

Unravelling the role of proline in glyphosate-mediated toxicity – tolerance mechanism or stress signal?

Pedro Francisco Couto Nadais

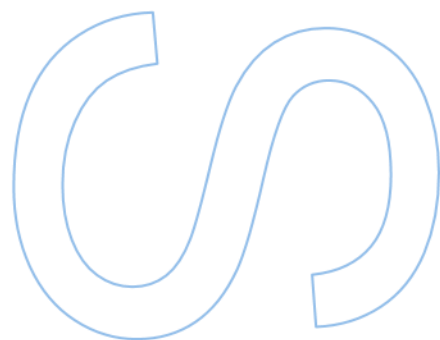
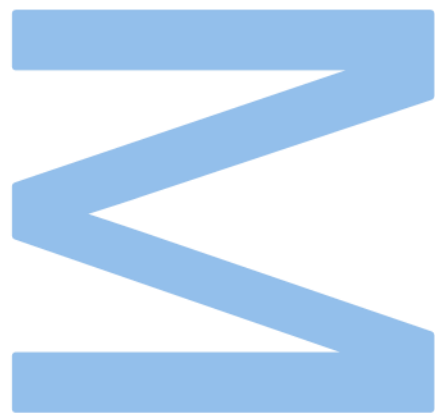
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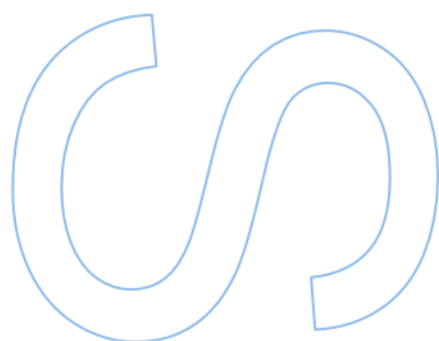
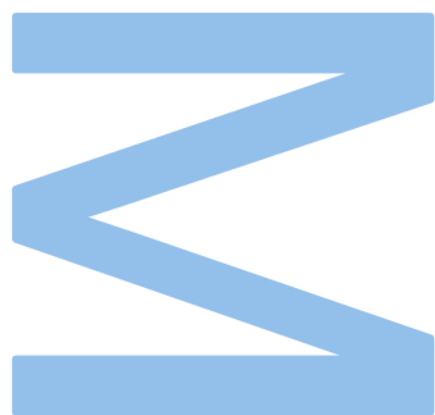
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Pedro Francisco Couto Nadais

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Resumo

Atualmente, o glifosato (GLY) é o herbicida mais utilizado em todo o mundo, e a sua utilização constante e cumulativa tem resultado na contaminação dos solos agrícolas, possivelmente impondo efeitos prejudiciais em organismos não alvo, tais como plantas de interesse comercial. Embora a ação deste herbicida não esteja diretamente relacionada com danos oxidativos, estudos recentes sugerem que um desequilíbrio na homeostase redox é frequentemente uma consequência indireta da exposição das plantas ao GLY. Como tal, uma boa regulação dos mecanismos antioxidantes deve ser orquestrada para melhorar o seu crescimento sob um ambiente contaminado com GLY. De todos os componentes desta maquinaria antioxidante (AOX), a prolina (Pro) deve ser destacada, pois os níveis elevados deste importante aminoácido são geralmente relatados em plantas com stresse imposto pelo GLY, mas ao contrário do que se poderia esperar, isto não é acompanhado por uma resposta de tolerância. Além disso, quando são aplicados bioestimulantes distintos (por exemplo silício, óxido nítrico, ácido salicílico) para mitigar a toxicidade induzida pelo GLY, os níveis Pro diminuem substancialmente. Portanto, a principal questão subjacente a este estudo é se a acumulação excessiva de Pro contra a toxicidade induzida pelo GLY pode ser interpretada como um mecanismo de tolerância ou um sinal de stresse. Para responder a esta questão, foram utilizadas linhas mutantes com inserção de T-DNA em *Arabidopsis thaliana* para genes envolvidos no metabolismo (*P5CS1*) e no catabolismo (*ProDH*) de forma a analisar a resposta das plantas tratadas com GLY com padrões de acumulação contrastantes de Pro. Em primeiro lugar, o conteúdo Pro foi determinado em plantas cultivadas em meio nutritivo com concentrações crescentes de GLY (0,5; 1; e 2 mg L⁻¹) durante 14 dias, para verificar se o GLY leva a um aumento de Pro e selecionar a dosagem adequada de GLY para ensaios subsequentes. Depois, foram selecionadas linhas mutantes homozigóticas e funcionalmente caracterizadas, nomeadamente *p5cs1-1*, *p5cs1-4*, e *prodh*, seguidas de experiências *in vitro* com todos os genótipos. Após 14 dias de exposição ao GLY (0,75 mg L⁻¹), observou-se uma diminuição geral da biomassa fresca após exposição ao herbicida em todos os genótipos, a que se seguiu uma acumulação excessiva de Pro. Contudo, os dados também mostraram que as plântulas do genótipo *prodh* eram as mais sensíveis, atingindo valores de inibição de crescimento em torno dos 80%, enquanto também apresentavam, como esperado, os níveis mais elevados de Pro. Ao avaliar a formação de espécies reativas de oxigénio e danos oxidativos, verificou-se que os genótipos com uma maior acumulação de Pro apresentaram um aumento significativo de peróxido de

hidrogénio e peroxidação lipídica em resposta ao GLY. Além disso, foi realizada uma avaliação exaustiva do sistema AOX das plantas, e, curiosamente, os mutantes com menor acumulação de Pro foram os que apresentaram uma ativação mais eficiente do sistema AOX, tanto a nível enzimático como não enzimático. De facto, os nossos dados revelaram níveis aumentados de glutatona e ascorbato nas plantas *p5cs1-1* e *p5cs1-4*, bem como uma maior atividade das enzimas catalase e ascorbato peroxidase nas plantas *p5cs1-4*. A exposição GLY também alterou os padrões de expressão genética, uma vez que os genes *P5CS1* e *P5CS2* foram desregulados por este herbicida, especialmente nos mutantes *p5cs1-1*. Por outro lado, verificou-se que os níveis de expressão *OAT* foram aumentados em plantas de tipo selvagem cultivadas sob a influência do GLY, bem como em mutantes *prodh*. Globalmente, estes resultados sugerem que a sobreacumulação de Pro em plantas expostas ao GLY está relacionada com a sensibilidade ao stresse e não com uma maior tolerância, uma vez que esta acumulação não ajuda a planta a defender-se contra o stresse imposto pelo herbicida. Sendo assim, podemos propor que a Pro seja utilizada como um sinal de alerta precoce de toxicidade induzida pelo GLY, que pode ser utilizada para calcular o risco de exposição do GLY a plantas não alvo, bem como para detetar a resistência das ervas daninhas.

Palavras-chave: glifosato, prolina, *P5CS1*, *P5CS2* *ProDH*, *OAT*, espécies reativas de oxigénio, sistema antioxidante.

Abstract

Nowadays, glyphosate (GLY) is the most extensively used herbicide around the world, and its constant and cumulative use has been resulting in the contamination of agricultural soils, possibly imposing detrimental effects on non-target organisms, such as crops. Although GLY's herbicidal action is not directly related to oxidative bursts, recent evidence suggests that a disbalance in the redox homeostasis is often an indirect consequence of GLY exposure. As such, a fine regulation of the plants' antioxidant mechanisms must be orchestrated to improve their growth under a GLY contaminated environment. Of all components of this antioxidant (AOX) machinery, proline (Pro) should be highlighted because high levels of this important amino acids are usually reported in GLY-stressed plants, but contrarily to what could be expected, this is not accompanied by a tolerance response. Moreover, when distinct biostimulants (e.g. silicon, nitric oxide, salicylic acid) are applied to mitigate GLY toxicity, Pro levels decrease substantially. Therefore, the main question underlying this study is whether the overaccumulation of Pro against GLY toxicity can be interpreted as a tolerance mechanism or a stress signal. To answer this, *Arabidopsis thaliana* T-DNA insertional mutant lines for genes involved in Pro metabolism (*P5CS1*) and catabolism (*ProDH*) were used to analyse the response of GLY-treated plants with contrasting Pro accumulation patterns. Firstly, Pro content was determined in plants growing on nutritive medium with increasing GLY concentrations (0.5, 1, and 2 mg L⁻¹) for 14 days, to verify that GLY leads to a Pro increase and select the adequate GLY dosage for subsequent assays. Then, homozygous mutant lines were selected and functionally characterized, namely *p5cs1-1*, *p5cs1-4*, and *prodh*, followed by *in vitro* experiments with all genotypes. After 14 days of exposure to GLY (0.75 mg L⁻¹), a general decrease in fresh biomass upon herbicide exposure was observed in all genotypes, this being followed by an overaccumulation of Pro. However, data also showed that *prodh* seedlings were the most sensitive, reaching growth inhibition values around 80% while also accumulating, as expected, the highest levels of Pro. When assessing reactive oxygen species formation and oxidative damages, it was found that the genotypes with a higher accumulation of Pro showed a significant increase in hydrogen peroxide and lipid peroxidation in response to GLY. Additionally, a thorough evaluation of the plants' AOX system was carried out, and, interestingly, mutants with lower Pro accumulation were the ones who presented a more efficient activation of the AOX system, both at an enzymatic and non-enzymatic level. Indeed, our data revealed increased levels of both glutathione and ascorbate in *p5cs1-1* and *p5cs1-4* plants, as well as an enhanced activity of the enzymes

catalase and ascorbate peroxidase in *p5cs1-4* plants. GLY exposure also altered gene expression patterns, as *P5CS1* and *P5CS2* were downregulated by this herbicide, especially in *p5cs1-1* mutants. On the other hand, *OAT* expression levels were found to be increased in wild-type plants grown under GLY's influence, as well as in *prodh* mutants. Overall, these findings suggest that Pro overaccumulation under GLY exposure is related to stress sensitivity rather than an increased tolerance, since this accumulation does not aid the plant defending itself against the GLY-imposed stress. As a result, we may propose that Pro be utilized as a GLY toxicity early-warning signal that can be used to calculate the risk of GLY exposure to non-target plants as well as to detect weed resistance.

Keywords: glyphosate, proline, *P5CS1*, *P5CS2*, *ProDH*, *OAT*, reactive oxygen species, antioxidant system.

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List of Abbreviations

2,4-D – 2,4-dichlorophenoxyacetic acid	ETC – Electron transport chains
ABA – Absciscic acid	gDNA – Genomic DNA
<i>ACT8</i> – <i>ACTIN 8</i>	GFP – Green fluorescent protein
ANOVA – Analysis of variance	GLY – Glyphosate
AOX – Antioxidant	GR – Glutathione reductase
APX – Ascorbate peroxidase	GSA – Glutamic-5-semialdehyde
AsA – Reduced ascorbate	GSH – Glutathione
AsA-GSH – Ascorbate-glutathione cycle	GSSG - Glutathione disulfide
BIP – 4,4'-bipyridyl	GST – Glutathione S-transferase
BSA – Bovine serum albumin	IAA – Indole-3-acetic acid
Car – Carotenoids	LB – Luria Broth medium
CAT – Catalase	LPr – Left primer
Chl – Chlorophylls	LP – Lipid peroxidation
CTL – Control	MDA – Malondialdehyde
DABs – Decolorized aniline blue	MDHA – Monodehydroascorbate
DCF-DA – 2',7'-dichlorodihydrofluorescein diacetate	MDHAR – Monodehydroascorbate reductase
DHA – Oxidized ascorbate	MS – Murashige and Skoog
DHAR – Dehydroascorbate reductase	NASC – Nottingham <i>Arabidopsis</i> Stock Center
DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid)	NEM – <i>N</i> -Ethylmaleimide
DTT – Dithiothreitol	OAT – Ornithine δ -aminotransferase
EDTA – Ethylenediaminetetraacetic acid	P5C – pyrroline-5-carboxylate
EPSPS – 5-enolpyruvylshikimate-3-phosphate synthase	P5CR – Δ 1-pyrroline-5-carboxylate reductase

P5CS – Δ^1 -pyrroline-5-carboxylate-synthetase

PCD – Programmed cell death

PEP – Phosphoenolpyruvate

PK – Potassium phosphate

PMSF – Phenylmethylsulphonyl fluoride

PQ – Plastoquinone

Pro – Proline

PSI – Photosystems I

PSII – Photosystem II

PVPP – Polyvinylpolypyrrolidone

ROS – Reactive oxygen species

RP – Right Primer

RT – Room temperature

RUB 1 – *RELATED TO UBIQUITIN 1*

RuBisCO – ribulose-1,5-biphosphate carboxylate-oxygenase

SB – Sodium Borate Buffer

SD – Standard Deviation

SDS – Sodium Dodecyl Sulfate

-SH – Thiol

SOD – Superoxide Dismutase

TBA – Thiobarbituric Acid

TCA – Trichloroacetic Acid

TET – Tetracycline

Wt – Wild-type

1. Introduction

1.1. AGROCHEMICALS

Natural or synthetic agrochemicals, which comprises pesticides, are widely used to protect crops from diseases and pests (Mandal et al., 2020). Depending on the group of target species, they can be grouped in a range of classifications, including herbicides, insecticides, rodenticides, fungicides, and bactericides, among others (Sharma et al., 2019). Before the creation of synthetic agrochemicals, only natural techniques were available to control the devastation caused by pests and disease vectors, even though historically the usage of pesticides dates back many thousand years (Tudi et al., 2021). The first synthetic pesticides were produced during World War II, marking the pesticide business's beginning (Bernardes et al., 2015; Tudi et al., 2021). Actually, despite dichlorodiphenyltrichloroethane being developed in the 1800's, it was only classified as an insecticide by Hermann Müller in 1939. It was initially employed for non-agricultural objectives, specifically to eradicate typhus, yellow fever, and malaria vectors (de O. Gomes et al., 2020). In addition to dichlorodiphenyltrichloroethane, throughout the 1940s of the 20th century, the synthetic auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D) was synthesized during the World War II and, due to its simple and affordable production, its commercialization expanded swiftly across the world (Hasanuzzaman et al., 2020). As weeds compete with crops directly for light, water, nutrients, and space as well as serve as natural reservoirs for disease vectors, the growth of undesired plants has long been a major hindrance for agricultural production (Swinton et al., 1994; Boydston et al., 2008; Ziska, 2016). Moreover, since weeds have a greater capacity than crops to adapt to new climatic and edaphic circumstances, they are projected to develop more quickly as a result of the increase in atmospheric carbon dioxide (CO₂). In fact, as they have not undergone artificial selection for advantageous developmental features, these species often exhibit a significantly higher genetic diversity (Korres et al., 2016). Therefore, and despite the present tendencies for conservation agriculture, it is anticipated that the widespread usage of herbicides will increase over the coming years, as weed interference can result in yield losses up to 70% (Hasan et al., 2021). Herbicides can be divided into categories based on a variety of characteristics, such as their mode of action, selectivity, target crops, and active ingredient. Atrazine, 2,4-D, and glyphosate (GLY) are some of the herbicides that are used most frequently on a global scale. Despite their function in the management of weeds, there is considerable debate regarding their potential non-target, environmental, and health impacts (Hasanuzzaman et al., 2020).

1.1.1. Glyphosate (GLY)

Despite the ever-growing knowledge in the diverse areas that comprises the development of new agrochemical products, such as biology and chemistry, and with the appearance of newer products, the most popular herbicide worldwide nowadays is still glyphosate (GLY) [N-(phosphonomethyl)glycine], and it has seen a significant increase in its use in agriculture since the development of glyphosate-resistant crops. It is believed that the GLY molecule was created for the first time in a small Swiss pharmaceutical business (based in Cilag), by Henri Martin; nevertheless, it was never tested or, at the very least, not patented for herbicidal application. Not only, until John E. Franz of Monsanto Co. formulated and tested, in 1970, GLY as an herbicide for the first time, where shortly after, it was granted a patent for herbicidal use, under the trade name of RoundUp® (Duke and Powles, 2008). This herbicide entered the market in 1974 as the isopropylamine salt of GLY, and as a post-emergence, broad-spectrum, non-selective systemic herbicide, used to completely control vegetation, and its use increased progressively (Duke and Powles, 2008; Gomes et al., 2014). Usually, GLY is sprayed on plant foliage in agricultural fields and urban contexts, but some of the applied doses may also be spilled to the ground or carried by the wind to nearby soils and surface waters, exposing non-target plants to the toxin (**Figure 1**). Additionally, GLY transfer to nearby agricultural fields can occur as a result of waterway contamination, particularly in cases where fields are irrigated by pumping water into surface water bodies. Exudation from the roots of plants sprayed with this herbicide and its release from dead plants are two additional significant sources of GLY exposure (Gomes et al., 2014) Once in the soil, GLY may be adsorbed onto soil particles, broken down by bacteria, or migrate through soil pores or root canals to deeper soil horizons.

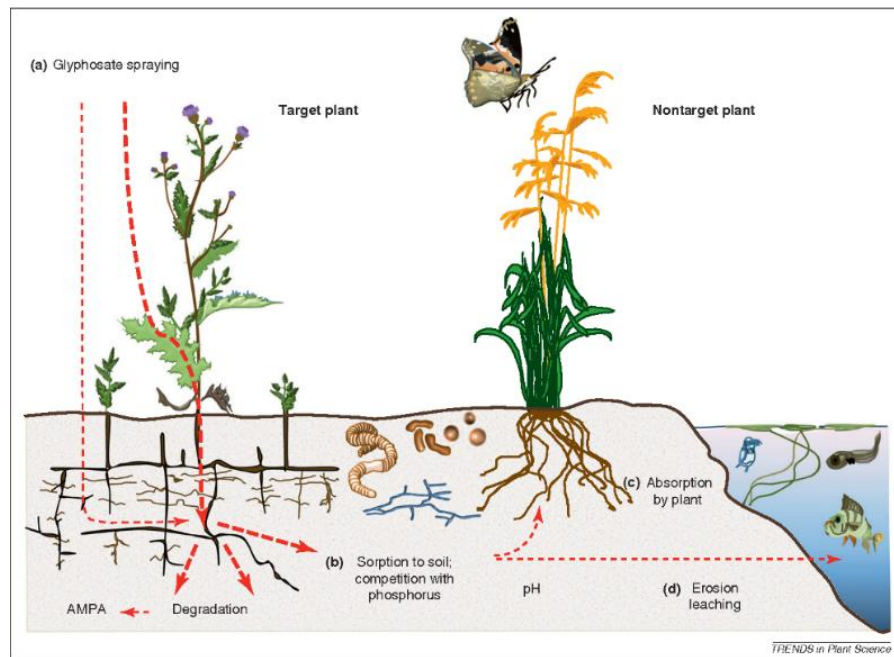


Figure 1 Mechanisms by which GLY can reach the soil and move to other environmental compartments. Upon treatment of the foliage of target plants. Retrieved from Helander et al. (2012)

However, some agricultural techniques, including phosphorous amendment, may resolubilize GLY in soils, making it available for leaching and non-target plants' rhizosphere (Borggaard and Gimsing, 2008; Gomes et al., 2014). In terms of mode of action, GLY was created to particularly obstruct the shikimate pathway (**Figure 2**), a metabolic pathway only present in plants and some microorganisms (Duke and Powles, 2008). Briefly, GLY directly competes with phosphoenolpyruvate (PEP), one of the substrates of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), due to their structural similarities. As a result, when GLY is present, EPSPS activity is inhibited, which causes an overabundance of shikimate and lower levels of the product of this enzyme, enolpyruvylshikimate-3-phosphate (EPSP), and chorismite, precursors of the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan, which are also involved in the biosynthesis of key specialized metabolites in plant growth, such as indole-3-acetic acid (IAA), anthocyanins, and flavonoids (Tzin and Galili, 2010; Vencill and Duke, 2020).

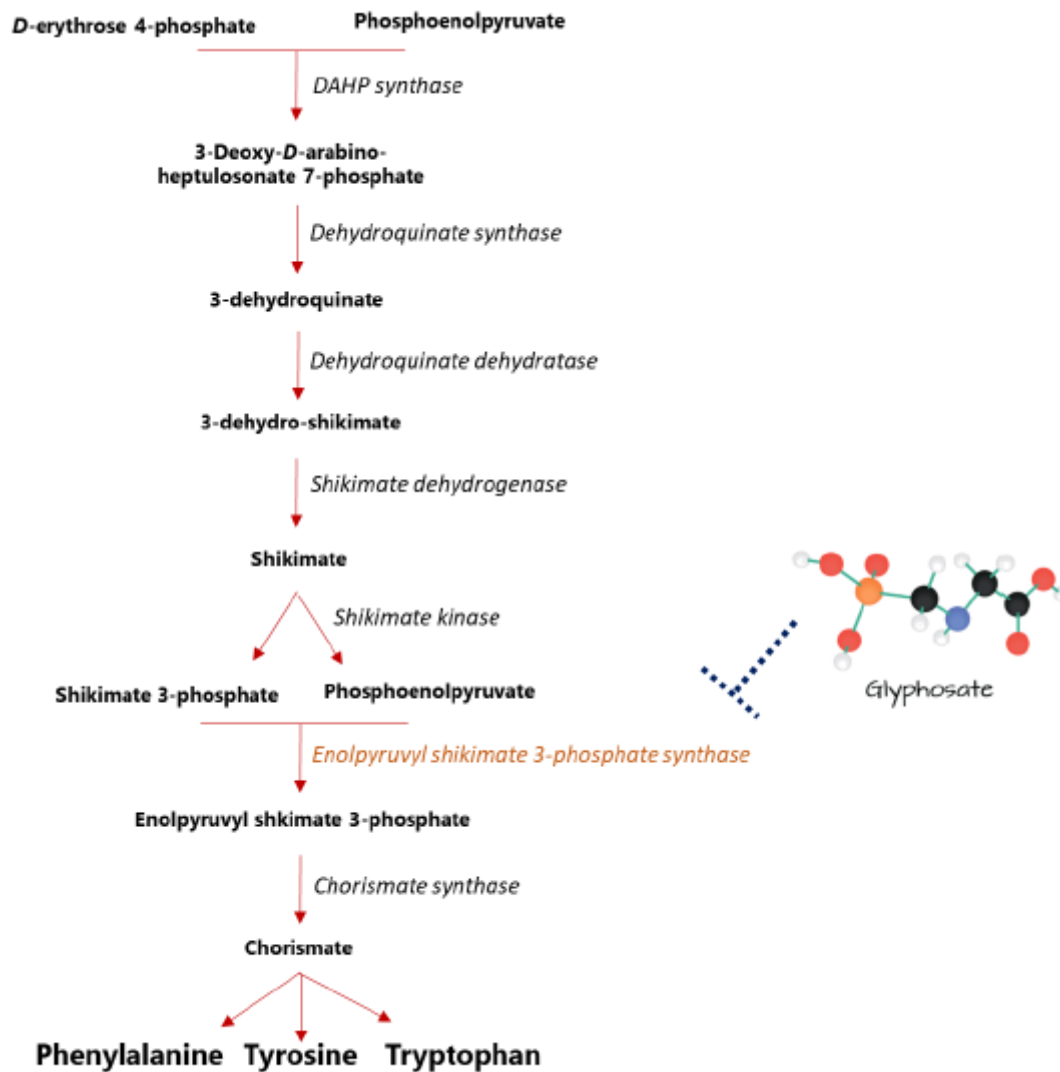


Figure 2 Schematic representation of the shikimate pathway showing the Glyphosate (GLY) interference with one of its biochemical steps, namely the inhibition of enolpyruvyl shikimate 3-phosphate synthase (EPSPS) (Soares, 2022).

At least three connected effects of EPSPS inhibition may result in phytotoxicity. The most evident one is the loss of aromatic amino acids and molecules derived from them, including IAA and plastoquinone (PQ), which are both crucial for plant metabolism. This way the inhibition of EPSPS compromises several mechanisms and functions in the plant, such as protein synthesis, which requires aromatic amino acids, IAA, which is produced from tryptophan, one of the major phytohormones, necessary during several stages of plant development and growth, and PQ, derived from tyrosine, essential for electrons transport during photosynthesis (Vencill and Duke, 2020). Phenylalanine and tyrosine are the precursors to several phenylpropanoids including lignin and certain phytoalexins. A plant may not be killed by preventing the production of just these latter

products of the shikimate pathway, at least not quickly enough to qualify as an herbicide, and by reducing the production of these compounds that are involved in pathogen resistance, plants are, thereby, more susceptible to plant pathogens. Another consequence of GLY's mode of action is related to the accumulation of toxic derivatives of the shikimic acid pathway, such as shikimate (Gomes et al., 2020). The increased intracellular abundance of shikimate can shift the carbon flux since the dysregulation of the shikimate pathway ultimately affects the negative feedback control process and results in a lower photosynthetic capacity (Duke & Powles, 2008). Because GLY is non-selective, non-target plants that encounter its residues typically experience growth retardation and morphological changes, which seriously affect plant development (Singh et al., 2020). Alongside conditioning the normal functioning of particular and specific cellular events, GLY-associated impacts on plant physiology are strongly correlated with its ability to induce oxidative damage, by enhanced the production of reactive oxygen species (ROS), and/or suppression of the antioxidant (AOX) defenses (Gomes et al., 2014; Hasanuzzaman et al., 2020). Although the precise mechanisms by which ROS and the AOX players are controlled by the herbicide are not always the same, it appears that GLY can have an impact on the redox homeostasis of plant cells. As demonstrated by several authors, subjecting plants to soil residues of GLY can cause severe redox abnormalities and increase levels of lipid and protein oxidation. A different study showed that after 28 days of growth, GLY-treated tomato plants suffered growth-related disorders in their roots and shoots, along with an excess accumulation of malondialdehyde (MDA) and superoxide anion ($O_2^{\cdot-}$), nonetheless these effects were diminished when plants were subjected to treatment with silicon in both nano and bulk forms (Soares et al., 2021).

1.2. REACTIVE OXYGEN SPECIES AND THE INDUCTION OF OXIDATIVE STRESS

Plants, unlike animals, are sessile organisms and therefore unable to escape harsh environmental conditions, being, in this way, exposed to a variety of unfavourable conditions that may restrict their ability to grow and survive. Although different types of stress, either abiotic (e.g. metal toxicity, pesticide exposure, salinity, drought, etc) or biotic (diseases and pests), can induce signature effects and lead to particular adaptations, it is recognized that the establishment of oxidative stress by an excess of reactive oxygen species (ROS) is one of the common features found in plants growing under stress (Gill and Tuteja, 2010). ROS are a natural by-product of aerobic organisms existing in an oxygen-derived atmosphere, and their formation is intimately tied to the

ability of molecular oxygen (O_2) to be used as the final acceptor in electron transport chains (ETC) of various organelles and plasma membranes. Additionally, ROS are continuously produced as by-products of several metabolic pathways, which are confined to certain cellular compartments including chloroplasts, mitochondria, and peroxisomes (Blokina and Fagerstedt, 2010; Gill and Tuteja, 2010; Heyno et al., 2011; Sharma et al., 2012; Soares et al., 2019a). Despite being frequently labelled as extremely toxic compounds, gathered knowledge over the past decades has now clearly elucidated that ROS play an important, yet contrasting, dual role: at low concentrations, they can operate as signalling agents, successfully activating the AOX system and modulating gene expression; at higher concentrations, these highly reactive compounds may damage proteins, carbohydrates, lipids, and DNA. It is important to mention that the delicate balance between ROS formation and scavenging at the appropriate site and time determines whether ROS formation will function as a signalling or a harmful agent, having the ability to modulate gene expression but also to induce damage to cells, oxidizing lipids, proteins and DNA, and eventually leading to plant death (Gill and Tuteja, 2010; Foyer, 2018; Soares et al., 2019a).

In terms of generation, two chemical processes are primarily responsible for the production of ROS: the transfer of energy to molecular oxygen (O_2), which results in the production of singlet oxygen ($^1O_2$), and the transfer of up to 3 electrons to oxygen, which results in the production of superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) (Gill and Tuteja, 2010; Soares et al., 2019a). The most common ROS, and the first to be produced as a result of the aerobic metabolism, is the $O_2^{\cdot-}$, and it is commonly acknowledged that its generation is mostly related to ETC, in the complexes I and III of the mitochondria and in photosystems I (PSI) and II (PSII) during the photosynthesis. Depending on the type of cell or cellular compartment, $O_2^{\cdot-}$ sets off a series of reactions that produce other ROS, either directly or mainly through enzyme- or metal-catalyzed processes. One electron or two protons can be taken up by $O_2^{\cdot-}$ to generate H_2O_2 . $O_2^{\cdot-}$ can be quickly dismutated into H_2O_2 , either nonenzymatically or through a reaction catalyzed by superoxide dismutase (SOD; EC 1.15.1.1) (Sharma et al., 2012; Soares et al., 2019a). H_2O_2 , despite being produced under normal conditions, can also be generated when plants are facing a wide variety of stressful conditions, such as drought, exposure to heavy metals, pathogens, and xenobiotics (Sharma et al., 2012). This reactive molecule, alongside $O_2^{\cdot-}$ is considered one of the primary ROS; however, because of its high stability and longer half-life, it can impose more severe oxidative stress when compared to $O_2^{\cdot-}$. The main source of H_2O_2 production in plant cells is ETC

in different organelles, such as mitochondria, chloroplasts, endoplasmic reticulum, and the plasma membrane, and β -oxidation of fatty acids and photorespiration in peroxisomes (Sharma et al., 2012; Soares et al., 2019a). This moderately reactive molecule can easily cross biological membranes and, for this reason, its targets can be located far from its production site. On the one hand, because of its high stability when compared to other ROS, and its ability to move through aquaporins, H_2O_2 , when present at low concentrations, gained substantial recognition as a signal molecule involved in the regulation of different biological processes and triggering tolerance mechanisms against a wide range of stresses, such as plant-pathogen interactions. Actually, a wide variety of physiological processes, including senescence, cell cycle, stomatal development, photorespiration, photosynthesis, growth, and development have also been found to be significantly regulated by H_2O_2 , thus gaining recognition as a secondary messenger for signals produced by ROS (Gill and Tuteja, 2010). On the other hand, when present at high levels, not only this molecule can inactivate enzymes by oxidizing their thiol (-SH) groups, and oxidize methionine and cysteine residues, but it can also exert toxic effects on plant cells leading to programmed cell death (PCD), and lipid peroxidation (LP) (Gill and Tuteja, 2010; Sharma et al., 2012). The reaction between O_2^- and H_2O_2 in the presence of redox-active metals like Cu and Fe results in the production of the most hazardous and reactive ROS, $\cdot\text{OH}$ (Soares et al., 2019). Since this ROS has an extremely short half-life of 1 ns, its primary targets and sites of action are positioned nearby to its production site (Sharma et al., 2012; Soares et al., 2019a). In addition to its chemical characteristics that result in high reactivity and consequently high toxicity, $\cdot\text{OH}$ can harm all organic molecules severely despite having a very brief life span, and these potential harms can be exacerbated by the lack of an enzymatic mechanism that would normally be in charge of its metabolism and degradation (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019a).

1.3. ANTIOXIDANT (AOX) SYSTEM

Due to the contrasting roles of ROS, cells have an urge to control their levels in an effort to prevent their overaccumulation and avoid oxidative injuries. The management of intracellular ROS levels is carried by a powerful AOX system, composed of enzymatic and non-enzymatic mechanisms, which enables effective ROS scavenging and regulation in plant cells and organelles (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019a). Regarding the enzymatic mechanism, the main AOX enzymes are SOD (EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), glutathione S-transferase (GST; EC 2.5.1.18), and enzymes present in the ascorbate-glutathione cycle (AsA-GSH) namely

ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.8.1.7). When it comes to the non-enzymatic AOX component, different metabolites are viewed as significant AOX agents, such as glutathione (GSH), ascorbate in its reduced form (AsA), proline (Pro), and many others (Soares et al., 2019a).

1.4. AOX SYSTEM – ENZYMATIC COMPONENTS

1.4.1. SOD (EC 1.15.1.1)

The reaction in which $O_2^{\cdot -}$ is dismutated into H_2O_2 and molecular oxygen, is catalysed by the AOX enzyme SOD. By influencing the levels of both $O_2^{\cdot -}$ and H_2O_2 , and limiting the oxidative damage brought on by high levels of $O_2^{\cdot -}$, SOD plays a crucial role in the detoxification of ROS. This AOX enzyme serves as a metalloenzyme in plants that can be divided into three classes, depending on the ion it carries Mn-SOD, Fe-SOD, and Cu/Zn-SOD (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019a).

1.4.2. CAT (EC 1.11.1.6)

With the ability to spontaneously neutralize H_2O_2 into H_2O and O_2 , CAT is a tetrameric heme-containing protein with enzymatic activity that is essential for ROS detoxification under stressful conditions (Gill and Tuteja, 2010). Even though several enzymes are involved in the breakdown of H_2O_2 , CAT plays a crucial role in the scavenging process because it does not require any reducing power. Additionally, among AOX enzymes, CAT has one of the greatest turnover rates, with one CAT molecule being capable of reducing 6 million H_2O_2 molecules per minute (Gill and Tuteja, 2010; Soares et al., 2019a). Despite the great specificity of CAT to H_2O_2 , its affinity for H_2O_2 is considerably lower than that of other enzymes, such as APX, and, as a result, this enzyme's activity is only effective when high amounts of H_2O_2 are present (Sharma et al., 2012; Soares et al., 2019a). CAT is mostly present in peroxisomes, which produce the majority of the H_2O_2 in plant cells through aerobic metabolism, therefore this enzyme scavenges ROS in the peroxisome during the photorespiration oxidation, β -oxidation of fatty acids, and purine catabolism (Sharma et al., 2012).

1.4.3. APX (EC 1.11.1.11)

APX is an enzyme found in several species, including plants, that uses two molecules of AsA to reduce H_2O_2 to water and two molecules of monodehydroascorbate (MDHA). This

enzyme is one of the most abundant AOX enzymes in plant cells, and it is part of a small multigenic family, whose transcription rates are controlled by a variety of factors, including redox signals and H_2O_2 concentration (Soares et al., 2019a). Since AsA serves as the electron donor for APX, its activity is highly dependent on AsA regeneration. Taking into consideration the differences found in the amino acid sequences, five distinct APX isoenzymes have been discovered, each with a unique subcellular location in plant cells. These are stromal, cytosolic, thylakoidal and peroxisomal isoforms (Sharma et al., 2012; Soares et al., 2019a). While the cytosolic APX removes H_2O_2 created in the cytosol, apoplast, or that which disseminates from organelles, APX located in the organelles scavenges H_2O_2 produced therein. In contrast to CAT, APX has a high affinity for H_2O_2 and can function even in the presence of low levels of this ROS, suggesting that it is in charge of regulating H_2O_2 levels that are necessary for signalling events, whereas CAT is primarily involved in preventing H_2O_2 -induced cellular damage by scavenging the excess of H_2O_2 (Mittler, 2002; Sharma et al., 2012; Soares et al., 2019a).

1.4.4. GST (EC 2.5.1.18)

One of nature's most adaptable enzymes, GSTs are a broad and varied group of enzymes, present in different types of organisms, that catalyses a range of processes involving the conjugation of electrophilic xenobiotic substrates with the tripeptide GSH to generate more soluble peptide derivations (Gill and Tuteja, 2010; Soares et al., 2019a; Hernández Estévez and Rodríguez Hernández, 2020). These enzymes play several roles in biotic and abiotic stresses as well as regulatory processes, such as detoxifying herbicides, signal transduction, protecting plants from ozone damage, heavy metals, and xenobiotics, transporting anthocyanins, detoxifying hydroperoxide, maintaining auxin homeostasis, regulating apoptosis and tyrosine metabolism (Gill and Tuteja, 2010; Hernández Estévez and Rodríguez Hernández, 2020). It has also been reported that GSTs have the ability to eliminate cytotoxic or genotoxic substances, which can react with or harm DNA, RNA, and proteins. In addition, GSTs can create scavengers of cytotoxic and genotoxic chemicals and reduce peroxidases with the cooperation of GSH, and its gene overexpression is associated with enhanced tolerance to various abiotic stresses (Gill and Tuteja, 2010).

1.5. AOX system – non-enzymatic components

1.5.1. Carotenoids (Car)

Plants have developed a myriad of ways to discharge extra energy from photosynthetic membranes in order to avoid light-induced damage. Car are a large group of lipophilic compounds, being one of the most abundant naturally occurring pigments present in both plants and microorganisms, and play a crucial role in photoprotection by either dissipating excess energy or scavenging ROS (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019a). Car can be grouped into two different classes: the oxygen-free carotenoids, called carotenes, and their oxygen-containing derivatives, called xanthophylls. In terms of main biological functions, Car performs three key tasks in plants: i) they absorb light within a wavelength between 400 and 550 nm of the visible spectrum; ii) they serve as an AOX, inhibiting the synthesis of $^1\text{O}_2$ in photosynthetic organs by quenching excited chlorophyll and triplet sensitizer, safeguarding the photosynthetic apparatus, and lowering LP; iii) they play a crucial role in thylakoid membrane stabilization, PSI assembly, and in the stabilization of the light-harvesting complex protein (Gill and Tuteja, 2010; Soares et al., 2019a). Additionally, Car also act as precursors to signalling molecules that modulate plant development and how they respond to biotic and abiotic stresses. Actually, Car are the precursors of two plant hormones, strigolactones and abscisic acid (ABA), making them essential in the regulation of developmental processes (Soares et al., 2019).

1.5.2. Glutathione (GSH)

One essential low-molecular weight non-protein thiol that is essential for intracellular protection against ROS-induced oxidative stress is the tripeptide GSH, which is found abundantly in its reduced form (Sharma et al., 2012). Despite GSH being found in all cell compartments, it is synthesized in the cytosol and chloroplast by specific enzymes, glutamylcysteine ligase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) (Sharma et al., 2012). Besides acting as an efficient ROS scavenger, GSH also serves as a cellular buffer, helping to keep a variety of cell components in their reduced state under stressful conditions. Moreover, because of its reducing power, GSH is connected to protein synthesis, enzymatic regulation, and expression of stress-responsive genes (Soares et al., 2019). GSH has numerous AOX properties since it can directly operate as a free radical scavenger by chemically reacting with $\text{O}_2^{\cdot-}$, $^{\cdot}\text{OH}$, and H_2O_2 . GSH can also function as a proton donor in the presence of ROS or organic free radicals, producing glutathione disulfide (GSSG), or it can form direct adducts with reactive electrophiles, which can protect macromolecules, such as proteins, lipids, and DNA. Through the AsA-GSH cycle, it can take part in the regeneration of a potent AOX, AsA (Sharma et al., 2012). One of the key elements in preserving the cellular redox state is the balance between GSH and

GSSG, where GSH is required to maintain the normal reduced state of cells to counteract the inhibitory effect of ROS-induced oxidative stress (Sharma et al., 2012; Soares et al., 2019).

1.5.3. Ascorbate – reduced (AsA) and oxidized (DHA) forms

Ascorbate, commonly known as vitamin C, is the most prevalent, low-molecular-weight AOX, and it plays a crucial defensive function against oxidative bursts. Under normal circumstances, the majority of AsA is found in its reduced form, whose pool is kept constant by the activity of MDHAR and DHAR. In terms of its biosynthesis, AsA is mainly generated in the mitochondria by L-galactono-lactone dehydrogenase (EC 1.3.2.3), though it can also be synthesized remainder from D-galacturonic acid, being later transferred to other organelles by active transport or facilitated (Sharma et al., 2012; Soares et al., 2019a). This water-soluble AOX is found in distinct cell types, organelles, and apoplast, but accumulates more intensely in photosynthetically active tissues. Nearly 90% of AsA which is in the cytoplasm is exported to the apoplast, and this apoplastic AsA is believed to be the first line of defence against oxidative damage (Soares et al., 2019a). AsA is regarded as a potent AOX, because of its capacity to donate electrons in a variety of enzymatic and non-enzymatic reactions, such as in the PX catalytic activity, where H_2O_2 is reduced generating MDHA, and also its ability to directly interact with different ROS, neutralizing the harmful effects of $^1\text{O}_2$, O_2^- and $\cdot\text{OH}$ (Sharma et al., 2012).

1.5.4. Proline (Pro)

Pro differs from other amino acids in its metabolism and structure. Not only is it the only proteogenic amino acid that has a secondary amine in the α -amino group, but also has a cyclized side-chain on to the backbone amine position, conferring conformational rigidity to this amino acid (Williamson, 1994; Verslues and Sharma, 2010). The large accumulation of Pro that occurs in response to a variety of abiotic stresses, especially during water stress, can be explained because this amino acid is the most water-soluble one, and also by existing in a zwitterionic state (Verslues & Sharma, 2010). This property of Pro is shared with other compounds, that are built up by an array of organisms in order to adjust their cellular osmolarity, commonly known as compatible solutes (Yancey, 2005). Given its distinctive and special features, it is not surprising that many biological roles have been attributed to Pro across decades. For instance, overaccumulation of Pro allows the plant cell to decrease its osmotic potential in cases of low water availability in the soil while ensuring the cell water content to be maintained. Moreover, due to its

chemical properties, Pro can also protect the cellular structure during dehydration, serve as a redox buffer and scavenge ROS, by reacting with and detoxifying these compounds. Aside from these, Pro can also act as a signal molecule (Verslues & Sharma, 2010). Not only does Pro accumulate during the occurrence of different types of stresses, but it has also been suggested to play roles in some non-stress conditions, more precisely, it occurs in reproductive organs, and is associated with flowering and other developmental processes such as in the development of pollen and fertilization in the model plant *Arabidopsis thaliana* (Mattioli et al., 2009). A clear role for Pro in development may be the damage prevention of developing cells against osmotic stress, particularly in developmental processes such as pollen formation and during embryogenesis, where tissues go through dehydration. Pro has been implied to act as a source of energy to support metabolically demanding programs, since the oxidation of one molecule of Pro yields 30 ATP, proposing this way that this amino acid is well suited to sustain high energy-requiring processes (Mattioli et al., 2009).

Regarding Pro metabolism, two main biosynthetic pathways – the glutamate and ornithine pathways, have been proposed, where glutamate and ornithine are used as precursors, respectively. Whilst the main Pro biosynthetic pathway is the glutamate one, ornithine is referred to as an alternative one. L-proline is mainly incorporated from glutamate and its metabolism comprises two enzymes, responsible for catalysing Pro production from glutamate in the cytoplasm or chloroplast, and other two enzymes, responsible for catalysing the catabolism of Pro back to glutamate, in the mitochondria (**Fig. 3**) (Hayat et al., 2012; Meena et al., 2019; Verslues & Sharma, 2010). One of the enzymes that catalyses the production of Pro is Δ^1 -pyrroline-5-carboxylate-synthetase (P5CS, EC 2.7.2.11; EC 1.2.1.41), and it is involved in the conversion of glutamate into glutamic semialdehyde, which goes through a spontaneous cyclization forming pyrroline-5-carboxylate (P5C). The last enzyme involved in Pro production is Δ^1 -pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2), being responsible for the transformation of P5C into Pro, utilizing 1 ATP and 2 NADPH per L-proline. Concerning the alternative pathway, the first step is catalysed by the enzyme ornithine δ -aminotransferase (OAT), in which the reversible transamination of ornithine into glutamic-5-semialdehyde (GSA) takes place. This intermediate is then spontaneously cyclized into P5C, and at the end, the P5C enzyme converts the intermediate into Pro, just as in the glutamate pathway (**Figure 3**) (Verslues & Sharma, 2010; Meena et al., 2019). Some of the enzymes present in **Figure 3** will be thoroughly described in the sections below.

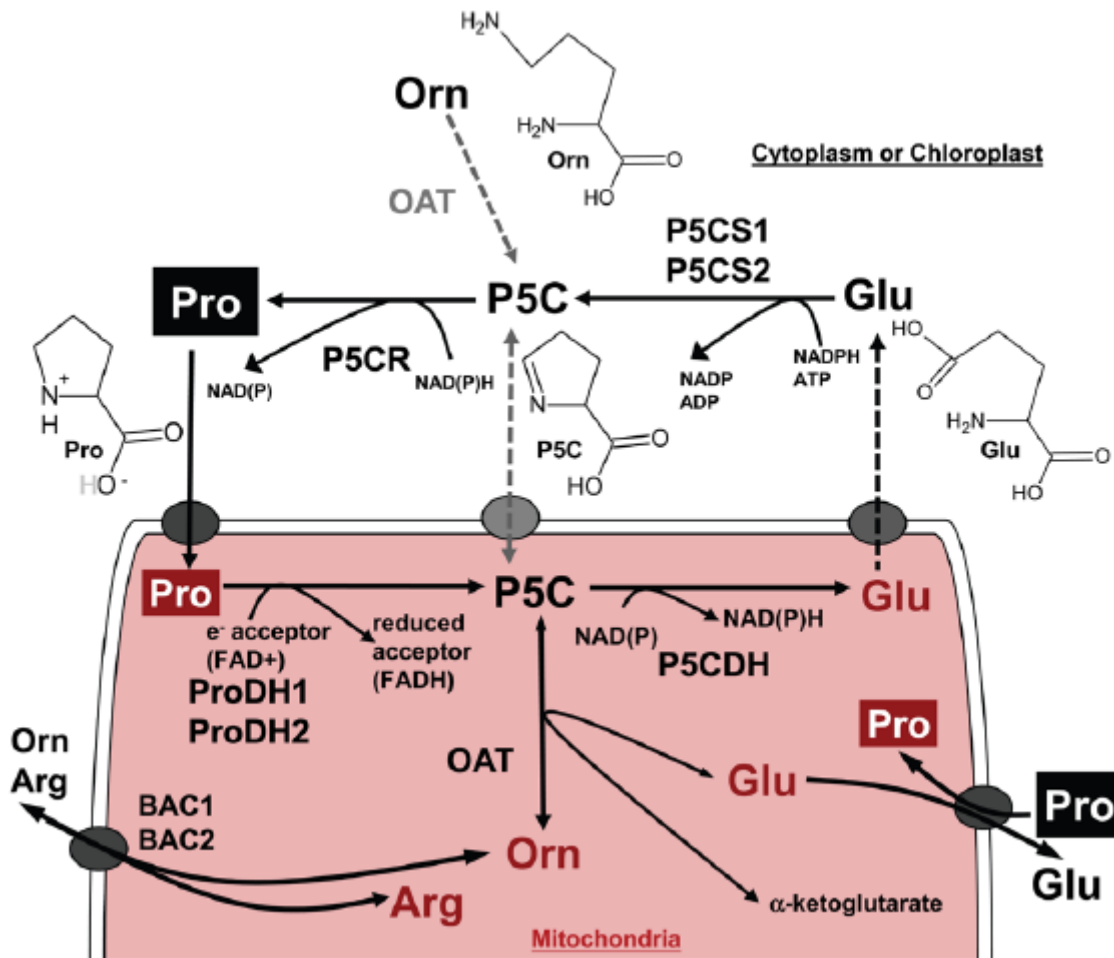


Figure 3 Core pathways (Glutamate and ornithine) of the proline metabolism (Verslues & Sharma, 2010)

1.5.4.1. Proline-5-Carboxylate-Synthetase (P5CS)

P5CS (EC 2.7.2.11/1.2.1.41) is a bifunctional enzyme that combines the activities of glutamate 5-kinase and γ -glutamyl phosphate reductase in a single polypeptide, that in a first step, catalyses the conversion of glutamate to the intermediate GSA which is then converted into P5C through a cyclization event. Similar to other plants, *Arabidopsis* possesses two P5CS isoforms, which are reportedly sub-functionalized in the stress responsive (P5CS1) and housekeeping (P5CS2) forms (Verslues and Sharma, 2010; Funck et al., 2020). In fact, different studies have shown that *P5CS1* is expressed mostly in shoot tissues and its transcription is highly induced under different conditions such as high salinity, cold, and dehydration. Unlike *P5CS1*, *P5CS2* expression exhibits little to no transcriptional up-regulation in response to stress, and all signs point to it playing a small role in Pro synthesis brought on by abiotic stress. Additionally, *P5CS2* is expressed more frequently in callus that are actively dividing, and in cell suspension cultures. *p5cs2* mutants are embryo lethal even when *P5CS1* is functioning, indicating that *P5CS2* plays

a particular developmental role (Székely et al., 2008). Analysis of green fluorescent protein (GFP) fusion proteins of both P5CS1 and P5CS2 revealed that both isoforms are mainly cytosolic, but, under stress conditions, they can be relocated to the chloroplast, especially P5CS1. For instance, Székely et al. (2008) noticed a re-localization of over 80% of the P5CS1-GFP signal from the cytosol to the chloroplast upon exposure to 200 mM NaCl in *Arabidopsis*. Studies testing an exogenous application of abscisic acid (ABA) and ABA-deficient mutants have shown that ABA partially controls Pro accumulation during stress, but that ABA alone is insufficient to enhance proline levels. Nitric oxide and light have both been reported to enhance the expression of *P5CS1* (Hayashi et al., 2000; Zhao et al., 2009), by contrast, the administration of brassinosteroids reduced *P5CS1* expression (Ábrahám et al., 2003).

1.5.4.2. Proline-5-Carboxylate Reductase (P5CR)

P5CR (EC 1.5.1.2) is the enzyme that mediates the transformation of the intermediary molecule P5C into Pro, being, in *Arabidopsis*, encoded by a single gene. It is usually believed that it does not play a rate-limiting role in the synthesis of Pro, mostly because its transcription lacks stress-induced regulation (Kishor et al., 2005). P5C reductase transcript accumulation and developmental regulation in *Arabidopsis* imply that it is crucial for Pro synthesis in cells that are quickly dividing and/or going through osmotic potential shifts. Even though both *P5CS* and *P5CR* transcripts were induced in response to stress, P5CR activity might not significantly contribute to Pro build-up, since it is assumed not to be a rate-limiting enzyme in Pro synthesis (Kishor et al., 2005; Verslues & Sharma, 2010). P5CR can utilize, as reductants, NADPH and NADH. Hua et al. (1997) concluded that, by affecting the level of NADPH reduction, P5CR activity may play a role in the metabolic regulation of cellular redox potential (Kishor et al., 2005). According to research on the subcellular localization of P5CR, through Western blot assay, the enzyme is mainly concentrated in the cytosol and plastid fractions in various plant sections. The presence of this enzyme in plastids raises the possibility that it could help to protect the ribulose-1,5-biphosphate carboxylate-oxygenase (RuBisCO) (EC 4.1.1.39) enzyme from photoinhibition damage in challenging conditions (Kishor et al., 2005).

1.5.4.3. Proline Dehydrogenase (ProDH)

Pro catabolic pathway occurs in the mitochondria and the first enzyme to act is ProDH (EC 1.5.5.2), an enzyme localized in the inner mitochondrial membrane, that catalyses

the oxidation of Pro to P5C (Meena et al., 2019, Verslues and Sharma, 2010). Low water potential, dehydration, and salinity, decrease the expression of *ProDH*, but stress release and exogenous Pro administration were found to restore it. Curiously, it is commonly acknowledged that one regulatory point that can enhance Pro build-up under stress is the downregulation of *ProDH* expression (Verslues & Sharma, 2010). However, it has been noted that *ProDH* was upregulated in response to stress, even while Pro levels were rising. Just like *P5CS*, *ProDH* has two isoenzymes, *ProDH1*, and *ProDH2*, where the last one is expressed at a lower level and can be induced by exogenous Pro and downregulated by exogenous sugar (Funck et al., 2010; Verslues & Sharma, 2010). When there are significant quantities of Pro present, *ProDH* is downregulated by salt stress or drought (Verslues and Sharma, 2010). Stress can therefore prevent *ProDH* from being induced by Pro (Miller et al., 2005). This arises the issue of whether the increase in *ProDH* following the removal of stress is merely the result of the stress-induced inhibition of *ProDH* being withdrawn, allowing the high level of Pro that has accumulated in the plant to stimulate it.

1.5.4.4. Δ^1 -Pyrroline-5-Carboxylate Dehydrogenase (P5CDH)

P5CDH (EC 1.5.1.12 or 1.2.1.88) is an enzyme, located in the mitochondria, encoded by a single-copy gene in *Arabidopsis*, responsible for catalysing the second step of Pro oxidation by converting the intermediate P5C into glutamate (Verslues and Sharma, 2010). Both environmental cues and developmental variables affect *P5CDH* transcription. *P5CDH* is expressed, in basal levels, in all tissues throughout development except flowers, where its expression rises. P5CDH stress regulation is more varied (Verslues & Sharma, 2010). Lack of P5CDH activity increased plants' vulnerability to heat stress and increased ROS generation in both dark and light environments when Pro was in excess (Miller et al., 2009). Therefore, maintaining a balance between mitochondrial ROS generation and enhanced Pro oxidation is essential to preventing Pro-related harmful consequences. Consequently, it is essential to have normal P5C oxidation to Glu by P5CDH in order to stop P5C-Pro intense cycling and block ROS overproduction from electron run-off (Miller et al., 2009).

1.5.4.5. Ornithine δ -aminotransferase (OAT)

OAT (EC 2.6.1.13) is the enzyme that catalyses the reversible transamination of the common intermediate P5C, in both Pro synthesis and catabolism of the ornithine

pathway (Verslues & Sharma, 2010). OAT functions as a transaminase, transferring the amino group from ornithine to α -ketoglutarate to produce GSA and glutamate (Anwar et al., 2018). Under stress conditions, Pro production occurs in the cytosol via the glutamate pathway, but under continuous stress, the ornithine pathway is upregulated. OAT has frequently been reported to be involved in Pro metabolism in drought-stressed cells, and there are numerous lines of evidence linking this enzyme to Pro build-up in osmotic stress-related cells (Xue and Hua, 2009). However, some earlier investigations have presented contradictory results (Delauney et al., 1993; Roosens et al., 1998; Anwar et al., 2018). Because of this, it is unclear how OAT contributes to stress-induced Pro accumulation in plants, and additional research is needed to validate its many functions during biotic and abiotic stress.

1.5.4.6. Impacts of pesticide-induced stress on Pro endogenous levels

In the last decade, a growing number of studies have been focusing their research on unravelling the effects of several agrochemicals on the growth and redox homeostasis, through the analysis of ROS production and AOX metabolism, of different plant species (e.g. Soares et al., 2019b; Ould said et al., 2021). Some of the pesticides considered in the aforementioned studies include the insecticides deltamethrin, imidacloprid, phosalone, emamectin benzoate, α -cypermethrin, chlorpyrifos, omethoate, and dimethoate; the fungicides metalaxyl, acetophenone, chlorothalonil, tricyclazole, and thiabendazole, and herbicides like bentazon, prometryn, butachlor, paraquat, fluroxypyr, clopyralid, atrazine, and glyphosate. A thorough comparison of the outcomes of these articles, highlights, in most cases, the accumulation of Pro as a common effect of pesticide-induced stress, regardless of the xenobiotic and plant species considered. At the same time, these increased Pro levels are often accompanied by enhanced activities of GST and SOD enzymes. Altogether, these results suggest that the accumulation of Pro in plants exposed to pesticide-induced stress seems to be tightly associated not with its AOX properties of membrane stabilization and ROS scavenging, but with its action as a signalling molecule, as observed by Shopova et al. (2021). From this perspective, the increased levels of Pro seem to act as a stress signal that triggers the expression of genes related to the AOX defence system and the xenobiotic detoxification pathway, in the attempt of counteracting two of the most common consequences of pesticide exposure: ROS overproduction and pesticide bioaccumulation in plant tissues, respectively. Although the existence of direct interaction between Pro accumulation and

pesticide toxicity is difficult to establish, a study conducted with *A. thaliana* mutant plants has recently shed some light on this matter (Han et al., 2015). The overexpression of a bacterial D-amino acid oxidase (DAAO), a glyphosate-degrading enzyme, in *A. thaliana* seedlings increased plant resistance to the herbicide, lowering the overaccumulation of ROS and significantly reducing the levels of Pro, in comparison with wild-type plants (Han et al., 2015), thereby allowing plant survival upon pesticide's exposure.

2. Aims

Plant exposure to GLY, either by foliar spray or soil contamination, often results in cellular redox disorders, favouring the occurrence of oxidative stress and the modulation of several AOX mechanisms. In fact, different studies have shown that GLY-induced toxicity results in an overaccumulation of Pro, a non-enzymatic AOX, in both resistant and sensitive plants (Soares et al., 2019b). However, when examining ecological approaches to reduce GLY-induced stress (e.g. exogenous application of biostimulants and growth regulators), Pro levels decrease, followed by an improved growth performance (Soares et al., 2019b). At this point, the main biological question underlying this project arises: is Pro accumulation in response to GLY a signal of tolerance or susceptibility? To achieve this, mutant lines of *Arabidopsis* plants were used, and studied focused on a set of specific objectives:

- functional characterization of the selected mutants under GLY exposure;
- comparison of GLY sensitivity between wild-type and mutant plants;
- assessment of the plant's physiological performance, by analysing the main ROS and AOX players;

3. Materials and Methods

3.1. Functional characterization of the mutants and obtention of homozygous seeds

3.1.1. Plant material and growth conditions

In this study, *Arabidopsis thaliana* (L.) wild-type seeds (Columbia 0 ecotype, Col 0) were obtained from FCUP's seed collection (available at SPRed Lab), while mutant seeds *p5cs1-1* (SALK_058000), *p5cs1-4* (SALK_063517), and *prodh* (SALK_081276) were obtained through the Nottingham *Arabidopsis* Stock Center (NASC). Seeds were germinated directly in pots filled with a commercial substrate – COMPO SANA® (pH 6, 97% organic matter; COMPO), and plants were grown in a growth chamber with controlled conditions [22 °C under long days (16 h light/8 h dark), photosynthetic active radiation (PAR) of 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and 60% relative humidity], for later genotyping and collection of homozygous seeds for each mutant line.

3.1.2. Mutant genotyping and characterization

3.1.2.1. gDNA extraction

Genomic DNA (gDNA) was extracted according to Edwards *et al.* (1991). Firstly, a small leaf disc was collected to a 1.5 mL microtube and ground using a small pestle for approximately 15 s; then, 400 μL of extraction buffer [200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5% (w/v) sodium dodecyl sulfate (SDS)] were added to the tube and briefly vortexed. After spinning the tube at full speed (14 000 g) in a microcentrifuge for 1 min to pellet debris, 400 μL of the supernatant (SN) were transferred to a new 1.5 mL microtube, where 350 μL of isopropanol were added and incubated at room temperature (RT) for 2 min. Afterwards, the tube was centrifuged for 5 min at full speed (14 000 g) to pellet the DNA; the SN was removed and the pellet dried gently at RT or by incubating the tubes at 37 °C for 20 - 30 min. Finally, 50 μL of ultra-pure water were added to each tube, and the pellet was dissolved by shaking it gently.

3.1.2.2. Genotyping

The genotype of all mutant lines was confirmed by employing a PCR-based approach, to confirm the T-DNA's insertion presence, using the gDNA of the different mutant plants.

To perform this technique, the enzyme *DreamTaq DNA polymerase* (Thermo Scientific) was used according to the manufacturer's indications. The first step in the PCR programs consisted of denaturation (2 min, 95 °C) followed by 30 to 35 cycles of denaturation (30 s; 95 °C), annealing (30 s, at the temperature specific for the annealing of the primers used in the reaction – Supplementary material, **Table 6**), and extension (1 min *per* 1kb, 72 °C), a final extension (5-10 min, 72 °C) and a step of cooling down (4 °C) by the end of the cycles. The reaction mixture, containing the components described in **Table 1**, was assembled up to a total of 25 µL. T100 Thermal Cycler (Bio-Rad) was used and programmed depending on the mutant line genotyped and in accordance with the conditions established in **Table 2**.

Table 1 Components of the PCR reactions using DreamTaq Polymerase (Thermo Scientific)

Components	Volume up to 25 µL	Final Concentration
Sterile H ₂ O	Until 25 µL	-
DreamTaq 10 x Buffer	2.5 µL	1x
dNTPs 2 mM	0.5 µL	0.2 mM
Primer 1	1 µL	0.2 µM
Primer 2	1 µL	0.2 µM
DNA	1 µL	0 pg – 1 pg
Taq DNA Polymerase	0.13 µL	1.25 U

Table 2 PCR program used for genotyping, using DreamTaq Polymerase (Thermo Scientific)

Program		
Initial denaturation		95 °C / 2 min
Denaturation	32 – 35 cycles	95 °C / 30s
Annealing		50 °C – 60 °C / 30s
Extension		72 °C / 1 min per Kb
Final extension		72 °C / 5 min
Cooling		4 °C / + ∞

Upon PCR, amplicons were separated by agarose (0.8% - 1% w/v) gel electrophoresis in 1x sodium borate buffer (SB) [47 g L⁻¹ boric acid, 200 mM sodium hydroxide (NaOH), pH 8.2], with 30 µL L⁻¹ of GreenSafe Premium (NZYTech) added to the gel before polymerization. The 1x Orange G loading dye [10 mM Tris-HCl (pH 7.6), 0.15% Orange

G dye, 60% (w/v) glycerol, 60 mM EDTA) was added to each sample before loading the gel. GeneRuler DNA Ladder mix (Thermo Scientific) was used as a molecular weight marker and SB as the running buffer. The electrophoretic separation procedure was conducted between 250 – 270 V, for 10 to 15 min. The GreenSafe fluorescence enabled the DNA visualisation in a UV transilluminator (302-365 nm). Images were acquired with a KODAK camera and analysed using the software Image Analysis KODAK 1D, version 3.5 (KODAK Scientific Imaging Systems).

In order to confirm the exact location of the T-DNA insertion in each mutant line under analysis, two reaction mixtures of 25 µL, containing the components described in **Table 1**, were assembled, to amplify the mutant band of each mutant line. A T100 Thermal Cycler (Bio-Rad) was used and programmed in accordance with the conditions established in **Table 2**. After the PCR, the two reaction mixtures for each mutant band were loaded into the same well of an agarose 0,8% gel and after electrophoresis as previously described in this section, a clean-up of each gel band was performed using GeneJet Gel Extraction Kit (Thermo Scientific) according to the manufacturers' instructions, sent for sequencing (<https://eurofinsgenomics.eu/>), and analyzed using ApE, a plasmid editor (<https://jorgensen.biology.utah.edu/wayned/ape/>).

3.1.2.3. Predicted expression pattern of *P5CS1* and *ProDH*

To identify the expression patterns of the *P5CS1* and *ProDH* genes, the BAR Expression Angler tool (<https://bar.utoronto.ca/>; Toufighi et al., 2005) was used. Expression profiling data was displayed using the HeatMapper Plus tool or Arabidopsis eFP browser tool at the Bar (Toufighi et al., 2005; Winter et al., 2007).

3.1.2.4. RNA Extraction

To extract the total RNA, young leaves of the different genotypes (homozygous and wild-type plants) were collected. The extraction was performed using the PureZOL™ RNA Isolation Reagent (Bio-Rad). Firstly, samples were homogenized with 700 µL of Purezol and incubated at RT for 5 min. After being centrifuged at 12 100 g for 10 min at 4 °C, the SN was transferred to a new microtube, and 140 µL of chloroform were added. Then, the reaction mixture was mixed vigorously by hand, for 15 s, and the tubes were incubated at RT for 5 min. Posteriorly, all samples were centrifuged at 12 100 g for 15 min at 4 °C, until three phases appeared. The superior aqueous phase was transferred to a new microtube and 350 µL of isopropanol were added. Upon manual mixing of the samples, they were left at RT for 5 min, followed by centrifugation at 12 100 g for 10

minutes at 4 °C. SN was discarded, and the pellet was then washed with 700 µL 75% (v/v) ethanol. After being vortexed and centrifuged at 7 500 *g* for 5 min at 4 °C, the SN was once again discarded, the pellet was resuspended in 40 µL of pure water, and samples were incubated at 55 °C for 10 min. Afterwards, RNA was quantified using a DS-11 Spectrophotometer/Fluorometer (Denovix). 0.5 µL of RiboLock RNase Inhibitor (Thermo Scientific) were added to each sample, which were stored at -80 °C until use.

3.1.2.5. cDNA synthesis

The reverse transcriptase (RT) reaction was conducted after the RNA extraction in accordance with the instructions in the kit *RevertAid first strand cDNA synthesis* (Thermo Scientific). For each PCR tube 17.5 µL of RNA (approximately 380 ng µL⁻¹) were added, 1 µL of 5x Reaction Buffer [250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT] and 1 µL of DNase I (Thermo Scientific). Then, the tubes were placed in a thermocycler for 30 min at 37 °C, and after that, 1 µL of 50 mM EDTA was added. Posteriorly, tubes were incubated for 10 min at 65 °C, followed by the addition of 1 µL of 100 µM Oligo(dT)₁₈ primers and a final incubation at 65 °C for 5 min. Lastly, 7 µL of a mix containing 4 µL of 5x buffer, 2 µL of 10 mM dNTP mix, and 1 µL of RevertAid RT (Thermo Scientific) was added to each tube. Thereafter, the tubes were heated at 42 °C for 60 min and 70 °C for 5 min. The obtained cDNA was tested for possible gDNA contaminations by a PCR using primers for the gene *ACTIN 8* (*ACT8*) (program presented in **Table 2**, and primers in Supplementary material **Table 7**).

3.1.2.6. RT-qPCR

Primers for analysing the expression level of the genes *P5CS1* (At2g39800), *P5CS2* (At3g55610), *ProDH* (At3g30775) and *OAT* (At5g46180) were designed by using the Primer 3 software (<https://primer3.ut.ee/>) and tested to verify their specificity by PCR. The cDNA was amplified utilizing the SSoFast™ SYBR® Green Supermix on an iQ5™ Real-Time PCR Detection System (Bio-Rad). Serial dilutions of cDNA from *Arabidopsis* ecotype Columbia were used to set up a calibration curve, which was used to quantify plant cDNA in each sample.

Specific primers previously tested for each gene and primers for the reference genes *ACT 8* and *RELATED TO UBIQUITIN 1* (*RUB 1*) were also used, as described in Supplementary material **Table 7**. Three technical replicates of each reaction were performed. After 3 min at 95 °C followed by a 10 s denaturation step at 95 °C, samples were run for 40 cycles of 10 s at 95 °C and 30 s at 60 °C. After each run, a dissociation

curve was acquired to check for amplification specificity by heating the samples from 60 to 95 °C, with 0.5 °C increments every 5 min. At the end of the PCR cycles, the data obtained were analysed using the program CFX Maestro™ Software (Bio-Rad).

3.1.2.7. Pollen Tube Staining with Decolorized Aniline Blue

Arabidopsis whole inflorescences, with flowers at different developmental stages, were collected and fixed in 10 % (v/v) acetic acid prepared in ethanol for approximately 16 h, followed by 3 washing cycles with water, 5 min each. Afterwards, flowers were softened with 1 M NaOH for 16 h, washed 3 times with water, 30 min each, followed by overnight incubation with 0.1% (w/v) decolorized aniline blue (DAB) at 4 °C. The sepals, petals, anthers, filaments, and valve tissues were removed carefully to place the naked pistils with exposed ovules attached to the septum on a glass slide under an anatomical microscope, and then a drop of DAB solution was added, and with the use of a Zeiss Axiophot D1 microscope, the samples were analysed. Using the Axiovision program, images were acquired with a Zeiss AxioCam MRc5 camera (version 4.1).

3.2. Biometric, biochemical and molecular assessment of GLY-induced stress in wt and mutant plants

3.2.1. Dose-response assays with glyphosate (GLY)

In order to determine the most appropriate concentration of GLY to study the role of proline against herbicide-induced stress, a preliminary assay was performed by testing a series of sequential concentrations of GLY (0, 0.25, 0.5, 1, and 2 mg L⁻¹) under *in vitro* conditions. For this purpose, media containing 0.5x MS (Murashige and Skoog, 1962) solidified with 0.625% (m/v) agar, supplemented or not with GLY at different concentrations (0.25, 0.5, 1 and 2 mg L⁻¹), were prepared. GLY was acquired in the form of RoundUp® Ultramax (Bayer, Germany), which is a commercial formulation containing 360 g L⁻¹ GLY in the form of potassium salt. From this formulation, a stock solution of GLY (1 g L⁻¹) was freshly prepared and used to obtain the final volume to be added to each medium. Afterwards, all media were autoclaved at 121 °C (0.1 MPa) and then immediately divided into Petri dishes (50 mL/plate). Seeds from wt *Arabidopsis* plants were surface disinfected with 70% (v/v) ethanol for 3 min and dried at RT in a laminar flow chamber (Bio II 12, ADS Laminaire). Then, seeds were sown in Petri dishes and incubated for 48 h in dark conditions at 4 °C for stratification. Then, all plates were transferred to a growth chamber with controlled conditions, as described in 2.1.1. For each concentration of GLY, including the control (0 mg L⁻¹), three biological replicates

(considered as a plate) were considered, with 40 seeds each. Fourteen days after germination, seedlings were photographed and collected for the quantification of proline (2.3.1.).

3.2.2. Main experiment with wildtype and mutant plants

Following the toxicological test, the adequate concentration of GLY was selected for the main experiment, where seeds of homozygous lines of all tested genotypes (wt, *p5cs-1.1*, *p5cs-1.4*, and *prodh*) were exposed to 0.75 mg L⁻¹ GLY. For this purpose, seeds from all genotypes were surface disinfected as previously detailed and sown in Petri dishes, containing 0.5x MS medium, solidified with 0.625% (m/v) agar, and supplemented, or not, with 0.75 mg L⁻¹ GLY. At this time, the following experimental groups were considered:

- **CTL wt** – wild-type *Arabidopsis* plants growing on nutritive medium without GLY;
- **CTL *p5cs1-1*** – *p5cs1-1* *Arabidopsis* mutant plants growing on nutritive medium without GLY;
- **CTL *p5cs1-4*** – *p5cs1-4* *Arabidopsis* mutant plants growing on nutritive medium without GLY;
- **CTL *prodh*** – *prodh* *Arabidopsis* mutant plants growing on nutritive medium without GLY;
- **GLY wt** – wild-type *Arabidopsis* plants growing on nutritive medium supplemented with 0.75 mg L⁻¹ GLY;
- **GLY *p5cs1-1*** – *p5cs1-1* *Arabidopsis* mutant plants growing on nutritive medium supplemented with 0.75 mg L⁻¹ GLY ;
- **GLY *p5cs1-4*** – *p5cs1-4* *Arabidopsis* mutant plants growing on nutritive medium supplemented with 0.75 mg L⁻¹ GLY ;
- **GLY *prodh*** – *prodh* *Arabidopsis* mutant plants growing on nutritive medium supplemented with 0.75 mg L⁻¹ GLY.

Upon 48 h for seed stratification, plates were incubated in the same growth chamber with the conditions described in 2.1.1 and grown for 14 days after germination. For each genotype and situation (CTL and GLY), a total of three replicates were prepared. The experiment was repeated 5 times to achieve enough biomass for the employed analyses. At the end of the assay, seedlings were collected and immediately processed for biometric assessment (fresh biomass production). Afterwards, plant material was frozen in liquid nitrogen and stored at -80 °C until posterior use for biochemical and molecular analysis.

3.2.3. Macroscopic analysis of plant growth and fresh biomass determination

At the end of the assay, after 14 days of growth, any eventual visual symptoms of phytotoxicity were recorded using a digital photograph camera. Moreover, the seedlings of each plate were collected, and their collective fresh biomass was recorded using a precision balance (KERN®; EWJ 300-3; KERN & SOHN GmbH, Balingen, Germany).

3.2.4. Analysis of physiological indicators

3.2.4.1. Extraction and quantification of photosynthetic pigments

The evaluation of chlorophylls (chl) and carotenoids (car) was performed based on the protocol of Lichtenthaler (1987). Aliquots from frozen seedlings of approximately 10 mg were homogenized in 80% (v/v) acetone, using a bead mill homogenizer Bead Ruptor 12 (Omni International Inc.), and centrifuged at 1400 *g* for 10 min. After collecting the SN, the absorbance was recorded at 470, 647, and 663 nm, and the concentrations of chl a, chl b, and car were calculated using the following formulas (Lichtenthaler, 1997):

- **Chl a** (mg L^{-1}) = $12.25 \times \text{Abs}_{663} - 2.79 \times \text{Abs}_{647}$
- **Chl b** (mg L^{-1}) = $21.50 \times \text{Abs}_{647} - 5.10 \times \text{Abs}_{663}$
- **Car** (mg L^{-1}) = $(1000 \times \text{Abs}_{470} - 1.82 \times \text{Chl a} - 85.02 \times \text{Chl b}) / 198$

Results were expressed as mg g^{-1} fresh mass (fm).

3.2.4.2. Extraction and quantification of soluble proteins

Total soluble proteins were extracted from *Arabidopsis* seedlings, using frozen aliquots of approximately 100 mg, in 1.5 mL of extraction buffer containing 100 mM potassium phosphate (PK) buffer (pH 7.3), 1 mM ethylenediaminetetraacetic acid (EDTA), for protection against metallic peroxidases, 8% (v/v) glycerol, a cryoprotectant used to increase protein stability, 1 mM phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor, 5 mM AsA, and 2% (w/v) polyvinylpyrrolidone (PVPP), which inhibits phenolic compounds from inactivating enzymes. Samples were homogenized in the bead mill homogenizer Bead Ruptor 12 (Omni International Inc.) using 5 beads per tube, in a series of 3 sequential cycles. To prevent sample overheating, after each homogenizing cycle, tubes were incubated on ice for 1 min. Afterwards, the extracts were

centrifuged at 16 000 *g* for 25 min at 4 °C, and the SN was used to quantify the total soluble proteins, and to measure the total activity of AOX enzymes (section 2.4). Total soluble proteins were quantified according to Bradford (1976), by recording the absorbance at 595 nm. A calibration curve, using different known concentrations of bovine serum albumin (BSA), was used to calculate the total protein content expressed in mg g⁻¹ fm.

3.2.5. Evaluation of oxidative stress markers

3.2.5.1. Lipid peroxidation (LP)

The membrane damage was evaluated through the determination of LP, by quantifying MDA levels according to the procedures described by Heath & Packer (1968). Fresh seedlings of approximately 100 mg were homogenized in 1 mL of 0.1% (m/v) trichloroacetic acid (TCA), using a bead miller [Bead Ruptor 12 Homogenizer (Omni International Inc.)], being then centrifuged at 16 000 *g* for 15 min. Subsequently, 1 mL of 0.5% (w/v) of thiobarbituric acid (TBA) in 20% (w/v) TCA was added to 250 µL of SN, and the blank tube was prepared by replacing the SN with 250 µL of 0.1% (w/v) TCA. In parallel, to minimize the impact of compounds present in the SN that could affect the absorbance reading, a different reaction was prepared without adding TBA, where 1 mL of 20% (w/v) TCA was added to 250 µL of SN. Another blank tube was also prepared the same way. Afterwards, all tubes were incubated at 95 °C for 30 min and then cooled on ice for 15 min, before being centrifuged one last time at 10 000 *g* for 7 min. In the end, the absorbances of each sample, with and without the TBA, were measured at 532 and 600 nm. The values of 600 nm were subtracted from the ones at 532 nm to minimize the effects of unspecific turbidity and, then, the values of the samples incubated without TBA were subtracted from those incubated with TBA. The MDA content was calculated using an ϵ of 155 mM⁻¹ cm⁻¹ and expressed as nmol g⁻¹ fm.

3.2.5.2. Quantification of H₂O₂

The quantification of H₂O₂ was performed according to the protocol described by de Sousa et al (2013). For this purpose, frozen aliquots (ca. 100 mg) were homogenized, on ice, with 1.2 mL of 50 mM PK buffer (pH 6.5), using a bead miller [Bead Ruptor 12 Homogenizer (Omni International Inc.)]. Between each homogenizing cycle, tubes were incubated for 1 min on ice to prevent overheating of the samples. After the centrifugation (10 000 *g*, 20 min, 4 °C), the SN was collected and 500 µL of SN were mixed with 500 µL of 0.1% (w/v) titanium sulphate (TiOSO₄) prepared in 20% (v/v) H₂SO₄. Upon mixing

in the vortex, new centrifugation took place at 6 000 *g* for 15 min at 4 °C. The absorbance values were recorded at 410 nm and the concentration of this ROS was calculated using an ϵ of 0.28 $\mu\text{M}^{-1} \text{cm}^{-1}$. Results were expressed on a fm basis.

3.2.6. Evaluation of non-enzymatic AOX metabolites

3.2.6.1. Proline (Pro)

Proline (Pro) was quantified by the ninhydrin-based colorimetric assay, described by Bates et al. (1973). Fresh seedlings (ca. 100 mg) were homogenized, on ice, in 1 mL of 3% (w/v) sulfosalicylic acid, using a bead miller [Bead Ruptor 12 Homogenizer (Omni International Inc.)] where between each homogenizing cycle, tubes were incubated for 1 min on ice to prevent overheating of the samples, and then centrifuged at 16 000 *g* for 15 min. Subsequently, 200 μL of SN were mixed with 200 μL of glacial acetic acid and 200 μL of acid ninhydrin. All reaction mixtures were incubated at 96 °C for 1 h and posteriorly cooled on ice. Then, 1 mL of toluene was added to each tube, followed by vigorous vortexing to extract the complex toluene-Pro. After the total separation of phases, the absorbance of the redish upper phase was measured at 520 nm, using toluene as a blank. The Pro content was estimated using a calibration curve obtained by different solutions of known Pro concentration, and the results were expressed in $\mu\text{g g}^{-1} \text{fm}$.

3.2.6.2. Glutathione (GSH)

The assessment of GSH levels was conducted in accordance with Soares et al. (2019b), using the same SN as for the Pro quantification, described before. Briefly, 50 μL of SN were added to 250 μL of deionized water and 750 μL of a reaction mixture containing 100 mM PK buffer (pH 7.0), 1 mM EDTA, and 0.1 M 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's Reagent). After vortexing, the samples were incubated in the dark at RT for 10 min; then, the absorbance was recorded at 412 nm. The levels of GSH were quantified using a calibration curve prepared with solutions of known GSH concentrations, and the results were expressed in $\mu\text{mol GSH g}^{-1} \text{fm}$.

3.2.6.3. Ascorbate – reduced (AsA) and oxidized (DHA) forms

The spectrophotometric quantification of ascorbate was performed based on an α - α' -bipyridyl-based colorimetric assay, as described by Gillespie & Ainsworth (2007) Frozen

seedling aliquots were homogenized in 6% (w/v) TCA, using the Bead Mill Homogenizer Bead Ruptor 12 from Omni International Inc. (5 beads per tube; 3 cycles of homogenization). Between each homogenizing cycle, tubes were incubated for 1 min on ice to prevent overheating of the samples. Following a 10 min centrifugation (15 000 g; 4 °C), SNs were used to quantify both total and reduced ascorbate. Briefly, for total ascorbate determination, 50 µL of 75 mM PK buffer (pH 7.0) and 50 µL of 10 mM dithiothreitol (DTT) were added to 100 µL of SN. The tubes were vortexed and incubated at RT for 10 min to allow DTT to reduce all the ascorbate present in the samples; then, 50 µL of 0.5% (w/v) *N*-Ethylmaleimide (NEM) were added to remove the excess of DTT. Tubes were once again vortexed and incubated at RT for, at least, 30 s. Regarding the quantification of AsA, the same procedure was followed, but the volume of DTT and NEM was replaced by deionized water. Afterwards, all samples were supplemented with 250 µL of 10% (w/v) TCA, 200 µL of 43% (v/v) phosphoric acid (H₃PO₄), 200 µL of 4% (w/v) 4,4'-bipyridyl (BIP) and 100 µL of 3% (w/v) iron chloride (FeCl₃), vigorously vortexed and incubated at 37 °C for 1 h. Finally, the absorbance of each sample was registered at 525 nm. The concentration of both total and reduced ascorbate was determined through a calibration curve obtained by the above described protocol and prepared with known concentrations of AsA. DHA was calculated by subtracting AsA from the total ascorbate. The results were expressed in terms of µmol g⁻¹ fm.

3.2.7. Evaluation of AOX enzymes

3.2.7.1. Catalase (CAT; EC 1.11.1.6)

The activity of CAT was measured spectrophotometrically following the protocol described by Aebi (1984), with an adaptation for UV microplates. The activity of this enzyme was determined by the temporal enzymatic degradation of H₂O₂. This assay was performed in a 96-well UV microplate, where 20 µL of protein extract were added to 160 µL of 50 mM PK buffer (pH 7.0) and 20 µL of 100 mM H₂O₂. CAT activity was measured, at 240 nm, for 2 min in intervals of 5 s, and the results were expressed in nmol of H₂O₂ min⁻¹ mg⁻¹ of protein, considering an ε value of 39.4 mM⁻¹ cm⁻¹.

3.2.7.2. Ascorbate peroxidase (APX; EC 1.11.1.11)

Similarly to CAT, APX activity was also evaluated spectrophotometrically in a 96-well UV microplate, through the oxidation of AsA. Following the protocol described by Murshed et al. (2008), 20 µL of the protein extract were added to each well, followed by 170 µL of reaction buffer [50 mM PK buffer (pH 7.0) supplemented with 0.6 mM AsA], the reaction

started with the addition of 10 μL of 254 mM H_2O_2 . The abs variances were recorded at 290 nm in 5 seconds intervals for 2 min, and the results were expressed as nmol ASA $\text{min}^{-1} \text{mg}^{-1}$, assuming an ϵ value of $2.8 \text{ mM}^{-1} \text{cm}^{-1}$

3.2.8. Semi-quantitative evaluation of H_2O_2 accumulation

The analysis of the presence of H_2O_2 was performed in seedlings with 12 days of growth using the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma-Aldrich), a method adapted from Sandalio et al., (2008). For this purpose, seedlings were selected and incubated for 30 min at 37 °C in a solution of 25 μM DCF-DA in 10 mM Tris-HCl buffer (pH 7.5), and for each situation, there was a negative control where plants were also incubated at 37 °C, but only in 10 mM Tris-HCl buffer (pH 7.5). The plant material was placed between a slide and a coverslip, in an aqueous solution, and viewed under a fluorescence microscope (Nikon Eclipse Ci-L). The images were captured with the same exposure between the various situations to allow comparison and quantification of the values obtained, and the images were analyzed and processed using the program ImageJ/Fiji (<https://imagej.net/Welcome>). In this program, the fluorescence quantification was conducted by measuring the mean signal intensity of each pixel, which regardless of the excitation and emission wavelengths, is recorded in the image on a grayscale, where black corresponds to the absence of signal and white to the maximum amount of signal, which translates the amount of probe that reacted positively to H_2O_2 .

3.3. Transcript accumulation of target genes by RT-qPCR

3.3.1. RNA extraction and cDNA synthesis

At the end of the assay, after 14 days of growth, seedlings from 3 replicates of each situation were collected and frozen for further RNA extraction and cDNA obtention. RNA extraction and cDNA synthesis were conducted as described in the sections (3.1.2.3. and 3.1.2.4., respectively).

3.3.2. qPCR conditions

The expression of several genes encoding different enzymes for the Pro pathway was evaluated through a RT-qPCR according to the conditions established in section 3.1.2.5.

Primers for the genes *P5CS1*, *P5CS2*, *ProDH*, and *OAT* were previously acquired, and their efficiencies were calculated the same way as it was described in section 3.1.2.5.

3.3.3. cDNA constructs

To evaluate how GLY affects the subcellular localization of some key enzymes involved in its toxicity (*ProDH*, *P5CS1*, *EPSPS*, *GSTF2*, and *GSTF4*), several molecular biology techniques were performed to create chimeras with the fluorescent protein mNeonGreen.

3.3.4. DNA amplification and cloning techniques

The expression vector pMDC38, was already available at the laboratory and maintained in *Escherichia coli* DH5 α . Plasmids were extracted using the NZYMiniprep kit (NZYTech[®]) following the manufacturer's guidelines. Two cloning strategies were used in this study, where the first one consisted in cloning directly the PCR product with blunt ends using the NZY-blunt PCR cloning kit (NZYTech[®]) following all the instructions presented by the manufacturer. This allowed to obtain the cDNA coding for each enzyme in an intermediate vector. The second strategy was designed to clone the cDNA directly into the binary vector pMDC83 carrying mNeonGreen, through the Gibson assembly method, where a reaction mixture, presented in **Table 3**, was performed and alongside with the cDNA was incubated for 1h at 50 °C (Gibson et al., 2009).

Table 3 Gibson Assembly reaction.

Components	Volumes
DNA to assemble	Equimolar volumes
5x IRB	4 μ L
Taq DNA Ligase	2 μ L
Phusion HF DNA Polymerase	0.25 μ L
T5 Exonuclease (prediluted in 1:30 1x IRB)	0.25 μ L
DdH₂O	Up to 20 μ L

cDNA from leaves was amplified with Phusion High-Fidelity PCR Master Mix (Thermo Scientific) using a reaction mix and a PCR program as described in the following tables (**Table 4** and **Table 5**, respectively), using primers previously designed for *P5CS1*, *EPSPS*, *ProDH*, *GSTF2* and *GSTF4*, described in Supplementary material (**Table 8**). PCR products were later confirmed by an electrophoresis gel and all products were

purified using NZYGelpure kit (NZYTech) according to the manufacturer and quantified in a DS-11 microvolume Spectrophotometer (Denovix) using 1 µL of DNA. To check for correct cDNA insertion in the plasmid, an enzymatic digestion was performed. The digestion reaction was prepared according to the instructions of the manufacturer (Anza line, Invitrogen). Briefly, a mix of 10 µL of DNA, 2 µL of 10x Anza Red Buffer, and 1 µL of each restriction enzyme were added to a final volume of 20 µL. Then, this reaction mixture was incubated for 15 min at 37 °C. After the digestion was completed, a clean-up using NZYGelpure kit (NZYTech), was done according to the manufacturer's instructions and quantified in a DS-11 microvolume Spectrophotometer (Denovix). The ligation was then performed with Anza T4 DNA Ligase Master Mix (Invitrogen), regarding the company specifications. For this reaction, a fixed quantity of 50 ng of the vector was used, and the amount of insert was calculated using the formula $\text{ng insert} = (\text{ng insert} \times \text{bps insert}) / (\text{bps vector})$, in order to have a 1:5 ratio of vector: insert.

Table 4 Components using Phusion Master Mix, for the amplification of cDNAs of interest

Components	Concentration	Volume
Primer forward	0.5 µM	1.25 µL
Primer reverse	0.5 µM	1.25 µL
Template DNA	5 ng	Variable
2x Phusion Master Mix	1x	25 µL
Nuclease-free water	-	Variable
Final volume	-	50 µL

Table 5 PCR program using Phusion Master Mix for the amplification of cDNAs of interest.

Program		
Initial denaturation		98 °C / 3 min
Denaturation	35 cycles	98 °C / 10s
Annealing		50 °C – 60 °C / 30s
Extension		72 °C / 30 s per Kb
Final extension		72 °C / 10 min
Cooling		4 °C / + ∞

3.3.5. Preparation of competent cells

3.3.5.1. Chemical *Escherichia coli* competent cells

In order to prepare chemical competent cells, isolated colonies from NZYStar (NZYTech) were inoculated in 25 mL of Luria Broth medium (LB) (Duchefa Biochemie) supplemented with 10 $\mu\text{L mL}^{-1}$ tetracycline (TET) and were grown overnight (ON) at 27 °C, with orbital shaking at 200 rpm. The cultures were added to 225 mL of SOB medium supplemented with 1 M MgCl_2 and 1 M MgSO_4 and were incubated at 37 °C with orbital shaking, until the optic density of the culture, at 600 nm (OD600), was close to 0.7. When the optimal OD was achieved, the culture was incubated on ice for 10 min and centrifugated at 3000 g for 5 min. The SN was removed, and the pellet was resuspended in 100 mL of RF1 (prepared with 1.51 g Rubidium Chloride (RbCl), 0.41 g Calcium chloride (CaCl_2), 21.55 mL 87% (v/v) glycerol, pH 5.8; after autoclaved 10 mL of 0.5 M Manganese Chloride (MnCl_2) and 10 mL of 0.3 M KAc were added) and left on ice for 15 min. Afterwards, the mix was centrifuged at 3000 g for 5 min, and the SN was discarded. The pellet was then resuspended in 16 mL of RF2 (prepared with 0.019 g RbCl , 0.396 g CaCl_2 , 0.033 g MOPS and 1.72 mL 87% (v/v) glycerol, pH 8.0). Finally, the mix was divided into 100 μL aliquots and stored at -80 °C.

3.3.6. Bacterial transformation

The final volume of ligations or Gibson assembly mixes were used to transform *E. coli* NZYStar chemically competent cells. The DNA was mixed with 100 μL of competent cells and incubated on ice for 15 min, and posteriorly transferred to a heating block at 42 °C. Then 1 mL of LB was added, to help bacteria to recover from the heat shock, followed by an incubation at 37 °C for 1 h, with orbital shaking. At the end, the cells were pelleted at 3 000 g , and 900 μL of the SN were discarded, the cell pellet was then resuspended in the remaining liquid, and it was then transferred to a plate containing 1.5 % (w/v) LB agar, that included 50 g mL^{-1} kanamycin and/or 10 g mL^{-1} tetracycline. At 37 °C, the plate was allowed to incubate ON.

3.4. Statistical analyses

Biometrical and biochemical analyses were carried out considering at least 3 biological replicates ($n \geq 3$). Results were expressed as $\pm$ standard deviation (SD). After checking the homogeneity of variances, data were subjected to a two-way analysis of variance (ANOVA), defining as fixed factors the “genotype” and the “GLY treatment”. In case of significant effects, two different *post-hoc* tests were used to compare means: Tukey’s *post-hoc* was used to analyse differences between different genotypes in the same

treatment (CTL or GLY); Sidak's multiple comparison test to evaluate the differences between treatments within the same genotype. All tests were used assuming 0.05 as a significance level (p). Statistical analyses were performed using the software GraphPad Prism® 8 (San Diego, CA, USA).

4. Results

4.1. Genotyping

In order to confirm if the different mutant lines under analysis contain the T-DNA insert at the predicted chromosomal location and whether the plants were homozygous or heterozygous for the mutation, a PCR based approach was used. Three different primers were used for this: one specific for the T-DNA insertion (LBb1.3), a right primer (RP), and a left primer (LPr), both specific for each gene (*P5CS1* and *ProDH*). One PCR reaction with the gene specific primers (RP and LPr), which spans the predicted T-DNA insertion site, detects the presence of a wild-type copy of the gene in the plant. A band of approximately 900-1100 bp (from LPr to RP) representing a wt copy of the gene generated in the absence of the T-DNA insert, appearing either in wt plants or heterozygous plants. A second PCR reaction with a gene specific primer (LPr or RP) and a T-DNA insert specific primer (LBb1.3) amplifies the T-DNA insert junction and a band with 410 + N (N = 0 – 300 bps) is generated in heterozygous and homozygous lines. For this purpose, two different reactions were prepared, one to detect the wt fragment and the other to detect the T-DNA insertion. Upon separation of the PCR products by agarose gel electrophoresis, and through observation of the bands pattern, it was possible to determine that *p5cs1-1* plants #2, #3, and 5# were homozygous for the T-DNA insertion (**Figure 4a**). Likewise, among the *p5cs1-4* segregating plants, 3 homozygous plants were detected (#1, #2, and #4), as can be clearly observed through the pattern of bands, appearing only one with approximately 700 bp (**Figure 4b**). Concerning the *prodh* genotyping, all plants that were genotyped were homozygous for the T-DNA insertion, except for sample 1 (**Figure 4c**). In order to confirm the exact location of the T-DNA insertion in each mutant line under analysis, samples from previously genotyped homozygous plants were sent for sequencing and analyzed using ApE, a plasmid editor. Therefore, the sequence of the PCR product for *p5cs1-1* and *p5cs1-4* was aligned against *P5CS1* sequence, and *prodh* PCR product was aligned against *ProDH* sequence. After analyzing the alignment (Supplementary material, **Figure 22, 23, and 24**) it was possible to conclude that both *p5cs1-1* and *prodh* had the T-DNA insertion in the expected location, whereas *p5cs1-4* the T-DNA insertion was located 17 bp upstream the expected insertion site, but was still in the same exon (**Figure 4d**).

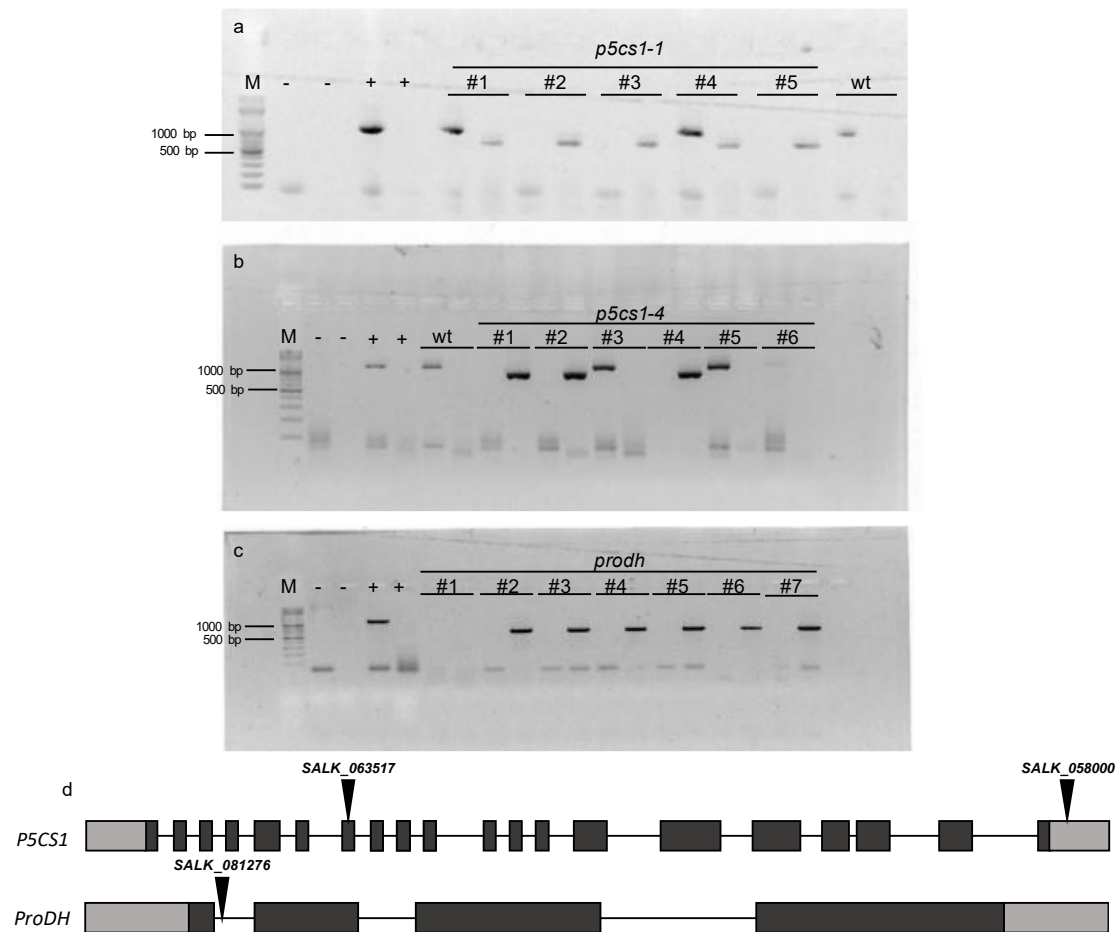


Figure 4 (a,b,c) For each plant genotype, two master mixes were prepared, one with the goal to amplify the wt fragment, and the other to amplify the region containing the T-DNA insertion. (a) Electrophoresis agarose gel regarding the PCR products corresponding to wt and mutant bands for the *p5cs1-1* plants. Gene ruler DNA ladder mix (M), negative control containing no DNA (-) and positive control (+) for each master mix, one with a wt fragment and the other with the mutant fragment. (b) Electrophoresis agarose gel regarding the PCR products corresponding to wt and mutant bands for the *p5cs1-4* plants. Gene ruler DNA ladder mix (M), negative control containing no DNA (-) and positive control (+) for each master mix, one with a wt fragment and the other with the mutant fragment. (c) Electrophoresis agarose gel regarding the PCR products corresponding to wt and mutant bands for the *prodh* plants. Gene ruler DNA ladder mix (M), negative control containing no DNA (-) and positive control (+) for each master mix, one with a wt fragment and the other with the mutant fragment. (d) Schematic diagrams of the *P5CS1* and *PROD*H gene structures and T-DNA insertion sites. Grey represents the untranslated region, and Black represents the exons.

4.2. Predicted expression patterns of *P5CS1* and *ProDH*

P5CS1 and *ProDH* expression patterns were analyzed using the *Arabidopsis* eFP browser tool. According to the microarray expression datasets for different tissues in the BAR eFP browser, the *P5CS1* shows high expression levels in sepals, reaching its maximum expression at the level of the stamens (**Figure 5a**). *P5CS1* also presents moderate expression levels in cotyledons and in the vegetative rosette (**Figure 5a**).

Regarding the expression levels of *ProDH*, this gene presents moderate levels of expression in the cotyledons, however in the mature pollen it reveals its highest expression levels (**Figure 5b**)

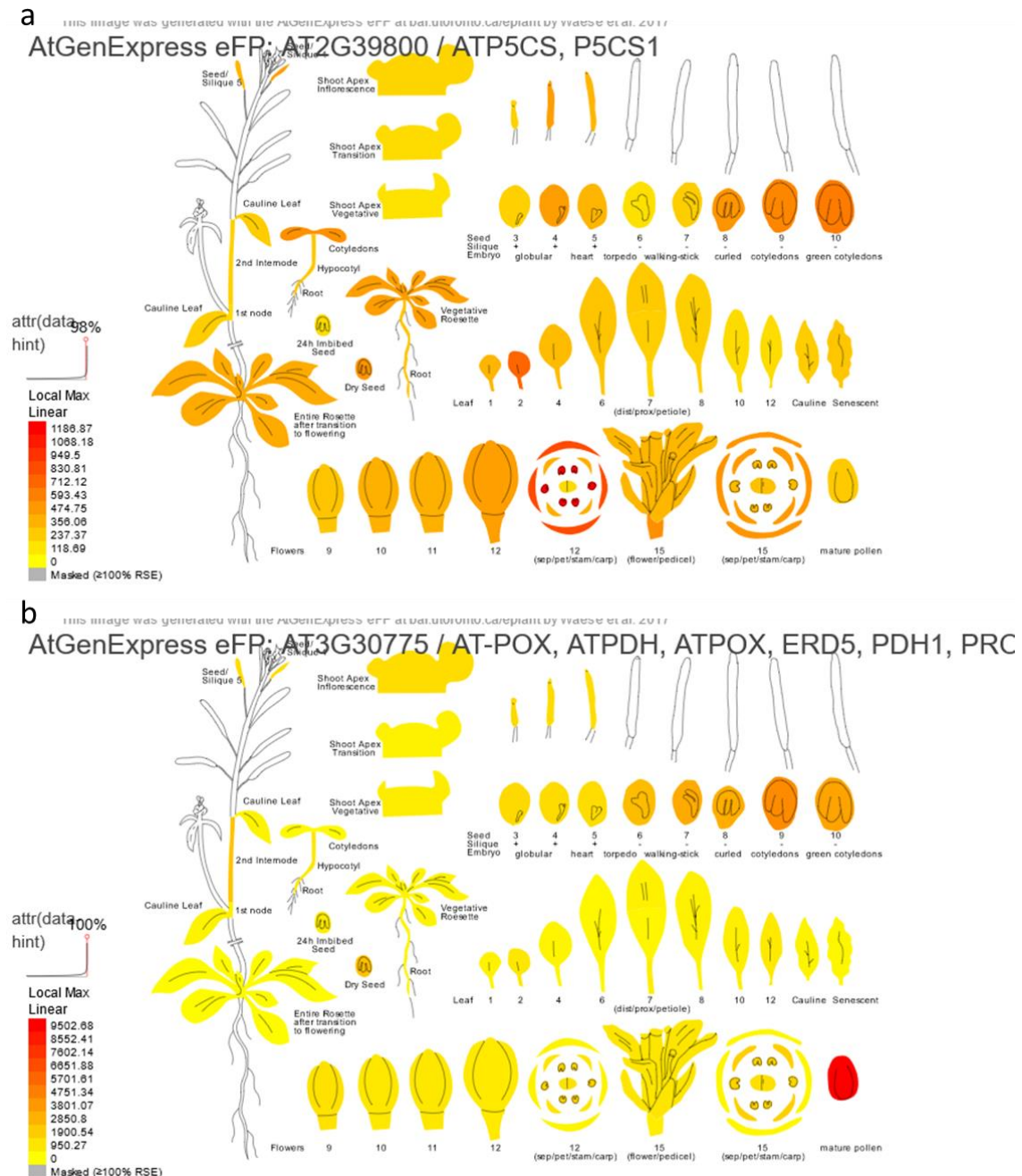


Figure 5 (a) Predicted expression pattern of *P5CS1* gene using the Arabidopsis eFP browser tool. (b) Predicted expression pattern of *ProDH* gene using the Arabidopsis eFP browser tool.

4.3. cDNA synthesis

cDNA was obtained from leaf tissues of wt plants and from the previously genotyped homozygous mutant lines, to verify if the homozygous lines for the different T-DNA insertions were indeed knock-out or knock-down lines. To confirm if there was gDNA contamination in the cDNA samples, a PCR was performed using primers for the housekeeping gene *ACT8*, flanking its intron, and the results were later analysed by agarose gel electrophoresis. Therefore, if the sample only contained cDNA, a 185 bp band was expected, whilst if there were gDNA contamination a band with a slightly higher size would appear, with 292 bp. By analysing **Figure 6**, it was possible to observe that none of the samples tested were contaminated by gDNA.

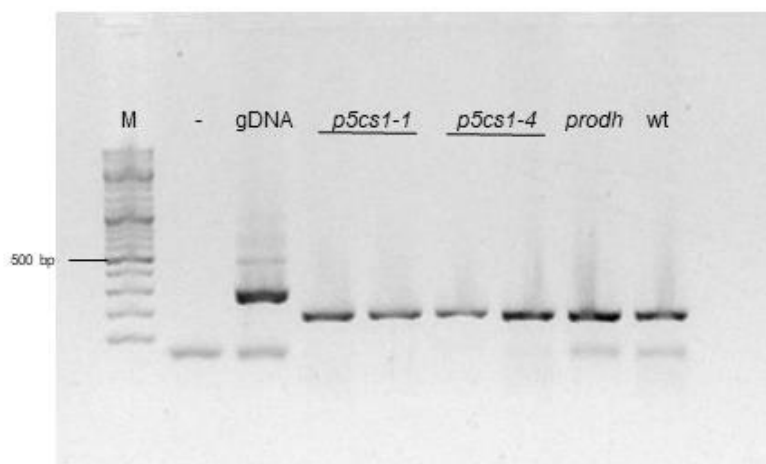


Figure 6 Agarose gel electrophoresis for the PCR products corresponding to *ACT8* gene to detect the presence or absence of genomic DNA contamination in the different cDNA samples for all the mutants. Gene ruler DNA ladder mix (M), negative control, containing no DNA (-) and gDNA positive control (gDNA).

4.4. RT-qPCR

A qPCR was performed to evaluate the expression levels of the genes *P5CS1* and *ProDH* in the analysed mutant lines. Through this technique, it was possible to verify that both *p5cs1-1* and *p5cs1-4* revealed a significant decrease in the transcription levels for the gene *P5CS1*, being knock-down lines for this gene. Concerning *ProDH* gene expression in *prodh* mutant plants, *ProDH* transcripts were not detected, and given this, *prodh* plants were considered knock-out lines for this gene (**Figure 7**).

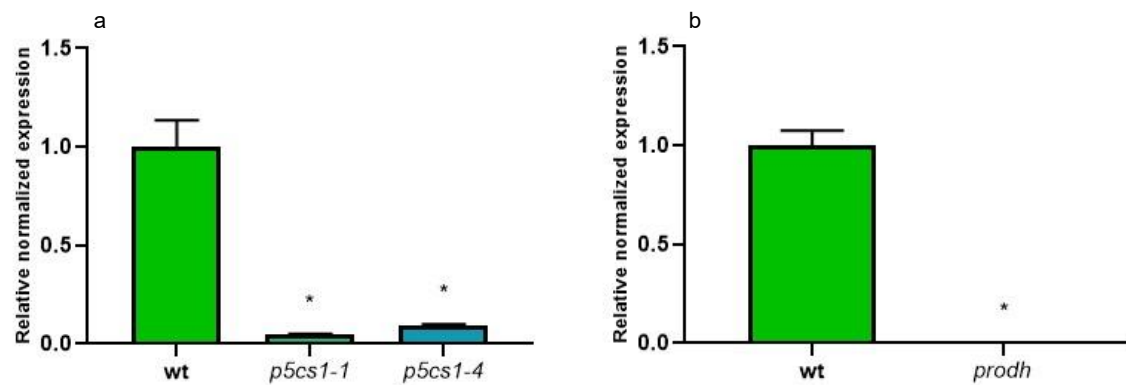


Figure 7 (a) Relative expression of the gene *P5CS1* in *wt*, *p5cs1-1* and *p5cs1-4* plants. (b) Relative expression of the gene *ProDH* in *wt* and *prodh* plants. Regarding the expression levels of *P5CS1*, the mutants *p5cs1-1* and *p5cs1-4* significantly decreased their expression levels. *ProDH* transcripts not detected in the mutant *prodh*. Transcript levels were normalized according to the reference genes *ACT8* and *RUB1*. Each bar represents the mean of three biological replicates; * represents statistical differences with a $p \leq 0.05$.

4.5. Aniline Blue staining

Aniline blue stains the callose wall of the pollen tubes growing through the different tissues of the pistil as well as the callose plugs formed along the pollen tube, thus allowing them to be viewed under the microscope. In this way, by observing 8 – 10 pistils from each genotype stained with DABs under the fluorescence microscope (**Figure 8**), it can be seen the normal growth of the pollen tubes and the successful fertilization of the ovules in the *wt* plants. When directly comparing the stained pistils of the different mutants with the *wt* ones, it can be noted that these mutants presented no abnormal phenotypes in the reproductive tissues, presenting a normal pollen tube growth, accompanied by the correct fertilization of the ovules.

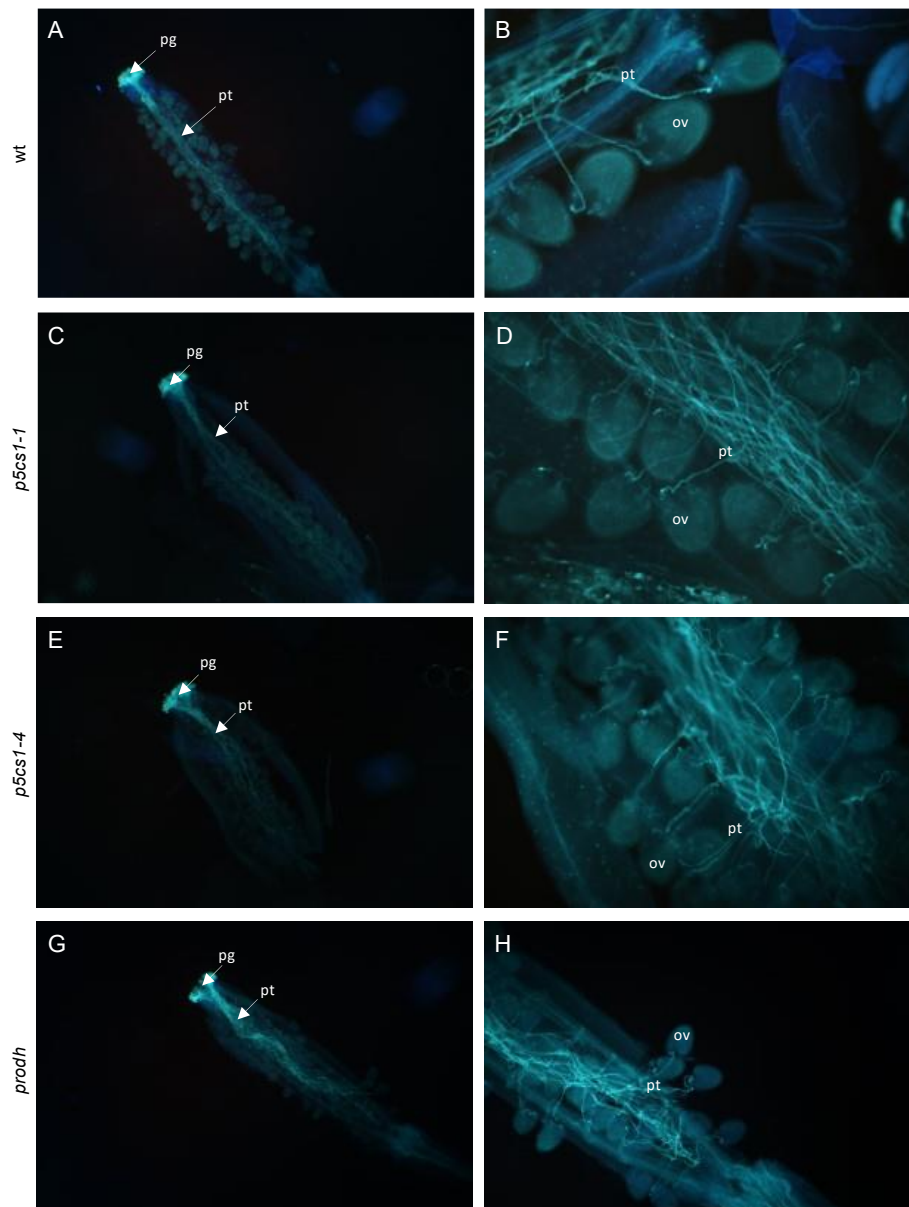


Figure 8 Decolorized aniline blue staining of pollen tubes in self-pollinated wt, *p5cs1-1*, *p5cs1-4*, and *prodh* plants. **A.** Pollen tubes growing along the transmitting tract towards the entrance of the embryo sac in a self-pollinated wt pistil. **B.** Detail of wt ovules showing pollen tubes growing towards its micropylar entrance. **C.** Pollen tubes growing along the transmitting tract towards the entrance of the embryo sac in a self-pollinated *p5cs1-1* pistils. **D.** Magnification of C detailing *p5cs1-1* ovules, with pollen tubes growing along the transmitting tract, and successfully turning its growth direction to reach the ovules. **E.** Pollen tubes growing along the transmitting tract towards the entrance of the embryo sac in a self-pollinated *p5cs1-4* pistils. **F.** Magnification of E detailing *p5cs1-4* ovules, with pollen tubes growing towards the ovules entrance. **G.** Pollen tubes growing along the transmitting tract towards the entrance of the embryo sac in a self-pollinated *prodh* pistils. **H.** Magnification of G detailing *prodh* ovules with pollen tubes growing towards its entrance. pg – pollen grains; pt – pollen tubes; ov – ovule.

4.6. Dose-response assay with GLY – Pro quantification and macroscopy analysis

Wt plants were exposed to increasing GLY concentrations, and after 14 days of growth, a macroscopic analysis was conducted as along with Pro quantification. As expected,

GLY leads to a major decline in both roots and shoots size as can be seen in **Figure 9a**, and also causes an increase in Pro levels in all tested concentrations (0-2 mg L⁻¹), especially at levels higher than 0.25 mg L⁻¹, where significant differences were recorded (**Figure 9b**). As can be observed, the exposure of wt *Arabidopsis* to 0.5 mg L⁻¹ and 1 mg L⁻¹ induced an increase of 1.7-fold; in its turn, the highest concentration led to a much more pronounced response, as Pro was acutely enhanced by 3.3-fold when compared to the control situation (0 mg L⁻¹).

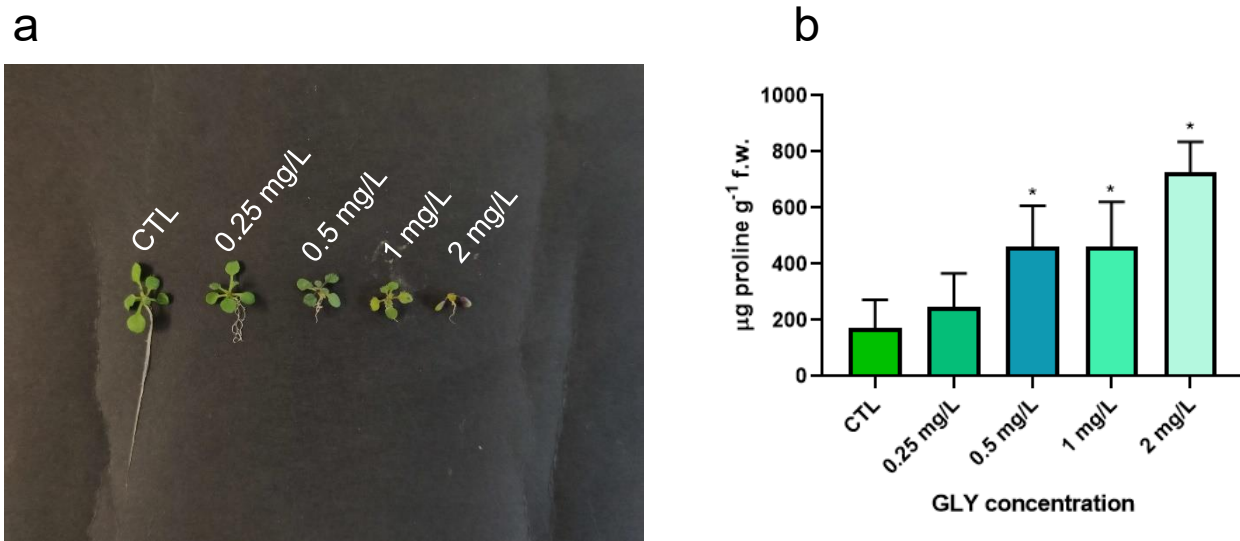


Figure 9 Effects of exposure to crescent concentrations of GLY in *Arabidopsis thaliana* seedlings biomass with 14 days of growth. (a) Macroscopic evaluation. (b) Proline quantification. * represents significant differences between the different concentrations, at $p \leq 0.05$.

4.7. Fresh biomass determination

Results referring to biomass production are presented in **Figure 10**. As detailed in Table 9 (Supplementary Material), significant differences were found for the treatment, being responsible for 83% of the variation. From the analysis of **Figure 10**, seedlings from all genotypes experienced a harsh reduction in terms of biomass production upon exposure to 0.75 mg L⁻¹ GLY. Yet the different genotypes showed distinct inhibition rates: while *p5cs1-4* was the one showing the lowest inhibition percentage (68%, when compared to its respective control), similarly to wt and *p5cs1-1*, *prodh* mutants were the ones being most affected by the presence of the herbicide, with inhibition values of higher than 80% (**Figure 10**).

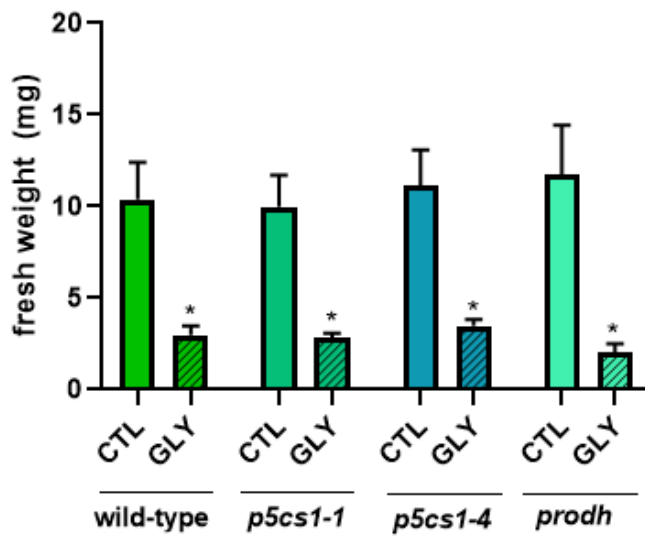


Figure 10 Fresh biomass of *Arabidopsis* seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and striped bars to GLY. * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$.

4.8. Photosynthetic pigments

The variation of the following results concerning the chl content can be explained by the genotype, treatment, and also by the interaction between the two factors, where the treatment factor was the one that caused a major source of differences (52%) (Table 9, Supplementary material). As can be observed in **Figure 11a**, chl content only showed a significant decrease when exposed to GLY, wt, and *prodh*, with 66% and 59% decrease, respectively. Nonetheless, when comparing the CTL situation between all genotypes, it was possible to notice that wt and *prodh* plants had similar levels of chl, whilst *p5cs1-4* had the highest values of chl content, and *p5cs1-1* had the lowest values of chl a + b of this photosynthetic pigment. Regarding the car content, the variation of the results can be elucidated by both of the factors (treatment and genotype) (Table 9, Supplementary material). Similarly to the chlorophylls, when exposed to the herbicide, all genotypes, besides *p5cs1-1* showed a tendency to decrease the car levels, with the only statistically significant decrease being with *prodh* mutants (45%; **Figure 11b**). When comparing the

CTL levels between all genotypes, *prodh* plants showed an enhanced car content when compared to the other genotypes.

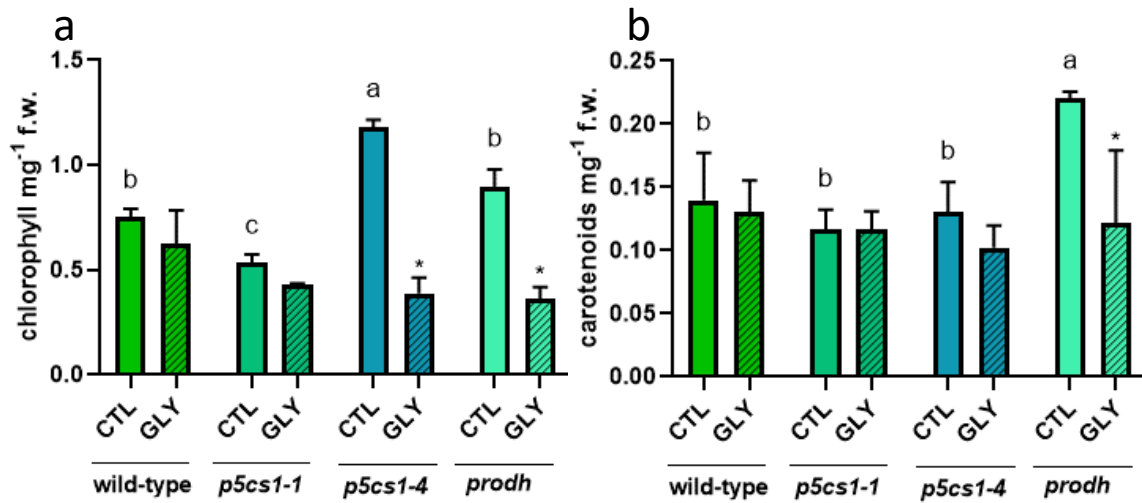


Figure 11 (a) Chlorophyll content and (b) carotenoids content of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and striped bars to GLY. Different letters represent differences between the CTL situation of every genotype and * represent significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$

4.9. Lipid Peroxidation

Lipid peroxidation, evaluated by the quantification of MDA, was found to be significantly affected by the treatment (30%), genotype (53%), and their interaction (42%) (Table 9, Supplementary Material). As shown in **Figure 12**, although under CTL conditions all genotypes presented similar levels of MDA, the presence of the herbicide affected this parameter in a genotype-dependent manner. Indeed, from all four genotypes, only wt and *prodh* seedlings had a statistically significant increase in LP, where GLY lead to a 0.9-fold increment of PL in wt plants, and 4-fold in *prodh* mutants, when compared to their respective controls.

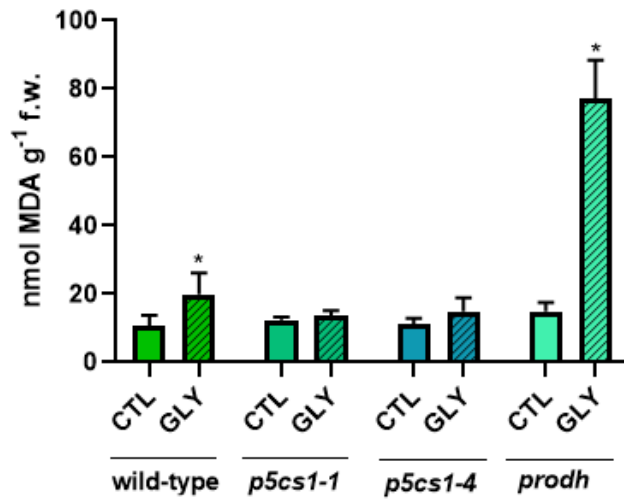


Figure 12 MDA content of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and striped bars to GLY. * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$

4.10. H₂O₂

Regarding H₂O₂ levels, the statistical analyses revealed significant differences for both studied factors (treatment and genotype) (Table 9, Supplementary Material). As can be observed (**Figure 13**), the content of H₂O₂ was stimulated by the presence of GLY in both wt and especially in *prodh* mutants, where a significant increase of 87% was found in relation to their respective controls. On the contrary, *p5cs1-1* and *p5cs1-4* did not alter their H₂O₂ levels in response to GLY (**Figure 13**).

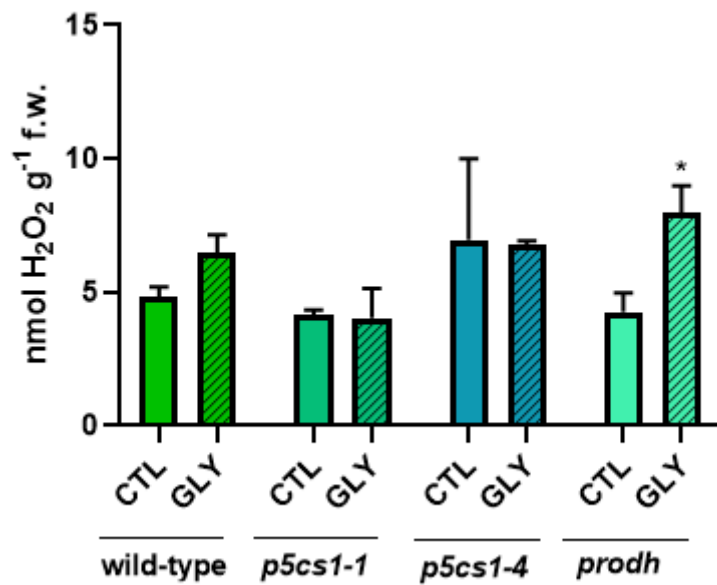


Figure 13 H₂O₂ content of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and striped bars to GLY. * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$.

4.11. Evaluation of H₂O₂ accumulation through fluorescent microscopy

To complement the results obtained in the biochemical quantification of H₂O₂ and obtain a more detailed analysis, a procedure adapted from Sandalio et al. (2008) was performed, allowing to compare the accumulation of this ROS between the various genotypes, in the two different experimental situations and in both leaves and roots. This way, and by directly comparing the images obtained from the two different situations by fluorescence microscopy, in leaves (**Figure 14a**), it was noted an increased accumulation of H₂O₂ in both *p5cs1-4* and *prodh* when were exposed to GLY, however, wt and *p5cs1-1* plants seemed to have no differences in the intensity of the fluorescent probe between the CTL and GLY situations. When comparing the fluorescence in the roots (**Figure 14b**), it appeared to be an increased intensity, indicating an enhanced accumulation of this ROS, when wt, *p5cs1-1*, and *prodh* plants were exposed to the herbicide. On the other hand, the mutants *p5cs1-4* appeared to have a similar accumulation of ROS in the roots when compared to the CTL situation of the same genotype.

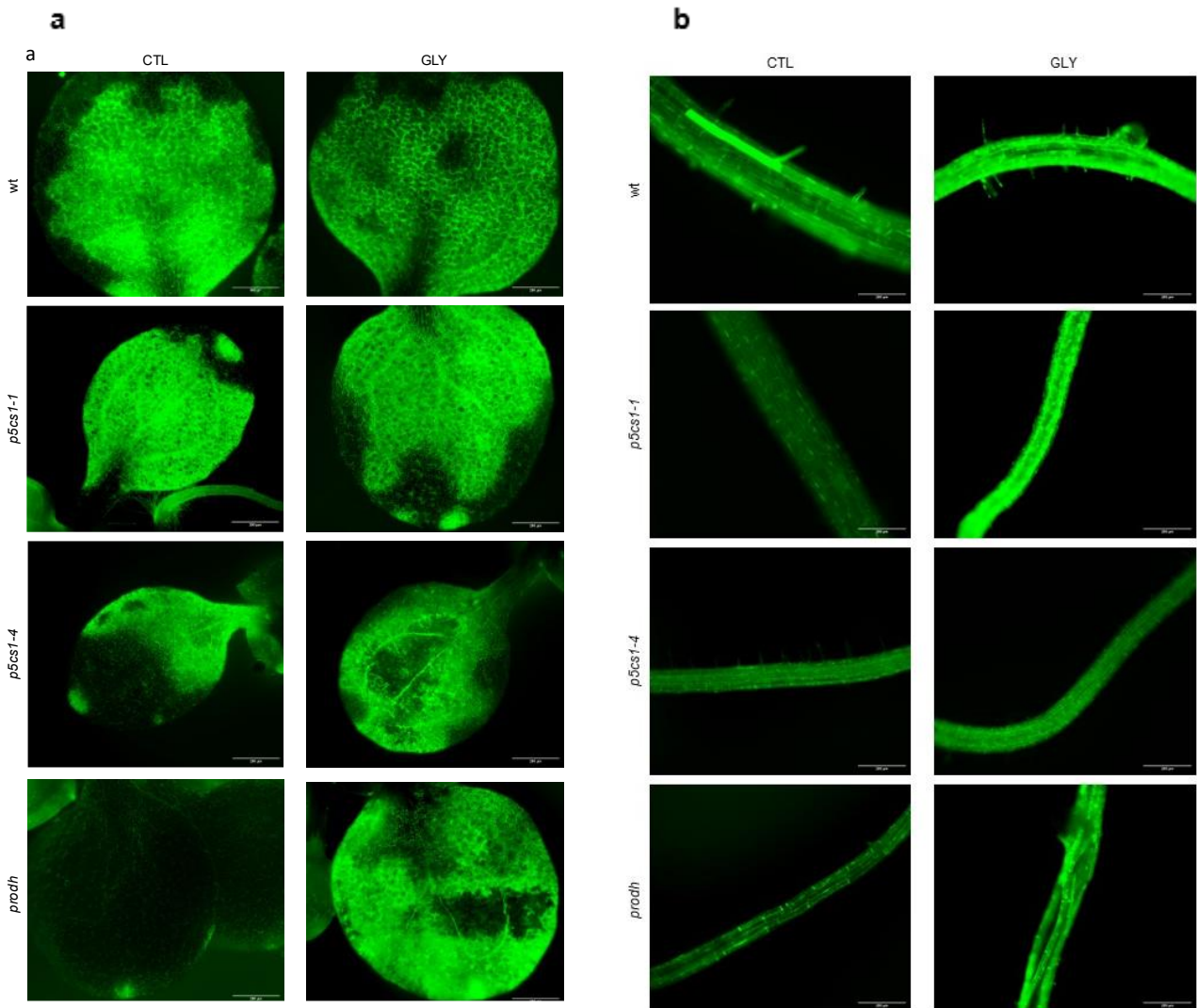


Figure 14 (a) Representative fluorescence microscopy images of the shoots of the different genotypes in both CTL and GLY situations of Arabidopsis seedlings leaves with 14 days of growth using the fluorescence probe DCF-DA; (b) Representative fluorescence microscopy images of the roots of the different genotypes in both CTL and GLY situations of Arabidopsis seedlings roots with 14 days of growth using the fluorescence probe DCF-DA

The mean intensity of the several images of both leaves and roots from each circumstance was calculated using the ImageJ/Fiji program, in order to directly compare the values obtained, clearing up any ambiguity regarding the interpretation of the existence of this ROS in the different situations. The measurements were performed on a large number of representative images. The variation of the mean intensity of H_2O_2 in roots and leaves,

was significantly modulated by both factors and also by the interaction between them (Table 10, Supplementary material). As shown in **Figure 15a**, the highest levels of intensity, in CTL situation, were found to be present in both *p5cs1-1* and *p5cs1-4* plants. However, upon GLY exposure, only wt and *prodh* plants showed a significant increase in the mean intensity of H₂O₂ of 44% and 72%, respectively. Regarding the graphic presented in **Figure 15b**, under CTL situation, both wt and *p5cs1-4* plants showed the highest values of mean intensity whilst *p5cs1-1* had the lowest values. When plants were grown in the presence of GLY, significant increases in the mean intensity were found in three different genotypes, wt, *p5cs1-1*, and *prodh*, where *p5cs1-1* plants showed the most pronounced augmentation of mean intensity levels, with an increase of 119%.

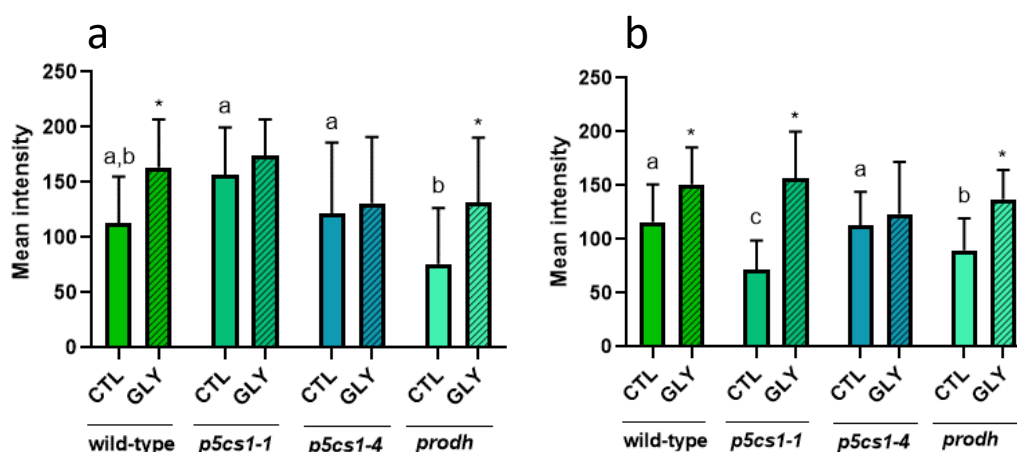


Figure 15 The analysis of the presence of H₂O₂ in the seedlings (a) leaves and (b) roots with 14 days of growth using the fluorescence probe DCF-DA. Coloured bars refer to the control situation and striped bars to GLY. * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$.

4.12. Proline

Pro levels were found to be modulated significantly by both factors (genotype and treatment), as well as by their interaction, explaining 29% of the total variation (Table 9, Supplementary Material). Under control conditions, all genotypes presented identical values of this amino acid. Moreover, and as expected, seedlings exposed to GLY (0.75 mg L⁻¹) suffered a substantial overaccumulation of Pro, regardless of the analysed genotype. Yet, from the analysis of the **Figure 16**, it is also clear that Pro accumulation was much more pronounced in the wt, but especially in the *prodh* mutants, where significant rises of 4.7- and 15-fold were found, respectively and in comparison with their controls; in contrast, the mutants *p5cs1-1* and *p5cs1-4* had the lowest amount of Pro content when exposed to the herbicide, with an increase around 3-fold.

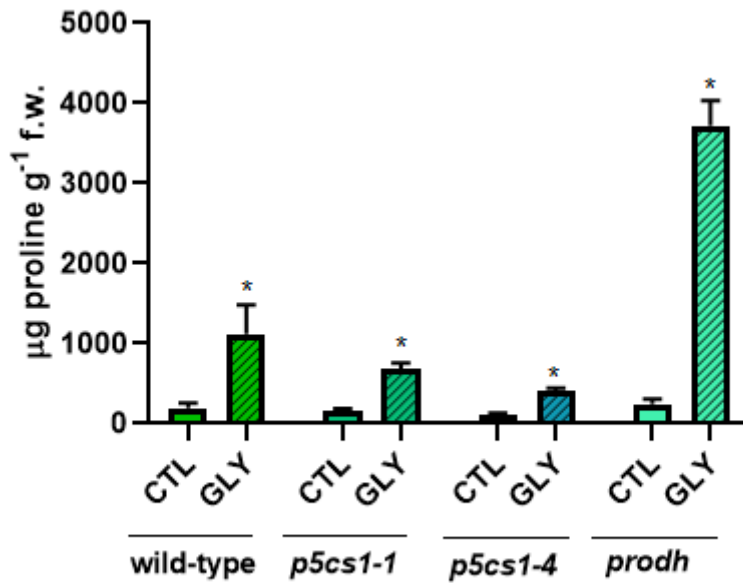


Figure 16 Pro content of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and striped bars to GLY. * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$.

4.13. Ascorbate

Results concerning total ascorbate and the ratio between reduced (AsA) and oxidized ascorbate (DHA) are presented in **Figure 17**. As detailed in Table 9, the two-way ANOVA identified a significant effect of the treatment, the genotype, and their interaction on the levels of total ascorbate (Supplementary Material). However, such effect only translated into a significant and clear increase of total ascorbate when *p5cs1-4* seedlings were exposed to GLY, where a 2.8-fold increment, when compared to the CTL situation, was recorded (**Figure 17a**). Regarding AsA/DHA, both factors caused significant differences, but there was also an interaction that explained 42% of the variation of the results (Table SX; Supplementary Material). Similarly, to total ascorbate, the AsA/DHA ratio followed the same pattern, with a significant rise in *p5cs1-4* individuals upon exposure to GLY (**Figure 17b**).

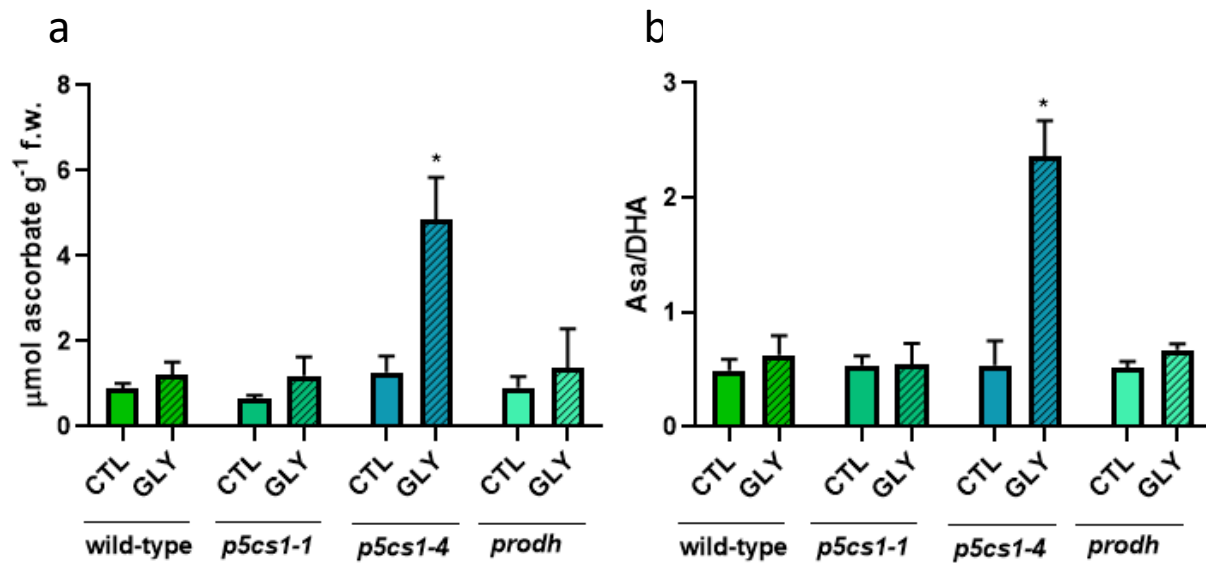


Figure 17 (a) Total ascorbate content and (b) AsA/DHA of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L^{-1} GLY. Coloured bars refer to the control situation and striped bars to GLY. * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$.

4.14. Glutathione

Regarding GSH accumulation, both factors caused a significant variation, as well as their interaction (Table 9, Supplementary Material). GSH production and accumulation appeared to follow a similar pattern to that of AsA. As shown in **Figure 18**, all genotypes showed an increase in GSH levels when grown under the influence of GLY, though statistical relevance was only achieved for *p5cs1-4* seedlings, where a 2-fold rise was registered, in comparison with its respective control.

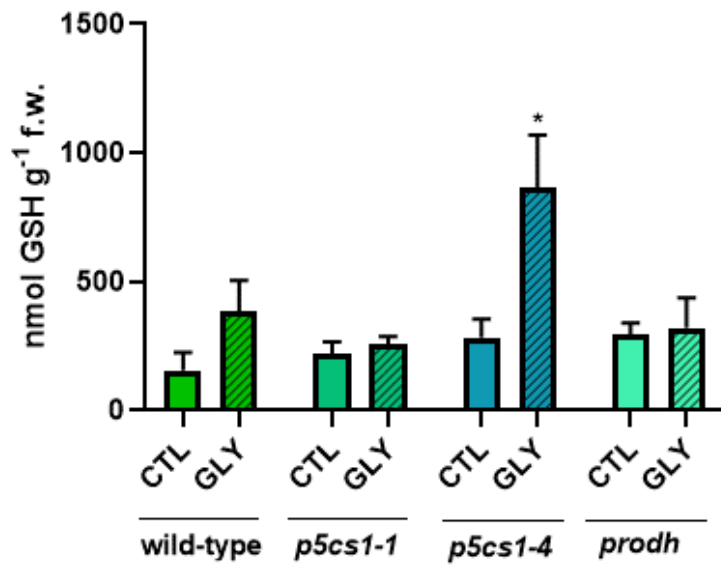


Figure 18 GSH content of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and striped bars to GLY. * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$.

4.15. APX

The variation of the APX activity was significantly modulated by the genotype, and by its interaction with GLY (Table 9; Supplementary Material). Under control conditions, the highest levels of APX activity were found in *p5cs1-1* mutants. Upon exposure to GLY, however, APX activity was decreased in *p5cs1-1* by 37%, but enhanced in *p5cs1-4* and *prodh*, when compared to their respective controls. No major differences were found in the wt seedlings (**Figure 19**).

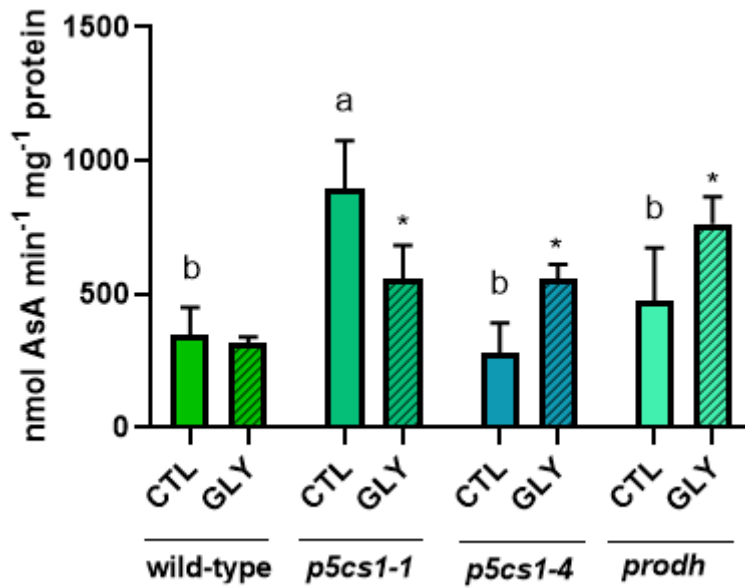


Figure 19 APX activity of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and striped bars to GLY. Different letters represent differences between the CTL situation of every genotype and * represents significant differences between the GLY and CTL situation within each genotype, at $p \leq 0.05$.

4.16. CAT

Regarding CAT activity, both factors as well as the interaction between them caused a significant variance in the results (Table 9, Supplementary Material). Results concerning CAT activity are illustrated in **Figure 20**. As can be observed, in the absence of GLY, wt, *p5cs1-1* and *prodh* seedlings presented similar activity values, while *p5cs1-4* exhibited a significantly lower CAT activity. Curiously, upon exposure to the herbicide, CAT activity was stimulated by 183% in *p5cs1-4* seedlings, compared with its respective control. No differences were found for wt, *p5cs1-1*, and *prodh* in response to the herbicide (**Figure 20**).

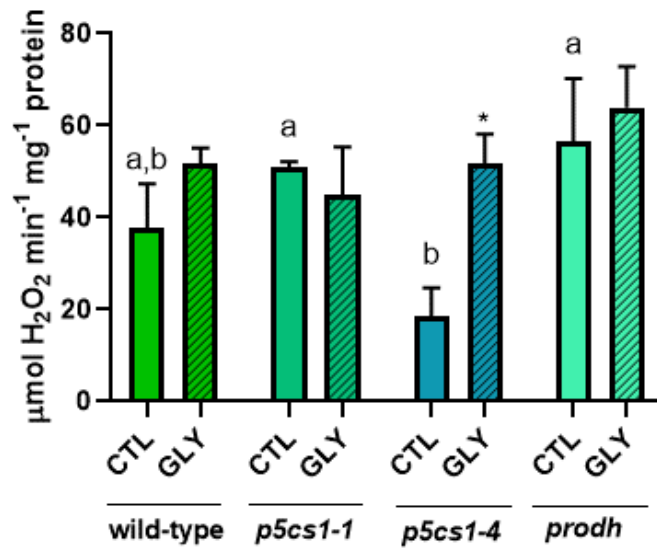


Figure 20 APX activity of Arabidopsis seedlings (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*) grown for 14 days under the presence or absence of 0.75 mg L⁻¹ GLY. Coloured bars refer to the control situation and stripped bars to GLY. Different letters represent differences between the CTL situation of every genotype and * represents significant differences between the GLY and CTL situation within each genotype. At $p \leq 0.05$.

4.17. Gene expression under GLY toxicity

In order to evaluate the effects of the GLY exposure on the expression levels of the genes *OAT*, *P5CS1*, *P5CS2*, and *ProDH*, which are involved in the Pro biosynthetic pathway, a qPCR was performed, utilizing cDNA of the different genotypes exposed or not to 0.75 mg L⁻¹ GLY. By analysing **Figure 21** and taking a closer look at the expression levels of *OAT*, there were no significant alterations in the transcript levels in plants grown under the CTL situation, despite presenting a tendency to decrease its expression levels when compared to wt plants. When exposed to the herbicide wt and *prodh* plants were the ones that showed a tendency to increase the *OAT* transcription levels (**Figure 21a**). Considering the *P5CS1* gene expression, no statistical differences were found despite being a clear tendency that GLY leads to an increase in *P5CS1* transcript levels (**Figure 21b**). *P5CS2* expression levels showed a tendency to decrease in wt, *p5cs1-1* and *p5cs1-4* plants in both situations, whereas *prodh* mutants appear to have a slight increase in the expression levels of *P5CS2* (**Figure 21c**). Regarding the accumulation of *ProDH* transcripts, no statistical differences were found, despite GLY exposure showing a tendency to decrease the levels of expression of this gene (**Figure 21d**).

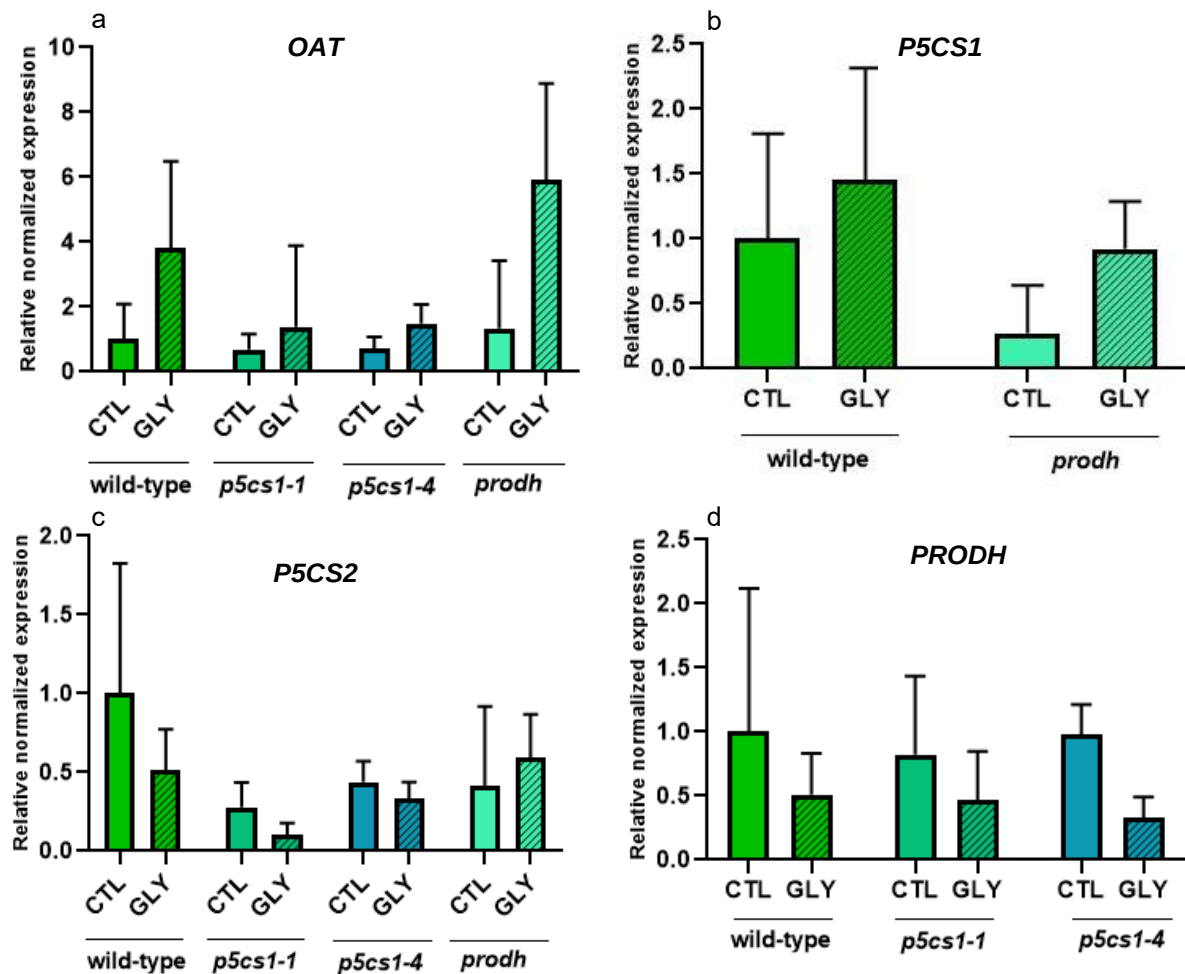


Figure 21 (a) Relative expression of the gene *OAT* in wt, *p5cs1-1*, *p5cs1-4*, and *prodh* plants grown during 14 days in the presence or absence of 0.75 mg L⁻¹ GLY. (b) Relative expression of the gene *P5CS1* in wt and *prodh* plants grown during 14 days in the presence or absence of 0.75 mg L⁻¹ GLY. (c) Relative expression of the gene *P5CS2* in wt, *p5cs1-1*, *p5cs1-4*, and *prodh* plants grown during 14 days in the presence or absence of 0.75 mg L⁻¹ GLY. (d) Relative expression of the gene *ProDH* in wt, *p5cs1-1*, *p5cs1-4*, and *prodh* plants grown during 14 days in the presence or not of 0.75 mg L⁻¹ GLY. Transcript levels were normalized according to the reference genes *ACT8* and *RUB1*. Coloured bars refer to the control situation and stripped bars to GLY. Each bar represents the mean of three biological replicates; * represents statistical differences with a $p \leq 0.05$.

5. Discussion

The ever-growing increase in world population, paired with the generalized use of agrochemicals ensures crop yields and faces the global food demand, efforts must be made to better understand the impacts of soil contamination by residues of these agrochemicals, since they can accumulate in the environment, posing a threat towards non-target organisms, including plants (Soares et al., 2019b; Spormann et al., 2019; Soares et al., 2021a; Soares et al., 2021b). Of all herbicides present in the market, GLY continues to be a standout, as it is the most used weed killer on a global scale, given not only its great efficacy but also its reduced price. This way, recently, there have been several studies that provided important findings regarding the GLY non-target toxicity focusing on the plant's redox homeostasis (Soares et al., 2019b; Spormann et al., 2019; Soares et al., 2021a). For a few years now, our research group has been carrying out work in this field, by exploring the risks of GLY contamination and developing new ways to mitigate the negative effects caused by GLY, from applying nanoparticles to organic compounds such as salicylic acid (Spormann et al., 2019; Soares et al., 2021a). A common discovery among these studies is that GLY causes a big augmentation in Pro levels, whilst the plant showed several symptoms of phytotoxicity. But when biostimulants were applied, GLY's toxic effects were less noticeable, and the Pro levels decreased. Han et al. (2015) conducted a different study in which the effects of D-amino oxidase (DAO) overexpression on GLY-induced toxicity in *Arabidopsis* were assessed. As a result, this gene was transformed into *Arabidopsis* and with the application of 10 mM GLY, after 9 days all wt plants perished, in contrast transgenic plants were able to grow normally. Apart from reporting that this gene is associated with enhanced resistance to GLY, Pro content was also found to be greatly increased in the presence of the herbicide where the transgenic plants presented a diminished accumulation when compared to wt plants. Therefore, the main goal of this study was to disclose if Pro overaccumulation during GLY exposure was connected to a signal of stress sensitivity or tolerance, by using T-DNA *Arabidopsis* mutants. After their functional characterization, Pro accumulation and biosynthetic-related genes were evaluated, along with the assessment of seedlings' physiological performance and redox status.

5.1. Genotyping – selection of homozygous mutant lines

The vast collection of T-DNA insertion mutants from *Arabidopsis thaliana* that have their sequences indexes are among the most significant resources to come from the genome

sequencing process. Phenotypes discovered with T-DNA-insertion mutants have been essential in improving both basic and applied plant research during the past ten years. These often-used mutants are a priceless resource for directly examining gene function. To identify homozygous plants for the analysis of the traits conferred by the insertional mutagenesis, a genotyping step is required because most lines are heterozygous for the insertion (O'malley and Ecker, 2010). Thus, to ensure that both copies of the gene are disrupted when using a T-DNA insertion line to study a trait, researchers often prefer to work with plants that are homozygous for the insertion. Since the majority of the lines are heterozygous, plants are genotyped to determine the homozygous individuals. To accomplish this, gDNA is taken from *Arabidopsis thaliana* leafs and amplified through a PCR using two primer pairs (O'malley and Ecker, 2010). Therefore, in this study, T-DNA insertion mutant lines were acquired and successfully genotyped, where from all the mutant lines seeds germinated, at least one plant was homozygous for the T-DNA insertion. After confirming the plants which were homozygous for the T-DNA insertion of the three mutant lines under analysis (*p5cs1-1*, *p5cs1-4*, and *prodh*), a qPCR was performed to verify if the T-DNA insertion was leading to an abolishment or a reduction on the transcript levels for the targeted genes. Though, *p5cs1-1* and *p5cs1-4* mutants had diminished *P5CS1* expression levels, *prodh* mutants clearly showed no expression levels of *ProDH* gene. This way, in the present study, for two of the lines used, the T-DNA insertion caused a knock-down effect on the gene expression, whilst *prodh* mutant lines were considered knockout mutants for the *ProDH* gene. For all lines, the region flanking the T-DNA insertion was sequenced and all were found to be relatively close to the predicted insertion site by TAIR SeqViewer tool (<https://seqviewer.arabidopsis.org/servlets/sv>) and by Salk T-DNA express (<http://signal.salk.edu/cgi-bin/tdnaexpress>).

5.2. Pollen tube growth – reproductive phenotype

In *Arabidopsis thaliana*, *P5CS1* and *ProDH* are genes that under normal conditions are expressed, in the reproductive organs and tissues, such as flower buds, pollen tubes, sepals, stamens and pollen grains (Yoshida et al., 1999; Winter et al., 2007; Verslues and Sharma, 2010). Therefore, we considered important to analyze if the impaired expression of the genes where the T-DNA was inserted were responsible for any alterations in the reproductive processes. Consequently, to analyze if there was any abnormal phenotype regarding the reproductive tissues, the callose wall of the pollen tube, and the callose plugs formed along the pollen tubes were stained by decolorized aniline blue. No differences could be observed when comparing the different mutants

with wt plants, despite *P5CS1* and *ProDH* presenting relatively higher transcript levels in flower buds (Yoshiba et al., 1999; Verslues and Sharma, 2010), more specifically in the anthers, as shown by the predictions made with the eFP Browser tool. In the case of *p5cs1-1* and *p5cs1-4*, it could be that its most closely related gene *P5CS2* (Mattioli et al., 2009b) could be acting redundantly with *P5CS1*. Also, for *ProDH*, a second isoform exists in *Arabidopsis*: *ProDH2* (At5g38710) with high homology to *ProDH1*. Funck et al., (2010) were able to show that the overexpression of a *ProDH2*-GFP construct in *Arabidopsis* was able to rescue the Pro-hypersensitive phenotype of the *pdh1-1* knockout mutant plants. Although this study claims that these two genes are acting non-redundantly, they still may play overlapping functions in some developmental processes such as the case of reproduction. Further analysis of double mutants, either for *P5CS1* and *PRODHDH* will be valuable to decipher these issues.

5.3. GLY-induced Pro accumulation – a preliminary assay

A preliminary assay was carried out in order to optimize the GLY concentration by evaluating the macroscopic effects of this herbicide and the accumulation of Pro, before the main experiment, where the effects of GLY exposure on the different genotypes were evaluated. *Arabidopsis thaliana* wt plants, when exposed to increasing GLY concentrations, showed to increase Pro accumulation in a concentration-dependent manner. In fact, this exacerbated accumulation of Pro has been described by several authors (Han et al., 2015; Soares et al., 2019b; Spormann et al., 2019). Therefore, it is not surprising that this amino acid showed a big increase in its levels, similarly, Han et al. (2015) when exploring the effects of the overexpression of D-amino oxidase (DAAO), a GLY degrading enzyme in *Arabidopsis*, reported a big accumulation of Pro in wt plants when they were exposed to 10 mM GLY. This way, in this preliminary assay, of all the different concentrations tested, only the ones equal to or higher than 0.5 mg L⁻¹ showed a statistically significant accumulation of Pro. Nonetheless, as the GLY concentration was increased, plants showed severe signs of phytotoxicity. These findings come in accordance with a study where the ultrastructure of *Solanum lycopersicum* leaves was analysed, where it was found that GLY in fact, affects leaf structure, cellular and subcellular homeostasis, by altering chloroplast structure and lowering photosynthetic pigments (Soares et al., 2020). Thus, for the final assays, the chosen concentration was 0.75 mg L⁻¹ because it showed a significant accumulation of Pro and at the same time the growth impairment wasn't the same as the highest concentrations tested.

5.4. Physiological performance of the different genotypes under GLY-induced stress

After adjusting the concentration to 0.75 mg L⁻¹ GLY, seedlings from all genotypes (wt, *p5cs1-1*, *p5cs1-4* and *prodh*) were grown in the presence of the herbicide for 14 days. In the end, a set of biometrical and physiological parameters, such as biomass and photosynthetic pigments, respectively, were evaluated. As expected, *A. thaliana* seedlings growing in the presence of GLY exhibited a big impairment in plant growth, which is in agreement with the literature available on the effects of GLY on the growth of several plant species (Spormann et al., 2019; Soares et al., 2021a). In fact, GLY competes with PEP, one of EPSPS substrates, and as result of the inhibition of this enzyme plants are not able to produce the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan, which are involved in the biosynthesis of different important metabolites (Tzin and Galili, 2010; Duke, 2020). Another effect of GLY's mode of action is the buildup of hazardous byproducts of the shikimic acid pathway, namely shikimate (Gomes, 2020). GLY-related effects on plant physiology are strongly correlated with its capacity to cause oxidative damage by increasing the production of ROS and suppressing AOX defenses, in addition to conditioning the normal functioning of particular cellular events (Gomes et al., 2014; Hasanuzzaman et al., 2020). Still, in spite of all genotypes decreasing their growth in response to the herbicide, different inhibition magnitudes were observed. For instance, while wt and the genotypes with a lower accumulation of Pro (*p5cs1-1* and *p5cs1-4*) had growth inhibition values of around 60-70%, seedlings from the *prodh* mutant which present a lower degradation of Pro, were the ones that had the highest inhibition rate (82%) in terms of growth, suggesting a higher susceptibility. Unlike other types of stress, such as drought and salinity, this big accumulation of Pro in response to GLY appears not to be helping the plant cope with the toxicity induced by this herbicide. In fact, Soares et al. (2019b) recently reported that, as the GLY concentration increases (0-30 mg kg⁻¹ GLY), *Solanum lycopersicum* fresh weight was inversely proportional to the Pro accumulation. Whereas Guerzoni et al. (2014) reported that by overexpressing *P5CS1* in sugarcane under salt stress (0-200 mM NaCl) transgenic plants, which accumulated more Pro, had an improved stress tolerance.

Besides causing a big decrease in biomass, it is well described that GLY affects cellular and subcellular homeostasis, by lowering photosynthesis-associated pigments, altering the chloroplast structure, and blocking the transcription of genes essential to

photosynthesis (Gomes et al., 2014; Soares et al. 2020). After evaluating the photosynthetic pigments, the results suggested that GLY, led to a major decrease in carotenoids and, especially, total chlorophylls in *A. thaliana*. A study conducted by Spormanm et al. (2019) observed that *Hordeum vulgare* L. plants, when exposed to 30 mg kg⁻¹ GLY, presented a slight tendency to have diminished content of chlorophylls and carotenoids. In fact, one of the many known indirect effects of GLY is the prevention of the formation and build-up of pigments like chlorophyll a and b and carotenoids. This can happen either because the shikimate metabolic pathway is blocked or because the secondary metabolite aminomethylphosphonic acid (AMPA) is also recognized as a potent phytotoxin, affecting chl biosynthesis (Kitchen et al., 1981; Wong, 2000; Gomes et al., 2014). Curiously, when taking into consideration the different genotypes, *p5cs1-4* and *prodh* mutants were the ones who got a major decrease in chlorophyll levels when exposed to GLY, but noticeably these mutants had also higher chlorophyll content in the control situation. However, *p5cs1-1* didn't show high levels of chlorophylls in the CTL situation, in fact, this mutant showed diminished levels of this photosynthetic pigment when compared to the CTL situation of the wt plants.

5.5. Wt and mutant plants overaccumulate Pro under GLY toxicity – a focus on Pro levels and its biosynthesis and degradation

As was already mentioned, oxidative stress is a frequent result of abiotic stress on plants. According to previous reports, GLY-induced stress can cause oxidative stress, which is primarily characterized by the buildup of ROS and oxidation byproducts like MDA. Therefore, the speed at which plants can activate their AOX defenses affects the severity of oxidative damage, thus being important to evaluate the AOX performance of a plant under stressful conditions (Soares et al., 2019a).

In addition, several AOX players were evaluated, and amongst them, Pro levels were quantified. By interacting and detoxifying these substances, Pro can also shield the cellular structure from dehydration, function as a redox buffer, and scavenge ROS. In addition to these, Pro can also function as a signal molecule and is included in a group of low molecular weight metabolites called compatible solutes (Verslues and Sharma, 2010). As expected, GLY led to a significant accumulation of this amino acid in wt seedlings, corroborating our previous data. Also, these findings come in accordance with several other studies on GLY toxicity, where a major common response to this herbicide was a big accumulation of Pro (Han et al., 2015; Spormanm et al., 2019, Soares et al.,

2021a). Moreover, significant increases in Pro levels were also noticed in mutant plants, namely *prodh*; despite *p5cs1-1* and *p5cs1-4* having lower *P5CS1* transcripts accumulation, one of the genes responsible for Pro synthesis, these mutants showed a significant increase in the levels of this amino acid, where such difference was not so expected. Still, while mutants with residual *P5CS1* gene expression were those accumulating Pro to a lesser extent, *prodh* seedlings were the ones with the most significant Pro augmentation when grown under GLY-induced toxicity. Indeed, these mutants do not have the ability to breakdown Pro with the enzyme ProDH, which is directly involved in the Pro metabolism by being the enzyme that transforms Pro into the intermediary P5C (Verslues and Sharma, 2010). With this enzyme being unable to degrade Pro, the big increase and disability to the breakdown of this proteogenic amino acid might be altering the redox homeostasis. The observed increases in Pro content when plants are exposed to this herbicide, support the idea that plant cells can detect GLY intracellularly and react in an effort to reduce its toxicity. However, as observed in the present study and discussed in the literature (Soares et al., 2021a), a rise in this AOX is not always an indication of improved tolerance but can be actually a sign of stress. Indeed, in *prodh* plants, the enhanced accumulation of Pro was accompanied by big growth inhibition when exposed to GLY, suggesting that the oxidative metabolism might be altered in this mutant line. To better understand the regulation loop behind Pro accumulation in response to GLY among the different genotypes, a qPCR analysis of different genes involved in the Pro biosynthetic pathway was performed. Briefly, it was possible to observe that GLY caused a slight alteration in the gene expression pattern, showing a tendency to increase the transcript accumulation of *P5CS1*, in wt plants exposed to GLY, which encodes an enzyme responsible for producing P5C, an intermediary compound of this amino acid; GLY exposure also resulted in a diminished expression of *ProDH*, partially explaining Pro overaccumulation. Regarding the transcript levels of *P5CS2*, GLY exposure tended to decrease its expression levels. In fact, *p5cs1-1* mutants growing both in CTL and GLY situations, presented very low expression levels of this gene. As in *p5cs1-1* mutants, wt and *p5cs1-4* plants, when exposed to GLY, showed a decrease in *P5CS2* expression levels, thus enforcing the idea that this gene is not a stress-induced isoform. As reviewed by Szabados and Savouré, (2010), the synthesis of Pro is governed, in plants, by two P5CS genes that encode one housekeeping (*P5CS2*) and one stress-specific isoform (*P5CS1*). *OAT* is a gene involved in the production of Pro through a secondary pathway that uses ornithine as a precursor, instead of glutamate. A study conducted by Funck et al., (2008) concluded that *OAT* does not contribute to stress-induced Pro accumulation, however, in the present study,

when plants were exposed to GLY, they showed an enhanced expression of *OAT* especially in wt and *prodh* plants. Thus, indicating that this alternative Pro biosynthetic route may be responsible for some of the Pro produced, since the expression levels of *P5CS1* and *P5CS2* were not as high as the ones for *OAT* in wt plants.

5.6. GLY-induced oxidative stress – do mutants and wt behave differently?

As was already stated, oxidative stress is a frequent result of abiotic stress on plants. Despite GLY's primary target is not related to redox disorders, according to several reports, GLY-induced stress can lead to oxidative stress, which is characterized by the overaccumulation of ROS. In order to evaluate the effects of GLY-induced stress, H₂O₂ generation and the level of LP in seedlings from all genotypes were measured. Corroborating the data obtained for the biometrical assessment, only *prodh* mutants showed a significant increase in H₂O₂ levels, despite wt plants showing a tendency to increase the levels of H₂O₂ when exposed to GLY. This increase of H₂O₂ upon GLY exposure was already reported by several authors (Spormanm et al., 2019; Soares et al., 2021a), however, contrasting information on H₂O₂ accumulation has been found, where *Arabidopsis* seedlings grown in the presence of 20 µM GLY were found to have decreased levels of H₂O₂ when compared to the CTL situation (de Freitas-Silva et al. 2017). Notwithstanding the wt plants showing a tendency to increase this ROS, the significant increase was only recorded in *prodh* mutants, raising the question if in fact, this large accumulation of Pro is leading to an increased H₂O₂ accumulation. Similarly to H₂O₂, the evaluation of LP revealed that seedlings from wt and *prodh* plants had a significant increase, especially the *prodh* mutant, suggesting that GLY induces LP. In this way, the pattern of results regarding the LP and H₂O₂ found in this study go hand in hand with each other, as H₂O₂ is a major source of LP (Soares et al., 2019b). Actually, several studies have reported that GLY induces LP; for instance, Spormanm et al. (2019) and Soares et al. (2021a) reported that *Hordeum vulgare* L. and *Solanum lycopersicum* L., when exposed to GLY (30 mg kg⁻¹ and 10 mg kg⁻¹) experienced a significant increase of LP in shoots. Interestingly, *p5cs1-1* and *p5cs1-4* seedlings showed little to no variation in their MDA content, suggesting, once again, that these plantlets might be less susceptible to GLY exposure, as these were the ones exhibiting the lowest growth inhibition rates. To complement this evaluation, H₂O₂ was also determined in an *in vivo* experiment using a fluorescent probe. It was noted that GLY leads to an increased

fluorescence, in leafs, especially in wt and *prodh* mutant lines, and in roots this augmentation was also noted in wt, *p5cs1-1*, and *prodh*, being this way in accordance with the data obtained through the biochemical quantification of this ROS. For instance, H_2O_2 levels remained equal to the CTL situation when *p5cs1-1* plants and were exposed to GLY. However, the fluorescence in this mutant increased substantially in roots exposed to this herbicide. Despite in general the microscopy results being in agreement with the biochemical quantification of this ROS, the technique and protocol used, have some limitations, that might be over- or underestimating the H_2O_2 detection. For this experiment, a fluorescence microscope (Nikon Eclipse Ci-L) was used, and with the utilization of this microscope, the presence of autofluorescence molecules present in plant tissues (Donaldson, 2020) might be interfering with the probe fluorescence leading to an over-quantification of H_2O_2 . This way, in order to obtain more reliable results without this autofluorescence, ideally, confocal laser scanning microscopy would be used in order to obtain a more detailed image. This microscopy technique has several advantages associated with it, such as the ability to detect only in-focus sections within a field, minimal sample preparation and the ability to do optical sectioning, acquiring this way a 3-D image of the original structure (Pedreschi and Aguilera, 2002). Despite the limitations presented by the technique used to evaluate the fluorescence, the protocol itself has some constraints. Even though it was initially believed that DCF was a fairly accurate H_2O_2 indicator, there are some questions about its actual validity (Keston and Brandt). Therefore, it is unclear which oxidative species is in charge of DCF's oxidation, in fact some studies have shown that DCF can react with other peroxides, primarily hydroperoxides. In this way, it is imperative to use H_2O_2 inhibitors, such as AsA, as a negative control, and also, as a positive control a known concentration of this ROS (Sandalio et al., 2008).

5.7. AOX responses from wt and mutant seedlings to GLY exposure

In order to see if there was a shift in the plant's defenses against GLY-induced toxicity, that could explain how *prodh* mutants were more sensitive to this herbicide, whilst the other mutant lines were more resistant, some components of the AOX machinery were assessed.

Regarding the levels of total AsA, GLY seemed to induce a slight increase in this non-enzymatic AOX, where the only significant enhancement of AsA levels was in *p5cs1-4* plants. A study assessing the impacts of silicon in GLY-induced toxicity in tomato plants,

also shared a very similar pattern of accumulation, where this herbicide, caused indeed an accumulation of the total AsA (Soares et al., 2021a). Interestingly, the *p5cs1-4* plants, which had a significant increase in total AsA were also the ones that had the lowest levels of Pro when grown in the presence of GLY, suggesting, a shift in the response of the plant AOX system, probably as a way to deal with GLY in a much more efficient way than over accumulating Pro. Working together with AsA, GSH levels presented a similar pattern of accumulation to the AsA, where *p5cs1-4* mutants were the only genotype that showed a significant increase, strengthening the hypothesis that, in the absence of such big Pro accumulation, plants employ other AOX mechanisms to deal with the presence of the herbicide. In fact, GSH is conjugated to electrophilic compounds by one of the most versatile enzymes, GST, being one of the key players in xenobiotic detoxification (Hernández Estévez and Rodríguez Hernández, 2020). Therefore, this augmentation in GSH levels might be suggesting that GLY is being degraded in an attempt to minimise the damages caused by this herbicide, which is in line with the results obtained in fresh biomass, where these mutants showed the lowest inhibition rate of all genotypes. In fact, GLY toxicity is also known to cause a major increase in its levels, as it was reported by Soares et al. (2019 and 2021), where the GLY treatment leads to a big accumulation of GSH in the roots.

Concerning the enzymatic component of the AOX system, the activity of APX and CAT, enzymes both involved in H₂O₂ detoxification, was evaluated. GLY-mediated impacts on the enzymatic AOX hub are rather uniform amongst works, so an easy comparison and assumptions are not straightforward and should be taken with precaution. For instance, when soil was contaminated with 30 mg kg⁻¹ GLY, *Hordeum vulgare* plants, presented an increase in CAT activity (Spormann et al., 2019); also, when *Arabidopsis* seedlings were exposed to 20 µM GLY an increased CAT activity was observed (de Freitas-Silva et al., 2017). Contrastingly, Soares et al. (2021) showed a significant decrease in this enzyme activity when tomato plants were exposed to 10 mg kg⁻¹ GLY. In the case of the present study, CAT activity appeared to be modulated by both factors (genotype and GLY). While wt plants showed a tendency to increase CAT activity, the only significant increase was noticed when *p5cs1-4* plants were exposed to GLY. Accordingly, seedlings from this mutant did not alter their H₂O₂ levels not LP, and stimulated their AOX defenses, namely GSH and AsA. Concerning the results obtained regarding the APX activity, it is noticeable that GLY leads to an augmentation of this enzyme activity in *p5cs1-4* and *prodh* mutants, whilst *p5cs1-1* mutants showed a significant decrease in its activity when grown in the presence of this herbicide. Just like the literature available on the effects of

GLY on CAT activity, different patterns of APX activity were found, whereas, on the one hand, de Freitas-Silva et al. (2017) found that APX activity in *Arabidopsis* seedlings was greatly increased in the presence of GLY, and on the other hand, Soares et al. (2021) found, APX activity, to be considerably lower when *Solanum lycopersicum* was exposed to GLY. Considering these results, *p5cs1-4* mutants were found to have a noticeable activation of both non-enzymatic and enzymatic components of the AOX, explaining this way the lower inhibition rate, H₂O₂ accumulation, and LP levels, suggesting this way that the lower amount of Pro accumulation found in the presence of this herbicide might induce plant defences against oxidative damages.

6. Conclusion and Future Perspectives

Taking all together the results obtained within this study, with analysis ranging from the evaluation of the AOX players to gene expression patterns of plants exposed to GLY, it is possible to conclude that:

- Crescent concentrations of GLY lead to a big increase in Pro levels, as well as a noticeable macroscopic growth impairment;
- The exposure of *Arabidopsis thaliana* seedlings to 0.75 mg L⁻¹ GLY caused a big decrease in fresh biomass;
- The different genotypes responded differently to the GLY-induced toxicity, where *prodh* had the highest inhibition rate, whereas *p5cs1-4* had the lowest decrease in fresh biomass;
- GLY exposure leads to a big increase in Pro levels in all genotypes, especially in wt and *prodh* plants. Despite *p5cs1-1* and *p5cs1-4* plants having a T-DNA insertion in a gene responsible for the production of Pro leading to a severe decrease in the expression of *P5CS1*, these plants were found to have, also, an increased Pro accumulation;
- The genotypes that had a higher Pro accumulation (wt and *prodh*) showed an augmentation in H₂O₂ levels and LP when grown in the presence of GLY, suggesting that this increase in Pro is not helping the plant to counteract the accumulation of this ROS and oxidative damages caused by it;
- Despite the big increase in Pro levels, especially in *prodh* plants, they did not show a decrease in LP;
- Car content was greatly decreased in plants that had the highest accumulation of Pro, namely *prodh* mutants;
- Plants that showed a diminished Pro accumulation, due to the T-DNA insertion in the gene *P5CS1*, presented a stronger activation of the non-enzymatic AOX machinery when exposed to GLY, showing increased levels in both AsA and GSH;
- GLY altered the expression levels of several genes involved in the Pro biosynthetic pathway, being the most notable change, the upregulation of *OAT*, a gene involved in a secondary route of Pro production, that in normal conditions contributes poorly to the pool of this proteogenic amino acid;
- *p5cs1-4* plants were the ones that presented a better activation of the AOX enzymes CAT and APX, being greatly induced in the presence of GLY;

- *P5CS1* and *P5CS2* transcript levels were, also, found to be altered by the exposure of *Arabidopsis* seedlings to GLY, where both genes were noticed to be downregulated by this herbicide, especially in *p5cs1-1* plants;
- *OAT* might be the gene responsible for the big accumulation of Pro under GLY-induced toxicity since the expression levels of *P5CS1* and *P5CS2* were not as high as the ones for *OAT*.

Overall, the results suggest that the strong accumulation of Pro that occurs when plants are facing oxidative stress imposed by GLY is not helping the plant to cope with the herbicide, being perceived as a stress signal rather than a defence mechanism. Therefore, we can suggest that Pro may be used as an early-warning signal of GLY toxicity, which can be applied to estimate GLY risks towards non-target plants, as well as in the identification of weed resistance.

Consequently, it is important to better understand the inner workings of the Pro biosynthetic pathway in response to GLY-induced stress and to find out whether this accumulation comes from the glutamate or the ornithine pathway, and if there are differences in the way plant perceives proline accumulation from the different biosynthetic pathways, and whether one is related to increased tolerance or a stress signal. Although this work has helped to clarify the role of Pro, there is still much to be done to fully understand the role of this amino acid in response to GLY. With this in mind, this study opens up a few avenues for future research on the role of Pro in GLY-induced stress, as it would be interesting to research the following topics:

- Perform in-depth studies focusing on the *p5cs1-4* and *prodh* mutants, since they were the ones who responded differently to the GLY-induced toxicity;
- Explore mutant lines of the gene *OAT*, in order to check if the response is the same as the mutants studied in this research;
- Evaluate the activity of the enzyme that GLY inhibits, EPSPS;
- Explore the subcellular localization of several enzymes, such as ProDH, P5CS1, EPSPS, and GST;
- Assess the activity of the enzymes involved in the Pro biosynthetic pathway;
- Obtain high order mutants with *p5cs1-4* and *OAT* mutant lines, in an attempt to completely block all biosynthetic routes to produce Pro or obtain multiple inducible knock-down mutant lines in order to avoid embryonic lethal phenotypes;
- Explore whether the Pro accumulation in response to GLY comes from the glutamate or ornithine pathway;

- Translate this study to plants with economic interest, such as tomato plants.

7. Bibliography

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8. Supplementary Material

Table 6 List of primers used in genotyping.

Gene	Mutant line	Primer	Sequence
<i>P5CS1</i>	<i>p5cs1-1</i>	1409 (LPr)	TTGTCTGCGGTTTAGTCCATC
		1408 (RP)	TTCATTTCGTCTCTAATGACATGC
	<i>p5cs1-4</i>	1411 (LPr)	CTCTGCAACTTCGTGATCCTC
		1410 (RP)	TGATCACTGATGCAATTCCAG
<i>ProDH</i>	<i>prodh</i>	1413 (LPr)	CGATTTCAGGCGTACTTGAGAG
		1412 (RP)	ATTTGGCGTGCTTATGAATTG
		LBb1.3	ATTTTGCCGATTTCGGAAC

Table 7 List of primers used in the qPCR technique.

Gene	Primer	Sequence
<i>P5CS1</i>	RT_P5CS1_fw	GAACCTTCCCTCGCAGCAAC
	RT_P5CS1_rv	AGGACTACGTGTTGGAACCT
<i>P5CS2</i>	RT_P5CS2_fw	ACGCCACCATAAGCTGCATT
	RT_P5CS2_rv	GGCCCTCCAAGTGATTCTACC
<i>ProDH</i>	RT_ProDH_fw	AACGACACCGAAACCAGAAC
	RT_ProDH_rv	GTTGGTGAGAGGGGCTTACA
<i>OAT</i>	RT_OAT_fw	GCAGTTTCGACACCTTCAGC
	RT_OAT_RV	GCAGGAACAAGTGGAGAAGC
<i>ACT8</i>	252.RT_861	CTCAGGTATTGCAGACCGTATGAG
	253.RT_862	CTGGACCTGCTTCATCATACTCTG
<i>RUB</i>	RUB1RTfw	CTGTTACGGAACCCAATTC
	RUB1RTrv	GGAAAAAGGTCTGACCGACA

Table 8 List of primers used for the Gibson assembly technique.

Gene	Primer	Sequence
<i>EPSPS</i>	Fwd_fimEPSPS_overlaplinker	TCCGGAGTCGACATGCTTTGTGATTCTTTCAAGGA
	Rev_linker_overlapimEPSPS	TCACAAAGCATGTCGACTCCGGACTCAGAT
	Fwd_pMDC_overlapInicioEPSPS	AGAAGACGCCATGTGAATTTCGGCCGAGGATAA
	Rev_InicioEPSPS_overlappMDC	GCCGAATTCACATGGCGTCTTCTCTCACTTC

<i>GSTF2</i>	Rev_GSTF2_overlaplinker	TCCGGAGTCGACCTGAACCTTCTCGGAAGCTG
	Fwd_linker_overlapGSTF2	AGAAGGTTTCAGGTCGACTCCGGACTCAGAT
	Rev_pMDC_overlapGSTF2	ATACCTGCCATGTGAATTCGGCCGAGGATAAT
	Fwd_GSTF2_overlapMDC	GGCCGAATTCACATGGCAGGTATCAAAGTTTTTCGG
<i>GSTF4</i>	Rev_GSTF4_overlaplinker	CCGGAGTCGACGTTCTTGGCTTATTAAACCAAG
	Fwd_linker_overlapGSTF4	ATAAGCCAAGGAACGTCGACTCCGGACTCAGATCC
	Rev_pMDC_overlapGSTF4	AAGCAGTCCATGTGAATTCGGCCGAGGATAA
	Fwd_GSTF4_overlapMDC	GGCCGAATTCACATGGACTGCTTACAGATGGTG
<i>P5CS1</i>	Rev_P5CS1_overlaplinker	TCCGGAGTCGACAGCTTGGATGGGAATGTCCTG
	Fwd_linker_overlapP5CS1	TCCCATCCAAGCTGTCGACTCCGGACTCAGAT
	Rev_pMDC_overlapP5CS1	AGCTCCTCCATGTGAATTCGGCCGAGGATAA
	Fwd_P5CS1_overlapMDC	GCCGAATTCACATGGAGGAGCTAGATCGTTCA
<i>ProDH</i>	Rev_ProDH_overlaplinker	TCCGGAGTCGACCGCAATCCCGGCGATTAATC
	Fwd_linker_overlapProDH	CCGGGATTGCGGTCGACTCCGGACTCAGAT
	Rev_pMDC_overlapProDH	CGGGTTGCCATGTGAATTCGGCCGAGGATAA
	Fwd_ProDH_overlapMDC	GCCGAATTCACATGGCAACCCGTCTTCTCCGAA

Table 9 Detailed ANOVA results concerning the biometric and biochemical assays of plants grown for 14 days with the presence of 0.75 mg L⁻¹ GLY, defining as fixed as fixed factors the treatment (CTL and GLY), and the genotype (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*). Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Endpoint	ANOVA		
	Treatment	Genotype	Interaction
Fresh biomass	F (1, 58) = 455.7; $p < 0.0001$	F (3, 58) = 1.140; $p = 0.3407$	F (3, 58) = 1.899; $p = 0.1397$
Chlorophylls	F (1, 10) = 122.9; $p < 0.0001$	F (3, 10) = 12.78; $p = 0.0009$	F (3, 10) = 39.40; $p < 0.0001$
Carotenoids	F (1, 19) = 7.318; $p < 0.014$	F (3, 19) = 3.982; $p < 0.0234$	F (3, 19) = 2.712; $p < 0.0738$
Lipid peroxidation	F (1, 34) = 208.5; $p < 0.0001$	F (3, 34) = 122.3; $p < 0.0001$	F (3, 34) = 98.21; $p < 0.0001$
H ₂ O ₂	F (1, 14) = 4,942; $p = 0.0432$	F (3, 14) = 4,405; $p < 0.0222$	F (3, 14) = 2.496; $p = 0.1023$
Pro	F (1, 41) = 677.4; $p < 0.0001$	F (3, 41) = 243.3; $p < 0.0001$	F (3, 41) = 213.5; $p < 0.0001$
Total ascorbate	F (1, 31) = 52.20; $p < 0.0001$	F (3, 31) = 29.11; $p < 0.0001$	F (3, 31) = 17.65; $p < 0.0001$
AsA/DHA	F (1, 34) = 111.8; $p < 0.0001$	F (3, 34) = 74.71; $p < 0.0001$	F (3, 34) = 70.79; $p < 0.0001$
GSH	F (1, 16) = 26.95; $p < 0.0001$	F (3, 16) = 13.35; $p = 0.0001$	F (3, 16) = 9.517; $p = 0.0008$
APX	F (1, 18) = 1.061; $p = 0.3165$	F (3, 18) = 13.33; $p < 0.0001$	F (3, 18) = 8.898; $p = 0.0008$
CAT	F (1, 16) = 12.50; $p = 0.0028$	F (3, 16) = 9.293; $p = 0.0009$	F (3, 16) = 5.725; $p = 0.0074$

Table 10 Detailed ANOVA results concerning the evaluation of H₂O₂ accumulation through fluorescent microscopy of plants grown for 12 days with the presence of 0.75 mg L⁻¹ GLY, defining as fixed as fixed factors the treatment (CTL and

GLY), and the genotype (wt, *p5cs1-1*, *p5cs1-4*, and *prodh*). Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold

Endpoint	ANOVA		
	Treatment	Genotype	Interaction
Leaf's mean intensity	F (1, 291) = 22.63; $p < 0.0001$	F (3, 291) = 16.86; $p < 0.0001$	F (3, 291) = 3,526; $p = 0.0154$
Root's mean intensity	F (1, 298) = 112,5; $p < 0.0001$	F (3, 298) = 5,399; $p = 0.0012$	F (3, 298) = 13,62; $p < 0.0001$


```

1  taatttggcgtgcttatgaattgagtttgatatttaaattgttcttctacttttagaaccactatcgattaagaattctccttcacatctccttctcttcc 100
657 ~~~~~GG-G-G-TTATGAATTGAGTTTGTATTAAATTGTTCTTCTACTTTTAGAACCACATCGATTAGAATTCTCCTTCATCTCCTTCTTCTTCC 567
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
101 tttttactcaacttgatttctgaccttatcatataactgcttcatgatcaggaatctttgttttatttgtttcaagctatctaatttttatttcacggca 200
566 TTTTACTCAACTTGATTCTGACCTTATCATATAACTGCTTCATGATCAGGAATCTTTGTTTATTGTTTCAAGCTATCTAATATTTTATTCACGGCA 467
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
201 aaagaaaaatgaagtaaaatatataaaaaagtaaaagcattttttattgataaggtgaatatacatattgtgaatgatataattgatagtaaaacatagtcc 300
466 AAAGAAAATGAAGTAAATATATAAAAAAGTAAAAAGCATTTTTATTGATAAGGTGAATATACATTTGTGAATGATATATTGATAGTAAAAACATAGTCC 367
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
301 ctcatgaaaaatatttagccacaggaatgttcttactcctactccttcttatattataatttttgttttcaatttcttaactacatagaagaattt 400
366 CTCATGAAAAATATTTAGCCACAGGAAATGTTCTTACCTACTCCATTTCTTATATTATAATTTTGTGTTTCAATTTCTTAACTACATAGAAGAAATTT 267
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
401 attcatctacatttcccaatttcatttaattggctccatactctctctTTACGCAATCCCGGCGATTAAATCTCCTCTTAAGTTCCATCctgcAtaacc 500
266 ATTCATCTACATTTCCCAATTTCAATTAATGGCTCCATACTCTCTCTTTACGCAATCCCGGCGATTAAATCTCCTCTTAAGTTCCATCCTGC----- 174
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
501 taccaacattagtaaaccaaatccaaaatctattaactaaactatatgggttaaagaactaatcaagggtgttaactacCTCATGAGTTGACGGTCATGAG 600
173 ----- 173

```

Figure 22 Alignment of the *prodh* PCR fragment sequenced against *ProDH* gene, where the T-DNA insertion location is highlighted in green.

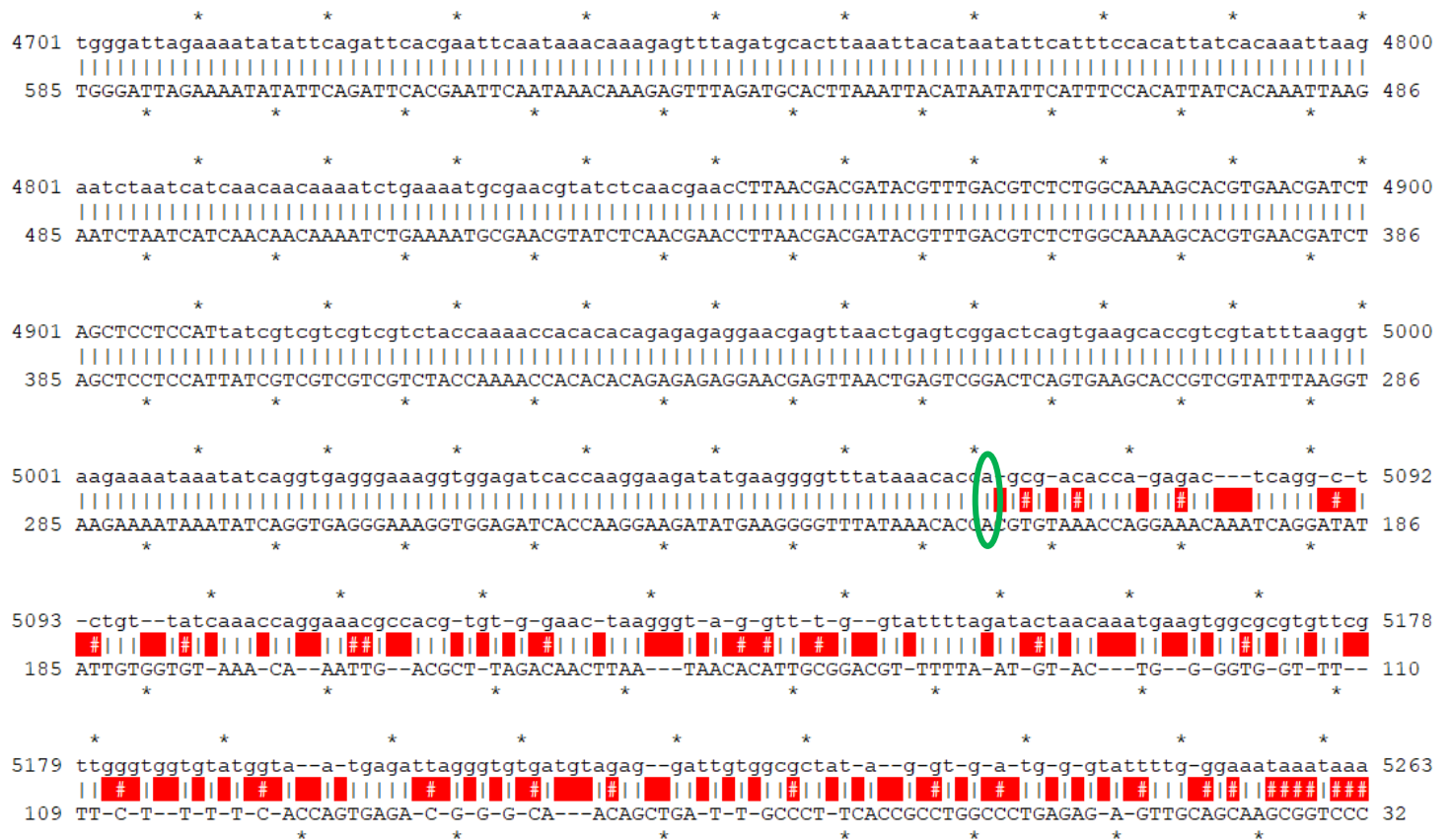


Figure 23 Alignment of the *p5cs1-1* PCR fragment sequenced against *P5CS1* gene, where the T-DNA insertion location is highlighted in green.

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      *      *      *      *      *      *      *      *      *      *
1200 ACAACTTCAACAGTGAAGCCTTGGCACAGTACTCATGGTTGAATGACCGTGCTTCTGGTATGTTTCTAGTATCTTACTTGCCCTTGGTCCACCATACAAAG 1299
      |
      |
      |
627 ACAACTTCAACAGTGAAGCCTTGGCACAGTACTCATGGTTGAATGACCGTGCTTCTGGTATGTTTCTAGTATCTTACTTGCCCTTGGTCCACCATACAAAG 528
      *      *      *      *      *      *      *      *      *      *

      *      *      *      *      *      *      *      *      *      *
1300 TGACTCctgtagtttaatatatctcatgagttatgccactctagaaatgttttcatgagatgaccattcaatatttataatcaaacaggctacatgtata 1399
      |
      |
      |
527 TGACTCCTGTAGTTTAATATATCTCATGAGTTATGCCACTCTAGAAATGTTTTCATGAGATGACCATTCAATATTTATAATCAAACAGGCTACATGTATA 428
      *      *      *      *      *      *      *      *      *      *

      *      *      *      *      *      *      *      *      *      *
1400 tctatgagacatccagatggaaggttaacaagaggtcaaataaatggttaagatagaccaacaaatgattgggccatttatgacgtacCATTGCTCTGCAG 1499
      |
      |
      |
427 TCTATGAGACATCCAGATGGAAGGTAACAAGAGGTCAAATAAATGGTTAAGATAGACCAACAAATGATTGGGCCATTATGACGTACCATTGCTCTGCAG 328
      *      *      *      *      *      *      *      *      *      *

      *      *      *      *      *      *      *      *      *      *
1500 AGCAAAAATAAGCTCATTAAAGCACAGCATTCTGCTCTAGATCCTTATGCACAAGAAGGGTTTCctgaaagagacgagagaagatgaaatgagtcagttga 1599
      |
      |
      |
327 AGCAAAAATAAGCTCATTAAAGCACAGCATTCTGCTCTAGATCCTTATGCACAAGAAGGGTTTCCTGAAAGAGACGAGAGAAGATGAAATGAGTCAGTTGA 228
      *      *      *      *      *      *      *      *      *      *

      *      *      *      *      *      *      *      *      *      *
1600 actttagaatctcccactgtattaattaaatctcgatggaaggttaaaagtctcttacCATCGCATTACAGGCTGCTGGATAGTCCAACCTTTGATCAG 1699
      |
      |
      |
227 ACTTTAGAATCTCCCACTGTATTAATTAATCTCGATGGAAGGTAAAAAGTTCTCTTACCATCGCATTACAGGCTGCTGGATAGTCCAACCTTTGATCAG 133
      *      *      *      *      *      *      *      *      *      *

      *      *      *      *      *      *      *      *      *      *
1700 AAATATGCGCTTTGCCATATCCGTATCACAAGCCTTGTCGACATATACATGACAGATTCCATctgcaagtacacaattaagccatcgctcacactctagc 1799
      |
      |
      |
132 ----- 132

```

Figure 24 Alignment of the *p5cs1-4* PCR fragment sequenced against *P5CS1* gene, where the T-DNA insertion location is highlighted in green.