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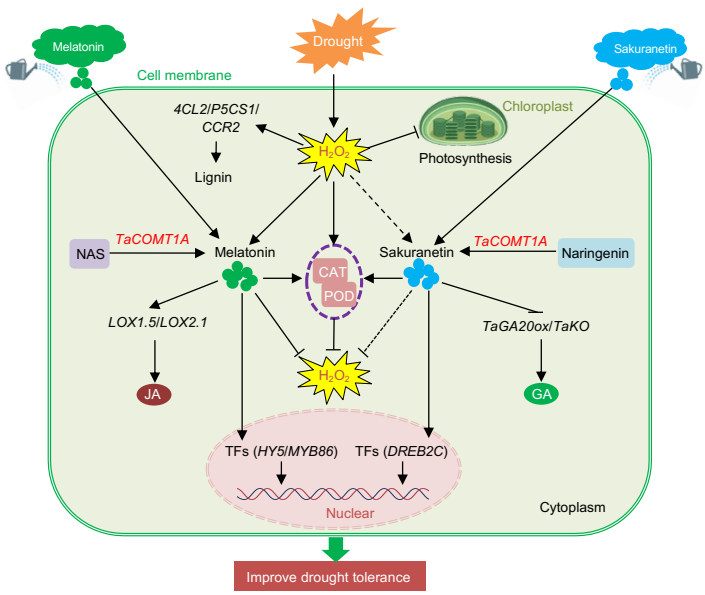
Drought tolerance in wheat: role of *TaCOMT1A* in melatonin and sakuranetin contents



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Drought tolerance in wheat: role of *TaCOMT1A* in melatonin and sakuranetin contents

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Promoteur(s) : Professeur Pierre Delaplace
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Abstract

Mingzhao Luo (2024). "Drought tolerance in wheat: role of *TaCOMT1A* in melatonin and sakuranetin contents" (PhD Dissertation in English).

Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liege.

183 pages, 48 figures, 13 tables.

Abstract: Drought stress significantly affects global food security, particularly in arid and semi-arid farming regions. Wheat (*Triticum aestivum L.*), one of the most critical staple crops worldwide, experiences substantial growth and yield losses under drought conditions. Caffeic acid O-methyltransferase (COMT) is a key enzyme in the phenylpropanoid metabolic pathway, playing a pivotal role in the biosynthesis of flavonoids, lignin, and melatonin, among other metabolites. Melatonin, a multifunctional signaling molecule, promotes plant growth and mitigates biotic and abiotic stresses, including drought, cold, and salinity. Sakuranetin, a methoxylated flavonoid functioning as a phytoalexin, is induced by environmental stimuli such as jasmonic acid (JA), UV light, and pathogen invasions. However, the role of COMT in wheat (*TaCOMT1A*) and the mechanisms by which melatonin and sakuranetin regulate drought tolerance remain poorly understood. This study systematically examines the multifunctionality of COMT and sakuranetin in plants and highlights recent advances in melatonin-mediated drought tolerance. To address key gaps, we designed three research directions:

(1) Overexpression of *TaCOMT1A* enhances drought tolerance in wheat. Transgenic wheat lines overexpressing *TaCOMT1A* exhibited enhanced drought tolerance during both seedling and maturation stages compared to wild-type (WT) plants. Under field drought conditions, these lines achieved a 15.21%–20.27% increase in grain weight per plant while exhibiting a 10.92%–12.47% reduction in plant height compared to WT. Metabolomic analysis revealed elevated levels of key flavonoids, including sakuranetin, isorhamnetin, vitexin, kaempferol, and narcissin in the overexpression lines. Biochemical studies showed that *TaCOMT1A* directly synthesizes sakuranetin and melatonin, underscoring its multifunctional enzymatic activity in drought tolerance.

(2) Melatonin enhances wheat drought tolerance. Exogenous application of melatonin (100 μM) significantly improved drought tolerance in wheat varieties such as "Chinese Spring," "Shi4185," and "Hanxuan10," but not "Chang6878." Transcriptomic and proteomic analyses demonstrated that melatonin enhances JA biosynthesis by upregulating *LOX1.5*, *LOX2.1*, and transcription factors *HY5* and *MYB86*. It also increased lignin biosynthesis (*4CL2*, *P5CS1*, *CCR2*) and starch metabolism (*PME53*, *SUS4*), while mitigating oxidative damage by maintaining low hydrogen peroxide levels. These findings suggest that melatonin enhances drought resilience by upregulating JA and lignin biosynthesis and offers a potential eco-friendly biostimulant for improving wheat productivity.

(3) Sakuranetin reduces plant height and improves drought tolerance. Exogenous application of sakuranetin also conferred drought tolerance and reduced plant height

in conventional wheat varieties. Transcriptomic analysis indicated that sakuranetin alleviates drought stress by upregulating photosynthesis-related genes and downregulating gibberellic acid (GA) biosynthesis genes, such as *TaGA2ox-5B* and *TaKO-7A*. Additionally, sakuranetin enhanced drought tolerance by upregulating the key drought-responsive gene *TaDREB2C-1A*. These results highlight sakuranetin as a promising candidate for improving drought tolerance and regulating plant height.

Overall, this study elucidates the role of *TaCOMT1A* in flavonoid biosynthesis and drought resilience, highlighting its enzymatic activities in producing melatonin and sakuranetin. It also establishes the potential of melatonin and sakuranetin as eco-friendly agents to enhance wheat drought tolerance and productivity, paving the way for sustainable agricultural practices under water-limited conditions.

Keywords: melatonin, sakuranetin, drought, caffeic acid O-methyltransferase, wheat, jasmonic acid, gibberellic acid

Résumé

Mingzhao Luo (2024). "Tolérance à la sécheresse chez le blé: rôle de *TaCOMT1A* dans les teneurs en mélatonine et sakuranétine" (Thèse de doctorat en anglais).

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183 pages, 48 figures, 13 tableaux.

Résumé: Le stress dû à la sécheresse a un impact significatif sur la sécurité alimentaire mondiale, en particulier dans les régions agricoles arides et semi-arides. Le blé (*Triticum aestivum L.*), l'une des cultures vivrières les plus importantes au niveau mondial, subit des pertes considérables de croissance et de rendement sous des conditions de sécheresse. La caféate O-méthyltransférase (COMT) est une enzyme clé de la voie métabolique des phénylpropanoïdes, jouant un rôle essentiel dans la biosynthèse des flavonoïdes, de la lignine et de la mélatonine, entre autres métabolites. La mélatonine, une molécule de signalisation multifonctionnelle, favorise la croissance des plantes et atténue les stress biotiques et abiotiques, notamment la sécheresse, le froid et la salinité. La sakuranétine, un flavonoïde méthoxylé fonctionnant comme phytoalexine, est induite par des stimuli environnementaux tels que l'acide jasmonique (JA), les rayons UV et les invasions pathogènes. Cependant, le rôle de la COMT chez le blé (*TaCOMT1A*) et les mécanismes par lesquels la mélatonine et la sakuranétine régulent la tolérance à la sécheresse restent mal compris. Cette étude examine de manière systématique la multifonctionnalité de la COMT et de la sakuranétine chez les plantes et met en lumière les progrès récents dans la tolérance à la sécheresse médiée par la mélatonine. Pour combler les lacunes existantes, nous avons conçu trois axes de recherche:

(1) La surexpression de *TaCOMT1A* améliore la tolérance à la sécheresse chez le blé. Les lignées transgéniques de blé surexprimant *TaCOMT1A* ont montré une tolérance accrue à la sécheresse aux stades de la plantule et de la maturation, comparativement aux plantes de type sauvage (WT). Dans des conditions de sécheresse sur le terrain, ces lignées ont obtenu une augmentation de 15,21 % à 20,27 % du poids des grains par plante, tout en affichant une réduction de 10,92 % à 12,47 % de la hauteur des plants par rapport au WT. L'analyse métabolomique a révélé des niveaux accrus de flavonoïdes clés, notamment la sakuranétine, l'isorhamnétine, la vitexine, le kaempférol et la narcissine dans les lignées surexprimées. Des études biochimiques ont montré que *TaCOMT1A* synthétise directement la sakuranétine et la mélatonine, soulignant son activité enzymatique multifonctionnelle dans la tolérance à la sécheresse.

(2) La mélatonine améliore la tolérance à la sécheresse chez le blé. L'application exogène de mélatonine (100 μM) a significativement amélioré la tolérance à la sécheresse chez des variétés de blé telles que « Chinese Spring », « Shi4185 » et « Hanxuan10 », mais pas chez « Chang6878 ». Les analyses transcriptomiques et protéomiques ont démontré que la mélatonine

stimule la biosynthèse du JA en régulant à la hausse *LOX1.5*, *LOX2.1* et les facteurs de transcription *HY5* et *MYB86*. Elle augmente également la biosynthèse de la lignine (*4CL2*, *P5CS1*, *CCR2*) et le métabolisme de l'amidon (*PME53*, *SUS4*), tout en atténuant les dommages oxydatifs en maintenant de faibles niveaux de peroxyde d'hydrogène. Ces résultats suggèrent que la mélatonine améliore la résilience à la sécheresse en régulant à la hausse la biosynthèse du JA et de la lignine, offrant un biostimulant écologique potentiel pour améliorer la productivité du blé.

(3) La sakuranétine réduit la hauteur des plants et améliore la tolérance à la sécheresse. L'application exogène de sakuranétine a également conféré une tolérance à la sécheresse et réduit la hauteur des plants chez plusieurs variétés conventionnelles de blé. L'analyse transcriptomique a révélé que la sakuranétine atténue le stress hydrique en régulant à la hausse les gènes liés à la photosynthèse et en régulant à la baisse ceux liés à la biosynthèse de l'acide gibbérellique (GA), tels que *TaGA2ox-5B* et *TaKO-7A*. De plus, la sakuranétine a renforcé la tolérance à la sécheresse en régulant à la hausse le gène clé *TaDREB2C-1A*. Ces résultats positionnent la sakuranétine comme un candidat prometteur pour améliorer la tolérance à la sécheresse et réguler la hauteur des plants.

Cette étude élucide le rôle de *TaCOMT1A* dans la biosynthèse des flavonoïdes et la résilience à la sécheresse, mettant en évidence ses activités enzymatiques dans la production de mélatonine et de sakuranétine. Elle établit également le potentiel de la mélatonine et de la sakuranétine comme agents écologiques pour renforcer la tolérance à la sécheresse et la productivité du blé, ouvrant la voie à des pratiques agricoles durables dans des conditions de ressources hydriques limitées.

Mots-clés: mélatonine, sakuranétine, sécheresse, caféate O-méthyltransférase, blé, acide jasmonique, acide gibbérellique

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Mingzhao Luo

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2-ODD	2-oxoglutarate-dependent dioxygenase
2-OHM	2-hydroxymelatonin
3-OHM	3-hydroxymelatonin
4-OHM	4-hydroxymelatonin
6-OHM	6-hydroxymelatonin
ABA	abscisic acid
AFMK	N1-acetyl-N2-formyl-5-methoxyknuramine
AMF	arbuscular mycorrhizal fungi
AMK	N-acetyl-5-methoxyknuramine
APX	ascorbate peroxidase
ASA	ascorbic acid
ASDAC	N-acetylserotonin deacetylase
ASMT	N-acetylserotonin methyltransferase
BR	brassinosteroids
CAT	catalase
COMT	caffeic acid O-methyltransferase
CTK	cytokinin
DAP	differentially abundant protein
DAS	day after sowing
DEG	differentially expressed genes
DREB	dehydration responsive element binding
DS	drought sensitive
DT	drought tolerance
eggNOG	evolutionary genealogy of genes: non-supervised orthologous groups
ET	ethylene
FASP	filter-aided sample preparation
FDR	false discovery rate
FLC	flowering locus C
FT	flowering locus T
GA	gibberellic acid
GAMT1	GA methyl transferase 1
GMP	geometric mean productivity
GO	gene ontology
GPCR	G protein-coupled receptor

GPX	glutathione peroxidase
H_2O_2	hydrogen peroxide
HIOMT	hydroxyindole-O-methyltransferase
HPLC	high-performance liquid chromatography
IAA	indole-3-acetic acid
IDO	indoleamine 2,3-dioxygenase
IPA	indole-3-pyruvate
IPTG	isopropyl-β-d-thiogalactoside
IR	increase ratio
JA	jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
K_m	Michaelis constant
KOG	eukaryotic orthologous groups
LC-MS/MS	liquid chromatography tandem mass spectrometry
LIR	limited irrigation
M2H	melatonin 2-hydroxylase
M3H	melatonin 3-hydroxylase
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MT	melatonin receptor
NAS	N-acetylserotonin
NOMT	7-O-methyltransferase
NR	non-redundant protein sequence database
PBZ	paclobutrazol
PCA	principal component analysis
PGRs	plant growth regulators
PMTR1	phytomelatonin receptor
POD	peroxidase
PS	photosystems
qRT-PCR	real-time fluorescence quantitative polymerase chain reaction
RNS	reactive nitrogen species
ROS	reactive oxygen species
SA	salicylic acid
SAM	S-adenosylmethionine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNAT	serotonin N-acetyltransferase

SOD	superoxide dismutase
SPSS	statistical package for the social sciences
SSI	stress susceptibility index
STI	stress tolerance index
SM	secondary metabolite
T5H	tryptamine 5-hydroxylase
TAA	tryptophan aminotransferase of Arabidopsis
TDC	tryptophan decarboxylase
TFs	transcription factors
UPLC-MS/MS	ultra-high-performance liquid chromatography-tandem mass spectroscopy
UV	ultraviolet
V_{max}	maximum reaction rate
VOC	volatile organic compounds
WIR	well-irrigated
WT	wild type
YUC	YUCCA, The *YUCCA* gene encodes a flavin monooxygenase-like enzyme

Chapter 1

Research background and current advances

This chapter summarizes the advances in drought tolerance, caffeine acid O-methyltransferase (COMT), melatonin, and sakuranetin in plants, especially in wheat. The contents of 1.3 were accepted in the *Tropical Plants* journal.

Abstract: Drought stress is a major environmental constraint affecting crop growth, development, and yield worldwide. Plants employ complex adaptive mechanisms, including the production of secondary metabolites and phytohormones, to mitigate drought-induced damage. Caffeic acid O-methyltransferase (COMT) is a pivotal enzyme involved in the biosynthesis of lignin, flavonoids, and melatonin, which are crucial for plant structural integrity and stress responses. Despite its known roles in other species, the functional characterization and enzymatic mechanisms of COMT in wheat (*Triticum aestivum* L.) under drought stress remain poorly understood. Melatonin, a multifunctional signaling molecule, enhances plant tolerance to abiotic and biotic stresses by scavenging reactive oxygen species (ROS), regulating germination, root development, stomatal closure, leaf senescence, and osmotic homeostasis. However, its molecular mechanisms and regulatory pathways in wheat drought tolerance have yet to be elucidated. Similarly, sakuranetin, a key flavonoid known to be induced by jasmonic acid, ultraviolet radiation, and pathogen attack, plays significant roles in plant defense and stress adaptation. Nevertheless, its broader biological functions and regulatory mechanisms, particularly in wheat, remain largely unexplored. This chapter comprehensively reviews the roles of COMT, melatonin, and sakuranetin in plant responses to drought stress, highlighting recent advances in their functional and mechanistic understanding. Additionally, it identifies key knowledge gaps and proposes scientific questions to investigate the contributions of these molecules to drought tolerance in wheat. This work aims to establish a foundation for future research and foster a deeper understanding of the molecular networks underlying wheat resilience to water deficit conditions.

Keywords: caffeic acid O-methyltransferase, melatonin, sakuranetin, drought, wheat

1.1. Recent advances in drought tolerance in wheat

1.1.1. Introduction

Drought stress remains one of the most significant environmental challenges to agricultural productivity, drastically reducing plant growth, development, and yield. Wheat (*Triticum aestivum* L.), as one of the most important staple crops globally, is particularly vulnerable to water deficit conditions. Approximately 36% of the world's land area and 43% of its arable land are classified as arid or semi-arid regions, emphasizing the scale of this challenge (Gupta et al. 2020). Moreover, projections suggest that by 2050, agricultural water demand could double, while freshwater availability is expected to decline by 50% due to climate change (Ullah et al. 2018). This alarming scenario underscores the critical need for understanding and enhancing drought tolerance in crops to secure food production for the rapidly growing global population. Drought tolerance in plants is a multifaceted trait encompassing two primary strategies: drought avoidance and dehydration tolerance (Avramova et al. 2015). Drought avoidance involves traits such as deep root systems, efficient water use, and adaptations to exploit available rainfall. Conversely, dehydration tolerance refers to a plant's capacity to endure desiccation and resume growth upon rehydration. At the reproductive level, drought stress disrupts critical developmental processes, leading to smaller spikes, reduced grain number, and lower grain weight, culminating in significant yield losses (Farooq et al. 2014; Guo et al. 2021). These impacts on agronomic traits highlight the urgency of developing drought-resilient wheat varieties to safeguard global food security.

Wheat production is particularly vulnerable to drought stress compared to other cereals, such as maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.), especially as most wheat is cultivated in rain-fed regions with limited irrigation infrastructure (Nyaupane et al. 2024). Rising temperatures, declining water resources, and increasing climate variability compound these vulnerabilities, posing a significant threat to wheat yields (Adel and Carels 2023). Moreover, adaptations such as accelerated reproductive stages, evidenced by earlier booting and heading under limited irrigation, are critical for improving drought resilience (Chowdhury et al. 2021). These adaptations underscore the importance of breeding programs targeting drought-responsive traits to enhance wheat's productivity and stability under water-limited conditions.

Plants have evolved a suite of complex drought response mechanisms across morphological, physiological, and biochemical domains to maintain growth and development under water deficit conditions. Morphological adaptations, such as increased root-to-shoot ratios, smaller leaves, reduced plant height, and earlier maturity, enhance water uptake efficiency and minimize water loss. Physiological responses, including the accumulation of osmoprotectants (e.g., proline and mannitol) and stress hormones like abscisic acid (ABA), are pivotal in mitigating the impacts of drought stress (Kumar et al. 2018). At the biochemical level, plants

enhance antioxidant defense systems by increasing the activity of enzymes such as superoxide dismutase (SOD) and catalase (CAT) to mitigate oxidative stress caused by reactive oxygen species (ROS) (Liu et al. 2022b). Additionally, the synthesis of soluble proteins and metabolites helps maintain cell membrane integrity and reduces ROS-induced damage (Seleiman et al. 2021; Juenger and Verslues 2023; Jiao et al. 2023).

Recent advances in genomics, transcriptomics, and biotechnology have facilitated deeper investigations into the molecular mechanisms underpinning plant drought responses. Researchers have identified numerous drought-responsive genes and regulatory pathways with significant potential for application in modern crop breeding programs (Takahashi et al. 2018; Xu et al. 2021; Tang et al. 2023). These studies have revealed how plants modulate the production of secondary metabolites (SMs) and phytohormones, such as ABA, melatonin, and flavonoids, to enhance drought tolerance. For instance, genes associated with osmotic adjustments, antioxidant systems, and hormone signaling pathways play pivotal roles in conferring drought resistance (Wei et al. 2020; Zhou et al. 2020b). In this study, we review the morphological, physiological, biochemical, and molecular strategies employed by plants to cope with drought stress, with a specific focus on wheat. This work aims to provide a comprehensive foundation for future research and breeding efforts targeting enhanced drought tolerance and sustainable crop production by summarizing recent progress in understanding wheat's adaptive mechanisms to water deficit conditions.

1.1.2. Plant response to drought in wheat

In plants, drought stress induces a wide range of morphological, physiological, biochemical, and molecular abnormalities, depending on the timing, severity, and developmental stage of water stress. These disruptions significantly impair crop growth and development, particularly during critical phenological stages such as tillering, jointing, booting, anthesis, and grain filling. Drought stress during these stages can result in yield losses of up to 69%, highlighting its crucial role in agricultural productivity. As one of the most significant environmental constraints on wheat production, drought necessitates the evolution of complex and integrated adaptive responses to sustain growth and yield under water-limited conditions. These responses encompass morphological changes, physiological adjustments, biochemical pathways, and molecular mechanisms enabling wheat plants to optimize water use efficiency, preserve cellular integrity, and reprogram metabolic activities (Figure 1-1). Understanding these adaptive strategies is essential for advancing the development of drought-resilient wheat varieties and addressing the global challenge of food security under changing climatic conditions. This section provides a comprehensive review of wheat's responses to drought stress, emphasizing the key traits and underlying mechanisms that confer tolerance to water scarcity.

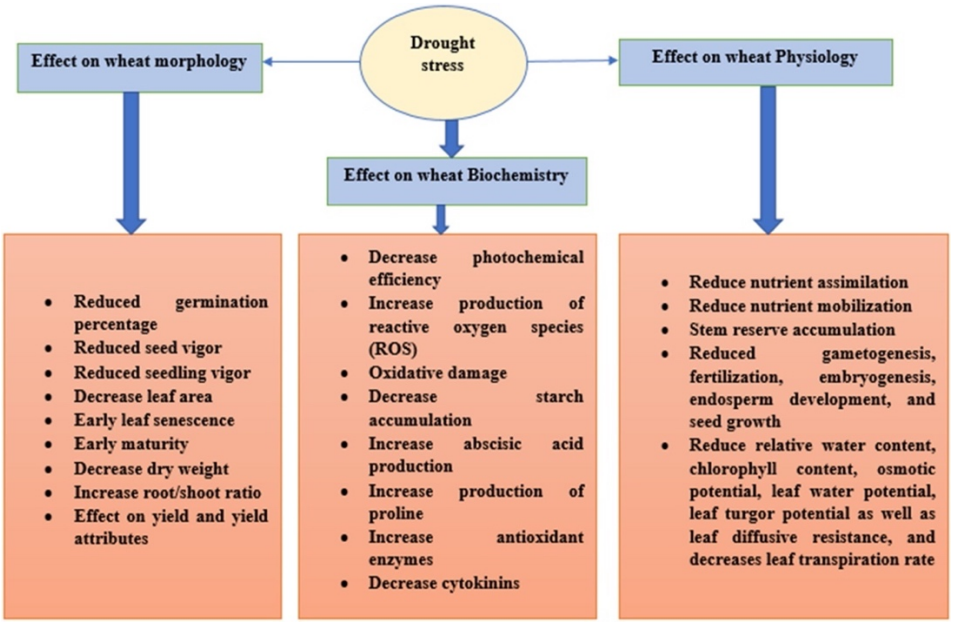


Figure 1-1 Effect of drought stress on wheat morphology, physiology, and biochemistry (Nyaupane et al. 2024).

1.1.2.1. Morphological changes of wheat under drought conditions

Morphological adjustments are among wheat plants' first lines of defense to cope with drought stress. One of the most apparent morphological responses is the reduction in leaf area. Wheat plants produce smaller and narrower leaves, reducing the surface area available for transpiration and conserving water (Yang et al. 2023). Furthermore, leaf rolling and folding are critical adaptations that protect the plant from excessive heat and minimize water loss by decreasing exposure to solar radiation (Jenks et al. 2001). While advantageous for water conservation, these modifications can limit photosynthetic activity, creating a trade-off between survival and growth.

Another vital morphological change is the thickening of leaves, often associated with enhanced cell wall development. This adaptation supports water retention and strengthens the plant's structural integrity under stressful conditions (Khasin et al. 2021). Moreover, specific wheat cultivars exhibit delayed leaf senescence, maintaining photosynthetic activity for extended periods and contributing to yield stability despite prolonged drought conditions (Pirasteh-Anosheh et al. 2016; Martínez-Vilalta and Garcia-Forner 2017; Liu et al. 2022b). Additionally, wax deposition on leaf surfaces reduces cuticular water loss, further enhancing drought tolerance (Jenks et al. 2001). These changes collectively improve the plant's ability to withstand water-limited environments by reducing water loss through leaf structure and function modifications.

The root system architecture also undergoes significant modifications in response to drought. Wheat plants develop deeper, more extensive root systems to access water from deeper soil layers (Rabello et al. 2008; Altaf et al. 2022). This adaptation is often accompanied by an increase in the root-to-shoot ratio, reflecting a strategic reallocation of biomass to prioritize water uptake over shoot growth (Altaf et al. 2022). Enhanced lateral root proliferation and root hair development increase the root's absorptive surface area, facilitating improved water extraction under drought conditions. These root-related traits are essential targets for breeding programs to enhance wheat drought tolerance.

1.1.2.2. Physiological and biochemical changes of wheat under drought conditions

Physiological and biochemical responses are central to wheat's ability to survive and maintain metabolic activities under drought stress. A key physiological response is the regulation of stomatal conductance, which minimizes water loss through transpiration while balancing CO_2 uptake for photosynthesis (Ahmad et al. 2022). However, stomatal closure under severe drought conditions can significantly impair photosynthetic activity, reducing biomass accumulation and yield (Ren et al. 2021).

Drought stress also induces oxidative stress in plants due to the overproduction of ROS, which can damage lipids, proteins, and nucleic acids (Cruz De Carvalho 2008). Wheat plants activate a sophisticated antioxidant defense system to mitigate these effects (Ma et al. 2014; Baozhu et al. 2022). Enzymatic antioxidants, including SOD, CAT, and peroxidase (POD), play pivotal roles in scavenging ROS and maintaining cellular redox homeostasis. For example, SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide, which is subsequently detoxified by CAT and POD (Ajithkumar and Panneerselvam 2014; Zhou et al. 2020a). Non-enzymatic antioxidants, such as glutathione (GSH), ascorbate (vitamin C), and α-tocopherol (vitamin E), complement enzymatic defenses by neutralizing ROS and protecting cellular components from oxidative damage (Hashmi et al. 2023). Additionally, wheat accumulates polyphenolic compounds, including flavonoids and phenolic acids, which possess potent antioxidant properties and contribute to overall stress tolerance (Ma et al., 2014; Baozhu et al., 2022).

Another critical biochemical response involves the accumulation of osmoprotectants, such as proline, glycine betaine, and soluble sugars. These molecules act as osmolytes, maintaining cellular turgor and protecting cellular structures under dehydration (Sharifi and Mohammadkhani 2016; Maghsoudi et al. 2016; Mathobo et al. 2017). Furthermore, drought stress triggers the production of detoxification metabolites and the enhancement of cell wall integrity, which collectively support plant survival under water-deficit conditions.

1.1.2.3. Molecular mechanism of drought tolerance in wheat

At the molecular level, drought tolerance in wheat is mediated by a network of signaling pathways, transcriptional regulators, and stress-responsive genes. Drought perception initiates a complex signaling cascade mediated by phytohormones like ABA, ethylene, and jasmonic acid (JA) (Mizoguchi et al. 2010; Cui et al. 2018; Kour and Zhawar 2018). ABA is particularly central to drought signaling. ABA

perception and signaling activate transcription factors such as AREB/ABFs and DREBs, which regulate the expression of genes involved in osmotic adjustment, antioxidant defenses, and cellular protection (Zhou et al. 2020b; Mei et al. 2022).

Aquaporins, which facilitate water transport across cellular membranes, are another key component of wheat's molecular response to drought. Aquaporins optimize water uptake and distribution by regulating water movement within the plant, ensuring that critical tissues receive adequate hydration (Adel and Carels 2023). SMs, including flavonoids, alkaloids, and terpenoids, further support cellular homeostasis by scavenging ROS and stabilizing cellular structures under drought stress.

Recent advancements in genomics and transcriptomics have identified numerous quantitative trait loci (QTLs) and candidate genes associated with drought tolerance in wheat. These discoveries have provided valuable insights into the genetic basis of drought resilience, enabling the development of molecular markers for use in breeding programs. For instance, genes encoding late embryogenesis abundant (LEA) proteins and other osmoprotectants have been extensively studied for their roles in protecting cellular components during dehydration (Khan et al. 2019; Ali et al. 2020).

The advent of genome editing technologies, such as CRISPR/Cas9, has opened new avenues for enhancing drought tolerance in wheat. By targeting specific genes involved in drought adaptation, researchers can precisely modify traits to improve resilience under water-limited conditions. Additionally, integrative approaches combining transcriptomics, proteomics, and metabolomics hold promise for uncovering novel mechanisms of drought tolerance and accelerating the development of resilient wheat varieties.

1.1.3. Conclusion

Drought stress poses a critical challenge to global wheat production, with far-reaching implications for food security and agricultural sustainability. Wheat's adaptive responses to drought encompass a diverse range of morphological, physiological, biochemical, and molecular strategies, each contributing to its survival and productivity under water-limited conditions. Despite significant progress in understanding these adaptive mechanisms, developing drought-tolerant wheat cultivars remains a formidable challenge. The complexity of the wheat genome, coupled with the multifaceted nature of drought tolerance, underscores the need for interdisciplinary approaches integrating traditional breeding, modern genomics, and advanced biotechnological tools.

Emerging technologies, such as genome editing and high-throughput phenotyping, offer unprecedented opportunities to accelerate the breeding of drought-resilient wheat. By leveraging these tools and expanding our understanding of the genetic and physiological basis of drought tolerance, researchers can develop cultivars capable of sustaining global food security in the face of climate change and water scarcity. Integrating these insights into breeding programs will be critical for ensuring the resilience and sustainability of wheat production systems in the 21st century.

Drought-induced losses in wheat significantly impact farmers' livelihoods, food

availability, and global economies, thereby threatening food security worldwide. However, recent advancements in genomic tools offer significant promise for enhancing wheat's drought tolerance. Exploring genetic diversity within wheat gene banks and identifying genome-wide quantitative trait loci (QTLs) have facilitated the discovery of potential drought-tolerant genes. Despite these advancements, the development of drought-resistant wheat cultivars has been limited, primarily because breeders have historically prioritized morphological traits over physiological drought tolerance.

Breeding for drought tolerance remains particularly challenging due to the complex nature of the wheat genome. Integrating physiological studies, omics technologies, and quantitative genetics is crucial for developing drought-tolerant wheat cultivars, although research initiatives adopting this comprehensive approach still need to be developed. Recent evaluation and trait breeding advances, led by novel genomic techniques, offer considerable potential for producing superior wheat cultivars. Functional genomics has revealed key drought-signaling molecules, aiding in identifying critical genes for future breeding efforts. Furthermore, CRISPR/Cas9 genome-editing technology presents opportunities for a deeper exploration of drought tolerance pathways. Integrating omics data with morpho-physiological responses is essential for accelerating the development of drought-tolerant wheat cultivars.

1.2. Multifunction of caffeic acid O-methyltransferase in plants

1.2.1. Introduction

In plants, the second metabolites, including flavonoids, lignin, and stilbenes, were O-methylated by converting hydroxy groups into methoxy groups to enhance their membrane penetration and bioavailability (Morreel et al. 2004; Du et al. 2015). O-methylation is a prevalent biochemical modification in plants that significantly impacts metabolites' physicochemical and functional properties. In addition, O-methylation of plant metabolites is usually catalyzed by S-adenosyl L-methionine (SAM)-dependent O-methyltransferases (OMTs). SAM is a methyl group donor in the methylation phase (Bureau et al. 2007; Balasubramani et al. 2021). This process can influence their biological activity, stability, and subcellular localization. For instance, the O-methylation of anthocyanins has been shown to enhance their structural strength and modify their chromatic properties, thereby contributing to the variation in pigmentation across plant tissues, including petals, seeds, and fruits. All the OMTs in plants were divided into two types according to their protein sequence homology, molecular weight, and cation dependency. Type I OMTs have low subunit sizes of 23–27 kD and are cation-dependent for activity; type II has high subunit sizes (38–43 kD) and do not require divalent cations (Lu et al. 2022). The caffeic acid OMTs (COMT) and 7-O-methyltransferase (NOMT) belong to type II OMT, and they can O-methylate the hydroxyl of multiple different substances (Zhang et al. 2021). The multifunctionality and diversity of OMTs are pivotal in synthesizing and

modifying plant metabolites, which play vital roles in growth and stress tolerance.

COMT, also called 5-hydroxyconiferaldehyde O-methyltransferase (CAldOMT), is a large gene family conserved in amino acid sequences (Figure 1-2) (Lam et al. 2024). In addition, previous studies have demonstrated that COMT has multifunction to generate different products by identifying different substrates (Wang et al. 2019b). Some COMT promotes biosynthesis of other compounds, including flavonoids, anthocyanins, lignins, etc., in the phenylpropanoid pathway, which helps plants to enhance lodging and stress tolerance by regulating cell wall, osmotic substances, and antioxidant enzyme activities (Liang et al. 2022). The cell walls of plants comprise primary and secondary cell walls according to the arrangement, flexibility, and structure of matrix polymers and the organization of microfibrils. The cell walls are mainly constructed of cellulose, which is crucial for providing shape and mechanical strength to withstand changing turgor pressures. Drought reduces the growth and productivity of plants by causing various physiological changes, including loss of turgor and reduce the flexibility of cell walls. In addition, a COMT involved in the biosynthesis of lignin can identify different substrates such as caffeic acid, 5-hydroxypinobanksyl aldehyde, and 5-hydroxypinobanksyl alcohol to produce other products such as ferulic acid, mustard aldehyde, and mustard alcohol, respectively (Li et al. 2022a). Recently, COMT was reported to increase grain yield (Huangfu et al. 2022), and increase abiotic stress tolerances, including salt, drought, and heat, etc, by involving in the biosynthesis of melatonin (Byeon et al. 2015; Zhao et al. 2021a). This section summarizes the enzyme activity of COMT and its function on drought tolerance in plants.

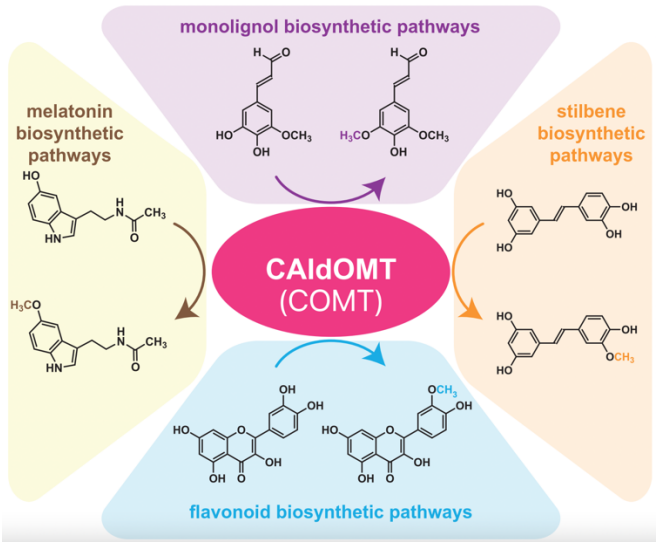


Figure 1-2 Current understanding of the roles of 5-hydroxyconiferaldehyde O-methyltransferases (CAldOMTs) in plant metabolism (Lam et al. 2024).

1.2.2. Function of COMT in plants

1.2.2.1. Biosynthesis of lignin

Vascular plants need lignin to provide mechanical support to cell walls, which allows plants to grow tall and transport water and nutrients during the entire life cycle (Freudenberg 1965; Vanholme et al. 2010). Moreover, plant cell expansion requires continuous deposition and modification of the cell wall. Lignin is an aromatic polymer with a three-dimensional network structure deposited in vascular plants' secondary cell walls (Weng and Chapple 2010). It is vital for lodging resistance, pathogen resistance, and abiotic resistance in plants (Jeon et al. 2023; Dwivedi et al. 2024). There are mainly three monolignols, including the units of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H), which are incorporated into lignin biomacromolecules (Barros et al. 2019). They have different degrees of meta-O-methylation at the 3 and 5 positions, and the type of monomers determines the lignin composition and influences the lignin properties in different plant lineages (Figure 1-3). For example, G-lignin and H-lignin monolignols were formed by 3-O-methylating coniferyl alcohol and a small amount of p-coumaryl alcohol, respectively, in both gymnosperms and angiosperms (Cesarino 2019). Additionally, S-lignin units were generated by incorporating 3,5-O-methylated sinapyl alcohol in angiosperm (Cesarino et al. 2016). Previous studies have shown that monolignol biosynthesis and polymerization to form lignin are tightly controlled in different cell types and tissues. Overall, the diversity of monomer types present determines the composition of lignin and significantly impacts its structural and functional properties across various plant lineages.

Many studies have demonstrated that COMT plays a crucial role in S-lignin biosynthesis in terrestrial plants (Abu-Omar et al. 2021). COMT-deficient mutants reduced the S/G unit ratio and decreased total lignin content. For example, *OsCOMT1* deficiency in rice (*Oryza sativa* L.) led to proportional reductions in *p*-coumaroylated and non-acylated S-lignin units, suggesting the involvement of COMT in S-lignin biosynthesis regardless of the acylation status of monolignols (Lam et al. 2019). In addition, expression of the *SmCOMT* from *Selaginella moellendorffii* increased S-lignin and sinapoyl glucose content, and it is a bifunctional phenylpropanoid O-methyltransferase that can methylate phenylpropanoid meta-hydroxyls at both the 3- and 5-position (Weng et al. 2011). Further studies demonstrated that COMTs participate in lignin biosynthesis by catalyzing different substrates in phenylpropanoid pathways, including the 3-O-methylation of caffeic acid, caffeyl-CoA, caffealdehyde, and caffeyl alcohol, as well as 5-O-methylation of 5-hydroxyferulic acid, 5-hydroxyferuloyl-CoA, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol, with aldehydes and alcohols as preferred substrates (Yokawa et al. 2014; Lu et al. 2022; Shamloo-Dashtpagerdi et al. 2022; Liang et al. 2022; Singh and Sharma 2023). Hence, the first and most widely studied function of COMTs is their function of O-methyltransferase to synthesize lignin.

Lignin is a key structural component of secondary cell walls in vascular plants, playing a critical role in plant growth, development, and mechanical support (Choi et al. 2023). In wheat, lignin accumulation has been shown to correlate negatively with lodging percentage and positively with stem strength and lodging resistance

index (FU et al. 2019). As a complex and heterogeneous polymer composed of phenylpropanoid subunits, lignin contributes to cell wall specialization, rendering them rigid and water-impermeable upon its deposition (Fujita et al. 2020; Ramachandran et al. 2021). Beyond its developmental functions, lignin is integral to plant defense responses against various biotic and abiotic stresses (Bhuiyan et al. 2009; Liu et al. 2020). While the relationship between lignin and drought stress has been observed in numerous plant species, many studies report lignin content and composition changes under drought conditions without delving into the molecular mechanisms involved (Sharma et al. 2020b). Recent evidence, however, highlights that increased lignin deposition, driven by transcriptional or enzymatic modifications, enhances drought tolerance (Figure 1-3) (Bang et al. 2019, 2022; Jung et al. 2022; Yadav and Chattopadhyay 2023). Master transcriptional regulators of lignin biosynthesis appear to be activated by drought stress and ABA through transcriptional regulation or post-translational modifications (Liu et al. 2021a, b; Chun et al. 2021). The accumulation of lignin in response to drought may result from deposition in newly formed xylem or pre-existing vascular cells, suggesting potential molecular mechanisms underpinning drought-induced xylem differentiation and lignin deposition (Lee et al. 2016; Ramachandran et al. 2021). Lignin deposition in vascular cells is essential for facilitating long-distance water transport and enabling plant recovery under drought conditions.

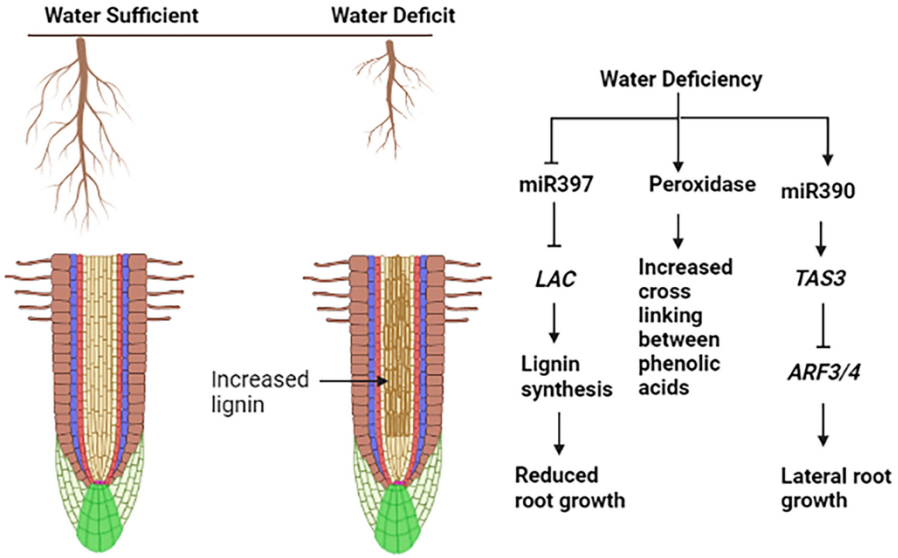


Figure 1-3 Plant's response to water deficit conditions (Yadav and Chattopadhyay 2023).

Plant response is regulated on several levels by phytohormones, microRNAs, and transcription factors. Plants exhibited shorter primary root lengths and fewer lateral roots in water-deficit conditions. Drought stress reduces the expression of miR397 in most of the crops. MiR397 targets laccase and leads to an increase in lignin production. Drought also

causes an increase in the activity of peroxidases. *MiR390* triggers TAS3 under stress, causing downregulation of auxin and the number of lateral roots. Note: *LAC = Laccase*, *ARF3/4 = Auxin response factors3/4*, *TAS3 = Trans-acting siRNA3*

In plants, lignin concentrations are notably higher in vascular tissues, particularly within the secondary cell walls, primary cell walls, and cell corners of xylem cells (Pesquet et al. 2019). It has been suggested that increased lignification within xylem cells is critical in enhancing drought tolerance. Under normal watering conditions, water movement occurs from the soil, through the plant, and into the atmosphere, driven by gradients in water potential. However, during drought, reduced soil water potential creates an imbalance between the roots' water supply and the leaves' demand (Cochard et al. 2004). Beyond lignin content, the composition of lignin polymers also appears to influence drought tolerance significantly (Moura et al. 2010; Lee et al. 2013). Specifically, G-lignin within cell walls has been identified as a key factor in promoting recovery from drought. Elevated levels of G-lignin enhance drought resilience by mitigating inward cell collapse and facilitating structural recovery of xylem cells (Ménard et al. 2022). These findings suggest that lignin content and the subunit composition of lignin polymers, particularly the ratio of G-lignin, are critical determinants of drought tolerance in plants. Further investigations into the effects of drought on lignin chemistry are needed to deepen our understanding of the physical adaptations plants employ under water-limiting conditions. This knowledge highlights the potential of targeting lignin biosynthesis and composition as a strategy for developing crop varieties with enhanced drought tolerance.

1.2.2.2. Biosynthesis of flavonoids and stilbenes

Flavonoids are a special type of phenolic metabolites in plants to help increase their tolerance to adverse environmental conditions, such as bacteria and fungi pathogens, as well as abiotic stresses, including drought, salt, and ultraviolet irradiation (Jan et al. 2021, 2022; Baozhu et al. 2022). In addition, flavonoids exhibit high efficiency in scavenging the ROS in plants (Agati et al. 2012). Like lignin biosynthesis pathways, flavonoids are also derived from phenylalanine in the Phenylpropane metabolic pathway (Fraser and Chapple 2011; Koeduka 2014; Dong and Lin 2021). The molecular skeleton of flavonoids has three rings (C6–C3–C6), consisting of two aromatic rings (A- and B-rings) and one pyrone ring (C-ring) (Shen et al. 2022). Approximately 8000 flavonoids were identified in plants, and they were divided into seven categories according to the hydroxylations, O-methylations, prenylations, and C- and O-glycosylations in A- and B- rings, including flavonols, flavones, isoflavones, anthocyanidins, flavanones, flavanols, and chalcones (Panche et al. 2016; Tohge et al. 2017; Shen et al. 2022). Previous studies demonstrated that the COMTs can catalyze 3'-O-methylation of eriodictyol, dihydroquercetin, quercetin, luteolin, catechin, and rhamnetin, as well as 3',5'-O-methylation of myricetin and tricetin, and 5'-O-methylation of selgin *in vitro* conditions (Lin et al. 2006; Zhou et al. 2008; Lam et al. 2024). For example, a *SbCOMT* in sorghum (*Sorghum bicolor* L.) was demonstrated to be involved in the biosynthesis of tricin using selgin as a substrate (Eudes et al. 2017). In addition, a

naringenin 7-OMT (OsNOMT), the ortholog of COMT in rice (*Oryza sativa* L.), can O-methylate the A-ring of flavonoids (flavanones, flavones, and flavonols), but cannot accept isoflavones and caffeic acid as substrates (Shimizu et al. 2012). Furthermore, two orthologous COMTs (*ZmFOMT2* and *ZmFOMT4*) were identified in maize, which can catalyze the O-methylation of diverse flavonoids, the substrates including flavanones (2-hydroxynaringenin, naringenin), flavonols (quercetin, kaempferol), and flavones (scutellarein, chrysin, luteolin, apigenin) *in vitro* (Förster et al. 2022). Unlike the constitutive expression of *COMT*, *OsNOMT* and *ZmFOMT* were induced by JA and pathogen invasion (Shimizu et al. 2012; Förster et al. 2022). Those findings suggested that flavonoids, as the crucial SMs in plants, are catalyzed by the COMT enzyme to increase environmental stress tolerance.

Stilbenes are a bunch of polyphenolic allelochemicals in plants, which can against pathogens, bacteria, and fungi invasion as phytoalexins (Chong et al. 2009; Al-Khayri et al. 2023). Similar to flavonoids, stilbenes also have two aromatic rings (A- and B-rings), but an ethylene moiety connects those two rings to form a basic molecular skeleton (C6–C2–C6) (Al-Khayri et al. 2023). O-methylation in the stilbenes biosynthesis pathways increases the diversity of stilbenes during environmental stress conditions. PsPMT2 is a COMT-like enzyme in *Scots pine* (*Pinus sylvestris* L.), which preferentially O-methylates pinosylvin (3,5-dihydroxystilbene, PS) into its monomethylether (3-methoxy-5-hydroxystilbene, PSME) *in vitro* and is specific to stilbenes as substrate (Paasela et al. 2017). In addition, the stilbene 3,5-O-methyltransferase (*SbSOMT*), an ortholog gene of *SbCOMT*, catalyzes the resveratrol to biosynthesis pinostilbene and pterostilbene under pathogen conditions in sorghum (Lui et al. 2023). These studies demonstrated that the COMT enhances stress tolerance by catalyzing the biosynthesis of diverse SMs.

Plants produce various SMs essential for survival under adverse environmental conditions. The flavonoids and stilbenes are important SMs in plants. These metabolites play key roles in interspecies communication, enzyme regulation, signaling, and defense (Ramakrishna and Ravishankar 2011). Although extensive research has been conducted on SMs, their full significance remains incompletely understood. However, advances in research methodologies and technologies have greatly enhanced our understanding of their functions. SMs have gained attention for their applications in medicine, nutrition, cosmetics, and stress physiology. They play vital roles in defense and signaling under biotic and abiotic stresses, including drought (Figure 1-4) (Jogawat et al. 2021; Yadav et al. 2021). Therefore, investigating the biosynthetic pathways of SMs could provide valuable insights into improving crop resilience to environmental stresses. Developing innovative approaches to harness SMs is crucial for enhancing crop drought tolerance.

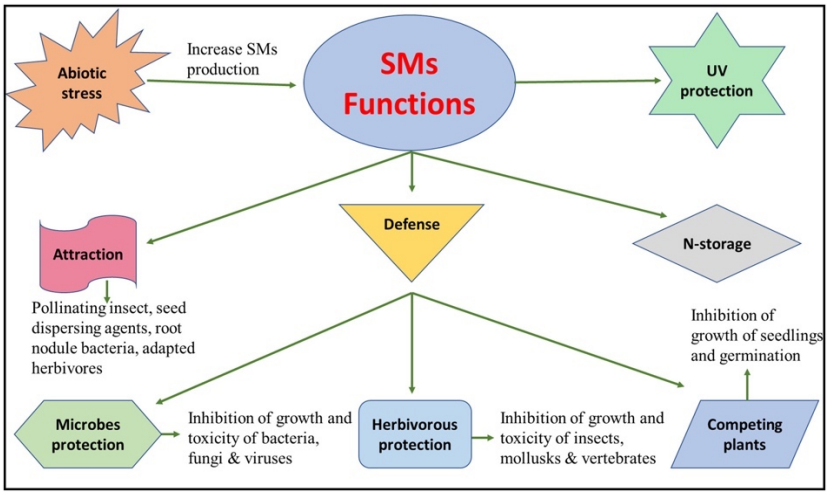


Figure 1-4 Schematic representation of secondary metabolites (SMs) role during abiotic stress, including drought stress (Yadav et al. 2021).

Abiotic stress induces the production of SMs, which in turn causes various metabolic pathways that alter plant physiology to mitigate stress.

1.2.2.3. Melatonin biosynthesis

Melatonin widely exists in animals and plants, and it plays a vital role in helping living beings cope with adverse environmental factors as an antioxidant (Rodriguez et al. 2004; Zhang and Zhang 2014; Michard and Simon 2020). Previous studies demonstrated that N-acetylserotonin methyltransferase (ASMT) is the key enzyme in the O-methylation of melatonin biosynthesis pathways both in animals and plants (Park et al. 2013; Botros et al. 2013). To investigate the biosynthesis enzyme of melatonin in plants, several studies identified that the enzyme activity of ASMTs in catalyzing melatonin biosynthesis in plants is significantly lower than that in animals. Then, Byeon et al. initially investigated the function in the biosynthesis of melatonin of 14 other O-methyltransferases, homologs with previous AMST enzyme activity genes. Their results found that one of the OMTs in Arabidopsis (AtCOMT), which is also known as COMT that catalyzes the conversion of caffeic acid to ferulic acid, can catalyze N-acetylserotonin (NAS) to melatonin with higher enzyme activity (K_m = 233 μm and V_{max} = 1800 pmol/min/mg protein) than that to ferulic acid (K_m = 103 μm and V_{max} = 564,000 pmol/min/mg protein) (Byeon et al. 2014a). Furthermore, they also demonstrated that AtCOMT is a multifunction enzyme that catalyzes O-methylation of different substrates, including caffeic acid, quercetin, and NAS (Figure 1-5). In addition, the phylogenetic analysis of ASMT and COMT suggested that COMTs in higher plants evolved from ASMT in primitive bacteria (Zhao et al. 2021b). And the phenolic substrate sites of COMTs have more highly conserved amino acids than in ASMT (Figure 1-6). Those studies illustrate the

reasons why AtCOMT has more significant enzyme activity than AtASMT in melatonin biosynthesis pathways (Figure 1-7) (Kang et al. 2013; Park et al. 2013; Byeon et al. 2014a, 2015, 2016). Further studies showed that melatonin biosynthesis in plants through multiple pathways, COMT is involved in the final step by catalyzing NAS to melatonin, and melatonin is mainly produced in mitochondria and chloroplasts of plant cells (Figure 1-8) (Tan et al. 2013; Back et al. 2016). After that, several studies demonstrated that overexpression of COMT increases melatonin content and enhances tolerance to biotic and abiotic stresses in different plant species (Sun et al. 2020; Chang et al. 2021; Huangfu et al. 2022; Li et al. 2022b). However, according to our knowledge, no reports have shown the melatonin biosynthesis gene in wheat before we started our project.

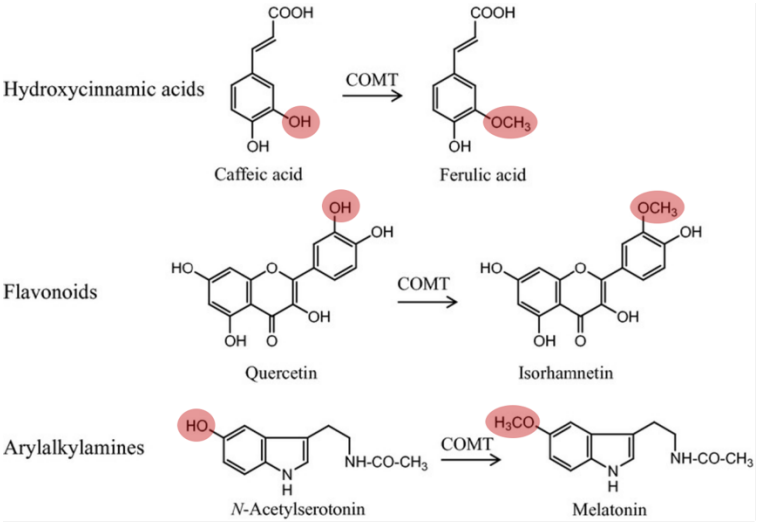


Figure 1-5 Schematic diagram of representative reaction products catalyzed by AtCOMT (Byeon et al. 2014a).

AtCOMT, *Arabidopsis thaliana* caffeic acid O-methyltransferase (GenBank accession number: *At5g54160*). Red circles represent the position of O-methylation.

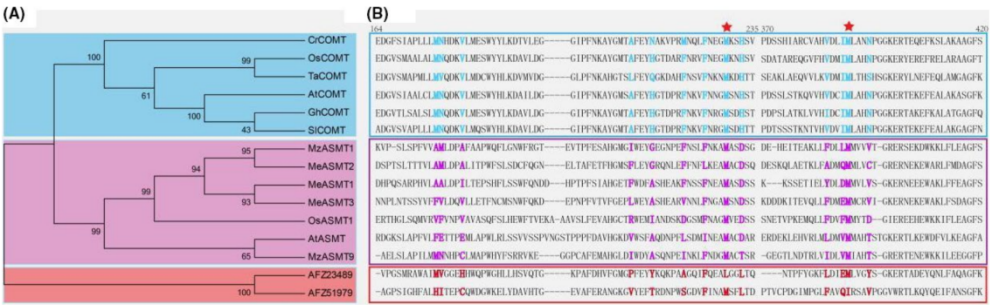
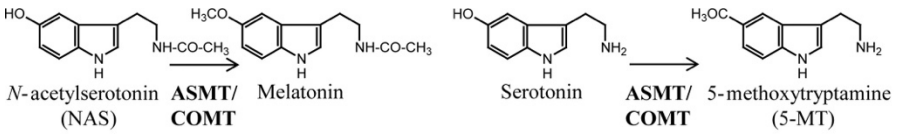


Figure 1-6 Phylogenetic analysis of well-defined ASMT/COMT and two bacterial ASMT proteins (Zhao et al. 2021b).

(A), and corresponding sequence alignment of substrate binding sites from these selected proteins (B). Shown are substrate binding sites of ASMT from *Oryza satica* (OsASMT1: *LOC_Os09g17560*), *Arabidopsis thaliana* (AtASMT: *AT4G35160*), *Malus zumi* (MzASMT1: *AIY62760*, MzASMT9: *AIZ00789*), *Manihot esculenta* (MeASMT1: *Manes.13G140900*, MeASMT2: Manes.17G050500, MeASMT3: Manes.13G140500); COMT from *Oryza satica* (OsCOMT: *LOC_Os08g06100*), *Arabidopsis thaliana* (AtCOMT: *AT5G54160*), *Solanum lycopersicum* (SlCOMT: XP_004235028), *Carex rigescens* (CrCOMT: *QDF21518*), *Triticum aestivum* (TaCOMT: *Traes_1AL_D9035D5E0*), *Gossypium hirsutum* (GhCOMT: *Gh_D12G2714*), *Cylindrospermum stagnale* PCC 7417 (*AFZ23489*), *Dactylococcopsis salina* PCC 8305 (*AFZ51979*). Residues involved in substrate binding from bacterial ASMT (red), well-defined ASMT (pink), and COMT (blue) are highlighted. The pentagram represents the high degree of conservation of residues in all ASMT/COMT proteins.



GenBank accession no.	Organisms	K_m (mmol L^{-1})	V_{max} (pkat mg^{-1} protein)	Temp (°C)	Refs
		N-acetylserotonin			
AK072740	*Oryza sativa* ASMT1	0.222/0.864[a]	150/0.21[a]	55	51, 70
KJ123721	*Malus zumi* ASMT1	nd	0.09[a]	nd	74
At4g35160	*Arabidopsis* ASMT1	0.456	0.11	37	75
At5g54160	*Arabidopsis* COMT	0.233	30	37	77
AK064768	*Oryza sativa* COMT	0.243	40	47	81
		Serotonin			
At5g54160	*Arabidopsis* COMT	3,396	8.8	37	55
At4g35160	*Arabidopsis* ASMT1	1,035	0.29	37	75

[a]Activity was measured at 30°C.

Figure 1-7 The enzymatic features of ASMT and COMT in plants (Back et al. 2016).

Representative ASMT and COMT enzymes characterized by various plant species are described. The values for V_{max} and K_m were obtained at various temperatures. ASMT: *N*-acetylserotonin methyltransferase; COMT: caffeic acid *O*-methyltransferase.

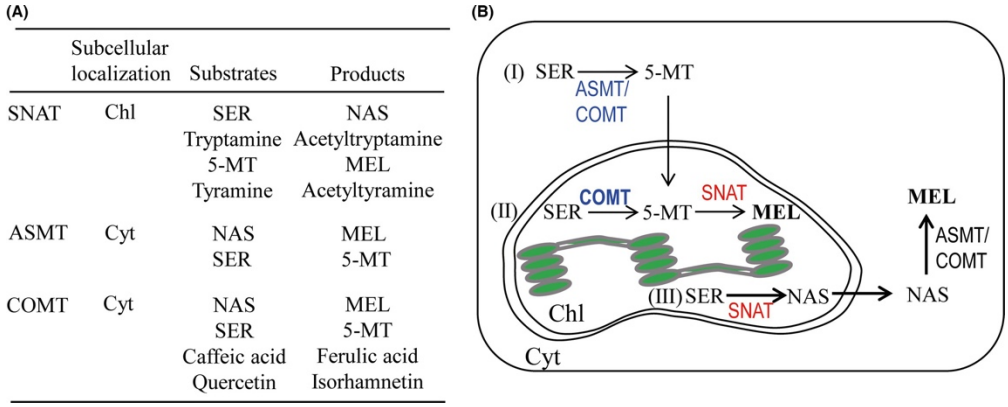


Figure 1-8 The multiple pathways of melatonin biosynthesis in plants (Choi et al. 2017).

(A) Enzymatic features of the enzymes involved in the last two steps in melatonin synthesis in plants and (B) schematic diagram of the melatonin biosynthetic pathway. Melatonin is believed to be synthesized by two pathways: the classical pathway (III) and an alternate pathway (I). Pathway II denotes the engineered pathway for efficient melatonin synthesis via 5-MT in chloroplasts. ASMT: *N*-acetylserotonin methyltransferase; SNAT: serotonin *N*-acetyltransferase; COMT: caffeic acid *O*-methyltransferase; Chl: chloroplasts; Cyt: cytoplasm; SER: serotonin; 5-MT: 5-methoxytryptamine; MEL: melatonin; NAS: *N*-acetylserotonin.

In addition, melatonin plays a crucial role in mitigating the detrimental effects of various abiotic stresses on plants. This review highlights the regulatory mechanisms underlying melatonin-mediated abiotic stress tolerance. Plant resilience is typically enhanced by melatonin through several key pathways. Firstly, melatonin functions as a direct scavenger of ROS and reactive nitrogen species (RNS), thereby enhancing the plant's antioxidant capacity. Additionally, melatonin acts as a signaling molecule, modulating the expression of stress-responsive genes, antioxidant production, and phytohormone pathways, including ABA, ethylene, and JA (Figure 1-9) (Zeng et al. 2022). Furthermore, melatonin significantly influences the redox network by interacting with nitric oxide (NO) to regulate redox homeostasis, ensuring the maintenance of antioxidant potential under abiotic stress conditions. This raises the interest for us to investigate the function and molecular mechanism of COMT in wheat melatonin biosynthesis and drought tolerance.

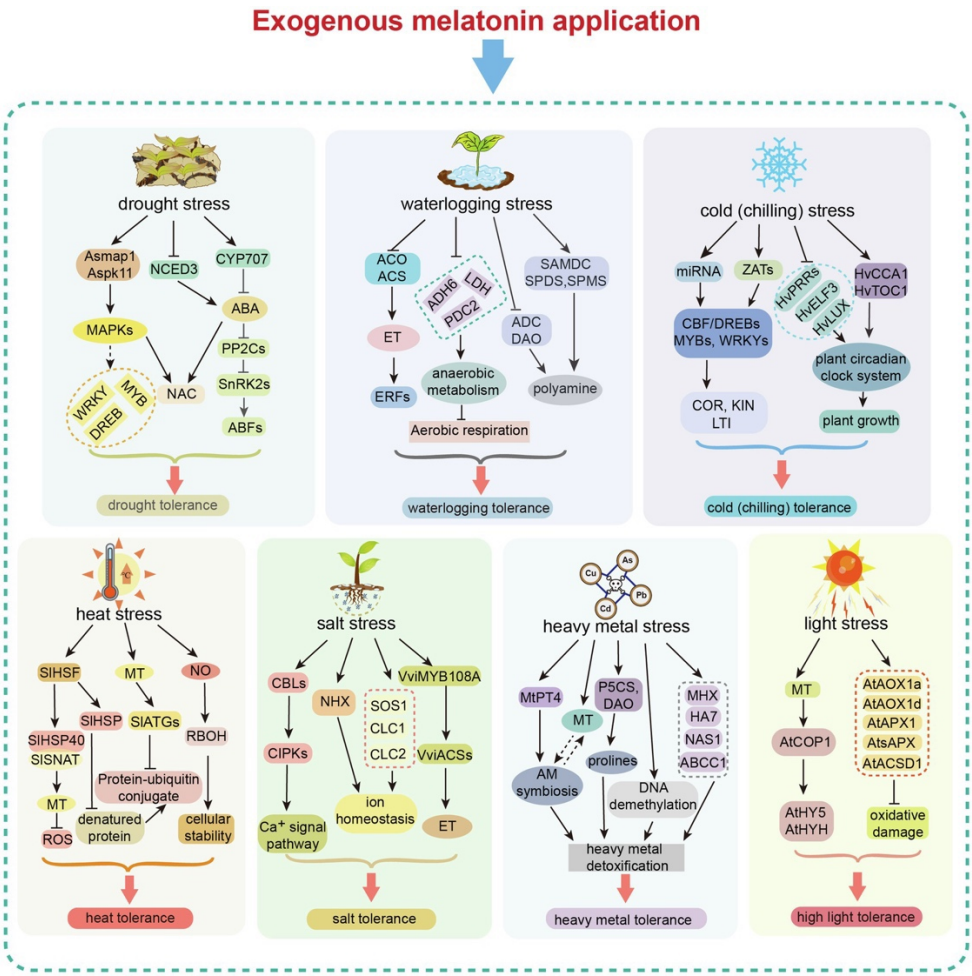


Figure 1-9 Exogenous melatonin-mediated regulation in response to different abiotic stresses (Zeng et al. 2022).

Exogenous melatonin application induces changes in gene expression in different abiotic stress response pathways, resulting in enhanced tolerance to major abiotic stresses (drought, waterlogging, heat, cold, salt, heavy metal toxicity, and light). MAPK: mitogen-activated protein kinase; NCED3: nine-cis-epoxycarotenoid dioxygenase 3; NAC: N-acetylcysteine; DREB: dehydration responsive element binding; CYP: cytochrome P450; PP2C: protein phosphatase 2C; SnRK: sucrose non-fermenting–related related kinase; ABF: abscisic acid responsive element-binding factor; ABA: abscisic acid; ACO: acyl-CoA oxidase; ACS: acetyl-CoA synthetase; ET: ethylene; ERF: ETS2 repressor factor; ADH: alcohol dehydrogenase; LDH: lactate dehydrogenase; PDC2: pyruvate decarboxylase-2; SAMDC: S-adenosylmethionine decarboxylase; SPDS: spermidine synthase; SPMS: spermine synthase; ADC: arginine decarboxylase; DAO: di-amine acid oxidase; CBF/DREB: dehydration

responsive element binding/C-repeat binding factor; ZAT: zinc transporter; COR: cold-regulated gene; KIN: Kin17 DNA and RNA binding protein; LTI: low-temperature-induced genes; PRRs: pseudo-response regulators; ELF3: E74 like ETS transcription factor 3; CCA1: circadian colck associated 1; TOC1: timing of CAB expression 1; HSF: heat shock facter; HSP: heat shock protein; SNAT: serotonin N-acetyltransferase; MT: melatonin; RBOH: respiratory burst oxidase homolog; ATG: autophagy-related; CBL: calcineurin B-like proteins; CIPK: CBL-interacting protein kinases; NHX: Na^+/H^+ exchanger protein; SOS1: SOS Ras/Rac guanine nucleotide exchange factor 1; CLC: chloride channel; PT4: phosphate transporter 4; AM: arbuscular mycorrhizal; P5CS: delta 1-pyrroline-5-carboxylate synthase; DAO: D-amino acid oxidase; MHX: magnesium/proton exchanger; NAS1: nicotianamine synthase 1; nicotianamine synthase 1; COP1: constitutive photomorphogenic 1; HY5: elongated hypocotyl 5; HYHAPX: hy5 homolog; APX: ascorbate peroxidase; AOX: aldehyde oxidase; and ACSD: 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase.

1.2.3. Conclusion

From the preceding discussion, it can be concluded that COMTs play a pivotal role in catalyzing the O-methylation of various hydroxylated substrates within the secondary metabolic pathway. Notably, the SMs produced through COMT activity not only enhance plant adaptation to adverse environmental conditions but also possess significant health benefits for humans. Thus, understanding and elucidating the functions and regulatory mechanisms of COMTs across different plant species is of great importance. Moreover, future research should focus on characterizing the biochemical properties and enzymatic activity of multifunctional COMTs to deepen our knowledge of their roles in plant metabolism and stress tolerance.

1.3. Recent advances in melatonin-regulating drought tolerance in plants

1.3.1 Introduction

Drought is a primary environmental factor that adversely affects crop production. Over half of crops are cultivated in arid and semi-arid regions, where drought significantly reduces both crop yield and quality (Gu et al. 2024). Under water deficit conditions, the excessive accumulation of reactive oxidative species (ROS) can severely impair plant growth and development (Gu et al. 2022). In response, plants have evolved numerous sophisticated mechanisms to counteract dehydration, including the regulation of growth and maturation, reduction in plant height, promotion of root development, and acceleration of both leaf senescence and flowering (Wang et al. 2013). For example, to mitigate drought stress, plants accelerate leaf senescence and promote flowering by reallocating water and nutrition from older to younger leaves and reproduction issues (Yang et al. 2023). Furthermore, plant hormones, flavonoids, and microbiomes contribute to the regulation of drought stress resilience (Salvi et al. 2022). During drought stress, ABA accumulation induces stomatal closure and leaf senescence through an increase in ROS levels (Huang et al. 2018). Therefore, plants modulate diverse molecular and

physiological pathways to improve resilience to drought stress.

Melatonin, also known as 5-methoxy-N-acetyltryptamine, was first discovered in the pineal gland of bovine (Lerner et al. 1958) and subsequently discovered in plants as early as 1995 (Dubbels et al. 1995; Hattori et al. 1995). This multifunctional molecule is involved in plant growth and development, including germination, root architecture, flowering time, leaf senescence, and fruit ripening (Pan et al. 2023). Moreover, melatonin has emerged as a critical regulator of plant resilience to various biotic and abiotic stresses, including salinity, extreme temperatures, heavy metals, UV radiation, and drought (Ahmad et al. 2023). Consequently, research investigating melatonin's functions and regulatory mechanisms in plants has expanded exponentially in recent years. Numerous studies indicated that melatonin alleviates drought stress by regulating various morphological, physiological, and biochemical processes, including seed germination, root and shoot development, leaf senescence, flowering, stomatal closure, photosynthetic efficiency, cell membrane integrity, osmotic stress, autophagy, etc (Supriya et al. 2024). For instance, seeds pretreated with melatonin had significantly improved germination under drought stress in various species, including hexaploid triticale (*Triticale hexaploid* L.), cotton (*Gossypium hirsutum* L.), and wheat (Hu et al. 2021; Luo et al. 2023). Moreover, melatonin plays a positive role in root growth and a negative role in shoot development, facilitating water absorption and enabling plants to cope with drought stress.

These findings suggested the crucial role of melatonin on drought stress tolerance. However, comparing these studies is challenging due to the complexity of phenotypes and physiological activities under varying drought conditions. Factors such as drought treatment methods (osmotic stress or soil drought stress), duration of drought stress (hours or days), sampling positions (roots, stem, leaves, or whole plants), and culture conditions (greenhouse or field conditions) add to this complexity. Therefore, further studies are needed to explore the underlying mechanisms by which melatonin enhances drought resilience. In this review, we critically evaluate the ROS-scavenging properties of melatonin and its metabolites under drought stress conditions, alongside its regulation of growth, development, and physiological activities during drought conditions. Then, we discuss how melatonin enhances drought tolerance by regulating plant hormones, microorganisms, and flavonoids. In addition, we propose several future research directions for melatonin that could advance agricultural production under drought conditions.

1.3.2. Boosting melatonin to combat drought stress in plants

1.3.2.1. Melatonin biosynthesis and its role in enhancing plant drought tolerance

Melatonin biosynthesis in plants has been extensively studied following its discovery. Initially, researchers utilized heterologous expression of animal biosynthetic genes, such as hydroxyindole-O-methyltransferase (HIOMT) from sheep, to enhance plant melatonin levels (Yuan et al. 2016). Subsequent

investigations revealed that melatonin can be biosynthesized in both the chloroplasts and mitochondria of plants (Tan et al. 2013; Back et al. 2016). In plants, both melatonin and indole-3-acetic acid (IAA) are synthesized from tryptophan. As shown in Figure 1-10, melatonin biosynthesis involves two distinct pathways that involve 6 enzymes, including tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), serotonin N-acetyltransferase (SNAT), N-acetylserotonin methyltransferase (ASMT), and caffeic acid O-methyltransferase (COMT) (Figure 1-10). Then, numerous studies have established that overexpression or knockout melatonin biosynthesis genes can directly modulate melatonin levels, thereby regulating plant stress tolerance (Table 1-1). In Arabidopsis, overexpression of TaCOMT enhanced drought tolerance by promoting root growth and increasing proline content (Yang et al. 2019). Therefore, further studies could use genetic modification techniques, such as CRISPR-Cas9, to improve plant drought tolerance by regulating melatonin biosynthesis genes in crops.

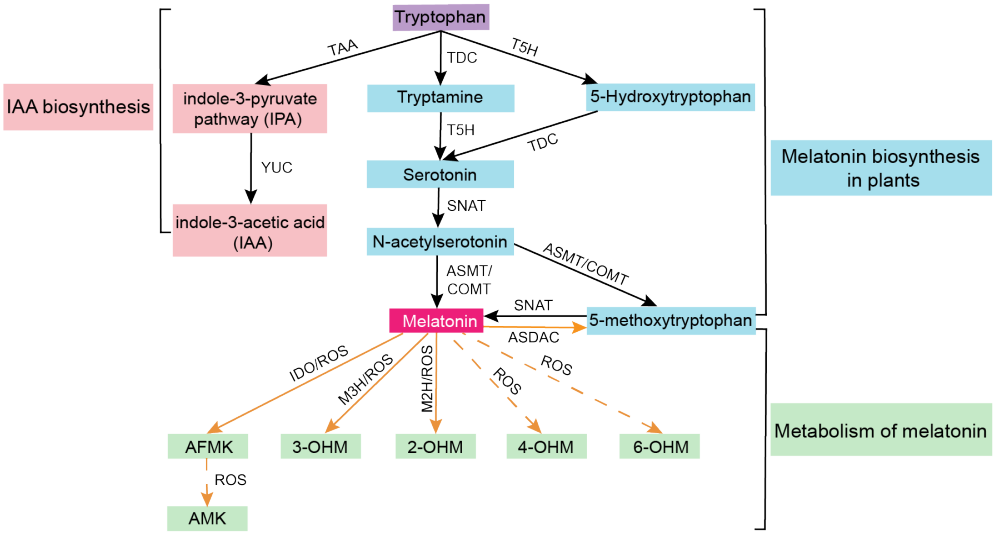


Figure 1-10 Biosynthesis and metabolism pathways of melatonin in plants.

Solid lines represent enzymes or oxidants identified in plants, while dash lines represent enzymes or oxidants identified in animals but not yet in plants. ASDAC: AFMK: N1-acetyl-N2-formyl-5-methoxyknuramine; AMK: N-acetyl-5-methoxyknuramine; ASDAC, N-acetylserotonin deacetylase; ASMT: N-acetylserotonin methyltransferase; COMT: caffeic acid O-methyltransferase; IAA: indole-3-acetic acid; IDO: indoleamine 2,3-dioxygenase; IPA: indole-3-pyruvate; M2H: melatonin 2-hydroxylase; M3H: melatonin 3-hydroxylase; ROS: reactive oxygen species; RNS: reactive nitrogen species; SNAT: serotonin N-acetyltransferase; TAA: tryptophan aminotransferase of Arabidopsis; TDC: tryptophan decarboxylase; T5H: tryptamine 5-hydroxylase; TPH: tryptophan hydroxylase; YUC: YUCCA, The *YUCCA* gene encodes a flavin monooxygenase-like enzyme; 2-ODD: 2-oxoglutarate-dependent dioxygenase; 2-OHM: 2-hydroxymelatonin; 3-OHM: 3-hydroxymelatonin; 4-OHM: 4-hydroxymelatonin; 6-OHM: 6-hydroxymelatonin.

In addition, several studies demonstrated that high melatonin content enhances stress tolerance by increasing plant flavonoid biosynthesis (Yue et al. 2023). On the contrary, some flavonoids, including morin and myricetin, can significantly decrease melatonin content by inhibiting the expression of *SNAT1* and *SNAT2* in rice (Bai et al. 2022). To date, only several studies indicate that heterogeneous overexpression of melatonin biosynthesis genes significantly enhances drought tolerance (Li et al. 2022b). However, no studies have demonstrated the regulation of melatonin biosynthesis genes by transcription factors under drought conditions. Hence, further studies can focus on identifying the upstream transcription factors that interact with melatonin biosynthesis genes during drought stress.

1.3.2.2. Exogenous application of melatonin enhances drought tolerance

Exogenous application of melatonin has been widely used to increase melatonin levels within the plant to enhance stress tolerance. Indeed, appropriate concentrations of melatonin can bolster drought tolerance in various plant species, including apple (*Malus domestica* Borkh.), tomato (*Solanum lycopersicum* L.), rice, cotton, wheat, maize, etc (Huang et al. 2019; Cao et al. 2023). Exogenous melatonin is typically applied through foliar spraying, root application, or seed priming (Table 1-2). However, most of these studies are investigating the application of melatonin in greenhouse conditions across various species. Hence, future studies should focus on developing melatonin-related biostimulants for sustainable agricultural production in field conditions. This may involve investigating optimal application methods, timing, dosage, and other relevant factors under drought conditions. Overall, one of the research directions is to incorporate melatonin and its metabolites into crop breeding programs to enhance food production and security.

1.3.3. Molecular mechanisms of melatonin signaling-mediated drought tolerance

Melatonin can stabilize membrane integrity by reducing ROS content under drought conditions. Previous studies demonstrated that melatonin plays a pivotal role in maintaining ROS homeostasis, both in regulating plant growth and enhancing plant stress resilience (Ahammed et al. 2024). On the one hand, melatonin reduces ROS levels by increasing antioxidant systems under stress conditions. On the other hand, melatonin also directly interacts with ROS/RNS to produce various metabolites, including 2-hydroxymelatonin (2-OHM), 3-hydroxymelatonin (3-OHM), 4-hydroxymelatonin (4-OHM), 6-hydroxymelatonin (6-OHM), 5-methoxytryptamine (5-MT), N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), and N-acetyl-5-methoxykynuramine (AMK) (Figure 1-10). In addition, several plant enzymes involved in melatonin metabolism have been identified, including melatonin 2-hydroxylase (M2H), melatonin 3-hydroxylase (M3H), indoleamine 2,3-dioxygenase (IDO), and N-acetylserotonin deacetylase (ASDAC), which produce 2-OHM, 3-OHM, AFMK, and 5-MT, respectively. Further studies have indicated that these metabolites exhibit higher antioxidant activity than melatonin itself, under stress conditions. For example, 2-OHM could mitigate cadmium stress in cucumber (*Cucumis sativus* L.) and increase drought stress tolerance by regulating the

antioxidant system (Lee and Back 2019a). Additionally, various transcription factors and flavonoids directly regulate melatonin biosynthesis genes in cassava (*Manihot esculenta* Crantz.). For example, the transcription factor MeWRKY20/75 interacts with three melatonin biosynthesis enzymes (MeTDC2, MeASMT2/3) to enhance melatonin levels (Wei et al., 2018b). These authors also revealed that *MeRAV1* and *MeRAV2* positively regulate melatonin biosynthesis genes to enhance disease resilience against cassava bacterial blight (Wei et al., 2018a). However, further studies are required to elucidate the function and the mechanisms by which these melatonin metabolites mitigate drought stress by decreasing ROS levels.

Furthermore, studies on drought also indicate that transcription factors, such as *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN* (*DREB*), *NAC*, *MYBs*, *bZIPs*, and *WRKYs*, play an important role in plant resilience (Li et al. 2024). Melatonin has been shown to modulate a variety of physiological processes and developmental growth to enhance plant stress tolerance by interacting synergistically with key phytohormones, such as ABA and gibberellic acid (GA) (Arnao and Hernández-Ruiz 2021a). These interactions are often mediated through intricate signaling networks and transcription factors, such as *NAC* and *MYB*, which regulate gene expression in response to various environmental factors (Liu et al., 2024). Additionally, several studies have reported that melatonin delays flowering time by stabilizing DELLA proteins or promoting the transcription of flowering locus C (FLC) to inhibit flowering locus T (FT) (Arnao and Hernández-Ruiz 2020). However, the regulatory mechanisms of melatonin on leaf senescence and flowering transition under drought conditions require further investigation. In addition, autophagy is activated to degrade and recycle drought-induced proteins, reducing their toxicity. Previous studies demonstrated that melatonin regulates autophagy by an ABA-independent pathway to enhance drought tolerance (Supriya et al. 2024). For example, Wang et al. initially discovered that melatonin regulates autophagy to mitigate the accumulation of oxidized proteins during oxidant stress (methyl viologen) (Wang et al. 2015). In wheat, rab-related proteins (RAB5C and RAB11A) related to autophagy activation were accumulated by applying melatonin during osmotic stress conditions (Cui et al. 2018). Subsequently, studies revealed that melatonin enhances drought tolerance by upregulating *ATG8* and *ATG8-PE* expression related to autophagosomes in cotton (Supriya et al. 2022). Hence, plants regulate ABA-dependent or independent pathways to improve drought stress tolerance.

Moreover, preliminary findings indicate that melatonin contributes to the regulation of stomatal conductance to enhance drought tolerance by accumulating the osmolytes and improving water use efficiency. This regulatory mechanism also underpins the observed increase in photosynthetic efficiency under drought conditions (Altaf et al. 2022). For example, melatonin can mitigate the reduction of stomatal conductance to enhance photosynthetic efficiency in various plants under drought conditions (Talaat 2023). In addition, melatonin was also shown to prevent disruption of the photosystems within the thylakoids and the reduction of chlorophyll, rubisco protein, etc., under drought conditions (Ahmad et al. 2021). For example, Ye et al. report that melatonin alleviates chlorophyll degradation and

disruption of fluorescence parameters caused by drought stress (Ye et al. 2016). Exogenous application of melatonin could improve photosynthetic efficiency and grain filling rate, thereby enhancing grain yield potential under drought stress conditions in soybean (*Glycine max* L.) (Zou et al. 2019) and maize (Ahmad et al. 2022). Melatonin can also protect the photosynthetic electron transport process in photosystems (PSI and PSII) and restore the fluorescence parameters to increase photosynthetic capacity (Guo et al. 2020). However, the regulatory mechanism of melatonin on photosynthesis under drought stress conditions remains largely unknown.

In addition, the identification of receptor proteins is vital for studying the regulation and signal pathways of melatonin in plants. The first phyto-melatonin receptor1 (Cand2/PMTR1), a putative G protein-coupled melatonin receptor-like protein, was identified in Arabidopsis (Wei et al. 2018a). In their study, melatonin was shown to regulate stomatal closure by inducing ROS and Ca^{2+} signaling pathways or activating mitogen-activated protein kinase (MAPK) cascades in guard cells (Wei et al. 2018a). The melatonin receptor mutant, *cand2*, exhibited reduced osmotic stress tolerance with ROS accumulation, and exogenous melatonin could not revert this osmotic stress phenotype in Arabidopsis (Wei et al. 2018a). Subsequently, homologs of PMTR1 have been identified in other plants. For instance, overexpressing *ZmPMTR1* in Arabidopsis enhanced osmotic stress tolerance by reducing the rate of transpiration (Wang et al. 2022b). However, Lee & Back argued that PMTR1 is not a G protein-coupled melatonin receptor as, under stress conditions, melatonin-activated MAPK (MPK3/6) cascades without the need for PMTR1 (Lee and Back 2020). In addition, it is noteworthy that previous studies have identified at least two receptors (MT1 and MT2) in animals (Zlotos et al. 2014). Therefore, further investigations are necessary to confirm the receptor function of PMTR1 or identify new melatonin receptors and elucidate its signal transduction pathways.

Additionally, MAPK cascades are essential signal transduction pathways that respond to various environmental stresses, such as drought, salt, and pathogens. Previous studies have demonstrated that melatonin activates MAPK signaling in response to pathogens in plants (Lee and Back 2017a). In addition, melatonin regulated chloroplast protein quality control (CPQC), which is critical for starch synthesis and reducing DNA damage by activating the plant MAPK pathways (Maity et al. 2022). Recent studies have further elucidated melatonin's role in MAPK pathways under stress conditions. For example, in apples, melatonin-activated *MdMPK3* and *MdMPK6* upregulate the melatonin biosynthesis gene, *MdASMT7*, by phosphorylating the transcription factor *MdWRKY17* (Song et al. 2023). Similarly, in Sour jujube (*Ziziphus jujuba* var. *spinose*), melatonin could enhance salt resilience by upregulating *MKK3* in the *MAPK* signaling pathway (Zhu et al. 2023). Despite these findings, the specific mechanisms by which melatonin activates the MAPK cascade to alleviate drought stress in plants remain underexplored. Therefore, further studies are needed to elucidate MAPK cascade pathways involved in regulating drought resilience.

1.3.4. Melatonin regulates other pathways to enhance drought tolerance

1.3.4.1. Melatonin cross-talks with other plant hormones to enhance drought tolerance

Furthermore, recent studies have shown that melatonin enhances drought tolerance by modulating other plant hormones, microbiome communities, and flavonoids. Plant hormones are vital for drought resilience in plants through their regulation of growth, development, and numerous physiological processes. In contrast, melatonin promotes stomatal reopening by decreasing ROS and ABA levels under drought conditions, and new studies have suggested that melatonin achieves this by downregulating ABA biosynthesis genes and upregulating ABA catabolism genes (Yang et al. 2023; Talaat 2023). Other studies have demonstrated that melatonin can inhibit ABA transport from roots to shoots during drought stress (Jahan et al. 2023). Furthermore, melatonin can synergistically interact with cytokinin (CTK) to increase drought tolerance by upregulating dehydration-responsive genes (*JUB1* and *DREB2A*) and alleviate drought-induced leaf senescence by increasing endogenous CTK content (Ma et al. 2018). Additionally, melatonin increased brassinosteroid (BR) content by upregulating biosynthesis genes, including *CYP750A1*, *CYP707A5*, *CYP707A7*, *CYP87A3*, and *CYP90D2*, thereby improving drought and cold tolerance (Fu et al. 2022). The exogenous application of melatonin increased the level of salicylic acid (SA), GA, and IAA under imposed water deficit conditions (Talaat 2023). Moreover, JA levels, which are increased by drought stress and contribute to drought tolerance, were positively regulated by melatonin during drought conditions (Luo et al. 2023). Overall, these studies demonstrate that melatonin plays a central role by modulating these plant hormones and reducing ROS concentration caused by water deficit conditions (Table 1-3). Although these studies illustrated that melatonin can regulate plant growth, development, and physiological processes to enhance drought resilience by changing plant hormones' content, its interactions with other plant hormones under drought conditions remain largely unexplored. Therefore, further detailed omics studies are essential to identify the transcription factors and regulatory elements involved in this signaling cascade.

1.3.4.2. Melatonin regulates microbes to enhance drought stress

Plants can manipulate and recruit beneficial microbial communities to produce various compounds, including phytohormones, osmolytes, antioxidants, and volatile organic compounds (VOCs), that mediate in alleviating drought stress (Afridi et al. 2022). Under drought conditions, plant microbiomes also influence the expression of drought-related genes. For instance, a *B. subtilis* strain can induce the expression of drought-related genes, such as *RD29* and *DREB2*, to mitigate drought stress in rapeseed (*Brassica napus* L.) and Arabidopsis (Poudel et al. 2021). Melatonin can also interact, synergistically, with arbuscular mycorrhizal fungi (AMF) to improve drought tolerance. Applying melatonin to roots can alter the composition of rhizosphere microbiomes, promoting bacteria involved in carbohydrate and carboxylate degradation, which was shown to help alleviate drought stress in barley (Ye et al. 2022). In addition, melatonin affects the structure of microbial

communities, including soil fungi and bacteria, leading to improved soil nitrogen content and utilization efficiency, thereby enhancing drought resilience (Du et al. 2022). Currently, the mechanism(s) by which melatonin interacts with the plant microbes to mitigate drought stress remains largely unknown. This is the new future research direction for improving plant drought tolerance. Future research should focus on elucidating the communication mechanisms between melatonin and microbial communities to mitigate drought stress in plants. Furthermore, identifying specific bacterial or fungal communities that enhance melatonin's function in improving drought resilience represents a promising direction for the enhancement of global agriculture.

1.3.4.3. Melatonin regulates flavonoids to enhance drought tolerance

Flavonoids, such as anthocyanins, kaempferol, and quercetin, are specialized SMs known for their roles as phytoalexins or antioxidants, through their ROS-scavenging trait. Such flavonoids are crucial for protecting plants against both biotic and abiotic stress, including drought, as their accumulation has been demonstrated to significantly enhance drought tolerance. In addition, both melatonin and flavonoids have multifunctional roles in the plant and interact with plant microbes to regulate drought tolerance, as previously established.

Metabolomics analyses have shown that melatonin can regulate a variety of metabolites in the flavonoid biosynthesis pathway during drought stress conditions. For example, melatonin improves tolerance to salt, drought, and heat stresses, by upregulating the luteolin biosynthesis genes (Song et al. 2022). It also enhances stem strength by increasing lignin content and secondary cell wall thickness in herbaceous peonies (Zhao et al. 2022). Here, in particular, melatonin can increase the accumulation of apigenin, luteolin, and quercetin, under drought-stress conditions. In tobacco (*Nicotiana tabacum* L.), kaempferol, a type of flavonol, has also been reported to interfere with melatonin's regulation of ROS and stomatal closure (Xiao et al. 2023). Therefore, these recent studies confirmed that melatonin accumulates several different flavonoids to enhance drought tolerance. However, further studies are warranted to explore the relationship between melatonin and flavonoids in regulating drought tolerance. Especially the lack of molecular mechanisms between melatonin and different flavonoids under drought conditions.

1.3.5. Conclusions

In conclusion, current evidence indicates that melatonin, whether through the regulation of its biosynthesis genes or exogenous application, primarily functions by scavenging ROS. This activity helps to maintain ROS homeostasis and alleviates drought-induced stress. This pivotal role protects plants from drought-induced damage, including compromised seed germination, inhibited root growth, stomatal closure, decreased photosynthesis, etc. Moreover, melatonin mediates drought tolerance through intricate signal transduction pathways, potentially interacting with membrane receptors such as PMTR1 or activating mitogen-activated protein kinases (MAPKs) like *MdMPK3* and *MdMPK6*. Additionally, recent investigations have elucidated melatonin's interactions with various phytohormones, plant microbes, and

flavonoids, which further augment its role in drought tolerance (Figure 1-11). For instance, melatonin synergizes with AMF to improve drought tolerance and modulates microbial community structures to enhance nutrient uptake and utilization. Metabolomics analyses have revealed that melatonin regulates differentially expressed metabolites in flavonoid biosynthesis during drought stress, increasing the accumulation of key flavonoid metabolites, such as apigenin, luteolin, and quercetin.

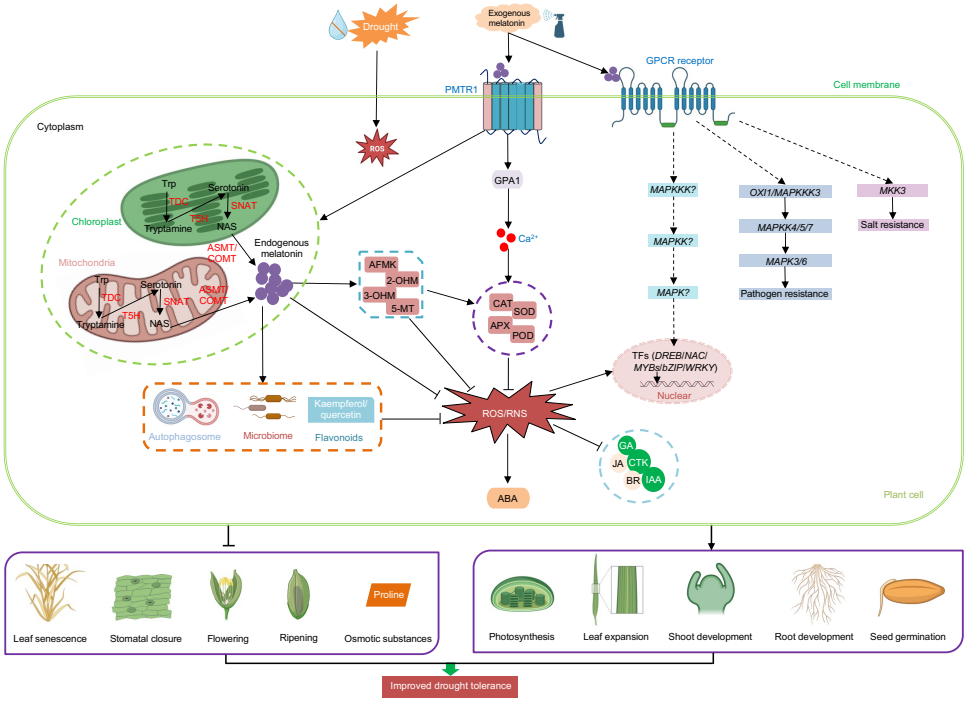


Figure 1-11 Growth, development, and physiological activity regulated by melatonin in plants under drought conditions.

The orange arrows represent upregulated or downregulated by drought stress, and the green arrows represent upregulated or downregulated by melatonin under drought stress conditions. ABA: abscisic acid; APX: ascorbate peroxidase; BR: brassinosteroids; CAT: catalase; CTK: cytokinin; GA: gibberellins acid; GPX: glutathione peroxidase; IAA: indole-3-acetic acid; JA: jasmonic acid; MAPK: mitogen-activated protein kinase; PMTR1: phytomelatonin receptor; ROS: reactive oxygen species; RNS: reactive nitrogen species; SA: salicylic acid; SOD: superoxide dismutase; TFs: transcription factors.

Despite these advancements, the mechanisms through which melatonin interacts with these various pathways to confer drought tolerance remain to be fully elucidated. Future research programs should focus on characterizing the detailed molecular and biochemical pathways involved in melatonin-mediated drought resilience. Identifying transcription factors and regulatory elements that interact with

melatonin, as well as the role of melatonin in modulating plant microbiomes and other phytohormones, are promising avenues for exploration. Hence, advanced omics approaches, such as transcriptomics, proteomics, and metabolomics, will be instrumental in uncovering these complex interactions.

In summary, melatonin is a multifaceted molecule with significant potential in enhancing plant drought tolerance. By deepening our understanding of its underlying mechanisms, we can better harness melatonin's capabilities to mitigate drought stress in agricultural settings, thereby improving crop resilience and productivity in the face of climate change.

Table 1-1 Regulation of melatonin biosynthesis genes to enhance stress tolerance in plants.

Object species name	**Melatonin biosynthesis genes**	**Function of melatonin**	**References**
Apple (*Malus domestica* Borkh.)	*MdASMT9*	Overexpression of *MdASMT9* also reduces ABA accumulation through promoting *MdWRKY33*-mediated transcriptional inhibition of *MdNCED1* and *MdNCED3*, thus inducing stomatal opening for better heat dissipation.	(Gao et al. 2024)
Apple (*Malus domestica* Borkh.)	*MdASMT7*	MdWRKY17 interacts with MdASMT7 to promote the expression of *MdASMT7* Melatonin treatment activates the mitogen-activated kinases (MPKs) *MdMPK3* and *MdMPK6*, which phosphorylate *MdWRKY17* to promote transcriptional activation of *MdASMT7.*	(Song et al. 2023)
Arabidopsis thaliana L.	*AtSNAT6*	*AtHY5* inhibits *AtSNAT6* expression directly, and overexpression of *AtSNAT6* decreases cotyledon opening via changing melatonin biosynthesis.	(Wang et al. 2022a)
Arabidopsis thaliana L.	*AtSNAT2*	*snat2* knockout mutant exhibits delayed flowering and reductions in leaf area and biomass.	(Lee et al. 2019)
Barley (*Hordeum vulgare* L.)	*HvCOMT1*, *HvCOMT2*, and *HvCOMT3*	Reducing thioacidolysis yields in the *COMT* RNAi lines are an indication of changes to lignin structure with a greater proportion of resistant bonds in the lignin.	(Daly et al. 2019)
Carex rigescens	*CrCOMT*	Overexpression of *CrCOMT* in Arabidopsis exhibits enhancement of growth and physiological performance under salt stress, such as higher lateral root numbers, proline level, and chlorophyll content.	(Zhang et al. 2019)

Cassava (*Manihot esculenta* Crantz)	*MeTDC2*, *MeASMT2*, and *MeASMT3*	MeATG8b/8c/8e interact with melatonin synthesis enzymes (MeTDC2, MeASMT2/3) and thus coordinate the dynamics of melatonin biosynthesis and autophagic activity in cassava.	(Wei et al. 2020)
Cassava (*Manihot esculenta* Crantz)	*MeTDC2*, *MeT5H*, *MeASMT1*, *MeTDC1*, *MeASMT2*, *MeASMT3*, and *MeSNAT*	*MeRAV1* and *MeRAV2* positively regulate 7 melatonin biosynthesis genes (*MeTDC2*, *MeT5H*, *MeASMT1*, *MeTDC1*, *MeASMT2*, *MeASMT3*, and *MeSNAT*) and the endogenous melatonin level in plant disease resistance against cassava bacterial blight.	(Wei et al. 2018b)
Cassava (*Manihot esculenta* Crantz)	*MeTDC2*, *MeASMT2*, and *MeASMT3*	MeWRKY20/75 interacts with 3 melatonin synthesis enzymes (MeTDC2, MeASMT2/3) and positively regulates endogenous melatonin accumulation.	(Wei et al. 2018c)
Grapevine (*Vitis vinifera* L.)	*VvASMT1*	Ectopic overexpression of *VvASMT1* in *Nicotiana benthamiana* significantly enhances melatonin production and increased tolerance to salt and osmotic stresses.	(Yu et al. 2022)
Grapevine (*Vitis vinifera* L.)	*VvSNAT2*	Overexpression of *VvSNAT2* in Arabidopsis results in greater accumulation of melatonin and chlorophyll and enhances resistance to powdery mildew.	(Yu et al. 2019)
Malus zumi Mats.	*MzASMT1*	Overexpression of *MzASMT1* increases melatonin levels and drought tolerance in *Arabidopsis.*	(Zuo et al. 2014)
Rice (*Oryza Sativa* L.)	*SNAT1* and *SNAT2*	Flavonoids, including myricetin and quercetin, dose-dependently and potently inhibited level of rice *SNAT1* and *SNAT2.*	(Lee et al. 2018)
Rice (*Oryza Sativa* L.)	*OaSNAT*	The *TS:OaSNAT* transgenic lines exhibits increasement of seminal root growth.	(Byeon et al. 2014b)
Rice (*Oryza Sativa* L.)	*OsASMT1*, *OsASMT2*,	All three *ASMT* mRNAs are simultaneously induced in	(Park et al. 2013)

	and *OsASMT3*	treatments with abscisic and methyl jasmonic acids.	
Rice (*Oryza Sativa* L.)	*OsMTS1*	Disruption of *OsMTS1*, which codes for a methyltransferase, can trigger leaf senescence as a result of decreased melatonin production.	(Hong et al. 2018)
Rice (*Oryza Sativa* L.)	*OsCOMT*	Overexpression of *OsCOMT* significantly delays leaf senescence at the grain-filling stage by inhibiting the degradation of chlorophyll and chloroplast. *OsCOMT* plays a positive role in the vascular development of rice. *OsCOMT* is a positive regulator of grain yield.	(Huangfu et al. 2022)
Rice (*Oryza Sativa* L.)	*OsSNAT1*	*OsSNAT1*-overexpressing rice plants increased resistance to cadmium and senescence stresses.	(Lee and Back 2017b)
Rice (*Oryza Sativa* L.)	*OsSNAT2*	*SNAT2* RNAi lines indicated a decrease in melatonin and a dwarf phenotype with erect leaves by exhibiting photomorphogenic phenotypes such as inhibition of internodes and increased expression of light-inducible CAB genes in the dark.	(Hwang and Back 2018)
Sheep (*Ovis aries*)	*OaAANAT*	Overexpression of *AANAT* played pivotal roles in modulating plant growth through its regulation of cell elongation, and regulating flowering through photoperiod and GA pathways in switchgrass.	(Huang et al. 2022)
Sheep (*Ovis aries*)	*OaAANAT* and *OaHIOMT*	*OaAANAT* overexpression in Micro-Tom tomato plants has higher melatonin levels and lower indoleacetic acid (IAA) contents, and *OaHIOMT* overexpression lines increased melatonin content and drought tolerance.	(Wang et al. 2014)
Tobacco (*Nicotiana*	*NtCOMT1*	Overexpression of *NtCOMT1* promoted drought resistance by	(Yao et al. 2022)

tabacum L.)		increasing melatonin content.	
Tomato (*Solanum lycopersicum* L.)	*SlSNAT*	HSP40, a DnaJ-type chaperone, was found to interact with SlSNAT in the chloroplast. HSP40 functions as a chaperone to protect the SNAT enzyme during melatonin synthesis under heat stress.	(Wang et al. 2020)
Tomato (*Solanum lycopersicum* L.)	*SlCOMT1*	Overexpression of *caffeic acid O-methyltransferase 1* (*SlCOMT1*) significantly increases both melatonin content and salt tolerance in the germinated seeds.	(Ge et al. 2023)
Tomato (*Solanum lycopersicum* L.)	*SlCOMT1*	Overexpression of *COMT1* significantly enhances the capacity of the tomato to reduce fungicide carbendazim phytotoxicity and residue.	(Choi et al. 2017)
Watermelon (*Citrullus lanatus* L.)	*ClCOMT1*	Overexpression of *ClCOMT1* enhances transgenic Arabidopsis tolerance against with cold, drought, and NaCl.	(Chang et al. 2021)
Wheat (*Triticum aestivum* L.)	*TaCOMT*	Overexpression of the wheat *TaCOMT* gene enhances drought tolerance and increases the content of melatonin in transgenic *Arabidopsis.*	(Yang et al. 2019)

Table 1-2 Exogenous melatonin enhanced drought tolerance in plants.

Species name	Melatonin concentration	Function of exogenous melatonin under drought stress	References
Alfalfa (*Medicago sativa* L.)	Foliar spray (100 μM)	Boosting antioxidant enzyme activities, improving photosynthetic performance, and accumulating total soluble sugar and proline content.	(Roy et al. 2021)
Apple (*Malus domestica* Borkh.)	Root application (100 μm)	Helping to maintain the better function of PSII and controlling the burst of hydrogen peroxide to delay the leaf senescence under drought.	(Wang et al. 2013)
Barley (*Hordeum vulgare* L.)	Root application (2 mM)	Exogenous melatonin increases the relative abundance of the bacterial community in carbohydrate and carboxylate degradation while decreasing the relative abundance in the pathways of fatty acid and lipid degradation and inorganic nutrient metabolism under drought.	(Ye et al. 2022)
Cotton (*Gossypium hirsutum* L.)	Foliar spray (100 and 200 μM)	Improving the translocation of carbon assimilates to drought-stressed anthers, regulating the carbohydrate balance of drought-stressed anthers to improve male fertility.	(Hu et al. 2020a)
Cotton (*Gossypium hirsutum* L.)	Foliar spray (100 μM)	Down-regulating chlorophyll degradation-related genes and senescence marker genes (*GhNAC12* and *GhWRKY27*/*71*). Improving photosynthetic efficiency, reducing chlorophyll degradation and ROS accumulation, and inhibiting ABA synthesis, thereby delaying drought-induced leaf senescence in cotton.	(Yang et al. 2023)
Cotton (*Gossypium hirsutum* L.)	Seed priming (10 μM)	Increasing photosynthetic activity, water-use efficiency, and nitrogen metabolism. Upregulating the expression of the autophagosome marker [lipidated (ATG8-PE)].	(Supriya et al. 2022)
Creeping	Foliar spray (20 μM)	Increasing visual quality, photochemical efficiency, chlorophyll	(Ma et al. 2018)

bentgrass (*Agrostis stolonifera*)		content, and relative water content. Up-regulating and chlorophyll-degradation genes, and cytokinin-signaling and synthesis genes.	
Kiwifruit (*Actinidia chinensis Planch*)	Root application (100 μM)	Improving photosynthesis by inhibiting stomatal closure, enhancing light energy absorption, and promoting electron transport in PSII.	(Liang et al. 2019)
Maize (*Zea mays* L.)	Foliar spray (100 μM)	Increasing the accumulation of flavonoid metabolites, particularly apigenin, luteolin, and quercetin. Upregulating the expression of genes related to flavonoid biosynthesis (*PAL*, *C4H*, *4CL*, *HCT*, *CHS*, *CHI*, *F3'5'H*, and *DFR*), activates drought-responsive transcription factors (*ERFs*, *NACs*, *MYBs*, and *bHLHs*).	(Wang et al. 2023)
Maize (*Zea mays* L.)	Foliar spray (100 μM) and root application (50 μM)	Reducing the reactive oxygen species burst and enhancing the photosynthetic activity by protecting it from damage.	(Ahmad et al. 2019)
Maize (*Zea mays* L.)	Root application (100 μM)	Improving the photosynthetic activities and alleviated the oxidative damages of maize seedlings under the drought stress.	(Huang et al. 2019)
Moldavian balm (*Dracocephalum moldavica*)	Foliar spray (100 μM)	Increasing soluble sugar content, malondialdehyde content, and lipoxygenase activity, non-enzyme antioxidants (including flavonoid, polyphenol compounds, and anthocyanin) under moderate and severe drought stress.	(Naghizadeh et al. 2019)
Naked oats (*Avena nuda* L.)	Foliar spray (100 μM)	Increasing the chlorophyll content and photosynthetic rate of leaves, increasing expression of *PYL*, *PP2C*, *ABF*, *SNRK2*, and *IAA*.	(Zhang et al. 2022b)
Pepper (*Capsicum annuum* L.)	Foliar spray (100 μM)	Reducing oxidative stress and improving nitrogen metabolism by activating various enzymes such as nitrate reductase, nitrite reductase, glutamine synthetase, and glutamine dehydrogenase	(Kaya and Shabala 2023)

		activities.	
Rapeseed (*Brassica napus* L.)	Root application (50 μM)	Alleviating the seedling growth inhibition and increasing the leaf area and fresh and dry weights of roots and shoots under drought stress.	(Li et al. 2018a)
Rice (*Oryza Sativa* L.)	Root application (100 μM)	Promoting root, shoot length, fresh and dry weight, and increasing chlorophyll contents.	(Khan et al. 2024)
Rice (*Oryza Sativa* L.)	Seed priming (100 μM)	Promoting the germination rate and improving the biomass of rice seed shoots and roots. Alleviating the oxidative damage of rice seeds caused by drought stress.	(Li et al. 2022c)
Soybean (*Glycine max* L.)	Foliar spray or root application (50 μM and 100 μM)	Improving photosynthetic activity, reduction of abscisic acid and drought-induced oxidative damage.	(Imran et al. 2021)
Tomato (*Solanum lycopersicum* L.)	Root application (150 μM)	Affecting stomatal conductance and the activity of ROS scavenging enzymes.	(Jensen et al. 2023)
Tomato (*Solanum lycopersicum* L.)	Root application (100 μM and 500 μM)	Carbon monoxide is a downstream signal molecule of melatonin-enhanced drought resistance by promoting chlorophyll biosynthesis.	(Liu et al. 2024b)
Tomato (*Solanum lycopersicum* L.)	Seed priming (100 μM)	Increasing stomatal conductance, photochemical efficiency, and the antioxidant system, and also reducing the cellular content of toxic substances.	(Liu et al. 2015)
Tomato (*Solanum lycopersicum* L.)	Root application (100 μM)	Improving the seedlings growth, root characteristics, leaf photosynthesis and antioxidant machinery.	(Altaf et al. 2022)
Tomato (*Solanum lycopersicum* L.)	Foliar spray (100 μM) and root application (100	Alleviating the inhibition of drought stress on the gas exchange parameters and the leaf net photosynthetic rate, protecting the thylakoid membrane from damage, and strengthening the ATP-	(Yang et al. 2017)

	μM)	synthase activity.	
Two Citrus cultivars	Foliar spray (100 μM)	Increasing total flavonoid and total phenolic contents under severe drought stress.	(Jafari and Shahsavar 2021)
Wheat (*Triticum aestivum* L.)	Root application (500 μM)	Decreasing membrane damage, more intact grana lamella of chloroplast, higher photosynthetic rate, and maximum efficiency of photosystem II, as well as higher cell turgor and water-holding capacity.	(Cui et al. 2017)
Wheat (*Triticum aestivum* L.)	Root application (100 μM)	Alleviating photosynthetic and cell membrane damage by maintaining low levels of hydrogen peroxide.	(Luo et al. 2023)
Wheat (*Triticum aestivum* L.)	Root application (100 μM)	Improving fine root, lateral root, and root hair, plant height, dry weight, net photosynthesis, and stomatal aperture of leaves.	(Zhang et al. 2023)

Table 1-3 Regulatory pathways by melatonin under drought conditions.

Species name	Regulation objects	Function of melatonin under drought conditions	References
Apple (*Malus domestica* Borkh.)	Decreased ABA	Melatonin scavenges H_2O_2 and reduces ABA by upregulating *MdNCED3* and downregulating *MdCYP707A1* and *MdCYP707A2* to re-open stomatal under drought conditions.	(Li et al. 2015)
Barley (*Hordeum vulgare* L.)	Increased SA, GA, CK, and IAA. Decreased ABA	Melatonin increases levels of SA, GA, CK, and IAA, as well as a decrease in ABA to enhance stress tolerance in barley.	(Talaat 2023)
Chinese hickory (*Carya cathayensis L.*)	Increased JA and GA, reduced ABA	Regulating key metabolic pathways such as phenylpropanoid, chlorophyll and carotenoid biosynthesis, carbon fixation, and sugar metabolism. Increasing JA and GA, reducing ABA during PEG treatment conditions.	(Sharma et al. 2020a)
Cotton (*Gossypium hirsutum* L.)	Decreased ABA	Melatonin can effectively enhance the antioxidant enzyme system, improve photosynthetic efficiency, reduce chlorophyll degradation and ROS accumulation, and inhibit ABA synthesis, thereby delaying drought-induced leaf senescence in cotton.	(Yang et al. 2023)
Cotton (*Gossypium hirsutum* L.)	Increased GA and reduced ABA	Increasing the germination rate, germination potential, radical length, and fresh weight, as well as the activities of superoxide dismutase (SOD), peroxidase (POD), and α-amylase. Melatonin alleviates drought stress by reducing ABA content and increasing GA_3 content.	(Bai et al. 2020)
Creeping bentgrass (*Agrostis stolonifera*)	Increased CTK	Melatonin synergistically interacts with cytokinins to suppress drought-induced leaf senescence. Increasing endogenous cytokinins content and upregulating cytokinins signal transduction genes and transcription factors.	(Ma et al. 2018)
Maize (*Zea mays* L.)	Reduced ABA	Reducing ABA accumulation and inducing stomatal reopening by	(Li et al. 2021b)

		inhibiting up-regulation of *NCED1*, and up-regulating ABA catabolic genes *ABA8ox1* and *ABA8ox3.*	
Maize (*Zea mays* L.)	Increased GA and IAA. Decreased ABA	Melatonin increases GA and IAA while reducing the ABA levels in leaves under drought conditions.	(Ahmad et al. 2022)
Maize (*Zea mays* L.)	Increased JA	Up-regulating JA biosynthesis genes and increasing JA contents to mitigate drought stress.	(Zhao et al. 2021a)
Perennial ryegrass (*Lolium perenne* L.)	Increased BR	Brassinosteroid plays a critical role in the melatonin-mediated mitigation of cold and drought stress by triggering antioxidant activities as well as enhancing the photosynthetic capacities. Brassinosteroid biosynthesis and it signaling pathway are induced by melatonin.	(Fu et al. 2022)
Potato (*Solanum tuberosum* L.)	ABA decreased in shoots and increased in roots	Melatonin hinders ABA transport from the root to the shoot system to enhance drought tolerance.	(El-Yazied et al. 2022)
Soybean (*Glycine max* L.)	Decreased ABA in leaves and increased ABA in roots	Melatonin-received plant leaves accumulates less ABA but roots content higher ABA. Melatonin significantly suppresses ABA biosynthesis and signaling gene expression in soybean exposed to drought stress.	(Jahan et al. 2023)
Wheat (*Triticum aestivum* L.)	Increased JA	Up-regulating JA biosynthesis genes and increasing JA contents to mitigate drought stress.	(Luo et al. 2023)

1.4. Function of sakuranetin

1.4.1 Introduction

Plants have evolved an array of defense mechanisms to counteract pathogen attacks, including fortifying local cell walls, modulating phytohormone signaling, activating innate immune responses, and synthesizing phytoalexins (Valletta et al. 2023). Among rice phytoalexins, which include diterpenoids, phenylamides, and flavonoids, sakuranetin stands out as a significant flavonoid phytoalexin. Beyond its critical role in enabling plants to withstand various stresses, sakuranetin offers numerous health-promoting benefits (Stompor 2020). For instance, sakuranetin exhibits anti-tumor, anti-viral, anti-parasitic, anti-inflammatory, and antioxidant activities. Previous research has demonstrated its anti-proliferative effects on ESCC, B16BL6 melanoma, and Colo 320 cancer cells.

Sakuranetin (5,4'-Dihydroxy-7-methoxyflavanone) is a natural plant flavonoid, one of the methoxylated flavanones, which consists of two fused rings (A rings and C ring) and a phenyl ring B (Kodama et al. 1992; Shimizu et al. 2012). In addition, sakuranetin was first discovered in the cherry tree bark (*Prunus* spp.) in 1908 (Asahina 1908). Subsequent studies isolated and characterized sakuranetin in other plant species, revealing its diverse biological roles. Research has shown that sakuranetin acts as a flavonoid phytoalexin in rice (*Oryza sativa* L.), protecting against fungal pathogen invasion (Hasegawa et al. 2014; Katsumata et al. 2018). In addition, UV light, JA, and $CuCl_2$ also induced the accumulation of sakuranetin in rice (Kodama et al. 1992; Ogawa et al. 2017). Recently, Liu et al. demonstrated that sakuranetin also protects rice from brown planthopper attack by depleting its beneficial endosymbionts in rice (Liu et al., 2023). Those findings indicated that sakuranetin plays a crucial role in pathogen and pest resistance in plants.

Beyond its biological roles in plant resistance to pathogens and pests, sakuranetin shows potential as a plant-derived antibiotic and a pharmaceutical agent (Stompor 2020). However, its full range of functions remains underexplored. In this section, we review the latest research on sakuranetin and identify existing knowledge gaps regarding its roles in plants, particularly its potential applications beyond pathogen and pest resistance.

1.4.2 Antioxidant properties of sakuranetin

Sakuranetin presents significant potential as a nutraceutical and pharmaceutical agent. It plays a vital role in diverse therapeutic properties, including antiviral, anticancer, anti-inflammatory, antiparasitic, antioxidant, and anti-allergic effects (Stompor 2020). Several studies have established the correlation between dietary phytochemicals, particularly flavonoids, and the prevention of lifestyle-related diseases such as cancer. This class of natural compounds is associated with minimal adverse effects and systemic toxicity, rendering them safe for human use. Sakuranetin has been shown to inhibit tumor growth via the apoptosis pathway in both in vitro and in vivo models. Its primary mode of action involves inducing cell

death through apoptosis, highlighting its potential as a targeted anticancer agent.

In addition to its therapeutic applications, sakuranetin and its derivatives exhibit anti-aging properties due to its ability to absorb ultraviolet (UV) radiation and its potent antioxidant activities (Kodama et al. 1992). These properties underscore its potential as a versatile compound for applications in health, medicine, and stress tolerance, warranting further investigation into its mechanisms of action and efficacy across diverse contexts.

1.4.3 Sakuranetin in plants

Flavonoids are widely distributed secondary metabolites in plants, recognized for their roles as medicinal agents, nutritional compounds, and pigments. They are integral to plant defense, providing UV protection and aiding responses to abiotic stresses (Shen et al. 2022). Some flavonoids also function as phytoalexins during plant–microbe interactions. For instance, sakuranetin, a methoxylated flavonoid, was first identified in UV-irradiated rice leaves and rice blast fungus (Magnaporthe oryzae)-infected tissues. It has since been established as a phytoalexin effective against fungal pathogens, while its precursor, naringenin, demonstrates antibacterial activity (Zhao et al. 2024). The accumulation of these flavonoids varies among rice cultivars, reflecting differences in defense mechanisms, with studies identifying flavonoid synthases involved in related pathways, such as maysin biosynthesis (Casas et al. 2016).

Discovered in 1908 from cherry tree bark (Prunus spp.) as the aglycone of sakuranin, sakuranetin has been confirmed in various plant species, including *Artemisia campestris*, *Baccharis* spp., *Betula* spp., and *Rhus* spp (Stompor 2020). These plants, traditionally used in herbal medicine to treat diabetes, inflammation, allergies, and cancer, reflect sakuranetin's potential health benefits. In *Ribes nigrum*, sakuranetin has been associated with powdery mildew resistance, while its absence correlates with susceptibility, emphasizing its significance in disease resistance (Atkinson and Blakeman, 1982) . In addition, sakuranetin's biosynthesis is induced by environmental stimuli, such as UV radiation and pathogen attacks, highlighting its ecological relevance. However, the enzymatic pathways responsible for its production in species beyond rice remain poorly understood. Additionally, emerging studies on its functions in other crops, such as wheat, underscore the need for further research to elucidate its molecular mechanisms and broader applications in agriculture and health sciences.

1.4.4 Biosynthesis of sakuranetin

To date, research on the *de novo* synthesis of sakuranetin in plants or other species has been limited. However, the biosynthesis of its precursor, naringenin, has been extensively studied. Naringenin biosynthesis originates from aromatic amino acids such as phenylalanine or tyrosine and involves four key enzymes: tyrosine ammonia lyase (TAL) or phenylalanine ammonia-lyase (PAL), 4-coumaric acid-CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI) (Sun et al. 2022). This process operates through the flavonoid branch of the phenylpropanoid pathway.

The naringenin was catalyzed to synthesize sakuranetin by an O-methyltransferase depending on the S-adenosylmethionine (SAM) .

Moreover, several studies have identified that naringenin can be enzymatically converted into sakuranetin in rice through the activity of an O-methyltransferase, specifically naringenin 7-O-methyltransferase (OsNOMT), which shares a high degree of sequence homology with OsCOMT (Figure 1-12) (Rakwal et al. 1996, 2000; Murata et al. 2020). While the role of sakuranetin in conferring pathogen resistance in rice leaves is well-documented, metabolomic studies have revealed its absence in mature rice seeds. Furthermore, the identification of biosynthetic genes and functional roles of sakuranetin in other plant species remain largely unexplored, highlighting the need for further investigation into this promising compound.

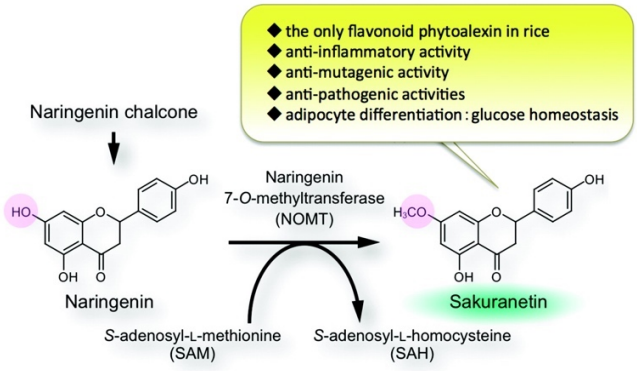


Figure 1-12 Biosynthetic pathway of sakuranetin from naringenin in rice (Shimizu et al. 2012).

Sakuranetin is a major rice phytoalexin and a potential pharmaceutical agent. SAM: S-adenosyl-L-methionine; SAH: S-Adenosyl-L homocysteine. Pink dots represent the position of O-methylation. Illustration taken from Shimizu et al. (2012)

1.4.5 Conclusion

In conclusion, sakuranetin demonstrates a wide range of biological activities, making it a compelling subject for further exploration, particularly in the context of its potential medical applications and role in enhancing plant resilience to stress conditions. While its antioxidant properties are well-established, its practical biological utility is limited by uncertainties surrounding its cytotoxic effects. The precise mechanisms and functions of sakuranetin under drought stress conditions remain poorly understood. Moreover, advancing research to increase sakuranetin content in crops could significantly improve resistance to environmental stressors while offering additional nutritional benefits. These investigations will deepen our understanding of the functional mechanisms of natural methoxyflavones such as sakuranetin and contribute to the development of innovative strategies for crop improvement and sustainable agriculture.

Chapter 2

Research objectives and roadmap

2.1. Research objectives

Caffeic acid O-methyltransferase (COMT) is a crucial multifunction enzyme in the biosynthesis of melatonin, lignin, and flavonoids (sakuranetin is one of the major flavonoids) in plants. Previous studies demonstrated that overexpression of COMT enhanced drought tolerance by increasing melatonin content (Li et al. 2016, 2022b). Wheat is one of the most important staple crops worldwide, and its plant growth and crop yield are affected by drought stress dramatically (Khadka et al. 2020; Bhandari et al. 2021; Hashmi et al. 2023). However, the function and mechanism of COMT in wheat drought tolerance remains unknown. Hence, we proposed investigating the drought tolerance and enzyme activity of TaCOMT1A, a homology of AtCOMT, in wheat. In addition, melatonin is a versatile small molecular that promotes plant growth and development, as well as improves tolerance to biotic stresses, such as viruses, bacterial invasion, and abiotic stresses, including cold, drought, salt, etc. (Zhao and Hu 2023; Aghdam et al. 2023; Colombage et al. 2023; Ahammed et al. 2024). Sakuranetin biosynthesized mainly in stress conditions, such as jasmonic acid, ultraviolet light, and pathogen invasion (Kodama et al. 1992; Ogawa et al. 2017; Yang et al. 2022). Furthermore, genomics, transcriptomics, proteomics, and metabolomics were applied to investigate the regulatory mechanism of melatonin with other plant hormones and stress factors (Zhao et al. 2021a; Xie et al. 2022; Liu et al. 2023a). Hence, we also performed omics analysis (transcriptomic and proteomic) to parse the regulatory pathways by exogenous melatonin and sakuranetin under drought conditions in wheat. In this study, our objectives are as follows:

(1) Investigating the phenotype and physiological of *TaCOMT1A* under drought conditions by overexpressing it in wheat (“fielder,” WT).

(2) Comparing the flavonoid metabolomic between *TaCOMT1A* overexpression lines and WT.

(3) Investigating the enzyme activities of TaCOMT1A *in vitro* by purifying TaCOMT1A protein from *Escherichia coli*.

(4) Investigating the phenotype and physiology of wheat by applying exogenous melatonin under drought stress conditions (“Chinese Spring,” “Shi4185,” “Chang6878,” “Hanxuan10”, and “Aikang58”).

(5) Investigating the regulatory mechanism of melatonin under drought conditions by analyzing transcriptomic and proteomic data in wheat (“Aikang58”).

(6) Investigating the phenotype and physiology of wheat by applying exogenous sakuranetin under drought stress conditions (“Chinese Spring,” “Shi4185,” “Chang6878,” “Hanxuan10”).

(7) Investigating the regulatory mechanism of sakuranetin under drought conditions by analyzing transcriptomic in wheat (“Shi4185,” “Chang6878,” “Hanxuan10”).

2.2. Research roadmap

To reach those objectives, we designed three routes, as shown in Figure 2-1.

Our study used six wheat varieties. The variety "fielder" was used for transgenic receptors. We selected "Aikang58" because it is the wild-type variety of our mutant library. Two drought-sensitive varieties ("Chinese Spring" and "Shi4185") and two drought-tolerance varieties ("Hanxuan10" and "Chang6878") were also used for exogenous melatonin and sakuranetin experiments.

Firstly, according to our previous study, one of the 14 COMT genes (*TaCOMT1A*) in wheat increases melatonin content and drought tolerance in Arabidopsis (Yang et al. 2019). Hence, we overexpressed it in "fielder" (Transgenic recipient wheat variety as WT). Then, we investigated phenotype (plant height, fresh weight, dry weight, etc.) and physiological activities (CAT, H_2O_2, MDA, etc.) of *TaCOMT1A* overexpression lines and WT under drought stress conditions. In addition, we used flavonoid metabolomics to compare the flavonoid contents in overexpression lines and WT. At the same time, we expressed and purified the His-TaCOMT1A protein *in vitro* to investigate the multifunctional enzyme activity in the biosynthesis of melatonin and other flavonoids. Overall, we planned to examine the function of TaCOMT1A under drought conditions and the enzyme activities in wheat in this part.

Secondly, according to the enzyme activity of TaCOMT1A in melatonin biosynthesis, we proposed investigating the function of melatonin on wheat drought tolerance ("Chinese Spring," "Shi4185," "Hanxuan10," "Chang6878," and "Aikang58") and the regulatory mechanism of exogenous melatonin on drought tolerance in wheat ("Aikang58"). We treated the wheat with four treatment groups: watering conditions (W), applying melatonin under watering conditions (MW) and drought conditions (D), and applying melatonin under drought conditions (MD). Then, we observed the phenotype and physiological activities under these four treatments. The transcriptomic and proteomic methods were used to parse the regulatory pathways of melatonin under drought conditions.

Thirdly, according to the enzyme activity of TaCOMT1A in sakuranetin biosynthesis, we also proposed investigating the function of sakuranetin on wheat drought tolerance ("Chinese Spring," "Shi4185," "Chang6878," and "Hanxuan10") and the regulatory mechanism of exogenous sakuranetin on drought tolerance in wheat ("Shi4185," "Chang6878," and "Hanxuan10"). We treated those wheat varieties with four treatment groups: watering conditions (W), applying sakuranetin under watering conditions (SW) and drought conditions (D), and applying sakuranetin under drought conditions (SD). We observed the phenotype and physiological activities under these four treatments. Then, we used transcriptomic analysis to illustrate the regulatory mechanism of sakuranetin in drought tolerance in wheat.

Overall, our study aimed to explore the molecular mechanism of melatonin and sakuranetin in drought tolerance using overexpression of *TaCOMT1A* or exogenous application of melatonin and sakuranetin in wheat.

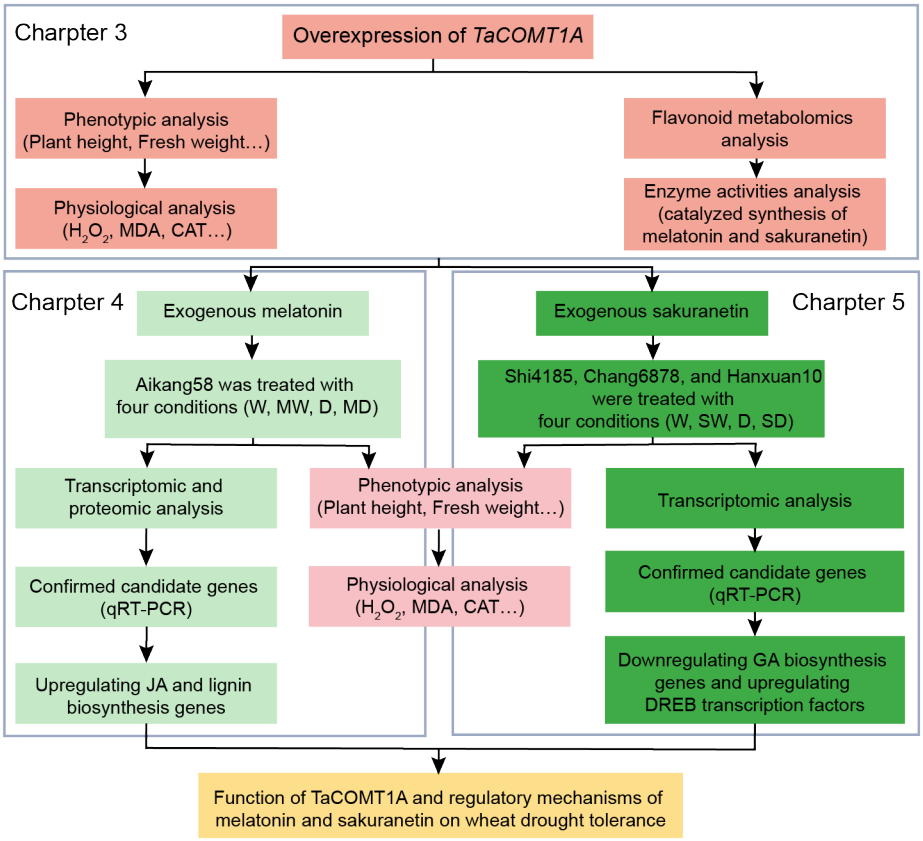


Figure 2-1 Research route of the whole thesis.

W: watering conditions; MW: applying melatonin under watering conditions; D: drought conditions; SW: applying sakuranetin under watering conditions; MD: applying melatonin under drought conditions; SD: applying sakuranetin under drought conditions

Chapter 3

Overexpression of *TaCOMT1A* enhances drought tolerance and decreases plant height in wheat (*Triticum aestivum* L.)

Under review by *Plant Biotechnology Journal*

In this chapter, we overexpressed the melatonin biosynthesis gene *TaCOMT1A* in wheat to observe its phenotype and physiological activities under drought conditions. Then, we applied flavonoid metabolomic analysis to compare the *TaCOMT1A* overexpression line with the receptor variety (WT). We also tested the enzyme activity of TaCOMT1A *in vitro* to investigate its function and regulatory mechanism in wheat.

Dr. Wenjing Yang constructed TaCOMT1A overexpression lines in our lab. The flavonoid metabolomic analysis was performed in Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China). Melatonin, sakuranetin, and GA_3 content were subjected by the Major Platform Center, Institute of Crop Science, Chinese Academy of Agricultural Sciences (Beijing, China). Mingzhao Luo carried out other experiments.

Abstract: Caffeic acid O-methyltransferase (COMT) is a crucial methylase in the phenylpropane metabolic pathway; it is one of the key enzymes that participate in the biosynthesis of flavonoids, lignin, melatonin, etc., in plants. In addition, COMTs have high conservation at the amino acid level and contribute to promoting plant growth and adapting to biotic and abiotic stresses. We previously isolated the *TaCOMT1A*, encoding a caffeic acid O-methyltransferase from wheat, and overexpression in Arabidopsis significantly improved the drought tolerance and melatonin content. However, the function and mechanism of *TaCOMT1A* in wheat (*Triticum aestivum* L.) drought tolerance remains unknown. In this study, we aimed to investigate the enzyme activity of *TaCOMT1A* and overexpress it in wheat to identify the function of drought tolerance. Our results demonstrated that TaCOMT1A can not only synthesize melatonin but, more importantly, can promote the synthesis of another important phytoalexin, sakuranetin, in wheat. The *TaCOMT1A* overexpression lines conferred drought tolerance compared to WT ("fielder") during the seedling and maturation stages. Especially under drought conditions in the field, the grain weight per plant of *TaCOMT1A* overexpression lines increased by 15.21%–20.27% compared to WT. Meanwhile, the plant height of *TaCOMT1A* overexpression lines decreased by 10.92%–12.47% compared to WT. In addition, *TaCOMT1A* overexpression lines decreased GA_3 content and decreased seed germination rate. Two GA biosynthesis genes (*TaGA2ox-5B* and *TaKO-7A*) and a transcription factor (*TaDREB2C-1A*) were downregulated in overexpression lines. Those results give new clues to illustrate the function of *TaCOMT1A* and provide alternation for plants adapting to adverse environmental conditions.

Keywords: caffeic acid O-methyltransferase, drought, plant height, wheat

3.1. Introduction

Drought stress, a major abiotic challenge exacerbated by global climate change, significantly impacts global agriculture, with 1.8 billion people projected to face severe water scarcity by 2025 (Hashmi et al. 2023). Responsible for 70% of crop yield losses, drought disrupts plant growth and development. Plants adapt through mechanisms such as early flowering, stomatal regulation, and expansive root systems, alongside physiological responses like maintaining tissue water status, producing osmolytes, and activating stress-responsive genes (Adel and Carels 2023). Phytohormones, such as abscisic acid (ABA) and Jacimonica acid (JA), play a key role by regulating stomatal closure, reducing water loss, and triggering drought-related gene expression (Bapela et al. 2022). Therefore, understanding these adaptive strategies is critical for developing drought-tolerant crops and ensuring food security.

Caffeic acid O-methyltransferase (COMT) is a large gene family that catalyzes the synthesis and metabolism of many secondary metabolites (SMs), including the synthesis of flavonoids, anthocyanins, lignin, etc. in the phenylpropanoid pathway, which helps plants to enhance lodging and stress tolerance by regulating cell wall, osmotic substances, and antioxidant enzyme activities (Zhang et al. 2021; Liang et al. 2022). In addition, the same COMT is involved in the biosynthesis of lignin by identifying different substrates, such as caffeic acid, 5-hydroxypinobanksyl aldehyde, and 5-hydroxypinobanksyl alcohol, to produce various products such as ferulic acid, mustard aldehyde, and mustard alcohol, respectively (Lam et al. 2024). Recently, the *OsCOMT* was reported to increase grain yield (Huangfu et al. 2022) and increase abiotic stress tolerances, including salt, drought, heat, etc., by being involved in the biosynthesis of melatonin in rice (Byeon et al. 2015; Zhao et al. 2021b). Hence, COMT is a multifunction enzyme that generates different products by identifying several substrates to improve stress tolerance.

Wheat (*Triticum aestivum* L.) is one of the important staple foods worldwide, and severe drought stresses significantly damage our food security and quality (Daryanto et al. 2016; Zhang et al. 2023). Drought stress inhibited root development and induced high reactive oxygen species (ROS) levels in plants. Drought also has an impact on photosynthesis and cell membranes. However, melatonin can reverse these conditions, promoting root development, decreasing ROS, and maintaining plants' photosynthesis and cell membrane integrity (Guo et al. 2020; Muhammad et al. 2023). Previous studies suggested that overexpression of COMT could enhance drought tolerance by increasing melatonin content in other plants (Li et al. 2016, 2022b). Our previous study found that a *TaCOMT1A* from wheat can improve the drought tolerance of overexpression Arabidopsis by increasing melatonin synthesis (Yang et al. 2019). However, the function and regulatory mechanism of the *TaCOMT1A*, a melatonin biosynthesis gene, remains unknown in wheat.

In this study, we transformed *TaCOMT1A* into wheat. *TaCOMT1A* improves the drought tolerance of overexpression lines in greenhouse conditions and increases grain yield under drought conditions in field conditions. More interestingly, we

demonstrated that TaCOMT1A can synthesize sakuranetin through metabolomic analysis of overexpression lines and *in vitro* enzyme activity experiments. Overexpression of *TaCOMT1A* in wheat and direct application of product sakuranetin can achieve the same goals, including increasing drought tolerance and reducing wheat plant height by regulating the expression of similar downstream genes in wheat. As a new plant-derived growth regulator, sakuranetin can improve the tolerance of wheat to drought stress and decrease plant height to reduce lodging risk, which suggests that sakuranetin has essential application value in wheat production in the field.

3.2. Materials and Methods

3.2.1. Vector construction and wheat genetic transformation

According to sequence blast results (https://phytozome-next.jgi.do.gov), we obtained the orthologous homeotic gene *TaCOMT1A* (*Traes_1AL_D9035D5E0*) of *AtCOMT* in wheat, and we demonstrated that overexpression of *TaCOMT1A* improved Arabidopsis improved the drought tolerance in a previous study (Yang et al. 2019). In this study, *TaCOMT1A* was inserted into vector pWMB190 with the ubiquitin promoter, and primers were listed in the supplemental table (Table 3-1). The recombinant plasmid pWMB190–*TaCOMT1A* was transformed in wheat variety "fielder" using Agrobacterium-mediated (strain GV3101) transformation (Zhou et al. 2022).

3.2.2. Plant growth and drought treatment conditions

The wheat variety "fielder" was used for the transgenic receptor material. All those wheat seeds were conserved in our lab. All experiments at the seedling stage were cultured in a climate chamber set at 24/16 °C day/night, 70% relative humidity, and a 14/10 h light/darkness photoperiod.

For the drought stress experiment of "fielder" and overexpression lines at the seedling stage, soil (Pindstrup, Denmark) and water were blended at 1:2 (g/mL). Then, a 1.8 kg soil mixture was put in a green shallow basin and laid flat. Geminated wheat seeds were planted in soils and covered with soil. All seedlings were stopped watering on the 1st day after sowing (DAS), and 1 L was rewatered on the 21st DAS in each green shallow basin. Five 15-day-old seedlings were mixed into a biological replicate sample and preserved at -80 °C for further study.

For the field experiment in the suburb of Beijing (40°13′49″N, 116°33′28″E), we planted "fielder" and *TaCOMT1A* overexpression lines in the field with furrow irrigation following previous field research protocols for drought stress conditions (Zhou et al. 2022). In brief, in limited irrigation (LIR), two rounds of irrigation were applied, including at the pre-wintering and jointing stages. In well-irrigated (WIR) conditions, three rounds of irrigation were used, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied to 750 m^3 of water per hectare area in the field.

Table 3-1 All primers for PCR in Chapter 3

Primer name	Primer sequence	Gene ID	Role of the primers
TaCOMT-1AL-AF1	AGATGAATGGGTGGATTCGTGTG	*Traes_1AL_D9035D5E0*	for gene cloning
TaCOMT-1AL-AR1	CTCCAGAAAATGATCCAAGTAAAAT		
TaDREB2C-1A-F	ACCACTCAGATGTTGCTTCTAA	*TraesCS1A02G221900*	for qPCR
TaDREB2C-1A-R	AGCATCAGGAACAGTGTCTTTA		
TaGA20ox-5B-F	TTAGCTGCTGAGACTGAGAAAA	*TraesCS5B02G560300*	for qPCR
TaGA20ox-5B-R	CCATGCTTCTTCGTACGTAGTA		
TaKO-7A-F	GTGCAACATGAACAAGAAGGAT	*TraesCS7A02G362300*	for qPCR
TaKO-7A-R	GAATGCCATGGTCTTGTACATG		
TaCOMT-1AL-qF	ATGGAGCATGTTCCCGCAATT	*Traes_1AL_D9035D5E0*	for qPCR
TaCOMT-1AL-qR	CTATTTTGTAAATTCAATAGCCCACGATCC		
TaActin1-F	AAATCTGGCATCACACTTTCTAC	*TraesCS1A01G274400*	for qPCR
TaActin1-R	GTCTCAAACATAATCTGGGTCATC		

3.2.3. Analysis of flavonoids and Gibberellic acid contents in wheat

The flavonoids and different forms of GA in "fielder" and overexpression line *#9* were extracted and analyzed using an ultra-high-performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) (HPLC, Shim-pack UFLC SHIMADZU CBM30A; MS, Applied Biosystems 4500 QTRAP) according to the previous study (Chen et al. 2013). Three replicates in "fielder" and overexpression line *#9*, respectively. Each replicate contains five seedling shoots. 158 flavonoids and their metabolic intermediates, as well as five forms of GA, were identified in this study. Those data were obtained from MetWare Biotech Co., Ltd., Wuhan, China.

3.2.4. Determination of physiological characteristics and lignin content

The activity of catalase (CAT) and the content of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in wheat seedlings were measured according to the corresponding kit protocol (Comin Biotechnology, Suzhou, China). The absorbance values were measured by a Thermo Scientific Microplate Reader (Thermo Fisher Scientific Inc., USA). The endogenous lignin content was extracted using an immunosorbent assay kit (Jianglai, Shanghai, China), and the lignin content was calculated by comparing the obtained spectrophotometric values to those in the standard curve.

We weighed approximately 0.1 g (W) of tissue and added 1 mL of phosphate-buffered saline (PBS) (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) to homogenize under low temperatures. The grinding mixtures were centrifuged at 8000 g at 4 °C for ten min. Then, we took the supernatant and measured it at 240 nm. We used distilled water as a control. After one minute, the initial absorption value A1 and the absorption value A2 were recorded. Calculate ΔA_{240} = A1 - A2. Catalytic one nmol H_2O_2 degradation per g tissue per minute is one unit of CAT enzyme activity. The W represents the fresh weight of the sample used for the determination. We measured the absorption value at 415 nm for H_2O_2. We measured the absorption value at 532 nm and 600 nm for MDA. $\Delta A_{MDA} = A_{532} - A_{600}$.The activity of CAT and the content of H_2O_2 and MDA followed the formulas below:

CAT (nmol/min/g (fresh weight)) = $918 \times \Delta A_{240} \div W$

H_2O_2 (μmol/g (fresh weight)) = $2.67 \times (A_{415} - 0.0006) \div W$

MDA (nmol/g (fresh weight)) = $25.8 \times \Delta A_{MDA} \div W$

3.2.5. Investigation of agronomic traits in fields

Some agronomic traits of "fielder" and overexpression lines were investigated in the field, 30 individual plants were randomly selected for each material, and three biological repeats were analyzed. Traits including grain weight per plant, biomass, thousand seed weight, seed length, seed width, plant height, panicle number per

plant, grain number per panicle, grain number per plant, and main panicle length were investigated. We calculated the stress susceptibility index (SSI), stress tolerance index (STI), and geometric mean productivity (GMP) according to the previous study (Zhou et al. 2020b).

Stress susceptibility index, SSI = [1 - (*Ys*)/(*Yp*)] / [1 - (*s*)/(*p*)],

Stress tolerance index, STI = $(Ys)(Yp)/(p)^2$,

Geometric mean productivity, GMP = $\sqrt{(Ys)(Yp)}$.

Where *Ys* and *Yp* represent the mean grain weights of 5 plants of "fielder" and overexpression lines evaluated under stress and normal conditions, respectively, and *s* and *p* are the mean grain weights of all the "fielder" and overexpression assessed lines under stress and non-stress conditions, respectively.

3.2.6. Analysis of wheat stem strength

To identify the stem strength of "fielder" and *TaCOMT1A* overexpression lines during the maturation stage, strength resistance to puncture and lodging was analyzed using a Handheld digital display push-pull force meter with two different types of probes (Handpi instruments, model number HP-200), which is measured the strength (Newton, N) when the probe push or pierce the wheat stem.

3.2.7. Determination of the sakuranetin and melatonin

This method referenced previous studies. Briefly, the shoot of different wheat samples (including different varieties and overexpression lines in this study) or enzyme-catalyzed reactants (including different concentrations of substrate NAS or naringenin with protein TaCOMT1A) in enzyme activity experiments were subjected to HPLC with an Ultra Violet detector system (Wooking, K2025, China). Sakuranetin, melatonin, and GA_3 were detected at 270 nm (Rakwal et al. 2000), 220 nm (Byeon et al. 2014a), and 210 nm(Cen et al. 2023), respectively.

3.2.8. In vitro expression and purification of TaCOMT1A protein

The coding sequences of *TaCOMT1A* were amplified and cloned into the vectors pET-28a (*Eco*RI/*Sal*I) for the heterologous expression of proteins in a prokaryotic system. The fused protein TaCOMT1A-His was purified using the His label affinity chromatography (Byeon et al. 2015). The Bradford method determined the protein concentration using a protein assay dye kit (Bio-Rad, USA). The purified protein was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the purified protein was stored at -20 °C for further studies.

3.2.9. In vitro enzyme activity analysis of TaCOMT1A

Substrate affinity (K_m) and maximum reaction rate (V_{max}) values of

TaCOMT1A were calculated according to Lineweaver-Burk plots at 37 °C based on the previous study (Byeon et al. 2015). In brief, 50 μg TaCOMT1A-His protein purified from *E. coli* was used for the *in vitro* enzyme activity analysis. The purified protein was incubated with different concentrations of substrate N-acetylserotonin (NAS) or naringenin (5,7,4'-Trihydroxyflavanone) (0.1, 0.2, 0.4, 0.8, and 1.6 $mmol \cdot L^{-1}$) in the reaction buffer for 60 min at 37 °C. The enzyme-catalyzed reactants were collected to further measure the content of sakuranetin and melatonin. Each experiment was repeated independently three times.

3.2.10. Constructing phylogenetic tree of COMT gene family in rice and wheat

We downloaded the amino acid sequence of 12 COMT in wheat from the phytozome (https://phytozome-next.jgi.doe.gov) and the amino acid sequence of 2 COMT in rice from the national rice data central (https://www.ricedata.cn/gene/). The phylogenetic tree of the COMT gene family was constructed by the MEGA11.0 (Molecular Evolutionary Genetics Analysis) software (Tamura et al. 2013).

3.2.11. Statistical analysis

To analyze the significant difference between *TaCOMT1A* overexpression lines and WT, we conducted a significance analysis via two-tailed *Student's t-tests*. For each treatment, the SD of the mean was calculated based on at least three biological replicates. To analyze the difference in phenotypic and physiological characteristic values among different concentrations of sakuranetin in drought stress conditions, Duncan's test ($P<0.05$) was performed to evaluate the significance of the difference using the SPSS 17.0 statistical software package. All histogram graphs were drawn using the GraphPad prism (Version 8.4.0), and the pictures using Adobe Illustrator 2021.

3.3. Results

3.3.1. Overexpression of TaCOMT1A enhanced overexpression lines drought tolerance both in greenhouse and field

Our previous study has demonstrated that *TaCOMT1A* (*Traes_1AL_D9035D5E0*) from wheat can improve the drought tolerance of overexpression Arabidopsis by increasing the synthesis of melatonin in the greenhouse (Yang et al. 2019). In this study, we transformed the *TaCOMT1A* gene into the wheat variety "fielder" using Agrobacterium-mediated methods (Figure 3-1A). Through PCR assay for T_0-T_2 generation, we obtained eight T_3 generation overexpression lines, including lines *#2*, *#3*, *#6*, *#7*, *#8*, *#9*, *#11*, and *#15* (Figure 3-1B). In addition, qRT-PCR analysis in T_3 generation suggested that the expression of TaCOMT1A in eight overexpression lines was significantly higher than wild wild-type (WT) wheat ($P<0.01$) (Figure 3-

1C). Among eight overexpression lines, *TaCOMT1A* expression in the *#6*, *#8*, and *#9* overexpression lines was higher than the other lines, and lines *#6*, *#8*, and *#9* were used for further functional analysis.

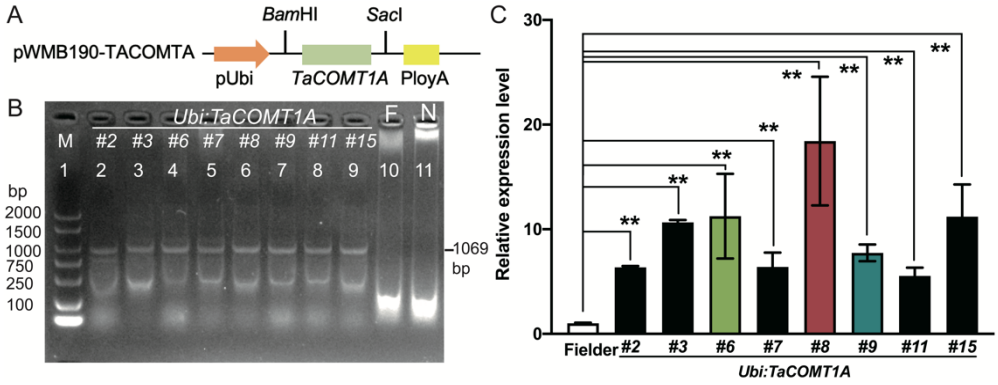


Figure 3-1 Overexpression of *TaCOMT1A* in transgenic receptor variety ("fielder").

(A) Diagram of the plant expression vector. (B) T_3 generation transgenic lines of *Ubi:TaCOMT1A*. Lane 1: M, marker 2000. Lane 2–9: positive transgenic plants of *TaCOMT1A*. Lane 10: F, "fielder." Lane 11: N, negative control (water). (C) Relative expression of *TaCOMT1A* in all positive transgenic lines and "fielder." *TaActin* was used as the internal control. All data represents means ± SD (n = 3). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (**$P<0.01$).

To investigate the drought tolerance of *TaCOMT1A* overexpression lines during the seedling stage, we cultured seedlings of *TaCOMT1A* overexpression lines and WT in boxes. We stopped watering on the 1st day after sowing (DAS) seeds. The soil relative water content decreased to around 62% and 46% on the 15th and 18th DAS, respectively (Figure 3-2D). Then, we rewatered on the 21st DAS, and the survival rate was counted on the 7th day after rewatering. Results showed that the growth of overexpression lines was better than WT under drought stress, especially after drought treatment for 15 DAS and rehydration (Figure 3-2A–3-2C), and the survival rate of overexpression lines was significantly ($P<0.01$) higher than WT after rewatering (Figure 3-2E). We investigated stress-related physiological characteristics of overexpression lines on 15th DAS. Results indicated that overexpression lines had significantly ($P<0.01$) higher fresh weight and relative water content of shoot compared to WT (Figure 3-2F–3-2G). At the same time, there were no significant differences in dry weight of the shoot between WT and overexpression lines (Figure 3-2H). In addition, results showed that the MDA and H_2O_2 content of overexpression lines were significantly lower than WT, and CAT activity of overexpression lines was significantly ($P<0.01$) higher than WT after drought treatment for 15 days during the seedling stage (Figure 3-2I–3-2K). Those results

suggested that overexpression of *TaCOMT1A* significantly enhanced overexpression lines' drought tolerance during a seedling stage in the greenhouse.

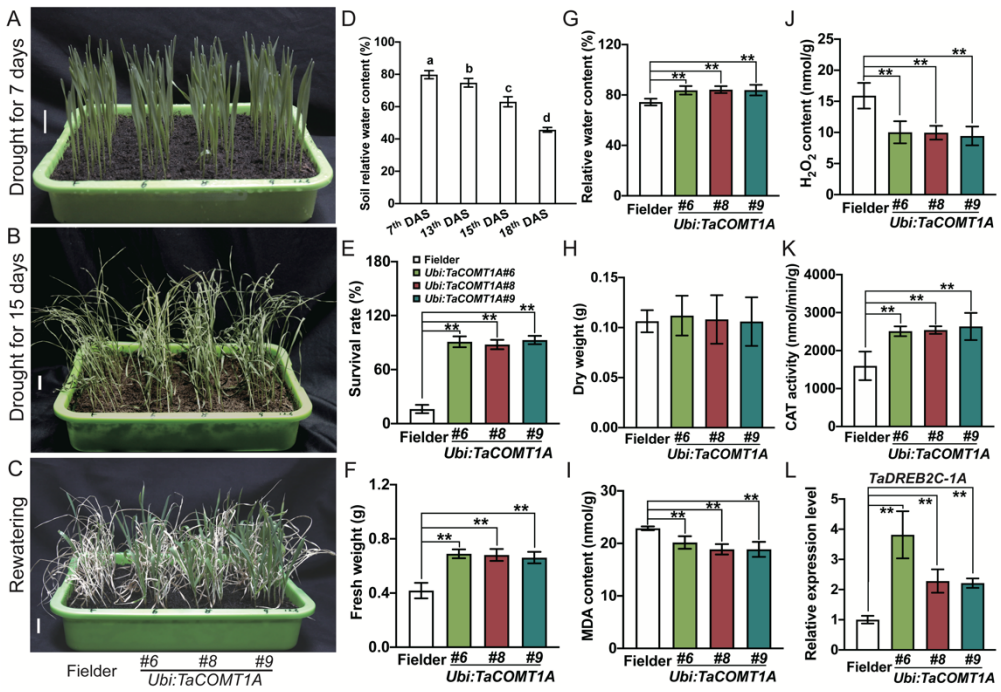


Figure 3-2 Phenotype of *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder") under drought conditions at the seedling stage.

(A) Seedlings of "fielder" and overexpression lines after drought treatment for 7 days, all pots were stopped watering from 1st day after sowing (DAS), and germinated seeds were planted in a soil mixture which was blended with soil (Pindstrup, Denmark) and water at a ratio of 1:2 (g/mL). (B) Overexpression lines grew better than "fielder" after drought treatment for 15 days at the seedling stage. (C) More seedlings survived in overexpression lines than in "fielder" after drought stress for 21 days and rewatering for one week. (D) Soil relative water content on the 7th, 13th, 15th, and 18th DAS. (E) Survival rates of "fielder" and overexpression lines after drought stress for 21 days and rewatering for one week. (F)–(H) Fresh weight (F), relative water content (G), and dry weight (H) of seedlings of "fielder" and overexpression lines after drought treatment for 15 days. (I)–(J) MDA (I) and H_2O_2 (J) content of seedlings of "fielder" and overexpression lines after drought treatment for 15 days. (K) CAT activity of "fielder" seedlings and overexpression lines after drought treatment for 15 days. (L) Relative expression of a drought-related transcription factor, *TaDREB2C-1A*, in "fielder" and transgenic lines. All data represents means ± SD (n = 3). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (***P*<*0.01*). Scale bars = 3 cm. DAS: days after sowing; H_2O_2: hydrogen peroxide; MDA: malondialdehyde; CAT: catalase.

We planted overexpression lines and "fielder" in the field to further identify the

drought tolerance of *TaCOMT1A* overexpression lines in the field. We treated them with two irrigation conditions, including WIR (well-irrigated, three rounds of irrigation throughout the entire growth period) and LIR (limited irrigation, two rounds of irrigation throughout the entire growth period) (Figure 3-3A–3-3B, Table 3-2–3-3). After the harvest, we found that the *TaCOMT1A* overexpression lines had larger grain sizes than "fielder" under WIR and LIR conditions (Figure 3-3C–3-3D). Results of grain characters investigation showed that seed length, seed width, and thousand seed weight of the overexpression lines were higher than WT under WIR and LIR conditions (Figure 3-3E–3-3G). There is no significant difference in panicle number per plant and grain number per panicle between overexpression lines and WT under WIR and LIR conditions (Figure 3-4A–3-4B), whereas, under LIR conditions, the grain number per plant of overexpression lines was significantly higher than that of WT ($P<0.05$) (Figure 3-4C). Finally, under the LIR conditions, grain weight per plant of overexpression lines was significantly higher than that of WT ($P<0.01$), while under the WIR conditions, those differences were not significant (Figure 3-3H). In addition, the biomass of overexpression lines plants was also higher than WT under the LIR conditions ($P<0.05$), while under the WIR conditions, those differences were not significant (Figure 3-3I). The plant height and main panicle length of overexpression lines significantly decreased compared with the "fielder" under WIR and LIR conditions (Figure 3-3J, Figure 3-4D). In addition, data in the year 2022 also showed the same results (Figure 3-5A–3-5J, Table 3-4–3-5). Based on the results of the grain weight per plant under WIR and LIR conditions, we calculate three parameters, the stress tolerance index, the geometric mean productivity, and the stress susceptibility index, to evaluate the drought tolerance of overexpression lines in the field. Finally, results showed that the stress tolerance index and geometric mean productivity of *TaCOMT1A* overexpression lines were significantly higher than "fielder" (Figure 3-3K–3-3L). In contrast, the stress susceptibility index of overexpression lines was considerably lower than in "fielder" (Figure 3-3M), which indicated that *TaCOMT1A* overexpression lines significantly improved grain weight per plant under drought conditions and had more substantial drought tolerance compared to WT in the field.

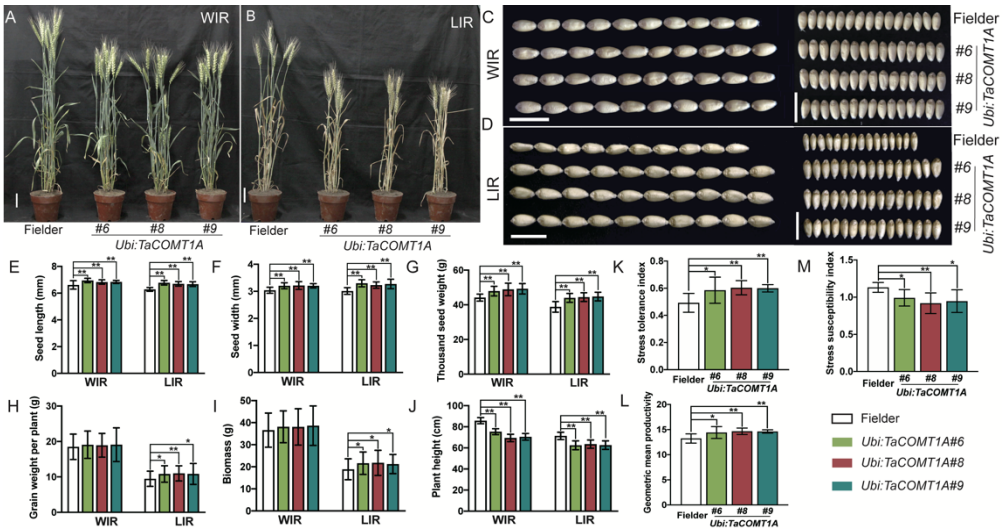


Figure 3-3 Agronomic traits of *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder") at the maturation stage in well-irrigated and limited irrigation field conditions.

(A)–(B) Phenotype of "fielder" and overexpression lines in WIR (A) and LIR (B) conditions. Scale bars = 10 cm. (C)–(D) Seed of "fielder" and overexpression lines in WIR (C) and LIR (D) conditions. Scale bars = 1 cm. (E)–(J) Seed length (E), seed width (F), thousand seed weight (G), grain weight per plant (H), biomass represents the fresh weight of the above-ground plant part. (I), and plant height (J) of "fielder" and overexpression lines in WIR and LIR conditions. (K)–(M) Stress tolerance index (K), geometric mean productivity (L), and stress susceptibility index (m) of "fielder" and overexpression lines in field conditions. All data represents means ± SD ($n \geq 6$). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*$P<0.05$; **$P<0.01$). LIR: limited irrigation, WIR: well-irrigated. Sowing density was set at 50 seeds per row (length of each row is 2 m), "fielder" and overexpression lines have three rows in each replicate, and three replicates were given in WIR and LIR. In LIR conditions, two rounds of irrigation were applied in total, including at the pre-wintering and jointing stages. In WIR conditions, three rounds of irrigation were applied, including at the pre-wintering, jointing, and filling stages. One round of irrigation was applied to 750 m^3 of water per hectare area in the field.

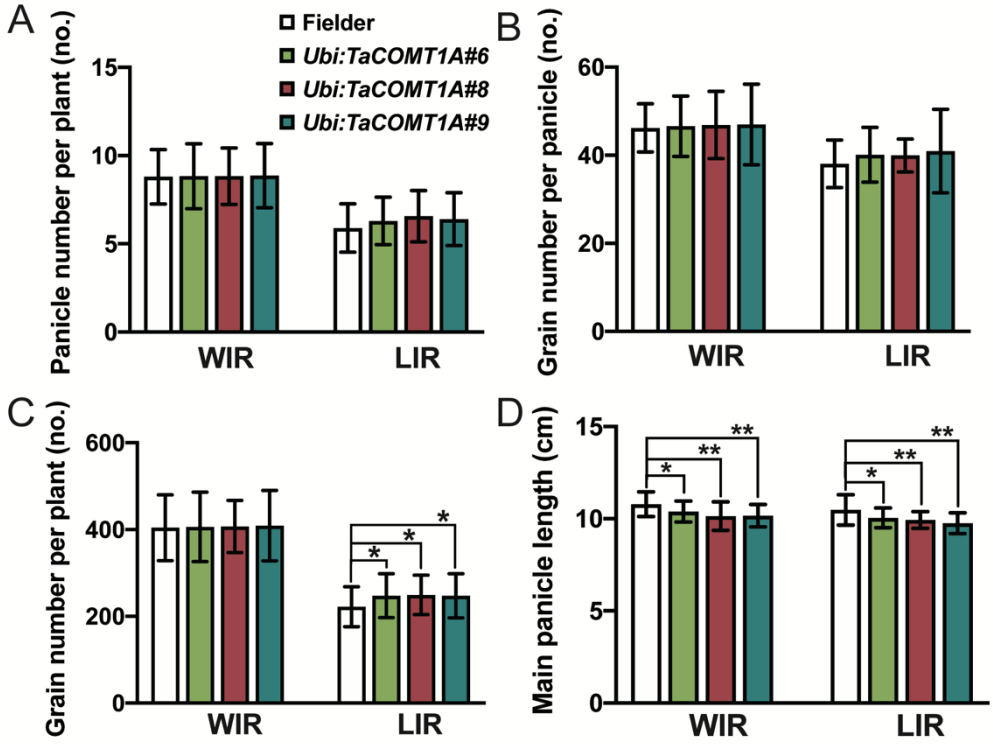


Figure 3-4 Agronomic traits of *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder") in well-irrigated and limited irrigation conditions in 2023.

(A)–(D) Panicle number per plant (A), grain number per panicle (B), grain number per plant (C), and main panicle length (D) of "fielder" and *TaCOMT1A* overexpression lines in WIR and LIR conditions, LIR: limited irrigation, WIR: well-irrigated. Sowing density was set at 50 seeds per row (length of each row is 2 m), "fielder" and overexpression lines have three rows in each replicate, and three replicates were given in LIR and WIR conditions. In LIR conditions, two rounds of irrigation were applied, including the pre-wintering and jointing stages. In WIR conditions, three rounds of irrigation were applied, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied 750 m^3 of water per hectare area in a field. All data represents means ± SD (n = 30). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*$P<0.05$, **$P<0.01$).

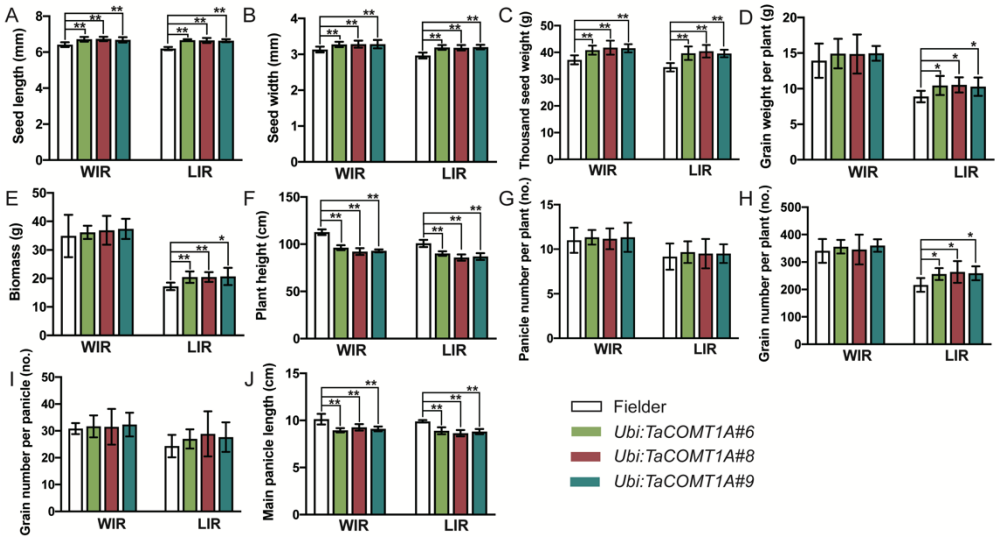


Figure 3-5 Agronomic traits of *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder") in well-irrigated and limited irrigation conditions in 2022.

(A)–(J) Seed length (A), seed width (B), thousand seed weight (C), grain weight per plant (D), biomass represents the fresh weight of the above-ground plant part (E), plant height (F), panicle number per plant (G), grain number per plant (H), grain number per panicle (I), and main panicle length (J) of "fielder" and *TaCOMT1A* overexpression lines in WIR and LIR conditions, LIR: limited irrigation, WIR: well-irrigated. Sowing density was set at 50 seeds per row (length of each row is 2 m), "fielder" and overexpression lines have three rows in each replicate, and three replicates were given in LIR and WIR conditions. In LIR conditions, two rounds of irrigation were applied, including the pre-wintering and jointing stages. In WIR conditions, three rounds of irrigation were applied, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied 750 m^3 of water per hectare area in a field. All data represents means ± SD (n = 6). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*$P<0.05$, **$P<0.01$).

3.3.2. Overexpression of TaCOMT1A inhibited plant height and increased stem strength in wheat

Further phenotype observation of *TaCOMT1A* overexpression lines and "fielder" during the maturation stage in the field, we found that three overexpression lines have more resistance to lodging than the WT in field conditions (Figure 3-6A). In addition, the plant length, spike length, and different internodes of overexpression lines were shortened to varying degrees, and the stem diameter became thicker than WT (Figure 3-6B–3-6C). The analysis results showed that the decrease in plant height of overexpression lines was mainly caused by the shortening of the 1st, 2nd, and 5th internode (from top to bottom) length of the main stem compared to WT

(P<0.01) (Figure 3-6D). Consistently, overexpression lines significantly decreased plant height at the seedling and heading stage compared to WT (Figure 3-7A–3-7D). In addition, overexpression lines have significantly wider internode diameters in the 2nd, 3rd, and 4th main stem internodes (Figure 3-6E).

Furthermore, we also found that the lignin content in overexpression lines was significantly higher than in "fielder" in the stem (P<0.01) (Figure 3-6F). The puncture resistance and lodging resistance capability of overexpression lines were considerably stronger than "fielder" (P<0.01) (Figure 3-6G–3-6H). Those results indicated that overexpression of *TaCOMT1A* can inhibit plant height and increase stem strength in wheat.

Table 3-2 Agronomic traits of "fielder" and overexpression lines under well-irrigated field conditions in 2023.

Traits	**"fielder"**	***Ubi:TaCOMT1A#6***		***Ubi:TaCOMT1A#8***		***Ubi:TaCOMT1A#9***	
	Mean ± SD	Mean ± SD	IR	Mean ± SD	IR	Mean ± SD	IR
Seed length (mm)	6.61 ± 0.31	6.95 ± 0.16**	5.04%	6.83 ± 0.16**	3.28%	6.84 ± 0.11**	3.43%
Seed width (mm)	3.04 ± 0.11	3.21 ± 0.11**	5.43%	3.22 ± 0.14**	5.74%	3.2 ± 0.08**	5.37%
Thousand seed weight (g)	44.15 ± 1.99	47.89 ± 2.71**	8.46%	48.86 ± 3.52**	10.66%	49.24 ± 2.96**	11.51%
Grain weight per plant (g)	18.49 ± 3.52	19.07 ± 3.79	3.15%	18.92 ± 3.29	2.33%	19.09 ± 4.67	3.24%
Biomass (g)	36.6 ± 7.61	38.13 ± 7.1	4.17%	38.07 ± 8.09	4.02%	38.63 ± 8.8	5.54%
Plant height (cm)	85.77 ± 2.88	75.34 ± 2.75**	-12.15%	69.4 ± 3.42**	-19.08%	70.49 ± 3.09**	-17.82%
Panicle number per plant (no.)	8.8 ± 1.51	8.83 ± 1.81	0.38%	8.83 ± 1.57	0.38%	8.87 ± 1.78	0.76%
Grain number per panicle (no.)	46.11 ± 5.32	46.56 ± 6.75	0.96%	46.81 ± 7.49	1.52%	46.95 ± 8.97	1.82%
Grain number per plant (no.)	404.17 ± 74.42	406.13 ± 78.81	0.49%	406.93 ± 59.03	0.68%	409 ± 79.67	1.20%
Main panicle length (cm)	10.79 ± 0.66	10.39 ± 0.56*	-3.71%	10.14 ± 0.76**	-6.00%	10.16 ± 0.6**	-5.78%

Sowing density was set at 50 seeds per row (length of each row is 2 m), "fielder" and overexpression lines have three rows in each replicate, and three replicates were given in LIR and WIR. In LIR conditions, two rounds of irrigation were applied, including the pre-wintering and jointing stages. In WIR conditions, three rounds of irrigation were used, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied to 750 m^3 of water per hectare area in the field. All data represents means ± SD (n = 30). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*$P<0.05$; **$P<0.01$). Biomass represents the fresh weight of the above-ground plant part. IR: increase ratio; SD: standard deviation; WIR: well-

irrigated.

Table 3-3 Agronomic traits of "fielder" and overexpression lines under limited irrigation field conditions in 2023.

Traits	**"fielder"**	***Ubi:TaCOMT1A#6***		***Ubi:TaCOMT1A#8***		***Ubi:TaCOMT1A#9***	
	Mean ± SD	Mean ± SD	IR	Mean ± SD	IR	Mean ± SD	IR
Seed length (mm)	6.28 ± 0.14	6.77 ± 0.17**	7.80%	6.7 ± 0.17**	6.63%	6.67 ± 0.18**	6.26%
Seed width (mm)	3.01 ± 0.12	3.3 ± 0.13**	9.49%	3.23 ± 0.12**	7.24%	3.27 ± 0.17**	8.62%
Thousand seed weight (g)	38.8 ± 3.02	43.99 ± 2.52**	13.36%	44.41 ± 2.5**	14.46%	44.76 ± 2.45**	15.36%
Grain weight per plant (g)	9.47 ± 2.1	10.83 ± 2.28*	14.28%	10.99 ± 2.1**	16.04%	10.84 ± 2.9*	14.43%
Biomass (g)	18.86 ± 4.67	21.6 ± 4.99*	14.53%	21.78 ± 5.6*	15.47%	21.24 ± 4.27*	12.59%
Plant height (cm)	71.35 ± 3.39	62.45 ± 4.12**	-12.47%	63.56 ± 3.84**	-10.92%	62.58 ± 4**	-12.29%
Panicle number per plant (no.)	5.9 ± 1.35	6.3 ± 1.32	6.78%	6.57 ± 1.43	11.30%	6.4 ± 1.47	8.47%
Grain number per panicle (no.)	37.93 ± 5.36	40.06 ± 6.16	5.62%	39.98 ± 3.69	5.40%	40.94 ± 9.31	7.94%
Grain number per plant (no.)	222.13 ± 45.19	247.53 ± 49.91*	11.43%	249.37 ± 44.55*	12.26%	247.47 ± 49.86*	11.40%
Main panicle length (cm)	10.48 ± 0.81	10.05 ± 0.52*	-4.13%	9.93 ± 0.44**	-5.22%	9.75 ± 0.55**	-6.97%

Sowing density was set at 50 seeds per row (length of each row is 2 m), "fielder" and overexpression lines have three rows in each replicate, and three replicates were given in LIR and WIR. In LIR conditions, two rounds of irrigation were applied, including the pre-wintering and jointing stages. In WIR conditions, three rounds of irrigation were used, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied to 750 m^3 of water per hectare area in the field. All data represents means ± SD (n = 30). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (**P<0.05*; ***P<0.01*). Biomass represents the fresh weight of the above-ground plant part. IR: increase ratio; SD: standard deviation; LIR: limited irrigation.

Traits	**"fielder"**	***Ubi:TaCOMT1A#6***		***Ubi:TaCOMT1A#8***		***Ubi:TaCOMT1A#9***	
	Mean ± SD	Mean ± SD	IR	Mean ± SD	IR	Mean ± SD	IR
Seed length (mm)	6.41 ± 0.12	6.72 ± 0.12**	4.81%	6.73 ± 0.11**	4.94%	6.68 ± 0.14**	-17.58%
Seed width (mm)	3.13 ± 0.07	3.28 ± 0.07*	4.52%	3.28 ± 0.09*	4.79%	3.28 ± 0.11*	-10.20%
Thousand seed weight (g)	37.2 ± 1.53	40.84 ± 1.55**	9.78%	41.77 ± 2.4**	12.27%	41.53 ± 1.4**	7.08%
Grain weight per plant (g)	13.93 ± 2.2	14.93 ± 1.89	7.14%	14.87 ± 2.5	6.72%	14.96 ± 0.95	3.03%
Biomass (g)	34.91 ± 6.78	36.16 ± 2.12	3.60%	36.87 ± 4.63	5.61%	37.38 ± 3.23	5.80%
Plant height (cm)	112.73 ± 2.71	96.27 ± 2.11**	-14.60%	92.18 ± 3.29**	-18.23%	92.91 ± 1.38**	7.39%
Panicle number per plant (no.)	11 ± 1.29	11.33 ± 0.75	3.03%	11.17 ± 1.07	1.52%	11.33 ± 1.49	11.64%
Grain number per panicle (no.)	31.05 ± 2.17	31.62 ± 3.81	1.86%	31.49 ± 6.28	1.43%	32.33 ± 4.36	4.16%
Grain number per plant (no.)	340.5 ± 39.42	355.92 ± 22.73	4.53%	345.58 ± 49.56	1.49%	360.25 ± 20.87	4.79%
Main panicle length (cm)	10.13 ± 0.51	8.95 ± 0.21**	-11.68%	9.27 ± 0.31**	-8.55%	9.1 ± 0.23**	4.13%

Table 3-4 Agronomic traits of "fielder" and overexpression lines under well-irrigated field conditions in 2022.

Sowing density was set at 50 seeds per row (length of each row is 2 m), "fielder" and overexpression lines have three rows in each replicate, and three replicates were given in LIR and WIR conditions. In LIR conditions, two rounds of irrigation were applied, including the pre-wintering and jointing stages. In WIR conditions, three rounds of irrigation were used, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied to 750 m^3 of water per hectare area in the field. All data represents means ± SD (n = 6). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using Student's t-test (*$P<0.05$; **$P<0.01$). Biomass represents the fresh weight of the above-ground plant part. IR: increase ratio; SD: standard deviation; WIR: well-irrigated.

Table 3-5 Agronomic traits of "fielder" and overexpression lines under limited irrigation field conditions in 2022.

Traits	**"fielder"**	***Ubi:TaCOMT1A#6***		***Ubi:TaCOMT1A#8***		***Ubi:TaCOMT1A#9***	
	Mean ± SD	Mean ± SD	IR	Mean ± SD	IR	Mean ± SD	IR
Seed length (mm)	6.18 ± 0.07	6.65 ± 0.04**	7.55%	6.63 ± 0.11**	7.21%	6.63 ± 0.07**	-13.80%
Seed width (mm)	2.97 ± 0.07	3.19 ± 0.06**	7.58%	3.18 ± 0.07**	7.30%	3.2 ± 0.06**	-10.94%
Thousand seed weight (g)	34.45 ± 1.43	39.68 ± 2.31**	15.17%	40.42 ± 2.15**	17.31%	39.59 ± 1.28**	20.23%
Grain weight per plant (g)	8.89 ± 0.74	10.45 ± 1.22*	17.50%	10.52 ± 0.98*	18.37%	10.28 ± 1.17*	3.64%
Biomass (g)	17.21 ± 1.2	20.46 ± 1.83**	18.87%	20.49 ± 1.56**	19.06%	20.69 ± 2.74*	19.55%
Plant height (cm)	100.82 ± 3.62	90.09 ± 1.57**	-10.64%	85.82 ± 1.89**	-14.88%	86.91 ± 3.54**	15.58%
Panicle number per plant (no.)	9.17 ± 1.34	9.67 ± 1.11	5.45%	9.5 ± 1.5	3.64%	9.5 ± 0.96	14.89%
Grain number per panicle (no.)	24.09 ± 3.77	26.78 ± 3.07	11.16%	28.79 ± 7.56	19.53%	27.65 ± 4.84	7.14%
Grain number per plant (no.)	216.5 ± 22.86	256.17 ± 19.69*	18.32%	263.67 ± 36.17*	21.79%	258.83 ± 23.33*	7.87%
Grain number per panicle (no.)	24.09 ± 3.77	26.78 ± 3.07	11.16%	28.79 ± 7.56	19.53%	27.65 ± 4.84	14.79%

Sowing density was set at 50 seeds per row (length of each row is 2 m), "fielder" and overexpression lines have three rows in each replicate, and three replicates were given in LIR and WIR. In LIR conditions, two rounds of irrigation were applied, including the pre-wintering and jointing stages. In WIR conditions, three rounds of irrigation were applied, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied to 750 m^3 of water per hectare area in the field. All data represents means ± SD (n = 6). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using Student's t-test (*$P<0.05$; **$P<0.01$). Biomass represents the fresh weight of the above-ground plant part. IR: increase ratio; SD: standard deviation; LIR: limited

irrigation.

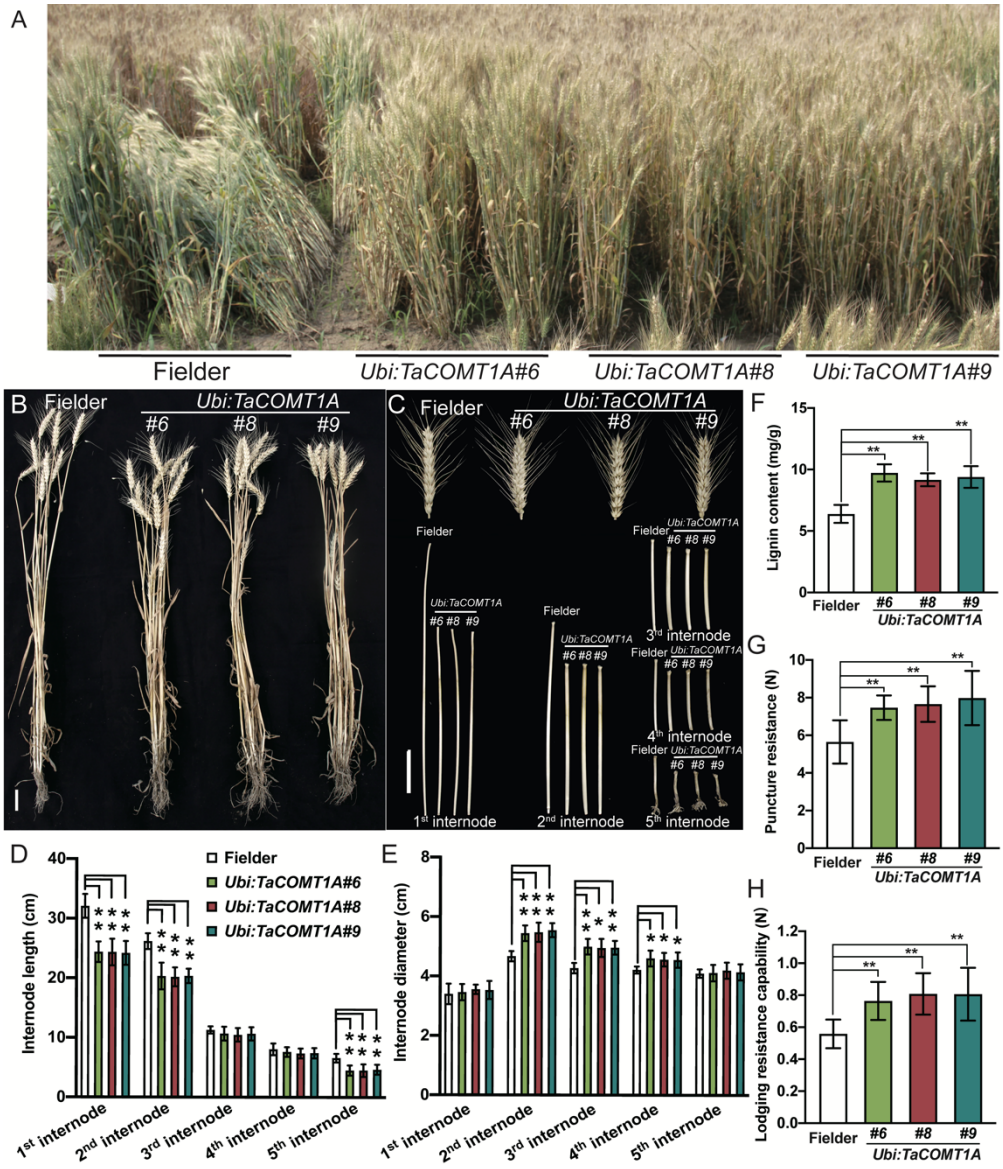


Figure 3-6 Plant height and stem strength of *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder") at the maturation stage.

(A) *TaCOMT1A* overexpression lines have higher lodging resistance. (B) Whole plant phenotype of "fielder" and *TaCOMT1A* overexpression lines at maturation stage. (C) The main panicle and internode phenotype of "fielder" and *TaCOMT1A* overexpression lines are at the maturation stage. (D) Internode length of "fielder" and *TaCOMT1A* overexpression lines at the maturation stage. (E) The internode diameter of the "fielder" and *TaCOMT1A*

overexpression lines are at the widest point of each internode at the maturation stage. (F) Lignin content in "fielder" and overexpression lines. (G)–(H) Puncture resistance (G) and lodging resistance capability (H) of "fielder" and *TaCOMT1A* overexpression lines at the maturation stage. All data represents means ± SD (n = 3). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*$P<0.05$; **$P<0.01$). Scale bars = 5 cm.

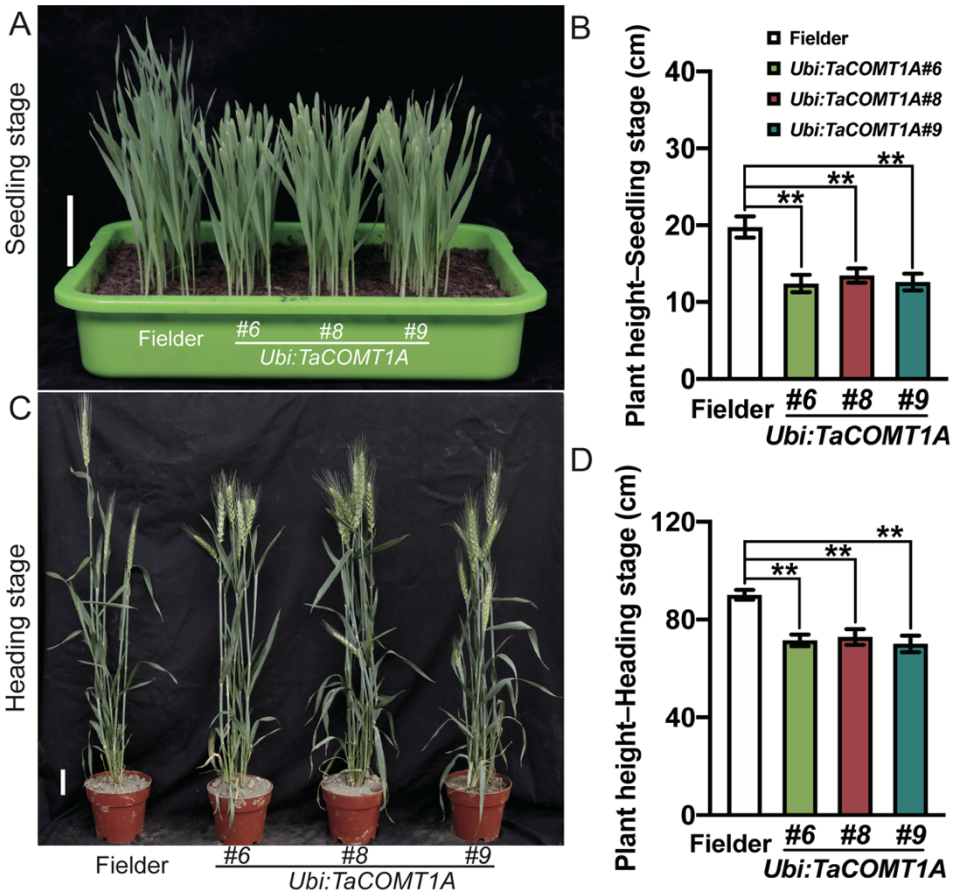


Figure 3-7 Plant height of *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder") both at the seedling and heading stage.

(A) Phenotype of "fielder" and *TaCOMT1A* overexpression lines at the seedling stage. (B) Plant height of "fielder" and *TaCOMT1A* overexpression lines at the seedling stage. (C) The phenotype of "fielder" and *TaCOMT1A* overexpression lines at the heading stage. (D) Plant height of "fielder" and *TaCOMT1A* overexpression lines at heading stage. All data represents means ± SD (n = 3). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (**$P<0.01$). Scale bars = 5 cm.

3.3.3 Overexpression of TaCOMT1A decreased GA_3 content by downregulating GA biosynthesis genes

To figure out whether overexpression of *TaCOMT1A* decreased plant height through inhibition of GA synthesis, the plants were sampled under normal conditions during the seedling stage (Figure 3-7A), and different forms of GA, including GA_1, GA_3, GA_7, GA_9, and GA_{15} were detected in the "fielder" and overexpression lines line *#9* by using the UPLC-MS/MS method. We found that the GA_1 and GA_3 content was significantly decreased in overexpression line *#9* than that in "fielder" (Figure 3-8A–3-8E), which was consistent with the shortening of overexpression lines compared to WT (Figure 3-7A). To further confirm that the dwarfing of *TaCOMT1A* overexpression lines plants was related to GA biosynthesis, we completed the GA_3 complementary hydroponic experiment (Figure 3-9A). Results illustrated that GA_3 content in overexpression lines *#6*, *#8*, and *#9* was significantly lower than in "fielder" in normal water conditions, whereas supplementing 10 μM GA_3 in hydroponic solution reduced plant height differences between overexpression lines and WT (Figure 3-9A). The GA_3 content analysis results showed that the endogenous GA_3 content of overexpression lines was significantly lower than that of WT before GA_3 treatment, and GA_3 treatment could induce an increase in endogenous GA_3 content in all plants. In contrast, the difference in endogenous GA_3 content between overexpression lines and WT became insignificant after GA_3 treatment (Figure 3-9B). Supplementing GA_3 decreased the difference in stem and root length between overexpression lines and WT (Figure 3-9C–3-9D), consistent with the results of GA_3 content analysis (Figure 3-9B). Moreover, we found that overexpression of *TaCOMT1A* can decrease significantly the germination rate after imbibition of 12 h in water conditions (Figure 3-10A, 3-10D, 3-10E). Supplementing 10 μM and 20 μM, GA_3 increased the germination rate of overexpression lines and decreased the difference between overexpression lines and WT (Figure 3-10B–3-10D, 3-10F–3-10G). These results suggested that overexpression of *TaCOMT1A* decreased GA_3 content, and exogenous GA_3 recovered the depletion.

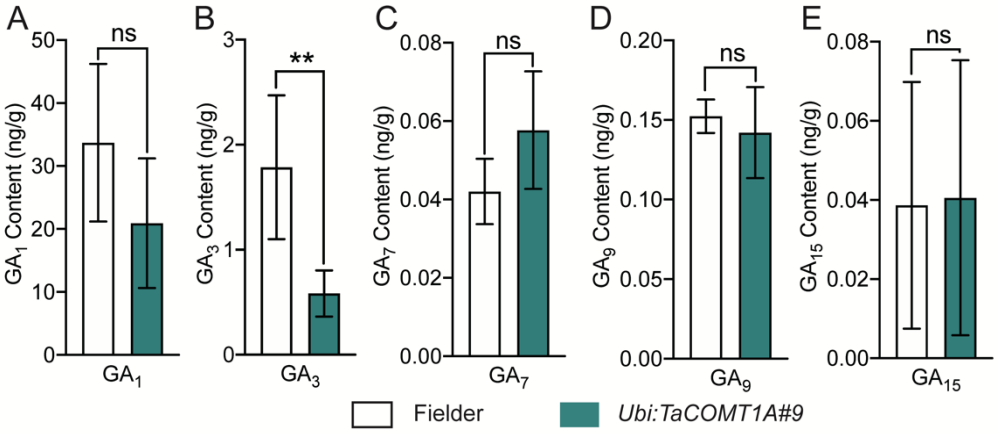


Figure 3-8 Different forms of GA content in *TaCOMT1A* overexpression line #9 and transgenic receptor variety ("fielder").

(A)–(E) Multiple GA forms (GA_1, GA_3, GA_7, GA_9, GA_{15}) content was determined using UPLC-MS/MS in "fielder" and overexpression line *#9*. All data represents means ± SD (n = 3). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (ns, no significant difference; **P<0.05*; ***P<0.01*). GA: gibberellic acid; UPLC: ultra-performance liquid chromatography.

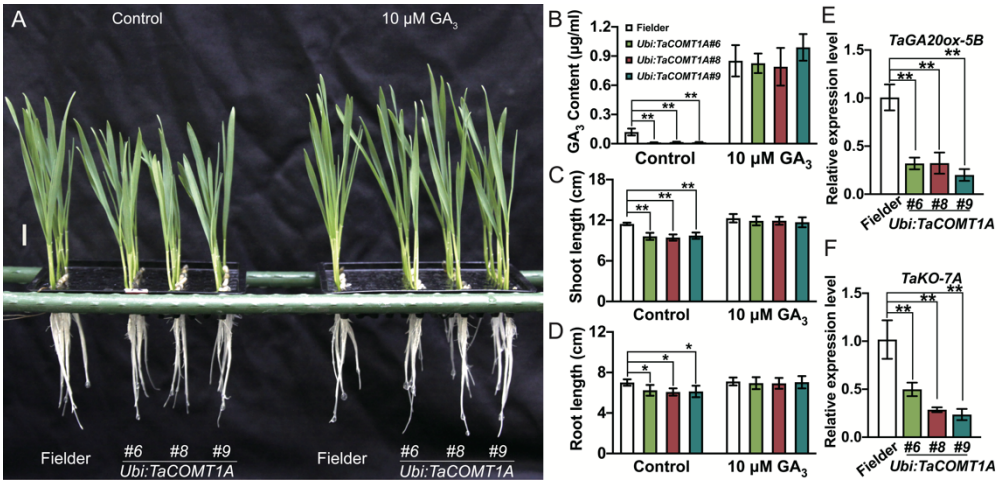


Figure 3-9 GA_3 content and GA biosynthesis genes in *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder").

(A) Phenotype of "fielder" and overexpression lines in the conditions of water (control) and 10 μM GA_3 for 7 days. (B)–(D) GA_3 content (B), shoot length (C), and root length (D) of "fielder" and overexpression lines in the conditions of water and 10 μM GA_3 treatment. (E)–(F) Relative expression of *TaGA20ox-5B* (E) and *TaKO-7A* (F), which are the key genes in the GA biosynthesis pathway, in "fielder" and overexpression lines.

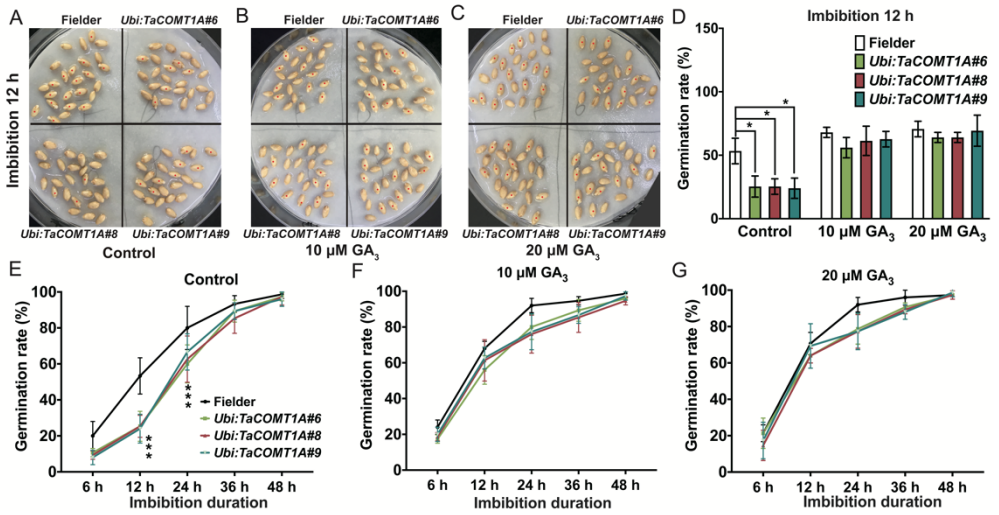


Figure 3-10 Seed germination rate of *TaCOMT1A* overexpression lines and transgenic receptor variety ("fielder").

(A)–(C) Germinated seed of "fielder" and *TaCOMT1A* overexpression lines under water as control (A), 10 μM GA_3 (B), and 20 μM GA_3 (C) conditions. (D) The germination rate of "fielder" and overexpression lines under different conditions after seeds imbibition for 12 h. (E)–(G) Gemination rate of "fielder" and *TaCOMT1A* overexpression lines during imbibition of water (E), 10 μM GA_3 F), 20 μM GA_3 (G) conditions. All data represents means ± SD (n = 3). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*P < 0.05). GA: gibberellic acid.

3.3.4. TaCOMT1A has the activity of synthesizing melatonin and sakuranetin

Previous studies have demonstrated that COMT is a multifunction enzyme that generates different products, some COMTs in the phenylpropanoid metabolite pathway produce different flavonoids (Byeon et al. 2014a; Zhao et al. 2021b). Then, we expressed and purified TaCOMT-His fusion protein using an *Escherichia coli* expression system, to further validate the enzyme activities of *TaCOMT1A in vitro* (Figure 3-11A). Our results showed that TaCOMT1A has the activity of catalyzing substrate, NAS to produce melatonin (K_m = 5.02 μm; V_{max} = 12.06 μm $min^{-1}mg^{-1}$ protein) (Figure 3-12A). Consistently, we observed that overexpression of *TaCOMT1A* significantly increased melatonin content in overexpression lines *#6*, *#8*, and *#9* compared to WT (*P*<0.01) (Figure 3-12B).

To further investigate the function of TaCOMT, ultra-high-performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) was conducted to quantify the flavonoid compounds in "fielder" and overexpression lines. We identified a total of 158 flavonoid compounds in *TaCOMT1A* overexpression lines

and WT. Among 158 flavonoids, the content of 55 flavonoids decreased and five flavonoids including 5,4'-Dihydroxy-7-methoxyflavanone (sakuranetin); isorhamnetin-3,7-O-diglucosid (isorhamnetin); vitexin-7-O-rutinoside (vitexin); kaempferol-3-O-(2'-O-acetyl) glucuronide (kaempferol); isorhamnetin-3-O-rutinoside (narcissin), were significantly increased in overexpression lines compared to WT. To analyze the possible contribution of these flavonoids to function on the drought tolerance of *TaCOMT1A*, we treated wheat with those five flavonoids and investigated drought tolerance during the seedling stage. The results showed that only three flavonoids, including sakuranetin, isorhamnetin, and vitexin, could improve the drought tolerance of wheat (The result is not displayed).

Then, we analyzed the activity of TaCOMT1A in synthesizing the above three flavonoids by using an *in vitro* enzyme activity experiment. The results showed that TaCOMT1A can only synthesize sakuranetin *in vitro* (K_m = 775.71 μm; V_{max} = 20.41 μm $min^{-1}mg^{-1}$ protein) and has no activity in synthesizing the other two flavonoids (Figure 3-11B). In addition, the metabolomic analysis results showed that the content of the substrate (naringenin) to synthesize sakuranetin decreased, and the content of sakuranetin increased in *TaCOMT1A* overexpression lines compared to WT (P<0.01) (Figure 3-11C–3-11D). Furthermore, we confirmed that the sakuranetin content in the three overexpression lines was significantly (P<0.05) higher than that in WT (Figure 3-11E). These results demonstrated that in addition to synthesizing melatonin, TaCOMT1A has the activity of synthesizing sakuranetin, which is a new function of TaCOMT1A enzyme.

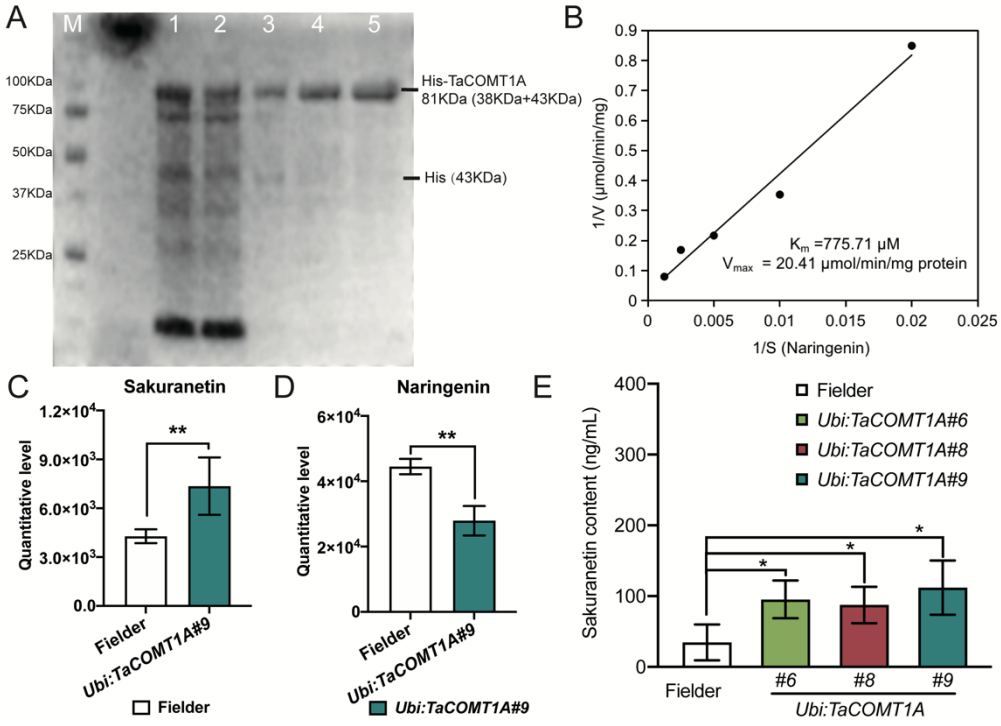


Figure 3-11 Purification of the TaCOMT1A protein and methyltransferase activity of TaCOMT1A in the sakuranetin biosynthesis pathway.

(A) Purification of His-tagged TaCOMT1A protein in *Escherichia coli*, purified protein was used for further determination of *in vitro* enzyme activity of TaCOMT1A. M: molecular protein standard; lane 1–2, total proteins in 15 μL aliquots of bacterial culture with IPTG; lane 3–5, purified His-TaCOMT1A proteins. (B) Determination of the K_m and V_{max} of TaCOMT1A for the biosynthesis of sakuranetin from naringenin. TaCOMT1A (1 μg) was incubated with different concentrations of substrate (naringenin) for 60 min at 37°C, and sakuranetin content was measured at different substrate conditions using HPLC with an Ultra Violet detector system (Wooking, K2025, China). The K_m and V_{max} values were determined using Lineweaver–Burk plots. (C) In overexpression line *#9, Sakuranetin content* was significantly higher than in "fielder." (D) Naringenin (substrate of biosynthesis to sakuranetin) content was significantly decreased in the overexpression line *#9* compared to "fielder." Sakuranetin and naringenin content in shoots of "fielder" and overexpression line *#9* were determined using an ultra-high-performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) (HPLC, Shim-pack UFLC SHIMADZU CBM30A; MS, Applied Biosystems 6500 QTRAP). (E) Sakuranetin content was determined using high-performance liquid chromatography (HPLC) with an Ultra Violet detector system (Wooking, K2025, China) in shoots of "fielder" and *TaCOMT1A* overexpression line *#6*, *#8*, and *#9*. All data represents means ± SD (n = 3). HPLC: high-performance liquid chromatography; IPTG: isopropyl-β-d-thiogalactoside; K_m: Michaelis constant; UPLC-MS/MS: ultra-high-performance liquid chromatography-tandem mass spectroscopy; V_{max}: maximum reaction rate.

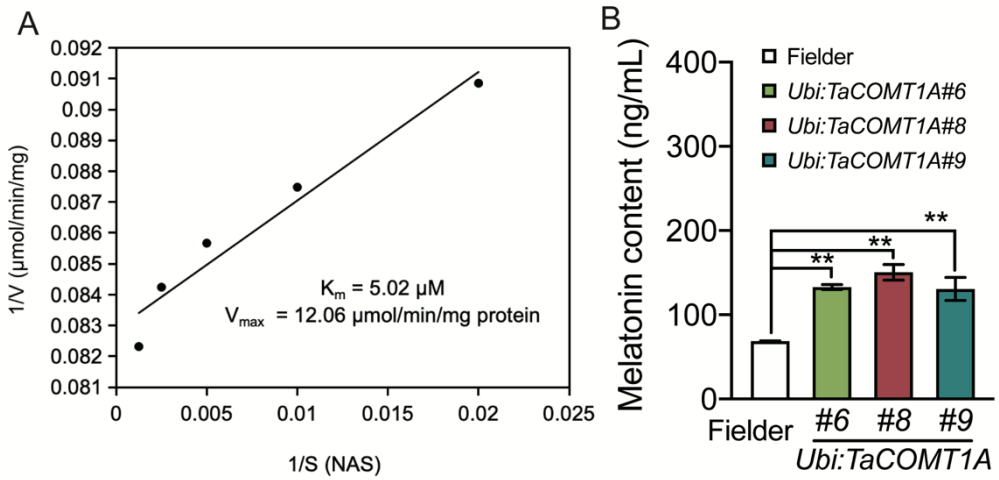


Figure 3-12 Methyltransferase activity of TaCOMT1A in melatonin biosynthesis pathway.

(A) Determination of the K_m and V_{max} of TaCOMT1A for N-acetylserotonin (NAS). TaCOMT1A (1 μg) was incubated with different substrate concentrations (NAS) for 60 min

at 37°C. The K_m and V_{max} values were determined using Lineweaver–Burk plots. N-acetylserotonin methyltransferase activity of purified TaCOMT1A protein by HLPC. (B) Melatonin contents were determined using ultra-high-performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) in "fielder" and *TaCOMT1A* overexpression lines *#6*, *#8*, and *#9*. All data represents means ± SD (n = 3). HPLC: high-performance liquid chromatography; IPTG: isopropyl-β-d-thiogalactoside; K_m: Michaelis constant; UPLC-MS/MS: ultra-high-performance liquid chromatography-tandem mass spectroscopy; V_{max}: Maximum reaction rate.

3.4. Discussion

3.4.1. TaCOMT1A is a multifunctional enzyme that can synthesize not only melatonin but also sakuranetin

In this study, we identified that the TaCOMT1A has enzyme activity by catalyzing the conversion of naringenin to sakuranetin and NAS to melatonin *in vitro* (Figure 3-11B and Figure 3-12A). In addition, overexpression of *TaCOMT1A* significantly enhanced endogenous sakuranetin and melatonin levels in "fielder" (Figure 3-11E and Figure 3-12B). All those results suggested that TaCOMT1A synthesizes sakuranetin and melatonin in wheat. The AtCOMT was involved in the biosynthesis of ferric acid and isorhamnetin before being identified in O-methylation, the precursor in melatonin biosynthesis pathways (Byeon et al. 2014a, 2015). In addition, the COMT can locate the different substrates, such as caffeic acid, 5-hydroxypinobanksyl aldehyde, and 5-hydroxypinobanksyl alcohol, to produce various products, such as ferulic acid, mustard aldehyde, and mustard alcohol, respectively (Byeon et al. 2015). In rice, sakuranetin can be biosynthesized from naringenin by a 7-O-methyltransferase (NOMT), not by the caffeic acid O-methyltransferase (COMT) (Rakwal et al. 2000; Shimizu et al. 2012; Murata et al. 2020; Yang et al. 2021), and the naringenin can be conversed to several different flavonoids, including sakuranetin, apigenin, kaempferol, quercetin, tricin, and anthocyanins, through hydroxylation, methylation, and additional modifications (Murata et al. 2020). Both NOMT and COMT belong to Type II OMT (O-methyltransferase). All the OMTs in plants were divided into two types according to their molecular weight and the cation dependency. Type I OMTs have low subunit sizes of 23–27 kD and have cation-dependent activity. Type II OMTs have high subunit sizes (38–43 kD) and have no cation-dependent activity (Bureau et al. 2007; Lu et al. 2022). Both type I and type II OMT can functionally catalyze the methyl of S-adenosylmethionine (SAM) transferred to the hydroxyl group of multiple different substances (Zhang et al. 2021). Those findings explain the multifunction TaCOMT1A in the biosynthesis of melatonin and sakuranetin.

According to the phylogenetic tree analysis of all *COMT* in wheat and *OsCOMT* and *OsNOMT* in rice, all those genes were divided into three groups. *TaCOMT1A* is more closely to *OsCOMT* but not *OsNOMT* in evolution, consistently, that TaCOMT1A methylate NAS to melatonin has higher enzyme activity than methylate naringenin to sakuranetin (Figure 3-13, Figure 3-11B and Figure 3-12A). Hence, this precisely means that TaCOMT1A is a multifunctional enzyme, and it has the

potential to synthesize both sakuranetin and melatonin in wheat, but how the biosynthesis of sakuranetin *in vivo* and the regulatory mechanism of this process need further investigation.

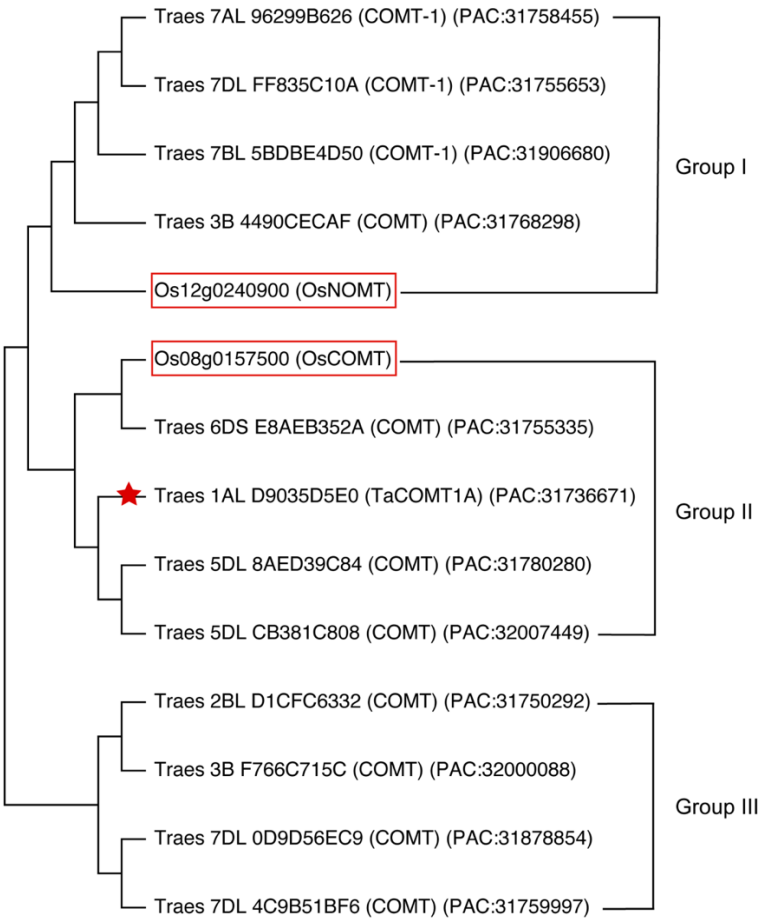


Figure 3-13 Phylogenetic tree of COMT gene family in wheat and rice.

We downloaded the amino acid sequence of 12 COMT in wheat from the phytosome (https://phytozome-next.jgi.doe.gov) and the amino acid sequence of 2 COMT in rice from the national rice data center (https://www.ricedata.cn/gene/). In this study, the red star marks TaCOMT1A, and the red underlines represent the OsNOMT and OsCOMT in rice.

3.4.2. TaCOMT1A enhances drought tolerance

In this study, overexpression of *TaCOMT1A* significantly enhanced drought tolerance at seedling and mature stages in the field conditions (Figure 3-2 and Figure 3-3). Several previous studies demonstrated that overexpression of COMT increases

melatonin contents to improve abiotic stress tolerance in other species (Pham et al., 2024; Yan et al., 2019a, 2019b). Their results suggested that overexpression of COMT has a significant positive function in scavenging ROS. We consistently find that overexpression lines have lower H_2O_2 contents under drought conditions (Figure 3-2J). In addition, several previous studies demonstrated that the DELLA protein promotes the expression of transcription factors related to drought stress by interacting with their promoter (Colebrook et al. 2014; Xie et al. 2016; Li et al. 2018b). Tan et al. reported that GA inhibits flavonol (quercetin) biosynthesis via DELLA proteins in Arabidopsis, and flavonol content in roots negatively correlates with the number of lateral roots (Tan et al. 2019). Those studies suggested that GA contents are negative with drought tolerance. Consistently, we identified that the overexpression lines decreased GA_3 (Figure 3-9B) content and enhanced drought tolerance at the seedling stage (Figure 3-2). However, a drought response transcription factor, dehydration-responsive element binding (DREB) (*TaDREB2C-1A*), was upregulated in overexpression lines compared to "fielder" (Figure 3-2L). Hence, further investigations are needed to explore which pathway plays the major role in TaCOMT1A increasing drought tolerance.

3.4.3 TaCOMT1A decreases plant height and GA content by disturbing GA biosynthesis

Our results suggested that overexpression of TaCOMT1A decreases plant height at seedling, heading, and mature stages (Figure 3-6 and Figure 3-7). It may relate to reducing GA_3 content by downregulating GA biosynthesis genes, including *TaGA2ox-5B* and *TaKO-7A* (Figure 3-9E–3-9F). Furthermore, overexpression of *TaCOMT1A* slows down the germination rate and exogenous GA_3 recovery of the germination rate (Figure 3-14). Previous findings indicated that GA promotes plant growth and development, such as seed germination, stem elongation, and leaf expansion, but it is also involved in abiotic stresses, including lodging resistance and drought tolerance (Plaza-Wüthrich et al. 2016; Shohat et al. 2021). For example, two OMTs, GA methyl transferase 1 (*GAMT1*) and *GAMT2*, de-active GA to increase drought stress tolerance in tomato (*Solanum lycopersicum* L.) (Varbanova et al., 2007; Nir et al., 2014). Combining our results, we deduced that *TaCOMT1A* de-active GA by downregulating GA biosynthesis in wheat. However, we must further investigate whether sakuranetin or melatonin plays a role in decreasing GA_3 content.

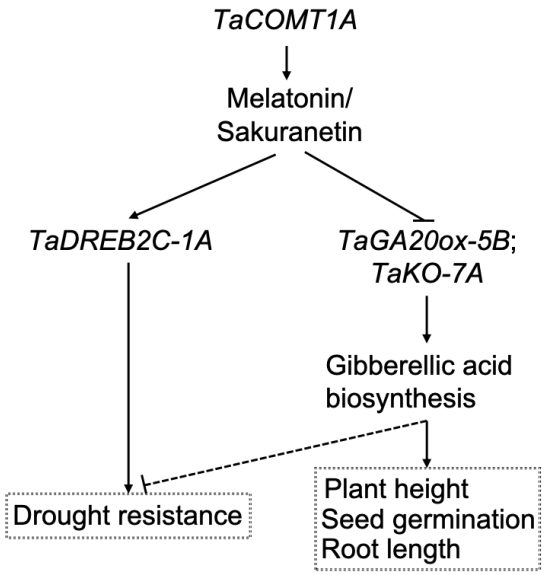


Figure 3-14 The schematic diagram of melatonin and sakuranetin regulates development and drought tolerance in wheat.

Chapter 4

Melatonin enhances drought tolerance by affecting jasmonic acid and lignin biosynthesis in wheat (*Triticum aestivum* L.)

Luo M, Wang D, Delaplace P, et al. Melatonin enhances drought tolerance by affecting jasmonic acid and lignin biosynthesis in wheat (*Triticum aestivum* L.)[J]. Plant Physiology and Biochemistry, 2023, 202: 107974. https://doi.org/10.1016/j.plaphy.2023.107974

In the previous chapter, we demonstrated that TaCOMT1A participates in the biosynthesis of melatonin and sakuranetin. Overexpression of *TaCOMT1A* increases drought tolerance and decreases plant height in wheat. Hence, in this chapter, we investigate the effect of exogenous melatonin on drought tolerance using transcriptomic and proteomic comparative analysis in wheat.

The transcriptomic analysis was performed in Biomarker Technologies Co, LTD (Wuhan, China). The Major Platform Center, the Institute of Crop Science, and the Chinese Academy of Agricultural Sciences (Beijing, China) conducted the proteomic analysis. Mingzhao Luo carried out other experiments.

Abstract: Drought severely affects the yield of wheat (*Triticum aestivum* L.), mainly grown in arid and semi-arid regions. Melatonin plays a vital role in various types of plant stress tolerance, including drought tolerance. However, the molecular mechanism of melatonin affecting drought tolerance remains largely unknown. This study revealed that melatonin (100 μM) significantly improved drought tolerance during the maturation stage of "Chinese Spring," "Shi4185," and "Hanxuan10" varieties, but not "Chang6878." Further physiological, transcriptomic, and proteomic data analysis at the wheat seedling stage revealed that melatonin increased jasmonic acid (JA) content, upregulating the expression of JA genes (*LOX1.5* and *LOX2.1*) and two transcription factors (*HY5* and *MYB86*) under drought conditions. It also upregulated genes related to lignin biosynthesis (*4CL2*, *P5CS1*, and *CCR2*) and starch and sucrose metabolism (*PME53* and *SUS4*). Additionally, melatonin alleviated photosynthetic and cell membrane damage caused by drought stress by maintaining low levels of hydrogen peroxide. The current results elucidate melatonin-regulated pathways in wheat and provide evidence for using melatonin as a potential biostimulant to improve wheat drought tolerance under field conditions.

Keywords: drought, hydrogen peroxide, jasmonic acid, lignin biosynthesis, melatonin, wheat

4.1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) was first isolated from the pineal glands of bovines in 1958 (Lerner et al. 1958). It was initially thought to play an important role in mammal physiology, including regulating neurohormones or antistress factors, circadian rhythm, sleep, and the immune system (Zhao et al. 2019; Mannino et al. 2021). Approximately 40 years later, in 1995, researchers found that melatonin was also present in medicinal plants (Dubbels et al. 1995; Hattori et al. 1995), whereafter studies on melatonin in plants exponentially increased (Murch and Erland 2021). Melatonin biosynthesis from tryptophan involves six types of enzymes and four steps, which occur in plants' chloroplasts and mitochondria (Figure 4-1) (Liu et al. 2022a). In addition, melatonin not only promotes growth and development but also enhances tolerance to various biotic and abiotic stresses in the plant kingdom, including tolerance to pathogen attack, drought, heavy metal, cold, salinity, and heat (Arnao and Hernández-Ruiz 2021b, a; Chen and Arnao 2022).

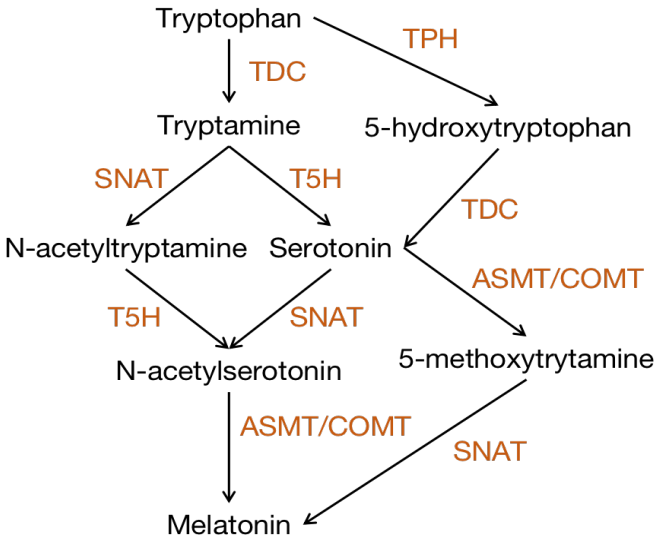


Figure 4-1 Melatonin biosynthesis pathway in plants.

ASMT: acetylserotonin methyltransferase; COMT: caffeic acid-O-methyltransferase; T5H: tryptamine 5-hydroxylase; SNAT: serotonin N-acetyltransferase; TDC: tryptophan decarboxylase; TPH: tryptophan hydroxylase. Illustration taken from Liu et al. (2022) (Liu et al., 2022).

Several studies have shown that exogenous melatonin application or overexpression of synthase genes enhances stress tolerance in many plant species, including Arabidopsis (*Arabidopsis thaliana* L.), cassava (*Manihot esculenta* Crantz), tomato (*Solanum lycopersicum* L.), cucumber (*Cucumis sativus* L.), apple

(*Malus domestica* Borkh.), cotton (*Gossypium hirsutum* L.), and rice (*Oryza sativa* L.) (Al-Huqail et al., 2020; Altaf et al., 2020; Karaca and Cekic, 2019). Overall, the central role of melatonin is that of maintaining reactive oxygen species (ROS) and reactive nitrogen species (RNS) homeostasis under stress conditions via the direct function of melatonin and its metabolites or indirectly through stimulating antioxidant enzyme activities (Lee and Back 2021). The pleiotropic effects of melatonin on plant stress tolerance have led researchers to understand the molecular mechanisms involved in these pathways (Zeng et al. 2022). The first potential phytomelatonin receptor, *CAND2/PMTR1*, was identified in Arabidopsis by Wei et al. (2018a) (Wei et al., 2018). Further studies demonstrated that it stimulates the release of G protein signaling pathway-derived calcium ions (Ca^{2+}) (Wang et al. 2021; Yu et al. 2021; Bai et al. 2022). Others have proposed the mitogen-activated protein kinase (*MAPK*) cascade as a downstream signaling pathway in melatonin-mediated tolerance (Lee and Back 2016, 2021; Maity et al. 2022b). In addition, some regulators enhance stress tolerance through direct interaction with melatonin biosynthesis and metabolism genes, such as *MeATG8b*/*8c*/*8e*, *MeWRKY79*, *MeHsf20*, *MeRAV1*, and *MeRAV2* (Cai et al., 2017; Wei et al., 2018b, 2018a).

Overall, melatonin is regarded as a master regulator of the crosstalk between other plant hormones, such as abscisic acid (ABA), salicylic acid (SA), cytokinin (CTK), jasmonic acid (JA), gibberellin (GA), ethylene (ET), brassinosteroid (BR), and indole-3-acetic acid (IAA) (Altaf et al. 2021; Chen and Arnao 2022). JA is a pleiotropic plant hormone, similar to melatonin, which modulates plant growth and development and contributes to tolerance against biotic and abiotic stresses in complex environments (Ali and Baek 2020; Gomi 2021). Several studies have shown that melatonin synergizes with JA to regulate tolerance to cold, copper, and ozone stress (Qiu et al. 2019; Hu et al. 2020b). However, further investigation is required to understand how melatonin regulates JA under stress conditions.

Wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide, with its growth and yield dramatically affected by drought stress (Savyata Kandel 2021; Adel and Carels 2023). Although several studies have reported that melatonin enhanced drought or osmotic stress tolerance in wheat, few have shown this effect during the entire growth period, and the mechanism of melatonin in regulating drought tolerance under soil drought conditions remains largely unknown (Cui et al. 2017; Shamloo-Dashtpagerdi et al. 2022; Zhang et al. 2023). In this study, we aimed to investigate the effects of melatonin on wheat yield and to elucidate the molecular mechanism of melatonin on wheat drought tolerance under soil drought stress conditions. We found that an appropriate melatonin concentration enhanced drought tolerance at both the seedling and maturation stages under water-deficit soil conditions. Furthermore, we demonstrated that exogenous melatonin upregulates genes related to JA and lignin biosynthesis under drought, consistently improving JA and lignin content. These results illustrate how exogenous melatonin regulates JA and lignin production to enhance wheat yield under drought-stress conditions.

4.2. Materials and methods

4.2.1. Plant materials and growth conditions

Melatonin (Biotech, Beijing, China) was dissolved in ethanol and diluted with water to different concentrations (50, 100, 300, 500, and 1000 μM). For hydroponic culture experiments, germinated "Aikang58" seeds were grown in water conditions for three days and then cultured in Hoagland nutrient solution with or without different melatonin concentrations for seven days under 20% PEG6000, according to previous reports. Experimental details are listed in Table 4-1 (Wang et al. 2019a).

Table 4-1 Hydroponics experiments under different treatment conditions were conducted to explore the appropriate concentration of exogenous melatonin to improve the osmotic stress tolerance of wheat.

"Aikang 58" was cultured in Hoagland nutrient solution with or without different melatonin concentrations under 20% PEG6000 conditions to screen for the optimal melatonin concentration for osmotic stress tolerance. M_0–M_{1000}: Seedling growth in 20% PEG6000 plus various melatonin concentrations (0 to 1000 μM) for seven days.

Group	Three days	Seven days
Water	Water	Hoagland nutrient solution
M_0	Water	Hoagland nutrient solution + 20% PEG6000
M_{50}	Water	Hoagland nutrient solution + 50 μM melatonin + 20% PEG6000
M_{100}	Water	Hoagland nutrient solution + 100 μM melatonin + 20% PEG6000
M_{300}	Water	Hoagland nutrient solution + 300 μM melatonin + 20% PEG6000
M_{500}	Water	Hoagland nutrient solution + 500 μM melatonin + 20% PEG6000
M_{1000}	Water	Hoagland nutrient solution + 1000 μM melatonin + 20% PEG6000

For soil drought stress experiments at the seedling stage, germinated "Aikang58" seeds were planted in flowerpots containing a 180 g soil mixture. The size of the flowerpot was as follows: outside diameter, 13 cm; height, 11 cm; bottom basin diameter, nine cm. The soil mixture was blended with soil (Pindstrup, Denmark) and water or melatonin solution (100 μM) at a ratio of 1:2 (g: mL). We sowed 22 seeds in each flowerpot and covered them with 30 g of soil. Whereafter, all pots were divided into four treatment groups (W, MW, D, and MD). Experimental details are listed in Table 4-2. Briefly, group W was cultured in a water soil mixture and administered 50 mL water once every five days. Group MW was cultured in a melatonin soil mixture and administered 50 mL water every five days. Group D was cultured in a water soil mixture, and we stopped watering it from the 1st day after sowing (DAS) for drought stress treatment. Group MD was cultured in a melatonin soil mixture, and we stopped watering it from the 1st DAS. The shoots of 18-day-old seedlings in the four treatments were collected for physiological and omics analyses. Shoot samples were rapidly frozen in liquid nitrogen and stored at -80 °C. Three biological replicates were performed in each experiment.

In the soil drought stress experiment during the whole growth period, germinated

seeds of “Chinese Spring,” “Shi4185,” “Hanxuan10,” and “Chang6878” were first subjected to vernalization for one month at 4 °C. Then, five seedlings of each variety were planted in a round flowerpot containing a 1.8 kg soil mixture. The size of the flowerpot was as follows: outside diameter, 22 cm; inside diameter, 20 cm; height, 16 cm; bottom basin diameter, 14.5 cm. The soil mixture was blended with field soil (soil from fields for growing wheat), soil (Pindstrup, Denmark), and water at a ratio of 1:1:3 (kg: kg:L). In the first two months, all pots were irrigated with 200 mL water once a week. Then, each variety was divided into four treatment groups at the joint stage (W', MW', D', and MD'). Group W' was provided with water (50 mL) daily for 14 days and then given 200 mL of water once a week for one month. Group MW' was administered 100 μM melatonin (50 mL) daily for 14 days and then given 200 mL of water once a week for one month. Group D' was provided with water (50 mL) daily for 14 days and then subjected to drought stress for one month. Group MD' was administered 100 μM melatonin (50 mL) daily for 14 days and then subjected to drought stress for one month. Experimental details are listed in Table 4-3. There were three pots for each variety, and two replicates were performed per experiment.

Table 4-2 Experimental conditions of increasing drought tolerance by applying melatonin at the seedling stage.

“Aikang58” was cultured in a soil mixture that was blended with soil (Pindstrup, Denmark) and water or melatonin solution (100 μM) at a ratio of 1:2 (g: mL) at the seedling stage. Samples were obtained on the 18th DAS to acquire the transcriptomic, proteomic, and physiological data. For the drought stress groups (D and MD), we stopped watering on the 1st day after sowing (DAS) the seeds. DAS: day after sowing; D: drought conditions; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

Group	Soil conditions	5th DAS	10th DAS	15th DAS	18th DAS
W	Soil: water = 1:2 (g:mL)	50 mL water	50 mL water	50 mL water	Rewatering
MW	Soil: water = 1:2 (g:mL)	50 mL water	50 mL water	50 mL water	Rewatering
D	Soil: water = 1:2 (g:mL)	0 mL water	0 mL water	0 mL water	Rewatering
MD	Soil: water = 1:2 (g:mL)	0 mL water	0 mL water	0 mL water	Rewatering

Table 4-3 The experimental conditions of the whole growth period of different wheat

varieties improved drought tolerance by applying melatonin.

Group	One month of vernalisation	Soil conditions	Two months	14 days	One month
W'	4 ℃ in the refrigerator	Field soil: nutrition soil: water = 1:1:3 (kg:kg:L)	Water 200 mL/week	Water (50 mL/day)	Water 200 mL/week
MW'	4 ℃ in the refrigerator	Field soil: nutrition soil: water = 1:1:3 (kg:kg:L)	Water 200 mL/week	100 μM melatonin (50 mL/day)	Water 200 mL/week
D'	4 ℃ in the refrigerator	Field soil: nutrition soil: water = 1:1:3 (kg:kg:L)	Water 200 mL/week	Water (50 mL/day)	Drought
MD'	4 ℃ in the refrigerator	Field soil: nutrition soil: water = 1:1:3 (kg:kg:L)	Water 200 mL/week	100 μM melatonin (50 mL/day)	Drought

"Chinese Spring," "Shi4185," "Hanxuan10," and "Chang6878" were cultured in different soil conditions during the maturation stage to investigate the effects of melatonin on yield traits under drought stress conditions. For the drought stress groups (D' and MD'), we stopped watering for one month. D': drought conditions; MD': applying melatonin under drought conditions; MW': applying melatonin under watering conditions; W': watering conditions.

4.2.2. Determination of relative water content

To observe the degree of soil drought on the 10^{th}, 12^{th}, 15^{th}, and 18^{th} DAS, soil samples were weighed before and after drying, and the soil relative water content was calculated using the formula below.

Relative water content (%) = $\frac{W1-W2}{W1} \times 100\%$,

W1 indicates the soil weight before drying treatment, and W2 indicates the soil weight after drying for 48 h in a temperature oven set at 60 ℃.

The shoot's relative water content was also calculated using the above formula. The shoots were obtained on the 18^{th} DAS. Their fresh weight was indicated as W1, and their dry weight was shown as W2.

4.2.3. Determination of biochemical parameters

We took 0.1 g of shoots from 18-day-old seedlings in each treatment to measure the hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and starch content. We also assessed the activity of catalase (CAT) and peroxidase (POD) following the protocol of corresponding assay kits. These kits were purchased from Comin Biotechnology Co., Ltd. (Comin Biotechnology, Suzhou, China). Leaf chlorophyll was extracted with 80% acetone for 12 hours under dark conditions. The extracts were detected by measuring the absorbance values at 645 nm and 663 nm using a Thermo Scientific Microplate Reader (Thermo Fisher Scientific Inc., USA), and the total chlorophyll content was calculated according to the previous study (Arnon 1949). Three replicates were performed, and each replicate sampling consisted of five seedlings.

4.2.4. Determination of the photosynthetic rate

The photosynthetic rate of the third fully expanded leaf was determined after drought treatment for 11 days using a LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, United States) following a previous study (Zhang et al. 2018). The leaf chamber environment was set according to the following parameters: CO_2 mixer = 400 ppm, lamp (light density) = 1500 lx, at least ten leaves were used to cover the entire leaf chamber at each measurement, and there were 15 replicates for groups W, MW, D, and MD.

4.2.5. Determination of lignin, JA, and melatonin content

Endogenous lignin, JA, and melatonin content were extracted following the protocol provided along with the corresponding immunosorbent assay kit (Jianglai, Shanghai, China) (Wei et al. 2018a). Briefly, 0.1 g of fresh shoot samples of "Aikang58" wheat seedlings were homogenized in phosphate buffer (PBS, pH 7.4), and the absorbance at 450 nm was measured using a Thermo Scientific Microplate Reader (Thermo Fisher Scientific Inc., USA).

4.2.6. Transcriptomic data analysis

A total of 12 samples, including three replicates for each of the four treatments (W, MW, D, and MD), were used for transcriptomic analysis. Transcriptomic data were obtained from Biomarker Technologies Co., Ltd. In brief, the RNA concentration and purity were measured using the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). Library preparations were sequenced on an Illumina platform, and Hisat2 tools software was used to map the reference genome (Kim et al. 2015). Differentially expressed genes (DEGs) between the treated and control samples (false discovery rate < 0.01 and fold-change ≥ 2) were identified using EBseq (Anders and Huber 2010). We performed transcriptomic analysis using the Genome-guided mRNA Sequencing Analysis tools on BMKCloud (www.biocloud.net).

4.2.7. Proteomic data analysis

Protein extraction was performed using the filter-aided sample preparation (FASP) method. Tryptic peptides were collected and quantified according to the obtained

OD280 values. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and label-free quantification data analysis were performed to identify and quantify proteins (Sun et al. 2016). We performed a comparative analysis of differentially abundant proteins (DAPs) with a fold change of ≤ 0.83 or ≥ 1.2 ($P < 0.05$) (Cui et al. 2018).

4.2.8. Functional annotation and omics data analysis

We identified the DEGs and DAPs between treatment groups ("MW vs. W," "D vs. W," "MD vs. D," and "MD vs. W"). All genes were annotated using Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG), Pfam, Swiss-Prot, evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG), non-redundant protein sequence database (NR), and clusters of orthologous groups for eukaryotic complete genomes (KOG) (Niu et al. 2023).

4.2.9. qRT–PCR analysis

Total RNA was extracted using a Plant Total RNA Kit (Zoman Biotechnology, Beijing, China) and used to synthesize first-strand cDNA using a FastQuant RT Kit (Vazyme, Nanjing, China). SuperReal PreMix kits (Vazyme) were used for qRT-PCR analyses. The actin gene was used as a housekeeping gene. Relative expression was determined as the level of amplification of the transcripts of a gene relative to that of the housekeeping gene. All primers used for gene expression assays are listed in Supplementary Table 4-4. Each expression analysis was performed independently at least three times. The data were analyzed with the $2^{-\Delta\Delta Ct}$ method.

Table 4-4 Primers for qRT-PCR in chapter 4.

Gene ID	Abbreviation	Fprimer	Rprimer	T_m
TraesCS2B02G555400	*LOX1.5*	TCTTCTTCTTGCAAGACGACTC	GGACATAGTTGTTCTTGCATGC	58
TraesCS5A02G007900	*LOX2.11*	GCATCTACGACTACGATGTGTA	CATGAACTTCTTGGTGTCGAAG	58
TraesCS3D02G129800	*HY5*	ATGACGGAGCTGGAGGTGAAGG	CAGTATCTGGCGGAGCGTGTTG	58
TraesCS6A02G266700	*4CL2*	GTCATGATCCTGCTCCAGAAC	GACTGGGTGACGATCATCTTG	58
TraesCS1A02G281400	*P5CS1*	CAGAGACCTTTCTACGTCAAGT	GCCATCGTGTAGTTAAAAGACC	58
TraesCS5B02G160700	*CCR2*	ATTTCTTTTCCTCCCTAAGGCA	ACACTTATGGGCTTGCCTATTA	58
TraesCS2D02G099900	*PME53*	CACACGTCGTCATGTAAAGAAG	CCCAAAAGAAGACTGCAAAAGA	58

TraesCS4D02G169800	*SUS4*	TGGCTTTTGGAAGTATGTTTCG	GTCGTCAGCTGCATCTTATTTC	58
TraesCS2B02G177900	*MYB86*	AACTGGTGCTTGAATTGAACTC	ACTCGCGTATACGTTACAATCT	58

4.2.10. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 17.0. Duncan's test ($P < 0.05$) evaluated treatment effects. Two groups were compared via the two-tailed Student's *t*-test. For each treatment, the standard deviation of the mean was calculated based on at least three biological replicates.

4.3. Results

4.3.1. Application of melatonin improved the drought tolerance of wheat during the maturation stage

To explain the melatonin-induced improvement in four varieties ("Chinese Spring," "Shi4185," "Hanxuan10," and "Chang6878") under normal and drought conditions, we investigated their agronomic traits (Table 4-4). We found that melatonin (100 μM) had a significant ($p<0.05$) effect on "Chinese Spring," "Shi4185," and "Hanxuan10," whereas it did not affect "Chang6878" during the maturation stage (Figure 4-2A–4-2B). When comparing the MD' and D' groups, our results showed that melatonin significantly reduced the damage caused by drought-induced plant damage. The plant heights of the "Chinese Spring," "Shi4185," and "Hanxuan10" varieties were approximately 11.52%, 17.30%, and 13.00% higher, respectively, in the MD' group (Figure 4-2C). The seed weight per panicle of "Chinese Spring," "Shi4185," and "Hanxuan10" was 35.48%, 38.27%, and 35.48% higher in the MD' group when compared with that in the D' group (Figure 4-2D). The thousand seed weights of "Chinese Spring," "Shi4185," and "Hanxuan10" were 9.81%, 9.50%, and 10.26% higher, respectively, in the MD' group when compared to that in the D' group (Figure 4-2E). The grain number per panicle of "Chinese Spring," "Shi4185," and "Hanxuan10" was 21.25%, 27.92%, and 27.04% higher, respectively, in the MD' group compared to that in the D' group (Figure 4-2F). However, the height of "Chang6878" was 5.42% lower in the MD' group. The seed weight per panicle, thousand seed weight, and grain number per panicle of "Chang6878" were not significantly different between the MD' and D' groups. These results indicated that melatonin enhanced drought tolerance during the growth period and that this effect was variety-dependent.

Table 4-5 Plant height, seed weight per panicle, thousand seed weight, and grain number per panicle of four wheat varieties ("Chinese Spring," "Shi4185," "Hanxuan10," and "Chang6878") under different treatments (W', MW', D', and MD').

Phenotype	Wheat varieties	W'	MW'	D'	MD'	Increase ratio (MD' vs. D')
Plant height (cm)	"Chinese Spring"	80.49±8.1 a	76.98±5.09 a	66.86±9.67 b	75.54±7.57 a	11.52%
	"Shi4185"	50.71±4.11 a	53.88±1.7 a	41.17±5.66 b	49.73±4.35 a	17.30%
	"Hanxuan10"	70.42±9.25 a	74.4±4.72 a	52.23±2.07 c	60.03±6.02 b	13.00%
	"Chang6878"	60.63±4.4 a	60.77±4.77 a	42.87±2.43 b	40.61±5.07 b	-5.42%
Seed weight per panicle (no.)	"Chinese Spring"	0.96±0.18 a	1.06±0.12 a	0.45±0.05 c	0.62±0.02 b	35.48%
	"Shi4185"	1.15±0.03 a	1.19±0.11 a	0.54±0.09 c	0.81±0.07 b	38.27%
	"Hanxuan10"	0.9±0.06 a	0.94±0.06 a	0.41±0.05 c	0.62±0.05 b	35.48%
	"Chang6878"	1.16±0.11 a	1.27±0.09 a	0±0 b	0±0 b	0
Thousand seed weight (g)	"Chinese Spring"	41.43±0.85 a	38.47±1.03 a	34.8±0.45 c	37.73±0.41 b	9.81%
	"Shi4185"	36.57±0.87 a	34.8±0.39 a	30.57±0.77 c	33.73±0.95 b	9.50%
	"Hanxuan10"	32.47±0.74 a	31.53±0.86 a	28.5±0.75 b	31.27±0.63 a	10.26%
	"Chang6878"	43.27±1.1 a	29.93±1.4 a	0 b	0 b	0
Grain number per panicle (no.)	"Chinese Spring"	23.67±5.25 a	27±2.94 a	12.67±1.25 c	16±0.82 b	21.25%
	"Shi4185"	32.33±1.89 a	32.67±3.09 a	17.33±2.87 c	24±1.63 b	27.92%
	"Hanxuan10"	27.67±2.49 a	28.67±3.09 a	14.33±1.25 c	19.67±1.89 b	27.04%
	"Chang6878"	26.67±2.05 a	28.33±2.05 a	0±0 b	0.33±0.47 b	

All data are presented as the mean ± standard deviation ($n \geq 3$). Different letters indicate significant differences among treatments ($P < 0.05$ according to the one-way analysis of variance (Duncan's multiple range test). D': drought conditions; MD': applying melatonin under drought conditions; MW': applying melatonin under watering conditions; W': watering conditions.

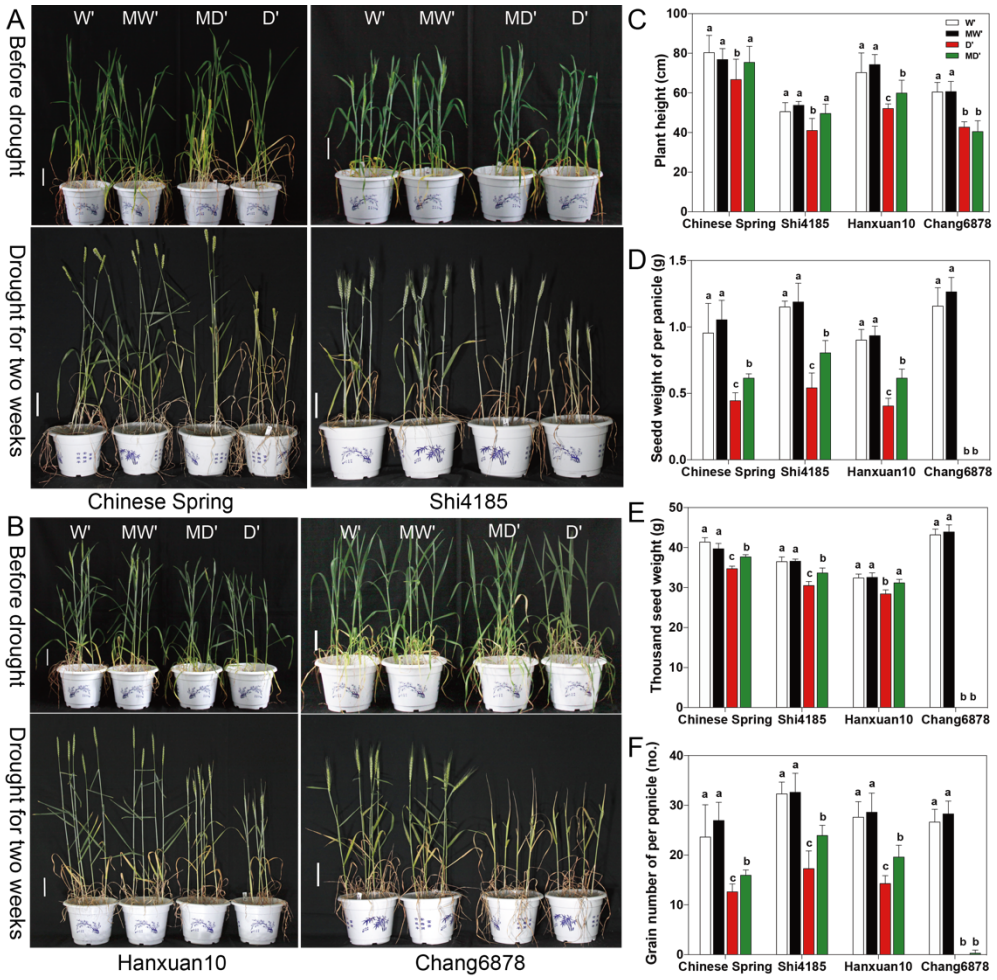


Figure 4-2 Melatonin-mediated mitigation of drought-induced damage in different varieties.

(A) Phenotypes of "Chinese Spring" and "Shi4185" under four different treatments (W', MW', D', and MD'). (B) Phenotypes of "Hanxuan10" and "Chang6878" under four different treatments (W', MW', D', and MD'). (C) Plant height of different varieties under four treatments (W', MW', D', and MD'). (D) Seed weight per plant of different varieties under four treatments (W', MW', D', and MD'). (E) Thousands of seed weights of different varieties under four treatments (W', MW', D', and MD'). (F) Grain number per panicle of different varieties under four treatments (W', MW', D', and MD'). Group W' was given water (50 mL) daily for 14 days and then 200 mL once a week for 1 month. Group MW' was provided with 100 μM melatonin (50 mL) daily for 14 days and then given 200 mL of water once a week for 1 month. Group D' was provided water (50 mL) daily for 14 days and then subjected to drought stress for 1 month. Group MD' was provided with 100 μM melatonin (50 mL) daily for 14 days and then subjected to drought stress for 1 month. All data are presented as the

mean ± standard deviation ($n \geq 3$). Different letters indicate significant differences among treatment groups ($P < 0.05$ according to one-way analysis of variance, Duncan's multiple range test). Scale bar = 3 cm. D': drought conditions; MD': applying melatonin under drought conditions; MW': applying melatonin under watering conditions; W': watering conditions.

4.3.2. Exogenous melatonin enhanced the drought tolerance of wheat seedlings under water-losing soil conditions by maintaining H_2O_2 homeostasis

To further investigate the effect of exogenous melatonin on wheat at the seedling stage, we treated "Aikang58" plants with different concentrations of melatonin plus stress treatment with 20% PEG6000 (Figure 4-3A, Table 4-1). We found that seedlings treated with 50–300 μM melatonin significantly increased in terms of fresh weight (Figure 4-3B), shoot length (Figure 4-3C), and primary root length (Figure 4-3D) while having a significantly lower H_2O_2 content (Figure 4-3E) when compared with untreated seedlings under osmotic stress conditions. The concentration of 100 μM melatonin was selected for further study as the optimal concentration to improve osmotic stress tolerance. To further investigate the function and mechanism of melatonin in wheat drought tolerance, we sowed "Aikang58" seeds on a soil mixture blended with water or melatonin (100 μM), dividing them into four treatment groups (W, MW, D, MD) (Table 4-2). Exogenous melatonin significantly enhanced drought tolerance, whereas it had no significant effect on seedlings under normal watering conditions (Figure 4-4A–4-4C). In the drought stress groups (D and MD), we stopped watering from the 1st DAS (Figure 4-4D). Significantly lower soil-relative water content was noted in these groups than in the normal watering groups (W and MW) from the 12th DAS (Figure 4-4E). The H_2O_2 content of seedling shoots was measured in all four treatment groups on the 12th to 18th DAS, revealing seedlings sustained a relatively lower H_2O_2 content with or without melatonin treatment under normal watering. In contrast, melatonin-treated seedlings showed no dramatic increase in H_2O_2 caused by drought stress on the 16th, 17^{th,} and 18th DAS (Figure 4-4F). Hence, all samples on the 18th DAS were used for further psychological and omics analysis. Taken together, exogenous melatonin enhanced the drought tolerance of wheat seedlings and maintained a lower H_2O_2 content under drought stress conditions.

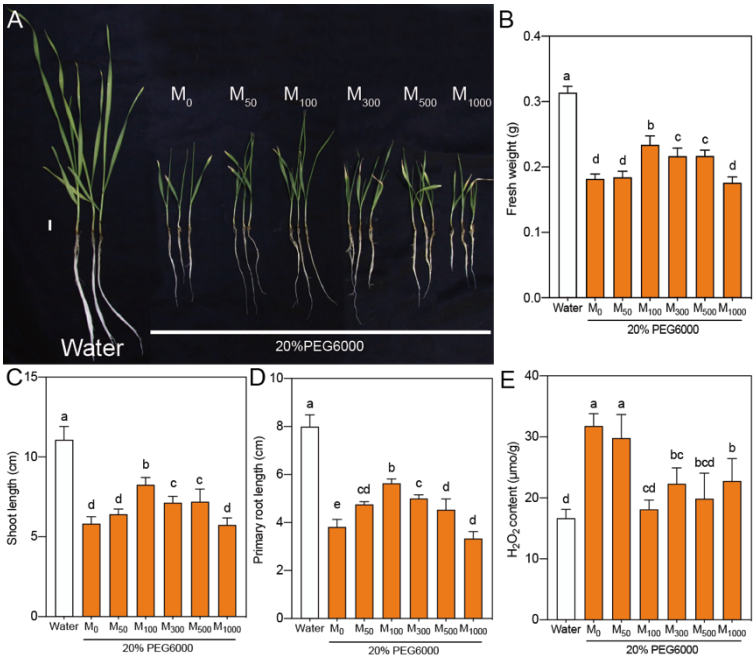


Figure 4-3 An appropriate melatonin concentration enhanced wheat osmotic stress tolerance at the seedling stage ("Aikang58" variety).

Germinated seeds were planted in normal water conditions for three days and then transplanted in different conditions (normal water conditions as control, containing 20% PEG6000 plus various concentrations of melatonin for osmotic stress) for 7 days. The 10-day-old seedlings were used to measure phenotype and physiological data. (A) Phenotype of 10-day-old seedlings. Scale bar = 1 cm. (B) Fresh weight of seedlings. (C) Primary root length of seedlings. (D) Shoot the length of seedlings. (E) H_2O_2 content in shoots of seedlings. Water: Seedling growth in the water conditions. M_0–M_{1000}: Seedling growth in 20% PEG6000 plus various melatonin concentrations (0 to 1000 μM) for 7 days. All data are presented as mean ± standard deviation (n = 3). Different letters indicate significant differences among treatments ($P < 0.05$) according to a one-way analysis of variance and Duncan's multiple-range test.

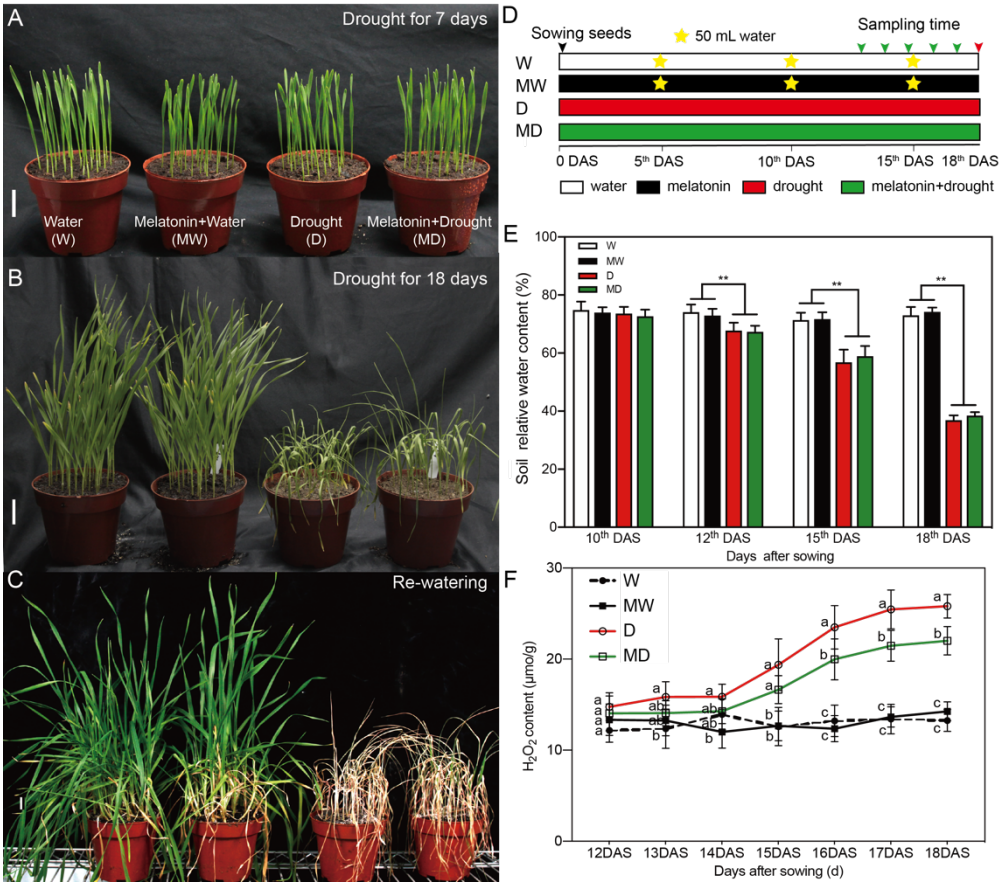


Figure 4-4 Exogenous melatonin improved "Aikang58" drought tolerance under soil conditions.

(A) Phenotype of "Aikang58" 7 days after sowing (DAS); we stopped watering all pots to induce drought stress on the 1st DAS. (B) Phenotype of seedlings subjected to drought for 18 days in four treatment groups. (C) The phenotype of rewatered drought-affected seedlings. (D) Diagram of soil conditions in the four treatment groups. The white frame represents the W group, the black frame represents the MW group, the red frame represents the D group, and the green frame represents the MD group. Yellow stars indicate the watering of each plastic pot with 50 mL water; the black arrow indicates the sowing time of seeds, while green and red arrows represent the sampling time. (E) Relative soil water content on different days after sowing seeds. (F) H_2O_2 content on the 12th to 18th DAS. Group W was cultured in a water soil mixture and given 50 mL water every 5 days. Group MW was cultured in a melatonin soil mixture and given 50 mL water every 5 days. Group D was cultured in a water soil mixture, and we stopped watering on the 1st DAS for drought stress treatment. Group MD was cultured in a melatonin soil mixture, and we stopped watering from the 1st DAS. The soil mixture was blended with soil (Pindstrup, Denmark) and water or melatonin

solution (100 μM) at a ratio of 1:2 (g:mL). All data are shown as the mean ± standard deviation (n ≥ 3). Different letters indicate significant differences among different treatments ($P < 0.05$ according to one-way analysis of variance, Duncan's multiple range test). Scale bar = 3 cm. D: drought conditions; DAS: days after sowing; H_2O_2: hydrogen peroxide; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

Our results showed that the survival rate, fresh weight, and shoot relative water content in the melatonin treatment group (MD) were significantly higher than those without melatonin treatment (D) under the drought stress conditions, whereas no difference in dry weight was found between the two groups (Figure 4-5A–4-5D). Moreover, melatonin-treated seedlings had a significantly lower MDA content under drought stress conditions relative to untreated seedlings (Figure 4-5E). Melatonin treatment significantly upregulated endogenous melatonin content as well as CAT and POD activity under drought stress conditions (Figure 4-5F–4-5H). These results indicated that exogenous melatonin improved drought tolerance by enhancing antioxidant enzyme activity.

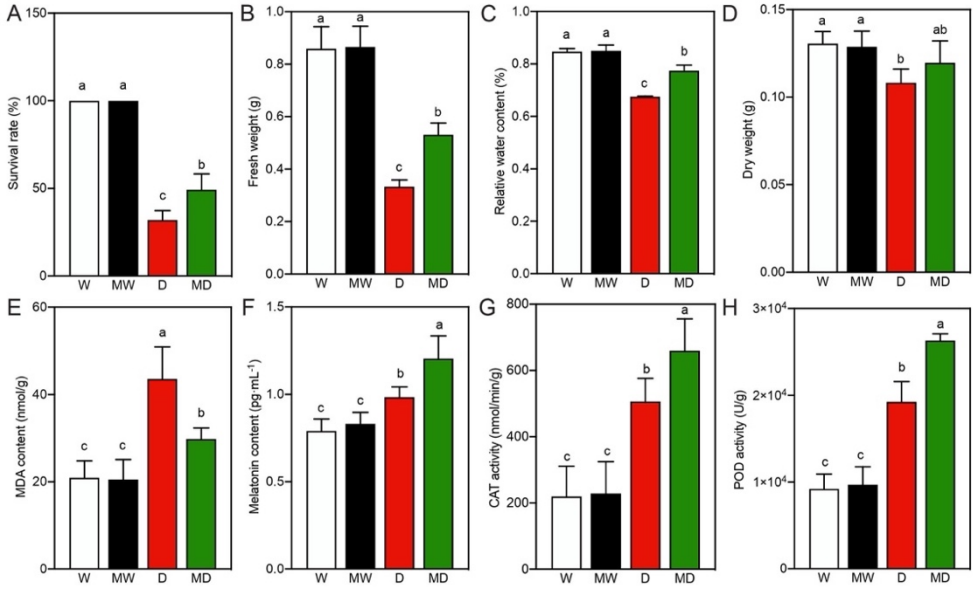


Figure 4-5 Physiological data of 18-day-old "Aikang58" seedlings treated with melatonin under drought stress conditions.

(A) Survival rate of 18-day-old seedlings after rewatering one month later. (B) Fresh weight. (C) Relative water content in the shoots. (D) Dry weight. (E) MDA content. (F) Melatonin content. (G) CAT activity. (H) POD activity. All samples were obtained on the 18th DAS in the four treatment groups (W, MW, D, and MD). CAT: catalase; D: drought conditions; MDA: malondialdehyde; MD: applying melatonin under drought conditions; MW: applying

melatonin under watering conditions; POD: peroxidase; W: watering conditions.

4.3.3. Transcriptomic data analysis and functional annotation of differentially expressed genes (DEGs)

To elucidate the mechanism of melatonin-mediated drought tolerance in wheat, we investigated the transcriptional changes in seedlings of the four treatment groups (W, MW, D, and MD), with three replicates for each treatment. Principal component analysis (PCA) and Pearson's correlation coefficients between all sample pairs revealed good reproducibility and reliability for each sample (Figure 4-6A–4-6B). After the RNA-sequencing of 12 samples, we obtained 130.43 Gb of clean data, with at least 10.16 Gb of clean data for each sample. We then mapped these clean data to the reference genome, with a mapping ratio from 89.46% to 93.51%. Finally, we identified 22,076 genes, 15,164 of which were functionally annotated in seven databases (NR, SwissProt, KEGG, KOG, GO, eggNOG, and Pfam). These results suggested that all transcriptomic data were qualified.

We determined the DEGs between melatonin- or drought-treated seedlings compared to the untreated watering control ("M vs. W," "D vs. W"). In contrast, the melatonin plus drought-treated seedlings were compared to drought-treated ("MD vs. D") or untreated watering controls ("MD vs. W") (|fold-change| > 2 and adjusted P value < 0.01). In total, 1466 (363 up- and 1103 down-regulated), 11,739 (5876 up- and 5863 downregulated), 2796 (586 up- and 2210 down-regulated), and 9054 (4000 up- and 5054 downregulated) DEGs were identified in the comparison of "MW vs. W," "D vs. W," "MD vs. D," and "MD vs. W," respectively (Figure 4-6C). Taken together, the number of DEGs induced by drought (with or without applying melatonin) ("D vs. W" and "MD vs. W") was significantly greater than that caused by melatonin (whether under normal or drought conditions) ("MW vs. W" and "MD vs. D"). Further, melatonin induced a greater number of DEGs under drought conditions ("MD vs. D") than under normal conditions ("MW vs. W") (Figure 4-6C).

We performed GO and KEGG enrichment analyses to elucidate the biological functions of these DEGs. Among the top six enriched GO terms for biological processes, the two that most commonly appeared in the four comparisons were 'protein phosphorylation' and 'defense response' (Figure 4-7A–4-7D). In addition, we analyzed the first 20 KEGG pathway terms according to the q-value (Fisher adjusted p-value) and identified several key pathways correlated with drought in each of the four comparisons, including 'cutin, suberin, and wax biosynthesis', 'linoleic acid metabolism', and 'plant-pathogen interaction' in "MD vs. D," 'phenylalanine metabolism', 'starch and sucrose metabolism', and 'plant hormone signal transduction' in "D vs. W," as well as 'phenylalanine metabolism', 'linoleic acid metabolism', and 'carotenoid biosynthesis' in "MD vs. W" (Figure 4-8A–4-8D). These results illustrated that the mechanism through which melatonin regulates drought tolerance is complex and overlaps several pathways compared to "MD vs. W" and "MD vs. D." Venn diagram analysis was used to narrow down the number of DEGs. We excluded DEGs that were only disturbed by melatonin or drought as well as common DEGs between the comparison of "MD vs. W" and "D vs. W,"

which were not identified in “MD vs. D” (Figure 4-6D). Therefore, we selected the DEGs induced by melatonin under drought conditions (“MD vs. D”) and those caused by melatonin plus drought (“MD vs. W”), but not by drought alone (“D vs. W”) (Figure 4-6D). In total, 4400 DEGs related to both melatonin and drought treatments were included for further examination.

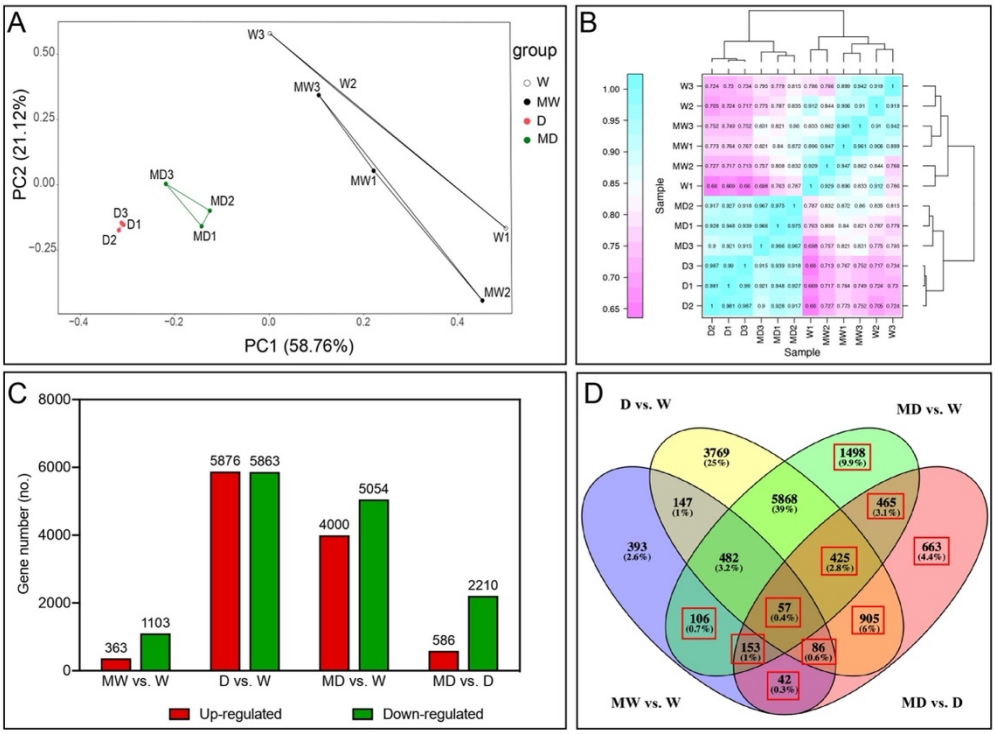


Figure 4-6 Overview and Venn diagram of DEGs identified via transcriptomic analysis in comparing the four treatment groups using cut-offs of |log2 (fold change)| > 1 and FDR < 0.05.

(A) Principal component analysis of the transcriptomic data for the four treatment groups. (B) Pearson’s correlation analysis of transcriptomic data from 12 different samples. (C) Total number of DEGs identified in the four comparisons (“MW vs. W,” “D vs. W,” “MD vs. W,” and “MD vs. D”); red bars represent genes with upregulated expression, whereas green bars represent genes with downregulated expression. (D) The Venn diagram of DEGs identified in the four comparisons; red frames represent DEGs that might be related to the melatonin-mediated regulation of drought stress tolerance. Overlapping regions indicate co-expressed DEGs among different datasets, with numbers in a single circle representing DEGs expressed in only one library. These samples were obtained on the 18th DAS in the four treatment groups (W, MW, D, and MD). D: drought conditions; FDR: false discovery rate; MD: applying melatonin under drought conditions; DEG: differentially expressed genes; MW: applying melatonin under watering conditions; W: watering conditions.

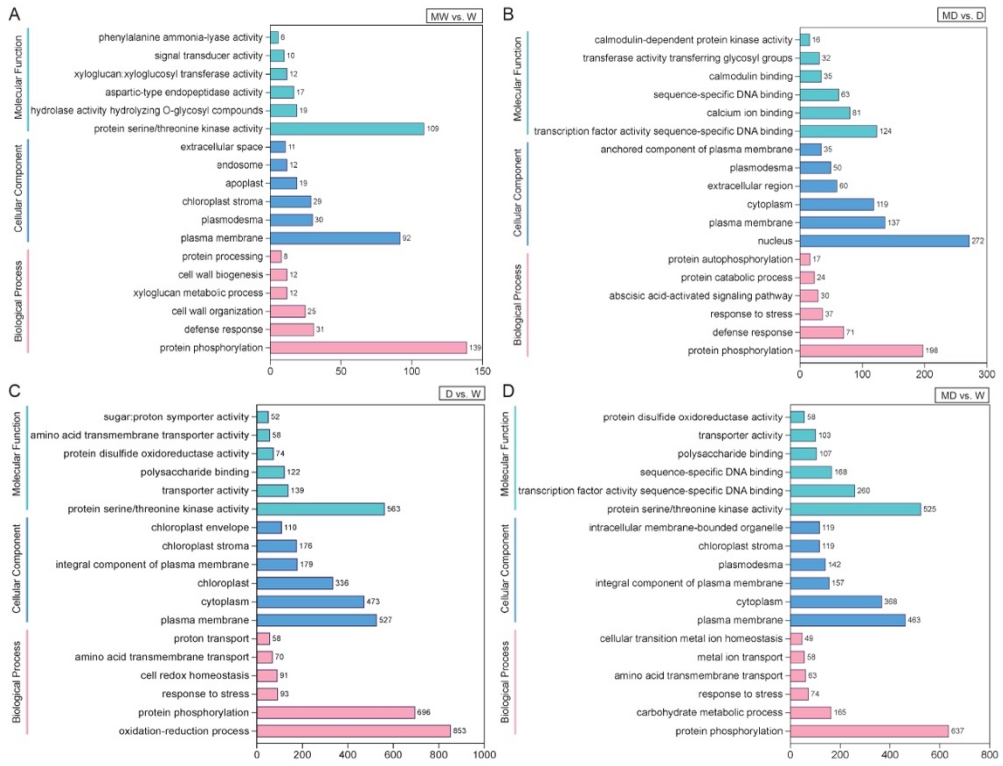


Figure 4-7 Major GO terms for DEGs identified in the comparisons of the four treatment groups.

(A) Major GO enriched terms for DEGs between the melatonin and water conditions ("MW vs. W"). (B) Major GO enriched terms for DEGs between the melatonin plus drought and drought stress conditions ("MD vs. D"). (C) Major GO enriched terms for DEGs between the drought stress conditions and water conditions ("D vs. W"). (D) Major GO enriched terms for DEGs between the melatonin plus drought and water conditions ("MD vs. W"). D: drought conditions; DEG: differentially expressed gene; GO: Gene Ontology; MD: applying melatonin under drought conditions; MW, applying melatonin under watering conditions; W: watering conditions.

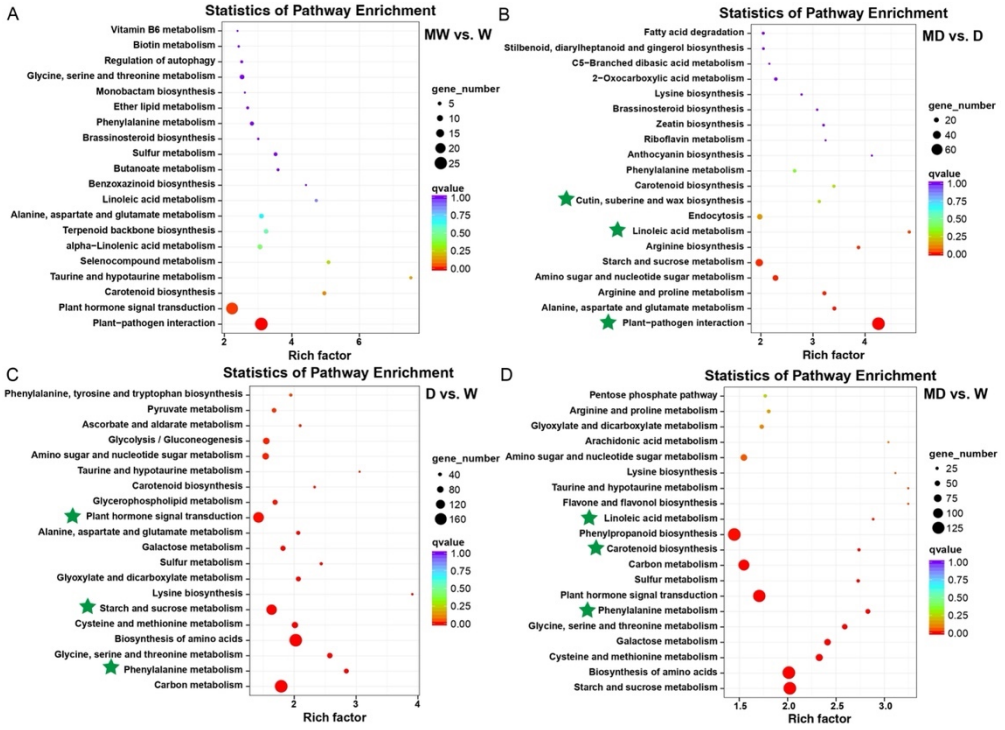


Figure 4-8 Major KEGG pathways for DEGs identified in comparisons between the four treatment groups (W, MW, D, and MD).

(A) Major KEGG pathways for DEGs in the comparison of "MW vs. W." (B) Major KEGG pathways for DEGs in the comparison of "MD vs. D." (C) Major KEGG pathways for DEGs in the comparison of "D vs. W." (D) Major KEGG pathways for DEGs in the comparison of "MD vs. W." Green five-pointed stars represent the key pathways that might be related to melatonin-regulated wheat drought tolerance. The q-values represent the Fisher adjustment of p-values. D: drought conditions; DEG: differentially expressed gene; KEGG: Kyoto Encyclopedia of Genes and Genomes; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

4.3.4. Transcriptomic analysis revealed that exogenous melatonin regulates drought tolerance via JA and lignin

To further ascertain the mechanism through which melatonin regulates drought tolerance of wheat at the seedling stage, we analyzed GO terms and KEGG pathways for 4400 DEGs. The top six GO enrichment categories for biological processes were 'protein phosphorylation' (GO:0006468), 'regulation of transcription, DNA-templated' (GO:0006355), 'defense response' (GO:0006952), 'cell wall organization' (GO:0071555), 'response to stress' (GO:0006950), and 'intracellular signal transduction' (GO:0035556) (Figure 4-9A). In addition, DEGs were enriched

for 116 KEGG pathways. According to the function and enrichment ratio for each pathway, we mainly focused on the top 10 pathways, including 'phenylpropanoid biosynthesis' (ko00940), 'plant hormone signal transduction' (ko04075), 'endocytosis' (ko04144), 'starch and sucrose metabolism' (ko00500), 'cutin, suberin, and wax biosynthesis' (ko00073), 'plant-pathogen interaction' (ko04626), 'zeatin biosynthesis' (ko00908), 'arginine and proline metabolism' (ko00330), 'brassinosteroid biosynthesis' (ko00905), and 'linoleic acid metabolism' (ko00591) (Figure 4-9B). These pathways were divided into three categories based on their function. First, several genes were involved in the biosynthesis and signal transduction of plant hormones, including the biosynthesis of JA, BR, and CTK (Figure 4-10A). Second, cell wall- and osmotic substance-related genes were differentially expressed in seedlings subjected to melatonin treatment and drought (Figure 4-10B). Third, the expression of some genes in 'endocytosis' and 'plant-pathogen interaction' was also affected by melatonin and drought (Figure 4-10C). Overall, melatonin mainly regulates other plant hormones, cell wall-related genes, osmotic substances, and autophagy responses under drought conditions at the transcriptomic level.

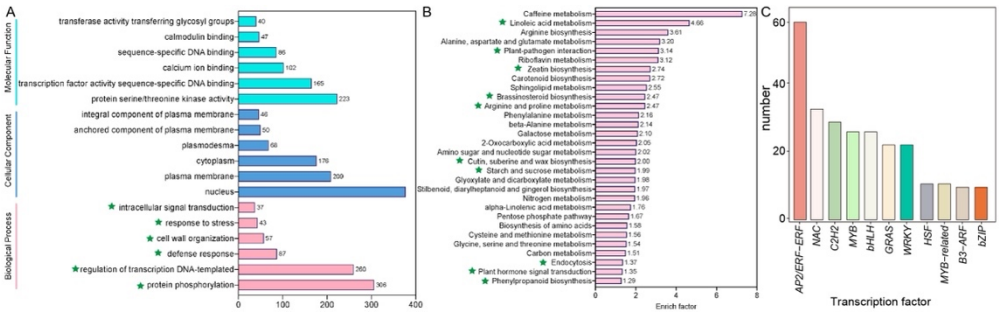


Figure 4-9 GO and KEGG enrichment analysis of 4400 DEGs implicated in melatonin-regulated wheat drought tolerance.

(A) Major GO enrichment term analysis of 4400 DEGs related to melatonin-regulated wheat drought tolerance. (B) Major KEGG pathway analysis of 4400 DEGs related to melatonin-regulated wheat drought tolerance. (C) The number of different types of predicted transcription factors among 4400 DEGs. Green five-pointed stars represent the key pathways that may be related to the regulation of wheat drought tolerance by melatonin. D: drought conditions; DEG: differentially expressed gene; KEGG: Kyoto Encyclopedia of Genes and Genomes; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

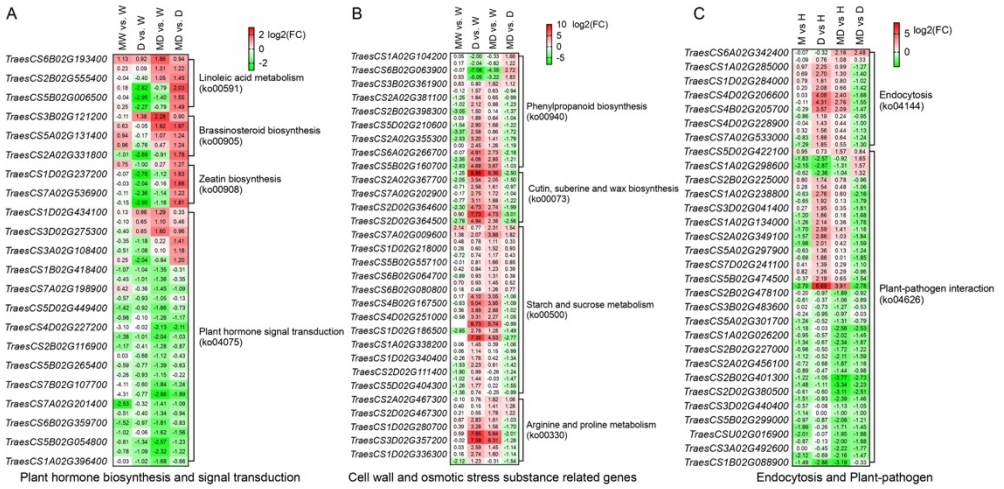


Figure 4-10 Major KEGG pathways related to melatonin regulation of drought tolerance.

(A) DEGs related to plant hormone biosynthesis and signal transduction. (B) DEGs are related to cell walls and osmotic substances. (C) DEGs related to endocytosis and plant-pathogen interactions. All of these samples were obtained on the 18th DAS in the four treatment groups (W, MW, D, and MD). D: drought conditions; DAS: days after sowing; DEG: differentially expressed genes; JA: jasmonic acid; KEGG: Kyoto Encyclopedia of Genes and Genomes; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

Transcription factors play a crucial role in various aspects of plant growth, development, and stress responses. A total of 389 transcription factors were identified among the 4400 DEGs, including 60 *AP/ERF–AP*, 34 *NAC*, 27 *MYB*, 23 *GRAS*, 23 *WRKY*, and 10 probable *bZIP* transcription factors (Figure 4-9C). Based on the expression of DEGs induced by melatonin and drought, we divided all these transcription factors into four types. Type I DEGs were upregulated by drought stress ("D vs. W" or "MD vs. W") and further upregulated by melatonin under drought stress conditions ("MD vs. D"). Type II DEGs were downregulated by drought stress but upregulated by melatonin under drought stress conditions ("MD vs. D") (Figure 4-11 A). Type III DEGs were upregulated by drought stress ("D vs. W" or "MD vs. W") but downregulated by melatonin under drought stress conditions ("MD vs. D"). Type IV DEGs were downregulated by drought stress and further downregulated by melatonin under drought stress conditions ("MD vs. D") (Figure 4-11B). Our results indicated that seven transcription factors, namely *MYB*, *bHLH*, *NAC*, *AP2*, *WRKY*, *HSF*, and *bZIP*, are mainly involved in melatonin-regulated drought tolerance in wheat.

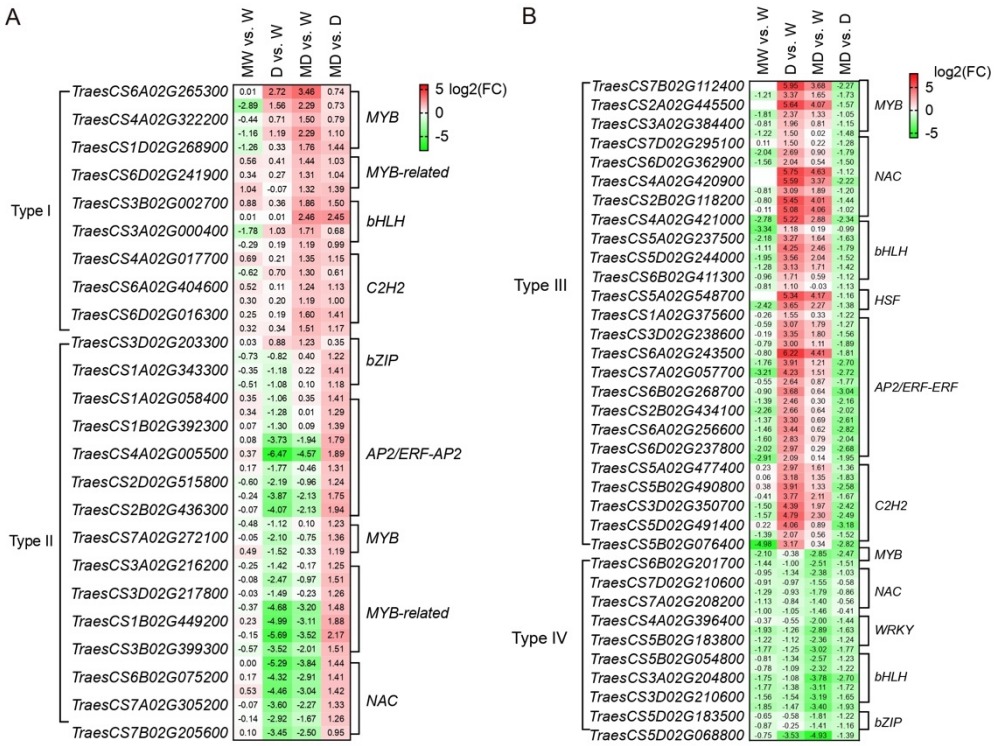


Figure 4-11 Key predicted transcription factors among the 4400 DEGs related to the regulation of wheat drought tolerance by melatonin.

(A) Melatonin-induced upregulation of predicted transcription factors under the drought conditions ("MD vs. D"). (B) Melatonin-induced downregulated expression of predicted transcription factors under the drought conditions ("MD vs. D"). Type I: DEGs with upregulated expression due to drought or melatonin plus drought ("D vs. W" and "MD vs. W") and upregulated expression due to melatonin under drought conditions ("MD vs. D"). Type II: DEGs with downregulated expression due to drought or melatonin plus drought ("D vs. W" and "MD vs. W") and upregulated expression due to melatonin under drought conditions ("MD vs. D"). Type III: DEGs with upregulated expression due to drought or melatonin plus drought ("D vs. W" and "MD vs. W") and downregulated expression due to melatonin under drought conditions ("MD vs. D"). Type IV: DEGs with downregulated expression due to drought or melatonin plus drought ("D vs. W" and "MD vs. W") and downregulated expression due to melatonin under drought conditions ("MD vs. D"). D: drought conditions; DAS: Day after sowing; DEG: differentially expressed gene; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

4.3.5. Proteomic analysis of melatonin-mediated drought tolerance

To verify the global proteomic changes in melatonin-induced drought tolerance, we used the same 12 samples as in the transcriptomic analysis (three replicates in each of the four treatments: W, MW, D, and MD) to obtain protein abundance data. In total, 3716 proteins were identified, and 2077 of them were common among all four treatments. We performed a comparative analysis of differentially abundant proteins (DAPs) (with a fold change of ≤ 0.83 or ≥ 1.2, $P < 0.05$) between treatment groups ("MW vs. W," "D vs. W," "MD vs. D," and "MD vs. W"). A total of 735 (338 increased and 397 decreased in abundance), 876 (424 increased and 452 decreased), 540 (311 increased and 229 decreased), and 991 (543 increased and 448 decreased) DAPs were identified in the comparisons of "MW vs. W," "D vs. W," "MD vs. D," and "MD vs. W," respectively (Figure 4-12A). Furthermore, Venn diagram analysis indicated that 890 DAPs were involved in melatonin-regulated drought tolerance (Figure 4-12B). In addition, GO and KEGG annotations of the 890 DAPs showed that melatonin had a major impact on various metabolic pathways under drought conditions, including 'oxidative phosphorylation' (ko00190), 'ribosome' (ko03010), 'carbon fixation in photosynthetic organisms' (ko00710), 'photosynthesis' (ko00195), 'carbon metabolism' (ko01200), 'porphyrin and chlorophyll metabolism' (ko00860), and 'linoleic acid metabolism' (ko00591) (Figure 4-12C–4-12E). Hence, melatonin mediates drought tolerance by disturbing protein translation and posttranslational modifications, photosynthesis, and JA biosynthesis at the proteomic level.

4.3.6. Melatonin increased endogenous JA content to enhance the drought tolerance of wheat seedlings

Based on the transcriptomic and proteomic data, we found that 208, 149, 19, and seven genes exhibited significant differences in the "MD vs. W," "D vs. W," "MD vs. D," and "MW vs. W" comparisons, respectively. We found that three genes (proteins), including *TraesCS2B02G555400* (A0A3B6CF89), *TraesCS2D02G528500* (A0A3B6DM57), and *TraesCS5A02G007900* (A0A3B6KC55) in linoleic acid metabolism (ko00591), which were related to JA biosynthesis, were commonly upregulated in "D vs. W" and "MD vs. D," both at the transcriptomic and proteomic levels. These results suggested that melatonin and drought stress might increase JA biosynthesis to enhance drought tolerance.

To further clarify the mechanism through which melatonin mediates drought tolerance, we selected some key related genes from the pathways mentioned above to verify their expression patterns in seedlings from the four treatment groups (W, MW, D, and MD). qRT–PCR and omics analysis revealed similar expression changes in several genes. Taken together, two genes related to JA biosynthesis (*LOX1.5* and *LOX2.1*) (Figure 4-13A–4-13B) and two transcription factors (*HY5* and *MYB86*) (Figure 4-13C–4-13D) were upregulated under conditions of drought stress and further upregulated by melatonin under drought conditions. In addition, three genes (*4CL2*, *P5CS1*, and *CCR2*) related to lignin biosynthesis (Figure 4-13E–4-

13G) and two genes (*PME53* and *SUS4*) involved in starch and sucrose metabolic pathways associated with the osmotic stress response (Figure 4-13H–4-13I) were induced by drought as well as by melatonin plus drought. These results revealed that melatonin mainly upregulates JA biosynthesis, lignin biosynthesis, and osmotic stress to enhance drought tolerance in wheat.

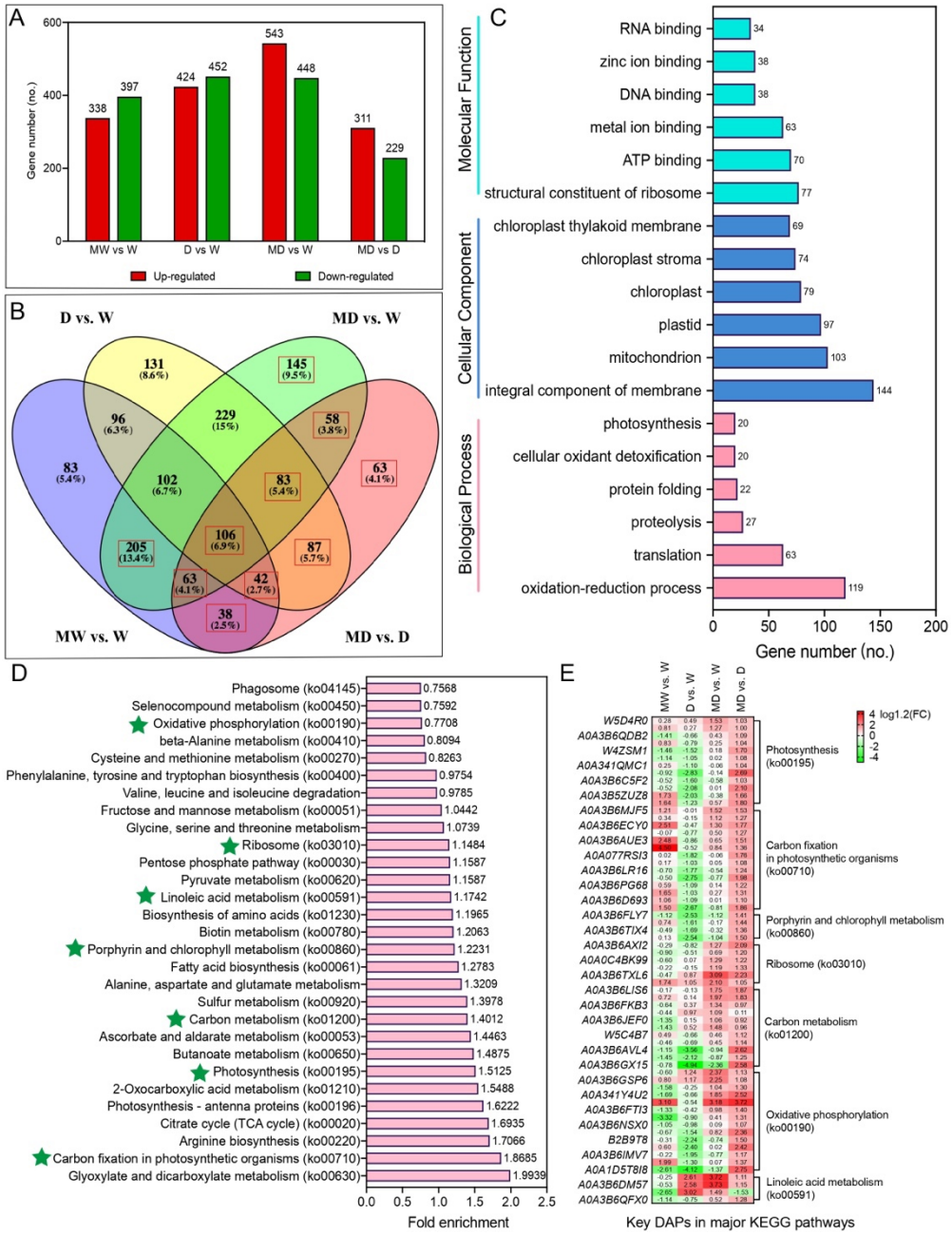


Figure 4-12 Overview and Venn diagram of DAPs identified via proteomic analysis in the four groups, with a threshold of |fold change| > 1.2 and FDR < 0.05.

(A) Total number of DAPs identified in the four comparisons ("MW vs. W," "D vs. W," "MD vs. W," and "MD vs. D"); red bars represent genes with upregulated expression, whereas green bars represent genes with downregulated expression. (B) The Venn diagram of the four groups. Red frames represent DAPs that might be related to the melatonin-mediated regulation of drought stress tolerance. Overlapping regions indicate co-expressed DAPs among different datasets, with numbers in a single circle representing DAPs expressed in only one library. (C) Major GO enrichment analysis of 890 DAPs related to melatonin-mediated regulation of drought tolerance. (D) Major KEGG analysis of 890 DAPs related to melatonin-mediated regulation of drought tolerance. (E) Key DAPs in major KEGG pathways of the 890 DAPs. Green five-pointed stars represent the key pathways potentially related to the regulation on drought stress by melatonin. D: drought conditions; DAP: differentially abundant protein; FC: fold change; FDR: false discovery rate; GO: Gene Ontology; KEGG: Kyoto encyclopedia of genes and genomes; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; TCA: tricarboxylic acid; W: watering conditions.

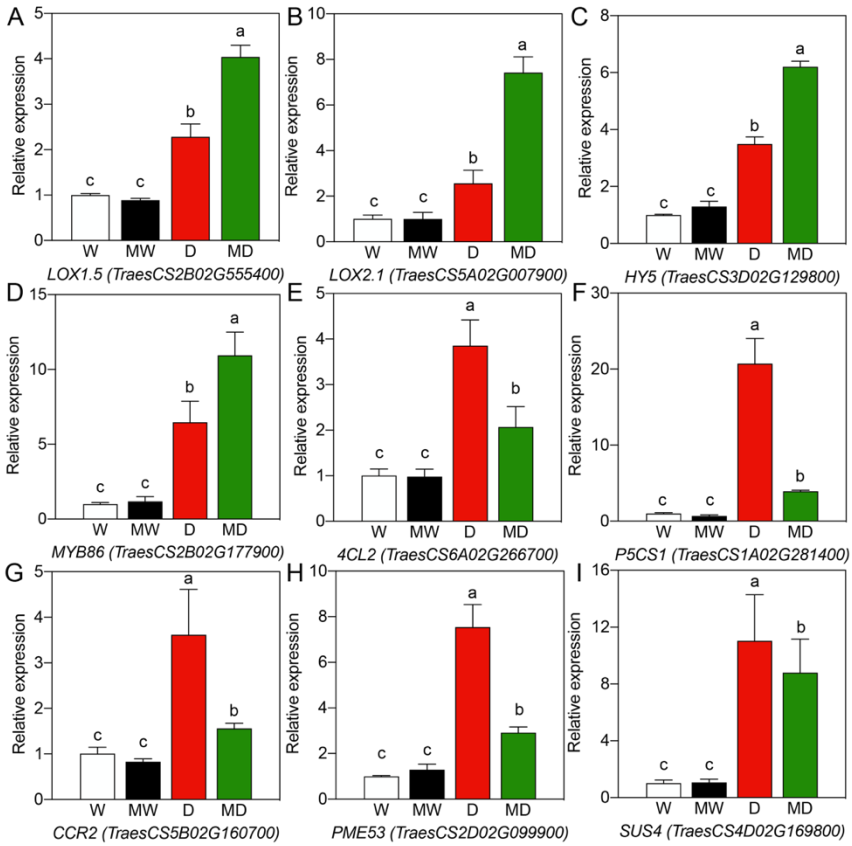


Figure 4-13 Verification of DEGs in key pathways related to melatonin-regulated wheat drought tolerance via qRT-PCR.

(A, B) The relative expression level of two jasmonic acid biosynthesis genes (*LOX1.5* and *LOX2.1*). (C, D) The relative expression level of two transcription factors (*HY5* and *MYB86*). (E, G) The relative expression level of three phenylpropane metabolic pathway-related genes (*4CL2*, *P5CS1*, and *CCR2*). (H, I) The relative expression level of cell wall-related genes (*PME53* and *SUS4*). These samples were obtained on the 18th DAS in the four treatment groups (W, MW, D, and MD). DAS: days after sowing; D: drought conditions; DEG: differentially expressed genes; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

To confirm how melatonin regulates physiological activity under drought conditions, we measured photosynthetic parameters, lignin, and JA content in 18-day-old seedlings under the four treatments. The net photosynthetic rate, stomatal conductance, and transpiration rate were significantly decreased, and, correspondingly, the level of intercellular CO_2 was significantly increased under drought conditions. At the same time, melatonin significantly relieved drought damage on photosynthesis (Figure 4-14A–4-14D). Furthermore, drought stress significantly decreased starch and chlorophyll content, whereas treatment with melatonin reduced drought stress-induced damage (Figure 4-14E–4-14F). In addition, drought and melatonin plus drought significantly increased JA and lignin content (Figure 4-14G–4-14H). These results suggested that melatonin has protective effects on photosynthesis under drought stress conditions, stimulating JA and lignin biosynthesis to enhance wheat drought tolerance.

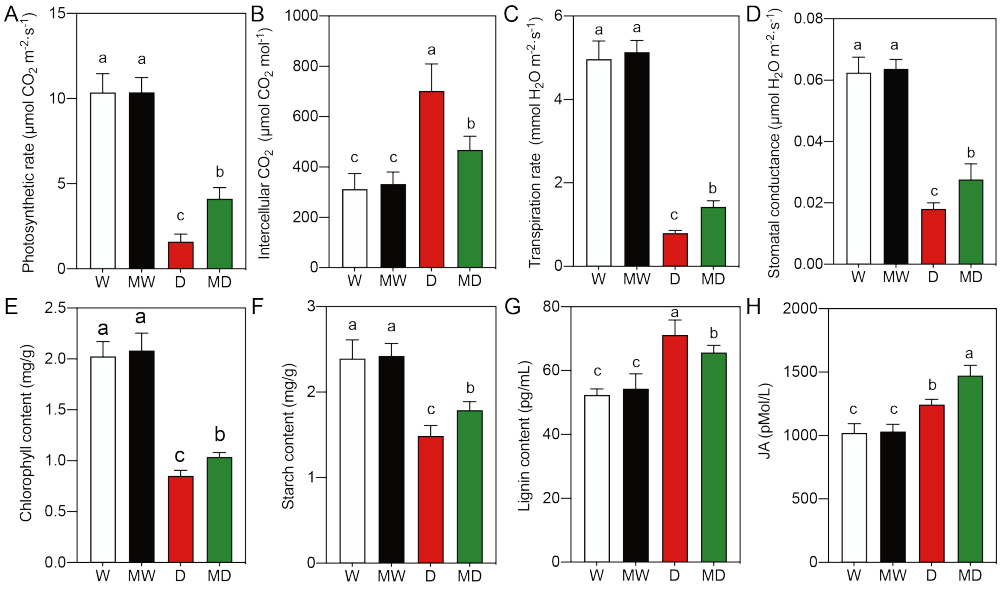


Figure 4-14 Response of photosynthetic data, lignin, and JA content to melatonin and drought stress.

(A) Net photosynthetic rate. (B) Intercellular CO_2 concentration. (C) Stomatal conductance. (D) Transpiration rate. (E) Starch content. (F) Total chlorophyll content. (G) Lignin content. (H) JA content. These samples were obtained on the 18th DAS in the treatment groups (W, MW, D, and MD). D: drought conditions; DAS: days after sowing; JA: jasmonic acid; MD: applying melatonin under drought conditions; MW: applying melatonin under watering conditions; W: watering conditions.

4.4. Discussion

4.4.1. Exogenous melatonin enhanced drought tolerance at both the seedling and maturation stages

In this study, we directly demonstrated that melatonin enhanced drought tolerance at the seedling and maturation stages under soil drought conditions (Figure 4-2 and Figure 4-4). This result further suggested that melatonin can act as a drought-resistant agent to increase the yield under drought stress. The improvement of drought tolerance by melatonin may be variety-dependent because exogenous melatonin increased the yield of "Chinese Spring," "Shi4185," and "Hanxuan10" but had no effect on the yield of "Chang6878" under soil conditions (Figure 4-2A–4-2F). Previous studies have reported that melatonin is vital in alleviating adverse effects in living organisms under various stress conditions (Chung and Deng, 2020). Among these, melatonin was also reported to improve osmotic stress tolerance in wheat plants (Cui et al. 2017, 2018). In addition, Li et al. (2019) demonstrated that melatonin improved seed germination and antioxidant metabolism in a drought-sensitive variety (JM22) compared with that in a drought-resistant variety (HG35) under osmotic stress conditions (Li et al. 2019). Hence, we hypothesized that the varietal dependence of melatonin on drought tolerance may be related to the different endogenous melatonin content in various varieties. This result might be associated with the different endogenous melatonin contents in different varieties because herbs with high melatonin content and overexpression plants overexpressing melatonin synthesis genes reportedly exhibit higher stress tolerance (Lee and Back 2019b; Wei et al. 2020; Liu et al. 2022a). In the future, exogenous melatonin might be directly applied with pesticides or chemical fertilizers to enhance the yield of cultivated plants under drought-stress conditions. However, the underlying reasons for the differential effects of melatonin on drought tolerance in different varieties require further study.

4.4.2. Melatonin increased JA biosynthesis to enhance drought tolerance

Here, we demonstrated that exogenously applied melatonin at an appropriate concentration significantly enhanced the drought tolerance of wheat seedlings under soil drought or osmotic stress conditions (Figure 4-2A, Figure 4-4A–4-4C). Further transcriptomic analysis revealed that melatonin induced multiple plant hormone pathways, including JA (*LOX1.5*, *LOX2.1*, and *LOX2.3*), ABA (*NCED5* and *NCED1*), BR (*CYP85A1*, *CYP90A4*, and *CYP90A4*), and CTX (*CKX9* and *CKX11*),

to improve drought tolerance. Furthermore, two *LOX* genes (*LOX1.5* [*TraesCS2B02G555400*] and *LOX2.1* [*TraesCS5A02G007900*]), which are involved in fatty acid metabolism and are key enzymes in JA biosynthesis, were consistently identified through transcriptomic and proteomic analysis (Figure 4-13A–4-13B). These findings are similar to previous reports that melatonin increased the JA content and activated the JA signaling pathway to enhance stress tolerance, such as disease tolerance in tomatoes (Liu et al. 2019) and cold tolerance in watermelon (Li et al. 2021a). However, combining transcriptomic and metabolomic analysis, Hu et al. reported that melatonin pre-treatment alleviated copper toxicity by inhibiting JA biosynthesis (Hu et al. 2020b). We hypothesized that the conflicting effects of melatonin on JA biosynthesis were attributable to different stress conditions or sampling times. In addition, we found that melatonin activated several transcription factors in plant hormone biosynthesis and signaling transduction pathways, including *SAUR3*, *IAA2*, *IAA12*, *IAA18*, *IAA19*, *ARF15*, *PIF3*, *EIL3*, *HY5*, and *MYB86* (Figure 4-11). Among them, *HY5* is known as a key regulator of light/dark transition and photomorphogenesis, exhibiting multifaceted roles as a transcriptional network hub in different processes, including growth, development, and abiotic stress (Zhang et al., 2020; Ding et al., 2022). Compared to previous results, overexpression of TaCOMT1A decreases GA_3 content in Chapter 3. We deduced that melatonin plays a central role in regulating the biosynthesis of other plant hormones. These results further show how melatonin regulates JA to enhance drought tolerance.

4.4.3. Exogenous melatonin influenced lignin, H_2O_2, and osmotic regulators to enhance drought tolerance

Based on our findings, we concluded that melatonin regulates drought stress in various ways, including upregulating genes related to JA biosynthesis, lignin content, osmotic substances, and several transcription factors (Figure 4-15). Interestingly, H_2O_2 has been identified as a key element in the melatonin-induced regulation of stress responses, with previous studies reporting that melatonin maintains low H_2O_2 levels either directly binding to H_2O_2 or indirectly by upregulating antioxidant enzyme levels (Tan et al. 2015; Back 2021). Our results also consistently indicated that melatonin maintained lower H_2O_2 content, which typically increased under drought stress (Figure 4-4F). This result is also consistent with previous results that exogenous melatonin or overexpressing the melatonin biosynthesis gene, *TaCOMT1A*, decreases H_2O_2 content and lignin content in Chapter 3 (Figure 3-2J and Figure 3-6F). In addition, melatonin significantly increased the activity of CAT and POD under drought stress (Figure 4-5G–4-5H). These results suggested that the first step through which melatonin helps plants delay or avoid drought stress responses is to control surges in H_2O_2 levels. Previous studies have indicated that plants dramatically increase the levels of osmotic substances, unsaturated fatty acids, lignin, and autophagy to alleviate cell damage induced by adverse environmental factors (Cui et al. 2017, 2018; Khadka et al. 2020). Accordingly, our study revealed that melatonin mitigated this drought-induced increase in lignin content and the up-regulation of genes related to biosynthesis, thereby improving drought tolerance in seedlings (Figure 4-13E–4-13G, 4-14G). However, further research on drought

stress under field conditions is required to demonstrate melatonin's potential role as a chemical agent for improving crop drought tolerance. Further, more wheat varieties should be investigated to understand better the connection between endogenous melatonin content and stress tolerance. Future experiments should investigate the overexpression or knockout of screened genes to confirm their involvement in melatonin-enhanced drought tolerance in wheat.

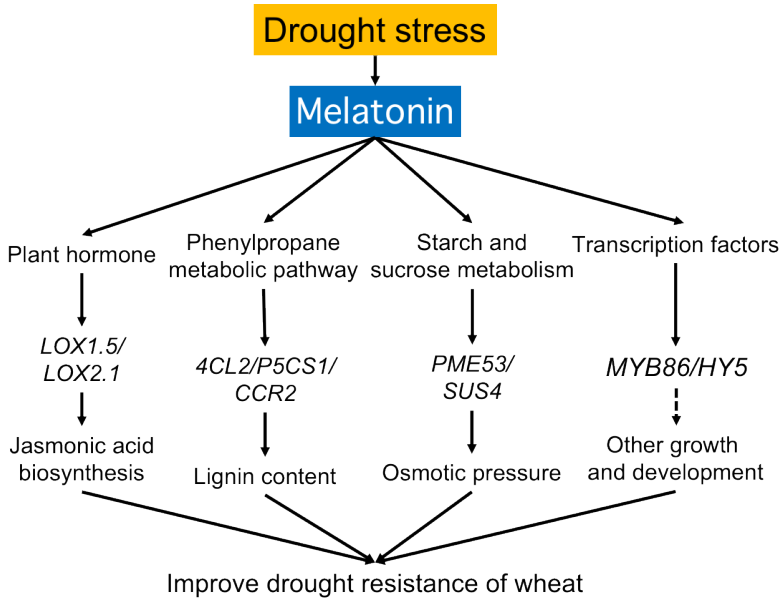


Figure 4-15 A proposed model for melatonin-mediated drought stress responses in wheat.

4.5. Conclusion

This study demonstrates that exogenous melatonin enhanced wheat drought tolerance at both seedling and maturation stages. This suggests the potential of melatonin as an agent for improving yield under drought conditions. Through the analysis of transcriptomic, proteomic, and physiological data, we identified several genes related to JA biosynthesis (*LOX1.5* and *LOX2.1*), lignin content, and osmotic substances, as well as transcription factors (*HY5* and *MYB86*). This information can help us to understand the mechanism through which melatonin enhances drought tolerance. To this end, overexpression lines and biochemical analyses should be performed in the future. Our findings suggest that melatonin alleviates cell membrane, chlorophyll, and photosynthesis damage caused by drought stress. In conclusion, our results highlight a new approach for improving wheat drought tolerance, revealing several candidate genes related to plant hormones and expression programs to lay a foundation for further studies on the mechanism of melatonin for improving drought tolerance.

Chapter 5

Exogenous sakuranetin enhances drought tolerance and decreases plant height in wheat (*Triticum aestivum* L.)

(Unpublished data)

In the previous chapter, we explored the regulatory mechanism of exogenous melatonin enhancing drought tolerance by transcriptomic and proteomic analysis. In this chapter, we further investigate the regulatory mechanism of exogenous sakuranetin in drought tolerance by using transcriptomic analysis in wheat.

The transcriptomic analysis was performed in OEbiotech Co, LTD (Shanghai, China). The content of GA_3 was subjected by the Major Platform Center, Institute of Crop Science, Chinese Academy of Agricultural Sciences (Beijing, China). Other experiments were carried out by Mingzhao Luo.

Abstract: Drought tolerance significantly influences wheat production and grain quality. Sakuranetin, a flavonoid and a key phenolic phytoalexin, is known to accumulate under biotic and abiotic stress conditions. However, its specific role and regulatory mechanisms in wheat remain unexplored. In this study, we demonstrated that sakuranetin application enhances drought tolerance in conventional wheat varieties during the seedling stage while simultaneously reducing plant height. Transcriptomic analysis revealed that sakuranetin upregulates photosynthesis-related genes and suppresses IAA and GA biosynthesis pathways under drought conditions. Moreover, similar to the effects observed with *TaCOMT1A* overexpression, sakuranetin application modulates key drought-related pathways, including the upregulation of *TaDREB2C-1A* and the downregulation of GA biosynthesis genes such as *TaGA2ox-5B* and *TaKO-7A*. These findings suggest that sakuranetin not only enhances drought tolerance but also reduces plant height, contributing to increased planting density, improved lodging resistance, and better adaptability. Therefore, sakuranetin presents potential for future applications in improving wheat production, especially under drought conditions.

Keywords: drought, plant height, sakuranetin, wheat, melatonin

5.1. Introduction

Dramatically increase in population boosted the demand for food production, whereas climate change developed a series of adverse environmental factors, such as drought and heat, which caused a great loss of crop production (Langridge and Reynolds 2021; Bapela et al. 2022). Plant growth regulators (PGRs) are commonly utilized in agriculture to augment overall plant growth (Zhang et al. 2022a). There are several classes of PGR, including auxin, abscisic acid (ABA), cytokinins, gibberellic acid (GA), salicylic acid (SA), jasmonic acid (JA), and ethylene, as well as more recently investigated brassinosteroids, strigolactones, polyamine, and triazole, etc (Franzoni et al. 2022). Among them, triazole as a growth regulator can not only regulate crop growth but also improve stress tolerance (Hasanuzzaman et al. 2021; Rezaei-Chiyaneh et al. 2023). Paclobutrazol (PBZ) as a triazole regulates plant growth by regulating plant hormones, especially negative regulating on GA biosynthesis (Maheshwari et al. 2022). Moreover, PBZ can alleviate drought stress by reducing glutathione and lipid peroxidation and increase grain yield by improving leaf number, stem diameter, root architecture, and decreasing plant height (Soumya et al. 2017; Desta and Amare 2021). PBZ has been applied in many aspects of agricultural production, including crops, vegetables, and forests. In recent years, due to concerns that residues of chemicals such as pesticides on agricultural products may threaten human health (Chen et al. 2020), some low environmental pollution compounds derived from plants have been developed for agricultural production (Rahimi et al. 2024). For example, seaweed extracts as biostimulants can enhance drought tolerance by positively regulating the ABA biosynthesis gene *NCED3* in soybean (*Glycine max* L.) and increasing the expression of flavonoids biosynthesis-related genes in Arabidopsis (Shukla et al. 2018). The application of new biostimulants will contribute to the healthy and sustainable development of agricultural production.

In this study, direct application of sakuranetin can increase drought tolerance and reduce wheat plant height. As a new plant-derived growth regulator, sakuranetin can improve the tolerance of wheat to drought stress and decrease plant height to reduce lodging risk, which suggests that sakuranetin has important application value in wheat production in the field.

5.2. Methods and materials

5.2.1. Plant growth and drought treatment conditions

Wheat varieties “fielder,” “Chinese Spring,” “Shi4185,” “Chang6878,” and “Hanxuan10” were used in this study. All those wheat seeds were conserved in our lab. All experiments at the seedling stage were cultured in a climate chamber set at a temperature of 24/16 °C day/night, 70% relative humidity, and a 14/10 h day/night photoperiod.

At the seedling stage, we prepared the mixture soil by blending the soil (Pindstrup,

Denmark) with water or different concentrations of sakuranetin (10 μM and 20 μM) at a ratio of 1:2 (g/mL) in blue shallow basins. We sowed wheat seeds on the mixture soil surface and covered it with soil. All seedlings were stopped watering on the 1st day after sowing (DAS) and rewatered it one L on the 15th DAS in each blue shallow basin. Five 12-day-old seedlings were mixed into a biological replicate sample and preserved at -80 °C for further study.

For the exogenous application of sakuranetin on wheat growth in hydroponic culture conditions. Several wheat varieties, including "Chinese Spring," "fielder," "Shi4185," "Chang6878," and "Hanxuan10," were cultured for 7 days underwater, 10 μM and 20 μM sakuranetin solution, respectively. The shoot length and root length were measured on the 7th DAS. The shoots of each variety were collected for determination of GA_3 content and extraction of RNA for qRT-PCR. Five 7-day-old seedlings were mixed into a biological replicate sample and preserved at -80 °C for further study.

For the field experiment conducted in the suburbs of Beijing (40°13′49″N, 116°33′28″E), we planted wheat variety "fielder" using furrow irrigation, adhering to established field research protocols under well-irrigated (WIR) conditions as described by Zhou et al. (2022) (Zhou et al. 2022). In brief, three rounds of irrigation were implemented, the pre-wintering, the jointing stage, and the filling stage. Each round consisted of applying 750 m³ of water per hectare. To investigate the effects of sakuranetin under field conditions, we sprayed "fielder" wheat with different concentrations of sakuranetin (20 μM and 100 μM) twice, during the jointing and heading stages. The plant height was measured at the maturation stage. The experiment included three replicate plots under WIR conditions, with each treatment comprising three rows.

5.2.2. Determination of physiological characteristics

The activity of catalase (CAT), and the content of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in wheat seedlings were measured according to the corresponding kit protocol (Comin Biotechnology, Suzhou, China). The absorbance values were measured by Thermo Scientific Microplate Reader (Thermo Fisher Scientific Inc., USA). All of these methods are the same as those in the previous two chapters.

5.2.3. Determination of the GA_3 content

This method was referencing previous studies (Cen et al. 2023). Briefly, the shoots of different wheat samples (including those treated with water and sakuranetin solution) were extracted with methyl alcohol. The supernatants were subjected to HPLC with an Ultra Violet detector system (Wooking, K2025, China). GA_3 content was detected at 210 nm. All measurements were reproduced in triplicate.

5.2.4. RNA sequencing and differential expressed genes analysis

A total of 36 samples, with 3 replicates for each of the 4 treatments (W, SW, D,

and SD) in 3 wheat varieties ("Shi4185," "Chang6878," and "Hanxuan10"), were used for transcriptomic analysis. OE Biotech Co., Ltd. (Shanghai, China) conducted the transcriptomic sequencing and analysis. The W group represents wheat seedlings cultured in watering conditions for 15 days, the SW group represents wheat seedlings treated with 20 μM sakuranetin and cultured in watering conditions for 15 days, the D group represents wheat seedlings cultured in drought conditions (without watering after sowing) for 15 days, and the SD group represents wheat seedlings treated with 20 μM sakuranetin and cultured in drought conditions (without watering after sowing) for 15 days. In brief, the total RNA was extracted using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then, the libraries were constructed using VAHTS Universal V6 RNA-seq Library Prep Kit according to the manufacturer's instructions. Differentially expressed genes (DEGs) between the treated and control samples (false discovery rate < 0.05 and fold-change ≥ 1.5) were identified using EBseq.

All genes were annotated using Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG), Pfam, Swiss-Prot, evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG), non-redundant protein sequence database (NR), and clusters of orthologous groups for eukaryotic complete genomes (KOG).

5.2.5. RNA extraction and qRT–PCR analysis

Total RNA was extracted using the Plant Total RNA kit (Zoman Biotechnology, Beijing, China) and used to synthesize first-strand cDNA using the Fast Quant RT Kit (Vazyme, Nanjing, China). SuperReal PreMix kits (Vazyme, Nanjing, China) were used for qRT–PCR. The *actin* gene was used as the control gene. All primers for gene expression assays are listed in Table 3-1 (Table 3-1). The data were analyzed with the $2^{-\Delta\Delta Ct}$ method. Each expression analysis was performed independently at least four times.

5.2.6. Statistical analysis

To analyze the difference in phenotypic and physiological characteristic values among different concentrations of sakuranetin in drought stress conditions, Duncan's test ($P < 0.05$) was performed to evaluate the significance of the difference by using the SPSS 17.0 statistical software package. All histogram graphs were drawn using the GraphPad prism (Version 8.4.0), and the pictures using Adobe Illustrator 2021.

5.3. Results

5.3.1. Exogenous sakuranetin improves drought tolerance and reduces the plant height in wheat

To investigate whether sakuranetin has the same effect as TaCOMT1A on drought tolerance and plant height, we treated with 10 μM and 20 μM sakuranetin on several wheat varieties, including "fielder" (drought-sensitive variety), the other two drought-sensitive varieties ("Chinese Spring" and "Shi4185"), and two drought-tolerance varieties ("Chang6878" and "Hanxuan10") (Figure 5-1A–5-1I). Under drought conditions, the water content in the box gradually decreased with the extension of treatment time. After 15 days of drought treatment, the soil's relative water content was approximately 45%, and then the plant survival rate was investigated after 7 days of rewatering (Figure 5-1J). Results showed that treatment with sakuranetin significantly increased the survival rate of all five wheat varieties compared with no sakuranetin treatment seedlings in different degrees, and application of 20 μM sakuranetin had a better effect on survival rate improvement than 10 μM sakuranetin (Figure 5-1K). Among these varieties, the difference in the survival rate of the "Chinese Spring" with and without treatment of sakuranetin was the largest, indicating that the effect of sakuranetin on "Chinese Spring" is the most significant (Figure 5-1K). In addition, treatment with sakuranetin significantly increased the relative water content and fresh weight of the shoot in comparison to no application with sakuranetin treatment in "Chinese Spring," "fielder," "Chang6878," and "Hanxuan10," while increasement was fewer in "Shi4185," suggesting that different wheat varieties have different sensitivities to sakuranetin treatment (Figure 5-1L–5-1M). We also found that treatment with sakuranetin decreased the MDA and H_2O_2 content significantly more than no treatment, and for "Shi4185," this change was also not significant (Figure 5-1N–5-1O). Those results indicated that applying sakuranetin on different wheat varieties can enhance drought tolerance, and the higher concentration of sakuranetin improves drought tolerance, and different wheat varieties have different sensitivity to sakuranetin.

Furthermore, to explore whether sakuranetin decreased plant height in wheat, we treated 10 μM and 20 μM sakuranetin on different wheat varieties through hydroponic experiments (Figure 5-2A). Results showed that treatment with two concentrations of sakuranetin solution inhibited the growth of shoots and roots of different wheat varieties compared with treatment with no sakuranetin solution during the seedling stage (Figure 5-2B–5-2C). The degree of inhibition of root length was more pronounced than that of the stem, and the degree of inhibition of stems varies among different varieties, while the degree of inhibition of "fielder" stems was insignificant (Figure 5-2B–5-2C). In addition, we found that exogenous sakuranetin decreases GA_3 content in five wheat varieties during the seedling stage (Figure 5-2D). In addition, the application of 20 μM sakuranetin significantly also decreased GA_3 content in wheat varieties (Figure 5-2F). The expression of two GA biosynthesis genes, *TaGA20ox-5B* and *TaKO-7A*, were downregulated considerably by sakuranetin in different wheat varieties (Figure 5-2E–5-2F). Treatment with sakuranetin also increased the expression of *TaDREB2C-1A* compared to that in the two drought-tolerant wheat varieties "Chang6878" and "Hanxuan10" (Figure 5-2G). Those results demonstrated that the application of sakuranetin significantly decreased plant height during the seedling stage by downregulating GA biosynthesis genes and decreasing GA_3 contents.

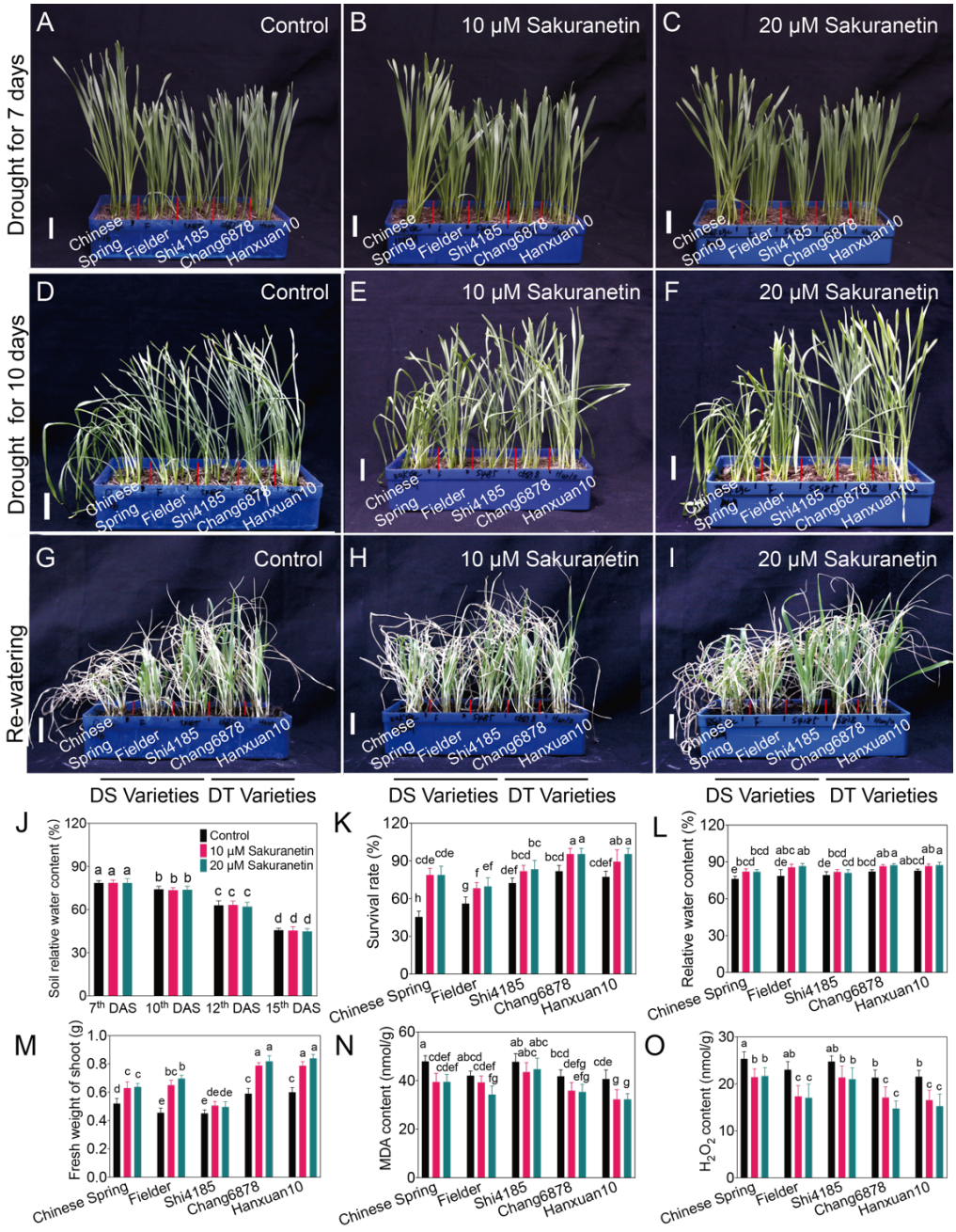


Figure 5-1 Exogenously sakuranetin increased the drought tolerance of different wheat varieties.

(A)–(C) Phenotype of 5 wheat varieties ("Chinese Spring," "fielder," "Shi4185,"

"Chang6878," "Hanxuan10") after drought treatment for 7 days, all pots were stopped watering from 1st day after sowing (DAS), and all germinated seeds were planted in the soil mixture which was blended with soil (Pindstrup, Denmark) and water (A), ten μM sakuranetin (B), and 20 μM sakuranetin (C) at the ratio of 1:2 (g/mL). (D)–(F) Phenotype of 5 wheat varieties after drought treatment for 10 days in the conditions of water (D), ten μM sakuranetin (E), and 20 μM sakuranetin (F). (G)–(I) Survival seedlings of 5 wheat varieties after drought treatment for 15 days in the conditions of water (G), ten μM sakuranetin (H), 20 μM sakuranetin (I) and rewatering for one week. (J) Soil relative water content in different mixed soil conditions on 7th, 10th, 12th, and 15th DAS. (K) Survival rates of 5 wheat varieties after treated for 15 days and rewatering for one week. (L)–(M) Relative water content (L) and fresh weight (M) of shoot of 5 wheat varieties after drought treatment for 12 days in different conditions of mixed soil. (N)–(O) MDA (N) and H_2O_2 content (O) of 5 wheat varieties after drought treatment for 12 days in different conditions of mixed soil. All data represents means ± SD ($n \geq 3$). Different letters indicate significant differences by analysis difference among different treatments ($P < 0.05$ according to one-way ANOVA, Duncan's multiple range test). Scale bars = 2 cm. DAS: days after sowing, DS: drought sensitive, DS varieties including "Chinese Spring," "fielder," and "Shi4185." DT: drought tolerance, DT varieties including "Chang6878" and "Hanxuan10."

We applied 100 μM sakuranetin on the "Shi4185" in the field under WIR and LIR conditions (Figure 5-3A–5-3B). Spraying with 100 μM sakuranetin significantly increased the yield of "Shi4185" under WIR and LIR conditions. The yield of "Shi4185" under WIR and LIR conditions increased by 13.22% and 16.08%, respectively, after 100 μM sakuranetin treatment (Figure 5-3C, Table 5-1). We investigated the three yield factors: panicle number per hectare, seed number per panicle, and thousand seed weight. The results showed that a significant increase in the number of spikes per hectare was the main reason for the sakuranetin increase in yield under LIR conditions (Figure 5-3D). However, applying 100 μM sakuranetin has no effects on seed number per panicle, thousand seed weight, or grain weight per panicle under WIR or LIR conditions (Figure 5-3E–5-3G), but it significantly decreased the plant height of "Shi4185" both under WIR and LIR conditions (Figure 5-3H). Hence, we concluded that applying sakuranetin significantly enhanced drought tolerance and inhibited plant growth during the seedling stage, and spraying sakuranetin in the field could improve yield and inhibit plant growth.

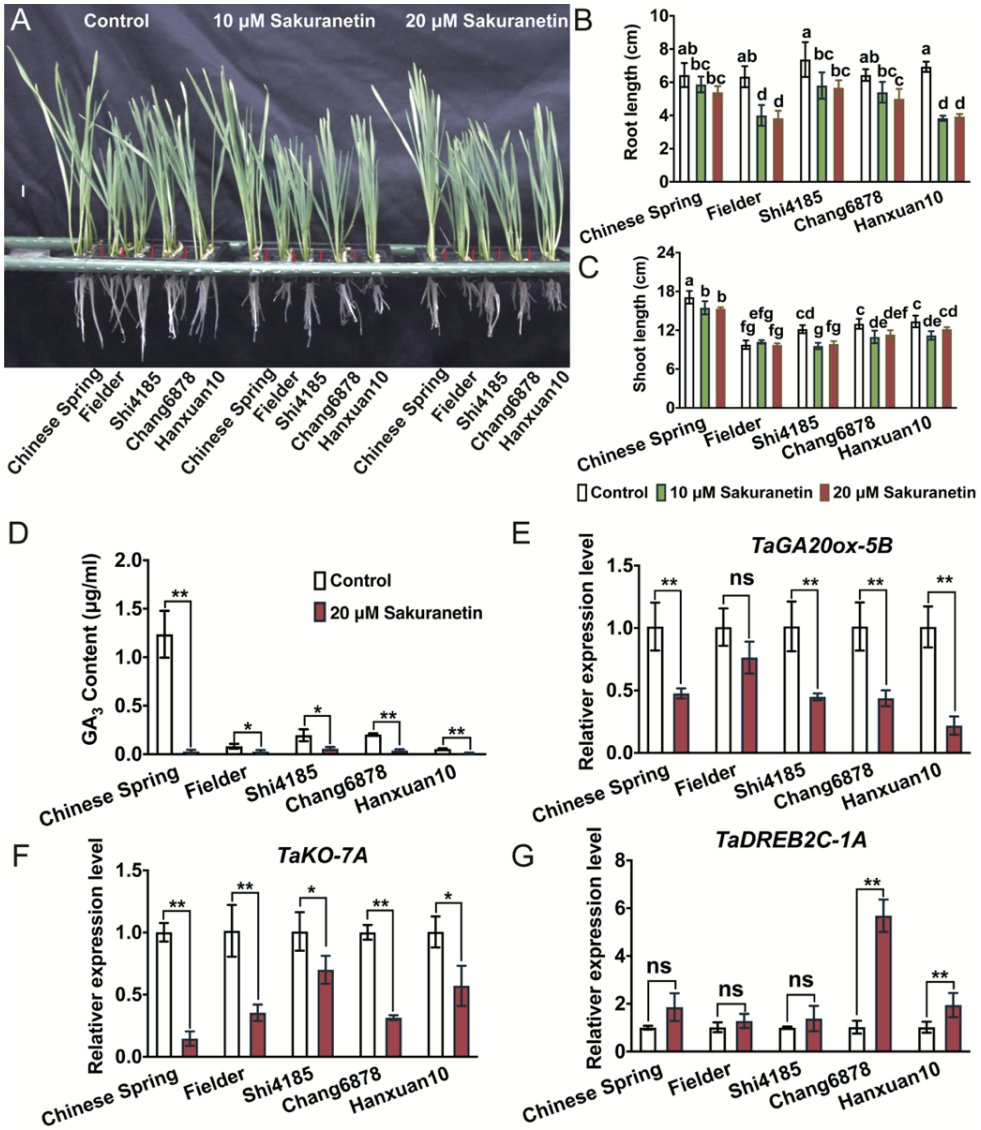


Figure 5-2 Phenotype of five wheat varieties treated with sakuranetin.

(A) Seedlings of five wheat varieties, that is, "Chinese Spring," "fielder," "Shi4185," "Chang6878," and "Hanxuan10" were cultured in water (Control), water plus 10 μM sakuranetin, water plus 20 μM sakuranetin for 7 days. (B)–(C) Root length (B) and shoot length (C) of five wheat varieties in different concentrations of sakuranetin for 7 days. (D) Gibberellic acid (GA_3) content in the shoot of five wheat varieties was measured with HPLC in an ultraviolet detector system (Wooking, K2025, China) in water and 20 μM sakuranetin for 7 days. (E)–(G) Relative expression of *TaGA20ox-5B* (E), *TaKO-7A* (F), and *TaDREB2C-1A* (G) under treatment with 20 μM sakuranetin. All data represent means ± standard

deviation (n ≥ 3). Different letters indicate significant differences by analysis difference among different treatments ($P < 0.05$ according to one-way analysis of variance (ANOVA), Duncan's multiple range test). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*$P < 0.05$; **$P<0.01$; ns, not significant). Scale bar = 1 cm. GA_3: gibberellic acid; HPLC: high-performance liquid chromatography.

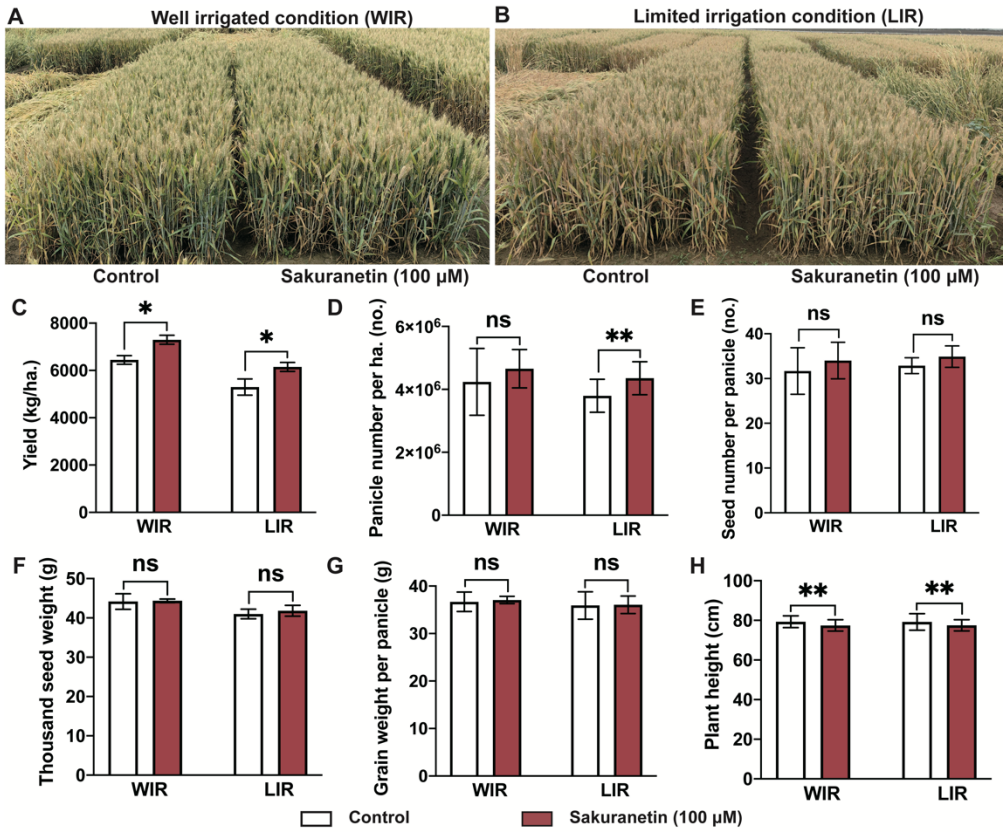


Figure 5-3 Phenotype of wheat variety "Shi4185" after spraying sakuranetin under well-irrigated and limited irrigation conditions in the field.

(A)–(B) Phenotype of "Shi4185" after spraying water (control) and 100 μM sakuranetin under well-irrigated conditions (WIR) (a) and limited irrigation conditions (LIR) (b), respectively. (C)–(H) Yield (C), panicle number per hectare (D), seed number per panicle (E), thousand seed weight (F), grain weight per panicle (G), and plant height (H), of "Shi4185" after spraying water (control) and sakuranetin during the maturation stage. All data represent means ± standard deviation (n ≥ 3). Asterisks indicate significant differences between overexpression plants and "fielder" plants at the same time points using *Student's t-test* (*$P<0.05$; **$P<0.01$; ns, not significant).

Table 5-1 Results of yield and agronomic traits after directly applying sakuranetin for wheat variety "Shi4185" under well-irrigated (WIR) and limited irrigation (LIR) conditions.

Field conditions	Well-irrigated (WIR)			Limited irrigation (LIR)		
"Shi4185"	Control	Sakuranetin	increase ratio	Control	Sakuranetin	Increase ratio
Yield (kg/ha.)	6444.44±181.40	7296.30±188.86	13.22%*	5296.30±343.47	6148.15±188.86	16.08%*
Panicle number per ha. (no.)	4237647±106295 7	4656471±60988 9	9.88%	3796667±52433 0	4355000±52565 0	14.71%**
Seed number per panicle (no.)	31.68±5.20	34.02±4.08	7.39%	32.87±1.78	34.90±2.40	6.18%
Thousand seed weight (g)	44.16±1.99	44.34±0.47	0.41%	41.00±1.20	41.82±1.39	2.00%
Grain weight per panicle (g)	36.69±2.05	37.06±0.77	1.01%	35.91±2.89	36.06±1.85	0.42%
Plant height (cm)	79.35±3.00	77.48±2.87	-2.36%**	79.26±4.18	77.52±2.82	-2.20%**

The sowing density was set at 120 kg per hectare, each plot was set at 1.5 m × 8 m, and each treatment had three replicates under LIR and WIR conditions. Under LIR conditions, two rounds of irrigation were applied, including the pre-wintering and jointing stages. Under WIR conditions, three rounds of irrigation were applied, including the pre-wintering, jointing, and filling stages. One round of irrigation was applied using 750 m^3 of water per ha in the field. All data represent means ± standard deviation (SD) ($n \geq 3$). Asterisks indicate significant differences between 100 μM sakuranetin-applied plants and water-applied (control) plants at the same time points using *Student's t-test* (*$P<0.05$; **$P<0.01$). SD, standard deviation; LIR, limited irrigation; WIR, well-irrigated.

5.3.2. Transcriptomic analysis of sakuranetin in regulating drought tolerance

To elucidate the molecular mechanism underlying sakuranetin-mediated drought tolerance in wheat, we conducted a transcriptomic analysis of seedlings across four treatment groups (W, SW, D, and SD) with three replicates per treatment in three wheat varieties ("Shi4185," "Chang6878," and "Hanxuan10"). Following mRNA sequencing of 36 samples, 250.88 Gb of clean data were obtained, with each sample producing at least 6.88 Gb of clean data. These reads were subsequently mapped to the reference genome, achieving mapping ratios ranging from 95.4% to 96.61%, confirming the quality of all transcriptomic datasets.

Differential expression analysis was performed to identify differentially expressed genes (DEGs) in response to sakuranetin and drought stress across the three wheat varieties. Specifically, DEGs were identified using criteria of |log1.5 fold-change| > 1 and adjusted P value < 0.05 in three comparisons: "SW vs. W," "SD vs. D," and "D vs. W." DEGs in "SW vs. W" and "SD vs. D" comparisons were regulated by sakuranetin under well-watered and drought conditions, respectively, while DEGs in "D vs. W" were indicative of responses to drought stress alone.

Consistent with phenotypic observations, the drought-sensitive variety "Shi4185" exhibited more DEGs in response to drought stress than the drought-resistant varieties "Chang6878" and "Hanxuan10." Conversely, "Shi4185" showed fewer DEGs in response to sakuranetin compared to "Chang6878" and "Hanxuan10." Specifically, in the "D vs. W" comparison, we identified 38,732 DEGs (17,425 up-regulated and 21,307 down-regulated) in "Shi4185," 26,570 DEGs (10,830 up-regulated and 15,740 down-regulated) in "Chang6878," and 26,376 DEGs (10,266 up-regulated and 16,110 down-regulated) in "Hanxuan10." In "SW vs. W," 48 DEGs (23 up-regulated and 25 down-regulated), 114 DEGs (67 up-regulated and 47 down-regulated), and 461 DEGs (244 up-regulated and 217 down-regulated) were identified in "Shi4185," "Chang6878," and "Hanxuan10," respectively. In "SD vs. D", 10 DEGs (8 up-regulated and 2 down-regulated), 28 DEGs (12 up-regulated and 16 down-regulated), and 83 DEGs (23 up-regulated and 60 down-regulated) were identified in "Shi4185," "Chang6878," and "Hanxuan10," respectively (Figure 5-4A).

To identify DEGs regulated by sakuranetin, we utilized Venn diagrams to refine the DEGs from three comparisons ("SW vs. W," "SD vs. D," and "D vs. W") across three wheat varieties ("Shi4185," "Chang6878," and "Hanxuan10"). Our analysis revealed that 87, 221, and 597 DEGs were regulated by both sakuranetin and drought in "Shi4185," "Chang6878," and "Hanxuan10," respectively (indicated by red squares plus white squares in Figures 5-4B to 5-4D). Additionally, we identified 9, 31, and 66 DEGs regulated by sakuranetin under normal watering conditions in "Shi4185," "Chang6878," and "Hanxuan10," respectively (represented by orange squares in Figures 5-4B to 5-4D).

These findings collectively illustrate that sakuranetin regulates a more significant number of genes in drought-tolerant varieties ("Chang6878" and "Hanxuan10") than in the drought-sensitive variety "Shi4185." Moreover, sakuranetin affects more

genes under well-watered conditions than under drought conditions.

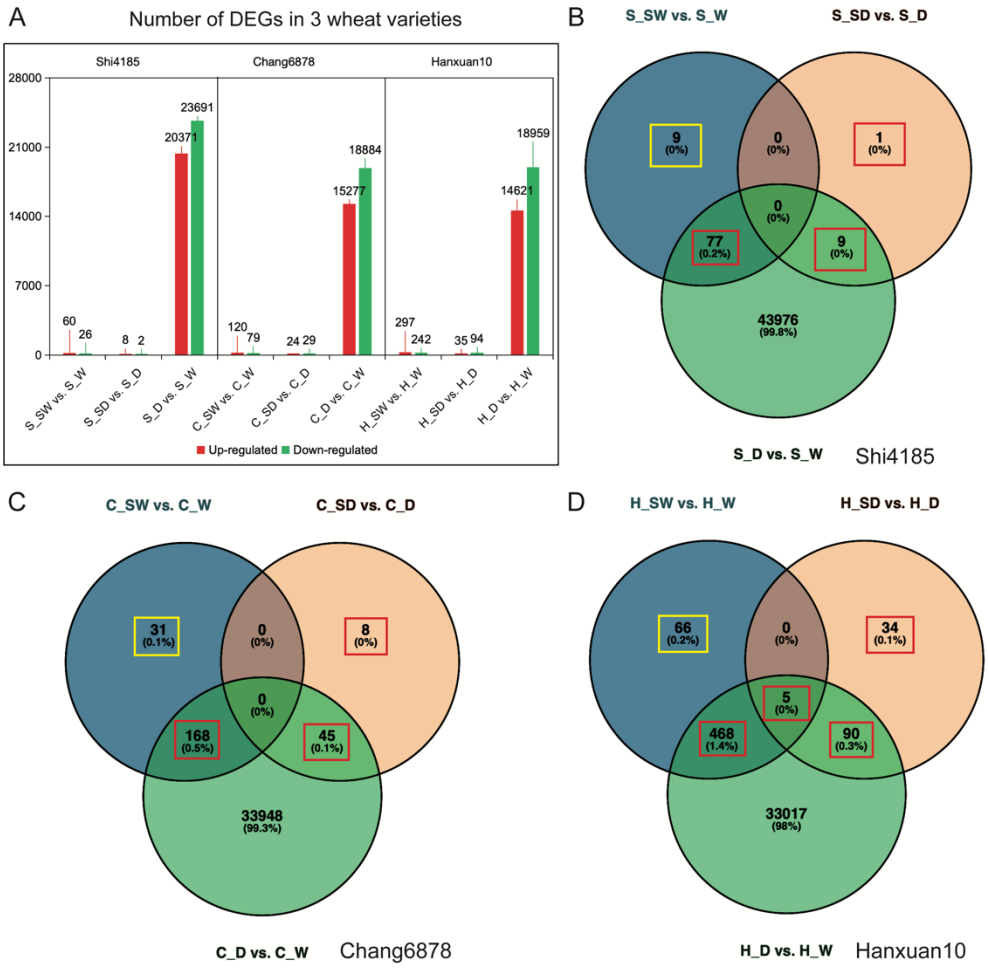


Figure 5-4 Differentially expression genes (DEGs) analysis in three comparisons ("SW vs. W," "SD vs. D," and "D vs. W") of 3 wheat varieties ("Shi4185," "Chang6878," and "Hanxuan10").

(A) The number of DEGs (including up-and down-regulated genes) in different wheat varieties. (B)–(D), The Venn diagram of DEGs identified in the three comparisons in "Shi4185" (B), "Chang6878" (C), and Haxuan10 (D). Overlapping regions indicate co-expressed DEGs among different datasets, with numbers in a single circle representing DEGs expressed in only one library. The red squares represent the DEGs regulated by sakuranetin in drought conditions. The yellow squares represent the DEGs regulated by sakuranetin in watering conditions. C_: "Chang6878"; D: drought conditions; DEGs: Differentially expression genes; H_: "Hanxuan10"; S_: "Shi4185"; SD: applying sakuranetin under drought conditions; SW: applying sakuranetin under watering conditions; W: watering

conditions.

To further understand the biological functions of these DEGs, we performed KEGG pathway functional annotations for each of the three wheat varieties. In all three varieties, genes related to photosynthesis (including those associated with chlorophyll, Rubisco, and photosystems) were downregulated by drought stress but upregulated by sakuranetin under both watering and drought conditions. In "Chang6878" and "Hanxuan10," genes involved in ABA biosynthesis and JA signal transduction were upregulated by drought stress and were further upregulated by sakuranetin under watering conditions but downregulated under drought conditions. Additionally, in "Hanxuan10," genes involved in IAA signal transduction and GA biosynthesis were downregulated by drought stress and further downregulated by sakuranetin under watering and drought conditions (Figure 5-5A–5-5C).

Furthermore, transcription factors also play a vital role in plant stress tolerance. Therefore, all transcription factors in those DEGs were analyzed in the next step. A large number of *WRKY*, *MYB*/*MYB* like, *NAC*, *MADS*, and *DREB* transcription factors were regulated by sakuranetin under drought stress conditions, and we also identified some transcription factors involved in GA biosynthesis and GA metabolism (Figure 5-6A–5-6C). Taken together, those results demonstrated that sakuranetin mitigates drought stress by upregulating photosynthesis genes and decreasing the expression of ABA and JA-related genes, and sakuranetin inhibited plant growth by increasing ABA and JA, as well as reducing IAA and GA.

5.4. Discussion

5.4.1. Sakuranetin improves drought tolerance

Here, we first found that exogenously application of sakuranetin enhanced drought tolerance (Figure 5-1). On account of other reports showed that sakuranetin as a flavonoid phytoalexin has health-beneficial effects in rice, and accumulation of sakuranetin increased antioxidant activities in response to external environmental factors, including pathogen invasion, UV light, and JA (Liu et al., 2023). Our study demonstrated that sakuranetin, like melatonin, improves drought tolerance at the seedling and mature stage in field conditions (Figure 5-1 and Figure 5-3). These results are consistent with the overexpression sakuranetin biosynthesis enzyme gene in Chapter 3 (Figure 3-2). Hence, sakuranetin can be developed into a water-saving, disease-resistant, and insect-resistant agent. However, the application of sakuranetin in the field conditions needs more investigation to be suitable for large-scale agricultural production.

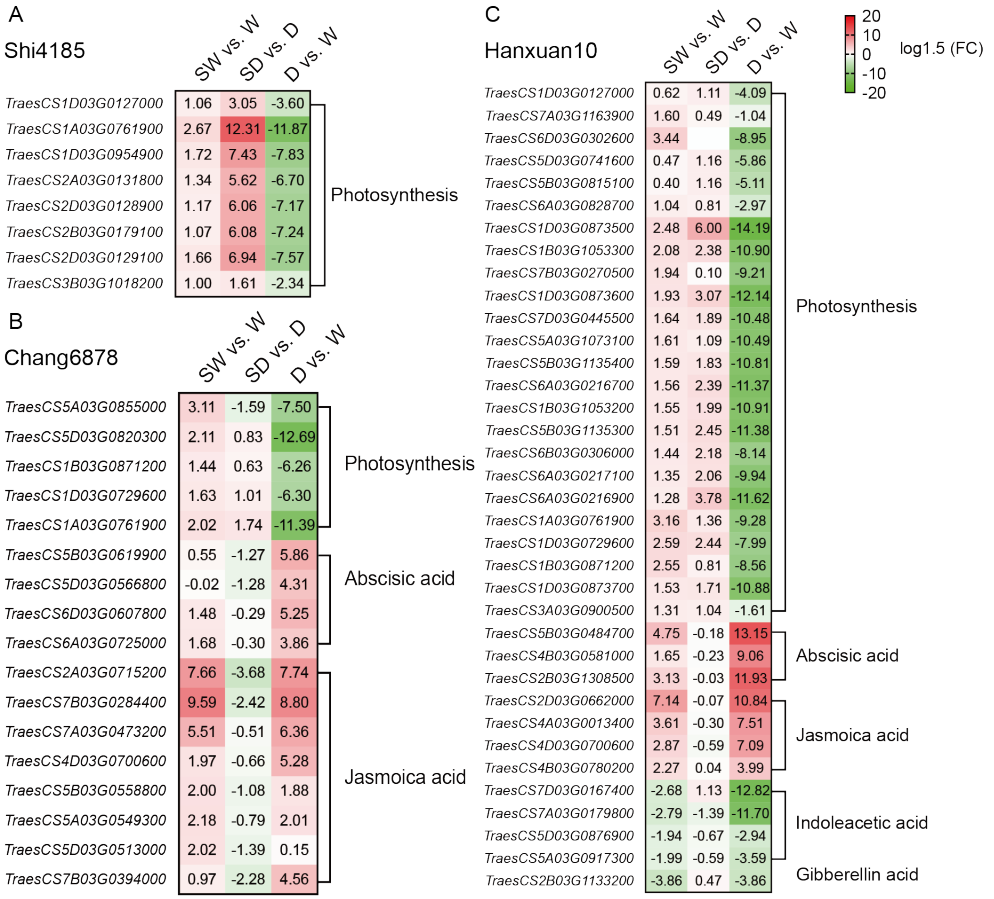


Figure 5-5 Key DEGs in significant pathways related to sakuranetin regulation of drought tolerance in 3 wheat varieties ("Shi4185," "Chang6878," and "Hanxuan10").

(A)–(C) Major KEGG pathways related to sakuranetin regulation of drought tolerance in "Shi4185" (A), "Chang6878" (B), and "Hanxuan10" (C). C_: "Chang6878"; D: drought conditions; DEG: Differentially expressed gene; H_: "Hanxuan10"; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK: mitogen-activated protein kinases; S_: "Shi4185"; SD: applying sakuranetin under drought conditions; SW: applying sakuranetin under watering conditions; W: watering conditions.

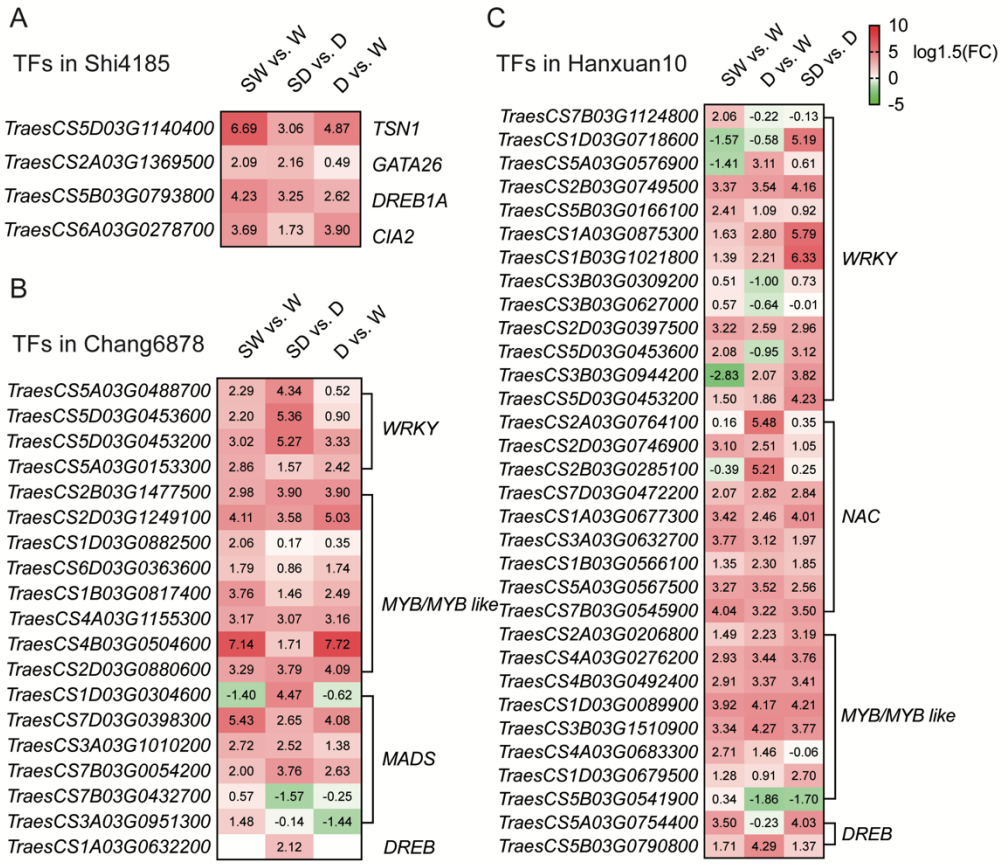


Figure 5-6 Key predicted transcription factors related to the regulation of wheat drought tolerance by sakuranetin in 3 wheat varieties ("Shi4185," "Chang6878," and "Hanxuan10").

(A)–(C) Major KEGG pathways related to sakuranetin regulation of drought tolerance in "Shi4185" (A), "Chang6878" (B), and "Hanxuan10" (C). D: drought conditions; DEG: Differentially expressed gene; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK: mitogen-activated protein kinases; SD: applying sakuranetin under drought conditions; SW: applying sakuranetin under watering conditions; TFs: transcription factors; W: watering conditions.

5.4.2. Sakuranetin decreases GA_3 content and downregulates GA biosynthesis genes.

Exogenous sakuranetin significantly decreases shoot and root development by reducing GA_3 content in several wheat varieties (Figure 5-2). In addition, sakuranetin decreases the expression of several genes in the GA biosynthesis pathways according to the transcriptomic data analysis and qRT-PCR results (Figure 5-2E–5-2F and Figure 5-5). Those results are consistent with overexpression

TaCOMT1A in Chapter 3 (Figure 3-9B). Hence, sakuranetin decreases Previous studies suggested that applying PBZ reduced plant height and ameliorated drought stress damage (Saleem et al. 2024). In addition, GA deficiency by applying PBZ also increased lodging resistance in rice (Plaza-Wüthrich et al. 2016). However, due to concerns about residues of PBZ and other chemicals, we prospected that sakuranetin may act as a new but more eco-friendly PBZ-like chemical in agricultural production. In the future, it will play an important role in intensive farming by decreasing plant height and increasing lodging resistance. Applying sakuranetin increased wheat yield by planting more seeds per unit area.

5.4.3 Sakuranetin upregulates photosynthesis under drought conditions

In this study, exogenous sakuranetin significantly increased the expression of photosynthesis-related genes in several wheat varieties ("Shi4185," "Chang6878," and "Hanxuan10") (Figure 5-5). These results are similar to transcriptomic and proteomic comparisons in melatonin-regulating genes under drought conditions in Chapter 4. Combining the results both melatonin and sakuranetin decrease H_2O_2 content under drought stress conditions (Figure 4-4F and Figure 5-1O). Therefore, we deduced that sakuranetin and melatonin protect the photosynthesis of wheat by maintaining homeostasis of H_2O_2 content during drought conditions. This is consistent with previous studies, that melatonin and sakuranetin have strong abilities to scavenge ROS under stress conditions (Shimizu et al. 2012; Zhou et al. 2020a). However, exogenous sakuranetin has no significant effect on JA biosynthesis genes in this study. This is a little contrary to the results in melatonin upregulating JA biosynthesis genes under drought conditions (Figure 4-13A–B, Figure 4-14H, and Figure 5-6). In addition, exogenous sakuranetin had no substantial influence on the ABA pathway under drought conditions. Those results suggested that sakuranetin improves wheat drought tolerance mainly by scavenging ROS in an ABA-independent way. But melatonin may also regulate other plant hormones to increase drought tolerance. However, limited to our results, further experiments needed to investigate the regulatory mechanism of sakuranetin on wheat drought tolerance.

Chapter 6

Discussion and conclusion of the whole thesis

6.1. TaCOMT1A is a multifunctional enzyme that can synthesize not only melatonin but also sakuranetin

Our research reveals the involvement of TaCOMT1A in regulating SMs, particularly melatonin and sakuranetin, which are essential for stress responses and plant defense. The increased levels of these compounds in *TaCOMT1A* overexpression lines suggest a connection between COMT-mediated pathways and stress adaptation in wheat. Overexpression of *TaCOMT1A* significantly increased wheat's melatonin and several flavonoids (including sakuranetin, vitexin, kaempferol, etc.) content. We also demonstrated that TaCOMT1A is a multifunctional enzyme that catalyzes the conversion of naringenin to sakuranetin and NAS to melatonin *in vitro*. Those results are consistent with previous studies that COMT is a multifunction enzyme by catalysis of different substrates. For example, Byeon et al. demonstrated that AtCOMT catalyzed the caffeic acid, 5-hydroxypinobanksyl aldehyde, and 5-hydroxypinobanksyl alcohol to produce ferulic acid, mustard aldehyde, and mustard alcohol, respectively, in Arabidopsis (Byeon et al. 2014a, 2015). This highlights the potential of genetic manipulation to enhance stress tolerance by upregulating the enzyme biosynthesis genes in crops.

In addition, our study provides insights into how wheat responds to drought stress, focusing on the function of TaCOMT1A in enhancing drought tolerance. We found that overexpression of *TaCOMT1A* improves wheat's ability to withstand drought conditions, leading to better survival rates and physiological performance under water-deficient conditions. However, this increased tolerance is associated with changes in plant height, indicating potential trade-offs between drought tolerance and growth traits (Figure 3-2 and Figure 3-7). Consistently, we demonstrated that exogenous sakuranetin has similar effects on several wheat varieties. It can enhance drought tolerance but also decrease plant height and GA_3 content (Figure 5-1 and Figure 5-2). According to these results, we deduced that TaCOMT1A can synthesize sakuranetin and de-active GA_3 to inhibit plant growth. GA promotes plant growth and development, such as seed germination, stem elongation, and leaf expansion, but is also involved in abiotic stress tolerance, including lodging resistance and drought tolerance(Plaza-Wüthrich et al. 2016; Shohat et al. 2021). Two OMTs, GA methyl transferase 1 (*GAMT1*) and *GAMT2*, which are de-active GA, increased drought stress tolerance in tomatoes (*Solanum lycopersicum*) (Varbanova et al., 2007; Nir et al., 2014).

Nevertheless, our study has limitations that require further investigation. Firstly, while our *in vitro* assays offer insights into TaCOMT1A's enzymatic activity, additional *in vivo* studies are necessary to confirm its physiological relevance and regulatory mechanisms in plants. For instance, we should construct the CRISPR-Cas9 knockout mutant and determine the melatonin and sakuranetin content in future studies. Melatonin and sakuranetin are versatile molecules for mitigating stress, such as cold. Previous study showed that WRKY transcription factors can directly interact with melatonin biosynthesis genes (Wei et al. 2018c). Generating

CRISPR-Cas9 knockout mutants of *TaCOMT1A* and examining their response to drought stress would provide valuable insights into its functional importance. Thirdly, we didn't obtain the downstream pathways of *TaCOMT1A* in drought conditions. We need further studies to find the possible transcription factors that can interact with TaCOMT1A. Additionally, the broader effects of *TaCOMT1A* overexpression on other stress responses, such as cold, disease, and salt tolerance, need to be explored to understand its full potential in improving stress resilience in wheat.

In summary, our findings advance our understanding of how wheat copes with drought stress and highlight TaCOMT1A as a promising target for enhancing crop stress tolerance. However, how to balance the growth and drought stress tolerance still need more investigations. Further research into the interactions between TaCOMT1A-mediated pathways and other stress signaling networks will be crucial for developing resilient wheat varieties capable of overcoming the challenges posed by climate change and ensuring food security.

6.2. Exogenous melatonin enhances drought tolerance by increasing JA biosynthesis

An appropriate melatonin concentration enhances wheat drought tolerance at the seedling and maturation stage. Our investigation has elucidated that exogenous melatonin application elevates the activity of antioxidant enzymes while concurrently reducing H_2O_2 levels under drought conditions, corroborating previous assertions regarding melatonin's efficacy in mitigating oxidative stress induced by diverse environmental stimuli (Bian ets al. 2021; Tiwari et al. 2021; Muhammad et al. 2023). These findings highlight melatonin's potential as a drought-ameliorating agent, bolstering agricultural productivity under water-deficient conditions.

Further elucidation through transcriptomic and proteomic analyses has unveiled melatonin-mediated upregulation of genes associated with osmotic stress, JA signaling, and lignin biosynthesis, fortifying wheat's drought resilience. Notably, the augmentation of JA biosynthesis genes (*LOX1.5* and *LOX2.1*) and transcription factors (*HY5*, *MYB86*, and *DREB2C*) following melatonin treatment under drought conditions underscores melatonin's multifaceted regulatory role in orchestrating stress-responsive pathways. Consistently, we found that overexpression of TaCOMT1A and exogenous sakuranetin significantly upregulate a drought response transcription factor, *TaDREB2C-A*. However, exogenous sakuranetin has no effect on JA biosynthesis genes under drought conditions in our study (Figure 5-5). Hence, we draw conclusions carefully that TaCOMT1A synthesized melatonin and then upregulated JA biosynthesis to enhance drought tolerance. In addition, TaCOMT1A synthesized sakuranetin and mainly upregulated DREB transcription factors to enhance drought tolerance. Both melatonin and sakuranetin can enhance drought tolerance, but mainly regulating two different pathways. ABA can enhance drought tolerance but has side effects on yield and plant growth (Liu et al. 2022b). In the present study, we found that *TaCOMT1A* catalyzes sakuranetin synthesis. Exogenous sakuranetin or overexpression of *TaCOMT1A* significantly enhanced drought

resistance through ABA-independent pathways, including upregulation of the expression of a drought resistance transcription factor (*TaDREB2C-1A*) and improved photosynthesis.

However, despite these compelling findings, our study is not without limitations. Primarily, the experimental settings were confined to greenhouse conditions, warranting validation through field trials to ascertain the translatability of our observations to real-world agricultural contexts. Secondly, future investigations should delve into the mechanistic underpinnings of specific transcription factors such as *HY5* and *MY86*, whose modulation by melatonin under drought stress warrants deeper scrutiny. Leveraging techniques like CRISPR-Cas9-mediated mutagenesis or heterologous expression coupled with protein-protein interaction assays could offer insights into their functional significance. Lastly, while our data suggest a role for melatonin in promoting JA biosynthesis to enhance drought tolerance, the precise molecular mechanisms governing this interaction remain elusive and necessitate further exploration at both transcriptional and post-translational levels.

In summary, while our study underscores melatonin's potential as a drought alleviator in wheat, comprehensive investigations integrating diverse methodologies are imperative to elucidate its mechanistic intricacies and optimize its agricultural applicability in combating drought stress.

6.3. Exogenous sakuranetin enhances drought tolerance and decreases plant height in wheat

With increasing concern about food and environmental safety, the search for novel biostimulants with low toxicity and environment-friendly properties for agricultural production has attracted increasing attention. Sakuranetin is a flavonoid phytoalexin that is widely resistant to pathogenic infections and pests (Shimizu et al., 2012b). We found that sakuranetin improved wheat tolerance to drought stress, reduced plant height, and increased stem strength in the greenhouse and field (Figures 5-1 and Figure 5-3). Sakuranetin can improve the adaptability of wheat under different biotic and abiotic stresses while appropriately reducing plant height and decreasing the risk of wheat lodging. Several compounds are used in crop production to regulate crop development. The application of PBZ reduces plant height and ameliorates drought stress damage (Davari et al. 2021; Maheshwari et al. 2022). GA deficiency caused by PBZ application increases lodging resistance in rice (Plaza-Wüthrich et al. 2016). Compared with PBZ, sakuranetin has many advantages, including being derived from plants, low toxicity, environmental friendliness, and resistance to both biotic and abiotic stresses (González-Pérez et al. 2022; De Diego and Spíchal 2022). Therefore, sakuranetin can serve as a novel, eco-friendly biostimulant for agricultural production.

Our study demonstrated that the exogenous application of sakuranetin, a plant flavonoid, significantly enhances drought tolerance and reduces plant height in wheat. This phenomenon has been observed across multiple wheat varieties. Transcriptomic analyses revealed that sakuranetin downregulated genes involved in

GA biosynthesis and upregulated DREB (dehydration-responsive element-binding) transcription factors, which are crucial for plant stress responses. These findings suggest that sakuranetin can reduce plant height and enhance drought tolerance in wheat. Consequently, we propose that sakuranetin and melatonin can be developed into eco-friendly agrochemicals suitable for large-scale agricultural production, particularly in arid and semiarid regions. These compounds can be co-applied with pesticides and fertilizers to improve yield and mitigate drought stress in future farming practices. Investigations into application methods, timing, dosage, and other relevant factors will be necessary for optimizing their use.

However, the current study has several limitations. Firstly, the observed effects of exogenous sakuranetin on drought tolerance and the reduction in root and shoot length were confined to the seedling stage of four wheat varieties; further research is needed to confirm these endogenous sakuranetin in different wheat varieties. Secondly, although our results indicated that sakuranetin reduces wheat root and shoot length by decreasing GA content and downregulating GA biosynthesis genes, it remains unconfirmed whether the enhancement of drought tolerance by sakuranetin is mediated through GA regulation. Sakuranetin affects the root and shoot growth, but we didn't measure the root architectures under drought conditions. The lateral root is the crucial for drought tolerance. Thirdly, the differential effects of exogenous sakuranetin on drought tolerance and plant height across various wheat varieties may be attributed to variations in endogenous sakuranetin levels between drought-sensitive and drought-tolerant varieties.

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Appendix

Publications

1. **Luo M**.*, Zhang D.*, Tang W.*, Delaplace P., et al. (2024). Recent advances in melatonin regulating drought tolerance in plants. (Accepted by *Tropical Plants*)
2. **Luo M**., Wang D., Delaplace P., Pan Y., et al. (2023). Melatonin enhances drought tolerance by affecting jasmonic acid and lignin biosynthesis in wheat (*Triticum aestivum* L.). *Plant Physiology and Biochemistry*, 202:107974. https://doi.org/10.1016/j.plaphy.2023.107974
3. **Luo M**., Zhang S., Tang C., Jia G., et al. (2018). Screening of mutants related to the C_4 photosynthetic kranz structure in foxtail millet. *Frontiers in Plant Science*, 9:1650. https://doi.org/10.3389/fpls.2018.01650
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6. Tang C.*, **Luo M**.*, Zhang S., Jia G., et al. (2023). Variations in chlorophyll content, stomatal conductance and photosynthesis in Setaria EMS mutants. *Journal of Integrative Agriculture*, 22(6):1618-1630. https://doi.org/10.1016/j.jia.2022.10.014 (* co-first author)
7. Xu C.*, **Luo M**.*, Sun X., Yan J., et al. (2022). *SiMYB19* from foxtail millet (*Setaria italica*) confers transgenic rice tolerance to high salt stress in the field. *International Journal of Molecular Sciences*, 23(2):756. https://doi.org/10.3390/ijms23020756 (* co-first author)
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12. Zhang S., Zhi H., Zhang W., Tang C., **Luo M**., et al. (2022). Phenotype analysis and low-resolution mapping of a stripe-leaf mutant *t122* in foxtail millet (*Setaria italica* L.). *Journal of Plant Genetic Resources*, 23(4):1076-1084. https://doi.org/10.13430/j.cnki.jpgr.20220211001 (Chinese periodicals)
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Unpublished papers

1. **Luo M.***, Hu L.*, Li W.*, Ge L., Qin Y., et al. (2022) Heterotrimeric G protein α subunit (GPA1) regulates the response to low-nitrogen stress in Arabidopsis by interacting with AtNRT1.4 and AtATG8a. bioRxiv 2022.01.27.478073 (Being resubmitted to Plant Science)
2. **Luo M.*** Xu C.*, Yang W. Zhou Y. et al. TaCOMT1A-mediated synthesis of sakuranetin: an effective approach to enhance drought tolerance and manage plant height in wheat (*Triticum aestivum* L.) (Under reviewed by Plant Biotechnology Journal)

Patents

1. Chen M., Ma Y., **Luo M**., Yang W., et al., (2023) Application of sakuranetin in improving drought resistance of wheat at the seedling stage. 202311003257.8. China Patent
2. Chen M., Ma Y., **Luo M.,** Yang W., et al., (2023) Application of vitexin in improving drought resistance of wheat at the seedling stage. 202311003256.3. China Patent
3. Diao X., Tang C., **Luo M**., Chen S., et al., (2016) A new incubator with automatic control of CO_2 concentration for basic scientific research. CN201610015521. Y. China Patent

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