorants in the food and cosmetic sectors, although studies have shown their neuroprotective [25], chemoprotective [26], and antimicrobial potential [24].

N-trans-feruloyltyramine, dopamine, noradrenaline, and oleraceins are alkaloids also identified in this plant species [27]. These nitrogen-containing compounds have reported immune-enhancing and neuroprotective effects, among others, and under- went studies for the prevention and treatment of neurodegenerative diseases [28–30]. The terpene content, which includes portulosides A-B, portulenes, and others, also contributes to potentializing antimicrobial and hepatoprotective effects of purslane extracts, and so on [27]. Other bioactive components include lignans, phenolic acids, and esters [31], and new molecules are constantly isolated from this plant through various methodologies [30, 32, 33].

Purslane is also a rich source of omega-3 and omega-6 fatty acids, thus contributing to its nutritional value [34]. These are the precursors of eicosapentaenoic and docosahexaenoic acids, which can reduce the risk of cardiovascular and cerebral diseases [12]. Studies on the development of functional food products from this plant are already available [35], as it also has considerable amounts of vitamins and dietary minerals [11]. Overall, each phytoconstituent mentioned contributes to establishing the ethnobotanical importance of purslane and supports the application of this plant in the pharmaceutical, food, and cosmetics industries.

2.2 A source of so many agricultural important traits

Besides being a source of many traits for the pharmaceutical and chemical industries, purslane is also a source of features of direct importance to the agricultural and agri-industrial sectors. Among the most important ones are phytoremediation, alleopathy, and tolerance to biotic and abiotic stress. Below we present some insights into some of those traits and then—more to the end of this chapter—report intensively on resistance to salinity stress.

Due to the purslane tolerance capacity for metal stress, it undergoes phytoremediation and biomonitoring studies in the field and closed conditions [36]. Phytoremediation is an economic process that exploits plants' capacity to accumulate heavy metals in polluted habitats by their harvestable parts [37], while biomonitoring is the capacity to monitor contaminated environments [36]. Mohammadzadeh and Hajiboland [38] reported a successful study using purslane in phytoremediation strategies to remove nitrate from nitrate-contaminated sites.

Allelopathy is the ability of a plant to suppress the germination, growth, survival, and reproduction of other plants in its surroundings. It produces and releases allelochemicals (secondary metabolites) that negatively affect other plants. Hamad [39] showed that aqueous extracts from purslane shoots and roots have allelopathic (inhibitor) effects on seed

germination and the growth of monocots and dicots. Rashidi et al. [40] investigated the allelopathic effect of purslane on seed germination and growth of several plant species and demonstrated its allelopathic potential against *Phaseolus vulgaris* L. and *Allium cepa* L. as it reduced their seed germination rate.

After studying the chemical composition and yield of six purslane genotypes, Petropoulos et al. [34] reported that the biomass yield (fresh weight) in the open field was affected by genotype, with the highest yield of the tested genotypes being 33 tons/hectare, and the lowest being 11.5, with an average of about 22.5 among these genotypes. Kong and Zheng [41] evaluated the potential of producing purslane in a hydroponic system by testing two distinct cultivars—Green and Golden. Both cultivars performed similarly, generating a marketable yield of approximately 5.75 kg per m^2 on a bimonthly basis, which might yield 345 tons/hectare/year if cultivated in a bimestrial regime. Alu'datt et al. [42] evaluated the effect of different soil-less substrates on the fresh yield of purslane over five harvest cycles during the growing season under closed conditions and reported productivity of approximately 27 kg per m^2 when using Tuff: Peatmoss (2:1) substrates; what might yield 270 tons/hectare/year.

Purslane is a succulent herbaceous halophyte plant classified as invasive and considered the eighth most common weed in the world; it grows in warm moist places during the summer and spring seasons and can grow in almost any unshaded area, including gardens, crop fields, and waste places [43]. Because of that, its outdoor production in extensive areas faces several concerns. However, the above-mentioned high productivity of purslane in the context of controlled-environment agriculture [44–46] can open many doors of opportunities for the purslane industry. Many of those might take advantage of having a highly efficient protocol for engineering/editing purslane genome.

3. Genetic engineering/editing of purslane: state of the art

Genetically modified/edited plants are usually developed by *in vitro* regeneration from single transformed cells, and because of that, using *in vitro* plant tissue culture- based methods is required. However, that is not the only way to develop such types of plants. Some strategies of plant transformation that do not depend on *in vitro* regeneration are available and are known as "*in planta*" transformation methods. The floral dip transformation method is the most well-known of them [47]; however, no report is available on its successful use in purslane.

3.1 Purslane in vitro tissue culture

Once the goal is the *in vitro* regeneration from single transformed cells, it is necessary to develop first a reliable and efficient purslane tissue culture protocol. Such a process may take advantage of the organogenesis or embryogenesis capability of the plant species in question and need to evaluate some factors such as the most appropriate- ate type of explant, culture medium, growth regulators, and cultivation conditions, among others [48]. Unfortunately, there are not many reports on purslane *in vitro* tissue culture. The few ones available will be reported in the next paragraphs.

Safdari and Kazemitabar [49] was the first report on *in vitro* regeneration of purslane plants, intending to determine the best hormonal treatment for the induction of embryogenic callus from leaf tissue, the best type of explant and hormones for plant regeneration, and root induction from regenerated shoots. Later, Sharma et al. [50] reported an attempt to establish

an efficient *in vitro* protocol for plant regeneration through organogenesis, using 1.5 cm long knots as explant, and achieving a stable efficiency of 70%.

Shekhawat et al. [51] reported an efficient *in vitro* regeneration method for purslane using a liquid medium, where the explants used were shoots with one and two nodes, obtaining a rooting efficiency rate of 96%. Oraibi et al. [52] reported success in efficiently inducing callus from purslane leaves, with subsequent production of extracts from the callus that presented antibacterial activity.

Purslane is sexually propagated, producing an enormous amount of seeds in a short period—within 60–90 days. Besides, purslane is also efficiently vegetatively propagated from cutting. The success of propagation (by seeds or cuttings) is prob- ably one of the reasons that justify that there are not many reports on purslane *in vitro* tissue culture. The lack of demands for eradicating pathogens could be another reason to explain it.

The demonstrated capacity for producing over a hundred tons of biomass per hectare per year under closed conditions [42] makes purslane an ideal candidate as the crop to produce its bio-molecules, reducing the risk associated with the fact it is a weed [43]. However, one cannot forget that the growth of purslane cell suspension using bioreactors [53] is another way ahead to produce such bio-molecules under a controlled environment. In such case, there is the need to develop protocols to obtain and maintain purslane cell suspension.

Consequently, there is no doubt that for purslane to become a model plant for functional genomics research, aiming to advance on the exploitation of so many of its bio-molecules—whether in the pharmaceutical sector, in the agronomical sector, or in other sectors—the scientific community must expand and deeper the studies in many of the frontlines of plant tissue culture, such as haploid/di-haploid production, cell suspension production and maintenance, and, of course, genetic modification/ editing. The results of the tissue culture survey on *Portulaca oleracea* are summarized in the table below (**Table 1**).

References	Explant	Multiplication technique	Efficiency	Objective
Safdari and Kazemitabar, [49]	Petioles, shoot tips, and leaves.	Callogenesis	70%	Test different hormones and explants.
Sharma et al. [50]	Nodal shoot segments	Organogenesis	70%	Development of an efficient regeneration protocol.
Shekhawat et al. [51].	Nodal shoot segments	Organogenesis	96%	Regeneration in liquid medium and implications of growth regulators.
Oraibi et al. [52]	Leaves	Callogenesis	80%	Antibacterial Activity.
Sedaghati et al. [54]	Seeds and leaves	Somatic embryogenesis	72.22%	Development of an efficient regeneration protocol.

Table 1.
Published findings on in vitro *tissue culture of purslane (Portulaca oleracea L.) - search done in December 2022.*

3.2 Genetic modification of purslane

The genetic transformation of plants involves the insertion, integration, and expression of exogenous genes into the genome of a plant species. One of the main focuses in obtaining transgenic cultures is incorporating new characteristics, studying primary biological processes, and producing bio-pharmaceutical proteins. Since the 1980s, different techniques became available for introducing heterologous genes into the genome, among which the

transformation mediated by Agrobacterium and biolistics stands out [55]. The sonication-assisted Agrobacterium-mediated gene transfer system increases the transformation efficiency [56], and studies using sonication associated with vacuum infiltration proved to be efficient when applied to different cultivars of economic importance [57].

Sedaghati et al. [54] aimed to develop an Agrobacterium-mediated transformation and regeneration system using somatic embryogenesis in purslane, obtaining an effi- ciency of 72.22% from leaf explants. Studies carried out by the same group in 2021, seeking to optimize this transformation process, used sonication associated with vacuum infiltration from seeds, obtaining a maximum efficiency of 39.25 ± 2.88%. Subsequently, Sedaghati et al. [58] carried a new study out where they applied purslane as a green bioreactor to evaluate the expression of the HSA human serum albumin gene.

Two reports describe success in transforming purslane using *Agrobacterium rhizogenes* [59, 60]. Tandon et al. [59] in an attempt to improve the phytoremediation of municipal waste water by increasing the root biomass, transformed four distinct plant species using *A. rhizogenes*, including *P. oleracea*. They used nodules and roots as explants for transformation, and achieved an increase in phytoremediation efficiency of transformed portulaca for the treatment of municipal waste water was observed
over the non-transformed plants. The study by Ahmadi Moghadam et al. [60] aimed to optimize and evaluate the production of dopamine in hairy roots of purslane, using rolB as the gene of interest; and roots, stems, leaves, and cotyledons as explants. It was found that the most suitable explants for the induction of hairy roots were the cotyle- dons, with an efficiency of 53.3%. The effect of methyl jasmonate and salicylic acid on the accumulation and biosynthesis of dopamine in hairy roots culture of purslane was investigated, showing that elicitation with the former resulted in about 4.3-fold higher dopamine yield compared to the control hairy root cultures. The results of the genetic modification survey on *Portulaca oleracea* are summarized in the table above (Table 2).

References	Explant	Technique	Efficiency	Gene
Tandon et al. [59]	Nodal segments and roots	*Agrobacterium rhizogenes*	42–68%	_
Moghadam et al. [60]	Cotyledon leaves	*Agrobacterium rhizogenes*	53.30%	*rolB*
Sedaghati et al. [54]	Stem and leaf explants	Agrobacterium-mediated	72.22%	*GUS*
Sedaghati et al. [57]	Seeds	Agrobacterium-mediated transformation using sonication and vacuum infiltration.	39.25%	uidA
Sedaghati et al. [58]	Seeds and leaves	Agrobacterium-mediated transformation.	100%	HSA

Table 2.
Published findings on genetic modification in purslane (Portulaca oleracea L.) - search done in December 2022. Genetic Engineering of Purslane (Portulaca oleracea L.)

4. **Purslane as a model plant to study resistance to abiotic stresses**

Purslane is a vegetable highly adaptable to various climates and adverse conditions (such as heat, drought, and salinity), giving it a competitive advantage over many other plant species produced [48]. It can grow in arid and saline soils, and it is consid- ered both a

halophyte (adapted to salty environments) and a xerophyte (adapted to dry ones); it is also listed as a halophyte plant in the eHaloph database [3, 61, 62].

Ren et al. [63] characterized the responses of 10 different purslane accessions to drought stress, and two African accessions were the most drought tolerant ones, Tokombiya and Egypitum. Drought tolerance was highly variable among the tested accessions at seed germination, seedling development, and adult stages. Jin et al. [64] evaluated the effect of drought and heat stress on purslane, individually and simultaneously. They demonstrated different survival strategies to physiological and metabolic stress, expressed as an increase in malondialdehyde, electrolyte leakage, O_2 and superoxide dismutase, and peroxidase activities, and a decrease in chlorophyll content. Furthermore, they suggested that combined stresses have a more severe effect on purslane plants compared to individual stresses. Another indication is that purslane is a promising candidate to be used in the recovery of ecosystems in arid and semi-arid regions [64].

Another very important fact about purslane is that, depending on the environmental condition, it can switch between the C4 photosystem and Crassulacean acid metabolism (CAM). Ferrari et al. [65] used purslane as a model system to investigate the involvement of the circadian clock, transcription factors, and plant hormones in coordinating the expression of C4 and CAM genes. They showed that, in general, the endogenous circadian clock coordinates and optimizes the daily time of regulation of the C4 and CAM genes in purslane leaves that are well-irrigated and under water stress [65].

There are studies of taxonomy, ecology, physiology, biochemistry, and genetics of purslane, including those characterizing its genetic variability. That information already available regarding the development of purslane, together with characteristics such as a short life cycle, small size, and relatively easy handle, reinforces the idea of turning purslane a research model plant for a better understanding of plant resistance to those two abiotic stresses [66, 67]. It is necessary to use different model plants, instead of the regular ones—Arabidopsis, for instance—as responses to those stresses are different between species [68].

Our research group developed a robust salt stress protocol (**Figure 2**) to characterize the morphophysiological responses of young purslane plants to salinity stress, along with multi-omics analyses, where it was possible to observe three distinct levels of salt stress by electrical conductivity gradients and water potential in substrate saturation extract [69]. The multi-omics integration studies showed 51 pathways having at least one enzyme and one metabolite differentially expressed in the leaves of purslane as a result of salt stress [69]. The characterization of the metabolomics, transcriptomics, and proteomics profiles on the leaves and roots of adult purslane plants were generated, and their analyses allowed further insights on the resistance of purslane to salinity stress (Rodrigues-Neto et al., unpublished).

Figure 2.
Purslane (Portulaca oleracea L.) submitted to salinity stress studies at Embrapa Agroenergia during seed germination, seedling development, and different adult stages. Source: Souza Jr., M. T.

Salinity tolerance is an important agronomic feature due to the great problem of salinized soil, which corresponds to 20% of the irrigated land in the world [48]. Studies carried out by Hamidov et al. [70] suggest the use of purslane to promote the rehabilitation of saline soils in the northern part of Uzbekistan, in addition to high- lighting its great nutritional quality and its efficiency in tolerating chloride salinity, which makes this species a potential candidate for biosaline agriculture [70].

Yang et al. [30] applied physiological and comparative proteomics to investigate the mechanisms underlying purslane's tolerance to high-temperature and high- humidity stresses, demonstrating that this plant species deploys multiple strategies to cope with these combined stressors. Several strategies, from the activation of several metabolisms to the transient development of a CAM-like metabolism, were responsible for increased adaptation to water stress and combined stress in purslane [71].

To become a reliable model plant in functional genomics studies aiming to characterize the genetic basis of the resistances to those different abiotic stresses, it would be helpful to avail a reference whole genome sequence, as well as an efficient and reliable genetic modification/editing protocol.

5. **Purslane as a source of bio-molecules of biotechnological importance**

5.1 Screening for bio-molecules in purslane biomass

The final composition of bioactive compounds in purslane is affected by harvest- ing period, soil characteristics, cultivation practices, biotic/abiotic stresses, and other environmental factors, besides the genetic background. Temperature conditions during storage also play an essential role in this plant's quality, as it may lead to the degradation of particular components. Moreover, the efficiency of the extraction pro- cess is highly dependent on the

plant's material, target metabolites, the temperature set, and the type of solvent used. Understanding these aspects that result in purslane's and its extract variability can allow for the management of specialized chemicals, thus leading to increased added-value products [12, 34, 72].

Novel bioactive compounds are continuously being isolated and identified from purslane through different extraction methods, including alkaloids [32, 73], flavo- noids [22, 31], lignans [31, 74], and so on. Furthermore, changes in the metabolism— caused by biotic and abiotic factors—can be analyzed through various analytical methods. Metabolomic profiling techniques can determine and compare the chemical content of cultivars grown in varying conditions [12]. For instance, studies have shown that certain levels of salinity treatment result in increased concentration of valuable metabolites in purslane. Salt, as abiotic stress, triggers the plant to potential- ize specific chemical pathways. However, it is essential to consider that these effects may also depend on the cultivar and geographic distribution, among other factors. Still, this indicates the potential for purslane's accessions to be explored for stress- induced augmented production of bioactive compounds [12, 48].

Moreover, fermentation can enrich the profile of active compounds found in purslane. During this specific process, enzymes derived from the metabolism of microorganisms promote the degradation of antinutritional factors and organic complexes. That gives rise to low-molecular-weight compounds—such as free amino acids [75] that can serve as substrates in the biosynthetic routes of secondary metabo- lites [76]. Lactic acid fermentation, for instance, is a suitable tool that explores the functional potential of purslane's biomass. It promoted increased bio-availability and profile modification of its specialized constituents and supplemented the matrix with active metabolites from the bacteria. Based on this, choosing a microbial group with high metabolic potential and versatility could lead to a selection of suitable strains for effective biotechnological processes [77, 78].

In summary, purslane is a rich source of specialized chemicals, and abiotic and biotic factors strongly influence their final content. Post-harvest treatments and extraction methods may also result in compound variability. Based on that, this species is the source of novel phytochemicals, and many techniques are employed to understand those aspects and compare cultivars. Stress-induced methods can lead to obtaining higher concentrations of bioactive compounds. Additionally, fermentation and biotechnological processes are promising strategies for exploring its functional potential.

5.2 Bio-molecules to control plant pathogens

The intensification of agricultural activity through the implementation of extensive areas of monoculture led to the emergence of pests at high levels, capable of reaching the threshold of economic damage. Groups of pathogens such as fungi, bacteria, viruses, nematodes, and insects are biotic agents whose infection to culti- vated plants drastically reduces productivity. In the case of phytonematodes, global losses reach values above US$ 100 billion annually [79], with emphasis on the spe- cies *Meloidogyne incognita*. Several methods can be used to control phytopathogens, and the integration of these different strategies results in the most effective form of management. However, under high population densities, the suppression of these pathogens is largely carried out by making use of synthetic chemical pesticides, products that represent a risk of intoxication for humans and animals, as well as contamination of the environment. Chemical pest control substantially burdens the production process and also incurs the risk of selecting resistant organisms [80]. In view of all the problems involved in the adoption of this control method, it is necessary to search for more sustainable measures as an alternative way to contain the attack of agricultural pests. A

trend that has shown promise and is gaining space in the field of research is the investigation of the activity of botanical extracts against plant parasites [81].

A vast number of species belonging to different botanical families can produce, through their secondary metabolism, several compounds from different chemical classes with the potential to suppress pest attacks. Chemical groups, such as alkaloids, terpenes, coumarins, flavonoids, among others, represent some of the elements respon- sible for inhibiting the action of various phytoparasites and, therefore, have competence to serve as a basis for the development of more eco-friendly pesticides, and mitigate the negative effects caused by pesticides [82]. Brazil has a mega diversity of plant resources with potential to be exploited in the prospection of biopesticides [83].

Botanical extracts can be produced from different structures, such as roots, flowers, seeds, and leaves, among others, and even from industrial co-products using different extracting solutions [84]. In this context, the use of these solutions with different physical-chemical characteristics allows obtaining a greater number of compounds, of different chemical classes with multiple applicability related to the medical, cosmetics, livestock, and agricultural areas [85]. Purslane is a plant of wide geographic distribution and highly attractive as an object of study for having a rich variety of phytochemicals with biocidal action. Furthermore, the use of plant residues as a green technology in pest control is a bi-sustainable possibility. This measure is characterized by the removal of materials from the industrialization process, for the production of sustainable pesticides, replacing conventional agrochemicals, whose molecules are generally classified as having a high toxicological degree [86].

Among the advantages of using phytochemicals in pest management is the possibility of associating extracts from different species, aiming at obtaining multifunctional technologies without generating incompatibility between the different molecules that participate in the compounds, which commonly happens in mixtures of synthetic chemicals [87]. Besides, the miscellany of extracts from different botanical families might result in the development of technologies that control two or more pathogens simultaneously, without negatively affecting the community of beneficial microorganisms in the soil.

Identified and characterized botanical compounds with biocidal effect can be used as tools in activities devoted to the discovery of genes and gene products involved in their synthesis routes. Such information is later used in metabolic engineering studies, which will allow the synthesis of the compound of interest in different parts of the plant, such as the root, for the control of soil pathogens. In addition, extracts and botanical compounds can also be used as basic constituents in the generation of green nanoparticles [88, 89]. Brazil has one of the greatest biodiversity on the planet and represents a relevant source of natural plant-based products. This fraction of biodiversity constitutes the so-called plant genetic resources. At the Brazilian Agricultural Research Corporation—Embrapa, a significant inter- and intraspecific genetic variability is conserved in active Germplasm Banks. These banks represent a source of diverse materials with numerous physicochemical characteristics and, therefore, an important and interesting reservoir for the prospection of extracts, fractions, and biocidal compounds. These materials might be used in the formulation of new eco-pesticides, following the global market trend that arises from society's growing demand for safer food and environmentally friendly practices [90].

The use of plant extracts, as a source of bioactive compounds in the suppression of phytopathogens, represents another possibility to be used in the management of agricultural pests [91]. However, some points deserve attention regarding the use of plant extracts as a basis for the development of natural pesticides. They are (i) the availability of plant material considering the seasonality of the crop in question, (ii) the search for biocidal action standardization showed by plants of the same species, but coming from different regions, (iii)

the obstacles in the production of extracts on a large scale, (iv) the low residual characterizing a shorter time of action and, there- fore, the demand for a greater number of applications, and (v) the restrictions that come up against the legislation concerning the registration of these products as they fall under the pesticide law.

In short, the implementation of natural pesticides in the production process, replacing synthetic agrochemicals whenever possible, is highly required. The plan- ning to reduce the population of plant parasites must be prioritized and carried out rationally. Furthermore, the biology of the pathogen, the different methods of control, and their respective tactics must still be considered and could contribute to the minimization of the environmental imbalance. The joint implementation of these actions may strengthen the implantation of preventive measures and restrict the use of curative agricultural practices.

Purslane extract can be used as a pesticide as a pest control measure [4, 92]. Studies have shown that the methanolic extract of *Portulaca oleracea* showed high contact toxicity and dichloromethane exhibited antifood toxicity against the *Aphis gossypii* pest that affects cotton [4]. Tayyab et al. [93] carried out a study with 40 species of indigenous plants, including purslane, evaluating the insecticidal potential of acetone extracts. About 41% death rate was observed for the cotton aphid using 10% acetone extract obtained from purslane leaves [93].

6. Conclusions and future perspectives

Purslane is a multipurpose plant species source of many known bio-molecules and features of interest to several distinct industries—pharmaceutical, chemical, food, and feed, just to name a few. It is one of the most used medicinal plants, according to the WHO; but, it is also the eighth most common weed in the world. As an invasive weed, purslane raises several concerns when considered to be grown outdoors, in open fields. Because of that, it has been subject of increasing interest in the context of controlled-environment agriculture, where it deliveries very high biomass productivity. Within the scope of controlled-environment, purslane could be produced in greenhouses, in containers (vertical farming systems), or in bioreactors (cell suspensions).

After performing a bibliometric analysis of purslane *in vitro* tissue culture and genetic modification/editing, we came across with very little scientific data available. To exploit the biotechnological potential of purslane, as a source of valuable bio- molecules for the many different industries, it is imperative to change this scenario. Among the key initiatives necessary to do so there are: (a) a Whole Genome Project to generate and make available a reference genome for this plant species; (b) a reliable and efficient protocol for genetic modification/editing, able to produce a large number (hundreds or thousands) of mutants (genetically modified/edited) per period (a few months or a year); and (c) to build up a multi-omics database harboring information regarding the response of this plant species to many environmental conditions.

Other initiatives, such as the generation of haploid/di-haploid individuals; the production and maintenance of cell suspensions; the production, maintenance, and characterization of purslane's extract library, would be also of great value to exploit the biotechnological potential of this important plant species.

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Conflict of interest

The authors declare no conflict of interest.

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Artigo 2 - Elaborado conforme a norma NBR 6022	
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UNIVERSIDADE FEDERAL DE LAVRAS

ARTIGO 2 - ANTIOXIDATIVE DEFENSE MECHANISM IN PORTULACA OLERACEA'S RESPONSE TO HIGH SALINITY STRESS: INSIGHTS FROM A TRANSCRIPTOMICS STUDY

O MECANISMO DE DEFESA ANTIOXIDANTE NA RESPOSTA DA PORTULACA OLERACEA AO ESTRESSE DE ALTA SALINIDADE: PERCEPÇÕES DE UM ESTUDO DE TRANSCRITÔMICA[2]

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[2] Artigo científico apresentado à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biologia.

Abstract: This study explored the role of the antioxidant defense system in *Portulaca oleracea* L. (purslane), an halophyte species resistant to high salinity stress. Mature purslane plants (B1 accession) were exposed to a harsh salinity challenge (2.0 g NaCl/100 g substrate) for 12 days. Roots and leaves from stressed and control plants were then harvested for RNA extraction and qRT-PCR analysis. We focused on a pre-selected group of genes encoding key antioxidant enzymes (4) and redox molecules (2) known for their role in the plant's antioxidant defense system. These genes were investigated using qRT- PCR, phylogenomic analysis, and further structural and functional characterization. Our findings revealed a significant positive regulation of all four antioxidant enzymes and both redox molecules specifically in salt-stressed leaves, not roots. This study represents a pioneering application of RNA-Seq and qRT-PCR to unravel the contribution of the antioxidant defense system to purslane's remarkable resilience against high salinity stress.

Keywords: purslane; resistance; enzymes; abiotic stress; transcriptome; qRT-PCR.

Resumo: Este estudo investigou o papel do sistema de defesa antioxidante na resistência da *Portulaca oleracea* L. (beldroega), uma espécie halófita resistente a elevado estresse salino. Plantas maduras de beldroega (acesso B1) foram expostas a um desafio de salinidade severa (2,0 g de NaCl/100 g de substrato) por 12 dias. Em seguida, raízes e folhas de plantas estressadas e controle foram coletadas para extração de RNA e análise de qRT-PCR. Nos concentramos em um grupo pré-selecionado de genes que codificam enzimas antioxidantes chave (4) e moléculas redox (2) conhecidas por seu papel no sistema de defesa antioxidante da planta. Esses genes foram investigados por meio de qRT-PCR, análise filogenômica e caracterização estrutural e funcional adicional. Nossos resultados revelaram uma regulação positiva significativa de todas as quatro enzimas antioxidantes e ambas as moléculas redox especificamente nas folhas estressadas pelo sal, não nas raízes. Este estudo representa uma aplicação pioneira de RNA-Seq e qRT-PCR para desvendar a contribuição do sistema de defesa antioxidante para a notável resiliência da purslane ao estresse salino elevado.

Palavras-chave: beldroega; resistência; enzimas; estresse abiótico; transcritoma; qRT-PCR.

1 INTRODUCTION

A significant portion of agricultural land, around one-fifth, faces challenges due to salty or sodic soils. This issue affects over 100 countries worldwide, and its economic impact is substantial, with estimates suggesting annual losses of 27.3 billion US dollars due to reduced agricultural productivity (Negacz *et al*., 2022; Shahid *et al*., 2018). Salt stress disrupts water uptake by plants, mimicking drought; and most crops haven't adapted to salty environments and struggle to grow, even dying in moderately salty soil (100-200 mM NaCl); however, some plants have develop resistance to salt, and such resistance results from the combination of various mechanisms, making such trait complex and due to the combined action of multiple genes (Chele *et al*., 2021; El-Ramady *et al*., 2024).

Purslane (*Portulaca oleracea* L.) is a tough dicot plant that thrives in harsh environments, being nicknamed a "super accumulator" because it can absorb high amounts of

salt (Rodrigues *et al*., 2023; Silva *et al*., 2022). It is an example of a xerohalophyte species, as it can grow in both dry deserts (xerophyte) and salty soils (halophyte) (Ferreira *et al*., 2023). The accumulated knowledge about the development of this plant, together with its characteristics such as short life cycle, small size and convenient management, suggest purslane as a useful model species for research aimed at better understanding the resistance of plants to salinity stresses (Borsai *et al*., 2018).

Salt is an environmental stressors that can generate harmful free radicals in plant cells. To combat this threat, some plants have developed a sophisticated antioxidant defense system that works in two main ways: a) Enzymatic defenders: specialized enzymes, like catalase and superoxide dismutase, act like cellular firefighters, neutralizing different types of free radicals before they can cause damage; and b) Non- Enzymatic protectors: Alongside the enzyme team, various antioxidant compounds, such as vitamin C and carotenoids, directly scavenge free radicals, providing an extra layer of defense. This combined enzymatic and non-enzymatic approach ensures plants can effectively neutralize free radicals and maintain cellular homeostasis under stress conditions (Abogadallah, 2010; García-Caparrós *et al*., 2019; Hasanuzzaman *et al*., 2021; Isayenkov, 2019; Mushtaq; Faizan; Gulzar, 2020).

The biochemical and molecular underpinnings of purslane's anti-oxidative response to salinity stress remain largely unexplored. While studies have documented changes in the activity of antioxidant enzymes like superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, and glutathione reductase, alongside fluctuations in oxidative stress markers and non-enzymatic antioxidants (Borsai *et al*., 2020; He; Leng; Qin, 2023; Lim *et al*., 2007; Tang *et al*., 2020; Xing *et al.,* 2019; Yazici *et al*., 2007), a comprehensive transcriptome analysis specifically targeting the anti-oxidative defense machinery in purslane under saline conditions is, to our knowledge, absent from the current literature.

Building on previous work establishing a robust salt stress protocol for purslane, this study investigates the role of the anti-oxidative defense mechanism in adult purslane resistance to high salinity stress. We leverage multi-omics approaches to analyze the response of this salt-tolerant species (Rodrigues *et al*., 2023; Silva *et al*., 2022). We focus on genes coding for antioxidant enzymes and redox molecules, known to be part of this defense mechanism, and previously identified as responsive to salinity stress in young plants. Their expression in adult plant roots and leaves under high salinity is evaluated using qRT-PCR and RNA-Seq. Genes with strong evidence for a role in stress resistance undergo further structural, functional, and evolutionary analysis.

2 MATERIALS AND METHODS

A) Generation and use of a Reference Transcriptome (RT) from Young Purslane Plants:

All sequencing data for the generation of the Reference Transcriptome (RT) came from a previous study done by our group (Silva *et al*., 2022). The raw sequence data have been uploaded in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the BioProject PRJNA575830 and BioSample SAMN12911623 (Portulaca oleracea_B1—TaxID: 46,147).

The analysis pipeline used leveraged OmicsBox 1.3 for transcriptome analysis, and was already described in Silva *et al*. (2022). Quality control steps, including read filtering and low-quality base removal, were performed using FastQC and Trimmomatic. To construct a reference-free transcriptome assembly, we employed Trinity v2.8.5 and Bowtie2 v2.3.5.1 with default settings. After assembling the RT, the TransDecoder tool was used to predict coding regions. STAR mapped RNA-Seq data to the assembled transcriptome. Finally, gene and transcript expression quantification was achieved using HTSeq v0.9.0. All steps in the pipeline through OmicsBox 1.3 used default parameters, exception made to Trimmomatic, where the reads were maintained only if their average quality exceeded Q30 (Phred score) and minimum length surpassed 75 bases. All complete open reading frames (ORFs) were submitted to the GhostKOALA tool (Kanehisa *et al*., 2016) for annotation.

B) Plant material - samples for qRT-PCR analysis:

All experiments were carried out at the Plants Genetics and Biotechnology Laboratory - Embrapa Agroenergia, in Brasília, DF, Brazil (S - 15,732º, W - 47,900º). The B1 accession of purslane (*P. oleracea* L.) used in this study belongs to the Purslane Collection of Embrapa Agroenergia. Leaves and roots from control and salt stressed plants came from the study previously reported by Rodrigues *et al.* (2023). Twelve samples were collected - six from leaves and six from roots, and out of three plants per treatment, immediately immersed in liquid nitrogen, and then stored at − 80 °C until total RNA extraction. Total RNA was isolated from purslane samples using the Qiagen RNeasy® Plant Mini kit (QIAGEN, CA, USA) following the manufacturer's protocol. RNA quantity was measured using a Nanodrop Qubit 2.0 Fluorometer (Life Technologies, CA, USA), and RNA quality was evaluated with an Agilent Bioanalyzer Model 2100 (Agilent Technologies, Palo Alto, CA, USA).

Primers for qRT-PCR (Table 1) were designed using an in-house Python script. The script utilizes the pandas, biopython, primer3, and pydna libraries, implementing a complete pipeline for primer design and validation. The pipeline initially receives a table containing the genes of interest and a FASTA file with the reference transcriptome sequences. Next, the primer design for the genes of interest is performed using the primer3 library. Finally, in the validation step, the pydna library is used to verify whether the primers specifically anneal to the gene of interest and do not anneal to other genes non-specifically. For each primer pair, the library simulates annealing with each gene in the complete list. The primer pair is considered valid if it anneals to the gene of interest in only one location and does not anneal to any other gene. At the end of the pipeline, a list containing the primers that passed the validation is generated.

qRT-PCR analysis was performed using the SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen®) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The cycling conditions were: 95°C for 120 seconds (denaturation), followed by 40 cycles of 95°C for 5 seconds (denaturation) and 60°C for 30 seconds (annealing/extension) with fluorescence measurement during the second step. A melting curve analysis (95°C for 15s, 60°C for60s, 95°C for 15s) was included for specificity verification. Fluorescence data was collected at each cycle using StepOnePlus™ Real-Time PCR and analyzed with StepOnePlus™ software v2.3 (Life Technologies). Three biological replicates with three technical replicates per sample (nine reads per gene/treatment) were used for target genes. Housekeeping gene analysis used three biological replicates with two technical replicates per sample. The ΔΔCt method was employed to calculate relative gene expression levels (Livak; Schmittgen, 2001).

Table 1 - Primer pairs used for differential expression analysis of the 12 genes investigated by qRT-PCR.

Gene_ID	Primer F	Length_fwd	Primer R	Length_rev	PCR_Length	Enzyme or Redox Type
Purslane_DN3589_c1_g1_i12.p1	5'-ATGACAAGCTCCTCCAGTGC-3'	20	5'-TCTCTTTCGGAATCACGGCC-3'	20	267	Catalase
Purslane_DN11599_c0_g1_i2.p1	5'-CGAGATCGATGAGGACGACG-3'	20	5'-CCACAAACACCACCGGAAAC-3'	20	119	Glutaredoxin
Purslane_DN17160_c0_g1_i2.p1	5'-TACAACGTGACCGAGATGGC-3'	20	5'-ACCTGATGGCCGATGACATC-3'	20	140	Glutaredoxin
Purslane_DN126243_c0_g1_i1.p1	5'-CGGGGTTTGCAGTCCCTTAC-3'	20	5'-TGAACCGTGCCTAGATTACC-3'	20	109	Glutathione
Purslane_DN26161_c0_g1_i3.p1	5'-AAAGGGAGAGGAACAAGCCG-3'	20	5'-CGATTTGCAGGCACCTCTTG-3'	20	230	Glutathione
Purslane_DN13217_c0_g1_i3.p1	5'-GCTACGCATGCACTTCCATG-3'	20	5'-GTAGGTTGACCTCACTGGCC-3'	20	299	Peroxidase
Purslane_DN18979_c0_g1_i8.p1	5'-CAGGTATCTGGTCGGCCATC-3'	20	5'-TTCGCAGCACTTTCACTTGC-3'	20	346	Peroxidase
Purslane_DN47528_c0_g1_i1.p1	5'-GATCCTAAAGTCCCGGCTCG-3'	20	5'-CGGTATCGTTTGCGCTTGAG-3'	20	316	Peroxidase
Purslane_DN109806_c0_g1_i1.p1	5'ACTGCAACTTGGTGTGGACC-3'	20	5'-CGTGTTCTTCTGCAGTTTCCG-3'	21	127	Thioredoxin
Purslane_DN306_c0_g1_i5.p2	5'-AGGCTAACAAGCTGGTGGTG-3'	20	5'-ACACGACATGGGGGAACTTC-3'	20	108	Thioredoxin
Purslane_DN8014_c0_g1_i12.p2	5'-TCCATTTGTACCGTGGAGCC-3'	20	5'-ATGTGCTGGAAGAAGGGTCG-3'	20	210	Thioredoxin
Purslane_DN2303_c0_g1_i8.p1	5'-CCCCTCAGTGGACCAAACTC-3'	20	5'-AGCATTGCCTGTGGTTTTGC-3'	20	108	Superoxide Dismutase
Purslane_DN3230_c0_g1_i2.p1	5'-AGTCGAAGAAGCTGGTGGTG-3'	20	5'-GGGCACTCTCGGTCGTATAC-3'	20	205	Superoxide Dismutase

Source: Author.

C) Phylogenomics Analysis:

The evolution of detoxifying enzymes in halophytes and glycophytes was performed using OrthoFinder (v2.5+). To infer phylogenetic relationships among gene sets, pairwise sequence similarities from 68 species with sequenced genomes (13 halophytes and 55 glycophytes) was analyzed (Supplementary Table 1). The software was run with the parameters "-M msa" and "-I 1.8" for the method used to infer the gene tree and MCL inflation parameter, respectively.

D) Structural & Functional annotation:

In an initial characterization of the genes, we utilized the InterProScan tool (Paysan-Lafosse *et al*., 2022) to analyze gene sequences for functional domains, Gene Ontology (GO) terms, and protein families. MotifScan software (Su *et al*., 2018), available at Expasy (Duvaud *et al*., 2021), was employed to further investigate functional domains, searching all available databases. Domain information was then used to generate illustrations with IBS Illustrator software (Liu *et al*., 2015).

For tertiary structure prediction, an interactive version of AlphaFold software was employed (Jumper *et al*., 2021). Finally, subcellular localization of each gene was predicted using DeepLoc-2.0 (Thumuluri *et al*., 2022) and Plant-mPLoc (Chou; Shen, 2010).

3 RESULTS AND DISCUSSION

Our analysis of RNA-Seq data from Silva *et al*. (2022) identified 56,667 complete ORFs within the Young Purslane's Reference Transcriptome (YPRT). All ORFs were captured in FASTA files, containing both nucleotide and protein sequences. These sequences facilitated both functional annotation of the proteins and primer design for targeted amplification of the corresponding nucleotide sequences.

The RNA-Seq data from Silva *et al.* (2022) was further utilized for differential expression (DE) analysis. This analysis compared the leaf transcriptome of stressed plants to controls at two time points: one and four days after initiating salinity stress. Additionally, DE analysis was performed within the control and stressed groups themselves, comparing gene expression at one day to four days. Importantly, only complete ORFs were included in the differential expression analysis. Finally, the annotated YPRT, having the DE analysis results linked to it, was employed for keyword-based searches.

Accordingly to Rajput *et al*. (2021), several enzymes, particularly those involved in the Haber-Weiss reaction and the Foyer-Halliwell-Asada pathway, help plants combat oxidative stress by scavenging harmful reactive oxygen species (ROS) like H_2O_2. These enzymes utilize the reducing power of NADPH to break down H_2O_2, preventing cellular damage, and are part of the so called antioxidant defense system in plants. Among those enzymes, the most well-known are: Catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione reductase (GR; EC 1.8.1.7), glutathione S-transferases (GST; EC 2.5.1.18), ascorbate peroxidase (APX; EC 1.11.1.11), peroxidase (POX; EC 1.11.1.7), and superoxide dismutase (SOD; EC 1.15.1.1).

A search for "Catalase" within the annotated YPRT identified five proteins across two genes. Three isoforms (variant forms) originated from one gene, and two isoforms stemmed from another. All five proteins shared the same K0 identifier, K03781. Differential expression analysis using RNA-Seq data revealed that the three isoforms from the first gene were positively regulated at day 1 but showed no change at day 4 after salinity stress. Conversely, the two isoforms from the second gene displayed positive regulation only at day 4, with no change at day 1. For further investigation using qRT- PCR, the protein with the highest expression increase was selected. qRT-PCR analysis on adult purslane plants under high salinity stress showed that this chosen protein's expression was halved in the roots but increased fourfold in the leaves (Figure 1).

The structural and functional annotation of this putative CAT enzyme positively regulated in the leaves of adult purslane plants under salinity stress revealed that its biological process is related to the response to oxidative stress (GO:0006979). Its molecular function is related to catalase activity (GO:0004096) and heme binding (GO:0020037). It is a member of the Heme-containing, monofunctional catalase protein family (IPR018028) and Heme-containing, monofunctional catalase, clades 1 and 3 (IPR024711). It had conserved domains related to the catalase enzyme (Figure 2). Both software indicated that this protein is located in the peroxisome with 95% certainty.

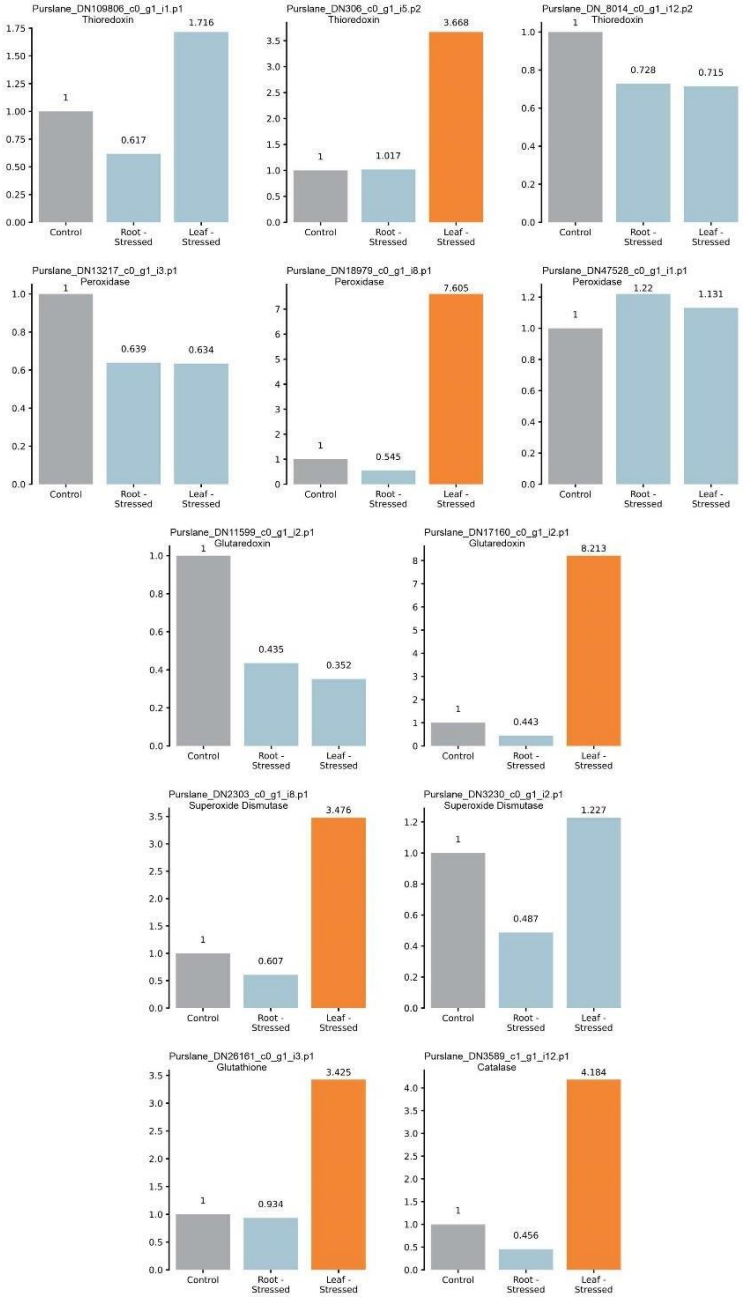
CAT, an antioxidant enzyme, exhibits diverse functions across plant tissues, all related to managing cellular oxidative stress (Vuosku *et al*., 2015). Within peroxisomes, organelles rich in ROS, CAT works alongside SOD, ascorbate, and monodehydroascorbate (MDA) to neutralize these damaging molecules (Vuosku *et al*., 2015). Interestingly, distinct CAT classes have specialized roles: Class I tackles H_2O_2 in photosynthetic tissues during photorespiration, Class II aids vascular development and lignification triggered by ABA and senescence, and Class III is concentrated in flowers and fruits with minimal leaf presence (Rajput *et al*., 2021;

Vuosku *et al*., 2015). Accordingly to the InterProScan tool, this putative CAT, positively regulated in adult purslane in response to salinity stress, represents clades 1 and 3 of the mono-functional, haem-containing catalases.

Glutathione exists in two different states, i.e., reduced (GSH) and oxidised (GSSG), and regulates the gene expression by interacting with another redox molecule, such as thioredoxin, glutaredoxin, peroxiredoxins and GSSH (Rajput *et al*., 2021). Glutathione peroxidase (GPX; EC 1.11.1.9), glutathione reductase (GSR; EC 1.8.1.7), and glutathione S-transferases (GST; EC 2.5.1.18) are well known antioxidant enzymes in plants (Hasanuzzaman *et al*., 2017; Rajput *et al*., 2021). A search for the keyword "Glutathione" within the annotated YPRT identified three GPX and four GSR, all non- differentially expressed. In the case of GST - glutathione S-transferase enzyme class (EC: 2.5.1.18), there were 67 positive hits. These GST proteins stemmed from 35 distinct genes, with the number of isoforms per protein ranging from one to six. Notably, 65 glutathione S-transferases shared the same K0 identifier, K00799; one was K23790 and the other K13299. Differential expression analysis revealed that 37 of these enzymes displayed changes in expression in response to the stress conditions. Among them, the protein exhibiting the most significant increase in expression was chosen for further analysis. The qRT-PCR results demonstrated a 3.4-fold positive regulation of this specific enzyme in the leaves, while root expression showed only a minor decrease (Figure 1).

Structural and functional analysis of a salinity-responsive GST protein in adult purslane leaves revealed its involvement in glutathione metabolism (GO:0006749) and glutathione transferase activity (GO:0004364). It had conserved domains related to the GST enzyme (Figure 2). Belonging to the Glutathione transferase (IPR040079) and Glutathione S-transferase Omega/Tau-like (IPR045073) families, this protein is likely localized in the cytoplasm (75% certainty). Notably, GSH plays a critical role in managing ROS levels and metabolizing electrophilic molecules (xenobiotics) through redox reactions of its thiol group (Ribas; García-Ruiz; Fernandez-Checa, 2014). Accordingly to the InterProScan tool, this putative GST is a member of the Glutathione S-transferase Omega/Tau-like (IPR045073) family.

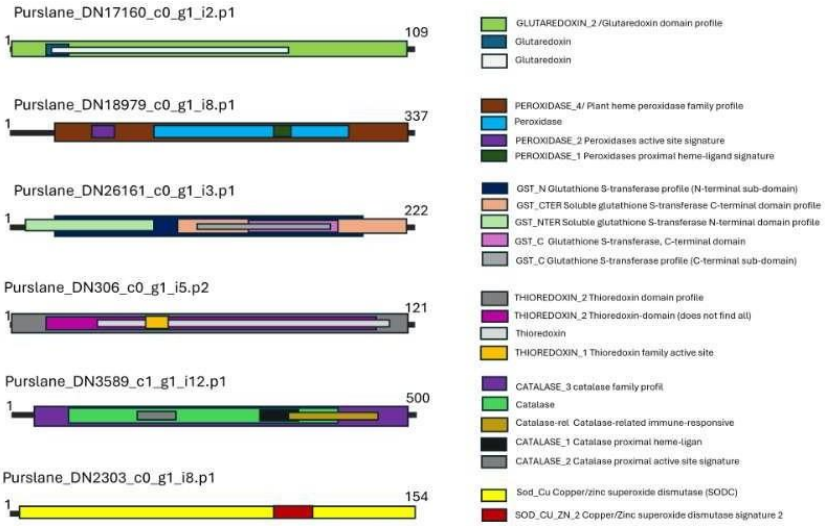
Figure 1 - Expression profile analysis of 12 salt stress-responsive genes selected from transcriptome analysis of young purslane (*P. oleracea*) plants, performed using qRT-PCR.



Note: Internal control gene: EgEfMPOB00119, 40S mRNA of ribosomal protein S23, a constitutive gene in *E. guineensis*.

Source: Author.

Figure 2 - Structural characterization of salt stress-responsive enzymes identified from the transcriptome of leaves of young purslane (*P. oleracea*) plants.



Note: Domains of the 6 proteins with significant expression differences. Each colored box represents a distinct domain and its position within the protein. The numbers on each line indicate the total amino acid length.

Source: Author.

The YPRT database identified 51 putative APX proteins upon searching for the EC number "EC:1.11.1.11." Interestingly, all shared the same K0 identifier (K00434), suggesting high sequence similarity. These proteins originated from 12 APX genes, with the number of isoforms per gene ranging from one to seven. DEA revealed significant changes in expression for eleven isoforms from six genes in the leaves of young purslane plants under salinity stress. Notably, four isoforms were upregulated, while eight were downregulated in response to salt stress. All isoforms downregulated experienced a decrease ≥70% after one day under stress, which remained at four days under stress. Among the upregulated ones, two isoforms from the same gene experienced large increase in expression after one day under stress, but such increase was almost completely lost three days afters, or four days under stress.

A search for peroxidases (POX) using the EC number "EC:1.11.1.7" in the YPRT database yielded 131 putative POX proteins. The majority (128) shared the K00430 identifier, while three had a distinct K19511 identifier. Interestingly, none of the K19511 isoforms showed DE in purslane leaves under salt stress. The remaining 128 K00430 isoforms originated from 41 putative POX genes, with a varying number of isoforms per gene (1-16). DE analysis identified 39 DE isoforms under salinity stress, with roughly equal numbers being upregulated (19) and downregulated (20). Three distinct POX isoforms were chosen for further investigation: two positively regulated and one negatively regulated. qRT-PCR analysis

revealed contrasting expression patterns: The first putative POX showed a substantial decrease in expression, down to about 60% in both roots and leaves. The second one exhibited a decrease in expression to 55% in roots, but a significant 7.6-fold increase in the leaves. The third protein displayed a modest increase in expression, by 22% and 13% in roots and leaves, respectively (Figure 1).

Functional analysis of the putative POX from purslane revealed its involvement in the response to oxidative stress (GO:0006979) through its hydrogen peroxide catabolic activity (GO:0042744). The presence of conserved heme binding (GO:0020037) and peroxidase activity (GO:0004601) domains (Figure 2), coupled with a predicted extracellular localization (65% certainty), suggests classification as a class III plant secretory peroxidase. These enzymes play diverse tissue-specific roles, including detoxification of ROS in the chloroplast and cytosol, oxidation of xenobiotics, and contribution to cell wall biosynthesis (Li; Poulos, 1994; Rajput *et al*., 2021). Accordingly to the InterProScan tool, this putative peroxidase is a member of the Plant peroxidase (IPR000823) family.

A search for "Superoxide Dismutase" in the annotated YPRT identified 20 proteins originating from eleven genes. The number of isoforms per protein ranged from one to five. These proteins belonged to different superoxide dismutase families, reflected by their varying K0 identifiers (K04565, K04564, and K16627). Differential expression analysis revealed that eight of these proteins showed changes in expression under salinity stress. Two of these differentially expressed proteins were chosen for further analysis – from K04565 and K04564. The qRT-PCR results demonstrated that both proteins displayed decreased expression in the roots, down to approximately 61% and 49% respectively. Conversely, their expression levels increased in the stressed leaves, with fold-changes of 3.48 and 1.23 (Figure 1).

Functional analysis of the putative salinity-responsive Cu/Zn superoxide dismutase (SOD) that showed the highest fold-change in purslane leaves revealed its involvement in the superoxide anion metabolic process (GO:0006801). This enzyme exhibits metal ion binding activity (GO:0046872), specifically binding copper ions (GO:0005507). Consistent with its function, the protein belongs to the Superoxide dismutase (Cu/Zn) / copper chaperone for superoxide dismutase (IPR024134) family and possesses conserved SOD enzyme domains (Figure 2). Subcellular localization prediction software identified the protein with a 78% certainty to be localized in both the cytoplasm and chloroplast compartments. Accordingly to the InterProScan tool, this putative SOD is a member of the Superoxide dismutase (Cu/Zn) / superoxide dismutase copper chaperone (IPR024134) family.

Superoxide is a byproduct of oxygen metabolism and, if not regulated, can cause various types of cellular damage. The compartmentalization of different SOD forms within the plant body allows for more effective stress neutralization. Cu-Zn SODs have significantly different electrical properties than the other classes of SOD (Valentine & Zittin Potter, 2005); being concentrated in the chloroplast, cytosol, and, in some cases, the extracellular space. Notably, Cu-Zn SODs provide less protection than Fe SODs when located outside the chloroplast, which is not the case for the enzyme found in this study.

Plant glutathione peroxidases (GPX) utilize the reducing power of a molecule called thioredoxin to convert harmful ROS like hydrogen peroxide and hydroperoxyl radicals into harmless water and lipid alcohols, respectively (Rajput *et al*., 2021). An analysis of the annotated YPRT identified 42 proteins upon searching for the keyword "Thioredoxin." These proteins originated from 23 genes, with the number of isoforms per protein ranging from one to eight. All 42 proteins shared the same K0 identifier, K03671. Among these, 32 showed no change in expression (non-differentially expressed) under any of the stress conditions tested. From the remaining ten, three distinct genes/proteins were chosen: one positively regulated and two negatively regulated by salt stress. qRT- PCR results revealed varying expression patterns: The first protein showed a decrease to 62% in roots, but a 72% increase in leaves. The second protein's expression remained unchanged in the roots but exhibited a 3.7-fold increase in the leaves of adult purslane plants under high salinity stress. The third protein displayed a decrease in expression to about 72% in both roots and leaves (Figure 1).

In the case of this third putative thioredoxin (Trx) from purslane, the functional analysis revealed protein-disulfide reductase activity (GO:0015035), characteristic of the Thioredoxin family (IPR005746). Subcellular localization prediction softwares suggest a cytoplasmic location with 65% certainty. Thioredoxins are ubiquitous small redox proteins (12 kDa) that play a critical role in maintaining cellular redox homeostasis in all living organisms. They achieve this by catalyzing the reduction and oxidation of disulfide bonds in other proteins through a rapid and reversible thiol-disulfide exchange reaction. This reaction is essential for regulating diverse physiological processes, including internal biological metabolism and the plant's response to environmental stress (Cook & Hogg, 2013). Therefore, this putative purslane Trx likely plays a role in stress tolerance by modulating protein function through redox signaling pathways.

Trxs function within a complex redox network, acting as electron donors for glutathione peroxidase (GPX), and facilitating its reduction of hydrogen peroxide. Subsequently, oxidized Trx is regenerated by thioredoxin reductase (TR) using NADPH as an electron source.

However, Trx activity can be modulated by glutathionylation, a process where a glutathione molecule is attached to a cysteine residue, leading to inactivation. Conversely, deglutathionylation by glutaredoxin reactivates Trx. These intricate interactions between Trxs, GPXs, TRs, glutaredoxins, and glutathione highlight the dynamic nature of the cellular redox regulatory network, warranting further investigation to fully understand their interplay in stress tolerance mechanisms (Cook; Hogg, 2013; Ribas; García-Ruiz; Fernandez-Checa, 2014). Our analysis of young purslane leaves under salinity stress did not reveal any differentially expressed GPX genes. Consequently, qRT-PCR analysis for GPX was not performed. Therefore, this study cannot establish a direct interaction between GPX and Trx.

At last, a search for the keyword "Glutaredoxin" in the annotated YPRT yielded 44 positive results, corresponding to 23 genes/proteins. The number of isoforms varied between one and five per protein. All 44 proteins shared the same K0 number, K03676. Of these, 25 showed no differential expression under any of the analyzed scenarios. Among the remaining 19, two distinct genes/proteins were selected, one positively and one negatively regulated in response to salt stress. qRT-PCR results revealed that both selected proteins had their expression reduced to 44% in the roots. However, in the leaves, one protein was negatively regulated to 35%, while the other showed an eightfold increase in adult purslane plants under high salt stress (Figure 1).

The results for the putative glutaredoxin protein did not reveal any GO terms despite demonstrating the presence of conserved domains related to the corresponding redox molecule Glutaredoxin (Figure 2). This protein was predicted to be localized in the cell membrane with 60% certainty. Additionally, the Plant-mPLoc software identified it as being present in the chloroplast. Glutaredoxins (GRXs) play a multifaceted role in cellular redox homeostasis in plants, acting as essential electron donors for ribonucleotide reductase in GSH-dependent deoxyribonucleotide synthesis, a critical process for DNA replication (Wu *et al*., 2017). Besides, it exhibits remarkable versatility, reducing a diverse array of substrates, including disulfides in proteins involved in metabolism and defense mechanisms. The redox state of the glutathione pool, GSH and its oxidized form GSSG, is a key regulator of gene expression. GSH interacts with other redox molecules, including peroxiredoxins (Prxs), Trxs, and GRXs themselves, influencing their activity and impacting various cellular processes. This intricate network involving GRXs and the GSH pool allows cells to tightly regulate gene expression and maintain redox balance essential for proper function.

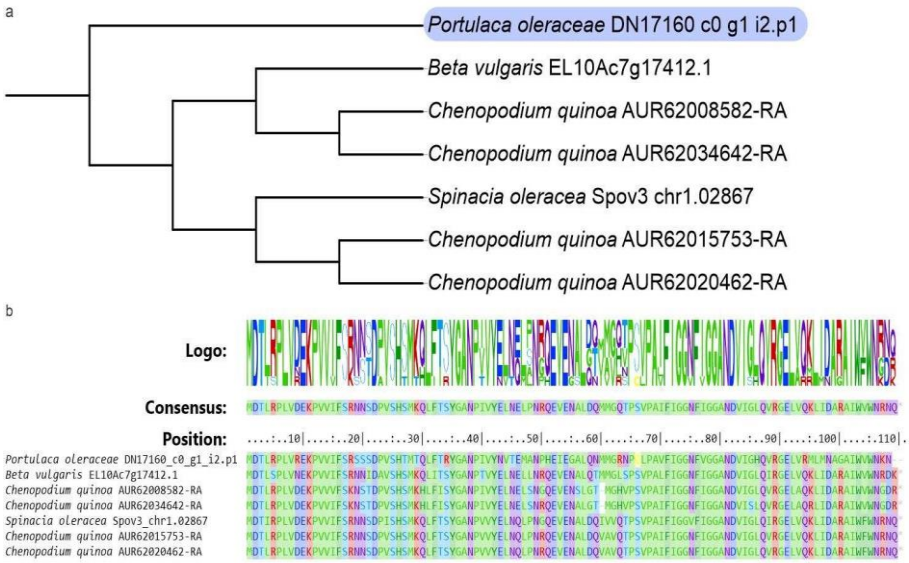
Our analysis of four antioxidant enzymes and two redox molecules (Supplementary Table 2) revealed that at least one protein from each category displayed a significant increase

in expression within the leaves of adult purslane plants under high salinity stress. This observation suggests that the plant's antioxidant defense mechanism plays a crucial role in maintaining cellular balance (homeostasis) in the leaves under these harsh conditions. Conversely, the data did not provide evidence for the involvement of this mechanism in root-level stress resistance.

A phylogenomic analysis using a database of proteins from 68 plant species was conducted. OrthoFinder software identified groups of genes (orthogroups) with a common ancestor. Notably, the putative GRX protein that showed an eightfold increase in adult purslane plants under high salt stress – Purslane_DN17160_c0_g1_i2.p1 - clustered with only four close relatives from *Chenopodium quinoa*, one from *Beta vulgaris* and one from *Spinacia oleracea*, suggesting a more distinct evolutionary path compared to other genes (Figure 3). Conversely, the class III plant secretory peroxidase that showed almost eightfold increase belonged to a much larger orthogroup, indicating a more conserved protein sequence across plant species. The GST and Trx proteins formed smaller clades within larger orthogroups, containing relatives from *C. quinoa*, *S. oleracea*, and *B. vulgaris*. This suggests a balance between shared ancestry and some unique evolutionary changes for these purslane genes. Similarly, the CAT protein clustered with *Carica papaya* and *Manihot esculenta* within a larger group, highlighting shared ancestry beyond the closely related Amaranthaceae family (including *C. quinoa*, *S. oleracea*, and *B. vulgaris*). Finally, the SOD protein formed a clade with proteins from *P. oleracea*, suggesting a closer evolutionary relationship within this species.

The analysis of protein tertiary structures revealed distinct structural features among the studied putative proteins. GRX protein is predominantly composed of α- helices (40%), with a minor contribution from β-sheets (around 3 folds). The remaining portion consists of irregular coils and extended segments. POX shares a similar composition, with a higher α-helix content (main component) and approximately 30% β- folds. Notably, POX possesses a heme group within its structure. GST adopts a more defined structure with three straight α-helix segments and three β-folds. Trx resembles GST, containing three straight α-helix segments but with a lower β-sheet content (10%). Both putative proteins have irregular coiled and extended segments. CAT exhibits a higher α-helix content (45%) compared to β-folds (around 5 folds) and possesses a significant portion of irregular regions (50%). Similar to POX, CAT also contains a heme group. Finally, SOD stands out for its complete lack of α-helices. It primarily consists of β-folds (35%) with the remaining portion being irregular coils and extended segments (Figure 4).

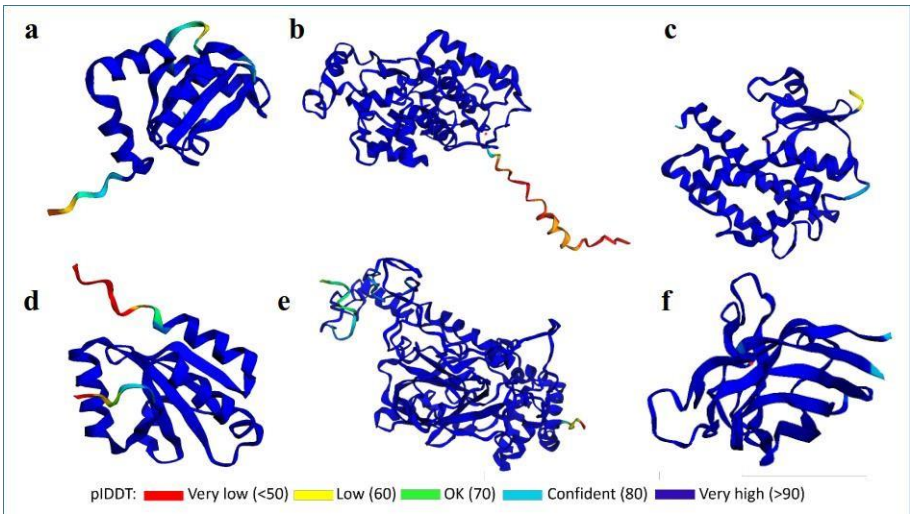
Figure 3 - Glutaredoxin (Purslane_DN17160_c0_g1_i2.p1) found in a single clade of an orthogroup exclusive to halophyte species.



Note: A – Exclusive cladogram of related species. B – Multiple sequence alignment of the corresponding sequences of each species.

Source: Author.

Figure 4 - Protein tertiary structure prediction using the platform AlphaFold.



Note: A – Glutaredoxin (Purslane_DN17160_c0_g1_i2.p1); B – Peroxidase (Purslane_DN18979_c0_g1_i8.p1); C – Glutathione (Purslane_DN26161_c0_g1_i3.p1); D – Thioredoxin (Purslane_DN306_c0_g1_i5.p2); E - Catalase (Purslane_DN3589_c1_g1_i12.p1); and F – Superoxido Dismutase (Purslane_DN2303_c0_g1_i8.p1).

Source: Author.

4 CONCLUSION

Catalase (EC 1.11.1.6), glutathione peroxidase (1.11.1.9), glutathione reductase (1.8.1.7), glutathione S-transferases (2.5.1.18), ascorbate peroxidase (1.11.1.11), peroxidase (1.11.1.7), and superoxide dismutase (1.15.1.1) are the most well-known groups of enzymes of the so called antioxidant defense system that helps plants combat oxidative stress by scavenging harmful ROS, and preventing cellular damage. The combination of RNA-Seq and qRT-PCR tools allowed to show that enzymes from four of those groups, and two redox molecules, displayed a significant increase in expression within the leaves of adult purslane plants under high salinity stress, but not in the roots. Such results are strong evidences that the plant's antioxidant defense mechanism plays a crucial role in maintaining cellular balance (homeostasis) in the leaves of adult purslane plants under these harsh conditions. Phylogenomic analysis suggests independent evolutionary trajectories for each of the four enzymes and two redox molecules. The putative GRX protein clustered with only four close relatives, all from plant species within the eHALOPH database (Santos *et al*., 2016). This database focuses on halophytes and other salt-tolerant plants. Compared to the other putative proteins analyzed, this suggests a more distinct evolutionary path for the gene coding for the GRX protein. Among the remaining proteins, two displayed a balance between shared ancestry with other species and unique evolutionary changes specific to their lineage, one shared a common ancestor beyond the closely related Amaranthaceae family, suggesting an even deeper evolutionary connection, and one belonged to a much larger orthogroup, indicating a more conserved protein sequence across plant species. The final one showed a remarkably close relationship with orthologs within the purslane genus itself, implying a more recent divergence.

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Supplementary Table 1. List of the 68 plant species, which genome was employed in the Phylogenomics analysis, indicating whether they are present or not in the eHaloph database (Santos *et al*., 2016).

Species	eHaloph	Species	eHaloph	Species	eHaloph	Species	eHaloph
Anacardium occidentale	No	*Cof fea arabica*	No	*Lotus japonicus*	No	*Prosopis cineraria*	Yes
Ananas comosus	No	*Cucumis sativus*	No	*Malus domestica*	No	*Prunus persica*	No
Apium graveolens	Yes	*Daucus carota*	Yes	*Manihot esculenta*	No	*Ricinus communis*	No
Arabidopsis thaliana	No	*Dioscorea alata*	No	*Medicago truncatula*	No	*Setaria italica*	No
Arachis hypogaea	No	*Elaeis guineensis*	No	*Musa acuminata*	No	*Sinapis alba*	No
Asparagus of ficinalis	Yes	*Eleusine coracana*	Yes	*Olea europaea*	No	*Solanum lycopersicum*	No
Beta vulgaris	Yes	*Eragrostis curvula*	Yes	*Oryza sativa*	No	*Solanum pennellii*	Yes
Brassica oleracea	Yes	*Eutrema salsugineum*	Yes	*Panicum virgatum*	Yes	*Solanum tuberosum*	No
Brassica rapa	No	*Fragaria vesca*	No	*Paspalum vaginatum*	Yes	*Sorghum bicolor*	No
Carica papaya	No	*Fragaria x ananassa*	No	*Phaseolus lunatus*	No	*Spinacia oleracea*	No
Carya illinoinensis	No	*Gliricidia sepium*	No	*Phaseolus vulgaris*	No	*Theobroma cacao*	No
Castanea dentata	No	*Glycine max*	Yes	*Phaseolus acutifolius*	No	*Triticum aestivum*	No
Caulanthus amplexicaulis	No	*Glycine soja*	No	*Phoenix dactylifera*	Yes	*Vaccinium darrowii*	No
Chenopodium quinoa	Yes	*Helianthus annuus*	No	*Poncirus trifoliata*	No	*Vigna unguiculata*	No
Cicer arietinum	No	*Hordeum vulgare*	Yes	*Populus euphratica*	Yes	*Vitis vinifera*	No
Citrus clementina	No	*Lactuca sativa*	No	*Portulaca oleraceae*	Yes	*Zea mays*	No
Citrus sinensis	No	*Lepidium sativum*	No	*Prosopis alba*	Yes	*Zostera marina*	Yes

Source: Author.

Supplementary Table 2. mRNA sequences of the four antioxidant enzymes and the two redox molecules from purslane that belongs to the antioxidative defense mechanism acting against salinity stress.

Transcript Name	Sequence
Purslane_DN306_c0_g1_i5.p2	ATGGCGGAAGAGGGACAAGTGATCGCTTGTCGGTCGACTGACCACTGGAAACAGCTTCTTGATCAAGCCAAACAGGCTAACAAGCTGGTGGTGGTTGATTTCACTGCTACATGGT GTGGCCCGTGTCGTTTCATTGCCCCTATTCTTGAAGATTTTGCAAAGAAGTTCCCCCATGTCGTGTTTGTCAAGGTGGATGTTGATGAGCAGAAGCCTATTGCTGAGGAGTGGAAG GTGGAGGCAATGCCAACCTTTGTCTTCCTGAAAGATGGACAGGAAATTGAGAGGATTGTCGGGGCCAACAAAGACGGGCTCTTGGCTGCCATAACAAAGCATGCAAGTGAGGCC ACAGTCAATGCTTAA
Purslane_DN18979_c0_g1_i8.p1	ATGAGGAACAATACCAACCTTCTTAACCCTCTTATCCTTGTTTCTTGTTGCTTTGCCCTCTCCTACACTCTACTAATTTCGGCCCAACTCGATTACAGATTCTACGACCAAACTTGCC CTAATCTAACCAGGATTGTCCGATCAGGTATCTGGTCGGCCATCTCTAACGACACTAGGATGGCCGCCTCTCTACTGCGACTTCACTTCCATGATTGCATCGTCAATGGTTGTGATG GGTCGATACTACTAGACGATACAAGCACAATGACAGGAGAAAAGGGCGCGAATCCGAACAAAAACTCAGCAAGAGGGTTCGAGGTGATCGATGCAATCAAGGTCGCGGTTGAG CAAGCTTGCCCTTCTACTGTGTCTTGCACTGATATCTTAGCTCTTGCTGCTAGGGAGTCTGTTTTCTTGACAGGAGGGCCATGCTACCAGTTACCCATGGGTCGTAGAGATGGGCTG ACAGCAAGTGAAAGTGCTGCGAATCAACAAATACCGTCGCCTTTTGAGCCCTTGGACAATATCACTCTCAAATTCACGTCCAAAGGACTTGACCAAAAAGACGTCGTTGTGCTCTC TGGTGCTCATACAATTGGATTTGCACAATGCATTACATTCAAATCAAGGCTCTTCGACTTCGACGGGACAGGGAAGGCAGACCCTTCCATGGATGCATCCTTGTTAAATAACCTCC AACAAACATGCCCTAATCAAGCCAACTCCAACACCAATTTGGCACCGTTGGATCGTCTAACGAAGAATAGATTCGACAATCTCTACTACAACAACCTCGTCAACAACTCCGGGGT GTTGCAATCAGACCAGGCTCTCATGGGGGATAATGACACTATGCCAATGGTGGTGAATTACAGCAAGTACCCTTTTTTGTTTTACAGGGACTTTGGAGTGTCGATGGCGAAATTGG GTAATGTGGGTGTTTTGACGGGGCAAAATGGGGAAATCAGGAAGAATTGTAGAGTTGTGAACAACTAA
Purslane_DN17160_c0_g1_i2.p1	ATGGATACCCTAAGGCCACTGGTGAGAGAAAAGCCAGTAGTGATATTCAGCCGGAGCAGTTCGGACCCAGTGAGCCACACCATGACCCAACTGTTCACAAGGTACGGTGCGAAC CCTATTGTTTACAACGTGACCGAGATGGCTAACCCGCACGAAATCGAGGGTGCTCTTCAGAACATGATGGGCCGTAACCCGTGCTTGCCGGCCGTCTTCATCGGTGGGAATTTCGT CGGTGGTGCTAACGATGTCATCGGCCATCAGGTCCGAGGGGAACTTGTTAGAATGCTCATGAATGCTGGAGCTATTTGGGTCTGGAACAAGAACTAA
Purslane_DN2303_c0_g1_i8.p1	ATGGGGAAGGCTGTTGCAGTCTTGACTGGCACTGAGGGTGTCTCTGGAACTGTCCAATTCACCCAAGAAGGAGATGGTCCCACAACTGTAACTGGAACCATCTCTGGTCTCAAGC CTGGGCTCCATGGATTCCACATTCATTCTCTTGGTGACACAACTAATGGCTGCATGTCAACTGGACCTCACTTCAACCCAGAAGGAAAAGAACATGGTGCTCCTGAGGATGATGTT CGCCATGCTGGTGACCTTGGAAATGTCACTGCTGGTGATGATGGTACTGCTACCTTCACAATAATTGACAAGCAGATTCCCCTCAGTGGACCAAACTCAATTGTTGGGAGAGCTGT TGTGGTCCATGCTGATCCTGACGATCTTGGGAGGGGTGGCCATGAACTCAGCAAAACCACAGGCAATGCTGGCGGAAGAGTGGCTTGCGGTGTTATCGGTCTTCAAGGTTAA
Purslane_DN26161_c0_g1_i3.p1	ATGGCGGGCGTTGTATTGCTTGACTGCTGGGCGAGCATGTTCGGAATGCGTGCCAGAATCGCCCTCGCTGAGAAAGGCGTCGACTACGAGTACAGAGAGGAAAATCTCCGCAACA AGAGCGATTTGCTACTCAAGATGAACCCGGTTCACACCAAAATCCCGGTTCTCATCCACAAGGACCGGCCCATCTGTGAGTCGCTCATCATTGTTCAGTACATCGATGAGGTTTGG AATCACAATAGCCCTTTGCTTCCTTCTGACCCTACCAGCGAGCACAGGCTCGATTCTGGGCTGATTTTGTTGATAAGAAGATATACGATAGCGGAAGAAGGATTTGGACAAGAAA GGGAGAGGAACAAGCCGCAGCAAGACAAGACTTCATAGACGCGCTAAAACTACTGGAGCAAGAGCTTGGGGACAAGCCTTACTTTGGAGGGGACAAGATCAACTACGTCGACAT CGCATTGGTGCCATTTTACAGCTGGTTTTACTCGTACGAGACATTCGGAAATTTCAAGGTAGAAGAGAGTTGTCCGAAACTGGCCCAATGGGCCAAGAGGTGCCTGCAAATCGAG AGTGTGGCTAAGGCTCTCCCTGATGACAAGAAGATTTATGAGTTCGTTGTTGAACTCAGGAAGAAGTATGGCATTGAGTGA
Purslane_DN3589_c1_g1_i12.p1	ATGGATCCCTCCAAGCATCGTCCATCAAGTTCATTCAATACCCCGTTCTGGACAACAAATTCTGGTGCACCAGTATGGAACAACACCTCTGCCATGAGCCTTGGATCGCGAGGACC GGTGCTACTGGAAGACTACCACCTGATCGAAAAAATCACCAACTTCCACCGCGAGCGCATCCCCGAGCGGATTGTCCACGCTCGGGGCGCCTCGGCAAAGGGCTTCTTCGAAGTA ACCCACGACATAACCCACCTAACATGCGCGGACTTTCTGCGCGCCCCGGGGGTCCAAACCCCCGTGATCGTCCGCTTCTCAACGGTCATCCACGAGAAAGGCAGCCCGGAGACGA TCCGCGACCCCCGGGGCTTCGCGACGAAGTTCTACACCCGGGAAGGGAACTTCGACATCGTCGGGAACAACTTCCCGGTGTTCTTCATCCGGGACGGGATGAAGTTTCCCGACGT CATCCACGCGTTCAAACCTAATCCTAAGTCCCACATCCAAGAGTACTGGAGAGTGTTGGATTTCTGCGCACATCATCCGGAGAGTTTGAATACGTTTGCGTGGCTGTTTGATGACG TCGGGGTGCCGACGGATTACAGGCATATGGAGGGGTTTGGGGTGAATACGTTTAGTTTGATTAATAAGGCTGGGAAAGTGCATTATGTCAAGTTTCATTGGAAGCCGACGTGTGG GGTGAAGAGTTTGTTGGAGGAGGAGGCTGTTAGGGTTGGGGGGTTGAATCATAGCCATGCTACTCAGGATCTATATGAGTCCATTGCTGCAGGAAACTACCCGGAATGGAAATTG TACATCCAGGTAATGGACCCAGCCGACGAGGACAAGTACGACTTCGACCCACTCGACATGACCAAGATCTGGCCGGAAGACCTGGTGCCCCTGCAACCCGTAGGGCGCATGGTGC TCAACCGGAATGTCGACAACTTCTTCGCCGAGAACGAGCAGCTGGCATTCAGCCCCGGGGTGGTGGTCCCCGGAATCTACTACTCCGATGACAAGCTCCTCCAGTGCCGTGTCTTC GCTTATGGCGACGCCCAGAGATACCGTCTTGGGGCTAACTACATGATGCTCCCTGTCAATGCACCCAAATGTGCCCACCATAACAACCAGTATGATGGTGTCATGAACTTCATGCA TAGGGATGAAGAGATTGACTATTTCCCGTCAAAGTTTGACCGTGTTCGTGAGGCGGAGAAATACCCCATTATTACTAAGGTTTACACTGGCAAGCGGGAAAAGGCCGTGATTCCG AAAGAGAACAACTTCAAGCAACCCGGAGATAGATATCGATCTTGGGACCCTGCTAGGCAAGAGAGGTACATTAAAAGATGGGTGGACGTTCTAGCTGATCCTCGTGTGACTCTTG AACTACGAAACATTTGGATCTCTTACTTGTCCGAGAGTGACAAATCTTTGGGGATGAAGGTAGCAACCAGACTGAACATGAGGCCAACCATGTGA

Source: Author.

Artigo 3 - Elaborado conforme a norma NBR 6022	
Título do artigo:	Unraveling the Intricate Antioxidant Response of *Gliricidia sepium* (Jacq.) Kunth to Extreme Salinity Stress: A Predominant Role of the Root System
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UNIVERSIDADE FEDERAL DE LAVRAS

ARTIGO 3 - UNRAVELING THE INTRICATE ANTIOXIDANT RESPONSE OF *GLIRICIDIA SEPIUM* (JACQ.) KUNTH TO EXTREME SALINITY STRESS: A PREDOMINANT ROLE OF THE ROOT SYSTEM

DESVENDANDO A INTRINCADA RESPOSTA ANTIOXIDANTE DE *GLIRICIDIA SEPIUM* (JACQ.) KUNTH AO ESTRESSE SALINO EXTREMO: UM PAPEL PREDOMINANTE DO SISTEMA RADICULAR

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Abstract: This study delves into the intricate interplay of the antioxidant defense system in *Gliricidia sepium* (Jacq.) Kunth acclimated to extreme salinity stress. RNA-Seq analysis of leaves revealed no significant changes in core antioxidant enzyme isoforms (APX, CAT, DHAR, GSR, SOD) but identified a down-regulated monodehydroascorbate reductase (MDHAR) isoform in adapted plants. Glutathione S-transferase (GST) isoforms displayed diverse expression patterns, with one exhibiting contrasting responses within a single gene (down-regulation in short-term stress, up-regulation in long-term stress). qRT-PCR validation of seven isoforms across various enzymes and redox molecules (performed in both leaves and roots under short-term and long-term stress) further unveiled the complexity. Peroxidase (POX) isoforms exhibited contrasting expression between leaves (down-regulation) and roots (up-regulation). Similarly, a GST isoform showed contrasting behavior between leaves and roots during adaptation. Notably, a glutathione peroxidase (GPX) isoform displayed reversed age-related down- regulation in adapted plant leaves, with qRT-PCR revealing upregulation in both leaves and roots under stress. Glutaredoxin 3 isoforms displayed contrasting down-regulation patterns in leaves, while both isoforms showed increased expression in roots under stress. Interestingly, a Thioredoxin isoform with remarkable upregulation in leaves according to RNA-Seq data exhibited strong down-regulation in both leaves and roots under all stress conditions. These findings underscore the intricate regulatory mechanisms governing antioxidant enzymes and redox molecules in gliricidia plants adapted to very high salinity stress, highlighting a predominant role for the root system in this response.

Keywords: gliricidia; resistance; enzymes; abiotic stress; transcriptome; qRT-PCR.

Resumo: Este estudo explora a complexa resposta do sistema de defesa antioxidante em *Gliricidia sepium* (Jacq.) Kunth aclimatada ao estresse salino extremo. A análise de RNA- Seq de folhas revelou que enzimas antioxidantes centrais (APX, CAT, DHAR, GSR, SOD) permaneceram relativamente estáveis, enquanto uma isoforma da monodeidroascorbato redutase (MDHAR) apresentou desregulação negativa em plantas adaptadas. As isoformas da glutationa S-transferase (GST) exibiram padrões de expressão diversos, com respostas contrastantes dentro do mesmo gene (negativa no estresse de curto prazo e positiva no de longo prazo). A validação por qRT-PCR de três enzimas e duas moléculas redox, realizada em folhas e raízes sob estresse de curto e longo prazo, aprofundou a compreensão da complexidade. As isoformas da peroxidase (POX) apresentaram expressão contrastante entre folhas (negativa) e raízes (positiva). Uma de GST exibiu comportamento contrastante entre folhas e raízes durante a adaptação; e uma da glutationa peroxidase (GPX) apresentou reversão da desregulação negativa relacionada à idade nas folhas de plantas adaptadas, com o qRT-PCR revelando regulação positiva em ambas as folhas e raízes sob estresse. As de glutaredoxina 3 apresentaram padrões contrastantes de desregulação negativa nas folhas, enquanto ambas as isoformas mostraram expressão aumentada nas raízes sob estresse. Uma de tioredoxina com regulação positiva notável nas folhas, de acordo com os dados do RNA-Seq, apresentou forte desregulação negativa em ambas as folhas e raízes sob todas as condições de estresse. Os resultados destacam os intrincados mecanismos regulatórios que governam as enzimas antioxidantes e as moléculas redox em gliricídia adaptada a um estresse salino muito alto, evidenciando um papel predominante do sistema radicular nesta resposta.

Palavras-chave: gliricidia; resistência; enzimas; estresse abiótico; transcritoma; qRT-PCR.

1 INTRODUCTION

With the global human population reaching an estimated 8.1 billion in early 2024 and projected to balloon to 11.2 billion by 2100 (UN, 2017), responsible food consumption and production have become one of the most pressing issues addressed in the Sustainable Development Goals (UN, 2017). Agriculture, heavily reliant on land and water conditions, is increasingly affected by climate change (Negacz *et al*., 2022). Salinization poses a major challenge for modern agriculture, with significant areas worldwide already impacted by salt (Negacz *et al*., 2022).

Salt stress disrupts water uptake by plants, mimicking drought conditions. Most crops, not adapted to salty environments, struggle to grow and may even die in moderately salty soil (100-200 mM NaCl). However, some plants exhibit salt resistance, a complex trait arising from the combined action of multiple genes (Chele *et al.,* 2021; El-Ramady *et al*., 2024). When exposed to this environmental stressor, plant cells experience a surge of harmful free radicals. To combat this threat, some plants have developed a remarkably sophisticated antioxidant defense system, operating on two key fronts: enzymatic and non-enzymatic mechanisms. Specialized enzymes like catalase and superoxide dismutase act as cellular warriors, neutralizing free radicals before they can inflict damage (enzymatic mechanisms). Additionally, various antioxidant compounds like vitamin C and carotenoids directly eliminate free radicals, providing an extra layer of protection (non-enzymatic mechanisms). This combined enzymatic and non-enzymatic approach ensures plants can efficiently neutralize these harmful molecules and maintain cellular stability (homeostasis) even under stressful salt conditions (García-Caparrós *et a*l., 2019; Hasanuzzaman *et al*., 2021; Mushtaq; Faizan; Gulzar*,* 2020).

Gliricidia (*Gliricidia sepium* (Jacq.) Kunth) is a medium-sized (10-15 m) legume from the Fabaceae family, native to Central America. This fast-growing tree is a highly valuable multipurpose tree. It is cultivated for improving soil fertility through nitrogen fixation, medicinal applications, use as timber and firewood, charcoal production, and shade in plantations (Rahman *et al.,* 2019). Economically, gliricidia is recognized for improving soil infiltration and increasing water holding capacity, reducing erosion and contributing to soil restoration and quality improvement, which results in higher crop yields (Diouf *et al*., 2017). Additionally, as reported in Carvalho e Silva *et al.* (2022), gliricidia is noteworthy for its ability to adapt to a variety of soils, including acidic, eroded, sandy, heavy clay, calcareous, and alkaline soils.

In a 45-day experiment with varying NaCl levels, gliricidia plants exhibited contrasting responses (Carvalho e Silva *et al*., 2022). Plants under low salinity stress (up to 15 dS m^{-1}) displayed tolerance, maintaining healthy aboveground parts (no wilting, yellowing, burning, or leaf drop) but with reduced biomass in both shoots and roots. In contrast, plants exposed to higher salinity (≥30 dS m^{-1} NaCl) rapidly lost all leaves within the first week. However, demonstrating remarkable resilience, they initiated new leaf growth from lateral meristems around two weeks later, which continued throughout the experiment. This response suggests an adaptation strategy in gliricidia to cope with extreme salt stress.

Despite existing knowledge of gliricidia's salt tolerance, the underlying biochemical and molecular mechanisms of its antioxidant response remain elusive. Building on previous multi-omics studies that characterized gliricidia's salinity stress response (Braga *et al.,* 2022; Carvalho e Silva *et al*., 2022), this work focuses on genes encoding known antioxidant enzymes and redox molecules. These genes, previously identified as responsive to salinity stress in gliricidia leaves, are key players in the plant's defense mechanism. The current study employs qRT-PCR and RNA-Seq to evaluate their expression in both roots and leaves under high salinity conditions, investigating both short-term and long-term responses. Genes with a strong correlation to the adaptation phenotype will undergo further structural, functional, and evolutionary analysis.

2 MATERIALS AND METHODS

A) Generation and use of a Reference Transcriptome (RT) from the leaves of gliricidia:

All sequencing data for the generation of the Reference Transcriptome (RT) came from a previous study done by our group (Carvalho da Silva *et al*., 2022). The raw sequence data have been uploaded in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the BioProject PRJNA634354 and BioSample SAMN14992839 (GS_BR01 - TaxID: 167663).

The analysis pipeline used leveraged OmicsBox v1.3 (BioBam, 2019) for transcriptome analysis, and was already described in Belo Silva *et al.* (2022). Quality control steps, including read filtering and low-quality base removal, were performed using FastQC and Trimmomatic. To construct a reference-free transcriptome assembly, we employed Trinity v2.8.5 and Bowtie2 v2.3.5.1 with default settings. After assembling the RT, the TransDecoder tool was used to predict coding regions. STAR mapped RNA- Seq data to the assembled transcriptome. Finally, gene and transcript expression quantification was achieved using HTSeq v0.9.0. All steps in the

pipeline through OmicsBox 1.3 used default parameters, exception made to Trimmomatic, where the reads were maintained only if their average quality exceeded Q30 (Phred score) and minimum length surpassed 75 bases. All complete open reading frames (ORFs) were submitted to the GhostKOALA tool (Kanehisa *et al.,* 2016) for annotation.

B) Plant material - samples for qRT-PCR analysis:

All experiments were carried out at the Plants Genetics and Biotechnology Laboratory - Embrapa Agroenergia, in Brasília, DF, Brazil (S - 15,732º, W - 47,900º). Leaves and roots from control and salt stressed plants came from the study previously reported by Braga *et al.* (2022). The experimental design was completely randomized and used five and a half-month-old gliricidia plants that were kept under control conditions or subjected to saline stress (27 dS/m of electric conductivity) for 55 days. Plant samples were collected at two (short-term stress) and 55 (long-term stress), immediately immersed in liquid nitrogen, and then stored at − 80 °C until total RNA extraction. Total RNA was isolated from purslane samples using the Qiagen RNeasy® Plant Mini kit (QIAGEN, CA, USA) following the manufacturer's protocol. RNA quantity was measured using a Nanodrop Qubit 2.0 Fluorometer (Life Technologies, CA, USA), and RNA quality was evaluated with an Agilent Bioanalyzer Model 2100 (Agilent Technologies, Palo Alto, CA, USA).

Primers for qRT-PCR (Table 1) were designed using an in-house Python script. The script utilizes the pandas, biopython, primer3, and pydna libraries, implementing a complete pipeline for primer design and validation. The pipeline initially receives a table containing the genes of interest and a FASTA file with the reference transcriptome sequences. Next, the primer design for the genes of interest is performed using the primer3 library. Finally, in the validation step, the pydna library is used to verify whether the primers specifically anneal to the gene of interest and do not anneal to other genes non-specifically. For each primer pair, the library simulates annealing with each gene in the complete list. The primer pair is considered valid if it anneals to the gene of interest in only one location and does not anneal to any other gene. At the end of the pipeline, a list containing the primers that passed the validation is generated. qRT-PCR analysis was performed using the SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen®) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The cycling conditions were: 95°C for 120 seconds (denaturation), followed by 40 cycles of 95°C for 5 seconds (denaturation) and 60°C for 30 seconds (annealing/extension) with fluorescence measurement during the second step. A

melting curve analysis (95°C for 15s, 60°C for 60s, 95°C for 15s) was included for specificity verification. Fluorescence data was collected at each cycle using StepOnePlus™ Real-Time PCR and analyzed with StepOnePlus™ software v2.3 (Life Technologies). Three biological replicates with three technical replicates per sample (nine reads per gene/treatment) were used for target genes. Housekeeping gene analysis used three biological replicates with two technical replicates per sample. The ΔΔCt method was employed to calculate relative gene expression levels (Livak; Schmittgen, 2001).

C) Phylogenomics Analysis:

The evolution of detoxifying enzymes in halophytes and glycophytes was performed using OrthoFinder (v2.5+). To infer phylogenetic relationships among gene sets, pairwise sequence similarities from 68 species with sequenced genomes (19 halophytes and 49 glycophytes) was analyzed (Supplementary Table 1). The software was run with the parameters "-M msa" and "-I 1.8" for the method used to infer the gene tree and MCL inflation parameter, respectively.

D) Structural & Functional annotation:

In an initial characterization of the genes, we utilized the InterProScan tool (Paysan-Lafosse *et al*., 2022) to analyze gene sequences for functional domains, Gene Ontology (GO) terms, and protein families. MotifScan software (Su *et al.,* 2018), available at Expasy (Duvaud *et al*., 2021), was employed to further investigate functional domains, searching all available databases. Domain information was then used to generate illustrations with IBS Illustrator software (Liu *et al.,* 2015).

For tertiary structure prediction, an interactive version of AlphaFold software was employed (Jumper *et al.,* 2021). Finally, subcellular localization of each gene was predicted using DeepLoc-2.0 (Thumuluri *et al.,* 2022) and Plant-mPLoc (Chou; Shen, 2010).

Table 1 - Primer pairs used for differential expression analysis of the seven genes investigated by qRT-PCR.

Gene ID	Primer F	Length fwd	Primer R	Length rev	PCR Length	Enzyme Type
GSepium_DN10039_c0_g3_i1.p1	5'-TGAGTCACACAGTGAAGGCTC-3'	21	5'-CGGCAACCAAGTTGAACCAG-3'	20	118	Glutaredoxin
GSepium_DN4224_c0_g1_i1.p1	5'-AGTGACAAGGTTGGCCTCTG-3'	20	5'-GGGATAGGTGTAGTGCAGCC-3'	20	189	Glutaredoxin
GSepium_DN1399_c0_g1_i21.p1	5'-TGCTATGTGTACAAGCGGGG-3'	20	5'-AAGCATCTTGTCCCTTGGGG-3'	20	289	Glutathione
GSepium_DN2011_c0_g1_i16.p1	5'-TGACCACCGAATCCACCAAG-3'	20	5'-AATAGCGACCCACCACTTGG-3'	20	450	Glutathione
GSepium_DN1151_c0_g1_i2.p1	5'-TGCGTGAAGTCGTAAGGGAC-3'	20	5'-AGGTTGAAGAAAGGAGCCGG-3'	20	382	Peroxidase
GSepium_DN1151_c0_g1_i11.p1	5'-TGCTTGCCAGTCTCGTTAGG-3'	20	5'-TAGTGCCAGGTCCACCATTG-3'	20	552	Peroxidase
GSepium_DN9643_c0_g1_i5.p2	5'-ACTCAACCGACGGCATCTAC-3'	20	5'-AGGCGCTATCATCCTGCATG-3'	20	176	Thioredoxin
EgEfMPOB00119	5'-AGCCATCTTGGAAACGAATG-3'	20	5'-CACTTTCGGATGGCAGAGTT-3'	20	429	Reference Gene

Source: Author.

3 RESULTS AND DISCUSSION

Analysis using the RNA-Seq data from Carvalho e Silva *et al.* (2022) identified 90,581 complete ORFs within the *Gliricidia sepium*'s Reference Transcriptome (GSRT). FASTA files captured all ORFs, containing both nucleotide and protein sequences. These sequences facilitated both functional annotation of the proteins and primer design for targeted amplification of the corresponding nucleotide sequences.

The RNA-Seq data from Carvalho e Silva *et al.* (2022) underwent further differential expression analysis (DEA). This DEA compared the leaf transcriptome of stressed gliricidia plants to controls at two time points: two and 45 days after initiating salinity stress. Additionally, DEA was performed within the control and stressed groups, comparing gene expression at two (Short Term Stress) to 45 (Long Term Stress) days. Importantly, only complete ORFs were included in the DEA. Finally, the annotated GSRT, linked with the DEA results, facilitated keyword-based searches.

In plants, enzymatic mechanisms play a crucial role in mitigating oxidative stress caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl (OH) and singlet oxygen ($^1O_2$). As reported by Rajput *et al*. (2021), several enzymes, notably those participating in the Haber-Weiss reaction and the Foyer- Halliwell-Asada pathway, efficiently scavenge ROS. These enzymes exploit the reducing potential of NADPH to break down H_2O_2, thereby protecting plant cells from oxidative damage. Collectively, these enzymes constitute the plant antioxidant defense system. Key enzymes involved in this process include ascorbate peroxidase (APX; EC:1.11.1.11), catalase (CAT; EC:1.11.1.6), dehydroascorbate reductase (DHAR; EC:1.8.5.1), monodehydroascorbate reductase (MDHAR; EC:1.6.5.4), glutathione peroxidase (GPX; EC:1.11.1.9), glutathione reductase (GSR; EC:1.8.1.7), glutathione S-transferases (GSTs; EC:2.5.1.18), peroxidases (POXs; EC:1.11.1.7), and superoxide dismutase (SOD; EC:1.15.1.1).

A search within the annotated GSRT using the EC number "EC:1.11.1.11" and the keyword "L-ascorbate peroxidase" yielded 39 putative APX proteins isoforms encoded by eight genes. The number of isoforms per gene ranged from one to seven. Notably, all identified APX isoforms shared the same K0 identifier, K00434. DEA using RNA-Seq data did not detect any significant changes in the expression of any of the 39 isoforms in the leaves of *G. sepium* plants subjected to salinity stress.

Scrutiny of the annotated GSRT using the EC number "EC:1.11.1.6" and the keyword "catalase" identified eleven putative CAT proteins isoforms encoded by four genes. Two genes

harbored single isoforms, while the remaining two displayed four and five each. Similar to the APX, all identified proteins isoforms shared the same K0 identifier, K03781. DEA using RNA-Seq data revealed no significant changes in the expression of any CAT isoforms in the leaves of *G. sepium* under salinity stress.

Analysis of the annotated GSRT using the EC number "EC:1.8.5.1" and the keyword "dehydroascorbate reductase" identified eight putative DHAR proteins isoforms encoded by four genes. The number of isoforms per gene ranged from one to three. All identified proteins isoforms shared the same K0 identifier, K21888. DEA using RNA-Seq data revealed no significant changes in the expression of any dehydroascorbate reductase isoforms in the leaves of *G. sepium* experiencing salinity stress.

A search within the annotated GSRT using the EC number "EC:1.6.5.4" and the keyword "monodehydroascorbate reductase" yielded 24 putative MDHAR proteins isoforms encoded by five genes. The number of isoforms per gene varied from one to thirteen. RNA-Seq analysis revealed a single MDHAR isoform, out of thirteen total, that was significantly down-regulated in the leaves of *G. sepium* under salinity stress. This finding contrasts with the behavior of DHAR isoforms. The down-regulated MDHAR isoform showed a remarkable 98.5% reduction in expression specifically within the leaves of the salt-adapted plants.

Accordingly to Smirnoff (2018), dehydroascorbate reductase and monodehydroascorbate reductase are enzymes crucial for maintaining a reduced pool of ascorbate (vitamin C) in plants. DHAR catalyzes the reduction of dehydroascorbate (DHA) using GSH as an electron donor, generating ascorbate and oxidized glutathione. MDHAR, on the other hand, specifically reduces monodehydroascorbate (MDHA) to ascorbate.

Plants utilize GSH as a crucial molecule for mitigating oxidative stress. GSH exists in a reduced state and can be oxidized to glutathione disulfide (GSSG). This redox couple plays a role in regulating gene expression through interactions with other redox signaling molecules such as thioredoxin, glutaredoxin, and peroxiredoxins (Rajput *et al*., 2021). GSR is a key enzyme responsible for maintaining the cellular redox balance by regenerating GSH from GSSG. Additionally, GPX and GST are well-characterized antioxidant enzymes in plants that utilize GSH in their detoxification processes (Hasanuzzaman *et al.,* 2017; Rajput *et al*., 2021).

Within the annotated GSRT, a search using the keyword "EC:1.8.1.7" identified six putative GSR protein isoforms encoded by two genes, with one gene containing two isoforms and the other harboring four. All identified isoforms shared the K0 identifier K00383, and DEA using RNA-Seq data revealed no significant changes in expression under salinity stress. In contrast, a search for "EC:2.5.1.18" identified 111 putative GST protein isoforms. DEA using

RNA-Seq data revealed seven differentially expressed isoforms in the leaves of salinity-adapted gliricidia plants. These isoforms belonged to a single K0 identifier group, K00799, with an interesting expression pattern of one upregulated and six downregulated. Under salinity stress, two isoforms of the same gene displayed contrasting behavior in gliricidia leaves. One isoform exhibited downregulation specifically during the transition from short-term to long-term stress, coinciding with the emergence of the adapted phenotype. The other isoform countered a negative regulatory effect triggered by age, restoring expression levels to those observed in control plants under short-term stress.

A search using the keyword "EC:1.11.1.9" yielded 14 putative GPX protein isoforms encoded by six genes. All proteins shared the K0 identifier, K00432. Notably, only one isoform from the four-isoform gene exhibited differential expression in the leaves of salinity-adapted gliricidia plants. According to the data, this specific isoform was expected to be downregulated due to age to roughly 7% of its original level. However, salinity stress reversed this trend, upregulating its expression in the leaves of adapted plants to a level similar to control plants.

Out of 123 identified POX (EC:1.11.1.7) isoforms in gliricidia leaves, only eleven (six upregulated and five downregulated) showed differential expression in salinity- adapted plants compared to controls, after accounting for age effects. Interestingly, all these isoforms belonged to the same functional class (K00430). Notably, one gene even displayed contrasting behavior, with two isoforms – one upregulated and the other downregulated – under salinity stress. In contrast, a search for superoxide dismutase (EC:1.15.1.1) revealed no differential expression for any of the 16 SOD1 (K04565) or 12 SOD2 (K04564) isoforms encoded by seven and six genes, respectively (with 1-5 isoforms per gene).

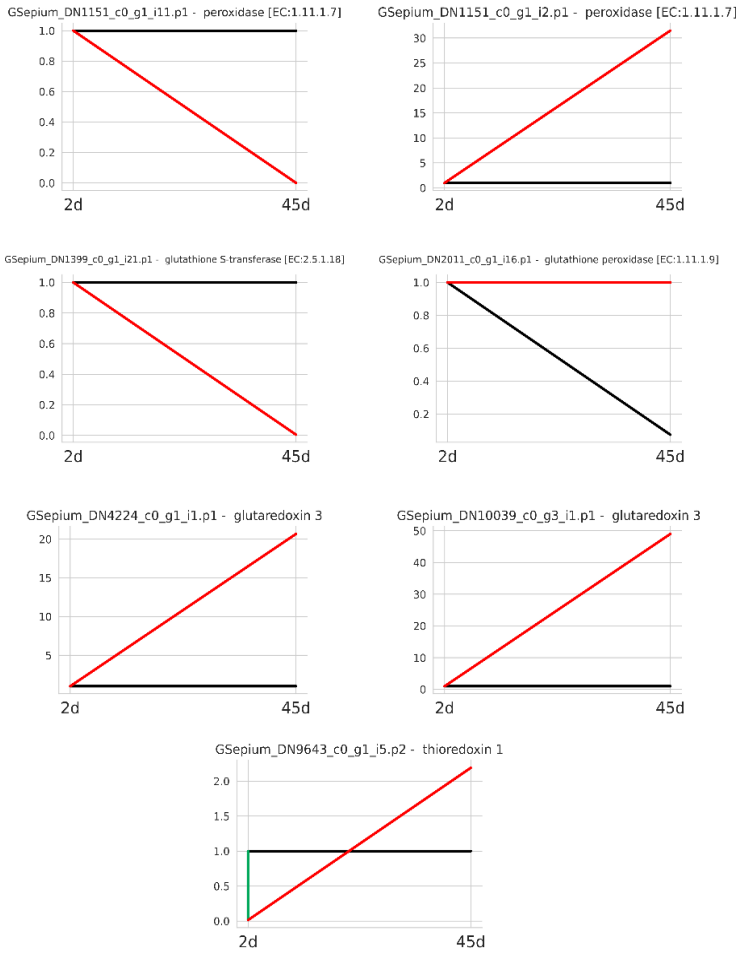
Plant GPX enzymes leverage the reducing power of thioredoxin to detoxify ROS like hydrogen peroxide and hydroperoxyl radicals into water and lipid alcohols, respectively (Rajput *et al*., 2021). Scrutiny of the annotated GSRT using the keywords "trxA and Thioredoxin 1" identified 35 putative protein isoforms encoded by 21 genes (1- 6 isoforms per gene). All shared the K0 identifier K03671. Notably, only one isoform, from a gene with six isoforms, exhibited differential expression. This specific isoform displayed a drastic response to salinity stress: downregulated by 99% in the short-term but upregulated a staggering 158-fold in the leaves of salinity-adapted gliricidia plants.

A search for the keywords "grxC and Glutaredoxin 3" in the annotated GSRT yielded 19 positive results, corresponding to 13 genes. The number of isoforms varied between one and three per protein. All proteins shared the same K0 number, K03676. Of these, only three were differentially expressed – and upregulated – in the leaf of salinity- adapted plants.

This study investigated the expression of seven isoforms from three enzymes and two redox molecules in gliricidia plants under salinity stress using qRT-PCR. These isoforms were initially identified in a leaf transcriptome from plants exposed to short-term (2 days) and long-term (45 days) stress (Figure 1). However, the qRT-PCR analysis focused on leaves and roots of plants subjected to short-term (2 days) and long-term (55 days) stress in a separate experiment (Braga *et al*., 2022). This approach aimed to achieve two objectives: a) Assess whether the isoforms identified in leaves from the first experiment exhibit similar expression patterns in leaves from the second experiment; and b) Evaluate potential differences in expression between leaves and roots for the selected isoforms in gliricidia plants under salinity stress. By comparing these two independent experiments, this study aims to determine the broader applicability of the initial findings.

Two isoforms of the same POX gene, exhibiting differing responses in gliricidia leaves under salinity stress (Figure 1), were chosen for further investigation. Surprisingly, qRT-PCR analysis did not reveal contrasting expression patterns for these isoforms in the leaves. Both isoforms displayed downregulation upon stress, with expression levels decreasing to 30% and 15% at short-term stress (STS) and 12% and 42% at long-term stress (LTS), respectively. These results suggest a potential absence of their role in the leaf's antioxidant defense system. Conversely, in the roots, both isoforms showed significant upregulation under both STS and LTS conditions (Figure 2). These contrasting findings between leaves and roots highlight the complex regulatory mechanisms of peroxidase (POX) isoforms in gliricidia plants during salinity stress.

Figure 1 - RNA-Seq differential expressed profiles of the seven isoforms selected for qRT-PCR analysis.



Note: Age Effect – Black line; Short-Term Stress – Green line; and Long- Term Stress – Red line. Measurement reported as Fold Change.

Source: Author.

Among the GST isoforms, one displayed a unique expression pattern (Figure 1). This isoform exhibited downregulation specifically during the transition from STS to LTS, coinciding with the emergence of the adapted phenotype in the leaves. qRT-PCR analysis showed that the isoform's expression increased in the leaves under STS but returned to normal levels at LTS (Figure 2). Interestingly, in the roots, this same isoform showed a significant upregulation (3.567-fold increase) during the transition to the adapted phenotype (Figure 2). This contrasting response between leaves and roots suggests a potential role for this specific GST isoform in regulating the adaptation process to salinity stress, particularly in the roots.

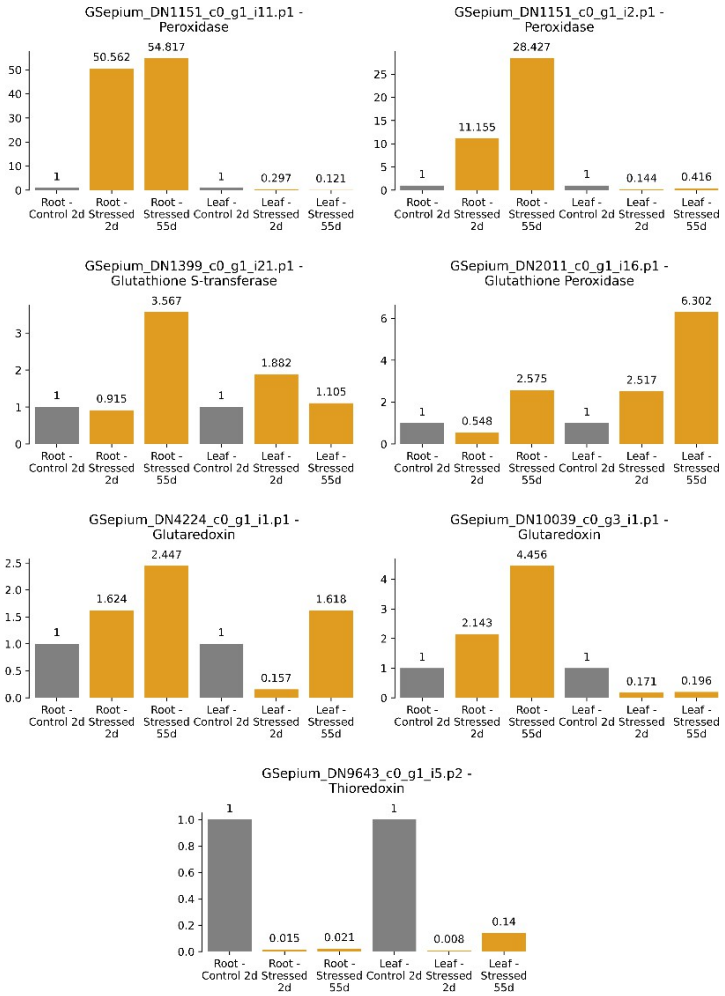
One GPX isoform, exhibiting reversed age-related downregulation under salinity stress in leaves from salinity-adapted gliricidia plants (Figure 1), was selected for further investigation. qRT-PCR analysis revealed an initial upregulation of this isoform in the leaves

under STS, which was further enhanced under LTS. In contrast, the same isoform in the roots displayed a decrease in expression at STS, followed by a significant increase (2.575-fold) at LTS (Figure 2). These contrasting expression patterns between leaves and roots suggest complex regulatory mechanisms of GPX isoforms in response to salinity stress.

Two isoforms of Glutaredoxin, encoded by different genes and upregulated in leaves of salinity-adapted gliricidia plants (Figure 1), were chosen for further investigation. qRT-PCR analysis revealed a contrasting response in their expression under salinity stress. Both isoforms displayed strong downregulation in the leaves under STS. However, one isoform maintained this low expression at LTS, while the other showed a tenfold increase between STS and LTS. In the roots, both isoforms exhibited increased expression at STS, and again at LTS (Figure 2). These findings highlight the complex regulatory mechanisms of Glutaredoxin 3 isoforms in response to salinity stress, with distinct patterns observed in leaves and roots.

One Thioredoxin isoform, exhibiting a remarkable 158-fold upregulation in the leaves of salinity-adapted gliricidia plants compared to control plants (Figure 1), was selected for further investigation. Interestingly, qRT-PCR analysis revealed a different pattern. This particular isoform displayed strong downregulation in both leaves and roots at both STS and LTS. These findings suggest that this specific isoform might not play a significant role in gliricidia's antioxidant defense system against salinity stress, despite the initial upregulation observed in adapted plants.

Figure 2 - Quantitative real-time PCR (qRT-PCR) analysis of the expression profiles of seven genes responsive to salt stress in *Gliricidia sepium* leaves.



Note: These genes were selected from RNA-Seq analysis. The housekeeping gene, EgEfMPOB00119, encoding the 40S ribosomal protein S23 mRNA, was used for normalization.
Source: Author.

The annotation results for the putative GST enzyme revealed its molecular function as glutathione transferase activity (GO:0004364) and its biological process as glutathione metabolic process (GO:0006749). It belongs to the Glutathione transferase family (IPR040079) and Glutathione S-transferase Omega/Tau-like (IPR045073). It had conserved domains related to the corresponding enzyme (Figure 3), and its subcellular location is in the cytoplasm with 65% certainty.

Accordingly to Mannervik (2012), GSTs are a versatile group of enzymes acting on a multitude of substrates. Their influence extends throughout the plant, functioning in both response to environmental challenges and in core regulatory processes; and even playing a role in signaling pathways, anthocyanin transport, auxin homeostasis, tyrosine metabolism, and

even programmed cell death regulation (apoptosis). This diverse range of functions highlights GSTs' crucial role in plant health and stress tolerance; from detoxifying harmful herbicides and xenobiotics to protecting against ozone damage and heavy metals, GSTs act as cellular guardians. Hao *et al*. (2021) investigated how common wheat responds to salt stress at the genetic level, and identified specific GST genes activated by salt stress, but interestingly, only at severe stressor concentrations. Additionally, the study by Hao *et al*. (2021) revealed that these specific GST genes primarily associated with salt response belong to the Tau and Phi classes.

The annotation of the putative GPX enzyme yielded the following description: Glutathione peroxidase (IPR000889). Its biological process is related to response to oxidative stress (GO:0006979); and its molecular function is associated with glutathione peroxidase activity (GO:0004602). It had conserved domains related to the corresponding enzyme (Figure 3). According to the software used, the subcellular location is situated in the chloroplast and mitochondria with 80% certainty.

The plant GPX family consists of multiple isoenzymes with distinct subcellular localizations that exhibit different tissue-specific expression patterns and responses to environmental stress (Bela; Riyazuddin; Csiszár, 2022). Additionally, GPX genes have been shown to be induced by oxidative stress (Yang *et al*., 2005), pathogen infections (Criqui *et al*., 1992), salt, cold treatments, drought, and metals (Bela *et al*., 2015; Diao *et al*., 2014; Fu, 2014; Gao *et al*., 2014). Two putative GPX genes from *Triticum aestivum* were overexpressed in *A. thaliana*, leading to altered transcription levels of genes involved in salt stress responses (SOS1 and RbohD) and ABA-related regulation (ABI1, ABI2), thus implicating the role of GPXs in salt and ABA signaling (Zhai *et al*., 2013).

The annotation results for the putative POX enzyme isoforms yielded the following description: Plant peroxidase (IPR000823). Its biological process is related to response to oxidative stress (GO:0006979) and hydrogen peroxide catabolic process (GO:0042744); molecular function related to heme binding (GO:0020037) and peroxidase activity (GO:0004601). It had conserved domains related to the corresponding enzyme (Figure 3), and the subcellular location is the extracellular space with 75% certainty.

POXs, as one of the main antioxidant enzymes, are widely distributed in nature and catalyze the oxidation of various electron-donating substrates concomitantly with the decomposition of H_2O_2 (Pandey *et al*., 2017). Non-animal plant POXs (class III peroxidase) are involved in various essential physiological processes of plant growth and development throughout their life cycle. In view of the ability of peroxidases to catalyze the redox reaction

for a wide range of substrates, they are considered one of the important enzymes from the point of view of their diverse medicinal, biochemical, immunological, biotechnological and industrial applications (Kidwai; Ahmad; Chakrabarty, 2020; Pandey *et al*., 2017).

In *A. thaliana*, overexpression of AtPRX3 has been shown to alleviate dehydration and increase salt tolerance. However, inhibition of AtPRX3 expression decreased dehydration and salt tolerance (Llorent *et al*., 2002). Additionally, class III PRX genes CrPrx1 and CrPrx in *Catharanthus roseus* have been reported to improve germination rate under salt stress in *Nicotiana tabacum* Kumar, Jaggi and Sinha (2012). Consistent with these results, the work recently performed by Su *et al*., (2020) proved consistent with the results of these previous reports, where they also demonstrated a positive regulator in wheat salt tolerance by TaPRX-2A.

The putative thioredoxin molecule belongs to the Thioredoxin (IPR005746) family. Its molecular function is protein-disulfide reductase activity (GO:0015035). It had conserved domains related to the corresponding enzyme (Figure 3), and the subcellular locations are the plastid and chloroplast region with 97% certainty.

Accordingly to Nikkanen and Rintamäki (2017), TRXs are oxidoreductase proteins that control the structure and function of cellular proteins by cleaving a disulfide bond between the side chains of two cysteine residues. Oxidized thioredoxins are reactivated by thioredoxin reductases (TRs) and a TR-dependent reduction of TRXs is called the thioredoxin system. Thiol-based redox regulation is an especially important mechanism for controlling chloroplast proteins involved in biogenesis, regulation of light capture and light energy distribution between photosystems, photosynthetic carbon fixation and other biosynthetic pathways, and plant stress responses (Nikkanen; Rintamäki, 2017).

Heterologous expression of AtTrx-h2 in *Brassica napus* conferred salt tolerance with plants grown on 50 mM NaCl having higher fresh weight and chlorophyll content compared to controls in the hydroponic growth system (Ji *et al*., 2020). Additionally, Ji and colleagues reported that AtTrx-h2-overexpressing transgenic plants exhibited lower levels of hydrogen peroxide and higher activities of antioxidant enzymes, including peroxidase, catalase, and superoxide dismutase, compared with plants expressing the empty vector control or AtTrx-h3. These results suggest that AtTrx-h2 is a promising candidate for engineering or breeding crops with enhanced salt stress tolerance (Ji *et al*., 2020).

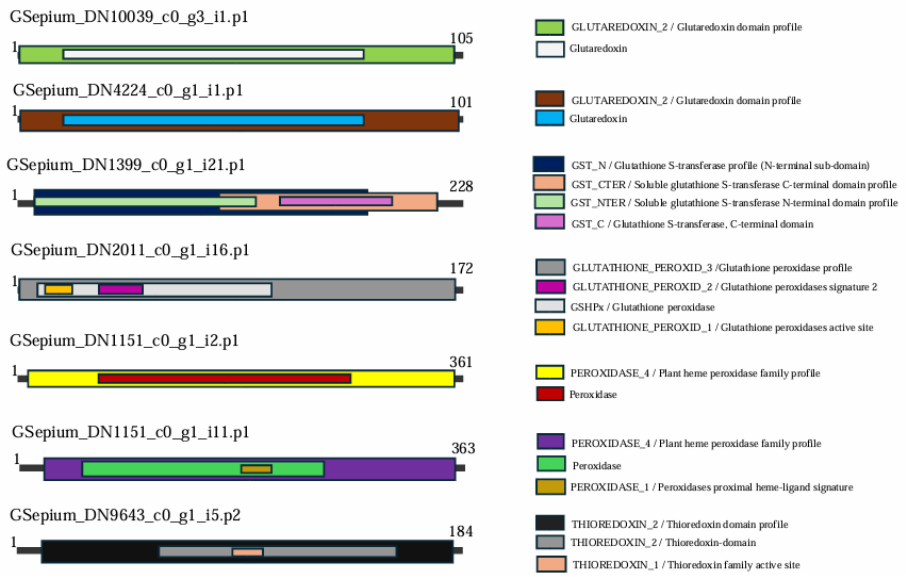
Both putative glutaredoxin molecules analyzed lacked specific Gene Ontology (GO) terms but shared the characteristic Glutaredoxin-like, plant II (IPR011905) family. Both possessed conserved domains related to their respective molecules (Figure 3), and the

subcellular localization analysis predicted their presence in the cytoplasm and chloroplast with varying confidence levels (57% and 78%, respectively).

Virus-induced silencing (VIGS) of the class II SlGRX1 gene in tomato results in increased sensitivity to salt and water stress, while overexpression of SlGRX1 in *A. thaliana* plants improves plant tolerance to drought and salt stress (Guo *et al*., 2010). Improvements in each abiotic stress alone are possible through the manipulation of individual stress-associated traits in different species. At the same time, there are few genetic engineering strategies for tolerance to these multiple abiotic stresses, so some members of the GRX family that have multiple functions in plants are strong candidates (Wu *et al*., 2017).

Tertiary structure analysis of the putative proteins revealed that the redox molecule glutaredoxin (Figure 4) is composed of 50% α-helix folds with about four β- folds, the rest of the molecule being irregular coiled and extended segments. The other glutaredoxin molecule has four α-helix folds, four β-folds, and three irregular coiled and extended segments (Figure 4). The glutathione enzyme is predominantly composed of α- helix folds, about 20% are β-folds, and the rest of the molecule are irregular coiled and extended segments. The glutathione transferase enzyme has predominantly β-folds and only two α-helix folds (Figure 4). The peroxidase enzyme has 80% α-helix folds, three irregular coiled and extended segments, and 15% β-folds. The other peroxidase protein found has two large irregular coiled and extended segments, 90% are α-helix folds, and 10% are β-folds. Finally, the redox molecule thioredoxin found has mostly irregular coiled and extended segments, followed by 5 β-folds and two α-helix folds.

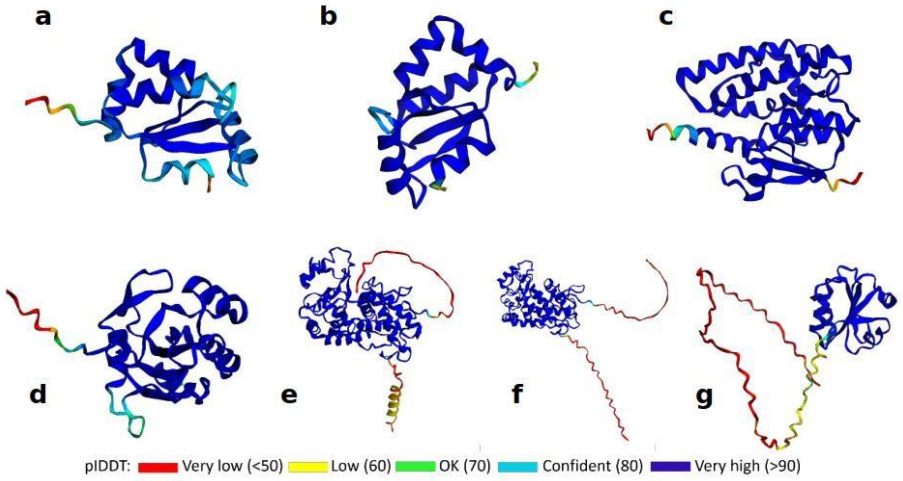
Figure 3 - *In silico* structural analysis of salt stress-responsive enzymes from *Gliricidia sepium* leaves.



Note: The analysis focused on the protein domains of the seven enzymes chosen for qRT-PCR analysis. A schematic representation is provided, where each colored box signifies a distinct protein domain and its relative position within the entire protein sequence. The numbers along the lines indicate the total amino acid length of each protein.

Source: Author.

Figure 4 - Predicted tertiary structures for salt stress-responsive enzymes from *Gliricidia sepium* leaves, generated using the AlphaFold protein structure prediction platform.

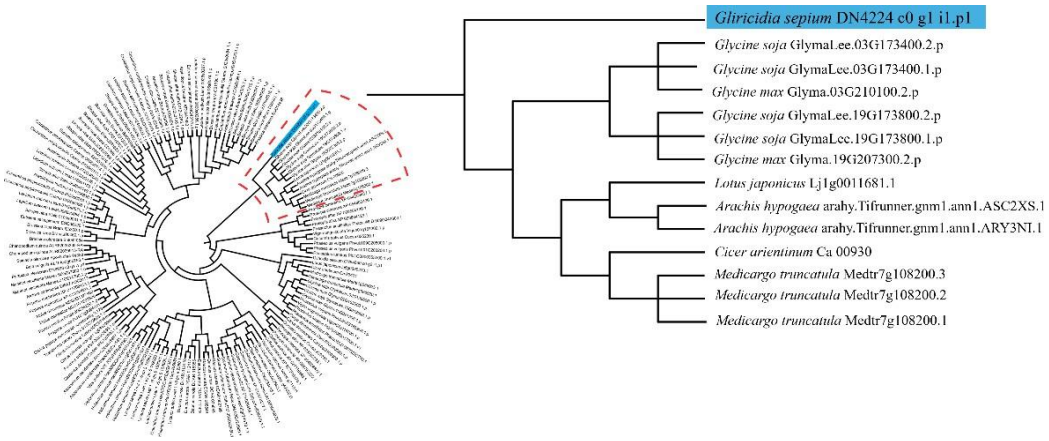


Note: The structures are presented for: (a & b) two glutaredoxins (GSepium_DN10039_c0_g3_i1.p1 and DN4224_c0_g1_i1.p1), (c) a glutathione S- transferase (DN1399_c0_g1_i21.p1), (d) a glutathione peroxidase (DN2011_c0_g1_i16.p1), (e & f) two peroxidases (DN1151_c0_g1_i2.p1 & DN1151_c0_g1_i11.p1), and (g) a thioredoxin (DN9643_c0_g1_i5.p2).

Source: Author.

To investigate the evolutionary relationships of the seven isoforms studied using qRT-PCR, a phylogenomic analysis was conducted. This analysis employed a protein database encompassing 68 plant species and utilized OrthoFinder software to identify orthogroups, which represent groups of genes derived from a single ancestral gene. Notably, all seven isoforms were assigned to six distinct orthogroups. Interestingly, the two POX isoforms clustered together within the same orthogroup, while the Glutaredoxin isoforms segregated into separate orthogroups. The number of proteins within each orthogroup displayed significant variation, ranging from 294 to 2,485. Importantly, all *Gliricidia sepium* isoforms resided within clades consistently containing proteins from other legume species (Figure 5). This observation suggests a potential shared evolutionary history among these isoforms and their counterparts in other legumes.

Figure 5. Circular dendrogram depicting all 294 protein sequences within the orthogroup.



Note: Notably, the *Gliricidia sepium* Glutaredoxin isoform (DN4224_c0_g1_i1.p1), highlighted in blue, forms a distinct clade containing exclusively legume-derived proteins.
Source: Author.

4 CONCLUSION

RNA-Seq analysis of *G. sepium* leaves exposed to high salinity stress revealed minimal changes in expression for core antioxidant enzyme isoforms (APX, CAT, DHAR, GSR, and SOD). In contrast, isoforms within MDHAR, GPX, POX, and GST displayed differential expression. This suggests a more prominent role for specific isoforms within these latter enzyme types in plant adaptation to salinity stress.

qRT-PCR analysis revealed tissue-specific regulation of POX, GST, GPX, and Glutaredoxin 3 isoforms. Notably, some isoforms displayed downregulation in leaves but

upregulation in roots under salinity stress. This suggests potentially distinct roles for these isoforms in different plant tissues.

Isoform-specific responses within gene families highlight the importance of studying isoforms beyond overall enzyme activity. This nuanced approach allows for a deeper understanding of the plant's stress response, as exemplified by the contrasting expression patterns observed in POX and Glutaredoxin 3 isoforms under salinity stress.

The dynamic changes in expression patterns of certain isoforms observed over time (STS vs LTS) suggest their potential involvement in regulating the adaptation process to salinity stress. This is particularly evident with the specific GST isoform in the roots, whose expression coincides with the emergence of the adapted phenotype, potentially playing a key role in the transition phase.

While RNA-Seq data initially suggested contrasting expression patterns for specific isoforms in leaves, qRT-PCR analysis revealed contrasting results in some cases. This underscores the importance of employing complementary techniques for robust validation of gene expression data. These findings highlight the potential complexities involved in accurately capturing the dynamic nature of transcript abundance.

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Supplementary Table 1. List of the 68 plant species, which genome was employed in the Phylogenomics analysis, indicating whether they are present or not in the eHaloph database (Santos *et al*., 2016).

Species	e H a loph	Species	e H a loph	Species	e H a loph	Species	e H a loph
Anacardium occidentale	No	*Cof fea arabica*	No	*Lotus japonicus*	No	*Prosopis cineraria*	Yes
Ananas comosus	No	*Cucumis sativus*	No	*Malus domestica*	No	*Prunus persica*	No
Apium graveolens	Yes	*Daucus carota*	Yes	*Manihot esculenta*	No	*Ricinus communis*	No
Arabidopsis thaliana	No	*Dioscorea alata*	No	*Medicago truncatula*	No	*Setaria italica*	No
Arachis hypogaea	No	*Elaeis guineensis*	No	*Musa acuminata*	No	*Sinapis alba*	No
Asparagus of ficinalis	Yes	*Eleusine coracana*	Yes	*Olea europaea*	No	*Solanum lycopersicum*	No
Beta vulgaris	Yes	*Eragrostis curvula*	Yes	*Oryza sativa*	No	*Solanum pennellii*	Yes
Brassica oleracea	Yes	*Eutrema salsugineum*	Yes	*Panicum virgatum*	Yes	*Solanum tuberosum*	No
Brassica rapa	No	*Fragaria vesca*	No	*Paspalum vaginatum*	Yes	*Sorghum bicolor*	No
Carica papaya	No	*Fragaria x ananassa*	No	*Phaseolus lunatus*	No	*Spinacia oleracea*	No
Carya illinoinensis	No	*Gliricidia sepium*	No	*Phaseolus vulgaris*	No	*Theobroma cacao*	No
Castanea dentata	No	*Glycine max*	Yes	*Phaseolus acutifolius*	No	*Triticum aestivum*	No
Caulanthus amplexicaulis	No	*Glycine soja*	No	*Phoenix dactylifera*	Yes	*Vaccinium darrowii*	No
Chenopodium quinoa	Yes	*Helianthus annuus*	No	*Poncirus trifoliata*	No	*Vigna unguiculata*	No
Cicer arietinum	No	*Hordeum vulgare*	Yes	*Populus euphratica*	Yes	*Vitis vinifera*	No
Citrus clementina	No	*Lactuca sativa*	No	*Portulaca oleraceae*	Yes	*Zea mays*	No
Citrus sinensis	No	*Lepidium sativum*	No	*Prosopis alba*	Yes	*Zostera marina*	Yes

Source: Author

Artigo 4 - Redigido conforme norma do periódico científico - Versão publicada	
Título do artigo:	Structural and functional analysis of stress inducible genes and their promoters selected from young oil palm (*Elaeis guineensis*) under salt stress
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ARTIGO 4 - Structural and functional analysis of stress inducible genes and their promoters selected from young oil palm (*Elaeis guineensis*) under salt stress

RESEARC Open Access

Structural and functional analysis of stress- inducible genes and their promoters selected from young oil palm (*Elaeis guineensis*) under salt stress

Thalita Massaro Malheiros Ferreira[1], Jaire Alves Ferreira Filho[2], André Pereira Leão[2], Carlos Antônio Ferreira de Sousa[3] and Manoel Teixeira Jr. Souza[1,2*]

Abstract

Background Soil salinity is a problem in more than 100 countries across all continents. It is one of the abiotic stress that threatens agriculture the most, negatively affecting crops and reducing productivity. Transcriptomics is a technology applied to characterize the transcriptome in a cell, tissue, or organism at a given time via RNA-Seq, also known as full-transcriptome shotgun sequencing. This technology allows the identification of most genes expressed at a particular stage, and different isoforms are separated and transcript expression levels measured. Once determined by this technology, the expression profile of a gene must undergo validation by another, such as quantitative real- time PCR (qRT-PCR). This study aimed to select, annotate, and validate stress-inducible genes—and their promoters— differentially expressed in the leaves of oil palm (*Elaeis guineensis*) plants under saline stress.

Results The transcriptome analysis led to the selection of 14 genes that underwent structural and functional annotation, besides having their expression validated using the qRT-PCR technique. When compared, the RNA-Seq and qRT-PCR profiles of those genes resulted in some inconsistencies. The structural and functional annotation analysis of proteins coded by the selected genes showed that some of them are orthologs of genes reported as conferring resistance to salinity in other species. There were those coding for proteins related to the transport of salt into and out of cells, transcriptional regulatory activity, and opening and closing of stomata. The annotation analysis performed on the promoter sequence revealed 22 distinct types of cis-acting elements, and 14 of them are known to be involved in abiotic stress.

Conclusion This study has helped validate the process of an accurate selection of genes responsive to salt stress with a specific and predefined expression profile and their promoter sequence. Its results also can be used in molecular-genetics-assisted breeding programs. In addition, using the identified genes is a window of opportunity for strategies trying to relieve the damages arising from the salt stress in many glycophyte crops with economic importance.

Keywords Abiotic stress, Salinity, Transcriptomics, qRT-PCR, RNA-Seq, Strategy

Background

Population growth and climate change affect the biomass production system on a regional, national, and global scale [1, 2]. The balance between demand and supply of food, fiber, feed, and bioenergy faces challenges of complex magnitude, demanding strong responses on several fronts—from scientists, policy analysts, and politicians, to mention a few [3]. Science is on the run to develop the necessary knowledge and technologies to guarantee this balance sustainably.

The molecular mechanisms of salt resistance were the object of extensive studies in Arabidopsis and agronomic plant species such as rice [4, 5, 6]. Salt stress directly alters biological and chemical compounds in plant cells, which activates the cellular response in glycophytic plants [7–9]. Furthermore, salt stress leads to ionic, secondary, osmotic, and oxidative stresses, triggering multiple complex signaling pathways [7].

Soil salinity is one of the abiotic stresses that threaten agriculture the most. Recently, it has gained momentum as a factor limiting the achievement of the sustainability goals in the Sustainable Development Agenda. Salinity stress causes numerous morphological, physiological, and biochemical changes in plants. Plants must maintain a high-water status in the face of water limitation and ionic toxicity to grow in saline conditions. As a result of salt stress, secondary stresses such as oxidative burst can occur, in which the production and accumulation of active radicals result in the oxidation of proteins and lip- ids and, eventually, the death of cells and plants [10].

In the ion transport process, there are two mechanisms conferring salinity resistance. First, the control of the influx and efflux of sodium, potassium, and calcium ions in the plant through the cell membranes in the roots to maintain the ionic balance of the cell and reduce the osmotic stress effect; and secondly, the transport and storage of ions within plant tissue, especially within the cell vacuole, by ionic and tonoplast membrane pumps to eliminate the effects of ionic toxicity [11]. If a plant can absorb water and excrete salt, it can grow and survive in saline conditions [12], and, given the growing saline lands, it seems necessary to consider strategies to increase salinity resistance to strengthen the biomass production system.

Recent studies on abiotic stress-related gene expressions enabled strategies to improve stress resistance in molecular breeding [13–15]. Gene expression analysis is commonly applied to understand molecular regulatory mechanisms and identify genes in current molecular biology [16, 17].

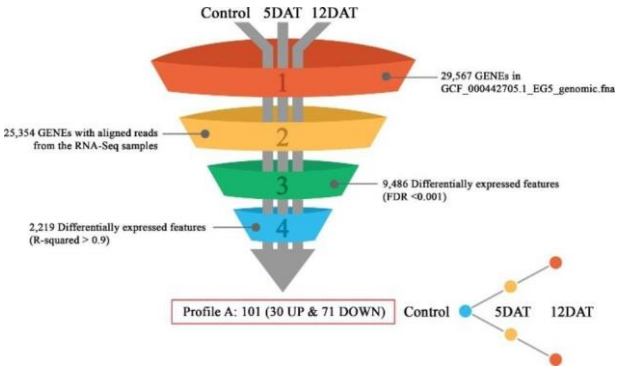

Fig. 1 Strategy applied to prospect a profile-specific salt stress-inducible set of genes and their promoters differentially expressed at the leaves of oil palm (*Elaeis guineensis*) plants under saline stress

Transcriptome analysis using RNA sequencing has become the most used approach to identify the genes and the mechanisms involved in resistance to abiotic stress, such as saline stress in non-model organisms [18]. RNA sequencing nowadays is a technology that utilizes the ability of NGS to obtain an overall picture of the presence and amount of RNA in a given time interval. With transcriptome analysis, most genes expressed in a particular scenario—under salinity stress, for instance— can be identified and their expression level measured [10, 19, 20].

At the end of the process, it allows the selection of candidate genes for validation and, most important, achieves the best pipeline to reduce false positives and increase gain in cost and time effectiveness [21]. That is possible from an experimental design that

represents the biological process of interest and allows better use of the data, followed by a robust bioinformatics pipeline that uses the most appropriate software according to the design. Both are needed to influence the achievement of the biological response as it is and ultimately achieve a high correlation between RNA-seq and quantitative real-time PCR (qRT-PCR) results. Thus, the present study aims to select a profile-specific salt stress-inducible set of genes, and their promoters, differentially expressed in the leaves of oil palm (*Elaeis guineensis*) plants under saline stress. Furthermore, the selected genes underwent qRT-PCR analysis to validate the expression profile seen in the RNA-Seq analysis. At last, the selected genes and their promoters underwent functional annotation analysis.

Results

Selection of profile-specific salt stress-inducible genes

The strategy used to select salt stress-inducible genes bearing the desired expression profile (Profile A) resulted in 101 genes—being 30 upregulated twice [from control to 05 days after treatment (DAT), and again from 05 to 12 days], and 71 downregulated twice (Fig. 1), out of the 29,567 genes present in the reference oil palm genome [22]. The same strategy used to select salt stress-inducible proteins resulted in 21 upregulated and 49 down- regulated ones out of the 43,551 proteins present in that genome. The first round of selection of differentially expressed genes used FDR (False Discovery Rate) ≤ 0.001 and R-squared ≥ 0.9.

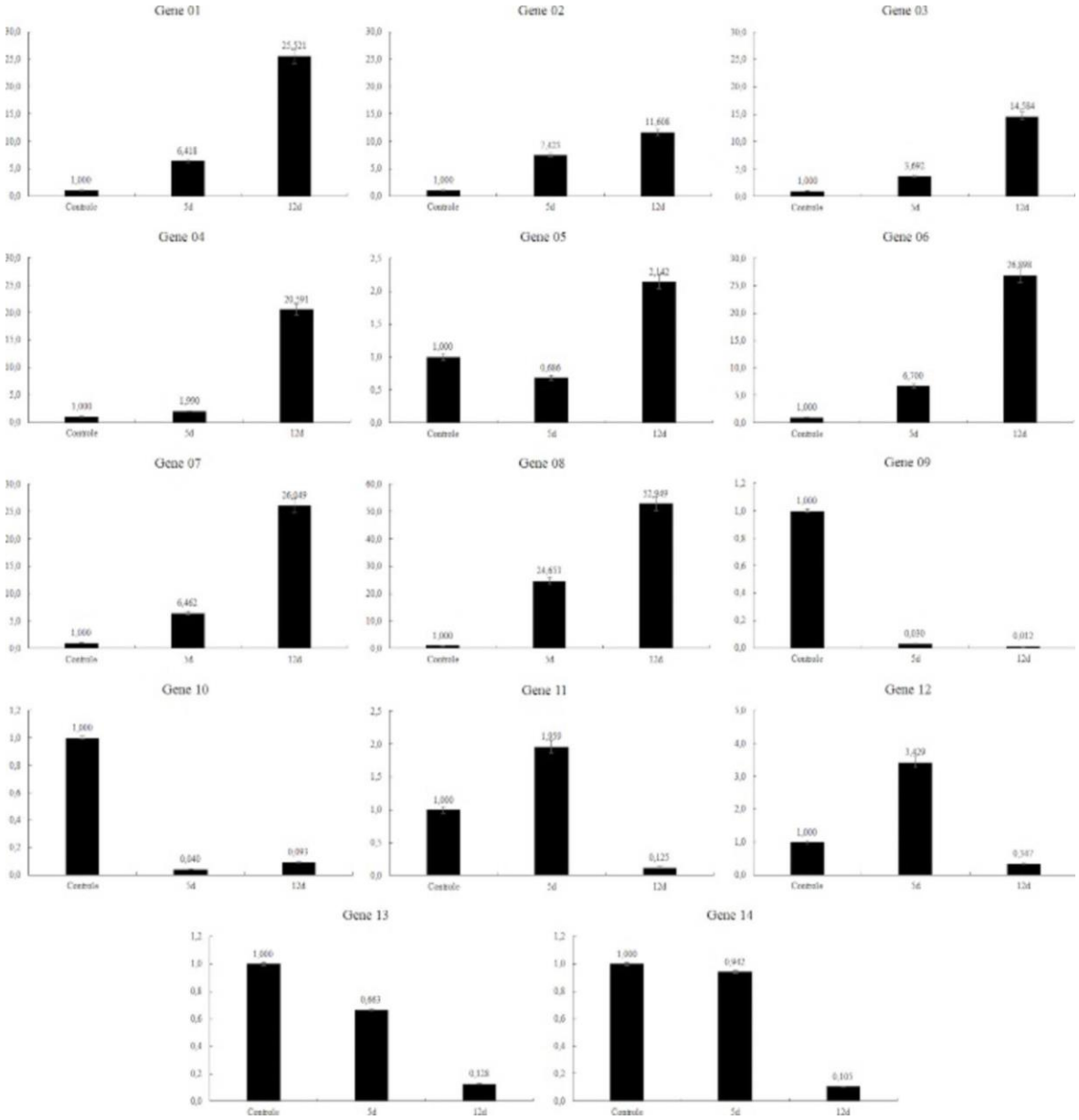

Fig. 2 qRT-PCR analysis of the expression profile of the 14 salt-stressed-responsive genes selected in the genome of oil palm (*E. guineensis*). Internal control gene: EgEfMPOB00119.

The second round of selection, which prioritized the Log_2(Fold Change) value (at 12 DAT in comparison with the control treatment), resulted in eight genes that upregulated from control to 05 days, and then again from 05 to 12 days, and six that downregulated twice. Those genes presenting the highest differences in expression level - in terms of fold change - went on to structural and functional annotation analysis.

Expression analysis through qRT-PCR analysis

The transcriptomics expression profile for each of the 14 selected genes is in Fig. 2. To compare control and stressed treatments, the expression level on the former was set up as 1.0, representing the initial transcription level. Among the genes tested, only genes 08, 11, and 12 presented an expression profile different than expected, in qualitative and quantitative terms—expression at 05 DAT was lower than at 12 DAT when upregulated or higher when downregulated.

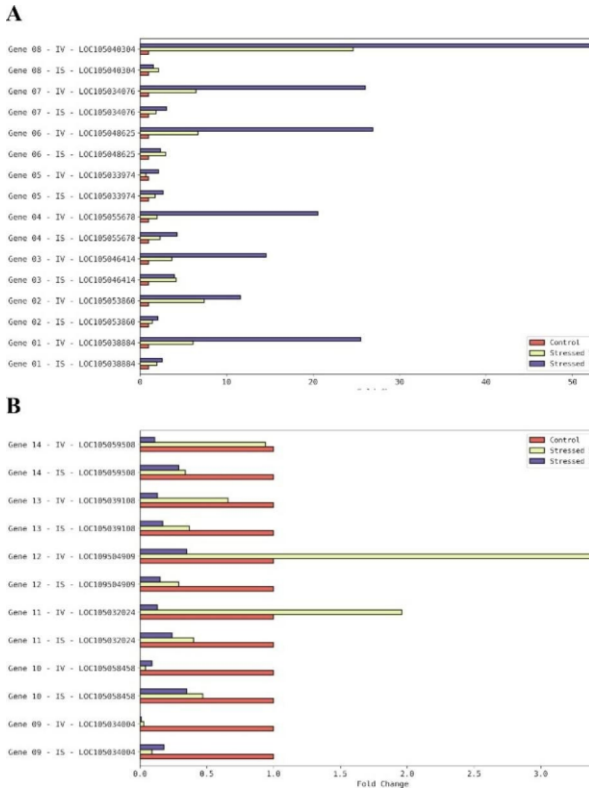

Fig. 3 Comparison of the RNA-Seq (IS) and qRT-PCR (IV) differential expression analysis of the 14 salt-stressed-responsive genes selected in the genome of oil palm (*E. guineensis*). A—upregulated genes; B—downregulated genes.

The quantitative differences between the RNA-Seq and qRT-PCR profiles are in Fig. 3. The upregulated genes showed expression levels at 12 DAT much higher for the latter than for the former, except for gene 05. These results corroborate the current knowledge that a strategy to select profile-specific stress-inducible genes and their promoters—for further use in molecular breeding—must not rely only on the RNA-Seq profile; the qRT-PCR vali- dation is a complementary must.

Structural and functional annotation of the coding region

Regarding the coding region structure, those 14 genes showed diversity in the number of exons present. Genes 1 and 12 have one exon; genes 2, 5, 6, 9, 10, 11, and 14 have two; genes 4 and 7 have three; gene 3 has four, and gene 13 has six exons (Fig. 4 A). Eleven genes coded for proteins having known domains in their coding region. Eighteen distinct domain types appeared on those proteins (Fig. 4B). The proteins coded by genes 1, 2, 3, 7, 12, and 13 showed only one domain, while those by genes 8, 9, and 11 had two, and the ones coded by genes 4 and 14 presented three.

All selected proteins underwent modeling analysis by RaptorX to help understand their functional mechanism. The tertiary structures of 11 proteins are in Fig. 5, from genes 1, 2, 3, 4, 5, 6, 7, 9, 10, 13, and 14. According to the secondary structure also predicted by RaptorX, protein 1 presents the distribution of the helix class in most of its residues, followed by the coil class and beta class. The secondary structure of protein 2 has the distribution of the coil class in most residues, followed by the beta class and the helix class. As for proteins 3, 4, and 5, the helix is the class that stands out among the amino acid residues, followed by the coil and by beta. For protein 6, the coil class is more distributed among the residues, followed by beta and helix. The beta class stands out in protein 7, followed by coil and helix. For protein 9, the prevalent distribution is that of the coil class, followed by helix and beta. The amino acid residues for protein 10 are mainly of the helix class, followed by the coil and beta. The helix class was also predominant for proteins 13 and 14, followed by the coil class for protein 13 and beta for protein 14.

After searching in the RaptorX software, according to the search for similarity with structures resolved with the PDB, it was possible to obtain several additional functional information of some of the proteins coded by the selected genes. The proteins that had similarities with tertiary structures resolved from the database were proteins 2, 3, 4, 7, 8, 9, and 13. The results of the structural and functional annotation for the 14 salt stress-inducible genes are in Table 1; Fig. 6. The proteins coded by those genes had positive hits to 13 biological processes, ten molecular functions, and six cellular components (Fig. 6). Protein binding (GO:0005515) was the molecular func- tion with the higher number of positive hits, with three hits (Table 1). The InterProScan Search

predicted that six of the 14 genes coded for an integral component of mem- branes (data not shown).

Structural and functional annotation of the promoter region

After submitting the putative promoter sequences—1,000 bp long sequence upstream of the initiation codon—of those 14 genes to PlantCARE to obtain cis-acting elements present in that region, it was possible to select ten promoters with positive hits in the PlantCare database. Twenty-two distinct cis-acting elements appeared in the promoter sequence of those genes (Fig. 7).

Eight out of the ten promoters presented the CAAT- box element, and seven had the MYB element, known to be related to abiotic stresses.

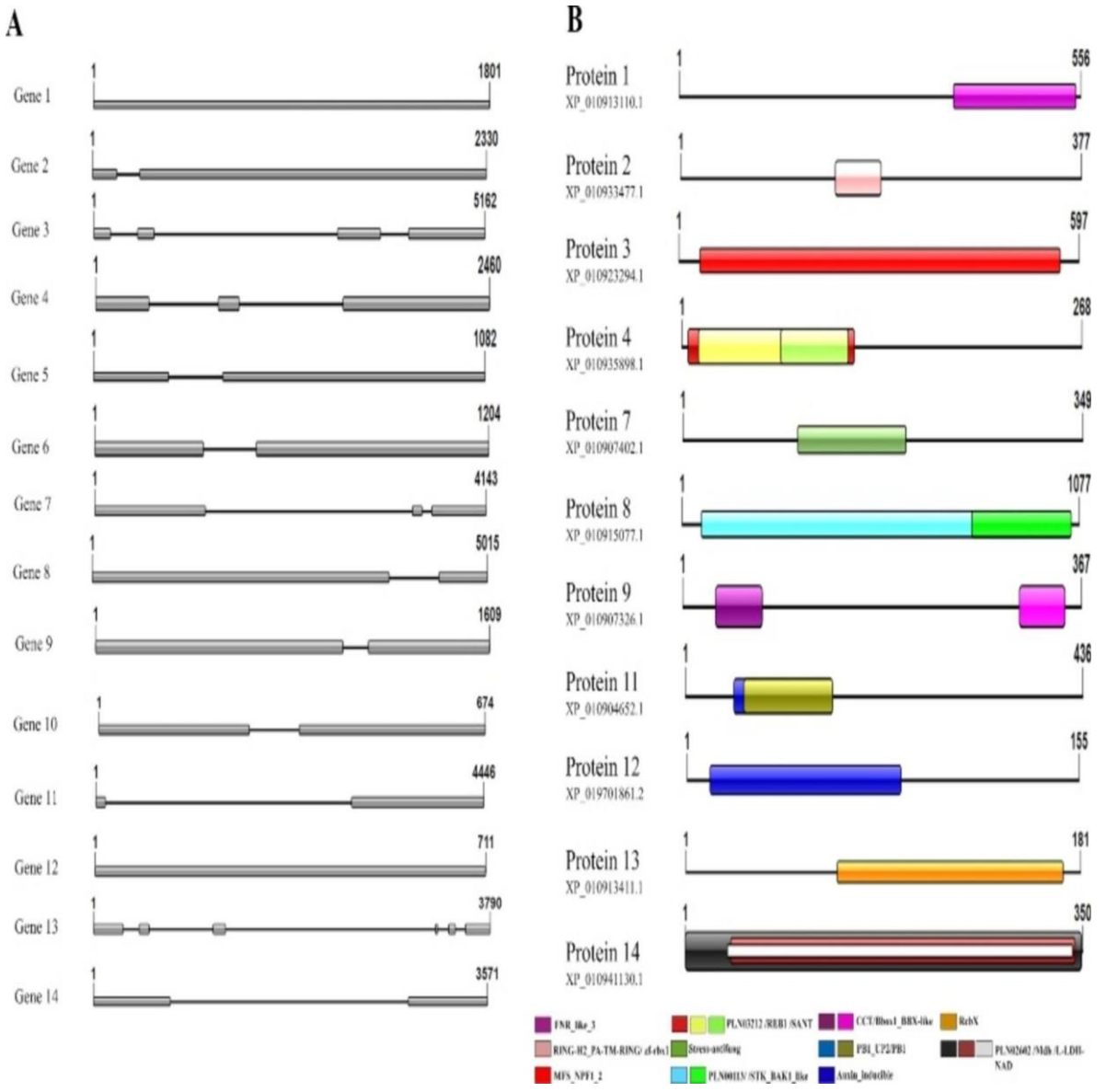

Fig. 4 Structural annotation of the salt-stressed-responsive genes selected in the genome of oil palm (*E. guineensis*). A—Intron and exon number and location. The gray boxes represent the number of exons and their location in the gene; B—Domains of the 11 proteins that had NCBI platform hit. Each colored box represents a different domain and its location in the protein. The numbers in each row represent the total size of both genes and proteins

Seven had the STRE cis-acting one, which undergoes activation by heat shock, low pH, lack of nutrients, and osmotic stresses. Six presented the ABRE and CGTCA, also related to abiotic stresses. Five showed the As-1, four showed the LTR and TGACG-motif elements, three the MBS and CAT-box elements related to abiotic stresses, and two the MYC and G-box elements related to transcription factors and different abiotic stresses. The cis-acting elements LTR, ABRE, ABRE 3a, ABRE4, G-Box, CAAT-box, CAT-box, MYB, MY-like sequence, MYB-binding site, STRE, TATA- box, MYC, and ARE, are found on the promoters of the selected genes and are linked to abiotic stresses [23, 24].

No pattern in the distribution of the cis-acting elements is evident when comparing the profile of these ten promoters evaluated, neither among those from upregulated nor downregulated ones. The 1,000 bp long sequence upstream of the initiation codon from genes 7 and 9 did not show the TATA-box element.

Discussion

Among the few studies reporting on the expression pro- file of oil palm genes under salinity stress [23, 24], neither carried out a strategy to prospect and characterize stress-inducible genes and their promoters nor compared RNA-Seq and qRT-PCR expression profiles.

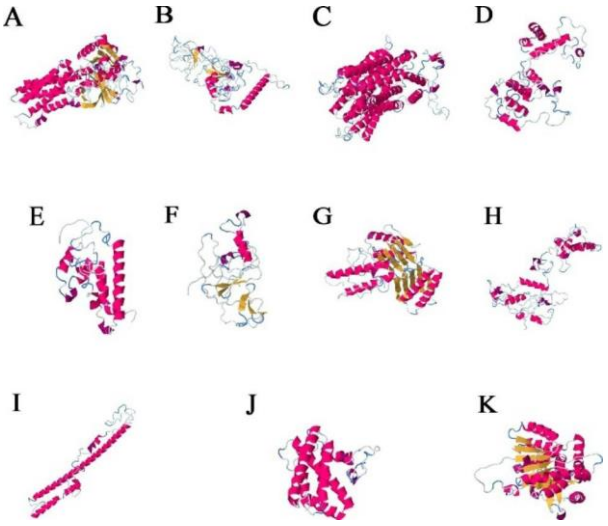

Fig. 5 Tertiary structure of the proteins that had similarity with re- solved structure of the PDB, obtained by the NCBI platform. A—Protein (XP_010913110.1) of gene 1; B—Protein (XP_010933477.1) of gene 2; C—Protein (XP_010923294.1) of gene 3; D—Protein (XP_010935898.1) of gene 4; E—Protein (XP_010907272.1) of gene 5; F—Protein (XP_010926289.1) of gene 6; G—Protein (XP_010907402.1) of gene 7; H— Protein (XP_010907326.1) of gene 9; I—Protein (XP_029124399.1) of gene 10; J—Protein (XP_010913411.1) of gene 13; K—Protein (XP_010941130.1) of gene 14. The models of proteins were obtained by the RaptorX online server. The α -helix, β -strand, and random coil are marked by red, yellow and blue, respectively.

Here we selected a profile-specific salt stress-inducible set of 14 genes and their promoter sequences by applying a time-course differential expression analysis. Eight genes were positively regulated twice, and six negatively regulated twice—initially from control to 05 DAT and later from 05 to 12 DAT. It is necessary to highlight the statistical criteria for selecting salt-responsive genes in oil palm. In the present study, we used a 99.9% reliability level and an R-squared ≥ 0.9, besides the fold change, to select such genes.

Research on gene expression mechanisms has contributed to unraveling the complex regulations in the life cycle of a plant, including how it responds to a saline environment. Transcription technology can reveal candidate genes and key pathways involved in salt resistance by analyzing

differentially expressed genes and performing their functional annotation [25]. Additionally, qRT-PCR is the most applied technique to determine the expression level of a target gene. This technique is one of the most sensitive, accurate, and reproducible techniques to quantify the expression of specific genes. It has become the most common method for validating RNAseq results, requiring a normalization method against reference genes to achieve reliable results [26, 27].

The performance of absolute quantification (gene expression correlation between RNA-Seq and qRT-PCR data) without assessing the performance of relative quantification (correlation of differential gene expression) has been the usual way of analysis, and the latter is the goal of most RNA-seq studies [26, 27]. Feng and colleagues [21] developed a series of performance parameters to evaluate RNA-seq quantification workflows when performing analyses in celery under abiotic stress and hormone treatment. Regarding the qualitative comparison of the expected expression profile for each gene selected in the present study, most genes presented the expression profile in the qRT-PCR analysis similar to the RNA-Seq differential expression study. On the other hand, genes 5, 11, and 12 did not repeat the RNA-Seq expression pro- file. That may be due to a version of a reference genome still needing improvement and an RNA-Seq experimental design with only three replicates per treatment. When analyzing the quantitative side of the comparison, some differences are evident. The increase in expression of genes 1, 4, 6, and 8 in RNA-Seq was lower than fivefold, while in qRT-PCR, it ranged from 20 to 50. Genes 10, 13, and 14 expressions dropped approximately 50–60% in the RNA-Seq analysis, and the qRT-PCR analysis showed a drop of about 90%.

This set of genes is *per se* a source of candidate genes to undergo future validation of their capacity to confer resistance to salinity stress via heterologous expression in model plants. Once this role is confirmed, a subsequent step would be the horizontal transfer (or gene editing) to economically important glycophyte plants [28]. For instance, gene 2, an E3 ubiquitin-protein ligase Os04g0590900 gene, came out as a member of the E3 ubiquitin-protein ligase RING1-like family. Many E3 ligase targets are proteins involved in abiotic stress responses, such as salt. Several E3 ligases regulate these responses to salinity stress by targeting and mediating the degradation of salt stress-related proteins [29]. Gene 4, a transcription factor MYB30, is another example. This gene codes for a protein that came out as one with family membership non-predicted and classified as an MYB family transcription factor in the Panther Classification System. MYB30 modulates plant salt resistance through the positive regulation of mitochondrial alternative oxidase AOX1a, and MYB30 mutants exhibit hypersensitivity to salt stress [30].

The remaining proteins selected in the present study stand out for their several distinct functions, found out by similarity in the PDB analysis, ranging from mem- brane proteins that may be related to the transport of salt into and out of cells, transport of ions, transcription related to transcriptional regulatory activity, and opening and closing of stomata. Some showed trans-membrane, cytoplasmic, and(or) non-cytoplasmic domains predicted (genes 1, 2, 3, 5, 7, and 10).

Table 1 Structural and functional characterization of the 14 stress-responsive genes prospected in the genome of oil palm (*Elaeis guineensis*)

SeqName	ID—NCBI	Description in NCBI	Chromosome or Scaffold Number	DNA Strand	Length (aa)	e-Value	sim mean	InterPro - Protein Family Membership	InterPro—GO terms	Enzyme Codes	Enzyme Names
Gene_01	LOC105038884	adenylate- forming reductase 06235-like	1	-	556	0.00E + 00	75.21	None predicted	No GO Terms	None	None
Gene_02	LOC105053860	E3 ubiquitin-protein ligase Os04g0590900-like	11	-	377	0.00E + 00	67.51	E3 ubiquitin-protein ligase RING1-like	No GO Terms	EC:2	Transferases
Gene_03	LOC105046414	protein NRT1/ PTR FAMILY 2.11-like	6	-	597	0.00E + 00	87.03	Proton-dependent oligopeptide transporter family	(GO:0055085)(GO:0022857)(GO:0016020)	EC:7	Translocases
Gene_04	LOC105055678	transcription factor MYB30-like	12	-	268	0.00E + 00	70.73	None predicted	No GO Terms	None	None
Gene_05	LOC105033974	uncharacter- ized protein LOC105033974	NW_011551084.1	+	149	4.01E-61	61.46	None predicted	No GO Terms	None	None
Gene_06	LOC105048625	putative mediator of RNA polymerase II transcription subunit 7	7	-	211	######	65.6	None predicted	No GO Terms	None	None
Gene_07	LOC105034076	plasmodesmata-located protein 7-like	NW_011551107.1	-	349	######	79.83	None predicted	No GO Terms	None	None
Gene_08	LOC105040304	probable LRR receptor-like serine/ threonine-protein kinase At1g34110	3	+	1077	0.00E + 00	89.57	None predicted	(GO:0006468)(GO:0004672)(GO:0005524) (GO:0005515)	EC:2.7.1	Transferring phos-phorus-containing groups
Gene_09	LOC105034004	zinc finger protein CONSTANS-LIKE 16-like	NW_011551090.1	-	367	0.00E + 00	59.34	None predicted	(GO:0008270)(GO:0005515)	None	None
Gene_10	LOC105058458	uncharacter- ized protein LOC105058458	15	-	195	######	65.8	None predicted	No GO Terms	None	None
Gene_11	LOC105032024	translation initiation factor IF-2	1	-	436	0.00E + 00	63.64	None predicted	(GO:0005515)	None	None
Gene_12	LOC109504909	auxin-responsive protein SAUR36-like	NW_011550957.1	+	155	######	78.74	Small auxin-up RNA	(GO:0009733)	None	None
Gene_13	LOC105039108	chaperonin-like RBCX protein 1, chloroplastic	2	-	181	######	86.79	Chaperonin- like RbcX	(GO:0110102)(GO:0044183)	None	None
Gene_14	LOC105059508	L-lactate dehydro-genase B-like	16	-	350	0.00E + 00	90.58	L-lactate/ malate dehydrogenase	(GO:0019752)(GO:0016616)	EC:1.1.1.27	L-lactate dehydroge-nase

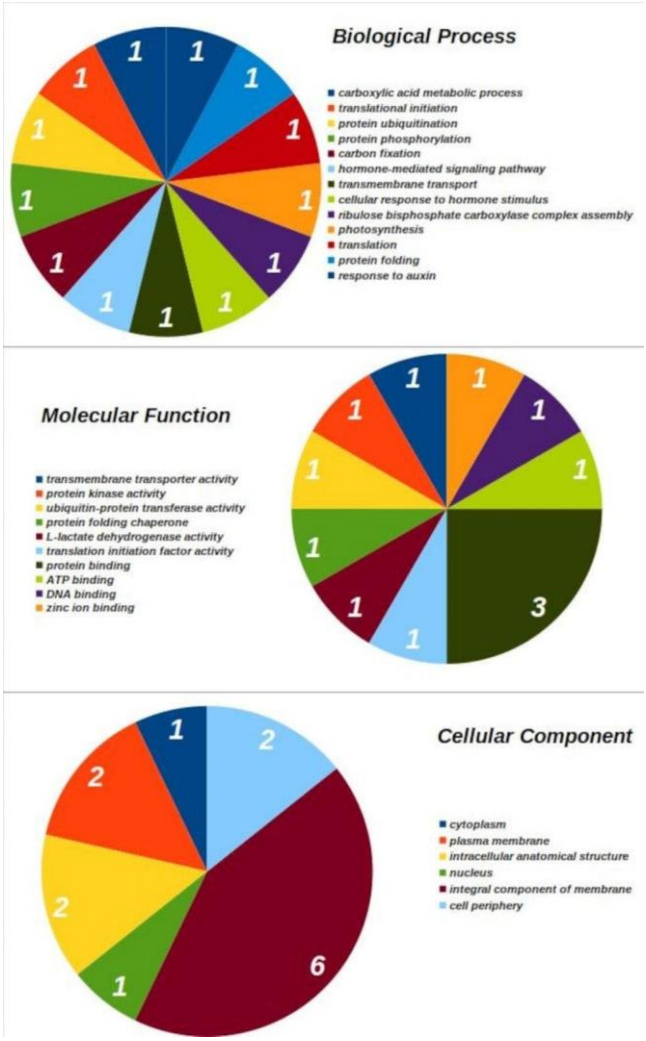

Fig. 6 Gene Ontology (GO) annotation classification statistics graph from the salt-stressed-responsive genes selected in the genome of oil palm (*E. guineensis*); classified accordingly to biological process, cellular component, and molecular function. Numbers represent the amount of positive hits

The α-helices, predominant in the proteins coded by the genes selected in this study, can serve as anchors to the lipid bilayer or form a channel through which various substances can pass. In the latter case, the α-helices have hydrophilic residues directed toward a channel and hydrophobic residues directed away from it and interacting with the lipid bilayer. In this way, many polar sub- stances that, in the absence of proteins, could not cross the membrane will be able to do so through these channels [31]. The protein from gene 3 has similarities to the crystal structure of the nitrate transporter NRT1.1, a transport and membrane protein from *A. thaliana*, which molecular functions are related to the transmembrane activity [32]. The response to nitrate and different stimuli, such as salt stress—stand out among the bio- logical processes [33]. The salt level linked to the low ability to exclude salt causes marked damage in older leaves of glycophyte plants. Such damage occurs due to the accumulation of ions and anions being superior to the compartmentalization capacity in the vacuoles of the cells, leading to cell death from salt intoxication or dehydration, depending on where such ions have accumulated—cytoplasm or cell wall. Transport and membrane proteins are critical to removing toxins inside cells [34, 35].

The protein coded by gene 13 has an affinity with the AtRbcX1 structure of *A. thaliana*, which is a chaperone. AtRbcX1 molecular function is protein folding, its biological process involves metabolic

processes and car- bon fixation, and its cellular component encompasses intracellular anatomical structure, chloroplast, stomatal complex, thylakoid, and plastid. A response commonly observed in plants under saline stress is stomatal closure, and in this condition, the amount of carbon dioxide gets compromised, inhibiting carbon fixation. Chloroplasts,

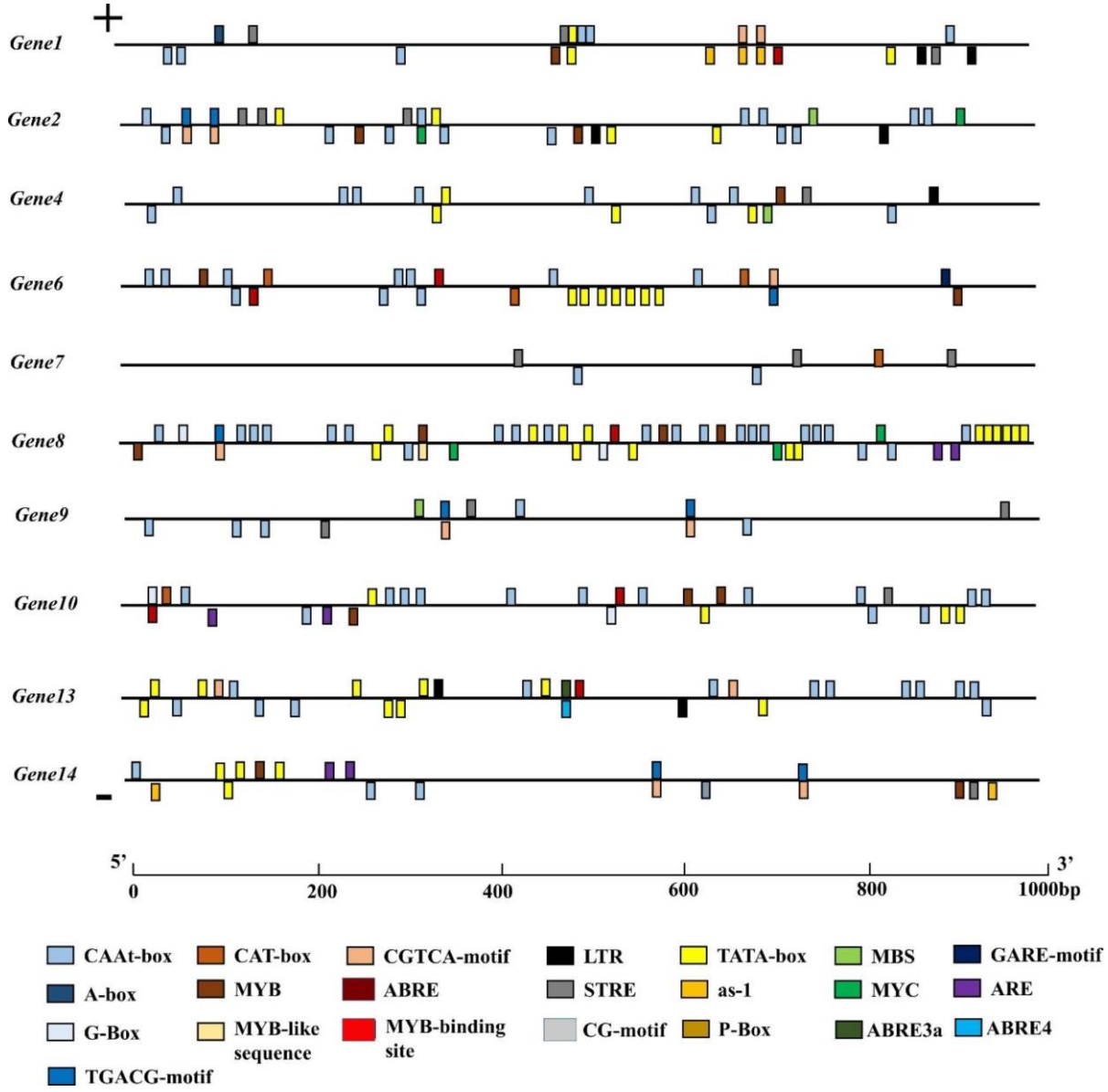

Fig. 7 Cis-Acting elements located in the 1,000 bp long sequence upstream of the initiation codon of the salt-stressed-responsive genes selected in the genome of oil palm (*E. guineensis*). Note: ABRE was involved in the abscisic acid responsiveness; LTR involved in low-temperature responsiveness; G- Box-motif was involved in light responsiveness; CAAT-box was involved in promoter and enhancer regions; GARE-motif, P-box and TATA-box involved in gibberellin-responsiveness; CGTCA-motif and TGACG-motif were involved in the MeJA responsiveness; MBS was involved in drought-inducibility; ARE was involved in light responsiveness; CAT-box was involved in related to meristem expression; MYB was involved in drought-inducibility; MYC was a transcription factor response element; STRE was a non-biological absorption of related reaction elements.

in turn, are exposed to excess energy, which increases the generation of reactive oxygen species [36].

The number and shape of cis-elements in promoter regions can play an essential role in regulating gene expression related to different metabolic pathways [37– 42]. Therefore, the 1000 bp upstream region of genes with similar expression profiles were subjected to a similarity search for the cis-acting elements

in common among them. Genes with STRE, MYB, MYC, and MYB-binding site cis-acting elements in their promoters showed increased expression when subjected to salt stress. Finally, regarding downregulated genes, when the gene has the promoter with cis-acting elements ABRE4, ARE, and MYB-like in the upstream region of the start codon, it experiences a reduction of expression when subjected to saline stress [38].

In the present study, among the cis-elements found in the promoter regions of the 14 genes, the MYC, G-Box, ABRE, and TATA-box are linked to salinity [37, 39, 42]. Although there are reports on TATA-less promoters in plant genomes [43–45], this not usual result seen in the promoter sequences from genes 7 and 9 can also be a result of the quality of the reference genome used. Further analysis is necessary to determine whether it is a case of the former or the latter.

Once looking for a specific distribution pattern of cis- acting elements in the putative promoter region, the present results confirm that it will be necessary to have many more sequences to find and consequently design markers for *in silico* genome-wide search for salinity- responsive genes. However, as it is known, one can select some of these cis-acting elements to target in an editing strategy aiming to interfere with the translation and, consequently, the expression of one or more proteins, which could change their function concerning saline stress. Therefore, they would become a target site for CRISPR-based testing for exact modifications in the cis-acting sequence, either to silence the gene or to place it in an upstream region of a not salt-responsive gene and then analyze the effect of that. The novel CRISPR–Cas9 genome editing system will be a factor in achieving a more precise horizontal transfer, reducing, to a certain extent, the need for some biosafety analysis demanded today for genetically modified plants [46].

The current study is another step in our work on characterizing the morphophysiological and molecular responses of oil palm plants to salinity stress [47–50]. Besides developing a salinization protocol successful in generating different levels of stress by gradients of electrical conductivity and water potential [47], the studies that followed up led to the identification of salt-responsive miRNAs and their putative target genes [48], as well as mapping the main pathways affected by this abiotic stress [49]. On top of that, they allowed the identification of several salt-responsive genes, proteins, metabolites, and promoter sequences [48, 49, 50, and the present report] which might open windows of opportunity to develop salt-tolerant oil palm genotypes via genetic engineering/ editing approaches.

Conclusion

Our study reports a strategy to select profile-specific salt stress-inducible genes and their promoters. This strategy employed RNA-Seq followed by time-course differential expression analysis. In addition, a quantitative and qualitative comparison study tried to validate the RNA-Seq results using qRT-PCR. Fourteen differentially expressed genes were selected and validated in this study. Some inconsistencies did appear when comparing the expression profiles—RNA-seq against qRT-PCR—that may have to do with the difference in sensitivity between them and the amount of biological and technical replicates used. This set of genes is *per se* a source of candidate genes to undergo future validation of their capacity to confer resistance to salinity stress. Regarding the promoters and their gene regulation regions, it was not yet possible to infer that a specific combination of cis-acting elements is a good candidate marker for future use in a genome-wide search for this type of profile-specific salt stress-inducible genes. However, one can consider some of the cis-acting seen as a target for gene editing by CRISPR–Cas9 technology.

Materials and methods

Oil palm transcriptome database

All oil palm plants used in the study were derived from embryogenic callus from genotype AM33, a Deli x Ghana from ASD Costa Rica (http://www.asd-cr.com). The AM33 genotype is a plant from a commercial field in the State of Pará, Brazil. Prof. Sergio Motoike, from the Universidade Federal de Viçosa—UFV, located in Minas Gerais, Brazil, supplied the embryogenic calluses from AM33. Young oil palm plants at the growth stage known as "bifid" seedlings were subjected to two different doses of NaCl in March 2018 and maintained under these conditions for 12 days in a completely randomized experimental design in a greenhouse in Brasília, DF, Brazil (15,732 ° S, 47,900° W, 1,030 m of altitude) [47].

The decision on which samples for transcriptomics analysis to use came after taking into consideration the morphophysiological responses of the young oil palm plants to salinity stress. Vieira et al. [47] characterized those responses by submitting the plants to different treatments (0.0, 0.5, 1.0, 1.5, and 2.0 g NaCl per 100 g substrate on a dry basis), with five replicates per treatment. For the present study, we collected the apical leaves from three stressed plants (2.0 g NaCl, or ~ 40 dS m^{-1} of electrical conductivity) at 05 and 12 days after the stress onset (DAT), together with the apical leaves from three control plants at 12 days (0.0 g NaCl, or ~ 2 dS m^{-1}), immediately frozen in liquid nitrogen and stored at − 80 °C until RNA-Seq. More details about the plant material, growth conditions, saline stress conditions, and the experimental design used in the study that generated this database were previously reported [47–50].

RNA-Seq data analysis

The raw data analyzed in this study are available in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information—BioProject PRJNA573093, BioSample SAMN12799239. The Omic-sBox Bioinformatics Platform [50] was employed to perform all RNA-Seq analyses, as described previously [48–50]. To run the time-course expression analysis among the treatments, we used the default parameters based on the software package maSigPro, from to the Bioconductor project, FDR (False Discovery Rate) ≤ 0.001, R-squared ≥ 0.9, without the use of a filter for low counts genes, and using the Trimmed mean of M values method of normalization [51]. Each treatment was represented in this RNA-Seq study by three replicates - or three plants.

Table 2 Pairs of primers designed and used for differential expression analysis of the 14 prospected genes by means of the qPCR technique

Gene	5′- end Primer	3′- end Primer
GENE_01	CTTCCAAGCCGAAACAACC	TACACCGTGAACAGTCCCT
GENE_02	AGAGCCAGTTGCTATCTCC	ATACTTGATGGCAGTG-GAAGG
GENE_03	TCAATTCAGGCTTGTTGAC	GAATGCGATCATAAACAGGT
GENE_04	CAAGCAAGCTGGTCTATT-GAG	TTCCTCCGAAGAATCCGTG
GENE_05	ATCTCCTCTGAGGAGAGG	AGACAGCAAGAGCAACAG
GENE_06	AATCCACCATCAGATGCA-CAG	ATCTGGCCTGACTTGCCA
GENE_07	AGACACGATCACGTTGAT-GAG	GAGAAGAGCATAATGGA-CAACAC
GENE_08	TTGCACCAGAGTATGGT-TACAC	AAATCTAC-CACCTTCAGGGAC
GENE_09	GACAACGCTATCACCTA-CACC	TCTCCCTATACCTCGTCACCT
GENE_10	ACTTATCCAATCGCACCT	TAATGCAATACCCACTCCAC
GENE_11	CTTCTGCCAATATG-TATTCCTCC	CTGGTACTTGATGGAGA-GTAGG
GENE_12	TAGTCACCACCAACCTCAG	GAGCCTTGTCCATATCCACAG
GENE_13	CGCAACTTGAGAGCTATA-ATCC	GTATACTCGTATGAAGGTG-GTG
GENE_14	ATGTGCAGGCCTACATGG	GGTTAGACTCCTCATCT-GTGAG
EgEfM-POB00119*	CCAGGGTTCAGCTGATTAAG	TCGTCCAAATCCAGCAATC

* Internal Control Gene

RNA extraction, reverse transcriptase-PCR and quantitative real-time PCR analyses

After being collected from young oil palm plants at 5 (stressed) and 12 (control and stressed) DAT, the apical leaves underwent immersion in liquid nitrogen and then stored at -80 °C until RNA extraction. Total RNA was isolated using the Qiagen Rneasy® Plant Mini kit (QIA- GEN, CA, USA) following the manufacturer's protocol, and RNA quantity was measured using the Nanodrop Qubit 2.0

Fluorometer (Life Technologies, CA, USA). After that, the extracted RNA was used as a template for reverse transcription to obtain cDNA using the commercial kit SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen®). The gene named EgEfMPOB00119 40 S ribosomal protein S23 mRNA, complete cds, mRNA sequence present in *E. guineensis*, was chosen as a positive control (constitutive gene).

The mRNA fasta files from the selected genes were downloaded from NCBI and exported to the PerlPrimer software [52] for designing the primers (Table 2). The qRT-PCRs were carried out in optical 96-well plats in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with an SYBR® GreenER™ qPCR Super- Mix Universal (Invitrogen®) (INVITROGEN, 2010), following the manufacturer's instructions. The thermal cycler was set as follows: (a) 95ºC for 34 s, 95ºC for 5 s, 60ºC for 34 s for 40 cycles, and at the second step of each cycle, fluorescence was obtained; (b) the dissolution curve was acquired as followed: 95ºC for 15s, 60ºC for 60s and 95ºC for 15s. Fluorescence readings were performed by StepOnePlus™ Real-Time PCR at each amplification cycle and, later, analyzed by StepOnePlus™ software v2.3—Life Technologies. Two biological replicates and three technical replicates were used for this study, generating six reads per gene per treatment. The method of $2^{-\Delta\Delta CT}$ was adopted to represent relative expression levels of the genes [26].

Structural & functional annotation

For the structural annotation of the sequences of the sen genes, the symbol, description, location on chromo- some, number of exons, type, and direction of each gene underwent analysis using the information available in the NCBI. The search for functional domains in the proteins used the NCBI Conserved Domain Search (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Parameter set- tings influencing the query execution results were the fol- lowing: E-value ≤ 1. With this, it was possible to produce the images by the IBS Illustrator Software [53]. The Soft- ware RaptorX (http://raptorx.uchicago.edu/Contact- Map) was used to investigate the tertiary structure of the proteins. In addition to the search for tertiary structures of proteins, a BLASTp was performed with the protein sequences in the BLAST database (Basic Local Alignment Search Tool) to search for similarity with resolved structures in the PDB.

Based on the oil palm reference genome [22], 1,000 bp upstream sequences of the selected genes were acquired. PlantCARE was used to analyze them with the default parameter (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) [54]. Parameter settings were the following: matrix score less than or equal to 5. The IBS Illustrator Software was again used to produce the images [53].

Abbreviations

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats
DAT days after imposition of the treatments
FDR False Discovery Rate
GO Gene Ontology
NCBI National Center for Biotechnology Information NGS Next-Generation Sequencing
PDB Protein Database
IBS Illustrator for Biological SequencesqPCR quantitive PCR

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Author contributions

C.A.F.S. and M.T.S.Jr. Designed the research; T.M.M.F. and A.P.L. performed the experiments; T.M.M.F., J.A.F.F., and M.T.S.Jr. Analyzed the data and drafted the manuscript. MTSJr have revised the manuscript. All authors read and approved the final manuscript.

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Data Availability
All RNA-seq fastq files used in this study have been uploaded in the SRA database of the NCBI under *Elaeis guineensis* Transcriptome_Drought and Salinity Stresses - BioProject PRJNA573093 (SUB6324604), BioSample
SAMN12799239 (SUB6325749), SRA submission SUB6335775 (accessions from SRR10219424 to SRR10219441). The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval
The species *Elaeis guineensis* is present in the annex of the Ministry of Agriculture, Livestock and Food Supply (MAPA) Normative Instruction No. 14, of 10/08/2021; so, this species is outside the scope of Law 13.123/2015
(Biodiversity Framework; Law on Access to Genetic Heritage) and, therefore, is not native from Brazil. The use of plant material and all methods applied were carried out in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest.

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THIRD PART

2 FINAL CONSIDERATIONS

Purslane (*Portulaca oleracea* L.) is a succulent and versatile plant, which stands out for its diverse characteristics of interest to different industries: pharmaceutical, chemical, food and feed, to name a few. Recognized by the WHO as one of the most used medicinal plants, it also ranks as the eighth most common weed in the world.

Purslane has been the subject of increasing interest in the context of agriculture in a controlled environment, where it has exceptional biomass productivity. In this scenario, different cultivation systems become viable: greenhouses, containers (vertical cultivation systems) and bioreactors (cell suspensions). However, there is still a need for biotechnological initiatives to maximize their potential, given the ability to provide a range of useful biomolecules for various sectors.

Our results provide a deeper understanding the role of the antioxidant defense system in purslane under high salinity stress, which demonstrated a high expression of the main groups of enzymes and redox molecules mainly in the leaves of adult purslane, providing a comprehensive view of the molecular mechanisms involved in the response of this halophytic plant. Differential expression studies provide a foundation of knowledge about this hitherto little studied mechanism. Our research can help future investigations to develop strategies in the context presented, in addition to studies to generate resistance to salinity.

In work on purslane, two enzymes exhibited a balance between shared ancestry with other species and unique evolutionary changes specific to their lineage, indicating a more conserved protein sequence among plant species. This shows that sequences have been maintained in evolution despite speciation. The longer the phylogenetic tree remains, the more conserved it is.

Gliricidia sepium (Jacq.) Steud., a member of the Fabaceae family and the Faboideae subfamily (Papilionoideae), is a tree species native to Mexico, with a distribution extending through Colombia, Venezuela, and the Guianas. In Brazil, it is classified as an introduced species, having been introduced to the Brazilian semi-arid region during the 1980s. *G. sepium* is distinguished by its rapid growth, high regenerative capacity, drought tolerance, and ease of propagation, both sexually and asexually.

This species is particularly notable for its nitrogen-fixing ability, which enhances soil fertility and has the potential to reduce or partially replace up to 45% of chemical fertilizer

usage. Furthermore, *G. sepium* contains a high crude protein content, which can reach as much as 23%. Its diverse array of applications has spurred increasing commercial and economic interest, particularly in tropical regions. The species' drought resistance, a key adaptive feature, facilitated its cultivation in the semi-arid regions of northeastern Brazil, where prolonged droughts are common. Additionally, the soils in this region are prone to salinization. Concerning salinity, research has demonstrated that *G. sepium* exhibits satisfactory growth and forage production under saline soil conditions, including those irrigated with brackish water. When grown with effluents from aquaculture systems, the plant shows enhanced production of both green and dry biomass.

RNA-Seq analysis of *G. sepium* leaves exposed to high salinity stress suggests a relevant role for specific isoforms within the MDHAR, GPX, POX and GST groups of enzymes in the plant adaptation process. Some isoforms exhibited downregulation in leaves but upregulation in roots under salt stress. This suggests potentially distinct roles for these isoforms in different plant tissues, giving important insight into the mechanism of plant adaptation. These findings cover possibilities for future research within the scope of the problem mentioned in this work. As with purslane, there is still a need for biotechnological initiatives to maximize the potential of gliricidia.

Portulaca oleracea (purslane), classified as a halophyte in the eHALOPH database, exhibits a gradual response to salt stress until senescence. In contrast, *Gliricidia sepium* (gliricidia) presents leaf abscission followed by regrowth. Additionally, purslane has a short life cycle, while gliricidia is a perennial legume. These characteristics may influence the expression and localization of proteins, especially enzymes, in each species.

Importantly, this doctoral thesis reports a significant strategy to select profile-specific salt stress-inducible genes in the three species studied. Furthermore, a quantitative and qualitative comparative study attempted to validate the RNA-Seq results using qRT-PCR to legitimize the research. The comparison of techniques proved to be inconsistent in many genes analyzed, both for purslane and oil palm. These differences may be related to the individual sensitivity of the techniques, the number of biological replicates and techniques used in each experiment.

Despite the inconsistencies, the set of genes identified in both studies both studies regarding the antioxidant defense system in purslane and gliricidia represent a valuable source of candidates for future research. These genes can be subjected to validation tests to determine their ability to confer resistance to salt stress. It is recommended to improve the phylogenetic analysis by including a larger number of species, allowing a more comprehensive understanding

of the evolutionary relationships between the genes studied. For tertiary analysis it would be of great value to focus on protein interactions.

In relation to oil palm, climate change and the expansion of the cultivation zone are likely to impose challenges on oil palm in the future that could negatively impact its productivity. Notably, among the abiotic stresses that affect crops, water availability and saline stress are among the most worrying.

Regarding promoters and their gene regulatory regions, it has not yet been possible to determine whether a specific combination of cis elements can be considered an ideal marker for future genome-wide research. However, some of the cis actions identified in this study can be considered targets for gene editing using CRISPR-Cas9 technology, opening the way for new and promising lines of research.

It was evident that the results of these studies represent important steps in understanding plant resistance to this environmental stress. With future research and the application of techniques such as CRISPR-Cas9, we can pave the way for the development of new plant varieties that are more tolerant to salt stress, ensuring food security and environmental sustainability.

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