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Stress effects in higher plants: case studies in *Arabidopsis* and *Cannabis*

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ABSTRACT

Plants exhibit complex stress responses to adapt to environmental changes involving metabolic pathways, hormonal signalling, and transcriptional regulation. These responses are fundamental for maintaining growth and development under stress conditions. Distress and eustress events play significant roles in plant homeostasis. In this project, different approaches were used to improve the knowledge of plant stress responses. In the model organism *Arabidopsis thaliana*, under cold stress, the pivotal role played by G6PD6 was demonstrated, providing reducing power as NADPH and supporting scavenger enzymes. In addition, regulating G6PD6 in cold conditions (4°C) is strictly connected to SK11 (ASKα) activity through phosphorylation (Dal Santo et al., 2012). In addition, alternative splicing events were investigated in 5'UTR regions of G6PD5. The expression of N-extended proteoforms is preferred in plants under cold stress. Furthermore, in an elicitation mechanism using yeast extract, the recruitment of G6PD6 and PAL (phenylalanine ammonia-lyase) increased in plants grown *in vitro*, confirming a switch-on of the primary and secondary metabolism in response to yeast extract. Based on a positive-stress mechanism, hemp (*Cannabis sativa*) varieties ([redacted]) were used under moderate salinity. At low doses, hemp plants showed an increase in biomass and phytochemical compounds. This hormetic effect underscores the potential of eustress in optimising crop performance. Furthermore, *calli* from different hemp varieties could rapidly screen different compounds and doses. Among the genotypes tested, [redacted] have shown a hormetic response to yeast extract, increasing biomass and flavonoids' synthesis genes. Finally, the impact of micro- and nano-plastic pollution is discussed, given that over 350 million tons of plastic are produced yearly. Plants' absorption of microplastics (MPs) and nanoplastics (NPs) significantly affects their growth, development, and photosynthesis, limiting crop yields and threatening natural biodiversity. This review summarises the physiological changes, reactive oxygen species (ROS) production, and other mechanisms triggered by MPs and NPs.

Dal Santo S., Jonak C., et al., Stress-Induced GSK3 Regulates the Redox Stress Response by Phosphorylating Glucose-6-Phosphate Dehydrogenase in Arabidopsis. 2012.The Plant Cell. doi/10.1105/tpc.112.101279

SUMMARY

General introduction

Plants' stress responses

Plants must appropriately respond to changes in environmental conditions by altering their growth and development. Topic research in *Arabidopsis*, the model organism in plant biology, and crop model systems, e.g. barley, provides insights into multilevel regulatory processes involved in stress responses. Under adverse environmental conditions, metabolic pathways, hormonal signalling, and transcriptional regulation orchestrate a sophisticated plant defence system.
Plants promptly handle stress damage by turnover/generation of regulatory proteins and post-translation modifications. In addition, molecular mechanisms are activated to address environmental changes, such as transcriptional regulation, promoting a specific transcription starting site, either through alternative splicing events in regulatory regions (UTRs) or changing the final product by choosing alternative exons. Hence, both transcription and translation mechanisms result in complex networks, which modulate membrane transport, chromatin remodelling, and cellular homeostasis (Haak et al., 2017). Among early events in plants to address injuries, the increase intracellular [Ca^{2+}] acts as a molecular signal, synthesising secondary signal molecules such as inositol phosphate, reactive oxygen species (ROS), and activating kinases Ca^{2+}-dependent. These signals are fundamental to activating specific transcription factors and enhancing expression levels of stress-response genes (Kumar et al., 2016). Further transcriptional regulation mechanisms and epigenetic modifications occur during stress response. Briefly, in the N-terminal region of histones, the tri-methylation of H3K4 activates the transcription, and the di-methylation of H3K9 and H3K27 represses transcription. These modifications could be permanent, ensuring the plant survives if it should again face a specific stress condition (Chinnusamy et al., 2011). Furthermore, stress conditions could damage cellular structures and impair physiological functions. For instance, alteration of cell wall and membrane architecture are translated as an internal signal of damage, and the cell responds by modulating metabolic pathways. Stress conditions activate and/or improve the synthesis of compounds that could act as osmoprotectants to maintain cell turgor (e.g. raffinose), stabilise proteins (e.g. proline), and enhance scavenging activity to handle ROS production (Jonak et al., 2012). Among the key metabolic pathways involved in the stress response, the Oxidative Pentose Phosphate Pathway (OPPP) plays an important role. The production of NADPH linked to the first oxidative steps of OPPP increases under stress conditions. Consequently, increased NADPH levels improve the scavenging enzymatic activities in

handling ROS (von Schaewen, 2003). Furthermore, phytohormones play important roles in signalling transduction in response to the development stage and stress conditions such as drought, salinity, and pathogens. Hormone signalling network consists of a complex web of molecular interactions to adapt the plant's responses to a stress condition (Kumar et al., 2016). Among phytohormones, the most involved mechanisms in stress response include abscisic acid (ABA), ethylene, and jasmonic acid (JA). In detail, ABA is the first activated in response to environmental changes (salinity, temperature, drought) due to the regulation of stomatal closure. Ethylene also influences stomata's sensitivity to ABA during drought conditions, enhancing the stomatal closure. Ethylene and JA are primarily activated in response to pathogens and mechanical damage; these signals cooperate synergistically to ensure plant survival. Following mechanical damage, ethylene can enhance JA signalling pathways, producing defence compounds.

Abiotic and biotic stressors

Plant stress events are generally due to external conditions that negatively affect metabolism, development, and survival. It is possible to distinguish between abiotic and biotic stressors. Abiotic stress consists of a wide range of environmental changes, such as light, water availability, temperature, and chemical pollution, as pest control compounds or metal ions which interfere with plant metabolism, growth and development. These stresses induce alterations in the physiology of plants, which manage new detrimental conditions by changing and/or specifically regulating metabolic pathways and gene expression on different targets based on the stressor. For example, cold exposure increases the C-repeat binding factor (CBF) gene family expression (Haak et al., 2017). In the crop industry, a hot focus is the search for sustainable pest control compounds. The herbicide glyphosate, commonly used in agriculture for pest control, has been shown to increase oxidative damage in plants (Corpas et al., 2017). Alternatively, damages due to living organisms are indicated as biotic stress. Insects, fungi, nematodes and/or bacteria can produce toxic organic compounds, damaging plant physiology and development. In response, plants can synthesise many compounds recruited in response to a biotic stressor, such as polyamines, acting as anti-microbial and anti-fungi molecules, enhancing plant resilience.

In conclusion, a specific external signal, both abiotic or biotic, activates metabolic and transcriptional pathways in plants. The intricate nature of molecular interactions makes this field of research compelling. By deepening our understanding of these interactions, we can significantly boost the productivity of plant species in agriculture and industry, empowering them to better respond to a wide range of environmental signals (Gull et al., 2019; Pereira, 2016).

Distress and eustress in plants

To ensure survival and development, plants use several mechanisms to coordinate metabolic processes and other molecular mechanisms. It is possible to distinguish different effects on plant physiology, named distress and eustress events. In plants, a distressing event indicates a negative-stress condition associated with harmful effects, such as prolonged cold exposure, excess UV radiation, and other phenomena that negatively affect plant development. Alternatively, "eustress" indicates a positive-stress condition; in this case, at a specific concentration, a harmful agent does not negatively alter plant development but enhances all processes involved in plant growth and response to an adverse condition (Saleh et al., 2019). For this reason, in plants exposed to a specific stressor agent, both distress and eustress phenomena are strictly linked to the dose: low doses enhance plant development, and high doses worsen plant development. Understanding how to take advantage of using the eustress phenomenon is still an exciting topic in plant research (Berni et al., 2024).

Elicitation mechanism and the Hormetic model

In response to external stimuli, the elicitation mechanism enhances the immunity system in plants, consisting of a complex network able to recognise a potential stressor, followed by signal transduction and gene expression regulation. Thus, the elicitation improves the synthesis of secondary metabolites, which act as defence molecules. The activation of the immunity system also increases ROS production, leading to enhanced activation of scavenging activities (Selwal et al., 2023). The elicitation can trigger hormetic mechanisms in plants. The hormesis is a complex process based on a low dose of a harmful stressor, such as a toxin or environmental factor, which stimulates a beneficial adaptive response in plants. In detail, growth and physiological adaptation are observed in plants exposed to a specific agent at low doses and at a specific time, inducing an increase in biomass and improving immunity responses. Based on the dose-effect correlation, a mathematical model was proposed to illustrate the hormesis (Belz et al., 2022). The hormetic model follows a biphasic response; here, a specific agent/compound at low doses resulted in enhancement and at high doses resulted in toxicity. In detail, it is possible to distinguish specific parameters to describe the hormetic model. Mainly the NOEL (No Observed Effect Levels), the dose at which no effects were observed, and the NOAEL (No Observed Adverse Effect Level), the highest dose at which no adverse effects were observed (Belz et al., 2022). The hormetic model proposed is based on statistical data; the model could differ concerning the species and growth condition tested (e.g., *in vitro*). Future research could be focused on improving experimental designs and verifying the application of existing models rather than developing a new

approach. For instance, standardising quantitative parameters across stressor compounds, species, and timing is recommended since a NOAEL effect can be predicted through statistical modelling of a biphasic dose-response curve (Rodrigues dos Reis et al., 2021).

In soil growth, *in vitro*, and cellular approaches to investigate plant responses under different stress conditions

The study of the physiology of stress in higher plants can involve several strategies to describe induction, response, resistance and resilience to adverse conditions. Therefore, testing different growth conditions could improve our knowledge of the molecular and physiological mechanisms activated. Soil-based cultivation is the traditional method of growing plants. Soil acts as a "natural buffer," regulating nutrient and water uptake and providing mechanical support for the entire plant. Since the soil condition is closer to a natural growth condition, the results can be highly variable among different experimental samples due to the extremely heterogeneous matrix. The hydroponic system is based on growing plants in a liquid solution with all the necessary nutrients for plant development. Using a hydroponic system ensures a precise and controlled environment for nutrients' availability, providing them solubilised and available to root tissue; hence, the growth rate in hydroponics rapidly increases if compared to soil-based methods. Although hydroponics offers improved growth conditions, stress tolerance remains lower than in soil-based methods. This is due to plants being adapted to a closed system, which makes them more susceptible to external agents. For this reason, the hydroponic method is mainly used to improve the knowledge about the plant's stress responses under adverse conditions (Hayes et al., 2012). Finally, *in vitro* growth conditions methods are based on the involvement of synthetic media (the most commonly used is Murashige and Skoog (Murashige, T., Skoog, F., 1962) characterised by specific nutrient mixture, requiring all steps to be performed under sterile conditions. The major advantage of the *in vitro* method is represented by uniform growth conditions, which provide the right amounts of nutrients that influence plant homeostasis. A cellular *in vitro* system utilising the growth of undifferentiated cells can enhance the understanding of plant biology and its several industrial applications. The "*callus*" indicates a multipotent cell mass which arises following an infection or physical damage, resulting in tumour formation. *Calli* are meristematic tissues, and different genomic regulation occurs. For instance, it is an optimal opportunity to understand better the molecular mechanisms besides development, gene expression, and effects following a specific treatment. Furthermore, starting from a *calli* tissue, the aim could be to obtain a stable cell line able to produce metabolites applied in different sectors (medical, pharmaceutic, cosmetic, food). These growth

strategies in plant biology provide extensive molecular information related to stress response mechanisms, thereby enhancing the production of specific compounds.

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Aims of the project

In this project, *in vitro* and soil-based methods have been used. The first part of the project was focused on understanding a specific abiotic stress condition (low temperature) in the model organism *Arabidopsis thaliana*, both *in vitro* and in soil growth conditions. Taking advantage of mutant knock-out plants, it has been possible to design an overview of plant responses after exposure to low temperatures in terms of both enzymatic and molecular adaptation. In addition, a biotic stressor (yeast extract) was used to verify a potential elicitation event. The second section of the project was focused on eustress responses in a plant of industrial interest: *Cannabis sativa*. *In vitro* plants' growth conditions under salinity stress were used. Different genotypes were selected to improve the expression profile in hemp under these conditions. Additionally, to enhance the knowledge regarding the application of hemp in the industry sectors, even *calli* were used to allow a massive compound screening prior to passing in a cell suspension culture. Finally, the third part of the project focused on the environmental effects of micro- and nano-plastics in higher plants regarding gene expression and physiological adaptation. A widespread bioinformatic analysis has been conducted, together with a review of recent bibliographic data, to depict the state-of-the-art research on micro- and nano-plastic pollution in the environment of crops. In conclusion, the project aimed to give a panoramic overview of molecular, enzymatic and physiological responses to different stressors in *Arabidopsis thaliana* and *Cannabis sativa*, connecting the model organism and an industry-interesting plant species.

Chapter 1

This chapter contains unpublished results.

Cold stress and yeast-mediated elicitation effects in *Arabidopsis thaliana* wild type and mutant strains, an overview of stress response mechanisms and the pivotal role of G6PD

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Introduction

The Oxidative Pentose Phosphate Pathway (the OPPP) and the role of G6PDH in *Arabidopsis thaliana*

The oxidative pentose phosphate pathway (OPPP) is a major source of reducing power and metabolic intermediates in biosynthetic processes. The OPPP is an essential source of reducing power in plants due to the reduction of $NADP^+$ in NADPH catalysed by two dehydrogenases (Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), leading to the synthesis of ribose-5-phosphate, a precursor for nucleotide biosynthesis. The whole pathway is divided into two distinct phases: the oxidative and non-oxidative phases. The oxidative phase generates NADPH and ribulose-5-phosphate, while the non-oxidative phase interconverts sugar phosphates to produce ribose-5-phosphate, and other metabolic intermediates (e.g. glycolysis, Calvin cycle, amino acids biosynthesis) (von Schaewen et al., 2003). The oxidative phase starts with the enzyme Glucose-6-phosphate dehydrogenase (G6PDH, *G6PD*), the pathway's first control checkpoint. G6PDH catalyses the conversion of glucose-6-phosphate to 6-phosphoglucono-δ-lactone, reducing $NADP^+$ to NADPH. The following steps are based on the hydrolysis of 6-phosphoglucono-δ-lactone to 6-phosphogluconate by 6-phosphogluconolactonase, followed by the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate, generating a second molecule of NADPH (Wakao et al., 2008), catalysed by the 6-phosphogluconate dehydrogenase (6PGDH). NADPH produced by the OPPP is used for various anabolic reactions, including fatty acid biosynthesis, nitrogen uptake, and the maintenance of reduced glutathione levels (Esposito, 2016; Landi et al., 2024). NADPH is also needed in chloroplasts for the Calvin cycle, which occurs during carbon fixation. The balance between the OPPP and glycolysis is tightly regulated to ensure an adequate supply of NADPH and ribose-5-phosphate according to the cell's metabolism (Noctor et al., 2011). The regulation of the OPPP in plants involves multiple mechanisms. Different signals modulate the activity of G6PDH: cellular redox state (in stress condition is active), NADPH concentrations (high levels of NADPH induce a switch off of the pathway), and accumulation of ribulose-5-P. Additionally, the expression of OPPP enzymes is influenced by developmental cues and environmental conditions, such as light, which directly affects photosynthetic activity and the demand for NADPH (von Schaewen, 2003). In the well-known model organism, *Arabidopsis thaliana* there were identified 5 G6PDH isoforms: one chloroplast (P1-G6PDH, *G6PD1*), two plastids (P2-G6PDH, *G6PD2* and *G6PD3*), two cytosolic (cyt-G6PDH, *G6PD5* and *G6PD6*), and the isoform P0-G6PDH in peroxisomes (*G6PD4*). Notably, *G6PD4* encodes a non-functional enzyme initially classified as a pseudogene. Further research indicates that this protein may play a key role in stress responses by the translocation

into peroxisomes of P1/P2-G6PD. Indeed, recent studies based on salinity stress conditions demonstrated an assembly of P1-G6PD/P0-G6PD in the cytosol (Dal Santo et al., 2012). The heterodimer is also characterised by a peroxisome target sequence (PTS) in the C-terminal region of the P0-G6PD subunit. In addition, specific 6PGLs and 6PGDHs have recently been found in *Arabidopsis* peroxisomes, thus triggering an additional OPPP into this organelle (von Schaewen et al., 2021). In chloroplasts, the OPPP is active in the dark. During light hours, the ferredoxin-thioredoxin system inactivates P1-G6PDH, reducing a regulatory disulfide bridge. In addition, the P1-G6PDH is inhibited by high concentrations of NADPH (Esposito, 2016). P2-G6PDH is mainly regulated by the NADPH/$NADP^+$ ratio, showing a 5–10-fold higher inhibition by NADPH than Cy-G6PDH and P1-G6PDH (Sakr et al., 2022). Additionally, the OPPP is essential in photosynthetic tissues, where the demand for NADPH is high for protection against oxidative stress. During periods of high photosynthetic activity, the non-oxidative phase of the PPP provides a flexible route for the redistribution of carbon skeletons, ensuring a balance between energy production and biosynthetic demands (Schulze et al., 1994). Additionally, the non-oxidative phase involves a series of reversible sugar phosphate interconversions that produce ribose-5-phosphate and xylulose-5-phosphate from ribulose-5-phosphate, involved in nucleotide biosynthesis, and fructose-6-phosphate and glyceraldehyde-3-phosphate, substrates for glycolysis reactions (Noctor et al., 2009).

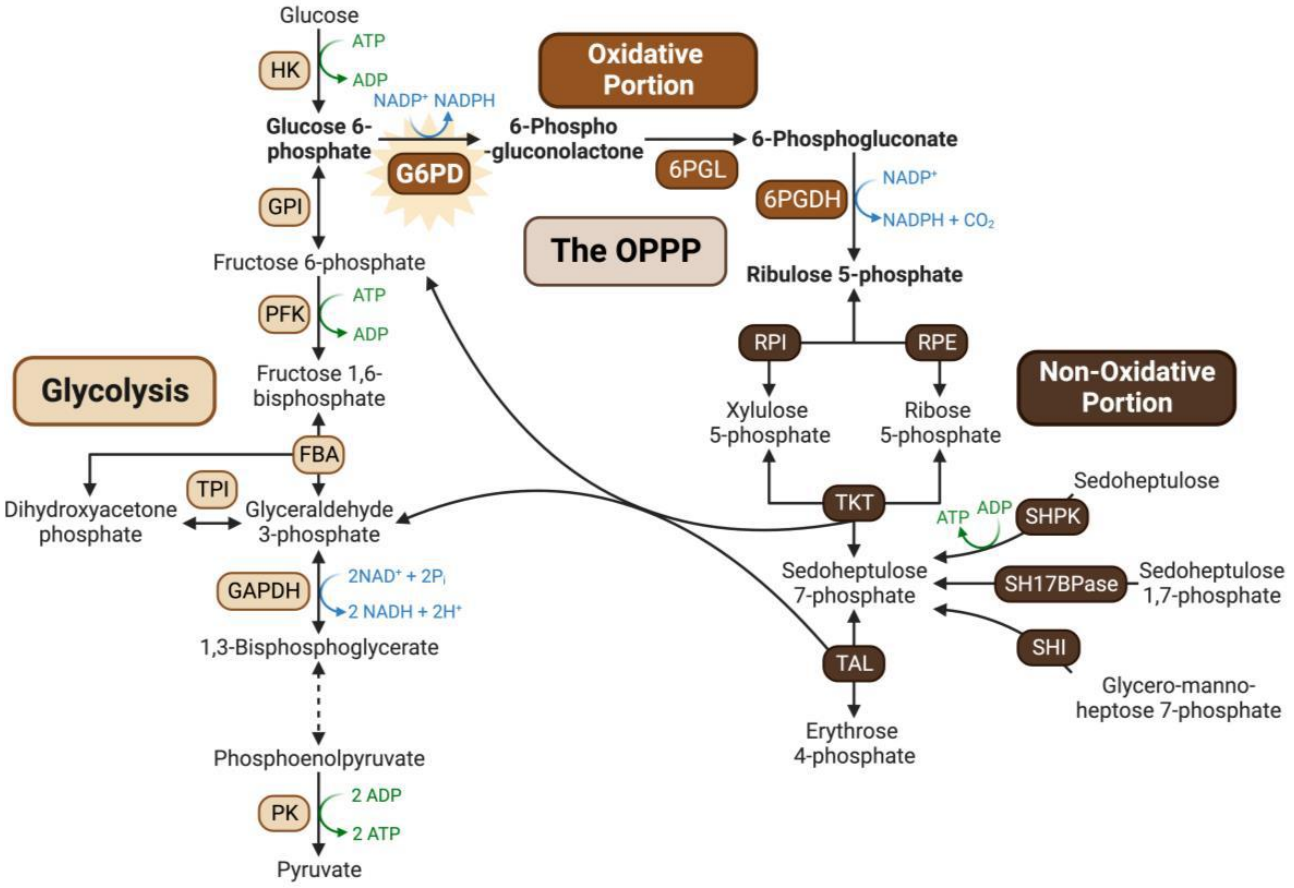


Figure 1 Schematic illustration of the Oxidative Pentose Phosphate Pathway. The first oxidative step is illustrated on the top, and below is the entire pathway with intermediate compounds produced. G6PD, glucose-6-phosphate dehydrogenase; 6PGL, 6-phosphogluconolactonase; 6PGDH, 6-phosphogluconate dehydrogenase; RPI, ribose 5-phosphate isomerase; RPE, ribulose 5-phosphate epimerase; TKT, transketolase; TAL, transaldolase; SHI, sedoheptulose 7-phosphate isomerase; SH17BPase, sedoheptulose 1,7-biphosphatase; SHPK, sedoheptulokinase; HK, hexokinase; GPI, glucose phosphate isomerase; PFK, phosphofructokinase; FBA, fructose biphosphate aldolase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PK, pyruvate kinase (Plaxton et al., 2024).

Finally, understanding these intricate networks of the OPPP in plants can offer insights into improving crop stress tolerance and metabolic efficiency.

Arabidopsis thaliana SHAGGY-related kinase 11

Plant Glycogen Synthase Kinase 3 family members (GSK3s) are highly conserved serine/threonine protein kinases essential for integrating various signalling pathways in plant development and responses to different stress conditions. In plants, GSKs are encoded by a gene family involved in different physiological responses (Dal Santo et al., 2012). These kinases are involved in several signalling pathways, including hormone signalling (e.g. brassinosteroids, abscisic acid) and environmental conditions response. GSKs act both in the nucleus and cytoplasm. In the nucleus, GSKs act as inhibitor transcriptional factors for the expression of brassinosteroid gene targets, while in the cytoplasm, they regulate several protein targets by phosphorylation. Moreover, GSKs are regulated

by post-translation modifications such as acetylation, phosphorylation and oxidation status (Li et al., 2021). GSKs play a pivotal role in plant metabolism and development, namely germination timing, root growth, protein activity and degradation by phosphorylation. Intriguingly, in *A. thaliana,* the activity of cytosolic G6PDH is regulated via phosphorylation catalysed by GSK3 (Dal Santo et al., 2012). In detail, it has been demonstrated that a specific GSK isoform, ASKα (SK11, AT5G26751), under abiotic stress conditions, catalyses the phosphorylation at position Thr-467 on the C-terminal region of G6PDH6, thereby increasing its enzymatic activity (Dal Santo et al., 2012; Landi et al., 2021). Additionally, mutant *SK11* plants under salt stress show a reduced germination rate and reduced root growth, confirming the primary activity of ASKα regarding the activation of G6PDH6 and its critical role in response to stress conditions (Dal Santo et al., 2012).

Cold as an abiotic stressor in plants, the role of Oxidative Pentose Phosphate Pathway

Cold is a significant abiotic stressor that alters plant growth, development, and survival. Like other abiotic stresses, cold represents a severe risk for plant productivity, limiting yields and quality of crops worldwide. To address low-temperature events, plants switch on a wide range of responses, activating scavenging enzymes, expression of chaperones, whole transcriptome reorganisation, photosynthetic performance regulation, and membrane composition modification. Furthermore, plants promote cold acclimation, which triggers several transcriptional and biochemical pathways to enhance the ability to survive (Landi et al., 2024). It is possible to distinguish two categories of cold stress: chilling stress (0-15 °C), which impacts membrane fluidity, enzyme activity, and cellular processes, and freezing stress (<0 °C), which favouring ice formation in plant tissues causes physical damage and disrupt cellular integrity, leading to cell death (Wang et al., 2021). Low temperatures induce the expression of cold-responsive (COR) genes, regulated by specific transcription factors like C-repeat binding factors (CBFs). Intriguingly, *G6PD1* was reported as a COR gene (Park et al., 2015), resulting in a down-regulation upon low temperature. In addition, the synthesis of defence compounds increases, enhancing secondary metabolic pathways, such as flavonoid biosynthesis (Chen et al., 2020). Moreover, cold stress stimulates the production of osmoprotectants (sugar and amino acids), regulating the cellular osmotic balance and the protection of cellular components (Li et al., 2024). In addition to molecular responses, the cell's architecture is compromised by low temperatures. Indeed, cell membrane permeability decreases upon cold stress, thus promoting physical damage with reduced enzyme activity. Among physical alterations, the photosynthetic system is compromised at low temperatures, affecting membrane fluidity and reducing the chlorophyll content (Landi et al., 2024; Yamada et al., 2019). Different metabolic pathways are finely regulated to address plant adaptation at low temperatures. Regarding these, the OPPP provides several metabolic intermediates, such as ribose-5-phosphate

(involved in the repair and synthesis of DNA and RNA) and erythrose-4-phosphate (substrate for the biosynthesis of aromatic amino acids and secondary metabolites), and reducing power by the production of NADPH to improve the antioxidant system (Tan et al., 2024; Landi et al., 2021). Additionally, low temperatures lead to the enhancing expression of *G6PD* (glucose-6-phosphate dehydrogenase) and *6PGD* (6-phosphogluconate dehydrogenase), coding for the first and second enzyme of OPPP, respectively (Landi et al., 2021). Notably, cyt- and P2-G6PD activity is critical in responding to low temperatures, ensuring the correct performance of stress response mechanisms (Landi et al., 2021; Landi et al., 2024). Following the production of NADPH, the OPPP, especially through the activation of G6PDH, facilitates the continuous detoxification of ROS, protecting plants from oxidative damage and enhancing their ability to withstand cold temperatures (Singh et al., 2024). Several genes associated with stress responses are strictly programmed at transcriptional and post-transcriptional levels. In plants, the prominent stress response mechanism is the activation of scavenger enzymes involved in the detoxification of ROS (Reactive Oxygen Species), such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Irimia et al., 2021; De Vita et al., 2012; Xiong et al., 2014). The dynamic interplay among ROS production, scavenging, and signalling transduction, along with regulating primary and secondary metabolism, underscores the complexity of plant defence strategies. In addition, under stress conditions, the demand for NADPH increases to supply the requirement of the glutathione reductase (GR) and to obtain reduced glutathione (GSH) starting from the oxidised form (GSSG). GSH is used by the glutathione peroxidase (GPX) that converts H_2O_2 to H_2O (Fischer et al., 2002). These reactions are fundamental for maintaining the right amount of reducing power in cells and avoiding oxidative damage to cell compartments.

Elicitation mechanism in *Arabidopsis thaliana* and the phenylpropanoid pathway

In plant biology, elicitation is a natural mechanism that activates the immune system in response to external stimuli named elicitors. These elicitors can be biotic, such as pathogens (e.g., bacteria, fungi, viruses), and abiotic, such as physical stress (e.g., wounding, drought, temperatures), or chemical signals (e.g. metal ions compound). Elicitors trigger signalling pathways that produce secondary metabolites and other defensive compounds to address adverse conditions. These secondary metabolites are natural compounds attractive for biotechnological applications. Therefore, plants handle their defence mechanisms, increasing the gene expression of specific targets, such as transcriptional factors and genes coding for enzymes involved in specific metabolic pathways, resulting in enhanced enzymatic activity (Zhou et al., 2022). Briefly, primary metabolism consists of photosynthesis, respiration, and synthesising biomolecules to provide chemical energy and intermediate compounds recruited in biosynthetic pathways. Secondary metabolism consists of a

complex network of metabolic processes that produce several compounds essential to the plant's interactions with the environment. Concerning secondary metabolism, the phenylpropanoid pathway plays a crucial role in plant growth, development, and immunity mechanisms due to the production of flavonoids and flavonols. The first key enzyme of the entire pathway is Phenylalanine Ammonia-Lyase (PAL), which converts the amino acid phenylalanine into cinnamic acid, followed by a series of hydroxylation, methylation, and glycosylation reactions that lead to several phenolic compounds, including flavonoids, lignin, and coumarins (Busatto et al., 2023).

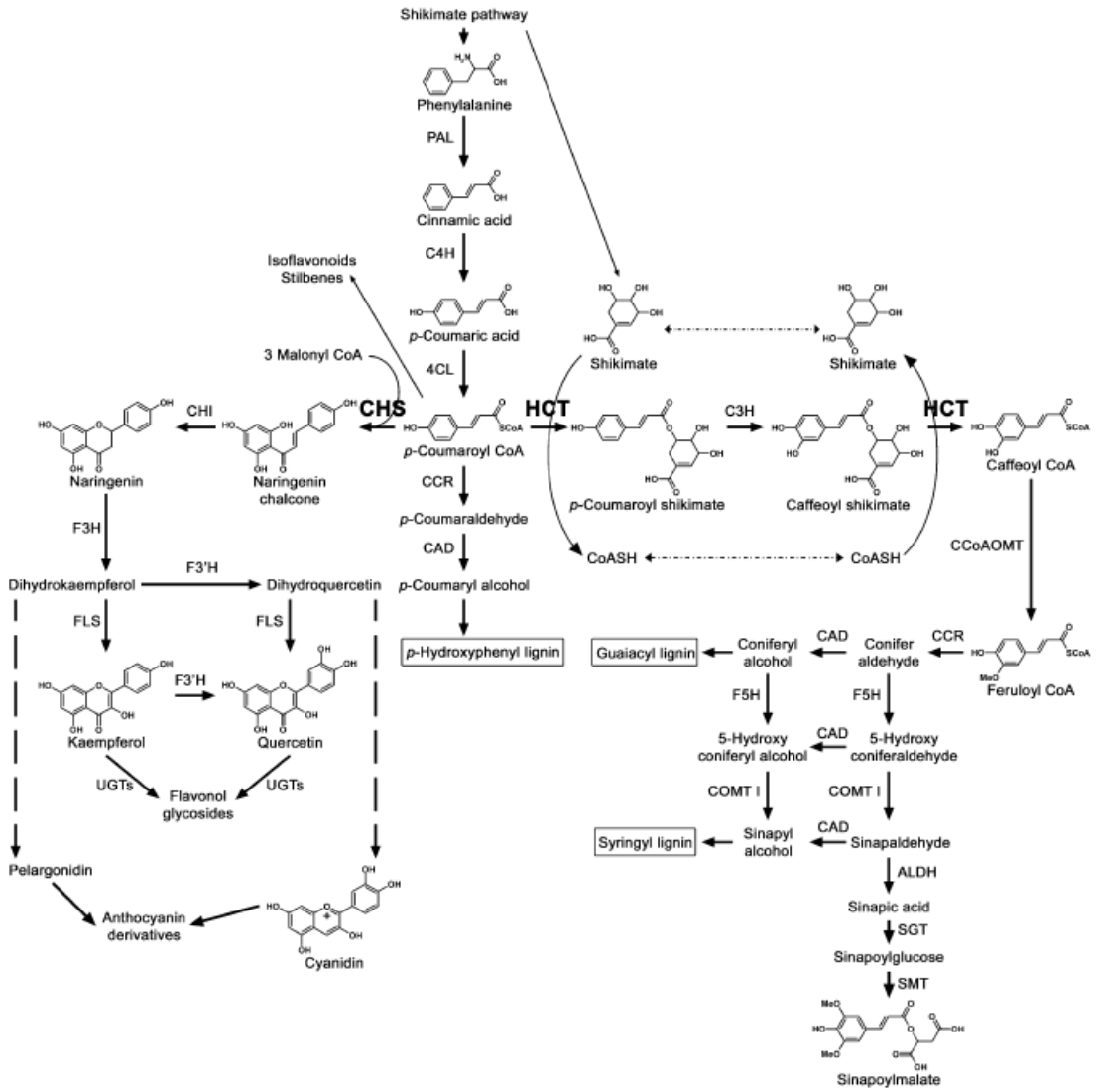


Figure 2 The phenylpropanoid pathway. P-coumaroyl CoA is at the crossroads of metabolic routes, leading to flavonoids, monolignols, and sinapoyl malate. CHS and HCT activities control the metabolic flux entering the two routes. ALDH, aldehyde dehydrogenase; C3H, C3-hydroxylase; C4H, C4-hydroxylase; F5H, ferulate 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CHI, chalcone isomerase; 4CL, 4-coumaroyl-CoA ligase; COMT I, caffeic acid O-methyltransferase of class I; F3H, flavanone 3-hydroxylase; F39H, flavonoid 39-hydroxylase; FLS, flavonol synthase; PAL, phenylalanine ammonia-lyase; SGT, sinapate UDP-glucose sinapoyl transferase; SMT, sinapoyl glucose malate sinapoyl transferase; UGTs, UDP sugar glycosyl transferases (Lengrand et al., 2007)

Flavonoids involve several processes, including UV protection, pigmentation, and defence against pathogens. Moreover, in elicitation response, the expression of phenylpropanoid pathway-related genes increases, together with the activity of the scavenging enzyme to counteract ROS accumulation (Bao et al., 2024). The phenylpropanoid pathway also provides lignin precursors, a complex polymer that contributes structural support and rigidity to plant cell walls. In *Arabidopsis*, lignin biosynthesis is finely regulated; the silencing of hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (*HCT*, AT5G48930), a lignin biosynthetic gene results in a substantial reduction of plant growth, however, the increased expression of chalcone synthase (*CHS*, AT5G13930) lead the plant to enhance the flavonoid biosynthesis (Legrand et al., 2007). Using *A. thaliana* in elicitation screening could provide new insights into crop productivity and industrial plant sectors.

Aim

This research project investigates responses in the SK11 knock-out strain of *A. thaliana* under cold stress. The primary objective is to understand how SK11 regulates the OPPP pathway and scavenging enzymes to counteract oxidative stress triggered by low temperatures. To achieve this, *Arabidopsis* plants WT (WT) and defective for the gene *AT5G267511* (*SK11*, KO-SK11) were grown using various experimental methods, including *in vitro* and soil-based approaches. Furthermore, a detailed analysis was carried out to elucidate the role of alternative splicing mechanisms in modulating the expression of *cyt-G6PDHs*, thereby enhancing the plant's ability to withstand cold stress conditions and different developmental stages (seedling and rosette). Additionally, an elicitation mechanism was carried out, involving the use of a biotic stressor (yeast extract), first in WT *in vitro*, then using WT and P1-G6PDH knock-out *A. thaliana* plants grown in soil, simulating exposure to a potential pathogenic agent. Ultimately, the project aims to comprehensively overview stress response mechanisms in different *A. thaliana* genotypes.

Results

Cold stress effects affect the activity of oxidative steps' enzymes of OPPP in mutant SHAGGY-related kinase 11 (SK11) *Arabidopsis thaliana*

To investigate the role of GSK3 upon cold stress, the relative expression of *SK11* in wild-type (WT) plants was tested. This preliminary screening was performed on plants grown *in vitro* and soil to verify whether the developmental stage and growth conditions could affect the expression level of *SK11*. Notably, early germinated WT plants grown at 4°C *in vitro* system showed an increased expression of *SK11*, resulting in an up-regulation after 1, 3 and 7 days of 5.05-fc, 7.68-fc and 16.65-fc, respectively (fc – **Fig. 3A**). On the contrary, in plants grown in soil the expression of *SK11* was up-regulated by 4.95-fold-change compared to control plant after one day upon cold temperatures. However, after one week (7 days) at 4°C, the expression of *SK11* resulted down-regulated in contrast to one-day treated plants. Nevertheless, *SK11* expression in 7 days of treated plants was higher than plants grown at 20°C, showing an up-regulation of 1.86-fc (**Fig. 3B**).

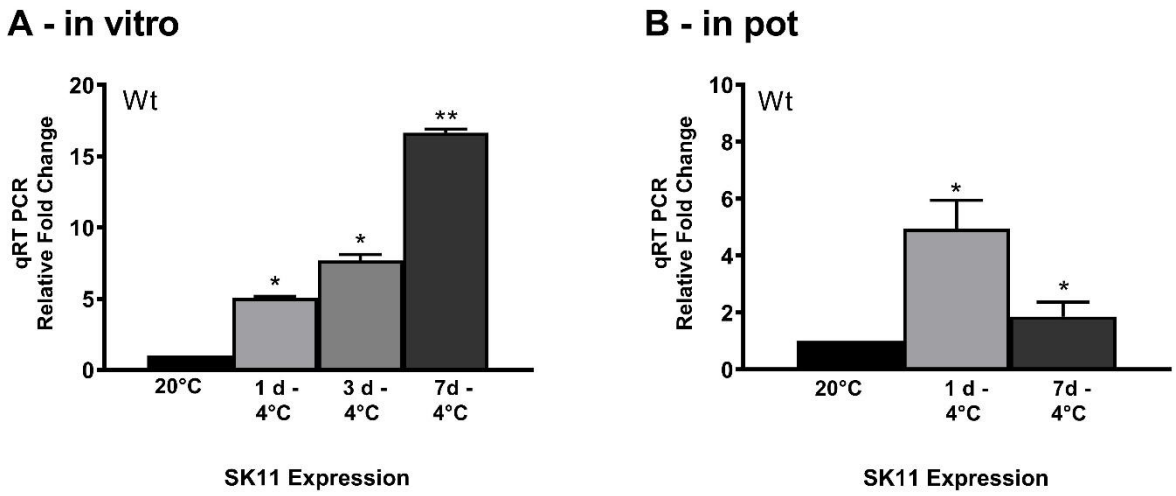


Figure 3 *qRT-PCR based on SK11 in A. thaliana WT strain growth in vitro (A) and in pot (B). Control plants grown at room temperature (20°C) and treated plants at 4°C. Asterisks indicate significant differences among treated groups (* $p < 0.05$, ** $p < 0.01$).*

Based on expression levels of *SK11* in two different development stages at low temperatures, the enzymatic activity of G6PDH and 6PGDH were measured in WT and KO-SK11 plants (**Fig. 4A**, **4B**). As shown in **Fig. 4A**, the activity of G6PDH *in vitro* plants showed no significant differences between WT and KO-SK11, both in control at 20°C and incubated at 4°C. The activity of 6PGDH showed few

differences between WT and KO-SK11, both at 20°C and 4°C until the third day of treatment, followed by a decreased activity in cold-treated KO-SK11 plants of 50% after one week (**Fig. 4B**).

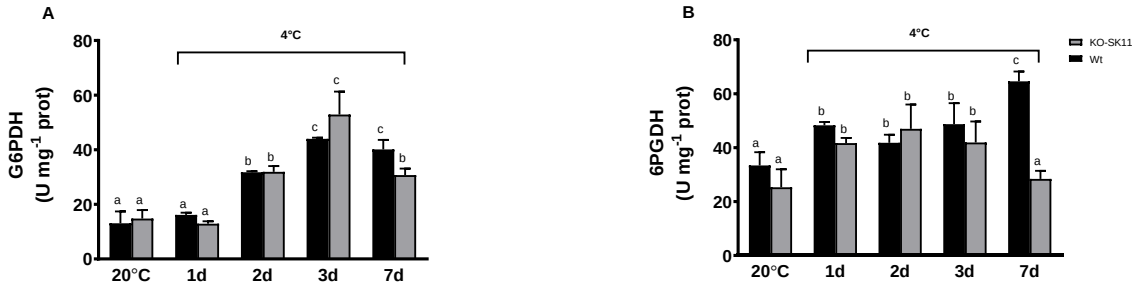


Figure 4 *Enzymatic activity of G6PDH (A) and 6PGDH (B) in leaves of A. thaliana WT (black column) and mutant SK11 (grey column) in vitro. Letters indicate the statistical significance among plants and treatments.*

Afterwards, the activity of G6PDH and 6PGDH was tested on *A. thaliana* plants in soil. As shown in **Fig. 5**, the activity of G6PDH strongly decreased in KO-SK11 both in control and 4°C, compared to the activity in WT, confirming the pivotal role of SK11 in regulating the cyt-G6PDH activity. In detail, KO-SK11 grown at 20°C showed a reduction in G6PDH activity of 60% compared to WT. In KO-SK11 growth at 4°C, after 3 days and 7 days, the activity was reduced by 40% compared to WT at 4°C. Instead, few differences were observed linked to the time of treatment at 4°C, both in WT and KO-SK11 (**Fig. 5A**). These data suggest that a loss of function of SK11 compromises the activity of G6PDH, confirming the pivotal role played by this kinase to in the regulation of G6PDH in cold stress conditions.

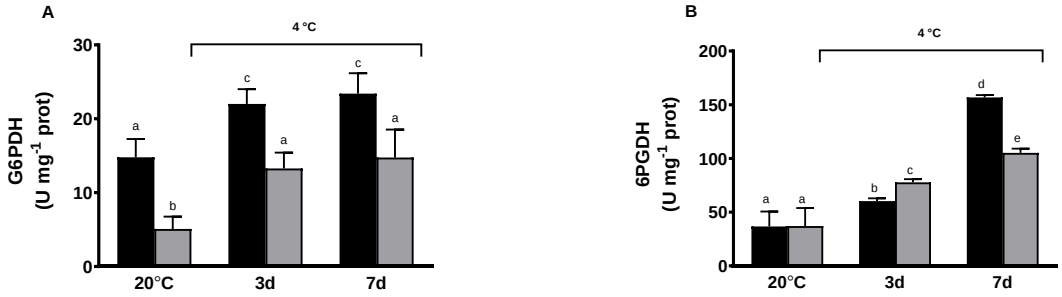


Figure 5 *Enzymatic activity of G6PDH (A) and 6PGDH (B) in leaves of A. thaliana WT (black column) and SK11 mutant (grey column) grown in soil. Letters indicate the statistical significance among plants and treatments.*

By contrast, 6PGDH activity showed no differences between WT and KO-SK11 grown at 20°C in soil. KO-SK11 plants incubated for 3 days at 4°C, the 6PGDH activity increased by 1.3-fc compared to WT. However, KO-SK11 plants collected after 7 days at 4°C showed a decrease in 6PGDH activity by 60%,

compared to WT (**Fig. 5B**). Hence, in KO-SK11 after one week of cold exposure, the activity of G6PDH and 6PGDH at 4°C were reduced both *in vitro* and in pot plants.

Relative expression analysis of genes involved in cold stress response in mutant SHAGGY-related kinase 11 (SK11) *Arabidopsis thaliana*

Since G6PD activity was reduced in KO-SK11 at 4°C and 20°C, a molecular approach was chosen to investigate the expression of different *G6PD* isoforms in plants grown in soil. To verify the transcriptional regulation of *G6PDs,* specific isoforms were selected: cytosolic (*cyt-G6PD6*), plastid (*p2-G6PD*), and peroxisomal (*p0-G6PD*). At 20°C, WT and KO-SK11 showed an equal expression level for each isoform tested (**Fig. 6A, B, C**). Moreover, WT plants grown at 4°C showed an up-regulation of all the isoforms, *cyt-G6PD* (**Fig. 6A**), *p2-G6PD* (**Fig.6B**), and *p0-G6PD* (**Fig. 6C**) of 10.4-fc, 3.9-fc, and 11.9-fc, respectively. By contrast, in KO-SK11, the expression level of *cyt-G6PD6* was unchanged among control temperature and 4°C, the *p2-G6PD* showed a reduction by 50% in plants grown at 4°C, and the *p0-G6PD* increased of 1.7-fc. These expression analysis results were consistent with the enzymatic activity observed in KO-SK11 in cold-stress conditions, suggesting that a transcriptional and/or post-transcriptional control occurs in addition to an enzymatic regulation.

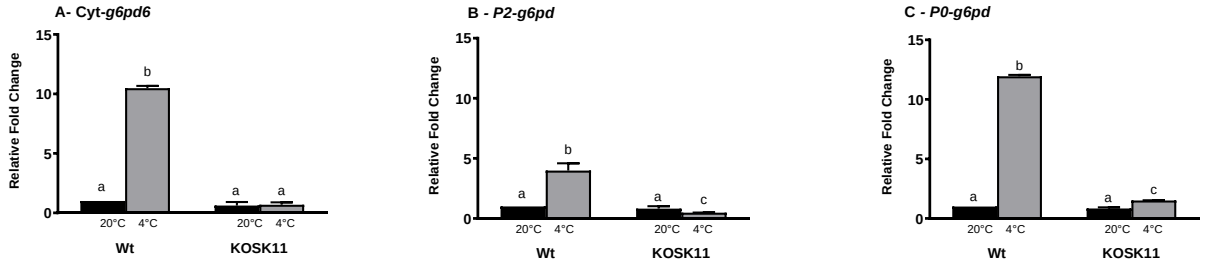


Figure 6 G6PDH qRT-PCR in WT and SK11 mutant A. thaliana grown in pot. A, cytosolic G6PD isoform; B, plastid G6PDH; C, peroxisomal G6PDH. Letters indicate the statistical significance among plants and treatments.

Thereafter, a screening of genes encoding scavenger enzymes was necessary. Among genes involved in stress response mechanisms, the analysis was centred on *APX*, *SOD*, and *CAT*. Notably, the *APX* is characterised by different compartmentalised isoforms: cytosolic (*Cyt-APX*), stromal (*S-APX*), and thylakoid-bound (*T-APX*). These latter isoforms are fundamental to protecting different chloroplast compartments from oxidative damage and ensuring the photosynthesis mechanism. However, in WT plants, it was observed that the cold treatment increased all targets' expression levels (**Fig. 7**), confirming the activation of stress response genes. On the other hand, a few genes resulted in an

increase in expression levels in KO-SK11 plants under cold stress, *S-APX* and *SOD* (**Fig. 7B, 7D**), which showed an up-regulation of 3.8-fc and 38-fc, respectively.

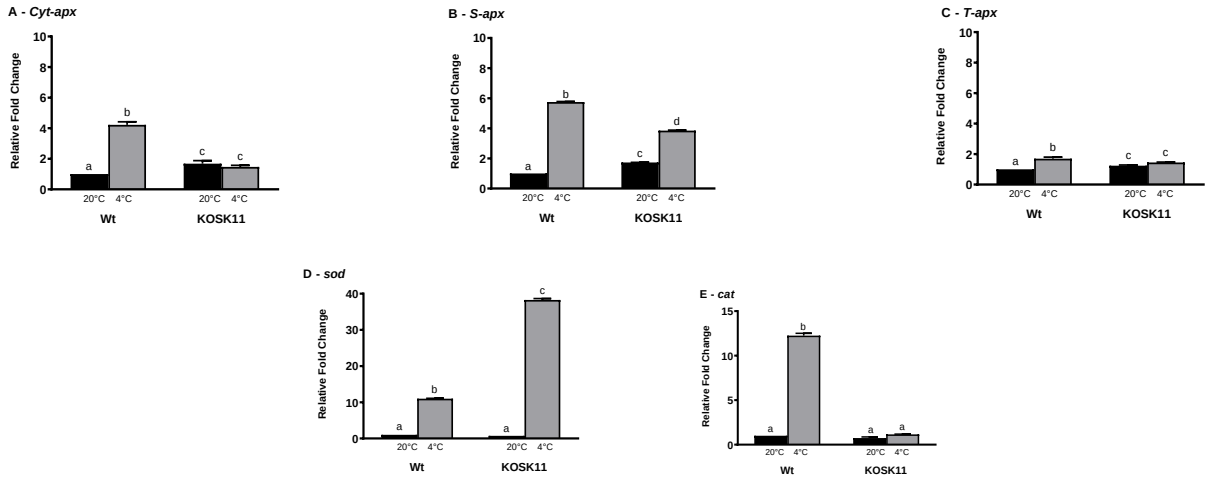


Figure 7 *Scavenger enzymes' relative expression in A. thaliana WT and mutant SK11 (KO-SK11) grown in pot. In black, control plants grew at room temperature (20°C); in grey, plants were treated at 4°C. A, Cyt-APX: cytosolic ascorbate peroxidase; B, S-APX: stromal ascorbate peroxidase; C, T-APX: thylakoid-bound ascorbate peroxidase; D, SOD: superoxide dismutase; E, CAT: catalase. Letters indicate the statistical significance among plants and treatments.*

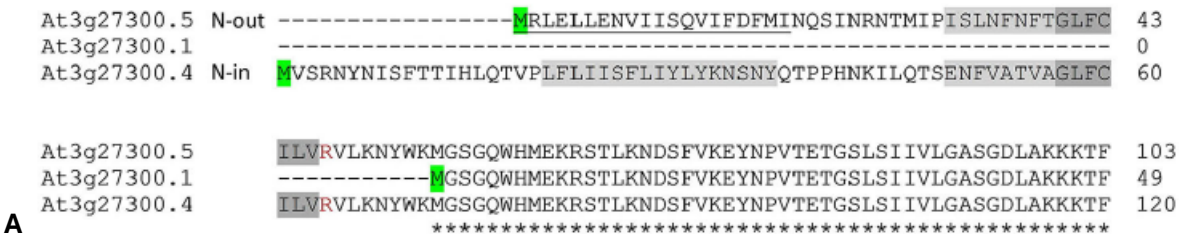
In addition, other genes involved in response to cold stress were tested: *gpt2* and *nced*. The *Glucose-6-phosphate translocator 2* (*gpt2*) is recognised by COR genes, coding for a chloroplast membrane protein involved in transporting glucose-6-phosphate to provide the substrate for the chloroplast OPPP. *9-cis-epoxy carotenoid dioxygenase* (*nced*) coding for an enzyme involved in the biosynthesis of abscisic acid (ABA), a plant hormone implicated in stress response mechanisms, well documented in abiotic stress conditions such as low temperature and drought conditions (Fuji, 2014; Sharma et al., 2017; Singh et al., 2023).

Figure 8 *Relative expression in A. thaliana leaves in control (black column) and treated plants (grey column) in WT and SK11 mutant (KOSK11) plants grown in pot. In A, the expression of gpt2 (Glucose-6-phosphate/phosphate translocator 2), and in B, nced (9-cis-epoxy carotenoid dioxygenase). Letters indicate the statistical significance among plants and treatments.*

Gpt2 resulted in an up-regulation in WT at 4°C (8.7-fc), suggesting the potential recruitment of substrates to enhance the OPPP in chloroplasts; in KO-SK11, the cold treatment did not affect the expression of *gpt2* (**Fig. 8A**). The expression of *nced* increased both in WT (30-fc) and KO-SK11 (4-fc) grown at 4°C compared to control conditions, confirming an increase in abscisic acid biosynthesis under cold stress in both genotypes (**Fig. 8B**).

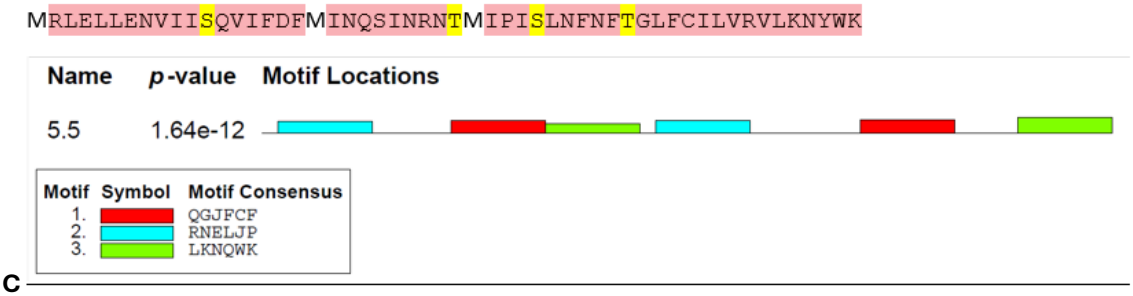
Glucose-6-phosphate dehydrogenase alternative splicing events in *A. thaliana*

In plants, the alternative splicing mechanism adapts the gene expression to environmental signals and development. For instance, using a bioinformatic approach, in *A. thaliana* different splicing events were identified in the 5' region of *G6PD* cytosolic isoforms. In detail, the *G6PD5* presents five alternative splicing isoforms in the 5' UTR (untranslated region), whose *G6PD5.4* and *G6PD5.5* coding for two different N-terminal regions. Instead, other alternative transcripts, *G6PD5.1*, *G6PD5.2,* and *G6PD5.3*, are characterised by the same coding sequence (CDS). The protein alignment of *A. thaliana* G6PDH5.4, G6PDH5.5 and G6PDH5.1 alternative N-terminal regions is displayed in **Figure 9A**, followed by the motif sequences and location in G6PDH5.4 (**Fig. 9B**) and G6PDH5.5 (**Fig. 9C**).



A

B



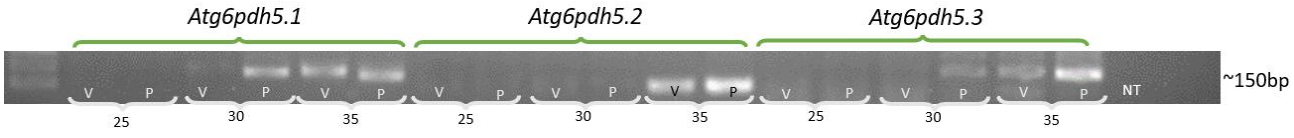
C

Figure 9 *A, Protein alignment in N-terminal region among AtG6PD5.4, AtG6PD5.5, and AtG6PD5.1 (Linnenbrügger et al., 2022). B, 5' region translation and putative motifs in AtG6PD5.4 isoform. C, 5' region translation and putative motifs in AtG6PD5.5 isoform. In yellow, phosphorylatable amino acid residues (bioinformatic tools used: Expasy https://web.expasy.org/translate/, MemeSuite https://meme-suite.org/meme/)*

Notably, the localisation of AtG6PDH5.4 and AtG6PDH5.5 in correspondence with the ER (endoplasmic reticulum) indicates the recruitment of NADPH-producing reactions to this organelle, which may have implications in cellular redox balance and plant metabolic processes (Linnenbrügger et al., 2022). Conversely, *AtG6PD6* presents two splicing variants in 5'UTR, renamed *G6PD6.1* and *G6PD6.2*, and coding for the same protein.
Based on these observations, a preliminary screening was performed to determine whether a splicing event regarding the cyt-*G6PD* expression occurs during the development stages in the *A. thaliana* wild-type strain. Hence, two development stages of *A. thaliana*, at seedling (14 days old, *in vitro*) and the "rosette" stage (3 weeks old, in soil), were selected. Since multiple splicing events occur in 5' UTRs of cytosol *G6PDs*, specific primers were designed on splicing junctions to obtain specific amplification products (see Material and Methods).

Therefore, a semi-quantitative RT-PCR was conducted to verify potential differences among isoforms of cytosolic G6PD in these two growth conditions: *in vitro* (V) and in pot (P). The amplification results are shown in **Fig. 10**. In **Fig. 10A** and **10B**, the amplification products of G6PD5 isoforms are shown. However, except for isoform 5.2, all the transcripts showed a higher expression in pot plants than *in vitro* plants, showing an amplification signal after 30 cycles, suggesting a predominant expression at this development stage. In **Fig. 10C**, the amplification of *G6PD6* reported a higher expression of isoform *6.1* in pot plants than *in vitro* plants; conversely, isoform *6.2* showed a similar expression between the two growth stages.

A



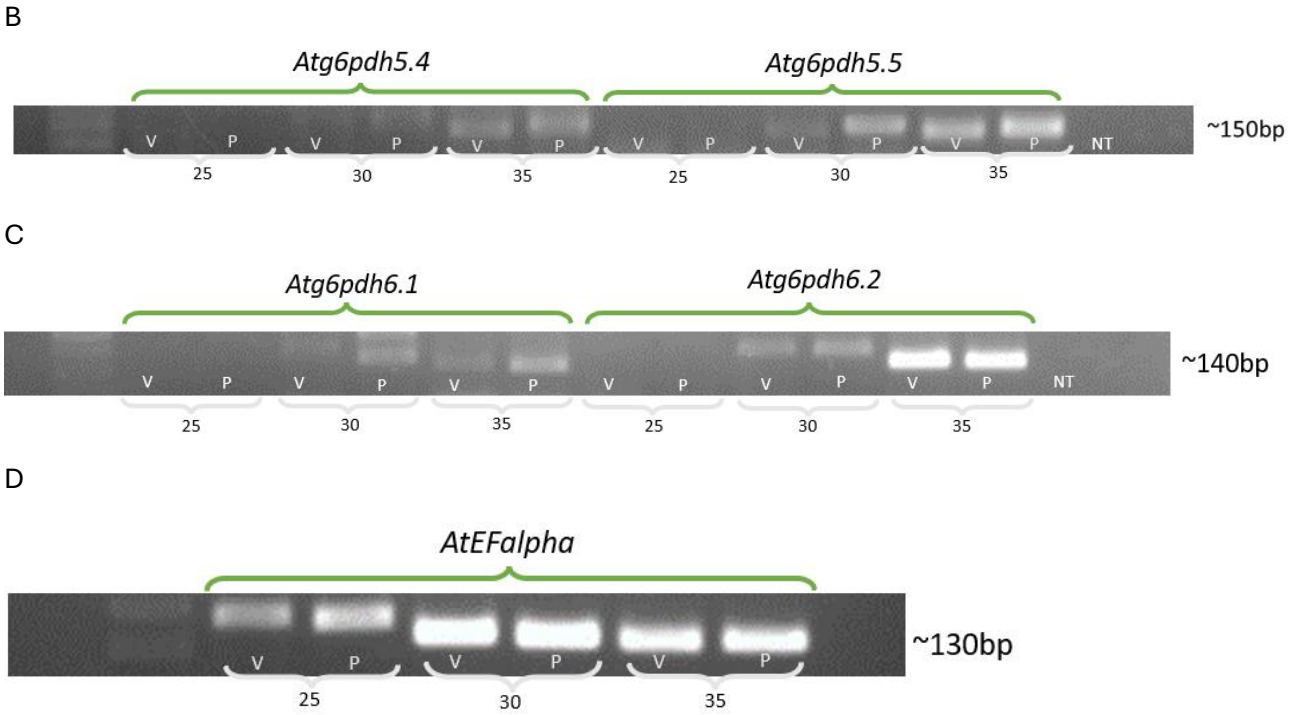


Figure 10 *Semi-quantitative RT-PCR to amplify G6PD cytosolic isoforms in vitro (V) and in pot (P) A. thaliana plants. A, amplification of G6PD5 isoforms 5.1, 5.2, and 5.3; B, amplification of G6PD5 isoforms 5.4 and 5.5; C, amplification of G6PD6 isoforms 6.1 and 6.2; D, amplification of EFalpha (Elongation Factor-1 alpha) as reference gene. Amplification products are shown. (NT, no template). Samples were loaded on Agarose 1,5% gel after 25, 30 and 35 amplification cycles.*

Finally, these results suggested an accurate regulation of splicing events in *G6PD* occurred in different growth conditions in *A. thaliana*, promoting specific 5' UTRs. In plants, selecting a specific 5' UTR may lead to the expression of cytosolic *G6PD* at higher levels, ensuring a better translation in protein and a higher enzymatic activity. Furthermore, it is not to be overlooked that the higher expression of cytosolic isoforms in pot plants is probably due to a significant external signal quantity that the plant had to manage to regulate the development. For this reason, plants grown *in vitro* conditions in a close environment with fewer external signals may express low levels of alternative *G6PD5* 5' UTRs, promoting the expression of *G6PD6* as the constitutive isoform.

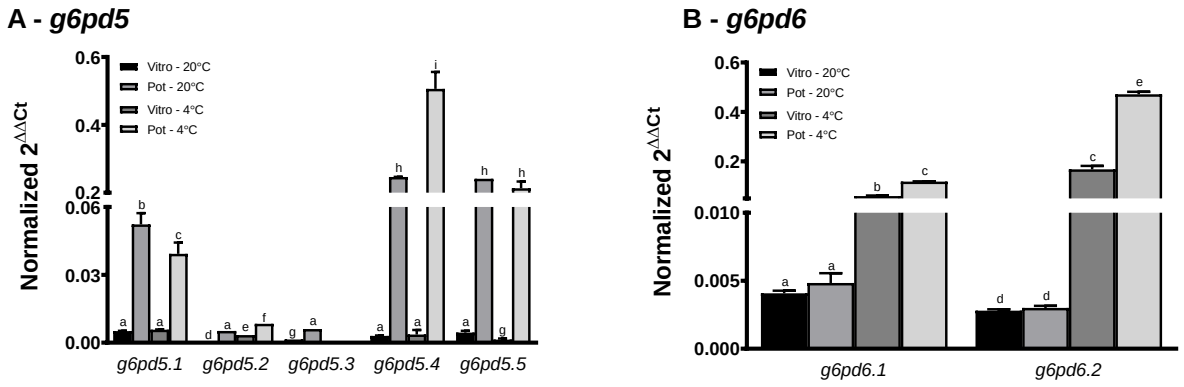


Figure 11 *qRT-PCR to amplify specific splicing isoforms of G6PDH5 (A), and G6PDH6 (B) in WT A. thaliana grown in vitro (14 days, seedling stage) and in soil ("pot", rosette stage) at 20°C and cold-stress conditions at 4°C. The primers used in this screening are illustrated in Table 1. Data obtained were normalised with the* $2^{\Delta\Delta Ct}$ *method. Letters indicate the statistical significance among plants and treatments.*

Therefore, to evaluate the expression levels among all the isoforms of cytosolic *G6PD*, qRT-PCR analyses were performed to compare WT plants grown at 4°C and 20°C at different development stages. Among the five different isoforms at the 5' region in *G6PD5* (**Fig. 11A**), plants at the seedling stage (*vitro*) grown at 20°C showed a reduced expression of isoforms *5.1, 5.4* and *5.5*, compared to higher expression in rosette stage at the same temperature. In detail, the amplification of *G6PD5.1* showed no significant differences *in vitro* plants at 20°C and 4°C, in contrast to rosette plants in which the expression of *G6PD5.1* showed a reduced expression by 70% in plants grown at 4°C. In addition, isoforms *G6PD5.2* and *G6PD5.3* were poorly expressed, showing few differences between the development stage and temperature-grown conditions. In both developmental stages, the isoform *G6PD5.3* was absent in plants grown at 4°C. Intriguingly, the cold-stress condition strongly affected the expression of *G6PD5.4*. In detail, the expression of *G6PD5.4* in plants grown in pot at 4°C was up-regulated by 2.04-fc compared to control plants. No significant differences were observed in *G6PD5.4* at the seedling stage between plants grown at 20°C and 4°C. In addition, the cold exposure did not affect the expression level of *G6PD5.5* in pot plants; instead, a reduced expression by 70% was observed *in vitro* plants treated at 4°C. Following the evaluation of *G6PD6*, no significant differences were observed in the expression of isoforms *G6PD6.1* and *G6PD6.2* between seedlings and rosette stages grew at 20°C (**Fig. 11B**). In cold stress conditions, plants at the seedling and the rosette stages had increased expression of *G6PD6.1* of 14.7-fc and 24.6-fc, respectively. The *G6PD6.2* isoform showed a similar expression level in plants *in vitro* and pot at 20°C. After the cold treatment, plants *in vitro* and in pot showed an up-regulation of *G6PD6.2* of 83-fc and 157-fc, respectively. This high increase in the expression of *G6PD6.2* suggests that it was the principal splicing variant selected in cold-stress responses, probably due to a better translation in a protein of the *6.2* than the *6.1* transcript.

Relative expression analysis of *G6PD5 and G6PD6* isoforms in mutant SHAGGY-related kinase 11 (SK11) *Arabidopsis thaliana* under cold stress

Finally, the expression of different 5' UTRs splicing isoforms of cytosolic *G6PD5* and *G6PD6* was investigated even in KO-SK11 plants grown at 20°C and 4°C. As previously discussed, the expression of splicing variants resulted in high levels in plants grown in soil and thus were used in this screening. Among all *G6PD5* isoforms analysed, the focus was on those mainly involved in cold-stress response: *G6PD5.4* and *G6PD5.5*.

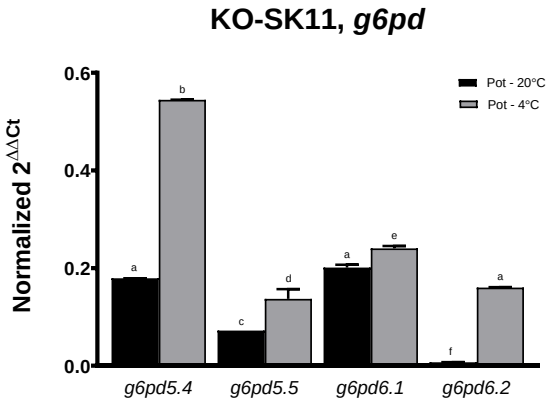


Figure 12 *Relative expression analysis of different splicing variants of G6PD5 and G6PD6 in SK11mutant rosette plants grown at room temperature (20°C, black columns) and in cold conditions (4°C, grey columns) for 7 days. Letters indicate the statistical significance among plants and treatments.*

A qRT-PCR was performed to verify the relative expression of *G6PD5.4* and *G6PD5.5*. The results showed a higher expression of all selected targets in cold-stressed plants than in control plants, confirming the involvement of these protein isoforms in cold-stress responses. In detail, all selected targets showed an up-regulation: *G6PD5.4* of 3-fc; *G6PD5.5* of 1.8-fc; *G6PD6.1* of 1.2-fc; and *G6PD6.2* of 22.8-fc (**Fig. 12**). Additionally, even other *G6PD5* splicing variants were tested (*5.1, 5.2*, and *5.3*), however, the expression was low in each condition (data not shown). Notably, in KO-SK11, the expression of the *G6PD6.1* was predominant compared to *G6PD6.2* in plants grown at 20°C, suggesting a better translation rate of the *6.1* transcript to obtain a functional G6PDH6 despite the absence of kinase SK11 in a control growth condition. However, in cold-stressed KO-SK11, *G6PD6.2* showed an enhanced expression (22.8-fc) at 4°C compared to 20°C, suggesting an intensification of the transcription rate to counteract cold damages.

Arabidopsis wild-type yeast extract treatment *in vitro* conditions

In plants, yeast extract (YE) is widely known as a biotic stressor, mainly due to its ability to elicit defence responses. In agricultural and industrial contexts, YE is used to enhance the production of secondary metabolites, which are essential to the plant's defence mechanisms (Rani et al.,2020; Świeca et al., 2016). The use of YE aimed to analyse the response of *Arabidopsis* in leaf tissue, testing the involvement of G6PD both transcriptionally and enzymatically. The YE induces an elicitation response in plants, enhancing the secondary metabolism. For this reason, the expression and activity of phenylalanine ammonia-lyase (PAL) were tested as an activation marker for this secondary pathway. Hence, a preliminary *in vitro* screening was performed using *Arabidopsis* WT plants grown with YE (1% YE, w/v). The activity of G6PDH and PAL has been measured to verify the activation of primary and secondary metabolism in response to YE. As shown in **Figure 13**, the activity of G6PDH was increased in treated plants (**Fig. 13A**), showing an up-regulation of 2.24-fc after 3 days and 2.57-fc after 7 days, compared to control plants. These observations confirmed the involvement of G6PDH in WT grown *in vitro* in response to YE treatment. In contrast, the PAL activity gradually increased with the incubation time (**Fig. 13B**). After 3 days, the PAL activity was up-regulated of 1.2-fc, and after 7 days of 1.3-fc, compared to the control.

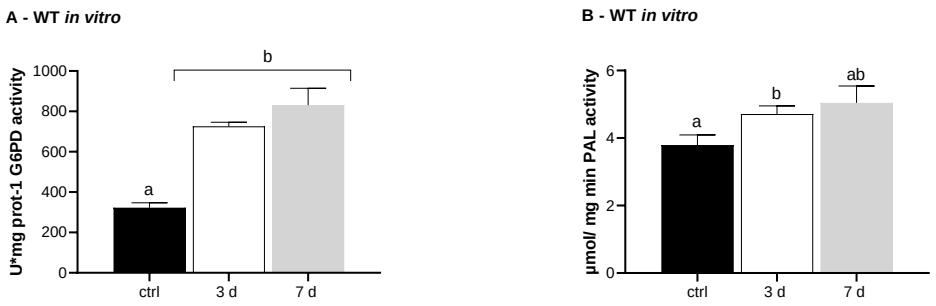


Figure 13 *In vitro, A. thaliana WT grown with 1% YE enzymatic activity. A, G6PD activity; B, PAL activity. Letters on the top of the graph indicate statistical significance among plants and treatment. "ctrl" indicates control plants; "3 d" 3 days treated plants; "7 d" 7 days treated plants.*

Therefore, a qRT-PCR analysis was performed to investigate the transcriptional activation of cytosol *G6PD6* and *PAL* in yeast-exposed plants. As shown in **Fig. 14**, after 7 days of incubation, the expression of *G6PD6* and *PAL* showed an up-regulation of 10.5-fc and 20.6-fc, respectively.

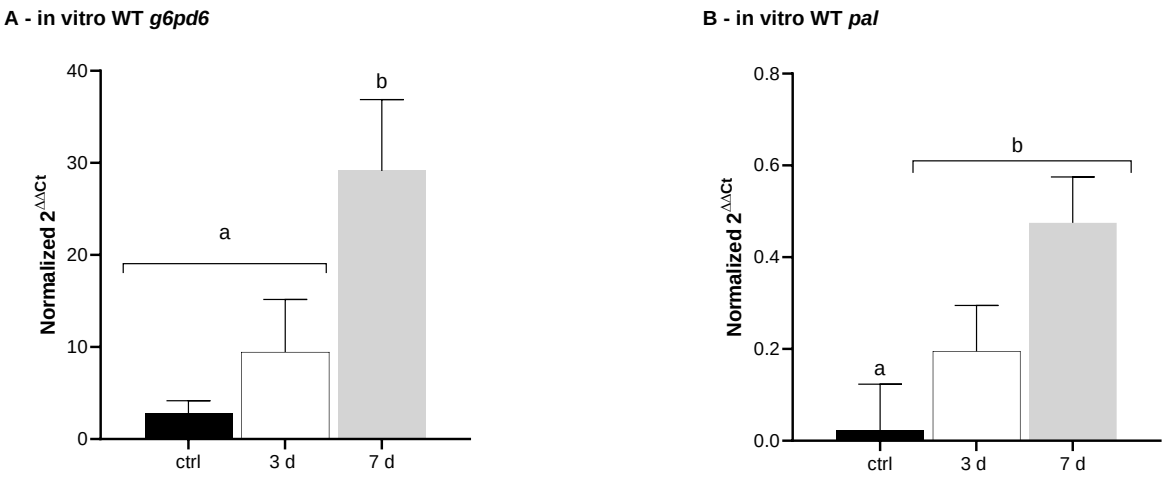


Figure 14 *In vitro, A. thaliana WT grown with 1% YE relative expression analysis. A, G6PD6 qRT-PCR; B, PAL qRT-PCR. Letters on the top of the graphs indicate statistical significance among plants and treatment. "ctrl" indicates control plants; "3 d" 3 days, and "7 d" 7 days treated plants.*

The expression analysis of *G6PD6* and *PAL* has confirmed the results obtained in enzymatic assays (**Fig. 14**), suggesting a proportional correlation between the expression and activity of these targets; in addition, one week seemed sufficient to observe the activation of the OPPP and phenylpropanoid pathway in plants grown *in vitro*.

Yeast extract treatment on *A. thaliana* growth in soil

Plants have to deal with a wide range of stress agents in natural environments. Based on preliminary data collected *in vitro* screening (see above), two *A. thaliana* genotypes, wild-type (WT) and KO-P1-G6PDH (KO-P1), were selected and grown in the soil. In this "in pot screening", choosing a wild-type and a mutant *A. thaliana* can improve the knowledge about the involvement of the OPPP and the phenylpropanoid pathway as a response to a positive-stress event in adult plants. In detail, the KO-P1 genotype is characterised by a loss of function of the chloroplast isoform of *G6PD1*, obtained after a T-DNA insertion. To verify the elicitor effects of YE on *Arabidopsis* leaves, a 1% solution (w/v, YE and Milli-Q water) was applied by brush to mimic the exposure to a potential pathogen agent. Control and treated plants were grown in different closed compartments to avoid any hormone-like signals among samples, e.g., the ethylene signalling pathway (Binder, 2020). The objective was to verify the potential continuous exposure to YE on leaf tissue *in vivo* plants, and the brushing was performed every 3 days. Moreover, as an additional control, some leaves were brushed with sterile water (UL, untreated leaves) in treated plants to verify whether the elicitation signal transduction occurred. For this reason, the

expression of *G6PD6* and *PAL* were tested in *Arabidopsis* leaves collected from the WT and KO-P1 (**Fig. 15**).

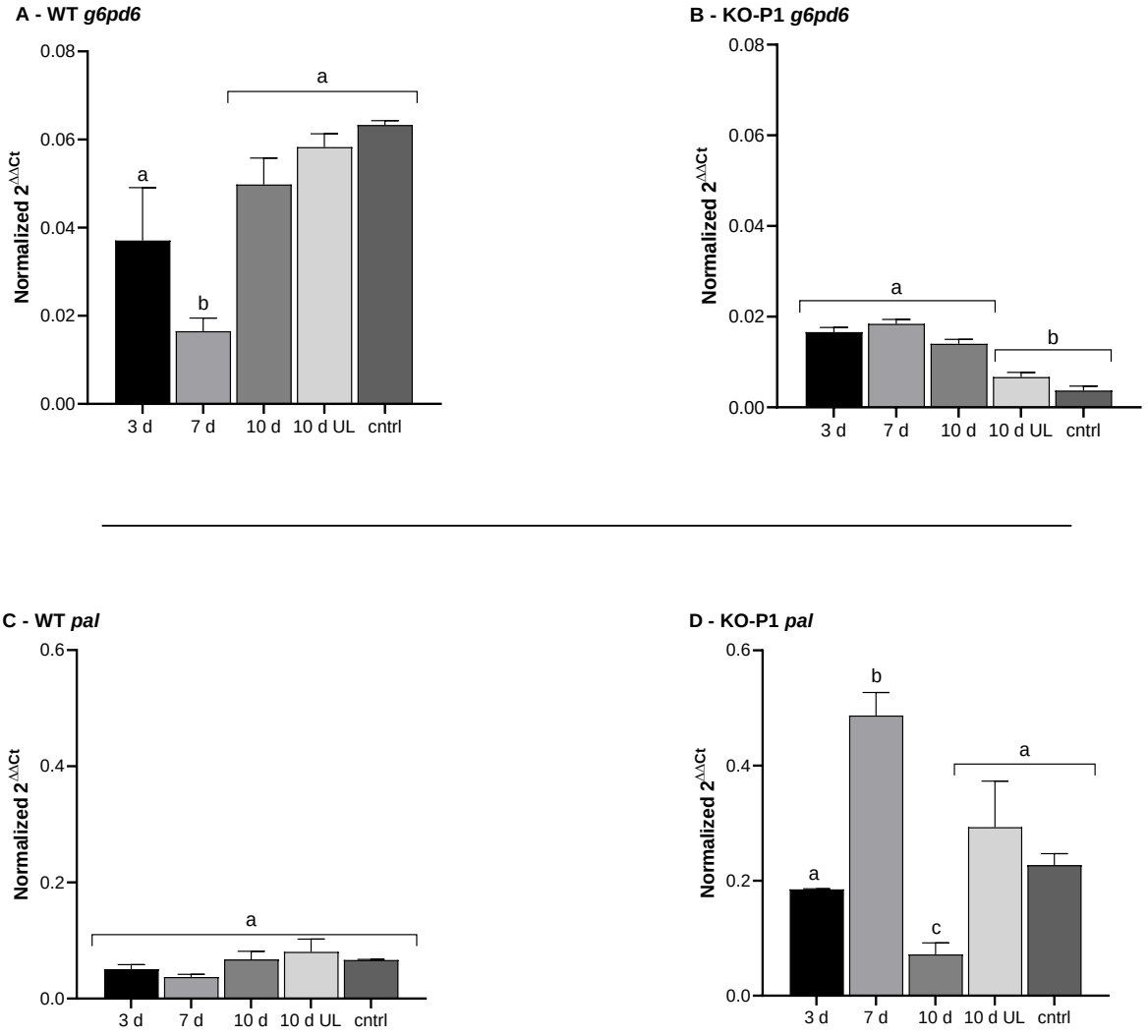


Figure 15 *qRT-PCR in WT and KO-P1 A. thaliana. Relative expression of G6PD6 is shown in WT (A) and KO-P1 (B). The relative expression of PAL is shown in WT (C) and KO-P1 (D). The letter "d" indicates "days" after the brushing;" UL", "Untreated Leaves". Letters on the top of the graphs indicate the statistical significance among plants and treatments.*

G6PD6 expression levels in WT showed a decrease in leaves collected after 7 days by 25% compared to control (**Fig. 15A**), probably due to a plant adaptation after one week of treatment. Interestingly, in leaves collected after 10 days of treatment, the *G6PD6* expression level in WT was similar to 10 UL and the control. Instead, in KO-P1, opposite results were observed: the expression level resulted higher in leaves treated for 3 (4-fc), 7 (4.5-fc), and 10 (3.5-fc) days than in untreated and control leaves (**Fig. 15B**). The up-regulation of *G6PD6* in KO-P1 treated leaves, suggested a positive eustress response after the YE application. Intriguingly, comparing the expression levels of *G6PD6* in WT and KO-P1, these were lower in the mutant, probably due to the different management of stress response mechanisms.

Furthermore, the expression of *PAL* (**Fig. 15C** and **15D**) was tested. The amplification level of *PAL* resulted unchanged in all treated samples from WT plants (**Fig. 15C**), suggesting no transcriptional activation of *PAL* in response to YE. Differently in KO-P1 plants, an increased *PAL* expression was observed in leaves collected after 7 days, resulting in 2.14-fc compared to control, suggesting an evident activation of the phenylpropanoid pathway after one week. However, leaves collected after 10 days of treatment showed a substantially decreased *PAL* expression by 20%, compared to 7 days-treated leaves (**Fig. 15D**), probably due to a switch-off of the pathway after more than one week and/or a feedback inhibition mechanism.

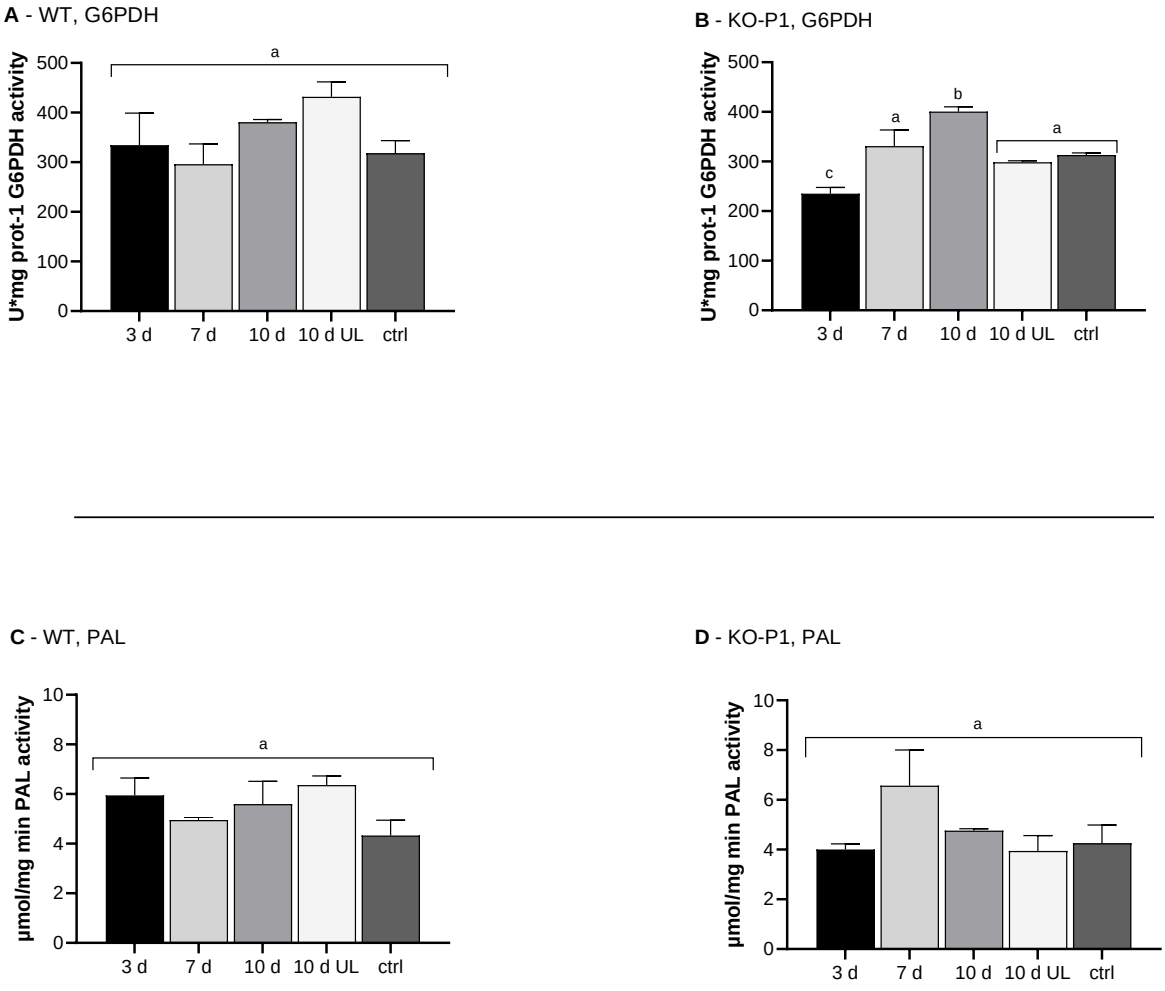


Figure 16 *Enzymatic activity in A. thaliana followed by YE treatment. G6PD activity is shown in WT (A) and KO-P1 (B). PAL activity is shown in WT (C) and KO-P1 (D). The letter "d" indicates "days" after the brushing;" UL", "Untreated Leaves". Letters on the top of the graphs indicate the statistical significance among plants and treatments.*

Together with the expression analysis, the enzymatic activity was measured. Compared to the expression analysis, the G6PDH activity showed no significant differences among treated groups of leaves in the WT (**Fig. 16A**). In KO-P1, the activity was gradually increased related to the timing of the treatment (**Fig. 16B**), confirming the expression analysis's results. In detail, the activity of G6PDH in KO-P1 leaves, collected after 10 days, resulted in an up-regulation (1.27-fc). Moreover, in KO-P1, the G6PDH activity showed the same trend observed in the expression analysis, suggesting a proportional activation in transcriptional and enzymatic regulation (**Fig. 15B** and **Fig. 16B**). Additionally, the enzymatic activity of G6PDH in KO-P1 was gradually increased linked to the treatment-time (**Fig. 16B**), confirming its role in response to YE. Finally, the PAL activity was tested. In WT leaves, PAL activity showed similar values in all groups (**Fig. 16C**), confirming the expression analysis (**Fig. 15C**), suggesting a weak involvement of PAL in response to YE in this genotype. In KO-P1 leaves, despite the lack of statistical significance, the PAL activity resulted higher after 7 days of treatment than in the other groups (**Fig. 15D**), following a similar trend observed in the expression analysis. However, KO-P1 leaves collected after 10 days showed PAL activity levels similar to those of untreated and control leaves, ensuring enzymatic activity despite the low expression level observed. Hence, a no-proportional regulation occurred in 10 days of YE-treated leaves in KO-P1, resulting in a switch-off at the transcriptional level, in contrast to enzymatic activity that resulted unchanged.

Discussion

Cold stress responses in *A. thaliana*, in wild-type and SK11 mutant, and the role of G6PD

Plants have developed several strategies to cope with environmental damage. Among stress conditions, low temperatures significantly affect plant growth, development, and productivity. Upon exposure to low temperatures, plants show physiological and biochemical changes, including membrane damage, reduced enzyme activity, and accumulating reactive oxygen species (ROS) (Irimia et al., 2021; De Vita et al., 2012). These stress conditions trigger the activation of various signalling pathways, including the oxidative pentose phosphate pathway (OPPP). Using a mutant *A. thaliana* strain (KO-SK11) defective for a regulating kinase (SK11), the pivotal role of this target in the regulation of enzymatic activity of cytosolic G6PDH6 (Dal Santo et al., 2012) under cold stress conditions (4°C) was demonstrated. In KO-SK11, the low expression of different *G6PD* isoforms reflected the low activity measured in cold-stressed plants, suggesting a potential correlation between the expression and activity of G6PD6 in the mutant SK11. Based on data collected in this screening, plants defective for SK11 may be more susceptible to stress damage, suggesting a deeper involvement of SK11 in development and stress responses. Comparing the two developmental stages (*in vitro*, at the seedling stage, and in soil, at the rosette stage) and cold-treated, a functional SK11 could be fundamental in regulating G6PDH activity in plants grown in soil under cold-stress conditions. Along with the low expression of different *G6PD* isoforms in KO-SK11, other genes associated with stress responses showed low expression, except for *SOD* and *stromal-APX,* in which the expression increased in cold conditions. These results suggest that SK11 could directly regulate enzymes involved in the response to oxidative damages and/or regulate transcriptional factors involved in the stress response mechanisms. This relates to the positive regulation of SK11 on G6PDH6 activity via phosphorylation (Dal Santo et al., 2012). Furthermore, SK11 plays an essential role in ensuring growth and development in osmotic stress response mechanisms. In *A. thaliana*, more than 1660 DEGs (Different Expressed Genes) were identified under osmotic stress conditions using a mutant strain of SK11. Several transcription factors involved in stress responses regulated by SK11were also identified, such as the xyloglucan endotransglucosylase/hydrolase protein gene (*XTH14*, AT3G49960), expressed in root hair cells, and TRANSPARENT TESTA 6 (*TT6,* AT3G51240), encoding a flavanone 3-hydroxylase involved in flavonoid biosynthesis, in knock-out strains, these targets resulted down-regulated (Dong et al., 2019). However, results collected on KO-SK11 showed an up-regulation under cold stress (4°C) of genes involved in glucose transport (*gpt2*) and abscisic acid biosynthesis (*nced*). The high expression of *gpt2* in KO-SK11 both at 20°C and 4°C may be due to the

defective regulation of cytosolic G6PDH6, thus increasing the enzymatic activity of plastid isoforms providing glucose-6-phosphate. In addition, different responses were observed when comparing plants grown *in vitro* and in pots. In plants grown in pots, the expression and activity of G6PDHs increased according to the time of cold incubation, in contrast to *in vitro* conditions in which the activity did not change. The important role of SK11 in cold stress response regulation was demonstrated, mainly through regulating G6PDH6 activity. It was demonstrated that the regulation of expression and activity of cytosolic G6PDH under cold stress conditions depended significantly on the developmental stage and the growth system used. Therefore, a qRT-PCR was performed to investigate alternative splicing events in cytosolic *G6PDs* (*G6PD5* and *G6PD6*) at two different developmental stages (seedling and rosette) and grown at different temperatures (20°C and 4°C) in wild-type plants. An increased expression of isoforms coding for N-terminal alternative protein forms was observed under cold stress. The expression of *G6PD5.4* and *G6PD5.5* significantly increased in plants grown in pots and exposed to cold, confirming the critical role played by cyt-G6PDH in cold stress responses providing the right amount of NADPH at the reticulum (von Schaewen et al., 2022) in plants grown in soil. Therefore, a molecular screening was performed on *G6PD5* and *G6PD6* alternative splicing events in KO-SK11. The expression of *G6PD5.4* and *G6PD5.5* increased in cold-treated plants compared to 20°C. Notably, in KO-SK11, the promoted expression of these alternative proteoforms is due to the assurance of a functional cytosolic G6PDH5 under cold stress. Furthermore, wild-types grown in pots at 4°C show high expression levels of *G6PD5.4* and *G6PD5.5*. A possible reason to justify these observations can be the alternative N-terminal regions, presenting potential phosphoryl-table amino acid residues that can be recognised by SK11 (von Schaewen et al., 2022) or other post-translation modifies. Moreover, despite the lack of SK11 that induces a positive regulation of *G6PD6*, in KO-SK11, the transcriptional levels of genes encoding cytosolic *G6PD6s* increase, suggesting a transcriptional adapting mechanism to handle cold stress responses in the absence of a functional SK11. Hence, using a mutant knock-out SK11, the pivotal role played by this kinase in cold-stress responses to regulate *G6PD6* and other stress-response targets was demonstrated.

Yeast extract treatment, an elicitation approach to evaluate the role of G6PD in *A. thaliana* wild-type and P1-G6PD mutant

Although G6PDHs are essential for managing stress damage in plant cells, an elicitation approach was chosen to investigate the potential involvement of this target in an eustress mechanism. Here, the yeast extract (Świeca et al., 2016) was selected to verify the involvement of cytosolic G6PDH in the elicitation system in *A. thaliana* wild-type and mutant KO-P1. Moreover, phenylalanine ammonia-lyase (PAL) activity and expression were analysed to follow the elicitation mechanism and the activation of anti-

inflammatory responses (Matsuura et al., 2017). PAL is a pivotal enzyme in plant secondary metabolism, catalysing the first reaction of the phenylpropanoid pathway that produces flavonoids, lignin and phenolic acids. Additionally, the expression of PAL genes increases in response to stress events, enhancing the production of defence compounds in plants (Zhang et al., 2020).

Firstly, an *in vitro* screening was performed on wild-type plants treated with yeast extract, and after one week, both *G6PD6* and *PAL* increased in terms of expression and activity. Data obtained demonstrate the connection between primary and secondary metabolisms upon yeast extract treatment. The activation of cytosolic G6PDH provides a correct amount of reducing power (NADPH) that could support the activity of C4H downstream of PAL in the phenylpropanoid pathway (Lengrand et al., 2007). In addition to the phenylpropanoid pathway, other secondary pathways could be enhanced using NADPH by activating G6PD. Among them is the glucosinolate pathway, which synthesises glucosinolates, sulphur-containing compounds in plant defence (Cabrerizo et al., 2023). Furthermore, cytosolic G6PDHs were up-regulated at the transcriptional level and in enzymatic activity in *Arabidopsis* haunted by Root-knot nematodes (RKNs, Meloidogyne spp.) (Moens et al., 2011). Mutant *A. thaliana* defective in *G6PD6* and *G6PD5* showed more susceptibility to RKNs infection than wild-type, suggesting a pivotal role played by cytosolic G6PDs under biotic stress conditions (Hu et al., 2018). Since the yeast extract is a widely used biotic stressor in elicitation mechanisms in cell culture systems (Park et al., 2016), it can be used as an immunity enhancer in plants grown *in vitro*. Based on these results, an "in vivo system" was chosen to verify the leaf application of the yeast extract in plants grown in soil. For instance, a leaf application by brush was performed to simulate a potential pathogen attack in a natural environment in rosette-stage plants. Despite the high expression and activity increase of selected targets (G6PD6 and PAL) *in vitro*, different results passing in the soil system were observed. Here, no differences in the WT related to the expression and activity of G6PDH6 and PAL were observed. In contrast, in KO-P1, the expression and activity of G6PD6 and PAL increased. After 7 days, the expression of *PAL* strongly increased, followed by a reduction at 10 days, probably due to different regulatory pathways activated in response to YE. Additionally, the expression of *G6PD6* in KO-P1 was up-regulated in treated leaves, suggesting a positive response to YE. In KO-P1 leaves, the G6PDH6 activity gradually increased with the treatment time, reaching higher activity after 10 days. Finally, the yeast extract treatment showed an up-regulation of G6PD6 and PAL as a confirmation of elicitation mechanisms activated in plants grown *in vitro*, inspiring new molecular interconnection of G6PD6 with secondary metabolism in *Arabidopsis*. In addition, the mutant P1-G6PDH (KO-P1) showed increased expression and activity of G6PD6 and a high up-regulation of PAL after one week. The developmental stage and growth conditions strictly connect these differences in expression and activity of G6PD6 and PAL: *in vitro*, the YE uptake occurs via roots in growing plants, and in vivo, the YE uptake occurs via leaf tissue in adult plants. Root uptake ensures an efficient transport mechanism to move absorbed nutrients to

other plant organs, passing through the xylem and phloem until leaf tissue (de Mello Prado, 2021). In conclusion, these data suggest that plants exposed to yeast extract activate G6PD6 in an elicitation response in wild-type (*in vitro*) and in KO-P1 (in soil). Finally, new molecular approaches will be necessary to evaluate the involvement of G6PD in elicitation responses and elucidate further possible connections with the secondary metabolism.

Material and Methods

Plant material

Arabidopsis thaliana Col-0 genotype was used as a wild-type (WT) strain. As mutant strains, SALK_014382 of SK11 (At5g26751), and P1-G6PDH SAIL_1252_E02 (G6PD1 – At5g35790 - Siddappaji et al., 2013), both provided by Nottingham Arabidopsis Stock Centre – NASC, were used. *A. thaliana* plants were grown in Petri dishes using Murashige and Skoog (MS) medium (Murashige, T., Skoog, F., 1962) (Sigma-Aldrich, Saint Louis, USA). Seeds were sterilised using a solution composed of water and bleach (2:1), after sowing on MS medium. Plants were kept in a growth cabinet at 20-22°C (16-h light/8-h dark photoperiod). Plants growth in soil (in pot) were positioned in the greenhouse.

Plant under cold conditions

To analyse the early-germinated development stage, after 7 days, *A. thaliana* WT and KO-SK11 plants were transferred into new, fresh plates and divided into two groups: control plants were maintained at 20-22°C, and stressed plants were grown at 4°C. Leaves were collected after 1, 2, 3, and 7 days after the cold stress induction. In addition, to analyse the mature development stage, after 7 days, plants were transferred into new fresh plates and then were grown in 20 cm diameter soil-filled plastic pots at 20°C, at 60-80% relative humidity, under 16h-light/8h-dark, and irrigated regularly. After 1 month, at the full expansion of the rosette, plants were subjected to 4°C stress. After 3 and 7 days, leaves from controls and cold-stress plants were collected and frozen in liquid nitrogen.

Plant sampling after yeast extract treatment *in vivo*

Arabidopsis thaliana WT (Col-0) and KO-P1 were grown in 20 cm diameter soil-filled plastic pots and incubated in the greenhouse; treated and control plants were located in two separate aquariums. Plants treated with Yeast Extract (1% w/v) through brushing were collected after 3 days, 7 days, and 10 days; brushing was performed every 3 days until the end of the treatment. In treated plants, two leaves were treated with Milli-Q water as an additional control.

Media preparation

Medium for growing plants comprised MS (Musharige & Skoog, 1962) and agar 8g/L. To obtain media for the elicitation screening, 1% yeast extract (SIGMA) was added to the MS medium described above. Petri dishes were used to prepare all conditions.

Bioinformatic tools

The TAIR database (https://www.arabidopsis.org/) was used. Primer sequences designed on splicing junctions of *G6PD5* and *G6PD6* are illustrated in the table below.

Primer name	**Sequence 5'→3'**
Fw_*G6PD5.1*	TATGCCATTGACCAGAACC
Fw_*G6PD5.2*	GGCTATAACATAACTGCGACGG
Rv_*G6PD5-1-2*	CTATCAAACTCCACCCCATAAC
Fw_*G6PD5.3*	CAATGATAGAGAGACTCCCAG
Rv_*G6PD5.3*	CCACCGTCGCAGAGTGTTA
Fw_*G6PD5.4*	GGTGGAGTTTGATAGTTCG
Rv_*G6PD5.4*	CTAACCTTACAATAATTCCCT
Fw_*G6PD5.5*	GGAATCATTGTGTTACGATTG
Rv_*G6PD5.5*	CGTCGCAGTTATGTTATAGCC
Fw_*G6PD6.1*	GGAGAAAATGACGGAAGCTA
Fw_*G6PD6.2*	GGATGATGAGATTCGATCTCTG
Rv_*G6PD6*	GAATCATTCCTAAACGTAGACC

Protein sequences were obtained using the Expasy web tool (https://web.expasy.org/translate/) and MEMEsuite to predict protein motifs (https://meme-suite.org/meme/). Sequence alignment was

performed with MUSCLE Multiple Sequence Alignment (MSA) (https://www.ebi.ac.uk/jdispatcher/msa/muscle?stype=protein). Accession numbers are shown in the table below.

Gene	**Accession number**
P1-G6PD	AT5G35790
P2-G6PD	AT5G13110
P0-G6PD	AT5G40760
G6PD6	AT5G40760
G6PD5	AT3G27300
Cyt-APX	AT3G09640
S-APX	AT4G08390
T-APX	AT1G77490
CAT	AT1G20630
SOD	AT1G08830
Gpt2	AT1G61800
Nced	AT3G24200
PAL4	AT3G10340
EFalpha-1	AT1G18070
ACT7	AT5G09810

RNA extraction

The RNA was extracted from 50 - 100 mg of fresh leaves, previously ground in liquid nitrogen, using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The total RNA amount was measured using a NanoDrop ND-1000 spectrophotometer (Thermofisher, Waltham, MA, USA).

RT-PCR and qRT-PCR

cDNA syntheses were made using the ThermoScript RT-PCR System. qRT-PCR carried out the gene expression analysis. Platinum SYBR Green qPCR SuperMix (Life Technologies, Carlsbad, CA, USA). The elongation factor was used as a reference gene (Punzo et al., 2020). The gene expression was quantised using the $2^{-\Delta\Delta Ct}$ method as in Livak & Schmittgen (2001). To perform semi-quantitative RT-PCR analysis, DreamTaq (Life Technologies, Carlsbad, CA, USA) was used, following the thermic protocol: 95°C, 5';

95°C, 30''; 58°C, 30''; 72°C 30''; 72°C, 5'. The amplification products were mixed with DNA-Dye NonTox solution (Applichem) (5:1 v/v) and then analysed using agarose gel electrophoresis.

Protein extraction

100 mg of fresh leaves were used for protein extraction, and the extraction buffer (Tris-HCl 50 mM, $MgCl_2$ 5 mM, sodium EDTA 4 mM, glycerol 10%) was added (2:1 v/w). The solution was incubated for 2 minutes, 5 times, in TissueLyserLT (Quiagen). The solution was centrifugated at 4°C, 13000 rpm, and the supernatant was collected. The last step was performed two times. The Bradford method was used to identify the total protein amount. Absorbance was monitored at 595 nm using a Cary 60 spectrophotometer (Agilent Technologies, USA).

Enzymatic assays

G6PDH activity was assayed according to the method described by Castiglia et al. (2015), which monitored $NADP^+$ reduction at 340 nm. The assay mixture contained 25 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 0.15 mM $NADP^+$, 3 mM G6P, and protein extract. PAL activity was measured according to the procedure described by El-Shora et al. (2002). The reaction mixture contained Tris–HCl buffer 100 mM (pH 8.8), l-phenylalanine 10 Mm and 100 – 50 µL of protein extract. After incubating at 30 °C for 15 min, the reaction was stopped by adding 100 µL of 6 M HCl, and the absorbance was measured at 290 nm. One unit of PAL activity was defined as the number of enzymes converting 1 mmol of l-phenylalanine into trans-cinnamic acid per minute. The synthesised trans-cinnamic acid was calculated using its molar extinction coefficient (9630/M/cm) and expressed as nmol trans-cinnamic acid/min/mg of protein. A Cary 60 spectrophotometer was used to monitor the absorbance.

Statistical analysis

Experiments were repeated two to five times in at least three biological replicates. Values were expressed as mean ± standard error (SE). The statistical differences were calculated through analysis of variance (ANOVA, $\alpha = 0.05$) and with the T-test. Differences between means were evaluated for significance using the Tukey–Kramer test. Prism v. 6.0 (GraphPad - https://www.graphpad.com) was used for statistical analyses.

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Chapter 2

This chapter contains unpublished results.

Eustress responses in textile hemp varieties

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Chapter 3

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Plastic in the Environment: A Modern Type of Abiotic Stress for Plant Physiology

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Review

Plastic in the Environment: A Modern Type of Abiotic Stress for Plant Physiology

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Abstract: In recent years, plastic pollution has become a growing environmental concern: more than 350 million tons of plastic material are produced annually. Although many efforts have been made to recycle waste, a significant proportion of these plastics contaminate and accumulate in the environment. A central point in plastic pollution is demonstrated by the evidence that plastic objects gradually and continuously split up into smaller pieces, thus producing subtle and invisible pollution caused by microplastics (MP) and nanoplastics (NP). The small dimensions of these particles allow for the diffusion of these contaminants in farmlands, forest, freshwater, and oceans worldwide, posing serious menaces to human, animal, and plant health. The uptake of MPs and NPs into plant cells seriously affects plant growth, development, and photosynthesis, finally limiting crop yields and endangering natural environmental biodiversity. Furthermore, nano- and microplastics—once adsorbed by plants—can easily enter the food chain, being highly toxic to animals and humans. This review addresses the impacts of MP and NP particles on plants in the terrestrial environment. In particular, we provide an overview here of the detrimental effects of photosynthetic injuries, oxidative stress, ROS production, and protein damage triggered by MN and NP in higher plants and, more specifically, in crops. The possible damage at the physiological and environmental levels is discussed.

Keywords: agriculture; microplastic; nanoplastic; oxidative; soil; transcriptomic; trace metals

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Introduction

In early 1900s, the innovation brought by the invention of plastics drastically revolutionized the world; in particular, after the end of World War II, plastics production boomed when military factories manufacturing plastic for war purposes revised their productions to civil use [1]. Currently, plastics are utilized for a wide range of items, from food packaging to technological applications, due to their resistance to corrosion, low density, and low electric and thermal conductivities [2]. In particular, one of the most significant advantages of the use of plastic material is the prolonged and complex degradation process, requiring from 250 to 1000 years. This aspect has become a significant environmental drawback, considering that, depending on their chemical structure, plastics decay over a long period and accumulate globally [3]. Since the early 1990s the dramatic implications of plastics for the environment have been proven, thus raising the concern of international institutions. In recent years, the presence of large quantities of microplastics (MPs) and nanoplastics (NPs) has been observed in the environment due to the degradation and dissemination of plastic waste [4].

More specifically, MPs are plastic particles with sizes from than 1 μm to 5 mm [1]. These particles include fragments smaller than 1 μm, which are considered NPs [4]. MPs

are divided into primary MPs, from chemically derived products used in pharmaceutical industries, and secondary MPs, formed when large plastics are broken down through physical, chemical, and biological processes [5,6]. MPs and NPs can exist as fragments, microfibers, spheres, films, and pellets [1].

Of course, both MPs and NPs can be classified based on the plastic polymers from which they originate, so there are MPs and NPs formed of polyamide (PA), polyethylene (PE), polyethylene terephthalate (PET), polyvinyl chloride (PVC), and polybutylene adipate terephthalate (PBAT). PE and PET are mainly used for packaging, bottles, and bags, while PVC is used for industry and construction [7]. PE and PET produce the most easily transportable MPs, especially through wind erosion, considering their low density [8].

Currently, MP and NP pollution is considered one of the most problematic environment-related issues, being widespread in deserts, tropics, deep oceans, and the Arctic and Antarctic poles [2]. Only considering aquatic environments, an annual release of 19–23 million tons of plastic residues has been estimated [1]. In the terrestrial environment, the release of plastic pollution, especially caused by poorly managed waste-disposal practices, is gaining increasing attention due the impact of plastic particles on plant growth, and animal and human health.

The aim of this work is to review the current knowledge and findings about the occurrences, effects, accumulation, and influences of MPs and NPs on different plant organisms. In particular, the detrimental effects triggered by MPs and NPs are summarized, including nutrient uptake, photosynthetic injuries, oxidative stress, ROS production, transcriptomic modification, and protein damage. The relationships between plastics and metals in the soil, how MPs can act as carriers of these pollutants, and the consequences for plant physiology are discussed.

Origin and Implications of Plastics in the Agricultural Environment

Soil is a heterogeneous habitat formed by a complex assemblage of minerals and organic matter, comprising a complex network of spaces filled with water and air [9]. MPs and NPs affect terrestrial environments, considering that these particles are solid contaminants similar in size and shape to those of the composing soil [10]. It has been observed that the presence of MPs and NPs can alter different physio-chemical properties of soil, such as bulk density, water retention capacity, pH, and organic matter (OM) content [10]. These aspects are critical for the soil system, impacting plant growth, development, and physiology. It is worth pointing out that soil properties are not only directly influenced by the presence of MPs and NPs, but they are also responsible for the bioavailability of the plastic particles influencing the activities of different protein receptors of microorganisms, animals, and plants [11].

MPs and NPs may also contain toxic compounds including pesticides, phthalates, endocrine-disrupting chemicals (EDCs), polycyclic aromatic hydrocarbons (PAHs), and trace metals. Therefore, MPs and NPs can act as vectors, transporting these compounds to the soil and causing them to become available depending on the temperature, ultraviolet radiation, pH, and oxygen content [11]. The presence of these toxic molecules in MPs and NPs negatively affects root growth, as well as disturbing the interaction between plants and their symbionts, damaging plant physiology and metabolism [12].

The utilization of plastics in agriculture has risen in recent years, with the specific aim of enhancing crop productivity and reducing food loss [13]. Plastics are commonly used for the construction of greenhouses and tunnels and to cover soil with mulch, shade cloth, pesticide containers, protective mesh, and irrigation tubing. Different estimations of the use of plastics in agriculture have been reported by several authors (Table 1).

Table 1. Estimation of plastic consumption for agricultural practices.

Year	Location	Plastic Estimation	Reference
2012	Worldwide	6.5 million tons/year	[14]
2014	China	1.4 million tons year *	[15]
2015	Worldwide	7 to 9 million tons/year	[16]
2019	EU	708.000 tons/year **	[1]

*= only for mulching; ** = not including plastic packaging for agricultural products.

One of the most widespread applications of plastic in agriculture consists of mulches mainly made of PE, PVC, and polypropylene (PP). Mulches effectively suppress weed growth, prevent soil erosion, and increase the soil temperature, leading to improved crop quality and yield [17]. Nevertheless, the potential advantages of plastic mulches and their widespread and prolonged use—coupled with a lack of proper management—result in the accumulation of plastic debris (MPs and NPs) in the soil. In the agricultural field, the removal of plastic films from soil at the end of the growing season is a labor-intensive process; moreover, it is impractical and cost-prohibitive to completely extract all the small fragments of the mulch film [18]. These residual plastic films result in the accumulation of MPs and NPs in the agroecosystems, increasing the effects of these toxic pollutants on plants and on the agricultural food chain [19].

In recent years, eco-friendly biodegradable plastics have been introduced. Their structural and surface characteristics allow for the attack of enzymes from the soil microbiota and biodeterioration, substantially reducing plastic pollution [20]. On the other hand, similar to conventional plastics, fragmentation of bioplastic mulches can occur by a range of environmental factors, thus releasing micro-bioplastics into agricultural soils [21].

Plastics as Abiotic Stress: General Effects on Plant Physiology and Metabolism

Is it possible to consider plastic contamination an abiotic stress for plants? Plants are not supposed to uptake or transfer MPs due their high molecular weight and large size; however, recent studies have highlighted that the presence of MPs can differentially affect plant growth, development, and physiology [4,22]. On the other hand, different crops have shown the ability to assimilate NPs penetrating into plant cell walls [23,24].

The effective impact of these particles on plant physiology is related to plastic quantities, cultivation strategies (e.g., soil or hydroponic), types, charges, and sizes of particles. In recent years, different authors have tested a range of MPs and NPs derived from PE, PP, PVC, and polystyrene (PS), showing critical effects on plants [24–27].

PS–MPs have shown detrimental effects on photosynthetic parameters, namely Fv/Fm, Fv/P_0, and chlorophyll content, both in soil and in hydroponic cultivation systems in *Brassica chinensis* and *Lemna minor* L. [25,26]. Interestingly, *L. minor* L. plants showed highly reduced effects when cultivated with 100 mg L^{-1} of PS-MPs, while serious disadvantages were noted when they were cultivated with 200 mg L^{-1} [26].

Pignatelli et al. [27] analyzed the effects in soil of PE–, PVC–, and PP–MPs with dimensions ≤ 0.125 mm on *Lepidium sativum* L. The authors reported detrimental effects for each plastic type analyzed, indicating that PE treatment induced the greater increase in H_2O_2, and PVC treatment showed the highest proline content [27]. These results suggest the activation of different abiotic stress response pathways to oxidative and osmotic damages, respectively [28,29].

The comparison of the effects of 10-µm PS–MPs and PVC–MPs on hydroponic rice showed PVC–MPs to be the most damaging [30]. In particularly, 3 mg L^{-1} of PVC–MPs reduced root and shoot weight, net photosynthesis, and stomatal conductance; unexpectedly, the use of PVC–MPs also induced a significant and enormous increase in water use efficiency (WUE) [30].

Furthermore, MPs and NPs alter the soil structure and properties interfering with the uptake of water and nutrients by crops and reducing their yield [9,10]. As a consequence, plastic residues in the soil induced detrimental effects on root architecture, impacting the

horizontal and vertical distributions of roots. For example, it was shown that root biomass and grain yield could be reduced by 30% and 9%, respectively, in *Zea mays* L. when the residual plastic films in soil reached 300 kg ha^{-1} [31].

Different studies have focused on the oxidative damages induced by MPs and NPs in higher plants, demonstrating that the toxic effects of MPs and NPs are dose- and size-dependent [32]. In particular, accumulation of NPs activates scavenging enzymes, such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX), which are generally involved in the response to abiotic stress [33]. These enzymes showed different behaviors depending on the model plant species and the type of used plastics (Figure 1).

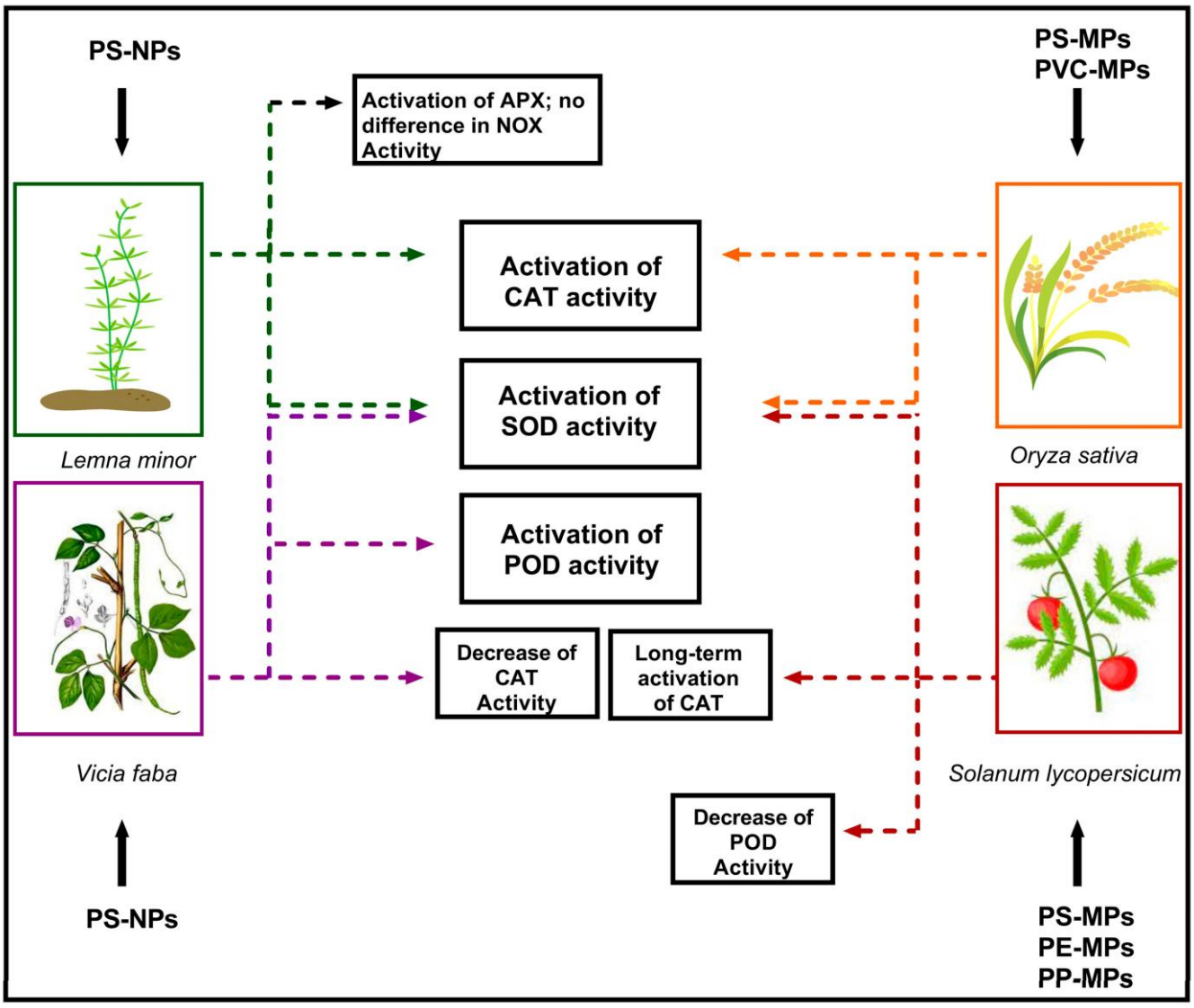

Figure 1. Scheme of the activation of scavenging enzymes SOD (superoxide dismutase), CAT (catalase), APX (ascorbate peroxidase), POD (peroxidase), and NOX (NADPH-oxidase) in different plant species exposed to MPs and NPs. Colors indicate different plant species.

In *Vicia faba* L., exposure to NPs is correlated with alterations in CAT, SOD, and POD enzymatic activities to counteract damage induced by the increase in ROS levels [33]. Exposure to 100 mg/L of PS–NPs with sizes of about 100 nm for 48 h induced strong increases in SOD activity [33]. Similar results were obtained for POD [30]. In contrast, CAT activity showed a significant decrease [33], likely correlated with the variation in H_2O_2 levels produced by SOD. Specific exposure to 100-nm NPs leads to the accumulation of

these particles in the root tissue, disrupting the nutrient import and probably causing both oxidative damage and genotoxic effects [33].

Similarly, tomato plants (*Solanum lycopersicum* L.) were tested for the toxic effects of different levels of PS–, PE–, and PP–MPs [34]. It was demonstrated that exposure to PE and PP (more than PS) affected seed germination, inducing oxidative stress in plants. The toxic effects of MPs in tomato plants are correlated with the alteration in SOD activity, with an initial increase and a subsequent decrease with the increase in MPs levels; POD showed a decrease in activity with any MPs dosage; and CAT played a major role in long-term response [34].

The treatment of *Oryza sativa* plants with plastic particles of higher dimensions also induced the activation of antioxidant enzymes to counteract the effects of ROS [30]. In particular, PS–MPs and PVC–MPs increased the activities of SOD, POD, and CAT using concentrations of about 0.5, 1.5, and 3 mg L^{-1}. Comparing the two different types of MPs, the PVC–MPs caused a faster and greater accumulation of H_2O_2 and malondialdehyde levels [30].

Arikan et al. [26] evaluated the potential effects of PS–NP exposure on the antioxidant system in *L. minor* L., exposing it to NPs in the medium (100 mg L^{-1} and 200 mg L^{-1}). These treatments increased the activity of SOD by 3-fold and 4.6-fold, respectively. Similarly, results were obtained for CAT and APX. Interestingly, the authors suggested that NADPH-oxidase (NOX)—a crucial enzyme responsible for O_2^- formation in plants—may be not involved in the response to NPs in *L. minor* L. [26].

In *Allium cepa*, NPs caused chromosome aberrations induced by the alteration of mitotic phases. This effect is related to the accumulation of ROS [35,36]. In particular, a gradual and dose-dependent decrease in mitotic index was demonstrated after 24 h, 48 h, and 72 h of incubation with 400 mg L^{-1} of NPs. The cytotoxicity induced by NPs is correlated with the down-regulation of cdc2, a fundamental gene coding for cell cycle regulation. The down-regulation of this gene is correlated with the mito-depressive activity of NPs by G2/M transition in living cells [37].

MPs and NPs Induce Transcriptomics Reorganization and Modify Plant Metabolism

Next-generation techniques, namely transcriptomics, proteomics, metagenomics, and metabolomics, have made available a number of datasets useful for exploring the relationships among different metabolic pathways in different plant species. Considering the serious environmental risk caused by MPs and NPs, these tools have been extensively used to investigate the effects of MPs and NPs in crops (Figure 2).

Oryza sativa plants treated with PS–NPs and graphene oxide nanosheets (GONs) were analyzed using RNA-seq [38,39], revealing a drastic re-organization of the transcriptome [38,39]. In particular, when plants were exposed to GON, 392 differentially expressed genes (DEGs) were identified (179 up-regulated and 213 downregulated). Plants treated with PS–NPs showed 592 DEGs (287 up-regulated and 305 down-regulated) [38]. Among these DEGs, specific genes were related to oxidative response and secondary metabolism (Figure 2). Furthermore, unusual expression of genes involved in carbon metabolism and the jasmonic acid pathway was reported in rice seedlings, together with a significant reduction in starch content and a corresponding increase in glucose levels [38].

The transcriptomic approach was used to investigate how genes involved in root development are regulated in presence of NPs [38]. PS–NPs regulate critical genes, namely *Os*MADS25, *Os*NAAT1, *Os*OFP2, and *Os*IRO2. *Os*MADS25 genes encodes for a transcription factor regulating lateral root development, associated with the nitrate signaling pathway [38]. Interestingly, *Os*NAAT1 and *Os*IRO2 are genes involved in the response to iron (Fe) uptake and deficiency, respectively [40,41]. These genes respond differently to NP treatment: the former is up-regulated, while the latter is down-regulated [38]. *Os*NAAT1 encodes for a nicotianamine aminotransferase (NA) that catalyzes the amino transfer of NA to produce an intermediate in the biosynthetic pathway of mugineic acid family phy-

tosiderophores (MAs) [40]. The gene *Os*IRO2 encodes for a basic helix–loop–helix (bHLH) transcription factor, and its expression is activated by an Fe deficiency [41]. On the other hand, the NPs down-regulated the *Os*OFP2 gene, which encodes for a transcription factor involved in lignin biosynthesis and hormone homeostasis [42].

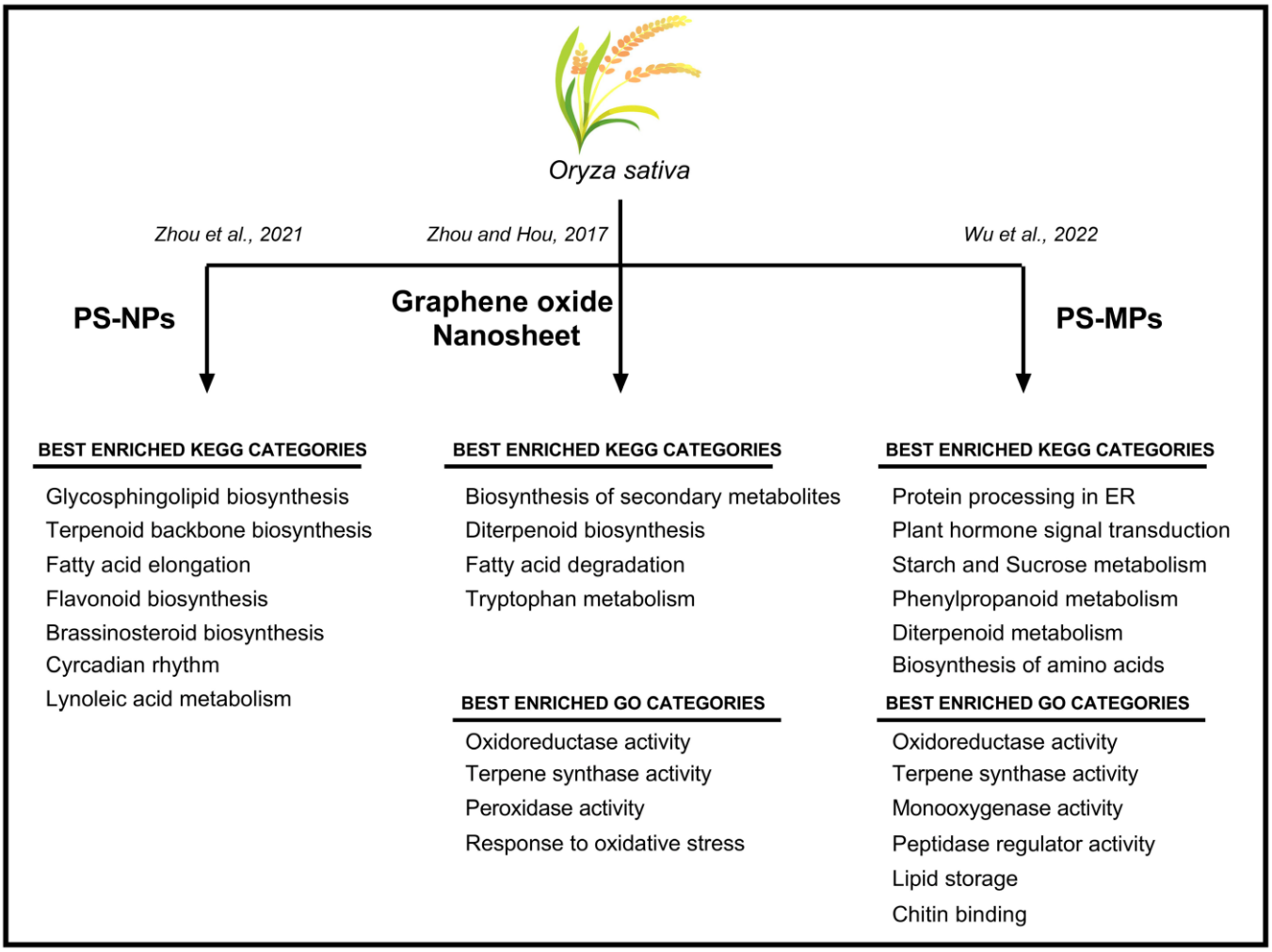

Figure 2. Scheme of the main GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Gene and Genome) categories enriched in *Oryza sativa* plants subjected to different types of plastics contamination [38,39,43].

Furthermore, integration of metabolomics and transcriptomics was used to analyze the effects of PS–MPs in two different rice genotypes. Wu et al. [43] identified 22.597 DEGs in genotype Y900 and 23.151 in XS123 genotype. Interestingly, the authors indicated a genotype-specific response to treatments [43]. Both genotypes showed dose-dependent activation of different pathways to respond to PS–MP stress. Y900 showed the regulation of DEGs related to polysaccharide metabolism after exposure to a low dose of MPs and to lipid biosynthesis after exposure to a high dose of MPs. Instead, exposure to both the low and high doses of PS-MPs induced in the XS123 genotype changes in DEGs related to amino–sugar and nucleotide–sugar metabolism and hydrolase activity. The two genotypes also showed contrasting behaviors in enzymes related to the TCA cycle. ATP citrate synthase (ACLY), isocitrate dehydrogenase (IDH1), dihydrolipoyllysine-residue succinyl transferase (OADH), and succinate dehydrogenase (SDH3-2) were differently expressed. The TCA cycle was strongly activated in the XS123 genotype and inhibited in the Y900 genotype [43].

The exposure to PE–MPs of *Zea mays* L. and *Nicotiana tabacum* L. [44,45] showed interesting results. PE–MPs of different dimensions (1.08 g cm^{-3} and 1.35 g cm^{-3}) on *Z. mays* L. roots affected 858 DEGs (624 up- and 234 down-regulated) and 1769 DEGs (1298 up- and 471 down-regulated), respectively. Transcriptomic analysis highlighted the activation of genes involved in the transport of different substrates as amino acids (similarly under different-sized MPs) and different sugars, namely galactose, glucose, and xylose, in a size-specific manner [44]. PE–MPs also affected the leaves of *N. tabacum* L. seedlings, influencing the expression of 5355 DEGs in plants grown in the presence of MPs (1000 g kg^{-1} in soil) [45]. The authors focused on photosynthesis, identifying that PE–MP stress significantly enriches different GO categories, particularly "photosynthesis, light harvesting in photosystem I", "photosynthesis, light harvesting", and "photosystem I reaction center".

An alternative approach to investigation of the effects of MPs and NPs is represented by metagenomics due to the impact of plastics in terrestrial environments on microbial communities and their relationships with plants [46]. However, comprehensive studies of the impacts of residual plastics have been scarce; in particular, in-depth studies on soil-rhizosphere microbe-plant interaction remain elusive. The importance of metagenomics studies should be based on the ubiquitous interactions among microorganisms, plants, and animals, while plastics and related compounds can produce multiple impacts on the environmental microbiomes [47]. These aspects would influence both the soil ecosystem and rhizosphere communities.

Thus, plants could play important roles in the shaping of soil and in the regulation of microbiomes growing in the rhizosphere, changing both the composition and amount of root exudates and recruiting specific soil microbiomes [9,10]. These modifications could influence the mobility and availability of MPs and NPs in the environment.

Curiously, damages induced by the presence of MPs and NPs in higher plants have been thoroughly investigated, but to our knowledge, no approach based on genetic engineering or synthetic biology has yet been suggested yet to overcome or mitigate plastic toxicity effects in plants.

Transcriptomics revealed the regulation of a large network of genes activated by the presence of MPs and NPs [38,39,43–45]. These catalogues of genes represent a powerful source to identify candidates able to improve plant tolerance to MPs and NPs.

From a physiological point of view, plants similarly respond to abiotic stress and plastic exposition by increasing the activities of ROS-detoxing enzymes, manipulating the expression of genes involved in nutrient uptake, as well as trace elements, and increasing the expression of chaperones and proteins able to guarantee the correct folding [48–50].

MPs and NPs Interaction with Trace Metals and Metalloids in Plants

Similar to other abiotic constraints, the contamination of soil by trace metals (TMs) and metalloids is a pressing issue for guaranteeing food safety, considering the dangerous effects of these elements on different aspects of plant physiology [48,51,52]. Plastic is an important factor regulating biological toxicity and the migration–transformation abilities of TMs in soil [53]. In particularly, MPs modify the abiotic properties of soil (namely pH value and cation exchange capacity), influence the chemical forms and bioavailability of nutrients and metals, impacting microbial, animal, and plant metabolism and their interactions [13,54]. The presence of MPs and NPs alters the bioavailability and content of TMs and nutrients [55]. Specifically, the effects of MPs and NPs can be contrasting, depending on the sizes, chemical types, and quantities, thus influencing the uptake of TMs and consequently affecting the plant physiology (Table 2).

Table 2. Effects of MPs and NPs on trace metal uptake.

Plant Species	Cultivation	Plastics Type	Concentration	Trace Metal (TM)	TM Concentration	Effects on HMs Uptake	Reference
Brassica chinensis	Soil	Polystyrene–MPs	0.5–2%	Cd	10 mg kg^{-1}	Decreased uptake	[25]
Oryza sativa	Soil	Polystyrene–MPs	0.25%	As	1.4, 24.7 and 86.3 mg kg^{-1}	Decreased bioavailability	[56]
Oryza sativa	Soil	Polytetrafluorethylene–MPs	0.5%	As	1.4, 24.7 and 86.3 mg kg^{-1}	Decreased bioavailability	[56]
Triticum aestivum	Soil	Polyethylene terephthalate–MPs	1 g in 20 mL	Pb	0.31 to 100.84 µg L^{-1}	Accumulation on PET–MPs	[57]
Triticum aestivum	Soil	Polyethylene terephthalate–MPs	1 g in 20 mL	Cd	5020.65 to 599,436.62 µg L^{-1}	Accumulation on PET–MPs	[57]
Brassica napus	Soil	Polyethylene–MPs	0.01 to 0.1% of soil weight	Pb	25–50 mg kg^{-1}	Increased bioaccumulation	[58]
Brassica napus	Soil	Polyethylene–MPs	0.01 to 0.1% of soil weight	Cu	50–100 mg kg^{-1}	Increased bioaccumulation	[58]
Lactuca sativa	Soil	Polystyrene–MPs	100 and 1000 ng kg^{-1}	Cu, Pb, Cd	82, 174.84, 42.0, 0.20 mg kg^{-1}	Increased uptake	[59]
Lactuca sativa	Soil	Polystyrene–NPs	100 and 1000 ng kg^{-1}	Cu, Pb, Cd	82, 174.84, 42.0, 0.20 mg kg^{-1}	Increased uptake	[59]
Zea mays	Hydroponics	Polystyrene–NPs	10 and 100 mg L^{-1}	Cd	2 mg/L and 10 mg L^{-1}	Antagonistic effects	[60]
Taraxacum asiaticum	Hydroponics	Polystyrene–MPs	1, 5 and 10 mg L^{-1}	Pb	10 mg L^{-1}	Increased uptake	[61]
Lemna minor	Hydroponics	Polystyrene–NPs	100 mg L^{-1}	As	100 µM	Decreased uptake	[62]
Lemna minor	Hydroponics	Polymethyl methacrylate–NPs	100 mg L^{-1}	As	100 µM	Decreased uptake	[62]
Lemne minor	Hydroponics	Combined–NPs	100 mg L^{-1}	As	100 µM	Decrease uptake	[62]
Oryza sativa	Hydroponics	Polystyrene–MPs	50 mg L^{-1}	As	250 µg L^{-1}	No effects	[63]
Oryza sativa	Hydroponics	Polystyrene–NPs	50 mg L^{-1}	As	250 µg L^{-1}	Promote accumulation	[63]

Pb and Cd accumulated on PET–MPs particles; these particles are able to adsorb these metals both in single-element and mixed solutions. In particular, the rate of Pb adsorption on PET particles was lower than that of Cd. The adsorbed TMs were then desorbed in the artificial rhizosphere zone of *Triticum aestivum*, demonstrating that PET particles can act as a carrier, increasing the uptake of TMs in the rhizosphere [57].

In *Brassica napus*, PE–MPs increased the bioaccumulation of Cu^{2+} and Pb^{2+} [58]. The concomitant presence of these metals and PS–MPs increased the oxidative damage with respect to that reported by the single presence of Cu^{2+} and Pb^{2+} in terms of malondialdehyde content and higher SOD and POD activities [58]. Likewise, combinatory treatments induced detrimental effects also for turnip quality, showing lower content in soluble sugars and vitamin C [58]

Similarly, PS–MPs and PS–NPs improved the uptake of Cu, Pb, and Cd in lettuce. In particular, plastic particles increase the abundance of bacteria (e.g., *Clostridium* sp.) able to improve the bioavailability of TMs. Both MPs and NPs increased the content of Cu, Zn, Pb, and Cd. In particular, PS-NPs (at 1000 mg kg^{-1} of soil) augmented the levels of these elements in plants up to 52.6, 174, 10.3, and 33.2 mg kg^{-1}, respectively [57]. Metabolomic analyses also showed that the simultaneous treatment with PS-NPs and TMs regulates different aspects of nutrient uptake, modifying the metabolism of ATP-binding cassette transporters and plant hormone signal transduction, therefore explaining the enhanced uptake in lettuce [59].

On the other hand, PSMPs and PSNPs can alleviate the toxic effects of Cd in *Brassica chinensis* L. and *Zea mays* L., respectively [25,60]. In contrast, the presence of different concentrations of PS–MPs in soil can affect different physiological parameters, such as biomass, photosynthesis, stomatal conductance, transpiration, and chlorophyll content, reducing the toxic effects of Cd. PS–MPs affect both Cd uptake and storage. In fact, plants exposed to Cd showed a concentration of 46.55 and 89.22 mg kg^{-1} in shoots and roots, respectively, while plants also treated with PS–MPs showed Cd content of about 31.30 and 75.98 mg kg^{-1} [25].

MPs and NPs are related to As and Pb uptake by plants [56,61,62]. The presence of PS–MPs and PTFE–MPs (polytetrafluorethylene) modify the bioavailability of arsenic (V) and arsenic (III) in soil [56]. The simultaneous presence of MPs and arsenic reduced the abundance of *Proteobacteria*, improving the occurrence of *Chloroflexi* and *Acidobacteria*; this outcome caused a reduction in the presence in soil urease, acid phosphatase, protease, dehydrogenase, and peroxidase, finally affecting the micro-environment of the rice rhizosphere. Therefore, PS–MPs and PTFE–MPs alleviate the effects of arsenic toxicity in soil, microbiomes, and plants [56].

Single or combined exposure of PS–NPs and polymers (such as polymethyl methacrylate nanoplastics, PMMA–NPs) showed the ability to modify different aspects of plant nutrition in *L. minor* L. [62]. Both single and combined treatments reduced the accumulation of micro- and macronutrients, namely N, P, K, Ca, Mg, and Mn. The effects of PS–NPs and PMMA–NPs indicated that the single-type particle treatment partially reduced As levels in plant tissues, but the combined treatment increased As uptake. Furthermore, As increased the content of PS–NPs and PMMA–NPs in *L. minor* L. [62]. Interestingly, Mamathaxim et al. [63] also showed comparable behavior in rice plants exposed to combined treatments with PS–MPs, PS–NPs, and As (III and V). No differences were reported comparing the uptake of As in the absence and presence of MPs. On the other hand, the presence of PS–NPs increased the presence of As (III) and As (V) in rice roots [63].

Plastics act as an effective abiotic stress, modifying the levels of different phytohormones, increasing the presence of abscisic acid (ABA), and decreasing the presence salicylic acid (SA) and jasmoic acid (JA) [62]. Interestingly, ABA showed the highest level in the presence of As, PS–NPs and PMMA–NPs, whereas under similar conditions, SA and JA showed the lowest levels [62].

The effects of NPs on phytohormone content reveal a possible role of these particles on plant signaling. The regulation of plant hormones affects gene expression (ABA or JA), contributing to the increase in chlorophyll biosynthesis (cytokinin) or optimizing the allocation of nitrogen (SA) [62]. Similarly, the effects on phytohormone regulation in the presence of PS were reported in rice [38] and tomato [64], but the mechanism regulating the role of NPs in this process has not yet been determined [65].

Crops Affected by MPs and NPs: A Threat for the Food Chain

Many studies have investigated the impact of ingestion of MPs and NPs on humans. These studies have shown a risk associated with plastic accumulation in human tissues, organs, and fluids. Their persistence induces specific biological responses and primarily affects the gastrointestinal tract and reproductive system. The accumulation of plastics in the organism can induce inflammation and immune and neurotoxic diseases [66–68]. In particularly, recent studies in human intestinal cells inoculated with fluorescent microspheres of PS–MPs showed significant accumulation of these particles in epithelial cells, with low cytotoxic effects but significant increases in ROS levels. These effects altered the correct cell morphology and function [68,69].

MPs and NPs are able to contaminate seeds, roots, culms, leaves, and fruits, depending on their size and type, including crops [70,71]. Considering this critical issue, different authors have investigated the effects of MPs and NPs in plants for human consumption and their effects on human health. Contamination by plastics negatively affects yields (Table 3), reducing crop productivity [72,73]. Examples include rice [74], spring onion [75], cucumber [76], and pea, which particularly showed a 30% yield reduction [75].

A further—and even worse—criticism is represented by the detection of MPs and NPs in edible fruits [73]. The presence and incidence of MPs and NPs in different vegetables and fruits purchased in both local and large distribution markets have been demonstrated [71]. Authors found MP and NP contamination in different edible species, namely two varieties of *Malus domestica* L., *Pyrus communis* L., and four vegetables: *B. oleracea* L., *Lactuca sativa* L., *Daucus carota* L., and *S. tuberosum* L.

Fruits accumulated up to 223,000 particles/gram (median value) in *M. domestica* and 176,500 particles/gram in *Pyrus communis* L. Conversely, *L. sativa* L. showed lower contamination, 52,050 particles g^{-1}. The sizes of MPs and NPs retrieved in these crops ranged from 1.4 to 3.2 µm [71], with higher levels of MPs and NPs in fruits. Therefore, it could be concluded that NP contamination affects vegetable quality.

Table 3. Effects of MPs and NPs on crop productivity. Abbreviations: primary polyamide (PA); polyester (PES); polyethylene high density (PEHD); polypropy- lene (PP); polystyrene (PS); polyethylene terephthalate (PET); polyvinyl chloride (PVC); polyethylene (PE); biodegradable microplastic (Bio–MPs); low-density polyethylene (LDPE–MPs).

Crops	Plastics Type	Effects	Yield	Reference
Cucumis sativus	PS	Reduction: soluble protein and sugar, vitamin C, and mineral element content; Increment: total soluble protein	50% reduction in vitamin C and sugar content	[76]
Pisum sativum	MP	Reduction: pods and beans for pod numbers; Increment: shoot growth Changes: amino acid profile	20.5% reduction in bean per pod number	[77]
Lactuca sativa	PS	Reduction: dry weight, height, leaf area, pigment content, shoot-to-root dry biomass ratios, macro- and micronutrient and amino acid content, photosynthetic performance, and chlorophyll and carotenoid content	Dry weight −27.3%; height −27.3%; leaf area −19.2%	[78]
Oryza sativa	PS	Reduction: seed weight, nutritional quality	Seed weight −3.45%	[79]
Arachis hypogaea	PS	Reduction: weight and total grain number per plant, nutritional quality	Empty shell number x plant +35.45% Seed-setting rate −3.02%,	[79]
Solanum lycopersicum	PET; PVC	Reduction: shoot growth, photosynthesis, fruit development and quality	No. of fruits x plant −28%; fruits' fresh weight −25%	[80]
Cucurbita pepo	PP; PE; PVC; PET	Reduction: shoot growth, leaf size, chlorophyll content; photosynthetic efficiency	Fresh weight of shoots −35%	[81]
Triticum aestivum	PS	Reduction: macro- and micronutrient content Increment: biomass and root elongation Changes: leaf metabolic profiles	Shoot biomass +87.1%; root biomass +116.5%, shoot root ratio −27.3%	[82]
Daucus carota	PS	Reduction: biomass; soluble protein, vitamin C, soluble sugar, α-carotene, and β-carotene and chlorophyll content		Increment: leaf area, root length, specific root nodules
Phaseolus vulgaris	LDPE	Reduction: chlorophyll content		

Phaseolus vulgaris	Bio–MPs	Reduction: root, shoot, and fruit biomass, chlorophyll content, leaf area. Increment: root length	Root biomass −12.7%; leaf biomass −21,4%	[83]
		Reduction: plant number, fruit production	No significant data obtained for fruit biomass	[84]
			Fruit biomass −43%	[84]
Solanum lycopersicum	PET; PVC	Increment: shoot and root biomass	No. of plants −25%; no mature tomatoes; shoot biomass increase +2.21 fold change; root biomass +2.89 fold change	[85]
Zea mays	PS	Reduction: biomass root and number of lateral roots	Root dry weight −49.4%	[86]
Lactuca sativa	PS	Reduction: root lengths and biomass, germination	Germination index −36.0%; root dry weight −50%	[86]

Foliar application of PS–NPs reduces the nutritional quality of *L. sativa* L. [78]. Significant reductions in total soluble proteins, total soluble sugars, and amino acids were observed in lettuce exposed to 1 mg L^{-1} of PS–NPs, together with changes in macro- and micronutrients [78]. The toxic effects of MPs and NPs were also observed in grains of *Oryza sativa* L. (*Poaceae*) and *Arachis hypogaea* L. (*Fabaceae*) [79]. In particular, peanut grains (developed underground) were more vulnerable to NPs. Both species showed the presence of PS–NPs in grain when cultivated in soil containing 250 mg kg^{-1} NPs, showing detrimental effects on grain quality. The presence of PS–NPs decreased peanut seed weight by 3.45%, while the rice empty-shell seeds increased by 35.45%. Furthermore, the presence of PS–NPs caused reductions in Ca, Mn, and Zn concentrations and in the levels of Tyr, Phe, Lys, and Arg. PS–NPs showed different effects on fatty acid content in rice and peanuts. MPs and NPs reduced the content of unsaturated fatty acid (UFAs) in rice grains, including C20:1 and C20:2, and saturated FAs (C12:0 and C17:0). Differently, exposure to 250 mg kg^{-1} PS–NPs in peanuts significantly reduced the levels of UFAs (C16:1, C18:3), but different SFAs (C10:0, C15:0, and C16:0) increased [79].

Plastic in the Environment: Research Gaps and Possible Strategies to Overcome Plastic Stress

Most of current knowledge about plastic pollution in the environment has been focused on aquatic ecosystems [2]. To limit damage, the utilization has been proposed of eco-sustainable and easily perishable materials in the marine environment. Since 2018, scientists have become increasingly interested in evaluating how plastic particles affect soil agricultural environments and crops. Therefore, this theme is relatively young.

The generation of plastic waste and the effects of these particles are pressing challenges in both industrial and developing countries, where they are related to a greater urbanization and higher economic growth [1].

The effects of globalization have shifted the environmental impacts to other countries around the world. Approximately 5–20% of imported plastic waste in emerging economies has no market value; therefore, this waste ends up in the environment or in open dumps or is burned [1]. As shown in this manuscript and the literature, plant exposure to MPs and NPs leads to toxicity symptoms, including growth inhibition, alterations in mineral homeostasis and photosynthesis efficiency, decreased cell division, and genotoxic effects [4,22,38,43]. These effects could vary as a function of plant species and therefore will possibly involve modifications to the composition of plant communities and primary production [12].

A number of questions and solutions about the toxic effects of MPs and NPs on the environment and plants remain unanswered. One of the most important questions is how plastic particles can gain entry into plants, How can plants activate a defense by closing off these particles? [87]. The identification of specific weights, sizes, types, and directions of MPs and NPs at different levels, such as in specific plant species, communities, rhizosphere communities, and whole ecosystems, will be challenging [12]. Similar to other abiotic stresses (drought, salinity, etc.), the identification of those genotypes (local or improved) able to tolerate or avoid the effects of plastic toxicity will be also critical. The genetic and phenotypic peculiarities must be investigated in their ability to avoid MP and NP uptake [12,87] or to retrieve the abilities of genotypes tolerant to typical abiotic constraints. Even though the effects of plastics on the terrestrial ecosystem have been recently documented, there have still been few studies exploring methods of plastic removal or degradation. In water environments, the removal of MPs and NPs is a difficult process considering their small size [88]. MPs remain stable during physical processes, such as coagulation, sedimentation, and screening, due their high polymeric flotation properties [89]. Other strategies involve different environmental factors that affect the physical structures of plastics or soil micro- and meso-fauna (microorganisms and invertebrates) [90,91]. In particular, the use of biotic processes would be an interesting alternative to overcome the presence of plastics in the soil environment and improve plants' tolerance of MPs and

NPs. However, the consumption of plastic by organisms and their degradation by host gut enzymes, related to their microbiome, require further confirmation [92].

Conclusions

The results and indications reported in this review summarize various aspects of MPs' and NPs' effects on agriculture soils and plant physiology. Currently, MPs and NPs, composed of different kinds of plastics, (polystyrene, polyvinyl chloride, polyethylene, polypropylene), impact various plant processes, including photosynthesis, nutrient uptake, growth, and development, inducing osmotic and oxidative damages similar to those reported with abiotic perturbations. The effects of plastics can be contrasting, depending on their dimensions, compositions, and concentrations. Examples are the relationships with trace metals, root morphology, or activation of the antioxidant systems. In fact, a beneficial impact of plastics has been reported in counteracting the detrimental effects of trace metals, but—generally—the presence of MPs and NPs in soil (or in hydroponics) induced the activation of typical stress response pathways, including oxidative burst and protein damage. Scavenging enzymes also differently respond to MPs and NPs. An emblematic case is catalase, showing a short-term activity increase in *Oryza sativa* L. and *Lemna minor* L., a long-term activity increase in *Solanun lycopersicum* L., and a long-term activity decrease in *Vicia faba* L. These results highlight the complexity and peculiarity of this specific anthropic stress.

Furthermore, MPs and NPs are able to regulate the expression of thousands of genes perturbing different metabolic pathways in important crops, such as rice, maize, and tobacco, similar to other abiotic stresses. This transcriptomic reorganization particularly influences biological processes, such as protein processing, biosynthesis of fatty acids and secondary metabolites, or the hormonal transduction pathways (ABA, SA, JA, etc.).

Finally, crop productivity and quality are seriously endangered by MPs and NPs. This endangerment will be especially true for agricultural production, considering the extensive and essential use of plastics. Consequently, in coming years, this issue could have critical effects on food demand, food quality, and human health. The "plastic problem" needs to be brought into the spotlight because it represents a critical challenge for plant and environmental scientists in the years to come.

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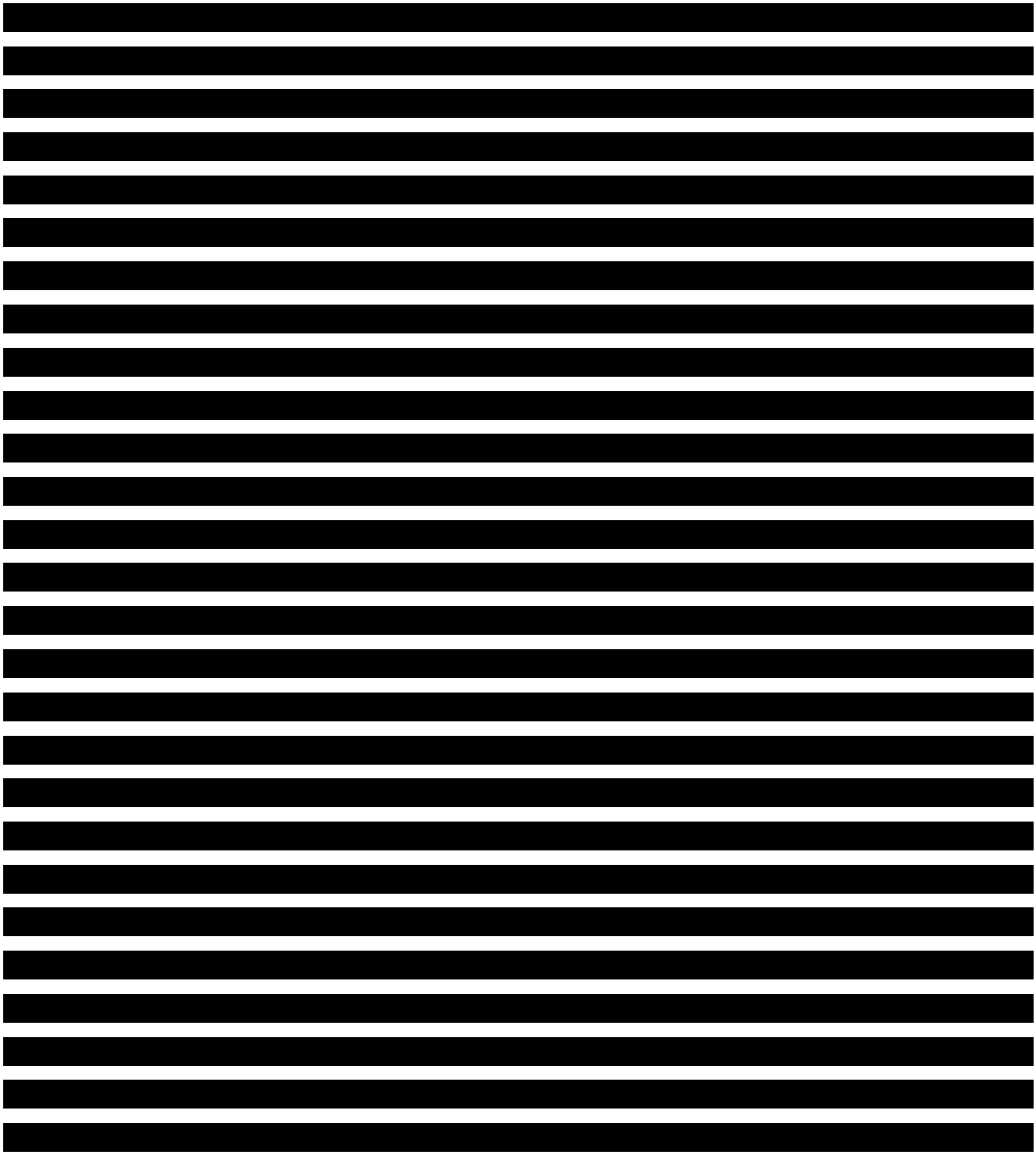
General conclusion

Climate change, pollution, and anthropic activities significantly impact plant growth, development, and crop productivity. However, plants have developed various strategies to counteract these environmental changes. For instance, plants can increase their production of antioxidant compounds, regulate the transcriptomic and metabolomic profile, and enhance defence mechanisms to counteract stress conditions. This project, through different experimental approaches and using different plant tissues and species, provides an overview of stress response mechanisms and the involvement of G6PD under distress and eustress conditions.

In the project's first section, the model organism *A. thaliana* wild-type and mutant strains were used. Upon cold stress conditions, a pivotal role was played by SK11 (ASKα) in regulating G6PD activity. SK11 is a serine/threonine protein kinase involved in the regulation of several targets (transcription factors, hormone signalling, and cytosolic G6PD) (Dal Santo et al., 2012; Dong et al., 2019; Li et al., 2021). A down-regulation of cytosolic G6PD was confirmed in mutant SK11 knock-out (KO-SK11) under cold stress conditions. In addition, other scavenger enzymes were down-regulated under cold stress in mutant SK11 plants, suggesting that this kinase could manage other protein activities, such as transcription factors or epigenetic modulators. This hypothesis is supported by the weak expression of different genes involved in stress response mechanisms in mutant plants. Further research will be necessary to identify potential SK11 targets under stress conditions (drought, salinity). It must be underlined that climate change will soon result in warmer winters and an extended growing season, which will help pests, pathogens, and invasive herbivores become more extensive and durable. Under this premise, we select to utilise yeast extract as a pathogen-like stressor. Since elicitation is a complex mechanism inducing the activation of the immunity system in plants, the expression and activity of phenylalanine ammonia-lyase (PAL) was identified as a key enzymatic activity (Zhang et al., 2020). PAL is the first enzyme in plants' phenylpropanoid pathway, synthesising several antioxidant compounds. These compounds act as a stress signal, increasing requests for reductants. Under this view, G6PD plays a central role in furnishing NADPH (Świeca et al., 2016; Moens et al., 2011). We found that G6PD expression and activity in an *in vitro* environment were significantly enhanced in response to yeast extract. These findings suggested a potential connection between the activation of the OPPP and the phenylpropanoid pathway following an elicitation mechanism in *A. thaliana* (Lengrand et al., 2007; Cabrerizo et al., 2023). By contrast, different results were observed in the *in vivo* system. In a soil-based approach, *A. thaliana* wild-type and P1-G6PDH mutant (KOP1-G6PDH) were used to improve our knowledge about the involvement of G6PDH in the elicitation mechanism. G6PDH and PAL expression and activity were tested. In wild-type *A. thaliana*, no significant differences were observed following the treatment in activating G6PDH and PAL. In KOP1-G6PDH mutants, yeast extract solution on leaves

increased expression and activity of cytosolic G6PDH after 10 days. The highest expression of PAL was reached after one week, followed by a substantial reduction after 10 days. These data suggested that the exposure to elicitors on the leaf affects the expression and activity of both G6PD and PAL in P1-G6PDH mutant plants. A hydroponic approach could improve our understanding of the link between yeast extract and G6PDH regulation. These results are discussed in Chapter 1, “Cold stress and yeast-mediated elicitation effects in *Arabidopsis thaliana* wild type and mutant strains, an overview of stress response mechanisms and the pivotal role of G6PD”.



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Finally, in Chapter 3, the impact of plastic pollution on higher plants and crops is discussed. This report summarises various aspects of MPs' (micro-plastics) and NPs' (nanoplastics) effects on agriculture soils and plant physiology. Currently, MPs and NPs, composed of different kinds of plastics (polystyrene, polyvinyl chloride, polyethylene, polypropylene), compromise many plant processes, including photosynthesis, nutrient uptake, growth, and development, also leading to osmotic and oxidative damage, similar to those reported under abiotic stress. The effects of MPs and NPs can differ depending on their dimensions, compositions, and concentrations. Examples are the relationships with trace metals, root morphology, or activation of the antioxidant systems. It is possible to distinguish different stress response patterns after exposure to MPs and/or NPs in various plant species. Indeed, a beneficial impact of plastics has been reported in counteracting the detrimental effects of trace metals, but - generally - the presence of MPs and NPs in soil (or in hydroponics) induced the activation of typical stress responses, including oxidative burst and protein damage. Scavenging enzymes also respond differently to MPs and NPs. A case study is represented by catalase, showing a short-term activity increase in *Oryza sativa L*. and *Lemna minor L.*, a long-term activity increase in *Solanum lycopersicum L.*, and a long-term activity decrease in *Vicia faba L.*. Furthermore, MPs and NPs act like abiotic stressors, regulating the expression of thousands of genes and affecting different metabolic pathways in important crops, such as rice, maise, and tobacco. This transcriptomic reorganisation mainly affects biological processes, such as protein processing, biosynthesis of fatty acids and secondary metabolites, or the hormonal transduction pathways (ABA, SA, JA, etc.). Finally, crop productivity and quality are seriously endangered by MPs and NPs. This endangerment will be especially true for agricultural production, considering the extensive and essential use of plastics. In the coming years, this issue could induce critical effects on food demand, food quality, and human health. The "plastic problem" needs to be brought into the spotlight, because it will undoubtedly represent a major and critical challenge for crops, plants and the environment in the years to come.

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