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Plant stress – a threat to food security

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Jagna Chmielowska-Bąk, Yuriy E. Kolupaev and Yaroslav B. Blume

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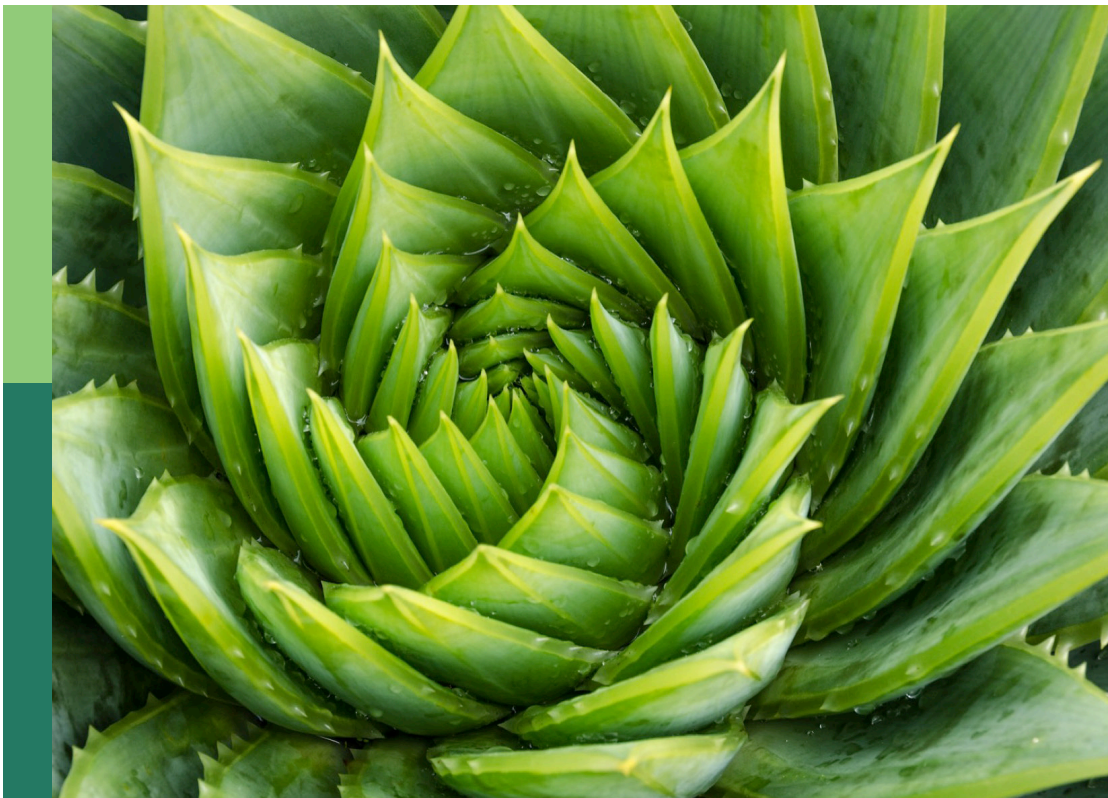

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Plant stress – a threat to food security

Topic editors

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Editorial: Plant stress – a threat to food security

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Editorial on the Research Topic
Plant stress – a threat to food security

The intensification of abiotic stress impacts on plants is becoming one of the main threats to global food security. According to predictions estimates, in the nearby future, the negative effects of abiotic stress factors on crop production may lead to food shortage for 1.8 billion people (Nyaupane et al., 2024). Drought, high temperatures, and soil salinization are the primary factors limiting yield growth and posing food security risks. Currently, drought-attributed crop losses range from 30% to 90% (Dietz et al., 2021), exceeding the total losses caused by all pathogens combined (Gupta et al., 2020). Another factor, often affecting plants in the same regions as drought, is soil salinization, which affects at least 20% of all irrigated areas (Mushtaq et al., 2021). Alongside drought and salinization, the impact of extremely high temperatures on food crops is also intensifying. It is predicted that the mean global temperature may be 1.8–4.0°C higher by the end of this century than in 2000 (Munaweera et al., 2022). These climate changes are, at least in part, associated with anthropogenic factors. The impact of heavy metals, metalloids, and other xenobiotics on plants and other groups of organisms is even more clearly related to human activities.

At the same time, plants possess enormous adaptive potentials, which are being continuously elucidated due to advances in studying the mechanisms of stress signal perception and transduction to the genetic apparatus, transcriptomic and proteomic changes, post-translational protein modifications, and epigenetic regulatory mechanisms. The Research Topic entitled "Plant Stress – A Threat to Food Security" brings together studies focused both on uncovering new fundamental mechanisms of adaptation, including functioning of macromolecules and signal-regulatory compounds, and on expanding the practical use of new bioregulators.

Several articles in this Research Topic are devoted to mechanisms of plant adaptation to drought, salinity, high temperatures, and induction of resistance to these factors through exogenous hormones and micronutrient nanoparticles. For example, Zhang et al. studied the heat shock protein 20 (Hsp20) gene family in *Lactuca sativa*. One of the most interesting findings was the identification of three types of cis-elements within the LsHsp20 family: (1) stress-responsive, (2) hormone-responsive, and (3) development-related. This expands our understanding of the Hsp20 functions in plants.

New hormones and compounds with hormone-like activities (Feng et al., 2024) hold significant potentials for enhancing plant tolerance to drought and associated stressors. Positive effects of one form of auxin (indole-3-butyric acid) were demonstrated by Zhou et al. in experiments on rice plants subjected to salt stress. The authors attribute the observed increase in shoot and root growth to modulated carbohydrate metabolism and activated syntheses of several secondary metabolites with stress-protective capacities. In the study by Cavalcante et al., the effectiveness of salicylic acid as a drought tolerance inducer in an important forage crop, *Vigna unguiculata*, was examined. In field experiments, the authors showed that salicylic acid enhanced antioxidant activity and osmolyte synthesis under drought, increasing the performance, although these effects appeared to be cultivar-specific. The need to pay close attention to species- and cultivar-specific peculiarities of adaptation of domestic plants to drought is also emphasized in the article by Zhou et al. The authors conducted the first bibliometric analysis of publications on drought tolerance of *Medicago sativa*. They concluded that for many years, two main directions have been prioritized: research into the mechanisms of alfalfa's response to drought and development of technological approaches to boost drought tolerance.

The review by Bao et al. analyses the potential use of nanomaterials to enhance plant tolerance to drought and high temperatures, with a focus on the activation of antioxidant systems by nanoparticles. Although antioxidant effects have been observed in plants treated with nanomaterials containing Ag, Zn, Ti, Se, and Mn compounds, the authors emphasize that the potential accumulation of these nanomaterials in the edible parts of agricultural plants may pose a threat to human health. Therefore, practical applications of nanomaterials must be preceded by thorough toxicological and ecological studies (Ekner-Grzyb et al., 2022). Nevertheless, it was revealed that treatment of plants with nanomaterials could considerably mitigate effects of toxic elements on plants. A striking example of the promising potential of such research is given in the study by Zeeshan et al., where zinc oxide nanoparticles (ZnO-NPs) significantly improved the growth of soybean plants exposed to arsenic. Co-treatment with ZnO-NPs led to hampered As uptake accompanied by enhanced growth and photosynthesis parameters. These effects could be a result of ZnO-NPs-dependent modulation of genes expression. The transcriptomic data revealed distinct expression of genes engaged in As transport, stress response, signaling and phytohormone metabolism under ZnO-NPs supplementation.

The topic of plant adaptation to toxicants is further developed in the review by El-Sappah et al. In particular, the review focuses on transporter proteins that transport heavy metal ions into vacuoles and on regulation of expression of genes encoding these proteins. This article describes the sources of metal contamination and pathways leading to their uptake by plants, transport within cells and distinct organs and activated defense mechanisms leading to their sequestration. In addition to the negative effects of metals/metalloids, some beneficial roles are presented, e.g. cofactors, metabolism modulators and anti-herbivore agents. Nonetheless, metals/metalloids in excess are harmful to plants and, thus, methods for alleviation of their toxicity are intensively studied.

Two other review articles are devoted to the general mechanisms of plant adaptation. In their short review, Wang et al. summarize recent findings on the role of histone acetylation in the regulation of plant responses to different abiotic stressors. The authors discuss technical aspects of studying this process and highlight the promising potential of evaluating histone acetylation's role in complex responses to multiple simultaneous stressors where histone acetyltransferases interact with several transcription factors and protein complexes, which are often co-regulated by other modifications such as methylation.

Close attention has been recently paid to the role in plant adaptation of several compounds that combine some properties of stress-related metabolites and phytohormones and/or components of signaling pathways. These include, in particular, gaseous signaling molecules (gasotransmitters) (Kolupaev et al., 2022) and melatonin, a hormone, which is well-studied in human and animal physiology but also synthesized in significant amounts by plants (Aghdam and Arnao, 2024). The review by Kolupaev et al. summarizes data indicating the involvement of nitric oxide, hydrogen sulfide, and carbon monoxide in melatonin's stress-protective functions in plants. It also discusses how melatonin modulates one of the most important effects of gasotransmitters and reactive oxygen species—post-translational protein modifications.

In conclusion, it should be noted that, despite its high relevance, the presented selection of articles cannot fully reflect the complexity of solving plant adaptation challenges in the context of food security. Nonetheless, the effectiveness of scientific efforts can be increased through interdisciplinary work, bridging studies at the molecular and cellular levels with studies on whole plants followed by translation of discovered effects into new biotechnologies.

Author contributions

JC: Conceptualization, Writing – original draft, Writing – review & editing. YK: Conceptualization, Writing – original draft, Writing – review & editing. YB: Supervision, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research on drought stress in *Medicago sativa L.* from 1998 to 2023: a bibliometric analysis

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Alfalfa (*Medicago sativa L.*) is one of the most important forage crops in the world. Drought is recognized as a major challenge limiting alfalfa production and threatening food security. Although some literature reviews have been conducted in this area, bibliometric reviews based on large amounts of published data are still lacking. In this paper, a bibliometric analysis of alfalfa drought stress from 1998–2023 was conducted using the Web of Science Core Collection database in order to assess global trends in alfalfa drought stress research and to provide new directions for future research. The results showed that the annual publication output maintained an increase in most years, with China and the United States contributing significantly to the field. Most of the journals published are specialized journals in botany, environmental science, soil science and crop science, as well as related agribusiness journals. "plant growth" and "yield" were the most frequently used keywords, reflecting the important purpose of research in this field. And two main research directions were identified: research on drought response mechanism of alfalfa and exploration of drought-resistant technology. In addition, physiological, biochemical, and molecular responses of drought tolerance and high yield in alfalfa, transgenics, and microbial fertilizer research have been hot research topics in recent years and may continue in the future. The ultimate goal of this paper is to provide a foundational reference for future research on alfalfa's drought resistance and yield optimization mechanisms, thereby enhancing the crop's application in agricultural production.

KEYWORDS

alfalfa, drought tolerance, response mechanism, yield, bibliometrics, research trends

1 Introduction

Recent years have seen global climate change inducing higher temperatures, which in turn has increased potential evapotranspiration and exacerbated drought conditions, particularly in semi-arid regions (Muluneh et al., 2014). Soil moisture, a critical factor for crop growth, is significantly compromised in these arid areas. The heightened water consumption by crops further aggravates soil drought, consequently diminishing crop productivity (Brookshire and Weaver, 2015). Drought is identified as a primary cause of reduced crop yields (Araya et al., 2012). Plant drought tolerance is described as the capacity of plants to sustain growth under less than optimal water supply conditions (Luo, 2010). Adaptation mechanisms to combat drought include osmotic regulation, production of protective metabolites, proteins, and systems for scavenging reactive oxygen species (ROS) (Ma et al., 2016). When soil water content is limited, plants experience alterations in their metabolism across developmental, physiological, and molecular levels. These changes lead to decreased growth and photosynthesis rates, modifications in photosynthetic proton and electron transport, and reductions in carbon oxidation cycling and photosynthetic carbon assimilation (Cornic, 2002; Zivcak et al., 2014; Putnam, 2021). Concurrently, drought stress can lead to a loss of cellular turgor and lower water content in cells (Hernandez-Santana et al., 2021), further impeding plant growth and accumulation of dry mass (Ogunkanmi et al., 2021; Wan et al., 2022). Notably, drought stress is a significant environmental challenge impeding the growth and yield of alfalfa globally (Diatta et al., 2021), characterized by its unpredictability and considerable adverse impacts on global crop production (Golldack et al., 2014; Anjum et al., 2017; Hussain et al., 2018). Water deficits inflict harm on plants by disrupting various physiological processes, such as carbon assimilation, cellular hydration, increased oxidative damage, and leaf gas exchange, culminating in lower yields (Chowdhury et al., 2016; Hussain et al., 2018). In the face of global warming, the development of drought tolerance in crops has become an increasingly pressing issue.

Alfalfa (*Medicago sativa L.*), often hailed as the "queen of forages," is a venerable wild plant originally found in the Mediterranean mountain forests of southwestern Asia. The term "alfalfa" stems from the Arabic "Al-Fasfasa," meaning "the father of all plants" (Lacefield et al., 1979). Renowned globally as a vital perennial legume forage (Lamb et al., 2006; Veronesi et al., 2010; Annicchiarico et al., 2014), alfalfa is celebrated for its high yield, superior quality, and rich protein content. Notably, it thrives on marginal lands (Brawley and Mathes, 1990), cementing its status as one of the most sought-after forage legumes due to its nutritional value and productivity (Basigalup et al., 2014). Alfalfa enhances soil structure through its deep-rooting system and leverages its symbiotic capability for biological nitrogen fixation with rhizobacteria (Carlsson and Huss-Danell, 2003), thereby boosting nitrogen availability for subsequent crops (Basigalup et al., 2014). Rhizosphere bacteria play a key role in improving the adaptability of alfalfa to drought stress (Fan et al., 2023). Exceptionally adaptable, alfalfa flourishes in various environments, particularly under drought conditions (Annicchiarico, 2007; Huang et al., 2018). Its deep root system contributes to its relative drought tolerance, especially beneficial in semi-arid regions (Ma et al., 2021). With numerous research papers on alfalfa under water stress published globally, effectively synthesizing this vast array of information is essential for researchers to gain a comprehensive understanding of the current research trends and future directions in a timely manner.

Bibliometric analysis employs statistical and visual techniques to dissect the complex characteristics of a body of published literature (Broadus, 1987; Okubo, 1997; Van Raan et al., 2010). This approach enables researchers to swiftly pinpoint relevant topics and directions amidst a vast array of literature, clarifying key information, contextualizing findings, and identifying the most active research frontiers and trends. Unlike traditional literature reviews and meta-analyses, bibliometric analysis provides a more holistic grasp of the current state, forefront, and potential future trends of a specific research field. To date, only a limited number of scholars have utilized bibliometric methods to visualize and analyze research on alfalfa drought tolerance. Thus, this study adopts a bibliometric approach, using tools such as VOSviewer and R, to dissect and elucidate the knowledge structure within alfalfa drought tolerance research. Our objective is to facilitate rapid access to the core research content and prevailing topics in this field.

2 Materials and methods

2.1 Data sources and screening

On November 23, 2023, we employed specific keywords to retrieve literature from the Web of Science Core Collection (WSCC) database at the University of Shanghai for Science and Technology (USST) Library. The search terms included combinations of "alfalfa or *Medicago sativa* or lucerne" with "drought tolerance or water stress or water deficit stress or drought-tolerant or drought persistence, et al." Recognizing that the earliest alfalfa-related literature in the Core Collection dates back to 1998, we selected a corpus of 1,081 articles and reviews covering the period from 1998 to 2023. For data export, we chose the "Full Record and Cited References" option, enabling the exportation of 500 articles at a time. Consequently, all literature was exported in three batches. The exported "text" files were initially processed using Co-Occurrence 9.9 (COOC) and subsequently converted into "excel" files for further analysis. To enhance the accuracy of our analysis, irrelevant keywords were omitted, and synonyms were consolidated. The selection and flow of this study are depicted in Figure 1.

2.2 Data visualization and analysis

The data for analysis, encompassing various metrics such as the number of publications by countries/regions, annual publications, journal contributions, citations, etc., were sourced from the WOS core dataset (Tan et al., 2021). We commenced with metric frequency analysis and interaction analysis to gain a preliminary understanding of the major contributing countries, institutions, and journals, as well as the extent of collaborative ties. This process was primarily

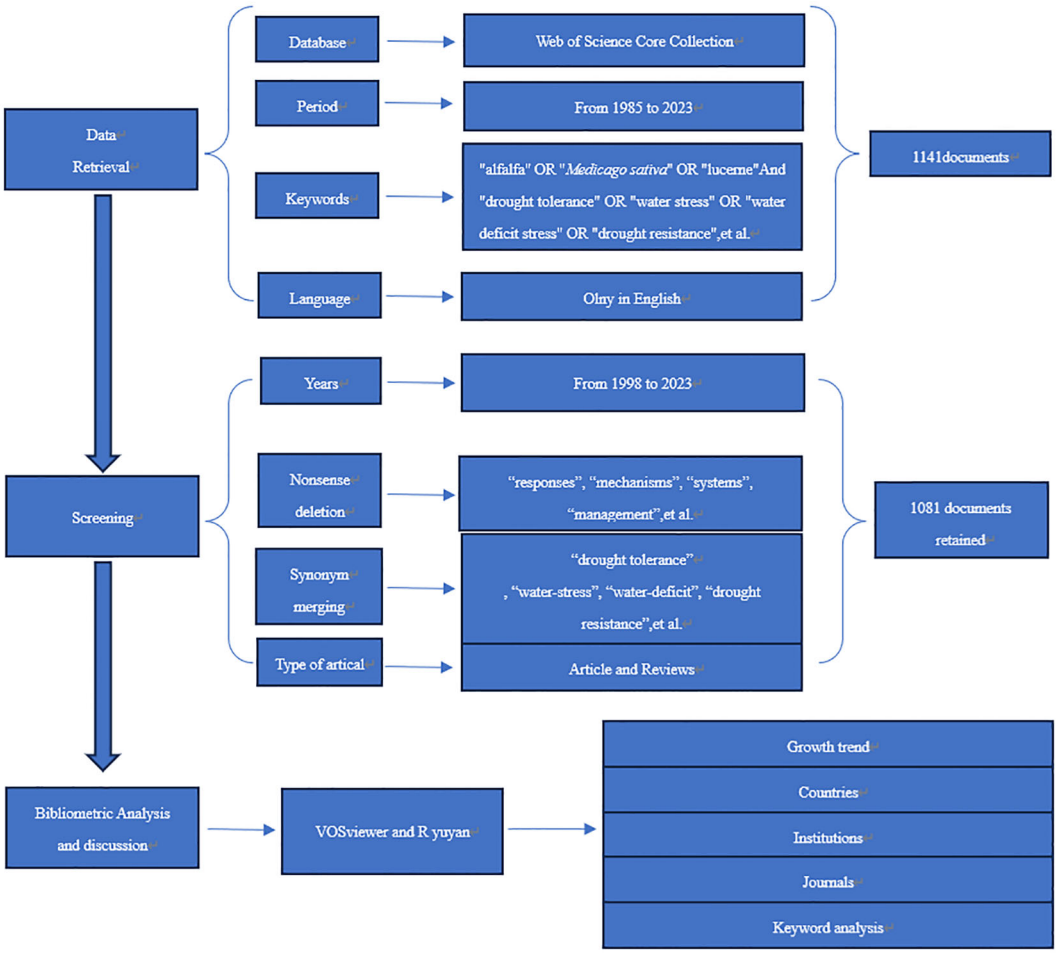

FIGURE 1
The research flowchart of present study.

conducted using VOSviewer (version 1.6.11) from the bibliometrics software suite, and R (version 4.3.0) for bibliometric analysis and visualization. The key operation involved using VOSviewer to extract information regarding the top 20 countries, institutions, and journals by publication count, the top 30 most cited documents, and the top 20 most recurrent Keywords Plus. Additionally, we created knowledge domain maps depicting co-authorship among countries, institutional coupling, journal co-citations, and keyword co-occurrences. In R, we generated knowledge domain maps for thematic keywords and trend topics. Given that author keywords tend to be highly subjective and lack statistical uniformity (Qin et al., 2021), we opted for Keywords Plus for its superior accuracy, authoritative nature, and statistical relevance for data analysis and visualization in this study.

3 Results

3.1 The publication trends

The temporal progression of publications pertaining to alfalfa drought research over the period from 1998 to 2023 is depicted in Figure 2. An examination of the figure shows a fluctuating increase in the number of related publications. However, there is an observable downward trend in the average total number of citations per article. This decline is likely attributable to factors such as the varying quality of article content and the timing of their publication. The coefficient of determination, R^2, is used to quantify the fit of the trend line representing changes in publication numbers over this 26-year span. In this study, R^2 is calculated to be 0.949. This high value suggests a strong correlation and indicates that the trend line closely aligns with the actual data, reflecting a significant and consistent increase in the volume of publications on alfalfa drought research over the studied period.

3.2 Countries and institutions distribution for the literature

The distribution and collaboration dynamics among countries and regions engaged in alfalfa drought research are elucidated in Figure 3. This figure, coupled with the data in Supplementary Table S1, displays the number of articles published by each of the 53 participating countries and the nature of their collaborative

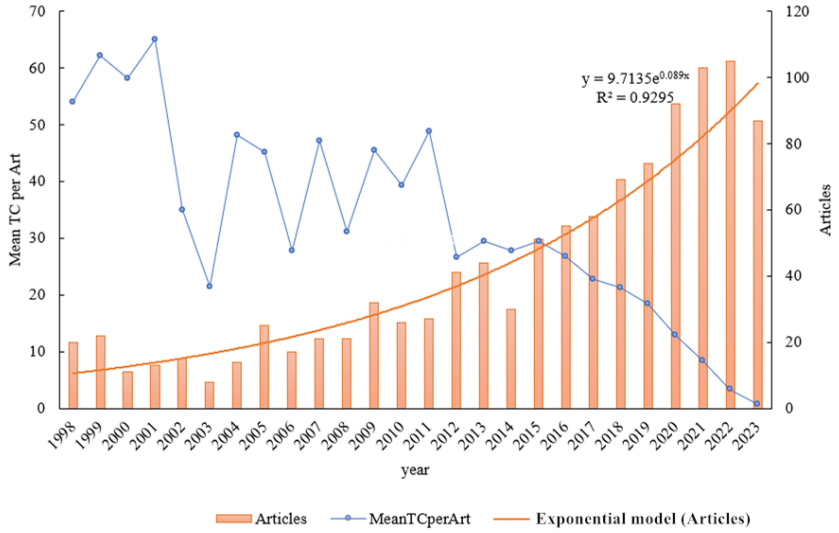

FIGURE 2
Temporal evolution of publications on drought stress in *Medicago sativa* from 1998 to 2023. Mean TC per Art -mean total citation per article.

relationships. China emerges as the most prolific contributor, with the largest node representing its 385 published articles. Furthermore, China has established collaborative ties with 26 countries, showcasing its closest collaboration with the United States (indicated by the highest Total Link Strength, TLS), followed by Australia. The United States, represented as the second largest node, has published 213 articles and has fostered collaborations with 39 countries. Overall, while China leads in article contribution, the United States demonstrates a broader spectrum of international collaborations. This suggests that China, despite its significant contributions, could benefit from enhancing its international cooperative efforts in this research domain.

The analysis of institutional collaboration offers insights into organizational contributions and inter-institutional interactions within this research topic. Figure 4, integrated with the details in Supplementary Table S2, presents an inter-institutional literature coupling analysis through scientific knowledge mapping. The Chinese Academy of Agricultural Sciences (CAAS) stands out as not only the most productive institution, with 64 publications, but also as a central node in the field, exhibiting the widest range of domain relationships (with the largest TLS). Following CAAS, the Chinese Academy of Sciences (CAS) with 59 publications and Lanzhou University (LU) with 56, both based in China, play significant roles in the scholarly network. The predominance of Chinese institutions in alfalfa drought research, as depicted in

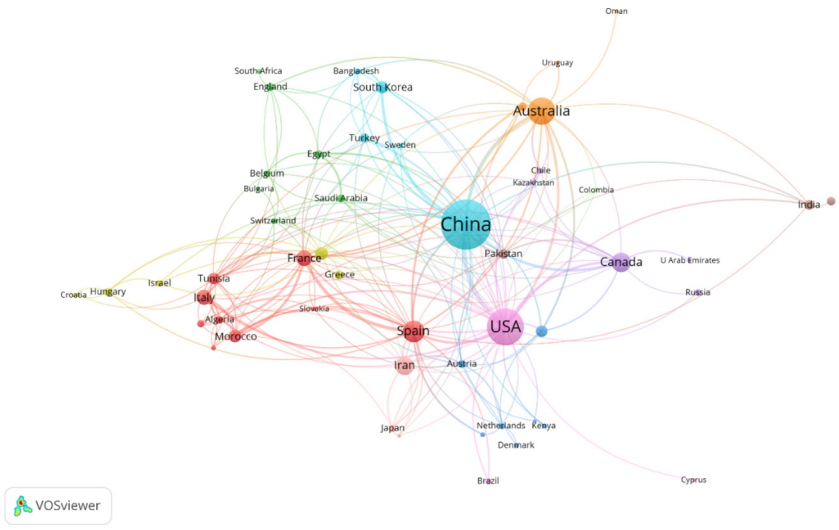

FIGURE 3
Mapping knowledge domain of co-authoring countries/regions on drought stress in *Medicago sativa* (frequency ≥ 2).

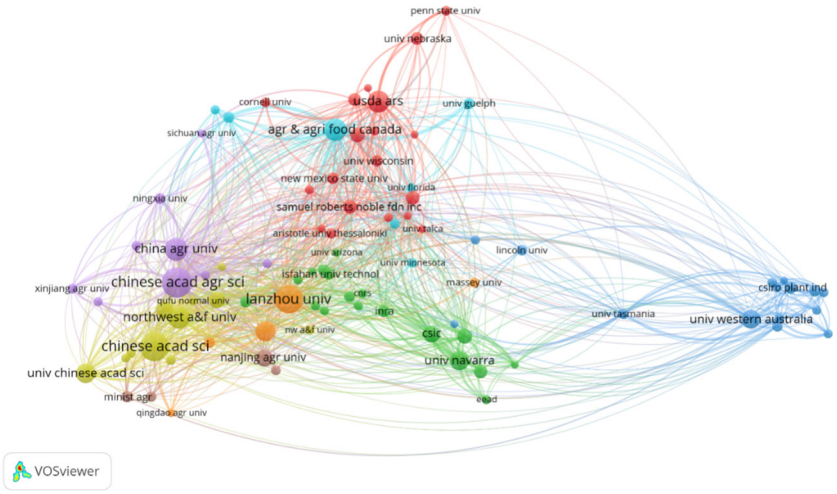

FIGURE 4
Bibliographic coupling analysis of institutions on drought stress in *Medicago sativa* (frequency ≥ 5).

Figure 4, aligns with the trends observed in the country-based analysis in Figure 3. This highlights China's leading position in the research of drought stress in *Medicago sativa*.

3.3 Main journals and most impacting papers

In this study, co-citation analysis was specifically applied to source journals to identify the core journals and foundational knowledge in alfalfa drought research. Figure 5, along with Supplementary Table S3, illustrates the knowledge map of 261 journal co-citations, shedding light on the scientific interrelationships among these journals. The largest node, representing Plant Physiology (plant physiol), with 2,268 citations, emerges as the most cited journal in this research area. Following closely are the Journal of Experimental Botany (j exp bot) with 1,313 citations and Crop Science (crop sci) with 1,272 citations. The thickest line between Plant Physiology and the Journal of Experimental Botany denotes the strongest co-citation link, indicating that articles from these two journals are frequently cited together. Similarly, notable connections are observed between Plant Journal (plant j) and Plant Cell. These four journals, especially Plant Physiology, hold significant citation counts, marking them as key publications in the field of alfalfa drought research. The division into four distinct clusters is evident: the green cluster, led by Plant Physiology, focuses on plant material metabolism, growth, and environmental interactions; the red cluster, anchored by Plant and Soil (plant soil), delves into plant biology and soil science interactions; the blue cluster, centered on Crop Science, investigates crop genetics and breeding; and the yellow cluster, headed by the Journal of Experimental Botany, publishes work on sustainable food, fuel, and renewable materials production. To summarize, alfalfa drought research predominantly features in specialized journals across plant science, environmental science, soil science, and crop science, as well as related agribusiness journals.

From the dataset, a total of 3,821 authors contributed to 1,081 articles on alfalfa drought tolerance. The top 30 cited articles were selected for a focused bibliometric analysis to determine their influence

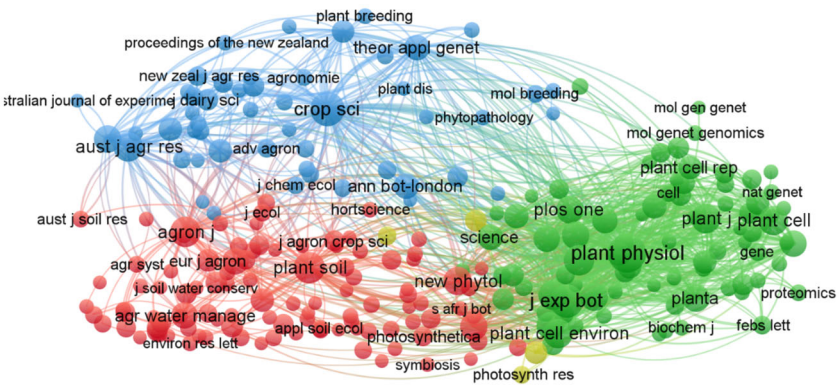

FIGURE 5
Mapped knowledge domains for journal co-citation on drought stress in *Medicago sativa* (frequency ≥ 30).

on the development of alfalfa drought research. The most cited article, with 312 citations, is by Zhang, JY et al., published in Plant Journal in 2005. The next two highly cited works are by Aranjuelo, Iker et al., in the Journal of Experimental Botany (2011), and by Antoniou, Chrystalla et al., in the Journal of Pineal Research (2011). These articles are highly recognized in the field, as evidenced by their citations. The analysis indicates that alfalfa drought research primarily involves collaborations among domestic institutions, highlighting a need for enhanced international collaboration.

According to the content of these 30 papers (Table 1), the research involving the field of drought stress in alfalfa was found to include: (1) Studies on the morphology of alfalfa leaves, epidermal wax content,composition or structure, and alkane content under drought stress; (2) Adaptation to drought by photosynthesis, osmotic regulation, antioxidant defense, and administration of exogenous hormones to the plant; (3) Excavation and identification of drought-resistant functional genes, and transgenic alfalfa research; (4) Variety selection and molecular breeding using molecular marker technology, etc.; (5) Irrigation, planting time adjustment, nutrient management and seed initiation in alfalfa. In general, it is morphology, physiology and biochemistry, molecular response mechanism research and drought-resistant breeding and cultivation technology research.

3.4 Research hotspots and evolution trend analysis

3.4.1 Topic keywords map and hotspot evolutionary trend

The co-occurrence of keywords in literature offers a gateway to understanding the intrinsic connections, research hotspots, and predictive development trends in an academic field. Table 2 shows the top 20 most frequently occurring keywords in the field of alfalfa drought tolerance research. Notably, “drought stress” and “*medicago-sativa*” are predominant, followed by “plant-growth” and “yield”. This pattern indicates the significant impact of drought on both the growth and yield of alfalfa, highlighting the importance of understanding the drought-plant growth-yield relationship as a foundational research area in this field. The

TABLE 1 Top 30 publications with the most citations on drought stress in *Medicago sativa*.

Rank	Title	Journal	Year	TC[c]	IF	CN	ON
(1)	Overexpression of *WXP1*, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*)	Plant Journal	2005	312	7.2	1	1
(2)	Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa L.*)	Journal Of Experimental Botany	2011	197	6.9	2	3
(3)	Melatonin systemically ameliorates drought stress-induced damage in *Medicago sativa* plants by modulating nitro-oxidative homeostasis and proline metabolism	Journal Of Pineal Research	2017	195	10.3	1	2
(4)	The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants	Plant Physiology	2007	160	7.4	2	3
(5)	MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa*) by silencing SPL13	Plant Science	2017	136	5.2	1	2
(6)	Biomass partitioning, morphology and water status of four alfalfa genotypes submitted to progressive drought and subsequent recovery	Journal Of Plant Physiology	2010	116	4.3	3	3
(7)	Effects of water stress on antioxidant enzymes of leaves and nodules of transgenic alfalfa overexpressing superoxide dismutases	Physiologia Plantarum	2002	111	6.4	2	3
(8)	System responses to long-term drought and re-watering of two contrasting alfalfa varieties	Plant Journal	2011	103	7.2	1	1
(9)	Response of alfalfa to putrescine treatment under drought stress	Biologia Plantarum	2006	96	1.5	1	3
(10)	Physiological and proteomic responses of contrasting alfalfa (*Medicago sativa L.*) varieties to PEG-Induced osmotic stress	Frontiers In Plant Science	2018	77	5.6	1	1
(11)	Soil water storage deficit of alfalfa (*Medicago sativa*) grasslands along ages in arid area (China)	Field Crops Research	2018	73	5.8	1	4

(Continued)

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TABLE 1 Continued

Rank	Title	Journal	Year	TC[c]	IF	CN	ON
(12)	The interplay between *miR156/SPL13* and *DFR/WD40–1* regulate drought tolerance in alfalfa	BMC Plant Biology	2019	68	5.3	2	3
(13)	Application of sewage sludge improves growth, photosynthesis and antioxidant activities of nodulated alfalfa plants under drought conditions	Environmental And Experimental Botany	2010	67	5.7	1	2
(14)	Drought tolerance in alfalfa (*Medicago sativa L.*) varieties is associated with enhanced antioxidative protection and declined lipid peroxidation	Journal Of Plant Physiology	2019	65	4.3	1	1
(15)	Improved drought stress response in alfalfa plants nodulated by an IAA over-producing *Rhizobium Strain*	Frontiers In Microbiology	2017	54	5.2	2	4
(16)	Water use efficiency, transpiration and net CO_2 exchange of four alfalfa genotypes submitted to progressive drought and subsequent recovery	Environmental And Experimental Botany	2011	54	5.7	3	3
(17)	Exploring the potential of nitric oxide and hydrogen sulfide (NOSH)-releasing synthetic compounds as novel priming agents against drought stress in *Medicago sativa* plants	Biomolecules	2020	53	5.5	2	3
(18)	Comparative physiological and transcriptional analyses of two contrasting drought tolerant alfalfa varieties	Frontiers In Plant Science	2016	52	5.6	2	2
(19)	Identification of loci associated with drought resistance traits in heterozygous autotetraploid alfalfa (*medicago sativa L.*) using genome-wide association studies with genotyping by sequencing	PLoS One	2015	51	3.7	1	2
(20)	Influence of arbuscular Mycorrhizae and *Rhizobium* on free polyamines and proline levels in water-stressed alfalfa	Journal Of Plant Physiology	1998	48	4.3	2	2
(21)	Influence of drought stress on afalfa yields and nutritional composition	PLoS One	2018	39	5.3	1	3
(22)	Maximizing productivity and water use efficiency of alfalfa under precise subsurface drip irrigation in arid regions	Irrigation And Drainage	2013	39	1.9	2	3
(23)	Physiological and morphological traits associated with adaptation of lucerne (*Medicago sativa*) to severely drought-stressed and to irrigated environments	Annals Of Applied Biology	2013	39	2.6	1	1
(24)	Transcriptome analysis of microRNA156 overexpression alfalfa roots under drought stress	Scientific Reports	2018	36	4.6	2	2
(25)	Physiological and biochemical changes in different drought-tolerant alfalfa (*Medicago sativa L.*) varieties under PEG-induced drought stress	Acta Physiologiae Plantarum	2018	36	2.6	1	2
(26)	Effects of water deficit on growth, nodulation and physiological and biochemical processes in *Medicago sativa*-rhizobia symbiotic association	Arid Land Research And Management	2016	36	1.4	2	3
(27)	Effects of engineered *sinorhizobium meliloti* on cytokinin synthesis and tolerance of alfalfa toextreme drought stress	Applied And Environmental Microbiology	2012	35	4.4	1	1

(Continued)

TABLE 1 Continued

Rank	Title	Journal	Year	TC[c]	IF	CN	ON
(28)	Seed osmopriming improves plant growth, nodulation, chlorophyll fluorescence and nutrient uptake in alfalfa (*Medicago sativa L.*) - rhizobia symbiosis under drought stress	Scientia Horticulturae	2016	34	4.3	1	3
(29)	Concerted changes in N and C primary metabolism in alfalfa (*Medicago sativa*) under water restriction	Journal Of Experimental Botany	2013	33	5.3	3	5
(30)	Leaf cuticular waxes and physiological parameters in alfalfa leaves as influenced by drought	Photosynthetica	2012	32	2.7	2	2

c, Total Citations; IF-Impact Factors; d, Avg. citations; e, Links; f, Total Link Strength; CN, Cooperative Nations Number; ON, Cooperative Organizations Number.

presence of "gene-expression" as a key keyword reflects the focus on genetic regulation as a means for alfalfa to combat drought stress. The inclusion of "arabidopsis" suggests its use as a model organism for gene function, genetics research, and related areas, pointing towards transgenic research in alfalfa as a current research hotspot. Based on these keywords, the current research in alfalfa drought tolerance encompasses (1) Enhancing plant growth and crop yield, and (2) Transgenic research, physiological and biochemical response mechanisms, and nutrient management as key research hotspots.

Keyword clustering analysis elucidates the relationships between keywords, revealing research hotspots. The visualization of this analysis in Figure 6 identifies 12 core clusters in alfalfa drought resistance research: (1) Red cluster focuses on alfalfa yield, plant physiological responses to drought, and water resource management. (2) Green cluster delves into alfalfa growth and forage quality studies. (3) Blue cluster explores alfalfa seedling drought resistance using polyethylene glycol, weed control, and regeneration management techniques. (4) Yellow cluster investigates stress signal transduction related protein genes

TABLE 2 Top 20 most frequently used keywords on drought stress in *Medicago sativa*.

Rank	Keyword Plus	Cluster	Occurrences	Links	TLS[f]
1	drought stress	1	555	302	2416
2	*medicago-sativa*	2	317	272	1425
3	plant-growth	9	203	194	911
4	yield	2	190	187	778
5	gene-expression	1	172	146	828
6	arabidopsis	1	105	108	516
7	nitrogen-fixation	7	65	105	341
8	abscisic-acid	1	63	104	336
9	oxidative stress	11	53	86	265
10	proline	1	50	85	268
11	photosynthesis	6	49	84	272
12	CO_2	5	48	88	254
13	water-use efficiency	3	47	91	213
14	n-fertilization	6	45	104	218
15	leaf	10	42	85	203
16	gas-exchange	1	41	76	184
17	transcription factors	7	38	78	210
18	root	8	37	74	173
19	lipid-peroxidation	1	36	63	185
20	chlorophyll	8	28	61	155

f, Total Link Strength.

involving Ca^{2+} as a second messenger molecule. (5) Purple cluster covers inter-root microbial research. (6) Light blue cluster relates to alfalfa in animal husbandry production and irrigation management. (7) Orange cluster deals with symbiotic nitrogen fixation, crossbreeding, and related transcription factors. (8) Brown cluster examines antioxidant defense mechanisms during water stress. (9) Aqua red cluster focuses on genetic diversity analysis in agronomic traits. (10) Light brown cluster investigates osmotic regulation and related gene regulation during water deprivation. (11) Light green cluster examines drought-resistant morphological characteristics and environmental interactions. (12) Gray cluster studies photosynthesis and leaf morphology adaptations to drought stress.

A comprehensive analysis of the results in Table 2 and Figure 6 underscores two main research aspects: alfalfa's response mechanisms to drought and drought-resistant technology research. These findings align with the conclusions derived from the analysis of the top 30 most influential papers, indicating a consistent focus within the field.

3.4.2 Identification of research frontiers

Figure 7 elucidates the categorization of research themes in the context of alfalfa drought resistance. The lower right quadrant, termed 'Basic Themes,' encompasses physiological and biochemical responses. These themes are pivotal to the field yet underdeveloped, signaling a need for further investigation. Contrarily, the upper right quadrant, 'Motor Themes,' comprises well-established and significant keywords, corroborating the findings presented in Table 2. 'Niche Themes' occupy the upper left quadrant, featuring keywords such as "drought stress," "gene-expression," "arabidopsis," "abscisic-acid," and "oxidative stress." Although their significance and developmental extent are marginally lower than those in the 'Motor Themes,' their importance remains substantial. The lower left quadrant represents 'Emerging or Declining Themes,' with keywords like "down-regulation" and "transgenic alfalfa." The inclusion of Figure 8 in the analysis aids in further delineating these research frontiers.

The 'Trend Topics' section facilitates a visual analysis and projection of research focal points. A notable concentration of research activity, represented by the largest nodes, occurred between 2017 and 2019, particularly in areas such as "drought stress" and "gene-expression." This trend highlights the significant attention devoted to these topics. Furthermore, the emergence of keywords like "regulator," "bacteria," and "gene-expression" up until 2022 suggests their potential as persistent research interests. Likewise, the appearance of terms such as "microbial biomass," "genome-wide identification," and "encodes" up to 2023 indicates their likelihood as future research directions. Cross-referencing Figures 7 and 8 reveals that the themes in the lower left quadrant of Figure 7 are gaining traction as emerging research areas. In conclusion, the current research frontiers in alfalfa drought resistance encompass gene expression studies, investigations into related microorganisms, and transgenic alfalfa research.

4 Discussion

Climate change, food shortages, water scarcity and population growth are some of the threatening challenges facing the world today. Drought stress (DS) is an ongoing challenge to agricultural crops and has been recognized as a serious constraint to global agricultural productivity, and its intensity and severity are expected to increase in the near future. Over the past two decades, the effects of drought stress on crop yield, growth and quality have increasingly become a major environmental issue (Wu et al., 2017) and a major limiting factor in alfalfa production (Ezura et al., 2015). Therefore, it is crucial to grasp the current status, frontiers and future research directions of the effects of drought stress on alfalfa in order to further improve

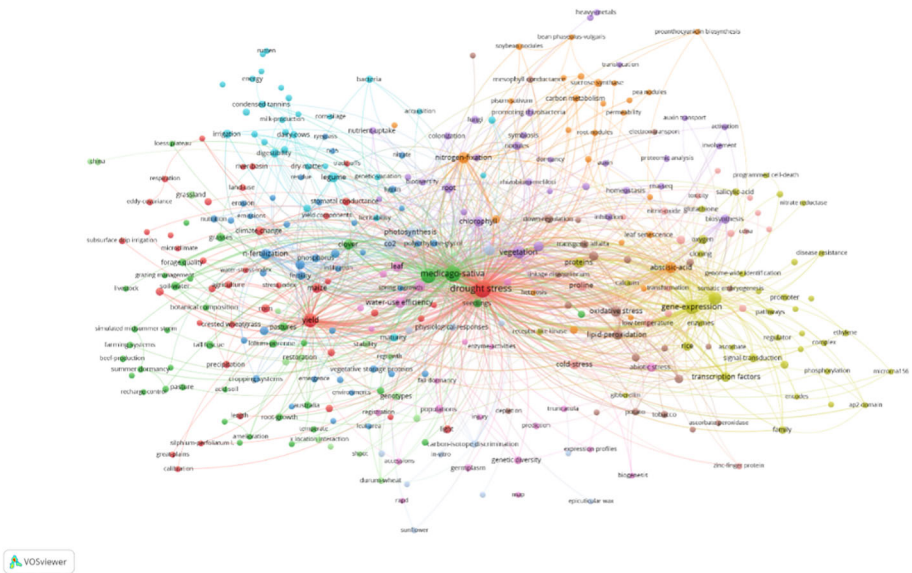

FIGURE 6
Knowledge domain map of the keywords co-occurrence network (frequency ≥3).

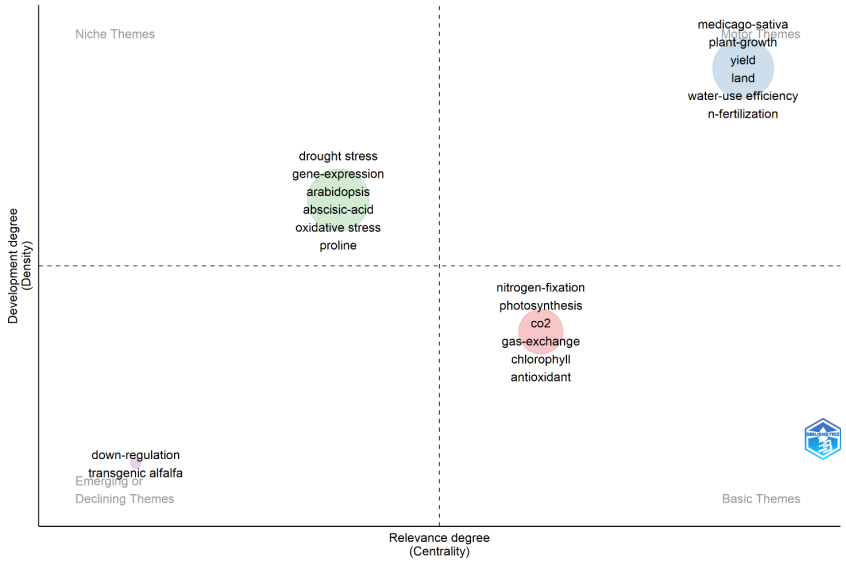

FIGURE 7
Thematic keywords on drought stress in *Medicago sativa* (frequency≥8).

alfalfa drought tolerance. In this paper, we collected and analyzed all the English academic publications on drought tolerance of alfalfa in the past 26 years for "article" and "review" by bibliometric analysis. Through bibliometric analysis, this study found that alfalfa drought stress research mainly includes two directions: response mechanism research and exploration of drought-resistant technology.

4.1 Drought resistance mechanisms

4.1.1 Morphological, physiological and biochemical responses to drought resistance mechanisms

Under drought conditions, alfalfa undergoes significant morphological adaptations to sustain growth and development, particularly in its leaves and root system. Firstly, leaves, being the primary interface with the external environment, are highly responsive to environmental changes. Drought stress alters leaf morphology in alfalfa, affecting metrics such as leaf area ratio (LAR), specific leaf area (SLA), and leaf weight ratio (LWR). SLA significantly decreases with decreasing moisture content (Gu and Wan, 2021). These changes in SLA or LAR can be instrumental in identifying drought-resistant alfalfa varieties (Erice et al., 2010). Secondly, the root system, a vital component of the soil-plant-atmosphere continuum, exhibits a positive correlation with drought tolerance. It plays a crucial role in adapting to drought-stressed environments. Under drought stress, as soil moisture diminishes, there is an increase in both the volume and length of alfalfa's root system, and a concurrent decrease in root diameter. This results in an expanded root-soil contact area, enhancing the plant's capacity

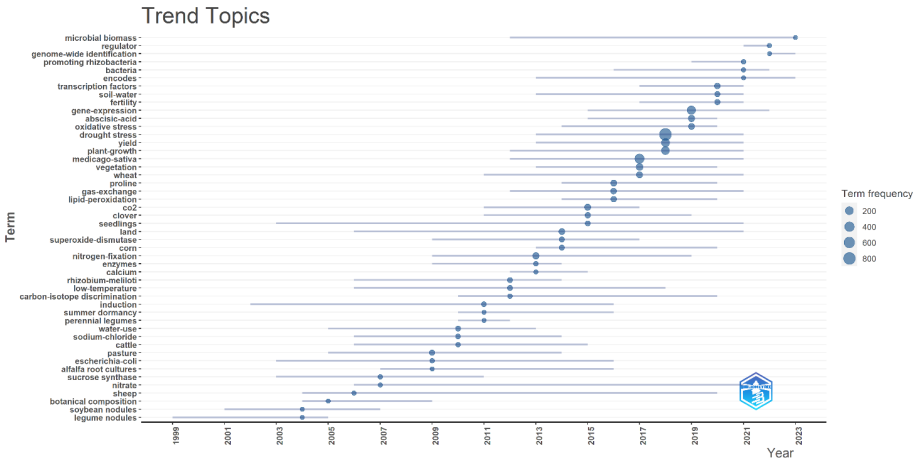

FIGURE 8
Keywords with the strongest occurrence bursts (frequency≥5).

to absorb and utilize water from deeper soil layers (Zhu et al., 2008). Recent studies have demonstrated that larger leaves and robust roots during drought contribute to the accumulation of higher biomass yield (Prince et al., 2022). Moreover, the root-crown ratio in alfalfa is particularly sensitive to drought stress. An increase in this ratio under water stress conditions is indicative of a reduction in water consumption and an enhancement in water uptake (Erice et al., 2010), serving as a significant morphological index for assessing alfalfa's drought resistance. The root-crown ratio stress index further reflects the drought resistance performance of alfalfa (Ma et al., 1993; Han et al., 2006).

In response to water deficits, alfalfa employs a range of physiological and biochemical strategies to mitigate drought stress. These responses can be categorized into five primary aspects.

(1) **Water metabolism:** Under drought conditions, there is a notable decrease in alfalfa's leaf water potential and relative water content (RWC), along with an increase in water saturation deficit. Varieties with robust drought resistance exhibit a more pronounced decrease in leaf water potential, a lesser rise in water saturation deficit, and a smaller decline in RWC (Han et al., 2008; Ren and He, 2010), thus maintaining higher aboveground RWC and water use efficiency (WUE) (Buddhaboon et al., 2011; Anower et al., 2015). Furthermore, leaf surface cuticle waxes play a pivotal role in enhancing plant water use efficiency during water deficits by minimizing cuticle water loss (Samdur et al., 2003). It is observed that the wax content is more sensitive to drought treatments than the total wax content (Ni et al., 2012), and an increase in alkane content is particularly significant in improving drought tolerance, serving as a potential indicator for selecting drought-resistant alfalfa varieties (Guo et al., 2011; Ni et al., 2012). The introduction of chitosan seeds can effectively regulate the water use process and improve the yield and quality of alfalfa (Mustafa et al., 2022).

(2) **Photosynthesis**: Drought stress adversely affects chlorophyll biosynthesis while accelerating its catabolism, resulting in a substantial reduction in chlorophyll content and consequently inhibiting photosynthesis (Fang and Xiong, 2014). Drought conditions have been shown to decrease chlorophyll a and b, as well as photochemical efficiency in alfalfa, along with a significant increase in leaf MDA and proline contents (Fan et al., 2014). A decrease in leaf water potential to -2.8 MPa inhibits photosynthesis and reduces CO_2 utilization due to stomatal closure (Irigoyen et al., 1992).

(3) **Compatible solutes and osmoregulation:** Research focusing on the accumulation of osmoregulatory substances in alfalfa under drought stress predominantly centers around organic osmoregulators such as proline, betaine, soluble protein (SP), and soluble sugar (SS) (Slama, 2013; Yang et al., 2013). These substances tend to increase with the duration and intensity of the drought (Yu et al., 2006; Li et al., 2007; Huo, 2010). Studies have indicated that drought-tolerant alfalfa cultivars accumulate higher levels of osmoregulatory substances compared to drought-sensitive varieties (Kang et al., 2011).

(4) **Antioxidant Defense:** Alfalfa's adaptability to drought stress is closely linked to its antioxidant capacity. The activities of antioxidant enzymes (such as SOD, POD, and CAT) are significantly enhanced under drought stress, contributing to the plant's increased resistance to drought (Han et al., 2005; Li et al., 2007; Huo, 2010; Quan et al., 2016). Studies have demonstrated that drought-resistant alfalfa cultivars exhibit lower electrolyte conductivity and MDA content under drought conditions, suggesting stronger resistance to oxidative stress (Hartung et al., 2001; Boldaji et al., 2011). Maghsoodi et al. confirmed a significant positive correlation between grass yield and peroxidase (POX), and a significant negative correlation with MDA, indicating that grass yield can serve as a marker for selecting drought tolerant varieties of alfalfa (Maghsoodi et al., 2017).

(5) **Plant endogenous (exogenous) hormones:** Abscisic acid (ABA) is a critical stress hormone playing a significant role in the plant's response to drought stress (Hartung et al., 2001). ABA regulates various physiological processes, including stomatal closure and the modulation of leaf expansion and cell division rates (Schachtman and Goodger, 2008; Tardieu, 2013). Research has shown that the ABA content in alfalfa varies between roots and leaves under drought conditions and is influenced by the duration of the stress (Ren and He, 2010; Gamal et al., 2016). The plants that were not sensitive to ABA during germination showed stronger drought tolerance (Xu et al., 2024). Additionally, the accumulation of ABA in alfalfa under drought stress is related to the dynamics of storage proteins, gene expression, and the accumulation of certain osmoregulatory substances in the primary roots (Li et al., 2015).Overall, these physiological and biochemical responses collectively enhance alfalfa's ability to withstand drought conditions, highlighting the complexity and adaptability of this crop in response to water stress.

4.1.2 Molecular responses to drought resistance mechanisms

In recent years, owing to the extensive application of molecular biology technology (Huang et al., 2022; Zhao et al., 2022a; Zhao et al., 2022b), research on alfalfa drought resistance has progressed to the molecular level, such as identification of relevant stress genes, transcriptomics, metabolomics, and proteomics (Zhang et al., 2014; Li et al., 2021). The primary focus of this molecular investigation is the study of functional genes (proteins) associated with drought resistance in alfalfa. Transcription factors have emerged as key players in the response of alfalfa to drought stress (Zhao et al., 2022c). MicroRNA156 (miR156), for instance, regulates the Squamosa promoter binding protein (SPL) gene family, which acts as transcription factors that subsequently modulate the

expression of downstream genes, thus regulating various plant growth and developmental networks (Cardon et al., 1999). Arshad and colleagues (Arshad et al., 2017) conducted experiments to explore the role of miR156d in alfalfa's response to drought stress. Their findings revealed that, in comparison to the wild-type control (WT), alfalfa genotypes overexpressing miR156 (referred to as miR1560E) exhibited significantly higher drought tolerance. The miR1560E genotype not only displayed increased survival rates and reduced water loss but also maintained higher stomatal conductance, enhanced accumulation of compatible solutes (e.g., proline), and elevated levels of abscisic acid (ABA) and antioxidants during drought stress, when compared to the WT. Similarly, alfalfa plants with reduced expression of miR156-targeted SPL13 demonstrated decreased water loss, enhanced stomatal conductance, elevated chlorophyll content, and improved photosynthetic assimilation. These results suggest that miR156 enhances drought tolerance in alfalfa, partially by suppressing the expression of SPL13. Expression analysis of transcription factor genes related to the endoplasmic reticulum stress signaling pathway in alfalfa confirmed that DTT treatment reduced the functionality of bZIP60 and bZIP28 genes involved in the ER stress pathway. The most critical time for plants to tolerate drought (PEG) is the 8th hour after treatment, during which bZIP60 plays a more active role than bZIP28 in the stress pathway; the leaf tissues were more affected than the root tissues (Oğuz et al., 2022).

Transcriptomics reflect the transcriptional expression and regulation of genes in different genotypes across specific cells, tissues, or organisms under various adversities and developmental stages, thereby identifying candidate genes and revealing interactions among different gene regulatory pathways (Mutz et al., 2013; Palovaara et al., 2013). Feyissa et al. further demonstrated that the miR156/SPL13 module alleviates drought stress in alfalfa through tissue-dependent regulatory molecules and physiological processes (Feyissa et al., 2020). Feyissa et al. further demonstrated that the miR156/SPL13 module alleviates drought stress in alfalfa through tissue-dependent regulatory molecules and physiological processes (Wan et al., 2021). Feyissa et al. further demonstrated that the miR156/SPL13 module alleviates drought stress in alfalfa through tissue-dependent regulatory molecules and physiological processes (Fang et al., 2023). Moreover, the sensitivity of alfalfa seeds to ABA is heritable, and screening for ABA during seed germination can help select alfalfa lines with better drought tolerance (Xu et al., 2024).

Metabolomics studies aim to identify specific traits associated with stress tolerance by examining the molecular phenotypes of plants under abiotic stress. Feyissa and colleagues (Feyissa et al., 2019) further elucidated the regulation of drought stress in alfalfa by coordinating gene expression with metabolites and physiological strategies involving miR156/SPL13 and WD40–1/DFR. Modest levels of miR156 overexpression led to the suppression of SPL13 and an increase in WD40–1, thereby fine-tuning DFR expression to enhance anthocyanin biosynthesis. This, in conjunction with the accumulation of other stress relief metabolites and physiological responses, contributed to improved drought tolerance. Dominant classes of differential metabolites include amino acids, organic acids, sugars, and alkaloids, such as 6-gingerol, salicylic acid (SA), indole-3-acetic acid (IAA), gibberellin A4 (GA4), abscisic acid (ABA), trans cinnamic acid, sucrose, L-phenylalanine, L-tyrosine, succinic acid, and nicotinic acid, essential for drought stress tolerance in alfalfa (Wang et al., 2024).

Proteomics studies have increasingly reported on the response of alfalfa to drought stress. A total of drought stress-responsive proteins that play a role in a variety of cellular functions in the alfalfa root system were identified in the study by Rahman and colleagues (Rahman et al., 2016). These functions include energy metabolism, signaling pathways, antioxidant mechanisms, stress defense mechanisms, transcriptional and translational processes, regulation of reactive oxygen species (ROS), abscisic acid (ABA) biosynthesis, calcium signaling and storage processes. Ma et al. investigated the proteomic changes during the germination stage of Zhongmu NO.3 seeds under 200 $mol{\cdot}L^{-1}$ NaCl and 180 $g{\cdot}L^{-1}$ PEG stress, identifying 17 differentially abundant proteins (DAPs) mainly involved in defense responses, energy metabolism, protein synthesis and degradation, oxidative stress, and carbohydrate metabolism. Osmotic stimulation, used as a pretreatment, accelerates germination and improves the uniformity of seedling growth (Ma et al., 2017).

4.2 Drought-resistant technologies include breeding and cultivation

Another critical aspect of alfalfa drought resistance research involves the exploration of drought-resistant technologies, encompassing both breeding and cultivation techniques. Given the looming threat of global warming, drought stands as a major limiting factor for present and future alfalfa production, emphasizing the necessity of developing drought-resistant alfalfa varieties.

4.2.1 Breeding techniques for drought resistance

Currently, conventional techniques continue to dominate the breeding of alfalfa varieties, with methods such as selective breeding and crossbreeding being the prevalent approaches. Simultaneously, molecular techniques have been progressively integrated into alfalfa breeding strategies. Selection breeding comprises various methods, including single selection, mixed selection, modified mixed selection, group selection, and rotational selection. For instance, Li et al (Li et al., 2011)successfully bred the drought- and cold-resistant Longmu 808 alfalfa by employing single-plant selection within the original population of Longmu 803 alfalfa, a process involving the selection of the best specimens while eliminating the least suitable.

Hybrid breeding, on the other hand, involves crossing individuals from different populations and genotypes to generate pure varieties through selection among their hybrid progeny. Notable Chinese registered varieties, such as Caoyuan No.1, Caoyuan No.2, and Gannong No.1, were bred through crosses between the tetraploid subsp. sativa and the diploid subsp. falcata (Shi et al., 2017). However, achieving a stable and mature variety through hybrid breeding requires multiple generations of backcrossing and faces challenges similar to standard crossbreeding.

An alternative approach is breeding based on male sterile lines, which offers a comprehensive method to produce genuine hybrids but is hampered by the difficulty of generating and maintaining stable male sterile lines. The CAU group employed a reverse genetic strategy, leveraging genome editing tools, to create genetically male sterile alfalfa and corresponding maintenance lines (Ye et al., 2022). Following ten generations of crossings between male sterile lines and maintenance lines, transgene-free male sterile lines were obtained, which can serve as parent materials for hybrid seed production.

Overcoming the challenge of cultivating purebred parents in alfalfa breeding is complicated due to its partial self-incompatibility and inbreeding suppression, whose molecular basis remains unclear. Molecular breeding, a contemporary approach, facilitates the rapid, stable, and directed creation of new varieties or species at the molecular level (Cai and Wang, 2003). As alfalfa genomics continues to advance, molecular marker technology can pinpoint drought-resistant genes or genes closely associated with drought resistance in alfalfa. This enables the swift identification of drought-tolerant varieties, shortening the breeding cycle and, in theory, reducing costs (Charles and Yu, 2018). However, its practical implementation hinges on the accuracy of marker data and thorough validation, particularly considering alfalfa's tetraploid nature and complex genetic characteristics.

In tandem with the progress in genetic engineering, transgenic technology has emerged as a novel avenue for enhancing alfalfa's drought resistance and improving variety traits. Zhang and colleagues (Zhang et al., 2005) reported the successful activation of alfalfa's waxy production and the conferment of drought tolerance through the introduction of the WXP1 gene from the model legume *Tribulus terrestris*. This resulted in a notable increase in leaf wax accumulation and enhanced drought tolerance in transgenic alfalfa.

Additionally, Nie et al. (Nie et al., 2017) observed increased proline accumulation in alfalfa through the overexpression of the AmDHN gene, a change that potentially induces the expression of genes related to the proline synthesis pathway, ultimately bolstering drought tolerance. Fehér-Juhász and colleagues (Fehér-Juhász et al., 2013) demonstrated that ectopic expression of the GsWRKY20 gene led to increased osmoregulatory substance accumulation, improved water-holding capacity, decreased membrane permeability, reduced MDA content, and enhanced drought tolerance in transgenic alfalfa. Mckersie and colleagues (McKersie et al., 2000) transplanted the Mn-SOD gene from blue snowy tobacco into alfalfa using *Agrobacterium*, with field experiments confirming the significant enhancement of cold and drought resistance in transgenic alfalfa plants.

These advancements in breeding and biotechnological approaches hold promise for mitigating the impact of drought on alfalfa production, offering potential solutions for sustaining alfalfa cultivation in the face of global climate challenges.

4.2.2 Cultivation techniques for drought resistance

Cultivation techniques aimed at enhancing water stress resistance in alfalfa encompass several strategies. The first strategy involves water conservation and drought resistance, which includes techniques such as seed triggering (Li et al., 2011; Antoniou et al., 2020), the utilization of irrigation systems (Annicchiarico et al., 2012; Ismail and Almarshadi, 2013; Huang et al., 2018), and adjustments in the timing of product harvest (Liu et al., 2018). Furthermore, the traditional application of organic fertilizers and other materials like sludge serves to protect alfalfa nodules from oxidative stress (Antolín et al., 2010). The application of plant growth-regulating substances, such as melatonin (Cardon et al., 1999), and the utilization of beneficial bacterial strains, including cytokinin-engineered bacteria (Xu et al., 2012), have been shown to improve drought resistance in alfalfa.

Cultivation of alfalfa is not limited to field management, and the establishment of a suitable alfalfa standardized growth and development patterns and designing ideal cultivation practices for different ecological zones is imperative. Alfalfa can be cultivated either as a pure stand or in rotation or intercropping systems, and a rational model necessitates a balanced relationship with companion crops. This presents both advantages and challenges (Buddhaboon et al., 2011; Grieder et al., 2021; Peng et al., 2022; Xu et al., 2022; Wang et al., 2023).

To enhance drought resistance, the cultivation process incorporates selecting appropriate alfalfa varieties and applying effective nutrient management, weed control, and pest management. These elements are pivotal in achieving optimal harvests and are crucial for sustaining alfalfa production under drought conditions (Putnam, 2021; Undersander, 2021; Graham et al., 2022; Wang et al., 2022; Yin et al., 2022). Additionally, mechanization of the entire production process is essential for alfalfa cultivation (Putnam, 2021). This requires alfalfa plants with an upright growth habit and robust resistance to lodging.

5 Conclusions

In this study, using alfalfa or *Medicago sativa* etc. as keywords, 1141 documents were collected in Web of Science database and 1081 were screened for bibliometric study. The findings revealed a steady increase in alfalfa drought tolerance research through the publication of articles in recent years. China contributed the most articles in this field, with 385 publications, followed by the United States and Australia. Notably, the journal "Plant Physiology" published the highest number of articles related to alfalfa drought tolerance research, with a total of 37 articles and 2082 citations. Through an analysis of the top 30 cited literature and keywords, the study identified the key developments in alfalfa drought stress research. The current research hotspots center around understanding the mechanisms by which alfalfa responds to water deficit, encompassing morphology, physiology, biochemistry, and molecular responses. Additionally, there is a growing emphasis on enhancing drought-related technologies, including drought-resistant breeding and optimization of cultivation techniques. Among these hotspots, research on the physiological, biochemical, and molecular response mechanisms of alfalfa, as well as investigations into transgenic alfalfa and nutrient management (such as microbial fertilizers), are expected to remain significant and likely become even more prominent in future research endeavors. The primary goal of this

study is to provide theoretical guidance for alfalfa drought tolerance research and to contribute to the enhancement of alfalfa crop yield.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

ZZ: Formal analysis, Methodology, Software, Writing – original draft. JL: Formal analysis, Visualization, Writing – review & editing. YG: Visualization, Writing – review & editing. XW: Formal analysis, Visualization, Writing – review & editing. RW: Supervision, Visualization, Writing – review & editing. HH: Supervision, Visualization, Writing – review & editing. YZ: Supervision, Visualization, Writing – review & editing. LZ: Formal analysis, Supervision, Visualization, Writing – review & editing. PW: Formal analysis, Funding acquisition, Methodology, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1406256/full#supplementary-material

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Salicylic acid improves cowpea productivity under water restriction in the field by modulating metabolism

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Introduction: Salicylic acid has shown promise in alleviating water stress in cultivated plants. However, there is a lack of studies confirming its effectiveness in cowpea plants grown in field conditions. Therefore, this research aimed to evaluate the use of salicylic acid as a water stress mitigator in cowpea cultivars under different irrigation depths in field conditions.

Methods: Four cowpea cultivars (BRS Novaera, BRS Tapaihum, BRS Pujante, and BRS Pajeú) were subjected to different treatments: control (W100: 100% replacement of crop evapotranspiration – ETc), W50 (50% of ETc), W50+SA2 (50% of ETc + 276 mg L^{-1} of SA), and W50+SA4 (50% of ETc + 552 mg L^{-1} of SA). The treatments were combined in a 4×4 factorial scheme with three replications, arranged in a randomized block design.

Results: Water restriction had a negative impact on the water status, growth, gas exchange, and production of the cultivars while also leading to changes in the antioxidant metabolism and osmolyte concentration. The application of SA enhanced antioxidant activity and the synthesis of osmotic adjusters under stress conditions. The most effective concentration was 276 mg L^{-1} in stage R2 and 552 mg L^{-1} in stage V7, respectively. The BRS Pujante cultivar showed increased productivity under water restriction with SA application, while the BRS Tapaihum was the most tolerant among the cultivars studied.

Discussion: In summary, our findings underscore the importance of using SA to mitigate the effects of water restriction on cowpea cultivation. These discoveries are crucial for the sustainability of cowpea production in regions susceptible to

drought, which can contribute to food security. We further add that the adoption of new agricultural practices can enhance the resilience and productivity of cowpea as an essential and sustainable food source for vulnerable populations in various parts of the world.

KEYWORDS

osmoprotection, antioxidant mechanism, photosynthesis, production, *Vigna unguiculata* (L.) Walp.

1 Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is a Fabacea widely grown in tropical and subtropical regions as well as is a staple in the diet of many populations due to its high nutritional value (Horn et al., 2022). Its cultivation is a key source of income for rural communities and a major contributor to grain production in these regions (Anago et al., 2021).

In the context, cowpea, being rich in nutrients, is a guarantee of food security, especially for vulnerable populations, in light of the Sustainable Development Goals (SDGs) of the United Nations 2030 Agenda, particularly SDG 2, which aims to eliminate hunger through food security and the promotion of sustainable agriculture (Omomowo and Babalola, 2021).

In Brazil, the cultivation of this fabaceae occurs predominantly in the North and Northeast regions on small and medium-sized rural properties and under rainfed conditions (Guimarães et al., 2020). Thus, despite its rusticity, some research has demonstrated that cowpea productivity in these regions is strongly affected by water deficits in at least one of its phenological phases (Silva et al., 2020; Souza et al., 2020a). According to data published by CONAB (2023), although the northeast region has the largest area cultivated with this species, its productivity (411 kg ha^{-1}) is lower than the central-south region (1,024 kg ha^{-1}), which presents greater technological innovations.

Water deficiency aggravates the loss of plant productivity by impeding development and restricting the net carbon assimilation. The inhibition of the photosynthetic process is caused by a restriction in stomatal conductance (Souza et al., 2020b), leading to reduced absorption of soil solution, hindered growth and development, and restricted production (Miri et al., 2021; Olorunwa et al., 2021). This restriction also promotes an increase in the concentration of reactive oxygen species (ROS), which can trigger oxidative stress (Khatun et al., 2021).

However, it is important to highlight that plants respond to water restriction by activating a series of mechanisms that help them mitigate the harmful effects of this stress. Osmotic adjustment is characterized by the accumulation of compatible organic solutes in the cell cytosol, which facilitates water absorption from the soil (Santos et al., 2022). Furthermore, the antioxidant enzyme system functions to eliminate ROS and regulate their levels in metabolism (Arif et al., 2023).

Additionally, the application of salicylic acid (SA) can be a promising strategy to mitigate water stress in cowpea plants (Sultana et al., 2024) because it can regulate metabolic pathways and enhance the plants' defense against water deficits (Ghahremani et al., 2023). The use of silicon has been shown to have positive effects in mitigating the impact of water stress on plants. It promotes an increase in relative water content (Carvalho et al., 2020), the concentration of organic solutes, and the activity of antioxidant enzymes (Shemi et al., 2021; Jales Filho et al., 2023). Silicon also regulates mechanisms that counteract oxidative stress (Arif et al., 2023) and increases the productivity of plants grown under water deficit (El-Sanatawy and Zedan, 2020).

Despite promising results, information regarding the interaction between water replacement levels and SA application in cowpea plants is still scarce under field conditions. Thus, considering its importance for semi-arid regions, such as the Brazilian Northeast, the present study was carried out with the objective of evaluating SA as a water stress attenuator in cowpea cultivars grown under irrigation levels under field conditions.

2 Materials and methods

2.1 Location and conduct of the experiment

The experiment was carried out in a agricultural area at the Center for Agricultural and Environmental Sciences (CCAA), Campus II of the State University of Paraíba (UEPB), Lagoa Seca-PB. The field conditions were observed from January to April 2020. The geographical coordinates of the location are latitude 7° 09'S, longitude 35° 52' W, and altitude 634 m. Biochemical analyses were conducted at the Ecophysiology of Cultivated Plants Laboratory (ECOLAB) at UEPB in Campina Grande-PB, Brazil. The laboratory is situated at latitude 07° 13' 50", longitude, 35° 52' 52", and altitude 551 m.

The research site has a tropical climate with a dry season, classified as type AS' by the Köppen system. The annual average temperature is 22°C, with a minimum of 19°C and a maximum of

26°C (Figure 1). The average annual precipitation is above 700 mm, with higher rainfall levels concentrated in the months of April to August. The average annual reference evapotranspiration is 500 mm, and the average annual relative humidity is 80%.

2.2 Treatments application and experimental design

Four cowpea cultivars (BRS Novaera, BRS Tapaihum, BRS Pujante, and BRS Pajeú) were subjected to control treatments (W100 – plants subjected to 100% replacement of crop evapotranspiration – ETc), W50 (50% of ETc), W50+SA2 (50% of ETc + 276 mg L^{-1} of SA) and W50+SA4 (50% of ETc + 552 mg L^{-1} of SA), combined in a 4×4 factorial design, with three replications. Each experimental unit measured 1.10 m wide and 2.0 m long, with five irrigation lines and 10 plants per line, arranged in a randomized block design. The salicylic acid (P.A. - $C_7H_6O_3$, molecular weight 138.12 g mol^{-1}) was from Sigma-Aldrich.

After applying the fungicide, the seeds were allowed to rest for 24 hours at room temperature and low light. Following this period, all seeds were manually sown, with one seed placed in each hole at a depth of 3.0 cm. The holes were spaced 10 cm apart, and there was a distance of 50 cm between the planting lines (Rocha et al., 2019).

The soil in the experimental area had the following characteristics: sand (86.04%), silt (12.05%), clay (1.91%), soil density (1.62 g cm^{-3}), particle density (2.69 g cm^{-3}), porosity (39.77%), calcium (2.31 $cmol_c$ dm^{-3}), magnesium (2.30 $cmol_c$ dm^{-3}), sodium (0.05 $cmol_c$ dm^{-3}), potassium (0.27 $cmol_c$ dm^{-3}), sulfur (4.93 $cmol_c$ dm^{-3}), hydrogen (0.89 $cmol_c$ dm^{-3}), aluminum (0.00 $cmol_c$ dm^{-3}), organic matter (1.10%), and pH (6.62).

Irrigations were applied daily according to crop evapotranspiration (ETc) from 7:00 to 8:00 AM. A drip irrigation system consisting of drip tapes with a wall thickness of 0.2 mm, an internal diameter of 16 mm, and self-compensating emitters with a flow rate of 1.6 L per hour and spaced every 10 cm between emitters and 0.5 m between lines. Water replenishment was calculated using the Penman-Monteith (FAO) method (Allen et al., 1998) based on climate data from the nearby agrometeorological station (7°09'26.1" S, 35°52'16.9W). The reference evapotranspiration (ETo), and total water level (TWL) were determined according to Equations 1, 2 respectively.

$$ET_0 = \frac{0.408\Delta(Rn - G) + \gamma \frac{900}{T+273} U_2(e_s + e_a)}{\Delta + \gamma(1 + 0.34U_2)} \quad (1)$$

Where: ETo = Reference evapotranspiration (mm day^{-1}); Rn = net radiation on the culture surface (MJ m^{-2} day^{-1}); G = soil heat flux (MJ m^{-2} day^{-1}); Δ= slope of the vapor pressure curve versus air temperature kg ha^{-1} (kPa $°C^{-1}$); U2 = wind speed measured at 2.0 m height (m s^{-1}); T = temperature (°C); es = water vapor saturation pressure (kPa); ea = real water vapor pressure (kPa); γ = psychrometric factor (MJ kg^{-1}).

$$TWL = ET_0 \times Kc \quad (2)$$

The maximum Kc used for each phenological stage was as follows: initial stage: 15 days (Kc = 0.8); growth stage: 25 days (Kc = 1.1); reproductive stage: 17 days (Kc = 1.4); final stage: 0.3, according to Bastos et al. (2008). The irrigation time was measured after the system reached a stable pressure of 0.8–1.0 kgf cm^{-2}. Pressure gauges in the secondary lines were used for measurement. The irrigation level for water deficit (W50) was set at 50% of the irrigation level without water restriction (W100).

Salicylic acid was applied at 18 and 36 days after sowing (DAS - stages V3 and V9, respectively) using 20 mL per plant with a knapsack sprayer at 40 psi pressure, targeting both sides of the leaves (adaxial and abaxial). Irrigation depths for water restrictions were determined the day after the first spraying (19 DAS).

At 10 and 18 DAS, DripSol MAP fertilizer (12% N and 65% P_2O_5) was applied through fertigation at a rate of 390 g diluted in 10 L of water per application, using a venturi fertilizer injector. At 37 DAS, 10 mL of Benevia® insecticide (100 g L^{-1}, a.i. Ciantraniliprole) was sprayed according to the manufacturer's recommendations, diluted in 20 L of water, and 4 mL of spreader-sticker. At 18 days after sowing (DAS), weed dry matter was uniformly added to the interrows of experimental area plots to form 5 cm layers (Maia Junior et al., 2019). Manual control of spontaneous plants was carried out throughout the experiment.

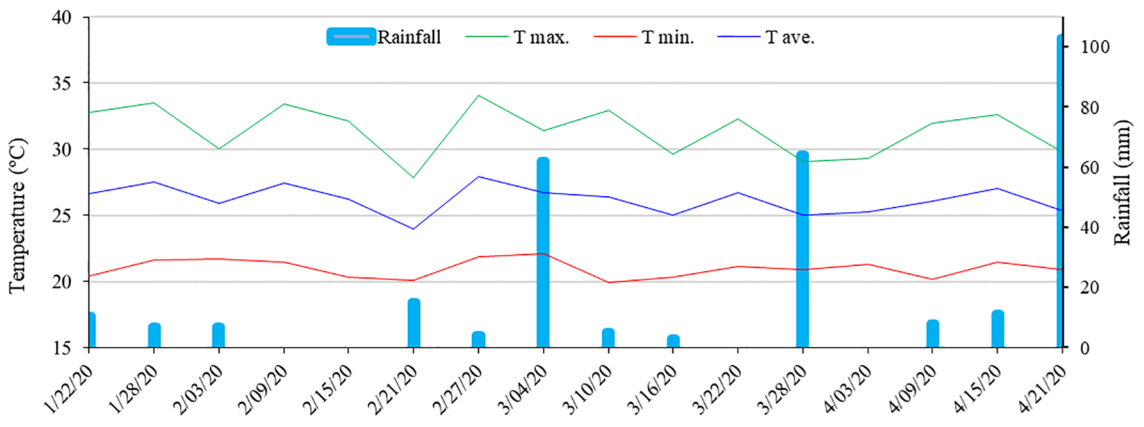

FIGURE 1
Data on maximum (T max.), minimum (T min.), average (T avg.) temperature (°C) and rainfall (mm) accumulated every six days during the experiment.

At 29 days after SA application (phenological stage V7), leaf gas exchange analyses were carried out using an infrared gas analyzer (IRGA - Infrared Gas Analyzer, GFS 3000 FL). Measurements included net photosynthesis (*A*) (μmol CO_2 m^{-2} s^{-1}) stomatal conductance (*gs*) (mmol m^{-2} s^{-1}) and transpiration (*E*) (mmol H_2O m^{-2} s^{-1}) in fully expanded leaves located at the median position of the plant. Three plants from each experimental plot were analyzed. Leaf tissue from one plant per plot was collected to determine free proline (FPR) content using the method by Bates et al. (1973) and total soluble sugars (TSS) content using the sulfuric phenol method by Dubois et al. (1956). The activity of ascorbate peroxidase (APX) and catalase (CAT) enzymes was determined following the methodologies by Nakano and Asada (1981) and Sudhakar et al. (2001), respectively. Results were expressed in nmol of ascorbate min^{-1} g^{-1} of fresh mass (FM) and μmol of H_2O_2 min^{-1} g^{-1} of FM.

Leaf water potential (Ψw) was measured using a Scholander-type pressure chamber (Scholander et al., 1965) on one plant per plot. The same chamber was used to calculate the total leaf area (TLA) with ImageJ software. Leaflets were digitized on an HP Deskjet Ink Advantage 2545 Multifunctional Printer at a two-centimeter scale. Total dry mass (TDM) was assessed in plants dried in an oven with air circulation at 70 °C for a period of 48 hours and then weighed on an analytical balance.

Plant evaluations were conducted at the R2 stage (51 DAS) and at the end of the crop cycle (R5 stage). The following agronomic characteristics were evaluated: a) the number of pods per plant (NPP) (by dividing the total number of pods by the number of plants in the plot); b) the number of grains per pod (NGP) (by counting the number of grains of 10 pods per irrigation line - 50 per plot); c) the weight of one hundred grains in grams (WOHG); and e) the grain yield (GY) (kg ha^{-1}). The experiment conduction in resume is show in the Figure 2.

The data were analyzed using ANOVA (F test at 5% probability) and Tukey's mean comparison test ($P \leq 0.05$) in SISVAR 5.6 software (Ferreira, 2019). Pearson correlation was performed on the variables using ggcorrplot package R software v. 4.2.3.

3 Results

3.1 Salicylic acid improves cowpea's water status and photosynthetic performance under water restriction

The study found that leaf water potential (Ψw) significantly decreased in the cultivars BRS Novaera (71.43%) and BRS Tapaihum (156%) under water restriction and absence of SA (W50) compared to W100 at stage V7 (Figure 3A). At the R2 phenological stage, in the same situation, a reduction was observed only in BRS Tapaihum (Figure 3B).

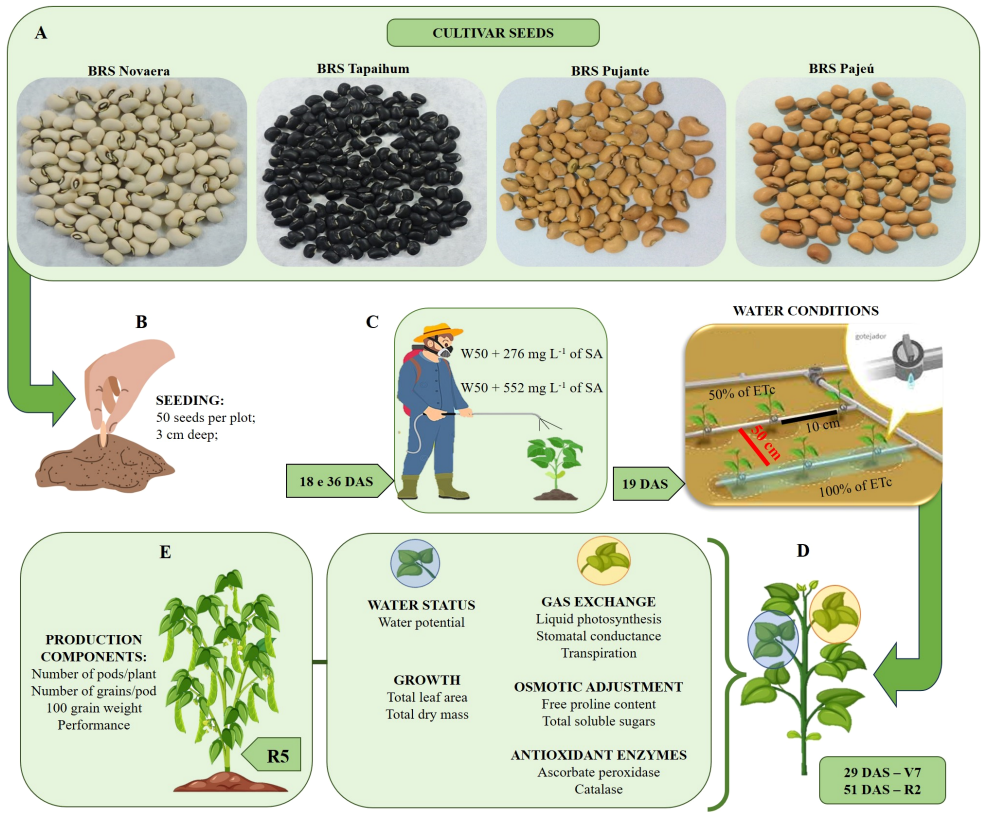

FIGURE 2
Experiment conducted and application of treatments. **(A)** cultivar seeds, **(B)** sowing, **(C)** application of SA concentrations at 18 and 36 days after sowing (DAS) and differentiation of irrigation depths (19 DAS), **(D)** variables analyzed in phenological stages V7 and R2, and **(E)** production variables analyzed at the R5 phenological stage.

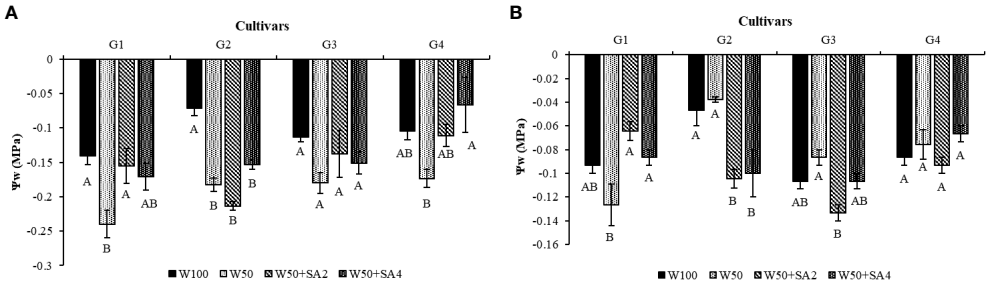

FIGURE 3
Leaf water potential (Ψw) in the phenological stages V7 **(A)** and R2 **(B)** of cowpea cultivars: BRS Novaera (G1), BRS Tapaihum (G2), BRS Pujante (G3), and BRS Pajeú (G4), subjected to control (W100), stress (W50), stress + 276 mg L^{-1} of SA (W50+SA2), and stress + 552 mg L^{-1} of SA (W50+SA4) treatments. Capital letters differentiate treatments within each cultivar (Tukey $P \leq 0.05$), and lowercase letters differentiate cultivars within each treatment (Tukey $P \leq 0.05$).

At the V7 phenological stage, even under stress conditions, applying 276 mg L^{-1} of SA to the BRS Novaera cultivar resulted in a 37.5% increase in leaf water potential, while applying 552 mg L^{-1} to BRS Pajeú resulted in a similar increase compared to W50 (Figure 3A). Notably, the plants treated with these concentrations had similar water potential to those receiving 100% water replacement (Figure 3A).

Water restriction significantly affected the gas exchange parameters evaluated in this study. However, the application of SA in some cultivars avoided these effects by maintaining the evaluated parameters in plants under stress at levels similar to those subjected to 100% water replacement.

In the absence of SA, water restriction reduced stomatal conductance (*gs*) and transpiration (*E*) of the BRS Tapaihum and BRS Pajeú cultivars compared to the control treatment (W100) at stage V7 (Figure 4A). Under stress conditions, applying 276 mg L^{-1} of SA increases *gs* by 68% and *E* by 50% in the BRS Tapaihum cultivar, while with 552 mg L^{-1}, it increases by 29% compared to W50 (Figure 4A).

At stage R2, the BRS Novaera cultivar exhibits a decrease in *gs* at 50% of the crop evapotranspiration (ETc) regardless of the treatment, compared to the W100 level. This behavior is also observed in the transpiration values of this cultivar (Figures 4B, D). Meanwhile, under stress, the cultivars BRS Tapaihum and BRS

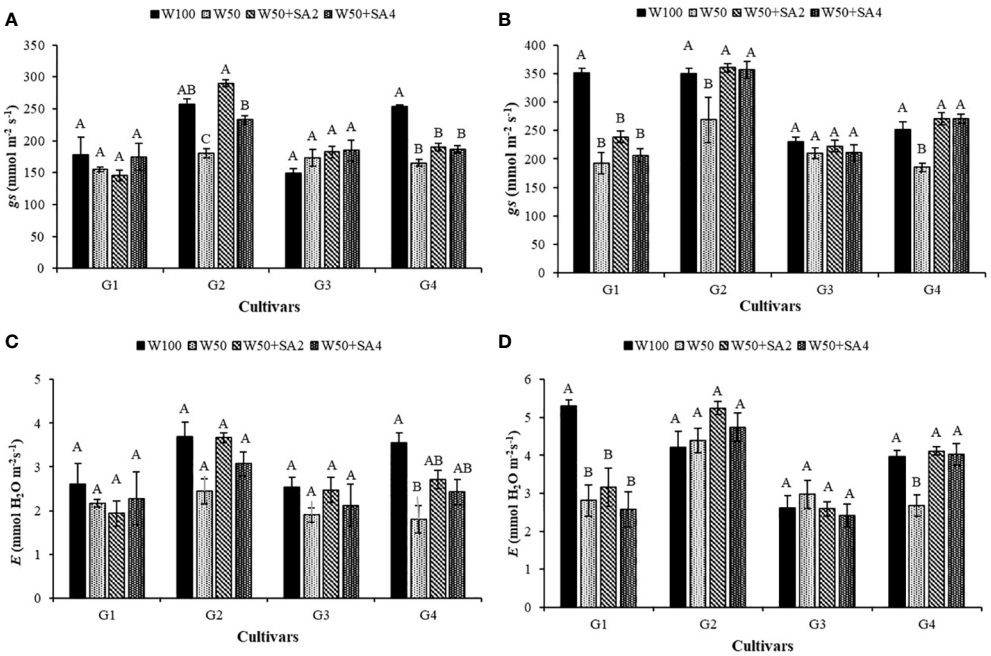

FIGURE 4
Stomatal conductance (*gs*) in phenological stages V7 **(A)** and R2 **(B)**, and transpiration (*E*) in stages V7 **(C)** and R2 **(D)** of cowpea cultivars: BRS Novaera (G1), BRS Tapaihum (G2), BRS Pujante (G3), and BRS Pajeú (G4), subjected to treatments control (W100), stress (W50), stress + 276 mg L^{-1} of SA (W50+SA2), and stress + 552 mg L^{-1} of SA (W50+SA4). Capital letters differentiate treatments within each cultivar (Tukey $P \leq 0.05$), and lowercase letters differentiate cultivars within each treatment (Tukey $P \leq 0.05$).

Pajeú only show a reduction in *gs* in plants that did not receive SA. However, this effect is reversed with the application of SA (treatments W50+SA2 and W50+SA4), where *gs* is significantly higher than in treatment W50 and equal to that observed in W100 (Figure 4B). It can also be highlighted that the BRS Pajeú cultivar presents similar behavior for *E*, which was reduced under water stress and in the absence of SA, as well as was significantly higher than plants subjected to SA application in the same water condition and at both concentrations (Figure 4D).

In Figure 5A, for photosynthesis (*A*), at the V7 stage and in the W50 treatment, there is a 19% reduction in the BRS Tapaihum cultivar and a 23% reduction in the BRS Pujante cultivar compared with the control (W100). However, the application of SA (276 mg L^{-1}) reversed the negative effect of water restriction on *A* for these cultivars. The plants subjected to the W50+SA2 treatment showed a higher photosynthetic rate than those in the W50 treatment and were statistically similar to the W100 treatment.

The cultivar BRS Tapaihum, exhibited a positive response to SA at a concentration of 552 mg L^{-1}. However, the cultivar BRS Pujante did not show a significant difference when compared to the W50 treatment at this concentration. BRS Pajeú, subjected to SA application, shows a significant reduction in *A* compared to the W100 treatment, while the W50 treatment does not differ statistically from this one (Figure 5A).

At the R2 stage and in the W50 treatment, a decrease in *A* was observed in the cultivars BRS Novaera, BRS Pujante, and BRS Pajeú, compared to W100 (Figure 5B). It is important to note that, except for the BRS Pujante cultivar, all other cultivars showed a reduction in *gs* at this stage, which may have contributed to the decrease in *A* observed.

At this phenological stage, the beneficial effect of SA can be verified by the increases in *A* observed under stress conditions and at the concentration of 276 mg L^{-1} for the cultivars BRS Novaera, BRS Tapaihum, and BRS Pujante. Further, with both concentrations, BRS Pajeú showed higher average *A* values than those observed in plants that did not receive this attenuator under the same water condition (Figure 5B).

3.2 Indicators of osmotic adjustment and the antioxidant mechanism of cowpea under water restriction are enhanced with salicylic acid

The BRS Tapaihum and BRS Pajeú cultivars showed an increase in the content of free proline (FPR) under water restriction and absence of SA in both phenological stages (Figures 6A, B) and in total soluble sugars (TSS) in the V7 stage (Figure 6C), with values higher than those observed in the W100 treatment. The FPR of the BRS Novaera cultivar did not differ between irrigation depths without SA treatment. The BRS Pajeú cultivar showed an increase in FPR at W50, only at the R2 stage (Figures 6A, B).

The BRS Tapaihum cultivar showed a 63% reduction in FPR with the 552 mg L^{-1} SA treatment compared to the W50 treatment. The BRS Pajeú cultivar had reductions of 60% and 52% with concentrations of 276 and 552 mg L^{-1} at stage V7, respectively (Figure 6A), and increased TSS content with both concentrations at the same phenological stage (Figure 6D). At stage R2 (Figures 6B, C), the BRS Tapaihum cultivar shows an increase in FPR corresponding to 42% in relation to the W50 treatment. At a concentration of 552 mg L^{-1} of SA, it maintained TSS levels similar to W50 with both SA concentrations. Meanwhile, the BRS Pajeú cultivar exhibits a 93% increase in FPR with 276 mg L^{-1} (Figure 6B) compared to the W50 treatment.

Under water restriction and without the application of SA, the BRS Novaera cultivar showed an 84.7% increase in APX activity compared to the control treatment (W100), while the cultivar BRS Pajeú exhibited a 231% increase in catalase enzyme (CAT) activity under the same condition and phenological stage (Figures 7A, C). Additionally, when plants are under inadequate irrigation, applying SA increases the activity of these enzymes, suggesting that this acid has a positive effect in reducing the impact of water restriction at this phenological stage. The BRS Pajeú cultivar showed an 88% increase in APX activity with a concentration of 276 mg L^{-1} and a variation between 172% and 107% in CAT activity, with 552 mg L^{-1} of SA in the BRS Novaera and BRS Pajeú cultivars, respectively (Figures 7A, C).

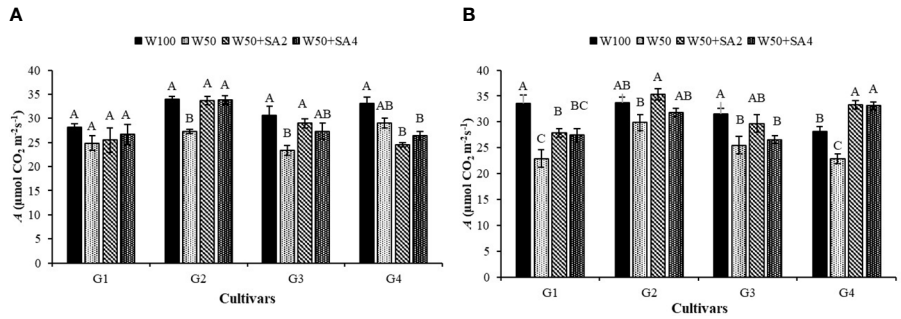

FIGURE 5
Photosynthesis (*A*) in the phenological stages V7 **(A)** and R2 **(B)** of cowpea cultivars: BRS Novaera (G1), BRS Tapaihum (G2), BRS Pujante (G3), and BRS Pajeú (G4), subjected to control (W100), stress (W50), stress + 276 mg L^{-1} of SA (W50+SA2), and stress + 552 mg L^{-1} of SA (W50+SA4) treatments. Capital letters differentiate treatments within each cultivar (Tukey $P \leq 0.05$), and lowercase letters differentiate cultivars within each treatment (Tukey $P \leq 0.05$).

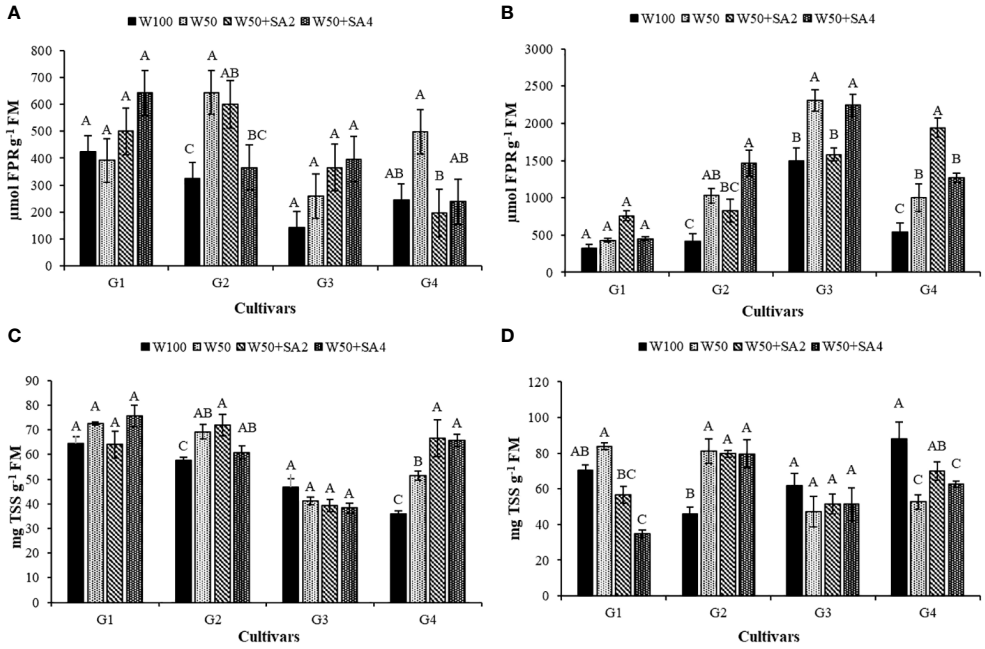

FIGURE 6
Free proline (FPR) content in phenological stages V7 **(A)** and R2 **(B)**; and total soluble sugars (TSS) in the V7 **(C)** and R2 **(D)** stages of cowpea cultivars: BRS Novaera (G1), BRS Tapaihum (G2), BRS Pujante (G3), and BRS Pajeú (G4), subjected to control (W100), stress (W50), stress + 276 mg L^{-1} of SA (W50+SA2), and stress + 552 mg L^{-1} of SA (W50+SA4) treatments. Capital letters differentiate treatments within each cultivar (Tukey $P \leq 0.05$), and lowercase letters differentiate cultivars within each treatment (Tukey $P \leq 0.05$).

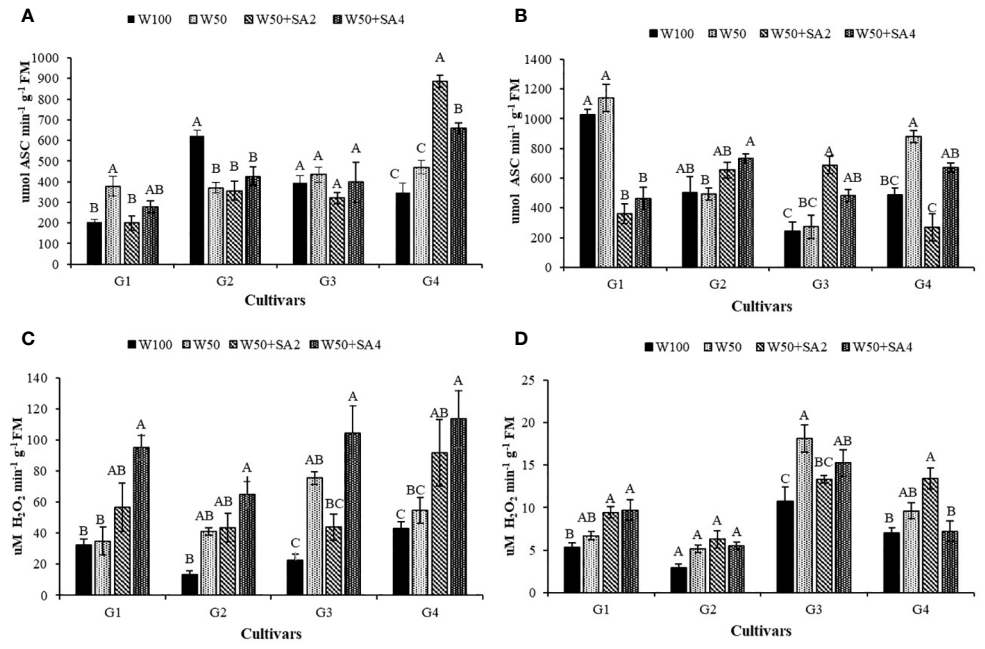

FIGURE 7
Activity of the enzyme ascorbate peroxidase (APX) in the phenological stages V7 **(A)** and R2 **(B)** and catalase (CAT) in the stages V7 **(C)** and R2 **(D)** of cowpea cultivars: BRS Novaera (G1), BRS Tapaihum (G2), BRS Pujante (G3), and BRS Pajeú (G4), subjected to treatments control (W100), stress (W50), stress + 276 mg L^{-1} of SA (W50+SA2) and stress + 552 mg L^{-1} of SA (W50+SA4). Capital letters differentiate treatments within each cultivar (Tukey $P \leq 0.05$) and lowercase letters differentiate cultivars within each treatment (Tukey $P \leq 0.05$).

At stage R2, when water was restricted and SA was absent, only the cultivar BRS Pajeú showed an increase in APX activity, which was 80% higher than that observed in W100 (Figure 7B). As for the catalase enzyme, this behavior was only observed in the cultivar BRS Pujante (Figure 7D). At this stage, the BRS Novaera cultivar has reduced APX activity at both SA concentrations, while the BRS Pajeú cultivar shows reduced enzyme activity with the application of 276 mg L^{-1}. However, the BRS Pujante cultivar shows 155% and 78% higher activity than that observed in W50, with concentrations of 276 and 552 mg L^{-1} of SA, respectively (Figure 7B). Regarding the CAT enzyme, a positive effect of SA is observed in the cultivars BRS Novaera and BRS Paje under stress conditions in treatments W50 +SA4 and W50+SA2, respectively, which supports the beneficial effect of SA in inducing resistance to water stress (Figure 7D).

In cultivar BRS Pajeú, the APX enzyme activity is positively correlated with net photosynthesis and growth variables at the V7 stage. Similarly, at stage R2, these variables also show a positive correlation with CAT activity. These results suggest that exposing plants to the concentration of 276 mg L^{-1} of SA in the early stages of stress can increase APX activity and allow for an improvement in net photosynthesis, which may have a positive impact on the growth of these cultivars. In the subsequent moments, this fact is ensured by the CAT enzyme, which has its activity increased in the R2 stage (Figure 8), positively correlating with photosynthesis and plant growth in this same phenological stage. These results demonstrate the significance of the activity of these enzymes in tolerating the damage caused by water stress, as well as elucidating the role of salicylic acid in this process.

3.3 Salicylic acid mitigates the effects of water deficit on the growth and production components of cowpea

At stage V7, water restriction negatively affected the total leaf area (TLA) of BRS Tapaihum and BRS Pajeú cultivars, regardless of SA application (Figure 9A). Meanwhile, at the R2 stage (Figure 9B), this behavior is observed in the cultivars BRS Novaera and BRS Pujante (W50 and W50+SA2).

At stage V7, a negative impact of water restriction was only seen in the BRS Novaera cultivar. However, this effect was reversed with the application of 552 mg L^{-1} of SA, leading to a 99.1% increase in total dry mass (TDM) (Figure 9C). At the R2 stage, a decrease in TDM was observed under water stress without SA in the cultivars BRS Tapaihum, BRS Pujante, and BRS Pajeú (Figure 9D).

Water deficit has a negative effect on the number of pods per plant (NPP) in the BRS Novaera and BRS Pajeú cultivars (Figure 10A). In contrast, the BRS Tapaihum and BRS Pujante cultivars showed greater resistance to water restriction, as they did not exhibit a difference in NPP between stressed plants and the control treatment (W100). The application of SA did not affect the NPP of tested cultivars because the plants subjected to the application of this attenuator presented values statistically similar to the W50 treatment (Figure 10A).

Water restriction and the absence of SA decreased the number of grains per pod (NGP) only in the BRS Pujante cultivar. However, applying concentrations of 276 and 552 mg L^{-1} of SA (Figure 10B) reversed this effect, increasing NGP by 19.7% and 15.5%,

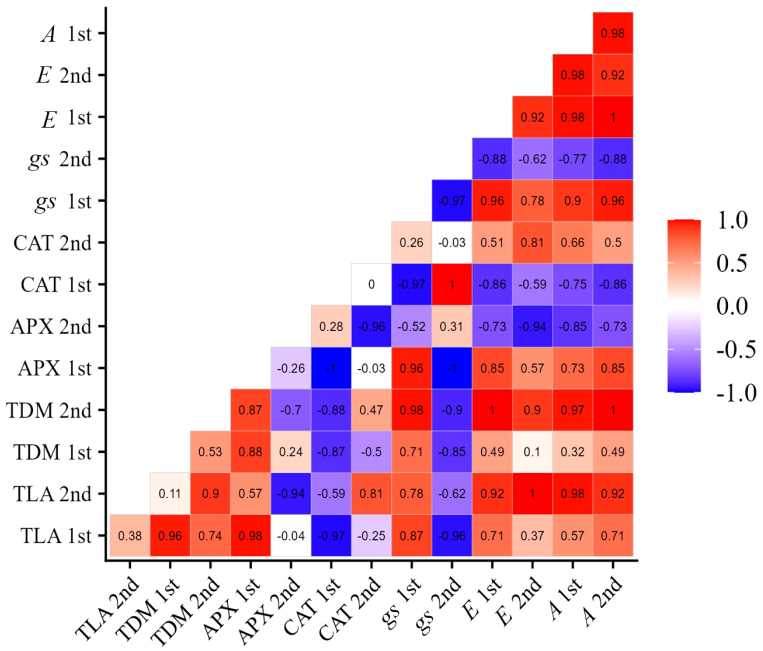

FIGURE 8
Correlation matrix of variables: net photosynthesis (*A*), transpiration (*E*), stomatal conductance (*gs*), catalase (CAT), ascorbate peroxidase (APX), total dry mass (TDM), and total leaf area (TLA), of cowpea cultivar BRS Pajeú, under water restriction (50% of ETc) and application of 276 mg L^{-1} of SA, in phenological stages V7 (1st) and R2 (2nd).

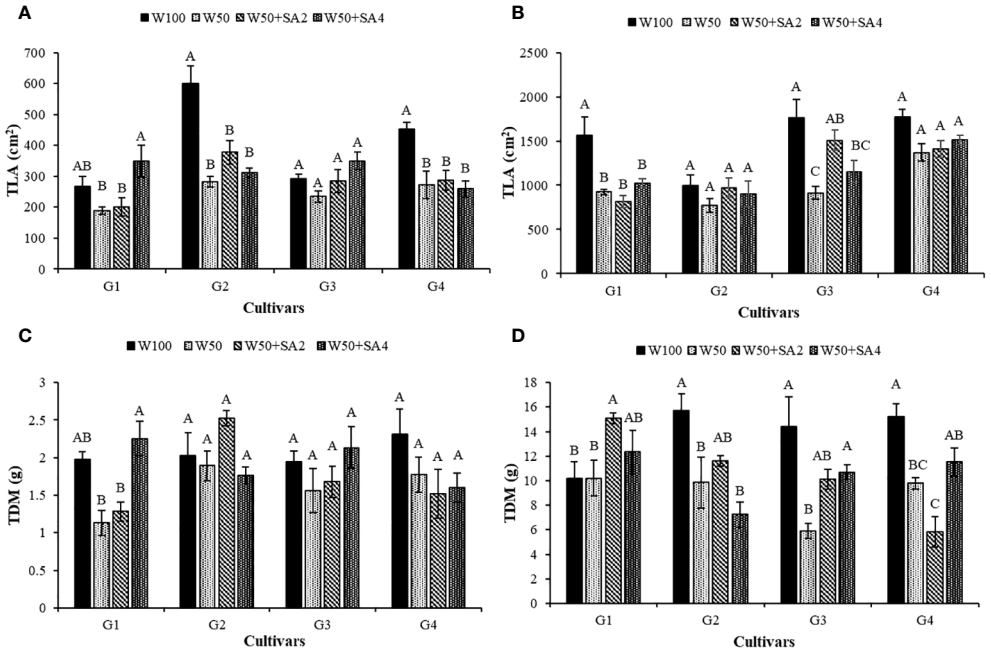

FIGURE 9
Total leaf area (TLA) at phenological stages V7 **(A)** and R2 **(B)**; and total dry mass (TDM) in stages V7 **(C)** and R2 **(D)** of cowpea cultivars: BRS Novaera (G1), BRS Tapaihum (G2), BRS Pujante (G3), and BRS Pajeú (G4), subjected to control (W100), stress (W50), stress + 276 mg L^{-1} of SA (W50+SA2) and stress + 552 mg L^{-1} of SA (W50+SA4) treatments. Capital letters differentiate treatments within each cultivar (Tukey $P \leq 0.05$) and lowercase letters differentiate cultivars within each treatment (Tukey $P \leq 0.05$).

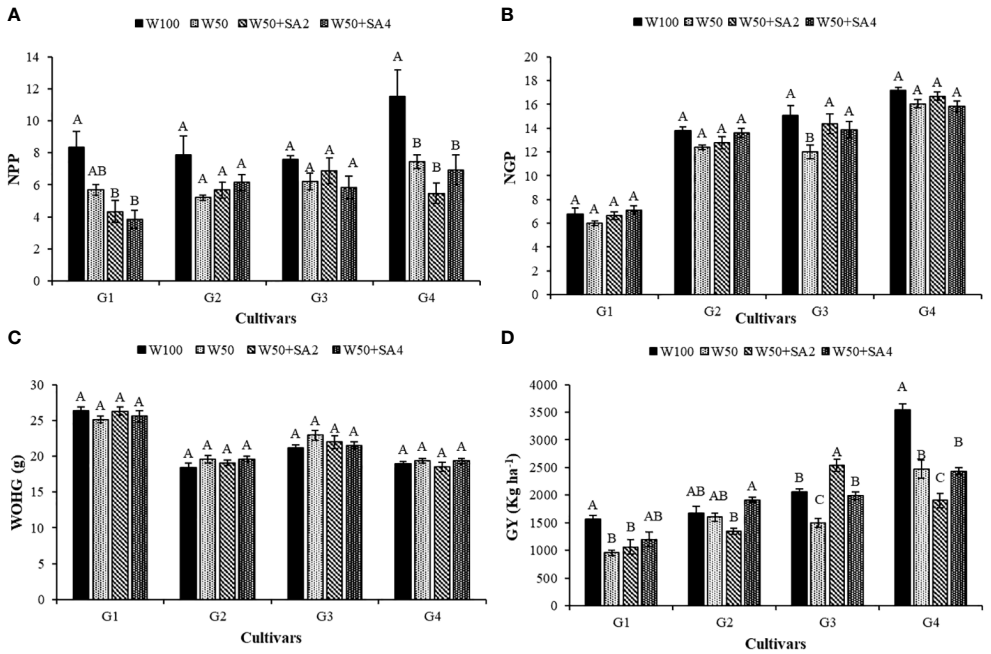

FIGURE 10
Number of pods per plant (NPP) **(A)**, number of grains per pod (NGP) **(B)**, weight of one hundred grains (WOHG) **(C)** and grain yield (GY) **(D)**, of cowpea cultivars: BRS Novaera (G1), BRS Tapaihum (G2), BRS Pujante (G3), and BRS Pajeú (G4) subjected to treatments control (W100), stress (W50), stress + 276 mg L^{-1} of SA (W50+SA2) and stress + 552 mg L^{-1} of SA (W50+SA4), at the R5 phenological stage. Capital letters differentiate treatments within each cultivar (Tukey $P \leq 0.05$) and lowercase letters differentiate cultivars within each treatment (Tukey $P \leq 0.05$).

respectively, compared to plants that were not treated with this attenuator.

The productivity of BRS Novaera and BRS Pajeú was negatively affected by water stress, even with SA application. BRS Pujante cultivar showed a reduced productivity only in the W50 treatment under water stress (Figure 10D).

The BRS Pujante cultivar's productivity decreased under water stress but increased by 69.3% with 276 mg L^{-1} of SA compared to the W50 treatment. It reached a productivity of 2,532.83 kg ha^{-1}, the highest grain yield (GY) for this cultivar (Figure 10D). This cultivar treated with 552 mg L^{-1} of SA showed similar productivity (1,996.27 kg ha^{-1}) to those receiving 100% water replacement. Productivity was 25% higher when compared to treatment W50 (1,496.20 kg ha^{-1}) (Figure 10D), suggesting that SA can maintain productivity in this cultivar under water deficit conditions.

The BRS Pajeú cultivar showed the highest productivity among the cultivars tested (Figure 10D), even though its yield decreased with the W50 depth. This highlights the cultivar's potential for high productivity in different conditions. BRS Tapaihum exhibited higher tolerance to water restriction in this study as its productivity did not differ between irrigation depths in the absence of SA.

Figure 11 shows that the increase in yield in the BRS Pujante cultivar presents a positive correlation with leaf water potential (1st and 2nd collections), ascorbate peroxidase enzyme activity (APX 1st and 2nd collections), net photosynthesis (1st and 2nd collections), and number of pods per plant. Additionally, it is possible to verify a positive correlation between leaf water potential and the content of osmotic adjusters (TSS and FPR) in the two phenological stages, which were also positively correlated with APX, *A*, and NPP.

4 Discussion

In the present study, the BRS Novaera and BRS Tapaihum cultivars showed a reduction in leaf water potential when subjected to water restriction. This restriction affected gas exchange and growth indicators, as evidenced by the reductions observed in stomatal conductance, transpiration (Figure 4), leaf area, and dry mass (Figure 9). Therefore, reducing leaf transpiration is a key

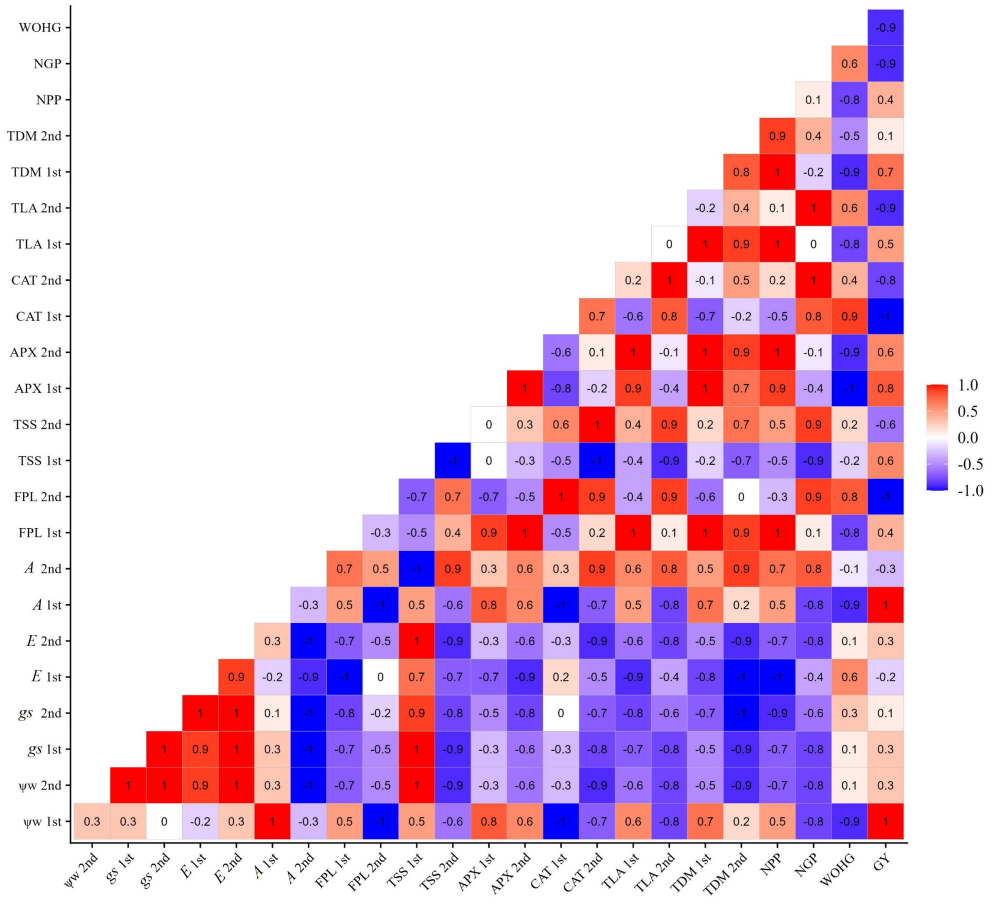

FIGURE 11
Correlation matrix of variables water potential (Ψw), total leaf area (TLA), total dry mass (TDM), free proline content (FPR), total soluble sugars (TSS), ascorbate peroxidase (APX), catalase (CAT), stomatal conductance (*gs*), transpiration (*E*), net photosynthesis (*A*), number of pods per plant (NPP), number of grains per pod (NGP), weight of one hundred grains (WOHG), and grain yield (cultivate bean- cowpea BRS Pujante), under water restriction (50% of ETc) and application of 276 mg L^{-1} of SA, in phenological stages V7 (1st) and R2 (2nd).

strategy plants use to prevent dehydration and survive drought conditions (Ferreira et al., 2021). When faced with water scarcity, plants typically lower transpiration rates by decreasing leaf area (Kapoor et al., 2020) and/or closing stomata (Souza et al., 2020b), as demonstrated in this study. While this mechanism is an interesting survival strategy in drought conditions, Ferreira et al. (2021) point out that it can restrict the influx of CO_2 and have a negative impact on photosynthesis. This report explains the reduction observed in net photosynthesis of the BRS Tapaihum and BRS Pajeú cultivars (Figure 5A).

It is important to note that a water deficit can lead to a decrease in chlorophyll content, which can inhibit photosynthesis (Parveen et al., 2021). This may have contributed to the observed reductions in BRS Pujante, as this cultivar did not exhibit a difference in stomatal conductance (*gs*) between different water conditions (Figure 4A).

Furthermore, under water restriction, it is also important to highlight that cowpeas can accumulate osmoprotective molecules, such as proline (Santos et al., 2022) and total soluble sugars (Jales Filho et al., 2023). These compounds favor water absorption, especially in low water availability soils, and are key mechanisms for plants to resist drought stress (Mohammadi et al., 2019; Santos et al., 2022; Gao et al., 2023; Jales Filho et al., 2023). In our study, we observed a similar response in the cultivars BRS Tapaihum, BRS Pujante, and BRS Pajeú. There were significant increases in FPR and TSS contents under water restriction (Figure 6).

Moura et al. (2016) reported that under water deficit conditions, the concentrations of TSS molecules change due to their impact on the translocation of photoassimilates in plants. This leads to a reduction in their utilization, causing them to accumulate as compatible organic solutes in the leaves. This accumulation contributes to the osmotic adjustment process (Coelho et al., 2018).

In plants under water restriction, applying SA can help increase leaf water potential to levels comparable to fully hydrated plants (Figure 3). This can help plants overcome the effects of water deficits by promoting stomatal conductance (Figure 4). These positive effects are linked to this elicitor's role in promoting the production of osmoprotective molecules (Sultana et al., 2024). Jales Filho et al. (2023) observed that applying SA to cowpea cultivars under water restriction increased organic solute content and maintained water status in the plants, as verified in the present study (Figures 6B–D).

Therefore, despite changes in water potential, it is evident that SA mitigated the damaging effects of the water deficit. The adjustments in Ψw observed with the application of this substance can enhance the stomatal opening mechanism (Carvalho et al., 2020), leading to increased photosynthetic rates (Figures 3B).

During water restriction, some cultivars exhibited elevated activity of antioxidant enzymes in the assessed phenological stages (Figure 7). Santos et al. (2022) reported that, under water stress, the heightened activity of these enzymes serves as a crucial defense mechanism against oxidative stress, aiding in the preservation of cell membrane integrity (Ghahremani et al., 2023). However, the activity of antioxidant enzymes in response to water stress varies dep ending on the tolerance level of each cultivar (Carvalho et al., 2019). This explains the diverse responses observed in the evaluated cultivars. It is remarkable that SA can act as a non-enzymatic antioxidant, helping to remove ROS and regulate their levels in cellular metabolism (Arif et al., 2023). This could explain the decrease in APX enzyme activity in BRS Novaera and BRS Pajeú cultivars during the R2 stage, even under water restriction (Figure 7B).

Additionally, under water restriction, the application of SA increased the activity of antioxidant enzymes such as APX and CAT, potentially helping to alleviate the negative impacts of water stress in the studied cultivars (Shemi et al., 2021; Sultana et al., 2024). Similar findings were reported by Oliveira et al. (2023) in the cowpea cultivar BRS Pajeú, where SA application at a concentration of 0.21 g L^{-1} led to an increase in APX activity. Jales Filho et al. (2023) also observed a similar response in the cowpea cultivar BRS Paulistão, highlighting the beneficial effects of SA in eliciting responses to water stress.

Water deficits can promote a series of physiological and morphological changes in cowpea plants, depending on the intensity, phenological stage, and cultivar resistance (Melo et al., 2022). During the reproductive phase, a water deficit can result in flower and pod loss, decreasing the number of pods per plant (Barros et al., 2021); this effect was observed in the BRS Novaera and BRS Pajeú cultivars (Figure 10A). For the BRS Pujante cultivar, the water deficit reduced NPP and impacted the production of photoassimilates necessary for grain production (Figure 10B), this result is corroborated by Martins et al. (2017).

There was no effect of treatments on cowpea cultivars for the weight of one hundred grains (WOHG) (Figure 10C). This result, as explained by Locatelli et al. (2014), is likely due to the high genetic heritability of WOHG, making it more resistant to environmental factors. Valeriano et al. (2019) found that this variable is associated with the movement of photoassimilates within the plant and is influenced by the source/sink relationship. It is important to highlight that the average WOHG values of the BRS Novaera and BRS Pujante cultivars meet the preferences of producers, buyers, and packers, who typically prefer grains weighing over 20 g per 100 grains, as reported by Públio Júnior et al. (2017).

The results demonstrate that water deficits can negatively impact cowpea production, and these effects can be directly linked to reductions in gas exchange caused by water restriction, particularly affecting stomatal conductance (*gs*) (Souza et al., 2020b). Stomatal closure limits the assimilation of CO_2 and affects the photosynthetic process, thereby impacting the production of photoassimilates necessary for grain formation (Ferreira et al., 2021).

In the BRS Pujante cultivar, the negative impact of the water deficit on productivity (Figure 10D) was mitigated by SA at a concentration of 272 mg L^{-1}. This suggests that the SA treatment increased the levels of osmotic adjusters, helping to maintain optimal leaf water potential and boosting APX activity in both phenological stages (Figure 11). These factors likely helped sustain net photosynthesis, leading to an increase in NPP (Figure 10A) and ultimately boosting the yield of the cultivar, even in conditions of water scarcity. Recent studies on wheat (El-Sanatawy and Zedan,

2020) and pea (Soni et al., 2021) plants have also demonstrated enhanced productivity in plants treated with SA under water stress.

5 Conclusions

Water restriction negatively affected water status, growth, gas exchange, and production of the four cultivars, as well as promoted changes in antioxidant metabolism and osmolyte concentration. The application of SA enhanced the antioxidant activity and the synthesis of osmotic adjusters, mitigating the effects of water restriction in cowpea. The concentration of 276 mg L^{-1} was more effective in stage R2 and 552 mg L^{-1} in the stage V7.

The BRS Pujante cultivar has increased its productivity under water restriction with SA application, and BRS Tapaihum is the most resistant among the cultivars studied.

Our findings underscore the importance of using SA to mitigate the effects of water restriction on cowpea cultivation. These discoveries are crucial for the sustainability of cowpea production in regions susceptible to drought, which can contribute to food security. We further add that the adoption of new agricultural practices can enhance the resilience and productivity of cowpea as an essential and sustainable food source for vulnerable populations in various parts of the world.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

IC: Investigation, Methodology, Visualization, Data curation, Formal analysis, Writing – original draft. AM: Investigation, Methodology, Visualization, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. RF: Investigation, Methodology, Visualization, Writing – review & editing. RA: Investigation, Methodology, Visualization, Writing – review & editing. GD: Investigation, Methodology, Visualization, Writing – review & editing. PV: Investigation, Methodology, Visualization, Writing – review & editing. MR: Visualization, Writing – review & editing. AN: Visualization, Writing – review & editing. FS: Visualization, Writing – review & editing. CL: Visualization, Writing – review & editing. PV: Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Genome-wide profile analysis of the Hsp20 family in lettuce and identification of its response to drought stress

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Heat shock protein 20 (Hsp20) plays a very important role in response to abiotic stressors such as drought; however, in lettuce (*Lactuca sativa* L.), this gene family is poorly understood. This study used bioinformatics methods to identify 36 members of the lettuce Hsp20 family, which were named *LsHsp20–1~LsHsp20–36*. Subcellular localization results revealed that 26 members of the LsHsp20 protein family localized to the cytoplasm and nucleus. Additionally, 15 conserved domains were identified in the LsHsp20 protein family, with the number of amino acids ranging from 8 to 50. Gene structure analysis revealed that 15 genes (41.7%) had no introns, and 20 genes (55.5%) had one intron. The proportion of the *LsHsp20* secondary structure was random coil > alpha helix > extended strand > beta turn. Chromosome positioning analysis indicated that 36 genes were unevenly distributed on nine chromosomes, and four pairs of genes were collinear. The Ka/Ks ratio of the collinear genes was less than 1, indicating that purifying selection dominated during *L. sativa* evolution. Thirteen pairs of genes were collinear in lettuce and *Arabidopsis*, and 14 pairs of genes were collinear in lettuce and tomato. A total of 36 LsHsp20 proteins were divided into 12 subgroups based on phylogenetic analysis. Three types of cis-acting elements, namely, abiotic and biotic stress-responsive, plant hormone-responsive, and plant development-related elements, were identified in the lettuce *LsHsp20* family. qRT-PCR was used to analyze the expression levels of 23 *LsHsp20* genes that were significantly upregulated on the 7th or 14th day of drought treatment, and the expression levels of two genes (*LsHsp20–12* and *LsHsp20–26*) were significantly increased by 153-fold and 273-fold on the 14th and 7th days of drought treatment, respectively. The results of this study provide comprehensive information for research on the LsHsp20 gene family in lettuce and lay a solid foundation for further elucidation of *Hsp20* biological functions, providing valuable information on the regulatory mechanisms of the LsHsp20 family in lettuce drought resistance.

KEYWORDS

gene family, lettuce, LsHsp20, drought, gene expression

1 Introduction

Heat shock proteins (Hsps) are an evolutionarily ancient, highly conserved group of intracellular molecules that are present in the cells of almost all organisms, including archaea, prokaryotes, and eukaryotes (Vierling, 1991; Waters, 2013). Heat shock proteins are responsible for assisting in the correct folding, assembly, and transport of proteins. They can maintain the conformational and functional stability of proteins by rebuilding normal proteins under stress conditions, participating in other stress response mechanisms, and playing an important role in protecting plants from abiotic stress (Wang et al., 2004). Research shows that when plants are subjected to various stresses, the Hsp protein content in the plant rapidly increases to help the plant resist stress (Zhang et al., 2015; Haq et al., 2019; Huang et al., 2022).

Ritossa first discovered heat shock protein in *Drosophila* salivary glands (Ritossa, 1962). Vierling was the first to discover Hsps in soybean plants, after which Nover et al. treated tomato cells at high temperatures and identified similar proteins related to high-temperature stress (Nover et al., 1983; Vierling, 1991). The functions of Hsps are also gradually being explored. Many studies have shown that Hsp expression is regulated by high temperatures as well as by abiotic stresses such as drought. Hsps can be divided into five categories according to their amino acid sequence homology and molecular weight: the high-molecular-weight protein families Hsp100, Hsp90, Hsp70, and Hsp60 and the low-molecular-weight protein family Hsp20. Hsp20 proteins are also called small heat shock proteins, and their molecular weights are between 15 and 42 kDa (Chen et al., 2018b; Zhao et al., 2018). The structure of the family of Hsp20 proteins includes an exogenous signal receiving end, an N-terminal variable domain, a C-terminal conserved domain, and an a-crystalline protein domain, which is also called ACD (Bondino et al., 2012; Lin et al., 2019). Moreover, these three regions play different roles: the N-terminal region is involved in regulating substrate binding and oligomerization, the C-terminal extension contains an amino acid motif that promotes homo-oligomerization and maintains organelle specificity, and the ACD has a conserved β-sheet structure and is composed of 80 to 100 amino acids that interact with the substrate (Waters, 2013; Peng et al., 2023). Hsp20 proteins have the largest number of members in plants (Hu et al., 2021). Hsp20 proteins are ATP-independent molecular chaperones with a strong ability to bind to denatured substrates and can maintain protein stability, prevent proteins from irreversibly aggregating or denaturing, and promote protein transport through membrane channels, thus preventing damage to proteins caused by abiotic stress (Haslbeck and Vierling, 2015). Therefore, Hsp20 proteins play an important role in improving plant tolerance and protecting plants from stress.

Since the discovery of the importance of *Hsp20* genes in coping with various biotic and abiotic stresses, Hsp20 gene family members have been identified in various plants; for example, 30, 48, 42, 38, and 42 Hsp20 gene family members have been identified in *Arabidopsis* (Huang et al., 2023), potato (Zhao et al., 2018), tomato (Yu et al., 2016), barley (Li and Liu, 2019), and peach (Lian et al., 2022), with 14, 12, 13, 7 and 11 subgroups, respectively. Studies have shown that *Hsp17.6B* overexpression in *Arabidopsis* significantly increases root elongation, plant survival rate, electrolyte leakage rate, and chlorophyll content under heat stress (Tawab et al., 2019). The overexpression of *CaHsp16.4* in pepper plants enhances the scavenging of reactive oxygen species produced under stress, leading to enhanced heat tolerance and drought resistance (Huang et al., 2019). In tomatoes, *Hsp20* genes can respond to high temperature, drought, and high salt stress to varying degrees. *Hsp20* genes are also involved in tomato fruit development. It is more actively expressed during the fruit maturity period than during other periods, and its expression increases as the fruit matures (Yu et al., 2016). Studies in peach plants have shown that overexpression of the *PpHsp20–32* gene is involved in the regulation of plant height and enhancing heat tolerance (Lian et al., 2022). The overexpression of *FaHsp17.4* in strawberry plants is also involved in the regulation of strawberry fruit growth and development (Li et al., 2016). The *Hsp20* genes in *Coix* can respond to high temperatures and drought to varying degrees and regulate growth and development (Hua et al., 2023). Taken together, these results indicate that the Hsp20 family plays an important positive role in improving plant immunity and alleviating abiotic stress.

Lettuce (*Lactuca sativa* L.) is one of the most widely planted vegetable species worldwide. Due to its shallow root distribution, poor water absorption capacity, and large water demand throughout its growth period, lettuce has high soil moisture requirements, and drought stress is one of the main factors affecting its growth and yield (Chen et al., 2018a; Balko et al., 2023; Ceylan et al., 2023). The lettuce Hsp20 gene family has not been systematically studied among the species in which it has been identified. Therefore, this study was based on lettuce genome information and used bioinformatics methods to identify members of the lettuce Hsp20 gene family and analyze their physical and chemical properties, conserved domains, and chromosomal locations. qRT-PCR technology was used to analyze the expression patterns of members of this gene family under drought stress, providing basic information for in-depth research on the function of the Hsp20 gene family in vegetables and its role in responding to adverse stress.

2 Materials and methods

2.1 Identification of LsHsp20 family members in lettuce

The lettuce reference genome and protein sequences were obtained from the Ensembl Plants database (https://plants.ensembl.org/index.html). The *Arabidopsis thaliana* Hsp20 protein family sequence was obtained from the TAIR database (https://www.arabidopsis.org). Hidden Markov model (HMM) files were obtained from the Pfam (PF00011) protein family database (http://pfam-legacy.xfam.org/). The *Arabidopsis* Hsp20 protein sequence was compared with the lettuce genome-wide protein sequence using the TBtools Blast function, and the resulting gene ID was determined in duplicate. To ensure that all candidate Hsp20 members contain ACD domains, the CDD (https://www.ncbi.nlm.nih.gov/structure/bwrpsb/bwrpsb.CGI), Pfam

(http://pfam-legacy.xfam.org/), and SMART (http://smart.embl-Heidelberg.de/) databases were used. The relative molecular weights of all candidate members were further screened and predicted, and the Hsp20 members without an ACD domain and with relative molecular weights greater than 15~42 kDa were removed. After identification and screening, the lettuce Hsp20 family members were obtained, and the genes were named *LsHsp20–1* to *LsHsp20–36* (Supplementary Tables S1, S2).

2.2 Basic information on the LsHsp20 gene family in lettuce

The ExPASy ProtParam (http://cn.expasy.org) program was used to analyze the molecular weights, theoretical isoelectric points, instability coefficients, and hydrophilicity indices of the proteins encoded by the lettuce LsHsp20 gene family. Furthermore, the online tool PSORT (https://wolfpsort.hgc.jp) was used to predict the subcellular localization of the Hsp20 proteins in lettuce.

2.3 Analysis of the structure and domain of the *LsHsp20* genes in lettuce

The exon and intron data for the lettuce *LsHsp20* genes were obtained from the database (http://cucurbitgenomics.org), and conserved protein motifs were analyzed with the MEME tool (http://meme.nbcr.net/meme). The maximum motif was set to 15, and the remaining parameters were set to the default values.

2.4 Secondary structure and three-dimensional structural model of the lettuce LsHsp20 proteins

The SOPMA (http://npsa-pbil.ibcp.fr) and SWISS-MODEL (https://swissmodel.expasy.org) online tools were used to determine the secondary structures and three-dimensional structural models of the lettuce LsHsp20 proteins.

2.5 Analysis of the chromosomal locations, collinearity, and evolutionary selection pressure of the lettuce *LsHsp20* genes

Chromosomal location information for the *LsHsp20* genes was obtained from the Ensembl Plants database (http://plants.bl.org/index.html), and the online tool MG2C V2.1 (http://mg2c.iask.in/mg2c_v2.1) was used to determine the chromosomal locations of the *Hsp20* genes. For the collinearity analysis of the Hsp20 gene family members, the TBtool software and its advanced Circos and Dual synteny plotter functions were used to perform intraspecies and interspecies collinearity analyses on the identified sequences, respectively.

2.6 Phylogenetic analysis and classification of the LsHsp20 family in lettuce

The Muscle function in MEGA 7.0 was used to perform multiple sequence alignment of Hsp20 proteins from five species: lettuce, *Arabidopsis* (Huang et al., 2023), tomato (Yu et al., 2016), rice (Ouyang et al., 2009), and barley (Li and Liu, 2019). After the alignment was completed, TBtools was used to trim the sequences. MEGA 7.0 maximum likelihood (ML) was used to construct a phylogenetic tree for lettuce, *Arabidopsis*, tomato, rice, and barley. The bootstrap value was set to 1,000, the gap was set to "pairwise deletion," and the "Poisson model" was used to verify that the tree was reliable. Based on the subcellular localization prediction for the LsHsp20 family in lettuce, the classification of Hsp20 proteins in other species, and the evolutionary structure of the phylogenetic tree, the LsHsp20 proteins were divided into different subgroups.

2.7 Analysis of cis-acting elements in the LsHsp20 gene family

The online prediction tool PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) was used to predict and analyze the 2,000-bp upstream sequence and main cis-acting elements of the lettuce *LsHsp20* genes. The cis-elements in the promoters of *LsHsp20* genes were also predicted.

2.8 Plant materials and drought stress treatment

The lettuce cultivar 'Yidali 151' (from Beijing Shuoyuan Seed Co., Ltd., Beijing, China) served as the plant material in this study and was cultured in an artificial climate chamber at Hebei Agricultural University. Seeds of full and consistent sizes were selected, a 72-hole plug tray was used for seedling cultivation using coconut peat:vermiculite:perlite at a ratio of 3:1:1 as the substrate, and the seeds were subjected to conventional seedling management at an indoor temperature of 25°C ± 2°C, humidity of 40%, and light intensity of 130 μmol·m^{-2}·s^{-1}. When the seedlings had six to seven true leaves, they were transplanted into the greenhouse. Drip lateral lines of 16 mm diameter were laid between the two rows, and when the seedlings survived, drought treatment was performed. The soil moisture content was monitored daily by a ZL6 data collector (METER Group, Inc., USA), which was inserted into a depth of 15 cm. The dripper discharge was 1.38 lph at a pressure of 0.1 MPa. When the soil moisture content was approximately 85%–95%, the sample was treated for 0 days (control) and irrigation was stopped, and when the substrate moisture content dropped to 60%–65%, which was marked as treatment, the moisture content of the substrate was maintained within this range. Samples were collected after 7 days, 14 days, and 21 days of drought treatment.

2.9 RNA extraction, cDNA synthesis, and real-time fluorescence quantitative PCR

Leaves from the normal and drought-treated lettuce groups were removed for RNA extraction and qRT-PCR analysis. Total RNA was extracted using an EasyPure® RNA Kit (TransGen, Beijing, China). A FastKing cDNA First-Strand Synthesis Kit (Tiangen, Beijing, China) was used to reverse transcribe total RNA to obtain cDNA. The NCBI database was used to design primers for 29 *LsHsp20* genes, and primer information was obtained (Supplementary Table S3). The serial number of the reference gene was *LSAT_8X116260*. Real-time fluorescence quantitative PCR was performed using the TransStart Top Green qPCR SuperMix kit (US Everbright, Suzhou, China).

2.10 Data analysis

SPSS·27.0 software was used for statistical analysis, and GraphPad Prism 8 software was used for mapping. The asterisks indicate the level of significance (* means $p < 0.05$, ** means $p < 0.01$) based on Duncan's multiple range test.

3 Results

3.1 Whole-genome identification and physical and chemical property analyses of the lettuce LsHsp20 family members

The presence of the ACD domain was confirmed via HMM by submitting the protein sequences to the CDD, Pfam, and SMART databases. After deleting sequences without typical ACD domains and sequences with molecular weights exceeding 15 to 42 kDa, 36 LsHsp20 family members were identified in the full lettuce genome, and their physical and chemical properties were analyzed (Table 1). The number of amino acids in the LsHsp20 proteins ranged from 137 (LsHsp20–16) to 331 (LsHsp20–18). The molecular weights of LsHsp20 proteins ranged from 15.62 kDa (LsHsp20–16) to 37.62 kDa (LsHsp20–18). The predicted p*I* of LsHsp20 proteins ranged from 4.94 (LsHsp20–6, LsHsp20–25) to 9.49 (LsHsp20–24), the instability index ranged from 24.34 (LsHsp20–27) to 70.9 (LsHsp20–35), the lipophilic index ranged from 62.86 (LsHsp20–19) to 92.65 (LsHsp20–2), and the overall average hydrophobicity index ranged from −0.865 (LsHsp20–3) to −0.207 (LsHsp20–16). Upon assessing subcellular localization, 26 LsHsp20 proteins were

TABLE 1 Physicochemical properties of the LsHsp20 proteins.

Gene	Gene ID	Number of amino acids	Molecular weight	Theoretical p*I*	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)	Subcellular location
LsHsp20–1	LSAT_5X168220	156	17,890.26	5.99	49.51	73.65	−0.694	Cytoplasm
LsHsp20–2	LSAT_8X130841	204	23,180.38	5.26	43.65	92.65	−0.49	Chloroplast
LsHsp20–3	LSAT_1X33281	211	24,441.67	7.89	50.11	68.82	−0.865	Nucleus
LsHsp20–4	LSAT_8X151001	235	26,595.23	8.95	39.23	67.19	−0.719	Chloroplast
LsHsp20–5	LSAT_5X171121	154	17,422.78	6.19	52.16	73.96	−0.606	Cytoplasm
LsHsp20–6	LSAT_4X107421	236	26,800.84	4.94	60.06	72.67	−0.828	Golgi apparatus
LsHsp20–7	LSAT_6X100581	242	27,788.84	6.13	45.6	81.65	−0.638	Cytoplasm
LsHsp20–8	LSAT_7X61061	164	18,279.92	6.1	36.32	80.18	−0.493	Cytoplasm
LsHsp20–9	LSAT_7X61001	163	18,371.88	6.44	33.18	74.11	−0.634	Cytoplasm
LsHsp20–10	LSAT_3X481	215	24,376.36	5.48	53.68	72.93	−0.728	Chloroplast
LsHsp20–11	LSAT_5X136741	161	17,777.49	6.84	60.24	88.88	−0.414	Nucleus
LsHsp20–12	LSAT_7X60201	163	18,433.05	5.97	34.53	77.12	−0.576	Cytoplasm

(Continued)

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TABLE 1 Continued

Gene	Gene ID	Number of amino acids	Molecular weight	Theoretical p*I*	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)	Subcellular location
LsHsp20–13	LSAT_7X108120	191	21,987.17	5.22	53.15	74.4	−0.386	Nucleus
LsHsp20–14	LSAT_9X81920	192	21,836.36	7.08	46.42	90.94	−0.482	Cytoplasm
LsHsp20–15	LSAT_7X60240	179	19,831.73	5.98	46.13	78.94	−0.522	Cytoplasm
LsHsp20–16	LSAT_7X17661	137	15,623.71	5.12	52.59	73.94	−0.207	Cytoplasm
LsHsp20–17	LSAT_7X61041	158	17,706.12	6.84	36.1	71.58	−0.642	Cytoplasm
LsHsp20–18	LSAT_9X82001	331	37,618.06	8.63	49.18	92.24	−0.319	Endoplasmic reticulum
LsHsp20–19	LSAT_2X28080	206	23,699.52	5.67	58.16	62.86	−0.8	Cytoplasm
LsHsp20–20	LSAT_2X28120	157	17,985.33	6.01	58.8	70.7	−0.735	Cytoplasm
LsHsp20–21	LSAT_2X120081	163	18,499.74	6.33	39.25	66.87	−0.663	Cytoplasm
LsHsp20–22	LSAT_2X120060	157	17,851.17	6.01	59.31	75.73	−0.687	Cytoplasm
LsHsp20–23	LSAT_1X25141	217	24,832.00	9.43	41.78	83.46	−0.4	Chloroplast
LsHsp20–24	LSAT_7X14200	264	29,265.61	9.49	42.87	78.6	−0.617	Cytoplasm
LsHsp20–25	LSAT_2X28000	210	23,811.85	4.94	56.19	74.24	−0.64	Cytoplasm
LsHsp20–26	LSAT_4X63061	223	25,019.76	8.84	37.61	79.91	−0.603	Cytoplasm
LsHsp20–27	LSAT_2X7120	198	21,114.95	5.17	24.34	80.66	−0.249	Cytoplasm
LsHsp20–28	LSAT_3X65081	141	15,645.93	6.85	31.12	86.17	−0.345	Peroxisome
LsHsp20–29	LSAT_1X56241	188	21,175.22	9.25	32.81	81.33	−0.424	Cytoplasm
LsHsp20–30	LSAT_7X61081	163	18,229.89	6.43	40.74	83.68	−0.49	Cytoplasm
LsHsp20–31	LSAT_6X91340	215	24,564.05	5.56	61.23	67.53	−0.629	Chloroplast
LsHsp20–32	LSAT_8X4541	233	26,148.58	7.61	48.7	71.5	−0.65	Chloroplast
LsHsp20–33	LSAT_8X72460	155	17,648.97	5.8	44.51	68.45	−0.693	Cytoplasm
LsHsp20–34	LSAT_8X72420	155	17,725.03	5.81	46.91	68.45	−0.715	Cytoplasm
LsHsp20–35	LSAT_4X59740	140	16,341.56	4.95	70.9	82.14	−0.321	Cytoplasm
LsHsp20–36	LSAT_9X40940	186	20,924.85	5.88	42.3	87.9	−0.511	Golgi apparatus

localized in the cytoplasm and nucleus, six were localized in chloroplasts, two were localized in the Golgi apparatus, one was localized in the peroxisome, and one was in the endoplasmic reticulum.

3.2 Analysis of the structures and domains of the *LsHsp20* genes in lettuce

By analyzing the conserved structural domains of the lettuce LsHsp20 family members (Figure 1A), a total of 15 conserved domains were identified, with the number of amino acids ranging from 8 to 50 (Supplementary Table S4). Among the identified motifs, motif 9 was 8 amino acids wide, while motifs 5, 8, and 14 had widths of 50 amino acids. The number of conserved domains per LsHsp20 protein ranged from 1 to 7. Most of the LsHsp20 contained three to seven conserved domains, while LsHsp20–13, LsHsp20–27, and LsHsp20–36 each contained one conserved domain. Motif 1 (83.3%) and motif 2 (97.2%) appeared more frequently among the lettuce LsHsp20 family members (Figure 1B). Based on the results of the Pfam and SMART analyses, motifs 1 and 2 are ACD structures and may play important roles in the stress response in lettuce. An analysis of the gene structure of the 36 identified lettuce LsHsp20 gene family members revealed that among the *LsHsp20* genes, 41.7% had no introns, 55.6% had one intron, and 2.7% had two introns (Figure 1C).

3.3 Secondary structure and three-dimensional structural model of the lettuce LsHsp20 protein family

An analysis of the secondary structures of the lettuce LsHsp20 proteins is shown in Supplementary Table S5. The secondary structures of the 36 proteins were composed of alpha helices, extended strands, random coils, and beta turns but were predominantly composed of random coils. The secondary structure proportion for the LsHsp20 protein family was as follows: random coil > alpha helix > extended strand > beta turn. Among them, alpha helices accounted for 10.68%~38.65%, extended strands accounted for 13.95%~25.76%, random coils accounted for 34.97%~60.52%, and beta turns accounted for 3.4%~9.31%. To determine the reasonable theoretical structures of LsHsp20 proteins, the three-dimensional structures of 36 lettuce LsHsp20 family members were predicted using the SWISS-MODEL homology modeling method (Figure 2), and the structure with the highest coverage score was selected as the best structure of the

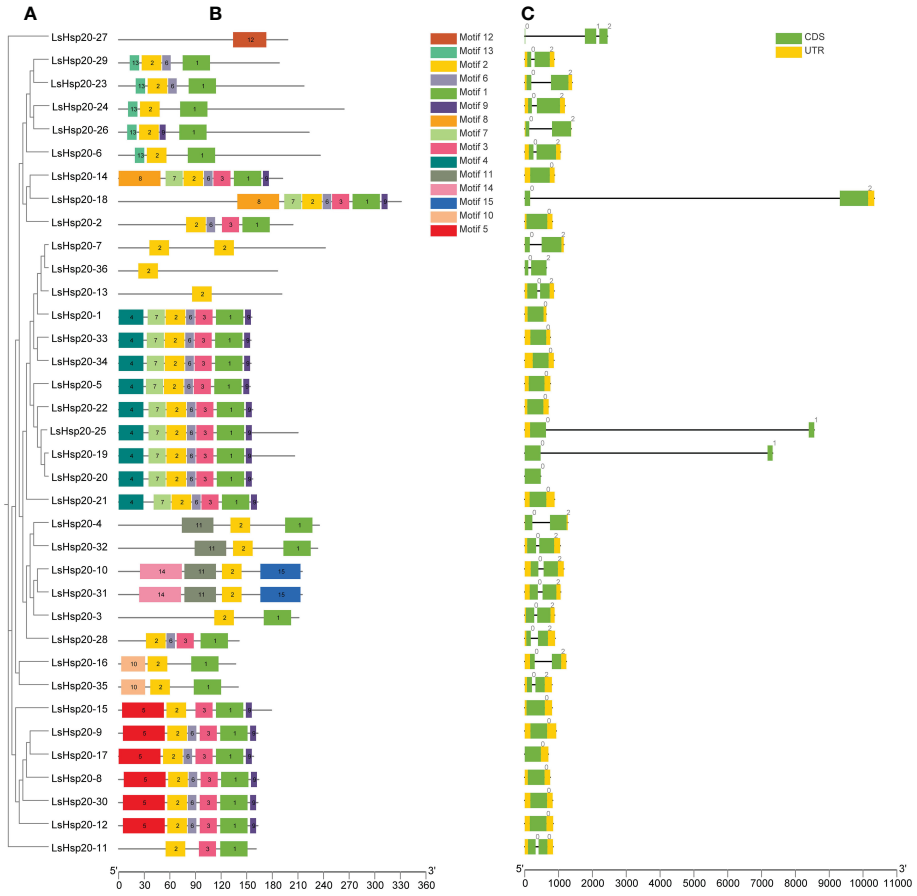

FIGURE 1
Evolutionary tree **(A)**, motif analysis **(B)**, and gene structure **(C)** of the lettuce LsHsp20 family.

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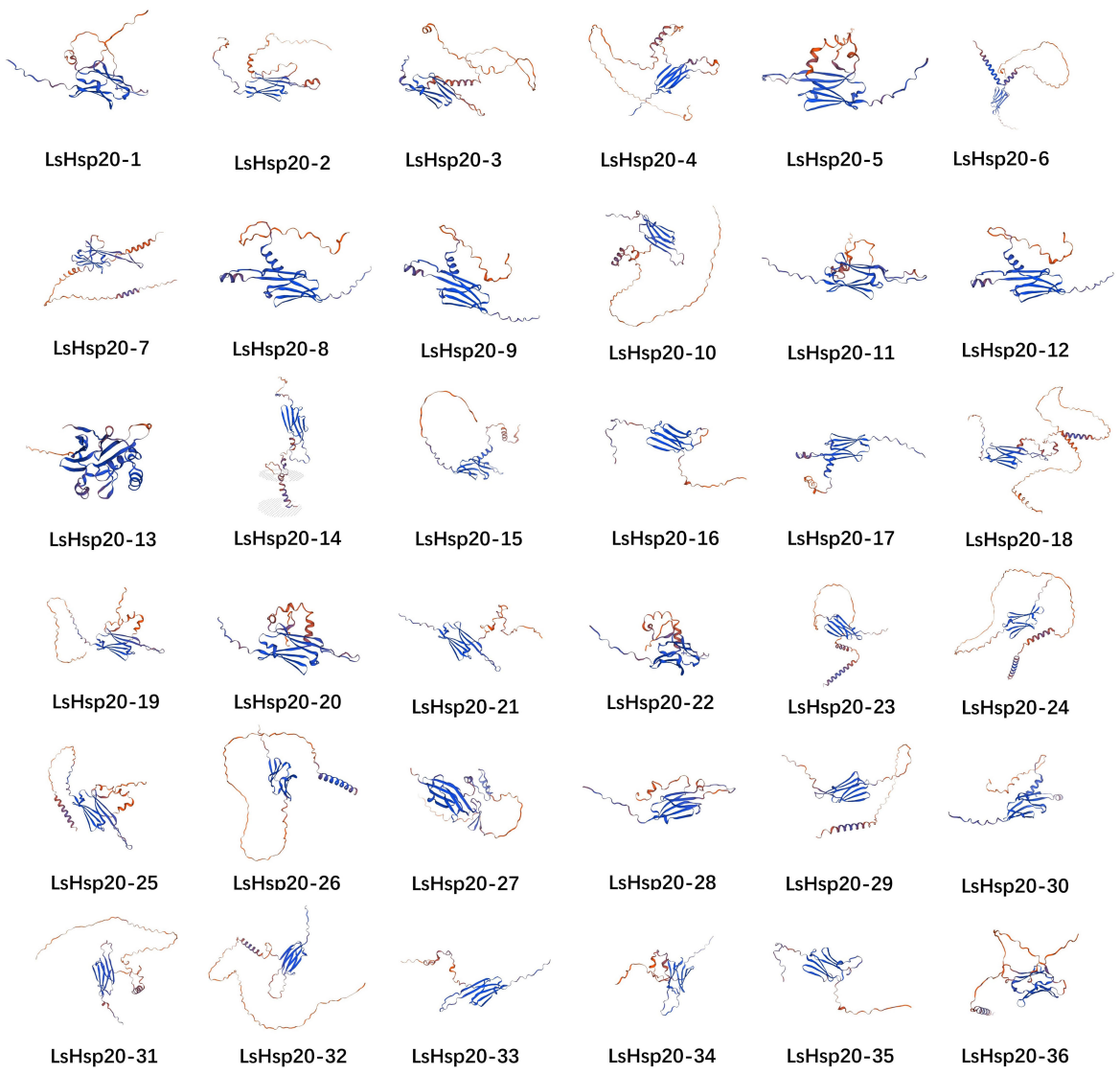

FIGURE 2
The tertiary structure of proteins of the LsHsp20 family. Using the protein homology modeling method based on the structures of LsHsp20 proteins in the SWISS-MODEL database, the structure with the highest score was chosen as the optimal structure for the LsHsp20 proteins.

LsHsp20 proteins. The results showed that all 36 LsHsp20 proteins were oligonucleotides and contained α folds, and 44% of the LsHsp20 protein models had no similarity.

3.4 Chromosomal location, collinearity analysis, and evolutionary selection pressure analysis of the lettuce *LsHsp20* genes

In this study, we further analyzed the chromosomal locations of 36 *LsHsp20* genes in lettuce. As shown in Figure 3, these genes were unevenly distributed on nine chromosomes. Chr 07 had up to nine genes, accounting for 25% of the *LsHsp20* genes in lettuce. Chr 02 had six genes; Chr 01, Chr 04, Chr 05, and Chr 09 each had three genes; Chr 02 had four genes; and Chr 03 and Chr 06 had at least two genes each. A total of five gene clusters were found in Chr 02, 07, 08, and 09. Furthermore, we analyzed the duplication events of the *LsHsp20* genes in lettuce (Figure 4). Chromosomal evolution and gene replication events inside lettuce were analyzed using collinearity within lettuce species. Four pairs of genes, namely, *LsHsp20–5* and *LsHsp20–34*, *LsHsp20–1* and *LsHsp20–34*, *LsHsp20–16* and *LsHsp20–35*, and *LsHsp20–24* and *LsHsp20–26*, had a collinear relationship and were segmented duplications, and the TBtools software was used to calculate the non-synonymous replacement rate (Ka) and the synonymous replacement rate (Ks). A Ka/Ks ratio greater than, equal to, or less than 1 represents positive, neutral, and purified options, respectively. The Ka/Ks ratios of the four pairs of collinear *LsHsp20* genes in lettuce were less than 1, indicating that the *LsHsp20* genes were mainly purified during *L. sativa* evolution (Table 2). To explore the evolutionary relationship of the LsHsp20 family in lettuce, a collinear map of

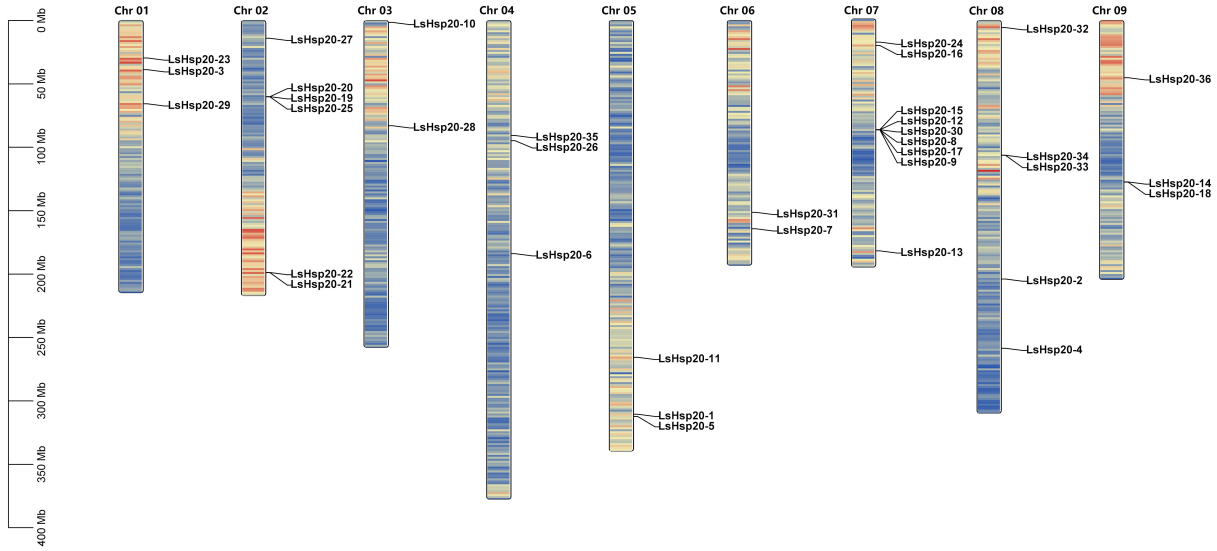

FIGURE 3
Chromosomal location of the LsHsp20 gene family in lettuce.

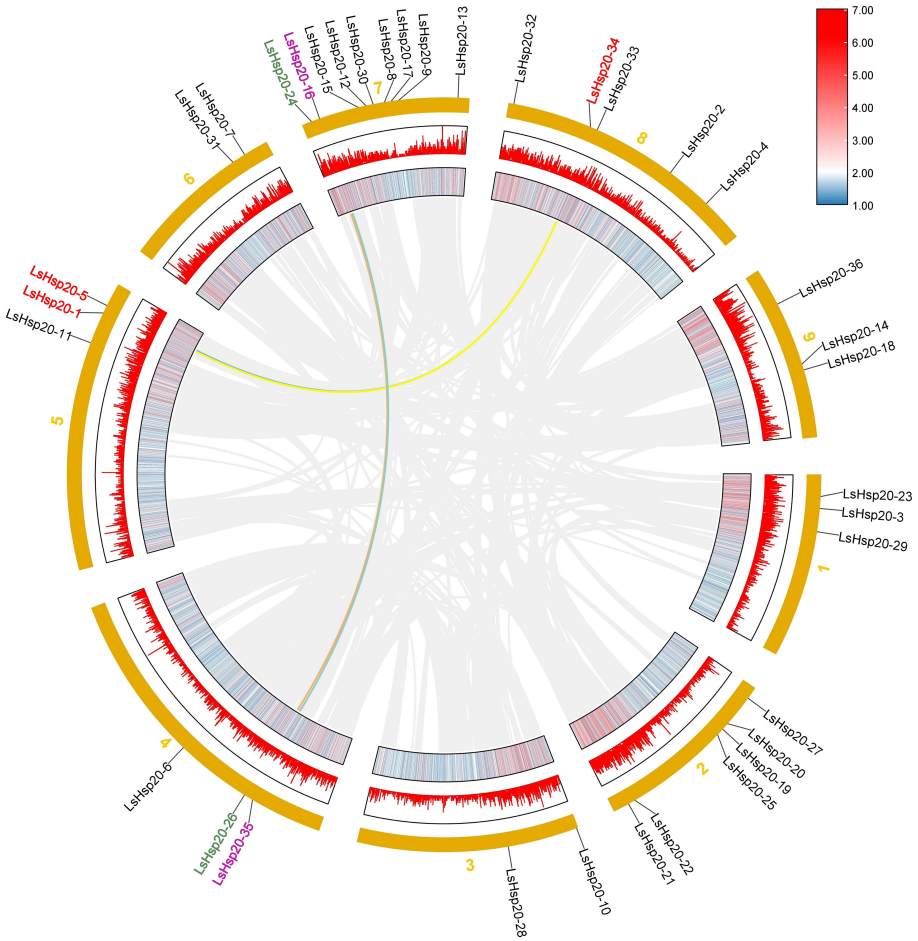

FIGURE 4
Collinearity analysis of lettuce.

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TABLE 2 Evolutionary selection pressure analysis of *LsHsp20*.

Duplicated gene pairs	Ka	Ks	Ka/Ks	Purifying selection
LsHsp20–35/LsHsp20–16	0.12	1.03	0.16	Yes
LsHsp20–26/LsHsp20–24	0.30	1.47	0.20	Yes
LsHsp20–1/LsHsp20–34	0.04	1.61	0.03	Yes
LsHsp20–5/LsHsp20–34	0.20	2.37	0.08	Yes

lettuce, *Arabidopsis*, and tomato plant was constructed. The results showed that 13 pairs of genes were collinear in lettuce and *Arabidopsis* (Figure 5), 14 pairs of genes were collinear in lettuce and tomato, and 7 pairs of genes were collinear in lettuce, *Arabidopsis*, and tomato.

3.5 Phylogenetic analysis of the LsHsp20 protein family in lettuce

To clarify the evolutionary relationship of the Hsp20 family, the sequences of lettuce and other species were compared to construct a phylogenetic tree. MEGA7.0 software was used to construct a phylogenetic tree (Figure 6). A total of 178 Hsp20 sequences, consisting of 27 *Arabidopsis thaliana* sequences, 42 tomato sequences, 35 rice sequences, 38 barley sequences, and 36 lettuce sequences, were used in the phylogenetic analysis (Supplementary Table S6). According to phylogenetic analysis, the lettuce LsHsp20 proteins can be divided into 12 subgroups (CI, CII, CIII, CIV, CV, CVI, CVII, CVIII, PO, CPI, CPII, and ER), among which 26 (72.2%) of the 36 LsHsp20 proteins belong to the CI–CVIII subgroups.

3.6 Analysis of cis-acting elements in the promoters of the lettuce LsHsp20 gene family

The 2,000-bp genomic sequence upstream of the *LsHsp20* genes was extracted to analyze the location and number of cis-acting elements. The 36 genes mainly included three categories: biotic and abiotic stresses, plant hormone responses, and plant growth and development. Five, five, and three cis-acting elements were identified, respectively (Supplementary Table S7). Analysis of the number of cis-acting elements in the LsHsp20 family in lettuce revealed that *LsHsp20–10* is the member with the largest number of cis-acting elements in its promoter, of which hormone response elements account for 78.95% and stress response-related elements account for 21.05%. *LsHsp20–3* is the member with the least number of cis-acting elements in the promoter, of which 50% are biotic and abiotic stress elements and 50% are plant hormone response elements. All cis-elements of *LsHsp20–9* are involved in the hormone response (Figure 7).

3.7 Expression patterns of the *LsHsp20* genes under drought stress

To study the response of LsHsp20 to drought stress, qRT-PCR was used to analyze the expression of LsHsp20 after drought treatment. There were differences in the relative expression levels of 28 *LsHsp20* genes under drought stress (Figure 8). The relative expression of *LsHsp20* exhibited fluctuations over the course of a 21-day drought treatment. Except for the *LsHsp20–5*, *LsHsp20–10*, *LsHsp20–13*, *LsHsp20–30*, and *LsHsp20–34* genes, the expression levels of other *LsHsp20* genes were significantly increased on the 7th and 14th days of drought treatment, and the expression levels of most genes were 2–60 times of the normal level. Notably, the expression of *LsHsp20–12* and *LsHsp20–26* genes was 153 and 273 times the normal level, respectively. The expression of most *LsHsp20* genes was downregulated on day 21 of drought treatment or had no significant difference compared with day 0. The expression levels of *LsHsp20–5*, *LsHsp20–10*, and *LsHsp20–30* were downregulated in each drought treatment period. In general, most *LsHsp20* genes are responsive to drought stress.

4 Discussion

Hsp20 proteins have the largest number of members in plants. They are not only involved in the regulation of plant growth and

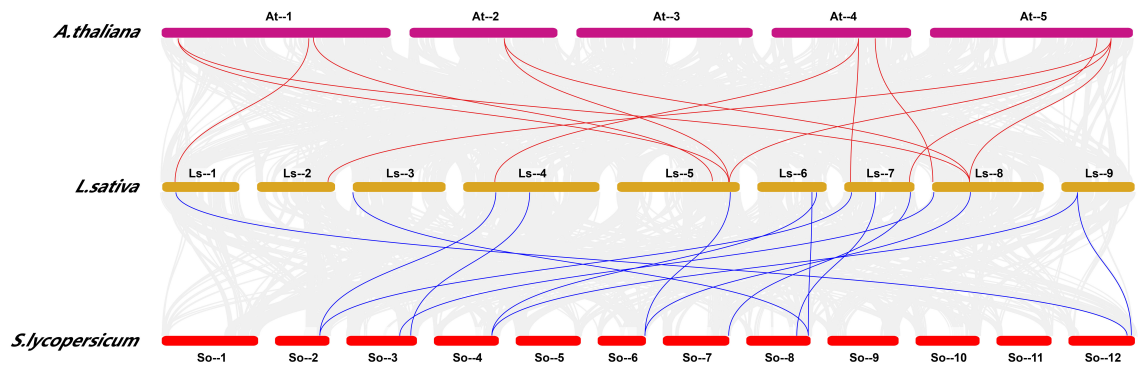

FIGURE 5
Analysis of the collinearity analysis of lettuce with *Arabidopsis* and tomato plants.

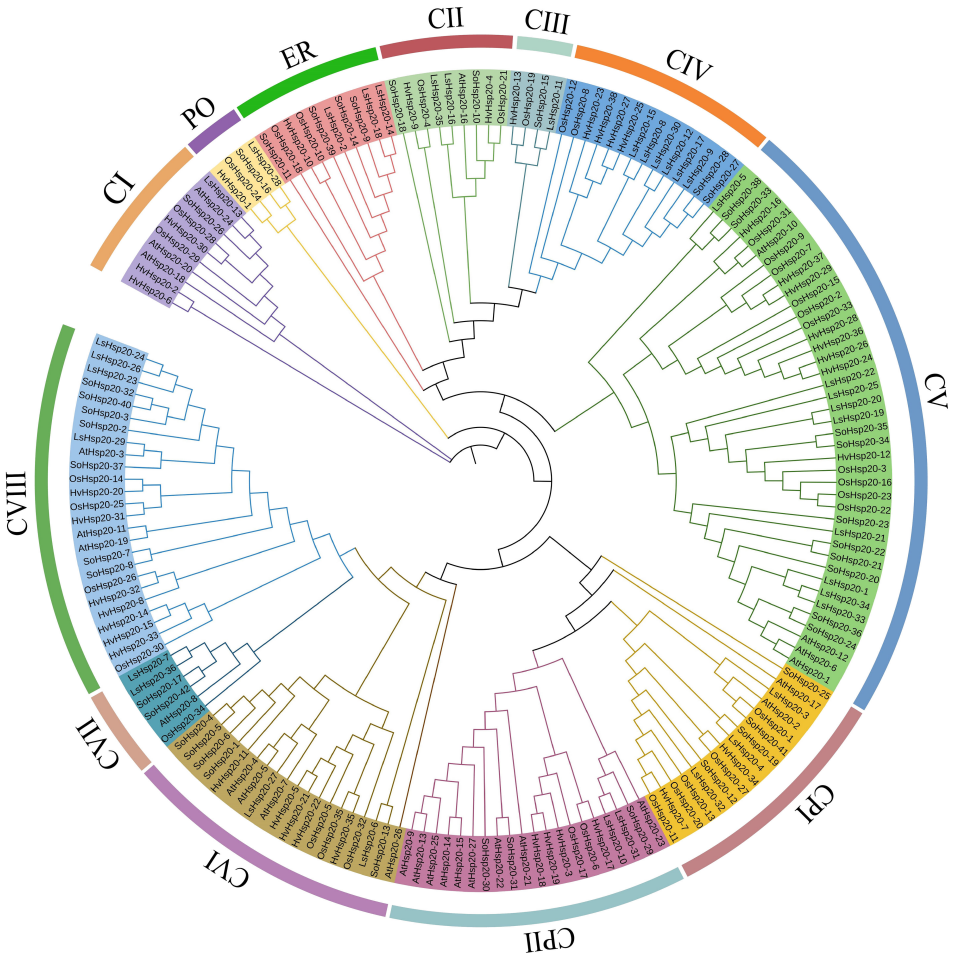

FIGURE 6
Phylogenetic tree analysis of the LsHsp20 protein family in lettuce and other plants. Ls, *Lactuca sativa*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; So, *Solanum lycopersicum*; Hv, *Hordeum vulgare*. CI–CVIII indicate that the protein is located in the cytoplasm or nucleus, PO indicates that the protein is located in the peroxisome, CP indicates that the protein is located in the chloroplast, and ER indicates that the protein is located in the endoplasmic reticulum.

development at specific developmental stages but also inhibit the irreversible aggregation of denatured proteins through molecular chaperone mechanisms when plants are subjected to abiotic stress, thereby enhancing plant resistance to adverse stress (Neto et al., 2020). To date, the Hsp20 gene family has been identified in multiple dicot and monocot species, such as rice (Ouyang et al., 2009), barley (Li and Liu, 2019), apple (Yao et al., 2020), peach (Lian et al., 2022), and pumpkin (Hu et al., 2021). However, no comprehensive study or identification of the lettuce Hsp20 family has been done.

The number of *Hsp20* genes may correlate with the different genome sizes of different plant species or to the expansion or reduction in the number of genes caused by genome duplication or loss during plant evolution (Guo et al., 2015; Qi et al., 2022). In this study, 36 *Hsp20* genes were identified in lettuce, which was less than those in potato (48 genes) (Zhao et al., 2018) and tomato (42 genes) (Yu et al., 2016) and more than in *Arabidopsis* (19 genes) (Siddique et al., 2008) and *Coix* (32 genes) (Hua et al., 2023). According to previous studies, a 200-kb chromosomal region containing two or more genes located on the same chromosome is defined as a tandem duplication (Ma et al., 2016). This study revealed that 36 *LsHsp20* genes were unevenly distributed on nine lettuce chromosomes. We identified five gene clusters, each containing at least two genes. In addition, in chromosome 7, many LsHsp20 family members clustered together (Figure 3). In the duplication event, we found that four pairs of genes had segmental duplications (Figure 5). These phenomena indicate that tandem duplication and segmental gene duplication events may have increased the number of LsHsp20 gene family members. This result is similar to the results obtained for pigeon pea and bread wheat (Muthusamy et al., 2017; Ramakrishna et al., 2022). To better understand the relationships between the *LsHsp20* genes and *Hsp20* genes from other species, we conducted a species collinearity analysis on lettuce, *Arabidopsis*, and tomato. Thirteen pairs of genes were collinear between lettuce and *Arabidopsis*, and 14 pairs of genes were collinear between lettuce and tomato, indicating that more than half of the *LsHsp20* genes had no collinear relationship with *Arabidopsis* and tomato, which indicates that the lettuce *LsHsp20* genes were relatively conserved during evolution (Ji et al., 2019; Gong et al., 2021; Qi et al., 2022).

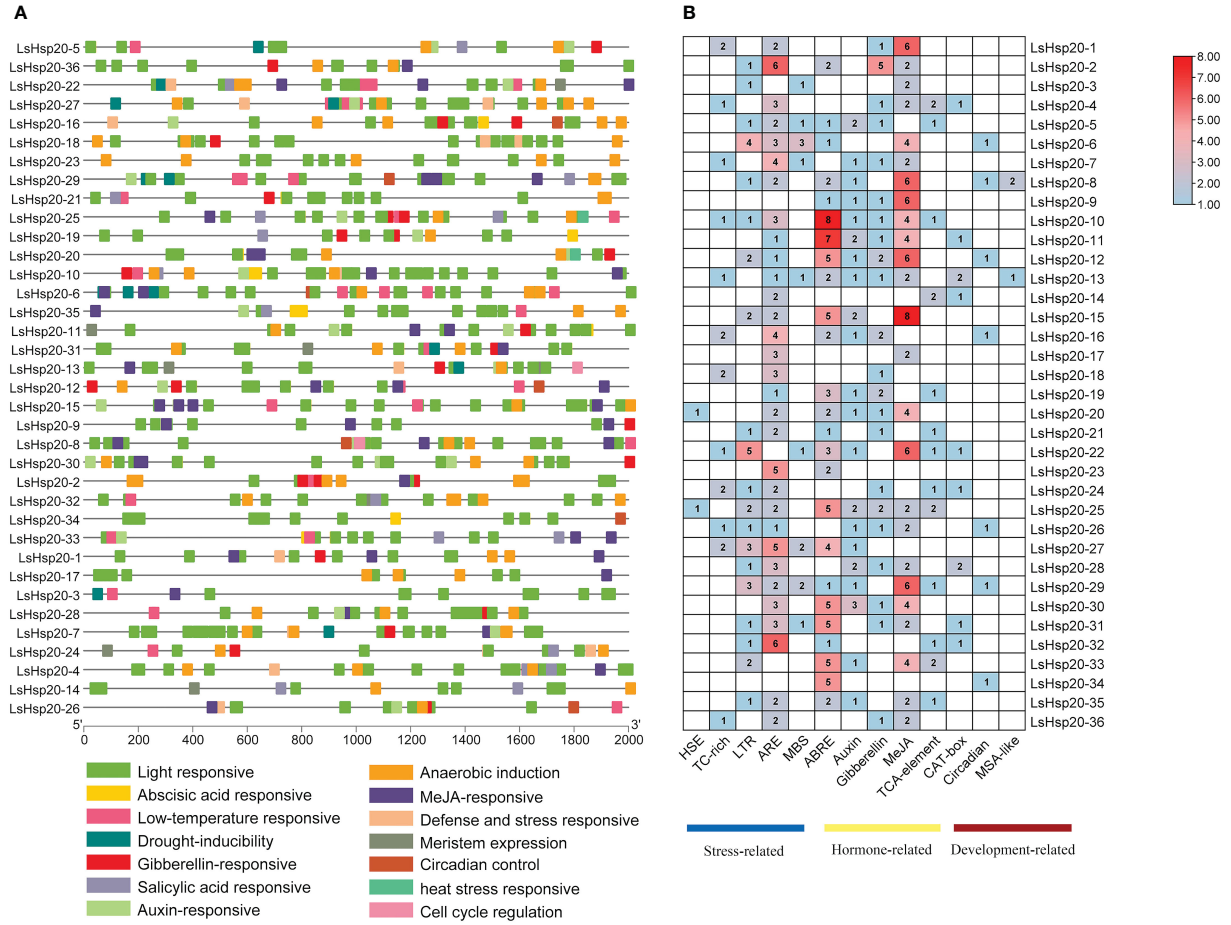

FIGURE 7
Prediction of the promoter cis-acting elements of the LsHsp20 gene family members in castor. Position **(A)** and number **(B)** of promoter cis-acting elements. HSE, heat stress-responsive cis-acting element; TC-rich, defense and stress signal response elements; LTR, low-temperature signal response element; ARE, anaerobic inducible element; MBS, drought induction element; ABRE, abscisic acid response cis-element; Auxin, auxin response cis-element; Gibberellin, gibberellin response cis-element; MeJA, jasmonic acid response cis-element; TCA-element, salicylic acid response cis-element; CAT-box, meristem expression cis-element; Circadian, circadian rhythm control cis-element; MSA-like, cell cycle regulation cis-element.

Gene structure plays a very important role in plants and facilitates a better understanding of the evolution of gene families in species (Silva et al., 2022). Studies have shown that when plants are subjected to biotic or abiotic stress, genes with fewer or no introns can be quickly activated to help plants cope with the stress, and genes with fewer or no introns have higher expression levels in plants (Ren et al., 2006; Wang et al., 2021). The gene structure analysis of the 36 identified lettuce LsHsp20 gene family members showed that 15 genes (41.7%) had no introns and 20 genes (55.6%) had one intron. *LsHsp20–27* has two introns, the LsHsp20 gene family shows similar motif arrangement in each phylogenetic subgroup, and genes in the same subgroup have the same intron phase, which indicates that the structure may be relatively conservative during evolution, which is similar to the finding of previous studies on apple and *Dendrobium catenatum* (Sarkar et al., 2009; Yao et al., 2020; Wang et al., 2024). The 36 LsHsp20 proteins were divided into 12 subfamilies, 26 of which were located in the cytoplasm and nucleus (CI, CII, CIII, CIV, CV, CVI, CVII, and CVIII), constituting the largest subgroup branch, indicating that the cytoplasm and nucleus may be the main functional sites of the LsHsp20 family. This phenomenon has also been confirmed in other species, such as tomato (Yu et al., 2016) and *Cannabis sativa* (Huai et al., 2022). Studies have shown that plants may have lost or reacquired new genes during the evolution. This study did not identify subgroups such as CX, CXI, CIX, MI, MII, and P from the lettuce LsHsp20 proteins. Similar findings have been reported for other species. In pumpkin, the Hsp20 family lacks subfamilies such as CVI, CVI, and CVIII (Hu et al., 2021). In cucumber, the Hsp20 family lacks CIII, CX, CXI, and other subfamilies (Huang et al., 2022). The loss of certain genes in the lettuce LsHp20 family during evolution may have led to a lack of subgroups.

Studies have shown that promoter cis-elements play important roles in plant physiological responses to biotic and abiotic stresses (Lopes-Caitar et al., 2013; He et al., 2018; Sun et al., 2022). Three major categories of response elements were identified in the promoter regions of the *Hsp20* genes, including hormone response elements, stress response elements, and plant

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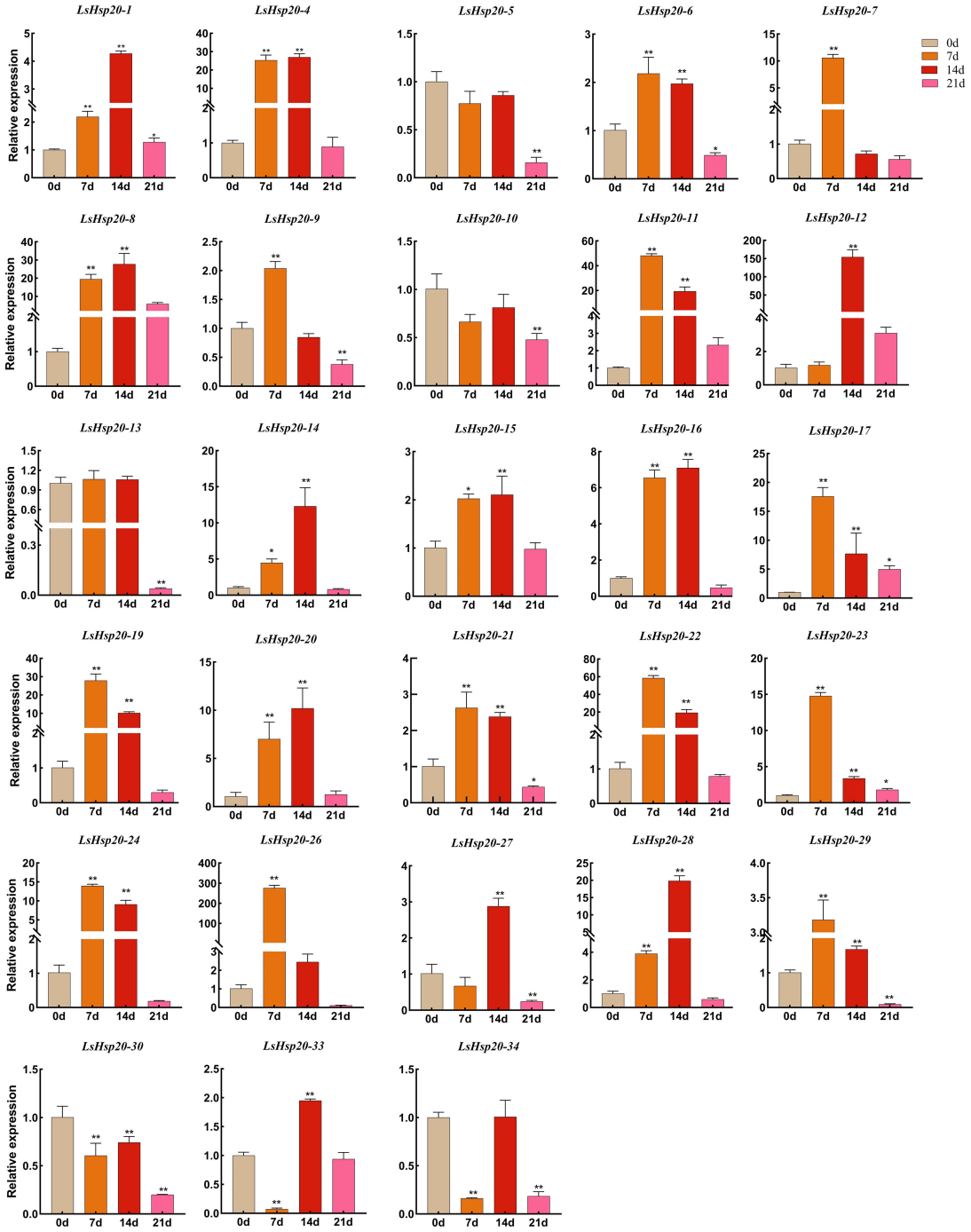

FIGURE 8
Expression levels of the *LsHsp20* genes under drought stress. All data shown are the means SD of three biological replicates. The asterisks indicate the level of significance (*$p < 0.05$, **$p < 0.01$) based on Duncan's multiple range test.

development-related elements (Figure 7), among which hormone response elements accounted for the greatest proportion. These findings indicate that the lettuce *Hsp20* genes have multiple or specific functions. Studies have shown that plant hormones can participate in the regulation of plant growth and development and can finely regulate environmental stress through interactions between different hormone signaling pathways (Sato et al., 2018). *AtHSP17.8* is involved in regulating ABA-mediated signaling by overexpressing genes in *Arabidopsis* and lettuce, resulting in a resistance phenotype when plants face environmental stress (Kim et al., 2013). Overexpression of *MsHsp16.9* increased ABA biosynthesis and accumulation in plants, suggesting that *MsHsp16.9* may act as a positive regulator of ABA signaling in *Arabidopsis* (Yang et al., 2017).

Most Hsp20 can be strongly induced under abiotic and biotic stress conditions, including high temperature, drought, salinity, low temperature, heavy metals, hypoxia, and some pathogenic bacteria, thereby enhancing plant tolerance (Peng et al., 2023; Jung et al., 2024). Under high-temperature stress in apples, the expression of 12 *Hsp20* significantly increased more than 1,000-fold after 4 h of heat stress (Yao et al., 2020). In potato, the expression of most *StHsp20* genes was upregulated under high temperatures, drought, and salt stress (Zhao et al., 2018). The overexpression of three *HvHsp20* genes in barley can improve plant resistance to heat stress and biotic stress (powdery mildew) (Li and Liu, 2019). In our study, under drought stress, the expression levels of 23 *LsHsp20* genes were significantly upregulated, among which the expression levels of *LsHsp20–12* and *LsHsp20–16* significantly increased by 153- and 273-fold on the 14th and 7th days of drought treatment, respectively, indicating that the *LsHsp20* genes of lettuce respond to drought stress. These two genes may be more responsive to drought stress and can be used as candidate genes for the selection of drought-tolerant lettuce varieties and their genetic improvement. This study detected the gene expression levels of LsHsp20 in lettuce under drought conditions and found that the gene expression levels of most LsHsp20 increased after drought stress.

5 Conclusion

In summary, we performed a genome-wide analysis of the LsHsp20 family in lettuce and identified 36 *LsHsp20* genes. These 36 *LsHsp20* genes are unevenly distributed across nine chromosomes, and the 36 LsHsp20 proteins are divided into 12 subfamilies based on phylogenetic tree and subcellular localization data. To better explore the evolutionary relationships among members of the LsHsp20 family, we analyzed the protein structure, gene structure, conserved motifs, cis-acting elements, and homology between lettuce and other species. The qRT-PCR data revealed significant upregulation of the expression levels of 23 *LsHsp20* genes on the 7th or 14th day of drought stress, indicating strong responsiveness of most LsHsp20 genes in lettuce to drought stress. This indicates that *LsHsp20* genes play an important role in the drought tolerance of lettuce. This study provides information on *LsHsp20* genes and a theoretical basis for the selection of drought-tolerant lettuce varieties and their genetic improvement.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

QZ: Writing – original draft, Software. BD: Writing – original draft, Formal analysis. MF: Writing – review & editing, Formal analysis. LY: Writing – review & editing, Methodology. CL: Writing – review & editing, Methodology. GH: Writing – review & editing, Data curation. XW: Writing – review & editing, Data curation. HG: Writing – review & editing, Conceptualization. JL: Writing – review & editing, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1426719/full#supplementary-material

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Plant responses to abiotic stress regulated by histone acetylation

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In eukaryotes, histone acetylation and deacetylation play an important role in the regulation of gene expression. Histone acetylation levels are reversibly regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Increasing evidence highlights histone acetylation plays essential roles in the regulation of gene expression in plant response to environmental stress. In this review, we discussed the recent advance of histone acetylation in the regulation of abiotic stress responses including temperature, light, salt and drought stress. This information will contribute to our understanding of how plants adapt to environmental changes. As the mechanisms of epigenetic regulation are conserved in many plants, research in this field has potential applications in improvement of agricultural productivity.

KEYWORDS

abiotic stress, epigenetic regulation, histone acetylation, histone acetyltransferase, histone deacetylase

Introduction

As sessile organism, the growth and development of plants are constantly affected by environmental conditions. Adverse environmental conditions severely affect the growth and productivity of crop plants. Abiotic stress disrupts the growth and development of crop plants, leading to the reduction of quality and yield, which is one of the main factors restricting the yield of crop plants in China (Upadhyay et al., 2019; Salava et al., 2021). Many studies have been made to reveal the mechanism of plants response to stress conditions.

Epigenetics explores heritable alterations in gene expression without changes to DNA sequence itself (Stam, 2009; Gao et al., 2021b). This field examines a variety of phenomena, including DNA methylation, genomic imprinting, gene silencing, RNA editing, defense against transposon proliferation, etc (He et al., 2011; Raissig et al., 2011; Nuñez et al., 2021). Additionally, in plants, epigenetic mechanisms play crucial roles in development and in responding to environmental stressors (Rando and Chang, 2012; Berry and Dean, 2015). In eukaryotic cells, DNA wraps around core histone proteins -H2A, H2B, H3, and H4 to form chromatin (Du et al., 2020). These histone proteins undergo various post-translational

modifications including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP ribosylation, impacting gene expression and activity (Ueda and Seki, 2020).

Histone acetylation is a crucial epigenetic modification, regulates gene expression in eukaryotes (Gan et al., 2021; Xu et al., 2022), affecting plant growth, development, and stress response (Supplementary Table 2). Histone acetylation levels are reversibly regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Jiang et al., 2020; Zheng et al., 2020) (Figure 1B). HATs add acetyl groups to specific lysine residues on N-termini of histone H3 and H4, which activates gene transcription (Figures 1B, C). This process neutralizes the positive charge on the histone tail, reducing the interaction between histone and DNA or other histones, thereby loosening chromatin structure. This allows transcription factors (TFs) easier access to target genes, facilitating the regulation of downstream gene expression. On the contrary, HDACs are associated to transcriptional repression and gene silencing by removing acetyl groups from lysine residues (de Rooij et al., 2020) (Figure 1C).

Histone acetylation typically regulates by recruiting TFs to modulate acetylation levels at various downstream gene promoter sites. It primarily occurs on conserved lysine residues at the N terminal of H3 and H4, with modification sites include H3 (K4, K9, K14, K18, K23, K27) and H4 (K5, K8, K12, K16). In plants, HATs are categorized into four families based on domain characteristics: GNAT (Gcn5-related N-acetyltransferases), MYST (MOZ-YBF2/SAS3-SAS2/TIP60), CBP (CREB-binding protein) and TAFII250 (TATA-binding protein-associated factor), also known as HAG, HAM, HAC, and HAF, respectively (Figure 1A). These HAT families possess distinct conserved domains granting them multifunctional capabilities (Supplementary Table 1). For example, HAG ELP3 (elongator complex protein 3) interacts with RNA Pol II, HAT1 (histone acetyltransferase 1) conducts histone acetylation, Znf-ZZ and Znf-TAZ facilitates protein-protein interactions, and the PHD domain enables HATs to interact with other histones. Thus, HATs form protein complexes to collaboratively regulate gene expression with TFs and other histone modifiers.

The number of HATs varies among plant species, with *Arabidopsis* having 12, rice 8, and tomato 32. Research indicates that histone acetylation plays a crucial role in plant responses to various stresses, including light (Perrella et al., 2020; Ageeva-Kieferle et al., 2021), temperature (Hu et al., 2015; Ohama et al., 2017), salt (Zheng et al., 2021; Feng et al., 2022) and ABA (Chen et al., 2010; Liao et al., 2016).

Accordingly, it is critically important to conduct research on the epigenetic regulation of crop plants under abiotic stress. This review encapsulates the advancements in understanding histone acetylation in plant responses to abiotic stress, providing key epigenetic insights for the genetic enhancement of future crops.

Functions of histone acetylation in plants response to abiotic stress

Salt stress

HATs/HDACs are pivotal in managing salt stress by regulating the expression of salt stress responsive genes. In *Arabidopsis*, histone acetyltransferase GCN5 regulated the expression of cellulose synthesis genes to maintain cell wall integrity by altering the acetylation level at H3K9 and H3K14, thereby improving salt stress tolerance (Zheng et al., 2019). In contrast, more histone deacetylases are involved in salt stress regulation. For example, HD2C interacted with another histone deacetylase HDA6, regulating ABA-responsive genes expression by changing histone H3K9 and H3K14 acetylation, responding to ABA and salt stress (Luo et al., 2012). Overexpression of the *HDA15* gene conferred

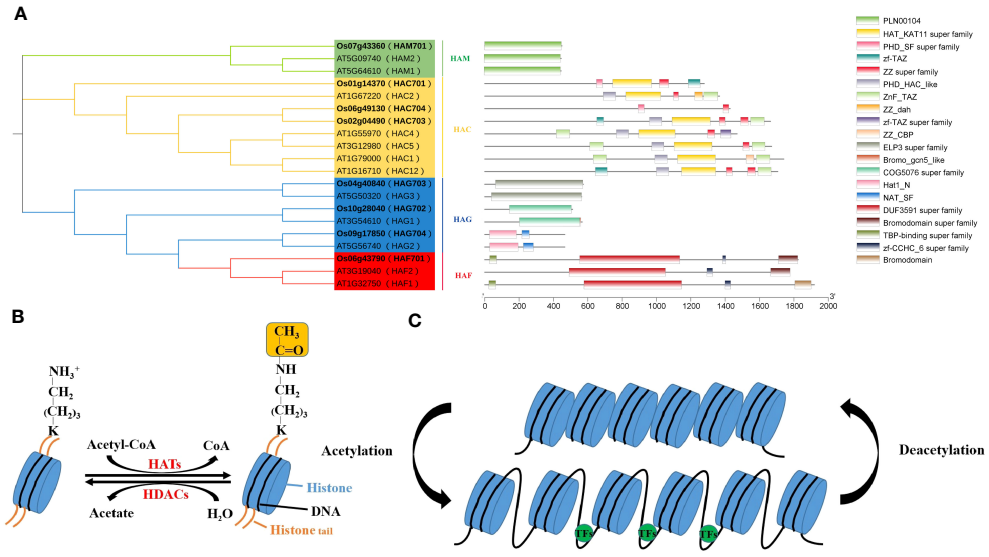

FIGURE 1
Conserved domain analysis of the AtHAT/OsHAT gene families **(A)** and regulation of histone acetylation dynamics **(B, C)**.

resistance to salt stress by regulating the H3K14ac and H4K16ac levels of *NCED3* (Ueda et al., 2017; Truong et al., 2021). Research indicates that WRKY53 and HDA9 play contrasting roles in regulating plant response to salt stress (Zheng et al., 2020). Moreover, HY5 was found to collaborate with HDA9 to regulate the transcription of *HsfA2* in response to salt stress (Yang et al., 2023b). Histone deacetylase AtSRT2 was shown to regulate salt tolerance during seed germination via repression of vesicle-associated membrane protein 714 (*VAMP714*) (Tang et al., 2022). The Histone Deacetylase Complex 1 (HDC1) modulated the response of salt-treated seedlings by changing the acetylation levels at histone H3 lysine 9 and 14 (H3K9ac/H3K14ac) (Perrella et al., 2023). SAP18 interacted with HDA1, exerting a negative regulatory effect on the adaption to salt stress (Song and Galbraith, 2006). Furthermore, MSI1, HDA19 and HDC1complex was identified to interact with SIN3-like proteins, collectively contributing to the intricate regulatory network that governs salt stress tolerance (Mehdi et al., 2016).

In rice, *OsHAC701* has been reported to respond to salt treatment (Liu et al., 2012). OsHDA706, a histone H4 deacetylase, could enhance the salt tolerance of rice by regulating the expression of *OsPP2C49* via H4K5 and H4K8 deacetylation (Liu et al., 2023). IDS1 interacted with histone deacetylase HDA1 to regulate rice salt tolerance by repressing the expression of *LEA1* and *SOS1* (Cheng et al., 2018). Another histone deacetylase, HDA710, controlled salt tolerance by regulating H4K5 and H4K16 acetylation on genes responsive to ABA (Ullah et al., 2020). HDA704 directly bound to *DST* and *ABIL2*, repressing their expression to positively regulate drought and salt tolerance (Zhao et al., 2021).

HATs/HDACs are also involved in salt stress regulation in other plants such as wheat, maize, soybean, cotton, poplar. In wheat, TaHAG1, a histone acetyltransferase, enhancing salt tolerance by modifying H3K9ac and/or H3K14ac at TSSs (Transcription Start Sites) (Zheng et al., 2021). Maize's *ZmHATB* and *ZmGCN5* boost salt tolerance by elevating H3K9ac levels at *ZmEXPB2* and *ZmXET1* promoters, causing root swelling (Li et al., 2014). GmPHD5 interacted with acetyltransferase GmGNAT to regulate salt responsive genes in soybean through H3K14ac (Wu et al., 2011). A transcription factor GsNAC83 in wild soybean, interacts with histone acetyltransferase GsMYST1 and GsSnRK1 kinase, to increase *COR15B* promoter activity for better tolerance to salt stress (Feng et al., 2022). Under salt stress, GmNFYA likely accumulated and competed with GmHDA13 for interaction with GmFVE, reducing H3K9ac at target loci and improving tolerance in soybean (Lu et al., 2021). Cotton expresses genes like *GhHAC1501* and *GhHAG1504* expressed higher under salt stress (Imran et al., 2019). Histone deacetylase gene *PtHDT902* negatively regulated salt stress tolerance in poplar (Ma et al., 2020). While expression changes in *SiHAT17*, *SiHAT23* and *SbHDACs* under salt stress indicated their role in stress management (Du et al., 2022; Xing et al., 2022).

Drought stress

Drought stress is one of the important abiotic stresses, which can cause serious harm to plants. Histone acetylation is widely involved in managing this stress. ABA-Responsive Element Binding Protein 1 (AREB1) and the ADA2b-GCN5 HAT complex regulate the expression of the drought-responsive genes (*PtrNAC006*, *PtrNAC007* and *PtrNAC120*) by enhancing H3K9ac under drought stress conditions, suggesting that transcription factors coordinated with histone acetylation to play important role in response to drought stress (Li et al., 2019). CRISPR/dCas9 -AtHAC1 fusion improves drought tolerance in *Arabidopsis* by activating *AREB1* and *RD29A* (Roca Paixão et al., 2019). Rice, wheat and Chinese cabbage show increased expression of various HAT genes under drought conditions, indicating their involvement in drought response (Fang et al., 2014; Eom and Hyun, 2018; Tan et al., 2019; Hou et al., 2021; Li et al., 2022).

GhHDT4D may enhance drought tolerance by suppressing *GhWRKY33* via reducing its H3K9ac, thereby activating the downstream drought response genes in cotton (Zhang et al., 2020a). *SlHDA1* and *SlHDA3* (Guo et al., 2022a; Guo and Wang, 2023a), and the interaction between HD2A and HD2C functions by H3K9ac in stomatal closure and root growth (Tahir et al., 2022), underscore HAT/HDAC's role in drought stress management. HDA9 interacted with PWR-ABI4 complex to promote drought tolerance, contrasting with WRKY53's effect under drought stress (Ali and Yun, 2020; Zheng et al., 2020). HDT4 worked with ENAP1-ENAP2-MYB44 complex to regulate drought responsive genes by altering H3K27ac (Zhao et al., 2022a). A histone deacetylase of *Brachypodium distachyon*, BdHD1 regulated the expression of *BdWRKY24* by changing H3K9ac to positively regulate drought response (Song et al., 2019b). Meanwhile, BdHD1 interacted with two drought-responsive transcription factors, BdWRKY24 and BdMYB22 to combat drought stress (Song et al., 2020). In conclusion, HAT/HDAC typically engages with ABA-related transcription factors such as AREB and WRKY members to participate in drought-related gene regulation. However, more regulatory elements, including additional transcription factors requires identification.

Temperature stress

Temperature stress, both high and low, hinders growth and severely affects their life processes. Plants have evolved mechanisms to adapt and attenuate the hazards of temperature stress, with HAT/HDACs playing a significant role. Heat stress increases the expression of HAT genes in rose, suggesting histone acetylation adjustments in response (Wu et al., 2022). GCN5 regulated heat stress responsive genes *HSFA3* and *UVH6* by facilitating H3K9ac and H3K14ac to maintain thermotolerance in *Arabidopsis* (Hu et al., 2015). In maize, the down-regulation of *ZmHO-1* and *ZmGSL1* was associated with the decrease of acetylation levels in their promoter regions under heat stress, indicating that histone acetylation was involved in the regulation of genes expression in response to heat stress (Zhang et al., 2018b). Studies also show significant changes in histone acetylation and methylation, indicating their combined involvement in maize's heat stress response (Hou et al., 2019; Yue et al., 2021). Wheat's TaHAG1 and TaNACL interaction enhances heat tolerance (Lin et al., 2022), while *Arabidopsis*'s HD2C and SWI/SNF complex interaction

suppresses heat-activated genes (Buszewicz et al., 2016). *Arabidopsis* also utilizes HD2B and HD2C with ARGONAUTE4 for heat tolerance through heterochromatin stabilization (Yang et al., 2023a). *ZmHDACs* downregulation and H3K9ac/H4K5ac upregulation in histones under heat suggests their critical role in heat stress response (Zhang et al., 2020b).

Heat stress is characterized by the adverse impact on plant growth and development when plants are exposed to high-temperature environments that surpass their optimal temperature range for normal physiological functioning. While ambient warm temperature conditions induce thermomorphogenesis, a process that shapes plant growth and development through a series of morphological adaptations. These adaptations include thermal acclimation, the development of thinner leaves, and the elongation of petioles and hypocotyls. Increasing evidence suggests that histone acetylation is also involved in thermomorphogenesis. MRG2 was shown to directly interact with the acetyltransferase HAM1/2, which is responsible for histone H4K5ac modification, thereby enhancing the transcription of thermal response genes such as *YUC8* and *SAUR19* (Zhou et al., 2024). One study showed that three HDACs (HDA9, HDA15, and HDA19) were involved in the thermomorphogenesis response of *Arabidopsis*. HDA15 was shown to be a direct repressor of plant thermal response process, while HDA9 and HDA19 promoted thermal response indirectly (Shen et al., 2019). Furthermore, HDA9 was reported to be involved in thermomorphogenesis in an auxin dependent manner (van der Woude et al., 2019).

Cold stress responses include HD2C degradation and the PWR-HOS15 complex recruiting CBF transcription factors and HATs to activate Cold Responsive (*COR*) gene transcription and freezing tolerance (Lim et al., 2020). In rice, cold stress induces H3K27ac but inhibits H3K27me3 to promote transcription of *COR* genes (Dasgupta et al., 2022). Cotton shows decreased acetyltransferase levels under cold (Truong et al., 2021). Under cold stress, the hyper-acetylation of H3K9 at the promoter and upstream region of the rice *dehydration responsive element binding protein 1b* (*OsDREB1b*) promoted chromatin remodeling and enhanced transcriptional activation (Roy et al., 2014). *Arabidopsis* recruits CBF factors to *COR* gene promoters, increasing H3 acetylation and activating *COR* genes (Pavangadkar et al., 2010). Similarly, the MaMYB4 factor in bananas represses *ω-3 MaFADs* transcription by modulating acetylation levels during cold stress (Song et al., 2019a). Overall, histone acetylation and methylation significantly impact plant responses to temperature stress, with HAT/HDAC regulating genes through interaction with temperature-related transcription factors.

Light signaling

Light stress is a significant abiotic stress, with both excessive and insufficient light having detrimental effects. Research indicates that HATs/HDACs play a crucial role in plant responses to light signals. For instance, variations in light intensity influence Nitric Oxide (NO) levels, correlating with changes in histone acetylation such as H3ac, H3K9ac and H3K14ac, regulated by HDA6 (Ageeva-Kieferle et al., 2021). GCN5-HD1-TAF1 complex regulated light-responsive gene expression by altering the acetylation level of H3K9, H3K27 and H4K12 in *Arabidopsis* (Benhamed et al., 2006). The up-regulation of the photoreceptor gene *PHYA* (*PHYTOCHROME A*) was associated with an increased pattern of histone acetylation at the *PHYA* locus, indicating that the expression of the photoreceptors themselves seemed to be regulated by histone acetylation (Jang et al., 2011). HAF2, from the TAFII250 family, activates light-dependent gene expression, affecting red/far-red and blue light responses via histone acetylation (Lee and Seo, 2018; Servet et al., 2010). Mutants of GCN5 and other chromatin factors affecting H2B ubiquitination, H3K36 trimethylation and H2A.Z removal impair photomorphogenesis and light adaptation (Bieluszewski et al., 2022).

The HOS15-EC-HDA9 complex reduces the activity of the *GIGANTEA*, which is crucial for initiating flowering based on day length (Park et al., 2019). SNL-HDA19 represses *HY5* and *BBX22*, affecting *Arabidopsis* photomorphogenesis (Jing et al., 2021). The HY5-HDA15 complex represses cell wall and auxin signaling genes by altering the levels of histone H4 acetylation to promote photomorphogenesis in a light-dependent manner (Zhao et al., 2019). HY5 also interacted with HDA9 to repress autophagy-related genes by changing H3K9ac and H3K27ac levels, in response to light-to-dark conversion (Yang et al., 2020). The PIF3-HDA15 protein complex negatively regulated the expression of photosynthetic genes by reducing the acetylation level of target genes (Liu et al., 2013). Furthermore, HDA19 and MED25 are recruited by PIF1/PIF3 to target gene promoters, thereby playing a negative role in photochrome signalings (Guo et al., 2023b). Recent study has revealed that the direct-target genes of PIF rapidly adjusted the level of H3K9ac in response to the light signal (Gonzalez et al., 2022). This finding complements the work of Willige et al (Willige et al., 2021), which showed that PIFs also changed the level of H3K9ac responding to the change of light quality, highlighting the intricate interplay between light perception and epigenetic regulation in plants. Further research is needed to uncover more proteins involved and to understand the intricate ways they regulate plant responses to light.

Phytohormone signaling

Phytohormones play important roles in plant growth and productivity. Recent studies suggest that several HATs/HDACs are involved in the signaling pathways of plant hormone such as ABA (Abscisic Acid), ethylene, JA (Jasmonic Acid), SA (Salicylic Acid) and BR (Brassinolide). HDA15 interacts with MYB96 to negatively regulate *RHO GTPASE OF PLANTS* (*ROP*) genes in ABA signaling via mediating the deacetylation of histone H3 and H4 (Lee and Seo, 2019). HDA15 also affects ABA responses by interacting with MAC3A/MAC3B to mediate splicing of introns (Tu et al., 2022). *Brachypodium* histone deacetylase BdHD1 regulates the expression of *BdWRKY24* by changing H3K9ac to regulate ABA and drought stress responses (Song et al., 2019b). AtHD2D interacted with CKA4 contributing to ABA response and root development (Zhang et al., 2022b). The MSI1-HDA19 complex repressed the expression of ABA-responsive genes by keeping low levels of histone H3K9ac to obtain decreased ABA sensitivity

(Mehdi et al., 2016). HAT/HDAC typically regulates the ABA signaling pathway by interaction with ABA-related TFs like WRKYs and MYBs, affecting plant senescence and stress response.

In ethylene signaling, SRT1, SRT2 and ENAP1 form a complex to suppress genes by reducing H3K9ac at their promoter regions (Zhang et al., 2018a). The MdERF4-MdTPL-MdHDA19 repressor complex participates in the epigenetic regulation of fruit ripening and ethylene production by facilitating H3K9 deacetylation (Hu et al., 2022). Histone deacetylase SlHDT1 regulates the genes related to ethylene and carotenoid biosynthesis to delay tomato fruit ripening by altering total histone H3ac level (Guo, 2022b). The SlERF.F12-TPL2-HDAs protein complex regulates ripening genes in ethylene signaling by changing the level of H3K9ac and H3K27ac (Deng et al., 2022). Thus, HATs are typically involved in the ethylene signaling pathway through interaction with histone binding proteins ENAP1 and transcription factors like TPL, affecting fruit development and ripening.

The GCN5-TPL-HDA6 module maintains the homeostasis of acetylated TPL to regulate JA signaling (An et al., 2022). JA and HDA6 altered the level of H4ac and H3K27me3 to allow adaptation to environmental challenges in *Arabidopsis* (Vincent et al., 2022). Thermomorphogenesis is induced by the phytohormone auxin, HDA9 was involved in auxin accumulation and thermomorphogenesis by mediating histone deacetylation at the *YUCCA8* locus (van der Woude et al., 2019). In rice, the histone deacetylase HDA703 interacts with OsBZR1 to regulate BR signaling, growth and heading date by regulating *Ghd7* expression via histone H4 deacetylation (Wang et al., 2020). Histone acetyltransferase HAM1 interacts with molecular chaperone DNAJA2 and confers immune responses by promoting H3K9ac and H4K5ac of salicylic acid biosynthetic genes in cassava (Zhao et al., 2023).

Conclusion and perspectives

The regulation of abiotic stress in plants involves a complex process where HATs/HDACs cannot work alone. They need to cooperate with transcription factors or protein complexes to regulate the expression of stress-responsive genes (Kim et al., 2010; Chen et al., 2019; Gao et al., 2021a; Deng et al., 2022; Zhao et al., 2023). Acetylation generally makes the chromatin structure more open, allowing genes to be more easily influenced by regulatory factors like transcription factors. However, whether a gene is turned on or off depends on whether the transcription factor acts as a positive or negative regulator. Therefore, identifying and understanding how transcription factors interact with HATs/HDACs (Figure 2) is crucial in regulation of crop plants under abiotic stress through histone acetylation.

HATs/HDACs interact with diverse DNA-binding transcriptional factors forming multiple protein complexes to regulate the chromatin structure and the gene expression in plant responses to stresses. Identifying the transcriptional factors that interact with HATs/HDACs through yeast two-hybrid screening, *in vivo* immunoprecipitation in combination with mass spectrometry (IP-MS) or pull down in combination with mass spectrometry (Pull down-MS) is vital for mapping out the protein-protein interaction networks in the regulation of abiotic stress responses (Hou et al., 2022). To further

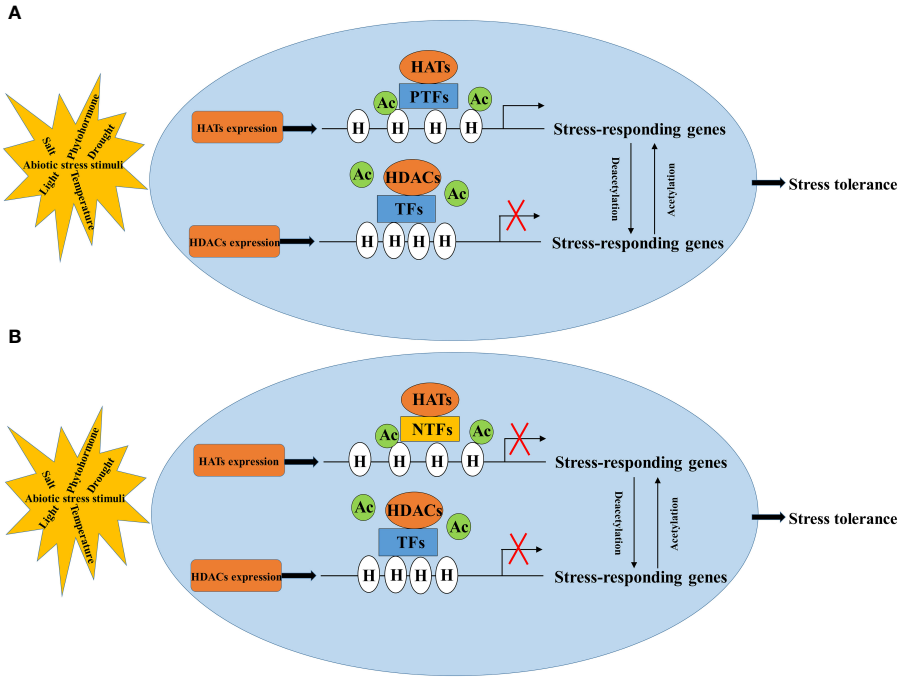

FIGURE 2
Regulatory pattern of histone acetylation in response to abiotic stress. NTFs, negative transcription factors **(B)**; PTFs, positive transcription factors **(A)**; HATs, histone acetylases; HDACs, histone deacetylases; H, histone; Ac, acetylation.

understand how HATs are involved in plant responses to abiotic stress, it is also important to identify the transcriptional regulatory network and the genome-wide binding site of HATs regulated histone modification by using RNA-seq and ChIP-seq approaches (Supplementary Table 3). Techniques like ChIP-qPCR, ChIP-PCR, and Western blotting can analyze acetylation levels and binding sites on gene promoters (Micsinai et al., 2012; Zhang et al., 2022a) (Supplementary Tables 2, 3). There are many other methods to detect histone acetylation besides ChIP-seq, ChIP-PCR and WB. For example, mass spectrometry is a commonly used method for acetylation modification detection. By hydrolyzing the acetylated protein into polypeptide fragments, the modification site and the quantity of acetylation on the protein can be determined by mass spectrometer analysis. Furthermore, novel histone modifications can also be identified by mass spectrometry. Due to the high sensitivity and accuracy of mass spectrometry, as well as large-scale analytical capabilities, it has been widely used in epigenetics research. CUT-TAG (Cleavage Under Targets and Tagmentation) developed on the basis of CUT-RUN (Cleavage Under Targets & Release Using Nuclease). CUT-TAG has emerged as a more user-friendly alternative compared to its predecessor, streamlining the process of identifying target genes. In the case of known modification sites, CUT-TAG can be used to study the enrichment of specific histone modification in the whole genome. DNase-seq (DNase I hypersensitive sites sequencing), MNase-seq (Micrococcal Nuclease digestion with deep sequencing) and ATAC-seq (Assay for Transposase-Accessible Chromatin with high throughput sequencing) also aimed to the study of histone acetylation. DNase-seq and MNase-seq require a large number of cells, and the enzyme digestion conditions are difficult to control. However, ATAC-seq requires a low number of cells and is highly sensitive. Compared with ChIP-seq and CUT-TAG, there is no need for specific antibodies such as transcription factors or histone modification antibody. In epigenetic studies, ATAC-seq can directly measure the degree of chromatin accessibility between two different samples. The level of histone acetylation and its modification site are usually determined by more than one method, making the results more convincing. Yeast one-hybrid (Y1H), dual-luciferase reporter system (Luc/Ren) and electrophoretic mobility shift assay (EMSA) can be used to identify the interaction relationships between the transcription factors and downstream genes in response to stress (Long et al., 2021; Wang et al., 2022).

Further research is required to investigate histone acetylation in more plants respond to abiotic stress beyond the commonly studied *Arabidopsis* and rice. It's important to study this process in various plants to understand the regulatory differences and similarities. Since plants face multiple stresses simultaneously, involving complex responses where HATs interact with multiple TFs and protein complexes, often co-regulated with other modifications like methylation. Therefore, advanced research and methods are necessary to uncover the intricate components and regulatory networks associated with HATs.

In recent years, epigenetic studies in biology, medicine and model plants have laid an important foundation for studying histone acetylation in plants (EPIC Planning Committee, 2012). Improved analytical techniques now allow for more precise insights into its regulatory roles. This knowledge offers promising ways to enhance plant stress resistance by managing histone acetylation. This review covers advances in histone acetylation for abiotic stress management, outlines common research methods, and highlights its potential in boosting agricultural productivity through better plant adaptation to environmental changes.

Author contributions

FW: Writing – original draft, Writing – review & editing. C-HL: Software, Writing – original draft. YL: Data curation, Software, Writing – original draft. L-FH: Software, Writing – original draft. PL: Investigation, Writing – original draft. J-XG: Investigation, Writing – original draft. NZ: Supervision, Writing – review & editing. BZ: Writing – review & editing. Y-DG: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1404977/full#supplementary-material

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Insights into the ameliorative effect of ZnONPs on arsenic toxicity in soybean mediated by hormonal regulation, transporter modulation, and stress responsive genes

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Arsenic (As) contamination of agricultural soils poses a serious threat to crop productivity and food safety. Zinc oxide nanoparticles (ZnONPs) have emerged as a potential amendment for mitigating the adverse effects of As stress in plants. Soybean crop is mostly grown on marginalized land and is known for high accumulation of As in roots than others tissue. Therefore, this study aimed to elucidate the underlying mechanisms of ZnONPs in ameliorating arsenic toxicity in soybean. Our results demonstrated that ZnOB significantly improved the growth performance of soybean plants exposed to arsenic. This improvement was accompanied by a decrease (55%) in As accumulation and an increase in photosynthetic efficiency. ZnOB also modulated hormonal balance, with a significant increase in auxin (149%), abscisic acid (118%), gibberellin (160%) and jasmonic acid content (92%) under As(V) stress assuring that ZnONPs may enhance root growth and development by regulating hormonal signaling. We then conducted a transcriptomic analysis to understand further the molecular mechanisms underlying the NPs-induced As(V) tolerance. This analysis identified genes differentially expressed in response to ZnONPs supplementation, including those involved in auxin, abscisic acid, gibberellin, and jasmonic acid biosynthesis and signaling pathways. Weighted gene co-expression network analysis identified 37 potential hub genes encoding stress responders, transporters, and

signal transducers across six modules potentially facilitated the efflux of arsenic from cells, reducing its toxicity. Our study provides valuable insights into the molecular mechanisms associated with metalloid tolerance in soybean and offers new avenues for improving As tolerance in contaminated soils.

KEYWORDS

abiotic stress, heavy metal, hub genes, *Glycine max*, WGCNA

1 Introduction

Soybean [*Glycine max* (L.) Merrill] belongs to the Fabaceae and accounted for ~60% of world oilseed production in 2020/21 (SoyStats, 2023). The crop had the fourth largest area of cultivation of all crops worldwide in 2021 and ranked seventh largest in terms of production (FAOSAT, www.fao.org). Soybean is thought to have originated in China (Lee et al., 2011) and is commonly known as the "miracle crop", as it is a rich source of high-quality oils and proteins containing 20 and 40% of these compounds, respectively (Clemente and Cahoon, 2009). The southern provinces of China belong to the tropical/subtropical, multi-season ecological zone and are considered suitable for soybean cultivation. However, mining activities in the area have led to pollution of the soil with arsenic (As) and other metals and/or metalloids, causing reduced production and endangering food safety.

Soybean crops are largely cultivated on marginalized and contaminated land (Vezza et al., 2022), which not only limits productivity but also facilitates the accumulation of metal(loids) into the food chain (Majumdar et al., 2021). Studies have reported that As-contaminated underground water is often used to irrigate soybean crops in many regions (Mariño et al., 2020; Singh and Srivastava, 2020). Arsenate (As(V)) is the predominant species of inorganic As in soils and enters root cells through the activity of phosphate (Pi) transporters due to their chemical structure similarity (Zhao et al., 2010). Its toxicity arises because it replaces phosphate ions in ATP synthesis, depriving cells of energy (Armendariz et al., 2016). Upon uptake, most As is accumulated in roots, then shoots (Wu et al., 2020). As contents in different parts of rice plants follows the order roots > stems and leaves > husks > grain (Seyfferth et al., 2016). The mechanisms of As uptake from the soil and long-distance transport to aerial plant tissue and detoxification are well known (Zhao et al., 2010; Zvobgo et al., 2018b). For example, multidrug and toxic compound extrusion (MATE), natural resistance-associated macrophage protein (NRAMP), and ATP-binding cassette (ABC) family transporters are reported to have a role in As uptake and distribution in different higher plant species (Yanitch et al., 2017; Zvobgo et al., 2018b).

Nano-enabled agrochemicals have attracted increasing interest due to their potential applications in the management of plant stress as sustainable alternatives to other techniques (Agathokleous et al., 2020). For example, seed priming with zinc oxide nanoparticles (ZnONPs) potentially improved the growth and biomass production of maize (Salam et al., 2022). Similarly, supplementation with ZnONPs improved the metal tolerance index, reduced As uptake, and promoted the accumulation of antioxidants in *Oryza sativa* (Wang et al., 2018; Wu et al., 2020). Studies have shown that ZnONPs release Zn^{2+} ions (Horie et al., 2012; Hu et al., 2013), and an antagonistic interaction between As(V) and Zn was observed when both were applied to a wheat crop (Gong et al., 2020). As(V) and Zn have been shown to be taken up by inorganic phosphate transporters (*PHT1*) (Huang et al., 2000; Jain et al., 2013; Khan et al., 2014). In this context, there is a need for investigating the mutual interaction of ZnONPs and AsV in soybean.

A number of studies have been made on the physiological mechanisms of tolerance to heavy metal(loids), including cobalt (Co) in *Zea mays* (Salam et al., 2022) and As in *Oryza sativa* (Wu et al., 2020) and soybean (Zeeshan et al., 2021, 2022) induced by ZnONPs. These studies have shown that supplementation with ZnONPs ameliorates reductions in chlorophyll and photosynthesis, maintains the integrity of membranes by reducing oxidative damage, and increases nutrient uptake. ZnONPs have also been shown to regulate phytohormones in *Arabidopsis* (Vankova et al., 2017) and As (V) to modulate levels in soybean (Vezza et al., 2022). This suggests the involvement of phytohormone signaling in the mitigation of metalloid stress by nanoparticles (NP). In addition, several metal homeostasis genes, such as zinc finger family proteins, heavy metal ATPase4 (HMA4), and heavy metal transport/detoxification superfamily proteins, have been found to be regulated upon individual applications of ether As(V) or Zn in barley (Huang et al., 2012; Zvobgo et al., 2019) and *Arabidopsis* (Landa et al., 2015). However, to our knowledge, no studies at the molecular level have been performed examining the mitigating effects of ZnONPs on As intoxication. In addition, compared to rice and *Arabidopsis*, transcriptomic data of soybean roots are scarce, and a systematic examination of the effects elicited by the ZnONPs on plant physiology at the molecular level under As stress is lacking.

The recent release of a transcriptomic atlas and the complete sequencing of the genome of the variety "Williams 82" (Libault et al., 2010; Schmutz et al., 2010) have opened new windows for research on soybean functional genomics. Therefore, to uncover the protective effects of ZnONPs on As(V) stress, the present study first focused on

investigating the role of ZnONPs as a nano-fertilizer on root architecture, plant biomass, photosynthetic attributes, hormonal regulation and As(V) uptake under As(V) stress in soybeans. We then examined the abundance of individual mRNAs of the whole transcriptome to identify differentially regulated genes. These data were subjected to weighted gene co-expression network analysis (WGCNA) to identify genes whose activities were coordinated into multi-gene, adaptive complexes (modules/clusters) thus gaining new insights into the effects of As(V) on transcription and the mechanisms by which ZnONPs mitigate its toxic effects.

2 Materials and methods

2.1 Experimental design and treatment detail

Soybean seeds (genotype ZhongHoung302) were germinated in sterile vermiculite for ten days. Uniform seedlings were then transferred to 10 L pots filled with modified half-strength Hoagland's solution as described by Sugiyama et al. (2016) and grown under ambient temperatures of 25–28°C and a 15 h/9 h day/night cycle with artificial lighting. When the plant reached the V2 growth stage (first two trifoliolate leaf nodes), the nutrient solutions were either left unsupplemented or supplemented with either 25 µmol L^{-1} of arsenate (Na_2HAsO_4) only or with arsenate plus ZnONPs at 25 µmol L^{-1} or 50 µmol L^{-1}, hereafter referred to as CK, AsV-only, ZnOA, and ZnOB, respectively. The unsupplemented pots receiving only nutrient solution were considered as controls. The Na_2HAsO_4 and ZnONPs were purchased from Sigma Aldrich, USA and used as received. The size of ZnONPs was 20 nm and their zeta potential was -16 to 23 mV in an aqueous solution as determined by a zeta potential analyzer (NanoBrook, Brookhaven, USA). The ZnONPs were characterized by X-ray diffraction and energy dispersion spectra mapping as described in Zeeshan et al. (2021). The stock solution of ZnONPs, (4.07 mg L^{-1}) was suspended in ddH_2O and the suspension was stirred using ultra-sonicator for 1 h to disperse the NPs before use. In addition, the pots in which the seedlings were grown were regularly stirred to discourage NP aggregation and to maximize their suspension in the nutrient solution. The pH of the nutrient solution was kept at ~5.8, and the solution was changed twice a week. After 10 days of treatment, data were recorded with respect to various physiological parameters, and the roots harvested, thoroughly washed with ddH_2O and assessed as described below then frozen in liquid nitrogen and stored at −80°C until determination of hormones and the extraction of RNA.

2.2 Observation of root phenotypes and determination of dry matter accumulation, relative water contents, and total As contents

After 10 days of treatment, roots samples were collected, scanned with an Epson Perfection V500 photo scanner (Nagano, Japan), and the root total lengths, root diameters and the number of lateral and secondary roots were measured using WinRhizo Pro (S) v. 2009a software (Regent Instruments Inc., Quebec City, QC, Canada). Dry matter (DM) accumulation by the seedlings was measured after drying the whole plant at 70°C for three days. Relative water contents (RWC) in fully expanded trifoliolate leaves were determined following procedure mentioned by Zeeshan et al. (2020).

Whole soybean seedlings were collected and separated into roots and shoots. The roots were placed in 0.01 M ethylenediamine tetra acetic acid for 15 min and then carefully washed with ddH_2O to remove As from the root surfaces. Then, the total As contents in the root samples was measured by atomic fluorescence spectrometry-mass spectrometry (AFS-MS). The samples were prepared by digesting 0.2 g dried roots samples in 5 mL of concentrated HNO_3 (4 mL) and $HCLO_4$ (1 mL) for 12 h at room temperature. A certified reference material [GBW10023 (GSB-14)] was used for calibration.

2.3 Photosynthetic efficiency, photosynthetic pigment contents and chlorophyll fluorescence

After treatment, changes in physiological factors affecting photosynthesis were determined by measuring gas exchange parameters and the contents of photosynthetic pigments. Parameters associated with photosynthesis (net photosynthesis (*Pn*), stomatal conductance (*Gs*), intercellular CO_2 concentration (*Ci*), transpiration rate (*E*), chlorophyll fluorescence (*Fv/Fm*), and non-photochemical chlorophyll fluorescence quenching (NPQ) were recorded using a portable infrared analyzer (LI-6800 System; Li-COR) as described in Zeeshan et al. (2021). For the determination of pigment contents, fresh leaf samples were placed in 80% acetone at room temperature overnight in the dark. When the leaves became colorless, the extracts were centrifuged at 4000 × g for 12 min, and the absorbances of the supernatants were measured spectrophotometrically at 470, 663, and 646 nm from which chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), and carotenoids concentrations were calculated following Lichtenthaler and Wellburn (1983).

2.4 Determination of endogenous phytohormones in root samples

The phytohormones, indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA_3), and methyl jasmonate (MeJA), were assayed following the methods modified by Ahmad et al. (2022). Briefly, roots samples (50 mg) were ground to a powder using liquid nitrogen in a tissue homogenizer. The internal standards of the phytohormones, d_5-IAA, d_6-ABA, d_2-GA_3, and MeH_2 JA, were made up in the extraction buffer that consisted of a ratio of 0.002:1:2 (v/v/v) of HCL, H_2O, and 2-propanol and were added into the ground samples. The tubes containing samples were placed in a shaker for 30 min at 100 rpm and 4°C after which each tube was again shaken for 30 min at 4°C after the addition of 1 mL CH_2Cl_2.

After centrifugation at 13,000 × g for 5 min, the supernatants were collected, dried with a nitrogen evaporator, and dissolved in 100 μL CH_3OH for analysis by HPLC-MS. The equipment setup and related procedures are as reported in Ahmad et al. (2022).

2.5 RNA extraction, library preparation and sequencing

RNA was extracted from root tissue using TRIzol® and purified from DNA contamination using RNase-free DNase I. The isolation of RNA was performed on two independent biological replicates for each treatment in the roots. RNA quality was verified using agarose gel electrophoresis and a Bioanalyser with quality control parameters. RNA concentration was assessed using a spectrophotometer. Library preparation and sequencing were then performed on a Majorbio sequencing platform following manufacturer's instructions using Illumina technology. Library preparation steps included mRNA isolation using oligo(dT) beads, fragmentation in fragmentation buffer and synthesis of double-strand cDNA using random hexamer primers and a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA). The cDNAs were then processed with end repair mix, 'A' base addition, and phosphorylation. The sequences were randomly fragmented into small pieces of ~300 bp and amplified using Phusion DNA polymerase. Paired-end sequencing was performed on the Illumina NovaSeq 6000 sequencer following the manufacturer's protocol.

2.6 Quality control of raw sequence data and reliability analysis

The raw sequencing data were filtered to obtain high-quality data (clean data) to ensure smooth subsequent bioinformatic analyses. This included the removal of connector sequences in reads and low-quality bases from both 3′ to 5′ and 5′ to 3′ (Q < 20) reads using Fastp (https://github.com/OpenGene/fastp). The filtered reads data were aligned by HISAT2 software (http://ccb.jhu.edu/software/hisat2/index.shtml, default parameters) to the *Glycine max* reference genome (version Wm82.a4; available at https://data.jgi.doe.gov/refine-download/phytozome?genome_id=508; accessed on Jan 10, 2023). The obtained aligned data/reads ranged from 86.58% to 93.32% of each library and were statistically analyzed by StringTie.

The reliability of the RNA-seq data was assessed using qRT-PCR, following the SYBR Green Mastermix protocol (Applied Biosystems, Waltham, MA, USA). A list of selected genes and gene specific primers used in this assay are provided in Supplementary Table S1. Primers were synthesized using primer premier 5.0 (Primer, Palo Alto, CA, USA). Two independent biological replicates and two technical replicates were used. The RNA templates were same to those previously used in library construction, whereas cDNA synthesis was carried out using the SuperMix First-Strand Synthesis kit (TransGen Biotech Co., Ltd, Beijing, China). The qRT-PCR assay was then performed and analyzed according to previously described methods (Zeeshan et al., 2021).

2.7 Differential expression analysis and functional enrichment

The expression levels of genes were quantified using RSEM software (http://deweylab.github.io/RSEM/) set with default parameters and were expressed as transcripts per million reads (TPM) to determine differentially expressed genes (DEGs) between samples. DEGs were identified using DESeq2 (http://bioconductor.org/packages/stats/bioc/DESeq2/) using the following screening criteria: q-values < 0.001 and $|\log2FC| \geqq 1$; if a gene met both criteria, it was considered to be a DEG. To assign functional annotation to the identified genes, bioinformatic analyses such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed using Diamond (GO, https://github.com/bbuchfink/diamond) and ID mapping (KEGG), respectively. Four other databases, NR, Pfam, COG/KOG, and SwissPort, were also searched for assigning functional annotations.

2.8 Construction of co-expression network and identification of hub genes

The identified DEGs were used for network construction and module identification using the R package "WGCNA". We used TPM values of DEGs to determine the correlation strength between the nodes by calculating an adjacency matrix. A soft threshold of β = 10 was chosen to make the whole network fit the scale-free topology. Module partition and gene clustering were determined using a dynamic cutting algorithm. Principal components and Pearson correlation were applied to calculate the module eigengene and module-phenotypes associations, respectively. We selected the top 10 genes as hub genes in selected modules after calculating each gene connectivity within a module. The hub genes networks were visualized by Cytoscape (version 3.6.1). Finally, we conducted a GO pathway enrichment analysis to identify the biological functions of genes in selected modules.

2.9 Statistical analysis

The data for the physiological indexes and phytohormones were expressed as means ± standard deviations and statistical analysis was performed with DPS software (Data Processing system) applying one-way ANOVA. Statistical differences among the treatments with $p < 0.05$ was expressed as significant. Graphs were visualized by OriginPro 2022, whereas heatmaps were generated by R programming language using library "pheatmap".

3 Results

3.1 Observation of root phenotypes, dry matter accumulation and relative water contents and As contents of germinating soybean seedling

The effect of ZnONPs on root morphology, DM and RWC of the As(V)-stressed seedlings are presented in Figures 1A-F. The results show that the AsV-only treatment inhibited the growth of the soybean roots by reducing total root lengths, root diameters and lateral and secondary branching. The high-dose ZnONP supplementation mitigated the changes in growth caused by As (V) to a large extent, as evidenced by longer and more vigorous roots than the AsV-only treatment (Figures 1A-D). DM and RWC decreased by 67% and 24% in the AsV-only treatment compared to the control plants. Decreases in these parameters in ZnONP-supplemented plants were less than in the AsV-only treatment; DM decreased by 47% and 26% and RWC decreased by 11%, and 6%, respectively, compared to the control treatment when supplemented with 25 µmol L^{-1} and 50 µmol L^{-1} ZnONPs. Supplementation of plants with ZnONPs into the As(V)-containing medium reduced the As contents in roots in a dose dependent manner (Figure 1G). There was a 23% decrease in As content in soybean roots under ZnOA treatment and a 55% decrease under ZnOB treatment, both when compared to the As-alone treatment.

3.2 Gas exchange parameters, photosynthetic pigment contents and chlorophyll fluorescence

The effect of As(V) alone and in combination with ZnONPs on photosynthetic parameters of soybean seedlings was investigated in the present study. Compared with the control, the AsV-only treatment reduced all photosynthetic parameters (*Pn*, *E*, *Ci*, and *gs*), and the application of ZnONPs restored the photosynthetic ability of As(V)-stressed plants to some extent (Figures 2A-D). For instance, the *Pn* values were 1.6 and 1.9 times, the *E* values were 4.9 and 7.2 times, the *Ci* values were 1.8 and 2 times, and the *gs* values were 1.6 and 2.3 times higher in the ZnOA and ZnOB treatments, respectively, compared to the AsV-only treatment. Treatment with the higher concentration of ZnONPs (ZnOB, 50 µmol L^{-1}) had a larger, positive impact on the photosynthesis-related parameters than the low concentration (ZnOA, 25 µmol L^{-1}).

Results pertaining to photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) and to chlorophyll fluorescence (*Fv/Fm* and NPQ) are presented in Figures 2E-J. Analysis showed that the contents of these pigments were significantly reduced in the AsV-only treatment compared to the controls; supplementation with ZnONPs mitigated these effects of As(V) in a dose-dependent manner. For instance, the increases in chlorophyll *a*, chlorophyll *b* and carotenoid contents in the ZnOA and ZnOB treatments compared to plants given the AsV-only treatment were 2.2-, 1.2-, 2.4-fold, and 3.1-, 1.5-, 2.3-fold higher, respectively. Similarly, *Fv/*

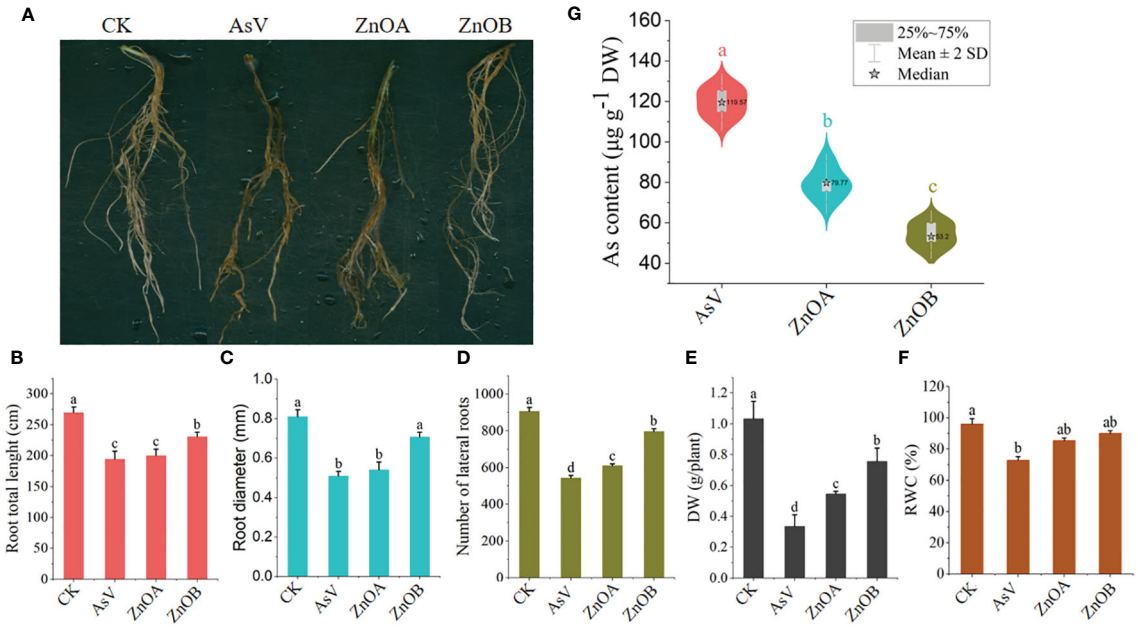

FIGURE 1
ZnONPs relieve AsV toxicity in soybean roots and inhibit As accumulation. **(A)** Root phenotypes, **(B)** total root lengths, **(C)** root diameters, **(D)** numbers of laterals roots, **(E)** dry weights (DW), **(F)** relative water contents (RWC); and **(G)** As contents of soybean germinating seedling 10 days after AsV and AsV + ZnONPs treatments. The different treatment groups were CK (control), AsV (25 µmol L^{-1} of arsenate), ZnOA (25 µmol L^{-1} of arsenate + 25 µmol L^{-1} of ZnONPs), and ZnOB (25 µmol L^{-1} of arsenate + 50 µmol L^{-1} of ZnONPs). Data are means ± SDs (n = 3). Different lowercase letters indicate significant differences using Tukey's *post-hoc* test, $p < 0.05$).

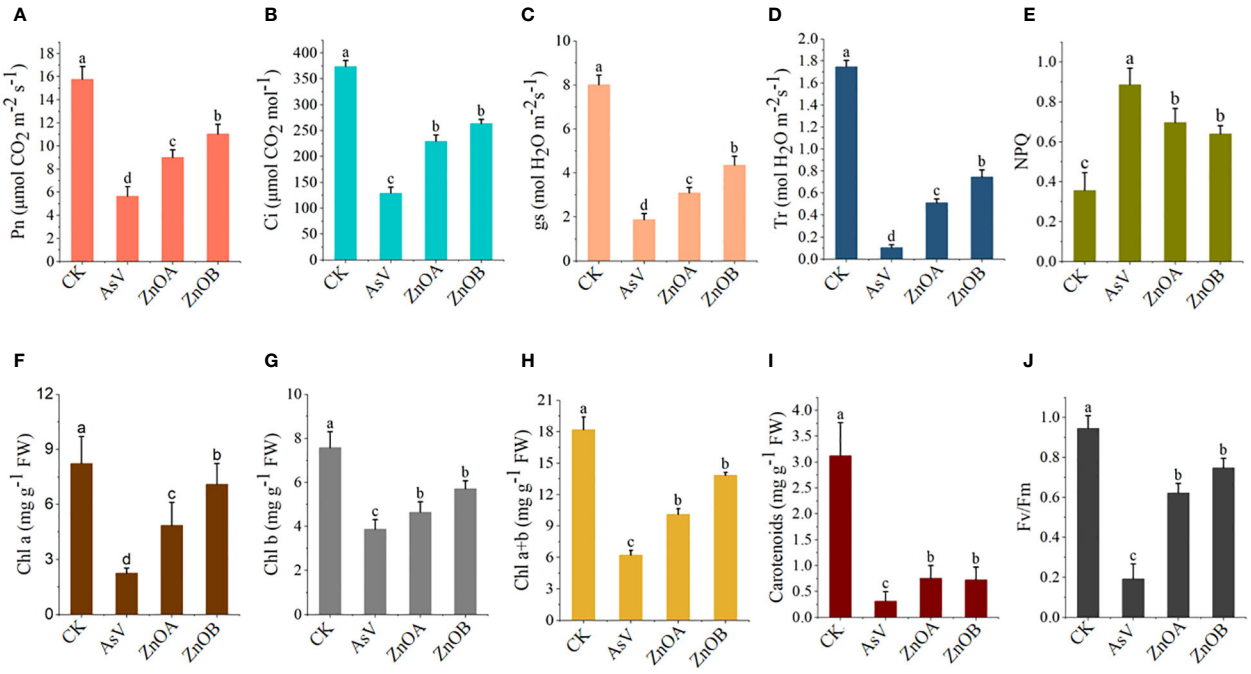

FIGURE 2
Responses of gas exchange parameter and photosynthetic pigmentation of soybean seedlings. **(A)** Net photosynthesis (*Pn*); **(B)** intercellular CO_2 concentrations (*Ci*); **(C)** stomatal conductance (*g*); **(D)** transpiration rates (*Tr*); **(E)** non-photochemical quenching (NPQ); **(F)** chlorophyll a (Chl *a*); **(G)** chlorophyll b (Chl *b*); **(H)** Chl *a+b*; **(I)** carotenoids; and **(J)** *Fv/Fm* of soybean seedlings 10 days after AsV and AsV + ZnONPs treatments. The different treatment groups were CK (control), AsV (25 μmol L^{-1} of arsenate), ZnOA (25 μmol L^{-1} of arsenate + 25 μmol L^{-1} of ZnONPs), and ZnOB (25 μmol L^{-1} of arsenate + 50 μmol L^{-1} of ZnONPs). Data are means ± SDs (n = 5). Different lowercase letters indicate significant differences using Tukey's *post-hoc* test, $p < 0.05$).

Fm and NPQ were substantially reduced when the plants were given AsV-only treatment and the effects were again mitigated by supplementation with ZnONPs.

3.3 Transcriptomic changes in soybean roots and expression validation

To obtain a genome wide view of the transcriptomic changes in soybean roots under As intoxication, alone and in response to supplementation with ZnONPs, four libraries were constructed from plants in the CK, AsV-only, ZnOA, and ZnOB treatments. RNA-seq of the libraries generated 55.3 GB raw reads which, after processing, comprised 54.2 GB clean reads across all libraries and with a base Q30 (the value of Phred>30) value of about 93%. The clean reads were mapped to the soybean reference genome. Only those reads that mapped to the soybean reference genome were processed further; unmatched reads were discarded. About 88 to 92% of clean reads mapped to the reference genome (Supplementary Figure S1A). The data pertaining to all samples were then subjected to principal component analysis (PCA) to visualize reproducibility indices (Supplementary Figure S1B). The results showed that there was a high degree of reproducibility between replicates and that there were differences in gene expression patterns among in the plants in the different treatments.

Furthermore, the differences in the abundance of DEGs identified by RNA-seq were further confirmed through qRT-PCR validation. From the treatment groups, twelve DEGs associated with stress response, hormonal regulation, and transporter functions were chosen for analysis. Regression analysis exhibited a positive linear correlation between the qRT-PCR and RNA-Seq data results i.e., AsV (R^2 = 0.9515), ZnOa (R^2 = 0.9667), and ZnOB (R^2 = 0.9371) as depicted in Supplementary Figures S2A-C. This analysis suggests that the RNA-seq data acquired here are reliable.

3.4 ZnONP supplementation modulates the transcriptomic signature of soybean roots by reversing As(V) toxicity

The expression profiles of the DEGs are presented in volcano plots (Figures 3A–C) and venn diagram (Figure 3D). Compared with the untreated group, the transcriptomic data of three treated groups suggested that: (i) 5045 genes were up-regulated and 4943 genes were down-regulated after treatment with AsV alone (Figure 3A); (ii) 2298 genes were up-regulated and 2241 genes were down-regulated after the ZnOA treatment (Figure 3B); and (iii) 3386 genes were differently expressed after the ZnOB treatment, with 1395 genes up-regulated and 1991 genes down-regulated (Figure 3C). The numbers of up-regulated DEGs were higher in the AsV-only and ZnOA treatments than were down-regulated, whereas the numbers of down-regulated DEGs were higher in the ZnOB treatment (Figure 3E). Furthermore, among the significantly regulated DEGs, 2329 genes were commonly

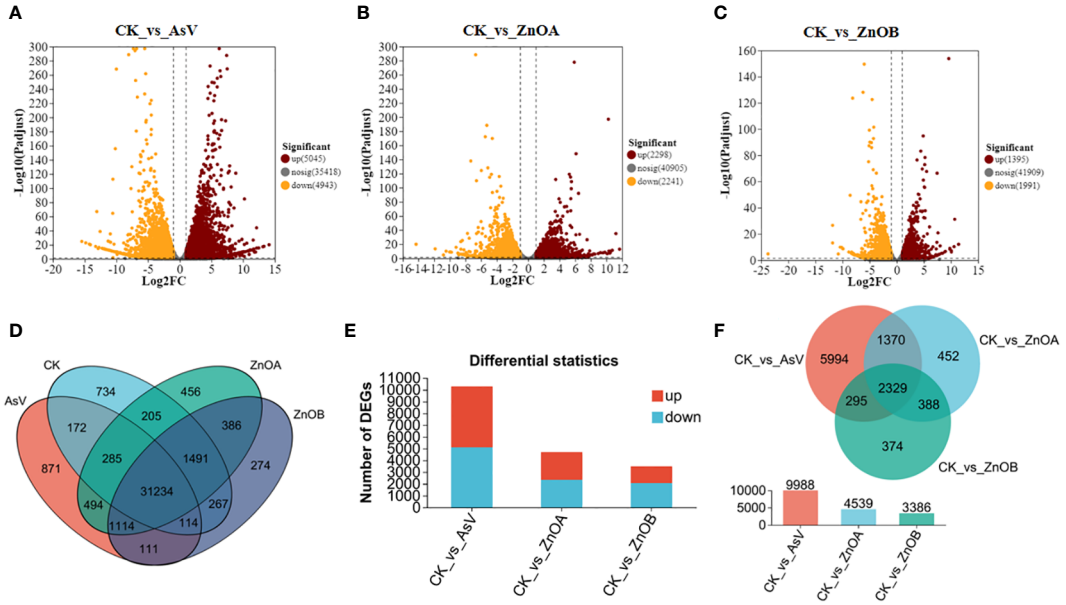

FIGURE 3
Different transcriptomic expressional patterns of soybean roots after treatment with AsV and ZnONPs. Volcano plots of differentially expressed genes (DEGs) in soybean roots between the following treatment combinations: **(A)** CK_vs_AsV; **(B)** CK_vs_AsV + ZnOA and **(C)** CK_vs_AsV + ZnOB relative to the control. Red dots in the plots represent upregulated DEGs, yellow dots represent downregulated DEGs, and black dots represent unchanged genes. **(D)** Venn diagram of the DEGs in the four treatment groups. **(E)** Barplots showing overall significantly up- and down-regulated DEGs. **(F)** Venn diagram of all significantly up-regulated and down-regulated DEGs among the three treatments groups. The different treatment groups were CK (control), AsV (25 μmol L^{-1} of arsenate), ZnOA (25 μmol L^{-1} of arsenate + 25 μmol L^{-1} of ZnONPs), and ZnOB (25 μmol L^{-1} of arsenate + 50 μmol L^{-1} of ZnONPs).

regulated within the three treatment groups, while 5994 DEGs were exclusively regulated in the As-only group and 452 and 374 DEGs were uniquely regulated in ZnOA and ZnOB treatments, respectively (Figure 3F). Some DEGs that were expressed in the treatments with As(V) alone and in combination with ZnONPs had significantly different expression trends.

3.5 As(V) tolerance due to supplementation with ZnONPs is associated with the mitigation of ROS and induction of stress-responsive genes

To explore the possible role of ZnONPs in the mitigation of As (V) toxicity, we examined the expression patterns of stress-responsive genes. Stress responsive genes putatively encoding peroxidase (*GmPOD*), glutathione S-transferase (*GmGST*), glutathione peroxidase (*GmGPX*), monodehydroascorbate reductase (*Gm*MDHAR), theriodoxin (*GmTrx*), lipoxygenase (*GmLOX*) and phenylalanine ammonia-lyase (*GmPAL*) were found to be differentially regulated among the treatment groups (Figure 4; Supplementary Table S2). For instance, three DEGs encoding *GmGPX*, two encoding Trx (*Glyma.02G023100.Wm82.a4.v1*, and *Glyma.06G266700.Wm82.a4.v1*), one encoding SOD (*Glyma.05G055000.Wm82.a4.v1*), one encoding MDHAR (*Glyma.08G017800.Wm82.a4.v1*) and four PAL-encoding DEGs showed dynamic expression patterns among the treatments (Figure 4A). In particular, the SOD-encoding DEG was only down-regulated in the AsV-only treatment but remained unchanged in plants supplemented with ZnONPs. The expression of 12 DEGs encoding lipoxygenases (*LOX*, *LOX3* and *LOX1-5*) were differentially modulated in the AsV-only treatment, whereas six genes encoding *LOX* enzymes in the two ZnONP treatments showed diverse expression patterns relative to the control (Figure 4B). Most of the GST-encoding DEGs were highly up-regulated by ZnONP supplementation compared to the AsV-only treatment group (Figure 4C). *Glyma.08G118800.Wm82.a4.v1*, encoding GST, was 9-fold more up-regulated in response to the ZnONPs supplementation relative to the AsV-only treatment. This suggests that under ZnONPs supplementation, GST-encoding genes play a key role in modulating As(V) tolerance and detoxification. Similarly, the peroxidase-related DEGs were mostly up-regulated in the AsV-only and As(V) +ZnONPs treatments relative to the control. The genes encoding peroxidases were more up-regulated under ZnONPs supplementation than the AsV-only treatment (Figure 4D).

3.6 ZnONPs-modulate metal transporters in soybean roots under As(V) stress

In this study, expression of 40 genes encoding various transporters was differentially regulated in the AsV-only and As (V)+ZnONPs treatments (Supplementary Figures S3A, B; Supplementary Table S3). Inorganic phosphate transporters play a major role in the uptake of AsV in plants and among the transporters identified in this study, five inorganic phosphate

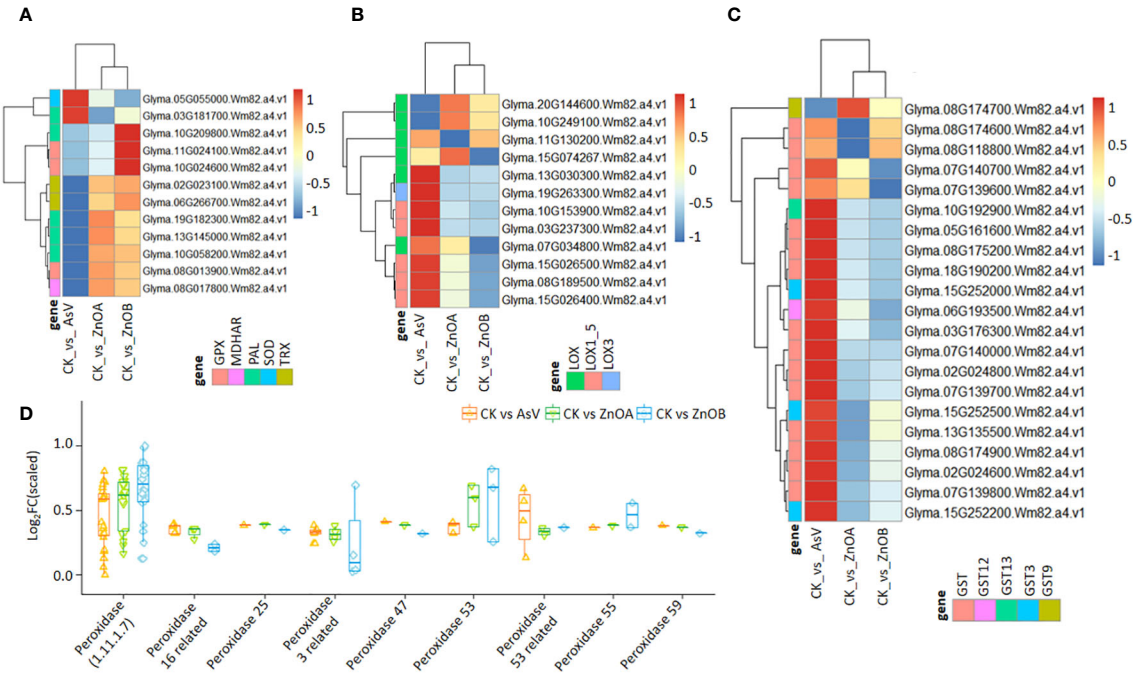

FIGURE 4
ZnONPs-modulated stress responsive DEGs under As stress in soybean roots. Heatmaps of the expression patterns of DEGs encoding: **(A)** GPX, MAHDR, PAL, SOD, and Trx; **(B)** LOX; and **(C)** GST. **(D)** Expression pattern of DEGs related to peroxidase. The scale represents normalized log2 fold change values. The different treatment groups were CK (control), AsV (25 μmol L^{-1} of arsenate), ZnOA (25 μmol L^{-1} of arsenate + 25 μmol L^{-1} of ZnONPs), and ZnOB (25 μmol L^{-1} of arsenate + 50 μmol L^{-1} of ZnONPs).

transporters were induced in the AsV-only treatment, but down-regulated in the ZnONPs group. It was noted that upon ZnONPs supplementation, lower As contents was observed in soybean roots, which can be linked with the reduced abundance of phosphate transporters in roots under ZnONPs supplementation. There were 13 DEGs encoding ZIP family (zinc/iron regulated-like protein) transporters that showed differential expression patterns due to the AsV-only and ZnONPs treatments, of which two ZIP family members, *Glyma.18G078600.Wm82.a4.v1*, and *Glyma.08G328000.Wm82.a4.v1* (both putative homologs of *At*ZIP11), were exclusively up-regulated in the AsV-only treatment. The other DEGs related to this family were mostly down-regulated in the treatments (Supplementary Figure S3A). Furthermore, 20 genes encoding ATP-binding cassette (ABC) transporters were differentially regulated in the AsV-only and As (V)+ZnONP treatments (Supplementary Figure S3B). Among them, 15 DEGs were up-regulated and five were down-regulated. Notably, the expression of up-regulated ABC transporters was higher in the As(V)+ZnONPs treatment than without ZnONP addition.

3.7 Alterations in plant hormone signaling under As stress in response to ZnONPs in soybean roots

Our data showed that 44, 19 and 27 DEGs related to the auxin signaling pathway (such as auxin transporter like-proteins/auxin influx carriers (*AUX1), AUX/IAA*, auxin response factor *(ARF), Small auxin up RNA (SUAR), Gretchen hagen (GH3)*, and *lateral organ boundaries domain (LBD*) were differentially regulated in the AsV-only, ZnOA and ZnOB treatments, respectively (Figure 5A; Supplementary Table S4). Upon imposition of As(V) stress, six AUX1 were down-regulated whereas their expression remained unchanged upon supplementation with ZnONPs. Similarly, most of the DEGs encoding AUX/IAA proteins, which are involved in auxin signal transduction, were induced in the AsV-only treatment whereas, with supplementation with ZnONP, only four and three DEGs were induced and two and three DEGs were suppressed, respectively, in the ZnOA and ZnOB treatments. Furthermore, only one gene encoding an auxin transcription factor (ARF) was induced in the AsV-only treatment, while four genes of this type were induced in the plants supplemented with ZnONPs. Other genes downstream of ARF involved in the auxin signaling pathway such as *SAUR* and *3GH3* were down-regulated due to As(V). In contrast, these genes were up-regulated or showed no changes in expression in the treatments supplemented with ZnONPs. Surprisingly, two *LBD* genes, which play a crucial role in stress tolerance and plant architecture, were up-regulated only in response to ZnONP supplementation. To further confirm the responses of the auxin signaling pathway, we determined the content of IAA in root tissues and found that IAA contents increased compared to the control by the addition of ZnONPs and reduced below concentrations in the controls by As(V) (Figure 5C). These results suggest that auxin signaling pathways are involved in the mitigation of As(V) stress by ZnONPs.

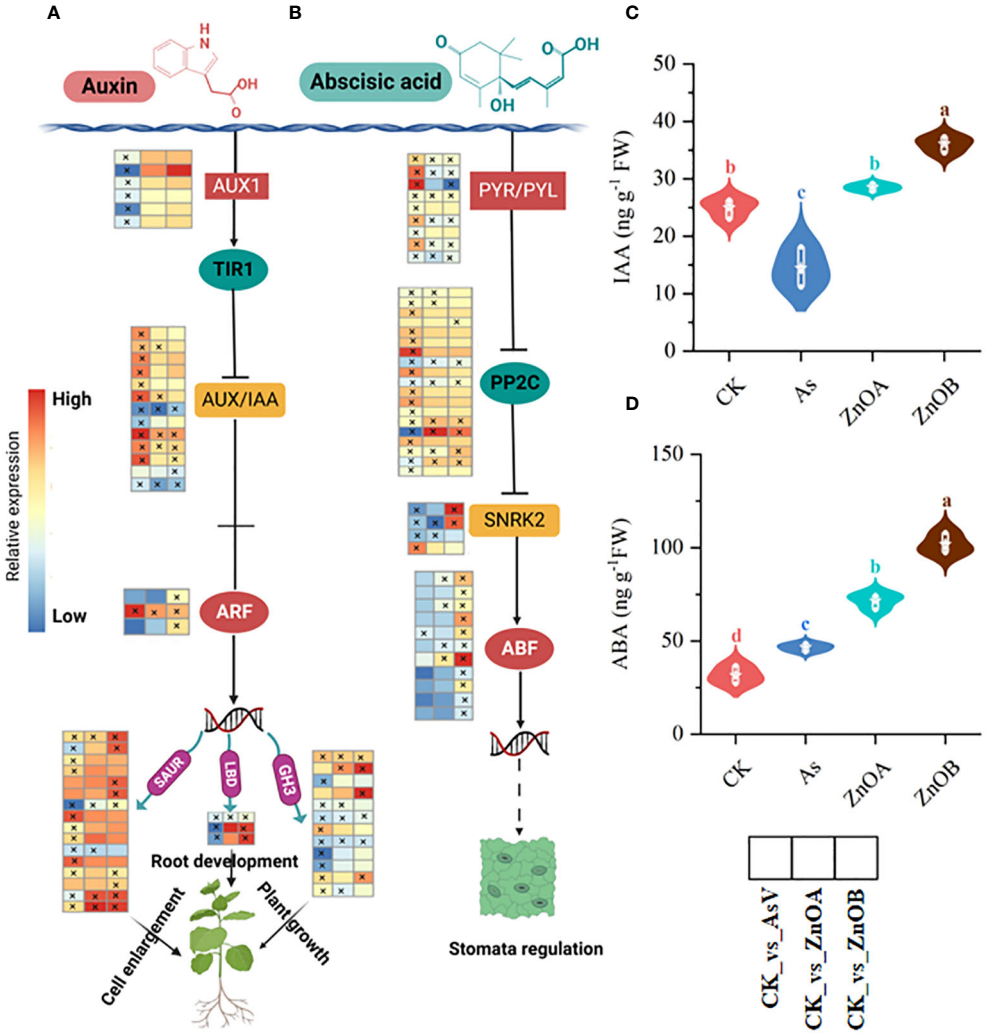

FIGURE 5
ZnONPs modulate auxin and abscisic acid signaling pathways under AsV intoxication. **(A)** Auxin signaling pathway. **(B)** Abscisic acid signaling pathway. **(C)** Indole acetic acid content (IAA). **(D)** Abscisic acid content (ABA). Heatmaps were generated from log2 fold change values of DEGs of respective treatment groups relative to controls and the asterisks in the heatmaps represent the DEGs in that specific treatment. The different treatment groups were CK (control), AsV (25 µmol L^{-1} of arsenate), ZnOA (25 µmol L^{-1} of arsenate + 25 µmol L^{-1} of ZnONPs), and ZnOB (25 µmol L^{-1} of arsenate + 50 µmol L^{-1} of ZnONPs). Results in the violin plots represent the means ± SDs (n = 3). Different lowercase letters represent significant differences Tukey's *post-hoc* test, $p \leq 0.05$.

Abscisic acid (ABA) is a core signaling molecule modulating plant growth and development under both non-stressed (Raghavendra et al., 2010) and stress conditions (Denancé et al., 2013; Zvobgo et al., 2018b). The core components of the ABA signaling pathway are pyrabatin resistance/pyrabatin resistance 1-like (PYR/PYL) ABA receptors, protein phosphate 2C (PP2C), sucrose nonfermenting-1-related protein kinase 2 (SnRK2s), and *ABSCISIC ACID-INSENSITIVE* (*ABI*). In this dataset, in response to the AsV-only treatment, we found that the expression of PYL/PYR-encoding genes were induced whereas the expression of PP2C-encoding genes was suppressed resulting in down-regulation of the SnRK2s family. In contrast, in response to treatment with ZnONPs, PYL/PYR-encoding DEGs were down-regulated resulting in the up-regulation of PP2C and SnRK2s and, subsequently, induced downstream ABF transcription factors (Figure 5B). ABA concentrations were lowest in the control plants, slightly raised in the AsV-only treatment but increased substantially by supplementation with ZnONPs (Figure 5D). Taken together, this indicates that ABA signaling and response are modulated in As(V)-stressed roots in response to the ZnONPs.

The GA signaling pathway was also significantly modulated by the AsV-only, ZnOA and ZnOB treatments (Figure 6A). For instance, DEGs involved in gibberellin signaling pathways such as *GIBBERELLIN INSENSITIVE DWARF1 GID1B* (*Glyma.03G148300.Wm82.a4.v1*), a gibberellin receptor, were significantly up-regulated, whereas most of the DELLA transcripts, which is a gibberellin repressor, and one phytochrome-interacting factor 3 (PIF3) transcription factor were down-regulated in the AsV-only treatment. None of these genes were differentially expressed under ZnONPs supplementation except for *Glyma.06G213100.Wm82.a4.v1* that encodes a DELLA protein. Other gibberellin signaling-related genes such as *gibberellin 2 oxidase* (*GA2ox*), which catalyzes the degradation of GA, was up-regulated in response to the AsV-only

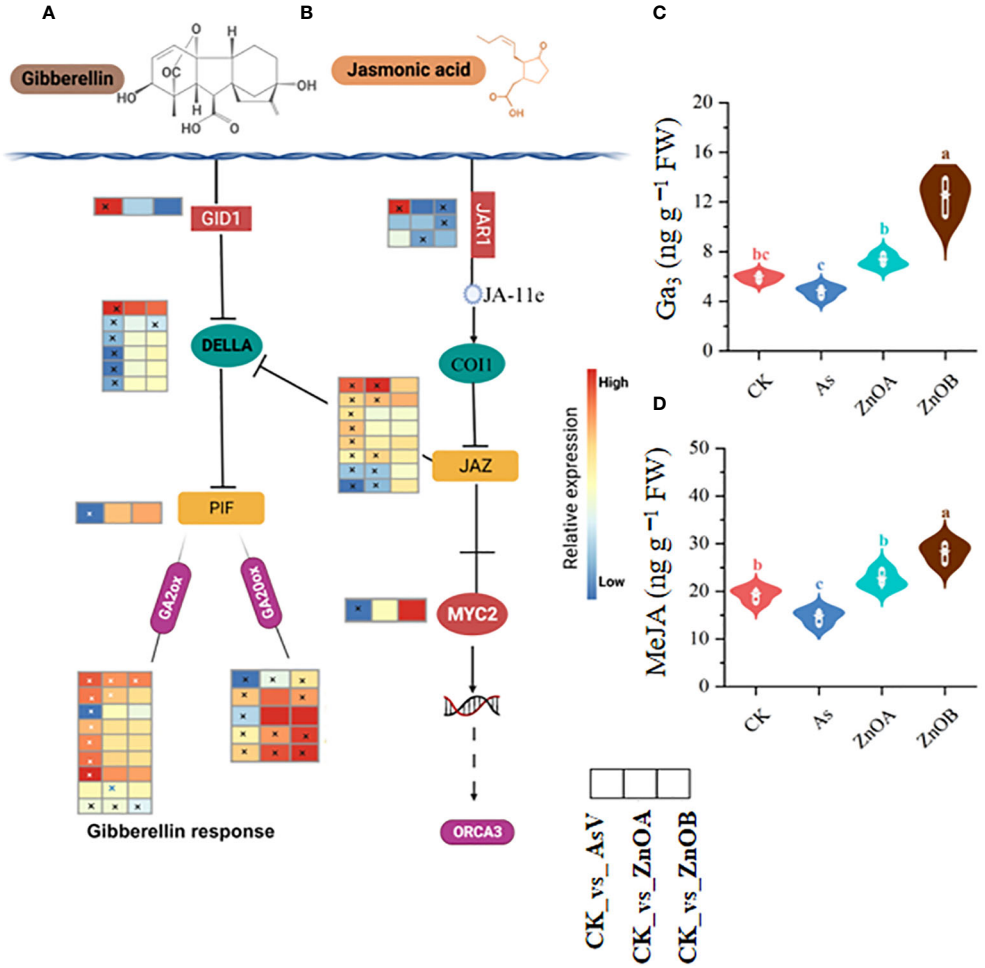

FIGURE 6
ZnONPs modulate the gibberellin and jasmonic acid signaling pathways under AsV intoxication. **(A)** Gibberellin signaling pathway. **(B)** Jasmonic acid signaling pathway. **(C)** Gibberellic acid contents (GA_3). **(D)** Methyl jasmonate contents (MeJA). Heatmaps were generated from log2 fold change value of DEGs of respective treatment group relative to controls and the asterisks in the heatmaps represent the DEGs in that specific treatment. The different treatment groups were CK (control), AsV (25 µmol L^{-1} of arsenate), ZnOA (25 µmol L^{-1} of arsenate + 25 µmol L^{-1} of ZnONPs), and ZnOB (25 µmol L^{-1} of arsenate + 50 µmol L^{-1} of ZnONPs). Results in the violin plots represent the means ± SDs (n = 3). Different lowercase letters represent significant differences using Tukey's *post-hoc* test, $p \leq 0.05$.

treatment; however, it showed diverse expression patterns in the ZnONPs treatments. It is important to note that the expression of most of the *gibberellin 20 oxidase* (*GA20ox*) genes remained unchanged in response to ZnONPs, suggesting that ZnONPs treatment relieved the As(V) stress and promoted the soybean growth without regulating GA-related genes. We also found higher GA_3 contents in soybean roots in response to ZnONPs treatment than in the control and AsV-only treatments (Figure 6C).

In the jasmonic acid signaling pathway, As(V) induced the expression of one gene encoding jasmonic acid-amido synthetase (*JAR1*), four TIFY proteins of the JAZ subfamily, but down-regulated two *JAR1*-encoding genes, one *MYC2* TF-related gene, and two *JAZ*-encoding genes. None of the *JAR1* encoding gene was up-regulated due to ZnONPs treatment, but we identified three TIFY proteins of the JAZ subfamily whose expression were up-regulated in response to the ZnOA treatment (Figure 6B). In addition, we analyzed MeJA contents in soybean roots and found that they were increased in ZnONP-treated samples compared to the controls and the AsV-only plants. There were 1.5- and 1.9-fold increases in the ZnOA and ZnOB treatments over the AsV-only treatment (Figure 6D). These results collectively suggested that ZnONP supplementation modulated phytohormone biosynthesis and signaling pathways, thereby mitigating the effects As (V) toxicity.

3.8 Co-expression network analysis reveals modules with different expression patterns and their association with physiological traits

Weighted gene co-expression network analysis (WGCNA) can provide an effective means of revealing the molecular mechanism of As(V) tolerance in response to ZnONPs by identifying key gene modules. The clusters (modules) were identified from the dendrogram in Figure 7A as each tree branch represents a

module and each leaf in a branch represent a gene. The dendrogram analysis classified the DEGs into a total of 10 modules (differentiated by color). The number of genes in each module is shown in Figure 7B. The highest number of DEGs (6370) were in the MEturquoise module followed by 3868 genes in the MEblue module; the lowest number of DEGs (47) were found in the MEmagenta module. Furthermore, the correlation of modules with the physiological traits revealed that five modules (MEpink, MEblue, MEbrown, MEturquoise and MEgreen) were significantly correlated with physiological traits (Figure 7D). Among these modules, MEblue and MEbrown have the highest, positive correlation with As contents. The MEturquoise and MEgreen modules have positive correlations with plant hormones, while the MEpink module correlates with RWC and DW. Moreover, through the integration of the identified modules with GO terms, we detected nine plant gene modules that formed an interactive network ($r > 0.6$, $p < 0.05$; Figure 7C). These gene modules represent different GO functions, e.g., signal transduction (MEgreen module), transmembrane transporter (MEpink module), stress response (MEbrown module), response to hormones (MEturquoise module).

3.9 Identification of hub genes in targeted modules

To identify the possible key regulators of As tolerance in response to ZnONPs supplementation, we found 37 major expression hub genes that showed a strong association with DEGs in targeted modules (Figure 8; Supplementary Table S5). Several of these hub genes are related to stress tolerance, transporters, phytohormone signaling, cellular signaling and cell death. For example, in the MEpink module, five hub genes were identified, of which the *NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER* (*NRT1/PTR*) homolog, *Glyma.03G122500.Wm82.a4.v1*, showed modulated regulation due to the AsV-only and As(V)+ZnONPs treatments. *PTR* genes are essential transporters for many substrates in plants, including nitrate, secondary metabolites, peptides, and hormones (Wen et al., 2020). *Glyma.02G082500.Wm82.a4.v1* encodes *vacuolar iron transporter homolog 3* (*VIT3*) and remained unchanged in the AsV-only and ZnOA treatments but was down-regulated in the ZnOB treatments. Another transcript, *Glyma.08G010000.Wm82.a4.v1*), of the MEpink module is related to trans-membrane transporter activity was down-regulated under ZnOB treatment.

Module MEyellow contains seven hub genes that are involved in transport and transmembrane transporter activity; all these genes showed diverse expression patterns due to the different treatments. Of these, two genes, *Glyma.11G195200.Wm82.a4.v1* and *Glyma.07G222700.Wm82.a4.v1*, belong to the MFS_1 (Major Facilitator Superfamily) superfamily and are associated with sugar transporters and high-affinity nitrate transporters. Two genes of the MATE/SLC47A family, *Glyma.10G239000.Wm82.a4.v1* (protein DETOXIFICATION 49) and *Glyma.13G275400.Wm82.a4.v1* (protein DETOXIFICATION 19), showed reduced expression under all treatment combinations. This family is a secondary

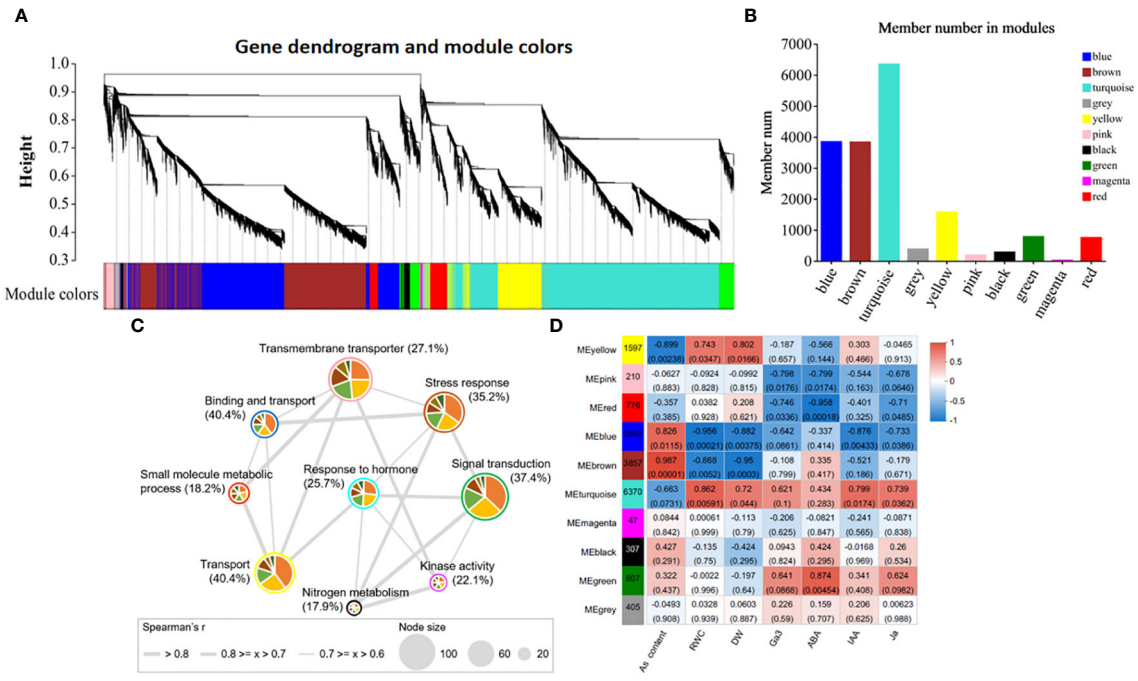

FIGURE 7
Weighted gene co-expression network analysis and correlation of trait-module analysis. **(A)** Clustering dendrogram tree showing co-expression modules. **(B)** The number of members in each module, **(C)** Interacted network-analysis of significantly enriched gene ontology (GO) terms among the 9 plant gene modules. **(D)** Physiological trait-module correlation analysis. The different colors circled around each node correspond to specific module and node size represents to the number of GO terms interacting in that specific module. The thickness of the edges represents the correlation strength.

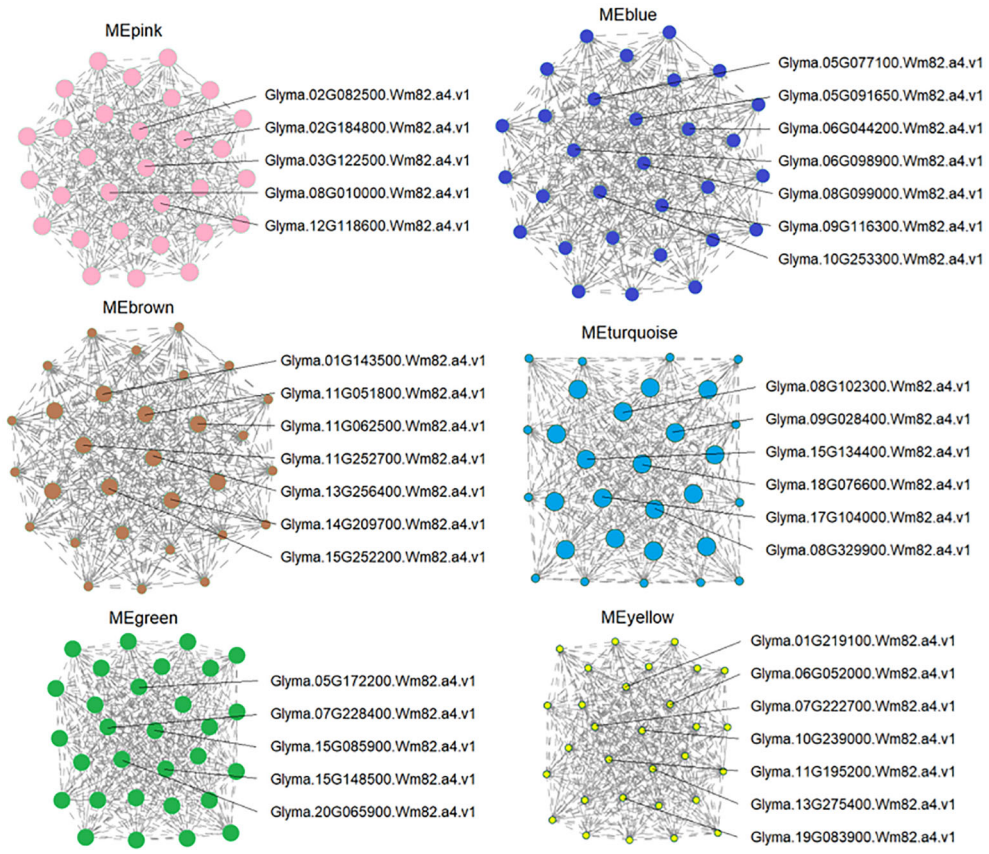

FIGURE 8
Candidate hub gene expression identified by co-expression networks. Candidate hub genes were selected based on those modules shown high correlation with physiological traits. The whole network of hub genes of each module was enlarged, and the hubs and surrounding nodes and edges were screen-captured and shown.

transport family mainly responsible for heavy metal/toxic compound detoxification (Wang et al., 2022). Three other homologs in this module with differential expression patterns are Cu^{+}-exporting ATPase (*Glyma.01G219100.Wm82.a4.v1*), a zinc transporter (ZIP) (*Glyma.06G052000.Wm82.a4.v1*), and an amino acid transporter (*Glyma.19G083900.Wm82.a4.v1*).

The annotation of the seven hub genes in MEblue showed they are related to kinase-like proteins (*Glyma.10G253300.Wm82.a4.v1*, *Glyma.06G098900.Wm82.a4.v1*, *Glyma.09G116300.Wm82.a4.v1*), *NRAMP2* (*Glyma.06G044200.Wm82.a4.v1*), the chloride channel protein, CLC-b (*Glyma.05G077100.Wm82.a4.v1*), transitional endoplasmic reticulum ATPase (*Glyma.05G091650.Wm82.a4.v1*), and hypothetical protein (*Glyma.08G099000.Wm82.a4.v1*). The regulation of hub genes of the three modules (MEpink, MEyellow, and MEblue) collectively suggest their participation in heavy metal transport and detoxification in soybeans.

Two hub genes in MEturquoise have functions related to plant hormones and signal development such as *Patellin-4* (*Glyma.09G028400.Wm82.a4.v1*, *Glyma.15G134400.Wm82.a4.v1*) and are associated with diverse signaling pathways, such as response to cytokinins (Černý et al., 2011) and auxins (Tejos et al., 2017). Their expression was suppressed in the AsV-only treatment while showing no change in expression in response to the application of ZnONPs. Furthermore, two candidate hub genes encoding *fasciclin-like arabinogalactan protein 1* (*FLAs*; *Glyma.08G329900.Wm82.a4.v1* and *Glyma.18G076600.Wm82.a4.v1*) were also do wn-regulated in the AsV-only treatment, but their expression was unchanged by ZnONPs supplementation.

4 Discussion

Soybean is a nutritionally rich crop but, unfortunately, it is often cultivated on marginal and arsenic-contaminated land in the southern provinces of China causing crops grown in this region to have poor yields and quality. Genomic resources for this important leguminous crop are lacking. Therefore, exploring the molecular mechanisms associated with arsenic tolerance is crucial for facilitating breeding soybean cultivars with high arsenic tolerance. The authors believe that this is the first study evaluating transcriptomic signatures in soybean roots after being subjected to As(V) stress with or without supplementation with ZnONPs.

4.1 ROS mitigation and regulation of stress responsive genes are associated with As(V) tolerance in response to ZnONP supplementation in soybean roots

Stress due to arsenic intoxication induces the accumulation of ROS that inhibit chlorophyll production and limit photosynthetic activity resulting in reduced growth (Thakur et al., 2019; Zeeshan et al., 2022). Increased production of ROS causes oxidative stress which reduces the integrity of cell membranes and causes changes to proteins, lipids, and DNA (Thakur et al., 2019; Zeeshan et al., 2022). Our data set showed that As(V) stress-induced *LOX2S* and *LOX1-5* in soybean roots, whereas simultaneous treatment with ZnONPs suppressed the expression of these genes in a dose-dependent manner. *LOX* genes have a role in catalyzing the peroxidation of unsaturated fatty acids of bio-membranes resulting in the generation of hydroperoxides and active oxygen species (You et al., 2011). Previously, we have found that amendment with ZnONPs and SeNPs, either alone or in combination, relieved oxidative stress in As(V)-treated soybean tissues (Zeeshan et al., 2021, 2022), results that can be explained by the low expression of *LOX* genes found in this study. Taken together, the responses to ZnONPs maybe through reduced oxidative stress resulting in maintaining the biosynthesis of photosynthetic pigments, water balance, and chloroplast structure.

To deal with oxidative stress, plants develop protective mechanisms to remove ROS. In this study, the RNA-seq data demonstrated that genes encoding GST, POD, GPX, Trx, SOD and MDHAR showed increased expression due to ZnONP-supplementation, and one GST-encoding gene (*Glyma.15G252200.Wm82.a4.v1*) is among the candidate hub genes in MEbrown module. Previously, we found that ZnONP-supplementation increased the expression of *GmSOD* and *GmCAT* genes whose functions are involved in the scavenging of ROS (Zeeshan et al., 2021, 2022).Plant GSTs have a major role in detoxifying hydroperoxides and xenobiotics and protect cells from lipid peroxidation (You et al., 2011). In this process, SOD catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2, which is then to converted to O_2 and water molecules by the action of CAT. Similarly, GPX and POD also play active roles in the removal of H_2O_2 by reducing it to oxygen (Herbette et al., 2007; Passaia et al., 2014). Thioredoxin (Trx) also has role in modulating hormone signaling, the production transcription factors, and DNA synthesis to protect cells from toxicants (Ouyang et al., 2018; Zhang H. et al., 2018). Taken together, these enzymes are key components of the plant defense system and through their coordination provide protection to cells against As(V) stress.

4.2 Dynamic expression of transporters in response to ZnONPs contribute to As (V) tolerance

Changes in arsenic translocation and sequestration into less sensitive cellular organs (such as vacuoles) are well-known tolerance mechanisms in plants (Zeeshan et al., 2023). Studies have shown that As(V) is taken up and transported by phosphate transporters (PHTs) in the roots (Zvobgo et al., 2018a; Zeeshan et al., 2022). In the current study, we identified five *inorganic phosphate transporter* (*PHTs*) genes, of which *Glyma.07G222700.Wm82.a4.v1* is a hub gene of the MEyellow module. The expression of these genes was induced upon As(V) stress; supplementation with ZnONPs reduced their expression. Phosphate transporters have either a low or high affinity and are responsible for As(V) uptake because of the similarity in chemical structure of arsenic with phosphate (Jian et al., 2008). Studies have found that two phosphate transporters were less induced in arsenic-tolerant genotypes than in sensitive ones during P starvation (Puckett et al., 2012; Zvobgo et al., 2018b) suggesting that P starvation promotes the uptake of As(V) via Pi transporters. Likewise, the over-expression of two soybean Pi transporters, *GmPT1* and *GmPT4*, enhanced As(V) uptake in *Arabidopsis* (Shin et al., 2004). This is consistent with higher concentrations of arsenic in soybean roots in the AsV-only treatment than in the other treatments. It is interesting to note that, in the current study, treatment with ZnONPs reduced the expression of Pi transporter genes in soybean roots. It is known that Zn deficiency in the root medium causes a loss of control transcription of Pi transporter genes in barley (Huang et al., 2000) and *Arabidopsis* (Jain et al., 2013; Khan et al., 2014) leading to accumulation of Pi, and Huang et al. (2000) suggest that Zn ions have a specific role in the regulation of genes encoding P transporters in plant roots. Supplementation with ZnONPs may, therefore, help control the transcription of PHT transporters reducing their expression and resulting in lower As(V) uptake; however, this needs further elucidation.

Zinc is the second largest trace element after iron and plays an important role in the modulation of different physiological and molecular processes and, in plants, its uptake is regulated by zinc transporters, particularly ZIP family proteins (Amini et al., 2022; Maharajan et al., 2023). ZIP family proteins are not only an important component of the Zn^{2+} uptake and transport system but are also involved in the uptake of other divalent metals such as cobalt, cadmium, copper, iron, and manganese (Pedas et al., 2009).In this study, most of *Gm*ZIP family members were down-regulated in the three treatments except for *Glyma.18G078600.Wm82.a4.v1* and *Glyma.08G328000.Wm82.a4.v1* that were up-regulated, compared to the controls, of which, one gene (*Glyma.06G052000.Wm82.a4.v1*) was identified as a hub gene in the MEyellow module. Previously, *At*ZIP family genes have shown reduced expression levels in roots and shoots of *Arabidopsis thaliana* under excessive supplementation with Zn^{2+} and ZnONPs (Nair and Chung, 2017). These authors attributed the reduced expression of these DEGs to Zn homeostasis via low uptake. Similarly, *ZIP*-encoding genes were also suppressed under excessive Zn^{2+} applications in various crop species (van de Mortel et al., 2006; Jain et al., 2013). In addition, three ZIP family proteins (*Hv*ZIP3, *Hv*ZIP5, and *Hv*ZIP8) showed high expression under Zn^{2+}-deficient conditions in barley (Pedas et al., 2009). Similarly, several ZIP transporters in *Arabidopsis* were also induced under Zn^{2+} depleted condition and reduced their expression when the plant were transferred to Zn^{2+} normal conditions (van de Mortel et al., 2006). In the current study, the reduced As(V) contents of the plants supplemented with ZnONPs may be due to the reduced expression of most of the genes encoding *ZIPs*.

The ABC transporter proteins play an important role as channels for the uptake of essential nutrients and toxic elements into plants (Kang et al., 2011; Zvobgo et al., 2018b). After uptake, As (V) is reduced into As(III) by the action of the enzyme, arsenate reductase, which then complexes with thiol compounds such as phytochelatins (PCs) and GSH (Kumar and Trivedi, 2018) and is transported to less sensitive cell organs by ABC transporter family proteins (Song et al., 2014; Kumar and Trivedi, 2018). In this study, we identified 21 ABC-type transporter genes belonging to the A, B, C and G subfamilies that were all up-regulated in all treatments; however, their expression was relatively higher in the As(V) +ZnONPs treatments than in the AsV-only treatment. The increased expression of ABC transporters due to ZnONP supplementation might be because these transporters are not only involved in the detoxification process (as mentioned earlier) but also facilitate the uptake of essential nutrients (Zvobgo et al., 2018b). In addition, in our previous study, supplementation of ZnONPs increased the production of PC contents in roots and shoots of As(V)-stressed soybean plants, and high accumulation of arsenic contents in vacuoles, suggesting its role in As(V) detoxification (Zeeshan et al., 2022). The lower accumulation of As in soybean roots in response to the ZnONPs treatments suggests a role for these transporters in As(V) tolerance.

4.3 Hormonal interplay in the presence of ZnONPs involves in As(V) tolerance

As sessile organisms, plants must evolve physiological and developmental adaptations to combat unfavorable conditions and use signaling molecules and mechanisms that mediate (re) patterning at the tissue and cellular level to adapt to the prevailing conditions. Phytohormones are involved in many aspects of plant development and are responsible for intra- and inter-cellular communication and modulation of cellular processes (Tejos et al., 2017). Our analyses showed that the AsV-only treatment and supplementation with ZnONPs modulated several hormone-related genes such as *GH3, AUX/IAA*, *LBD*, and *SAUR*. *AUX/IAA* is a negative regulator of auxin transduction by suppressing the ARF transcription factor (Liu et al., 2020), whereas *SAUR* acts as a positive regulator of the auxin signaling pathway (Stortenbeker and Bemer, 2019). In *Arabidopsis*, high concentrations of auxin suppress the AUX/IAA protein and increase transcription of ARF which directly regulates the expression of *LBD* family genes (Goh et al., 2012). Auxin response factor 3 (*ARF3*) was specifically up-regulated in the ZnOB treatment, whereas, it remains unchanged in the AsV-only and ZnOA treatments, indicating its involvement in the modulation of the auxin signaling of As(V)-stressed soybean roots supplemented with a high dose of ZnONPs. Three genes of the *LBD* family, which encode auxin-responsive *lateral organ boundaries* (*LOB*) gene and are responsible for lateral roots development (Majer and Hochholdinger, 2011) were downregulated in the AsV-only treatment but remained unchanged in the As(V)+ZnONPs treatments, suggesting that ZnONP supplementation relieved the As(V) stress by promoting the lateral root development as evidenced by improved root architecture observed during this study (Figures 1A–D).

This study identified several DEGs encoding the three principal components of the ABA signaling pathway, PYR/PYL, PP2C and SnRK2s, in the AsV-only and As(V)+ZnONPs treatment groups. PYR/PYL proteins are ABA receptors that upon activation release the PP2C protein which in turn regulates SnRK2s and activation of downstream targets (Raghavendra et al., 2010; Denancé et al., 2013). In the presence of ABA, the PYR/PYL complex tightly links with PP2C, thereby inhibiting PP2C-mediated dephosphorylation of SnRK2. This, in turn, allows activated SnRK2s to relay the ABA signal (Manohar et al., 2017). In a previous study, the expression of genes of the ABA signaling pathway module PYR/PYL-PP2C-SnRK2s were differentially genes due to treatments As(V)-stressed plants either in the presence or absence of an addition of P in the roots of an As-tolerant genotype *Hordeum vulgare*, showing this module plays a role in As tolerance (Zvobgo et al., 2018b). The results of this study confirm this conclusion, as we found higher ABA contents in soybean roots treated with ZnONPs and a smaller number of DEGs of the ABA signaling pathway in plants given the ZnOB treatment.

Jasmonic acid (JA) is lipid-derived signaling hormone and is involved in protecting plants against (a)biotic stresses (Zhang P. et al., 2018). The core components of the JA signaling pathway, such as TIFY10 of the JAZ subfamily and the transcription factor MYC2, were, respectively, up- and down-regulated in the AsV-only treatment. In contrast, the expression of JAZ subfamily genes was altered in the ZnONB treatment. The induction of JAZ family genes represses MYC2 which results in the suppression of JA-responsive gene transcription (Yang et al., 2012). However, it was found that a JAZ protein was suppressed and *MYC2* was induced in tolerant and sensitive genotypes of *Hordeum vulgare* upon imposition of As(V) stress (Zvobgo et al., 2018b), suggesting JA signaling is a complex process that shows variable responses in different crop species. This might be because JA exhibits synergistic and antagonistic crosstalk with auxin, ethylene (Wasternack, 2007), and especially with GA (Yang et al., 2012). It was previously noted that JA signaling was inhibited by the antagonistic effects of GA through JAZ-DELLA interactions and/or DELLA-JAZ interactions (Hou et al., 2010; Yang et al., 2012). In this study, we found low expression of the DELLA protein in response to the AsV-only treatment, whereas its expression was unaltered under ZnONPs supplementation. It seems that the induction of JAZ not only suppressed the MYC2 TF in AsV-only treatment but also inhibited the DELLA protein through its antagonist crosstalk. Also, there was a high concentration of GA in the treatment groups relative to the control; however, we only found a high expression level of GID1B (GA receptor) in the AsV-only treatment. In the presence GA, a GID1B makes a bond with GA, which facilitates the interaction with the DELLA protein (Achard and Genschik, 2009) resulting in the suppression/ degradation of the DELLA protein. In addition, GA2-oxidase, a catabolic enzyme usually activated under stress conditions, reduces bioactive GA level and suppress plant growth and was found to be upregulated in the AsV-only treatment, whereas supplementation with ZnONPs reduced its expression level. Also, the GA synthesis gene, *GA20ox*, was down-regulated in the ZnONP treatments

compared with AsV-only treatment. Therefore, these results suggest that GA signaling may be involved in As(V)-induced repression of soybean root growth. Further study is needed to explore the specific role of hormonal interplay in soybean under the concurrent application of As(V) and ZnONPs.

4.4 Co-expression network analysis and identification of hub genes by WGCNA showed diverse expression patterns of modules in soybean roots

WGCNA is a progressive data mining approach in which DEGs are divided into different co-expression modules. Genes in each module/cluster are highly interconnected and have similar expression patterns and performing similar physiological functions (Ye et al., 2020). Each module is then checked for its correlation with physiological traits and endogenous hormones. In this study, the DEGs were classified into 10 modules (as shown by different colors) with hierarchical clustering based on an unsigned co-expression network (Figure 7). GO analysis showed that DEGs in these modules were highly enriched in biological processes such as response to stress, signal transduction, trans-membrane transport, and response to hormones. To further narrow the range of arsenic tolerance genes related to these biological processes, we identified the hub genes in the five most significant modules having the highest correlation among the modules. We identified 37 principal hub genes in order to determine candidate genes responsible for arsenic tolerance that were regulated by ZnONPs.

Transport-related DEGs are crucial factors of As(V) stress tolerance. Several hub genes encoding transporters showed dynamic expression in the MEPink, MEyellow, and MEblue modules. Of these, NRT1/PTR 3.1 (NPF), a dual-affinity nitrate transporter, was suppressed only in response to the ZnOB treatment. Recently, studies also revealed its role in the transport of ABA, auxin and GA (Chiba et al., 2015). Another hub gene, an uncharacterized membrane protein (*Glyma.02G082500.Wm82.a4.v1*) and a homolog of *Arabidopsis vacuolar iron transporter* (*VIT*), plays a dominant role in iron transport and detoxification in protists, fungi and plant (Slavic et al., 2016). Study revealed that *VIT* also plays a significant role in nitrogen fixation in soybean, as this gene is also homologous to *Lotus japonicus* SEN1 (*LjSEN1*) (Brear et al., 2020). However, given this study, its specific role needs to be revaluated. Another gene, *BIDIRECTIONAL SUGAR TRANSPORTER SWEET10* (*Glyma.08G010000.Wm82.a4.v1*), also known as sugar/sucrose efflux transporter was induced in plants given the ZnOB treatment whereas its expression remained unchanged in the other treatments. The Sugars Will Eventually be Exported Transporter (SWEET) proteins play crucial roles in plant development by translocating sugars from one cell in more distant transport between organs (Wang et al., 2020). Sucrose is the main carbon energy source in plants. The sugars derived from sucrose metabolism provide tolerance against abiotic stresses (Misra and Mall, 2021), and the high expression of the *SWEET* gene positively contributes to sugar accumulation (Wei et al., 2014). Knockout of *GmSWEET10* reduced the oil content and seed size in soybean (Wang et al., 2020).The gene *NRAMP2* (*Glyma.06G044200.Wm82.a4.v1*), is a hub gene in the MEblue module and was induced in the AsV-only treatment. Initial work on the NRAMP gene family suggested that they have a role in Fe uptake, since then they have been linked with the uptake of several other metals (Tiwari et al., 2014 and the references therein) and they have subsequently been shown have potential roles in metal (loid) tolerance by sequestering ions in tolerant cell organs (Ma et al., 2021). The study by Tiwari et al. (2014) also suggested that the overexpression of the *OsNRAMP* gene in a yeast mutant and *Arabidopsis* roots affected As and Cd uptake. The *HvNRAMP5* gene was down-regulated by treatments with As(V) and As(V)+P in barley roots, suggesting its ambiguous role under As(V) stress (Zvobgo et al., 2018b). These genes may play cross-functional roles in tolerance to As(V) stress in response to the ZnONPs supplementation in soybean roots.

Two hub genes encoding *protein DETOXIFICATION 49* and *protein DETOXIFICATION 19* of the MATE family in MEyellow module showed reduced expression in the AsV-only and As(V) +ZnONP treatments. The MATE protein family plays an important role in plant development by modulating plant hormones and providing tolerance against (a)biotic stresses by scavenging toxic substances and secondary metabolites (Li et al., 2002). Generally, in plant cells, MATE proteins are localized in plasma membrane thereby facilitating the efflux of toxic substance from the cytoplasm, and the *Arabidopsis protein DETOXIFICATION 1* gene has been shown to be involved in the efflux of Cd^{2+} from the cytoplasm (Li et al., 2002). Also, in *Arabidopsis*, protein DETOXIFICATION 19 was expressed in root epidermal cells thereby protecting roots from hazardous compounds in the soil (Zheng et al., 2023). Others DETOXIFICATION genes of MATE family also play substantial role such as *AtDTX30* promote aluminum tolerance and regulate root hair roots (Ali et al., 2021). In this study, the downregulation of these genes suggest that they may not play significant role in As(V) tolerance and that soybean has developed a different strategy to detoxify/efflux the As(V) from cell in the presence of ZnONPs.

Two *PATELLINS* (*PATL*) genes are candidate hub genes in the MEturquoise module. In *Arabidopsis*, this protein is associated with plasma membrane mainly particularly in lateral roots, primary roots, embryos, and developing stomata (Tejos et al., 2017) and its function is mainly associated with various phytohormone signaling responses (Černý et al., 2011). qRT-PCR, found that four *PATL* genes showed diverse expression patterns in response to the IAA treatment suggesting that *PATL* genes might be involved in auxin signaling (Tejos et al., 2017). The current study suggests the same maybe the case and that high auxin accumulation and auxin signaling under ZnONPs supplementation might have regulated the two *PATL4* genes.

Two hub genes encoding fasciclin-like arabinogalactan-proteins (FLAs), were identified in the MEturquoise module and were down-regulated in the AsV-only treatment. Previous studies inferred that

FLAs genes are expressed in various plant tissues such as roots, leaves, stems and flowers and play a crucial function in plant development as well as in adaptation (Deng et al., 2022). For instance, the *ZeFLA11* gene and its homolog in *Arabidopsis* induces secondary cell wall thickening (Dahiya et al., 2006), *AtFLA18* promotes roots elongation (Allelign Ashagre et al., 2021) whereas *OsFLA1* is expressed in anthers and promotes pollen development in rice (Deng et al., 2022). In our previous study, we found that As stress strongly induced ROS accumulation causing lipid peroxidation resulting in cell wall disruption and cell death (Zeeshan et al., 2021). Therefore, the downregulation of two *FLAs* genes under As(V) stress indicates that As(V) stress negatively affects cell wall formation in soybeans. Surprisingly, the *GmPATL* and *GmFLA* genes showed less response to ZnONPs and suggests less need for these genes to contribute in As(V) tolerance in soybean germinating seedling.

5 Conclusions

This study was designed to elucidate the physiological changes, hormonal regulation and differential expression of the transcriptome in response to ZnONP supplementation in soybean roots under As(V) stress. Our datasets showed that supplementation with ZnONPs increased photosynthesis efficiency, induced the stress responsive genes, *GmPOD, GmGST, GmTrx, GmGPX* and *GmPAL*, and reduced the oxidative stress generated by As(V) thereby promoting plant growth. ZnONPs also ameliorated As(V) stress by limiting its uptake and facilitated its sequestration as evidenced by down-regulation of *PHT* and *NRAMP* genes and up-regulation of ABC transporters. Furthermore, high contents of phytohormones (IAA, GA_3, MeJA, and ABA) and the differential expression genes related to the signaling pathways of these hormones were found in response to ZnONP supplementation. Importantly, although this study found DEGs that were common to the ZnONP and AsV-only treatments, the number of DEGs were lower under ZnONP supplementation than in AsV-only treatment. Although this study has given new insights into the mechanisms of As(V) tolerance in soybeans, it will be necessary to functional characterization these DEGs to clarify their roles in biological pathways involved in As(V) metabolism in soybeans.

Data availability statement

The original contributions presented in the study are publicly available. The data presented in the study are deposited in the Sequence Read Archive (SRA) repository, accession number PRJNA1120636.

Author contributions

MZ: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. CS: Software, Visualization, Writing – review & editing. XW: Data curation, Writing – review & editing. YH: Visualization, Writing – review & editing. HW: Data curation, Writing – review & editing. SL: Methodology, Writing – review & editing. AS: Writing – review & editing. SZ: Data curation, Investigation, Writing – review & editing. AK: Writing – review & editing. PH: Writing – review & editing. MA: Funding acquisition, Writing – review & editing. ME: Funding acquisition, Writing – review & editing. ZZ: Funding acquisition, Resources, Supervision, Writing – review & editing. PZ: Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1427367/full#supplementary-material

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Nanobiotechnology-mediated regulation of reactive oxygen species homeostasis under heat and drought stress in plants

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Global warming causes heat and drought stress in plants, which affects crop production. In addition to osmotic stress and protein inactivation, reactive oxygen species (ROS) overaccumulation under heat and drought stress is a secondary stress that further impairs plant performance. Chloroplasts, mitochondria, peroxisomes, and apoplasts are the main ROS generation sites in heat- and drought-stressed plants. In this review, we summarize ROS generation and scavenging in heat- and drought-stressed plants and highlight the potential applications of plant nanobiotechnology for enhancing plant tolerance to these stresses.

KEYWORDS

drought stress, heat stress, plant nanobiotechnology, chloroplasts, mitochondria, apoplast, ROS homeostasis

1 Introduction

Water scarcity is a constraint on agriculture, and global warming is exacerbating the problem. Drought caused by high temperature has become one of the major abiotic stresses threatening crop yield globally (Lippmann et al., 2019; Dietz et al., 2021). The United Nations report shows, the global surface temperature over 2011–2020 was about 1.1°C higher than that over 1850–1900, and the land surface temperature was 0.71°C higher than the sea surface temperature (Calvin et al., 2023).According to a report released by the Food and Agriculture Organization of the United Nations (FAO) and World Meteorological Organization (WMO), the number of people threatened by food security worldwide has increased significantly from 25.3% in 2019 to 29.6% in 2022 (FAO, 2023), and the heat and drought in 2023 have affected the food security of millions of people (Climate change indicators reached record levels in 2023: WMO, n.d). Lifting the constraints imposed on agriculture by high temperatures and drought can further unleash the potential of agricultural production to effectively respond to the food crisis.

Reactive oxygen species (ROS) in plant cells mainly include superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) (Dat et al., 2000; Halliwell, 2006; Mittler, 2017). Normally, ROS are key signaling molecules in plant cell cycle regulation and programmed death (Gapper and Dolan, 2006; Livanos et al., 2012; Petrov et al., 2015; Qi and Zhang, 2020), and they interact with signaling molecules such as salicylic acid, jasmonic acid, ethylene, and abscisic acid and are the upstream or downstream regulators of many metabolic pathways in plants (Kwak, 2003; Jammes et al., 2009; Denness et al., 2011; Steffens, 2014; Herrera-VÃ¡squez et al., 2015). The excessive accumulation of ROS under high temperature and drought stress can have toxic effects on plants (Suzuki and Mittler, 2006; Djanaguiraman et al., 2018b; Hussain et al., 2019). High levels of ROS can lead to DNA breakage as well as the inactivation of photosystem II (PSII) in the chloroplasts, thereby inhibiting photosynthesis (Kruk et al., 2005). Excessive ROS levels can impair plant growth and development, while maintaining ROS homeostasis is beneficial to plant growth (Dat et al., 2000; Mittler, 2017). The ROS in plant cells are mainly formed in the chloroplasts, mitochondria, peroxisome, and apoplasts. Plants also have antioxidant systems (Mittler et al., 2004; Impa et al., 2012), including both enzymatic and non-enzymatic systems (Hernández et al., 2012; Rajput et al., 2021), that protect cells from the excessive accumulation of ROS. The antioxidant enzyme system includes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) (Alscher et al., 2002; Laxa et al., 2019)

Non-enzymatic systems include small organic molecules with antioxidant capabilities, such as polyphenols, carotenoids, vitamin C, glutathione, melatonin and polyamines (Hernández et al., 2012; Kolupaev et al., 2023). Maintaining ROS homeostasis can improve crop resistance to high temperature and drought stress (Awasthi et al., 2015; Verma et al., 2019).

In recent years, it has been reported that nanoparticles improve plant abiotic stresses tolerance. For example, iron oxide nanoparticles ameliorated the cadmium and salinity stresses in wheat plants (Manzoor et al., 2021). Application of ZnO nanoparticles could alleviate the low temperature damage on the early growth of rice (Mai et al., 2024). Emerging studies focuse on the use of plant nanobiotechnology to regulate plant ROS homeostasis to improve plant resistance to stress (Zhao et al., 2020; Liu et al., 2021a).To date, several nanomaterials, such as CeO_2 (Khan et al., 2021), and Mn_3O_4 (Liu et al., 2023) were reported as an ROS scavenger in plant abiotic stress tolerance. For example, cerium oxide nanoparticles enable plants to withstand drought and saline-alkali stress (Djanaguiraman et al., 2018b; Liu et al., 2021). The high temperature tolerance of plants is improved with nano-selenium treatment (Djanaguiraman et al., 2018a). In addition, some nanomaterials can maintain ROS homeostasis and enhancing plants heat and drought tolerance (Wu et al., 2017).

Nanobiotechnology can effectively maintain ROS homeostasis and improve plant resistance to heat and drought stress. Therefore, in this paper, we presented a overview of how plants generate and scavenge ROS during heat and drought stress. Also show the role of nanobiotechnology in maintaining ROS homeostasis in crops, the underlying potential mechanism in enhancing crop resistance and ensuring sustainable agriculture.

2 Effect of heat and drought stress in plants

Drought and heat stress affect the plant morphology and physiological functions. For instance, both stresses retard the photosynthetic machinery and limit photosynthesis (Carmo-Silva et al., 2012), cause ROS over-accumulation (Das and Roychoudhury, 2014), and hinder plant growth and development (Yadav et al., 2022).

Combined heat and drought exacerbates the adverse effects on plants compared to individual stresses. Drought typically causes stomatal closure (Buckley, 2019), whereas heat stress results in stomatal opening (López et al., 2022). During concurrent heat and drought stress, drought can exacerbate thermal damage during heatwaves (Marchin et al., 2022). Both stresses lead to ROS over-accumulation. For example, it was reported that heat and drought stress cause ROS accumulation and suppress seed germination and growth in rice (Liu et al., 2019). Therefore, maintaining ROS homeostasis is a must to do physiological process in plants to ensure survival. This could be achieved through modulating the activity of ROS scavenging pathways and recruiting antioxidant enzyme activities. However, heat and drought significantly decrease the activities of antioxidant enzymes such as SOD, POD, and CAT (Hu et al., 2023), which is detrimental to maintaining ROS homeostasis in plants. Hence, investigating options to assist plants performance under harsh conditions it is particularly important task to explore.

3 ROS generation in plants

3.1 ROS in the mitochondria

The mitochondria are one of the ROS production sites. In plant cells, most of the ROS produced under light come from the chloroplasts or peroxisomes, but in non-green tissues or darkness, mitochondrial ROS production dominates (Navrot et al., 2007) (Figure 1). This production is an inevitable result of aerobic metabolism, and about 1–5% of the oxygen consumption of plants is due to H_2O_2 production (Černý et al., 2018). The main site of ROS formation in the mitochondria is the respiratory electron transport chain (RETC), which involves the reduction of oxygen to H_2O. Different pathways are involved in this reduction process. Some enzyme systems, such as cytochrome c oxidase (COX), directly reduce oxygen, while others reduce oxygen in steps, i.e., oxygen accepts only one electron, resulting in the formation of $O_2^{\bullet-}$. Nicotinamide adenine dinucleotide (NAD^+) is an important coenzyme in RETC in the inner mitochondrial membrane. Also, it is an important electron acceptor in the RETC. NADH, ubiquinone oxidoreductase (Complex I), and the cytochrome C reductase complex (Complex III) is the main site of ROS production, Complex I mainly produces a small amount of $O_2^{\bullet-}$, and Complex

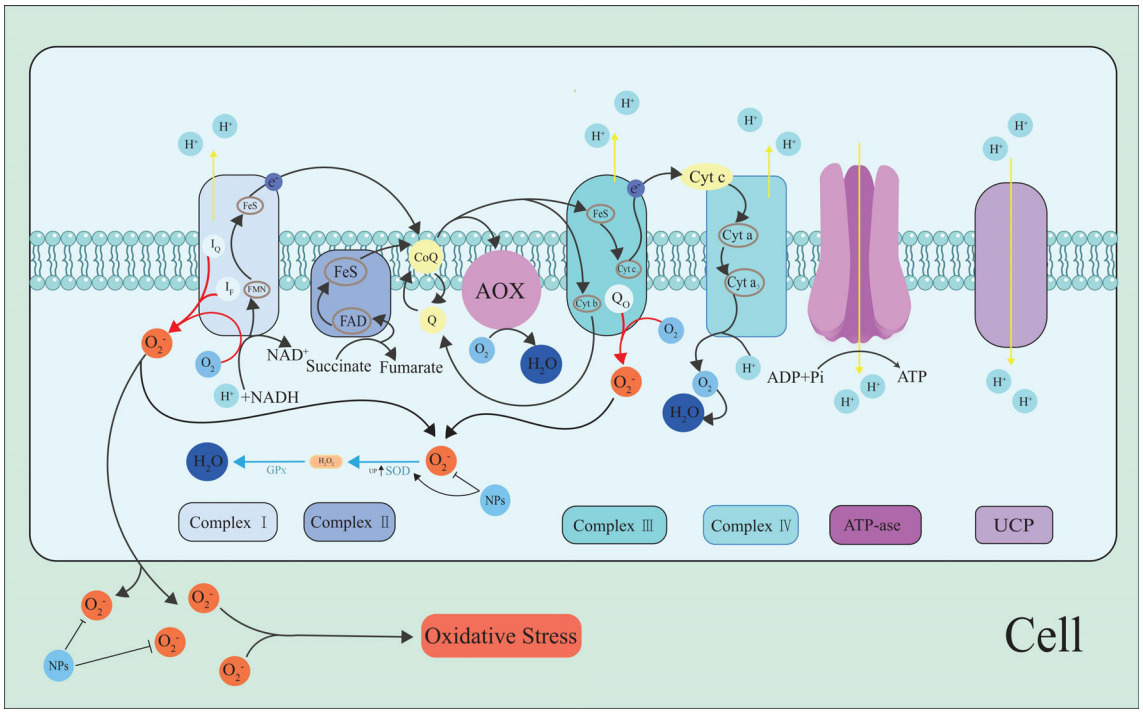

FIGURE 1
ROS generation along the RETC pathway. $O_2^{\bullet-}$ is formed upon single-electron reduction of O_2 (Chenna et al., 2022). Complex I and III in the RETC are the major sites of $O_2^{\bullet-}$ production. UCP and AOX in RECT. The energy of the proton gradient drives the ATP synthesis of or can be consumed by UCPs. NPs inhibit the ROS generation and scavenge ROS, maintain cell ROS homeostasis. The black arrow indicates the electron pathway or ROS transfer, and the red arrows represent $O_2^{\bullet-}$ generation.

III produces $O_2^{\bullet-}$ which is then converted to H_2O_2 (Gakière et al., 2018; Dourmap et al., 2020).

Mitochondrial damage caused by oxidative stress can trigger the release of metals in mitochondrial proteins (Mittler et al., 2004). In the presence of high levels of $O_2^{\bullet-}$ and H_2O_2, the release of metals accelerates the reaction to generate $^{\bullet}OH$, thus causing membrane damage through lipid peroxidation (Tan et al., 2010; Mittler, 2017; Suzuki, 2023). Some studies have shown that the content of Cu and Fe in the mitochondria is reduced by 40% after oxidative stress, which indicates that the complex containing these two metals in the electron transport chain on the mitochondrial membrane is damaged (Tan et al., 2010). This may lead to an increase in free ferrous ions, with H_2O_2 and Fe^{2+} forming highly toxic $^{\bullet}OH$, which damages plants (Mittler, 2017). As these complexes are sensitive to abiotic stresses, their protection is important for maintaining ROS homeostasis.

Uncoupling protein (UCP) is a membrane albumen located in the inner membrane of the mitochondria that belongs to the mitochondrial anion operating family. One study found that the increased expression of UCP in the tobacco mitochondria may lead to greater tolerance of drought stress (Begcy et al., 2011). Further research on the mechanism of UCP in maintaining mitochondrial ROS homeostasis may unearth the great potential of plants in the face of abiotic stresses.

Alternation oxidase (AOX) is another mitochondrial protein involved in maintaining cellular metabolism and energy balance, and its role is even more important under stress (Kumari et al., 2019; Garmash, 2022).AOX reduces excess oxygen in mitochondria by providing additional electron consumption pathways, thereby reducing ROS production and maintaining ROS homeostasis (Maxwell et al., 1999; Analin et al., 2023).In addition, AOX plays an important role in ethylene-induced drought resistance and improves drought resistance in tomato seedlings by balancing ROS levels in autophagy production (Zhu et al., 2018).Under osmotic and temperature stress, AOX pathway regulation of ROS through redox couples related to malate valve and antioxidant system (Dinakar et al., 2016). A study found that a lack of AOX under drought stress led to an increase in cell damage and a decrease in the ability of tobacco plants to recover quickly once the water supply was restored (Wang and Vanlerberghe, 2013). In another study AOX metabolites alongside the activation of several AOX enzymes relieve impact of combined heat and salt stresses on tomato (Sousa et al., 2022).

3.2 ROS in the chloroplasts

As the main site of photosynthesis, chloroplasts are important organelles in plants, and the photosynthetic electron transport chain (PETC) on the thylakoid membrane is crucial for plant photosynthesis. Chloroplasts not only carry out photosynthesis but also sense and transmit stress signals under abiotic stresses such as high temperature and drought (Foyer and Shigeoka, 2011; Hu et al., 2020b; Somal et al., 2023). They are also the most

important plant parts for producing ROS under stress. Photosystem I (PSI) and PSII in the PETC on the thylakoid membrane are the main sites for ROS production (Takahashi and Asada, 1988; Roach and Krieger-Liszkay, 2014) (Figures 2, 3).

The Mehler reaction is the main pathway of ROS production in the chloroplasts. During plant stress, the Mehler reaction reduces O_2 to $O_2^{\bullet-}$ by PETC in PSI (Kozuleva et al., 2020a, Kozuleva et al., 2020b). PSII also produces ROS such as H_2O_2 (Pospíšil, 2009). Water-water cycling (WWC) is a highly efficient photoprotection strategy that can effectively scavenge $O_2^{\bullet-}$ and H_2O_2 from the thylakoids to protect plants from oxidative damage (Asada, 1999, Asada, 2000; Rizhsky et al., 2003; Awad et al., 2015; Huang et al., 2019). Additional findings suggest that WWC enhancement at high temperatures protects PSI, while cyclic electron flow enhancement at low temperatures compensates for WWC deactivation, both of which together protect plants from ROS damage (Sun et al., 2020; Jiang et al., 2021). Maintaining chloroplast ROS homeostasis by regulating WWC may be an effective way to improve plant stress resistance.

Nicotinamide adenine dinucleotide phosphate (NADP) is one of the end electron acceptors in the electron transport chain of photosynthesis. The reduced nicotinamide adenine dinucleotide phosphate (NADPH) is an important reducing force in chlorophyll synthesis and the Calvin cycle. NAD kinases (NADK) are involved in many plant life activities, such as maintaining intracellular REDOX balance and responding to environmental stress. Studies have shown that NADK activity mediates response mechanisms to regulate PSI biosynthesis (Ji et al., 2022). Because NADP can provide the main reduction power for the ROS scavenging system (Mittler, 2002).In addition, NADK deficiency results in decreased ROS stress and drought tolerance in plants (Sun et al., 2017; Wang et al., 2020; Chaomurilege et al., 2023).

Non-photochemical Chlorophyll fluorescence quenching (NPQ) is a process that occurs in plants where excess absorbed light energy is converted into heat energy (Demmig-Adams et al., 2014). The relationship between NPQ and ROS homeostasis is complex. When the NPQ generation process is inhibited, such as by thermal inactivation of APX, the formation of ROS increases, which can lead to plant damage. It was found that heat treatment could significantly stimulate NPQ, while increasing the temperature almost completely inhibited NPQ, and was accompanied by the generation of ROS (Hideg et al., 2008). This indicates that the NPQ generation and ROS clearance processes are closely linked. According to Liu et al. (2022) the significantly higher non-photochemical quenching (NPQ) in bundle sheath chloroplasts compared to mesophyll chloroplasts under drought stress could

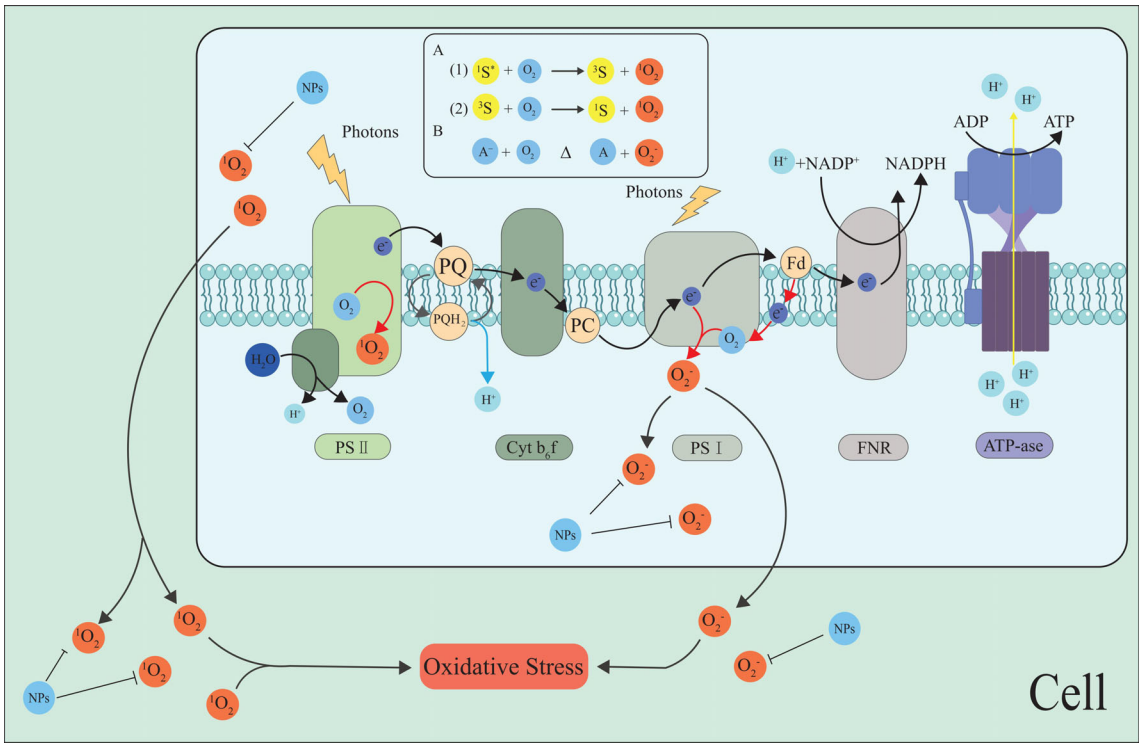

FIGURE 2
The generation of ROS in the photosynthetic electron transport chain. Photoexcitation of chlorophyll at PSII and PSI drives electron transport, but in the absence of acceptors, excitation may transfer to O_2 at the PSII reaction center, forming a $^1O_2$ and $O_2^{\bullet-}$. NPs inhibit the ROS generation and scavenge ROS, maintain cell ROS homeostasis. The most common mechanism of $^1O_2$ generation is photosensitization, i.e., the reaction of O2 with a photoexcited sensitizer dye (S*). Produced by spin-conserved Reactions (A1) and (A2). $O_2^{\bullet-}$ is mainly formed by the interaction of O_2 with reducing compounds (A) with low redox potential (B) (Khorobrykh et al., 2020).The black arrow indicates the electron pathway or ROS transfer, and the red arrows represent $O_2^{\bullet-}$ generation.
Fd, ferredoxin; FNR, ferredoxin-NADP reductase; PC, plastocyanin; PQ, plastoquinone; and Cytb$_6$f, Cytochromeb$_6$fcomple.

be a primary reason for the lower accumulation of ROS in bundle sheath chloroplasts. Studying the relationship between NPQ and ROS homeostasis and the light damage resistance of chloroplasts is important for improving plant stress resistance.

3.3 ROS in the apoplasts

Apoplasts are composed of intercellular space, a cell wall, and an extracellular matrix, and they play an important role in the transport of water, mineral nutrients, and organic nutrients, as well as the production of signaling molecules in response to stress (Hoson, 1998; Sattelmacher, 2001; Koch et al., 2003; Qi et al., 2017). Although the level of ROS production in the apoplasts is much lower than that of the chloroplasts and mitochondria, as the site of ROS production, it is no less important to plants than the chloroplasts and mitochondria (Jubany-Mari et al., 2008; Barceló and Laura, 2009). It plays an important role in the plant perception of external stresses (Mittler, 2017; Mittler et al., 2022).

In the apoplasts, ROS are mainly produced by NADPH oxidase (Podgórska et al., 2017). Respiratory burst oxidase homologs (RBOHs) are the main sites of ROS production in apoplasts, and they transfer electrons from cytosolic NADPH to O_2 and then produce $O_2^{\bullet-}$ in apoplast bodies, following which they generate relatively stable H_2O_2 catalyzed by SOD and then enter the cell as a signaling molecule (Sagi and Fluhr, 2006; Marino et al., 2012; Hu et al., 2020a). NADPH acts as a fuel for RBOHs and provides sufficient substrate for ROS production when plants encounter stress (Wu et al., 2023a). ROS waves are crucial to the acquired adaptation of plant systems (Zandalinas et al., 2020). It was reported that the dependent *D*(*rbohD*) gene inhibits signal propagation by inhibiting ROS accumulation away from the start site, suggesting that ROS waves are RBOHD-dependent (Miller et al., 2009; Mittler and Blumwald, 2015).

The H_2O_2 produced by NADPH oxidase acts as a secondary messenger to activate the MAPK, a conserved intracellular pathway in plants consisting of MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK). These kinases are activated step by step through phosphorylation and dephosphorylation. In this process, MAPKKK is first activated; it then activates the downstream MAPKK, and finally, MAPKK will activate the most downstream MAPK, this cascade reaction allows the signal to be amplified and transmitted within the cell (Jonak, 2002). The MAPK cascade pathway is highly correlated

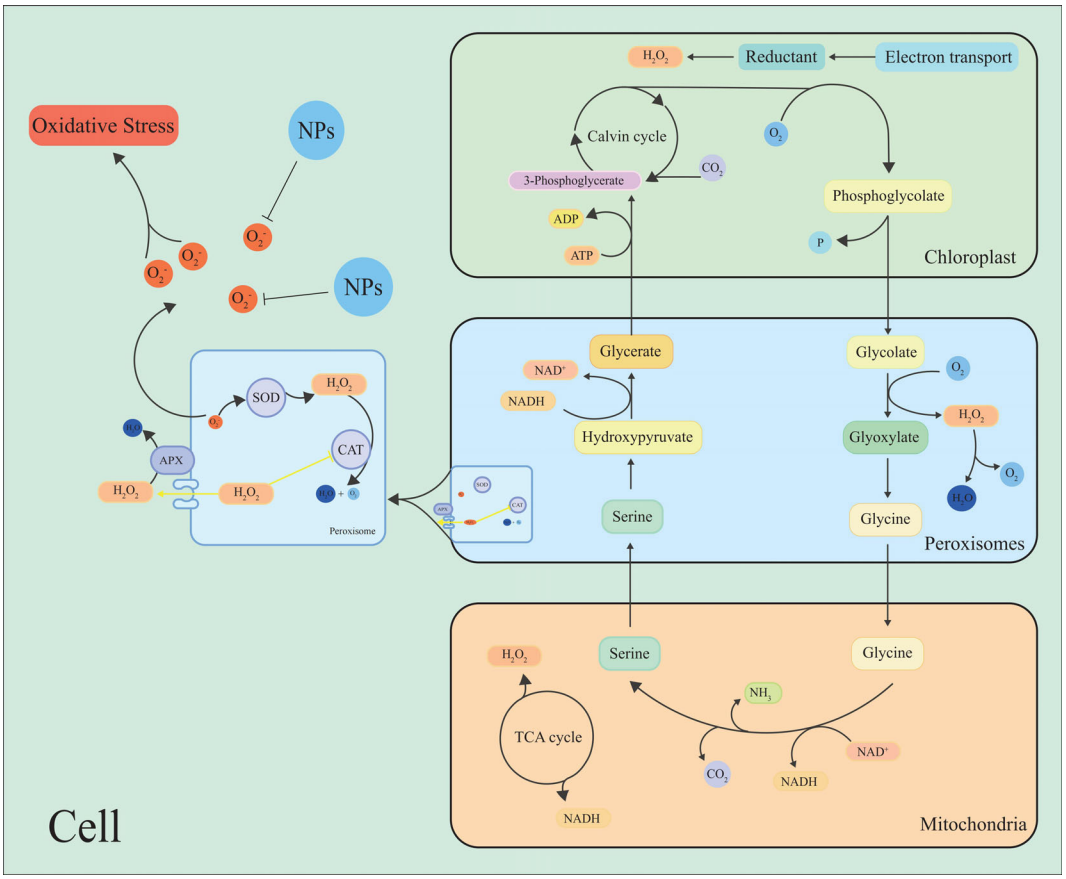

FIGURE 3
Simple model for the production or removal of H_2O_2 by plants through photorespiration and peroxisomal metabolism. During photorespiration, when oxalates are oxidized to glyoxylate by enzymes called glycolates, H_2O_2 is produced as a by-product. NPs inhibit the scavenge ROS, maintain cell ROS homeostasis.

with abiotic stresses such as high temperature and drought; for example, the expression level of MAPK was significantly correlated with the relative biomass of plants in response to drought (Liu et al., 2015). Another study showed that *PlMAPK1* plays a positive regulatory role in the plant response to heat stress (Qian et al., 2023). ROS can induce MAPK activation, and interfering with the MAPK cascade can modulate ROS production and response. With further climate change, it is important to investigate the relationships and interaction mechanisms of this pathway with ROS, phytohormones, and other substances in the apoplasts to improve plant resistance to unfavorable conditions. Extracellular peroxidase (ECPOX) is a protein that induces ROS production and detoxification in apoplasts, It is closely related to the formation of extracellular $O_2^{\bullet-}$ (Li et al., 2010; Gautam et al., 2017).Studies have found that the activity of ECPOX is related to the production of ROS, in the apical cell wall of pea seeds, the activity of ECPOX increases simultaneously with the production of $O_2^{\bullet-}$ (Kranner et al., 2010).In wheat roots, the production of $O_2^{\bullet-}$ sensitive to peroxidase inhibitors was taken as evidence indicating that ECPOX was involved in ROS production (Minibayeva et al., 2009).

Polyamines are important regulatory factors of ROS homeostasis (Saha et al., 2015; Kolupaev et al., 2019). H_2O_2 in plant cell walls can be produced by the metabolism and recovery of polyamines, of which diamine oxidase (DAO) and polyamine oxidase (PAO)are key to polyamine metabolism (Moschou et al., 2008). PAOs used polyamine as substrate to catalyze the formation of smaller polyamines, as well as a molecule of amino aldehyde and H_2O_2. The other was DAO dependent on $Cu2^+$ and using pyridoxal phosphate as coenzyme. DAO catalyzes putrescine to produce H_2O_2, ammonia and 4-aminobutyraldehyde (Kusano et al., 2007, Kusano et al., 2008). Another characteristic of tomato slpao3 mutant was found to be tolerant to drought stress, indicating polyamine helps to improve drought resistance (D'Incà et al., 2024).

Oxalate oxidase (OXO) in the cell wall catalyzes the oxidation of oxalic acid with molecular oxygen to form CO_2 and H_2O_2 (Verma and Kaur, 2021). Studies have shown that high levels of OXO expression produce a large amount of H_2O_2 that also has a direct harmful effect on plants, inducing cell death (Delisle et al., 2001).

3.4 ROS in the peroxisomes

Peroxisomes are important sites to produce ROS by oxidative metabolism in plant cells, such as $O_2^{\bullet-}$, H_2O_2, and $^1O_2$, which participate in different physiological processes and play an important role in maintaining cellular redox homeostasis (Río et al., 2006; Choudhury et al., 2016) (Figure 3). Photorespiration has a substantial impact on plant metabolism and is the pathway of ROS production and clearance in peroxisomes (Noctor, 2002; Foyer et al., 2009; Del Río, 2020; Timm and Hagemann, 2020) (Figure 3).

Hydroxy pyruvate reductase 1 (HPR1), the main metabolic enzyme in the photorespiration cycle, can be inhibited by high light intensity, which exacerbates the production of ROS (Wang et al., 2022d). It has been found that the ROS metabolism of photorespiration has a dual role, and xanthine oxidase can mediate the production of $O_2^{\bullet-}$ (Tewari et al., 2009). Xanthine dehydrogenase 1 (XDH1) produces ROS with NADPH oxidase in leaf epidermal cells, and the clearance of H_2O_2 in mesophyll cells by XDH1 protects plants from oxidative damage (Ma et al., 2016). Researchers have found that photorespiratory metabolism was downregulated in *Arabidopsis* after hypoxia or the addition of aminooxyacetic acid (a photorespiratory inhibitor), while ROS levels were significantly elevated (Saini, 2023). Another study found that the photorespiration metabolism of rice was upregulated under high temperature and iron stress to ensure survival under stress (De Souza et al., 2024). These studies suggest that the regulation of photorespiration is an important way to maintain ROS homeostasis.

It was found that the H_2O_2 content in quinoa increased under high temperature and drought conditions, and Peroxisome abundance was positively correlated with H_2O_2 content in the leaves (Hinojosa et al., 2019). The *OsAPX4* gene encoding peroxisomes in rice is thought is thought to affect plant development and resilience by regulating ROS signaling, suggesting that the peroxisome plays a significant role in maintaining ROS homeostasis in plant cells (Ribeiro et al., 2017).

4 ROS detoxification

4.1 Enzymatic system

Antioxidant enzymes such as CAT, SOD, APX, and glutathione reductase (GR) constitute the ascorbate-glutathione cycle (ASA-GSH cycle), which can effectively scavenge ROS such as H_2O_2 in plants. This pathway plays a vital role in helping plants cope with stresses such as high temperature and drought (Tiwari et al., 2017; Del Río, 2020). There are other antioxidant enzymes, such as GPX, glutathione S-transferase (GST), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR), which together form a complex component of the antioxidant defense system and can effectively scavenge ROS to maintain ROS homeostasis in plants (Zhou et al., 2017, Zhou et al., 2018; Sarker and Oba, 2018; Hasanuzzaman et al., 2019).

SOD can be divided into Fe-SOD, Mn-SOD, and Cu/Zn-SOD isoenzymes according to the different metal co-groups, which can catalyze $O_2^{\bullet-}$ to H_2O_2 (Alscher et al., n.d.). H_2O_2 can be further broken down by other antioxidant enzymes such as CAT or APX; CAT can split H_2O_2 into water and oxygen, and APX uses ascorbic acid to scavenge H_2O_2 under drought stress (Hasanuzzaman et al., 2012; Tiew et al., 2015). A study showed that CAT activity increased significantly under severe drought conditions, while mild and severe drought altered the expression patterns of CAT1 and CAT2. These results suggest that complex regulation at the level of CAT mRNA translation controls the accumulation of H_2O_2 in leaves (Luna, 2004). At high temperatures, *SmAPX2* with APX activity can catalyze the oxidation of ascorbic acid by H_2O_2 *in vitro* and reduce the accumulation of H_2O_2 under high temperature stress *in vivo* through the temporary expression of *SmAPX2*. These results indicate that *SmAPX2* plays an active role in plant responses to high temperature stress (Shen et al., 2024).

The GR-mediated ASA-GSH cycle in the peroxisome has special significance in ROS clearance and tolerance to abiotic stress

(Foyer and Noctor, 2011). One study showed that the accumulation of GR gene transcripts under heat stress was 3.1 times that of untreated samples, and the gene was upregulated by various stresses (Chanda Roy and Chowdhary, 2023). *OsNAC092* mutant rice had higher ROS clearance ability and maintained a higher GSH/GSSG ratio and reduction level under drought conditions, thus protecting cells from oxidative stress (Wang et al., 2022a).

A study by explored the response of maize varieties with different heat tolerances to the ASA-GSH cycle under high temperature stress. The results showed that high temperature resulted in increased ROS and increased levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in all varieties and significantly increased GST activity. The enzyme activities of APX, MDHAR, and DHAR were decreased in sensitive varieties, while the GR enzyme activities were significantly increased in heat-resistant varieties, and the AsA levels were increased in heat-resistant varieties and decreased in sensitive varieties (Tiwari and Yadav, 2020). Another study on wheat under high temperature stress found that both the GR and APX activities increased with increasing heat stress. The GR and APX activities of wheat varieties with higher heat resistance were the highest (Mohi-Ud-Din et al., 2021). Furthermore, when cotton was stimulated by H_2O_2, the activity of MDHAR significantly increased. Additionally, SOD, APX and CAT activities also increased significantly (Madhu et al., 2024). These results suggest that *GhMDHAR* can participate in the H_2O_2 response and plays an important role in maintaining ROS homeostasis. Recent studies have shown that the *TaMDHAR* gene was significantly upregulated in wheat under drought, high temperature, and high salt conditions. The activity of *TaMDHAR* increased significantly under high temperature and drought stress, while that of MDHAR decreased. It was also found that the MDHAR protein mainly interacted with Asc, DPN, $NADP^+$, NADPH, CoA, FAD^+, MgATP, H_2O_2, FMN, MgADP, and GSSG (Madhu et al., 2024). The researchers found that high temperatures increased MDHAR activity in two rice varieties, IR-64(heat sensitivity) and Huanghua Station (heat tolerance), and CAT, SOD, and POD activities were also affected. Moreover, the contents of H_2O_2 and MDA in rice were increased under high temperature, and the concentrations of cytokinin and auxin in different plant sites in the tested heat-sensitive and heat-tolerant cultivars were also affected. The study also found that the MDHAR activity was increased following the application of plant growth regulators such as methyl jasmonate and ascorbic acid (Al-Zahrani et al., 2022).

In conclusion, the ASA-GSH cycle plays an important role in plants during stress, and MDHAR has a non-negligible effect on the ASA-GSH cycle and even the antioxidant system of the entire plant. Studies on the ASA-GSH cycle, the enzyme system in the peroxisome, and the regulatory effects of exogenous substances on the ASA-GSH cycle are of great significance for improving crop stress resistance.

4.2 Non-enzymatic system

Small-molecule antioxidants exist in plant cells. Some highly toxic ROS, such as $^1O_2$ and $^{\bullet}OH$, that cannot be scavenged by the antioxidant enzyme system can neutralize free radicals or produce a relatively harmless free radical by donating electrons or hydrogen atoms by antioxidants, which are later effectively scavenged by other antioxidant systems (Hernández et al., 2009). Antioxidants in plants include AsA, GSH, flavonoids, tocopherols, and carotenoids, among others (Hernández et al., 2009, Hernández et al., 2012; Dangles, 2012; Shen et al., 2018).

Tapetum development and functional defects1 (TDF1) can inhibit tapetum cell division. The downstream target of TDF1, SKS18, was found to encode a polycopper oxidase-like protein with ascorbate oxidase activity. Moreover, TDF1 negatively controls an AsA biosynthetase VTC1 to regulate AsA biosynthesis and maintain appropriate AsA content. SKS18 knockdown or VTC1 overexpression can increase AsA concentration and reduce ROS accumulation, thereby balancing cell division and cell differentiation in the felt layer (Wu et al., 2023b). Flavonol synthetase (FLS) is one of the key enzymes in the synthesis of flavonoids. Under drought stress, overexpression of *EkFLS* in *Arabidopsis* resulted in the increased accumulation of flavonoids and significantly enhanced POD and SOD activities (Wang M. et al., 2021).

Carotenoids and α-tocopherol, as components of the non-enzymatic antioxidant system, can also scavenge ROS. Studies have shown that *NtCCD1* is a negative regulator of carotenoid content in tobacco, and silencing *NtCCD1* by virus-induced gene silencing (VIGS) can increase carotenoid contents and decrease ROS levels (Du et al., 2023). Another study in tomatoes identified a *VTE5* gene that plays a key role in alpha-tocopherol production. When *VTE5* is silenced, the production of α-tocopherol is affected, which reduces the plant's ability to resist high temperature and light stress. However, the content of α-tocopherol almost doubled under combined stress compared to exposure to high temperatures or high light alone (Spicher et al., 2017).

With the furthering of biological and agronomic research in recent years, the application of exogenous substances for improving the antioxidant capacity and enhancing the stress resistance of plants has become increasingly prevalent. For example, the exogenous application of AsA and α-tocopherol is used to maintain ROS homeostasis and improve drought resistance (El-Beltagi et al., 2022; Zafar et al., 2024). Melatonin was first discovered in the pineal gland of animals, and it is thought to be a free radical scavenger that can be synthesized *in vivo* (Hevia et al., 2014; Vielma et al., 2014; Hardeland, 2017; Zhao et al., 2019). The application of exogenous melatonin in plants has also gained increasing research interest. Studies have shown that exogenous melatonin can effectively reduce the oxidative stress caused by iron deficiency in sweet pepper and enhance antioxidant defense mechanisms. In addition, the chlorophyll content and photosynthetic efficiency of plants under salt stress can be improved (Kaya et al., 2020). Melatonin and hydrogen sulfide regulate the antioxidant defense system under chromium stress, and the supplementation of melatonin to tomato seedlings under stress eliminates excess ROS, thus maintaining ROS homeostasis and improving the stress resistance of tomato seedlings (Khan et al., 2023). Studies on the effects of exogenous melatonin under high temperature and drought stress showed that supplementation with exogenous melatonin could regulate the ASA-GSH system and affect the

activity of antioxidant enzymes, thereby protecting plants from oxidative damage caused by drought (Khan, 2023).Other studies have shown that exogenous melatonin can alleviate the negative effects of excess ROS, significantly increase the activity of antioxidant enzymes, stabilize chloroplast structure, and improve the drought resistance of maize seedlings (Muhammad et al., 2023). Moreover, melatonin can enhance the heat resistance of rice seeds by enhancing the activity of antioxidant enzymes and significantly reducing the content of malondialdehyde (Yu et al., 2022). In addition, some studies have shown that the application of exogenous melatonin can increase the activity of antioxidant enzymes and the concentration of metabolites involved in osmoregulation and ion homeostasis in mung bean under drought and high temperature stress, as well as improve the physiological and yield traits of mung bean under combined stress in the reproductive stage (Kuppusamy et al., 2023).

5 Nanobiotechnology regulate ROS homeostasis and heat and drought tolerance in plants

5.1 Reported nanomaterials maintain ROS homeostasis

Maintaining plant ROS homeostasis can improve plant stress tolerance. With increased research interest in nanotechnology in recent years, the relationship between nanomaterials and plants has attracted increasing attention.

Nanomaterials (NMs) are defined as materials with at least one dimension in the nanoscale (1–100 nm) (Auffan et al., 2009). Due to their small size effect, NMs have unique properties and can penetrate through cell walls and enter plant cells to regulate the complex physiological and biochemical processes of plants, thus regulating plant growth and development. Researchers are taking advantage of the special properties of NMs to study the interaction between NMs and plants (Shi et al., 2014; Ajmal et al., 2023). Researchers alter the properties of NMs by applying different modifications to their surfaces. As a nano-mimetic enzyme, polyacrylic acid-modified manganous tetroxide nanoparticles (PMO) have both SOD and CAT activities, and they can effectively scavenge excess ROS, including H_2O_2 (Liu et al., 2023). Studies have shown that under drought stress conditions, PMO can effectively scavenging excess ROS through their nanase activity and alleviate the mitotic activity inhibited by drought by maintaining ROS homeostasis (Sun et al., 2023). Mn_3O_4 NPs can enhance the endogenous antioxidant defense ability of plants and relieve the oxidative stress of cucumber plants (Lu et al., 2020). Cerium oxide nanoparticles are also known to be an effective ROS scavenger. The principle of ROS scavenge is to catalyze ROS decomposition by utilizing the oxygen vacancy in their lattice structure (Giraldo et al., 2014). Researchers have found that Poly(acrylic acid) nanoceria (PNC) can scavenge a variety of ROS, including $^{\bullet}OH$, protect plant photosynthesis, and reduce the damage to plants caused by abiotic stress (Wu et al., 2017). CeO_2 nanoparticles can also improve the SOD activity and salt tolerance of cotton and rape, Se-NPs scavenge ROS in sorghum under drought stress, relieve oxidative stress, and protect the photosynthesis and grain yield of sorghum (Djanaguiraman et al., 2018b; Liu et al., 2021; Li et al., 2022).

Nanozymes are nanomaterials with enzyme characteristics. Nanoparticles not only directly scavenge ROS but also indirectly improve the ability of plants to scavenge ROS by affecting the activity of antioxidant enzymes (Singh, 2019; Wu et al., 2019). Spraying nano-selenium under drought and high temperature stress increases the antioxidant enzyme activity of wheat seedlings, thereby improving the ability of wheat to resist high temperature and drought stress (Omar et al., 2023). Other nanoparticles such as Ag NPs can improve wheat resistance to high temperatures, while Si NPs help wheat mitigate drought stress, and TiO_2 NPs alleviate the effects of drought stress on tomato (Iqbal et al., 2019; Boora et al., 2023; Cevik, 2023). Currently, Mn NPs, Ce NPs, Si NPs, Se NPs, Ag NPs, Ti NPs, and other nanoparticles have been applied to wheat, cucumber, rape, sorghum, and other plants to circumvent the negative effects of high temperature and drought stress on plants (Djanaguiraman et al., 2018b; Iqbal et al., 2019; Boora et al., 2023; Cevik, 2023; Omar et al., 2023; Sun et al., 2023; Nauman Khan et al., 2024) (Figure 4; Table 1).

Research shows that effects of NMs on plants under stress are diverse. Research shows, that PNC catalytic scavenging of $^{\bullet}OH$ influences the activity of key plasma membrane channels controlling plant cell K^+ retention, thereby alleviating the damage of salt stress to *Arabidopsis* (Wu et al., 2018). In addition, carbon dots NMs also have ROS scavenging functions (Dong et al., 2022; Innocenzi and Stagi, 2023). *Salvia miltiorrhiza*-derived the synthesized carbon dots have a scavenging effect on intracellular ROS in plants (Wang et al., 2022d) (Table 1). Soil application of carbon dots enhanced the N-fixing ability, the expression of genes related to nitrogen transport and water absorption was enhanced of nodules enhanced N and water uptake in soybeans under drought stress, promoted the growth and nutritional quality of soybeans (Wang et al., 2022c).

Recently, researchers synthesized manganese oxide nanoparticles (MnO_2 MFs) and cerium oxide nanoparticles (CeO_2 NPs) and regulated the interaction between the two by polydiallyldimethylammonium chloride surface modification, forming Ce–PMn and Mn–PCe composite materials. The formed composites exhibited a variety of enzymatic activities, such as SOD and CAT functions, and the composites Ce–PMn and Mn–PCe retained the nanozymatic activities of individual metal oxides to a certain extent (Alsharif et al., 2023). This suggests that maintaining ROS homeostasis to improve stress resistance may not be limited to a single nanomaterial. The application of composite materials is also an important focus.

In addition to maintaining ROS homeostasis and improving plant stress resistance, the special properties of NMs can also be used as tools for conveying agricultural chemicals, such as nano-fertilizers, and nano-pesticide (Ahmadian et al., 2021; Wang et al., 2022d, Wang et al., 2023; Farajollahi et al., 2023). Based on the special properties of NMs, nano fertilizers also show better utilization efficiency. For example, the application of nano-silica and nano-iron oxide fertilizers under drought stress can improve

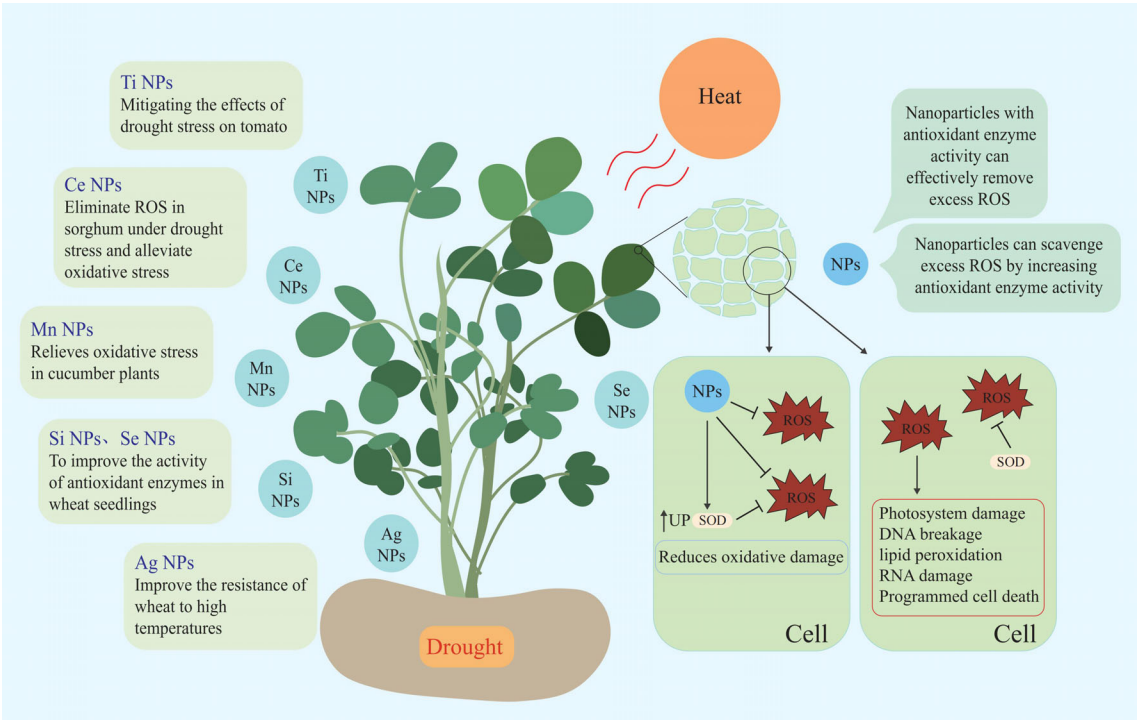

FIGURE 4
Nanoparticles used to stress tolerance and improve plant growth.

the activity of antioxidant enzymes and reduce the harmful effect of drought stress on wheat and soybeans, and the application of nano-iron oxide can also increase the activity of soybean nitrogenous enzyme (Ahmadian et al., 2021; Farajollahi et al., 2023). The use of NMs to improve the ability of biological nitrogen fixation not only uses the properties of NMs to target specific organelles for precise and effective delivery but also avoids the risks associated with transgenic methods (Li et al., 2023). In addition to nano fertilizers, nano pesticides show a better control effect than commercial synthetic pesticides in field trials, providing a new and sustainable approach for the control of plant diseases (Wang et al., 2022b). Recent studies have used nanotechnology to design a self-assembled nano bioprotectant based on double-stranded RNA and plant inducers, which enhances endocytosis and amplifies plant system defense responses, effectively helping plants cope with potato late blight infection (Wang et al., 2023).

For a long time, researchers try to investigate the possible mechanisms of nanomaterials enhance plant abiotic stress tolerance. Maintaining ROS homeostasis is the main idea of plant nanobitechology, and the downstream signaling pathway is also being studied. However, few attention has been paid to the material science mechanism.

Nanomedicine has some similarities with plant nanobitechology, and its material science mechanism can be used as reference. Cerium oxide nanoparticles (nanoceria), for instantance, was widely reported could enhance crop abiotic tolerance. Because the surface of NC has two valence states of Ce^{3+} and Ce^{4+}, creating an oxygen vacancy (Boghossian et al., 2013), nanoceria can scavenge ROS effectively and enhance the salt resistance ability of plants. Further study on the nanoceria crystal structure shows that the catalytic efficiency of nanoceria with different crystal structures on ROS is different (Wang Z. et al., 2021; Yuan et al., 2023)The functional groups modified on the surface may also affect nanomaterials biological effects (Cheng et al., 2021). But there are no standardized guidelines for agricultural nanomaterials synthesis. Thus, in general, the mechanisms of nanomaterials maintaining plant ROS homeostasis is through Redox reaction from botany, but material science mechanisms need further study.

5.2 Issues in nanomaterials and plants interaction

The first step in the interaction between nanomaterials and plants is the cell. The cell wall and membrane are hindrances for nanomaterials entering plant cells. The cell wall has been reported to have pores (Zeng et al., 2017) and negative potential (Fry, 2011). Normally, a particle size smaller than the pore's diameter and with positive potential can cross the cell wall more easily. Different sizes and potentials affect how nanomaterials enter plant cells. By exploring different leaf structures, researchers have found that they are dependent on a suitable size. However, in both cotton and maize, positive potential nanomaterials have better delivery efficiency. Problems usually have a secondary solution. The cell wall consists of pectin and cellulose; thus, cellulase can damage the cell wall and enable the entry of nanomaterials into the cell (Serag et al.,

TABLE 1 Nanoparticles used to stress tolerance and improve plant growth.

Nanomaterials	Mechanisms	Reference
PAA (polyacrylic acid) coated Mn_3O_4	scavenge excess ROS, maintaining ROS homeostasis	Liu et al., 2023; Sun et al., 2023
Mn_3O_4 NPs	Enhance the endogenous antioxidant defense ability	Lu et al., 2020
Cerium oxide nanoparticles	ROS scavenge is to catalyze ROS decomposition by utilizing the oxygen vacancy in their lattice structure	Giraldo et al., 2014
Poly(acrylic acid) nanoceria (PNC)	Scavenge of ROS and protect plant photosynthesis	Wu et al., 2017
Poly(acrylic acid) nanoceria (PNC)	Maintain cytosolic K^+/Na^+	Liu et al., 2021
Poly(acrylic acid) nanoceria (PNC)	Maintain plant cell K^+ retention	Wu et al., 2018
CeO_2 nanoparticles	Alleviate membrane oxidative damage	Li et al., 2022
Se-NPs	Enhance the endogenous antioxidant defense ability	Djanaguiraman et al., 2018b
Nano-selenium	Enhance the antioxidant enzyme activity	Omar et al., 2023
Ag NPs	Improving morphological growth.	Iqbal et al., 2019
Si NPs	Scavenge of ROS, increases the antioxidant enzyme activity	Boora et al., 2023
Salvia Miltiorrhiza-Derived the synthesized Carbon Dots	scavenge of ROS	Li et al., 2021
Zn NPs	Improve the activity of antioxidant enzymes and drought resistance of wheat	Yang et al., 2018
TiO_2 NPs	Increase wheat yields	Jaberzadeh et al., 2013
ZnO-NPs	Mitigating the effects of drought stress on sorghum	Dimkpa et al., 2019

2012). Groups and ions in nanomaterials are likely to have a decisive influence on the interaction between nanomaterials and plants. Pectin was reported as an key factor for nanomaterials access cell wall (Zhu et al., 2023).

As mentioned above, nanomaterials with a positive potential may have better delivery efficiency, but bio-functions can be quite the opposite. Nanoceria is considered to have a good ROS-scavenging ability but with uncertain bio-functions. Wu et al. (2017) studied different potential ceria bio-functions and indicated that negative potential ceria had the best performance in *Arabidopsis*. This means that the design of nanomaterials also needs to consider their application.

The effects of nanomaterials on plants have been reported with both negative and positive implications. With the development of nanotechnology research interest, too many nanoparticles are emitted into the environment and have imposed a negative impact on plants (Hossain et al., 2015). The way NPs are used need further understanding and determination. Existing application methods, such as foliar sprays and root applications, result in a significant release of nanomaterials to the environment. For food production, a large amount of NPs is absorbed by arable land (Prakash et al., 2021). It is reported that the soil microbial community's diversity and composition have been altered upon exposure to 100 mg/kg tungsten disulfides nanomaterials (Shi et al., 2022). Another study quantified the movement of NPs through the food chain, starting with NPs adhering to algae, then transferring to Daphnia, and ultimately ending up in fish. The study revealed that NPs were present in fish tissues, with the highest levels found in the brain (Abdolahpur Monikh et al., 2021). It is an important question to study how NPs are applied and how their concentrations affect plants and the environment to create a balance. When NPs are constantly being discharged, concerns about the potential accumulation of nanomaterials in edible parts of crops and subsequent accumulation in the food chain becomes a huge concern, which can pose risks to human health.

Achieving precise delivery of nanomaterials to plants and reducing the impact on the environment is the way forward. Moreover it is essential to establish thorough regulatory frameworks for the utilization of nanomaterials in agriculture and the environment. Creating regulatory guidelines and standardized procedures can help mitigate potential risks and ensure the safe use of nanotechnology for environmental sustainability.

6 Conclusion and prospects

The aim of the research and development of plant nanobitechology is to improve plant tolerance to stress and improve crop yield and quality. Murali et al. reviewed the uptake, transport, and bioaccumulation of NMs in plants, as their bioaccumulation has an impact on the food chain via their transportation and accumulation in the edible tissues of plants (Murali et al., 2022). While NMs have the potential to improve agricultural sustainability in crop production, their widespread manufacture and release into the environment are a growing concern. It is necessary to further explore the mechanism by which plant resistance is improved through plant nanobiology and to develop NMs whose production cost is affordable and on par with agricultural products. In addition, it is important that more environmentally friendly NMs are explored to determine the best NMs for application in different crops. The promotion of NMs from the experimental stage to real production applications is also a key issue.

Author contributions

LB: Conceptualization, Writing – original draft, Writing – review & editing. JL: Validation, Writing – review & editing. TM: Methodology, Writing – review & editing. LZ: Software, Writing – review & editing. DW: Conceptualization, Writing – review & editing. YZ: Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Plants' molecular behavior to heavy metals: from criticality to toxicity

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The contamination of soil and water with high levels of heavy metals (HMs) has emerged as a significant obstacle to agricultural productivity and overall crop quality. Certain HMs, although serving as essential micronutrients, are required in smaller quantities for plant growth. However, when present in higher concentrations, they become very toxic. Several studies have shown that to balance out the harmful effects of HMs, complex systems are needed at the molecular, physiological, biochemical, cellular, tissue, and whole plant levels. This could lead to more crops being grown. Our review focused on HMs' resources, occurrences, and agricultural implications. This review will also look at how plants react to HMs and how they affect seed performance as well as the benefits that HMs provide for plants. Furthermore, the review examines HMs' transport genes in plants and their molecular, biochemical, and metabolic responses to HMs. We have also examined the obstacles and potential for HMs in plants and their management strategies.

KEYWORDS

agricultural productivity, cross-tolerance, genotoxicity, hormesis, molecular responses, transport genes

1 Introduction

Plants, similar to other living species, are vulnerable to high levels of heavy metals (HMs) in the atmosphere, resulting from both human activities and environmental factors (El-Sappah and Rather, 2022; Li et al., 2023). The poisoning of the environment with HMs is mostly caused by intensive mining operations, fast industrialization, and widespread agricultural activities (Adnan et al., 2024). The presence of high levels of HMs in soil and water is a notable illustration of human activities that have a substantial impact on the environment and constitute a considerable hazard (El-Sappah et al., 2021a; Hama Aziz et al., 2023). HMs may be transferred over long distances in both gaseous and particle

forms, leading to their rapid buildup in biological systems, water, and sediment (Mishra et al., 2017).

A total of 53 elements have been classified as HMs based on their density, which exceeds 5 g/cm^3 (Ali and Khan, 2018). For the essential metabolic operations of plant cells, a total of 17 elements are required. However, only six of these elements are classified as HMs: copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), molybdenum (Mo), and nickel (Ni). The macroelements are carbon (C), oxygen (O), hydrogen (H), magnesium (Mg), sulfur (S), nitrogen (N), calcium (Ca), phosphorus (P), and potassium (K), while the microelements are Cu, Zn, Mn, Mo, boron (B), and chlorine (Cl) (Fan et al., 2021). In plants, the macro- and microelements are essential for the regulation of numerous physiological and biochemical processes, such as chlorophyll formation, photosynthesis, nucleic acid metabolism, protein modification, intra-compartmental redox reactions, carbohydrate metabolism, and N fixation (Dutta et al., 2018; Zayed et al., 2023).

It is intriguing that while numerous HMs function as microelements, others, such as aluminum (Al), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg), have detrimental effects on plants. These consequences include impaired photosynthesis, chlorosis, decreased biomass output, disrupted water balance, and impaired nutrient absorption (Angulo-Bejarano et al., 2021). The unpreceded use of agrochemicals, long-term application of municipal sewage effluent, industrial waste disposal, waste incineration, and vehicle exhausts are the primary sources of HMs in agricultural soils (Mishra et al., 2017).

Plants ingest and accumulate HMs in soil with high concentrations, which subsequently reach human nutrition through the food chain (Angon et al., 2024). The absorption of HMs by both underground and above-ground surfaces of plants can have a direct or indirect impact on plant health (Emamverdian et al., 2015). The inhibition of cytoplasmic enzymes and the injury to cell structures are the direct consequences of oxidative stress (Jadia and Fulekar, 2009). An often observed result of HM toxicity is the overabundance of reactive oxygen species (ROS) and methylglyoxal (MG), both of which can lead to lipid peroxidation, protein oxidation, enzyme deactivation, DNA damage, disruption of ionic balance in plant cells, and/or interaction with other essential components of plant cells (Hossain et al., 2012; Jomova et al., 2023).

Some HMs indirectly impose oxidative stress through a variety of mechanisms, including the depletion of glutathione, the binding of sulfhydryl groups of proteins (Jaishankar et al., 2014), the inhibition of antioxidative enzymes, or the induction of ROS-producing enzymes such as NADPH oxidases (Bielen et al., 2013). Regardless of whether it is direct or indirect, plants that are exposed to high levels of HMs experience a reduction or even the complete cessation of all metabolic activities (Singh et al., 2015).

Plant cells react to the toxicity caused by HMs via complex and interrelated systems that operate at many levels and include both immediate and long-lasting processes (Dutta et al., 2018). The immediate or short-term reactions include the rapid modification of the rates at which hundreds or even thousands of genes are transcribed, followed by alterations in physiological and metabolic processes (Dutta et al., 2018). On the other hand, genetic alterations and epigenetic modifications are associated with enduring reactions (Ryu et al., 2015). The control of gene expression, which is an essential part of the plant's response to stress, usually involves making changes to the levels of stress-responsive genes in a way that is both common to all plants and specific to each individual plant (Gallo-Franco et al., 2020).

Therefore, it is logical to expect that plants will react to HMs' toxicity, which causes both oxidative and genotoxic effects, by organizing and combining different elements of stress perception and signaling networks, with the possibility of communication at different stages, depending on the circumstances (Dutta et al., 2018). The environmental, ecological, and genetic effects of HMs on plants, as well as their resources, occurrence, and agroecological ramifications, were discussed in the current review. The challenges and prospects of HMs' impacts on plants, as well as the methods for mitigating them, have also been the subject of discussion.

2 HMs in plants: resources, occurrence, and agroecological ramifications

The presence of HMs in soil may have negative effects on human and animal health as well as on soil quality, fertility, and agricultural productivity (Rashid et al., 2023). Given the fast-paced changes in the economy and culture, many hazardous materials found in polluted soil constitute a risk to both the general people and the environment (Hajam et al., 2023). Cd, Hg, Cu, Zn, Ni, Pb, Cr, and arsenic (As) are often detected as contaminants in soil settings (Priya et al., 2023). This kind of pollution poses a significant biological risk, is widely spread, and is prevalent in the soil environment (Adnan et al., 2024). The concentration of hazardous materials in the soil is beyond the acceptable threshold in five million areas worldwide (Rodríguez Eugenio et al., 2018).

According to the Environmental Protection Agency (EPA) (Goyer et al., 2004), Hg, Pb, Cd, and As are the most dangerous metals/metalloids in the environment. Human activities, such as the use of fertilizers in agriculture, the manufacturing of compounds, and the extraction of minerals, are the main causes of the creation of hazardous substances in soil (Tang et al., 2019). Multiple studies have shown that natural sources of HMs in the environment are often of lesser importance when compared to human activities (Dixit et al., 2015). There are two main origins of HMs: natural and anthropogenic (Angon et al., 2024). The HMs are mostly derived from volcanic and sedimentary minerals, making them the most abundant natural sources (Alengebawy et al., 2021).

The main origin of HMs in soils is the parent material from which they were first generated (Angon et al., 2024). Sedimentary rocks make up around 5% of the Earth's mantle, whereas igneous elements make up 95% (Sarwar et al., 2017). On the other hand, the phrase "anthropogenic" usually refers to sources that are created by humans. Anthropogenic activities, such as burning fossil fuels for electricity, disposing of municipal waste, applying fertilizer, using pesticides, and irrigating with effluent, increase the levels of HMs in agricultural soil settings (Angon et al., 2024).

Effective soil management is a crucial aspect of sustainable agriculture, with soil biology playing a vital role in this context (Srivastava et al., 2017). Soil microorganisms are essential components of the ecosystem (Jiang and Li, 2020). Microorganisms play a crucial role in maintaining soil fertility by breaking down organic matter and cycling nutrients (Wu et al., 2024). However, stressors such as excessive temperature, pH, salinity, and chemical pollution might have a negative impact on them (Paz-Ferreiro and Fu, 2016). As the quantity of HMs grows, the capacity of microorganisms to survive declines (Igiri et al., 2018).

The addition of Pb–Cu slurry, Pb–Cu dust, Pb–Zn dust, and Cd–Pb–Zn to forest soil resulted in a reduction in the number of colony-forming units (CFUs) of bacteria and fungi (Srivastava et al., 2017). Generally, the impact of low levels of HMs on soil respiration is minimal (Verma et al., 2010). However, when HMs' pollution or toxicity intensifies, this effect becomes less significant. The introduction of HMs may either enhance or hinder N-mineralization, which can be related to differences in the experimental approach, variances in soil parameters, and substrate concentrations (Dai et al., 2004). HMs' pollution generally has a negative impact on N transformation processes, which, in turn, affects N-mineralization (Dai et al., 2004; Hamsa et al., 2017). The bioavailability of metals in soils is influenced by factors such as metal concentrations, soil pH, organic matter, and sediment content (Rieuwerts et al., 1998). HMs play a crucial role in controlling the activities of various soil enzymes, such as arylsulfatase, alkaline phosphatase, b-glucosidase, cellulase, dehydrogenase, invertase, protease, and urease (Aponte et al., 2020). Figure 1 depicts the many sources of HMs (Angon et al., 2024).

3 Plant response to HM exposure

The persistence of toxic HMs in the soil ecosystem is a considerable hazard for living animals and plants (Kraemer, 2009; Abd Elnabi et al., 2023). The plant roots serve as the main points of contact for terrestrial plants to be exposed to harmful HMs (Podar and Maathuis, 2022). Plants have developed new, adaptive, and precise methods to withstand the harmful effects of HM stress (Tiwari and Lata, 2018). This mechanism involves various strategies such as immobilization, exclusion outside the plasma membrane, restriction of absorption and transport, synthesis of specific HM transporters, induction of stress proteins, and chelation and sequestration by specific ligands (Clemens, 2001; DalCorso et al., 2008; Adrees et al., 2015).

To maintain a low concentration of metal ions in the cytoplasm, it is feasible to block the transport of hazardous metals across the plasma membrane, which is the cellular mechanism for HMs' tolerance (Hall, 2002). Here are two direct approaches. The objective may be achieved by either augmenting the attachment of metal ions to the cell wall or expelling the metal from the cell using active efflux pumps. Another approach to detoxification includes the process of chelation or modifying the concentration of harmful metal ions to a lower level, thereby rendering them inactive (Tong et al., 2004). Several factors, such as plant structure, plant life cycle, plant vigor, soil pH, root system depth, temperature, partial oxygen pressure, carbohydrate level, respiration rate, nutrient interface, and microbial presence, have a significant impact on the accumulation of metals in plants (Chen et al., 2006).

Plants have the ability to cause HMs to form negatively charged particles by changing the pH of the soil around their roots or by releasing negatively charged ions such as PO_4^{3-}. During the process of adsorption, the surface of the root has the ability to bind a substantial amount of HMs. The accumulation of these HMs [Cd, Ni, strontium (sr), and Pb] in plant root tissues happens quickly (Hossain et al., 2012). The plants have been categorized into three categories based on their survival strategies under adverse conditions: accumulators, excluders, and indicators (Baker, 1981). Plants undergo hyperaccumulation of HMs, resulting in the accumulation of metals exceeding 0.1%–1% of the dry weight. The term "hyperaccumulator" was used by Baker and Brooks (1989) to refer to plants that have a leaf nickel concentration above 1,000 mg/g. Hyperaccumulator species refer to plants that have the ability to collect more than 100 mg of Cd per kilogram or more than 500 mg of Cr per kilogram in dried plant leaf tissue (Kumar et al., 2019). A plant with a hyperaccumulator trait is capable of accumulating and enduring significant levels of metal pollution.

Some plant species have the ability to flourish in soil that is polluted with HMs and may collect substantial levels of metals in such soil (Lombi et al., 2002). The primary methods involved in the hyperaccumulation of toxic metals in plants include bio-activation of HMs in the rhizosphere through root microbe interfaces, enhanced activity of metal conveyor proteins in cell membranes, detoxification of metals by restricting them to apoplasts, chelation of HMs in the cytoplasm by multiple ligands, and sequestration of metals into the vacuole by multiple ligands (Kumar et al., 2019).

4 The performance of seeds and seedlings under HM stress

While the seed coat initially offers some defense against metal stress before germination, it will gradually rupture or become more porous throughout the germination process (Kranner and Colville, 2011). Current data suggests that metals have two distinct impacts on seed germination: their overall toxicity and their ability to hinder the uptake of water (Osman and Fadhlallah, 2023). Pb significantly affects the physical and biological characteristics of seeds, hindering their ability to sprout, the roots to grow, the seedlings to develop, the plants to grow, water to be transported, chlorophyll to be produced, and protein to be synthesized (Collin et al., 2022).

Pb also hampers the production of ATP, causes the oxidation of lipids, and leads to DNA damage, culminating in an accumulation of ROS (Pourrut et al., 2011; Ur Rahman et al., 2024). Soils contaminated with Pb hinder the growth of seedlings by causing an increase in lipid peroxidation and the activation of enzymes such as superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase,

FIGURE 1
Various origins of heavy metals. Coal combustion, mining, refining, soil erosion, and volcanic eruptions are all sources of antimony. Sources of arsenic include smelting, mining, atmospheric deposition, pesticides, and geological sedimentation. Volcanic dust and coal and hydrocarbon combustion are sources of beryllium as well. Sources of cadmium include plastic, fertilizer, pesticides, refining, and welding. Sources of chromium include textiles, dyeing, electroplating, paint manufacturing, steel fabrication, and tanning. Copper is obtained through mining, refining, painting, plating, and printing. Coal combustion, electroplating, battery manufacturing, mining, paint, and pigments are all lead sources. Batteries, coal combustion, geothermal activities, mining, paint and paper industries, volcanic eruptions, and geological weathering are all sources of mercury. Sources of nickel include porcelain enameling, electroplating, non-ferrous metals, and pigments. Sources of selenium include coal combustion and mining. Sources of silver include the production of batteries, mining, photographic processing, and smelting. Production of cement, combustion of fossil fuels, metal smelting, and hydrocarbon refining are all sources of thallium. Brass manufacturing, mining, hydrocarbon refining, and plumbing are all sources of zinc. This figure was made using BioRender.

and glutathione (GSH)–ascorbate cycle enzymes (Sethy and Ghosh, 2013).

Cd is known for its capacity to inhibit seed germination via several methods. It has a negative impact on metabolic reactivation by decreasing the number of hydrolyzing enzymes, hindering starch mobilization, and preventing seed imbibition. It may also influence signaling via Ca, mitogen-activated protein kinases (MAPKs), and transcription factors (TFs) as well as the levels of phytohormones such abscisic acid (ABA), auxin (AUX), gibberellic acid (GA), and ethylene (ET) (Rahoui et al., 2010; Vijayaragavan et al., 2011). Cd toxicity also induces the upregulation of glutathione peroxidase (Gpx) expression and decreases the activity of glutathione reductase (Branca et al., 2020). Additionally, Cd toxicity hinders the proper functioning of mitochondria (Genchi et al., 2020).

On the other hand, Co triggers DNA methylation in *Vicia faba* seeds (Rancelis et al., 2012) while Cu is harmful to young sunflower plants, causing oxidative stress by producing ROS and reducing catalase activity (García et al., 1999; Pena et al., 2011). Under stress conditions, the germination rate is decreased and there is a stimulation of biomass mobilization, which hinders the breakdown of starch and sucrose in reserve tissue (Sethy and Ghosh, 2013).

Cu poisoning induces oxidative stress by increasing the expression of antioxidant and stress-related proteins, hence altering metabolic processes (Liu et al., 2020). Ni is a noxious agent that impacts plant species by altering enzyme function and hindering seed germination and growth (Pandolfini et al., 2006; Sethy and Ghosh, 2013). It impacts the process of breaking down and moving food reserves in plants, resulting in decreased plant height, root length, fresh and dry weight, chlorophyll content, enzyme carbonic anhydrase activity, malondialdehyde content, electrolyte leakage, and photosynthetic pigments (Alam et al.,

2007). Ni stress has a detrimental impact on *Brassica nigra* seeds, resulting in a substantial decrease in growth, leaf water potential, pigments, and photosynthetic machinery (Yusuf et al., 2012).

5 The beneficial roles of metals in plants

Plants need six HMs, namely, Cu, Zn, Mn, Mo, Fe, and possibly Ni. Cu is a metallic element that is essential for the process of photosynthesis and is present in numerous enzyme systems (Festa and Thiele, 2011). Cu also improves the flavor and color of fruits, vegetables, and flowers by increasing the sugar content in plants (López-Vargas et al., 2018). Furthermore, Cu is essential for the respiration of plants and is involved in the production and formation of seeds (Chen et al., 2022). Zn, on the other hand, is a component of the enzymatic system and plant metabolism (Hamzah Saleem et al., 2022). It is essential for the synthesis of RNA and protein as well as the production of chlorophyll and carbohydrates (Umair Hassan et al., 2020; Costa et al., 2023). Additionally, it is involved in the production of growth hormones, which are responsible for the regulation of plant growth and stem elongation (Saboor et al., 2021).

In addition, Zn permits plants to endure frigid temperatures (Kudo et al., 2023). Mn is essential for photosynthesis and respiration (Alejandro et al., 2020). The availability of N, P, and Ca to the plant is enhanced by Mn, which facilitates their decomposition (Yang et al., 2021b). Additionally, Mn activates numerous enzyme systems, some of which are responsible for safeguarding plants from specific environmental stressors, such as drought, winter cold, salt damage, and ozone damage as well as specific soil-borne diseases and fungal leaf diseases (Alejandro et al., 2020). Additionally, it facilitates pollen tube development and pollen germination (Sawidis et al., 2021). It is also essential for the production of chlorophyll and protein (Mousavi et al., 2011).

Conversely, the plant necessitates Mo to convert nitrates into ammonia, a form that it can assimilate (Liu et al., 2022). Mo is also indispensable for specific microorganisms, such as rhizobia, which have a symbiotic relationship with legumes and contribute to the fixation of atmospheric N in legumes (Bursakov et al., 2023). Mo also facilitates the conversion of inorganic forms of phosphorus into organic forms that are able to be absorbed by the plant (Seeda et al., 2020). In contrast, Fe is critical to the plant's development and health. It is vital in metabolic activities such as DNA synthesis, energy transmission, photosynthesis, and respiration (Rai et al., 2021; Ning et al., 2023). Finally, Ni is essential for the biological fixation of atmospheric N in legumes and the metabolism of N in plants (Mendes et al., 2023). It is involved in the metabolism, iron assimilation, senescence, and disease resistance of plants (Begum et al., 2022). The beneficial effects and toxicity of critical HMs in various plants are reviewed in Figure 2.

Metal ions can cause hormetic reactions in plants (Salinitro et al., 2021). Hormesis is a biphasic reaction to diverse chemicals in living organisms that likely produce an adaptive stress response (Mattson, 2008). It is likely an adaptive stress response induced by a disturbance of homeostasis caused by low levels of biotic or abiotic stimuli (Vargas-Hernandez et al., 2017). HMs stimulate plant hormetic responses, which may impact nutrient absorption, activate particular defense processes, create ROS, activate antioxidant responses, and improve photosynthetic system efficiency, leading to increased biomass. This mechanism is thought to be an adaptive reaction to stress (Salinitro et al., 2021).

On the other hand, plants that efficiently protect themselves against one kind of stress may increase their tolerance to other types of stress (Perincherry et al., 2021). Cross-tolerance is a phenomena that highlights plants' capacity to swiftly adjust to changing environments, exhibiting their well-developed and durable defensive regulatory networks (Foyer et al., 2016). HMs may endanger herbivores, and HMs have been shown in studies to have an effect on fungi and insects, particularly aphids (Chen et al., 2020a). Air and soil pollution, especially HMs, may alter plant–insect or plant–disease connections. This pollution may be harmful, causing a hormesis effect and influencing these species' behavior and metabolism (Perincherry et al., 2021).

6 HMs–Transporters in plants

Metal ions are transported by a diverse array of transporters in organelles. Recent advancements in molecular and genetics research have found many crucial gene families that play a role in metal transport (Drew et al., 2021) (Table 1). These gene families have the potential to greatly contribute to HMs' tolerance in hyperaccumulator plants. So far, scientists have discovered many different types of metal transporter proteins in plants (Singh et al., 2015). These transporters are responsible for the safe storage of ions and the assistance of plants in recovering from the adverse effects of metal stress. These proteins have important roles in absorbing, transporting, sequestering, and storing metals in particular parts of the cell. Additionally, they have a noteworthy impact on the regulation of metal levels inside plant cells (Drew et al., 2021).

Metal transporters can be classified into six main groups: natural resistant-associated macrophage protein (NRAMP) (Li et al., 2024), Zn-regulated, Fe-regulated transporter-like proteins (ZIP) (Shuting et al., 2022), cation diffusion facilitator (CDF) transporters (Kolaj-Robin et al., 2015; El-Sappah et al., 2021b; El-Sappah et al., 2023), yellow stripe-like (YSL) proteins (Islam et al., 2020), and P1B-type HMs ATPases (HMAs) (Batool et al., 2023).

Cell organelles include specialized compartments dedicated to certain processes, including photosynthesis, respiration, phytohormone production, and metal detoxification (Jogawat et al., 2021). Among these transporters, the vacuole plays a crucial role in the accumulation and compartmentalization of metals for their detoxification. This is a significant strategy for lowering metal stress-related ailments in plants (Emamverdian et al., 2015; Sharma et al., 2016). Chloroplasts and mitochondria need transition metals to carry out essential operations such as photosynthesis, electron transport system, photoprotection, and other processes. Maintaining metal homeostasis is crucial for the optimal functioning and structural integrity of chloroplasts and mitochondria (Nouet et al., 2011).

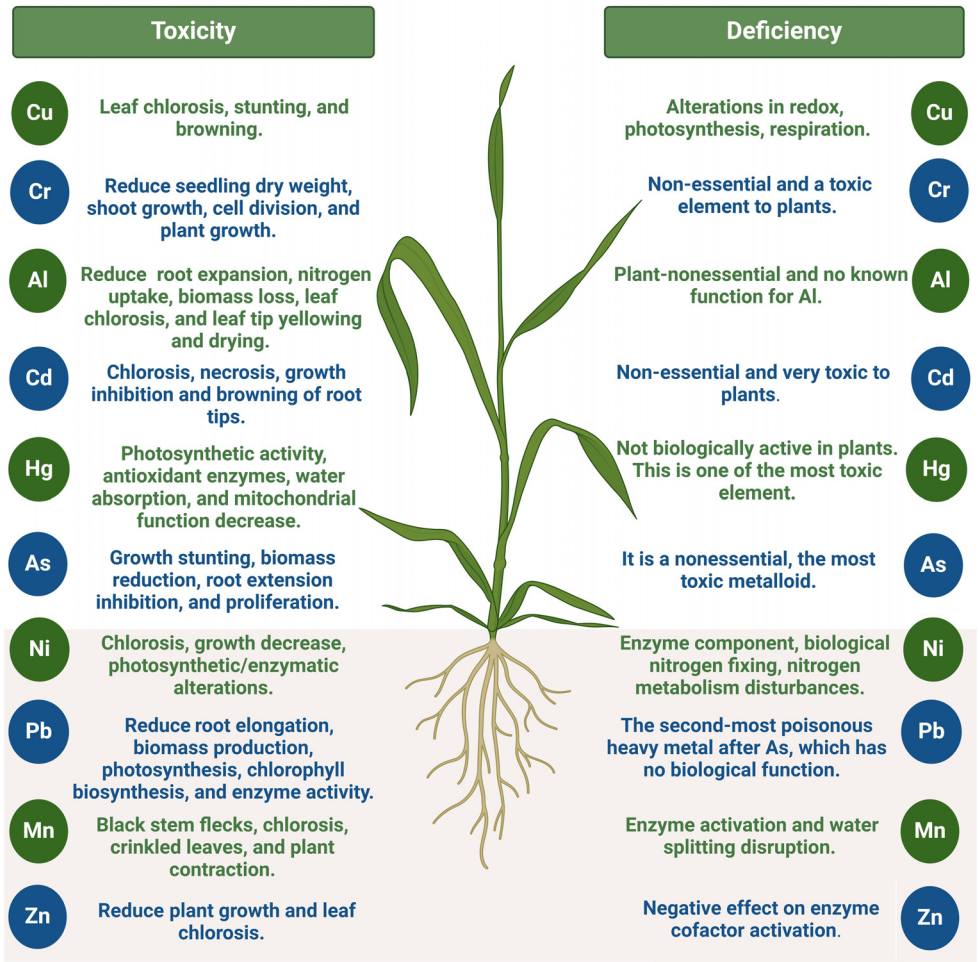

FIGURE 2
Toxic and inefficient impact of heavy metals on plants. This figure was made using BioRender.

Metal and nonmetal ions are primarily stored in vacuoles within plant cells. Additionally, the neutralization and mitigation of the detrimental effects of metal ions are contingent upon their storage in the vacuole (Hall, 2002). Tonoplasts (vacuolar membrane) contain a variety of transport proteins, such as MTPs, ABC transporters (ABCCs, ABCGs), HMAs, Ca^{2+} exchangers (CAXs), and NRAMPs. These proteins either remove metals from the cytosol or deposit them in the vacuolar area (Zhang et al., 2018a).

Chloroplasts have a crucial role in hosting transition metals due to their involvement in the process of photosynthesis and the breakdown of water molecules (Schmidt et al., 2020). Chloroplasts play a crucial role in reducing the harmful effects of metal toxicity by capturing metal ions to facilitate metal detoxification. The double membrane structure of the cellular envelope allows for the protection of its integrity and the detoxification of metals in the intermembrane gap. Various transporters located on the inner and outer membranes are necessary to maintain the homeostasis of chloroplastic metals (Nouet et al., 2011).

Mitochondria are a crucial cellular organelle occasionally referred to as the cell's powerhouse due to their substantial involvement in chemosmosis. This is because certain metals are essential for the correct functioning of mitochondria, as they serve as cofactors for critical enzymes and are also involved in the composition of electron transport molecules (Jogawat et al., 2021). Therefore, it is imperative to maintain the equilibrium of metals within the mitochondria. ATMs and mitochondrial iron transporters (MITs) are the two primary categories of transporters that modulate metal levels in mitochondria (Nouet et al., 2011).

Golgi apparatus is an essential component of the endomembrane system, which plays a crucial role in directing membrane-bound proteins (such as transporters) to either the plasma membrane or organelles (Jogawat et al., 2021). The major purpose of this is to regulate the balance of metals in the body. Metal transporters are also found in the Golgi apparatus to detect and import metals for their arrangement (Bressler et al., 2007). When exposed to metal stress, the Golgi apparatus system responds by reorganizing the endomembrane system and storing excess metals in vesicles (De Caroli et al., 2020). This process involves reducing the number of metal transporters at the plasma membrane. This substantially reduces the detrimental impacts of metals (De Caroli et al., 2020).

TABLE 1 List of gene families that play a role in metal transport.

Gene family	Genes	Plant	Roles	References
ZIP	*AtIRT1*, *AtIRT2*, and *AtIRT3*	*Arabidopsis thaliana*	*AtIRT1* is involved in the uptake of vital metals including iron, zinc, manganese, nickel, and cobalt as well as non-essential elements such as cadmium from the soil into the cells of the root epidermis. *AtIRT2* has a role in the segregation of iron and zinc into internal storage vesicles to mitigate the harmful effects of excessive metal levels. *AtIRT3* promotes the absorption of iron and zinc but does not improve the uptake of cadmium and manganese	(Grotz et al., 1998; Guerinot, 2000)
ZIP	*OsIRT1* and *OsIRT2*	*Oryza sativa*	They enhance the absorption of iron, zinc, and cadmium	(Nakanishi et al., 2006)
ZIP	*ZmIRT1*	*Zea mays*	It has a role in the transportation of iron and zinc	(Li et al., 2015)
ZIP	*NcZNT1*	*Nicotiana caerulescens*	It is a critical zinc-regulated, iron-regulated transporter-like proteins that is implicated in the hyper-accumulation and tolerance of zinc and cadmium. It is also responsible for the translocation of metals to the shoot through xylem-mediated processes	(Lin et al., 2016)
ZIP	*AtZIP4*	*Arabidopsis thaliana*	It is accountable for the translocation of metals to the shoot through xylem-mediated mechanisms	(Lin et al., 2014, Lin et al., 2016)
ZIP	*IRT3, ZIP3, ZIP6, ZIP9,* and *ZIP12*	*Arabidopsis halleri*	They ensure the homeostasis of heavy metals in the entirety of plants	(Chiang et al., 2006)
CDF	*ZAT1* (*MTP1*)	*Arabidopsis thaliana*	The primary expression of ZAT is found throughout the plant, and its expression is augmented by an increase in zinc concentration	(van der Zaal et al., 1999)
CDF	*AtMTP1* and *AtMTP3*	*Arabidopsis thaliana*	They are accountable for the vacuolar transport of zinc	(Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006)
CDF	*AhMTP1-A1* and *AhMTP1-A2*	*Arabidopsis halleri*	Under conditions of excessive zinc stress, they were upregulated in the roots	(Dräger et al., 2004; Shahzad et al., 2010)
CDF	*AhMTP1-B1*	*Arabidopsis halleri*	It manifested in roots when zinc deficiency was present	(Shahzad et al., 2010)
CDF	*AhMTP1-C* and *AhMTP1-D*	*Arabidopsis halleri*	Under conditions of excessive zinc stress, they were downregulated in the shoot and roots	(Shahzad et al., 2010)
CDF	*AtMTP11*	*Arabidopsis thaliana*	It is upregulated in the presence of a zinc deficiency	(Delhaize et al., 2007; Peiter et al., 2007)
CDF	*NtMTP1-A* and *NtMTP1-B*	*Nicotiana tabacum*	They upregulate in response to deficiencies in zinc and copper	(Shingu et al., 2005; Ricachenevsky et al., 2013)
CDF	*OsMTP1*	*Oryza sativa*	It is upregulated in the shoot and root in the presence of an excess of zinc, cobalt, nickel, cadmium, and iron	(Yuan et al., 2012; Menguer et al., 2013)
CDF	*PtMTP11.1* and *PtMTP11.2*	*Populus trichocarpa*	They upregulate under manganese deficiency	(Peiter et al., 2007)
COPT	*COPT1*, *COPT2*, and *COPT6*	*Arabidopsis thaliana*	They are indispensable elements of the primary pathway for cellular high-affinity uptake of copper	(Sancenón et al., 2003; Yuan et al., 2011; Garcia-Molina et al., 2013)
COPT	*COPY2*	*Arabidopsis thaliana*	This is a cell surface transporter that is primarily found in all regions of plants, with a particular abundance in roots, young leaves, apical meristems, trichomes, and anthers	(Perea-García et al., 2013)
COPT	*COPT5*	*Arabidopsis thaliana*	It is thought to have a role in maintaining balance inside cells	(Garcia-Molina et al., 2011)

(Continued)

TABLE 1 Continued

Gene family	Genes	Plant	Roles	References
ABC	*AtABCC1* and *AtABCC2*	*Arabidopsis thaliana*	They have been linked via vacuolar sequestration to phytochelatin-mediated cadmium and mercury detoxification	(Park et al., 2012)
ABC	*OsABCB14*	*Oryza sativa*	It is shown to be in charge of iron homeostasis	(Xu et al., 2014)
ABC	*OsABCG43*/ PDR5	*Oryza sativa*	Induced in rice roots during cadmium stress, it may be implicated in cadmium detoxification by compartmentalizing cadmium into organelles	(Oda et al., 2011; Xu et al., 2014)

Finally, endoplasmic reticulum (ER) is also part of the endomembrane system and has a vast lumen to store and utilize metals for different purposes (De Caroli et al., 2020). Broad-specificity transporters for Cd, Cu, and Zn are identified on the ER membrane and are sometimes found to be localized on the plasma membrane. This common localization might be due to the continuum of the endomembrane system as part of the secretory pathway (Jogawat et al., 2021).

7 Molecular, biochemical, and metabolic plant responses toward HMs

7.1 Negative impact of metals on plants

Exposure to HMs causes several reactions in plants, including physiological, biochemical, and agricultural production responses (Singh et al., 2020). The toxicity of HMs is influenced by a variety of factors, such as the plant species, the concentration of the individual metal, its chemical structure, soil composition, and pH level (Abd Elnabi et al., 2023). Certain HMs, including Cu and Zn, are essential for the vegetative plant growth (Arif et al., 2016). HMs can participate in enzyme processes by forming complexes with enzymes and substrates, acting as cofactors and activators (Witkowska et al., 2021). Trace metal nutrients have a vital role in redox reactions, electron transportation, and structural functions in nucleic acid processing (Sunda, 2012).

Additionally, HMs have various effects on the functioning of the photosynthetic system at different levels of organization (Ventrella et al., 2009). HMs directly affect plants by interfering with the PS I and PS II processes and indirectly affect photosynthesis, growth, and yield (Singh et al., 2015). Additionally, certain HMs, including Cd and Hg, possess phytotoxic properties that impede metal-sensitive enzymes, resulting in growth retardation and the mortality of organisms (Alengebawy et al., 2021). HMs can be classified into two categories based on their ability to undertake redox reactions: redox-active and redox-inactive (Ercal et al., 2001). The redox reaction within cells is facilitated by redox-active transition metals, including Fe, Cu, Cr, and Co (Kostenkova et al., 2022). This process leads to the production of superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) (Collin, 2019). The oxidative stress is induced by the indirect interactions with the antioxidant defense system, disruption of the electron transport chain, and induction of lipid peroxidation, which are the results of exposure to redox-inactive HMs (Bhattacharyya et al., 2014).

In plants, HMs are involved in the production and release of free radicals through chemical reactions, metabolic pathways, and physiological processes (Emamverdian et al., 2015). The ROS are generated by biological systems through the production of radicals that are centered on oxygen, S, N, and carbon (Phaniendra et al., 2015). The lipid content of thylakoid membranes is altered in plants that are exposed to HMs stress, which leads to membrane degradation and lipid peroxidation (Emamverdian et al., 2015). The primary site of lipid peroxidation is polyunsaturated fatty acids, and it is comprised of three distinct phases: initiation, advancement, and termination. The functionality of PS II is impeded by HMs, which leads to an increase in the formation of $O_2^{\bullet-}$ in leaves and an increase in lipid peroxidation (Hasanuzzaman et al., 2020). Recent research has revealed that HMs can damage numerous physiological systems by generating ROS that induce lipid peroxidation (Shahid et al., 2014). Furthermore, the rate of photosynthesis and PS II can be significantly affected by the by-products of lipid peroxidation (Pospíšil and Yamamoto, 2017).

Chlorophylls (Chl) and carotenoids are essential pigments that are involved in the conversion of solar energy to chemical energy during photosynthesis (Hashimoto et al., 2016). The production of photosynthetic compounds is specifically influenced by HMs (Ventrella et al., 2009). Chlorosis and plant growth retardation are frequently observed in metal-contaminated environments. These findings suggest that the biosynthesis of photosynthetic compounds has been disrupted (Yadav, 2010). Consequently, these variables influence the proliferation of plastids, the efficiency of photosynthesis, and the overall metabolism. Additionally, HMs inhibit the accumulation of photosynthetic compounds (Ventrella et al., 2009).

7.2 The mechanism of HM uptake and tolerance

The development of plants as phytoremediation agents is contingent upon an understanding of the genetic basis and interrelated network of physiological and molecular mechanisms that govern plant tolerance to specific HMs (Hossain et al., 2012).

Different plant species may have developed distinct mechanisms to tolerate excessive HMs, and even within a single plant species, multiple mechanisms may be in operation (Asiminicesei et al., 2024). To endure excessive HMs, plants possess both constitutive and adaptive mechanisms (Hasanuzzaman et al., 2013). To identify the underlying mechanisms of HMs' accumulation, tolerance, and adaptive mechanisms to contend with HM stress, physiological, biochemical, and molecular approaches are still being employed (Mashabela et al., 2023).

Among the adaptive mechanisms that tolerant plants have evolved are the synthesis of particular phytochelatins (PCs) and metallothioneins (MTs), induction of mechanisms opposing the effects of ROS and MG, induction of stress proteins, the biosynthesis of proline (Pro), polyamines (PAs), and signaling molecules like salicylic acid (SA) and nitric oxide (NO) (Hossain et al., 2012; Hasanuzzaman et al., 2019). Figure 3 demonstrates the process of HMs' sequestration in plant cells, specifically within the vacuoles. HMs are absorbed by plants through root interception, entrance into roots, and translocation to the stem (Khan et al., 2023). The entrance of HMs into the organism is contingent upon the sort of HM (Yimer et al., 2024).

The prevention of superfluous HMs from infiltrating the plant is one method of reducing or preventing the toxic effects of HMs. Plants can accomplish this by precipitating or complexing HMs in the root environment (Riyazuddin et al., 2021). Plants can precipitate HMs by either increasing the pH of the rhizosphere or excreting anions, such as phosphate (Hinsinger et al., 2003; Chen et al., 2017). In response to Al stress, root exudation of phosphate has been observed in maize (Calderón-Vázquez et al., 2011). Additionally, malate exudation from the roots of sorghum and citrate exudation from the roots of maize have also been documented in response to Cd stress (Piñeros et al., 2002). These results lend credence to the hypothesis that the HM-binding capabilities of root exudates may serve as a critical mechanism for stabilizing HMs in the vicinity of the root, thereby rendering them unavailable to the plant and reducing the toxicity encountered by the plant (Hossain et al., 2012).

There must also be other processes since certain tolerant and hyperaccumulator plants really absorb more HMs than sensitive plants. An essential adaptive strategy for HM tolerance in plants is the cellular exclusion of HMs (Riyazuddin et al., 2021). The apoplastic space is the location of a significant proportion of HMs in plant roots, which implies an exclusion mechanism (Sattelmacher, 2001). The cell wall–plasma membrane interface has the potential to serve as a site of HM tolerance, as it accumulates substantial amounts of HMs (Danouche et al., 2021). The plant

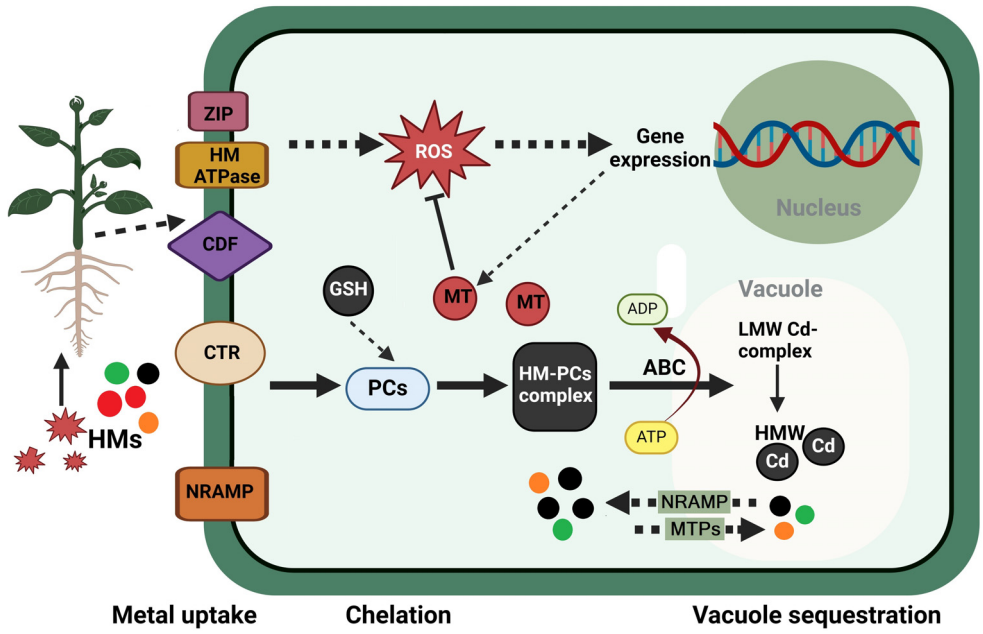

FIGURE 3
Sequestration of heavy metals in plant cells within the vacuoles. The uptake of heavy metal (HMs) ions is facilitated by a variety of transporters, such as the cation diffusion facilitator (CDF) family, the heavy-metal-transporting ATPase (HM ATPase), the copper transporter (CTR), the zinc-regulated, iron-regulated transporter-like proteins (ZIP), and the natural resistance-associated macrophage protein (NRAMP). For instance, HMs, such as Cd^{2+}, enter the cytosol and initiate the production of phytochelatins (PCs) after being transported by members of the ZIP family. PCs are produced through a transpeptidation reaction from reduced glutathione (GSH) in a non-translational manner. The primary function of PCs is to bind cytosolic HMs, which results in the formation of the HM–PC complex. In the case of Cd^{2+} ions, these bind with low-molecular-weight (LMW) complex and form the LMW Cd-complex. This complex is subsequently transported into the vacuole by a tonoplast-localized ATP-binding-cassette (ABC) transporter. The LMW Cd-complex is collected and converted into a high-molecular-weight (HMW) complex within the vacuole. This complex contains supplementary Cd^{2+} ions. The tonoplast-localized cation/proton exchanger (CAX) transporters facilitate the direct interaction between these HMW complexes and protons, thereby enabling them to access the vacuole. Metal tolerance proteins (MTPs) and NRAMPs are transporters that are present in the tonoplast. They are accountable for the migration of metal ions to facilitate compartmentalization or remobilization. Organic acids, amino acids, and metallothioneins (MTs) are among the chelators that contribute to the regulation of metal levels in the cytosol to a safe and low level. ROS, reactive oxygen species. This figure was made using BioRender.

cation exchange capacity (CECs) of sensitive wheat cultivars is substantially lower than that of tolerant cultivars (Masion and Bertsch, 1997). This implies that tolerant cultivars utilize a high CEC to complex HMs at the cell wall and obstruct their entry into the cell (Bortoloti and Baron, 2022).

Once HMs infiltrate the cell, plants employ a variety of strategies to mitigate their toxicity (Pande et al., 2022). One approach is to transport or sequester HMs into the vacuole, which serves as an appropriate storage reservoir for excessively accumulated HMs (Peng and Gong, 2014). Vacuolar assimilation of the majority of solutes is stimulated by two vacuolar proton pumps: a vacuolar proton-ATPase (V-ATPase) and a vacuolar proton pyrophosphatase (V-Ppase) (Hossain et al., 2012). Grasses have the capacity to actively transport Zn into vacuoles, with more tolerant clones being able to maintain the process at higher external Zn levels than sensitive clones (Brookes et al., 1981). Either channels or transporters can facilitate uptake. Genetic and molecular techniques have identified a variety of genes (Table 2) that are involved in the uptake of transition HM ions into cells, the sequestration of HMs in the vacuole, the remobilization of HMs from the vacuole, the loading of HMs in the xylem, and the discharge of HMs (Gill et al., 2021).

Zn-regulated transporter (ZRT), Fe-regulated transporter (IRT)-like protein ZIP family, ATP-binding cassette (ABC) transporters, the P-type metal ATPases, NRAMP family, multidrug resistance-associated proteins (MRP), CDF family of proteins, copper transporter (COPT) family proteins, pleiotropic drug resistance (PDR) transporters, YSL transporter, and CAX are among the well-characterized HM transporter proteins (Toyoda et al., 2008; Pittman and Hirschi, 2016; Wang et al., 2021a; Pacheco et al., 2023). MTs and PCs are two forms of peptide metal-binding ligands that are essential for the detoxification and tolerance of HMs in plants that are subjected to HM stress (Faizan et al., 2024).

PCs are synthesized from GSH and are induced by a variety of HMs, including Cd, Hg, Ag, Cu, Ni, Au, Pb, As, and Zn (Faizan et al., 2024). The activity of ABC transporters accumulates them in the vacuole, thereby restricting the circulation of free Cd^{2+} within the cytosol, and they complex Cd ions through the thiolic group (–SH) of cysteine (Salbitani et al., 2023). PCs are produced by both HM-resistant and HM-sensitive plants; however, certain reports have concluded that PCs are not the primary cause of the hyperaccumulation of Zn, Ni, or Pb (Hossain et al., 2012). The chelation of HM ions is not the sole mechanism of the HM detoxification process (Gulcin and Alwasel, 2022). The HM ion complex is transported to the vacuole and stabilized, thereby forming a complex with sulfides or organic acid, following the activation of PC synthase by HM ions and HM chelation by the synthesized PCs (Faizan et al., 2024). Nevertheless, the HM specificity or species specificity of hyperaccumulation is not adequately elucidated by the formation of HM complexes. Consequently, the precise function of PCs in the HM tolerance mechanism at the cellular level is yet to be ascertained (Emamverdian et al., 2015).

Plant MTs are polypeptides that are cysteine-rich, low molecular weight, and capable of engaging HMs through their cysteine residues (Freisinger, 2011). Their physiological functions encompass the protection against intracellular oxidative damage, the sequestration of toxic HMs, and the maintenance of essential transition HM homeostasis (Subramanian Vignesh and Deepe, 2017). The cysteine residue arrangement has resulted in the division of plant MTs into three classes, which are diverse (Guo et al., 2003). The organization of cysteine residues confers distinct MT isoforms and their capacity to bind and sequester distinct HM ions for the purposes of detoxification and homeostasis (Ruttkay-Nedecky et al., 2013).

Factors such as hormones, cytotoxic agents, and HMs induce MT biosynthesis, which is regulated at the transcriptional level (Thirumoorthy et al., 2007). Gene expression studies have demonstrated that MT genes are differentially regulated in response to a variety of HM stresses (Qu et al., 2024). The role of MTs in HM detoxification and homeostasis has been demonstrated by a variety of data (Ruttkay-Nedecky et al., 2013). However, the metal-inducibility of plant MTs has not always been demonstrated. Additional information regarding the structures and properties of MTs could provide a more comprehensive understanding of their functions and mechanism(s) of action (Hossain et al., 2012). The molecular mechanisms of HM transport, trafficking, tolerance, and homeostasis in plants are likely to be further elucidated through the use of a model system and a model hyperaccumulator, such as *Arabidopsis halleri* and particularly *Thlaspi* species (Pasricha et al., 2021).

In plants, metal chelation can be classified into two categories: internal tolerance and external exclusion. During the external detoxification process, organic acids expelled from plant roots may combine with HM ions to create stable HM–ligand complexes, which may change the HM ions' mobility and bioavailability (Sabreena et al., 2022). This obstructs the entry of HM ions into plants and prevents their accumulation in sensitive root sites. Organic acids may chelate with HM in the cytosol during internal HM detoxification, resulting in the transformation of the ions into a less toxic or nontoxic form (Gasic and Korban, 2006). Plants generate a variety of ligands for Al, Cd, Cu, Ni, Co, and Zn. Potential ligands for HMs include carboxylic and amino acids, including citrate, malate, and oxalate, histidine and nicotianamine, and phosphate derivatives (phytate), which are involved in detoxification and tolerance (Hossain et al., 2012). Citrate has a significant affinity for chelating HM ions, and other HMs, including Cd, Ni, Co, and Zn, also exhibit a high affinity for citrate (Gasic and Korban, 2006; Hossain et al., 2012).

At low Cd concentrations, citric acid is a significant ligand and contributes to the accumulation and tolerance of Zn (Najeeb et al., 2011). HMs such as Al are also detoxified, and oxalate is secreted by the roots (Zheng et al., 2005). In response to Al stress, buckwheat (*Fagopyrum esculentum* Moench.) secretes oxalic acid from the roots and accumulates nontoxic-Al-oxalate in the leaves (Feng Ma et al., 1998). Consequently, detoxification occurs both internally and externally (Hall, 2002). Histidine and nicotianamine are also involved in the chelation of HM ions in the xylem fluid and within plant cells (Zhakypbek et al., 2024). Nicotianamine is a nonproteinogenic amino acid that is mobile within the plant and has been identified in phloem fluid as well as in root and leaf cells (Klatte et al., 2009). It is suggested that it may be involved in the regulation of HM transfer within plant cells (Takahashi et al., 2003).

TABLE 2 Compilation of genes exhibiting variable expression in response to various heavy metals.

Plant	Gene	Metal	Response	References
Vigna radiata	*irt1*and *irt2*	Cadmium	Treatments involving cadmium under conditions of sufficient iron	(Muneer et al., 2014)
Arabidopsis thaliana	*AtABCC3* and *AtABCC6*	Cadmium	The phenomenon of tolerance during seedling development mediated by phytochelatin	(Brunetti et al., 2015)
Arabidopsis thaliana	*CAX2* and *CAX4*	Cadmium	It plays a role in the storage of cadmium in the vacuoles, which gives it the ability to tolerate heavy metals	(Koren'Kov et al., 2007; Mei et al., 2009)
Arabidopsis thaliana	*AtHMA4*	Cadmium	The expression of the gene decreased when exposed to cadmium stress	(Xu et al., 2010)
Arabidopsis thaliana	*AtNHX1*	Cadmium	It is accountable for the storage of metabolites in vacuoles and enhances tolerance	(Yao et al., 2020; Riyazuddin et al., 2021)
Oryza sativa	*cadA* and *bmtA*	Cadmium	The accumulation of cadmium and the production of cadmium-nanoparticles have been found to enhance tolerance by reducing oxidative stress	(Shi et al., 2020)
Nicotiana tabacum	*TaMT3*	Cadmium	It resulted in an elevation of superoxide dismutase activity and provided tolerance	(Zhou et al., 2014)
Hibiscus cannabinus L.	*WRKY*, *GRAS*, *MYB*, *bHLH*, *ZFP*, *ERF*, and *NAC*	Cadmium	The molecular mechanism underlying enhanced tolerance	(Chen et al., 2020b)
Fragaria vesca	*FvABCC11*	Cadmium	Enhancement of tolerance by the utilization of ATP binding cassette (ABC) transporters	(Shi et al., 2020)
Brassica napus	*BnaABCC3* and *BnaABCC4*	Cadmium	Augmentation of stress tolerance	(Zhang et al., 2018b)
Triticum aestivum	*TaABCC*	Cadmium	Unique molecular manifestation and heightened resilience	(Bhati et al., 2015)
Oryza sativa	*OsHMA3* and *OsABCC9*	Cadmium	Participating in the study of plant cadmium tolerance	(Sasaki et al., 2014; Yang et al., 2021a)
Oryza sativa	*OsMYB45*, *OsCATA* and *OsCATC*	Cadmium	*OsMYB45* induces upregulation of *OsCATA* and *OsCATC* receptors, enhances catalase activity in plants, and reduces rice's susceptibility to cadmium	(Hu et al., 2017)
Populus alba	*PyWRKY75*	Cadmium	The upregulation of *PyWRKY75* resulted in enhanced tolerance to cadmium	(Wu et al., 2022)
Oryza sativa	*OsNAC15*,*OsZIP7* and *OsZIP10*	Cadmium	The regulation of zinc and cadmium tolerance is mediated by *OsNAC15* by its interaction with the ZDRE motif located in the promoters of *OsZIP7* and *OsZIP10*, resulting in the inhibition of their transcription	(Zhan et al., 2022)
Arabidopsis thaliana	*MAN3*	Cadmium	The glutathione-dependent pathway is responsible for the regulation of cadmium tolerance	(Chen et al., 2015)
Arabidopsis thaliana	*AtFC1*	Cadmium	The control of both antioxidants and antioxidant enzymes is attributed to several factors	(Hossain et al., 2012)
Arabidopsis thaliana	*MYB40* and *PCS1*	Arsenic	The expression of *PHT1;1* was directly suppressed to decrease the absorption of As(V) into plant cells, while the expression of *PCS1* was directly increased to increase the amount of PCs, which formed complexes with As(III). The expression of *PHT1;1* was suppressed, while the expression of *PCS1* was upregulated	(Sung et al., 2009; Castrillo et al., 2013)
Triticum aestivum	*TaCATs*	Arsenic	Stress tolerance	(Tyagi et al., 2021)
Oryza sativa	*OsABCC1*	Arsenic	Sequestration can manifest in various parts of rice plants, including roots, stems, leaves, and husks. The presence of vacuoles plays a crucial role in mitigating the distribution of arsenic within rice grains	(Song et al., 2014)
Oryza sativa	*OsLsi1* and *OsLsi2*	Arsenic	They can lead to an augmentation in the absorption of arsenate by roots	(Pan et al., 2020a)

(Continued)

TABLE 2 Continued

Plant	Gene	Metal	Response	References
Oryza sativa	*OsLsi3/OsLsi6*	Arsenic	They result in a significant rise in the buildup of arsenic in shoots during the stages of heading to milk	(Pan et al., 2020a)
Arabidopsis thaliana	*ATQ1*	Arsenic	ATQ1 deficiency results in a reduced outflow of arsenic in roots and a notable rise in arsenic accumulation in shoots	(Chao et al., 2017)
Oryza sativa	*CCoAOMT*	Copper	Increased lignin synthesis and improved tolerance	(Su et al., 2020)
Nicotiana tabacum	*EhMT1*	Copper	It results in reduced hydrogen peroxide production and enhanced tolerance	(Xia et al., 2012)
Arabidopsis thaliana	*MT2a* and *MT3*	Copper	They are significantly stimulated by copper exclusively in the root tips and young leaves	(Guo et al., 2003)
Imperata cylindrica	*CRK10*, *SDI1*, *PHO1*, *PHT1-11*, *VIT1*, *VTC2*, *PAE7*, *SWEET3*, and *REX4*	Copper	Genes that are expressed differently in shoots under situations of copper stress	(Vidal et al., 2021)
Imperata cylindrica	*Mn-SODs*, *SOD1*, *ATOX1*, *HEPHL1*, *HMA5*, *NBR1*, *ACT1*, *Act87E*, *Arp2*, and *Actobindin-A*	Copper	These genes exhibit a correlation with copper-tolerant systems in roots	(Vidal et al., 2021)
Jatropha curcas	*JcMT2a* and *JcPAL*	Lead	The process of antioxidant accumulation, such as the presence of flavonoids and phenolics, as well as metal detoxification	(Pan et al., 2020b)
Nicotiana tabacum	*tCBP4*	Lead	Increased tolerance	(Sunkar et al., 2000)
Arabidopsis thaliana	*ACBP1*	Lead	Increased gene expression and improved tolerance	(Xiao et al., 2008; Du et al., 2015)
Linum usitatissimum	*LuACBP1* and *LuACBP2*	Lead	The transcript level was elevated in the transgenic group, resulting in enhanced tolerance	(Pan et al., 2020b)
Medicago sativa	Sucrose synthase, P5CS, and δ-OAT	Lead	Lead resistance	(Wang et al., 2021b)
Medicago sativa	YUCCA, 4CL, CCR, F5H, and COMT	Lead	Their increased expression results in the growth of roots during lead-induced stress	(Wang et al., 2021b)
Medicago sativa	NRAMP, MATE, HIPPs, MTP, and ABC transporter	Lead	They were subjected to lead stress	(Wang et al., 2021b)
Pogonatherum crinitum	*CAT*, *SOD*, and *POD*	Lead	Their antioxidant enzyme activities are consistent with the evolving trend of roots	(Zhu et al., 2022)
Oryza sativa	*OsSTAR1* and *OsSTAR2*	Aluminum	Reduced concentration of aluminum in the cell wall and increased tolerance	(Huang et al., 2021)
Arabidopsis thaliana	*AtALMT1* and *STOP1*	Aluminum	The transcription factor *STOP1t* plays a crucial role in the regulation of *ALMT1* expression, which is essential for the development of aluminum tolerance	(Daspute et al., 2017)
Arabidopsis thaliana	*AtBCB*	Aluminum	It provided a certain level of resistance to aluminum	(Ezaki et al., 2000)
Nicotiana tabacum	*parB*, *NtPox* and *NtGDI1*	Aluminum	It provided a certain level of resistance to aluminum	(Ezaki et al., 2000)
Oryza sativa	*ART1*, *Nrat1*, *OsFRDL4*, *OsALS1*, *OsMGT1*, *ASR5* and *ART2*	Aluminum	They have crucial functions in the resistance to aluminum poisoning	(Bhattacharjee et al., 2023)

Plants that are HM-tolerant frequently prevent HMs from being transmitted from root to stem by either detoxifying them through chelation or storage or retaining them in root cells (Singh et al., 2015). Nevertheless, a unique group of plants known as hyperaccumulators effectively transport HMs to the shoot through the xylem, a process that is likely facilitated by transpiration. They are capable of accumulating HMs from modest external concentrations, with the majority of them being

translocated to the shoot (Yang et al., 2005). Hyperaccumulators exhibit an unusually high uptake of HM at the root membrane level, which may be attributed to the presence of a high expression of an HM transporter in the plasma membrane (Skuza et al., 2022). Efficient intracellular compartmentalization and chelation may be the cause of this high HM tolerance.

A complex network of biochemical adaptive strategies, known as the antioxidant system, is present in plants to detoxify a variety of ROS (Dumanović et al., 2020). In general, this system can be divided into two categories. The first group comprises enzymes, including superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase, that eliminate oxygen radicals and their metabolites (Rajput et al., 2021). Nonenzymatic compounds such as glutathione, ascorbate, and phenolics comprise the second group. These compounds have the ability to neutralize ROS without transforming into deleterious radicals themselves (Zandi and Schnug, 2022).

The presence of the two HMA proteins, hma2/hma4, is essential for the uptake of Cd in the shoot via the xylem (Kraemer, 2009). When cultivated on regular soil, the shoot of the hma2/hma4 double-mutant shows severe signs of Zn shortage, as previously shown (Haydon and Cobbett, 2007). This suggests that both HMA2 and HMA4 are required for the movement of Zn from the roots to the shoots in regular soils (Claus et al., 2013). On the other hand, the heat shock proteins (HSPs) are molecular chaperones that are essential for the protection and repair of proteins under stress conditions as well as for the folding and assembly of proteins (El-Sappah et al., 2017; Abbas et al., 2022). In response to Cd stress, they can enhance the accumulation of large HSPs, such as HSP70, and are induced by transition metals (Zn, Cu, Cd, Hg, Al, and Cr) (Hasan et al., 2017).

Additionally, HSPs can prevent irreversible protein denaturation as a result of oxidative stress or facilitate proteolytic degradation (Kumar et al., 2022). Nevertheless, the extent of their involvement in HM tolerance is still mainly obscure. Enzymes that modify metal oxidation states or facilitate the incorporation of HMs into organic molecules are known as metal-modifying enzymes (Kaczmarek et al., 2009). After being supplied with Cr(VI) in nutrient culture, *Eichhornia crassipes*, a water hyacinth, accumulated innocuous Cr(III) in its root and branch tissues (Giri and Patel, 2011). This implies that *E. crassipes* detoxified Cr (VI) during root assimilation and transported a portion of the detoxified Cr to leaf tissues. A reductase at the root cell membrane in dicots reduces Fe and potentially Cu prior to assimilation (Cohen et al., 1997).

Plants may reduce harmful substances (e.g., HMs) through a process called *in situ* reduction, which can be advantageous for phytoremediation by helping to detoxify the environment (Kafle et al., 2022). Metal-responsive transcription factor 1 (MTF-1) plays a crucial role in the cellular response and tolerance to HM stress by activating genes important for HM uptake, transport, and detoxification (Wang et al., 2004). The TFs involved in the reaction to HM stress and tolerance have been reported in several plant species (Niekerk et al., 2024). On the other hand, oxidative stress and antioxidative defense systems are induced by HM stress (Mansoor et al., 2023). These systems are constituted of free-radical-scavenging molecules, such as ascorbate (AsA) and GSH, and the enzymes involved in their biosynthesis and reduction (Rajput et al., 2021).

During times of stress, specifically HM stress, SA interacts with many plant hormones, including AUX, ABA, and GA, to promote the synthesis of antioxidant chemicals and enzymes. This interaction serves to notify and assist plants treated with HM, helping to alleviate the stress caused by HM (Sharma et al., 2020). SA is a natural signal molecule that is crucial for the regulation of physiological and biochemical processes, thereby enhancing the resistance of plants to biotic and abiotic stresses (Mishra et al., 2024).

On the other hand, Pro accumulation in response to HM stress has also been extensively documented (Hayat et al., 2012). Enhanced protection against Cd stress is provided by increased Pro levels in microalgae (Siripornadulsil et al., 2002). Pro plays a vital role in mitigating the harmful effects of Cd stress by protecting against damage caused by free radicals and maintaining a controlled reducing environment within the cell rather than just isolating Cd (Hayat et al., 2012). However, PAs are organic cations that exist naturally and possess nonenzymatic antioxidant characteristics. They are thought to function as second messengers in regulating plant growth and development processes (Raychaudhuri et al., 2021).

PAs and Pro are components of the "general adaptation syndrome" (GAS) response to environmental adversities, including nutrient scarcity, HMs, and low temperatures (Gill et al., 2012). Engineered plants that overexpress genes involved in the biosynthesis of PAs exhibit an enhanced ability to withstand a range of environmental stressors, including HMs (Kajla et al., 2023). By modulating the level and toxicity of ROS and hormones, NO, a ubiquitous bioactive signaling molecule, serves a critical function in a wide range of physiological processes in plants (Jomova et al., 2023). By governing the general mechanisms for cellular redox homeostasis and promoting the transformation of $O_2^{\bullet-}$ to H_2O_2 and O_2, NO safeguards plants from oxidation damage (Huang et al., 2019).

NO may also safeguard cells from oxidative processes by promoting the synthesis of GSH (Lu, 2013), in addition to its direct ROS scavenging activity and the modulation of lipid peroxidation through lipoxygenase (LOX) inhibition. Under HM stress, exogenous NO can effectively induce tomato seedlings to modify their physiological and biochemical mechanisms to protect against Cu toxicity, thereby preserving their metabolic capacity and normal growth capabilities (Singh et al., 2015).

8 Challenges and prospective

It is crucial to examine the interactions between different HMs in plants. Certain HMs may have synergistic effects, whereby their collective presence amplifies toxicity beyond what would be anticipated based on individual doses (Angon et al., 2024). Conversely, some combinations may have antagonistic effects, where one metal reduces the toxicity of another. In order to assess and control risks, it is important to acknowledge these

linkages. Subsequent research should strive to measure the combined impacts of HMs on the physiological processes, growth, and reproductive capabilities of plants. In order to do this, researchers may examine the correlations between dosage and response, the patterns of bioaccumulation, and the effects, particularly on different tissues. There has been a scarcity of research on agricultural genetic diversity and the mechanisms of plant adaptation. The sensitivity and tolerance of plants to HMs might vary depending on the species and genotypes (Emamverdian et al., 2015).

Studying the way various plant species and genotypes react to environmental difficulties might impede the progress of creating metal-tolerant agricultural cultivars. It is crucial to bridge this gap in order to comprehend the ability of plants to withstand challenges, provide guidance for agricultural activities in polluted areas, and assist in environmental remediation efforts. Furthermore, investigating the transfer of HMs to crops is crucial for the development of sustainable agriculture. Gaining insight into the mechanisms by which HMs persist and migrate throughout successive plant generations may provide valuable guidance for addressing pollution in affected areas. This knowledge can be particularly useful in developing phytoremediation systems that effectively eliminate toxins from contaminated soil.

The current scientific investigations have mostly concentrated on the detection and analysis of microplastics in soil. Nevertheless, there is an increasing curiosity in comprehending the mechanisms by which these minute plastic particles might carry HMs and influence their dispersion and accessibility. It is crucial to enhance several techniques that aid in reducing and managing the stress caused by harmful substances in plants. Using a single technique is ultimately unrealistic and inadequate for effectively restoring soil that has been polluted with HMs (Priya et al., 2023). There have been numerous methods developed to mitigate or prevent HM pollution and to reestablish vegetation in polluted soil (Priya et al., 2023).

Restoring the flora of soil that has been contaminated with HMs is a highly promising approach known as phytoremediation (Yan et al., 2020). Public acceptance has been achieved, and it offers numerous advantages over other physicochemical treatments (Mitra et al., 2022).

The most effective and cost-efficient approach is the recent emergence of the introduction of nanoparticles (NPs) into plants to increase their tolerance to HM toxicity and facilitate the cleansing of these toxic elements (Zhou et al., 2020). Genetic engineering is a valuable method for modifying plants to manifest specific characteristics, including rapid growth, high biomass output, strong tolerance and accumulation of HMs, and adaptability to a variety of climatic and geological conditions (Yan et al., 2020). As a result, it will be essential to have a thorough understanding of the processes by which plants absorb, transport, and eliminate HMs as well as the identification and analysis of a variety of molecules and signaling pathways in order to create genetically engineered plant species that are optimal for phytoremediation (Priya et al., 2023). Enhanced tolerance or accumulation of HMs in plants may be achieved by manipulating genes associated with HM absorption, translocation, sequestration, and tolerance. In addition, the bioavailability of HMs may be improved by the use of chelating compounds and microorganisms, which, in turn, facilitates their accumulation in plants (Olaniran et al., 2013). In addition, they can be employed to improve the health of the soil and to further encourage the growth and fitness of plants. Several hyperaccumulator plants have been identified, and the most direct method for phytoremediation is the use of HM hyperaccumulators (Skuza et al., 2022). However, there are certain limitations that impede the utilization of these natural hyperaccumulators in phytoremediation (Yan et al., 2020).

Due to their capacity to penetrate plants extensively, exhibit superior adsorption, and deliver targeted effects, they may be instrumental in the regulation of photosynthesis and the detoxification of ROS (Rasheed et al., 2022). Subsequently, they can substantially improve the germination, growth, and yield of plant seeds (Kornarzyński et al., 2020). NPs also facilitate plant growth by modulating the movement and distribution of both mobile and immobile forms of HMs (Zhou et al., 2020). The potential of NPs to significantly improve the remediation of metal-contaminated soils in the future is suggested by the positive results observed in the use of NPs, particularly in the enhancement of plants' resistance to HMs and the facilitation of their development.

9 Conclusion

HMs are defined as metals with densities more than 5 g cm^3. HMs account for 53 of the almost 90 elements found in nature. Plant nutrition is thought to need a grand total of 18 components. Some of its parts are thought to have positive effects. HMs in the soil can have a negative impact on human and animal health, soil quality, fertility, and agricultural productivity. Plants have developed ways to deal with HM stress, such as immobilization, exclusion outside the plasma membrane, limited absorption and transport, production of specific HM transporters, activation of stress proteins, and chelation and sequestration by specific ligands.

Metals have two distinct impacts on seed germination: their overall toxicity and their ability to hinder water uptake. Plants absorb and store HMs using concentration gradients and selective absorption. These chemicals influence enzymes, cellular metabolism, and the production of nucleic acids, proteins, and pigments for photosynthesis. Recent molecular and genetic research has identified gene families that play a role in metal transport, contributing to metal tolerance in hyperaccumulator plants. Understanding the interactions between different HMs in plants is crucial for assessing and controlling risks.

Research on agricultural genetic diversity and plant adaptation mechanisms is also essential for developing metal-tolerant agricultural cultivars and reducing the harmful impact of contaminated soil on crop growth and productivity. Finally, the current review examined the locations in plants where HMs are found. It discussed the benefits of HMs to plants, the consequences on seed performance, and the initial plant reaction to HM exposure. This review also looks at the existence of HM transport genes in

plants and how plants react to HMs on a molecular, biochemical, and metabolic level, respectively. Methods for managing HMs in plants, together with the associated challenges and opportunities, have been also examined.

Author contributions

AE-S: Conceptualization, Funding acquisition, Validation, Visualization, Writing – original draft, Writing – review & editing. YZ: Visualization, Writing – review & editing. QH: Funding acquisition, Visualization, Writing – review & editing. BC: Writing – review & editing. SS: Writing – review & editing. MA: Writing – review & editing. KY: Conceptualization, Writing – review & editing. JL: Conceptualization, Funding acquisition, Writing – review & editing. KE-T: Conceptualization, Funding acquisition, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Potassium indole-3-butyric acid affects rice's adaptability to salt stress by regulating carbon metabolism, transcription factor genes expression, and biosynthesis of secondary metabolites

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Soil salinity pollution is increasing worldwide, seriously affecting plant growth and crop production. Existing reports on how potassium indole-3-butyric acid (IBAK) regulates rice salt stress adaptation by affecting rice carbon metabolism, transcription factor (TF) genes expression, and biosynthesis of secondary metabolites still have limitations. In this study, an IBAK solution at 40 mg L^{-1} was sprayed on rice leaves at the seedling stage. The results showed that the IBAK application could promote shoot and root growth, decrease sucrose and fructose content, increase starch content, and enhance acid invertase (AI) and neutral invertase (NI) activity under salt stress, indicating altered carbon allocation. Furthermore, the expression of TF genes belonging to the ethylene responsive factor (ERF), WRKY, and basic helix-loop-helix (bHLH) families was influenced by IBAK. Many key genes (*OsSSIIc*, *OsSHM1*, and *OsPPDKB*) and metabolites (2-oxoglutaric acid, fumaric acid, and succinic acid) were upregulated in the carbon metabolism pathway. In addition, this study highlighted the role of IBAK in regulating the biosynthesis of secondary metabolites pathway, potentially contributing to rice stress adaptability. The results of this study can provide new sustainable development solutions for agricultural production.

KEYWORDS

potassium indole-3-butyric acid, rice, transcriptome, metabolome, salt

1 Introduction

Saline soils contain sufficient soluble salts to impair their productivity (Chhabra, 2004). Excessive salt accumulation in the root zone negatively affects plant growth (Pearson and Bauder, 2006). Due to the high salinity in the soil, the osmotic pressure increases, weakening plants' water absorption capacity (dos Santos et al., 2022). When the osmotic pressure of plant cells is less than that of soil solution, plants encounter difficulty absorbing water. In addition, salt stress causes plants to absorb more sodium and chloride ions (Ketehouli et al., 2019). The intracellular ion homeostasis is destroyed, resulting in ion toxicity and mineral nutrient deficiency, affecting the normal growth of plants (Guo et al., 2020). In recent years, with the increasing population pressure, attention has been paid to developing and utilizing widely distributed saline-alkali wastelands to alleviate the food crisis.

Plant growth regulators are a class of substances that have similar physiological and biological effects to plant hormones. They have been widely used in field crops (Fahad et al., 2016; Amoanimaa-Dede et al., 2022), fruit trees (Bons and Kaur, 2020), and vegetables (Rahman et al., 2015) because of their significant and efficient regulating effects, which have played a particular role in promoting agricultural production. For example, diethyl aminoethyl hexanoate prolongs dormancy of storage organs, kinetin promotes seed germination (Araújo et al., 2019), paclobutrazol promotes rooting (Li et al., 2023), and gibberellin inhibits flower formation (Yamaguchi et al., 2014). Auxin is also a widely studied plant hormone that improves plant resistance to abiotic stress. Previous studies found that exogenous application with auxins such as indole-3-acetic acid (IAA) can effectively improve several crops' salt stress resistance (Ashraf and Foolad, 2005; Ashraf, 2010; Kaya et al., 2013; Ribba et al., 2020).

Potassium indole-3-butyric acid (IBAK), whose chemical formula is $C_{12}H_{12}KNO_2$, is a water-soluble plant growth regulator. It can act on vigorous growth parts, such as roots, buds, and fruits, and shows strong cell division and growth promotion on specific treatment sites. A recent study showed that spraying an IBAK solution at 80 mg L^{-1} on rice leaves at the jointing stage can regulate K^+ and Na^+ contents, increase net photosynthetic rate, increase catalase (CAT) activity and glutathione (GSH) content, and regulate the expression of genes related to carbohydrate metabolism under salt stress, indicating that IBAK played a role in enhancing the tolerance of rice to salt stress (Zhou et al., 2023). Zhou et al. (2023) preliminary revealed IBAK had a regulatory effect on rice carbohydrate metabolism. However, the changes in key enzymes and metabolites related to carbon metabolism still need to be further explored. In addition, because one of the functions of transcription factors is to improve plant stress resistance (Liu et al., 2023); secondary metabolites participate in protective functions in response to abiotic stress (Akula and Ravishankar, 2011). We speculated that IBAK may play a role in regulating TF gene expression and biosynthesis of secondary metabolites, which are also the focus of this study.

Rice is particularly sensitive to salt stress at the seedling stage (Moradi and Ismail, 2007). Based on previous research, this study decided to reveal the regulatory role of IBAK in carbon metabolism, TF genes expression, and biosynthesis of secondary metabolites under salt stress at the seedling stage through comprehensive transcriptome, metabolome, and physiological perspectives, filling the research gap and providing a new sustainable development solution for agricultural production.

2 Materials and methods

2.1 Experimental design

This study was conducted in the outdoor greenhouse of Guangdong Ocean University in 2021. The entire process was conducted under natural light, with day/night temperatures of 30/25 ± 2°C and 60% relative humidity. The rice variety used in this study was Xiangliangyou900. The concentration of the IBAK solution was 40 mg L^{-1}, and distilled water was used as a control. First, the sterilized seeds were soaked in water for 24 h at room temperature. Subsequently, the seeds were primed for 24 h at room temperature. 57 germinated seeds were sown in flower pots of 19 cm × 14 cm × 17 cm containing 2.65 kg of brick-red soil. The physical and chemical properties of the soil were as follows: pH 7.23; available phosphorus, 4.05 mg kg^{-1}; rapidly available potassium, 48.37 mg kg^{-1}; alkali-hydrolyzale nitrogen, 37.10 mg kg^{-1}; and organic matter, 32.44 mg kg^{-1}. NaCl treatment was started on the eight day after sowing (4.82g NaCl was evenly integrated into the soil). The leaves were sprayed with IBAK solution 3 days after the salt treatment. Samples were collected 7 days after IBAK treatment. Except for the first leaf, all the remaining leaves were collected to detect carbohydrate content and enzyme activity related to carbon metabolism. The latest fully expanded leaves were collected for transcriptomic, metabolomic, and quantitative real-time PCR detection. There were three treatments in this study: CK0 (freshwater treatment), CK03 (salt treatment), and IBAK03 (IBAK, salt treatment). Three replicates were set for each treatment.

2.2 Determination of plant height, fresh weight, dry weight, leaf area, root shoot ratio, and moisture content

After harvest, the plant height, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, shoot moisture content, root moisture content, leaf area, and root shoot ratio were measured. Leaf area was obtained by YMJ-PC leaf area analysis system. Moisture content was calculated according to formula 1; root shoot ratio was calculated using formula 2.

Moisture content = (fresh weight − dry weight)/fresh weight (Formula 1)

Root shoot ratio = root dry weight/shoot dry weight (Formula 2)

2.3 Determination of carbohydrate content

The contents of soluble sugar, sucrose, fructose, and starch were determined according to the description of Meng et al. (2023).

Leaf samples (0.5 g) were ground into homogenate with an 80% ethanol solution (v/v). The mixture was then centrifuged at 4,000 rpm for 5 min at 80°C after 20 min in a water bath. The supernatant was collected and fixed to 25 mL to determine fructose, soluble sugar, and sucrose content. The sediment was used to measure starch content.

2.4 Determination of the activity of enzymes related to carbon metabolism

AI and NI activity were determined according to the description of Wang et al. (2024) and Meng et al. (2023).

2.5 Transcriptome sequencing

Shenzhen BGI Technology Co., Ltd., performed transcriptome detection work.

The secondary structure of the RNA sample was opened, and oligo(dT) magnetic beads were used to enrich the mRNA. A fragmentation reagent was added to the obtained mRNA to fragment the mRNA. The reaction system was prepared to synthesize one-strand cDNA and two-strand cDNA. The double-stranded cDNA ends were repaired, and an A base was added to the 3′ end. An adapter ligation reaction system was prepared to ligate adapters to cDNA. The PCR reaction system was prepared to amplify the product. The library was quality checked. After denaturing the PCR product into a single-stranded product, a cyclization reaction system was prepared. The single-stranded circular product was obtained, and the uncirculated linear DNA molecules were digested. Finally, sequencing was performed by combined probe-anchored polymerization technology (according to the method description provided by Shenzhen BGI Technology Co., Ltd.).

2.6 Metabolome detection and analysis

Shenzhen BGI Technology Co., Ltd., performed the metabolome detection work.

In this project, untargeted metabolomics analysis was performed using liquid chromatography with tandem mass spectrometry (LC-MS/MS), and high-resolution mass spectrometer Q Exactive (Thermo Fisher Scientific, USA) was used for data acquisition in positive-ion and negative-ion mode, respectively, to improve the metabolite coverage. LC-MS/MS data processing by Compound Discoverer 3.1 (Thermo Fisher Scientific, USA) mainly included peak extraction, peak alignment, and compound identification. The BGI's own metabolomics software package metaX and metabolome information analysis process was used for data preprocessing, statistical analysis, metabolite classification, and functional annotation. The dimensionality of the original data of multiple variables was reduced by principle component analysis so as to analyze the grouping, trend (similarities and differences within and between sample groups), and outliers (whether there were abnormal samples) of the observed variables in the data set. The project used variable importance in projection (VIP) values of the first two principal components in multivariate partial least squares-discriminant analysis (PLS-DA) model, combined with fold change and Student's t-test of univariate analysis to choose differentially expressed metabolites. Differential metabolites screening criteria: (1) the VIP values of the first two PCs of the PLS-DA model ≥1; (2) fold change ≥1.2 or ≤0.83; and (3) p-value <0.05.

2.7 Quantitative real-time PCR

Quantitative real-time PCR experiment was performed by Sangon Biotech (Shanghai) Co., Ltd. The primers used for quantitative real-time PCR analysis were listed in Supplementary Table S1.

3 Results

3.1 Effects of IBAK on morphology, fresh weight, dry weight, root shoot ratio, and moisture content of rice seedlings under salt stress

As shown in Figure 1, salt stress significantly inhibited rice growth; compared with CK0, the plant height, fresh weight, dry weight, leaf area, and root moisture content of CK03 were significantly reduced. At the same time, this study found that the application of IBAK under salt stress had a certain alleviation effect; for example, the root fresh weight, root dry weight, and shoot dry weight of IBAK03 were significantly higher than those of CK03 by 82.81%, 41.68%, and 23.38%, respectively; other indicators were also improved to a certain extent but did not reach a significant level, such as shoot fresh weight, root shoot ratio, root moisture content, and leaf area.

In addition, salt stress significantly reduced rice root length, root surface area, and root volume and significantly increased the average root diameter. Compared to CK03, spraying IBAK under salt stress significantly increased the root length and surface area by 78.97% and 65.99%, respectively, promoting rice root growth (Figure 2).

3.2 Effect of IBAK on carbohydrate content and carbon metabolism-related enzyme activity under salt stress

As shown in Figure 3, salt stress can significantly increase the sucrose, fructose, soluble sugar, and starch contents in rice leaves. Compared with CK03, the sucrose, fructose, and soluble sugar contents of IBAK03 were significantly reduced by 13.74%, 39.23%, and 13.51%, respectively; the starch content was significantly increased by 64.13%.

As shown in Figure 4, the NI activity of CK03 was significantly improved compared with CK0. The activities of AI and NI were

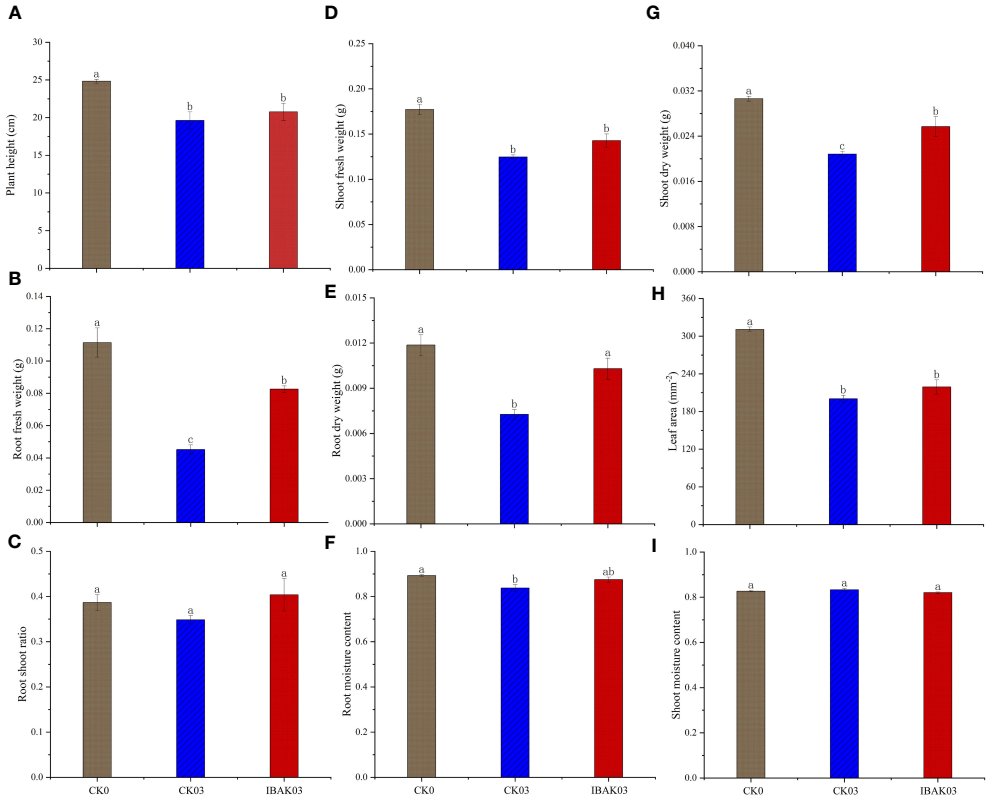

FIGURE 1
Effects of IBAK on morphology, fresh weight, dry weight, root shoot ratio, and moisture content of rice seedlings under salt stress. **(A)** plant height; **(B)** root fresh weight; **(C)** root shoot ratio; **(D)** shoot fresh weight; **(E)** root dry weight; **(F)** root moisture content; **(G)** shoot dry weight; **(H)** leaf area; **(I)** shoot moisture content.

significantly increased by 51.08% and 93.34% after using IBAK under salt stress compared with CK03.

3.3 Transcriptome

3.3.1 Quality control

This study had nine samples, each producing an average of 6.62 Gb of data (Supplementary Table S2). The clean reads Q30 was greater than 89.63% (Supplementary Table S2). Reference genome alignment showed a total mapping rate of over 82.63% and a uniquely mapping rate of over 80.89% (Supplementary Table S3). Reference gene alignment showed a total mapping rate of over 70.13% and a uniquely mapping rate of over 66.21% (Supplementary Table S4). To verify the accuracy of the transcriptome data, quantitative real-time PCR was used to detect the expression levels of several randomly selected differentially expressed genes (DEGs). The results showed that expression patterns were similar to the transcriptome data, demonstrating the reliability of the RNA-seq data (Supplementary Table S1).

3.3.2 DEGs statistics

In this study, 773 DEGs were detected in the CK03/CK0 comparison group, of which 431 were upregulated and 342 were down-regulated. In the IBAK03/CK03 comparison group, 783 DEGs were detected, of which 300 were upregulated and 483 were down-regulated (Figure 5C; Supplementary Tables S5, S6).

3.3.3 DEGs analysis

This study focused on analyzing 783 DEGs in the IBAK03/CK03 comparison group. Kyoto encyclopedia of genes and genomes (KEGG) annotation analysis found DEGs were mainly classified into transport and catabolism in Cellular Processes; signal transduction in Environmental Information Processing; folding, sorting and degradation in Genetic Information Processing; carbohydrate metabolism, global and overview maps, and amino acid metabolism in Metabolism; and environmental adaptation in Organismal Systems (Figure 5B).

At the same time, this study conducted gene ontology (GO) annotation analysis on these 783 DEGs. The abundant DEGs were classified into cellular process and metabolic process in Biological Process; cell and cell part in Cellular Component; and binding and catalytic activity in Molecular Function (Figure 6A).

KEGG enrichment analysis was performed to understand these genes' functions further. The results showed that photosynthesis - antenna proteins, nitrogen metabolism, circadian rhythm - plant, biosynthesis of amino acids, glyoxylate and dicarboxylate metabolism, carbon metabolism, and histidine metabolism were the most significant pathways enriched by DEGs; (Figure 5A).

In addition, GO enrichment analysis found that plastoglobule, chloroplast thylakoid membrane, photosystem I, photosystem II,

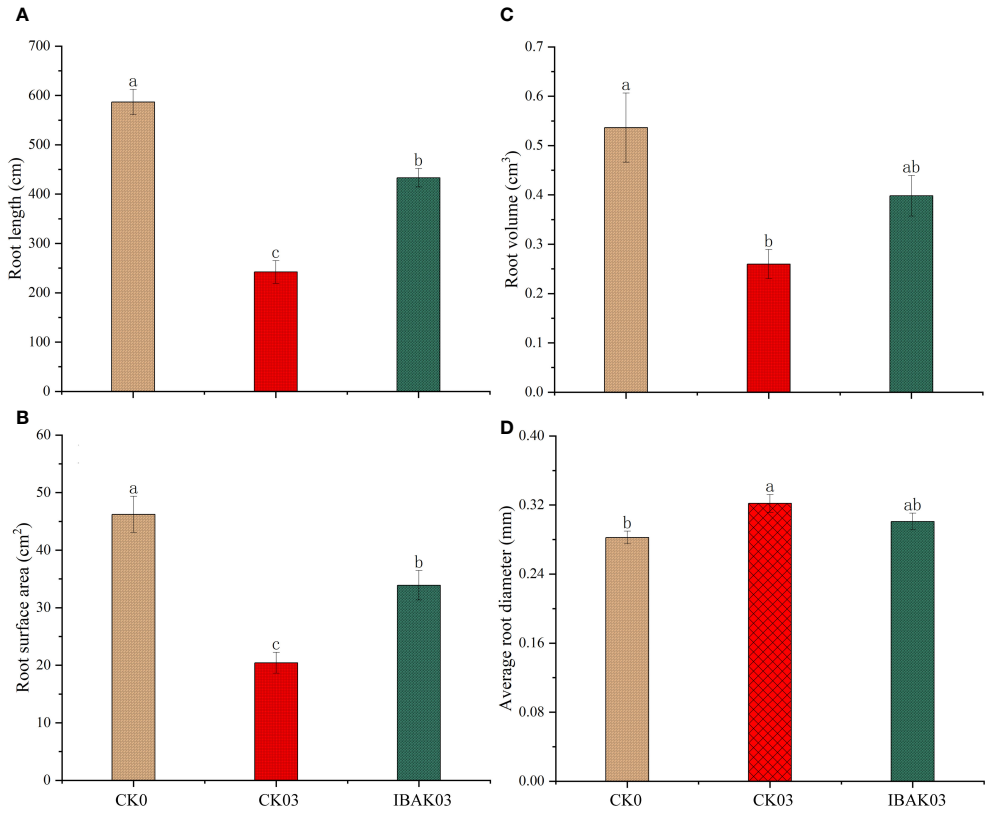

FIGURE 2
Effects of IBAK on root morphology under salt stress. **(A)** root length; **(B)** root surface area; **(C)** root volume; **(D)** average root diameter.

chloroplast envelope, plastid, thylakoid, nucleus, and nucleosome were the significantly enriched terms in Cellular Component in this study; among these significantly enriched terms, the most abundant genes were enriched in nucleus. Chlorophyll binding, nucleosomal DNA binding, glutamate synthase activity, and "3 iron, 4 sulfur cluster binding" were significantly enriched GO terms in Molecular Function. In Biological Process, a total of 19 GO terms were significantly enriched; among these significantly enriched GO terms, the most abundant gene was classified into protein ubiquitination (Figures 6B–D).

Finally, transcription factor classification was performed on the 783 DEGs detected in the IBAK03/CK03 comparison group. This study detected a total of 56 TF genes, and the abundant TF genes were classified into ERF, WRKY, and bHLH families (Figure 5D).

3.4 Metabolome

3.4.1 Principal component analysis

The principal component analysis showed that the six samples of each treatment were within the 95% confidence interval. The six samples of CK0 were basically distinguished from those of CK03; the six samples of CK03 were completely separated from those of IBAK03, indicating differences between the two treatments (Figure 7).

3.4.2 Partial least squares-discriminant analysis

PLS-DA is a statistical method for supervised discriminant analysis that best reflects the differences between taxonomic groups. The PLS-DA results showed significant differences between CK0 and CK03; similarly, CK03 and IBAK03 were completely separated. To judge the quality of the model, 200 response permutation testing were performed on the model of PLS-DA. In the positive or negative ion mode, the Q^2 of the two comparison groups were all less than 0, indicating no overfitting phenomenon, and the model was reliable (Supplementary Figures S1, S2; Supplementary Table S7).

3.4.3 Statistics and analysis of differential metabolites

In the positive ion mode, a total of 60 differential metabolites were detected in the CK03/CK0 comparison group, of which 30 were upregulated and 30 were down-regulated; in the negative ion mode, 34 differential metabolites were detected, of which 14 were upregulated, and 20 were down-regulated. For the IBAK03/CK03 comparison group, 120 differential metabolites were detected in the positive ion mode, of which 81 were upregulated, and 39 were down-regulated; 60 differential metabolites were detected in the negative ion mode, of which 31 were upregulated, and 29 were down-regulated (Supplementary Figure S3; Supplementary Table S8).

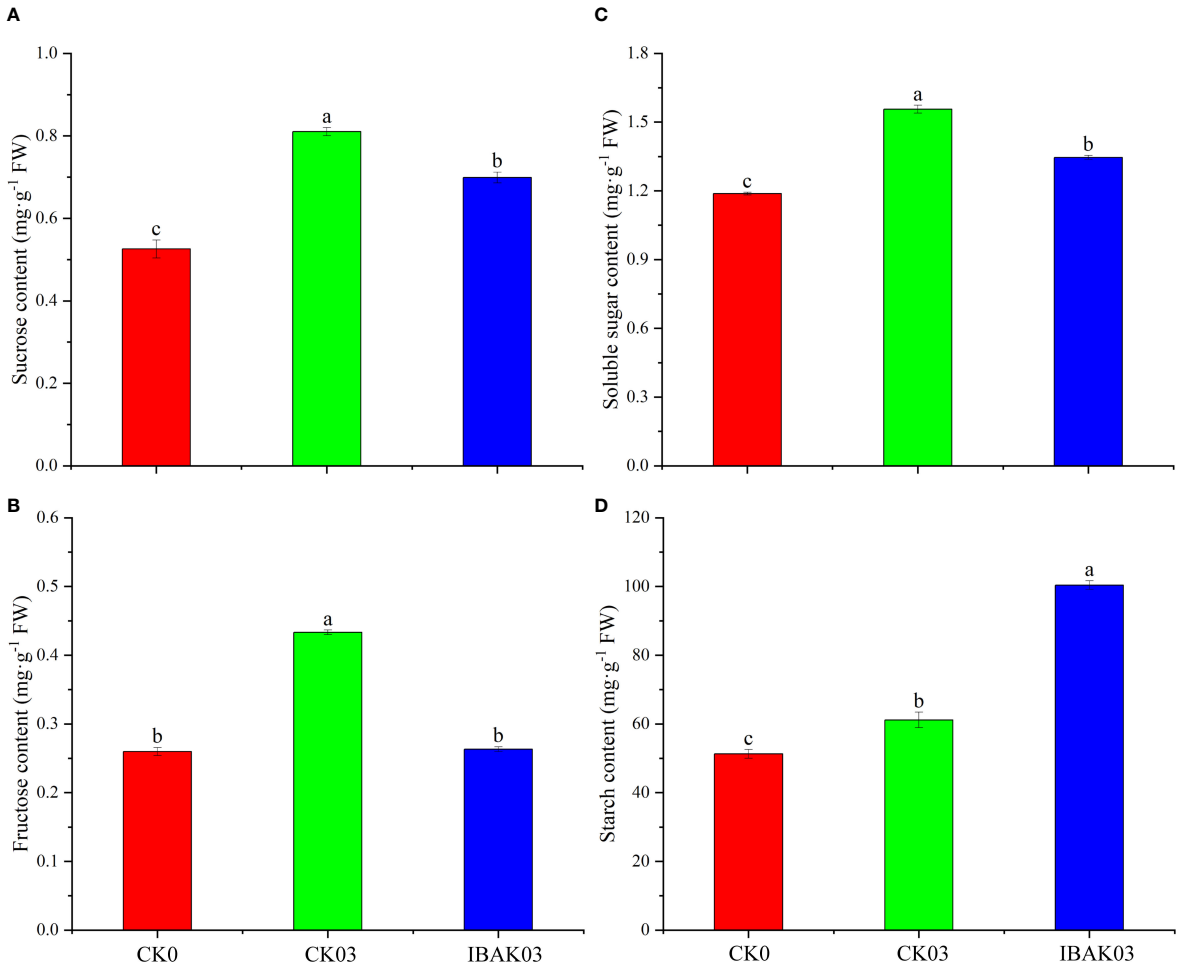

FIGURE 3
Effect of IBAK on sucrose, fructose, soluble sugar, and starch contents in rice leaves under salt stress. **(A)** sucrose content; **(B)** fructose content; **(C)** soluble sugar content; **(D)** starch content.

In order to further understand the functional properties of metabolites, this study conducted KEGG functional annotation on the differential metabolites identified in the IBAK03/CK03 comparison group. In negative ion mode, most differential metabolites were classified into global and overview maps, amino acid metabolism, biosynthesis of other secondary metabolites, metabolism of cofactors and vitamins, and carbohydrate metabolism in Metabolism. In positive ion mode, the abundant differential metabolites were classified into global and overview maps, lipid metabolism, amino acid metabolism, and biosynthesis

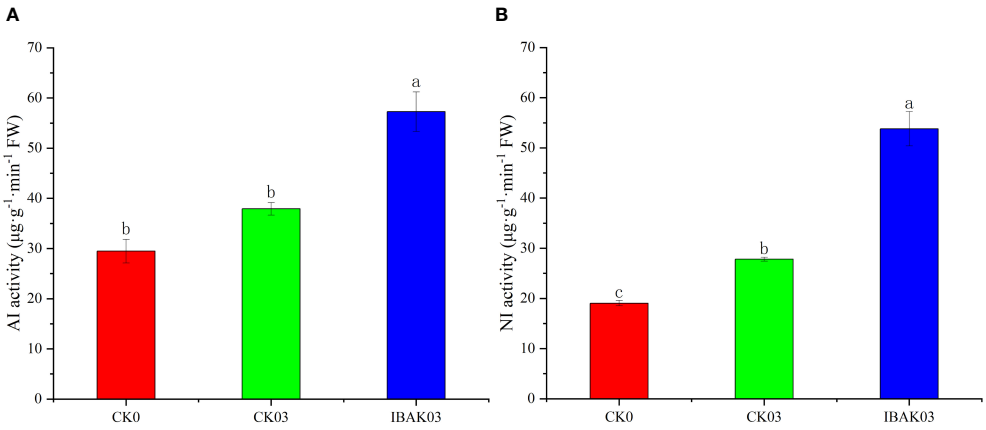

FIGURE 4
Effect of IBAK on AI and NI activity under salt stress. **(A)** acid invertase; **(B)** neutral invertase.

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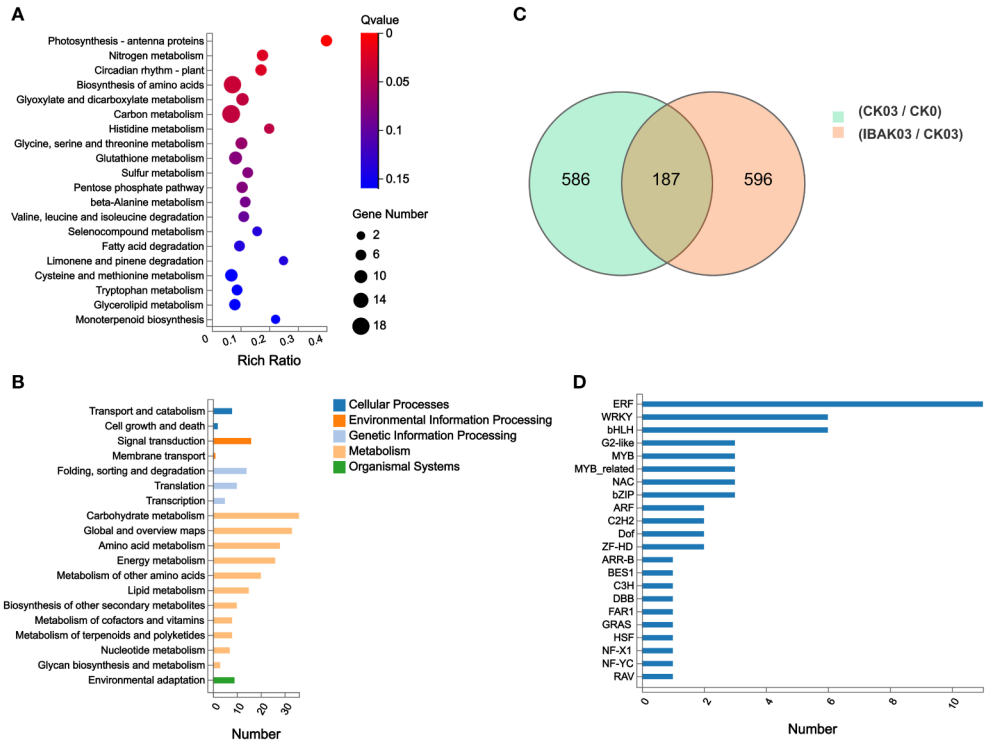

FIGURE 5
DEGs statistics, KEGG annotation analysis, KEGG enrichment analysis, and transcription factor genes statistics. **(A)** KEGG enrichment analysis; **(B)** KEGG annotation analysis; **(C)** DEGs statistics; **(D)** transcription factor genes statistics.

of other secondary metabolites in Metabolism (Supplementary Figure S4A).

In addition, KEGG enrichment analysis found a total of 31 significantly enriched KEGG pathways were found in negative ion mode in the IBAK03/CK03 comparison group; the differential metabolites were mainly enriched in phenylalanine metabolism, citrate cycle (TCA cycle), tyrosine metabolism, "alanine, aspartate and glutamate metabolism," butanoate metabolism, biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, and carbon metabolism pathway. In the positive ion mode, a total of 5 KEGG

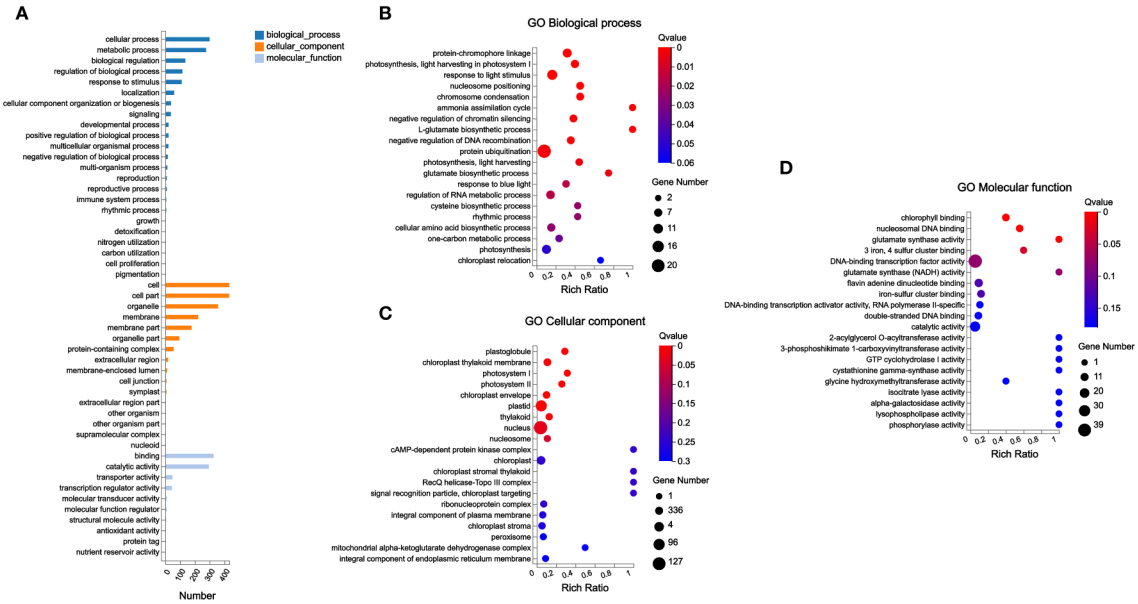

FIGURE 6
GO annotation analysis and GO enrichment analysis of DEGs. **(A)** GO annotation analysis; **(B–D)** GO enrichment analysis.

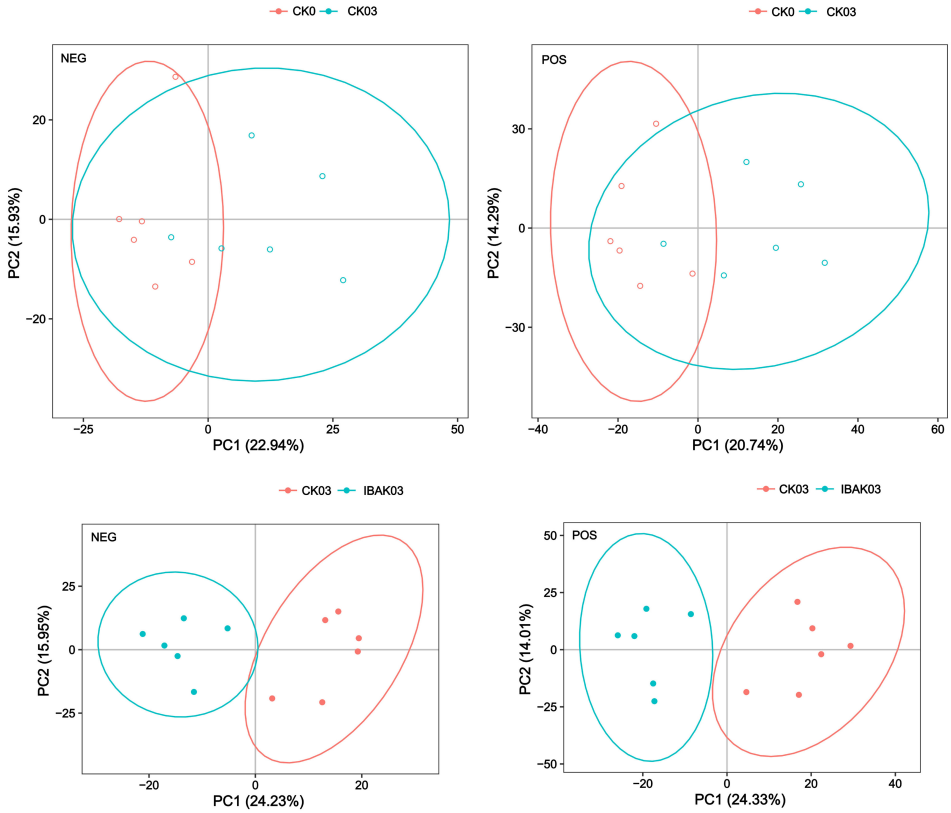

FIGURE 7
Principal component analysis.

pathways were significantly enriched, namely, phenylpropanoid biosynthesis, linoleic acid metabolism, phenylalanine metabolism, biosynthesis of secondary metabolites, and sphingolipid metabolism (Supplementary Figure S4B).

4 Discussion

IBAK is the potassium salt form of indole-3-butyric acid (IBA). IBA occurs naturally in many plants (Blommaert, 1954; Bayer, 1969; Sutter and Cohen, 1992). A previous study found that IBA can increase the number of lateral roots in the seminal root in rice, but IAA cannot, and the study pointed out that the signal transduction pathway for IBA was at least partially different from that for IAA (Wang et al., 2003). The auxin action of IBA has been suggested to be due to its conversion to IAA (Chhun et al., 2004). However, a study found that the stimulatory effect of IBA on lateral root development was not through its conversion to IAA in rice (Chhun et al., 2004). These studies indicated that there are differences between the response mechanisms of rice to IAA and IBA. IBA was reported to play a positive role in alleviating abiotic stress in plants (Tammam, 2009; Li et al., 2018). However, compared with IBA, the use of IBAK introduces potassium ions. Therefore, the action mechanism of IBAK on plants may be more complex. The results of this study showed that the root dry and fresh weight, root length, and root surface area were significantly increased after applying IBAK under salt stress, proving that IBAK had the function of promoting root growth. These results are similar to the findings by Jemaa et al. (2011) in *Arabidopsis*; their study found that IBA still retained the ability to stimulate lateral root growth under salt stress. Furthermore, the dry matter weight of the shoot in IBAK03 was higher than CK03. These results indicated that IBAK promoted rice growth under salt stress.

4.1 IBAK regulated carbon metabolism under salt stress

Carbon metabolism refers to the process in which plants convert carbon dioxide into organic substances (Dusenge et al., 2019), and it is the foundation of plant life activities. Zhou et al. (2023) found that spraying IBAK on rice leaves at the jointing stage can significantly upregulate two starch synthase genes: *OsSSIIb* (LOC4330709) and *OsGBSSII* (LOC4343010). The difference is that no significant changes were found in the expression levels of these two genes in the IBAK03/CK03 comparison group in this study. We speculated that this difference may be due to the different responses of rice at various growth stages to external stimuli or that different concentrations of IBAK solution may lead to different biological effects. Starch content was increased in IBAK03 compared with CK03. This may be related to another significantly upregulated starch synthase gene *OsSSIIc* (LOC4348711) detected in this study.

In addition, Zhou et al. (2023) reported that IBAK treatment upregulated the gene *OsUgp1* (LOC4347800), which is a UDP-glucose pyrophosphorylase gene, and its upregulation may contribute to the synthesis of sucrose. However, the expression level of this gene did not change significantly in this study. Meanwhile, this study found that sucrose, fructose, and soluble sugar were significantly reduced in IBAK03 compared with CK03. Therefore, we speculated that using IBAK (40 mg L^{-1}) under salt stress may adjust rice carbon allocation and improve starch storage at the seedling stage. Rice may use more carbon for starch synthesis instead of sucrose and fructose synthesis. In addition, the activities of AI and NI increased after using IBAK under salt stress. The increase in the activities of these enzymes may be one of the reasons for the decrease in sucrose content in this study. These two enzymes are involved in decomposing sucrose into fructose and glucose. Increasing AI and NI activity can regulate rice energy supply under salt stress by accelerating sucrose decomposition.

KEGG enrichment analysis on DEGs in the IBAK03/CK03 comparison group found that spraying IBAK upregulated the gene *OsSHM1* (LOC4334048) enriched in the carbon metabolism pathway. Wang et al. (2015) observed the growth status of *osshm1* mutant seeds in the greenhouse and found that the newly grown leaves after seed germination showed a decrease in the contents of total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids; *osshm1* produced higher ROS and increased O_2^- and H_2O_2 accumulation. Using IBAK to upregulate *OsSHM1* may be beneficial in reducing oxidative stress damage to rice under salt stress. In addition, gene *OsPPDKB* (LOC4338750) was upregulated in the IBAK03/CK03 comparison group. This gene is an essential regulator of carbon flux during starch and fat biosynthesis during rice filling (Kang et al., 2005).

In addition, spraying IBAK upregulated 2-oxoglutaric acid, fumaric acid, and succinic acid in the carbon metabolism pathway under salt stress (Supplementary Table S9). 2-oxoglutarate is a crucial metabolite at the crossroads of carbon/nitrogen metabolism as it is required for ammonia assimilation (Hodges, 2002; Araújo et al., 2014). Succinic acid has already been shown to be a stimulant of plant respiration (Bennet-Clark and Bexon, 1943; Turner and Hanly, 1949). In TCA cycle, 2-oxoglutaric acid is located after isocitrate and before succinyl coenzyme A. Succinic acid undergoes a series of reactions to finally generate oxaloacetate. The upregulation of these metabolites may increase the activity of the TCA cycle.

4.2 IBAK regulated biosynthesis of secondary metabolites pathway under salt stress

Secondary metabolites participate in protective functions in response to abiotic stress conditions (Akula and Ravishankar, 2011). In this study, many differential metabolites were enriched in the biosynthesis of secondary metabolites pathway in the IBAK03/CK03 comparison group. Specifically, this study found that 7-methylxanthine, 2-oxoglutaric acid, fumaric acid, succinic acid, L-phenylalanine, AICA ribonucleotide, 2-hydroxycinnamic acid, cinnamic acid, and 12-oxo phytodienoic acid were upregulated after using IBAK under salt stress (Supplementary Table S9, S10). 2-oxoglutarate (2-OG) is a crucial organic acid of the TCA (Lancien et al., 2000; Scheible et al., 2000; Araújo et al., 2014). It is also an obligatory substrate in a range of oxidative reactions catalyzed by 2-OG–dependent dioxygenases (Araújo et al., 2014). Fumaric acid is another essential component of the TCA cycle and can be metabolized to produce energy and carbon skeletons for the production of other compounds (Chia et al., 2000). 12-oxo-phytodienoic acid is the major precursor of (-)-jasmonic acid, playing a role in activating and fine-tuning defense responses, as well as plant growth processes (Liu and Park, 2021). These changes may reflect the impact of IBAK on the metabolic regulation and defense mechanisms of plants under salt stress.

4.3 IBAK regulated TF gene expression under salt stress

In this study, abundant TF genes were classified into ERF, WRKY, and bHLH families in the IBAK03/CK03 comparison group. These transcription factor families play essential roles in plant responses to abiotic stress. This study found that 11 TF genes belong to the ERF family. According to reports, several ERFs have been shown to be involved in plant stress-response processes, such as salt and drought (Zhang et al., 2009; Zhang and Huang, 2010; Yang et al., 2011; Cheng et al., 2019; Li et al., 2019; Huang et al., 2020). The bHLH family is also one of the largest transcription factor families in plants and plays an essential role in plant response to salt stress (Qian et al., 2021). LOC4345984 (transcription factor bHLH130), belonging to the bHLH family, was upregulated in this study. Previous studies reported that MdbHLH130 acts as a positive regulator of water stress response by regulating stomatal closure and reactive oxygen species (ROS)-scavenging in tobacco (Zhao et al., 2020; Qian et al., 2021). These results provided new clues for further research on the mechanism of IBAK regulating rice stress resistance by affecting TF gene expression.

5 Conclusion

The changes in multiple indicators in this study proved that IBAK can promote rice growth under salt stress. This study indicated that IBAK can change carbon allocation, increase starch reserves, and promote sucrose decomposition. Meanwhile, IBAK upregulated many key metabolites related to plant stress response and growth regulation in the biosynthesis of secondary metabolites pathway. In addition, multiple TF genes were differentially expressed, especially those in the ERF, WRKY, and bHLH families, which provided new clues for the mechanism of IBAK regulating rice resistance to salt stress.

Data availability statement

Transcriptome and metabolome raw data have been uploaded to online repositories. Transcriptome raw data was deposited in the Genome Sequence Archive in National Genomics Data Center,

China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences that are publicly accessible at https://ngdc.cncb.ac.cn/gsa accession number GSA: CRA018370. Metabolome raw data was deposited in the OMIX, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences: https://ngdc.cncb.ac.cn/omix with accession no. OMIX007146. 1. The Genome Sequence Archive Family: Toward Explosive Data Growth and Diverse Data Types. Genomics, Proteomics & Bioinformatics 2021, 19(4):578-583. https://doi.org/10.1016/j.gpb.2021.08.001 [PMID=34400360] 2. Database Resources of the National Genomics Data Center, China National Center for Bioinformation in 2024. Nucleic Acids Res 2024, 52(D1):D18-D32. https://doi.org/10.1093/nar/gkad1078 [PMID=38018256] 3 Database Resources of the National Genomics Data Center, China National Center for Bioinformation in 2022. Nucleic Acids Res 2022, 50(D1):D27-D38. https://doi.org/10.1093/nar/gkab951 [PMID=34718731].

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1416936/full#supplementary-material

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Functional interaction of melatonin with gasotransmitters and ROS in plant adaptation to abiotic stresses

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Melatonin is considered a multifunctional stress metabolite and a novel plant hormone affecting seed germination, root architecture, circadian rhythms, leaf senescence, and fruit ripening. Melatonin functions related to plant adaptation to stress stimuli of various natures are considered especially important. One of the key components of melatonin's stress-protective action is its ability to neutralise reactive oxygen species (ROS) and reactive nitrogen species directly. However, many of its effects are related to its involvement in the signalling network of plant cells and its influence on the expression of a large number of genes important for adaptation to adverse factors. Insights into the functional relationships of melatonin with gasotransmitters (GT) – gaseous molecules performing signalling functions – are still emerging. This review has analysed and summarised the experimental data that testify to the participation of the main GTs – nitric oxide, hydrogen sulfide, and carbon monoxide – in the implementation of the protective effect of melatonin when plants are exposed to abiotic stimuli of various nature. In addition, modulation by melatonin of one of the most important components in the action of GTs and ROS – post-translational modifications of proteins and the influence of ROS and GTs on melatonin synthesis in plants under stress conditions and the specific physiological effects of exogenous melatonin and GTs have been reviewed. Finally, the prospects of the GTs' practical application to achieve synergistic stress-protective effects on plants have been considered.

KEYWORDS

melatonin, ROS, nitric oxide, hydrogen sulfide, carbon monoxide, cell signalling, protein post-translational modifications, abiotic stress

Introduction

World agricultural statistics show that abiotic stresses have become the main factors limiting crop production in recent decades. They are responsible for more than a 50% reduction in the productivity of most crops (Kul et al., 2019). Climate change trends increase the importance of research aimed at developing new biotechnological tools to improve plant resistance. Enhancing the resistance of plants is actively pursued combining selection-genetic (traditional methods of selection and new means of genetic engineering) and physiological approaches. The latter includes a wide range of plant hormones, their synthetic analogues, as well as signalling mediators and stress metabolites, which are often collectively referred to as 'bioregulators' (Srivastava et al., 2016; Zulfiqar and Ashraf, 2021). Expanding the knowledge of compounds involved in the regulation of plant adaptive responses opens new opportunities both for the use of effective exogenous treatments of plants with new physiologically active substances and the control of their synthesis in plants by genome editing methods (Raza et al., 2022).

The regulation of adaptive responses and plant growth under stress conditions is mediated by a highly complex network of molecules. These include plant hormones (auxins, gibberellic acid, cytokinins, abscisic acid (ABA), salicylic acid, jasmonates, ethylene) (Ali et al., 2024), key signalling mediators (calcium ions, reactive oxygen species (ROS), nitric oxide, cyclic nucleotides) (Srivastava et al., 2016; Roy Chowdhury et al., 2019), and several compounds that combine the properties of stress metabolites and plant hormones and/or signalling network participants. The last group of compounds is currently the subject of the most in-depth research, resulting in the accumulation of a huge amount of experimental data.

The main representatives of this group of compounds turned out to be the so-called plant neurotransmitters, substances that act as mediators of nerve impulse transmission in animals (melatonin, serotonin, dopamine, acetylcholine, and gamma-aminobutyric acid) (Akula and Mukherjee, 2020). Within this group of compounds, the greatest research interest has been focused on melatonin (N-acetyl-5-methoxytryptamine) (Colombage et al., 2023; Taboada et al., 2023). For instance, an analysis of publications in the Web of Science™ database revealed that melatonin was the fourth most studied exogenous substance for mitigating plant stress caused by the most common factor, drought, over the past 24 years, preceded only by the major plant stress hormone ABA, signalling mediator hydrogen peroxide, and antioxidant glutathione (Feng et al., 2024). Owing to its low toxicity and environmental safety, melatonin (Pardo-Hernández et al., 2021) is regarded as a promising natural biostimulant for sustainable crop production under adverse environmental conditions (Manzoor et al., 2023).

The impact of melatonin on various stages of plant ontogenesis, including circadian rhythms, seed germination, root development, leaf senescence, flowering, seed formation, and fruit maturation, has been demonstrated (Pan et al., 2023; Zhao and Hu, 2023; Wang et al., 2024). Numerous data have also been accumulated on increasing the resistance of plants to abiotic stresses of various types under the influence of exogenous melatonin. These data are the subject not only of analytical reviews but also of meta-analyses (Agathokleous et al., 2021; Wang et al., 2022b; Plokhovska et al., 2023).

Melatonin is involved in the complex signalling network of plant cells through several mechanisms. One of the key components of this network is gasotransmitters (GTs) (Yao et al., 2019; Kolupaev et al., 2022). This term unites small gaseous molecules that are synthesised by living organisms and perform signalling functions. In contrast to hormones, their effects are not receptor-dependent; rather, they act on multiple intracellular targets of protein nature (Dey et al., 2024). Nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) are considered to be the main GTs of plant cells (Yao et al., 2019; Kolupaev et al., 2022; Dey et al., 2024).

Post-translational modification (PTM) of protein thiol groups is an important regulatory mechanism of GTs such as NO and H_2S (Zhang and Liao, 2019; Aroca et al., 2021; Martí-Guillén et al., 2022). The same groups are easily oxidised by ROS, especially by H_2O_2 (Valderrama et al., 2019; Xuan et al., 2020). There are reasons to believe that melatonin, as a redox-active molecule, can affect protein PTM processes induced by ROS and GTs. However, despite the intensive accumulation of data on the involvement of both melatonin and GTs in the regulation of plant adaptive responses, the data on their functional interaction remain poorly systematised (Wang et al., 2022a). On the other hand, data on the relationship between melatonin and two other important GTs, H_2S and CO, in the formation of adaptive responses in plants are still limited and have not been analysed from the point of view of the functioning of the signalling network as a whole. Taking into account the above mentioned, this paper presents a review and analysis of data on the mechanisms of participation of the main GTs in the realisation of the protective effects of melatonin under the influence of abiotic stresses on plants.

A brief summary of melatonin synthesis and metabolism in plant cells

In plants, melatonin is thought to be synthesised predominantly in chloroplasts (Zeng et al., 2022) (Figure 1). First, tryptophan decarboxylase (TDC) converts tryptophan to tryptamine by removing the carboxyl group. Then, under the influence of tryptamine 5-hydroxylase (T5H), tryptamine is hydroxylated to serotonin (5-hydroxytryptamine, 5-HT). The latter is converted to melatonin in two steps by serotonin N-acetyltransferase (SNAT) and N-acetylserotonin O-methyltransferase (ASMT, also known as caffeic acid O-methyltransferase, COMT) (Rehaman et al., 2021). However, the order of the transformations can vary depending on environmental conditions (Zeng et al., 2022). Under standard conditions, SNAT-catalysed acetylation of serotonin to N-acetyl 5-hydroxytryptamine (aHT) occurs first, followed by its O-methylation by ASMT/COMT, leading to melatonin (Ye et al., 2019). In contrast,

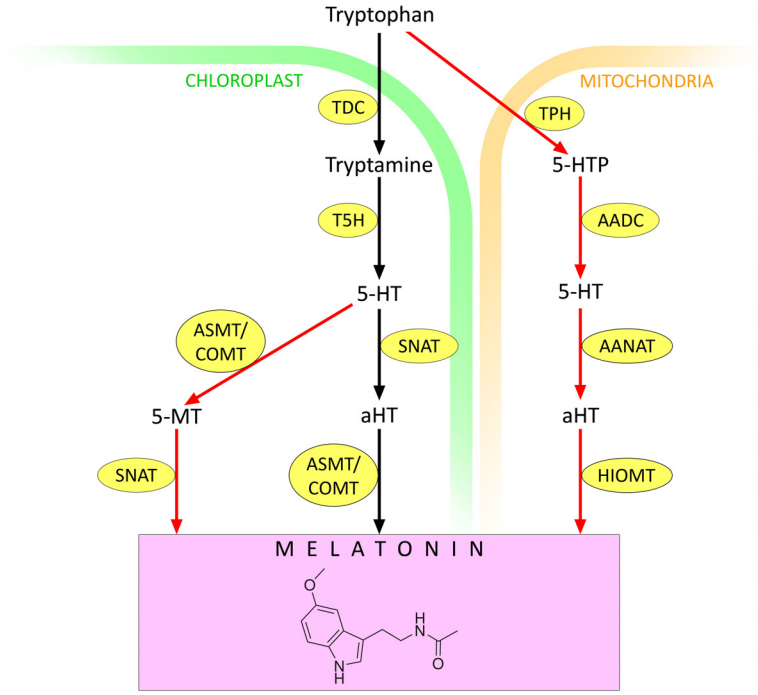

FIGURE 1
Melatonin synthesis in plants. 5-HT – 5-hydroxytryptamine (serotonin); 5-MT – 5-methoxytryptamine; AADC – aromatic amino acid decarboxylase; AANAT – arylalkylamine N-acetyltransferase (arylamine N-acetyltransferase); aHT – N-acetyl 5-hydroxytryptamine; ASMT – N-acetylserotonin O-methyltransferase; COMT – caffeic acid O-methyltransferase; HIOMT – hydroxyindole O-methyltransferase (N-acetylserotonin O-methyltransferase); SNAT – serotonin N-acetyltransferase; T5H – tryptamine 5-hydroxylase; TDC – tryptophan decarboxylase; TPH – tryptophan hydroxylase. Red arrows indicate melatonin synthesis pathways that are activated under stressful conditions. Other explanations in the text.

abiotic stress induces the expression of different isoforms of ASMT/COMT so that serotonin is first O-methylated to 5-methoxytryptamine (5-MT). Subsequently, 5-MT is acetylated by SNAT to form melatonin (Tan and Reiter, 2020).

In the mitochondria, tryptophan can be converted to 5-hydroxytryptophan by the action of tryptophan hydroxylase (TPH). Then 5-HT is formed under the action of aromatic amino acid decarboxylase (AADC). 5-HT is converted to aHT by arylalkylamine N-acetyltransferase (AANAT), also known as arylamine N-acetyltransferase. Finally, aHT is converted to melatonin by the action of hydroxyindole O-methyltransferase (HIOMT), also known as N-acetylserotonin O-methyltransferase (Zeng et al., 2022) (Figure 1). There is evidence that the mitochondrial contribution to melatonin synthesis increases under stress conditions (Zeng et al., 2022). Therefore, depending on the environment, both the contribution of different chloroplast pathways to melatonin synthesis and the ratio of melatonin synthesis in chloroplasts and mitochondria may change.

To date, a rather extensive phenomenology of melatonin synthesis enhancement in plants in response to stress stimuli of different natures has been accumulated. For example, an almost twofold increase in melatonin content was observed in wheat and rice plant organs in response to heating (Byeon and Back, 2014; Buttar et al., 2020). Similar effects at high temperatures were found in *Arabidopsis* (Shi et al., 2015) and tomato plants (Xu et al., 2016). Under salt, osmotic, and heat stress, an increase in endogenous melatonin levels was detected in grapes, barley, and lupin (Hassan et al., 2022).

The mechanisms of melatonin perception by plant cells have not been fully studied. Plant cells, similar to animal cells, contain three subunits of the G-protein complex (Gα, Gβ, and Gγ) and several putative G-protein-coupled receptors (GPCR) (Cannon and Chapman, 2021). In plants, CAND2 and CAND7 proteins have been found to interact with GPCR (Jin et al., 2012). It is the CAND2 protein that has been proposed as the phytomelatonin receptor1 (PMTR1) in 2018 (Wei et al., 2018). Experimental data have been obtained that implicate the CAND2/PMTR1 protein in the activation of Ca^{2+} influx and K^{+} efflux during melatonin-induced stomatal closure. The *Arabidopsis AtCand2* mutant, in contrast to wild-type plants, did not respond to melatonin treatment with stomatal closure (Wei et al., 2018). However, confocal microscopy data indicate that the CAND2 protein is localised in cytosol (Lee and Back, 2020). It is therefore, suggested that CAND2 might not be the G-protein that mediates melatonin-induced effects. Recently, receptor-like kinases (RLKs) have been considered as alternative candidates for melatonin receptors in plants (Back, 2021). *Arabidopsis* is known to have more than 600 RLK homologs. Since the MAP kinase cascade was activated in response to melatonin action, it is likely that one of the 600 RLKs in *Arabidopsis* functions as a melatonin receptor (Back, 2021). Nevertheless, recent experimental evidence points to a role for PMTR1 in melatonin perception and action in drought, salinity, and pathogen response in alfalfa, tobacco, and maize (Khan et al., 2023). For example, osmotic stress was shown to significantly increase *PMTR1* transcript levels in plants in a mannitol dose-

dependent manner (Wang et al., 2021). In addition, it was recently shown that overexpression of the *MePMTR1* gene isolated from the tropical cassava plant (*Manihot esculenta* Crantz) in *Arabidopsis* plants resulted in an increased resistance to dark-induced senescence compared to the wild type (Cheng et al., 2024). However, the presence and cellular localisation of melatonin receptors in higher plants still need further investigation (Corpas et al., 2022a).

Functional interaction of melatonin with ROS

ROS are essential signalling molecules that play diverse roles in the rapid response of plants to environmental stimuli (Ahammed et al., 2024). However, ROS generally act as signalling mediators when their levels are increased in individual cell compartments in a short-term and cell-controlled manner (Kolupaev et al., 2019; Dvořák et al., 2021; Li and Kim, 2022). Excessive production of ROS can cause programmed cell death, and large-scale stochastic ROS production can lead to uncontrolled destructive cell changes (Kacperska, 2004; Hasanuzzaman et al., 2020).

ROS are generated in one-, two-, and three-electron oxygen reduction reactions as a result of spontaneous and enzymatic oxidation of various substrates, as well as in photoinduced reactions (Sachdev et al., 2021). In higher plant cells, the primary sources of ROS are the electron transfer chains present in chloroplasts and mitochondria, but various ROS-generating enzymes are present in subcellular compartments (Taboada et al., 2023).

ROS generation in chloroplasts

Photosynthesis is one of the major sources of ROS in green plant cells. Superoxide anion radicals $O_2^{\bullet-}$ are generated by photosystem I and II electron transport chain (ETC) function (Li and Kim, 2022). The photoinduced generation of ROS mainly depends on environmental conditions and the physiological state of photosynthetic apparatus (Foyer and Shigeoka, 2011). When CO_2 fixation is limited under various stressors (e.g., drought, salinity, and high temperature), the NADPH pool is only slightly consumed, resulting in electron "leakage" from ferredoxin to molecular oxygen to form $O_2^{\bullet-}$. Superoxide anion radicals formed in chloroplasts are readily converted to the more stable ROS, hydrogen peroxide H_2O_2, under the influence of superoxide dismutase (SOD).

Singlet oxygen is also formed in chloroplasts, primarily as a consequence of the transition of chlorophyll P_{680} to the triplet state within the reaction centre of photosystem II and/or the light-harvesting complex. The likelihood of singlet oxygen formation, as well as other ROS, increases when the ETC is over-reduced, which is characteristic of stressful conditions (Li and Kim, 2022).

ROS generation in mitochondria

Mitochondria, similar to chloroplasts, contain a large number of electron carriers. Their inadvertent interaction with molecular oxygen can lead to a one-electron reduction of O_2 to $O_2^{\bullet-}$. The main electron leakage sites in plants, as in animals, are considered to be complexes I and III of the ETC (Cvetkovska and Vanlerberghe, 2013). However, the ETC of plant mitochondria also involves an alternative electron transport pathway. It includes two molecules of NAD(P)H dehydrogenase on the outer and inner sides of the inner mitochondrial membrane, alternative oxidase, and an uncoupling protein, UCP, which limit ROS generation (Sachdev et al., 2021). The functions of these proteins are enhanced under stressful conditions.

ROS synthesis in peroxisomes

Peroxisomes are also among the compartments that generate significant amounts of ROS in processes such as photorespiration and β-oxidation of fatty acids mediated by acyl-CoA oxidase (Sachdev et al., 2021). ROS generation in peroxisomes may also be related to the activities of flavin oxidase, urate oxidase, xanthine oxidase and other enzymes (Hasanuzzaman et al., 2020). Under abiotic stress, photorespiration is initiated in the chloroplast because of the limited availability of CO_2 and increased solubility of O_2, which competitively accelerates the oxygenation of ribulose-1,5-biphosphate to form glycolate. The latter is exported to peroxisomes, where it is oxidized by glycolate oxidase to form H_2O_2 (del Río et al., 2003; Das and Roychoudhury, 2014).

ROS generation in the apoplast

The apoplast is the site of generation of a large amount of ROS, which may be a component of cell signalling. For example, NADPH oxidase, known as the Respiratory Burst Oxidase Homologs (RBOH), is localised on the plasma membrane (Marino et al., 2012). This enzyme complex reduces molecular oxygen to form superoxide anion radical. *Arabidopsis* genome contains 10 members of the RBOH membrane-bound (catalytic) subunit gene family, and the involvement of some of them in signalling processes has been confirmed by molecular genetic methods (Torres et al., 2002; Hasanuzzaman et al., 2020). A significant pool of signalling ROS appears to be also generated in the apoplast by the oxidase activity of class III heme peroxidases (Gautam et al., 2017).

Involvement of melatonin in ROS regulation

As shown previously, melatonin affects cellular content of ROS as a direct antioxidant and as a probable participant in the signalling network and inducer of other components of the antioxidant system (Ahammed et al., 2019).

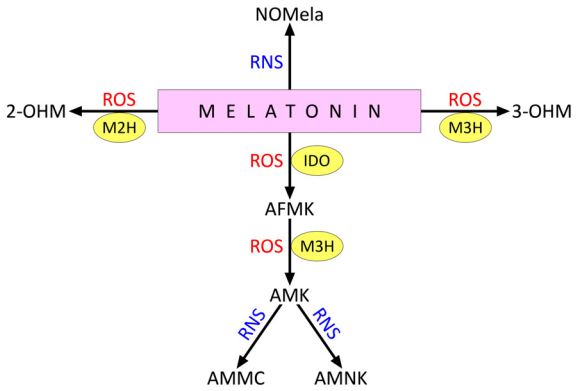

FIGURE 2
Direct interaction of melatonin with ROS and RNS. 2-OHM, 2-hydroxymelatonin; 3-OHM, 3-hydroxymelatonin; AFMK , cyclic N1-acetyl-N 2-formyl-5-methoxykynuramine; AMK , N1-acetyl-5-methoxykynuramine; AMMC , acetamidomethyl-6-methoxycinnolinone; AMNK , N1-acetyl-5-methoxy-3-nitrokynuramine; IDO , indoleamine 2,3-dioxygenase; M2H , melatonin 2-hydroxylase; M3H , melatonin 3-hydroxylase; NOMela , N-nitrosomelatonin; ROS , reactive oxygen species; RNS , reactive nitrogen species. Other explanations in the text.

Melatonin can directly interact with ROS such as $^{\bullet}OH$, H_2O_2, and $^1O_2$ (Galano and Reiter, 2018). Melatonin has been reported to be more active than glutathione and mannitol in binding hydroxyl radicals (Arnao and Hernández-Ruiz, 2019). The chemical mechanisms of melatonin interaction with different ROS and their corresponding constants have been described in several reviews (Galano and Reiter, 2018; Arnao and Hernández-Ruiz, 2019).

The catabolism of melatonin by ROS is one of the mechanisms by which melatonin exerts its direct antioxidant action. In addition, melatonin metabolites also possess direct and indirect antioxidant properties (Taboada et al., 2023).

Melatonin catabolism can occur with or without enzyme involvement (Back, 2021; Zeng et al., 2022). One pathway involves the conversion of melatonin to cyclic N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) by indoleamine 2,3-dioxygenase (IDO) (Wang et al., 2024) (Figure 2). AFMK is later converted to N^1-acetyl-5-methoxykynuramine (AMK) by the action of melatonin 3-hydroxylase (M3H). Melatonin can also be converted to 2-hydroxymelatonin (2-OHM) and 3-hydroxymelatonin (3-OHM) by interaction with ROS; these processes involve M2H and M3H, respectively (Back, 2021). 2-OHM and 3-OHM are the predominant melatonin metabolites in plants (Back, 2021; Khan et al., 2023). In addition, the formation of 4-hydroxymelatonin and 6-hydroxymelatonin is possible under the influence of radical ROS, the latter being more characteristic of melatonin degradation in animals (Mannino et al., 2021).

Melatonin also interacts with RNS, particularly when it reacts with peroxynitrite or nitrogen dioxide to form N-nitrosomelatonin (NOMela) (Figure 2). The possible physiological functions of this compound are discussed below in the section on the functional interactions between melatonin and NO. In addition, AMK formed by the interaction of AMFK with ROS can react with RNS to form 3-acetamidomethyl-6-methoxycinnolinone (AMMC) or N^1-acetyl-5-methoxy-3-nitrokynuramine (AMNK) (Mannino et al., 2021).

The melatonin hydroxymetabolites 2-OHM and 3-OHM are present in plant cells at much higher levels than melatonin itself. For example, it has been reported that in an intact rice leaf, the content of 3-OHM and 2-OHM exceeds the content of melatonin by 300 and 100 times, respectively (Byeon et al., 2015; Taboada et al., 2023). Even higher concentrations of melatonin hydroxymetabolites were recorded in rice under stress conditions, particularly under cadmium influence and salinity stress (Lee et al., 2017; Choi and Back, 2019).

The hydroxymetabolite 3-OHM, formed from melatonin as a result of M3H activity, has intrinsic antioxidant activity targeting hydroxyl and hydroperoxyl ($^{\bullet}OOH$) radicals (Tan et al., 2014). In reaction with 1,1-diphenyl-2-picrylhydrazyl, 3-OHM was shown to have 15 times more antioxidant activity than melatonin (Lee and Back, 2022).

The indirect effect of melatonin and its derivatives on the redox homeostasis of plant cells is even more complex. Several studies have reported increased gene expression and activity of antioxidant enzymes in plants of different taxonomic groups under normal and stress conditions (Magri and Petriccione, 2022; Zhang et al., 2022; Kolupaev et al., 2023a; Taboada et al., 2023). Such effects suggest the formation of ROS signalling under the influence of melatonin, which induces the antioxidant system (Figure 3). Indeed, some evidence has been obtained for the involvement of ROS generated by NADPH oxidase in the realisation of the stress-protective effect of melatonin (Gong et al., 2017; Wei et al., 2018; Sun et al., 2021). One of the mechanisms for the increase in NADPH oxidase activity under the action of melatonin may be related to the decrease in S-nitrosation of cysteine residues in its catalytic subunit (Gong et al., 2017). NO is known to participate in the S-nitrosation reaction of NADPH oxidase at Cys890, leading to its inhibition of ROS generation (Yun et al., 2011). Melatonin, which has the ability to bind NO, reduces S-nitrosation in NADPH oxidase molecules. Using *Solanum lycopersicum* as an example, it was found that treatment with exogenous melatonin first led to the accumulation of endogenous melatonin, which then removed the generated NO molecules, causing denitrosation of Cys890 residues in RBOH and increasing its activity (Gong et al., 2017) (Figure 3). Thus, activation of RBOH and increased ROS generation have been shown to be critical for melatonin to enhance plant resistance to drought, heat, and osmotic stress. Inhibition of RBOH or chemical removal of H_2O_2 by its scavenger significantly downregulated melatonin-induced plant defence responses, including decreased expression of several stress-related genes (*CDPK1*, *MAPK1*, *TSPMS*, *ERF4*, *HSP80*, and *ERD15*), and caused decreased activity of antioxidant enzymes (SOD, catalase, and ascorbate peroxidase) (Gong et al., 2017). The role of ROS generated by different molecular forms of NADPH oxidase in the stomatal closing effect induced by exogenous melatonin in *Arabidopsis* and dependent on the synthesis of NO was demonstrated (Wang et al., 2023a, see below).

Nevertheless, the mechanisms underlying the increase in NADPH oxidase activity upon melatonin treatment of plants do not seem to be limited to its effect on S-nitrosation of Cys890 residues. In particular, there is reason to believe that melatonin may

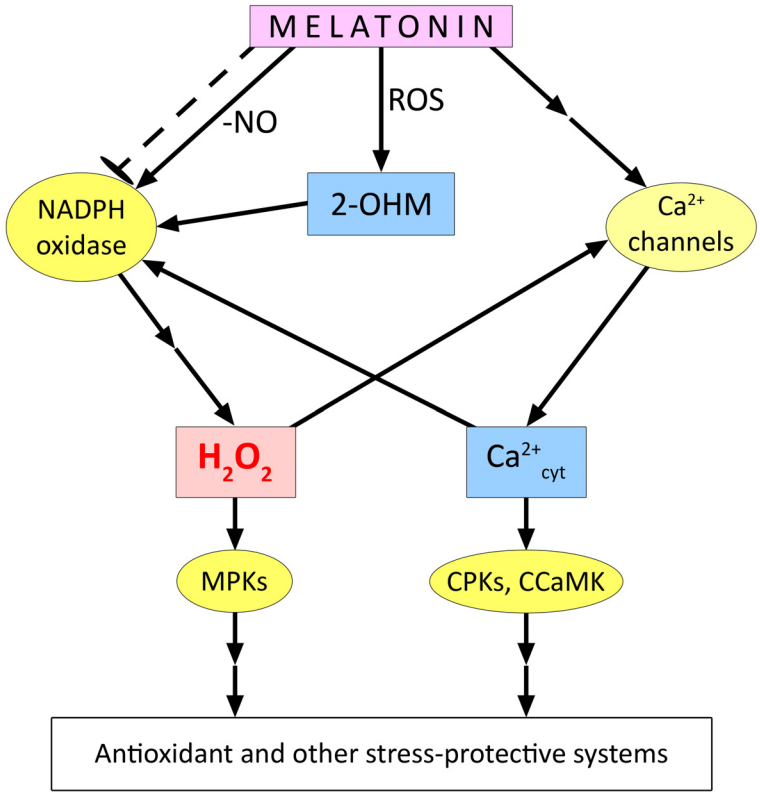

FIGURE 3
Functional interaction of melatonin with ROS in plant cells. 2-OHM – 2-hydroxymelatonin; CPKs, Ca^{2+}-dependent protein kinases; CCaMK, Ca^{2+}/calmodulin-dependent protein kinase; ROS, reactive oxygen species. The dashed arrow with blunt end indicates the possible inhibitory effect of melatonin on the expression of genes encoding certain molecular forms of NADPH oxidase. Other explanations in the text.

activate NADPH oxidase by affecting Ca^{2+} homeostasis. For example, in watermelon plants, it was shown that the recruitment of ROS generated by NADPH oxidase and calcium ions entering the cytosol through cyclic nucleotide-gated ion channels realise the protective effect of melatonin during cold stress (Chang et al., 2021). Our experiments showed that the melatonin-induced increase in heat tolerance of wheat seedlings was abolished by the H_2O_2 scavenger dimethylthiourea, the NADPH oxidase inhibitor imidazole, and various calcium antagonists (Kolupaev et al., 2024b). It is noteworthy that the activation of H_2O_2 accumulation under the action of melatonin was not manifested in the case of seedlings treated with EGTA, a chelator of extracellular calcium, or with neomycin, an inhibitor of phospholipase C, which is involved in formation of inositol 1,4,5-phosphate and thus in opening of intracellular Ca^{2+} channels. There is reason to believe that under the above experimental conditions, melatonin-induced changes in Ca^{2+} homeostasis are primordial in relation to activation of NADPH oxidase and increased generation of ROS, which act as signalling mediators (Figure 3). According to one model, calcium activates a Ca^{2+}-dependent protein kinase that phosphorylates the N-terminal region of the membrane-associated subunit (RBOH) of NADPH oxidase and causes its conformational change to facilitate the binding of its cytosolic component, the Rop protein (GTPase). This results in the formation of an active dimer, leading to increased ROS generation (Wong et al., 2007). In a form of the membrane-bound subunit of rice NADPH oxidase (OsRBOHB), the presence of EF arms in the N-terminal region was found, indicating the formation of a dimer involving Ca^{2+} (Oda et al., 2010). Thus, the increase in NADPH oxidase activity with the involvement of calcium may be related not only to its activation of the protein kinase, but also to the direct interaction of Ca^{2+} with the catalytic subunit. In this context, it is possible that ROS and cytosolic Ca^{2+}, as mediators of melatonin effects, may functionally interact according to the principle of a mutually reinforcing "signalling loop".

In general, several experimental data were obtained on the participation of NADPH oxidase in signalling processes necessary for the realisation of the stress-protective effect of melatonin. For example, the role of NADPH oxidase in the melatonin-induced development of tolerance to salinity has been demonstrated in *Arabidopsis*. It was found that melatonin treatment could enhance antioxidant defence in stressed wild-type plants but not in the *atrbohF* mutant (Chen et al., 2017). Exogenous melatonin treatment significantly reduced the phytotoxic effects of the pesticide chlorothalonil on tomato plants. Moreover, the melatonin-induced increase in the activity of glutathione cycle enzymes involved in chlorothalonil detoxification was abolished by treating plants with NADPH oxidase inhibitor and H_2O_2 scavenger. This suggests a role for ROS generated by NADPH oxidase in the realisation of the stress-protective effect of melatonin (Peng et al., 2023). The activating effect of ROS and Ca^{2+} on the

enzymatic antioxidant system is thought to be mediated mainly by MAP kinase signalling cascade, with the involvement of calmodulin-dependent protein kinases (Dvořák et al., 2021) (Figure 3). However, there is evidence for the involvement of the MAPK signalling cascade in shaping melatonin-mediated responses to abiotic and biotic stressors (Lee and Back, 2016; 2017; Mansoor et al., 2024). For example, the expression of MPK3 and MPK6 in Arabidopsis has been shown to be induced by melatonin and 2-OHM (Lee and Back, 2016). Thus, it is conceivable that different kinases are involved in the transduction of ROS and Ca^{2+} signals activated by melatonin and are required for its induction of the antioxidant system and possibly other plant defence responses (Figure 3).

However, melatonin may not only have an activating effect on NADPH oxidase and ROS generation. It has been shown that melatonin can also suppress the expression of genes encoding plasma membrane-bound NADPH oxidase (*TaRbohD*, *TaRbohF*) in wheat. Such data were obtained while studying the effect of priming wheat seeds and seedlings of *Brassica juncea* with melatonin, which mitigated the subsequent toxic effects of chromium (Lei et al., 2021; Kour et al., 2024). The authors consider this effect as an additional mechanism to mitigate oxidative stress. Similar effects were found in a study on the protective effects of melatonin on *Vicia faba* plants exposed to arsenic toxicity (Siddiqui et al., 2020). Melatonin treatment reduced the effects of increased NADPH oxidase activity and $O_2^{\bullet-}$ and H_2O_2 generation induced by the treatment. At the same time, no inhibitory effect of melatonin on NADPH oxidase activity and ROS generation was observed in the absence of stress stimuli. It is still unclear how the effects of NADPH oxidase activation and suppression by melatonin recorded in different objects relate to each other. A different set of mediators may be involved in the regulation of NADPH oxidase under stress conditions than under optimal conditions. Moreover, the regulation of redox homeostasis during the action of exogenous melatonin on plants may be even more complex because of its transformation into hydroxy derivatives. For example, Lee and Back (2021) obtained experimental data suggesting a more important role of 2-OHM in enhancing ROS generation than melatonin. It was shown that treatment of tobacco and *Arabidopsis* leaves with 2-OHM resulted in a significantly greater enhancement of superoxide anion radical generation compared to melatonin treatment.

Recently, more significant effects of 2-OHM on *Arabidopsis* seed germination than melatonin have been reported (Lee and Back, 2022). These effects are attributed to ROS-mediated induction of gibberellin synthesis. Molecular genetic studies using the knockout mutant *m2h* and plants with overexpression of the M2H gene confirmed the importance of increasing ROS generation in the implementation of the effect of 2-OHM on the expression of the gibberellin synthesis enzyme gene and seed germination (Lee and Back, 2022). Undoubtedly, data on the participation of 2-OHM and other metabolites of melatonin in cellular signalling processes are still insufficient. It is still very difficult to distinguish the physiological effects of these compounds from those of melatonin itself.

Functional relationships between melatonin and NO

GT NO, together with ROS, is considered a very important signalling mediator of plant cells, somehow related to almost all known signalling molecules (Santolini et al., 2017). One of the main ways of realising the signalling potential of NO molecules is through the PTM of proteins. The most common NO-mediated PTM is S-nitrosation, a reversible redox modification based on the addition of a nitroso group to the thiol group (SH) of a specific cysteine residue (Cys) to form S-nitrosothiol (SNO), which can induce conformational changes and, consequently, alter the activity or function of the corresponding protein (Lamotte et al., 2015). To date, hundreds of proteins that are regulated by S-nitrosation have been identified (Sánchez-Vicente et al., 2019). The second most abundant NO-mediated PTM is tyrosine nitration (Blume et al., 2013; Sánchez-Vicente et al., 2019; Zhu et al., 2019).

The biological significance of this process is still difficult to interpret, as it is considered one of the markers of nitrosative stress (Ischiropoulos, 2003). It is likely that tyrosine nitration is also important in plants for regulating the activity of individual proteins. For example, nitration of tyrosine residues in molecules of the major microtubule protein α-tubulin has been found, which may be related to the regulation of their dynamic properties and participation in the growth and division of plant cells (Blume et al., 2013).

NO is considered to be one of the main regulators of Ca^{2+} homeostasis. It affects several types of Ca^{2+} channels and promotes Ca^{2+} entry into the cytosol from the extracellular space and intracellular compartments (Jeandroz et al., 2013; Santolini et al., 2017). NO, through S-nitrosation, affects the functional activity of many proteins involved in Ca^{2+} signalling, including protein kinases (Courtois et al., 2008) and calmodulin (Astier et al., 2012; Jeandroz et al., 2013). It is also evident that the targets of NO-mediated PTM are also proteins involved in Ca^{2+} channel opening. Studies using agonists or antagonists of cGMP and cADP-ribose (cADPR) have shown that these secondary cytosolic messengers play a central role in the realisation of the effects of NO on Ca^{2+} channels (Santolini et al., 2017). There is also considerable experimental evidence for NO activation of the mitogen-activated protein kinase (MAPK) cascade, which in turn is tightly coupled to many other components of the signalling network (Zhu et al., 2019).

The signalling potential of NO is also realised by its functional and chemical interaction with ROS, in particular with $O_2^{\bullet-}$ and H_2O_2. This interaction of key signalling mediators can be either synergistic or antagonistic (Farnese et al., 2016). NO-induced PTMs of individual protein molecules can induce rapid and long-lasting effects that may have different directions. For example, inhibition of individual antioxidant enzymes by the direct action of NO may lead to the formation of a signal that induces gene expression of these enzymes (Corpas et al., 2022b; Kolupaev et al., 2023b). On the other hand, NO-induced PTMs can reduce the activity of enzymes involved in ROS generation, such as glycolate oxidase and NADPH oxidase, allowing the cell to maintain redox homeostasis (Farnese et al., 2016). To date, there is evidence that NO is also

involved in the realisation of the physiological effects of most known plant hormones (Zhou et al., 2019; Shang et al., 2022; Kolupaev et al., 2024a). Melatonin is a new plant hormone, but there is already considerable evidence of its functional and chemical interactions with NO (He and He, 2020). This interaction is manifested by melatonin binding NO to form nitrosomelatonin, influencing S-nitrosation processes, and inducing NO synthesis (Corpas et al., 2022a; Ahmad et al., 2023).

Pathways of nitric oxide synthesis in plants

It is now generally accepted that there are two main pathways for the synthesis of NO in plants: a reductive pathway from nitrates and nitrites, which is mainly driven by nitrate reductase (NR) activity, and an oxidative pathway associated with the conversion of L-arginine by an enzyme with activity similar to NO synthase (NOS), so called because its activity requires the same biochemical conditions as animal NOS (Astier et al., 2018; Corpas et al., 2022a). In higher plants, the main cellular compartments of NO generation are the cytosol, peroxisomes, chloroplasts, and mitochondria. Experimental data indicate that NO can be synthesized in the cytosol with the participation of NR, a multifunctional enzyme involved in nitrogen assimilation and metabolism. *In vivo* and *in vitro* experiments have shown that NR can catalyse the reduction of nitrate to NO and its derivative peroxynitrite ($ONOO^-$) (Khator et al., 2024). The activity of different molecular forms of NR can be regulated by phosphorylation involving MAP kinases, whose activity can be modulated by ROS (Wang et al., 2010; Zhu et al., 2019).

NO can be synthesized in mitochondrial membranes *via* the reductive pathway, but with the participation of other catalytic systems. NR as well as the enzymatic complexes of the electron transport chain cytochrome oxidase (CIII) and cytochrome reductase (CIV) can be involved in this process (Gupta and Kaiser, 2010; Farnese et al., 2016; Khator et al., 2024). The oxidative pathway of NO synthesis is considered to be as important as the reductive pathway, although the nature of the enzymatic systems that provide this pathway in higher plants has been debated for nearly three decades (Farnese et al., 2016). Nevertheless, enzymatic oxidation of L-arginine to citrulline and NO has been shown to be possible in leaf peroxisomes and chloroplasts of green algae and vascular plants (Hancock and Neill, 2019). This enzymatic activity has been named NOS-like because, as in the case of the animal enzyme, it has been reported to be strictly dependent on the presence of arginine and NADPH as well as several NOS cofactors (NADPH, FAD, FMN, Ca^{2+}, and calmodulin) (Corpas and Barroso, 2014; Farnese et al., 2016). However, molecular genetic evidence of the presence of the corresponding protein in higher plants is still lacking (Astier et al., 2018). Currently, there is a hypothesis that there are polypeptides with redox-active domains that can be assembled into a single enzymatic complex that catalyses the reactions of arginine-dependent NO formation in higher plants (Kolbert et al., 2019). Partial overlap of cellular compartments where NO is synthesised with those for melatonin synthesis (chloroplasts and mitochondria) is considered by some authors as a fact that indirectly indicates a possible relationship between melatonin and NO (Wang et al., 2022a).

Nitric oxide conjugates and their functional relationship to melatonin

A peculiarity of NO is its short half-life (approximately 30 s) (Martínez-Lorente et al., 2022). In this respect, its physiological effects depend largely on its transformation into more stable conjugated compounds. Among these, S-nitrosothiols are well known. The most abundant S-nitrosothiol is S-nitrosoglutathione (GSNO), which is formed by the interaction of NO with reduced glutathione (GSH) (Corpas et al., 2013). GSNO levels are regulated by S-nitrosoglutathione reductase, which reduces GSNO to glutathione sulfinamide ($GS(O)NH_2$) using NADH (Martínez-Lorente et al., 2022). Melatonin may affect the GSNO pool by inhibiting S-nitrosoglutathione reductase (Wen et al., 2016).

An even more important mechanism of interaction between NO and melatonin may be the formation of NOMela, which occurs in an aerobic environment and at physiological pH values (Martínez-Lorente et al., 2022). This interaction is considered one of the mechanisms of NO binding to prevent the development of nitrosative stress (Singh et al., 2016). At the same time, NOMela is considered one of the natural NO donors, like GSNO or S-nitrosocysteine (Mukherjee, 2019). Both nitrosothiols and NOMela can participate in protein PTM as NO sources. The ability of NOMela to efficiently transnitrosate cysteine residues in proteins has been demonstrated using an *in vitro* system (Singh et al., 2016). In particular, NOMela was found to be 10 times more effective than S-nitrocysteine in the reaction of S-nitrosation of catalytically active cysteine residues by glyceraldehyde-3-phosphate dehydrogenase (Kirsch and de Groot, 2008).

NOMela may be a conjugate that enables long-distance transport of both melatonin and NO at the whole-plant level (Mukherjee, 2019). It has been suggested that GSNO and NOMela may compete for binding sites for long-distance transport in plants, although direct experimental evidence for this hypothesis is still lacking (Singh et al., 2016). However, NOMela has been shown to be more efficient than GSNO in transporting NO from roots to leaves of *Arabidopsis* in a model experiment (Singh et al., 2021).

Effect of melatonin on NO synthesis in the regulation of plant resistance to stress

Several studies have reported an increase in NO synthesis during melatonin treatment of plants and the role of this effect in the induction of plant resistance to various stress stimuli (Table 1; Figure 4). For example, treatment of tomato plants with melatonin, which induces the development of heat tolerance, resulted in an NR-dependent increase in leaf NO content (Jahan et al., 2019). Data on the participation of NO as a mediator of the action of melatonin were also obtained when studying the induction of cold resistance in

TABLE 1 Examples of the involvement of nitric oxide in the realisation of the effect of melatonin on plant resistance to abiotic stresses.

Stressor	Plant species	Physiological effects of melatonin	Experimental evidence of NO involvement in melatonin effects	Source
High temperatures	*Solanum lycopersicum* L.	Increased heat resistance	Increased NO levels in leaves due to increased NR activity and enhanced *NR* gene expression	Jahan et al., 2019
Low temperatures	*Brassica napus* L.	Increased resistance to low temperatures	Increase in cytosolic Ca^{2+}, NO content in leaves with subsequent increase in MAPK3/6 expression. Elimination of melatonin effects in the background of NOS (L-NAME) and NR (tungstate) inhibitors and Ca^{2+} channel blocker $LaCl_3$ or Ca^{2+} chelator EGTA	Ma et al., 2022
	S. lycopersicum	Improvement of fruit storage at low temperatures	Enhanced *NOS1* gene expression and increased NOS-like activity	Aghdam et al., 2019
Salinisation	*B. napus*	Mitigation of growth inhibition, reduction of oxidative damage, preservation of ionic homeostasis	Increased NO levels in seedlings, increased levels of S-nitrosated proteins. Elimination of stress protective effect of melatonin by NO scavenger PTIO	Zhao et al., 2018
	Glycine max L.	Reduction of growth inhibition, enhancement of isoflavone accumulation	Increase in NO levels in seedlings, accompanied by increased expression of *NR1*, *NR2* and *NOA1* genes. Elimination of melatonin effects on plant growth and secondary metabolite accumulation in the presence of cPTIO	Yin et al., 2022
UV-B	*G. max*	Reduction of growth inhibition, activation of antioxidant system, accumulation of isoflavones, enhancement of gene expression of enzymes involved in their synthesis	Increased NR activity and enhanced *NR1* and *NR2* gene expression. Elimination by cPTIO treatment of the effects of increased gene expression of isoflavone synthesis enzymes and accumulation of these secondary metabolites.	Yin et al., 2023
Cd toxicity	*Triticum aestivum* L.	Mitigation of oxidative stress manifestations	Enhancement of NO synthesis, elimination of stress-protective effect of melatonin by plant PTIO treatment	Kaya et al., 2019
	T. turgidum L.	Reduction of seedling growth inhibition, mitigation of oxidative damage	Increase in endogenous NO levels and NR and nitrite reductase activities	Aloui et al., 2024
	Catharanthus roseus L.	Mitigation of oxidative damage, increased proline synthesis, increased activity of antioxidant enzymes	Elimination of melatonin stress-protective effect by cPTIO	Nabaei and Amooaghaie, 2019

plants, in particular rape seedlings (Ma et al., 2022). The increase in NO and cytosolic Ca^{2+} was more significant in melatonin-treated leaves in response to cold than in untreated leaves (Table 1). Notably, co-treatment of melatonin-treated plants with NO or Ca^{2+} antagonists also inhibited melatonin-induced MAPK3/6 expression under cold stress conditions (Ma et al., 2022). Thus, there is reason to believe that Ca^{2+} and NO are at the beginning of the melatonin-induced signalling cascade leading to the development of cold tolerance in rapeseed.

In addition, the involvement of NO in the preservation of melatonin-treated tomato fruits during storage at low temperatures was demonstrated (Aghdam et al., 2019).

One of the effects of exogenous melatonin, which is important for plant resistance to drought and other stresses associated with plant desiccation, is stomatal closure. These processes depend on NO and its functional interaction with ROS. In *Arabidopsis* plants, it was shown that exogenous melatonin-induced stomatal closure was reversed by the NO scavenger cPTIO (carboxy-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) (Wang et al., 2023a). In addition, lines mutant for the genes for NO synthesis enzymes, nitrate reductase 1 and 2 (*nia1/nia2*) and NO-associated 1 (*noa1*), were found to be unable to close stomata when exposed to melatonin. Melatonin-induced NO production was also impaired in plants mutant for NADPH oxidase genes (*rhohC* and *rbohD/F*). This indicates that NO is downstream of ROS in the melatonin-inducible signalling chain (Wang et al., 2023a) (Figure 4).

NO may also mediate the induction of salt tolerance by exogenous melatonin in rapeseed (Table 1). In addition, it was shown that mutants defective in NR genes had increased sensitivity to salt stress and that melatonin did not affect their salt tolerance (Zhao et al., 2018).

Using soybeans as an example, it has been shown that when melatonin induces salt tolerance and UV-B resistance NO is involved in an important defence response such as the accumulation of isoflavones (Yin et al., 2022, 2023) (Table 1).

The involvement of NO was also shown in the protective effect of melatonin on plants of two wheat species and *Catharanthus roseus* exposed to cadmium toxicity (Table 1). Notably, treatment of *C. roseus* plants with an NO donor, as with melatonin, increased proline content and antioxidant enzyme activities in roots under Cd stress (Nabaei and Amooaghaie, 2019). These melatonin-induced responses were inhibited by the NO scavenger, cPTIO. However,

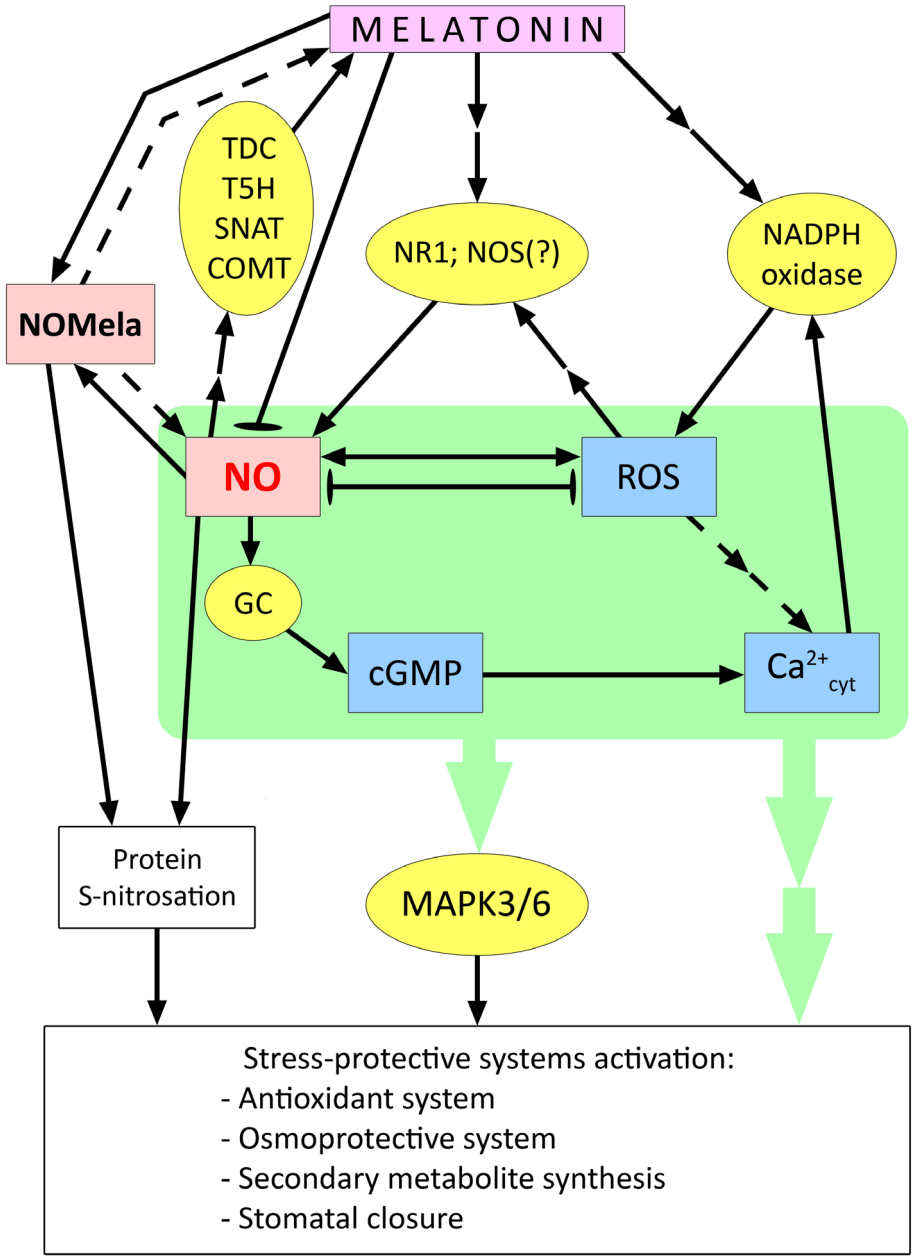

FIGURE 4
Involvement of NO in the activation of stress protection systems in plant cells under the action of melatonin. cGMP – cyclic guanosine monophosphate; COMT – caffeic acid O-methyltransferase; GC – guanylate cyclase; MAPK3/6 – mitogen activated protein kinases 3/6; NOMela – N-nitrosomelatonin; NOS – NO synthase; NR1 – nitrate reductase 1; ROS – reactive oxygen species; SNAT – serotonin N-acetyltransferase; T5H – tryptamine 5-hydroxylase; TDC – tryptophan decarboxylase. Dashed arrows indicate connections between signalling mediators without clear experimental evidence; blunt-ended arrows indicate antagonistic interactions between signalling mediators. Other explanations in the text.

different results were obtained when the effect of melatonin on the Cd resistance in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) was studied (Wang et al., 2021). It was shown that treatment of the plants with melatonin significantly reduced the Cd content in them. At the same time, treatment with the NO scavenger cPTIO had the same effect on the Cd content in the plants. The authors showed that NO enhances the expression of the Cd transporter gene IRT1, thereby increasing Cd uptake and aggravating stress in plants, while exogenously added melatonin can inhibit NO synthesis, thereby reducing Cd levels and alleviating stress caused by its toxic effect (Wang et al., 2021).

The effect of reducing NO levels in plants treated with melatonin has also been reported in some other studies. There are reasons to believe that melatonin, in addition to inducing the synthesis of NO as a signalling mediator necessary for the activation of stress-protective systems, can prevent the accumulation of excessive amounts of NO and the development of nitrosative stress in plants.

Apparently, there are complex direct and inverse relationships between melatonin and NO. In addition to the facts of melatonin modulation of NO synthesis discussed above, data have also been obtained on the ability of NO to act as a signal inducing melatonin synthesis. Thus, it has been shown that NO mediated by cGMP can activate the expression of genes for the melatonin synthesis enzymes TDC, T5H, SNAT, and COMT, which leads to an increase in the endogenous level of melatonin (Wang et al., 2022a; Aghdam and Arnao, 2024). The NO scavenger cPTIO was found to disrupt Cd-induced melatonin synthesis by decreasing the expression of TDC and COMT genes in rice (He and He, 2020). Thus, plants may possess mechanisms for the mutual enhancement of melatonin and NO synthesis. At the same time, melatonin, which is synthesized in chloroplasts and mitochondria, may directly reduce the toxic effect of RNS in these organelles (Wang et al., 2022a).

Summarising the available data, it can be assumed that cytosolic Ca^{2+}- and ROS-dependent enhancement of NO formation is a tool for melatonin activation of other components of the signalling network (in particular, the MAP-kinase cascade) and subsequent changes in the expression of genes that control rather universal stress-protective systems (antioxidant complex, synthesis of osmoprotectants, secondary metabolites, stomatal closure, etc (He and He, 2020; Khan et al., 2023; Plokhovska et al., 2023; Aghdam and Arnao, 2024) (Figure 4). On the other hand, the functional interaction between melatonin and NO also includes mechanisms that limit the effects of NO, in particular the formation of NOMela conjugate, as well as the negative effect of melatonin on the gene expression of NO synthesis enzymes, the mechanisms of which remain unclear. Thus, melatonin may prevent the development of nitrosative stress in plants. At the same time, it remains unclear how the transition from a synergistic interaction between melatonin and NO to an antagonistic one can occur. The complexity of the functional interaction between melatonin and NO is further complicated by the ambiguous and not yet well-understood role of NOMela, which may be both a product of excess NO binding and a source of NO for S-nitrosation processes of some proteins (Figure 4).

Functional relationships of melatonin and hydrogen sulfide in plants

Hydrogen sulfide (H_2S) in animal cells is considered the third GT in terms of time of discovery and the importance after NO and CO (Liu et al., 2024). However, in the context of plant cell function, evidence for the role of H_2S and related signalling processes is accumulating more dynamically than for the action of CO. Numerous studies have shown that H_2S acts as a gaseous signalling molecule that enhances plant adaptation/tolerance to various abiotic stresses, including drought, salinity, temperature extremes, heavy metal effects, and other adverse factors (Zhang et al., 2021; Liu et al., 2024). H_2S has also been found to be directly or indirectly involved in a wide range of physiological processes that occur under normal conditions, including seed germination, root development, stomatal movement, and fruit ripening (Corpas and Palma, 2020). At the same time, H_2S is closely related to other components of the signalling network, primarily ROS, calcium ions, and NO (Corpas et al., 2022b; Khan et al., 2022; Liu et al., 2024). One of the most studied ways in which H_2S is involved in signalling processes is through a PTM of proteins known as persulfidation (Liu et al., 2024). This process involves the conversion of protein thiol groups (RSH) to persulfide groups (RSSH). It is suggested that such a reversible PTM is not only important for signalling processes but is also one of the mechanisms of the direct protective effect of H_2S on proteins under conditions of oxidative stress (Wang et al., 2021). The mechanism of protein persulfidation is not fully understood. It is believed that H_2S or its ionic forms, HS^- and S_2^-, cannot react directly with protein thiols. Such interaction requires the presence of oxidizing agents (Aroca et al., 2021).

Persulfidation is likely a part of the toolkit for gene expression regulation. A transcriptome study performed on *Arabidopsis* plants showed that treatment with exogenous H_2S induced significant changes in the expression of genes encoding different transcriptional regulators (Aroca et al., 2017). Persulfidation is also one of the mechanisms regulating the activity of several antioxidant enzymes, and it can cause both increased and decreased catalytic activity and protect protein molecules from oxidative degradation (Li et al., 2020; Shivaraj et al., 2020). In addition, the same enzymatic proteins can be targets for persulfidation and S-nitrosation, which is a prerequisite for functional interaction between H_2S and NO (Kolupaev et al., 2023b). For example, the possibility of cross-regulation of activity by persulfidation and nitrosation has been demonstrated for several molecular forms of ascorbate peroxidase (Kolupaev et al., 2023b; Li et al., 2024). It was also shown that persulfidation of two cysteine residues increased the activity of one of the molecular forms of NADPH oxidase (Shen et al., 2020), suggesting a functional interaction between H_2S and ROS in signalling processes. On the other hand, as mentioned above, NO can inhibit NADPH oxidase through S-nitrosylation. Thus, ROS, NO, and H_2S form a complex signalling and regulatory network that controls redox modifications of proteins (Kolupaev et al., 2023b).

Another integral component of such a network is calcium as a universal intracellular messenger. In particular, the possibility of persulfidation of Ca^{2+} channels with an increase in the concentration of H_2S in the submembrane space is considered (Li et al., 2024). In many works, the dependence of induction of physiological reactions by exogenous H_2S (its donors) on Ca^{2+} homeostasis has been shown by inhibitor methods (Li et al., 2012; Kolupaev et al., 2017).

Brief overview of H_2S synthesis in plants

In plant cells, H_2S synthesis occurs predominantly in chloroplasts and to a lesser extent in other subcellular spaces such as the cytoplasm and mitochondria (Pandey et al., 2024). L-cysteine desulfhydrase (LCD) is considered to be the major enzyme of H_2S synthesis. It is localised in cytoplasm, plastids and mitochondria as reported by (Riemenschneider et al., 2005). The synthesis of H_2S from D-cysteine is also possible through the action of D-cysteine

TABLE 2 Examples of the involvement of hydrogen sulfide in the realisation of the effect of melatonin on plant resistance to abiotic stresses.

Stressor	Plant species	Physiological effects of melatonin	Experimental evidence of H_2S involvement in melatonin effects	Source
High temperatures	*Triticum aestivum* L.	Increased plant heat tolerance, photosynthetic activity and carbohydrate content	Elimination of melatonin effects by H_2S scavenger hypotaurine	Iqbal et al., 2021
Drought	*Solanum lycopersicum* L.	Increased drought tolerance with increased activity of mitochondrial enzymes and increased synthesis of stress proteins HSP17.6 and HSP70	Elimination of melatonin effects by H_2S synthesis inhibitor propargylglycine	Khan et al., 2024a
	Arabidopsis thaliana L.	Activation of drought defence systems: increased expression of genes for the transcription factors CBF2, CBF3, RD29A, DREB2A and DREB2B, as well as genes related to K^+ channels of stomatal closing cells	Increase in endogenous H_2S levels and increase in expression of genes for H_2S synthesising enzymes, *LCD* and *DES1*. Almost complete absence of melatonin stress protective effect in *lcd*, *des1*, and *lcd/des1* mutants	Wang et al., 2023b
Salinisation	*S. lycopersicum*	Mitigation of stress-induced growth inhibition and membrane damage	Increase in activity and appearance of new L-DES isoforms, increase in H_2S content	Mukherjee and Bhatla, 2021
		Maintenance of K^+/Na^+ homeostasis in seedling roots, increase in antioxidant enzyme activity, mitigation of oxidative damage	Increase in L-DES activity and H_2S content. Elimination of melatonin protective effects by H_2S scavenger hypotaurine	Siddiqui et al., 2021
	Satureja hortensis L.	Mitigation of growth inhibition, maintenance of K^+/Na^+ homeostasis, protection of photosynthetic apparatus from toxic effects of ions, enhancement of essential oil biosynthesis	Elimination of melatonin protective effects by H_2S scavenger hypotaurine and their enhancement by H_2S donor	Khalofah et al., 2024
	T. aestivum	Mitigation of salinity stress by increased activity of antioxidant system and upregulated expression of Na^+ transport genes (*SOS1*, *SOS2*, *SOS3*, *NHX1*), improved photosynthetic parameters	Elimination of melatonin effects by H_2S synthesis inhibitor propargylglycine	Khan et al., 2024c
Combined effects of drought and salinity	*S. lycopersicum*	Activation of enzymatic and non-enzymatic antioxidant systems, accumulation of osmolytes, maintenance of ion homeostasis	Elimination of melatonin protective effects by H_2S scavenger hypotaurine, synergistic effect of melatonin and H_2S donor on functioning of stress-protective systems	Khan et al., 2024b
As toxicity	*Capsicum annuum* L.	Mitigation of As-induced growth inhibition and oxidative stress, As localisation in vacuoles	Increased H_2S content, reduced protective effect of melatonin by H_2S scavenger hypotaurine and its enhancement by H_2S donor NaHS	Kaya et al., 2022
	S. lycopersicum	Reduction of As uptake, activation of the synthesis of phenolic and flavonoid compounds and phytochelatins	Inhibition of melatonin effects by hypotaurine and their enhancement by H_2S donor NaHS	Ghorbani et al., 2024
Cr toxicity	*S. lycopersicum*	Enhancement of phytochelatin synthesis, increase in H^+-ATPase activity, stabilisation of ionic homeostasis	Inhibition of melatonin effects by H_2S scavenger hypotaurine	Khan et al., 2023
Pb toxicity	*Carthamus tinctorius* L.	Decrease in stress-induced lipoxygenase activation, decrease in ROS and lipid peroxidation products; increase in antioxidant enzyme activity and increase in phytochelatins synthesis	Enhancement of melatonin effects by H_2S donor NaHS	Haghi et al., 2022

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desulfhydrase localised in the cytoplasm (Guo et al., 2016). In recent years, the desulfhydrase AtDES1 has been considered another novel H_2S synthesising enzyme in the *Arabidopsis* cytoplasm, which is believed to be a protein similar to cysteine synthase, but mainly exhibits activity related to the degradation of L-cysteine, accompanied by the formation of H_2S (Zhang et al., 2021). Experimental evidence has been provided for the role of this enzyme in the synthesis of H_2S in response to abiotic stresses, particularly drought (Zhang et al., 2021).

In chloroplasts, H_2S can also be synthesized by sulfite reduction involving sulfite reductase when the photosynthetic sulfate assimilation pathway is active (Pandey et al., 2024). In mitochondria, H_2S formation is possible during cyanide detoxification. This process is mediated by β-cyanoalanine synthase, an enzyme that catalyses the conversion of cyanide to β-cyanoalanine with the consumption of cysteine (Feng et al., 2022). The putative compartmentalisation of the synthesis of a significant pool of H_2S in mitochondria and chloroplasts, compartments in which melatonin is mainly synthesised, is considered the basis for studying the functional interaction between melatonin and H_2S (Wang et al., 2022b).

Involvement of H_2S in realisation of stress-protective effect of melatonin on plants

At present, there is a lot of data indicating the participation of H_2S as a mediator in the realisation of the protective effects of melatonin when plants are exposed to stress factors of various nature (Table 2). In particular, it was shown that the increase in heat resistance of wheat plants under the influence of melatonin was eliminated by the H_2S scavenger hypotaurine (Iqbal et al., 2021). The dependence of melatonin enhancement for drought tolerance in tomato plants on H_2S synthesis has also been reported (Khan et al., 2024a) (Table 2).

In *Arabidopsis* plants, molecular genetic methods have shown that H_2S is involved in many of the stress-protective effects of melatonin under drought (Wang et al., 2023b) (Table 2). It was also found that both melatonin and H_2S affected the level of transcription of genes associated with K^+ channels involved in maintaining stomatal closure (Wang et al., 2023b).

There is reason to believe that not only the effect of melatonin on stomatal status depends on H_2S synthesis, but conversely, H_2S-induced stomatal closure depends on melatonin synthesis (Wang

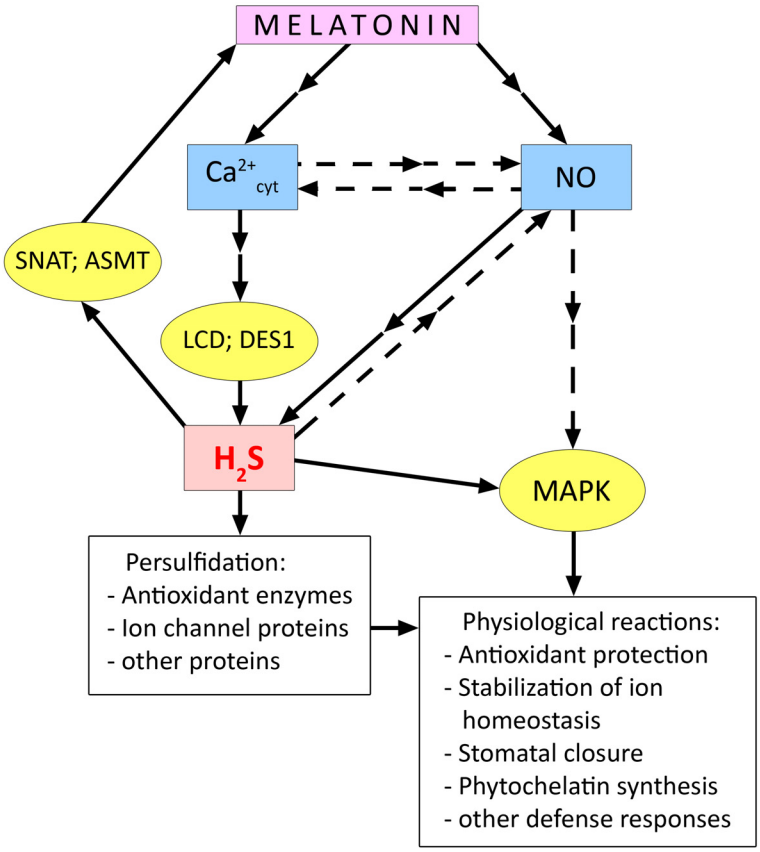

FIGURE 5
Relationship between melatonin and hydrogen sulfide during activation of plant cell stress defence systems. ASMT, N-acetylserotonin O-methyltransferase; DES1, cysteine synthase-like desulfhydrase 1; LCD, L-cysteine desulfhydrase; MAPK, mitogen activated protein kinases; SNAT, serotonin N-acetyltransferase. Dashed arrows indicate connections between signalling mediators without clear experimental evidence. Other explanations in the text.

et al., 2022a). For example, *Arabidopsis* plants showed that exogenous H_2S increased transcript levels of the melatonin synthesis enzymes SNAT, ASMT, and COMT1 in leaves and caused persulfidation of SNAT and ASMT. At the same time, H_2S was found to have little effect on the condition of stomata in mutants of the melatonin synthesis enzymes *snat*, *comt1*, and *asmt* (Wang et al., 2022a). These findings suggest that there may be some kind of signal amplifying loop between melatonin and H_2S (Figure 5).

This hypothesis is also supported by data on the synergistic interaction between melatonin and H_2S, obtained in the study of plant adaptation to various stresses. Thus, the involvement of endogenous H_2S and the LCD enzyme was found in the melatonin-induced increase in salt tolerance of tomato (Table 2). Synergism of the joint protective effect of melatonin and H_2S was also observed, which was expressed in the prevention of the development of oxidative cell damage by increasing the activity of antioxidant enzymes (Siddiqui et al., 2021). Data indicating the involvement of H_2S as a mediator in the realisation of stress-protective effects of melatonin were also obtained when studying its effect on salt tolerance of *Satureja hortensis* L. (Table 2).

The protective effect of melatonin on the state of antioxidant and osmoprotective systems of tomato plants under the combined action of drought and salinity was not manifested in the presence of the H_2S scavenger hypotaurine but was enhanced by simultaneous treatment of plants with a hydrogen sulfide donor (Khalofah et al., 2024; Table 2).

H_2S also appears to be involved in realising the protective effects of melatonin on pepper and tomato plants exposed to arsenic toxicity (Table 2). These studies (Kaya et al., 2022; Ghorbani et al., 2024) also found not only attenuation of the stress-protective effect of melatonin by the H_2S scavenger, but also enhancement of the protective effects when melatonin was used together with the H_2S donor NaHS.

Data were obtained on the participation of H_2S as a mediator in the development of specific protective reactions of plants induced by melatonin and in adaptation to the action of other heavy metals (Table 2). Thus, the melatonin-induced increase in the resistance of tomato seedlings was attenuated by an H_2S scavenger (Khan et al., 2023). Treatment of safflower plants with melatonin and the H_2S donor NaHS attenuated the toxic effect of lead (Haghi et al., 2022). The combined action of melatonin and NaHS was particularly effective (Table 2), with a significant increase in the activity of enzymes responsible for the ascorbate-glutathione cycle and increased synthesis of metal-binding ligands.

It is still unclear how H_2S as a participant of signal transduction induced by melatonin is related to other key signal mediators. At the same time, some experimental data suggest the role of interaction of H_2S with Ca^{2+} and NO in the manifestation of melatonin-induced stress-protective responses. Mukherjee et al. (2023) showed that enhancement of synthesis of H_2S required for melatonin to strengthen sunflower resistance to salt was suppressed in the presence of the Ca^{2+} chelator EGTA and the Ca^{2+} channel blocker verapamil. The involvement of Ca^{2+} signalling in the activation of H_2S synthesis was also confirmed in the study of melatonin activation of seed germination in plants of different species (Wang et al., 2024).

Exogenous melatonin caused an increase in salt tolerance of cucumber seedlings, improved the function of the photosynthetic apparatus and antioxidant system, and increased MAPK activity under stress conditions (Sun et al., 2021). All the above-mentioned stress-protective effects of melatonin were eliminated by treating plants with NO scavenger (cPTIO) and H_2S scavenger (hypotaurine). In contrast, the MAPK inhibitor (U0126) had no effect on H_2S and NO synthesis. The authors suggest that H_2S acts as a mediator of the physiological effects of melatonin and is upstream of the MAPK cascade in the signalling chain (Figure 5). Kaya et al. (2020) provided evidence that the melatonin-induced increase in H_2S synthesis in pepper (*Capsicum annuum* L.) plants was mediated by an increase in NO levels. The authors found that the melatonin-induced increase in H_2S content was suppressed by the action of both the H_2S scavenger hypotaurine and the NO scavenger cPTIO. In contrast, hypotaurine did not eliminate the effect of increased NO in plants. This work also showed the involvement of NO and H_2S in the realisation of the protective effect of foliar treatment of pepper plants with melatonin under iron deficiency and salinity stress (Kaya et al., 2020).

Thus, few experimental data indicate the joint involvement of NO and H_2S in transducing melatonin-induced signals leading to the development of plant resistance to certain stresses such as salinity (Kaya et al., 2020; Sun et al., 2021) (Figure 5). At the same time, it has been shown that treatment of etiolated seedlings of *Triticum turgidum* L. with melatonin, which increases their resistance to cadmium, was accompanied by an increase in NO levels but a decrease in H_2S levels and the activity of its synthetic enzymes L- and D-cysteine desulfhydrases (Aloui et al., 2024). It is difficult to interpret such data on the antagonistic interaction between melatonin and H_2S because they are sporadic and may be due to specific features of the object of study or the experimental design. In this regard, it should be noted that a recent work by Zulfiqar et al. (2024) reported a synergistic effect of melatonin and the H_2S donor NaHS on the resistance of *Matthiola incana* L. plants to the toxic effect of cadmium. When used together, melatonin and H_2S donor more effectively protected plants from the development of oxidative stress by increasing the activity of antioxidant enzymes and one of the key enzymes of the synthesis of secondary metabolites phenylalanine ammonia-lyase.

Possible relationship of melatonin and carbon monoxide in plant adaptation to abiotic stresses

The mechanisms underlying the biological activity of CO are significantly different from those of NO and H_2S. It is assumed that they are largely due to the formation of coordination bonds between CO and metals in the active centres of proteins, primarily heme-containing proteins (Feelisch and Olson, 2013). In general, however, the question of the molecular targets of CO action involved in the manifestation of certain physiological effects in plants remains open. Nevertheless, it is now clear that CO has a positive effect on seed germination, root development, fruit ripening

and maturation, and stomatal closure (Cao et al., 2007; Zhang et al., 2014; Gahir et al., 2020; Hong et al., 2024). In recent years, the involvement of CO in the adaptation of plants to the action of stress factors of different nature and the possibility of increasing plant resistance with the help of CO donors have been studied particularly intensively (Yao et al., 2019; Kolupaev et al., 2022).

Hemoxygenase, which catalyses the stereospecific conversion of heme to biliverdin-IXα (BV-IXα) with the release of Fe^{2+} and CO, is considered to be the major enzyme responsible for CO synthesis in both animals and plants (Shekhawat and Verma, 2010). This reaction requires NADPH as an electron source and molecular oxygen (Bilban et al., 2008). Plant hemoxygenases are represented by a family of four genes, of which *HO-1* is the most highly expressed (Matsumoto et al., 2004). In plant cells, the enzymatic protein HO-1 is found in chloroplasts and mitochondria (Shekhawat and Verma, 2010; Dixit et al., 2014). Thus, the localisation of CO synthesis, as well as other GTs, coincides with the subcellular localisation of melatonin formation in plants.

The data on the mechanisms of CO signal transduction in the genetic apparatus of plant cells, as well as on its relationship with other signal mediators, are still insufficient. It is known that CO can bind to the Fe atom of the heme fragment of guanylate cyclase in animal cells, thereby activating the enzyme and the synthesis of the secondary intracellular messenger cGMP (He and He, 2014). The presence of guanylate cyclase activity and cGMP in cells has been demonstrated in a number of plant species in recent years. It has been suggested that CO, like NO (Neill et al., 2008), may affect Ca^{2+} homeostasis *via* cGMP.

There are also data in the literature suggesting a functional relationship between CO and NO. For example, enhancement of lateral root formation in rapeseed induced by CO donor treatment was dependent on NO synthesis (Cao et al., 2007). Root hair development under the action of exogenous CO was accompanied by an increase in endogenous NO (Guo et al., 2009). It was also shown that stomatal closure effect induced by exogenous CO was accompanied by an increase in NO in the closing cells and was eliminated by inhibitors of NO synthesis (Song et al., 2008). The increase in heat resistance of wheat seedlings induced by the CO donor hemin was accompanied by a transient increase in NO levels in root cells associated with NR activation (Shkliarevskyi et al., 2021). Subsequently, H_2O_2 generation was enhanced by activation of extracellular peroxidase. However, both the effect of increased NO and the effect of increased H_2O_2 were supressed by various Ca^{2+} antagonists. It has been suggested that Ca^{2+} and NO are upstream of ROS in the signalling chain when heat resistance of wheat seedlings is induced by CO (Shkliarevskyi et al., 2020; 2021).

The relationship between CO and melatonin in plants has not been experimentally confirmed until recently. A review by Wang et al. (2022a) suggested a possible effect of melatonin on *HO-1* gene expression by analogy with the existence of such a mechanism in animal cells. More recently, the involvement of CO as a signalling mediator in melatonin-induced drought tolerance in tomato was investigated (Liu et al., 2024). It was shown that melatonin-induced alleviation of the growth inhibitory effects of drought was eliminated by the CO scavenger hemoglobin. Hemoglobin also eliminated the beneficial effect of melatonin on the expression of several genes related to chlorophyll and heme synthesis. Thus, based on the inhibitor analysis data, the authors suggest that CO is involved in the realisation of the stress-protective effect of melatonin on plants under drought. However, there are no direct experimental evidence confirming the effect of melatonin on *HO-1* gene expression and activity of this enzyme in plants. The connection of CO as a possible mediator in the realisation of melatonin action with other signalling molecules (ROS, NO, Ca^{2+} ions), which, as mentioned above, are involved in the signal transduction of CO when it induces stress-protective reactions in plants, remains unexplored. The potential effect of CO on melatonin synthesis in plants also remains unexplored. However, it has been shown in animal models that CO can stimulate pineal gland cells to produce melatonin (Romerowicz-Misielak et al., 2018).

Conclusion and prospects

Melatonin is now considered a novel plant hormone and a promising regulator of plant growth and adaptation (Aghdam, and Arnao, 2024; Arnao et al., 2022). At the same time, as the authors of a recent review wittily pointed out (Aghdam and Arnao, 2024), only a decade and a half of intensive research has moved phytomelatonin from being studied as a key player in intracellular signalling to being a player in the global horticulture market. The number of articles on phytomelatonin in leading journals has increased from 33 in 2007 to over 500 in 2023 (Aghdam and Arnao, 2024).

The accumulated data show that melatonin exerts its stress-protective effect mainly through its involvement in the general signalling network of plant cells (Murch and Erland, 2021). Data have been obtained indicating the ability of melatonin to influence the state of Ca^{2+} channels, and the melatonin-induced increase in Ca^{2+} influx into the cytosol from the extracellular space and intracellular compartments may be one of the first stages of its involvement in the signalling network. Equally important appears to be the involvement of melatonin in ROS signalling. Despite the potent direct antioxidant effect of melatonin, its exogenous entry

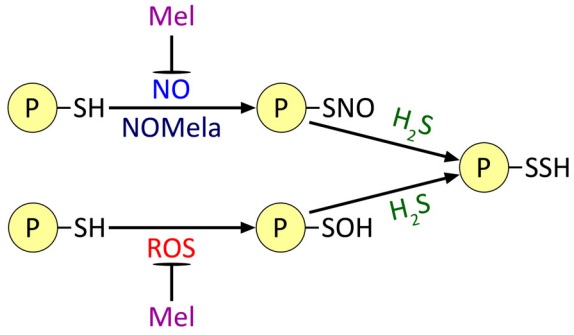

FIGURE 6
Possible effect of melatonin on the processes of post-translational modification of proteins by gasotransmitters and ROS. Mel, melatonin; NOMela, N-nitrosomelatonin; ROS, reactive oxygen species. Other explanations in the text.

into plant cells results in a transient increase in ROS generation, which is likely primarily associated with the activation of NADPH oxidase.

The involvement of GTs in plant cell signalling and adaptation to various stresses has become one of the main directions in experimental plant biology, along with the topic of melatonin (Yao et al., 2019; Kolupaev et al., 2022). However, it is only in the last few years that knowledge has begun to accumulate, which allows us to build, at least in a general way, models of the functional interaction between melatonin and GTs. Thus, in a review published two years ago (Wang et al., 2022a), the discussion of possible functional relationships between melatonin and GTs in plant cells was mainly based on analogies with the mechanisms of their interaction in animal cells. At present, experimental data have been obtained indicating that for the manifestation of many stress-protective effects of melatonin it is necessary to enhance NO synthesis in plants. Using inhibitor analysis and partly molecular genetic methods, information on the involvement of H_2S in the realisation of melatonin effects has also been obtained.

At the same time, the elucidation of the functional interaction of the two most important GTs, H_2S and NO, in the realisation of melatonin's regulatory effects is only beginning. The ability of both GTs to induce protein PTM and the ability of melatonin to bind NO and ROS, which react with thiol groups (Figure 6), suggest that melatonin may act as a regulator of the PTM of at least some proteins. However, the effects of melatonin on these processes are far from clear. For example, as mentioned above, NOMela formed by the interaction of NO with melatonin may be more efficient than NO in S-nitrosation reactions of thiol groups. On the other hand, the reaction product of melatonin with ROS 2-OHM can significantly activate one of the main ROS-generating enzymes, NADPH oxidase (Figure 3). Considering that groups previously subjected to S-nitrosation or oxidation are involved in persulfidation reactions by H_2S (Aroca et al., 2021), it is conceivable that melatonin may also play a prominent role in modulating the process of protein persulfidation. The hypothetical pathways outlined above only consider the direct interaction of melatonin with PTM inducers. However, there is no doubt that these *in vivo* processes are superimposed by the involvement of melatonin in the general signalling network and, as a consequence, its influence on the gene expression of many proteins. Unfortunately, the regulatory functions of melatonin as an agent potentially affecting protein PTMs involving ROS, NO and H_2S remain largely unexplored. Individual examples of proteins whose state is affected by melatonin and its derivatives (NADPH oxidase and glyceraldehyde-3-phosphate dehydrogenase) have been given above. On the other hand, the functional interaction of NO and H_2S GTs in protein PTM is better understood and there are considerably more examples of proteins regulated by them, including in a competitive manner (for reviews, see Arora and Bhatla, 2015; Correa-Aragunde et al., 2015; Corpas et al., 2019a, 2019; Mishra et al., 2021; Kolupaev et al., 2023b). Thus, a detailed study of the effect of melatonin on the processes of protein PTM by GTs may become one of the important areas of research that can contribute to the elucidation of the mechanisms of its stress-protective action in plant cells.

Finally, there is a clear lack of experimental data on the functional interaction between melatonin and CO in plant cells. The latter is a recognised GT of plant cells, but due to the lack of cheap and available CO donors, it has not yet found practical application in crop production. Nevertheless, elucidation of the role of CO as a likely link in the physiological effects of melatonin in plants is also necessary to fully understand the mechanisms of action of this new plant hormone.

Author contributions

YK: Formal analysis, Writing – original draft. AY: Methodology, Writing – review & editing, Writing – original draft. TY: Formal analysis, Writing – original draft, Writing – review & editing. YB: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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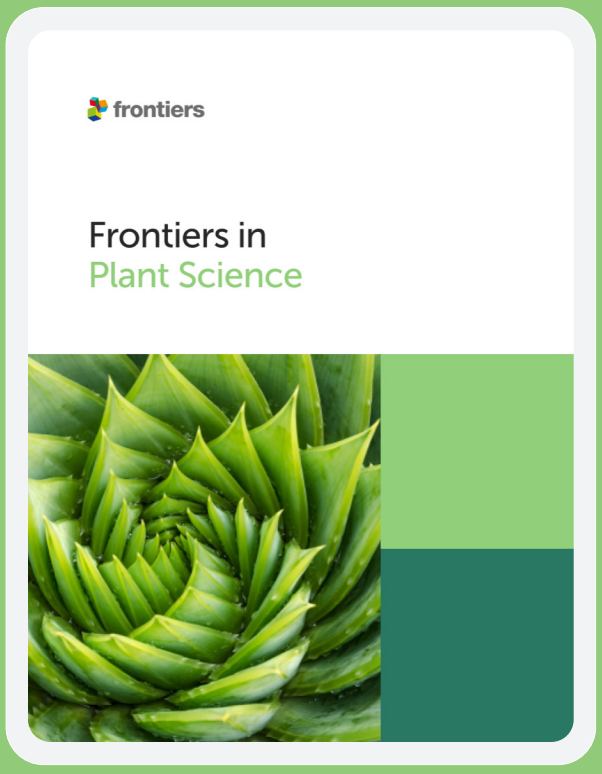

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