

Molecular insights into abiotic stress responses in barley and Arabidopsis

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Table of contents

1.	List of publications used in this thesis	4
2.	Introduction	5
3.	Results	18
	3.1. Publication 1	18
	3.2. Publication 2	21
	3.3. Publication 3	23
	3.4. Publication 4	25
4.	Summary	28
5.	References	33
6.	Acknowledgement	42
7.	List of all publications	43
8.	Appendix	44

1. List of publications used in this thesis

The chapters of this thesis have been published in several peer-reviewed scientific journals or have been made available as a pre-print by an authorized pre-print server, comprising the full extent of this cumulative thesis.

Part 1: Oxidative stress and Ca²⁺ signalling in barley

1. **Bhattacharyya, S.**, Giridhar, M., Meier, B., Peiter, E., Vothknecht, U.C., and Chigri, F. (2023). Global transcriptome profiling reveals root- and leaf- specific responses of barley (*Hordeum vulgare L.*) to H₂O₂. *Frontiers in Plant Science*, 14 (2023). DOI: <https://doi.org/10.3389/fpls.2023.1223778>.
2. **Bhattacharyya, S.**, Bleker, C., Meier, B., Giridhar, M., Rodriguez, E.U., Braun, A.M., Peiter, E., Vothknecht, U.C., Chigri, F. (2025). Ca²⁺-dependent H₂O₂ response in roots and leaves of barley - a transcriptomic investigation. *BMC Plant Biology* 25, 232. DOI: <https://doi.org/10.1186/s12870-025-06248-9>.

Part 2: Drought stress regulation in *Arabidopsis thaliana* by *GASA3* and *AFP1*

3. Mahmud, S., Ullah, C., Kortz, A., **Bhattacharyya, S.**, Yu, P., Gershenzon, J., Vothknecht, U.C. (2022). Constitutive expression of *JASMONATE RESISTANT 1* induces molecular changes that prime the plants to better withstand drought. *Plant Cell Environment*, 45(10): 2906-2922. DOI: <https://doi.org/10.1111/pce.14402>.
4. **Bhattacharyya, S.**, Turysbek, B., Lorenz, S., Rosales, D.C., Shoaib, Y., Gutbrod, K., Dörmann, P., Chigri, F., Vothknecht, U.C., (2025). Loss-of-function of the drought-induced genes *GASA3* and *AFP1* confers enhanced drought tolerance in *Arabidopsis thaliana*. bioRxiv, DOI: <https://doi.org/10.1101/2025.04.03.647048>.

2. Introduction

2.1. Oxidative stress and Ca^{2+} signalling in *Hordeum vulgare*

2.1.1. H_2O_2 in plants

Due to the immobile nature of plants, their growth, productivity, and survival are continuously shaped and determined by environmental stresses. To acclimatize to short-term detrimental conditions, plants have evolved efficient molecular and cellular machineries to respond to environmental cues. One of the initial responses of a plant to many forms of stress involves the generation of reactive oxygen species (ROS) as a signal to modulate crucial aspects of plant growth, development and stress adaptation (Baxter et al., 2014). ROS also include by-products of aerobic metabolism that under normal growth conditions are produced at a low level (Smirnoff and Arnaud, 2019); however, disruption of metabolic pathways during stressful environmental conditions might result in an unprecedented increase in their rate of production. Hydrogen peroxide (H_2O_2) is a stable ROS involved in plant signalling and stress responses. Unlike other ROS, such as superoxide radicals or hydroxyl radicals, H_2O_2 exhibits low reactivity and a longer half-life, making it well-suited for signalling functions (Mittler et al., 2011). It is produced under normal conditions and in response to environmental stressors, including drought, salinity, heavy metals, and pathogen attack (Neill et al., 2002; Mhamdi and Van Breusegem, 2018). H_2O_2 serves as a secondary messenger that modulates gene expression and defense mechanisms and is primarily generated in organelles, including chloroplasts, mitochondria, and even peroxisomes as well as in the apoplast (Foyer and Noctor, 2003). The NADPH oxidase family of enzymes, encoded by Respiratory Burst Oxidase Homologs (RBOH), plays major role in H_2O_2 production, particularly in biotic and abiotic stress responses (Torres et al., 2006). To prevent oxidative damage, plants have evolved scavenging mechanisms, including enzymatic and non-enzymatic antioxidants (Gill and Tuteja, 2010). Enzymes like catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and peroxiredoxins (PRXs) regulate intracellular H_2O_2 levels (Dumanović et al., 2021). Additionally, low molecular weight antioxidants such as ascorbic acid, glutathione, and flavonoids prevent harmful ROS accumulation (Hasanuzzaman et al., 2019; Shen et al., 2022). An overview of ROS perception and signalling has been provided in Figure 1.

H_2O_2 plays a central role in plant development by influencing processes such as seed germination, root elongation, and cell wall remodelling. During seed germination, controlled H_2O_2 levels disrupt seed dormancy and promote radicle emergence by regulating ABA and gibberellin (GA) signalling pathways (Wojtyla et al., 2016). In root systems, H_2O_2 regulates the balance between cell division and elongation, hence influencing root architecture (Liu et al., 2024). H_2O_2 also contributes to cell wall loosening and lignification (Schopfer, 2001; Liskay et al., 2004). These modifications reinstate plant structural integrity under stress conditions (MILLER et al., 2010). Under stressful conditions, plants increase H_2O_2 production to activate defense responses. H_2O_2 also functions as a signalling molecule in systemic acquired resistance (SAR) and hypersensitive response (HR), instigating programmed cell death (PCD) to reduce pathogen attacks (Lamb and Dixon, 1997; Torres et al., 2006). Additionally, H_2O_2 intermingles with important

hormonal signalling pathways like salicylic acid (SA) and jasmonic acid (JA) pathways to enhance resistance against microbial pathogens (Kwak et al., 2006). In abiotic stress responses, H₂O₂ modulates stomatal closure through ABA signalling, reducing water loss during prolonged periods of water-deficit (Pei et al., 2000). It also regulates antioxidant enzyme expression to mitigate oxidative damage caused by heavy metals and extreme temperatures (Gill and Tuteja, 2010). In staple crops like rice (*Oryza sativa*), wheat (*Triticum aestivum*), and barley, H₂O₂ signalling plays a crucial role in stress tolerance and yield improvement. In rice, H₂O₂ mediates root development and drought tolerance by interacting with auxin and ABA pathways (Zhao et al., 2012). In wheat, H₂O₂ influences grain filling and seed dormancy through GA and ABA signalling networks (Wang et al., 2021). It also plays a protective role against cold stress by modulating SA signalling (Wang et al., 2018).

Exogenous application of H₂O₂ has been demonstrated to increase plant tolerance to abiotic stress by regulation of multiple stress-responsive pathways and gene expression, including several heat shock proteins and genes involved in ABA biosynthesis (Wahid et al., 2007; Terzi et al., 2014). The activation of ROS-dependent signalling by H₂O₂ creates accumulation of defense proteins, such as ROS-scavenging enzymes, transcription factors, and stress-response regulators (Hossain et al., 2015), thereby enhancing plant resilience to abiotic stressors. Furthermore, certain HEAT SHOCK TRANSCRIPTION FACTORS (HSFs) have been proposed to function as sensor molecules that perceive H₂O₂ and regulate oxidative stress response genes (MILLER and MITTLER, 2006). One of the earliest transcriptomic studies investigating the effects of H₂O₂ was carried out with cell-suspension cultures in *Arabidopsis thaliana*. It revealed that various TFs, hormone-associated pathways, and key metabolic processes, including photosynthesis and fatty acid biosynthesis, were influenced by H₂O₂ treatment (Desikan et al., 2001). However, despite the great importance of H₂O₂ as a ROS and signalling molecule in plants, studies concerning the molecular especially the transcriptional effects of H₂O₂ upon exogenous addition in barley, remains scarce.

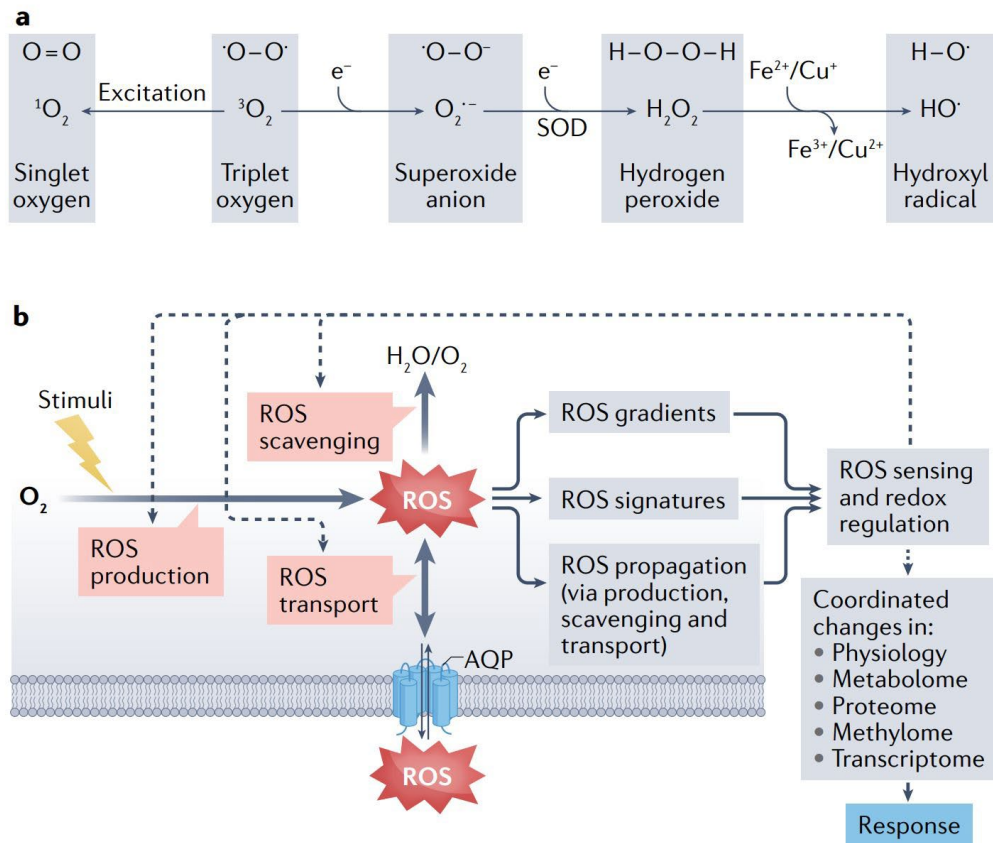


Figure 1: ROS generation, perception and signalling in plants (Mittler et al., 2022)

(a) Reactive oxygen species (ROS) are generated either by the excitation or reduction of atmospheric oxygen.

(b) Cellular ROS levels are tightly controlled through three key mechanisms: production, scavenging, and transport. In response to external or internal stimuli, ROS levels fluctuate. Cells detect and interpret these changes through modifications in the redox state of specific proteins, triggering coordinated responses. Beyond their localized roles, ROS-related processes can spread across membranes, between organelles, or even between cells, ultimately influencing the overall ROS balance in the plant. Dashed arrows indicate that ROS production, scavenging, and transport are modulated based on the redox state of the cell. AQP-aquaporin, SOD - superoxide dismutase.

2.1.2 Ca^{2+} signalling in plants

Calcium ions (Ca^{2+}) are secondary messengers playing fundamental roles in transducing environmental and developmental signals in higher plants. Ca^{2+} signalling integrates responses to abiotic and biotic stresses, hormone signalling, and developmental processes, making it a central component of plant cellular regulation (Hetherington and Brownlee, 2004). Plants regulate Ca^{2+} homeostasis through an entangled network of transporters, including Ca^{2+} channels, pumps, and exchangers located in various cellular compartments and organelles. Ca^{2+} influx into the cytosol is mediated by plasma membrane and calcium-permeable channels, such as cyclic nucleotide-gated channels (CNGCs) and glutamate receptor-like channels (GLRs) (McAinsh and Pittman, 2009). Efflux mechanisms like Ca^{2+} -ATPases and $\text{Ca}^{2+}/\text{H}^+$ exchangers, restore basal cytosolic Ca^{2+} levels, thereby ensuring controlled regulation of Ca^{2+} signalling (Costa et al., 2023). Ca^{2+} signals, often referred to as " Ca^{2+} signatures," are characterized by specific amplitude, frequency, and duration. These

signatures vary depending on the type of stimulus, allowing plants to distinguish between a diverse array of environmental stimuli (Pirayesh et al., 2021). For instance, drought stress induces oscillations in cytosolic Ca^{2+} levels compared to pathogen attacks, leading to specific downstream responses (Knight et al., 1997, 1998). The mechanism of Ca^{2+} signalling further proceeds through interactions with Ca^{2+} -binding proteins such as calmodulins (CaMs), Ca^{2+} -dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs), which decode Ca^{2+} signals to target cellular pathways (Luan et al., 2002). Plants encounter various abiotic stresses, including drought, salinity, and temperature fluctuations, where Ca^{2+} signalling plays a vital role in stress perception and adaptation. Under drought stress, Ca^{2+} signalling facilitates the activation of ABA-dependent pathways, causing stomatal closure to minimize water loss (Kim et al., 2010). In response to salt, Ca^{2+} signalling mediates ion homeostasis through the salt overly sensitive (SOS) pathway, where CBL-CIPK (CBL-interacting protein kinase) complexes regulate efflux of Na^+ ions from cells (Ma et al., 2020). Similarly, temperature stress instigates Ca^{2+} transients that modulate heat-shock protein expression, enhancing thermotolerance in plants (Ding and Yang, 2022). Figure 2 conglomerates the major molecular components of Ca^{2+} signalling in plants.

Plant immunity largely relies on Ca^{2+} -dependent signalling pathways that activate defense mechanisms against pathogens. Recognition of pathogen-associated molecular patterns (PAMPs) initiates a rapid influx of Ca^{2+} into the cytosol, activating several downstream responses such as ROS production and the expression of defense-associated genes (Lecourieux et al., 2006). CDPKs play a crucial role in mediating immune responses by phosphorylating key TFs involved in defense signalling (Boudsocq and Sheen, 2013). Furthermore, Ca^{2+} signalling contributes to SAR, creating long-term immunity in plants (Dubiella et al., 2013). It interacts with various phytohormones, including ABA, auxins, cytokinins, and JA, to mediate growth and stress responses. In ABA signalling, Ca^{2+} acts as a secondary messenger in guard cells, where it controls stomatal movements via Ca^{2+} -dependent activation of ion channels (Pei et al., 2000; Kim et al., 2010). In auxin-governed responses, Ca^{2+} signalling influences root development and elongation by modulating auxin transport and perception (Vanneste and Friml, 2009). JA-induced Ca^{2+} transients have been shown to play crucial roles in herbivory, thereby linking Ca^{2+} signalling to plant defense mechanisms (Hu et al., 2022). Beyond stress responses, Ca^{2+} is integral to various aspects of plant growth and development. Ca^{2+} regulated major developmental processes include pollen tube growth, root hair formation, and cell division (Hepler, 2005). Pollen tube elongation relies on Ca^{2+} transients that guide directional growth, leading to successful fertilization (Iwano et al., 2015; Scheible and McCubbin, 2019). Also, important developmental processes like root hair formation is similarly regulated by localized Ca^{2+} oscillations that control cell expansion (Bibikova et al., 1997).

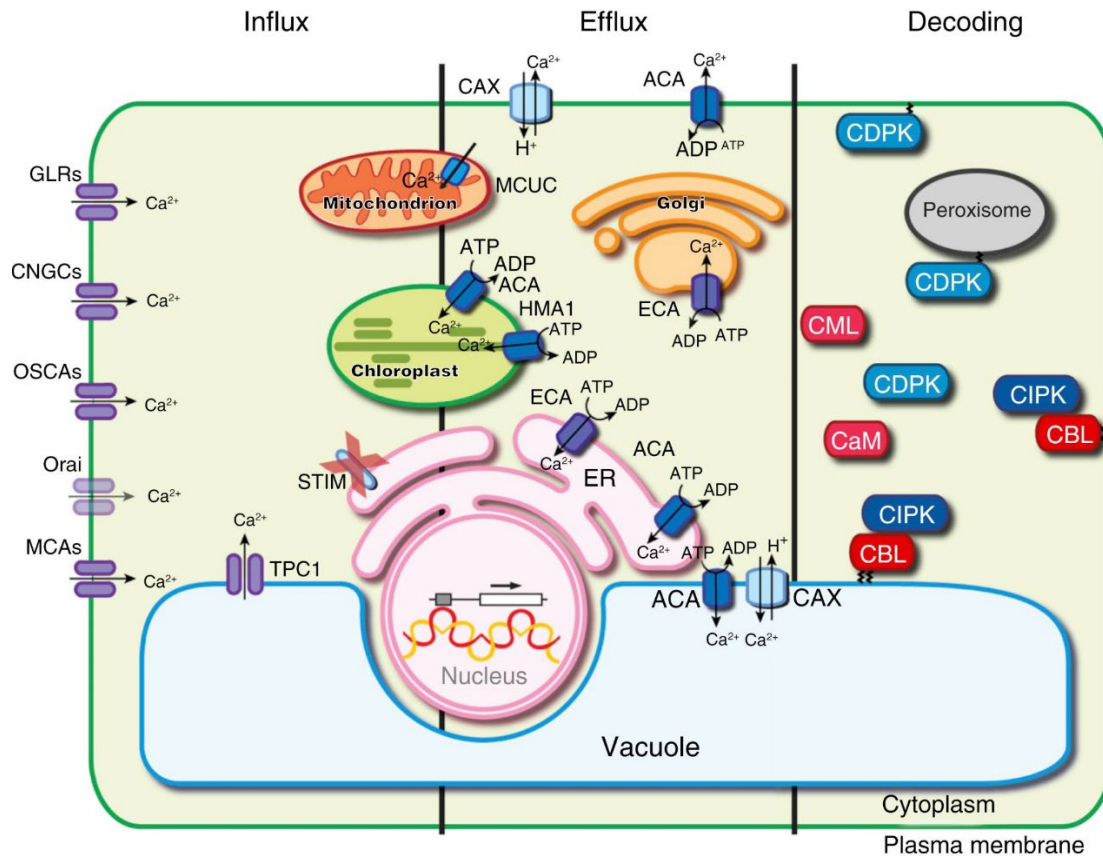


Figure 2: Molecular players of Ca^{2+} signalling (Edel et al., 2017)

The generation and processing of stimulus-induced Ca^{2+} signals rely on three key components: influx, efflux, and signal decoding. Ca^{2+} influx occurs through various channels, including cyclic nucleotide-gated channels (CNGCs), glutamate receptor-like channels (GLRs), two-pore channels (TPCs), mechanosensitive channels (MCAs), and reduced hyperosmolality-induced Ca^{2+} increase channels (OSCs). Once Ca^{2+} enters the cell, efflux systems regulate its concentration to ensure proper signal processing. These systems include autoinhibited Ca^{2+} -ATPases (ACAs), endoplasmic reticulum-type Ca^{2+} -ATPases (ECAs), P1-ATPases (HMA1), the mitochondrial Ca^{2+} uniporter complex (MCUC), and Ca^{2+} exchangers (CAX). Finally, Ca^{2+} signals are decoded by various protein families, many of which contain EF-hand motifs encoded in the Arabidopsis genome. Key players in this process include Ca^{2+} -dependent protein kinases (CDPKs), calcineurin B-like (CBL) protein kinases (CIPKs), and calmodulin (CaM) along with calmodulin-like proteins (CMLs).

2.1.3 H_2O_2 and Ca^{2+} crosstalk

The role of Ca^{2+} and H_2O_2 as central signalling molecules in plant responses to environmental stimuli has been widely studied. These two messengers orchestrate a wide range of physiological and biochemical processes that allow plants to acclimatize to abiotic and biotic stresses (Niu and Liao, 2016). The interaction between Ca^{2+} and H_2O_2 signalling pathways has been observed in response to various abiotic and biotic stresses (Ravi et al., 2023). However, the mechanisms underlying their mutual regulation remain unclear. Several studies indicate that Ca^{2+} functions as an upstream regulator in H_2O_2 signalling by modulating its synthesis. In plants, the enzymes RBOHs harbour a cytosolic N-terminal regulatory domain with Ca^{2+} -binding EF-hand motifs and Ca^{2+} -dependent phosphorylation sites targeted by CDPKs or CPKs, which are essential for RBOH activation and subsequent H_2O_2 generation (Kobayashi et al., 2008; Dubiella et al., 2013).

Beyond CPKs, additional Ca^{2+} sensors are thought to influence ROS production via RBOHs, either directly or indirectly. For instance, Ca^{2+} has been shown to activate via CaM, the NAD kinase, which may enhance NADPH levels required for RBOH activity (Harding et al., 1997). Inversely, evidence from previous studies suggests that H_2O_2 can act as an upstream signal regulating Ca^{2+} signalling. H_2O_2 -induced cytosolic Ca^{2+} level increases have been documented in various plant responses, including stomatal closure and stress adaptation (Rentel and Knight, 2004). This Ca^{2+} release is likely mediated by the direct activation of Ca^{2+} -permeable channels (Figure 3). Proposed H_2O_2 -activated Ca^{2+} channels include annexins, cyclic nucleotide-gated channels, and mechanosensitive ion channels (MSLs) (Demidchik et al., 2018; Fichman et al., 2022). A recent study characterized HPCA1 (H_2O_2 -induced Ca^{2+} increases 1) as a plant H_2O_2 sensor that mediates H_2O_2 -induced Ca^{2+} channel activation in guard cells, leading to increased cytosolic Ca^{2+} levels and stomatal closure (Wu et al., 2020). This sensor is also essential for systemic ROS and Ca^{2+} cell-to-cell signalling, which involves the Ca^{2+} -permeable channel MSL3, the Ca^{2+} sensor CBL4, and its interacting kinase CIPK26 (Fichman et al., 2022). However, despite the extensive body of research, the precise mechanisms by which H_2O_2 and Ca^{2+} signalling regulate each other, the factors determining the directionality of their crosstalk, and the integration of these pathways to generate a synchronized and coordinated response remain unclear.

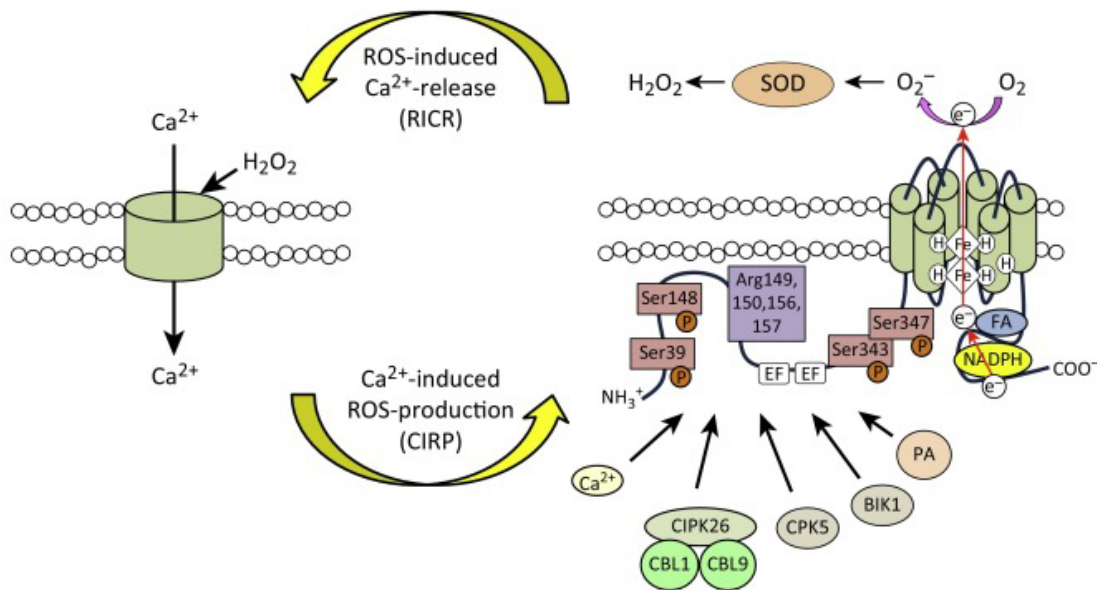


Figure 3: Integration of Ca^{2+} and ROS signalling (Gilroy et al., 2014)

ROS can modulate Ca^{2+} channel activity, either activating or inhibiting them, leading to the formation of distinct ROS-induced Ca^{2+} signalling patterns—a process known as ROS-induced Ca^{2+} release (RICR). Conversely, Ca^{2+} can influence ROS production either directly or indirectly through the activation of RBOH proteins, which generate superoxide radicals. These radicals subsequently undergo spontaneous or superoxide dismutase (SOD)-mediated conversion to H_2O_2 , a process termed Ca^{2+} -induced ROS production (CIRP). CBL1- CALCINEURIN-B-LIKE-PROTEIN-1, CBL9: CALCINEURIN-B-LIKE-PROTEIN-9; CIPK26- CALCINEURIN-B-LIKE-PROTEIN-INTERACTING-PROTEIN-KINASE 26; CPK5- CALMODULIN-DOMAIN-PROTEIN-KINASE-5; BIK1- BOTYRITIS-INDUCED-KINASE-1; FAD-Flavin adenine dinucleotide; NAD- Nicotinamide adenine dinucleotide; PA- Phosphatidic acid.

2.1.4. RNA-Seq and data analyses

The onset of omics technologies has unprecedentedly transformed biological research, allowing high-throughput data generation to study complex biological processes and systems at an unfathomable scale. Among these, transcriptomics enabled by RNA sequencing (RNA-Seq) has emerged as a powerful tool for investigating gene expression patterns, alternative splicing events, etc. RNA-Seq uses next-generation sequencing (NGS) technologies to provide a comprehensive picture of the transcriptome, offering greater sensitivity and dynamic range compared to traditional microarray-based techniques. The Illumina system is a popular platform, which is known for its high accuracy and cost-effectiveness (Modi et al., 2021). The sequencing process begins with library preparation, involving mRNA enrichment, ribosomal RNA depletion along with fragmentation, reverse transcription, and adapter ligation. One of the major steps in RNA-Seq analysis is read alignment, where sequencing reads are mapped to a reference transcriptome. TopHat2, a widely used read aligner, is specifically designed for spliced-read alignment and maps reads across exon-exon junctions (Trapnell et al., 2012). It uses the Bowtie2 algorithm (Langmead and Salzberg, 2012) for high-speed accurate mapping while allowing for gapped alignments to accommodate splicing events. The output of TopHat2 is stored in binary alignment map (BAM) format, a binary representation of alignment data that is essential for downstream analyses. Following alignment, the next step in RNA-Seq analysis is the quantification of gene expression levels. FeatureCounts, a widely used tool, facilitates the assignment of aligned reads to genetic features such as exons and genes, producing a set of raw read counts (Liao et al., 2014). These raw counts serve as the basis for differential expression analysis, which is commonly performed using DESeq2, a statistical package designed to identify DEGs (Love et al., 2014). It employs a negative binomial distribution model to account for biological and technical variability, providing proper statistical interpretation of expression changes between various experimental conditions. Normalization algorithms, such as variance stabilizing transformation (VST) or regularized logarithm (rlog) transformation, are used to correct for library size differences and sequencing biases, ensuring accurate comparisons across samples. Along with differential expression analysis, RNA-Seq data can be used for clustering techniques to identify co-expressed gene groups with common regulatory mechanisms. A popular clustering algorithm is k-means clustering, which partitions genes into distinct clusters based on their expression profiles (Ikotun et al., 2023). This approach relies on an iterative optimization procedure that minimizes the within-cluster variance while maximizing between-cluster differences.

2.1.5. Research goals

The goal of this part of the study was to elucidate the molecular link between H₂O₂-induced Ca²⁺ signalling and the resulting cellular responses in barley, which remains poorly understood. To achieve this, we investigated transcriptomic changes in barley roots and leaves following H₂O₂ treatment with and without blocking of the H₂O₂-induced cytosolic Ca²⁺ transient using RNA-Seq analysis. Comparing transcriptomic profiles under these conditions (H₂O₂ vs. H₂O₂ + LaCl₃) along with in depth data analyses, this study sought

to identify differentially expressed genes (DEGs) associated with oxidative stress and to uncover key crosstalk mechanisms between oxidative stress responses and Ca²⁺-mediated signalling in barley.

2.2. Drought stress regulation in *Arabidopsis thaliana* by *GASA3* and *AFP1*

2.2.1 Drought stress in plants

Drought stress consists of extended periods of water scarcity, significantly disrupting various aspects of plant growth and development, with a direct impact on crop production, which relies heavily on water availability. Climate-induced fluctuations in rainfall patterns may soon threaten food supply, making it imperative to develop effective strategies to counteract these challenges. Under conditions of water deficiency, plants exhibit a well-coordinated yet complex response, engaging a range of physiological, cellular, and molecular mechanisms to achieve water deficit tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007; Farooq et al., 2009). These physiological adaptations include restricted growth, reduced photosynthesis and transpiration, and increased respiration. At the cellular and molecular levels, plants accumulate various organic solutes, commonly referred to as osmolytes, or osmoprotectants, such as polyols, sulfonium compounds, sugars, and amino acids, alongside specific proteins that aid in stress resistance (Hasan et al., 2020). Plants generally exhibit similar physiological and biochemical responses to drought, categorized into three distinct strategies: i) drought escape, wherein plants complete their life cycle before the onset of severe drought; ii) drought avoidance, where plants enhance water retention by increasing root biomass or reducing evapotranspiration through stomatal closure and leaf curling; and iii) drought tolerance, which involves enduring water scarcity by minimizing biomass accumulation (Kooyers, 2015). The molecular basis of plant responses to stress and the mechanisms mitigating cellular damage have been extensively studied (Ingram and Bartels, 1996; Haghpanah et al., 2024). Investigating transcript-level variations in gene expression under drought, provides insights into plant stress responses (Alexandersson et al., 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). Several genes, including those encoding aquaporins, seed proteins, heat shock proteins (HSPs), dehydrins, membrane transporters, and late embryogenic abundant (LEA) proteins, have been implicated in stress adaptation (Shinozaki et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 2007; Harb et al., 2010). Drought conditions induce the production of the phytohormone ABA, which in turn promotes stomatal closure and triggers the expression of various stress-related genes. These genes include TFs belonging to both ABA-dependent and ABA-independent signalling pathways (Liu et al., 2018) as depicted in Figure 4. Additionally, other hormones such as JA (Riemann et al., 2015), GA (Shohat et al., 2021), and SA (Khalvandi et al., 2021) also contribute to plant adaptation to drought. Drought-induced gene expression plays a central role in plant adaptation to water deficit conditions. Several TFs, including members of the DREB, NAC, MYB, and WRKY families, regulate drought-responsive genes that enhance stress tolerance proteins (Singh and Laxmi, 2015), particularly DREB1 and DREB2, are known to mediate ABA-independent drought responses by binding to the dehydration-responsive element (DRE) within the promoter regions of stress-inducible genes

such as *RD29A* (*RESPONSE-TO-DESSICATION-29-A*), *RD29B* (*RESPONSE-TO-DESSICATION-29-B*), and *COR15A* (*COLD-RESPONSIVE-15-A*) (AKHTAR et al., 2012; Zhang and Xia, 2023). NAC TFs, such as SNAC1 and NAC029, also contribute to drought resistance by regulating stomatal movement and root architecture (Hu et al., 2006; Huang et al., 2015). Additionally, MYB TFs, such as MYB96 and MYB44, influence ABA-mediated drought responses by modulating gene networks involved in osmotic adjustment (Seo et al., 2009; Zhao et al., 2022).

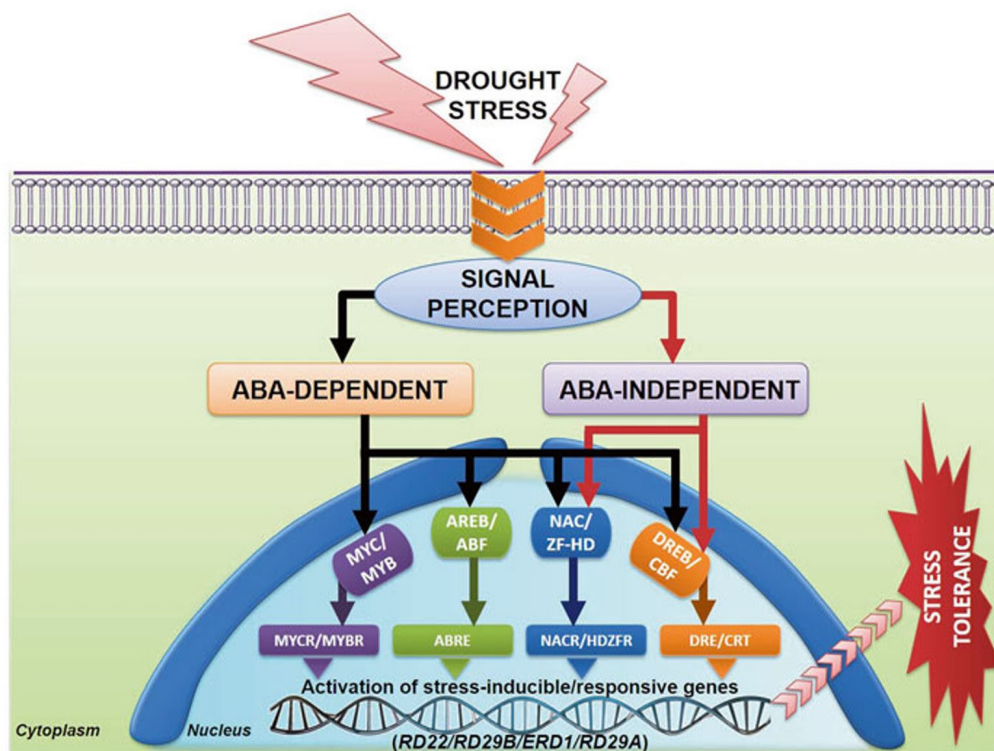


Figure 4: Major transcriptional components of drought stress signalling in plants (Lata et al., 2015)

Broad overview of the transcriptional machinery of drought signalling in plants, along with depiction of ABA- dependency and independency. ABRE- ABA RESPONSE ELEMENTS, DREB- DROUGHT-RESPONSE-ELEMENT-BINDING, DRE- DROUGHT-RESPONSE-ELEMENT, CBF-COLD-BINDING-FACTOR, CRT-COLD-RESPONSE-ELEMENT

Also, the WRKY TFs family has been demonstrated to play crucial roles in drought stress regulation in plants (Khoso et al., 2022). The upregulation of LEA proteins and HSPs is another major component of drought-induced gene expression. LEA proteins, such as LEA14 and LEA76, function as molecular chaperones that protect cellular structures and enzymes from dehydration-induced damage (Battaglia et al., 2008). HSPs, including HSP70, aid in protein stabilization under drought stress, ensuring proper protein folding and

preventing aggregation (Aghaie and Tafreshi, 2020). Furthermore, aquaporin genes such as *PIP1* and *PIP2* enhance water uptake efficiency by regulating membrane permeability in response to water deficiency (Kapilan et al., 2018). The activation of antioxidant-related genes, such as SOD (superoxide dismutase), APX or CAT contributes to the mitigation of oxidative stress generated during drought conditions (Mittler et al., 2004; Laxa et al., 2019).

Anthocyanins are a group of water-soluble flavonoid pigments that are widely distributed in plants and are responsible for the red, purple, and blue colors in many fruits, flowers, and leaves (Khoo et al., 2017). These pigments are synthesized via the flavonoid biosynthetic pathway and are known for their significant role in plant defense mechanisms, particularly under abiotic stresses. In recent years, growing evidence has emphasized the potential of anthocyanins in enhancing plant drought tolerance, with their protective functions during water deficit conditions (Cirillo et al., 2021; Dabravolski and Isayenkov, 2023). Several studies have shown that anthocyanin accumulation in drought-stressed plants is correlated with increased antioxidant enzyme activity, which helps in scavenging ROS and protecting plant cells from oxidative damage (Kaur et al., 2023). Additionally, other studies have suggested that anthocyanins may interact with other phytohormones, such as ABA, which plays a central role in regulating plant responses to drought stress. By modulating ABA signalling pathways, anthocyanins may enhance the plant's ability to respond to drought stress more effectively, thus improving its overall resilience (González-Villagra et al., 2019).

Understanding the genetic mechanisms behind drought regulation has therefore practical applications in developing drought-resistant crops through various engineering approaches. The integration of modern-day technologies like multi-omics approaches, including proteomics and metabolomics, further expands our understanding of drought-responsive networks and facilitates the identification of novel targets for improving plant resilience to water stress.

2.2.2. Role of phytohormones - key players of plant abiotic stress responses

ABA is a crucial plant hormone in abiotic stress responses, particularly in drought, salinity, and cold stress. It mediates stomatal closure, osmotic balance, and downstream gene expression in response to environmental factors. Abiotic stressors particularly lead to ABA accumulation in plant tissues, where it binds to PYR/PYL/RCAR receptors, inactivating PP2C phosphatases (Yoshida et al., 2010; AKHTAR et al., 2012; Liu et al., 2018; Ali et al., 2020, 2022; Fidler et al., 2022). This results in the activation of SnRK (SNF1-RELATED-KINASE) kinases, which phosphorylate transcription factors like AREB/ABFs (ABA-RESPONSE-ELEMENT-BINDING/ABA-RESPONSE-ELEMENT-BINDING-FACTOR), leading to the up-regulation of stress-responsive genes such as *RD29A* (*RESPONSE-TO-DESSICATION-29A*) and *COR15A* (*COLD-REGULATED-15A*). The physiological effects of ABA include the induction of stomatal closure via ion channel regulation, increased osmolyte production, and enhancement of antioxidant defenses (Araújo et al., 2011; Bharath et al., 2021). JA is primarily known for its role in biotic stress responses (Suza and Staswick, 2008; Hu et al., 2022), but it also participates in abiotic stress tolerance, particularly oxidative, drought, and salinity stress. Abiotic stresses trigger JA biosynthesis via the octadecanoid pathway, leading

to the binding of JA-Ile to COI1 (CORONATINE-INSENSITIVE-1), which results in the degradation of JAZ repressors (Suza and Staswick, 2008; Schaller and Stintzi, 2009; Riemann et al., 2015). This allows the activation of MYC2 transcription factors, which up-regulate stress-responsive genes (Kazan and Manners, 2013). JA also participates with ABA and ethylene pathways to enhance stress tolerance (Anderson et al., 2004). Its physiological effects include the induction of ROS-scavenging enzymes, osmotic adjustments, and secondary metabolite production. SA is an important player in plant defense against oxidative and thermal stress, modulating stress-related genes and antioxidant responses (Khan et al., 2015). Environmental stress increases SA levels, leading to the activation of NPR1 (NONEXPRESSOR-OF-PR-GENES-1) (Olate et al., 2018). The physiological effects of SA include the up-regulation of antioxidant enzymes, and modulation of ion transport under abiotic stresses (Yang et al., 2023).

GA primarily regulates growth and development, but it also plays a role in abiotic stress responses by modulating stress-related gene expression (Gupta and Chakrabarty, 2013). Under stress conditions, GA biosynthesis is generally suppressed, leading to an accumulation of DELLA proteins, which act as growth repressors. When GA binds to GID1 (GIBBERELLIN-INSENSITIVE-DWARF-1) receptors, DELLA repressors are degraded, allowing the modulation of growth-regulatory genes under stress conditions (Achard et al., 2006, 2008). GA interacts with ABA to balance stress adaptation and growth (Colebrook et al., 2014). The physiological effects of GA include enhanced root growth under drought conditions (Liao et al., 2023), modulation of seed germination under salinity stress (Kim et al., 2008), and maintenance of chlorophyll content under temperature stress (Nagar et al., 2021).

2.2.3. Impact of drought on plant growth

Among the most discernible phenotypic changes induced by drought are modifications in stomatal behaviour and flowering patterns (Takeno, 2016; Kollist et al., 2019) which are critical processes for maintaining growth and reproductive success during periods of stress. Under normal conditions, stomata open to allow for CO₂ uptake for photosynthesis (Lawson and Vialet-Chabrand, 2019). However, when plants face water-deficit, closing stomata minimizes transpiration and prevents undesirable amounts of water loss (Araújo et al., 2011; Agurla et al., 2018). Stomatal closure is tightly modulated by various signalling pathways, including those involving the phytohormone ABA, which accumulates in response to drought. ABA induces the closure of stomata by promoting the movement of K⁺ ions out of guard cells, leading to a decrease in turgor pressure and stomatal closure (Dietrich et al., 2001; Bharath et al., 2021). This process helps to judiciously conserve water and alleviate the damaging effects of water-deficit on plant tissues (Daszkowska-Golec and Szarejko, 2013; Agurla et al., 2018).

Flowering is a critical stage in the plant life cycle, and it is highly sensitive to environmental conditions, including water availability (Chen et al., 2023). Under drought stress, plants often delay or inhibit flowering as a means of conserving energy and resources. Since flowering requires considerable metabolic investment, and under water-limited conditions, it may be more advantageous for plants to delay reproduction until favourable conditions are restored (Shavrukov et al., 2017). In some plants, drought-induced delays in

flowering are an effect of altered hormone signalling, particularly involving ABA, which regulates many aspects of plant development, including flower initiation and development (Martignago et al., 2020; Mukherjee et al., 2023). Flowering time is also influenced by the interaction between drought stress and other environmental factors, such as temperature and light. For instance, high temperatures in combination with drought can aggravate the negative effects on flowering, leading to earlier or more severe delays in flower development (Kazan and Lyons, 2016). Furthermore, drought stress can alter the expression of genes involved in flowering time regulation (Chen et al., 2023). These changes in gene expression may contribute to the altered phenotypic responses observed under drought stress, often leading to delayed or reduced flowering. Understanding the genetic and molecular mechanisms behind drought-induced changes in flowering time is therefore critical for developing crops with improved drought tolerance and stable yields under water-limited conditions.

2.2.4. The GASA and AFP gene family

The GIBERELLIC-ACID-STIMULATED-ARABIDOPSIS (GASA) gene family comprises low-molecular-weight peptides also known as SNAKINs characterized by a conserved 60-amino-acid C-terminal domain containing 12 conserved cysteine residues (Bouteraa et al., 2023). Members of the GASA/SNAKIN family have been implicated in diverse developmental processes and responses to environmental stimuli (Sun et al., 2023). However, their precise functions and mechanisms of action remain largely unexplored. To date, 15 GASA members have been identified in Arabidopsis, though their specific functions remain unknown. Despite being plant-specific, the developmental roles of the GASA gene family are not yet fully understood. Among them, *GASA1* and *GASA4* are the most extensively studied proteins (Rubinovich and Weiss, 2010; Zhang et al., 2017). Some GASA proteins have also been suggested to promote GA responses, particularly in flowering and seed germination, and may also be involved in redox reactions due to their conserved cysteine-rich domain (Rubinovich and Weiss, 2010).

ABI5-BINDING-PROTEIN (AFPs) belong to a small, plant-specific protein family which are primarily associated with the regulation of ABA response through their interactions with ABI5-SENSITIVE 5 (ABI5), a key member of the small bZIP TFs subfamily in Arabidopsis (Vittozzi et al., 2024). AFPs enhance ABI5 proteolysis, thereby modulating ABA signalling and stress responses in *A. thaliana* (Lopez-Molina et al., 2003). Expression of AFPs is induced by ABA and/or dehydration stress, predominantly in seeds and young seedlings, where they co-expressed with ABI5 (Garcia et al., 2008). Transcripts of *AFPI* are present at lower levels in dry seeds but become more abundant following stratification, with their expression significantly increasing under stress conditions in mature plants (Garcia et al., 2008). Notably, AFPs can interact with other members of their family and also form homo- or heterodimers, suggesting a complex regulatory network (Garcia et al., 2008).

2.2.5. Research goals

This part of the study aimed to investigate the roles of *GASA3* and *AFPI* in drought stress adaptation in *Arabidopsis thaliana*. Our objectives include confirming the drought-induced expression of *GASA3* and *AFPI* under different durations of progressive drought and assessing their functional relevance. To achieve this, loss-of-function lines will be characterized along with evaluation of their associated drought-related phenotypes. Further validation will be conducted by generating and analysing transgenic lines, including double mutants and constitutive overexpression lines. Additionally, the role of ABA in *GASA3*- and *AFPI*-mediated drought responses will be investigated through hormone analyses and RT-qPCR. Finally, by examining their potential genetic interactions, this study aims to provide deeper insights into the physiological relationship between *GASA3* and *AFPI* in drought stress regulation.

3. Results

This section is divided into four chapters (corresponding to appendices 1, 2, 3 and 4) all of which have been published as different peer-reviewed articles or as authorized pre-prints. A dedicated materials and methods section is not included since all relevant information was included into the detailed methodology sections of the publications. The different chapters are ordered in a content-wise meaningful way.

3.1. Publication 1

Global transcriptome profiling reveals root- and leaf-specific responses of barley (*Hordeum vulgare* L.) to H₂O₂

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The study on “Global transcriptome profiling reveals root- and leaf- specific responses of barley (*Hordeum vulgare* L.) to H₂O₂” was published in the open-access journal Frontiers in Plant Science, in 2023, with me as the first author.

For this publication, I conducted the RNA-Seq analyses and also designed and executed follow-up experiments like the RT-qPCR assisted validation of differentially expressed genes (DEGs). Additionally, I developed coding algorithms, performed clustering analyses, generated graphs, and drafted the initial manuscript version.

My task in this project was first to decipher the nature of the DEGs in response to exogenous application of H₂O₂ in roots and leaves of barley. Firstly, processing of raw RNA-Seq data was carried out, obtained from RNA extracted from roots and leaves of 5-day-old barley seedlings either treated with H₂O (control) or with 10 mM H₂O₂. After alignment and generation of gene counts, the differential expression analyses were performed against the control. A total of 2,884 DEGs were detected across both tissues, with H₂O₂ application leading to more pronounced transcriptional changes in roots than in leaves. Among the 1,883 DEGs identified in roots, 701 were up-regulated and 1,182 were down-regulated, whereas in leaves, 1,001 DEGs were detected, with 546 up-regulated and 455 down-regulated. Notably, 75 genes were commonly up-regulated and 134 were commonly down-regulated in

both tissues, while 37 showed opposite expression patterns (counter-regulated). Gene Ontology (GO) analyses was used to identify the most significantly enriched biological processes found among the DEGs. In roots, many GO terms were related to oxidative stress, including H₂O₂ catabolism, glutathione and ROS metabolism, and cellular oxidant detoxification, in addition to processes involved in cell wall. A substantial number of DEGs were associated with H₂O₂ detoxification, particularly peroxidases and genes involved in glutathione metabolism. Surprisingly, our results showed a clear down-regulation of several GLUTATHIONE-S-TRANSFERASES (GSTs) and GLUTATHIONE PEROXIDASES (GTPs), along with other key components of H₂O₂ detoxification, such as orthologs of Arabidopsis *ASCORBATE PEROXIDASE 1 (APX1)* and *CATALASE 1 (CAT1)*. Additionally, putative *DETOXIFICATION EFFLUX CARRIERS/MULTIDRUG AND TOXIC COMPOUND EXTRUSION (DXT/MATE)* proteins were up-regulated in roots. MATE family of proteins facilitate the efflux of various compounds, including hormones and flavonoids, which contribute to stress adaptation. The group of genes severely affected by H₂O₂ treatment included the class III plant-type peroxidases. They play a well-established role in plant defense against biotic and abiotic stresses which are essential for maintaining cellular redox balance during stress, catalysing the oxidation of diverse substrates, and contributing to cell wall stability through polymerization of lignin and suberin. The up-regulation of these peroxidases in roots aligns with the increased expression of genes associated with cell wall metabolism in our study. In leaves, the top GO terms associated with up-regulated genes were linked to protein complex oligomerization, response to H₂O₂, and JA signalling. Notably, several enriched gene families in the leaves include heat shock proteins (HSPs) and various genes involved in phytohormonal signalling. The HSPs identified in our dataset belong to subfamilies with close orthologs in Arabidopsis, including HSP17.6, 15.4, etc. Especially HSP17.4 proteins have been shown to exhibit increased transcript levels under abiotic stress in Arabidopsis, suggesting that their induction may contribute to enhanced oxidative stress tolerance in barley leaves. Furthermore, our data showed the down-regulation of multiple genes involved in JA signalling, including an ortholog of Arabidopsis 12-OXOPHYTODIENOATE REDUCTASE-2 (OPR2), belonging to family of enzymes participating in JA biosynthesis, which was found to be down-regulated in leaves. Recent research has uncovered an alternative pathway for JA biosynthesis that operates independently of *OPR3*, an enzyme *which converts* 12-OXOPHYTODIENOATE (cis-OPDA) to the intermediate OPC8:0, that eventually lead to the formation of jasmonate. This route involves OPR2 and proceeds through intermediates such as dinor-OPDA (dnOPDA) and 4,5-didehydro-JA, which are ultimately converted into JA. In contrast, genes encoding *ALLENE OXIDE CYCLASE (AOC)* and *ALLENE OXIDE SYNTHASE (AOS)* were up-regulated in leaves, enabling the synthesis of both cis-OPDA and dnOPDA. Another crucial component of JA signaling identified in our dataset included TIFY domain-containing proteins, which were induced in response to H₂O₂. The TIFY domain is present in JASMONATE ZIM DOMAIN (JAZ) transcriptional repressors, which play a regulatory role in jasmonate signaling.

The findings in this study demonstrated that H₂O₂ plays a key role in regulation of gene expression across the barley genome, offering initial insights into its significant impact on cellular activity in barley. Many of the identified genes have previously been linked to stress responses in barley or, through their

orthologs, in Arabidopsis and other crops, highlighting a high level of conservation in plant responses to high H₂O₂ levels. The dataset from this study, became an important starting point for the next study which was to elucidate the role of Ca²⁺ in the H₂O₂ induced gene expression in barley tissues.

3.2. Publication 2

Ca²⁺-dependent H₂O₂ response in roots and leaves of barley - A transcriptomic investigation

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The study, “Ca²⁺-dependent H₂O₂ response in roots and leaves of barley – A transcriptomic investigation,” was published in *BMC Plant Biology* in 2025 as an open-access article. The research was based on findings from the previous study discussed in section 5.1. As first author of this publication, my primary contributions included conducting the initial processing of the raw RNA-Seq data and subsequent identification of Ca²⁺-dependent H₂O₂-responsive genes through list-based comparisons. These genes were further analyzed by our collaborators at the National Institute of Biology in Slovenia, where Dr. Carissa Bleker, the study’s second author, carried out network-based mapping using the SKM tool. Additionally, I performed H₂DCFDA staining analyses, confirming that LaCl₃ did not interfere with H₂O₂ penetration but specifically blocked Ca²⁺ channels. I also contributed to graph generation, data visualization, coding and drafting the initial manuscript. Furthermore, I also conducted RT-qPCR confirmations of the various Ca²⁺ dependent H₂O₂ induced genes.

Previous studies in our group have explored cytosolic Ca²⁺ transients induced upon treatment with exogenous H₂O₂ in barley (Giridhar et al., 2022). To investigate how Ca²⁺ signalling influences H₂O₂-induced transcriptomic changes, RNA-Seq was performed under conditions that inhibited H₂O₂-triggered Ca²⁺ transients by pre-treating barley seedlings with LaCl₃, a plasma membrane Ca²⁺ channel inhibitor, before H₂O₂ application. Additional RNA-Seq experiments were performed on plants treated with LaCl₃ or ddH₂O alone. DEGs were identified by comparing treatments to the ddH₂O control, using an FDR < 0.01 threshold. All other genes were classified as unchanged transcript levels (UCs). The H₂O₂+LaCl₃ treatment resulted in a quite similar number of up- and down-regulated genes in leaves (1,006 DEGs) and roots (1,344 DEGs). To process these results further, DEGs found under LaCl₃ alone treatment were excluded, leaving 989 DEGs in leaves and 1,001 in roots that are unique to the H₂O₂+LaCl₃ conditions. Although leaves and roots had comparable total number of DEGs, leaves

exhibited more down-regulated genes, whereas roots had more upregulated DEGs. A comparison was made with all the expressed genes under H₂O₂+LaCl₃, and the H₂O₂ induced DEGs to identify the Ca²⁺-dependent H₂O₂-responsive genes. The results revealed 331 and 1321 H₂O₂-responsive genes in leaves and roots, respectively, rely on Ca²⁺ to alter their expression. The clustering analysis of Ca²⁺-dependent H₂O₂-responsive genes resulted in five distinct clusters (L1–L5) in leaves and four (R1–R4) in roots. In leaves, clusters L1 and L2 include genes that were up-regulated and downregulated in response to H₂O₂. However, when treated with both H₂O₂ and LaCl₃, their expression levels remained unchanged compared to the control, suggesting that their response to H₂O₂ is strictly dependent on Ca²⁺ signalling. Clusters L3 and L4 had genes whose up-regulation and down-regulation in response to H₂O₂ were attenuated when the Ca²⁺ transient was inhibited by LaCl₃, yet their transcript levels remained significantly higher or lower than the control. This indicates that these clusters contain H₂O₂-responsive genes that exhibit partial dependence on Ca²⁺. Cluster L5 comprised genes that initially responded to H₂O₂ with up-regulation but switched to down-regulation upon Ca²⁺ transient inhibition, along with three genes whose down-regulation was further intensified. In roots, clusters R1 and R2 consisted of genes whose up-regulation and down-regulation, respectively, were entirely reliant on Ca²⁺ signalling. Unlike in leaves, no genes exhibited partial up- or down-regulation were identified. Instead, clusters R3 and R4 contain numerous H₂O₂-responsive genes that reversed their expression patterns - switching from up-regulation to down-regulation and vice versa - when the Ca²⁺ signal was inhibited.

To investigate various regulatory connections between known components of Ca²⁺ signalling networks and the identified Ca²⁺-dependent H₂O₂-responsive genes, CKN (comprehensive knowledge map) was used from the recently developed SKM resource (Bleker et al., 2024). Since the CKN is based on existing knowledge from *Arabidopsis*, our analysis focused only on the Ca²⁺-dependent H₂O₂-responsive genes identified in barley leaves and roots, respectively, that had recognizable orthologs in *Arabidopsis*. The shortest directed pathways were extracted by linking known Ca²⁺ signalling-related genes (source set) to the Ca²⁺-dependent H₂O₂-responsive genes identified in our transcriptomic analysis (target set). By combining these results, we identified several major network hubs that connected multiple Ca²⁺ signaling components to various target genes in both leaves and roots. In both leaves and roots, these hubs were predominantly represented by four TFs: HY5 (HYPOCOTYL-5), AGL15 (AGAMOUS-LIKE-15), PIF4 (PHYTOCHROME-INTERACTING-FACTOR-4), and EIN3 (ETHYLENE-INSENSITIVE-3). The Ca²⁺ signalling components within these networks primarily consisted of CaMs/CMLs and CDPKs/CPKs, along with CaM-interacting proteins. It is important to note that the CKN data used in our network modelling is derived from existing knowledge of *Arabidopsis*. Out of the 331 and 1,334 Ca²⁺-dependent H₂O₂-responsive genes identified in barley leaves and roots, respectively, only 192 and 894 genes were analyzed using CKN. This highlights the need for more experimental data from barley and other crops to bridge this significant knowledge gap. While many response mechanisms are conserved across land plants, some are species-specific. A deeper understanding of crop-specific responses is essential for accurate stress modelling and for leveraging this knowledge to enhance crop breeding strategies.

3.3. Publication 3

Constitutive expression of *JASMONATE RESISTANT 1* induces molecular changes that prime the plants to better withstand drought

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The study, “Constitutive expression of *JASMONATE RESISTANT 1* induces molecular changes that prime the plants to better withstand drought,” was published in Plant, Cell & Environment in 2022 as an open-access article. In this publication I was a co-author and my tasks included mostly experiments associated with investigation of phenotyping, screening of overexpression lines, and fluorescence microscopy. I also participated in editing and proof-reading of the final version of the manuscript.

Plants are constantly exposed to various biotic and abiotic stressors, necessitating a fine-tuned balance between growth and defense mechanisms. JA signalling is a crucial pathway that orchestrates plant development and stress adaptation. In this study, we investigated the effects of manipulating endogenous JA-Ile levels using a T-DNA insertion mutant in the *JAR1* (*JASMONATE RESISTANT 1*) locus (*jar1-11*) and a transgenic Arabidopsis overexpression line (*35S::JAR1*), which expresses *JAR1.1-YFP* under the control of the 35S promoter. Altered *JAR1* transcript levels and JA-Ile content in these transgenic lines led to distinct phenotypic differences compared to WT plants, even in the absence of external stress factors.

The *35S::JAR1* plants exhibited a dwarf phenotype, characterized by smaller rosettes and delayed flowering under normal conditions. Under drought stress, these plants maintained a higher relative water content (RWC) than WT, whereas *jar1-11* mutants exhibited the most significant RWC loss. This variation was associated with differences in stomatal closure responses, observed even under non-stressed conditions. Furthermore, RNA-Seq analysis revealed distinct patterns of DEGs among *jar1-11*, WT, and *35S::JAR1* under drought conditions. Many of these DEGs were linked to drought responses,

including water transport and general stress adaptation, while others might reflect secondary effects stemming from the varying drought phenotypes observed in the different genotypes.

Interestingly, even under non-stressed conditions, 35S::*JAR1* plants displayed down-regulation of specific drought-responsive genes, such as *RD29A* and *ERD7* (*EARLY-RESPONSIVE-TO DEHYDRATION-7*), as well as cold-responsive genes like *COR15B* (*COLD-REGULATED-15B*), suggesting a complex regulatory interplay that may influence drought resistance. Additionally, 35S::*JAR1* plants exhibited improved control over drought-induced ROS accumulation compared to WT. Further findings of this study indicate that exogenous jasmonate application suppresses methyl viologen (MeV)-induced H₂O₂ production in WT but not in *jar1-11*, likely due to the mutant's inability to convert JA into its bioactive form, JA-Ile. Moreover, genes involved in the ascorbate-glutathione (GSH) cycle, such as *DHAR1* (*DEHYDROASCORBATE-REDUCTASE-1*) and *GRI* (*GLUTATHIONE-REDUCTASE-1*), were up-regulated in WT under drought conditions, facilitating the interconversion of GSH and its oxidized form GSSG, a crucial process for maintaining redox homeostasis. Notably, under control conditions, 35S::*JAR1* plants did not show significant alterations in ascorbate-GSH cycle gene expression despite their elevated JA-Ile levels. However, under drought stress, *DHAR1* and *GRI/2* expression patterns diverged between *jar1-11* and 35S::*JAR1*, suggesting that rather than broadly activating the ascorbate-GSH cycle, JA-Ile may fine-tune its activity in response to drought stress, contributing to a more efficient redox balance.

In conclusion, this study demonstrates that modifying JA homeostasis enhances drought resistance in *Arabidopsis* by influencing key morphological and physiological traits. Elevated JA-Ile levels contribute to both priming and direct stress responses, reinforcing the enhanced drought resilience observed in 35S::*JAR1* plants. While targeting JA homeostasis presents a promising strategy for improving plant drought tolerance, potential trade-offs such as reduced growth and altered life cycle duration under optimal conditions should be carefully evaluated to maximize agricultural benefits without compromising overall plant fitness.

3.4 Publication 4

Loss-of-function of the drought-induced genes *GASA3* and *AFPI* confers enhanced drought tolerance in *Arabidopsis thaliana*

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bioRxiv (2025), <https://doi.org/10.1101/2025.04.03.647048>.

The study " Loss-of-function of the drought-induced genes *GASA3* and *AFPI* confers enhanced drought tolerance in *Arabidopsis thaliana* " is available as a preprint on the bioRxiv server, with me as the first author.

In this research, I identified *GASA3* and *AFPI* as highly drought-responsive genes and parts of potential new drought regulon. I contributed to the conceptual development of the study, selected single mutant lines, generated constitutive expression lines, and performed crossing experiments to create double mutants. And together with students supervised by me, I performed all phenotypic analyses. This project involved a collaboration with Dr. Katharina Gütbrod and Prof. Peter Dörmann from IMBIO, University of Bonn, utilizing a HPLC-ESI-MS platform to analyze ABA levels. I was responsible for plant samples preparation for these analyses. Furthermore, I performed RT-qPCR experiments and contributed to writing, editing, and data visualization.

The GASA (GIBERELLIC-ACID-STIMULATED (GAST)-HOMOLOG) family in *Arabidopsis* shares structural similarities with the original GAST proteins found in tomato (*Solanum lycopersicum*), which are defined by a consensus region at the C-terminus containing cysteine residues. Since their discovery, these proteins have been implicated in various aspects of plant development. For example, *AtGASA4* has been found to enhance heat stress tolerance, while *AtGASA5* negatively affects thermotolerance by making plants more susceptible to heat stress. Additionally, *AtGASA14* plays a positive role in salt stress tolerance by reducing reactive oxygen species (ROS) accumulation. However, *AtGASA3* has only been linked to increased transcript levels in seeds and has yet to be fully characterized in terms of its role in

abiotic stress, particularly drought. On the other hand, the AFP (ABI5-BINDING-PROTEIN) family consists of proteins that interact with the bZIP transcription factor ABI5 (ABSCISIC-ACID-INSENSITIVE 5), promoting its degradation and thereby negatively regulating ABA signalling. In Arabidopsis, four functional AFPs have been characterized. Except for AFP4, the other AFPs are known to inhibit the expression of ABA-regulated genes.

In the study shown in 5.3. involving transcriptomic analysis of WT plants subjected to 14 days of drought stress, *GASA3* and *AFPI* were found to be strongly up-regulated. The roles of these genes were here further investigated in the context of drought stress responses. Our findings suggest that the expression of these genes is linked to drought susceptibility in Arabidopsis. Firstly, a drought-responsive increase in *GASA3* and *AFPI* transcript levels could be reported from RT-qPCR analyses, confirming the RNA-Seq data from Mahmud et al. 2022. Furthermore, both *GASA3* and *AFPI* were confirmed as ABA-responsive genes, as their expression was highly induced by exogenous ABA treatment. This result could be further confirmed using the *aba2* mutant, which is impaired in ABA biosynthesis. In this mutant, neither *GASA3* nor *AFPI* showed significant induction under drought stress, showing that their expression depends on endogenous ABA availability. Notably, neither gene was induced by other hormones such as GA or JA, confirming that despite its name *GASA3* is not responsive to GA.

Phenotypic analyses of loss-of-function mutants (*gasa3* and *afp1*, *gasa3afp1*) and overexpression lines (*35S::GASA3* and *35S::AFPI*) showed that both genes act as negative regulators of drought tolerance in Arabidopsis. The mutants *gasa3* and *afp1* were more resilient to water with-holding whereas the constitutive overexpression lines showed earlier symptoms of wilting than the WT. The double mutants exhibited even greater tolerance than the single mutants, suggestion that the negative impact on drought tolerance of *AFPI* and *GASA2* to a certain degree is additive. The increased drought tolerance may be attributed to a smaller stomatal aperture, which reduces water loss through transpiration. This is supported by increase in the transcript of *SLAC1* (*SLOW-ANION-CHANNEL-1*), an anion channel participating in stomatal closure. It was also found that *gasa3* and *afp1* mutants exhibited significantly higher ABA accumulation under drought stress compared to WT plants. However, analysis of ABA biosynthesis genes like *ZEP* (*ZEAXANTHIN-EPOXIDASE*) and *ABA2* (*ABSCISIC ACID 2*) revealed that their expression was actually higher in WT plants under drought than in *gasa3* and *afp1* mutants. Instead, the mutants displayed increased expression of *BG2* (*BETA-GLUCOSIDASE-2*) under drought conditions, suggesting that ABA accumulation in these mutants results from the release of conjugated ABA-GE form stored in the vacuole through BG2 rather than *de-novo* biosynthesis. With the increased ABA levels, *gasa3* and *afp1* plants showed up-regulation of prominent ABA-responsive genes such as *ABF2* and *ABF3* (*ABA-RESPONSE-ELEMENT-BINDING-FACTOR-2 and 3*), which are key regulators of ABA-induced transcriptional networks. Furthermore, *RD29A* (*RESPONSE-TO-DESSICATION-29A*), a key component of ABA-mediated drought responses and *ABI5*, an important ABA signalling regulator, were significantly up-regulated in *gasa3* and *afp1* mutants under drought. Together these results suggest an enhanced ABA response, facilitating better adaptation to water deficit conditions.

Interestingly, *GASA3* induction was significantly reduced in the *afp1* mutant under drought, whereas *AFPI* expression remained unchanged in the *gasa3* mutant. This indicates that *GASA3* expression is dependent on *AFPI*, suggesting that *AFPI* may modulate drought responses by positively regulating *GASA3*. Despite higher *AFPI* expression in *gasa3* mutants under drought conditions, these mutants still displayed drought resilience, suggesting that *GASA3* is the primary effector of drought susceptibility, while *AFPI* plays a role by amplifying the expression of *GASA3* transcripts under drought stress. Taken together, our results identified a novel regulatory pathway involving *GASA3* and *AFPI* that negatively affect the drought tolerance in *Arabidopsis*.

4. Summary

The investigations considered different aspects of the molecular responses to environmental stress. In barley, the study focused on the transcriptomic effects of H₂O₂ and the interlink with Ca²⁺ signalling. In Arabidopsis, the research elucidated the role of a novel ABA-related regulon, comprising *GASA3* and *AFPI*, in the drought susceptibility.

4.1. Oxidative stress and Ca²⁺ signalling in *Hordeum vulgare*

In plants, H₂O₂ serves a dual purpose, acting both as a detrimental by-product of cellular metabolism and as a crucial secondary messenger affecting growth and development of plants (Hossain et al., 2015; Niu and Liao, 2016). Its interaction with various signalling molecules, including Ca²⁺ ions and hormones, plays a fundamental role in regulating diverse biological functions, particularly in response to biotic and abiotic stressors (Gilroy et al., 2014). Despite its significance, limited information is available regarding H₂O₂-induced transcriptomic changes in barley. The study shown in appendix 1 employed next-generation sequencing to analyse the barley transcriptome's response to H₂O₂. The RNA-Seq analysis revealed that under these conditions, H₂O₂ induced more pronounced transcriptional changes in roots than in leaves. Most DEGs were unique to the two tissues, highlighting tissue-specific responses. Only about 10% of DEGs were commonly up-regulated or down-regulated in roots and leaves with some showing opposing regulatory patterns. While differences in H₂O₂ penetration between organs might contribute to these differences, it is likely that each tissue exhibits a distinct response to H₂O₂ signalling and oxidative stress. The differential response is also reflected in the GO terms associated with the identified DEGs, which demonstrated minimal overlap, where we see in leaf, mostly top GO terms associated with abiotic stresses and signal transduction, whereas the root exhibited a much higher prevalence of ROS detoxification and oxidative stress terms. Since photosynthesis naturally produces a substantial amount of ROS, leaf tissues likely maintain a more robust, constitutive detoxification system. In contrast, roots may require the induction of such systems in response to H₂O₂ accumulation, which likely explains it's necessity to have relatively higher amount of differentially expressed transcriptional machinery concerning oxidative stress, as compared to the leaves.

This idea is supported by the observation that numerous genes associated with oxidative stress and detoxification are up-regulated in roots following H₂O₂ exposure. The findings of this study indicate that H₂O₂ significantly influences the expression of numerous genes within the barley genome. These results provide novel insights into H₂O₂'s role in modifying cellular activities in barley, though the mechanisms coordinating these genetic responses to stress-induced H₂O₂ accumulation require further investigation. Many identified genes have previously been linked to stress responses in barley or through their orthologs in Arabidopsis and other crops, suggesting a conserved mechanism among plant species in managing elevated H₂O₂ levels, whether as a stress-induced by-product or as a signalling molecule. However, some genes identified in this study had not been previously associated with stress responses.

Given the central role of ROS, particularly H_2O_2 , in cellular signalling, development, and stress adaptation, understanding its impact on gene expression offers valuable insights into oxidative stress responses in barley. The H_2O_2 induced DEGs from this study were therefore used in a subsequent study that looked more deeply into the crosstalk between H_2O_2 and Ca^{2+} signalling in plants.

In order to understand the contribution of Ca^{2+} signalling in the H_2O_2 response of barley, another RNA-Seq was performed using the same experimental set-up but the H_2O_2 induced cytosolic Ca^{2+} transient was blocked with La^{3+} (appendix 2). Based on a comparison between the two data sets, it could be determined that 30% and 70% of the total H_2O_2 -responsive genes in barley leaves and roots, respectively were found to be dependent on the H_2O_2 induced Ca^{2+} transient. In the leaves, the majority of Ca^{2+} -dependent H_2O_2 -responsive genes, showed a strict Ca^{2+} dependency, suggesting that Ca^{2+} signalling downstream of H_2O_2 is absolutely required to either activate or repress gene expression. A much smaller set of DEGs shows partial Ca^{2+} dependency, indicating that H_2O_2 and Ca^{2+} signals influence gene expression in an additive manner. Here, gene activation or repression by H_2O_2 does not rely entirely on H_2O_2 -induced Ca^{2+} transients, but Ca^{2+} serves to amplify the response. Intriguingly, leaf was the only tissue where such a Ca^{2+} -dependent attenuation of the H_2O_2 induced differential expression could be observed. By contrast, around 60 % of the H_2O_2 -responsive genes show a strict dependency in roots and all the remaining DEGs were counter-regulated. This means, the H_2O_2 -induced Ca^{2+} transient counteracts the effect of H_2O_2 on gene expression, inhibiting activation or repression while simultaneously triggering an opposite response. Such counter-regulation occurred only in seven genes in leaves. Overall, these results further substantiate the strong differences in the stress response observed between roots and leaves. To further explore the molecular pathways underpinning the Ca^{2+} -dependency in H_2O_2 -induced transcriptional responses, we modelled potential interactions between previously established Ca^{2+} -signalling components and the identified Ca^{2+} -dependent H_2O_2 -responsive genes using SKM. This merged network revealed key regulatory hubs linking multiple known Ca^{2+} -signalling components to various target genes in both leaves and roots. The most prominent hubs, shared between both tissues, were defined by only four TFs, namely AGL15, HY5, PIF4, and EIN3.

AGL15 is a member of the MADS-box family of TFs, which play crucial roles in various aspects of plant development, particularly during embryogenesis and seed development (Joshi et al., 2022). In addition to its developmental roles, AGL15 has emerged as a potential modulator of plant responses to abiotic stress (Guo et al., 2016). Recent studies indicate that it interacts with CaM (Popescu et al., 2007), suggesting a role in Ca^{2+} -mediated signalling pathways. Moreover, AGL15 has been implicated in hormonal cross-talk, particularly in the regulation of auxin and ethylene pathways (Joshi et al., 2022), which are crucial for plant adaptation to stress conditions. These findings match with our model, where indeed it was shown to interact with CaMs and related proteins like CML10.

HY5 is a bZIP-type TF recognized as a master regulator of photomorphogenesis in plants (Lee et al., 2021). By directly binding to light-responsive elements in the promoters of its target genes, HY5

controls the expression of genes involved in chlorophyll biosynthesis, photosynthesis, and seedling development (Xiao et al., 2022). It has been shown to modulate the homeostasis of ROS, aiding in tolerance to oxidative stress (Li et al., 2024). Additionally, HY5 interacts with other components of Ca^{2+} signalling, to modulate Ca^{2+} -dependent photomorphogenesis (Abbas et al., 2014). This interaction demonstrates HY5's capability as a signalling hub that integrates diverse environmental inputs. Our model reveals interactions between HY5 and CAM7 along with kinases such as CDPK7 (and MPK7 (MAPK-TYPE-PROTEIN-KINASE-7), suggesting post-translational regulation that adds complexity to its activity under stress.

PIF4 is a critical basic bHLH TF that plays a central role in integrating light and temperature signals to drive plant growth and development (Xu and Zhu, 2021). Positioned downstream of phytochrome B, PIF4 is essential for processes such as shade avoidance, thermomorphogenesis, and the elongation of hypocotyls (Lee et al., 2021). Emerging evidence indicates that PIF4 expression is modulated by ROS signalling during salt and drought stress, although the precise regulatory mechanisms are yet to be fully elucidated (Liu et al., 2022). Our innovative model highlights the upstream regulation of PIF4 by CAM5 and CPK1. This revelation suggests new and powerful layers of regulation that link Ca^{2+} signalling directly to growth control under stress conditions. Furthermore, CAM5 and CPK1 are closely associated with RGL2 (RGA-LIKE-2), a DELLA protein intricately involved in ROS generation and hormonal regulation, bolstering PIF4's role in facilitating stress adaptation (Stamm et al., 2012). Although PIF4 has not been viewed as a stress-responsive TF, its regulatory versatility and our recent findings supporting its involvement in oxidative signalling reveal broader functional roles.

EIN3 is a central TF in the ethylene signalling pathway and plays a crucial role in integrating developmental processes and environmental stress responses in plants (Dolgikh et al., 2019). Upon the perception of ethylene, EIN3 accumulates in the nucleus and activates a wide range of ethylene-responsive genes by binding to EIN3-binding sites (EBS) in their promoters. These target genes are involved in various processes, including fruit ripening, leaf senescence, root elongation, and pathogen defense. It also interacts with other signalling molecules, such as Ca^{2+} and ROS, particularly H_2O_2 , which can influence its transcriptional activity (Ludwig et al., 2005). In Arabidopsis, ethylene signalling involving EIN3 has been linked to H_2O_2 - Ca^{2+} signalling during salt stress (Lang et al., 2020), indicating a cross-regulation between ethylene and second messenger pathways. Our model suggests that this regulation may involve the CaM-binding protein IQD6 (IQ67 DOMAIN CONTAINING PROTEIN 6), which is recognized for its role in development and stress adaptation (Abel et al., 2005; Kumari et al., 2021).

We also found regulatory genes involved in phytohormone pathways - including ethylene, JA, ABA, SA, brassinosteroids, GA, and auxins as well as other signalling cascades - to be targets of the aforementioned TF hubs. This further reinforces the picture of large, intricate signaling networks that define the appropriate responses to environmental stress. Numerous studies have shown that both biotic and abiotic stress can trigger H_2O_2 accumulation and fluctuations in Ca^{2+} levels, therefore, these TFs

represent promising targets for further research in barley and other crops to elucidate the molecular mechanisms underlying H₂O₂-associated Ca²⁺ signal transduction. The knowledge gained through our study may contribute to improving stress resistance in barley and other crops, optimizing productivity under current and future climatic conditions.

4.2. Drought stress regulation in *Arabidopsis thaliana* by *GASA3* and *AFPI*

Drought is one of the most critical environmental stressors affecting global agricultural productivity, causing significant yield losses and threatening food security (Farooq et al., 2009). Plants have evolved diverse biochemical and molecular mechanisms to mitigate the adverse effects of drought stress, ensuring survival under water-deficient conditions. We identified two genes, *GASA3* and *AFPI* and characterized them as drought-induced genes participating in a pathway that appear to ultimately enhance drought susceptibility (appendix 4). Phenotypic characterization using loss-of-function mutants and overexpression lines demonstrated that both genes act as negative regulators of drought tolerance in *Arabidopsis*. Single-gene mutants for *GASA3* and *AFPI* exhibited enhanced drought tolerance, whereas overexpression lines displayed increased drought susceptibility. Interestingly, the *gasa3afp1* double mutants exhibited even greater drought tolerance than the single mutants suggesting the presence of an additive effect between the expression of these two genes. Further analyses also demonstrated that *GASA3* and *AFPI* are ABA-responsive genes, which themselves then attenuate the ABA-response by enhancing negative feed-back loops.

This study contributes to a deeper understanding of ABA-regulated drought tolerance by highlighting an alternative regulatory mechanism in *gasa3* and *afp1* mutants. While ABA is a well-established hormone involved in drought responses primarily through regulation of stomatal closure and water conservation (Bharath et al., 2021), our findings reveal that increased ABA levels in these mutants do not result from enhanced *de-novo* biosynthesis. Instead, ABA accumulation appears to be driven by the hydrolysis of vacuole-stored ABA-GE, as supported by the strong up-regulation of *BG2* under drought conditions. This shift from biosynthesis to mobilization reflects a potentially more rapid and energetically favourable strategy for ABA availability in response to stress.

In addition to elevated ABA levels, *gasa3* and *afp1* mutants exhibit a pronounced induction of core ABA-responsive genes indicating a more efficient activation of downstream signalling pathways. These results suggest that the mutants not only accumulate more ABA but also exhibit heightened sensitivity to it, leading to more robust drought adaptation. Furthermore, these observations align with current models of ABA signalling (see Figure 7 of Appendix 4), in which ABA-triggered phosphorylation of SnRK2 promotes activation of ABRE-binding factors and downstream effectors such as SLAC1, thereby facilitating stomatal closure. Interestingly, the loss of *AFPI* and *GASA3* also seems to reduce the ABA-dependent activation of PP2CA (PROTEIN-PHOSPHATASE-2C-A), thereby minimizing negative feedback on SnRK2 and further enhancing ABA signal propagation. Taken together, these findings suggest that *gasa3* and *afp1* mutants provide a valuable system to study non-canonical ABA

regulation. Future work should aim to identify the upstream as well as downstream regulators of these two genes and explore their roles within the broader hormonal and stress response networks in plants. Our data furthermore suggests that *AFPI* positively regulates *GASA3* expression, positioning *GASA3* as the primary effector of drought susceptibility, with *AFPI* playing a modulatory role by enhancing *GASA3* expression. Despite this regulatory relationship, the drought resilience of *gasa3* mutants, even with elevated *AFPI* expression highlights the dominant role of *GASA3* in mediating drought susceptibility. AFPs act as negative regulators of ABA signalling by suppressing the activity of the ABI5, a bZIP TF (Vittozzi et al., 2024). In support of this role, *AFPI* and its close homolog AFP2 have been shown to inhibit bZIP-mediated activation of specific ABA-responsive (ABRE-containing) genes (Lynch et al., 2022). *AFPI* can bind to phosphorylated ABI5, targeting it for proteasomal degradation (Vittozzi et al., 2024). Our results indicate an added level of regulation in the ABA signalling pathway, highlighting *GASA3* as a possible transcriptional target of *AFPI*, which leads to a reduction in ABA signalling and thus increases susceptibility to drought. However, the role of ABI5, being a degradation target of *AFPI*, in regulating *GASA3* expression still remains unclear, and therefore will need subsequent experimental validations.

Importantly, we show this *AFPI*–*GASA3* regulatory relationship specifically in leaf tissue, marking a significant shift from earlier research that mainly concentrated on function of *AFPI* in seeds and young seedlings. This tissue-specific aspect of the role of *AFPI* is a new dimension of its function and broadens its recognized regulatory profile. Going forward, additional studies into the spatial dynamics of *AFPI* and *GASA3* signalling, especially in vegetative tissues, will be essential. Such research could provide greater understanding of the molecular interactions between them and clarify how this regulatory module affects ABA signalling and drought response at various developmental stages and across different tissues. Future research should also focus on elucidating the precise molecular interactions between *GASA3*, *AFPI*, and ABA-responsive elements to better understand the regulatory networks governing drought stress responses. Additionally, exploring the roles of these genes in other plant species, particularly staple crops, could provide broader insights into their conservation and functionality in different agricultural contexts. By expanding our understanding of drought-responsive pathways, this study contributes to the long-term goal of developing climate-adaptive crop varieties capable of sustaining productivity under increasingly unpredictable environmental conditions.

5. References

- Abbas, N., Maurya, J. P., Senapati, D., Gangappa, S. N., and Chattopadhyay, S. (2014). Arabidopsis CAM7 and HY5 Physically Interact and Directly Bind to the HY5 Promoter to Regulate Its Expression and Thereby Promote Photomorphogenesis. *The Plant Cell* 26, 1036–1052. doi: 10.1105/tpc.113.122515
- Abel, S., Savchenko, T., and Levy, M. (2005). Genome-wide comparative analysis of the IQD gene families in Arabidopsis thaliana and Oryza sativa. *BMC Evolutionary Biology* 5, 72. doi: 10.1186/1471-2148-5-72
- Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., et al. (2006). Integration of Plant Responses to Environmentally Activated Phytohormonal Signals. *Science* 311, 91–94. doi: 10.1126/science.1118642
- Achard, P., Renou, J.-P., Berthomé, R., Harberd, N. P., and Genschik, P. (2008). Plant DELLAs Restrict Growth and Promote Survival of Adversity by Reducing the Levels of Reactive Oxygen Species. *Current Biology* 18, 656–660. doi: 10.1016/j.cub.2008.04.034
- Aghaie, P., and Tafreshi, S. A. H. (2020). Central role of 70-kDa heat shock protein in adaptation of plants to drought stress. *Cell Stress and Chaperones* 25, 1071–1081. doi: 10.1007/s12192-020-01144-7
- Agurla, S., Gahir, S., Munemasa, S., Murata, Y., and Raghavendra, A. S. (2018). “Mechanism of Stomatal Closure in Plants Exposed to Drought and Cold Stress,” in *Survival Strategies in Extreme Cold and Desiccation: Adaptation Mechanisms and Their Applications*, eds. M. Iwaya-Inoue, M. Sakurai, and M. Uemura (Singapore: Springer Singapore), 215–232. doi: 10.1007/978-981-13-1244-1_12
- Akhtar, M., Jaiswal, A., Taj, G., Jaiswal, J. P., Qureshi, M. I., and Singh, N. K. (2012). DREB1/CBF transcription factors: their structure, function and role in abiotic stress tolerance in plants. *Journal of Genetics* 91, 385–395. doi: 10.1007/s12041-012-0201-3
- Alexandersson, E., Frayse, L., Sjövall-Larsen, S., Gustavsson, S., Fellert, M., Karlsson, M., et al. (2005). Whole Gene Family Expression and Drought Stress Regulation of Aquaporins. *Plant Molecular Biology* 59, 469–484. doi: 10.1007/s11103-005-0352-1
- Ali, A., Pardo, J. M., and Yun, D.-J. (2020). Desensitization of ABA-Signaling: The Swing From Activation to Degradation. *Frontiers in Plant Science* 11. Available at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.00379>
- Ali, F., Qanmber, G., Li, F., and Wang, Z. (2022). Updated role of ABA in seed maturation, dormancy, and germination. *Journal of Advanced Research* 35, 199–214. doi: 10.1016/j.jare.2021.03.011
- Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., et al. (2004). Antagonistic Interaction between Abscissic Acid and Jasmonate-Ethylene Signaling Pathways Modulates Defense Gene Expression and Disease Resistance in Arabidopsis. *The Plant Cell* 16, 3460–3479. doi: 10.1105/tpc.104.025833
- Araújo, W. L., Fernie, A., Alisdair R., and Nunes-Nesi, A. (2011). Control of stomatal aperture. *Plant Signaling & Behavior* 6, 1305–1311. doi: 10.4161/psb.6.9.16425
- Battaglia, M., Olvera-Carrillo, Y., Garcarrubio, A., Campos, F., and Covarrubias, A. A. (2008). The Enigmatic LEA Proteins and Other Hydrophilins. *Plant Physiology* 148, 6–24. doi: 10.1104/pp.108.120725
- Baxter, A., Mittler, R., and Suzuki, N. (2014). ROS as key players in plant stress signalling. *Journal of Experimental Botany* 65, 1229–1240. doi: 10.1093/jxb/ert375
- Bharath, P., Gahir, S., and Raghavendra, A. S. (2021). Abscissic Acid-Induced Stomatal Closure: An Important Component of Plant Defense Against Abiotic and Biotic Stress. *Frontiers in Plant Science* 12. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2021.615114>
- Bibikova, T. N., Zhigilei, A., and Gilroy, S. (1997). Root hair growth in Arabidopsis thaliana is directed by calcium and an endogenous polarity. *Planta* 203, 495–505. doi: 10.1007/s004250050219
- Boudsocq, M., and Sheen, J. (2013). CDPKs in immune and stress signaling. *Trends in Plant Science* 18, 30–40. doi: 10.1016/j.tplants.2012.08.008

- Bouteraa, M. T., Ben Romdhane, W., Baazaoui, N., Alfaifi, M. Y., Chouaibi, Y., Ben Akacha, B., et al. (2023). GASA Proteins: Review of Their Functions in Plant Environmental Stress Tolerance. *Plants* 12. doi: 10.3390/plants12102045
- Chen, M., Zhang, T.-L., Hu, C.-G., and Zhang, J.-Z. (2023). The Role of Drought and Temperature Stress in the Regulation of Flowering Time in Annuals and Perennials. *Agronomy* 13. doi: 10.3390/agronomy13123034
- Cirillo, V., D'Amelia, V., Esposito, M., Amitrano, C., Carillo, P., Carputo, D., et al. (2021). Anthocyanins Are Key Regulators of Drought Stress Tolerance in Tobacco. *Biology* 10. doi: 10.3390/biology10020139
- Colebrook, E. H., Thomas, S. G., Phillips, A. L., and Hedden, P. (2014). The role of gibberellin signalling in plant responses to abiotic stress. *Journal of Experimental Biology* 217, 67–75. doi: 10.1242/jeb.089938
- Costa, A., Resentini, F., Buratti, S., and Bonza, M. C. (2023). Plant Ca²⁺-ATPases: From biochemistry to signalling. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1870, 119508. doi: 10.1016/j.bbamcr.2023.119508
- Dabravolski, S. A., and Isayenkov, S. V. (2023). The Role of Anthocyanins in Plant Tolerance to Drought and Salt Stresses. *Plants* 12. doi: 10.3390/plants12132558
- Daszkowska-Golec, A., and Szarejko, I. (2013). Open or Close the Gate – Stomata Action Under the Control of Phytohormones in Drought Stress Conditions. *Frontiers in Plant Science* 4. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2013.00138>
- Demidchik, V., Shabala, S., Isayenkov, S., Cuin, T. A., and Pottosin, I. (2018). Calcium transport across plant membranes: mechanisms and functions. *New Phytologist* 220, 49–69. doi: 10.1111/nph.15266
- Desikan, R., A.-H.-Mackerness, S., Hancock, J. T., and Neill, S. J. (2001). Regulation of the Arabidopsis Transcriptome by Oxidative Stress. *Plant Physiology* 127, 159–172. doi: 10.1104/pp.127.1.159
- Dietrich, P., Sanders, D., and Hedrich, R. (2001). The role of ion channels in light-dependent stomatal opening. *Journal of Experimental Botany* 52, 1959–1967. doi: 10.1093/jexbot/52.363.1959
- Ding, Y., and Yang, S. (2022). Surviving and thriving: How plants perceive and respond to temperature stress. *Developmental Cell* 57, 947–958. doi: 10.1016/j.devcel.2022.03.010
- Dolgikh, V. A., Pukhovaya, E. M., and Zemlyanskaya, E. V. (2019). Shaping Ethylene Response: The Role of EIN3/EIL1 Transcription Factors. *Frontiers in Plant Science* 10. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2019.01030>
- Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C.-P., et al. (2013). Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proceedings of the National Academy of Sciences* 110, 8744–8749. doi: 10.1073/pnas.1221294110
- Dumanović, J., Nepovimova, E., Natić, M., Kuća, K., and Jaćević, V. (2021). The Significance of Reactive Oxygen Species and Antioxidant Defense System in Plants: A Concise Overview. *Frontiers in Plant Science* 11. Available at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.552969>
- Edel, K. H., Marchadier, E., Brownlee, C., Kudla, J., and Hetherington, A. M. (2017). The Evolution of Calcium-Based Signalling in Plants. *Current Biology* 27, R667–R679. doi: 10.1016/j.cub.2017.05.020
- Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., and Basra, S. M. A. (2009). Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable Development* 29, 185–212. doi: 10.1051/agro:2008021
- Fichman, Y., Zandalinas, S. I., Peck, S., Luan, S., and Mittler, R. (2022). HPCA1 is required for systemic reactive oxygen species and calcium cell-to-cell signaling and plant acclimation to stress. *The Plant Cell* 34, 4453–4471. doi: 10.1093/plcell/koac241
- Fidler, J., Graska, J., Gietler, M., Nykiel, M., Prabucka, B., Rybarczyk-Płońska, A., et al. (2022). PYR/PYL/RCAR Receptors Play a Vital Role in the Absciscic-Acid-Dependent Responses of Plants to External or Internal Stimuli. *Cells* 11. doi: 10.3390/cells11081352

- Foyer, C. H., and Noctor, G. (2003). Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum* 119, 355–364. doi: 10.1034/j.1399-3054.2003.00223.x
- Garcia, M. E., Lynch, T., Peeters, J., Snowden, C., and Finkelstein, R. (2008). A small plant-specific protein family of ABI five binding proteins (AFPs) regulates stress response in germinating Arabidopsis seeds and seedlings. *Plant Molecular Biology* 67, 643–658. doi: 10.1007/s11103-008-9344-2
- Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48, 909–930. doi: 10.1016/j.plaphy.2010.08.016
- Gilroy, S., Suzuki, N., Miller, G., Choi, W.-G., Toyota, M., Devireddy, A. R., et al. (2014). A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. *Trends in Plant Science* 19, 623–630. doi: 10.1016/j.tplants.2014.06.013
- Giridhar, M., Meier, B., Imani, J., Kogel, K.-H., Peiter, E., Vothknecht, U. C., et al. (2022). Comparative analysis of stress-induced calcium signals in the crop species barley and the model plant Arabidopsis thaliana. *BMC Plant Biology* 22, 447. doi: 10.1186/s12870-022-03820-5
- González-Villagra, J., Cohen, J. D., and Reyes-Díaz, M. M. (2019). Absciscic acid is involved in phenolic compounds biosynthesis, mainly anthocyanins, in leaves of Aristotelia chilensis plants (Mol.) subjected to drought stress. *Physiologia Plantarum* 165, 855–866. doi: 10.1111/ppl.12789
- Guo, X., Chen, G., Cui, B., Gao, Q., Guo, J.-E., Li, A., et al. (2016). Solanum lycopersicum agamous-like MADS-box protein AGL15-like gene, SIMBP11, confers salt stress tolerance. *Molecular Breeding* 36, 125. doi: 10.1007/s11032-016-0544-1
- Gupta, R., and Chakrabarty, S. K. (2013). Gibberellic acid in plant. *Plant Signaling & Behavior* 8, e25504. doi: 10.4161/psb.25504
- Haghpanah, M., Hashemipetroudi, S., Arzani, A., and Araniti, F. (2024). Drought Tolerance in Plants: Physiological and Molecular Responses. *Plants* 13. doi: 10.3390/plants13212962
- Harding, S. A., Oh, S., and Roberts, D. M. (1997). Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species. *The EMBO Journal* 16, 1137–1144. doi: 10.1093/emboj/16.6.1137
- Hasan, H., Uzma, Gul, A., Amir, R., Ali, M., Kubra, G., et al. (2020). “Chapter 13 - Role of osmoprotectants and drought tolerance in wheat,” in *Climate Change and Food Security with Emphasis on Wheat*, eds. M. Ozturk and A. Gul (Academic Press), 207–216. doi: 10.1016/B978-0-12-819527-7.00013-3
- Hasanuzzaman, M., Bhuyan, M. H. M. B., Anee, T. I., Parvin, K., Nahar, K., Mahmud, J. A., et al. (2019). Regulation of Ascorbate-Glutathione Pathway in Mitigating Oxidative Damage in Plants under Abiotic Stress. *Antioxidants* 8. doi: 10.3390/antiox8090384
- Hepler, P. K. (2005). Calcium: A Central Regulator of Plant Growth and Development. *The Plant Cell* 17, 2142–2155. doi: 10.1105/tpc.105.032508
- Hetherington, A. M., and Brownlee, C. (2004). THE GENERATION OF Ca²⁺ SIGNALS IN PLANTS. *Annu. Rev. Plant Biol.* 55, 401–427. doi: 10.1146/annurev.arplant.55.031903.141624
- Hossain, M. A., Bhattacharjee, S., Armin, S.-M., Qian, P., Xin, W., Li, H.-Y., et al. (2015). Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. *Frontiers in Plant Science* 6. Available at: <https://www.frontiersin.org/articles/10.3389/fpls.2015.00420>
- Hu, C., Wu, S., Li, J., Dong, H., Zhu, C., Sun, T., et al. (2022). Herbivore-induced Ca²⁺ signals trigger a jasmonate burst by activating ERF16-mediated expression in tomato. *New Phytologist* 236, 1796–1808. doi: 10.1111/nph.18455
- Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., et al. (2006). Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proceedings of the National Academy of Sciences* 103, 12987–12992. doi: 10.1073/pnas.0604882103

- Huang, Q., Wang, Y., Li, B., Chang, J., Chen, M., Li, K., et al. (2015). TaNAC29, a NAC transcription factor from wheat, enhances salt and drought tolerance in transgenic *Arabidopsis*. *BMC Plant Biology* 15, 268. doi: 10.1186/s12870-015-0644-9
- Ikotun, A. M., Ezugwu, A. E., Abualigah, L., Abuhaija, B., and Heming, J. (2023). K-means clustering algorithms: A comprehensive review, variants analysis, and advances in the era of big data. *Information Sciences* 622, 178–210. doi: 10.1016/j.ins.2022.11.139
- Ingram, J., and Bartels, D. (1996). THE MOLECULAR BASIS OF DEHYDRATION TOLERANCE IN PLANTS. *Annual Review of Plant Biology* 47, 377–403. doi: <https://doi.org/10.1146/annurev.arplant.47.1.377>
- Iwano, M., Ito, K., Fujii, S., Kakita, M., Asano-Shimosato, H., Igarashi, M., et al. (2015). Calcium signalling mediates self-incompatibility response in the Brassicaceae. *Nature Plants* 1, 15128. doi: 10.1038/nplants.2015.128
- Joshi, S., Paul, P., Hartman, J. M., and Perry, S. E. (2022). AGL15 Promotion of Somatic Embryogenesis: Role and Molecular Mechanism. *Frontiers in Plant Science* 13. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2022.861556>
- Kapilan, R., Vaziri, M., and Zwiazek, J. J. (2018). Regulation of aquaporins in plants under stress. *Biological Research* 51, 4. doi: 10.1186/s40659-018-0152-0
- Kaur, S., Tiwari, V., Kumari, A., Chaudhary, E., Sharma, A., Ali, U., et al. (2023). Protective and defensive role of anthocyanins under plant abiotic and biotic stresses: An emerging application in sustainable agriculture. *Journal of Biotechnology* 361, 12–29. doi: 10.1016/j.jbiotec.2022.11.009
- Kazan, K., and Lyons, R. (2016). The link between flowering time and stress tolerance. *Journal of Experimental Botany* 67, 47–60. doi: 10.1093/jxb/erv441
- Kazan, K., and Manners, J. M. (2013). MYC2: The Master in Action. *Molecular Plant* 6, 686–703. doi: 10.1093/mp/sss128
- Khalvandi, M., Siosemardeh, A., Roohi, E., and Keramati, S. (2021). Salicylic acid alleviated the effect of drought stress on photosynthetic characteristics and leaf protein pattern in winter wheat. *Heliyon* 7, e05908. doi: 10.1016/j.heliyon.2021.e05908
- Khan, M. I. R., Fatma, M., Per, T. S., Anjum, N. A., and Khan, N. A. (2015). Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Frontiers in Plant Science* 6. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2015.00462>
- Khoo, H. E., Azlan, A., Tang, S. T., and Lim, S. M. (2017). Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *fnr* 61. Available at: <https://foodandnutritionresearch.net/index.php/fnr/article/view/1257> (Accessed March 23, 2025).
- Khoso, M. A., Hussain, A., Ritonga, F. N., Ali, Q., Channa, M. M., Alshegaihi, R. M., et al. (2022). WRKY transcription factors (TFs): Molecular switches to regulate drought, temperature, and salinity stresses in plants. *Frontiers in Plant Science* 13. Available at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.1039329>
- Kim, S.-G., Lee, A.-K., Yoon, H.-K., and Park, C.-M. (2008). A membrane-bound NAC transcription factor NTL8 regulates gibberellic acid-mediated salt signaling in *Arabidopsis* seed germination. *The Plant Journal* 55, 77–88. doi: 10.1111/j.1365-313X.2008.03493.x
- Kim, T.-H., Böhmer, M., Hu, H., Nishimura, N., and Schroeder, J. I. (2010). Guard Cell Signal Transduction Network: Advances in Understanding Abscissic Acid, CO₂, and Ca²⁺ Signaling. *Annual Review of Plant Biology* 61, 561–591. doi: <https://doi.org/10.1146/annurev-arplant-042809-112226>
- Knight, H., Brandt, S., and Knight, M. R. (1998). A history of stress alters drought calcium signalling pathways in. *The Plant Journal* 16, 681–687. doi: 10.1046/j.1365-313x.1998.00332.x
- Knight, H., Trewavas, A. J., and Knight, M. R. (1997). Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *The Plant Journal* 12, 1067–1078. doi: 10.1046/j.1365-313X.1997.12051067.x
- Kobayashi, F., Maeta, E., Terashima, A., Kawaura, K., Ogihara, Y., and Takumi, S. (2008). Development of abiotic stress tolerance via bZIP-type transcription factor LIP19 in common wheat. *Journal of Experimental Botany* 59, 891–905. doi: 10.1093/jxb/ern014

- Kollist, H., Zandalinas, S. I., Sengupta, S., Nuhkat, M., Kangasjärvi, J., and Mittler, R. (2019). Rapid Responses to Abiotic Stress: Priming the Landscape for the Signal Transduction Network. *Trends in Plant Science* 24, 25–37. doi: 10.1016/j.tplants.2018.10.003
- Kooyers, N. J. (2015). The evolution of drought escape and avoidance in natural herbaceous populations. *Plant Science* 234, 155–162. doi: 10.1016/j.plantsci.2015.02.012
- Kumari, P., Dahiya, P., Livanos, P., Zergiebel, L., Kölling, M., Poeschl, Y., et al. (2021). IQ67 DOMAIN proteins facilitate preprophase band formation and division-plane orientation. *Nature Plants* 7, 739–747. doi: 10.1038/s41477-021-00923-z
- Kwak, J. M., Nguyen, V., and Schroeder, J. I. (2006). The Role of Reactive Oxygen Species in Hormonal Responses. *Plant Physiology* 141, 323–329. doi: 10.1104/pp.106.079004
- Lamb, C., and Dixon, R. A. (1997). THE OXIDATIVE BURST IN PLANT DISEASE RESISTANCE. *Annual Review of Plant Biology* 48, 251–275. doi: <https://doi.org/10.1146/annurev.arplant.48.1.251>
- Lang, T., Deng, C., Yao, J., Zhang, H., Wang, Y., and Deng, S. (2020). A Salt-Signaling Network Involving Ethylene, Extracellular ATP, Hydrogen Peroxide, and Calcium Mediates K⁺/Na⁺ Homeostasis in Arabidopsis. *International Journal of Molecular Sciences* 21. doi: 10.3390/ijms21228683
- Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9, 357–359. doi: 10.1038/nmeth.1923
- Lata, C., Muthamilarasan, M., and Prasad, M. (2015). “Drought Stress Responses and Signal Transduction in Plants,” in *Elucidation of Abiotic Stress Signaling in Plants: Functional Genomics Perspectives, Volume 2*, ed. G. K. Pandey (New York, NY: Springer New York), 195–225. doi: 10.1007/978-1-4939-2540-7_7
- Lawson, T., and Vialet-Chabrand, S. (2019). Speedy stomata, photosynthesis and plant water use efficiency. *New Phytologist* 221, 93–98. doi: 10.1111/nph.15330
- Laxa, M., Liebthal, M., Telman, W., Chibani, K., and Dietz, K.-J. (2019). The Role of the Plant Antioxidant System in Drought Tolerance. *Antioxidants* 8. doi: 10.3390/antiox8040094
- Lecourieux, D., Ranjeva, R., and Pugin, A. (2006). Calcium in plant defence-signalling pathways. *New Phytologist* 171, 249–269. doi: 10.1111/j.1469-8137.2006.01777.x
- Lee, S., Wang, W., and Huq, E. (2021). Spatial regulation of thermomorphogenesis by HY5 and PIF4 in Arabidopsis. *Nature Communications* 12, 3656. doi: 10.1038/s41467-021-24018-7
- Li, J., Zeng, J., Tian, Z., and Zhao, Z. (2024). Root-specific photoreception directs early root development by HY5-regulated ROS balance. *Proceedings of the National Academy of Sciences* 121, e2313092121. doi: 10.1073/pnas.2313092121
- Liao, Y., Smyth, G. K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930. doi: 10.1093/bioinformatics/btt656
- Liao, Z., Zhang, Y., Yu, Q., Fang, W., Chen, M., Li, T., et al. (2023). Coordination of growth and drought responses by GA-ABA signaling in rice. *New Phytologist* 240, 1149–1161. doi: 10.1111/nph.19209
- Liszkay, A., van der Zalm, E., and Schopfer, P. (2004). Production of Reactive Oxygen Intermediates (O₂^{•-}, H₂O₂, and [•]OH) by Maize Roots and Their Role in Wall Loosening and Elongation Growth. *Plant Physiology* 136, 3114–3123. doi: 10.1104/pp.104.044784
- Liu, H., Mu, Y., Xuan, Y., Wu, X., Wang, W., and Zhang, H. (2024). Hydrogen Peroxide Signaling in the Maintenance of Plant Root Apical Meristem Activity. *Antioxidants* 13. doi: 10.3390/antiox13050554
- Liu, S., Lv, Z., Liu, Y., Li, L., and Zhang, L. (2018). Network analysis of ABA-dependent and ABA-independent drought responsive genes in Arabidopsis thaliana. *Genetics and Molecular Biology* 41.
- Liu, Z., Guo, C., Wu, R., Hu, Y., Zhou, Y., Wang, J., et al. (2022). FLS2–RBOHD–PIF4 Module Regulates Plant Response to Drought and Salt Stress. *International Journal of Molecular Sciences* 23. doi: 10.3390/ijms23031080
- Lopez-Molina, L., Mongrand, S., Kinoshita, N., and Chua, N.-H. (2003). AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. *Genes & Development* 17, 410–418. Available at: <http://genesdev.cshlp.org/content/17/3/410.abstract>

- Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15, 550. doi: 10.1186/s13059-014-0550-8
- Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S., and Gruissem, W. (2002). Calmodulins and Calcineurin B-like Proteins : Calcium Sensors for Specific Signal Response Coupling in Plants. *The Plant Cell* 14, S389–S400. doi: 10.1105/tpc.001115
- Ludwig, A. A., Saitoh, H., Felix, G., Freymark, G., Miersch, O., Wasternack, C., et al. (2005). Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. *Proceedings of the National Academy of Sciences* 102, 10736–10741. doi: 10.1073/pnas.0502954102
- Lynch, T., Née, G., Chu, A., Krüger, T., Finkemeier, I., and Finkelstein, R. R. (2022). ABI5 binding protein2 inhibits ABA responses during germination without ABA-INSENSITIVE5 degradation. *Plant Physiology* 189, 666–678. doi: 10.1093/plphys/kiac096
- Ma, X., Li, Q.-H., Yu, Y.-N., Qiao, Y.-M., Haq, S. U., and Gong, Z.-H. (2020). The CBL–CIPK Pathway in Plant Response to Stress Signals. *International Journal of Molecular Sciences* 21. doi: 10.3390/ijms21165668
- Martignago, D., Siemiakowska, B., Lombardi, A., and Conti, L. (2020). Absciscic Acid and Flowering Regulation: Many Targets, Different Places. *International Journal of Molecular Sciences* 21. doi: 10.3390/ijms21249700
- McAinsh, M. R., and Pittman, J. K. (2009). Shaping the calcium signature. *New Phytologist* 181, 275–294. doi: 10.1111/j.1469-8137.2008.02682.x
- Mhamdi, A., and Van Breusegem, F. (2018). Reactive oxygen species in plant development. *Development* 145, dev164376. doi: 10.1242/dev.164376
- Miller, G., and Mittler, R. (2006). Could Heat Shock Transcription Factors Function as Hydrogen Peroxide Sensors in Plants? *Annals of Botany* 98, 279–288. doi: 10.1093/aob/mcl107
- Miller, G., Suzuki, N., Ciftci-Yilmaz, S., and Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell & Environment* 33, 453–467. doi: 10.1111/j.1365-3040.2009.02041.x
- Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends in Plant Science* 9, 490–498. doi: 10.1016/j.tplants.2004.08.009
- Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., et al. (2011). ROS signaling: the new wave? *Trends in Plant Science* 16, 300–309. doi: 10.1016/j.tplants.2011.03.007
- Mittler, R., Zandalinas, S. I., Fichman, Y., and Van Breusegem, F. (2022). Reactive oxygen species signalling in plant stress responses. *Nature Reviews Molecular Cell Biology* 23, 663–679. doi: 10.1038/s41580-022-00499-2
- Modi, A., Vai, S., Caramelli, D., and Lari, M. (2021). “The Illumina Sequencing Protocol and the NovaSeq 6000 System,” in *Bacterial Pangenomics: Methods and Protocols*, eds. A. Mengoni, G. Bacci, and M. Fondi (New York, NY: Springer US), 15–42. doi: 10.1007/978-1-0716-1099-2_2
- Mukherjee, A., Dwivedi, S., Bhagavatula, L., and Datta, S. (2023). Integration of light and ABA signaling pathways to combat drought stress in plants. *Plant Cell Reports* 42, 829–841. doi: 10.1007/s00299-023-02999-7
- Nagar, S., Singh, V. P., Arora, A., Dhakar, R., Singh, N., Singh, G. P., et al. (2021). Understanding the Role of Gibberellic Acid and Paclobutrazol in Terminal Heat Stress Tolerance in Wheat. *Frontiers in Plant Science* 12. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2021.692252>
- Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D., and Hancock, J. T. (2002). Hydrogen peroxide and nitric oxide as signalling molecules in plants. *Journal of Experimental Botany* 53, 1237–1247. doi: 10.1093/jxb/53.372.1237
- Niu, L., and Liao, W. (2016). Hydrogen Peroxide Signaling in Plant Development and Abiotic Responses: Crosstalk with Nitric Oxide and Calcium. *Frontiers in Plant Science* 7. Available at: <https://www.frontiersin.org/articles/10.3389/fpls.2016.00230>
- Olate, E., Jiménez-Gómez, J. M., Holuigue, L., and Salinas, J. (2018). NPR1 mediates a novel regulatory pathway in cold acclimation by interacting with HSFA1 factors. *Nature Plants* 4, 811–823. doi: 10.1038/s41477-018-0254-2

- Pei, Z.-M., Murata, Y., Benning, G., Thomine, S., Klüsener, B., Allen, G. J., et al. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406, 731–734. doi: 10.1038/35021067
- Pirayesh, N., Giridhar, M., Ben Khedher, A., Vothknecht, U. C., and Chigri, F. (2021). Organellar calcium signaling in plants: An update. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1868, 118948. doi: 10.1016/j.bbamcr.2021.118948
- Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Seay, M., Gerstein, M., et al. (2007). Differential binding of calmodulin-related proteins to their targets revealed through high-density Arabidopsis protein microarrays. *Proceedings of the National Academy of Sciences* 104, 4730–4735. doi: 10.1073/pnas.0611615104
- Ravi, B., Foyer, C. H., and Pandey, G. K. (2023). The integration of reactive oxygen species (ROS) and calcium signalling in abiotic stress responses. *Plant, Cell & Environment* 46, 1985–2006. doi: 10.1111/pce.14596
- Rentel, M. C., and Knight, M. R. (2004). Oxidative Stress-Induced Calcium Signaling in Arabidopsis. *Plant Physiology* 135, 1471–1479. doi: 10.1104/pp.104.042663
- Riemann, M., Dhakarey, R., Hazman, M., Miro, B., Kohli, A., and Nick, P. (2015). Exploring Jasmonates in the Hormonal Network of Drought and Salinity Responses. *Frontiers in Plant Science* 6. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2015.01077>
- Rubinovich, L., and Weiss, D. (2010). The Arabidopsis cysteine-rich protein GASA4 promotes GA responses and exhibits redox activity in bacteria and in planta. *The Plant Journal* 64, 1018–1027. doi: 10.1111/j.1365-313X.2010.04390.x
- Schaller, A., and Stintzi, A. (2009). Enzymes in jasmonate biosynthesis – Structure, function, regulation. *Phytochemistry* 70, 1532–1538. doi: 10.1016/j.phytochem.2009.07.032
- Scheible, N., and McCubbin, A. (2019). Signaling in Pollen Tube Growth: Beyond the Tip of the Polarity Iceberg. *Plants* 8. doi: 10.3390/plants8060156
- Schopfer, P. (2001). Hydroxyl radical-induced cell-wall loosening in vitro and in vivo: implications for the control of elongation growth. *The Plant Journal* 28, 679–688. doi: 10.1046/j.1365-313x.2001.01187.x
- Seo, P. J., Xiang, F., Qiao, M., Park, J.-Y., Lee, Y. N., Kim, S.-G., et al. (2009). The MYB96 Transcription Factor Mediates Abscisic Acid Signaling during Drought Stress Response in Arabidopsis. *Plant Physiology* 151, 275–289. doi: 10.1104/pp.109.144220
- Shavrukov, Y., Kurishbayev, A., Jatayev, S., Shvidchenko, V., Zotova, L., Koekemoer, F., et al. (2017). Early Flowering as a Drought Escape Mechanism in Plants: How Can It Aid Wheat Production? *Frontiers in Plant Science* 8. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2017.01950>
- Shen, N., Wang, T., Gan, Q., Liu, S., Wang, L., and Jin, B. (2022). Plant flavonoids: Classification, distribution, biosynthesis, and antioxidant activity. *Food Chemistry* 383, 132531. doi: 10.1016/j.foodchem.2022.132531
- Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany* 58, 221–227. doi: 10.1093/jxb/erl164
- Shohat, H., Cheriker, H., Kilambi, H. V., Illouz Eliaz, N., Blum, S., Amsellem, Z., et al. (2021). Inhibition of gibberellin accumulation by water deficiency promotes fast and long-term ‘drought avoidance’ responses in tomato. *New Phytologist* 232, 1985–1998. doi: 10.1111/nph.17709
- Singh, D., and Laxmi, A. (2015). Transcriptional regulation of drought response: a tortuous network of transcriptional factors. *Frontiers in Plant Science* 6. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2015.00895>
- Smirnoff, N., and Arnaud, D. (2019). Hydrogen peroxide metabolism and functions in plants. *New Phytologist* 221, 1197–1214. doi: 10.1111/nph.15488
- Stamm, P., Ravindran, P., Mohanty, B., Tan, E. L., Yu, H., and Kumar, P. P. (2012). Insights into the molecular mechanism of RGL2-mediated inhibition of seed germination in Arabidopsis thaliana. *BMC Plant Biology* 12, 179. doi: 10.1186/1471-2229-12-179
- Sun, B., Zhao, X., Gao, J., Li, J., Xin, Y., Zhao, Y., et al. (2023). Genome-wide identification and expression analysis of the GASA gene family in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). *BMC Genomics* 24, 668. doi: 10.1186/s12864-023-09773-9

- Suza, W. P., and Staswick, P. E. (2008). The role of JAR1 in Jasmonoyl-l-isoleucine production during Arabidopsis wound response. *Planta* 227, 1221–1232. doi: 10.1007/s00425-008-0694-4
- Takeno, K. (2016). Stress-induced flowering: the third category of flowering response. *Journal of Experimental Botany* 67, 4925–4934. doi: 10.1093/jxb/erw272
- Terzi, R., Kadioglu, A., Kalaycioglu, E., and Saglam, A. (2014). Hydrogen peroxide pretreatment induces osmotic stress tolerance by influencing osmolyte and abscisic acid levels in maize leaves. *Journal of Plant Interactions* 9, 559–565. doi: 10.1080/17429145.2013.871077
- Torres, M. A., Jones, J. D. G., and Dangl, J. L. (2006). Reactive Oxygen Species Signaling in Response to Pathogens. *Plant Physiology* 141, 373–378. doi: 10.1104/pp.106.079467
- Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols* 7, 562–578. doi: 10.1038/nprot.2012.016
- Vanneste, S., and Friml, J. (2009). Auxin: A Trigger for Change in Plant Development. *Cell* 136, 1005–1016. doi: 10.1016/j.cell.2009.03.001
- Vittozzi, Y., Krüger, T., Majee, A., Née, G., and Wenkel, S. (2024). ABI5 binding proteins: key players in coordinating plant growth and development. *Trends in Plant Science* 29, 1006–1017. doi: 10.1016/j.tplants.2024.03.009
- Wahid, A., Gelani, S., Ashraf, M., and Foolad, M. R. (2007). Heat tolerance in plants: An overview. *Environmental and Experimental Botany* 61, 199–223. doi: 10.1016/j.envexpbot.2007.05.011
- Wang, W., Wang, X., Huang, M., Cai, J., Zhou, Q., Dai, T., et al. (2018). Hydrogen Peroxide and Abscisic Acid Mediate Salicylic Acid-Induced Freezing Tolerance in Wheat. *Frontiers in Plant Science* 9. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2018.01137>
- Wang, X., Li, Q., Yang, M., Zhang, J., Huang, M., Cai, J., et al. (2021). Crosstalk between hydrogen peroxide and nitric oxide mediates priming-induced drought tolerance in wheat. *Journal of Agronomy and Crop Science* 207, 224–235. doi: 10.1111/jac.12458
- Wojtyla, Ł., Lechowska, K., Kubala, S., and Garnczarska, M. (2016). Different Modes of Hydrogen Peroxide Action During Seed Germination. *Frontiers in Plant Science* 7. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2016.00066>
- Wu, F., Chi, Y., Jiang, Z., Xu, Y., Xie, L., Huang, F., et al. (2020). Hydrogen peroxide sensor HPCA1 is an LRR receptor kinase in Arabidopsis. *Nature* 578, 577–581. doi: 10.1038/s41586-020-2032-3
- Xiao, Y., Chu, L., Zhang, Y., Bian, Y., Xiao, J., and Xu, D. (2022). HY5: A Pivotal Regulator of Light-Dependent Development in Higher Plants. *Frontiers in Plant Science* 12. Available at: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2021.800989>
- Xu, Y., and Zhu, Z. (2021). PIF4 and PIF4-Interacting Proteins: At the Nexus of Plant Light, Temperature and Hormone Signal Integrations. *International Journal of Molecular Sciences* 22. doi: 10.3390/ijms221910304
- Yang, W., Zhou, Z., and Chu, Z. (2023). Emerging Roles of Salicylic Acid in Plant Saline Stress Tolerance. *International Journal of Molecular Sciences* 24. doi: 10.3390/ijms24043388
- Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., et al. (2010). AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *The Plant Journal* 61, 672–685. doi: 10.1111/j.1365-313X.2009.04092.x
- Zhang, L., Geng, X., Zhang, H., Zhou, C., Zhao, A., Wang, F., et al. (2017). Isolation and characterization of heat-responsive gene TaGASR1 from wheat (*Triticum aestivum* L.). *Journal of Plant Biology* 60, 57–65. doi: 10.1007/s12374-016-0484-7
- Zhang, Y., and Xia, P. (2023). The DREB transcription factor, a biomacromolecule, responds to abiotic stress by regulating the expression of stress-related genes. *International Journal of Biological Macromolecules* 243, 125231. doi: 10.1016/j.ijbiomac.2023.125231
- Zhao, B., Shao, Z., Wang, L., Zhang, F., Chakravarty, D., Zong, W., et al. (2022). MYB44-ENAP1/2 restricts HDT4 to regulate drought tolerance in Arabidopsis. *PLOS Genetics* 18, e1010473. doi: 10.1371/journal.pgen.1010473
- Zhao, F.-Y., Han, M.-M., Zhang, S.-Y., Wang, K., Zhang, C.-R., Liu, T., et al. (2012). Hydrogen Peroxide-Mediated Growth of the Root System Occurs via Auxin Signaling Modification and

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7. List of all publications

- Mahmud, S., Ullah, C., Kortz, A., **Bhattacharyya, S.**, Yu, P., Gershenzon, J. (2022). Constitutive expression of *JAR1* induces molecular changes that prime plants to better withstand drought. *Plant Cell Environ.*, 45(10):2906-2922.
- **Bhattacharyya, S.**, Giridhar, M., Meier, B., Peiter, E., Vothknecht, U.C., Chigri, F. (2023). Global transcriptome profiling reveals root- and leaf-specific responses of barley to H₂O₂. *Front. Plant Sci.*, 14:1223778.
- Mahmud, S., Kamruzzaman, M., **Bhattacharyya, S.**, Alharbi, K., Moneim, D.A.E., Mostofa, M.G. (2023). Acetic acid positively modulates proline metabolism for mitigating PEG-mediated drought stress in Maize and Arabidopsis. *Front. Plant Sci.*, 14:1167238.
- Suresh, K., **Bhattacharyya, S.**, Carvajal, J., Ghosh, R., Zeisler-Diehl, V.V., Böckem, V., Nagel, K.A., Wojciechowski, T., Schreiber, L. (2024). Effects of water stress on apoplastic barrier formation in soil-grown vs hydroponically grown roots. *Plant Cell Environ.*, 47:4917-4931.
- **Bhattacharyya, S.**, Bleker, C., Meier, B., Giridhar, M., Rodriguez, E.U., Braun, A.M., Peiter, E., Vothknecht, U.C., Chigri, F. (2025). Ca²⁺ dependent H₂O₂ response in roots and leaves of barley: a transcriptomic investigation. *BMC Plant Biol.*, 25,232(2025).
- **Bhattacharyya, S.**, Turysbek, B., Lorenz, S.D., Rosales, D.C., Shoaib, Y., Gutbrod, K., Dörmann, P., Chigri, F., and Vothknecht, U.C. (2025). Loss-of-function of the drought-induced genes *GASA3* and *AFPI* confers enhanced drought tolerance in *Arabidopsis thaliana*. bioRxiv, DOI: <https://doi.org/10.1101/2025.04.03.647048>
- Ma, X., Hasan, S., Anjam, M., Mahmud, S., **Bhattacharyya, S.**, Vothknecht, U.C., Mendy, B., and Gründler, F., Marhavy, P. (2025). Ca²⁺ Waves and Ethylene/JA Crosstalk Orchestrate Wound Responses in Arabidopsis Roots. *EMBO Repo.* 26:3187-3203.
- Letia, S., **Bhattacharyya, S.**, Mendy, B., Vothknecht, U.C., Reuss, S.V., Inada, M., Gründler, F., Hasan, S. (2025). Ascr#18 Promotes Plant Defense by Repressing Auxin Signaling. *Physiol.Plantar.* 177(4): e70386.

8. Appendix

The appendices are categorized from number 1 to number 4. They contain publications from 1 to 4.

Appendix 1



OPEN ACCESS

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Global transcriptome profiling reveals root- and leaf-specific responses of barley (*Hordeum vulgare* L.) to H₂O₂

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In cereal crops, such as barley (*Hordeum vulgare* L.), the ability to appropriately respond to environmental cues is an important factor for yield stability and thus for agricultural production. Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), are key components of signal transduction cascades involved in plant adaptation to changing environmental conditions. H₂O₂-mediated stress responses include the modulation of expression of stress-responsive genes required to cope with different abiotic and biotic stresses. Despite its importance, knowledge of the effects of H₂O₂ on the barley transcriptome is still scarce. In this study, we identified global transcriptomic changes induced after application of 10 mM H₂O₂ to five-day-old barley plants. In total, 1883 and 1001 differentially expressed genes (DEGs) were identified in roots and leaves, respectively. Most of these DEGs were organ-specific, with only 209 DEGs commonly regulated and 37 counter-regulated between both plant parts. A GO term analysis further confirmed that different processes were affected in roots and leaves. It revealed that DEGs in leaves mostly comprised genes associated with hormone signaling, response to H₂O₂ and abiotic stresses. This includes many transcription factors and small heat shock proteins. DEGs in roots mostly comprised genes linked to crucial aspects of H₂O₂ catabolism and oxidant detoxification, glutathione metabolism, as well as cell wall modulation. These categories include many peroxidases and glutathione transferases. As with leaves, the H₂O₂ response category in roots contains small heat shock proteins, however, mostly different members of this family were affected and they were all regulated in the opposite direction in the two plant parts. Validation of the expression of the selected commonly regulated DEGs by qRT-PCR was consistent with the RNA-seq data. The data obtained in this study provide an insight into the molecular mechanisms of oxidative stress responses in barley, which might also play a role upon other stresses that induce oxidative bursts.

KEYWORDS

barley, H₂O₂, oxidative stress, RNA-sequencing, reactive oxygen species (ROS), transcriptome profiling, stress response

1 Introduction

In aerobic organisms, reactive oxygen species (ROS) are generated as by-products of certain metabolic pathways in plant organelles such as chloroplasts, mitochondria, and peroxisomes (Huang et al., 2019; Smirnov and Arnaud, 2019). Because of their high reactivity with cellular components, aerobic organisms have developed systems for enzymatic ROS removal based on the activity of ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT) as well as non-enzymatic antioxidative systems such as ascorbic acid, proline, and glutathione (GSH) (Foyer and Noctor, 2003; Ahmad et al., 2010). Plants also actively produce ROS as part of signaling cascades that coordinate the appropriate responses to environmental stimuli and contribute to stress tolerance (Pei et al., 2000; Zhu, 2016; Mohanta et al., 2018). It is proposed that systemic communication *via* redox systems is very fundamental to all photosynthetic organisms.

The ROS species hydrogen peroxide (H_2O_2) has been shown to play a role in various processes such as cell differentiation, senescence, and cell wall formation (Kärkönen and Kuchitsu, 2015; Ribeiro et al., 2017; Zeng et al., 2017). It is generated from superoxide in various cellular compartments as well as the apoplast as a result of a highly conserved superoxide dismutation reaction (Smirnov and Arnaud, 2019). H_2O_2 is also known to be transported across the cell membrane by specific aquaporins (Bienert et al., 2007) and to participate in long distance cell signaling (Mittler et al., 2011). Exogenous treatment with H_2O_2 has been shown to increase the tolerance of plants to abiotic stress by regulating multiple stress-responsive pathways and expression of genes including heat shock proteins and genes involved in abscisic acid (ABA) biosynthesis (Wahid et al., 2007; Terzi et al., 2014). An activation of ROS-dependent signaling by H_2O_2 causes the accumulation of defense proteins such as ROS-scavenging enzymes, transcription factors (TFs), and other response factors (Hossain et al., 2015), and it thus increases the tolerance of plants to abiotic stress. For example, certain HEAT SHOCK TRANSCRIPTION FACTORS (HSFs) have been suggested to serve as sensors that perceive H_2O_2 and regulate the expression of oxidative stress response genes (Miller and Mittler, 2006).

An early transcriptomic approach pursued to elucidate the effect of H_2O_2 was performed in *Arabidopsis thaliana* cell suspension cultures and showed that various TFs, hormone-associated pathways, and genes associated with other vital metabolic pathways like photosynthesis and fatty acid biosynthesis were affected (Desikan et al., 2001). Other studies revealed the role of H_2O_2 as a signaling molecule in a variety of plant species and under various conditions. For instance, H_2O_2 is involved in the response of plants to a variety of environmental cues, such as salt stress in tomato (Li et al., 2019), heat stress in rice (Wang et al., 2014), chilling stress in mung beans and manila grass (Yu et al., 2003; Wang et al., 2010), copper stress in maize and mung bean (Guzel and Terzi, 2013; Fariduddin et al., 2014), and many more (Khan et al., 2018).

Barley is one of the oldest cultivated cereal crops and has a high tolerance to stresses like salt, drought, and heat (Munns et al., 2006; Rollins et al., 2013; Gürel et al., 2016). Whereas changes in the

barley transcriptome upon those stresses have been analyzed (Janiak et al., 2018; Osthoff et al., 2019; Nefissi Ouertani et al., 2021), a global transcriptome analysis in response to H_2O_2 has not been performed yet.

In the present study, we used RNA sequencing (RNA-Seq) to analyze changes in the transcriptome of barley roots and leaves upon application of H_2O_2 . This analysis identified a total of 1001 and 1883 differentially expressed genes (DEGs) in response to H_2O_2 in leaves and roots, respectively. Comparative and quantitative analyses of gene expression patterns revealed commonly regulated key genes related to H_2O_2 stress between both tissues, nine of which were further confirmed by qRT-PCR analysis. The data obtained in this study contribute to the understanding of molecular mechanisms of oxidative stress response in barley, which might also play a role upon other stresses that induce oxidative bursts.

2 Materials and methods

2.1 Plant material and growth conditions

Barley plants (*Hordeum vulgare* cultivar Golden Promise) were grown in pots filled with water-soaked vermiculite in a climate-controlled growth chamber under long-day conditions with 16 h light at 20°C and a light intensity of 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Philips TLD 18W of alternating 830/840 light color temperature) and 8 h darkness at 18°C for five days.

2.2 H_2O_2 application and RNA isolation

Five-day-old seedlings were harvested and washed carefully to remove any remaining vermiculite prior to submersion in 10 mM H_2O_2 (Carl Roth, Germany) or ddH₂O (control) for three hours. The duration of H_2O_2 treatment was selected based on previous studies, which showed that at this time point H_2O_2 induced the strongest changes in the expression of most of the H_2O_2 -responsive genes (Desikan et al., 2001; Stanley Kim et al., 2005; Hieno et al., 2019). Subsequently, seedlings were carefully rinsed with ddH₂O and dissected into roots and leaves. Samples were shock-frozen in liquid nitrogen and homogenized using a sterile, ice-cold mortar and pestle. Total RNA was extracted using the Quick-RNA miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. The yield and purity of extracted RNA was determined with a NABI Nanodrop UV/Vis Spectrophotometer (MicroDigital, South Korea). The integrity of the extracted RNA was verified by separation of the 28S and 18S rRNA bands on a 1% agarose gel.

2.3 RNA-sequencing and data analyses

RNA sequencing was performed on three biological replicates for each treatment. Each replicate furthermore consisted of pooled material from three plants. Library preparation and transcriptome sequencing (3' mRNA sequencing) were carried out at the NGS

Core Facility (Medical Faculty at the University of Bonn, Germany) using a NOVASEQ 6000 (Illumina, USA) with a read length of 1x100 bases and an average sequencing depth of >10 million raw reads per sample (Table 1). 3' end sequencing libraries were prepared using the QuantSeq protocol (Moll et al., 2014). Briefly, oligo dT priming were followed by synthesis of the complementary first strand without any prior removal of ribosomal RNA. After successful introduction of Illumina specific adapter sequences, the resulting cDNA was further purified with magnetic beads. The unpaired reads were processed for quality control using fastQC and cutAdapt (Martin, 2011) in order to trim any remaining adapter sequences. They were then aligned using Tophat2 software (Trapnell et al., 2012) against a *H. vulgare* IBSC v2 reference genome obtained from Ensembl (<http://plants.ensembl.org/info/data/ftp/index.html>) using a Bowtie index (Langmead and Salzberg, 2012) created with the help of the reference genome (in FASTA format; the individual FASTA files of the chromosomes were concatenated using the “cat” command in UNIX shell). The alignment with Tophat2 was performed on an Ubuntu 18.04 LTS operating system, in a UNIX shell environment. Every step after alignment was performed using R 4.0.0 (R Core Team, 2020). Gene counts from the aligned BAM files were generated using featureCounts function in RStudio (Liao et al., 2014). Differential gene expression analyses was carried out using DESeq2 (Love et al., 2014). The p-values were corrected using the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995) and subsequently the FDR and the log₂FC cutoffs were set to 0.01 and 1, respectively. Principal Component Analyses (PCA) plots were prepared with the raw gene counts for all samples and replicates using the tidyverse and ggplot2 packages. The volcano plots and heatmaps were generated using the EnhancedVolcano and Pheatmap packages, respectively. In addition, transcript per million (TPM) values of each gene were calculated using a separate function designed in the

R environment (Supplementary Table S1). With common regulated DEGs, a clustering was performed with four predefined clusters based on FDR and log₂FC cutoffs of 0.01 and 0.5, respectively. The first and second cluster consisted of commonly down- and up-regulated genes, respectively, while the third and fourth cluster contained counter-regulated genes between leaves and roots of barley. The clusters were then represented as heatmaps using the pheatmap package and line plots using the ggpubr package.

Gene ontology (GO) and enrichment analyses were carried out using shinyGO (Ge et al., 2020). Categories were chosen as significant if the FDR was less than 0.05 (Benjamini and Hochberg, 1995). Homology searches against the *A. thaliana* genome were carried out using the BaRT (Barley Reference Transcript) tool available on www.ics.hutton.ac.uk (Mascher et al., 2017) based on a E-value cutoff of 1e⁻³⁰.

2.4 Quantification of transcript levels by qRT-PCR

qRT-PCR was performed with three replicates for each sample. Each replicate consisted of the pooled RNA material from three different plants. Synthesis of first strand cDNA for qRT-PCR was carried out from at least 1 µg of total RNA using the RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, USA) with oligo-dT₁₈ primers following the manufacturer's instructions. The quality of cDNA was assessed using a NABI UV/Vis Nanodrop Spectrophotometer. Gene expression was quantified in 48-well plates using a BioRad CFX 96 real-time PCR detection system (BioRad, Germany) and a SYBR Green PCR master mix (Thermo Fisher Scientific, USA). All forward and reverse primers used for qRT-PCR are listed in Supplementary Table S2. Data were quantified using the BioRad CFX Maestro software, and the

TABLE 1 Summary of total reads and aligned reads in the RNA-seq samples from barley roots and leaves obtained under H₂O₂ treatment and control conditions.

Sample	Replicate	Total Reads	Aligned Reads	% Aligned Reads
root control	RC1	15222810	12333400	81.02
	RC2	13555021	10223311	75.42
	RC3	12544002	9988003	79.62
leaf control	LC1	12392862	9242908	74.58
	LC2	14067426	10125991	71.98
	LC3	12314839	9224084	74.90
root + H ₂ O ₂	RT1	12123370	8559783	70.61
	RT2	13079745	9303393	71.13
	RT3	12698432	10154310	79.97
leaf + H ₂ O ₂	LT1	13222658	11555866	87.39
	LT2	14555200	12333012	84.73
	LT3	12220331	10214419	83.59

For each treatment three biological replicates were performed, each containing the combined RNA from three plants. LC-Leaf control, LT-Leaf H₂O₂ treated, RC-Root control, and RT-Root H₂O₂ treated.

expression was estimated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) after normalization against the two reference genes *HvACTIN* and *HvGAPDH*, as the C_q values of both genes were unchanged upon H_2O_2 treatment. Data were analyzed statistically with one-way analysis of variance (ANOVA) and Tukey' Post-Hoc HSD test using the agricolae and tidyverse packages, respectively. Graphs were prepared using the ggpubr package.

2.5 H_2O_2 staining and microscopic analyses

Staining of hydrogen peroxide in barley leaves and roots was performed with 2',7'-dichlorodihydrofluorescein diacetate (H_2 -DCFDA; Thermo Fisher Scientific, USA) based on a modified protocol (Kaur et al., 2016). Briefly, five-day-old barley seedlings were treated with either 10 mM H_2O_2 or dd H_2O (control) for 3 hours. Afterwards, the seedlings were briefly rinsed and treated with 10 μ M H_2 -DCFDA prepared from a 4 mM stock dissolved in DMSO for 1 hour in the dark. After staining, seedlings were washed, and roots and leaves were mounted separately on a microscopy slide. 2',7'-Dichlorofluorescein (DCF) fluorescence was analyzed using a Leica SP8 Lightning confocal laser scanning microscope (Leica

Microsystems, Germany). For excitation, an argon laser with a wavelength of 488 nm was used, and emission of 517–527 nm was detected using a HyD Detector. Fluorescence intensity was quantified in regions of interest (ROI) using the integrated LASX software.

3 Results

3.1 Differential gene expression in leaves and roots of barley in response to application of H_2O_2

To investigate the transcriptomic modulation in barley (*Hordeum vulgare* cv. Golden Promise) in response to oxidative stress, five-day-old plants were exposed for three hours to 10 mM H_2O_2 or to dd H_2O as control (Figure 1A). H_2 -DCFDA staining confirmed that H_2O_2 penetrated both roots and leaves (Figures 1B, C and Supplementary Figure 1). RNA was then extracted separately from roots and leaves, and RNA-seq analysis was carried out on three biological replicates per tissue and treatment, each comprising the pooled RNA from three different plants (Supplementary Table S1). On average approximately 13 million total reads were obtained per sample. About 75–85% of these reads could be aligned to the

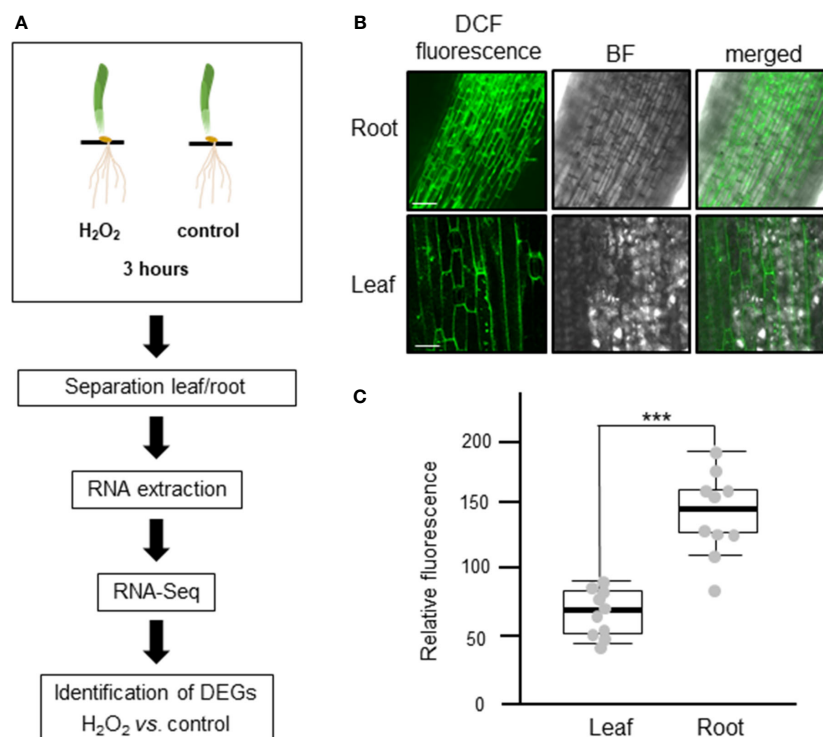


FIGURE 1

Experimental design to analyze the transcriptional changes of barley plants to oxidative stress. (A) Schematic representation of the study design. Five-day-old barley plants were treated with either 10 mM H_2O_2 or water (control) for three hours. After the treatment, leaves and roots were separated, RNA was extracted, and three independent biological replicates, each containing the pooled RNA from three plants, were submitted to RNA-Seq analyses. The raw reads obtained were subjected to quality control and aligned against the barley reference genome. Based on raw gene counts, a differential expression analysis was carried out using DESeq2. (B) Uptake of H_2O_2 in roots (upper panel) and leaves (lower panel) visualized by H_2 -DCFDA. Green fluorescence of the 2',7'-Dichlorofluorescein (DCF) was observed using a Leica SP8 lightning confocal laser scanning microscope. BF: bright field; bar: 100 μ m. (C) Quantification of fluorescence intensity of H_2 -DCFDA relative to untreated control tissues. Each dot represents the average of five regions of interests (ROIs). ROIs were taken from two independent images from three biological replicates ($n=6$). Statistical analysis was carried out using the two-tailed t-test (*** = $P<0.001$).

barley reference genome (Table 1). To assess the main variances within the dataset, a principal component analysis (PCA) was performed. The result showed that PC1 (X-axis), which separates the samples by tissue, represents the largest variation in our dataset compared to PC2 (Y-axis), which separates the samples by treatment (Figure 2A). Consequently, the differential gene expression analysis was separately performed for the leaf and root samples.

Differentially expressed genes (DEGs) between H₂O₂-treated and control samples were identified based on fold change (FC) $|\text{Log}_2\text{FC} \geq 1|$ and FDR < 0.01 (Supplementary Table S3). A total number of 2884 DEGs were detected across both tissues. H₂O₂ application clearly resulted in stronger transcriptional changes in roots compared to leaves (Figure 2B). Of the 1883 DEGs detected in roots, 701 were up- and 1182 were down-regulated, while in leaves 1001 DEGs were identified with 546 up- and 455 down-regulated (Figure 2C). Among all DEGs only 75 and 134 were commonly up- and down-regulated, respectively, in both tissues, while 37 were counter-regulated.

3.2 Gene ontology analyses

GO classification was used to identify the 20 most significant biological process categories within the DEGs. The results show that

not only the number of genes, but also the biological processes affected by H₂O₂ were clearly different between leaves and roots (Figure 3). In leaves, GO terms associated with genes that showed the highest fold change were related to protein complex oligomerization, response to H₂O₂ and jasmonate. Further categories with lower fold change but often higher number of genes comprised quite global stress effects associated with different, mostly abiotic stimuli, but also wounding (Figure 3A). In roots, many of the enriched GOs were associated with response to oxygenic stress including H₂O₂ catabolism, glutathione and ROS metabolism, or cellular oxidant detoxification as well as with cell wall modulation (Figure 3B).

3.2.1 Differentially expressed genes in barley leaves in response to H₂O₂

In barley leaves, the most highly enriched GO term category upon exposure to H₂O₂ was the response to H₂O₂ and protein complex oligomerization (Figure 3A). Both categories consist of the same SMALL HEAT SHOCK PROTEINS (SHSP domain-containing proteins) (Table 2). SHSPs are ubiquitous in prokaryotic and eukaryotic organisms and function as chaperone proteins involved in the response to many abiotic stresses (Basha et al., 2012; Waters, 2013). Their expression levels were shown in different plant species to increase upon stress and to enhance stress tolerance. Here, barley leaves exposed to H₂O₂ showed an increased

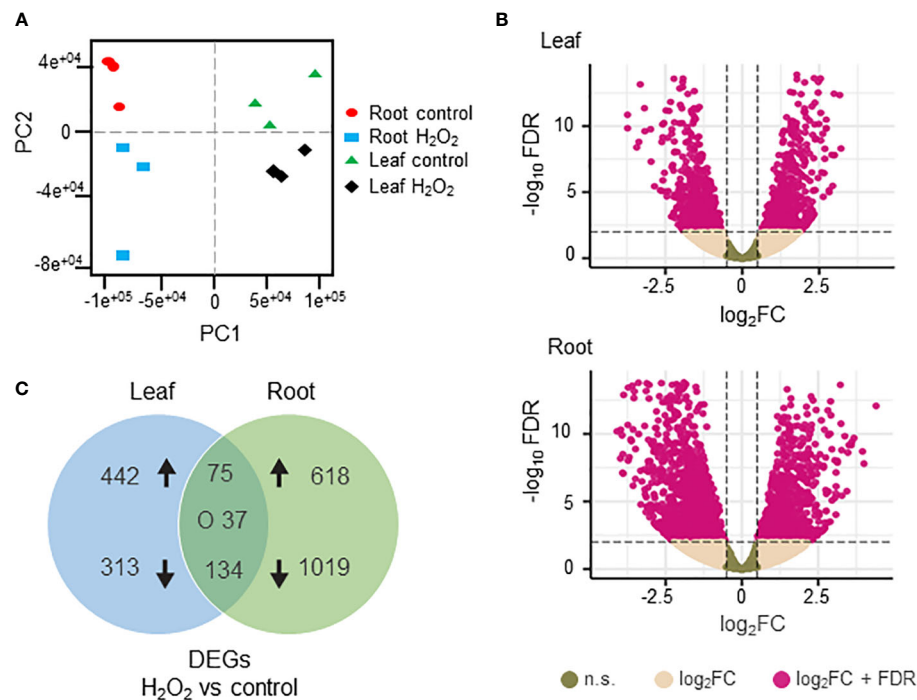
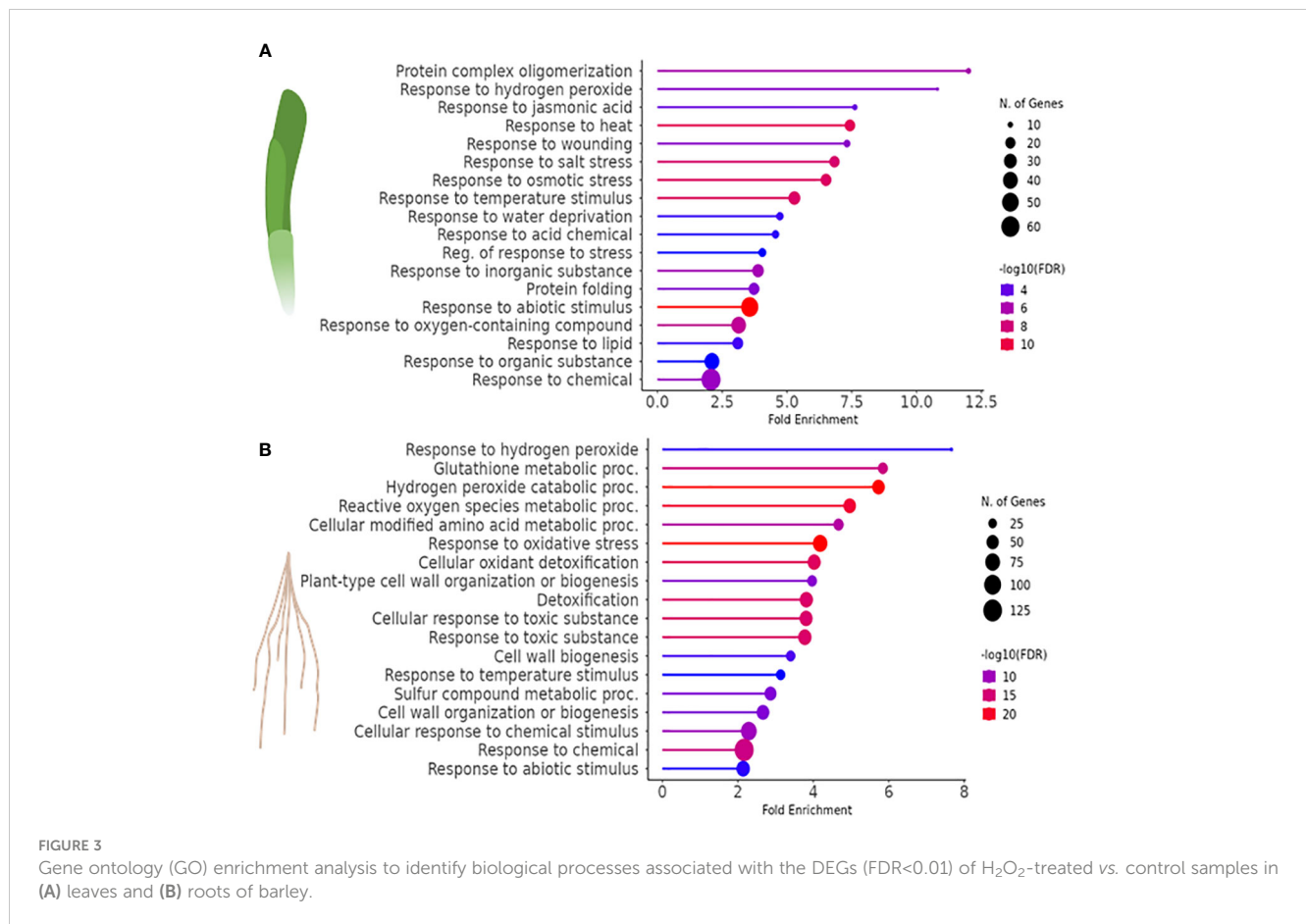


FIGURE 2

Differentially expressed genes (DEGs) in H₂O₂-treated and untreated barley plants. (A) Principal component analysis of the RNAseq data showing the homogeneity of the different samples. PC1 (X axis) separates the samples by tissue while PC2 (Y axis) separates the samples by treatment. (B) Volcano plots of the DEGs in leaves (upper panel) and roots (lower panel). The X axis represents the fold change (Log₂FC) of the DEGs (H₂O₂ vs. control), whereas the Y axis represents the statistical significance (log₁₀FDR). Pink dots indicate genes that fit the DESeq criteria of FDR and $|\text{Log}_2\text{FC}| \geq 1$, while beige dots represent DEGs that fit only Log₂FC. N.S.: not significant (C) Venn diagram representing DEGs (DESeq, adjusted to FDR < 0.01 and $|\text{Log}_2\text{FC}| \geq 1$) between H₂O₂-treated and untreated samples in leaves and roots. Arrows indicate up- and down-regulation. 'O' indicates counter-regulated genes.



expression of SHSPs, except for the 18.8 kDa class V heat shock protein (HORVU2Hr1G046370), which was down-regulated. All of the differentially regulated SHSPs have close orthologs in Arabidopsis (Li and Liu, 2019) with the majority being orthologous to *AtHSP17.6II* (At5g12020).

An enrichment was also found for genes involved in hormone biosynthesis and signaling, especially jasmonate, auxin, and abscisic acid (ABA). Jasmonate-related DEGs were represented by the specific GO-term category 'response to jasmonic acid'. This category comprised two up-regulated TIFY domain-containing proteins with no direct homologs in Arabidopsis (Table 2). The TIFY domain is also known as ZIM domain which is present in members of the transcriptional repressor JASMONATE ZIM-domain (JAZ) family, key elements in the jasmonate signaling pathway (Chung and Howe, 2009; Pauwels and Goossens, 2011). This category also includes genes that encode for enzymes involved in jasmonate biosynthesis (Schaller and Stintzi, 2009; Bittner et al., 2022) such as ALLENE OXIDE CYCLASE (AOC), and OXOPHYTODIENOATE-REDUCTASE (OPR) as well as ALLENE OXIDE SYNTHASE (AOS) but with a FC less than 2 (FC 1.69, Log₂FC=0.76). By contrast, genes related to other hormone signaling pathways were found redundantly interspersed in the two GO terms 'response to abiotic stimulus' and 'response to salt stress' (Figure 3A). With regard to auxin, a number of orthologs to auxin-responsive genes from Arabidopsis, especially IAA-type TFs, were found. Similar to the jasmonate signaling pathway, H₂O₂

seems to affect the auxin pathway differentially since both, up- and down-regulated DEGs, were identified. All components related to the phytohormone ABA were up-regulated and those related to APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) domain-containing proteins, known to be involved in abiotic stress responses and associated with various hormones, were down-regulated. Similar to the GO term categories related to auxin, both sets comprise mostly orthologs to TFs or co-regulators known in Arabidopsis (Table 2).

In leaves, genes associated with photosynthesis light harvesting in photosystem I, were also affected, however, the category did not appear in the top GOs since for several of the genes the FC was less than 2 but mostly higher than 1.5 (Table 2; Log₂FC between 0.5 and 1). This category contained mostly down-regulated DEGs, including several orthologs of Arabidopsis LHCII trimer components, i.e., genes encoding for LHCb1 and LHCb3, and the LHCa1 protein. It furthermore comprised orthologs to the photosystem I subunits PSAF and PSAL but also the oxygen evolving complex subunit PSBP-1 and the large subunit of RIBULOSE-1,4-BISPHOSPHATE-CARBOXYLASE/OXYGENASE (Rubisco) (Table 2).

3.2.2 Differentially expressed genes in barley roots in response to H₂O₂

In barley roots, the most enriched GO terms are associated with response to oxidative stress and detoxification (Figure 3B). This is

TABLE 2 Selected DEGs associated with top GO terms in leaves of barley in response to H₂O₂.

Category	Gene ID	log ₂ FC	Functional protein	Predicted ortholog in <i>A. thaliana</i>
Response to H₂O₂/ protein complex oligomerization				
	HORVU2Hr1G046370	-3.74	SHSP domain-containing protein	AT4G21870 (AtHSP15.4)
	HORVU3Hr1G020500	2.24	SHSP domain-containing protein	AT5G12020/AT5g12030 (AtHSP17.6)
	HORVU3Hr1G020490	3.03	SHSP domain-containing protein	AT5G12020/AT5g12030 (AtHSP17.6)
	HORVU3Hr1G020390	1.267	SHSP domain-containing protein	AT5G12020/AT5g12030 (AtHSP17.6)
	HORVU0Hr1G020420	1.54	SHSP domain-containing protein	AT5G37670 (AtHSP15.7)
	HORVU3Hr1G020520	1.84	SHSP domain-containing protein	AT5G12020/AT5g12030 (AtHSP17.6)
	HORVU6Hr1G082360	2.98	SHSP domain-containing protein	At1G54050 (AtHSP17.4)
Response to jasmonic acid				
	HORVU5Hr1G062290	2.34	TIFY domain-containing protein	AT1G74950 (AtJAZ12)
	HORVU4Hr1G076850	1.80	TIFY domain-containing protein	no homolog
	HORVU5Hr1G098090	1.21	Uncharacterized protein	AT1G13280 (AtAOC4)
	HORVU7Hr1G118010	-1.44	Oxidored FMN domain-containing	AT1G76680 (AtOPR1)
	HORVU2Hr1G004230	-1.55	Oxidored FMN domain- containing	AT1G76690 (AtOPR2)
	HORVU6Hr1G081000	0.76	Allene oxide synthase	AT5G42650 (AtCYP74A/AtAOS)
Response to abiotic stimulus/ osmotic stress/ hormones				
Auxin				
	HORVU7Hr1G084940	1.81	Auxin responsive protein	AT4G14550 (AtIAA14/AtSLR)
	HORVU5Hr1G087880	1.48	Auxin responsive protein	AT5G65980 (AtPILS7)
	HORVU7Hr1G033820	1.22	Auxin responsive protein	AT1G19220 (AtARF19)
	HORVU1Hr1G086070	1.00	Auxin responsive protein	no homolog
	HORVU1Hr1G086070	1.00	Auxin responsive protein	no homolog
	HORVU6Hr1G058890	-1.52	Auxin response factor	AT4G30080 (AtARF16)
	HORVU7Hr1G077110	-1.62	Auxin responsive protein	no homolog
	HORVU5Hr1G093580	-2.40	Auxin responsive protein	AT3G04730 (AtIAA16)
Abcisic acid				
	HORVU7Hr1G085130	2.34	Multiple protein bridging factor	AT3G24500 (AtMBF1c)
	HORVU7Hr1G035500	1.58	bZIP domain-containing protein	AT4G34000 (AtABF3/AtbZIP37)
	HORVU3Hr1G069590	1.37	HSF_domain-containing protein	AT3G24520 (AtHsfC1)

(Continued)

TABLE 2 Continued

Category	Gene ID	log ₂ FC	Functional protein	Predicted ortholog in <i>A. thaliana</i>
	HORVU6Hr1G028790	1.30	WRKY domain-containing protein	AT4G31800 (AtWRKY18)
	HORVU5Hr1G115100	1.03	GRAM domain-containing protein	At5G13200 (AtGEM5/AtGER5/AtGRE5)
other	HORVU5Hr1G097560	1.62	HTH MYB domain-containing protein	AT2G38090
	HORVU3Hr1G085180	1.26	MYB domain-containing protein	no homolog
	HORVU6Hr1G091700	-1.13	Ethylene receptor domain-containing protein	AT3G04580 (AtEIN4)
	HORVU4Hr1G077310	-1.31	AP2/ERF domain-containing protein	no homolog
	HORVU4Hr1G000700	-1.92	AP2/ERF domain-containing protein	AT3G23240 (AtERF092/AtERF1b)
	HORVU3Hr1G010190	-3.31	AP2/ERF domain-containing protein	AT1G68840 (AtEDF2/AtRAV2/AtTEM2)
Photosynthesis				
	HORVU6Hr1G091660	-1.67	Chlorophyll a-b binding protein	AT2G34420 (AtLHCb1.5)
	HORVU1Hr1G088920	-1.37	Chlorophyll a-b binding protein	AT2G34420 (AtLHCb1.5)
	HORVU7Hr1G040370	-1.16	Chlorophyll a-b binding protein	AT2G34420 (AtLHCb1.5)
	HORVU6Hr1G047870	-1.11	Ribulose biphosphate carboxylase LSU	ATCG00490 (RubisCo LSU)
	HORVU5Hr1G109250	-1.07	Chlorophyll a-b binding protein	AT1G29930 (AtLHCb1.3)
	HORVU5Hr1G109260	-0.93	Chlorophyll a-b binding protein	AT2G34420 (AtLHCb1.5)
	HORVU2Hr1G040780	-0.92	Chlorophyll a-b binding protein	AT5G54270 (AtLHCb3)
	HORVU1Hr1G078380	-0.91	Chlorophyll a-b binding protein	AT2G34420 (AtLHCb1.5)
	HORVU2Hr1G060880	-0.87	PsbP domain-containing protein	AT1G06680 (AtPsaP1)
	HORVU5Hr1G100140	-0.81	PSI-F	AT1G31330 (AtPsaF)
	HORVU7Hr1G046320	-0.72	Chlorophyll a-b binding protein	AT3G54890 (AtLHCA1)
	HORVU3Hr1G009210	-0.71	PSI subunit V	AT4G12800 (AtPsaL)
	HORVU1Hr1G088870	-0.68	Chlorophyll a-b binding protein	AT2G34430 (AtLHCb1.4)

also evident by the fact that many DEGs within those GO terms are class-III peroxidases, catalases, or genes related to glutathione metabolism, which were grouped together as a category named 'Detoxification of H₂O₂' (Table 3). In plants, class-III peroxidases have been described in association with a wide variety of biotic and abiotic stresses along with plant defense mechanisms (Almagro et al., 2009; Shigeto and Tsutsumi, 2016). While most peroxidases

were up-regulated, some were down-regulated along with a number of glutathione transferases, an ascorbate peroxidase (APX), and CATALASE 1. We also found strong up-regulation of the genes for two putative detoxification efflux carriers/multidrug and toxic compound extrusion (DTX/MATE) transporters. These metabolite transporters have been described to be associated with plant stress responses and overexpression of a gene encoding a cotton DXT

TABLE 3 Selected DEGs associated with top GO terms in roots of barley in response to H₂O₂.

Category	Gene ID	log ₂ FC	Functional annotation	Predicted ortholog in <i>A. thaliana</i>
Response to H₂O₂				
	HORVU0Hr1G020420	-1.21	SHSP domain containing protein	AT5G37670 (AtHSP15.7)
	HORVU2Hr1G077710	-1.59	SHSP domain containing protein	AT4G10250 (AtHSP22)
	HORVU3Hr1G006940	-2.24	SHSP domain containing protein	No ortholog
	HORVU3Hr1G020390	-1.92	SHSP domain containing protein	AT5G12020 (AtHSP17.6II)
	HORVU3Hr1G020490	-2.79	SHSP domain containing protein	AT5G12020 (AtHSP17.6II)
	HORVU3Hr1G020520	-2.96	SHSP domain containing protein	AT5G12020 (AtHSP17.6II)
	HORVU4Hr1G015170	-3.2	SHSP domain containing protein	AT4G10250 (AtHSP22)
	HORVU4Hr1G060720	-1.34	SHSP domain containing protein	AT3G46230 (AtHSP17.4)
	HORVU4Hr1G060760	-2.88	SHSP domain containing protein	AT1G53540 (AtHSP17.6C)
	HORVU6Hr1G008640	-2.55	Catalase	AT1G20630 (AtCAT1)
	HORVU7Hr1G014870	-1.54	ABC transporter domain containing protein	AT1G31770 (AtABCG14)
Detoxification of H₂O₂				
H₂O₂ catabolism				
	HORVU7Hr1G039550	3.97	Peroxidase	AT1G05260 (AtPRX3)
	HORVU2Hr1G026640	3.65	Peroxidase	AT1G05260 (AtPRX3)
	HORVU7Hr1G010280	3.598	Peroxidase	AT4G11290 (AtPRX39)
	HORVU1Hr1G016730	2.96	Peroxidase	AT2G18140 (AtPRX14)
	HORVU2Hr1G018550	2.91	Peroxidase	AT5G05340 (AtPRX52)
	HORVU7Hr1G039590	2.74	Peroxidase	AT1G05260 (AtPRX3)
	HORVU2Hr1G018530	2.60	Peroxidase	AT5G05340 (AtPRX52)
	HORVU7Hr1G039570	2.21	Peroxidase	AT1G05260 (AtPRX3)
	HORVU0Hr1G002840	2.17	Peroxidase	AT4G11290 (AtPRX39)
	HORVU2Hr1G100610	2.07	Peroxidase	AT5G17820 (AtPRX57/AtPRXR10)
	HORVU1Hr1G016770	2.01	Peroxidase	AT4G11290 (AtPRX39)
	HORVU2Hr1G026590	1.93	Peroxidase	AT4G11290 (AtPRX39)
	HORVU2Hr1G026520	1.84	Peroxidase	AT4G11290 (AtPRX39)

(Continued)

TABLE 3 Continued

Category	Gene ID	log ₂ FC	Functional annotation	Predicted ortholog in <i>A. thaliana</i>
	HORVU2Hr1G026540	1.83	Peroxidase	AT4G11290 (AtPRX39)
	HORVU6Hr1G026600	1.67	Peroxidase	AT5G05340 (AtPRX52)
	HORVU7Hr1G039560	1.52	Peroxidase	AT1G05260 (AtPRX3)
	HORVU1Hr1G016870	-1.84	Peroxidase	AT5G66390 (AtPRX72/AtPRXR8)
	HORVU2Hr1G124930	-1.99	Peroxidase	AT1G71695 (AtPRX12/AtPRXR6)
	HORVU4Hr1G022280	-2.15	Peroxidase	AT5G05340 (AtPRX52)
Glutathione metabolism	HORVU6Hr1G063830	-1.47	Glutathione peroxidase	AT4G11600 (AtGPX6/AtGPXL6)
	HORVU5Hr1G006330	-1.17	Glutathione transferase	no homolog
	HORVU1Hr1G049230	-1.28	Glutathione transferase	AT2G29470 (AtGSTU3)
	HORVU1Hr1G021140	-1.36	Glutathione transferase	AT3G62760 (AtGSTF13)
	HORVU6Hr1G011120	-2.16	GST_C terminal domain-containing protein	AT4G19880
	HORVU5Hr1G006330	-1.17	Glutathione transferase	no homolog
	HORVU1Hr1G049070	-2.86	GST_N terminal domain-containing protein	AT1G10370 (AtGSTU17)
	HORVU4Hr1G057170	-1.31	APX domain-containing protein	AT1G07890 (AtAPX1/AtC3H)
Response to ROS / Detoxification	HORVU6Hr1G008640	-2.55	Catalase	AT1G20630 (AtCAT1)
	HORVU4Hr1G011690	2.26	DTX/MATE metabolite transporter	AT3G26590 (AtDTX29)
	HORVU0Hr1G022350	-4.09	DTX/MATE metabolite transporter	AT5G52450 (AtDTX16)
Cell wall				
	HORVU4Hr1G028720	2.70	Xyloglucan endotransglucosylase/ hydrolase	AT5G13870 (AtXTH5/AtXTR12)
	HORVU2Hr1G010800	2.37	ExpansinA11	AT1G20190 (AtEXPA11)
	HORVU3Hr1G116470	2.07	Pectin acetylesterase	no homolog
	HORVU3Hr1G016820	2.04	Xyloglucan endotransglucosylase/ hydrolase	AT5G57550 (AtXTH25)
	HORVU2Hr1G120100	1.47	Endoglucanase	AT1G48930 (AtGH9C1/AtCEL6)
	HORVU3Hr1G016800	1.44	Xyloglucan endotransglucosylase/ hydrolase	AT5G57550 (AtXTH25)
	HORVU5Hr1G118270	1.43	Cellulose synthase	AT5G64740 (AtCESA6/AtIRX2)
	HORVU7Hr1G093680	1.27	Expansin	AT4G38210 (AtEXPA20)

(Continued)

TABLE 3 Continued

Category	Gene ID	$\log_2FC$	Functional annotation	Predicted ortholog in <i>A. thaliana</i>
	HORVU7Hr1G098370	1.55	Xyloglucan endotransglycosylase	AT4G25810 (AtXTH23/AtXTR6)
	HORVU3Hr1G091360	257	Pectin esterase	AT5G09760 (AtPME51)

protein in Arabidopsis reduced stress-induced levels of H_2O_2 (Lu et al., 2019).

As in leaves, the most highly enriched GO term category in roots upon exposure to H_2O_2 was the response to H_2O_2 , albeit with very few genes (Figure 3B). Similar to leaves, this category includes several SHSP domain-containing proteins, but in contrast to leaves, they were down-regulated (Table 3). All of the differentially regulated SHSPs have close orthologs in Arabidopsis, with several of them being orthologous to AtHSP17.6. This category contains also down-regulated catalase and ABC transporter containing domain proteins.

H_2O_2 treatment also induced up-regulation of components of cell wall biogenesis and modulation, such as xyloglucan endotransglucosylase/hydrolase, expansin, endo-1,4-beta glucanase, pectin acetyl esterase, and cellulose synthase (Table 3) that were found interspersed in several GO term categories. Indeed, H_2O_2 and peroxidases were shown to be involved in cell wall remodeling upon environmental stress (Tenhaken, 2015).

3.3 Common DEGs of leaves and roots in response to H_2O_2

As described above, we identified a total of 246 common DEGs between leaves and roots of barley when using a $|\log_2FC| \geq 1$ cutoff (Supplementary Table S3, Figure 2C). For several genes, we noticed that they were differentially regulated in both tissues, however, in one tissue they showed an expression with a $FC > 2$ ($|\log_2FC| \geq 1$) while in the other tissue a FC less than 2 but higher as 1.5. Thus, for ($|\log_2FC|$ between 1 and 0.5) was detected. determination of commonly regulated genes in leaves and roots we used a cutoff of $\log_2FC \geq 0.5$ and listed these genes separately in Supplementary Table S3. Using this cut-off, a total 349 common DEGs were identified between roots and leaves of barley (Supplementary Figure S2; Supplementary Table S3). Of these, 116 and 176 genes were up- and down-regulated, respectively, while 58 genes showed counter-regulation. These common DEGs were organized in four clearly distinguishable clusters (Figure 4A), with either commonly down- (cluster 1) and up-regulated (cluster 2) genes or genes up-regulated in leaves but down-regulated in roots (cluster 3) and *vice versa* (cluster 4). Heat maps and line plots were constructed to visualize the changes in gene expression pattern for each cluster (Figures 4A, B).

3.3.1 Commonly up- and down-regulated genes

Cluster 1 contains DEGs commonly down-regulated in leaves and roots upon H_2O_2 treatment (Supplementary Table S3), among them members of important transcription factors such as *AP2/ERF*,

WRKY, *CBF1*, *NAC*, and *HD-ZIP HOMEBOX* (Supplementary Table S4, Figure 5A). Cluster 1 also comprises orthologs to the Arabidopsis sugar transporters *SWEET10* and *SWEET5*. Other transporters were orthologs to the phosphate transporter *PHT1;7* and the aquaporin *TIP4;1*. TIP aquaporins in plants had been shown to not only transport water molecules but also other molecules like H_2O_2 (Kurowska et al., 2020). In addition to components of oxidative stress, detoxification or cell wall biogenesis and modification that were already discussed in chapter 3.2.2, cluster 1 also contained several kinases including orthologs to the *CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASES* (CRKs), *CRK29* and *CRK25*. CRKs are presented in Arabidopsis by a large gene family with over 40 members and have been associated with various abiotic and biotic stresses (Bourdais et al., 2015).

Cluster 2 contains DEGs commonly up-regulated in leaves and roots (Supplementary Table S3). Interestingly, it contains TFs of similar families as cluster 1, like *WRKY* and *AP2/ERF* but also orthologs of the LOB DOMAIN CONTAINING PROTEIN 41 (*LBD41*) from Arabidopsis (Supplementary Table S4; Figure 5B). DEGs associated with primary metabolism like amino acid and nucleic acid metabolism were also found in cluster 2. Genes associated with primary metabolism were also shown to be up-regulated in other transcriptome studies associated with abiotic stress (Hirai et al., 2004; Wang et al., 2014) and DEGs found in cluster 2 do not seem to be related to any specific metabolic pathway. Two MITOGEN-ACTIVATED PROTEIN KINASEs (MAPKs) identified in cluster 2 are orthologs to *AtMAPKKK16* and *AtMAPKKK17*, both of which were shown to be regulated by ABA (Wang et al., 2011).

3.3.2 Counter-regulated genes

Cluster 3 consists of 42 DEGs up-regulated in leaves and down-regulated in roots of barley upon H_2O_2 treatment (Supplementary Table S3). Nine of these DEGs are orthologs to different small heat shock proteins from Arabidopsis (Supplementary Table S4; Figure 6). The cluster furthermore comprises an assorted set of genes whose orthologs in Arabidopsis are connected with various metabolic pathways and hormone signaling.

Cluster 4 consists of only 15 genes and no common functional categories were found (Supplementary Table S4). However, they include genes, whose Arabidopsis orthologs have been associated with hormones, or cell wall modification, i.e. the *COPPER-CONTAINING AMINE OXIDASE 3* (*CUAO3*) that was suggested to be involved in stress response since it was up-regulated upon treatment with several hormones or flagellin (Planas-Portell et al., 2013).

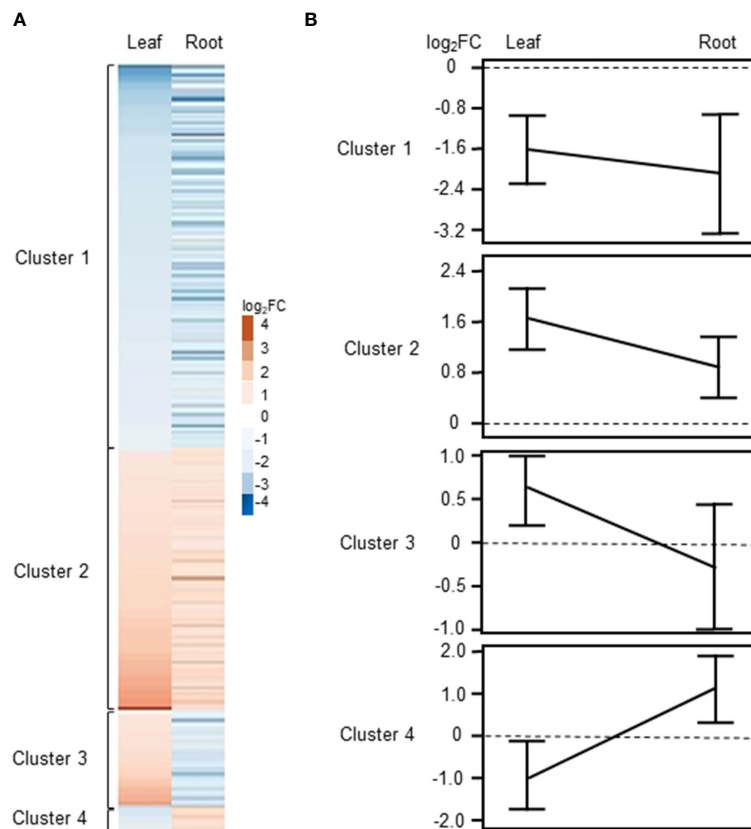


FIGURE 4

Clustering of DEGs commonly regulated or counter-regulated in leaves and roots of barley upon H₂O₂ treatment ($|\text{Log}_2\text{FC}| \geq 0.5$ and $\text{FDR} < 0.01$). (A) Heat map showing the Log₂FC associated with each gene in leaves and roots. (B) Line plot showing the mean $\pm$ SE of the $|\text{Log}_2\text{FC}|$ associated with each cluster in leaves and roots.

Overall, clusters 3 and 4 show very few genes previously described to be associated with oxidative stress.

3.4 qRT-PCR confirmation of selected DEGs

In order to confirm the results obtained from RNA-seq analyses, we performed quantitative RT-PCRs (qRT-PCR) on some of the identified DEGs. For these, we selected several DEGs that showed common regulation in leaves and roots in our dataset and which, based on their functional annotation, could be related to oxidative stress (Supplementary Table S5). Orthologs to some of them had already been shown to play an important role in H₂O₂ and ROS-related signaling not only in Arabidopsis but also in important crops like wheat, maize, and rice (Polidoros et al., 2005; Mylona et al., 2007; Steffens, 2014; Dudziak et al., 2019). They also represent different levels of regulation, some being among the most highly up- or down-regulated genes and other showing a much more subtle response. These genes represent different gene ontologies, and encode for a catalase, a peroxidase, a glutathione S-transferase, several TFs, a MAPKKK, and a xyloglucan endotransglucosylase, a protein involved in cell wall modification. As shown in Figure 7 and in Supplementary Table

S5, the log₂FC changes observed with the different techniques were often quite close and, in all cases, the results of the qRT-PCR matched the trend observed in the RNA-seq data.

4 Discussion

In plants, H₂O₂ is a crucial ROS which plays a dual role as a harmful by-product of cell metabolism and as a secondary messenger that affects development and growth. Complex cross-talk between H₂O₂ and other signaling molecules, such as Ca²⁺ ions and hormones, plays a key role in regulating different biological processes that contribute to the response to various biotic and abiotic stresses (Peiter, 2016; Saxena et al., 2016). Despite its importance, very little is known about H₂O₂-induced changes of the transcriptome in barley. In this study, an analysis of the barley transcriptome in response to H₂O₂ was performed using next generation sequencing. First, a suitable concentration of H₂O₂ that was shown to initiate a stress response in barley was selected on basis of previously performed experiments (Dodd et al., 2010; Giridhar et al., 2022). An increase in cytosolic Ca²⁺ ([Ca²⁺]_{cyt}) is one of the first responses of plants to most biotic and abiotic stresses (Dodd et al., 2010) that in turn leads to downstream stimulus-specific cellular responses. H₂O₂ was shown to induce such

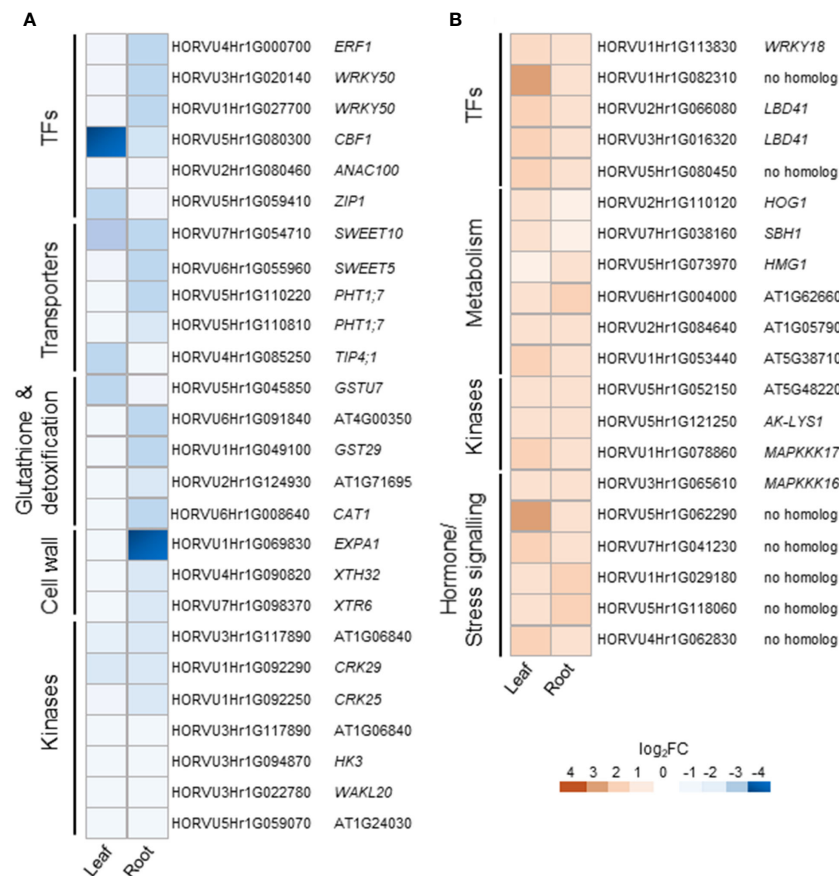


FIGURE 5

Selected DEGs commonly regulated in leaves and roots of barley upon H_2O_2 treatment. Down-regulated (A) and up-regulated (B) genes are grouped by functional category and presented with their Arabidopsis orthologs. TFs, transcription factors.

transient changes of $[Ca^{2+}]_{cyt}$ with 10 mM eliciting the highest response in barley roots and leaves (Giridhar et al., 2022). Staining of intact plants with the ROS indicator H_2 -DCFDA confirmed that the exogenously applied H_2O_2 penetrated into both organs (Figures 1B, C, Supplementary Figure 1). To exclude natural degradation of RNA and changes of the transcriptome driven by processes such as senescence or tillering, five-day-old barley plants were used. Growth of monocotyledonous leaves is initiated from the base and the leaf blade shows developmental gradients, i.e., disappearance of poly (A+) RNA levels along the developing blade (Hellmann et al., 1995). Moreover, plant senescence is a natural process known to be initiated by ROS that in turn activates transcription factors interacting with senescence associated genes (Bieker et al., 2012; Shimakawa et al., 2020). Thus, the growth conditions and plant age used in the analysis ensure as much as possible a solely treatment-dependent change of the transcriptome.

Overall, the RNA-seq analysis showed that under the chosen conditions H_2O_2 caused more transcriptional changes in roots compared to leaves (Figure 2). Most of the identified DEGs were found exclusively in one of the two plant parts, further confirming organ-specific responses. While this difference may be in part due to a difference in H_2O_2 penetration into roots and leaves, it is more likely caused by differential response of the two tissues to H_2O_2 signals and/or oxidative stress. Only about 10% of the DEGs were

found to be up- and down-regulated in leaves as well as in roots, some of which showed counter-regulation. This difference in response is also mirrored by the GO terms associated with the identified DEGs that only showed a minor overlap (Figure 3).

4.1 Leaf-specific transcriptomic changes in response to H_2O_2

Our data showed that several genes encoding for small heat shock proteins (SHSPs) were up-regulated by H_2O_2 in barley leaves (Table 2). In barley, the roles of several HSPs in response to a diverse range of abiotic stimuli have been characterized (Hlaváčková et al., 2013; Chaudhary et al., 2019; Landi et al., 2019). HSPs have also been shown to play crucial roles during abiotic stresses such as cold and heat in other important crop genera, like rice, maize, and wheat (ul Haq et al., 2019). SHSPs are a subgroup of HSPs defined by their size and a conserved α -crystalline C-terminal domain. They are known to form oligomeric complexes and prevent denatured proteins from aggregation until they can be refolded by other HSPs. They have been speculated to interact with transcription factors of the HEAT SHOCK FACTOR (HSF) family to create the HSP-HSF complex, alteration of which can drive essential reactions in response to ROS

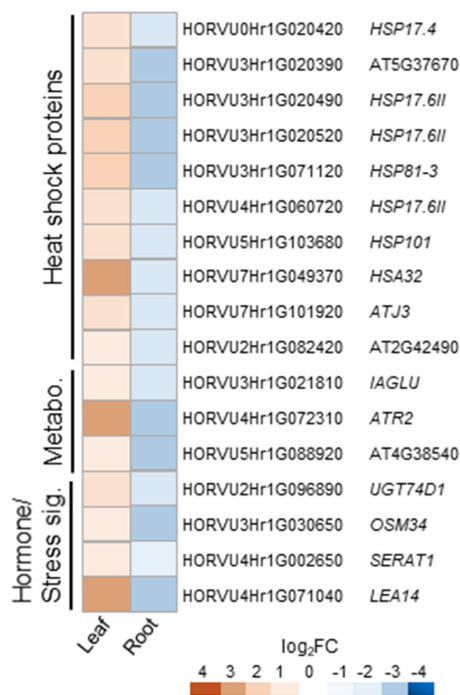


FIGURE 6

Selected counter-regulated DEGs in leaves and roots upon H_2O_2 treatment. Genes up-regulated in leaves and down-regulated in roots are grouped by functional category and presented with their Arabidopsis orthologs. Metabo., metabolism; sig., signaling.

(Driedonks et al., 2015). The SHSPs in our data set belong to subfamilies with close orthologs in Arabidopsis, i.e. HSP17.6, 15.4, 15.7, and 17.4 (Li and Liu, 2019). HSP17.6 and HSP15.7 have been shown to be localized in the peroxisomes in Arabidopsis (Ma et al., 2006; Li et al., 2017). Peroxisomes are one of the main subcellular compartments in which ROS are produced by processes such as β -oxidation and photorespiration, and which are crucial for antioxidant defense (Sandalio et al., 2013; del Río and López-Huertas, 2016). Additionally, HSP17.4 and 17.6 have been shown to exhibit increased transcript levels during periods of abiotic stress in Arabidopsis (Swindell et al., 2007). Thus, the induction of these HSPs points to a potential role of these proteins in increasing the tolerance to oxidative stress also in barley leaves. The single down-regulated SHSP is an ortholog to *AtHSP15.4*, for which this contrary behavior upon stress was already described (Siddique et al., 2008).

Not surprising, considering the well-established juxtaposition between ROS production and photosynthesis, the application of H_2O_2 negatively affected several photosynthetic components (Table 2). The most affected group represents chlorophyll a/b binding proteins orthologous to various light-harvesting complex proteins of the LHCb-type and to a component of the light-harvesting complex I, LHCA1, of Arabidopsis. Down-regulation of LHCb-type proteins upon oxidative stress has been previously described (Staneloni et al., 2008). It is likely part of an established photoprotection mechanism to alleviate increased ROS levels generated when the photosynthesis reaction becomes unbalanced, e.g., under high light conditions.

The role of phytohormones like ABA and jasmonate in response to several biotic and abiotic stimuli has been extensively studied in plants

(Verma et al., 2016). In our data, several genes related to jasmonate signaling were found to be down-regulated (Table 2), including an ortholog of Arabidopsis *12-OXOPHYTODIENOATE REDUCTASE* (OPR). The OPR3 protein of Arabidopsis has been denoted as one of the most crucial enzymes in jasmonate synthesis, which converts 12-oxophytodienoic acid (*cis*-OPDA) to OPC8:0 in peroxisomes (Bittner et al., 2022). However, recent studies highlighted the role of an OPR3-independent pathway for jasmonic acid (JA) biosynthesis, involving an OPR2-mediated alternative bypass *via* dinor-OPDA (dnOPDA) and 4,5-didehydro-JA, which is then converted to JA (Chini et al., 2018). Interestingly, we found a down-regulation of the barley ortholog of OPR2 in leaves, the consequence of which remains speculative due to the unclear role of the OPR3-independent bypass pathway. By contrast, genes coding for *ALLENE OXIDE CYCLASE* (AOC) and *ALLENE OXIDE SYNTHASE* (AOS) were up-regulated in leaves. These enzymes catalyze the generation of both *cis*-OPDA and dnOPDA, which in turn would increase OPDA production for both pathways. This is interesting, because OPDA is believed to have an independent regulatory function both on transcription (similar to JA-Ile), but also on protein activity by OPDadylation. Moreover, OPDA-mediated signaling seems closely associated with thiol metabolism and redox-mediated processes (Böttcher and Weiler, 2007; Ohkama-Ohtsu et al., 2011; Bittner et al., 2022). Also related to jasmonate signaling are two TIFY domain-containing proteins that were induced in response to H_2O_2 (Table 2). The TIFY domain is found in members of the JASMONATE ZIM DOMAIN (JAZ)-type transcriptional repressors involved in jasmonate signaling (Chung and Howe, 2009; Pauwels and Goossens, 2011). However, no regulation of TFs associated with jasmonate signaling was detected in our data set.

By contrast, many of the genes associated with other phytohormones, e.g. auxins and ABA, encode TFs or other proteins involved in transcription regulation (Table 2). Several of these genes belong to the large family of AP2/ERF-type TFs, members of which have been associated with environmental stresses including hypoxia and oxidative stress. While mostly associated with ethylene, AP2/ERF function is also connected to ABA, gibberellic acid, cytokinin, and brassinosteroids (Xie et al., 2019). The largest group of genes associated with hormones relates to auxin (Table 2), the role of which is mostly associated with development and growth. However, experimental evidence linked auxin also to oxidative stress, especially auxin-mediated stress-dependent cell proliferation including the RSL-type TF ROOT HAIR DEFECTIVE SIX-LIKE4 (RSL4) that targets NADPH oxidases also known as respiratory burst oxidase homologs (RBOHs) and secreted plant-specific type III peroxidases that impact apoplastic ROS homeostasis and in turn stimulate root hair cell elongation (Pasternak et al., 2005; Iglesias et al., 2010; Mangano et al., 2017).

4.2 Root-specific transcriptomic changes in response to H_2O_2

In roots, many DEGs were found to be associated with the detoxification of H_2O_2 (Table 3), especially peroxidases and genes related to glutathione metabolism. *GLUTATHIONE TRANSFERASES* (GSTs) and *GLUTATHIONE PEROXIDASES*

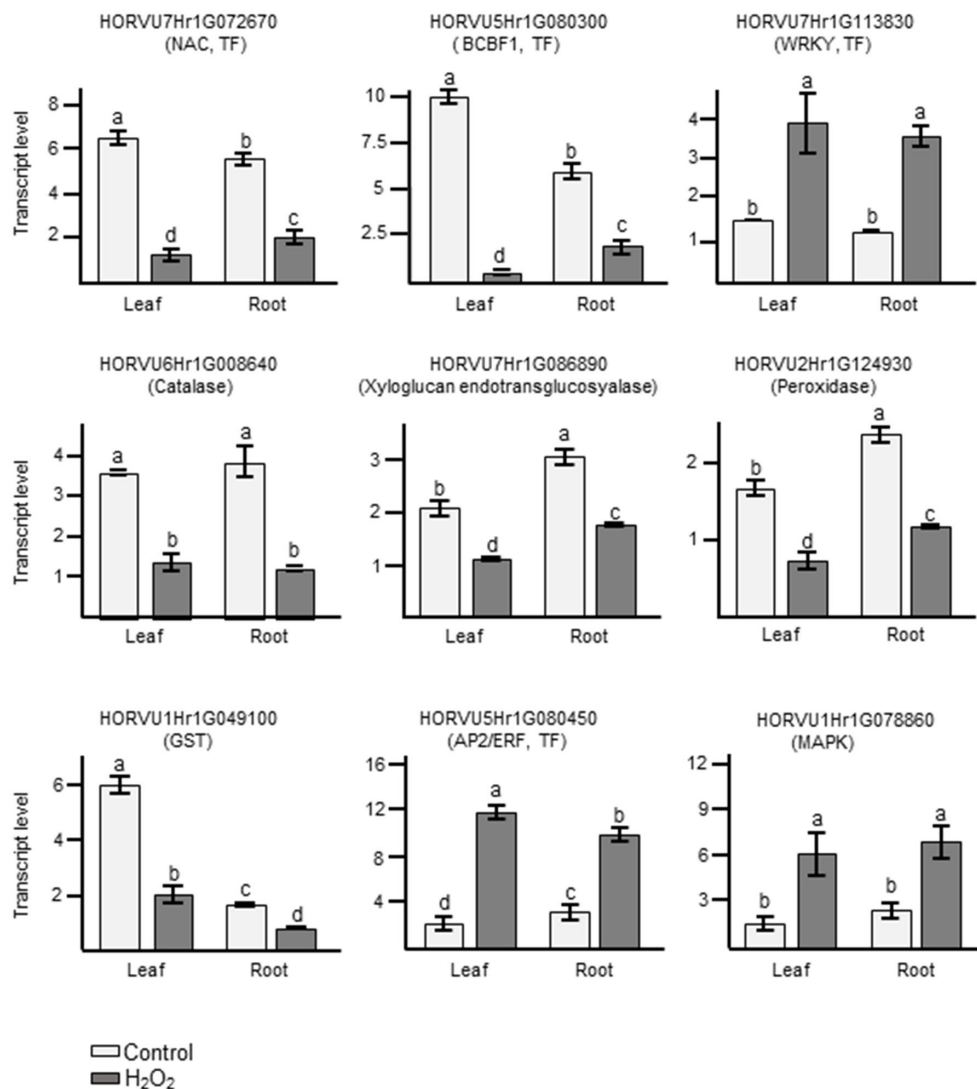


FIGURE 7

Analyses of transcript levels for selected candidate genes by qRT-PCR. Data represent means $\pm$ SE of three biological replicates ($n=3$), each having two technical repeats. Transcript levels were normalized to *HvACTIN* and *HvGAPDH*. Letters represent significant differences estimated using one-way ANOVA and Tukey's Post-Hoc HSD test ($P<0.05$). Potential functions of the genes inferred from orthologous genes in Arabidopsis are indicated in brackets.

(GTPs) have both been shown to be involved in plant stress responses (Bela et al., 2015; Nianiou-Obeidat et al., 2017). However, somewhat surprisingly, our data showed clear down-regulation of several GSTs and GTPs along with other key players associated with H₂O₂ detoxification such as orthologs of Arabidopsis *ASCORBATE PEROXIDASE 1* (APX1) and *CATALASE 1* (CAT1). Moreover, two putative *DETOXIFICATION EFFLUX CARRIERS/MULTIDRUG AND TOXIC COMPOUND EXTRUSION* (DXT/MATE) proteins were strongly up-regulated in roots. The MATE family proteins facilitate the efflux of various compounds including substances, such as hormones or flavonoids, that improve adaptation to stress (Ku et al., 2022).

The largest set of genes whose expression was affected in response to H₂O₂ belongs to class III plant type peroxidases (Table 3), whose role in plant defense mechanisms in response to

a wide variety of biotic and abiotic stresses is well established. They play an important role in the cellular redox homeostasis upon stress. In addition, they also catalyze the oxidation of a variety of substrates and have been linked to processes involved in cell wall stability, including lignin and suberin polymerization in response to stress (Kidwai et al., 2020). Thus, the up-regulation of these peroxidases in roots upon H₂O₂ treatment is in line with the up-regulation of genes involved in cell wall metabolism observed in this study. Some components of the cell wall architecture, particularly the xyloglucans, have been shown to play an important role in imparting abiotic stress tolerance by coordinating with hormonal and other signaling cascades. For example, a xyloglucan galactosyl transferase from Arabidopsis, *SHORT ROOT IN SALT MEDIUM 3* (RSA3), was shown to play a crucial role under salt stress by assembling actin microfilaments and thus preventing ROS

accumulation induced by disruption of actin microfilaments (Cho et al., 2006; Li et al., 2013). Also the role of xyloglucan modifying enzymes along with expansins in loosening and expanding the cell wall network upon abiotic stresses has already been described (Tenhaken, 2015).

4.3 Commonly and counter-regulated DEGs in responses to H₂O₂

Overall, leaves and roots showed very unique transcriptional responses upon H₂O₂ treatment. Not only the number of DEGs was much higher in roots compared to leaves, the change in transcription also affected a quite different set of genes (Figures 2, 3). Nevertheless, there are DEGs that were found in both plant parts (Figure 4). These 349 DEGs were further divided into four clusters, depending on their expression pattern. Looking at the two larger clusters, the commonly up- or down-regulated DEGs (Figure 5, Supplementary Table S3 and S4), certain patterns in the functional categories can be observed. Both clusters include TFs from different families. This is not unexpected and highlights their versatility in differentially regulating genes as an important part of all stress responses (Javed et al., 2020). However, of the TFs identified in this study, only few have previously been associated with oxidative stress, such as an Arabidopsis ortholog to *HORVU2Hr1G066080* and *HORVU3Hr1G016320*, the *LOB DOMAIN CONTAINING PROTEIN 41 (LBD41)*, that was previously identified in relation with low-oxygen endurance or high-light-induced increase in H₂O₂ (Mustroph et al., 2009; Vanderauwera et al., 2011). However, some were found associated with stresses, such as herbivory, that include ROS-mediated signaling or mutations that cause increased levels of ROS (Paudel et al., 2013; Garcia et al., 2016).

Several transporters were found commonly down-regulated (Supplementary Table S4 and Figure 5A). The aquaporin encoded by *HORVU4Hr1G085250* is orthologous to the *TONOPLAST INTRINSIC PROTEIN 4;1 (TIP4;1)* of Arabidopsis and rice. Aquaporins not only transport water but also other molecules including H₂O₂. *TIP4;1* from barley was shown to be up-regulated by ABA in roots and gibberellic acid in shoots (Ligaba et al., 2011). Moreover, its expression was also up-regulated upon drought (Kurowska et al., 2019). Also sugar transporters of the SWEET-type and PHT1.7 phosphate transporters have been demonstrated to play a role in abiotic stress tolerance and showed variable expression patterns under stress conditions (Cao et al., 2020; Gautam et al., 2022).

We also found common down-regulation of orthologs to *RECEPTOR-LIKE PROTEIN KINASES (RLKs)* from different subfamilies, i.e., WAK, LLR, CRK and RLCK (Supplementary Table S4 and Figure 5A). Experimental evidence suggests that RLKs are a vital part of the growth-defense trade-off, i.e. by facilitating the cross-talk between different phytohormones (Zhu et al., 2023). However, of the specific *RLKs* found commonly down-regulated in barley leaves and roots, only the pepper ortholog of *WAKL20* was described in relation to stress (Zhu et al., 2023). DEGs connected to various facets of primary metabolism were found commonly up-regulated

(Supplementary Table S4 and Figure 5B). While several of them are involved in pathways that play a role in stress responses, an obvious connection between these specific DEGs is lacking. Overall, even if no clear connection to oxidative stress exists, many of the commonly regulated DEGs have been described or postulated previously to be involved in stress tolerance mechanisms.

A very small number of DEGs was found counter-regulated upon treatment with H₂O₂ (Supplementary Table S4 and Figure 6), the majority of which showing up-regulation in leaves and down-regulation in roots. Several of those genes are connected to aspects of metabolism and hormone signaling, and some orthologous genes of other plant species, such as *SERAT1*, *OSM34*, and *UGT74D1* of tomato, grapevine and Arabidopsis have been previously connected to stress, ABA signaling, or auxin (Tavares et al., 2015; Jin et al., 2021; Park and Kim, 2021; Liu et al., 2022). Remarkably, this cluster also includes a group of nine *HSPs*, and this different expression in leaves and roots raises questions about their specific role in stress response in the different tissues.

5 Conclusions

Plant adaptation to changing environmental cues requires acclimation, enabling them to fulfil their lifecycle. This adaptation is based to a large extent on substantial changes on transcriptional level. Our data reveal that H₂O₂ modulates the expression of a wide range of genes within the barley genome. The results provide first insights into the significant role of H₂O₂ in altering cellular activities in this important crop species. However, in which manner all these genes are coordinated within the cell to provide an appropriate response during stress-induced H₂O₂ increase is an important question that needs to be addressed in further research. Many of them have previously been associated to stress responses in barley or more often *via* their orthologs in Arabidopsis or other crops. This reveals a high degree of similarity in the responses of these plants to situations where cellular H₂O₂ levels increase either as a toxic by-product of stress or as a dedicated signaling molecule. Other genes identified in this screen have so far not been associated with stress. As important redox molecules participating in plant cell signaling, developmental processes stress responses, as well as causing oxidative damage, uncovering the effect of ROS generally and H₂O₂ specifically on gene expression provides good insights into the molecular mechanisms of oxidative stress responses in barley. Such understanding might increase our ability to improve stress resistance in barley and other crops to optimize crop performance and productivity in present and future environmental climate challenges. Particularly, the highest up- or down-regulated genes in our dataset in both tissues were mostly uncharacterized and information on the exact nature of the genes is missing. These data can be used to guide future studies aimed to functionally characterize novel stress-related genes using state-of-the-art experimental designs including generation of mutants and ectopic expression lines. This will enable us to better understand H₂O₂ mediated regulation of adaptive processes not only in barley but also in other crops and might thus support targeted breeding of more resilient crops.

Data availability statement

The datasets presented in this study can be found in online repositories (<https://www.ncbi.nlm.nih.gov/sra/PRJNA973626>).

Author contributions

SB contributed to conceptualization, investigation (responsible for most experimental work), formal analysis (responsible for all bioinformatic analysis), validation, visualization, and writing - original draft as well as review & editing. MG contributed to investigation. BM contributed to validation (qRT-PCR) and writing - review & editing. EP contributed to supervision and writing - review and editing. UV contributed to conceptualization, validation, funding acquisition, project administration, supervision, and writing - review & editing. FC contributed to conceptualization, formal analysis, validation, visualization, supervision, and writing - original draft as well as review & editing. All authors contributed to the article and approved the submitted version.

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References

- Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., and Sharma, S. (2010). Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. *Crit. Rev. Biotechnol.* 30, 161–175. doi: 10.3109/07388550903524243
- Almagro, L., Gómez Ros, L. V., Belchi-Navarro, S., Bru, R., Ros Barceló, A., and Pedreño, M. A. (2009). Class III peroxidases in plant defence reactions. *J. Exp. Bot.* 60, 377–390. doi: 10.1093/jxb/ern277
- Basha, E., O'Neill, H., and Vierling, E. (2012). Small heat shock proteins and α -crystallins: dynamic proteins with flexible functions. *Trends Biochem. Sci.* 37, 106–117. doi: 10.1016/j.tibs.2011.11.005
- Bela, K., Horváth, E., Gallé, Á., Szabados, L., Tari, I., and Csiszár, J. (2015). Plant glutathione peroxidases: Emerging role of the antioxidant enzymes in plant development and stress responses. *J. Plant Physiol.* 176, 192–201. doi: 10.1016/j.jplph.2014.12.014
- Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x
- Bieker, S., Riestler, L., Stahl, M., Franzaring, J., and Zentgraf, U. (2012). Senescence-specific Alteration of Hydrogen Peroxide Levels in Arabidopsis thaliana and Oilseed Rape Spring Variety Brassica napus L. cv. MozartF. *J. Integr. Plant Biol.* 54, 540–554. doi: 10.1111/j.1744-7909.2012.01147.x
- Bienert, G. P., Möller, A. L. B., Kristiansen, K. A., Schulz, A., Möller, I. M., Schjoerring, J. K., et al. (2007). Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes*. *J. Biol. Chem.* 282, 1183–1192. doi: 10.1074/jbc.M603761200
- Bittner, A., Cieśla, A., Gruden, K., Lukan, T., Mahmud, S., Teige, M., et al. (2022). Organellar and phytohormones: a network of interactions in plant stress responses. *J. Exp. Bot.* 73, 7165–7181. doi: 10.1093/jxb/erac384
- Böttcher, C., and Weiler, E. W. (2007). cyclo-Oxylipin-galactolipids in plants: occurrence and dynamics. *Planta* 226, 629–637. doi: 10.1007/s00425-007-0511-5
- Bourdais, G., Burdiak, P., Gauthier, A., Nitsch, L., Salojärvi, J., Rayapuram, C., et al. (2015). Large-scale phenomics identifies primary and fine-tuning roles for CRKs in

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2023.1223778/full#supplementary-material>

- responses related to oxidative stress. *PloS Genet.* 11, e1005373. doi: 10.1371/journal.pgen.1005373
- Cao, M., Liu, H., Zhang, C., Wang, D., Liu, X., and Chen, Q. (2020). Functional analysis of stPHT1;7, a solanum tuberosum L. Phosphate transporter gene, in growth and drought tolerance. *Plants* 9, 1384. doi: 10.3390/plants9101384
- Chaudhary, R., Baranwal, V. K., Kumar, R., Sircar, D., and Chauhan, H. (2019). Genome-wide identification and expression analysis of Hsp70, Hsp90, and Hsp100 heat shock protein genes in barley under stress conditions and reproductive development. *Funct. Integr. Genomics* 19, 1007–1022. doi: 10.1007/s10142-019-00695-y
- Chini, A., Monte, I., Zamarreño, A. M., Hamberg, M., Lassueur, S., Reymond, P., et al. (2018). An OPR3-independent pathway uses 4,5-didehydrojasmonate for jasmonate synthesis. *Nat. Chem. Biol.* 14, 171–178. doi: 10.1038/nchembio.2540
- Cho, S. K., Kim, J. E., Park, J.-A., Eom, T. J., and Kim, W. T. (2006). Constitutive expression of abiotic stress-inducible hot pepper CaXTH3, which encodes a xyloglucan endotransglucosylase/hydrolase homolog, improves drought and salt tolerance in transgenic Arabidopsis plants. *FEBS Lett.* 580, 3136–3144. doi: 10.1016/j.febslet.2006.04.062
- Chung, H. S., and Howe, G. A. (2009). A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in arabidopsis. *Plant Cell* 21, 131–145. doi: 10.1105/tpc.108.064097
- del Rio, L. A., and López-Huertas, E. (2016). ROS generation in peroxisomes and its role in cell signaling. *Plant Cell Physiol.* 57, 1364–1376. doi: 10.1093/pcp/pcw076
- Desikan, R., A.-H.-Mackerness, S., Hancock, J. T., and Neill, S. J. (2001). Regulation of the arabidopsis transcriptome by oxidative stress. *Plant Physiol.* 127, 159–172. doi: 10.1104/pp.127.1.159
- Dodd, A. N., Kudla, J., and Sanders, D. (2010). The language of calcium signaling. *Annu. Rev. Plant Biol.* 61, 593–620. doi: 10.1146/annurev-arplant-070109-104628
- Driedonks, N., Xu, J., Peters, J. L., Park, S., and Rieu, I. (2015). Multi-level interactions between heat shock factors, heat shock proteins, and the redox system regulate acclimation to heat. *Front. Plant Sci.* 6. doi: 10.3389/fpls.2015.00999

- Dudziak, K., Zapalska, M., Börner, A., Szczerba, H., Kowalczyk, K., and Nowak, M. (2019). Analysis of wheat gene expression related to the oxidative stress response and signal transduction under short-term osmotic stress. *Sci. Rep.* 9:2743. doi: 10.1038/s41598-019-39154-w
- Fariduddin, Q., Khan, T. A., and Yusuf, M. (2014). Hydrogen peroxide mediated tolerance to copper stress in the presence of 28-homobrassinolide in *Vigna radiata*. *Acta Physiol. Plant* 36, 2767–2778. doi: 10.1007/s11738-014-1647-0
- Foyer, C. H., and Noctor, G. (2003). Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol. Plant* 119, 355–364. doi: 10.1034/j.1399-3054.2003.00223.x
- Garcia, L., Welchen, E., Gey, U., Arce, A. L., Steinebrunner, I., and Gonzalez, D. H. (2016). The cytochrome c oxidase biogenesis factor AtCOX17 modulates stress responses in *Arabidopsis*. *Plant Cell Environ.* 39, 628–644. doi: 10.1111/pce.12647
- Gautam, T., Dutta, M., Jaiswal, V., Zinta, G., Gahlaut, V., and Kumar, S. (2022). Emerging roles of SWEET sugar transporters in plant development and abiotic stress responses. *Cells* 11, 1303. doi: 10.3390/cells11081303
- Ge, S. X., Jung, D., and Yao, R. (2020). ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* 36, 2628–2629. doi: 10.1093/bioinformatics/btz931
- Giridhar, M., Meier, B., Imani, J., Kogel, K.-H., Peiter, E., Vothknecht, U. C., et al. (2022). Comparative analysis of stress-induced calcium signals in the crop species barley and the model plant *Arabidopsis thaliana*. *BMC Plant Biol.* 22, 447. doi: 10.1186/s12870-022-03820-5
- Gürel, F., Öztürk, Z. N., Uçarlı, C., and Rosellini, D. (2016). Barley genes as tools to confer abiotic stress tolerance in crops. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.01137
- Guzel, S., and Terzi, R. (2013). Exogenous hydrogen peroxide increases dry matter production, mineral content and level of osmotic solutes in young maize leaves and alleviates deleterious effects of copper stress. *Bot. Stud.* 54, 26. doi: 10.1186/1999-3110-54-26
- Hellmann, A., Meyer, C. U., and Wernicke, W. (1995). Tubulin gene expression during growth and maturation of leaves with different developmental patterns. *Cell Motil.* 30, 67–72. doi: 10.1002/cm.970300108
- Hieno, A., Naznin, H. A., Inaba-Hasegawa, K., Yokogawa, T., Hayami, N., Nomoto, M., et al. (2019). Transcriptome analysis and identification of a transcriptional regulatory network in the response to H₂O₂. *Plant Physiol.* 180, 1629–1646. doi: 10.1104/pp.18.01426
- Hirai, M. Y., Yano, M., Goodenowe, D. B., Kanaya, S., Kimura, T., Awazuhara, M., et al. (2004). Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci.* 101, 10205–10210. doi: 10.1073/pnas.0403218101
- Hlaváčková, I., Vitámvás, P., Šantrůček, J., Kosová, K., Zelenková, S., Prášil, I. T., et al. (2013). Proteins Involved in Distinct Phases of Cold Hardening Process in Frost Resistant Winter Barley (*Hordeum vulgare* L.) cv Luxor. *Int. J. Mol. Sci.* 14, 8000–8024. doi: 10.3390/ijms14048000
- Hossain, M. A., Bhattacharjee, S., Armin, S.-M., Qian, P., Xin, W., Li, H.-Y., et al. (2015). Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. *Front. Plant Sci.* 6. doi: 10.3389/fpls.2015.00420
- Huang, H., Ullah, F., Zhou, D.-X., Yi, M., and Zhao, Y. (2019). Mechanisms of ROS regulation of plant development and stress responses. *Front. Plant Sci.* 10. doi: 10.3389/fpls.2019.00800
- Iglesias, M. J., Terrile, M. C., Bartoli, C. G., D'Ippolito, S., and Casalongué, C. A. (2010). Auxin signaling participates in the adaptive response against oxidative stress and salinity by interacting with redox metabolism in *Arabidopsis*. *Plant Mol. Biol.* 74, 215–222. doi: 10.1007/s11103-010-9667-7
- Janiak, A., Kwasniewski, M., Sowa, M., Gajek, K., Żmuda, K., Kościelniak, J., et al. (2018). No time to waste: transcriptome study reveals that drought tolerance in barley may be attributed to stressed-like expression patterns that exist before the occurrence of stress. *Front. Plant Sci.* 8. doi: 10.3389/fpls.2017.02212
- Javed, T., Shabbir, R., Ali, A., Afzal, I., Zaheer, U., and Gao, S.-J. (2020). Transcription factors in plant stress responses: challenges and potential for sugarcane improvement. *Plants* 9, 491. doi: 10.3390/plants9040491
- Jin, S., Hou, B., and Zhang, G. (2021). The ectopic expression of *Arabidopsis* glucosyltransferase UGT74D1 affects leaf positioning through modulating indole-3-acetic acid homeostasis. *Sci. Rep.* 11, 1154. doi: 10.1038/s41598-021-81016-x
- Kärkönen, A., and Kuchitsu, K. (2015). Reactive oxygen species in cell wall metabolism and development in plants. *Mem. G. Paul Bolwell Plant Cell Wall Dyn.* 112, 22–32. doi: 10.1016/j.phytochem.2014.09.016
- Kaur, N., Dhawan, M., Sharma, I., and Pati, P. K. (2016). Interdependency of Reactive Oxygen Species generating and scavenging system in salt sensitive and salt tolerant cultivars of rice. *BMC Plant Biol.* 16, 131. doi: 10.1186/s12870-016-0824-2
- Khan, T. A., Yusuf, M., and Fariduddin, Q. (2018). Hydrogen peroxide in regulation of plant metabolism: Signalling and its effect under abiotic stress. *Photosynthetica* 56, 1237–1248. doi: 10.1007/s11099-018-0830-8
- Kidwai, M., Ahmad, I. Z., and Chakrabarty, D. (2020). Class III peroxidase: an indispensable enzyme for biotic/abiotic stress tolerance and a potent candidate for crop improvement. *Plant Cell Rep.* 39, 1381–1393. doi: 10.1007/s00299-020-02588-y
- Ku, Y.-S., Cheng, S.-S., Cheung, M.-Y., and Lam, H.-M. (2022). The roles of multidrug and toxic compound extrusion (MATE) transporters in regulating agronomic traits. *Agronomy* 12, 878. doi: 10.3390/agronomy12040878
- Kurowska, M., Małgorzata, Fahad, S., Saud, S., Chen, Y., Wu, C., and Wang, D. (2020). “TIP aquaporins in plants: role in abiotic stress tolerance,” in *Abiotic stress in plants* (Rijeka: IntechOpen). doi: 10.5772/intechopen.94165
- Kurowska, M. M., Wiecha, K., Gajek, K., and Szarejko, I. (2019). Drought stress and re-watering affect the abundance of TIP aquaporin transcripts in barley. *PLoS One* 14, e0226423. doi: 10.1371/journal.pone.0226423
- Landi, S., Capasso, G., Ben Azaiez, F. E., Jallouli, S., Ayadi, S., Trifa, Y., et al. (2019). Different Roles of Heat Shock Proteins (70 kDa) During Abiotic Stresses in Barley (*Hordeum vulgare*) Genotypes. *Plants* 8, 248. doi: 10.3390/plants8080248
- Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923
- Li, W., Guan, Q., Wang, Z.-Y., Wang, Y., and Zhu, J. (2013). A bi-functional xyloglucan galactosyltransferase is an indispensable salt stress tolerance determinant in *Arabidopsis*. *Mol. Plant* 6, 1344–1354. doi: 10.1093/mp/sst062
- Li, X., Li, Y., Ahammed, G. J., Zhang, X.-N., Ying, L., Zhang, L., et al. (2019). RBOH1-dependent apoplastic H₂O₂ mediates epigallocatechin-3-gallate-induced abiotic stress tolerance in *Solanum lycopersicum* L. *Revisiting Role ROS RNS Plants Change Environ.* 161, 357–366. doi: 10.1016/j.envexpbot.2018.11.013
- Li, G., Li, J., Hao, R., and Guo, Y. (2017). Activation of catalase activity by a peroxisome-localized small heat shock protein Hsp17.6CII. *J. Genet. Genomics* 44, 395–404. doi: 10.1016/j.jgg.2017.03.009
- Li, J., and Liu, X. (2019). Genome-wide identification and expression profile analysis of the Hsp20 gene family in Barley (*Hordeum vulgare* L.). *PeerJ* 7, e6832. doi: 10.7717/peerj.6832
- Liao, Y., Smyth, G. K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930. doi: 10.1093/bioinformatics/btt656
- Ligaba, A., Katsuhara, M., Shibasaki, M., and Djira, G. (2011). Abiotic stresses modulate expression of major intrinsic proteins in barley (*Hordeum vulgare*). *C. R. Biol.* 334, 127–139. doi: 10.1016/j.crv.2010.11.005
- Liu, D., Li, M., Guo, T., Lu, J., Xie, Y., Hao, Y., et al. (2022). Functional characterization of the Serine acetyltransferase family genes uncovers the diversification and conservation of cysteine biosynthesis in tomato. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.913856
- Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262
- Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550. doi: 10.1186/s13059-014-0550-8
- Lu, P., Magwanga, R. O., Kirungu, J. N., Hu, Y., Dong, Q., Cai, X., et al. (2019). Overexpression of cotton a DTX/MATE gene enhances drought, salt, and cold stress tolerance in transgenic *Arabidopsis*. *Front. Plant Sci.* 10. doi: 10.3389/fpls.2019.00299
- Ma, C., Haslbeck, M., Babujee, L., Jahn, O., and Reumann, S. (2006). Identification and characterization of a stress-inducible and a constitutive small heat-shock protein targeted to the matrix of plant peroxisomes. *Plant Physiol.* 141, 47–60. doi: 10.1104/pp.105.073841
- Mangano, S., Denita-Juarez, S. P., Choi, H.-S., Marzol, E., Hwang, Y., Ranocha, P., et al. (2017). Molecular link between auxin and ROS-mediated polar growth. *Proc. Natl. Acad. Sci.* 114, 5289–5294. doi: 10.1073/pnas.1701536114
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal* 17 (1), 10–12. doi: 10.14806/ej.17.1.200
- Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S. O., Wicker, T., et al. (2017). A chromosome conformation capture ordered sequence of the barley genome. *Nature* 544, 427–433. doi: 10.1038/nature22043
- Miller, G., and Mittler, R. (2006). Could heat shock transcription factors function as hydrogen peroxide sensors in plants? *Ann. Bot.* 98, 279–288. doi: 10.1093/aob/mcl107
- Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., et al. (2011). ROS signaling: the new wave? *Trends Plant Sci.* 16, 300–309. doi: 10.1016/j.tplants.2011.03.007
- Mohanta, T. K., Bashir, T., Hashem, A., Abd-Allah, E. F., Khan, A. L., and Al-Harasi, A. S. (2018). Early events in plant abiotic stress signaling: interplay between calcium, reactive oxygen species and phytohormones. *J. Plant Growth Regul.* 37, 1033–1049. doi: 10.1007/s00344-018-9833-8
- Moll, P., Ante, M., Seitz, A., and Reda, T. (2014). QuantSeq 3' mRNA sequencing for RNA quantification. *Nat. Methods* 11, i–iii. doi: 10.1038/nmeth.f.376
- Munns, R., James, R. A., and Läuchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. *J. Exp. Bot.* 57, 1025–1043. doi: 10.1093/jxb/erj100
- Mustroph, A., Zanetti, M. E., Jang, C. J. H., Holtan, H. E., Repetti, P. P., Galbraith, D. W., et al. (2009). Profiling translomes of discrete cell populations resolves altered cellular priorities during hypoxia in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 106, 18843–18848. doi: 10.1073/pnas.0906131106

- Mylona, P. V., Polidoros, A. N., and Scandalios, J. G. (2007). Antioxidant gene responses to ROS-generating xenobiotics in developing and germinated scutella of maize. *J. Exp. Bot.* 58, 1301–1312. doi: 10.1093/jxb/erl292
- Nefissi Ouertani, R., Arasappan, D., Abid, G., Ben Chikha, M., Jarak, R., Mahmoudi, H., et al. (2021). Transcriptomic analysis of salt-stress-responsive genes in barley roots and leaves. *Int. J. Mol. Sci.* 22, 8155. doi: 10.3390/ijms22158155
- Nianiou-Obeidat, I., Madesis, P., Kissoudis, C., Voulgari, G., Chronopoulou, E., Tsaftaris, A., et al. (2017). Plant glutathione transferase-mediated stress tolerance: functions and biotechnological applications. *Plant Cell Rep.* 36, 791–805. doi: 10.1007/s00299-017-2139-7
- Ohkama-Ohtsu, N., Sasaki-Sekimoto, Y., Oikawa, A., Jikumaru, Y., Shinoda, S., Inoue, E., et al. (2011). 12-oxo-phytodienoic acid–glutathione conjugate is transported into the vacuole in arabidopsis. *Plant Cell Physiol.* 52, 205–209. doi: 10.1093/pcp/pcq181
- Osthoff, A., Donà dalle Rose, P., Baldauf, J. A., Piepho, H.-P., and Hochholdinger, F. (2019). Transcriptomic reprogramming of barley seminal roots by combined water deficit and salt stress. *BMC Genomics* 20, 325. doi: 10.1186/s12864-019-5634-0
- Park, E.-J., and Kim, T.-H. (2021). Arabidopsis OSMOTIN 34 functions in the ABA signaling pathway and is regulated by proteolysis. *Int. J. Mol. Sci.* 22, 7915. doi: 10.3390/ijms22157915
- Pasternak, T., Potters, G., Caubergs, R., and Jansen, M. A. K. (2005). Complementary interactions between oxidative stress and auxins control plant growth responses at plant, organ, and cellular level. *J. Exp. Bot.* 56, 1991–2001. doi: 10.1093/jxb/eri196
- Paudel, J., Copley, T., Amirizian, A., Prado, A., and Bede, J. (2013). Arabidopsis redox status in response to caterpillar herbivory. *Front. Plant Sci.* 4. doi: 10.3389/fpls.2013.00113
- Pauwels, L., and Goossens, A. (2011). The JAZ proteins: A crucial interface in the jasmonate signaling cascade. *Plant Cell* 23, 3089–3100. doi: 10.1105/tpc.111.089300
- Pei, Z.-M., Murata, Y., Benning, G., Thomine, S., Klüsener, B., Allen, G. J., et al. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406, 731–734. doi: 10.1038/35021067
- Peiter, E. (2016). The ever-closer union of signals: propagating waves of calcium and ROS are inextricably linked. *Plant Physiol.* 172, 3–4. doi: 10.1104/pp.16.01037
- Planas-Portell, J., Gallart, M., Tiburcio, A. F., and Altabella, T. (2013). Copper-containing amine oxidases contribute to terminal polyamine oxidation in peroxisomes and apoplast of Arabidopsis thaliana. *BMC Plant Biol.* 13, 109. doi: 10.1186/1471-2229-13-109
- Polidoros, A. N., Mylona, P. V., Pasentsis, K., Scandalios, J. G., and Tsaftaris, A. S. (2005). The maize alternative oxidase 1a (Aox1a) gene is regulated by signals related to oxidative stress. *Redox Rep.* 10, 71–78. doi: 10.1179/135100005X21688
- R Core Team. (2020). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for statistical computing. Available at: <https://www.R-project.org>.
- Ribeiro, C. W., Korbes, A. P., Garighan, J. A., Jardim-Messeder, D., Carvalho, F. E. L., Sousa, R. H. V., et al. (2017). Rice peroxisomal ascorbate peroxidase knockdown affects ROS signaling and triggers early leaf senescence. *Plant Sci.* 263, 55–65. doi: 10.1016/j.plantsci.2017.07.009
- Rollins, J. A., Habte, E., Templer, S. E., Colby, T., Schmidt, J., and von Korff, M. (2013). Leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (*Hordeum vulgare* L.). *J. Exp. Bot.* 64, 3201–3212. doi: 10.1093/jxb/ert158
- Sandalio, L. M., Rodríguez-Serrano, M., Romero-Puertas, M. C., and del Río, L. A. (2013). “Role of peroxisomes as a source of reactive oxygen species (ROS) signaling molecules,” in *Peroxisomes and their key role in cellular signaling and metabolism*. Ed. L.A. del Río (Dordrecht: Springer Netherlands), 231–255. doi: 10.1007/978-94-007-6889-5_13
- Saxena, I., Srikanth, S., and Chen, Z. (2016). Cross Talk between H₂O₂ and Interacting Signal Molecules under Plant Stress Response. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.00570
- Schaller, A., and Stintzi, A. (2009). Enzymes in jasmonate biosynthesis – Structure, function, regulation. *Jasmonates Stress Responses Dev.* 70, 1532–1538. doi: 10.1016/j.phytochem.2009.07.032
- Shigeto, J., and Tsutsumi, Y. (2016). Diverse functions and reactions of class III peroxidases. *New Phytol.* 209, 1395–1402. doi: 10.1111/nph.13738
- Shimakawa, G., Roach, T., and Krieger-Liszka, A. (2020). Changes in photosynthetic electron transport during leaf senescence in two barley varieties grown in contrasting growth regimes. *Plant Cell Physiol.* 61, 1986–1994. doi: 10.1093/pcp/pcaa114
- Siddique, M., Gernhard, S., von Koskull-Döring, P., Vierling, E., and Scharf, K.-D. (2008). The plant sHSP superfamily: five new members in Arabidopsis thaliana with unexpected properties. *Cell Stress Chaperones* 13, 183–197. doi: 10.1007/s12192-008-0032-6
- Smirnov, N., and Arnaud, D. (2019). Hydrogen peroxide metabolism and functions in plants. *New Phytol.* 221, 1197–1214. doi: 10.1111/nph.15488
- Staneloni, R. J., Rodriguez-Batiller, M. J., and Casal, J. J. (2008). Absciscic acid, high-light, and oxidative stress down-regulate a photosynthetic gene via a promoter motif not involved in phytochrome-mediated transcriptional regulation. *Mol. Plant* 1, 75–83. doi: 10.1093/mp/ssm007
- Stanley Kim, H., Yu, Y., Snesrud, E. C., Moy, L. P., Linford, L. D., Haas, B. J., et al. (2005). Transcriptional divergence of the duplicated oxidative stress-responsive genes in the Arabidopsis genome. *Plant J.* 41, 212–220. doi: 10.1111/j.1365-3113.2004.02295.x
- Steffens, B. (2014). The role of ethylene and ROS in salinity, heavy metal, and flooding responses in rice. *Front. Plant Sci.* 5. doi: 10.3389/fpls.2014.00685
- Swindell, W. R., Huebner, M., and Weber, A. P. (2007). Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. *BMC Genomics* 8, 125. doi: 10.1186/1471-2164-8-125
- Tavares, S., Wirtz, M., Beier, M. P., Bogs, J., Hell, R., and Amâncio, S. (2015). Characterization of the serine acetyltransferase gene family of Vitis vinifera uncovers differences in regulation of OAS synthesis in woody plants. *Front. Plant Sci.* 6. doi: 10.3389/fpls.2015.00074
- Tenhaken, R. (2015). Cell wall remodeling under abiotic stress. *Front. Plant Sci.* 5. doi: 10.3389/fpls.2014.00771
- Terzi, R., Kadioglu, A., Kalaycioglu, E., and Saglam, A. (2014). Hydrogen peroxide pretreatment induces osmotic stress tolerance by influencing osmolyte and abscisic acid levels in maize leaves. *J. Plant Interact.* 9, 559–565. doi: 10.1080/17429145.2013.871077
- Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7, 562–578. doi: 10.1038/nprot.2012.016
- ul Haq, S., Khan, A., Ali, M., Khattak, A. M., Gai, W.-X., Zhang, H.-X., et al. (2019). Heat shock proteins: dynamic biomolecules to counter plant biotic and abiotic stresses. *Int. J. Mol. Sci.* 20, 5321. doi: 10.3390/ijms20215321
- Vanderauwera, S., Suzuki, N., Miller, G., van de Cotte, B., Morsa, S., Ravanat, J.-L., et al. (2011). Extranuclear protection of chromosomal DNA from oxidative stress. *Proc. Natl. Acad. Sci.* 108, 1711–1716. doi: 10.1073/pnas.1018359108
- Verma, V., Ravindran, P., and Kumar, P. P. (2016). Plant hormone-mediated regulation of stress responses. *BMC Plant Biol.* 16, 86. doi: 10.1186/s12870-016-0771-y
- Wahid, A., Gelani, S., Ashraf, M., and Foolad, M. R. (2007). Heat tolerance in plants: An overview. *Environ. Exp. Bot.* 61, 199–223. doi: 10.1016/j.envexpbot.2007.05.011
- Wang, Y., Li, J., Wang, J., and Li, Z. (2010). Exogenous H₂O₂ improves the chilling tolerance of manilagrass and mascarenegrass by activating the antioxidative system. *Plant Growth Regul.* 61, 195–204. doi: 10.1007/s10725-010-9470-0
- Wang, R., Liu, S., Zhou, F., and Ding, C. (2014). Exogenous ascorbic acid and glutathione alleviate oxidative stress induced by salt stress in the chloroplasts of oryza sativa L. *Z. Naturforsch C J Biosci* 69, 226–236. doi: 10.5560/znc.2013-0117
- Wang, R.-S., Pandey, S., Li, S., Gookin, T. E., Zhao, Z., Albert, R., et al. (2011). Common and unique elements of the ABA-regulated transcriptome of Arabidopsis guard cells. *BMC Genomics* 12, 216. doi: 10.1186/1471-2164-12-216
- Waters, E. R. (2013). The evolution, function, structure, and expression of the plant sHSPs. *J. Exp. Bot.* 64, 391–403. doi: 10.1093/jxb/ers355
- Xie, Z., Nolan, T. M., Jiang, H., and Yin, Y. (2019). AP2/ERF transcription factor regulatory networks in hormone and abiotic stress responses in arabidopsis. *Front. Plant Sci.* 10. doi: 10.3389/fpls.2019.00228
- Yu, C.-W., Murphy, T. M., and Lin, C.-H. (2003). Hydrogen peroxide-induced chilling tolerance in mung beans mediated through ABA-independent glutathione accumulation. *Funct. Plant Biol.* 30, 955–963. doi: 10.1071/FP03091
- Zeng, J., Dong, Z., Wu, H., Tian, Z., and Zhao, Z. (2017). Redox regulation of plant stem cell fate. *EMBO J.* 36, 2844–2855. doi: 10.15252/emboj.201695955
- Zhu, J.-K. (2016). Abiotic stress signaling and responses in plants. *Cell* 167, 313–324. doi: 10.1016/j.cell.2016.08.029
- Zhu, Q., Feng, Y., Xue, J., Chen, P., Zhang, A., and Yu, Y. (2023). Advances in receptor-like protein kinases in balancing plant growth and stress responses. *Plants* 12, 427. doi: 10.3390/plants12030427

Appendix 2

RESEARCH

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Ca²⁺-dependent H₂O₂ response in roots and leaves of barley - a transcriptomic investigation

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Abstract

Background Ca²⁺ and H₂O₂ are second messengers that regulate a wide range of cellular events in response to different environmental and developmental cues. In plants, stress-induced H₂O₂ has been shown to initiate characteristic Ca²⁺ signatures; however, a clear picture of the molecular connection between H₂O₂-induced Ca²⁺ signals and H₂O₂-induced cellular responses is missing, particularly in cereal crops such as barley. Here, we employed RNA-seq analyses to identify transcriptome changes in roots and leaves of barley after H₂O₂ treatment under conditions that inhibited the formation of cytosolic Ca²⁺ transients. To that end, plasma membrane Ca²⁺ channels were blocked by LaCl₃ application prior to stimulation of barley tissues with H₂O₂.

Results We examined the expression patterns of 4246 genes that had previously been shown to be differentially expressed upon H₂O₂ application. Here, we further compared their expression between H₂O₂ and LaCl₃ + H₂O₂ treatment. Genes showing expression patterns different to the previous study were considered to be Ca²⁺-dependent H₂O₂-responsive genes. These genes, numbering 331 in leaves and 1320 in roots, could be classified in five and four clusters, respectively. Expression patterns of several genes from each cluster were confirmed by RT-qPCR. We furthermore performed a network analysis to identify potential regulatory paths from known Ca²⁺-related genes to the newly identified Ca²⁺-dependent H₂O₂ responsive genes, using the recently described Stress Knowledge Map. This analysis indicated several transcription factors as key points of the responses mediated by the cross-talk between H₂O₂ and Ca²⁺.

Conclusion Our study indicates that about 70% of the H₂O₂-responsive genes in barley roots require a transient increase in cytosolic Ca²⁺ concentrations for alteration in their transcript abundance, whereas in leaves, the Ca²⁺ dependency was much lower at about 33%. Targeted gene analysis and pathway modeling identified not only known components of the Ca²⁺ signaling cascade in plants but also genes that are not yet connected to stimuli-associated signaling. Potential key transcription factors identified in this study can be further analyzed in barley and other crops to ultimately disentangle the underlying mechanisms of H₂O₂-associated signal transduction mechanisms. This could

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aid breeding for improved stress resistance to optimize performance and productivity under increasing climate challenges.

Keywords ROS, Stress, RNA-Seq, Ca^{2+} signaling, Crosstalk, *Hordeum vulgare*

Introduction

To withstand short-term detrimental conditions, plants have evolved complex and efficient molecular machineries to monitor and respond to environmental cues. An early plant response to many forms of stress involves reactive oxygen species (ROS) as a purposefully generated signal to modulate crucial aspects of plant growth, development, and stress adaptation [1]. ROS also constitute inevitable by-products of aerobic metabolism that under normal physiological conditions are mainly produced at a low level; however, disruption of metabolic pathways during stress often results in a dramatic increase in their rate of production [2, 3]. Hydrogen peroxide (H_2O_2), a very stable ROS, is generated within different cellular compartments such as chloroplasts, mitochondria, and peroxisomes, as well as extra-cellularly in the apoplast [4]. H_2O_2 is generated either passively by metabolic pathways such as photosynthesis, photorespiration and respiration, or produced actively by oxidases like the respiratory burst oxidase homologs (RBOHs) [3]. Also, H_2O_2 can be transported between different cellular compartments, cells or even tissues for the purpose of removal or accumulation, and is now considered as an important player in long-distance-signaling [5, 6].

At low levels, H_2O_2 can be beneficial for the plant and act as a signal transduction molecule to achieve stress tolerance; however, it can cause cellular damage and programmed cell death at higher concentrations [7]. Hence, a strict balance between production and scavenging of H_2O_2 is essential to prevent its accumulation to toxic levels and to ensure its function as a signaling molecule. Plants have thus evolved a complex array of enzymatic and non-enzymatic detoxification systems to adjust the H_2O_2 homeostasis in all subcellular compartments [8, 9]. As signaling molecule, H_2O_2 is involved in the regulation of various developmental and physiological processes such as root system development [10, 11], flowering [12], seed germination [13], senescence [14] and stomatal aperture [15]. Additionally, studies have uncovered key roles for H_2O_2 as a second messenger in the signaling pathways associated with environmental stress responses in *Arabidopsis thaliana* and crop species such as drought [16, 17], salinity [18], heat [19, 20], UV radiation [21], ozone [22], chilling [23], heavy metal [24], and pathogens [25, 26]. Various stimuli can induce increases of H_2O_2 levels, known as the “oxidative burst”, which is subsequently sensed and transmitted to activate downstream processes including transcriptional reprogramming to elicit

appropriate adaptive stress responses [27]. Moreover, H_2O_2 can activate other signaling cascades involving secondary messengers such as nitric oxide, phytohormones, and Ca^{2+} .

Ca^{2+} also plays a pivotal role in the regulation of various developmental processes and response to environmental stresses. Changes in cytosolic free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_{\text{cyt}}$) are one of the earliest cellular responses observed in plants to almost every biotic and abiotic stress that has been investigated, including salt [28, 29], cold [30, 31], drought [32–34], heat [35, 36], heavy metals [37], and pathogens [38, 39]. The transient changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ are sensed and decoded by a toolkit of Ca^{2+} sensor proteins like calmodulins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs), and CBL-interacting protein kinases (CIPKs) as well as Ca^{2+} -dependent protein kinases (CPKs/CDPKs) [40]. Like H_2O_2 , Ca^{2+} signaling affects different cellular processes including regulation of gene transcription and associated downstream responses [41].

A crosstalk between Ca^{2+} and H_2O_2 signaling pathways has been shown in response to various abiotic and biotic stresses [42, 43]. A number of studies indicated that Ca^{2+} acts as an upstream component in H_2O_2 signaling by regulating H_2O_2 production. In plants, RBOHs possess a cytosolic N-terminal regulatory domain containing Ca^{2+} -binding EF-hand motifs and Ca^{2+} -dependent phosphorylation sites as targets for CPKs that are necessary for RBOH activation [44–46]. By contrast, there is also evidence that H_2O_2 acts as an upstream signal by inducing $[\text{Ca}^{2+}]_{\text{cyt}}$ transients involved in plant responses such as stomatal closure, programmed cell death, and other stress adaptation [47–49]. H_2O_2 -induced Ca^{2+} release is likely due to the direct regulation of Ca^{2+} -permeable channels. Annexins, cyclic nucleotide gated channels (CNGCs), and mechanosensitive ion channels (MSLs) have been proposed to function as H_2O_2 -activated Ca^{2+} channels that mediate cellular Ca^{2+} influxes [50, 51]. In a recent study a H_2O_2 -sensor in plants, H_2O_2 -INDUCED Ca^{2+} INCREASES 1 (HPCA1) was identified that mediates H_2O_2 -induced activation of Ca^{2+} channels in guard cells leading to elevation in $[\text{Ca}^{2+}]_{\text{cyt}}$ and in turn initiation of stomatal closure [52]. Intriguingly, it has been shown that HPCA1 is required for systemic ROS- and Ca^{2+} -mediated cell-to-cell signaling and that this includes the Ca^{2+} permeable channel MSL3 as well as the Ca^{2+} sensor CBL4 and its interacting protein kinase CIPK26 [51]. However, despite the large volume of reports and studies, it remains unclear how H_2O_2 and Ca^{2+} signals regulate

each other, what determines the directionality of the crosstalk, and what connects both signaling pathways to achieve their synergistic response.

We thus intended to identify the contribution of cytosolic Ca^{2+} signals to H_2O_2 -induced transcriptomic changes in leaves and roots of barley. Barley is an important global feed and food source and has been widely studied as a model for monocot crops due to its diploid nature and ease of cross-breeding [53, 54]. The effect of H_2O_2 on the transcriptome was recently elucidated in barley leaves and roots [55], revealing common as well as tissue-specific changes in transcript abundance of over 4000 genes including various transcription factors (TFs), genes associated with hormone pathways, and other vital functions such as photosynthesis, cell wall biogenesis, and H_2O_2 detoxification. It has also been shown that barley, as other plants, reacts to H_2O_2 application with a transient elevation in $[\text{Ca}^{2+}]_{\text{cyt}}$ [56]. For the comparative approach carried out in the current study, Ca^{2+} transients were pharmacologically inhibited by the well-known plasma membrane Ca^{2+} channel blocker LaCl_3 . RNA-seq analyses revealed that 1652 of the previously identified H_2O_2 responsive genes were fully or partially dependent on Ca^{2+} signals for their regulation since their differential expression was altered when the Ca^{2+} signal was inhibited by LaCl_3 . Subsequent network analyses provided testable hypotheses on the molecular mechanisms of the crosstalk between oxidative stress and Ca^{2+} signaling. Ultimately, understanding the underlying molecular processes of this crosstalk might increase our ability to improve stress resistance in barley and other crops to optimize performance and productivity under increasing climate challenges.

Materials and methods

Plant material, growth conditions, and stress treatment

Barley plants (*Hordeum vulgare* cultivar Golden Promise) were grown for five days in pots filled with water-soaked vermiculite in a climate-controlled growth chamber under long-day conditions with 16 h light at 20 °C and a light intensity of 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Philips TLD 18 W of alternating 830/840 light color temperature) and 8 h darkness at 18 °C. For stress treatments, five-day-old barley seedlings were removed from the pots and incubated in ddH₂O with or without 10 mM LaCl_3 for one hour, briefly rinsed and then treated with ddH₂O with or without 10 mM H_2O_2 for three hours. Seedlings were thoroughly rinsed before subsequent analyses.

H_2O_2 staining and microscopic analyses

A modified protocol from [57] was used to stain H_2O_2 in barley leaves and roots with 2',7'-dichlorodihydrofluorescein diacetate (H_2 -DCFDA; Thermo Fisher Scientific, USA). After stress treatment as described above,

the seedlings were washed carefully and treated with 10 μM H_2 -DCFDA in 0.25% DMSO in the dark for one hour, followed by vacuum infiltration for 1 min in a desiccator. Approximately 5 mm segments of both tissues were mounted on a slide using tape. The fluorescence of 2',7'-Dichlorofluorescein (DCF) was analyzed using a Leica SP8 Lightning confocal laser scanning microscope (Leica Microsystems, Germany) with an excitation wavelength of 488 nm and emission between 517 and 527 nm which was detected using a HyD Detector. Fluorescence signals were quantified in regions of interest (ROIs) using the integrated LASX software (Leica Microsystems, Germany).

Ca^{2+} measurements using genetically encoded

APOAEQUORIN

Effects of LaCl_3 on Ca^{2+} signals were analysed as previously described [56]. Hv-AEQ_{cyt} plants expressing APOAEQUORIN were grown for five days on water-soaked vermiculite as described above, and 5 mm sections from the tip of leaves and primary roots were reconstituted in 2.5 μM coelenterazine (Carl Roth, Germany) in ddH₂O in 96-well plates for 16 h in the dark. After reconstitution, the coelenterazine solution was replaced by ddH₂O with or without 1 mM LaCl_3 , and samples were placed for one hour in light before measurements. Baseline luminescence was recorded for 90 s with an integration time of 1 s in a plate luminometer (Mithras LB940, Berthold Technologies, Germany) before injection of an equal volume of a 2-fold-concentrated solution of H_2O_2 (final concentration 10 mM). Changes in luminescence were recorded for another 600 s before the injection of a 2-fold-concentrated discharge solution (final concentration 1 M CaCl_2 in 10% ethanol) and a subsequent recording of luminescence for 300 s. $[\text{Ca}^{2+}]_{\text{cyt}}$ was calculated as described in [48]. To calculate $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$, the mean of $[\text{Ca}^{2+}]_{\text{cyt}}$ derived from 10 s of baseline prior to treatment was subtracted from the maximum increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ obtained after injection.

RNA-sequencing and data analyses

After stress treatments as described above, plants were carefully washed with ddH₂O several times before roots and leaves were separated and ground into a fine powder under liquid nitrogen using mortar and pestle. Total RNA was isolated from the tissues using the Quick-RNA miniprep Kit (ZymoResearch, USA) following the manufacturer's instructions. The quality of RNA was assessed using a NABI Nanodrop UV/Vis Spectrophotometer (MicroDigital, South Korea). Integrity of the extracted RNA was confirmed by separation of the 28 S and 18 S rRNA bands on a 1% agarose gel.

RNA-seq was performed on three biological replicates for each treatment. Each replicate consisted of pooled

material from three plants. 3' mRNA sequencing including synthesis, labelling, and hybridization of cDNA was performed at the NGS core facility (Medical Faculty at the University of Bonn, Germany) using a NovaSeq6000 (Illumina, USA). cDNA library preparation was done using the QuantSeq protocol [58], where oligo dT priming was followed by complementary strand synthesis without any prior removal of ribosomal RNA. All further steps of data processing and alignment were performed as previously described [55]. Gene counts were approximated from the aligned files using the FeatureCounts function from the Rsubread package [59]. Differential expression analyses using the normalized counts were carried out using the DeSeq2 package [60], with default parameters for variance stabilizing transformations. The False Discovery Rate (FDR) cutoff for inclusion of data was set to 0.01. Principal Component Analyses (PCA) plots were generated with the gene counts for each sample using the princomp() function, in order to analyze and map the different variances obtained in this study. The volcano plots were made using ggplot2 and ggrepel packages of RStudio. A homology search against the genome of the model organism *A. thaliana* (TAIR 10) was performed using the Barley Reference Transcript (BaRTv1.0) dataset [61] available at www.ics.hutton.ac.uk with an E-value cutoff of $1e^{-30}$. K-means clustering analyses [62, 63] was carried out using the base k-means function on RStudio with the help of pre-defined clusters determined with the help of the gap statistic method [64]. The clustering analyses were performed separately for leaf and root tissues. The clusters were then represented as heatmaps using the pheatmap function.

Network analyses

Stress Knowledge Map is a plant molecular interaction resource, containing the Comprehensive Knowledge Network (CKN), a large, condition agnostic knowledge graph of molecular interactions in *A. thaliana* [65]. CKN was used to identify potential upstream regulators of the Ca^{2+} -dependent H_2O_2 responsive genes. The network was first filtered to only reliable interactions (rank 0 - highest reliability, rank 1, and rank 2 edges), and GoMapMan (GMM) [66] annotations used to extract genes known to be involved in Ca^{2+} signaling (171 nodes annotated with GMM terms “30.3 - signaling.calcium”, “34.21 - transport.calcium”, or “34.22 - transport.cyclic nucleotide or calcium regulated channels”) or known to be involved in redox signalling (119 nodes annotated with GMM terms “21.1 - redox.thioredoxin”, “21.2 - redox.ascorbate and glutathione”, “21.4 - redox.glutaredoxins”, or “21.5 - redox.peroxiredoxin”). Shortest paths from the known Ca^{2+} involvement (“source”) set to *A. thaliana* homologs of the newly identified Ca^{2+} -dependent H_2O_2 responsive genes (“target” set), with a maximum path length of

three were extracted from CKN. To improve the biological plausibility of the extracted paths, we required that only a single transcriptional regulatory interaction was present in each path, and it directly regulates the target. The shortest paths were filtered to the closest source(s) per target, and merged. The same approach was taken to identify paths from the known redox related (source) set to the *A. thaliana* homologs of the Ca^{2+} -independent H_2O_2 responsive genes. The analysis was performed in Python using Stress Knowledge Map (SKM) tools [65], the networkX library [67], and graph-tools [68]. Results were visualised in Cytoscape [69] using the py4cytoscape library [68, 70]. Code for the network analyses is available on GitHub (see Availability of data and materials). The Cytoscape session file is available as an additional file (Additional File 1).

cDNA synthesis and RT-qPCR

Synthesis of cDNA was carried out with 0.5–1 µg of total RNA using the ThermoFisher first strand cDNA synthesis kit with oligo-dT₁₈ primers (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The cDNA synthesis reaction was terminated by heating at 70 °C for five minutes. 1:5 dilutions of the cDNAs were used for amplification, with 2 µl of the diluted cDNA added to a total reaction volume of 10 µl. RT-qPCR was carried out on a BioRad CFX 96 real-time PCR detection system (Biorad, USA) with a reaction mixture consisting of SYBR Green PCR Mix (ThermoFisher Scientific, USA), forward and reverse primers (Table S1), ddH₂O, and the template cDNA. Transcript levels were calculated using the $2^{-\Delta\Delta C_t}$ method [71] after normalization against *HvACTIN* and *HvGAPDH*. Data analyses, including preparation of bar graphs followed by ANOVA and Tukey's Post-Hoc multi comparison tests, were performed using the tidyverse and agricolae packages, respectively, in RStudio. Linear regression analyses were also performed for the RT-qPCR. The base lm () function was used for the analyses. Correlation analysis was additionally carried out with the Karl Pearson method, using the cor.test () function.

Results

Analysis of the transcriptional effects of H_2O_2 and $LaCl_3$ treatment in barley leaves and roots

In barley, it has been shown that the application of exogenous H_2O_2 induces increases in $[Ca^{2+}]_{cyt}$ in both leaves and roots [56]. To investigate the contribution of Ca^{2+} signaling in the H_2O_2 -induced transcriptomic changes, we performed RNA-seq analyses under conditions that inhibited H_2O_2 -induced Ca^{2+} transients. For that end, barley seedlings used for RNA-seq were pre-treated with the plasma membrane Ca^{2+} channel blocker $LaCl_3$ before application of H_2O_2 . Additionally, RNA-seq was

also performed on plants treated solely either with LaCl_3 or with ddH_2O . H_2O_2 -DCFDA staining revealed increased H_2O_2 levels inside both leaves and roots of barley compared to control plants and that the pre-treatment with LaCl_3 had no effect on the H_2O_2 increase in both tissues (Fig. 1A–C). Furthermore, the inhibitory effect of LaCl_3 on H_2O_2 -induced changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ was confirmed using transgenic barley reporter lines expressing the *APOAEQUORIN* reporter gene (Fig. 1D) in line with already published data [56].

RNA-seq analysis was carried out on three biological replicates per tissue and treatment, each comprising the pooled extracted RNA from three different plants. Approximately 13–15 million raw reads were

obtained and aligned against the barley reference genome (BaRTv1.0). The total alignment rate averaged from 70 to 80% across all the samples used in this study (Table 1). The aligned reads were used for differential expression analyses between the treatments and the ddH_2O -treated control. The homogeneity of the gene counts along with their associated variance across tissues and treatments was represented as a principal component analysis (PCA) plot (Fig. 2A). The highest percentage of variance was associated with the different tissues (PC1, X-axis), with slightly lesser variance associated with the treatments (PC2, Y-axis).

Differentially expressed genes (DEGs) between treatments and control (ddH_2O) were defined through

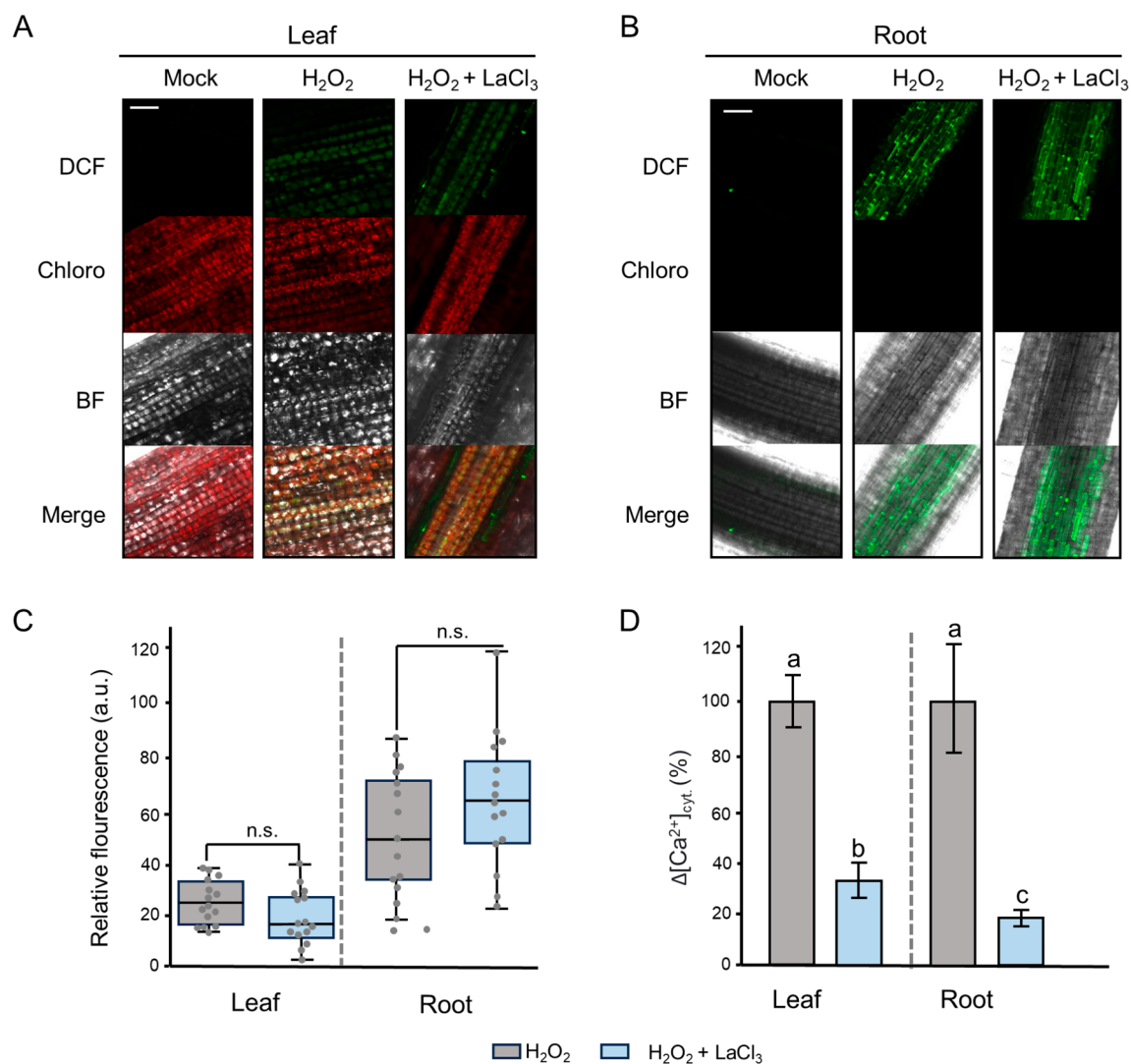


Fig. 1 Effects of LaCl_3 on the penetration of H_2O_2 and on H_2O_2 -induced Ca^{2+} signals in barley. Plants were pre-treated either with or without 1mM LaCl_3 before application of 10mM H_2O_2 . For visualization of H_2O_2 in (A) leaves and (B) roots of barley, H_2DCFDA staining was employed. BF: bright field, Chloro: Chlorophyll autofluorescence, DCF: Dichlorofluorescein, scale bar: 50 μm . (C) Quantification of relative DCF fluorescence using the LASX software. Values represent means $\pm$ SE of three independent replicates with 5 ROIs each ($n = 15$). n.s.: non-significant changes, a.u.: arbitrary units. (D) Inhibition of H_2O_2 -induced Ca^{2+} signals in barley leaf and root tips under the effect of LaCl_3 . Values represent means $\pm$ SE of three biological replicates ($n = 3$). Significances were estimated with one-way ANOVA and Tukey's Post-Hoc HSD analyses at $P < 0.05$ cutoff

Table 1 Summary of reads and alignment statistics. RNA-sequencing was carried out with three independent replicates. After quality control, reads were aligned against the barley reference genome (BaRTv1.0), and alignment files in bam format were then used for further processing

Sample	Replicate	Total Reads	Aligned Reads	Aligned Reads (%)
leaf LaCl ₃ + H ₂ O ₂	1	13,297,596	10,033,011	75.44
	2	13,122,889	10,246,998	78.08
	3	13,201,445	10,022,100	75.91
leaf LaCl ₃	1	12,787,648	9,420,291	73.70
	2	12,541,411	9,415,802	75.10
	3	14,111,932	10,538,682	74.70
root LaCl ₃ + H ₂ O ₂	1	14,455,626	10,715,747	74.12
	2	13,699,232	10,435,889	76.17
	3	13,599,945	10,166,184	74.75
root LaCl ₃	1	13,690,522	10,610,155	77.50
	2	12,208,414	9,302,812	76.20
	3	11,154,444	8,745,084	78.30

filtering with a cut-off of FDR<0.01, while the other genes were considered as genes with unchanged transcript levels (UCs) (Table S2). Volcano plot analyses showed that combined H₂O₂+LaCl₃ treatment resulted in a quite similar number of up- and down-regulated genes in leaves and roots with a total number of 1006 and 1344 DEGs detected, respectively (Fig. 2B; Table S2). From these DEGs we next omitted all the genes that showed similar differential expression upon treatment with LaCl₃ alone (Fig. S1; Table S2). Overall, this analysis identified 989 and 1001 DEGs in leaves and roots of barley, respectively, which are unique for the H₂O₂+LaCl₃ treatment (Fig. 2C, Table S2). While the overall number of DEGs was similar for both tissues, the leaves had slightly more down- and the roots considerably more up-regulated DEGs.

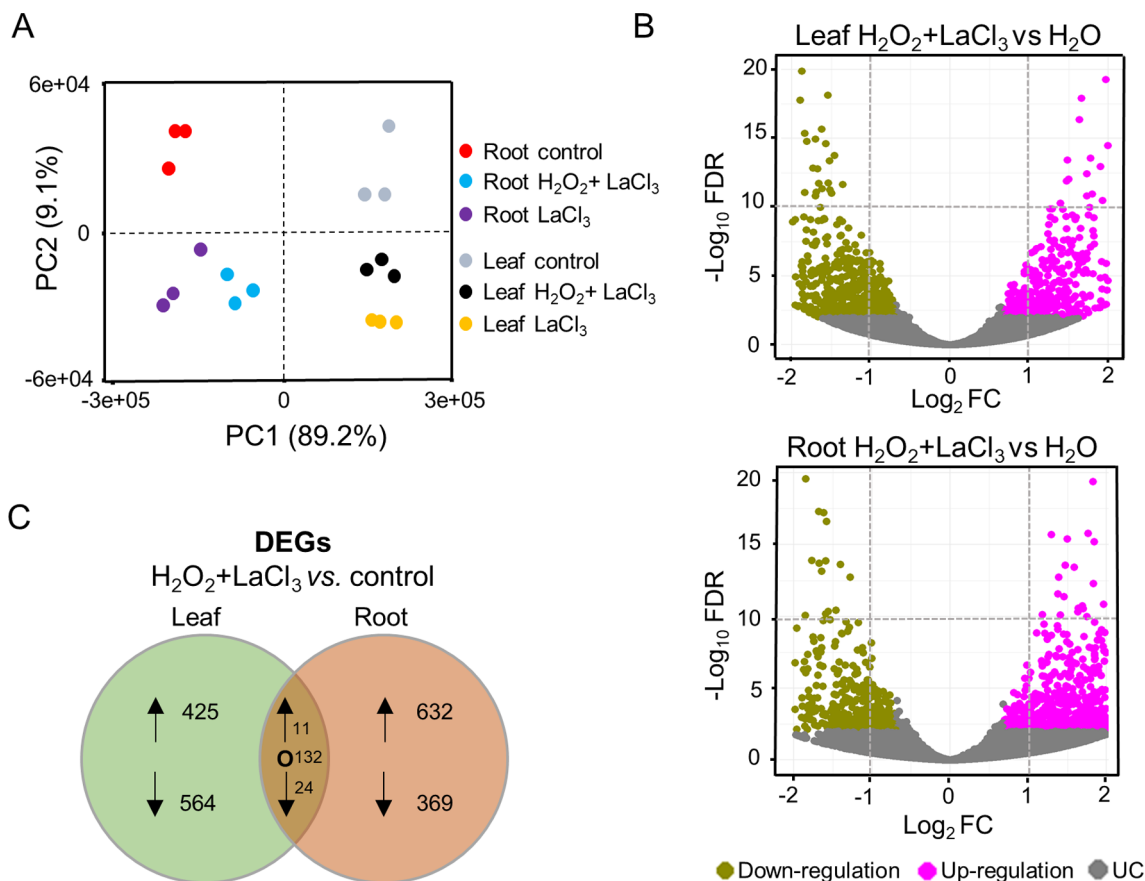


Fig. 2 Differentially expressed genes (DEGs) in H₂O₂+LaCl₃ treated vs. control plants. **(A)** PCA plot illustrating the homogeneity of the gene counts obtained with the various treatments and tissues. PC1 (X-axis) separates the samples by tissue and PC2 (Y-axis) by treatment. **(B)** Volcano plots depicting DEGs obtained in leaves (upper panel) and roots (lower panel). The X-axis shows the fold change (log₂FC) and the Y-axis represents the statistical significance (-log₁₀FDR). DEGs (FDR<0.01) are represented as up (magenta dots) and down (green dots) regulated, whereas genes with unchanged levels (UC) (FDR>0.01) are indicated as grey dots. **(C)** Bubble charts representing the unique DEGs (FDR<0.01,|log₂FC|≥0.5) of leaves and roots, after omitting DEGs shared between the H₂O₂+LaCl₃ and the LaCl₃ treatment. Genes found in both tissues are also indicated. Arrows indicate up (↑) and down (↓) regulation. O indicates unchanged expression

Identification of Ca^{2+} -dependent H_2O_2 -responsive genes in leaves and roots of barley

A previous transcriptome analysis of barley had shown that 1001 and 1883 genes in leaves and roots, respectively, were differentially expressed upon H_2O_2 treatment [55]. These H_2O_2 -DEGs were selected based on $\log_2\text{FC} \geq 0.5$ and $\text{FDR} < 0.01$ and were obtained by RNA-seq of samples obtained under the same experimental conditions as in the current study. To identify those H_2O_2 -DEGs that depend on the H_2O_2 -induced Ca^{2+} signals for their differential regulation, a comparative analysis between the transcriptomes in response to H_2O_2 [previously published data, 55] and to $\text{H}_2\text{O}_2 + \text{LaCl}_3$ was performed. More precisely, we selected those DEGs from the H_2O_2 treatment that either showed an unchanged

expression (UCs) under $\text{H}_2\text{O}_2 + \text{LaCl}_3$ treatment or which were DEGs under both treatments but their expression level differed significantly ($\Delta\log_2\text{FC} \geq 1$; corresponding to a fold change difference ≥ 2) when H_2O_2 treatment was compared to $\text{H}_2\text{O}_2 + \text{LaCl}_3$ treatment (Fig. 3A). $\Delta\log_2\text{FC}$ thus represents the difference between $\log_2\text{FC}$ s obtained under two conditions, i.e., H_2O_2 vs. H_2O and $\text{H}_2\text{O}_2 + \text{LaCl}_3$ vs. H_2O .

All in all, about 33% and 70% of the H_2O_2 -responsive genes in leaves and roots, respectively, were considered as Ca^{2+} -dependent H_2O_2 -responsive genes in barley (Fig. 3B). Of those, 295 genes in leaves and 799 genes in roots showed a strict dependency (DEGs- H_2O_2 vs. UCs- $\text{H}_2\text{O}_2 + \text{LaCl}_3$) on Ca^{2+} signals (Fig. 3B; Table S3 and S4). 36 genes in leaves and 522 genes in roots were either

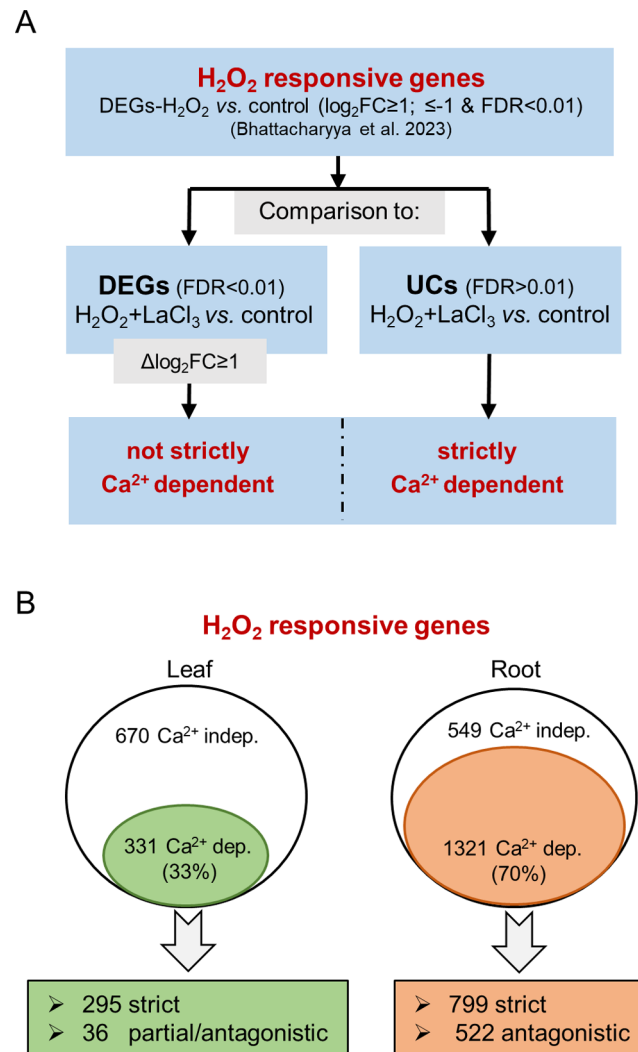


Fig. 3 Identification of Ca^{2+} -dependent H_2O_2 -responsive genes. **(A)** Schematic representation of the bioinformatic analysis steps to identify Ca^{2+} dependent H_2O_2 -responsive genes in leaves and roots of barley. UCs: genes with unchanged expression between $\text{H}_2\text{O}_2 + \text{LaCl}_3$ and control. $\Delta\log_2\text{FC}$ represents the difference between $\log_2\text{FC}$ s obtained under two conditions, i.e. H_2O_2 vs. control and $\text{H}_2\text{O}_2 + \text{LaCl}_3$ vs. control. **(B)** Egg-shaped representations of the comparison between Ca^{2+} -dependent and Ca^{2+} -independent H_2O_2 -responsive genes in leaves and roots of barley. The Ca^{2+} -dependent genes were further divided in strict and partial/antagonistic with regards to Ca^{2+}

partially dependent on Ca^{2+} signals (altered up- or down-regulation levels), or even displayed a counter-regulation from up to down or vice versa.

GO analyses of Ca^{2+} -dependent H_2O_2 -responsive genes

GO enrichment analyses were performed with the obtained Ca^{2+} -dependent H_2O_2 -responsive genes in leaves and roots of barley (Fig. 4). In leaves, the top biological terms were related to jasmonate (JA) signaling and wounding. Further enrichment was observed for terms related to abiotic stresses in general and salt, osmotic stress, and temperature in particular. Further GO terms were related to hormones and oxygen-containing compounds (Fig. 4A). By contrast, the root gene set yielded mostly GO terms associated with ROS/ H_2O_2 response and metabolism, response to oxidative stress,

and detoxification but also to cell wall biogenesis and organisation (Fig. 4B).

Clustering analysis of Ca^{2+} -dependent H_2O_2 -responsive genes

Clustering analysis of the Ca^{2+} -dependent H_2O_2 -responsive genes provided five clusters, L1-L5, for leaves and four clusters, R1-R4, for roots (Fig. 5, Fig. S2). In leaves, cluster L1 and L2 comprise genes which were up- and down-regulated under H_2O_2 , respectively, however, in the presence of $\text{H}_2\text{O}_2 + \text{LaCl}_3$ their expression level was unchanged compared to control conditions (Fig. 5A, Table S3). This indicates a strict dependence of their response to H_2O_2 on Ca^{2+} signals. The genes in cluster L3 and L4 showed a reduced up- and down-regulation in response to H_2O_2 , respectively, when the Ca^{2+} transient

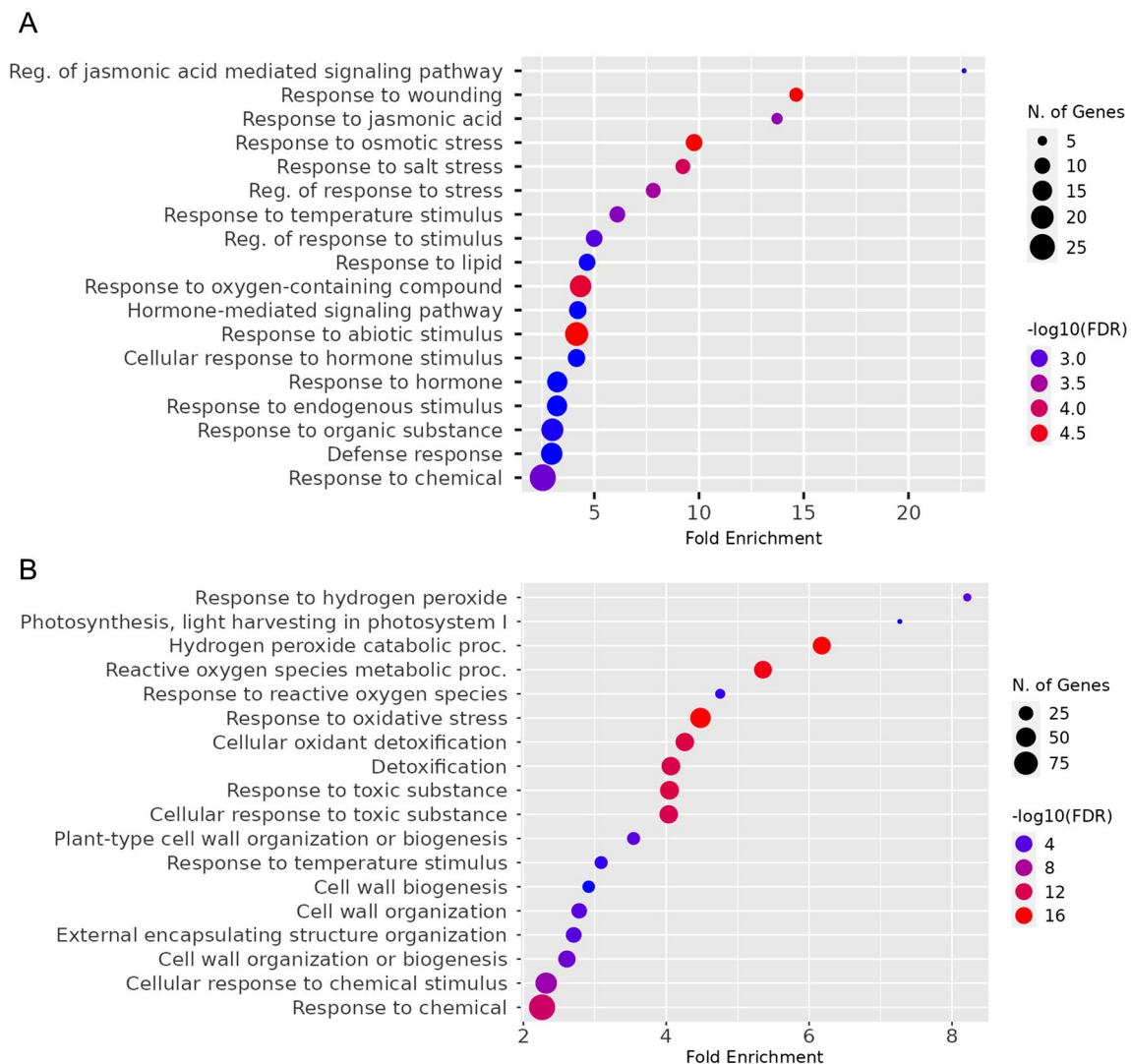


Fig. 4 Gene ontology enrichment analysis of Ca^{2+} -dependent H_2O_2 -responsive genes. The diagrams of enriched GO terms indicate total number of genes associated with various biological processes and their fold enrichment (relative to their overall occurrence in the genome) in (A) leaves and (B) roots of barley

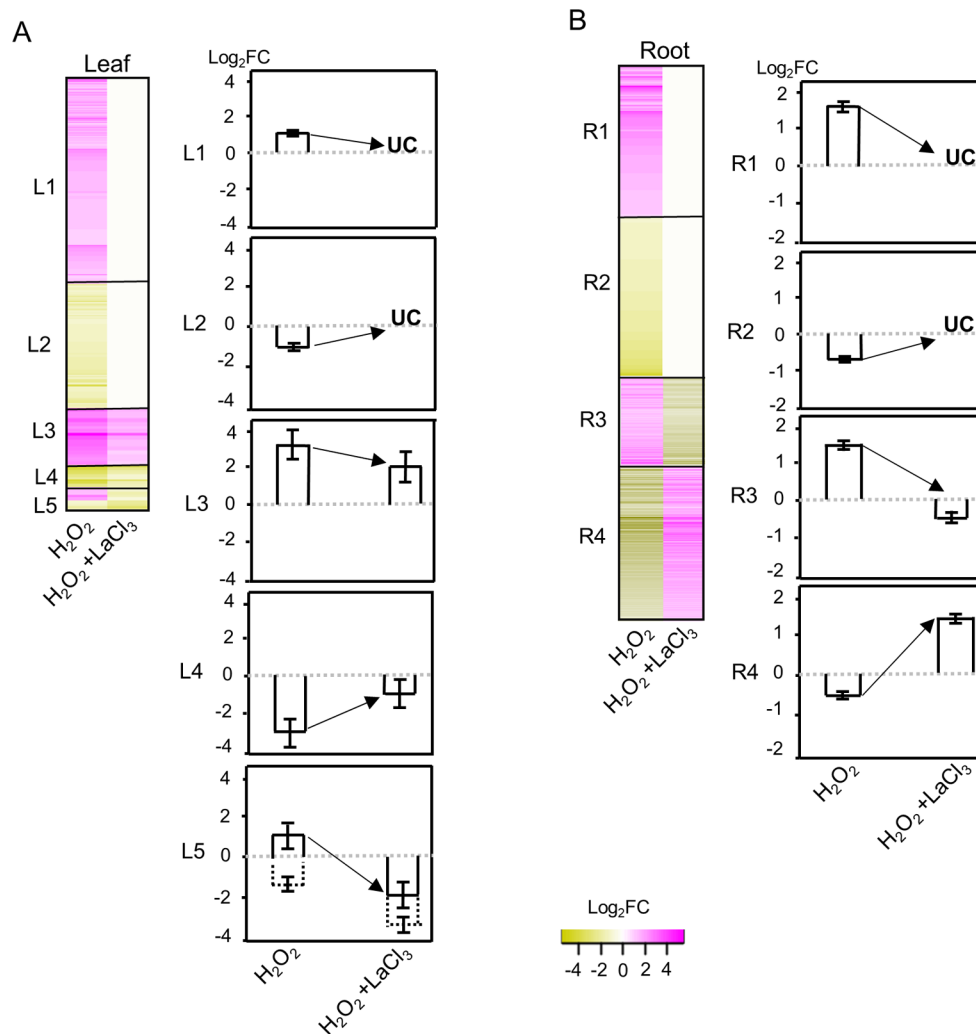


Fig. 5 Clustering analysis of the Ca^{2+} -dependent H_2O_2 -responsive genes. Gene clustering was used to group the Ca^{2+} -dependent H_2O_2 -responsive genes with similar expression patterns. The results provided five clusters in leaves (A) and four clusters in roots (B). Left panels of each subpart represent the heatmap of the genes in the clusters, and the right panel shows a bar chart representation of the mean $\pm$ SE of the Log_2FC of the genes in each cluster. UC: genes with unchanged expression between $\text{H}_2\text{O}_2 + \text{LaCl}_3$ and control

was blocked by LaCl_3 , however, transcript levels were still significantly higher or lower compared to the control. Thus, cluster L3 and L4 represent H_2O_2 -responsive genes with partial dependence on Ca^{2+} . Cluster L5 contains H_2O_2 -responsive genes that went from up- to down-regulation upon inhibition of the Ca^{2+} transient but also three genes for which their down-regulation was enhanced. Remarkably, in roots cluster R1 and R2 represent many genes with a strict dependence on the Ca^{2+} transient for their up- or down-regulation, respectively, however, in contrast to leaves, no partial up- and down-regulation was observed. Instead, clusters R3 and R4 comprise many H_2O_2 -responsive genes which upon inhibition of the Ca^{2+} signal went from up- to down-regulation and vice versa (Fig. 5B, Table S4).

To verify the accuracy of the RNA-seq data and clustering analysis, the expression levels of two randomly

selected genes from each cluster were re-evaluated by RT-qPCR (Figs. 6 and 7). For all candidate genes tested, the transcript levels determined by RT-qPCR showed similar trends as observed in the RNA-seq data. Linear regression analysis showed a correlation coefficient of >0.7 , indicating a positive correlation between RT-qPCR and RNA-seq data for all treatments and tissues (Fig. S3).

Cluster L1

Cluster L1 (up-regulation is strictly dependent on a Ca^{2+} signal) has a total of 196 genes, over 20 of which encode members of TF families (Table S3). Several of these TFs belong to the AP2/ERF (APETALA2/ethylene response factor) family, which has been associated with a wide variety of environmental stresses including hypoxia, cold, oxidative, and flooding stress not only in Arabidopsis but also in other plant species [72, 73]. Originally

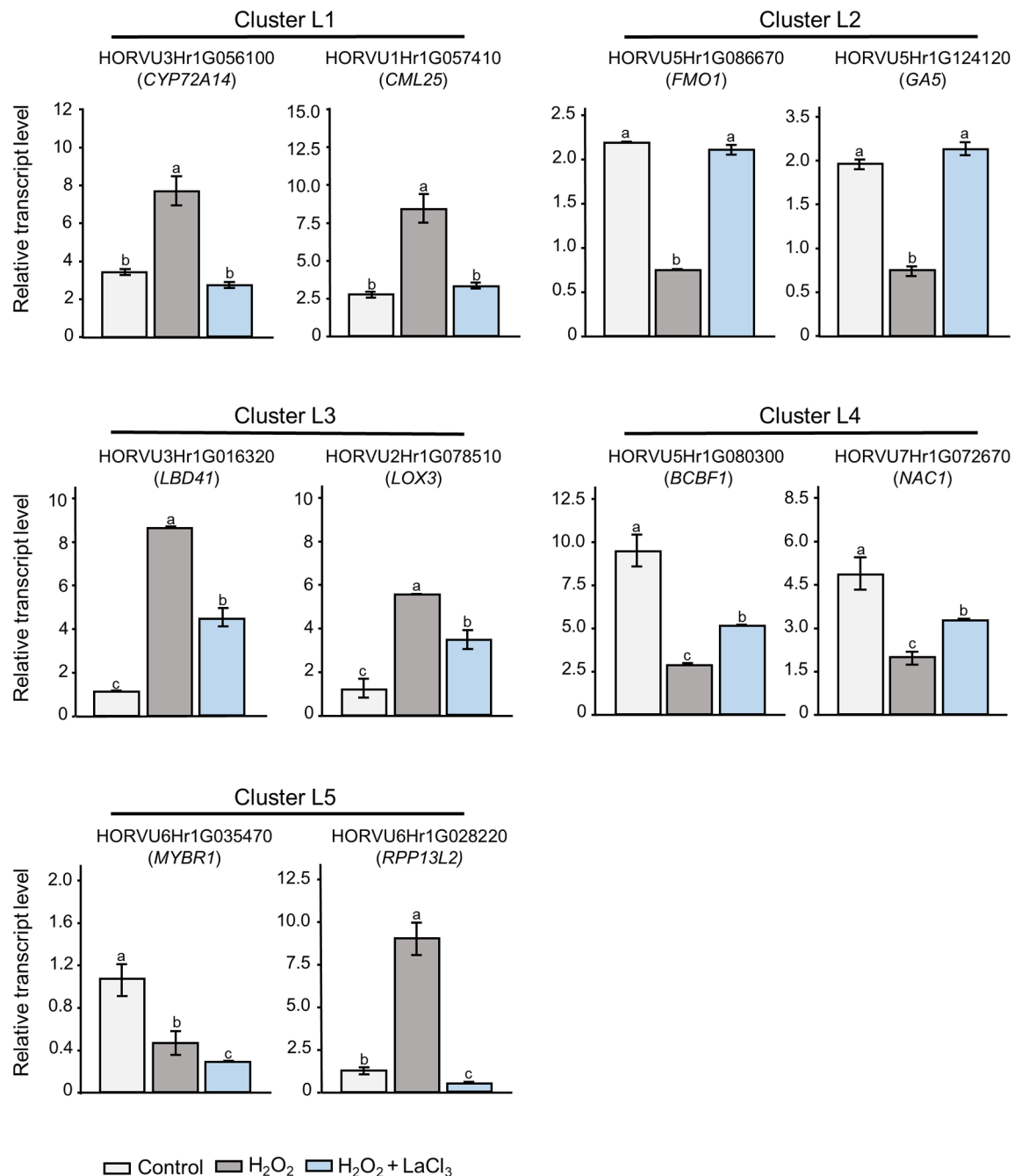


Fig. 6 RT-qPCR analyses of transcript levels in leaves. Two Ca²⁺-dependent H₂O₂-responsive genes from each leaf cluster were randomly selected. Data represent mean ± SE of three independent biological replicates and two technical repeats (*n* = 3). The transcript levels were normalized to the reference genes *HvACTIN* and *HvGAPDH*. Statistical significances were obtained using one-way ANOVA and Tukey's Post-Hoc HSD test (*P* < 0.05). The letters represent different levels of significance. Orthologous genes in Arabidopsis are indicated in brackets

associated with ethylene signaling, AP2/ERF TFs have also been connected to other hormones like abscisic acid (ABA), gibberellic acid (GA), and cytokinin [74]. Genes associated with these hormones were also found in this cluster. Other important TFs in cluster L1 belong to the WRKY, NAC, and F-BOX domain-containing TF families. These TF families have been shown to function

ubiquitously in a variety of abiotic and biotic stimuli by intercepting the ROS signaling [75–77]. Cluster L1 furthermore contains several genes related to Ca²⁺ signaling such as orthologs of genes encoding the calmodulin-like proteins AtCML11, AtCML25, or OsCML26 (LOC_Os12g01400.1), as well as AtCIPK1 (CBL-interacting protein kinase 1). It furthermore includes genes coding

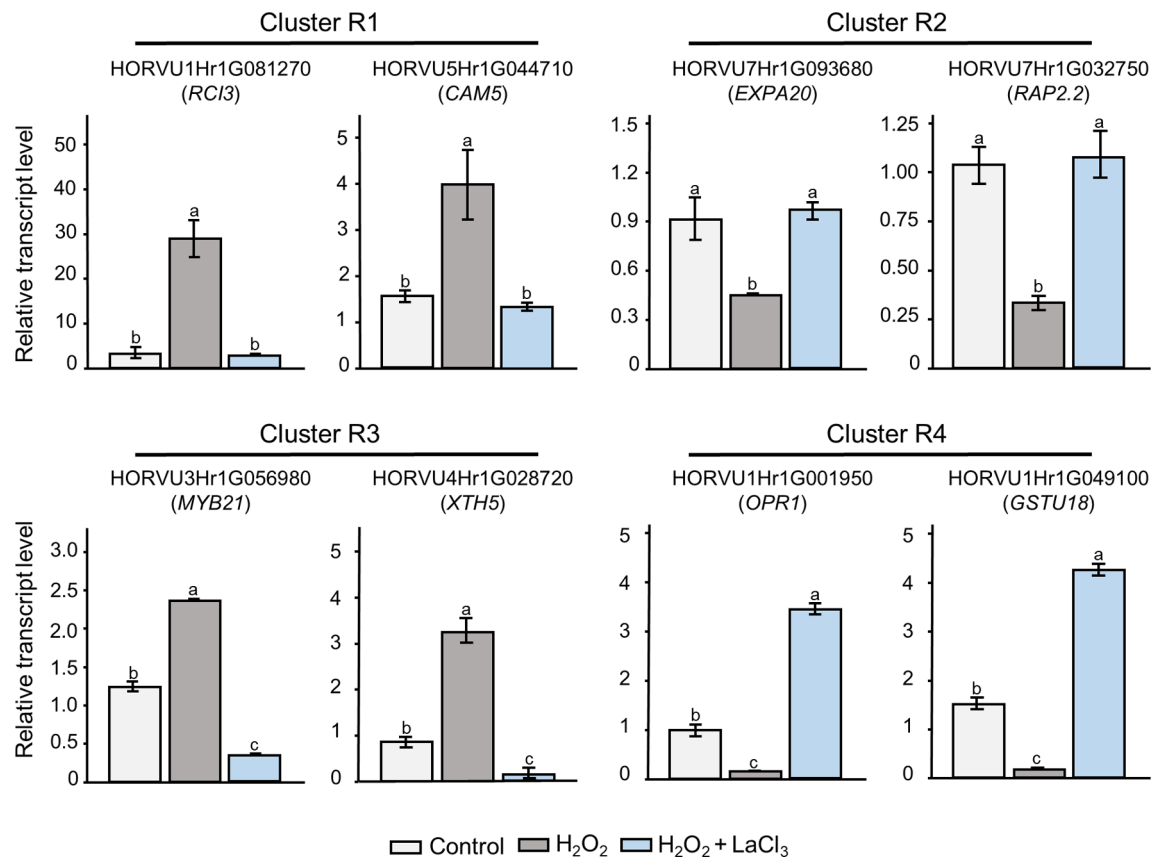


Fig. 7 RT-qPCR analyses of transcript levels in roots. Two Ca^{2+} -dependent H_2O_2 -responsive genes from each root cluster were randomly selected. Data represent mean $\pm$ SE of three independent biological replicates and two technical repeats ($n=3$). The transcript levels were normalized to the reference genes *HvACTIN* and *HvGAPDH*. Statistical significances were obtained using one-way ANOVA and Tukey's Post-Hoc HSD test ($P < 0.05$). The letters represent different levels of significance. Orthologous genes in Arabidopsis are indicated in brackets

for members of the MAPK (mitogen activated protein kinase) and MAPKKK (MAPK kinase kinase) family. With regard to hormone signaling, genes found in cluster L1 encode negative regulators of the JA pathway including proteins involved in the degradation of the biologically active form of jasmonate, JA-Ile [78, 79]. Genes encoding proteins involved in catabolic function were also found for GA, cytokinin and ABA. We furthermore identified three auxin responsive genes, only one of which has an ortholog in Arabidopsis (*AtIAA22*).

Cluster L2

Cluster L2 (down-regulation is strictly dependent on a Ca^{2+} signal) comprises a total of 99 genes. It also includes genes coding for various TFs of the AP2/ERF, WRKY, OVATE, or F-BOX families (Table S3). The AP2/ERF TFs were orthologs of *AtERF1* which has been associated with both JA and ethylene signaling [80], and *AtRAV2* which has been proposed to be involved in touch stimuli induced signaling [81]. Additionally, several genes encoding kinases associated with signal transduction events were identified including orthologs of the cysteine receptor kinase 28 (*AtCRK28*), which was associated with

ROS-related stress responses [82]. Cluster L2 includes three genes encoding class III plant peroxidases, particularly orthologs of *AtPRX52* and *AtPRX72* [83]. Interestingly, L2 was the only cluster in leaves that includes a group of genes encoding transport proteins, such as orthologs of the ABC domain containing JA/JA-Ile transporter *AtABCG16/JAT1* [84] and of AZA-RESISTANT GUANINE 2 (*AtAZG2*), a member of the AZG purine transporter family that has been shown to function in transportation of cytokinin [85]. Additionally, this cluster contains a number of other genes that play important roles in different stress pathways in plants such as orthologs of the *FLAVIN MONO-OXYGENASE 1* (*AtFMO1*), which is positioned downstream of SA induced Systemic Acquired Resistance (SAR) and related signaling pathways [86] and has also been associated with *AtCDPK5* a target of Ca^{2+} signals [87, 88].

Cluster L3

Cluster L3 (up-regulation is partially dependent on a Ca^{2+} signal) consists of 16 genes, most of which have no functional annotation and only six have a clear ortholog in Arabidopsis (Table S3). Of these genes, one encodes

an ortholog of the TF AtLBD41, a class IIA LBD protein that was previously identified in relation to low-oxygen endurance or high-light-induced increase in H_2O_2 in Arabidopsis [89, 90] as well as flooding response in soybean [91]. Another one encodes an ortholog of the 13 S-lipoxygenase 3 (AtLOX3), an enzymes that catalyze the first step in the biosynthesis of JA [92]. LOX3 was shown to play an important role in vegetative growth restriction after wounding [93], parasitic nematode infection [94], and salt stress [95], responses all of which include H_2O_2 and Ca^{2+} signaling.

Cluster L4

Cluster L4 (down-regulation is partially dependent on Ca^{2+} signal) comprises only 10 genes, Similar to cluster L3 many have no assigned function and only three have known orthologs in Arabidopsis (Table S4). Three TFs were found including HORVU3Hr1G010190, which is a different ortholog of AtRAV2 than the one found in cluster L2. Thus, RAV2-encoding genes show both strict and partial dependence on Ca^{2+} in their H_2O_2 -induced down-regulation. In this cluster we also found the gene HORVU1Hr1G063780, which is an ortholog of AtGA20OX2, which plays an important role in the rate-limiting steps of GA biosynthesis [96]. The GA20 oxidases, AtGA20OX1 and 2 are supposed to have a partially redundant function; however, we found the barley ortholog of AtGA20OX1 within the up-regulated genes (in cluster L1).

Cluster L5

Cluster L5 combines genes with two different types of regulation pattern. Three of the 10 genes showed enhanced down-regulation when Ca^{2+} signals were inhibited by $LaCl_3$. The other seven displayed counter-regulation going from up-regulation by H_2O_2 to down-regulation under combined $H_2O_2 + LaCl_3$ treatment. For only five genes an Arabidopsis ortholog and thus a potential function was identified (Table S3) and none of the genes in cluster L5 have so far been linked to H_2O_2 or Ca^{2+} signaling. One gene with enhanced down-regulation encodes an ortholog of AtMYBR1, also called MYB44, a TF that has been shown to negatively regulate ABA signaling by interacting with the nuclear ABA receptor PYR1-LIKE 8 [97]. It has also been associated with other hormone responses, i.e. to JA and SA [98].

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Cluster R1

Cluster R1 (up-regulation is strictly dependent on a Ca^{2+} signal) contains a total of 389 genes, including several TFs mostly belonging to sub-families like AP2/ERF, WRKY, MYB, OVATE, bHLH, HOMEBOX, F-BOX, GATA, and LEA (Table S4). Cluster R1 also contains genes encoding proteins related to glutathione metabolism and other forms of detoxification. By far the largest functional group are anti-oxidant enzymes with the majority being class III plant type peroxidases. Nine of these encode different barley orthologs of AtRCI3 and seven include orthologs to the secretory peroxidase AtPRX39 both of which has been associated with cold stress and tolerance [99, 100]. Also, genes related to Ca^{2+} signaling were identified such as orthologs of AtCAM5 [101] and the Ca^{2+} -dependent NADPH oxidase RBOHD [45, 102], AtCPK5 [103], and AtMPK9, a MAP kinase shown to positively regulate ROS-mediated ABA signaling downstream of Ca^{2+} signals [104]. Other kinases include orthologs of the cytoplasmic histidine kinase AtAHK5, the mutation of which leads to reduced stomatal closure in response to H_2O_2 [105]. The gene HORVU5Hr1G046020 encodes an ortholog of AtPBL8, a member of the subfamily VII of receptor-like cytoplasmic kinases (RLCK), other members of which were found in all root clusters and in leaf clusters L1 and L2. Several RLCKs play a role in pattern-triggered immune signaling, and the higher order mutant *atpbl8/16/17* showed increased flg22-triggered H_2O_2 generation [106].

Cluster R2

Cluster R2 (down-regulation is strictly dependent on a Ca^{2+} signal) is the largest cluster with 410 genes (Table S4). Again, a number of TFs belonging to different families were found in this cluster, including an ortholog of *AtERF1*, albeit a different one to the ortholog found in cluster L2. Similar to cluster R1, this cluster also contains genes encoding proteins involved in ROS metabolism and detoxification, such as another ortholog of *AtPRX52*. The cluster R2 contains several genes coding for proteins with Ca^{2+} -binding EF-hand domains, one of them being an ortholog of *AtCML39*. Interestingly in this cluster we found six genes related to photosynthesis, encoding orthologs of the Arabidopsis chlorophyll-binding proteins of the LHCA and LHCb type as well as *AtPSB28* and *AtPSAK*. Cluster R2 also comprises orthologs of several genes involved in hormonal signaling.

Cluster R3

Cluster R3 (counter-regulation from up to down) contains 128 genes. As in most clusters, we found genes belonging to major TF families (Table S4). We also found two peroxidases, orthologous of Arabidopsis *AtPRX71* and *AtRCI3*, the ortholog of TPR like thioredoxin *AtTTL1*, and genes associated with various aspects of hormone signaling. Additionally, several components of Ca^{2+} signaling pathways were present in this cluster such as orthologs of the Ca^{2+} sensor *AtCML25* and the Ca^{2+} associated protein kinases *AtCPK13*.

Cluster R4

Cluster R4 (counter-regulation from down to up) contains in total 394 genes, again with several members of different TF families (Table S4). Interestingly, this cluster contains an ortholog of vascular plant one-zinc finger 1 (*AtVOZ1*), which has been implicated in heat stress response in plants and acting as a repressor of *DREB2C* [107]. Cluster R4 also encompasses genes related to glutathione metabolism and detoxification, including four orthologs of the glutathione transferase *AtGSTU18*, for which orthologs were also found in cluster L2 and R2, and three for *AtGSTF13*. Many genes encoding for phi (GSTF) and tau (GSTU) glutathione transferases are upregulated under environmental stress and Arabidopsis plants overexpressing *VvGSTU13* showed enhanced tolerance to a variety of abiotic stress conditions like cold and salt [108]. This cluster contains further anti-oxidant enzymes, including three orthologs of *AtPRX52*, all of them encoded by barley paralogs different from those present in clusters L2, R1, and R2. Cluster R4 exhibits the largest number of HSPs, most of which were small HSPs (SHSPs) as well as HSPs mapping to the Arabidopsis orthologs *AtHSP81-1*, *AtHSP101*, and *AtHSP70*. Also in this cluster we found 14 genes related to photosynthesis.

Transcription factors as key regulators of Ca^{2+} -dependent H_2O_2 -responsive genes in barley

We next modelled potential connections from known components of Ca^{2+} signaling networks to the identified Ca^{2+} -dependent H_2O_2 -responsive genes (Fig. S4) using CKN of the recently described SKM resource [65]. The information in the CKN is based on present knowledge from Arabidopsis, thus only 192 and 894 Ca^{2+} -dependent H_2O_2 -responsive genes found in leaves and roots of barley, respectively, with identifiable orthologs in Arabidopsis were considered for analysis (Tables S3 and S4). We extracted the directed shortest paths from known Ca^{2+} signaling related genes (source set) to the Ca^{2+} -dependent H_2O_2 -responsive genes identified in our transcriptomic analysis (target set). We additionally required that the final edge regulating the target gene was a transcriptional regulatory interaction. Merging of the results revealed several major network hubs connecting multiple Ca^{2+} signaling components to multiple targets in leaves and roots (Figs. 8A and 9A). The most dominant of these hubs (by number of times they occur in a path as well as number of targets) were depicted separately (Figs. 8B-E and 9B-E). In both, leaves and roots these hubs were defined by the TFs *AGL15*, *HY5*, *PIF4*, and *EIN3* as key nodes regulating several targets (Figs. 8 and 9, orange nodes). The Ca^{2+} signaling components in these networks were mostly CaMs/CMLs and CDPKs/CPKs but also CaM-interacting proteins such as IQD6.

Ethylene insensitive 3 (*EIN3*)

Downstream of *EIN3*, the targets in both tissues include a unique mosaic of genes from different signaling pathways (Figs. 8B and 9B), with a greater prevalence of genes from cluster L1 in leaves (strict positive dependence on cytosolic Ca^{2+} signals) whereas in roots the target genes were interspersed from all the clusters. Noteworthy is the *ERF1* gene, encoding an AP2/ERF transcription factor, which is present in our data as a down-stream target of *EIN3* in both tissues (Figs. 8B and 9B). This is in line with a previous study that identified *ERF1* as a downstream component of the ethylene signaling pathway, whose expression is regulated by *EIN3* binding to the *ERF1* promoter in vivo [109]. *ERF1* was shown to integrate JA and ethylene signalling pathways in a synergistic manner during plant defense [80]. This crosstalk fits to other *EIN3*-regulated targets found in our dataset such as the JA catabolic protein *CYP94C1* and the ethylene biosynthetic protein 1-aminocyclopropane 1-carboxylate oxidase 5 (*ACO5*), which is known to have *EIN3* binding sites [110].

Hypocotyl 5 (*HY5*)

All downstream targets of *HY5* in leaves belong to cluster L1 (Fig. 8C), thereby suggesting a pre-dominant strict

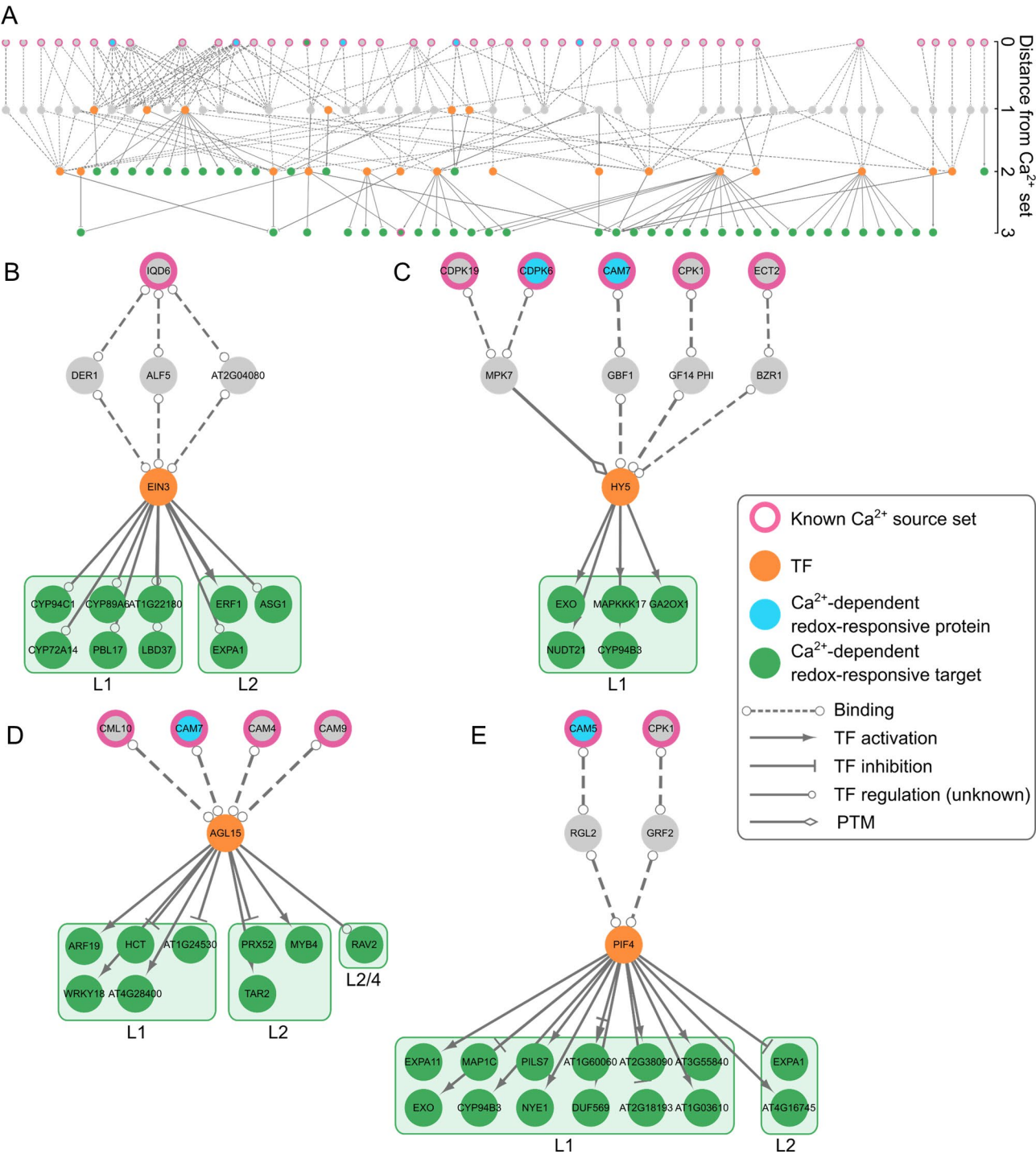


Fig. 8 Network analyses of Arabidopsis orthologs of the Ca^{2+} -dependent H_2O_2 -responsive genes found in barley leaves. **(A)** All shortest paths identified in KGN starting from known Ca^{2+} -related genes (sources, pink-bordered nodes) to Ca^{2+} -dependent H_2O_2 -responsive genes identified by RNA-seq (targets, green-filled nodes) merged into a single network. Sub-networks were extracted from the merged network with focus on **(B)** EIN3, **(C)** HY5, **(D)** AGL15 and **(E)** PIF4. Ca^{2+} -related components identified in a previous proteomic study as H_2O_2 -regulated in Arabidopsis leaves [65] are presented by a light blue-filling. Nodes are labelled with their short names, when available. The targets are ordered by corresponding clusters (L). PTM: post-translational modification, TF: transcription factor. Complete networks are provided in additional file 1

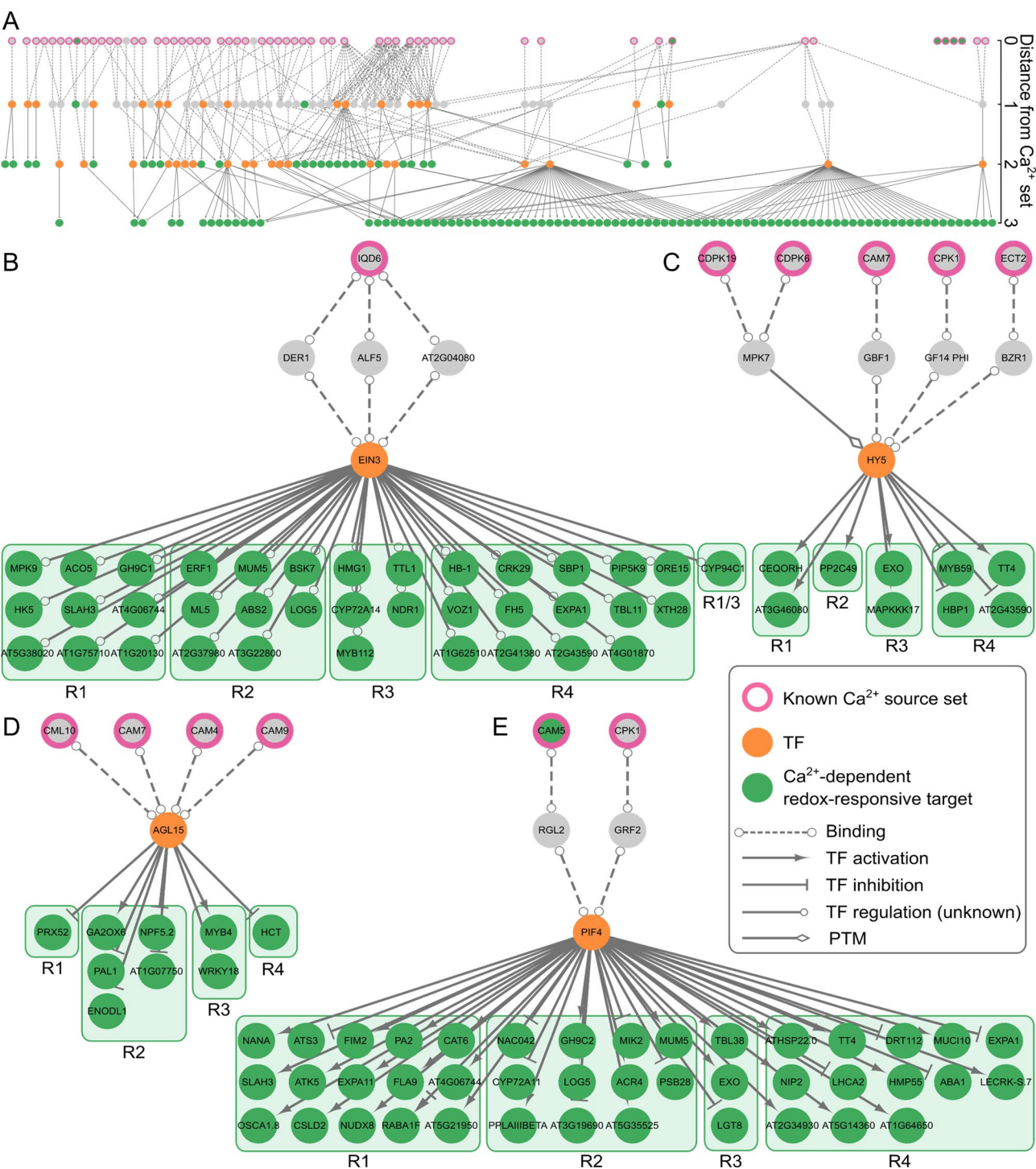


Fig. 9 Network analyses of Arabidopsis orthologs of the Ca^{2+} -dependent H_2O_2 -responsive genes found in barley roots. **(A)** All shortest paths identified in CKN starting from known Ca^{2+} -related genes. (sources, pink-bordered nodes) to Ca^{2+} -dependent H_2O_2 -responsive genes identified by RNA-seq (targets, green-filled nodes) merged into a single network. Sub-networks were extracted from the merged network with focus on **(B)** EIN3, **(C)** HY5, **(D)** AGL15 and **(E)** PIF4. Nodes are labelled with their short names, when available. The targets are ordered by corresponding clusters (R). PTM: post-translational modification, TF: transcription factor. Complete networks are provided in in additional file 1

dependency on Ca^{2+} signals for up-regulation, while in roots this TF again had downstream targets in all clusters (Fig. 9C). The targets in leaves include genes like the MAPKK kinase *MAPKKK17*, involved in plant herbivory responses [111], the phosphatase *PP2C49*, a negative regulator of salt stress tolerance in *Arabidopsis* [112], the *ceQORH* protein, a long-chain fatty acid reductase whose allocation between cytosol and chloroplasts is depending on CaM-binding [113], and the TF *MYB59* already established in negative regulation of Ca^{2+} signaling and homeostasis [114]. *HY5* is known to play a role in plant thermomorphogenesis in coordination with another TF, *PIF4* [115], which is also present in our network as a nodal hub (see below).

Agamous like 15 (AGL15)

Again, the largest group of *AGL15* downstream targets in leaves include genes from cluster L1 and L2 (Fig. 8C), representing a strict dependence on Ca^{2+} signals. In roots, the targets of *AGL15* include mostly genes from cluster R2 (Fig. 9C), thereby also showing strict dependency on Ca^{2+} . Common between leaf and root targets is the TF *MYB4*, which has an established role in protection against oxidative stress during cadmium stress [116] and flavonoid biosynthesis [117]. The targets also include an ortholog of the peroxidase *PRX52*, which has a number of orthologs in barley and is present in different clusters.

Phytochrome interacting factor 4 (PIF4)

The downstream targets of *PIF4*, also called *SRL2*, in leaves include mostly genes from cluster L1 (strict dependence on Ca^{2+} signals for H_2O_2 induced up-regulation), most of them without a direct relationship to ROS, Ca^{2+} signalling or stress. In roots, downstream targets were found in all clusters and included genes encoding for the Ca^{2+} channel *OSCA1.8* involved in osmotic stress induced Ca^{2+} signatures [118], the RAB GTPase *RABA1f* involved in salt stress response [119], and the TF *NAC042* previously shown to be involved in salt and drought stress [120, 121]. Furthermore, targets of *PIF4* include genes coding for proteins involved in detoxification of ROS.

Discussion

Our comparative analysis between the already published transcriptome changes induced by H_2O_2 [55] and those observed under a combined application of $\text{H}_2\text{O}_2 + \text{LaCl}_3$ (this study) showed that the H_2O_2 -induced Ca^{2+} signals affected the transcript abundance of many H_2O_2 -responsive genes. The transcriptome changes were not due to an interference with Ca^{2+} homeostasis per se, since only those genes from the $\text{H}_2\text{O}_2 + \text{LaCl}_3$ set that displayed changes under H_2O_2 alone but no changes with LaCl_3 alone were considered. Overall, in roots more

H_2O_2 -responsive genes showed a dependency on the H_2O_2 -induced Ca^{2+} signals compared to those in leaves (Fig. 3). This is in line with the higher number of genes for which transcriptional changes were observed after H_2O_2 treatment alone in roots [55]. However, even considering these differences in total numbers, expression of only 33% of the H_2O_2 -responsive genes in leaves, but about 70% of those in roots, was affected by LaCl_3 -sensitive Ca^{2+} signals (Fig. 3B). Moreover, most of the identified Ca^{2+} -dependent H_2O_2 -responsive genes were found only in one of the two tissues, indicating a clear tissue specificity of the response. H_2O_2 is not only generated in response to biotic attacks but also by imbalances in energy metabolism. Obviously, photosynthesis is a process generating a large amount of ROS and thus, leaf tissue simply might have a higher prevalence of detoxification systems already in place while they need to be induced upon the accumulation of H_2O_2 in roots. This would be in line with the observation that many genes related to oxidative stress and detoxification were observed in response to H_2O_2 in roots [55]. We also observed minor differences in H_2O_2 penetration (Fig. 1B) and a slightly stronger inhibition of the Ca^{2+} signal (Fig. 1C) by LaCl_3 in roots which might further affect the transcriptome changes.

The issues discussed above notwithstanding, strict and partial/antagonistic Ca^{2+} dependency of the H_2O_2 -responsive transcriptome was observed in both tissues (Figs. 3 and 5). Strict dependency (clusters L1, L2, R1, and R2) means that genes with significant changes in transcript level upon H_2O_2 application no longer showed significant changes after LaCl_3 pre-incubation when compared to the control. The most likely scenario for these genes is that a Ca^{2+} signal evoked by H_2O_2 is required to activate a transcription activator or repressor (Fig. 10, strictly). This can occur either more directly, e.g., by proteins such as Ca^{2+} -dependent TFs or CAM-TAs [122], or as the consequence of a longer signalling cascade that involves Ca^{2+} activated proteins such as CDPKs, CaMs, or CBLs [123, 124]. Such strictly Ca^{2+} -dependent H_2O_2 -responsive genes were strongly dominant in leaves (~90%) and also the majority in roots (~60%). Partially dependent genes showed a difference in transcript abundance between control and $\text{H}_2\text{O}_2 + \text{LaCl}_3$ treatment; however, the abundance was significantly different from H_2O_2 treatment alone. Of these cases, genes in cluster L3 showed a reduced up-regulation in the absence of an H_2O_2 -induced Ca^{2+} transient, while genes in cluster L4 show reduced down-regulation (Fig. 10, additive). Interestingly, this kind of additive regulation of H_2O_2 and Ca^{2+} was completely absent in roots. For genes in these clusters H_2O_2 affects changes in transcript abundance both independently and via a Ca^{2+} signal, and both regulations occur in the same direction. Even in the absence of the H_2O_2 -induced Ca^{2+} transient, the direct

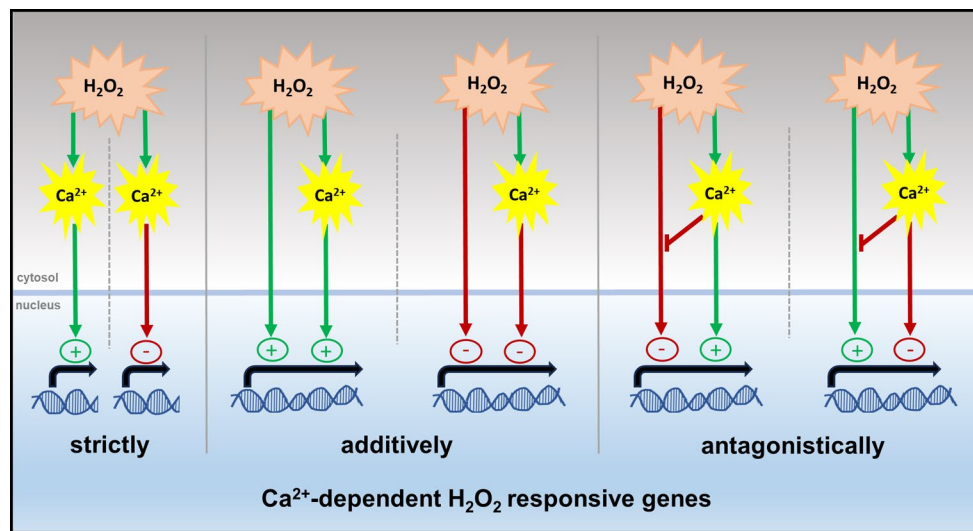


Fig. 10 Representative models of Ca^{2+} -dependent H_2O_2 -responses. Strict Ca^{2+} -dependency means that Ca^{2+} signaling operates down-stream of H_2O_2 to induce either activation or repression of gene expression. Partial dependency is seen when H_2O_2 and Ca^{2+} signals modulate gene expression in an additive way. In that case, the H_2O_2 activation/repression of gene expression is not fully dependent on the H_2O_2 -induced Ca^{2+} transient, but Ca^{2+} amplifies this regulation. In the antagonistic model, the H_2O_2 -induced Ca^{2+} transient inhibits the H_2O_2 -induced activation/repression while at the same time inducing an opposite response. Lack of the H_2O_2 -induced Ca^{2+} transient thus results in a changes of transcript abundance from up to down and vice versa. The arrowheads indicate activation (green) or repression (red) and the red T-headed arrows indicate inhibition

regulation by H_2O_2 remains. More complex is the regulation of those genes from cluster L5, R3, and R4, for which inhibition of the H_2O_2 -induced Ca^{2+} transient results in changes of transcript abundance from up to down and vice versa. The regulation of these genes can be explained by an antagonistic model (Fig. 10, antagonistic), where Ca^{2+} -dependent and independent pathways act in the opposite direction and Ca^{2+} signaling in addition inhibits or attenuates the Ca^{2+} -independent H_2O_2 induced activation/repression. Similarly, three genes in cluster L5 that show an increased reduction in transcript abundance in the absence of the Ca^{2+} transient could be regulated by multiple pathways in a Ca^{2+} -dependent and -independent manner; however, in this case Ca^{2+} signaling attenuates the H_2O_2 response, so that it becomes stronger in its absence (Fig. S5). It should be noted that for all clusters more complex models can be envisioned. Also, transcript abundance is not necessarily defined by gene expression, however, the models can easily be adapted for changes in transcript stability or degradation.

Indeed, our results reinforce the notion of complex, interacting pathways that define the ultimate response to a certain stimulus. While the responses are specific with regards to many factors such as type of stimulus, timing, tissue or developmental stage, they are variances of very similar patterns. After stimuli perception, the information is forwarded through the cell by signaling cascades involving components such as secondary metabolites, ions like Ca^{2+} , hormones, kinases, etc., to ultimately affect gene transcription, translation and/or protein activity. The latter is either due to novel synthesis, degradation or

alteration of activity that catalyses the molecular changes required. This cascade of event allows for multiple points of regulation and ensures a cross-talk of signals coming from different internal and external stimuli. Many of the intermediate players will be ready and in place to receive a stimulus; nevertheless, stimulus-induced transcriptional regulation of sensors, signaling kinases or TFs can occur to enhance the response or to initiate priming and long-term adaptation. Thus, it is not surprising, that TFs were found in all clusters. It is not uncommon to have TF cascades, in which an initially activated TF affects the transcription of multiple other TFs [125]. Also, different stresses can lead to binding of the target from TFs of different gene families to induce or repress the expression, e.g. the redox-related LEA protein *SAG21* binding to ERF (pathogen stress), WRKY (H_2O_2 stress), and NAC (wounding stress) TF [126].

Phytohormones have been repeatedly demonstrated to interact with each other at various points through versatile TF families, thereby eliciting a common, synchronized, and holistic change in the molecular and biochemical landscape of the plant in response to diverse stimuli [127]. Moreover, the study of interactions between phytohormones and secondary messengers like Ca^{2+} has gained momentum over the years; particularly the CDPKs have been closely linked to phytohormones such as GA, ABA, or JA in regulating crucial plant processes related to growth and development, flowering, and also responses and acclimation to a variety of biotic and abiotic stresses [128]. Other kinases, such as RLKs, were proposed to play crucial roles during growth-defense

trade-off, i.e. by intermingling with different phytohormone signal transduction pathways [129]. The presence of these kinases in different clusters is thus in line with a differential regulation through Ca^{2+} signals, but also suggest them as potential hubs which have the potential to transduce downstream signals crucial to the H_2O_2 - Ca^{2+} crosstalk by interacting with other major signaling pathways like phytohormones.

There are several remarkable differences between the response of leaves and roots. In general, the roots show a higher variety of GO terms compared to leaves (Tables S3 and S4). As mentioned above, roots show more changes in genes related to oxidative stress and detoxification. This is marked by a strong Ca^{2+} -dependent regulation of class III peroxidases with a total of 42 peroxidases present across all clusters. Also genes belonging to the GO term cell wall are more abundant in roots compared to leaves. Cell wall metabolism plays important roles in shaping plant responses to stress acclimation [130]. Several reactions associated with crosslinking of cell wall components, like hemicellulose and xyloglucans, along with crucial processes, like polymerization and depolymerization of cell walls, have previously been related to ROS production and anti-oxidant enzyme activities, which is a characteristic feature when plants are challenged with abiotic stress conditions [131]. For instance, the transcription factor short root (SHR) is involved in plant organogenesis including periclinal division in the root cortex that depends on an optimal H_2O_2 balance. On one hand, SHR activates H_2O_2 production by RBOHs and on the other hand induces SA signaling that increases H_2O_2 levels by repressing CATALASE 2 [132].

In roots, we also found a much larger and diverse group of membrane transporters as in leaves, i.e. the wall-associated-transporter-1-like (WAT1) and SWEET-type transporters, but also aquaporins. Aquaporins have been shown to be involved in dynamic ROS changes under stresses [133] and WAT1 was identified as a downstream target of RBOH-mediated ROS generation during parasitic infections [134]. More surprisingly, we could identify 20 genes involved in photosynthesis including LHC proteins and photosystems components to be regulated by H_2O_2 -induced Ca^{2+} signals in roots. The presence of photosynthesis-related genes in roots might seem a controversial result, but it could be hypothesized that the exposure of the roots to light for five days lead to such a phenomenon. Moreover, it was also proposed that root plastids might be involved in the process of anti-oxidative damage control under stress conditions which generate oxidative bursts [135, 136]. This has also been suggested in another study based on fluorescence spectra of Arabidopsis roots that showed a capacity of root plastids to form larger antenna complexes [137]. Our results therefore might point to a crucial and “less-known” role played

by the H_2O_2 - Ca^{2+} crosstalk in the induction of LHC-encoding genes and other genes related to photosynthesis in roots.

In an attempt to decipher the molecular basis of the Ca^{2+} -dependency of the H_2O_2 -induced transcriptional responses, we modelled potential connections between known components of the Ca^{2+} -signaling network and the Ca^{2+} -dependent H_2O_2 -responsive genes identified in this study. The Ca^{2+} -signaling components in this network included many CaMs, CMLs, and CDPKs, several of which, had been shown in a recent study in Arabidopsis leaves to undergo Ca^{2+} -dependent changes in protein level upon H_2O_2 application [65] (Fig. 8, light blue nodes). Moreover, the network analyses showed TFs, especially EIN3, AGL15, PIF4, and HY5, downstream of the Ca^{2+} components as hubs/nodal points regulating multiple Ca^{2+} -dependent H_2O_2 -responsive genes in different clusters in leaves and roots of barley (Figs. 8 and 9). These TFs are known from Arabidopsis to be involved in different physiological and developmental processes including phytohormone signaling and catabolism, photosynthesis, detoxification, cell wall metabolism, and cellular transport. EIN3 is a positive downstream regulator of the ethylene signalling pathway that affects various facets of plant development, several stress responses, and phytohormone pathways [138]. So far, ethylene signaling involving EIN3 has been related to Ca^{2+} and H_2O_2 during salt stress response in Arabidopsis [139]. According to our model, this H_2O_2 - Ca^{2+} regulation might be mediated by the CaM-binding protein IQD6 (IQ67 Domain Containing 6) (Figs. 8B and 9B), which is known to play a crucial role in plant growth and development [140]. HY5 is a bZIP type master transcriptional regulator of photomorphogenesis, also shown to be involved in other processes such as response to abiotic stresses [141]. It was also shown that HY5 participates in ROS homeostasis [142, 143] and to interact with CAM7 to regulate Ca^{2+} -dependent photomorphogenesis in plants [144]. Indeed, in our network CAM7 is connected to HY5 via the G-box-binding factor GBF1 (Figs. 8C and 9C), which was shown to play a role in plant defense upstream of SA [145]. We also obtained a connection with CDPK7 and MPK7, which possibly regulate HY5 expression through post-translational modifications. H_2O_2 was also shown to directly increase kinase activity of MPK7, underscoring the complexity of the signaling cross-talk [146]. AGL15 is a member of the MADS box TF family and was shown in vitro to bind CaM [147]. This is in line with our network analyses suggesting connections between AGL15 and multiple CaMs as well as CML10 (Figs. 8D and 9D). As for HY5, AGL15 regulation might also be controlled by CAM7.

PIF4, a member of the bHLH TFs family, has so far very little association with Ca^{2+} and ROS signaling, although

a recent report showed a connection to RBOHD-mediated up-regulation under salt stress [148]. RBOH is not present in our model since it was only shown that *PIF4* expression is attenuated in a *rboh* mutant. However, our model suggests regulation of *PIF4* by *CAM5* and *CPK1*, which have never been shown to be involved in any stress signaling pathways. Downstream, *CAM5* and *CPK1* were connected to *RGL2* (RGA-Like2), which is a member of the DELLA protein family and has previously been shown to be involved in ROS generation and phytohormonal signaling [149–151]. *GRF2* is a member of the 14-3-3 protein family. Although specific data linking *GRF2* to signaling or stress pathways is missing, 14-3-3 proteins have been previously linked to plant stress, Ca^{2+} signaling, and hormone signal transduction [152, 153].

However, it should be noted that the information in CKN used for our network modelling is based on current knowledge from Arabidopsis, so only those barley Ca^{2+} -dependent H_2O_2 -responsive genes with identifiable orthologs in Arabidopsis were considered for analyses. Thus, of the 331 and 1334 Ca^{2+} -dependent H_2O_2 -responsive genes in leaves and roots of barley, respectively, only 192 and 894 genes were used in CKN analyses. This clearly reinforces that there is an urgent need for more experimental data to be obtained from barley and other crops to close this vast knowledge gap. While multiple responses are conserved between different land plants, others are more specific. We will need to know the specific responses of crops for accurate stress for modeling and to use this information for improved crop breeding.

Conclusion

H_2O_2 is an indispensable ROS, which is generated as a toxic by-product of biological metabolic processes, but also functions as a signaling molecule that can influence plant growth and development. Moreover, it has an established potential to intermingle with signaling cascades associated with second messengers like Ca^{2+} . In this study, using transcriptomic analysis, the molecular landscape behind the tissue-wide H_2O_2 - Ca^{2+} crosstalk in the crop species barley was elucidated. Our data expands the knowledge on stress response in barley but also strengthen the relevance of findings in model plants such as Arabidopsis for barley. They reveal genes which have never been implicated in any canonical stress response pathway, and therefore may be used as candidates in future studies to further expand our understanding of this crosstalk. Similarly, network analyses suggested nodal TFs which in turn regulate the expression of genes involved in phytohormone pathways including ethylene, JA, ABA, SA, brassinosteroids, GA, and auxin, as well as in MAPK signaling cascades. Several studies have reported that both, biotic and abiotic stress, can lead to the accumulation of H_2O_2 and fluctuations in Ca^{2+} levels

which imply an enhancement in the vitality of plants to withstand those environmental stress. Hence, deciphering the molecular mechanisms underlying the H_2O_2 - Ca^{2+} crosstalk will ultimately provide more understanding of stress acclimation not only in barley but also in other crop species.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-025-06248-9>.

Supplementary Material 1: **Additional File 1:** Raw cytoscape output sessions (.cys) of the SKM network analyses in the roots and leaves of barley. Figures 8, 9 and S5 were prepared from the cytoscape files

Supplementary Material 2: **Additional File 2: Fig. S1** Unique and overlapping DEGs between $\text{H}_2\text{O}_2 + \text{LaCl}_3$ and LaCl_3 treatment alone vs. control treatment. Venn diagram of DEGs (FDR < 0.01) from (A) leaves and (B) roots. Only the unique DEGs from the $\text{H}_2\text{O}_2 + \text{LaCl}_3$ treatment were used for further analyses. **Fig. S2** Determining the number of clusters for Ca^{2+} -dependent H_2O_2 -responsive genes in (A) leaves and (B) roots. Gap statistics analysis was used for the calculation, with a total of 100 iterations. `set.seed(123)` function was used before running this function to reduce randomness and inconsistencies in the number of clusters generated. The number of clusters predicted by this analysis was used to perform k-means clustering analyses in figure 5. **Fig. S3** Validation of RNA-seq results by RT-qPCR. Linear regression analysis between transcript level ratios derived from RNA-seq and RT-qPCR data under different treatments in leaves (A and B) and roots (C and D). C: correlation coefficient, P: P-value, R2: R-regression coefficient. **Fig. S4** CKN analysis of H_2O_2 signaling based on Arabidopsis orthologs of the genes identified in barley. All paths identified in CKN leading from known Ca^{2+} -involved genes (pink-bordered nodes) to Ca^{2+} -dependent H_2O_2 responsive genes (green nodes), and from known redox-related genes (blue-bordered nodes) to Ca^{2+} -independent H_2O_2 -responsive genes (yellow nodes), obtained by RNA-seq, merged into a single network in (A) leaves and (B) roots. Transcription factors are indicated as orange nodes. Complete networks are provided in additional file 1. **Fig. S5** Two potential models for an increased reduction in transcript abundance in the absence of the H_2O_2 -induced Ca^{2+} transient. This could either occur by a regulation of Ca^{2+} -dependent and -independent pathways, which act in opposite directions with different strength of regulation (left panel). Alternatively, the H_2O_2 -induced Ca^{2+} signals might attenuate the H_2O_2 response, so that it becomes stronger in its absence. The arrowheads indicate activation (green) or repression (red)

Supplementary Material 3: **Additional File 3: Table S1** List of primer sequences used for RT-qPCR analyses in this study. Wherever applicable, the corresponding Arabidopsis orthologs are indicated in brackets

Supplementary Material 4: **Additional File 4: Table S2:** Differentially expressed genes (DEGs) between either $\text{H}_2\text{O}_2 + \text{LaCl}_3$ or LaCl_3 and control samples. Differential expression analysis was carried out with the genes using DESeq2. Attached here are the output files obtained after comparing $\text{LaCl}_3 + \text{H}_2\text{O}_2$ treated samples with control samples, in the leaf and the root, along with the DESeq2 output files obtained after comparing LaCl_3 treated samples. DEGs were identified based on adjusted FDR < 0.01 and are listed separately for leaves and roots. Further genes with FDR > 0.01, were considered as genes with unchanged expression (UCs) compared to control samples. Furthermore, the genes commonly regulated between $\text{H}_2\text{O}_2 + \text{LaCl}_3$ and LaCl_3 treatments were excluded to obtain the genes which are unique for $\text{H}_2\text{O}_2 + \text{LaCl}_3$ treatment and presented separately for leaves and roots of barley

Supplementary Material 5: **Additional File 5: Table S3** A comparison of the obtained unique genes in Leaf $\text{H}_2\text{O}_2 + \text{LaCl}_3$ (Table S2) to leaf H_2O_2 -DEGs obtained in our former study (Bhattacharyya et al 2023). For the shared genes between both treatments a selection based on a $\log_2\text{FC}$ difference ($\Delta \log_2\text{FC}$) was performed and only genes showing a $\Delta \log_2\text{FC} > 1$ was considered for further analyses. A clustering of the obtained genes resulted in a total of five clusters. control: dd H_2O ; UCs: genes with

unchanged expression vs control

Supplementary Material 6: **Additional File 6: Table S4** A comparison of the obtained unique genes in root H_2O_2 +LaCl₃ (Table S2) to root H_2O_2 -DEGs obtained in our former study (Bhattacharyya et al 2023). For the shared genes between both treatments a selection based on a log₂FC difference (delta log₂FC) was performed and only genes showing a delta >1 was considered for further analyses. A clustering of the obtained genes resulted in a total of five clusters. control: ddH₂O; UCs: genes with unchanged expression vs control

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Author contributions

SB contributed to conceptualization, investigation (responsible for most experimental work), formal analysis (responsible for bioinformatic analysis), validation, visualization, and writing - original draft as well as review & editing. EUR and AMB contributed to investigation (RT-qPCR). CB contributed to investigation (network analysis). BM contributed to investigation (Ca²⁺ measurements) and writing - review & editing. MG contributed to investigation. EP contributed to supervision and writing - review and editing. UCV contributed to conceptualization, validation, visualization, funding acquisition, project administration, supervision, and writing - review & editing. FC contributed to conceptualization, formal analysis, validation, visualization, supervision, and writing - original draft as well as review & editing. All authors contributed to the article and approved the submitted version.

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Data availability

Raw RNA-sequencing data used in this study are available in the SRA (Sequence Read Archive) repository from NCBI (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1061386> and <https://www.ncbi.nlm.nih.gov/sra/PRJNA973626>). Code for the network analyses is available on GitHub (<https://github.com/NIB-SI/skm-h2o2-ca2-barley>).

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Farooq MA, Niazi AK, Akhtar J, Saifullah, Farooq M, Soury Z, et al. Acquiring control: the evolution of ROS-induced oxidative stress and redox signaling pathways in plant stress responses. *Plant Physiol Biochem*. 2019;141:353–69.
2. Huang H, Ullah F, Zhou D-X, Yi M, Zhao Y. Mechanisms of ROS Regulation of Plant Development and stress responses. *Front Plant Sci*. 2019;10.
3. Smirnov N, Arnaud D. Hydrogen peroxide metabolism and functions in plants. *New Phytol*. 2019;221:1197–214.
4. Choudhury FK, Rivero RM, Blumwald E, Mittler R. Reactive oxygen species, abiotic stress and stress combination. *Plant J*. 2017;90:856–67.
5. Waszczak C, Carmody M, Kangasjärvi J. Reactive oxygen species in Plant Signaling. *Annu Rev Plant Biol*. 2018;69:209–36.
6. Mittler R, Zandalinas SI, Fichman Y, Van Breusegem F. Reactive oxygen species signalling in plant stress responses. *Nat Rev Mol Cell Biol*. 2022;23:663–79.
7. Mittler R. ROS are good. *Trends Plant Sci*. 2017;22:11–9.
8. Mhamdi A, Van Breusegem F. Reactive oxygen species in plant development. *Development*. 2018;145:dev164376.
9. Dumanović J, Nepovimova E, Natić M, Kuča K, Jačević V. The significance of reactive oxygen species and antioxidant defense system in plants: a concise overview. *Front Plant Sci*. 2021;11.
10. Liao W, Xiao H, Zhang M. Role and relationship of nitric oxide and hydrogen peroxide in adventitious root development of marigold. *Acta Physiol Plant*. 2009;31:1279–89.
11. Hernández-Barrera A, Velarde-Buendía A, Zepeda I, Sanchez F, Quinto C, Sánchez-Lopez R, et al. Hyper, a Hydrogen Peroxide Sensor, indicates the sensitivity of the Arabidopsis Root Elongation Zone to Aluminum Treatment. *Sensors*. 2015;15:855–67.
12. Liu J, Macarasin D, Wisniewski M, Sui Y, Droby S, Norelli J, et al. Production of hydrogen peroxide and expression of ROS-generating genes in peach flower petals in response to host and non-host fungal pathogens. *Plant Pathol*. 2013;62:820–8.
13. Barba-Espin G, Diaz-Vivancos P, Job D, Belghazi M, Job C, Hernández JA. Understanding the role of H₂O₂ during pea seed germination: a combined proteomic and hormone profiling approach. *Plant Cell Environ*. 2011;34:1907–19.
14. Liao W-B, Zhang M-L, Huang G-B, Yu J-H. Hydrogen peroxide in the vase solution increases vase life and keeping quality of cut Oriental Trumpet hybrid lily 'Manissa'. *Sci Hort*. 2012;139:32–8.
15. Ge X-M, Cai H-L, Lei X, Zhou X, Yue M, He J-M. Heterotrimeric G protein mediates ethylene-induced stomatal closure via hydrogen peroxide synthesis in Arabidopsis. *Plant J*. 2015;82:138–50.
16. Hameed A, Iqbal N. Chemo-priming with mannose, mannitol and H₂O₂ mitigate drought stress in wheat. *Cereal Res Commun*. 2014;42:450–62.
17. Ashraf MA, Rasheed R, Hussain I, Iqbal M, Haider MZ, Parveen S, et al. Hydrogen peroxide modulates antioxidant system and nutrient relation in maize (*Zea mays* L.) under water-deficit conditions. *Arch Agron Soil Sci*. 2015;61:507–23.
18. Sathiyaraj G, Srinivasan S, Kim Y-J, Lee OR, Parvin S, Balusamy SRD, et al. Acclimation of hydrogen peroxide enhances salt tolerance by activating defense-related proteins in Panax ginseng C.A. Meyer. *Mol Biol Rep*. 2014;41:3761–71.
19. Wang Y, Zhang J, Li J-L, Ma X-R. Exogenous hydrogen peroxide enhanced the thermotolerance of *Festuca arundinacea* and *Lolium perenne* by increasing the antioxidative capacity. *Acta Physiol Plant*. 2014;36:2915–24.
20. Wu D, Chu H, Jia L, Chen K, Zhao L. A feedback inhibition between nitric oxide and hydrogen peroxide in the heat shock pathway in Arabidopsis seedlings. *Plant Growth Regul*. 2015;75:503–9.
21. Czégény G, Wu M, Dér A, Eriksson LA, Strid Å, Hideg É. Hydrogen peroxide contributes to the ultraviolet-B (280–315nm) induced oxidative stress of plant leaves through multiple pathways. *FEBS Lett*. 2014;588:2255–61.
22. Oksanen E, Häikiö E, Sober J, Karnosky DF. Ozone-induced H₂O₂ accumulation in field-grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity. *New Phytol*. 2004;161:791–9.
23. Xu A, Cheng F, Zhou S, Hu H, Bie Z. Chilling-induced H₂O₂ signaling activates the antioxidant enzymes in alleviating the photooxidative damage caused by loss of function of 2-Cys peroxiredoxin in watermelon. *Plant Stress*. 2022;6:100108.
24. Wen J-F, Gong M, Liu Y, Hu J-L, Deng M. Effect of hydrogen peroxide on growth and activity of some enzymes involved in proline metabolism of sweet corn seedlings under copper stress. *Sci Hort*. 2013;164:366–71.
25. Shetty NP, Jørgensen HJL, Jensen JD, Collinge DB, Shetty HS. Roles of reactive oxygen species in interactions between plants and pathogens. *Eur J Plant Pathol*. 2008;121:267–80.

26. Nadarajah KK. Defensive strategies of ROS in Plant–Pathogen interactions. In: Verma PK, Mishra S, Srivastava V, Mehrotra S, editors. *Plant Pathogen Interactions*. Singapore: Springer Nature Singapore; 2023. pp. 163–83.
27. Hu C-H, Wang P-Q, Zhang P-P, Nie X-M, Li B-B, Tai L et al. NADPH oxidases: the vital performers and Center hubs during Plant Growth and Signaling. *Cells*. 2020;9.
28. Manishankar P, Wang N, Köster P, Alatar AA, Kudla J. Calcium signaling during salt stress and in the regulation of ion homeostasis. *J Exp Bot*. 2018;69:4215–26.
29. Seifikalhor M, Aliniaefard S, Shomali A, Azad N, Hassani B, Lastochkina O, et al. Calcium signaling and salt tolerance are diversely entwined in plants. *Plant Signal Behav*. 2019;14:1665455.
30. Yuan P, Yang T, Poovaiah BW. Calcium signaling-mediated plant response to cold stress. *Int J Mol Sci*. 2018;19.
31. Iqbal Z, Memon AG, Ahmad A, Iqbal MS. Calcium mediated cold acclimation in plants: Underlying Signaling and Molecular mechanisms. *Front Plant Sci*. 2022;13.
32. Knight H, Trewavas AJ, Knight MR. Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J*. 1997;12:1067–78.
33. Knight H, Brandt S, Knight MR. A history of stress alters drought calcium signalling pathways in. *Plant J*. 1998;16:681–7.
34. Shao H-B, Song W-Y, Chu L-Y. Advances of calcium signals involved in plant anti-drought. *CR Biol*. 2008;331:587–96.
35. Guihur A, Rebeaud ME, Goloubinoff P. How do plants feel the heat and survive? *Trends Biochem Sci*. 2022;47:824–38.
36. Kang X, Zhao L, Liu X. Calcium Signaling and the response to heat shock in crop plants. *Int J Mol Sci*. 2024;25.
37. Jalmi SK, Bhagat PK, Verma D, Noryang S, Tayyeba S, Singh K et al. Traversing the links between Heavy Metal Stress and Plant Signaling. *Front Plant Sci*. 2018;9.
38. Aldon D, Mbengue M, Mazars C, Galaud J-P. Calcium signalling in plant biotic interactions. *Int J Mol Sci*. 2018;19.
39. Negi NP, Prakash G, Narwal P, Panwar R, Kumar D, Chaudhry B et al. The calcium connection: exploring the intricacies of calcium signaling in plant-microbe interactions. *Front Plant Sci*. 2023;14.
40. Ranty B, Aldon D, Cotellet V, Galaud J-P, Thuleau P, Mazars C. Calcium sensors as key hubs in plant responses to biotic and abiotic stresses. *Front Plant Sci*. 2016;7.
41. Ren H, Zhang Y, Zhong M, Hussian J, Tang Y, Liu S, et al. Calcium signaling-mediated transcriptional reprogramming during abiotic stress response in plants. *Theor Appl Genet*. 2023;136:210.
42. Marcec MJ, Gilroy S, Poovaiah BW, Tanaka K. Mutual interplay of Ca^{2+} and ROS signaling in plant immune response. *Plant Sci*. 2019;283:343–54.
43. Ravi B, Foyer CH, Pandey GK. The integration of reactive oxygen species (ROS) and calcium signalling in abiotic stress responses. *Plant Cell Environ*. 2023;46:1985–2006.
44. Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, et al. Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *Plant Cell*. 2007;19:1065–80.
45. Ogasawara Y, Kaya H, Hiraoka G, Yumoto F, Kimura S, Kadota Y, et al. Synergistic activation of the *Arabidopsis* NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. *J Biol Chem*. 2008;283:8885–92.
46. Dubiella U, Seybold H, Durian G, Komander E, Lassig R, Witte C-P, et al. Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proc Natl Acad Sci*. 2013;110:8744–9.
47. Pei Z-M, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, et al. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*. 2000;406:731–4.
48. Rentel MC, Knight MR. Oxidative stress-Induced Calcium Signaling in *Arabidopsis*. *Plant Physiol*. 2004;135:1471–9.
49. Lecourieux D, Ranjeva R, Pugin A. Calcium in plant defence-signalling pathways. *New Phytol*. 2006;171:249–69.
50. Demidchik V, Shabala S, Isayenkov S, Cuin TA, Pottosin I. Calcium transport across plant membranes: mechanisms and functions. *New Phytol*. 2018;220:49–69.
51. Fichman Y, Zandalinas SI, Peck S, Luan S, Mittler R. HPCA1 is required for systemic reactive oxygen species and calcium cell-to-cell signaling and plant acclimation to stress. *Plant Cell*. 2022;34:4453–71.
52. Wu F, Chi Y, Jiang Z, Xu Y, Xie L, Huang F, et al. Hydrogen peroxide sensor HPCA1 is an LRR receptor kinase in *Arabidopsis*. *Nature*. 2020;578:577–81.
53. Gürel F, Öztürk ZN, Uçarlı C, Rosellini D. Barley genes as tools to Confer Abiotic stress tolerance in crops. *Front Plant Sci*. 2016;7.
54. Holubová K, Hensel G, Vojta P, Tarkowski P, Bergougnoux V, Galuszka P. Modification of Barley Plant Productivity through Regulation of Cytokinin Content by Reverse-Genetics approaches. *Front Plant Sci*. 2018;9.
55. Bhattacharyya S, Giridhar M, Meier B, Peiter E, Vothknecht UC, Chigri F. Global transcriptome profiling reveals root- and leaf-specific responses of barley (*Hordeum vulgare* L.) to H_2O_2 . *Front Plant Sci*. 2023;14.
56. Giridhar M, Meier B, Imani J, Kogel K-H, Peiter E, Vothknecht UC, et al. Comparative analysis of stress-induced calcium signals in the crop species barley and the model plant *Arabidopsis thaliana*. *BMC Plant Biol*. 2022;22:447.
57. Kaur N, Dhawan M, Sharma I, Pati PK. Interdependency of reactive oxygen species generating and scavenging system in salt sensitive and salt tolerant cultivars of rice. *BMC Plant Biol*. 2016;16:131.
58. Moll P, Ante M, Seitz A, Reda T. QuantSeq 3' mRNA sequencing for RNA quantification. *Nat Methods*. 2014;11:i–iii.
59. Team RC. Version 4.3.2., 2023. <https://www.r-project.org/>
60. Love MI, Huber W, Anders S. Moderated estimation of Fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550.
61. Mascher M, Gundlach H, Himmelbach A, Beier S, Twardziok SO, Wicker T, et al. A chromosome conformation capture ordered sequence of the barley genome. *Nature*. 2017;544:427–33.
62. Li Y, Wu H. A clustering method based on K-Means Algorithm. *Physics Procedia*. 2012;25:1104–9.
63. Handhayani T, Hiryanto L. Intelligent Kernel K-Means for Clustering Gene expression. *Procedia Comput Sci*. 2015;59:171–7.
64. Tibshirani R, Walther G, Hastie T. Estimating the number of clusters in a data set via the gap statistic. *J Royal Stat Society: Ser B (Statistical Methodology)*. 2001;63:411–23.
65. Bleker C, Ramšak Ž, Bittner A, Podpečan V, Zagorščak M, Wurzing B, et al. Stress knowledge map: a knowledge graph resource for systems biology analysis of plant stress responses. *Plant Commun*. 2024;5:100920.
66. Ramšak Ž, Baebler Š, Rotter A, Korbar M, Mozetič I, Usadel B, et al. GoMapMan: integration, consolidation and visualization of plant gene annotations within the MapMan ontology. *Nucleic Acids Res*. 2014;42:D1167–75.
67. Hagberg AA, Schult DA, Swart PJ. Exploring network structure, dynamics, and function using NetworkX. In: *Proceedings of the 7th Python in Science Conference (SciPy2008)*. Pasadena, California. pp. 11–5.
68. Peixoto TP. The graph-tool python library. 2014.
69. Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, et al. Integration of biological networks and gene expression data using Cytoscape. *Nat Protoc*. 2007;2:2366–82.
70. Gustavsen J, Pai S, Isserlin R, Demchak B, Pico A. RCy3: Network biology using Cytoscape from within R [version 3; peer review: 3 approved]. *F1000Research*. 2019;8.
71. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\text{CT}}$ method. *Methods*. 2001;25:402–8.
72. Bui LT, Giuntoli B, Kosmacz M, Parlanti S, Licausi F. Constitutively expressed ERF-VII transcription factors redundantly activate the core anaerobic response in *Arabidopsis thaliana*. *Plant Sci*. 2015;236:37–43.
73. Gibbs DJ, Conde JV, Berckhan S, Prasad G, Mendiondo GM, Holdsworth MJ. Group VII Ethylene Response Factors Coordinate Oxygen and Nitric Oxide Signal Transduction and stress responses in plants. *Plant Physiol*. 2015;169:23–31.
74. Xie Z, Nolan TM, Jiang H, Yin Y. AP2/ERF Transcription Factor Regulatory Networks in hormone and abiotic stress responses in *Arabidopsis*. *Front Plant Sci*. 2019;10.
75. Rushton DL, Tripathi P, Rabara RC, Lin J, Ringler P, Boken AK, et al. WRKY transcription factors: key components in abscisic acid signalling. *Plant Biotechnol J*. 2012;10:2–11.
76. Nuruzzaman M, Sharoni AM, Kikuchi S. Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Front Microbiol*. 2013;4.
77. Khoso MA, Hussain A, Ritonga FN, Ali Q, Channa MM, Alshegaih RM et al. WRKY transcription factors (TFs): molecular switches to regulate drought, temperature, and salinity stresses in plants. *Front Plant Sci*. 2022;13.
78. Koo AJK, Cooke TF, Howe GA. Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. *Proceedings of the National Academy of Sciences*. 2011;108:9298–303.
79. Heitz T, Widemann E, Lugan R, Miesch L, Ullmann P, Désaubry L, et al. Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone jasmonoyl-isoleucine for catabolic turnover. *J Biol Chem*. 2012;287:6296–306.

80. Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R. ETHYLENE RESPONSE FACTOR1 integrates signals from Ethylene and Jasmonate pathways in Plant Defense. *Plant Cell*. 2003;15:165–78.
81. Kagaya Y, Hattori T. Arabidopsis transcription factors, RAV1 and RAV2, are regulated by touch-related stimuli in a dose-dependent and biphasic manner. *Genes Genet Syst*. 2009;84:95–9.
82. Bourdais G, Burdiak P, Gauthier A, Nitsch L, Salojärvi J, Rayapuram C, et al. Large-scale Phenomics identifies primary and fine-tuning roles for CRKs in responses related to oxidative stress. *PLoS Genet*. 2015;11:e1005373.
83. Hoffmann N. Setting the stage for lignin deposition: spatial distribution of enzymes directing lignification in *Arabidopsis thaliana*. Text. 2019.
84. Wang F, Yu G, Liu P. Transporter-mediated subcellular distribution in the metabolism and signaling of Jasmonates. *Front Plant Sci*. 2019;10.
85. Tessi TM, Brumm S, Winklbauer E, Schumacher B, Pettinari G, Lescano I, et al. Arabidopsis AZG2 transports cytokinins in vivo and regulates lateral root emergence. *New Phytol*. 2021;229:979–93.
86. Hartmann M, Zeier T, Bernsdorff F, Reichel-Deland V, Kim D, Hohmann M, et al. Flavin Monooxygenase-generated N-Hydroxypipicolinic acid is a critical element of plant systemic immunity. *Cell*. 2018;173:456–e46916.
87. Czarnocka W, Fichman Y, Bernacki M, Różańska E, Sańko-Sawczenko I, Mittler R et al. FMO1 is involved in excess light stress-induced Signal Transduction and Cell Death Signaling. *Cells*. 2020;9.
88. Guerra T, Schilling S, Hake K, Gorzalka K, Sylvester F-P, Conrads B, et al. Calcium-dependent protein kinase 5 links calcium signaling with N-hydroxy-L-pipecolic acid- and SARD1-dependent immune memory in systemic acquired resistance. *New Phytol*. 2020;225:310–25.
89. Gasch P, Fundinger M, Müller JT, Lee T, Bailey-Serres J, Mustroph A. Redundant ERF-VII transcription factors bind to an evolutionarily conserved cis-motif to regulate hypoxia-responsive gene expression in Arabidopsis. *Plant Cell*. 2016;28:160–80.
90. Klecker M, Gasch P, Peisker H, Dörmann P, Schlicke H, Grimm B, et al. A shoot-specific hypoxic response of Arabidopsis sheds light on the role of the phosphate-responsive transcription factor PHOSPHATE STARVATION RESPONSE1. *Plant Physiol*. 2014;165:774–90.
91. Valliyodan B, Van Toai TT, Alves JD, De Fátima P, Goulart P, Lee JD, Fritsch FB, et al. Expression of Root-Related Transcription Factors Associated with flooding tolerance of soybean (*Glycine max*). *Int J Mol Sci*. 2014;15:17622–43.
92. Bittner A, Cieřla A, Gruden K, Lukan T, Mahmud S, Teige M, et al. Organelles and phytohormones: a network of interactions in plant stress responses. *J Exp Bot*. 2022;73:1765–81.
93. Yang T-H, Lenglet-Hilfiker A, Stolz S, Glauser G, Farmer EE. Jasmonate Precursor Biosynthetic enzymes LOX3 and LOX4 control wound-response growth restriction. *Plant Physiol*. 2020;184:1172–80.
94. Ozaivo R, Cabrera J, Escobar C, Christensen SA, Borrego EJ, Kolomiets MV, et al. Two closely related members of Arabidopsis 13-lipoxygenases (13-LOXs), LOX3 and LOX4, reveal distinct functions in response to plant-parasitic nematode infection. *Mol Plant Pathol*. 2014;15:319–32.
95. Ding H, Lai J, Wu Q, Zhang S, Chen L, Dai Y-S, et al. Jasmonate complements the function of Arabidopsis lipoxygenase3 in salinity stress response. *Plant Sci*. 2016;244:1–7.
96. Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, Griffiths J, Powers SJ, Gong F, et al. The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *Plant J*. 2008;53:488–504.
97. Jaradat MR, Feurtado JA, Huang D, Lu Y, Cutler AJ. Multiple roles of the transcription factor AtMYB1/AtMYB44 in ABA signaling, stress responses, and leaf senescence. *BMC Plant Biol*. 2013;13:192.
98. Shim JS, Jung C, Lee S, Min K, Lee Y-W, Choi Y, et al. AtMYB44 regulates WRKY70 expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. *Plant J*. 2013;73:483–95.
99. Llorente F, López-Cobollo RM, Catalá R, Martínez-Zapater JM, Salinas J. A novel cold-inducible gene from Arabidopsis, RCI3, encodes a peroxidase that constitutes a component for stress tolerance. *Plant J*. 2002;32:13–24.
100. Kim BH, Kim SY, Nam KH. Genes encoding plant-specific class III peroxidases are responsible for increased cold tolerance of the brassinosteroid-insensitive 1 mutant. *Mol Cells*. 2012;34:539–48.
101. Al-Quraan NA, Locy RD, Singh NK. Expression of calmodulin genes in wild type and calmodulin mutants of Arabidopsis thaliana under heat stress. *Plant Physiol Biochem*. 2010;48:697–702.
102. Evans MJ, Choi W-G, Gilroy S, Morris RJ. A ROS-Assisted calcium Wave Dependent on the AtrBOHD NADPH oxidase and TPC1 Cation Channel propagates the systemic response to salt stress. *Plant Physiol*. 2016;171:1771–84.
103. Liu N, Hake K, Wang W, Zhao T, Romeis T, Tang D. CALCIUM-DEPENDENT PROTEIN KINASE5 associates with the truncated NLR protein TIR-NBS2 to contribute to exo70B1-Mediated immunity. *Plant Cell*. 2017;29:746–59.
104. Jammes F, Song C, Shin D, Munemasa S, Takeda K, Gu D, et al. MAP kinases MPK9 and MPK12 are preferentially expressed in guard cells and positively regulate ROS-mediated ABA signaling. *Proc Natl Acad Sci*. 2009;106:20520–5.
105. Desikan R, Horák J, Chaban C, Mira-Rodado V, Witthöft J, Elgass K, et al. The histidine kinase AHK5 integrates endogenous and Environmental Signals in Arabidopsis Guard cells. *PLoS ONE*. 2008;3:e2491.
106. Rao S, Zhou Z, Miao P, Bi G, Hu M, Wu Y, et al. Roles of receptor-like cytoplasmic kinase VII members in pattern-triggered Immune Signaling. *Plant Physiol*. 2018;177:1679–90.
107. Song C, Lee J, Kim T, Hong JC, Lim CO. VOZ1, a transcriptional repressor of DREB2C, mediates heat stress responses in Arabidopsis. *Planta*. 2018;247:1439–48.
108. Sylvestre-Gonon E, Law SR, Schwartz M, Robe K, Keech O, Didierjean C et al. Functional, structural and biochemical features of plant serinyl-glutathione transferases. *Front Plant Sci*. 2019;10.
109. Solano R, Stepanova A, Chao Q, Ecker JR. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev*. 1998;12:3703–14.
110. Houben M, Vaughan-Hirsch J, Pattyn J, Mou W, Roden S, Martinez Roig A, et al. 1-Aminocyclopropane-1-carboxylic acid oxidase determines the fate of ethylene biosynthesis in a tissue-specific way to fine-tune development and stress resilience. *bioRxiv*. 2024. 2024.02.01.578397.
111. Romero-Hernandez G, Martinez M. Plant Kinases in the Perception and Signaling Networks Associated with Arthropod Herbivory. *Front Plant Sci*. 2022;13.
112. Chu M, Chen P, Meng S, Xu P, Lan W. The Arabidopsis phosphatase PP2C49 negatively regulates salt tolerance through inhibition of AtHKT1;1. *J Integr Plant Biol*. 2021;63:528–42.
113. Moyet L, Salvi D, Bouchnak I, Miras S, Perrot L, Seigneurin-Berny D, et al. Calmodulin is involved in the dual subcellular location of two chloroplast proteins. *J Biol Chem*. 2019;294:17543–54.
114. Fasani E, DalCorso G, Costa A, Zenoni S, Furini A. The Arabidopsis thaliana transcription factor MYB59 regulates calcium signalling during plant growth and stress response. *Plant Mol Biol*. 2019;99:517–34.
115. Lee S, Wang W, Huq E. Spatial regulation of thermomorphogenesis by HY5 and PIF4 in Arabidopsis. *Nat Commun*. 2021;12:3656.
116. Agarwal P, Mitra M, Banerjee S, Roy S. MYB4 transcription factor, a member of R2R3-subfamily of MYB domain protein, regulates cadmium tolerance via enhanced protection against oxidative damage and increases expression of PCS1 and MT1C in Arabidopsis. *Plant Sci*. 2020;297:110501.
117. Wang X-C, Wu J, Guan M-L, Zhao C-H, Geng P, Zhao Q. Arabidopsis MYB4 plays dual roles in flavonoid biosynthesis. *Plant J*. 2020;101:637–52.
118. Yuan F, Yang H, Xue Y, Kong D, Ye R, Li C, et al. OSCA1 mediates osmotic-stress-evoked Ca²⁺ increases vital for osmosensing in Arabidopsis. *Nature*. 2014;514:367–71.
119. Asaoka R, Uemura T, Ito J, Fujimoto M, Ito E, Ueda T, et al. Arabidopsis RABA1 GTPases are involved in transport between the trans-golgi network and the plasma membrane, and are required for salinity stress tolerance. *Plant J*. 2013;73:240–9.
120. Tak H, Negi S, Ganapathi TR. Banana NAC transcription factor MusaNAC042 is positively associated with drought and salinity tolerance. *Protoplasma*. 2017;254:803–16.
121. Alshareef NO, Wang JY, Ali S, Al-Babili S, Tester M, Schmöckel SM. Overexpression of the NAC transcription factor JUNGBRUNNEN1 (JUB1) increases salinity tolerance in tomato. *Plant Physiol Biochem*. 2019;140:113–21.
122. Noman M, Aysha J, Keteouli T, Yang J, Du L, Wang F, et al. Calmodulin binding transcription activators: an interplay between calcium signalling and plant stress tolerance. *J Plant Physiol*. 2021;256:153327.
123. Perochon A, Aldon D, Galaud J-P, Ranty B. Calmodulin and calmodulin-like proteins in plant calcium signaling. *Biochimie*. 2011;93:2048–53.
124. Dekomah SD, Bi Z, Dormatey R, Wang Y, Haider FU, Sun C et al. The role of CDPKs in plant development, nutrient and stress signaling. *Front Genet*. 2022;13.
125. Liu L, White MJ, MacRae TH. Transcription factors and their genes in higher plants. *Eur J Biochem*. 1999;262:247–57.
126. Evans KV, Ransom E, Nayakoti S, Wilding B, Mohd Salleh F, Gržina I, et al. Expression of the Arabidopsis redox-related LEA protein, SAG21 is regulated by ERF, NAC and WRKY transcription factors. *Sci Rep*. 2024;14:7756.

127. Verma V, Ravindran P, Kumar PP. Plant hormone-mediated regulation of stress responses. *BMC Plant Biol.* 2016;16:86.
128. Xu W, Huang W. Calcium-dependent protein kinases in Phytohormone Signaling pathways. *Int J Mol Sci.* 2017;18.
129. Zhu Q, Feng Y, Xue J, Chen P, Zhang A, Yu Y. Advances in receptor-like protein kinases in balancing plant growth and stress responses. *Plants.* 2023;12.
130. Le Gall H, Philippe F, Domon J-M, Gillet F, Pelloux J, Rayon C. Cell wall metabolism in response to Abiotic Stress. *Plants.* 2015;4:112–66.
131. Tenhaken R. Cell wall remodeling under abiotic stress. *Front Plant Sci.* 2015;5.
132. Liu W-C, Han T-T, Yuan H-M, Yu Z-D, Zhang L-Y, Zhang B-L, et al. Functions for seedling postgerminative growth by scavenging HO and stimulating ACX2/3 activity in. *Plant Cell Environ.* 2017;40:2720–8.
133. Kaplan R, Vaziri M, Zwiazek JJ. Regulation of aquaporins in plants under stress. *Biol Res.* 2018;51:4.
134. Chopra D, Hasan MS, Matera C, Chitambo O, Mendy B, Mahlitz S-V, et al. Plant parasitic cyst nematodes redirect host indole metabolism via NADPH oxidase-mediated ROS to promote infection. *New Phytol.* 2021;232:318–31.
135. Lohse S, Schliemann W, Ammer C, Kopka J, Strack D, Fester T. Organization and Metabolism of Plastids and Mitochondria in Arbuscular Mycorrhizal roots of *Medicago truncatula*. *Plant Physiol.* 2005;139:329–40.
136. Janiak A, Kwasniewski M, Sowa M, Gajek K, Żmuda K, Kościelniak J et al. No time to Waste: Transcriptome Study reveals that Drought Tolerance in Barley May be attributed to stressed-like expression patterns that exist before the occurrence of stress. *Front Plant Sci.* 2018;8.
137. Kobayashi K, Sasaki D, Noguchi K, Fujinuma D, Komatsu H, Kobayashi M, et al. Photosynthesis of Root chloroplasts developed in Arabidopsis Lines Overexpressing GOLDEN2-LIKE transcription factors. *Plant Cell Physiol.* 2013;54:1365–77.
138. Dolgikh VA, Pukhovaya EM, Zemlyanskaya EV. Shaping Ethylene response: the role of EIN3/EIL1 transcription factors. *Front Plant Sci.* 2019;10.
139. Lang T, Deng C, Yao J, Zhang H, Wang Y, Deng S. A Salt-Signaling Network Involving Ethylene, Extracellular ATP, Hydrogen Peroxide, and Calcium mediates K⁺/Na⁺ homeostasis in Arabidopsis. *Int J Mol Sci.* 2020;21.
140. Kumari P, Dahiya P, Livanos P, Zergiebel L, Kölling M, Poeschl Y, et al. IQ67 DOMAIN proteins facilitate preprophase band formation and division-plane orientation. *Nat Plants.* 2021;7:739–47.
141. Xiao Y, Chu L, Zhang Y, Bian Y, Xiao J, Xu D. HY5: A Pivotal Regulator of Light-Dependent Development in higher plants. *Front Plant Sci.* 2022;12.
142. Bellegarde F, Maghiaoui A, Boucherez J, Krouk G, Lejay L, Bach L et al. HNI9 and HY5 maintain ROS homeostasis under high nitrogen provision in Arabidopsis. *bioRxiv.* 2018;479030.
143. Li J, Zeng J, Tian Z, Zhao Z. Root-specific photoreception directs early root development by HY5-regulated ROS balance. *Proceedings of the National Academy of Sciences.* 2024;121:e2313092121.
144. Abbas N, Maurya JP, Senapati D, Gangappa SN, Chattopadhyay S. Arabidopsis CAM7 and HY5 physically interact and directly bind to the HY5 promoter to regulate its expression and thereby promote photomorphogenesis. *Plant Cell.* 2014;26:1036–52.
145. Giri MK, Singh N, Banday ZZ, Singh V, Ram H, Singh D, et al. GBF1 differentially regulates 2 and 4 transcription to promote pathogen defense in Arabidopsis thaliana. *Plant J.* 2017;91:802–15.
146. Dóczy R, Brader G, Pettkő-Szandtner A, Rajh I, Djamei A, Pitzschke A, et al. The Arabidopsis Mitogen-activated protein kinase kinase MKK3 is Upstream of Group C Mitogen-Activated Protein Kinases and participates in Pathogen Signaling. *Plant Cell.* 2007;19:3266–79.
147. Popescu SC, Popescu GV, Bachan S, Zhang Z, Seay M, Gerstein M et al. Differential binding of calmodulin-related proteins to their targets revealed through high-density Arabidopsis protein microarrays. *Proceedings of the National Academy of Sciences.* 2007;104:4730–5.
148. Liu Z, Guo C, Wu R, Hu Y, Zhou Y, Wang J et al. FLS2–RBOHD–PIF4 Module regulates Plant Response to Drought and Salt stress. *Int J Mol Sci.* 2022;23.
149. Stamm P, Ravindran P, Mohanty B, Tan EL, Yu H, Kumar PP. Insights into the molecular mechanism of RGL2-mediated inhibition of seed germination in *Arabidopsis thaliana*. *BMC Plant Biol.* 2012;12:179.
150. Ali F, Qanmber G, Li F, Wang Z. Updated role of ABA in seed maturation, dormancy, and germination. *J Adv Res.* 2022;35:199–214.
151. Puglia GD. Reactive oxygen and nitrogen species (RONS) signalling in seed dormancy release, perception of environmental cues, and heat stress response. *Plant Growth Regul.* 2024;103:9–32.
152. Denison FC, Paul A-L, Zupanska AK, Ferl RJ. 14-3-3 proteins in plant physiology. *Semin Cell Dev Biol.* 2011;22:720–7.
153. Yang Z, Wang C, Xue Y, Liu X, Chen S, Song C, et al. Calcium-activated 14-3-3 proteins as a molecular switch in salt stress tolerance. *Nat Commun.* 2019;10:1199.

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Appendix 3

ORIGINAL ARTICLE

Constitutive expression of *JASMONATE RESISTANT 1* induces molecular changes that prime the plants to better withstand drought

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Abstract

In this study, we investigated *Arabidopsis thaliana* plants with altered levels of the enzyme *JASMONATE RESISTANT 1* (*JAR1*), which converts jasmonic acid (JA) to jasmonoyl-L-isoleucine (JA-Ile). Analysis of a newly generated overexpression line (35S::JAR1) revealed that constitutively increased JA-Ile production in 35S::JAR1 alters plant development, resulting in stunted growth and delayed flowering. Under drought-stress conditions, 35S::JAR1 plants showed reduced wilting and recovered better from desiccation than the wild type. By contrast, *jar1-11* plants with a strong reduction in JA-Ile content were hypersensitive to drought. RNA-sequencing analysis and hormonal profiling of plants under normal and drought conditions provided insights into the molecular reprogramming caused by the alteration in JA-Ile content. Especially 35S::JAR1 plants displayed changes in expression of developmental genes related to growth and flowering. Further transcriptional differences pertained to drought-related adaptive systems, including stomatal density and aperture, but also reactive oxygen species production and detoxification. Analysis of wild type and *jar1-11* plants carrying the roGFP-Orp1 sensor support a role of JA-Ile in the alleviation of methyl viologen-induced H₂O₂ production. Our data substantiate a role of JA-Ile in abiotic stress response and suggest that JAR1-mediated increase in JA-Ile content primes *Arabidopsis* towards improved drought stress tolerance.

KEYWORDS

JA-Ile, jasmonic acid, phytohormones, plant development, RNA-seq, ROS

1 | INTRODUCTION

Jasmonic acid (JA) and its derivatives, collectively known as jasmonates, are phytohormones involved in the regulation of plant growth, development and stress responses (for recent reviews, see

Koo, 2018; Wasternack & Song, 2017). In the octadecanoid pathway, jasmonate biosynthesis is initiated from α -linolenic acid released from plastidial galactolipids through different lipoxygenases (13-LOXs) (Bell et al., 1995). Subsequently, ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASES generate the first committed

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precursor, 12-oxo-phytodienoic acid (*cis*-OPDA), which in peroxisomes is converted into JA by OPDA REDUCTASE 3 (OPR3) and β -oxidation. In the cytosol, JA is modified or conjugated to different derivatives, including the most bioactive form jasmonoyl-L-isoleucine (JA-Ile) (Koo, 2018; Westernack & Song, 2017). JA-Ile content seems to be tightly controlled via different regulatory loops, including potential autoregulation of jasmonate synthesis (Hickman et al., 2017). Moreover, catabolic derivatives of JA and JA-Ile might play a role in maintaining jasmonate homeostasis.

JASMONATE RESISTANT 1 (JAR1), a member of the GH3 family enzymes, holds a key position in jasmonate biosynthesis, because it catalyses the formation of JA-Ile from JA (Staswick & Tiryaki, 2004). JA-Ile can form a complex with the F-box protein CORONATINE INSENSITIVE 1 (COI1), various members of the transcriptional repressor JASMONATE ZIM-domain family (JAZ) and other components to form the SCF^{COI1} complex (Koo, 2018; Westernack & Song, 2017). Below a certain threshold level of JA-Ile, JAZ proteins interact with various transcription factors (TFs) that act as activators or repressors and ultimately regulate hundreds of genes. Accumulation of JA-Ile and formation of the SCF^{COI1} complex targets the JAZ proteins for degradation through the 26S proteasome, thus releasing suppression of jasmonate responsive genes. The bHLH-type TF MYC2 is considered a master regulator of jasmonate signalling (Dombrecht et al., 2007). Induced by JA-Ile, MYC2 regulates the transcription of jasmonate-responsive genes such as *VEGETATIVE STORAGE PROTEINS* (*VSP1* and *VSP2*), shown to participate in plant development and defence (Devoto et al., 2005; Westernack & Song, 2017). MYC2 also plays a role in terminating the jasmonate response via a negative feedback mechanism (Liu et al., 2019).

Drought is considered one of the major abiotic stresses that negatively affect plant growth and development (Yang et al., 2010). In *Arabidopsis*, exogenous MeJA application was shown to induce drought-responsive genes, whereas, vice versa, the exposure to drought induces jasmonate biosynthesis leading to JA-Ile accumulation (de Ollas et al., 2015a, 2015b; Harb et al., 2010; Zander et al., 2020). This relationship between jasmonate and drought stress was also reported for several crops (Creelman & Mullet, 1995; Du et al., 2013; Gao et al., 2004; Tayyab et al., 2020; Wang et al., 2021). Moreover, Marquis et al. (2022) showed recently that an *Arabidopsis* mutant in the *JASMONATE OXIDASE 2* (*JAO2*) gene locus, which is affected in jasmonate homeostasis, was more resistance to drought. Its drought resistant phenotype was dependent on JA-Ile signalling. The *jao2* mutant plants showed changes in the expression of defence-related genes already in unchallenged mutant leaves and also in the formation of defence-related metabolites. However, the allocation of metabolic resources to synthesize plant defence compounds is often associated with reduced growth and biomass accumulation (Züst & Agrawal, 2017).

Tolerance mechanisms to drought comprise a wide range of cellular processes. Among other things, reactive oxygen species (ROS) production is a common reaction to drought stress (Noctor et al., 2014). To cope with oxidative damage, jasmonate signalling was found to be involved in activating antioxidant mechanisms, such

as regulating the ascorbate-glutathione (GSH) cycle and synthesis of polyphenols (Dombrecht et al., 2007; Savchenko et al., 2019). At the same time, stress adaptation relies on the interplay of multiple signalling pathways to integrate different environmental and developmental signals. Abscisic acid (ABA) is the hormone most closely associated with drought and it was shown that JA-Ile and ABA signalling interact under water stress conditions (de Ollas et al., 2015a, 2015b).

In this study, we used *Arabidopsis* lines with altered *JAR1* expression to change the endogenous JA-Ile content. We could show that alteration in JA-Ile content affects plant growth even under non-stress conditions. Furthermore, a reduced JA-Ile content makes plants more susceptible to progressive drought, while constitutively increased JA-Ile content strongly alleviates the deleterious effects of drought, making plants less susceptible and more likely to recover. In depth analysis of RNA-sequencing (RNA-seq) data obtained under control and early drought conditions provided insight into the transcriptional reprogramming caused by the alteration in JA-Ile content. Based on these data, the connection between *JAR1*-dependent changes in gene expression and differences in *Arabidopsis* growth and drought response phenotypes are discussed.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

If not otherwise stated, experiments in this study were performed on *Arabidopsis thaliana* (ecotype Columbia; Col-0) plants or transgenic lines created in the Col-0 background (Supporting Information: Figure S1A). The T-DNA insertion lines *jar1-11* (SALK_034543) and *jar1-12* (SALK_011510) were obtained from NASC (RRID: SCR_004576) and plants homozygous for the T-DNA insertion were identified by PCR screening (Supporting Information: Figure S1B). Primers are listed in Supporting Information: Table S1. Plants were grown either in standard plant potting soil pretreated with Confidor WG 70 (Bayer Agrar) or on ½ Murashige and Skoog medium (½ MS medium; Duchefa Biochemie) with 1% (w/v) sucrose and 0.6% (w/v) phytigel (Sigma-Aldrich, Inc.). Plants grown on ½ MS were stratified for 2 days at 4°C in the dark. Plants were cultured in climatized growth chambers (equipped with Philips TLD 18 W of alternating 830/840 light colour temperature) at 22°C under long-day conditions (16 h light/8 h dark) with 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

2.2 | Generation of JAR1-YFP overexpression lines

To generate plants expressing *JAR1.1* as a fusion protein with yellow fluorescent protein (YFP) under the control of the 35S promoter (35S::*JAR1.1*-YFP), the entire coding sequence of the *JAR1.1* variant was cloned into the pBIN19 vector (Datla et al., 1992) in frame with the YFP sequence using *Apal* and *NotI* restriction sites. The resulting construct (Supporting Information: Figure S1C) was stably

transformed into Col-0 using the floral dip method. Three independent homozygous T-DNA insertion lines (35S::JAR1) were obtained each in the F3 generation. JAR1.1-YFP expression was confirmed through RT-qPCR (Figure 1a), confocal microscopy (Supporting Information: Figure S1D) and western blotting using an antibody against green fluorescent protein (GFP; Supporting Information: Figure S1E).

2.3 | Plant phenotyping

For analysis of soil-grown plants, seeds were directly planted in potting soil. Five days later, young seedlings were transplanted to fresh pots containing 100 g potting soil (either one or four seedlings per pot). This was denoted as Day 1. Plants were then grown for 18 days with regular watering using identical volumes of tap water. Afterward, plants were either watered normally or exposed to drought stress conditions by withholding watering for up to 14 days. During the drought-stress treatment, pot weights were measured regularly. The relative soil water content (SWC) calculated as $\{(\text{pot weight at the time of measurement}) - (\text{empty pot weight})\} / \{(\text{initial pot weight}) - (\text{empty pot weight})\} \times 100$ was adjusted between plant lines to ensure a similar drought stress level. After SWC dropped to 10%, plants were rewatered with equal volumes of tap water and survival rates of plants were calculated after 24 h and 7 days. The positioning of all pots in the climate chamber was randomized throughout the experiments. Photographs were taken at regular intervals and corresponding whole rosette leaves were collected for biochemical and RNA-seq analyses on Day 32.

For root growth assays, plants were grown on $\frac{1}{2}$ MS plates with and without the addition of 50 μM MeJA. The root length was measured on Day 14.

2.4 | Stomatal aperture, density and relative water content (RWC) measurements

Stomatal aperture diameters and density were measured from the 6th leaf of 21-day-old plants grown under control conditions by collecting the leaf epidermis as described previously (Hossain et al., 2011). The RWC of leaves was calculated according to Barrs and Weatherley (1962).

2.5 | In vivo redox imaging

In vivo redox imaging was performed on the leaves of 7–9-day-old seedlings as described in (Meyer et al., 2007) using a Leica SP8 lightning (Leica Mikrosysteme). After pre-incubation in imaging buffer (10 mM MES, 10 mM MgCl_2 , 10 mM CaCl_2 , 5 mM KCl, pH 5.8), seedlings were transferred into a perfusion chamber (QE-1, Warner Instruments) to allow the exchange to different treatment solutions under constant imaging. Pinhole was adjusted to 3. After each run,

representative samples were calibrated with 10 mM dithiothreitol (DTT; ratio = 0.18) and 10 mM H_2O_2 (ratio = 1.20). Data were processed using the integrated LASX software (RRID:SCR_013673) with the 'quantify' mode and the ratiometric image of 405/488 nm was calculated based on a standardization using 10 mM DTT and 10 mM H_2O_2 .

2.6 | Anthocyanin measurements

Anthocyanin content was measured by adding 300 μl extraction buffer (1% [v/v] HCl in MeOH) to 100 mg of liquid N_2 ground leaf tissue, mixed with 200 μl H_2O and 500 μl chloroform, and placed overnight at 4°C. After centrifugation, supernatants were collected and re-extracted with 400 μl of 60% methanol, 1% HCl. The absorbance was taken at 530 nm (anthocyanin) and 657 nm (background), and anthocyanin content was expressed as (A530-A657) per gram fresh weight.

2.7 | Western blot analysis

For extraction of total proteins, 100 mg finely ground leaf tissues were mixed with 100 μl 4 $\times$ sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) solubilizing buffer, vortexed and then incubated at 96°C for 10 min. After centrifugation for 10 min at 14,000g, proteins in the supernatant were separated on 10% SDS–PAGE gels and blotted onto nitrocellulose membranes. Western blot analysis was performed by a standard protocol using an antibody against GFP (α -GFP; Roche, Cat# 11814460001, RRID:AB_390913) and a secondary antibody coupled with alkaline phosphatase (ThermoFisher Scientific Cat# 31320, RRID:AB_228304).

2.8 | Phytohormone analysis

Flash-frozen whole rosette leaves from three plants per sample were ground to a fine powder in liquid N_2 . Approximately 50 mg of each sample was extracted with 1 ml methanol containing 30 ng D_6 -JA, 6 ng D_6 -JA-Ile (HPC Standards GmbH) and 30 ng D_6 -ABA (Santa Cruz Biotechnology) as internal standards. The extracts were vortexed vigorously for 4–5 s and incubated for 2 min at 25°C under constant agitation at 1500 r.p.m. in a heating block. After 5 min centrifugation at 13000g and 4°C, ~900 μl of the supernatant was transferred to fresh microcentrifuge tubes. The residual tissues were reextracted using 750 μl 100% methanol without standards. The supernatants (1650 μl in total) were completely dried under a flow of N_2 at 30°C and redissolved in 300 μl 100% methanol.

Phytohormone analysis was performed on an Agilent 1260 high-performance liquid chromatography system (Agilent Technologies) attached to a QTRAP 6500 tandem mass spectrometer (Sciex) equipped with a turbo spray ion source operated in the negative ionization mode (Ullah et al., 2019, 2022). The concentrations of

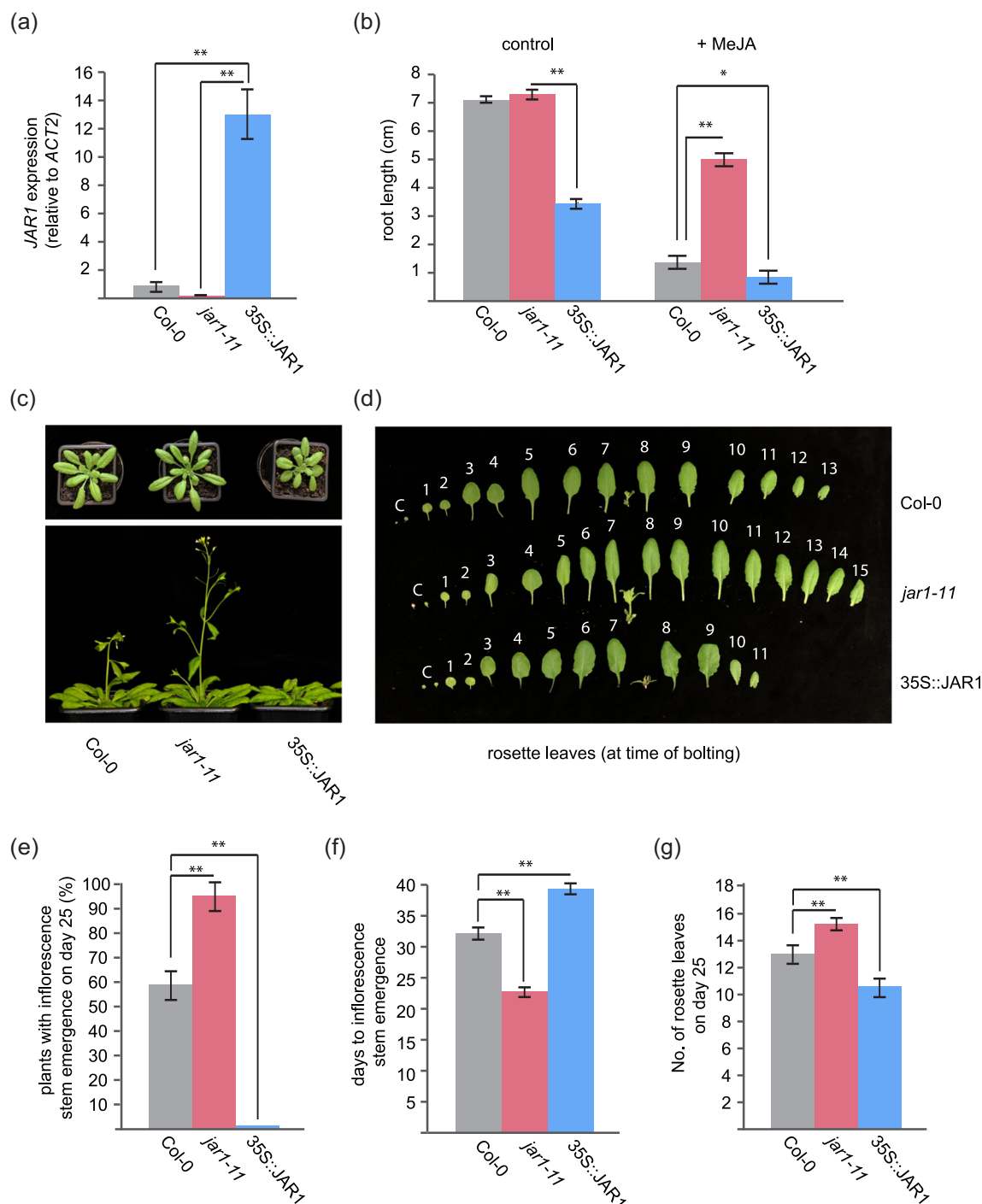


FIGURE 1 Alteration in *JASMONATE RESISTANT 1* (*JAR1*) expression affects *Arabidopsis* leaf growth and flowering time. (a) *JAR1* transcript levels, relative to *ACT2*, in Col-0, *jar1-11* and 35S::*JAR1* determined by RT-qPCR using rosette leaves of 25-day-old plants grown on soil. Data were analysed by one-way analysis of variance (ANOVA) (** $p < 0.01$) followed by multiple comparison analysis (Tukey's honest significant difference [HSD] test). Data represent means $\pm$ SE from three biological replicates ($n = 3$). (b) Root length of Col-0, *jar1-11* and 35S::*JAR1* plants grown on $\frac{1}{2}$ Murashige and Skoog medium ($\frac{1}{2}$ MS) medium with or without 50 μ M MeJA (see also Supporting Information: Figure S2). Data were analysed by one-way ANOVA (* $p < 0.05$, ** $p < 0.01$) followed by multiple comparison analysis (Tukey's HSD test). Data represent means $\pm$ SE from three biological replicates ($n = 3$), each containing >10 seedlings. (c) Representative photographs showing the growth phenotype of Col-0, *jar1-11* and 35S::*JAR1* plants after 25 days (upper panel) and 32 days (lower panel). (d) Detached rosette leaves at the time of inflorescence stem emergence (~ 1 cm stem length). Leaves were detached at Day 32 (Col-0), Day 25 (*jar1-11*) and Day 40 (35S::*JAR1*). (e) Percentage of plants with emerged inflorescence stem of at least 1 cm at Day 25. Data represent means $\pm$ SE from five biological replicates ($n = 5$), each containing a minimum of five individual plants. (f) Average day by which inflorescence stems had emerged. Data represent means $\pm$ SE from five biological replicates ($n = 5$), each containing a minimum of five individual plants. (g) Rosette leaf numbers at Day 25. Data represent means $\pm$ SE from five biological replicates ($n = 5$), each containing a minimum of five individual plants. Data (e–g) were analysed by one-way ANOVA (** $p < 0.01$) followed by multiple comparison analysis (Tukey's HSD test)

ABA, JA and JA-Ile were determined relative to the corresponding internal standards of D₆-ABA, D₆-JA and D₆-JA-Ile, respectively. Content of *cis*-OPDA was determined using D₆-JA, applying an experimental response factor (RF) of 1.0. Levels of 12-OH-JA-Ile and 12-COOH-JA-Ile were quantified relative to D₆-JA-Ile, applying an experimental RF of 1.0.

2.9 | RNA extraction, cDNA synthesis and RT-qPCR

Total RNA was extracted from the whole rosette leaves using the Quick-RNA Miniprep Kit (Zymo-Research). RNA quality and quantity were determined using a Nabi UV/Vis Nano Spectrophotometer (LTF Labortechnik). For RT-qPCR analysis, cDNA was prepared from 1 µg of messenger RNA (mRNA) with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, ThermoFisher Scientific). Gene expression was quantified using the Power SYBR Green PCR Master Mix in 48-well plates in a StepOne™ Real-Time PCR Thermocycler (Applied Biosystems, ThermoFisher Scientific) and the expression level was normalized to *ACTIN2* (*ACT2*) to express as relative quantity ($2^{-\Delta\Delta C_t}$). Primers used for RT-qPCR are listed in Supporting Information: Table S1.

2.10 | RNA-seq analysis

For each RNA-seq sample, the RNA extracted from three plants was pooled and the quality of RNA was checked by determining the RNA integrity number using a TapeStation 4200 (Agilent). For each line and experimental conditions, three independent pool samples were analysed. The library preparation and sequencing were performed by the NGS Core Facilities at the University of Bonn, Germany. Approximately 200 ng of RNA was used for library construction. Sequencing libraries were prepared using the QuantSeq 3' mRNA-Seq Kit (Lexogen) and sequenced on an Illumina HiSeq. 2500 V4 platform with a read length of 1 × 50 bases. For each of the samples, three biological replicates were sequenced with an average sequencing depth of 10 million reads.

CLC Genomics Workbench v.12.03 (RRID:SCR_011853) was used to process the raw sequencing data. Quality control and trimming were performed on FASTQ files of the samples. Quality trimming was performed based on a quality score limit of 0.05 and a maximum number of two ambiguities. To map the additional JAR1 reads from the JAR1.1-YFP lines, an additional chromosome comprising the YFP sequence was added to the Araport 11 (Cheng et al., 2017) genome and the annotation file. The FASTQ samples were then mapped to the modified Araport 11 genome, while only classifying reads as mapped, which uniquely matched with ≥80% of their length and shared ≥90% identity with the reference genome. For the mapping to the gene models, reads had to match with ≥90% of their length and share ≥90%

similarity with a maximum of one hit allowed. Further steps were completed using the R programming language (R Core Team, 2020). Gene Ontology (GO) term enrichment analysis was performed with the topGO package (RRID:SCR_014798). Additionally, transcripts per million (TPM) values were calculated based on the read counts. For individual genes, TPM values were compared by performing an analysis of variance (ANOVA) (RRID:SCR_002427) and a Tukey's honest significant difference (HSD) test with a confidence interval of 0.95 (Tukey & Hamner, 1949). Figures and plots were created using Venn Diagram, heatmap, ggpubr and EnhancedVolcano included in the R package.

2.11 | Statistical analyses

Data were analysed statistically with ANOVA followed by multiple comparisons (Tukey's HSD test) in R. One-way ANOVA was used for all parameters except hormonal data where two-way ANOVA was applied. For additional experiments, a two-tailed *t*-test was used. Bar plots with error bars were generated in Microsoft Excel. Real-time monitoring of the roGFP2-Orp1 sensor was done using the XY-simple linear regression with 95% confidence level in GraphPad Prism v.9.0.0. (RRID:SCR_002798).

2.12 | Data availability

A list of accession numbers is provided in Supporting Information: Data Set_1. The RNA-seq data are deposited in the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>, RRID:SCR_005012) under the submission number GSE196602.

3 | RESULTS

To investigate the effect of JA-Ile on plant growth, we used the *Arabidopsis* T-DNA insertion line *jar1-11* (Supporting Information: Figure S1A and S1B) and a newly generated line expressing the YFP-tagged JAR1.1 splice variant under control of the 35S promoter (35S::JAR1.1-YFP) in a Col-0 background, which we refer to as 35S::JAR1 (Supporting Information: Figure S1C). RT-qPCR analysis of rosette leaves under normal growth conditions detected very low expression of JAR1 transcripts in *jar1-11* (Figure 1a), confirming that it is a knockdown for JAR1 (Suza & Staswick, 2008). By contrast, 35S::JAR1 plants showed strongly elevated expression of JAR1 (Figure 1a). Fluorescence microscopy and western blot analysis with a GFP antibody furthermore confirmed the presence of high levels of JAR1.1-YFP protein in rosette leaves of the 35S::JAR1 line (Supporting Information: Figure S1D and S1E). Thus, these lines are a great resource to study the effects of varying internal JA-Ile levels on plant growth and stress responses.

3.1 | JAR1 expression levels affect JA-Ile content and alters growth and flowering time

When tested on ½ MS medium, *jar1-11* plants grew similar as Col-0, whereas 35S::JAR1 plants exhibited a retarded growth phenotype (Figure 1b and Supporting Information: Figure S2). As was shown before, exogenous MeJA application strongly reduced root growth and shoot development in Col-0. MeJA can be taken up by the plant and in the presence of JAR1 is converted to JA-Ile. Consequently, the *jar1-11* plants were much less affected and developed quite well, whereas 35S::JAR1 plants were most severely affected by MeJA treatment. Upon extended growth on soil, *jar1-11* plants displayed a slightly larger rosette size than Col-0, whereas 35S::JAR1 plants showed slightly stunted growth with shorter and somewhat wider leaf blades (Figure 1c,d). Moreover, *jar1-11* plants were a few days ahead in bolting and flowering compared with Col-0, whereas 35S::JAR1 plants lagged behind by about 8–10 days (Figure 1c,e,f). The number of rosette leaves at the bolting stage also varied, with the highest in *jar1-11* (~14–16) and the lowest in 35S::JAR1 (~10–11) (Figure 1d,g). No significant differences were observed with other parameters related to reproductive success, such as the number and length of siliques, number of seeds per silique or germination rate (Supporting Information: Table S2).

Analysis of various jasmonates (Figure 2a–g, blue bars) in rosette leaves of the different plant lines grown on soil showed that JA-Ile content in Col-0 was low and in *jar1-11* plants virtually absent (Figure 2d). The 35S::JAR1 plants accumulated elevated levels of JA-Ile, indicating that substantial amounts of JA-Ile were synthesized and retained in the presence of constitutively elevated JAR1 protein. On the other hand, content of JA did not change much (Figure 2a). With regard to catabolic derivatives of JA and JA-Ile, 12-OH-JA, 12-OH-JA-Ile and 12-COOH-JA-Ile showed a substantial increase in the 35S::JAR1 plants (Figure 2b,e,f), suggesting that increased JA-Ile production in these plants also leads to an increased formation of catabolic products.

Plants of the *jar1-12* line, also containing significantly lower JAR1 transcript levels, match the *jar1-11* phenotype of faster growth and early flowering, whereas two additional JAR1.1 overexpression lines support the stunted growth and late flowering observed in 35S::JAR1 (Supporting Information: Figure S3A and S3B). The early flowering phenotype seen in *jar1-11* is also found in other mutants related to jasmonate (Supporting Information: Figure S3C) where the pathway is blocked before JA-Ile production either at the synthesis of OPDA (*aos*) or JA (*opr3*). Together, our data indicate that changes in JAR1 transcript levels alter JA-Ile content and that this alteration is the decisive factor for the observed difference in growth and development.

3.2 | Morphological differences between *jar1-11* and 35S::JAR1 are reflected in the expression of growth- and flowering-related genes

Global transcriptional differences in the rosette leaves of 32-day-old soil-grown plants were elucidated by RNA-seq analyses (Supporting

Information: Data Set S1 and Figure S4). We found only four differentially expressed genes (DEGs) between *jar1-11* and Col-0 (Figure 3a and Supporting Information: Data Set S2), all of which were down-regulated. By contrast, we found 339 DEGs between 35S::JAR1 and Col-0 (Figure 3a,b and Supporting Information: Data Set S3) in line with the much stronger phenotypic difference observed between 35S::JAR1 and Col-0 compared with *jar1-11* under these growth conditions (Figure 1c).

The three genes down-regulated in *jar1-11* (but not 35S::JAR1) comprise JAR1 itself, AT1G22480 (a potential uclacyanin; cupredoxin superfamily protein) and the well-known jasmonate responsive VSP1 gene (Figure 3c and Supporting Information: Data Set S2). Although the closely related VSP2 showed only a slight, nonsignificant decrease in *jar1-11*, expression of both VSP1 and VSP2 was upregulated in 35S::JAR1 plants (Figure 3c). In line with the high levels of JA and JA-Ile derivatives, transcript levels of IAA-LEUCINE RESISTANT (ILR)-LIKE GENE 6 (ILL6) and JASMONATE-INDUCED OXYGENASES 3 (JOX3) were remarkably higher in 35S::JAR1. ILL6, a negative regulator of JA signalling, hydrolyses JA-Ile and 12-OH-JA-Ile to JA and 12-OH-JA, respectively (Bhosale et al., 2013; Widemann et al., 2013). JOX3 is involved in the oxidation of JA to 12-OH-JA (Smirnova et al., 2017).

Although it is described that JA-Ile accumulation releases transcriptional repression of MYC2, we found only a nonsignificant increase in MYC2 expression in the 35S::JAR1 plants (Supporting Information: Data Set S4). This indicates that increase in JA-Ile alone is not sufficient to alter the expression of this postulated master regulator of jasmonate signalling. It also indicates that VSP1 and VSP2 expression can increase in a JA-Ile-dependent manner independent of MYC2. Expression of MYC4, a TF that was suggested to work additively to MYC2 in some jasmonate-mediated responses (Fernández-Calvo et al., 2011), was significantly decreased in 35S::JAR1 (Figure 3c). Interestingly, MYC4 was suggested to regulate the transcription of genes such as G1F1, a gene involved in the regulation of leaf expansion that was found to be increased in 35S::JAR1 (Supporting Information: Data Set S4). Furthermore, several of the DEGs upregulated in 35S::JAR1 as compared with Col-0 are involved in cell cycle control, for example, SYP111 (KNOLLE), FBL17, CYCA3;2 and CYCB1;2 (Supporting Information: Data Set S4), and play a role in leaf growth and expansion (Vercruysse et al., 2020).

Although *jar1-11* plants showed early and 35S::JAR1 plants delayed flowering compared with Col-0 (Figure 1c), we found no variation in major photoperiod-related floral responsive genes such as FT, LEAFY or APETALA2 (Kinoshita & Richter, 2020). However, a heat map shows enhancement of FLOWERING LOCUS C (FLC) expression in 35S::JAR1 (Figure 3d), a major player of the autonomous flowering-time pathway (Wu et al., 2020). Early flowering inhibition by FLC involves repression of SOC1 (Michaels & Amasino, 2001), whose expression was decreased in 35S::JAR1, as was the expression of the early flowering inducers MAF1 (Ratcliffe et al., 2001) and SPL4 (Wu & Poethig, 2006). On the other hand, expression of MYROSINASE BINDING PROTEIN 2 (MBP2; F-ATMBP), which is related to flowering regulation through the COI1 receptor (Capella et al., 2001), was enhanced (Figure 3D).

Overall, the results suggest that the higher JA-Ile level in 35S::JAR1 causes changes in the expression of growth and flowering-related genes

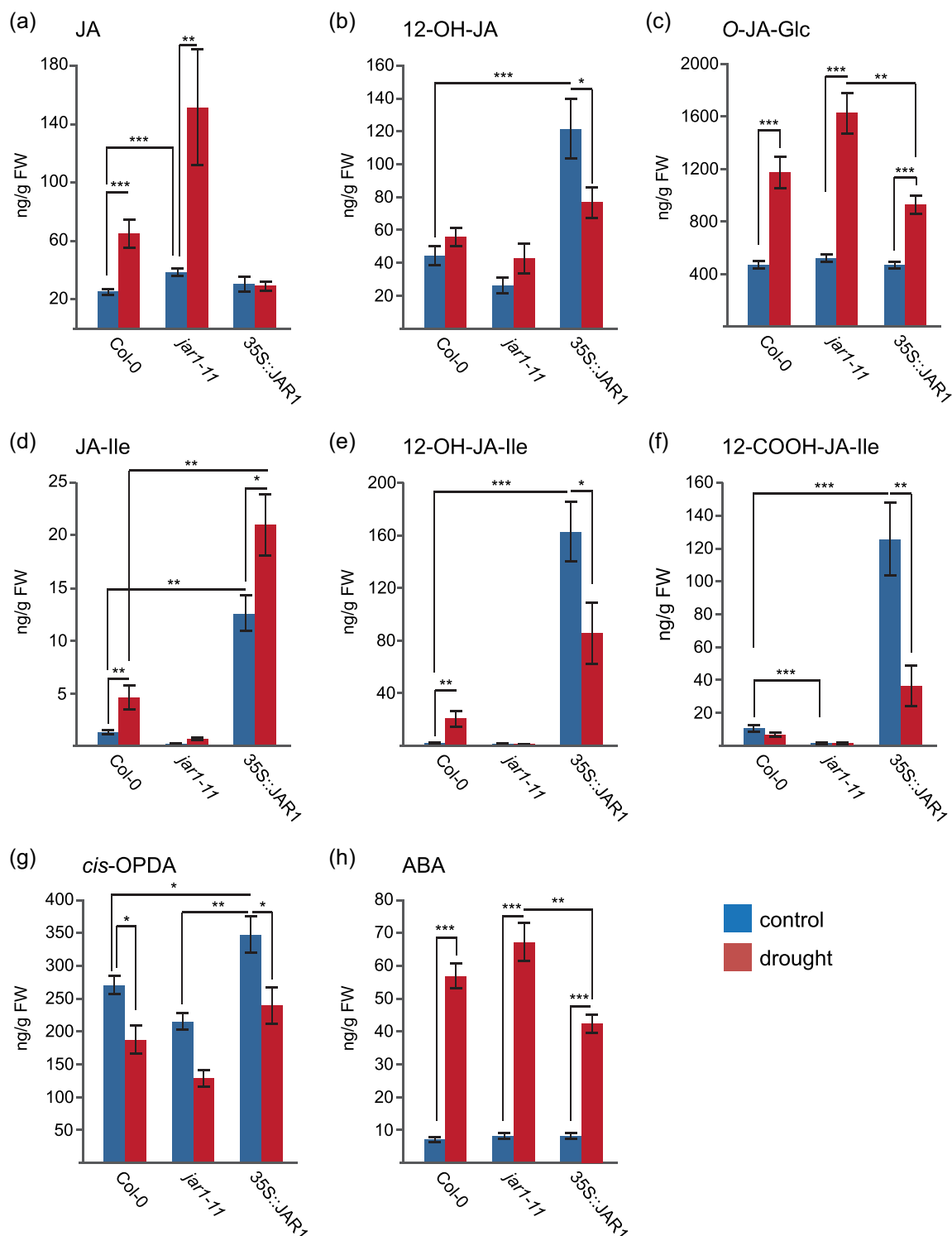


FIGURE 2 JASMONATE RESISTANT 1 (*JAR1*)-dependent changes in the contents of jasmonates and abscisic acid (ABA). The contents of different jasmonates (a–g) and ABA (h) were determined in rosette leaves of 32-day-old plants from wild type (Col-0), *jar1-11* and 35S::JAR1 grown under control and drought stress conditions. Compounds measured were jasmonic acid (JA), 12-hydroxy-jasmonic acid (12-OH-JA), 12-hydroxyl-jasmonoyl-glucoside (12-O-Glc-JA), jasmonoyl-L-isoleucine (JA-Ile), 12-hydroxy-jasmonoyl-isoleucine (12-OH-JA-Ile), 12-carboxy-jasmonoyl-isoleucine (12-COOH-JA-Ile), 12-oxo-phytodienoic acid (*cis*-OPDA) and ABA. Data represent means $\pm$ SE from six replicates ($n = 6$), each containing pooled extracts from three plants. Data were analysed by two-way analysis of variance (ANOVA) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) followed by multiple comparison analysis (Tukey's honest significant difference [HSD] test).

resulting in rosettes with shorter but wider leaves and a delay in transition from vegetative to reproductive mode.

3.3 | JAR1 expression levels affect drought tolerance of *Arabidopsis*

We next performed progressive drought experiments by withholding water from 18-day-old well-watered plants (Figure 4a). After 2 weeks

of water withholding (Day 32), the first indications of drought effects occurred (Figure 4b and Supporting Information: Figure S5A). Hypersensitivity of *jar1-11* to drought became clearly visible at Day 36, with *jar1-11* plants displaying severe signs of wilting compared with Col-0. Three days later, both Col-0 and *jar1-11* plants had reached a state of unrecoverable wilting and re-watering at this stage resulted in 0% survival. By contrast, 35S::JAR1 plants displayed an extended drought tolerance and showed first signs of wilting only at Day 39, which could be fully recovered by re-watering (Figure 4b and Supporting Information: Figure S5A). In line with the visible effects, 35S::JAR1 plants retained about 80% RWC at Day 36, whereas the RWC of Col-0 and *jar1-11* plants dropped to 50% and 30%, respectively (Figure 4c). The drought-susceptible phenotype of *jar1-11* could also be confirmed in the *jar1-12* line (Supporting Information: Figure S5B).

To ensure that the better performance of 35S::JAR1 plants under drought was not a direct effect of the reduced biomass and thus lower water uptake from the soil, Col-0, *jar1-11* and 35S::JAR1 plants were grown together in the same pot. With four plants in the same size pot, drought effects were slightly more severe also in 35S::JAR1, but as before, the 35S::JAR1 plants showed lesser wilting and recovered after only 1 day of re-watering, with no recovery seen for Col-0 and *jar1-11* plants (Supporting Information: Figure S5C). In a separate drought stress experiment, Col-0 plants were treated with a foliar spray of MeJA on Day 11, before the start of water withholding (Day 18). Similar to 35S::JAR1, MeJA-treated Col-0 plants showed stunted growth together with better drought resistance and recovery (Supporting Information: Figure S5D).

At Day 32, already before the onset of any severe drought effects, JA-Ile content increased significantly in Col-0 and 35S::JAR1, confirming that the plants already experience water deficiency and react by inducing jasmonate biosynthesis (Figure 2d). By contrast, JA-Ile content remained virtually absent in *jar1-11* even under these conditions. However, JA content in *jar1-11* was strongly increased (Figure 2a), likely because jasmonate biosynthesis is induced but the

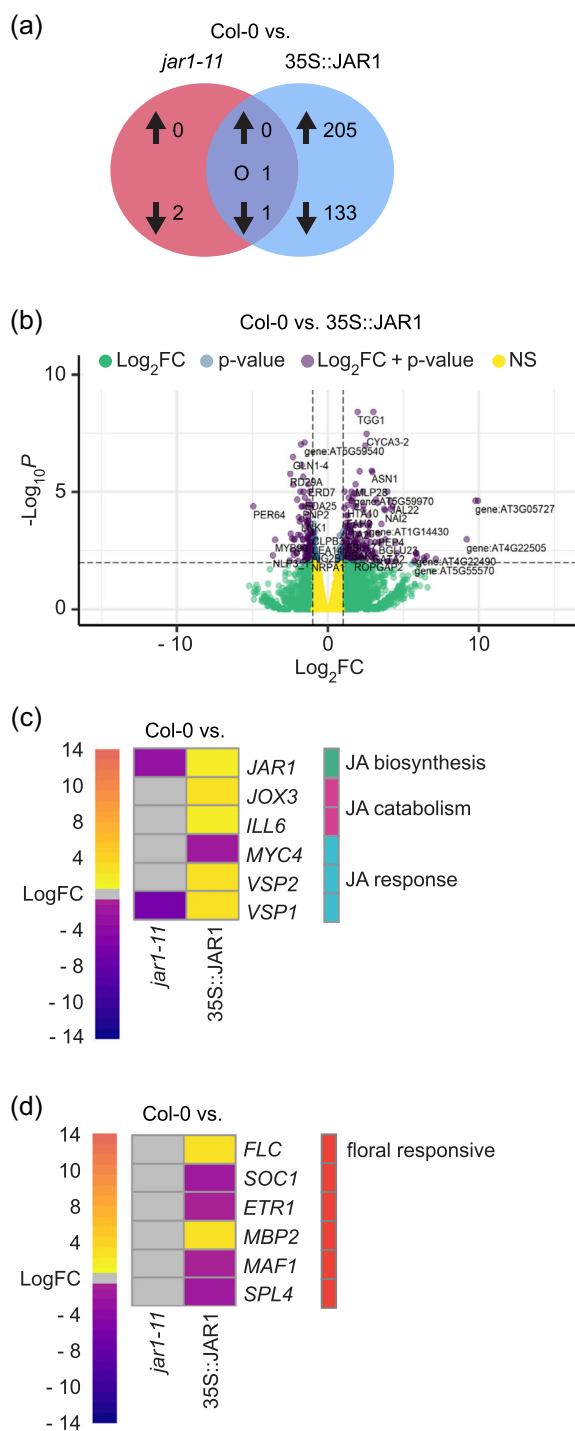


FIGURE 3 JASMONATE RESISTANT 1 (JAR1)-dependent changes in gene expression in rosette leaves under normal growth conditions. (a) Venn diagram showing differentially expressed genes (DEGs; DESeq, adjusted to false discovery rate (FDR) < 0.01 and $|\log_2FC| \geq 1$) in *jar1-11* and 35S::JAR1 compared with Col-0 in 32-day-old plants under normal growth conditions. Arrows indicate up- and downregulation. 'O' indicates counter-regulated genes. (b) Volcano plot showing statistical significance ($-\log_{10}P$) versus magnitude of change ($\log_2FC$) of DEGs between Col-0 and 35S::JAR1. Violet dots indicate genes that fit the DESeq criteria of FDR < 0.01 and $|\log_2FC| \geq 1$, whereas green and blue dots represent DEGs that fit only $\log_2FC$ or FDR, respectively. (c, d) Heat maps of genes involved in JA biosynthesis, catabolism and signalling response (c) or flowering responsive genes (d). Expression was compared between Col-0 and *jar1-11* or 35S::JAR1. Data were analysed using a cut-off of FDR < 0.05 and $|\log_2FC| \geq 0.5$.

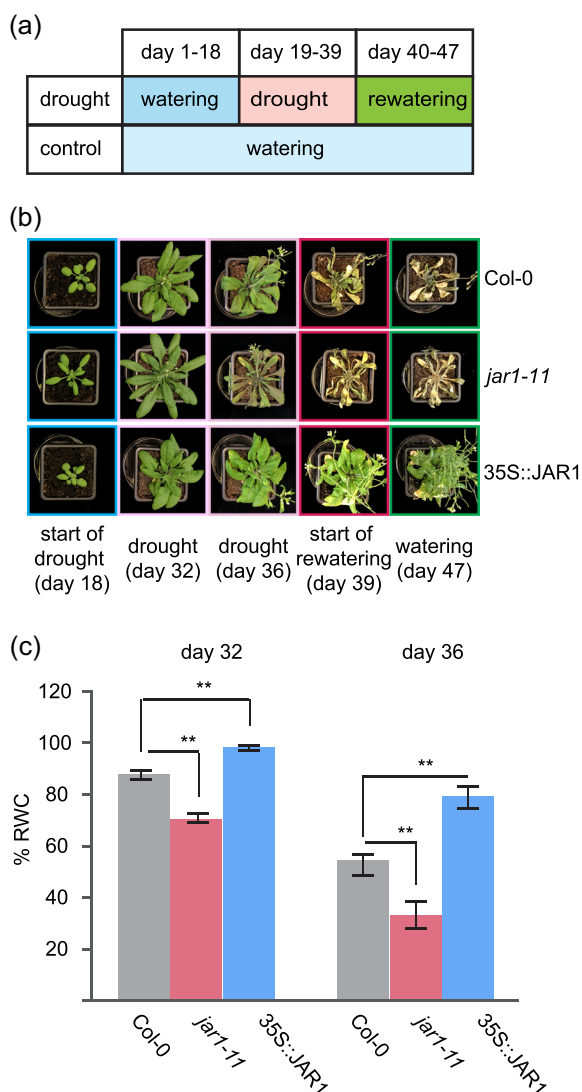


FIGURE 4 Increased JASMONATE RESISTANT 1 (JAR1) expression positively affects drought stress tolerance. (a) Schematic representation of the progressive drought stress experiment. Watering was stopped on Day 18. Drought exposed plants were watered again at Day 39. (b) Representative photographs showing plant phenotypes throughout the progressive drought stress experiment (see also Supporting Information: Figure S4A). (c) Leaf relative water content (% RWC) of drought-treated plants on Days 32 and 36. Data represent means $\pm$ SE from five biological replicates ($n = 5$), each containing five individual plants. Data were analysed by one-way analysis of variance (ANOVA) ($**p < 0.01$) followed by multiple comparison analysis (Tukey's honest significant difference [HSD] test).

pathway to JA-Ile is blocked. Content of the committed precursor *cis*-OPDA decreased in all lines under drought (Figure 2g) at levels in line with the formation of JA, JA-Ile and derivatives thereof. Especially O-JA-Glc levels, which were quite similar under control conditions, markedly increased in all lines upon drought (Figure 2c). Compared with Col-0, the increase was higher in *jar1-11* and lower in 35S::JAR1 (Figure 2C). Similarly, the contents of ABA, which did not differ statistically under control conditions, increased upon exposure to

drought with the highest increase in *jar1-11* and lowest in the 35S::JAR1 plants (Figure 2h).

3.4 | JAR1-mediated JA-Ile formation regulates genes related to drought resistance and responses mechanisms

We also performed RNA-seq analysis on Day 32 in plants grown under drought conditions (Supporting Information: Figure S4 and Data Set S2). In Col-0, we identified 3401 DEGs (Figure 5a) between control and drought-treated plants. By comparison, *jar1-11* plants showed a much higher (6139) and 35S::JAR1 a lower number (2025) of DEGs. The higher number of DEGs observed in the *jar1-11* plants supports that already at this point they experience a higher level of drought stress even though plants of the different lines still looked similar.

A comparison of the RNA-seq data between the different plant lines under drought conditions revealed 2411 DEGs between Col-0 and *jar1-11* and 998 DEGs between Col-0 and 35S::JAR1 (Figure 5b and Supporting Information: Data Set S3). Of these, 391 DEGs were counter-regulated between *jar1-11* and 35S::JAR1. GO enrichment analysis confirmed a reciprocal trend between *jar1-11* and 35S::JAR1 for a number of genes (Supporting Information: Data Set S5). Several of the genes involved in jasmonate biosynthesis upstream of JAR1 showed a lower expression in *jar1-11* under drought compared with Col-0, whereas their expression was similar or higher than Col-0 in 35S::JAR1 plants (Figure 5c). A similar pattern was observed for the expression of the jasmonate-related TF MYC2, the jasmonate-dependent genes VSP1 and VSP2, as well as most JAZ genes (Figure 5c). Remarkably, two of the JAZ genes show an opposite trend.

The majority of genes with decreased expression in *jar1-11* and increased expression in 35S::JAR1 were related to photosynthesis (Supporting Information: Data Set S5). On the other hand, the majority of genes with increased expression in *jar1-11* and decreased expression in 35S::JAR1 included various groups of genes responding to abiotic stresses and other hormones. Not surprisingly, genes known to be responsive to drought and ABA signalling were enriched in the upregulated gene sets of all three lines upon drought (Supporting Information: Data Set S3 and S4). However, compared with Col-0 and 35S::JAR1, *jar1-11* plants showed a stronger upregulation of several genes involved in the ABA signalling pathway (Figure 5c).

To further investigate the differential expression in response to drought compared with control conditions, we applied hierarchical clustering to all DEGs among Col-0, *jar1-11* and 35S::JAR1 (Supporting Information: Data Set S6). These clusters can be categorized into two sets, with the first set (Clusters 1-4) representing mechanisms to withstand drought stress effects (Figure 6). We found a decreased expression after drought stress in all lines in Clusters 1-4, albeit to a lesser extent in 35S::JAR1 compared with Col-0 and especially with *jar1-11*. Many genes in

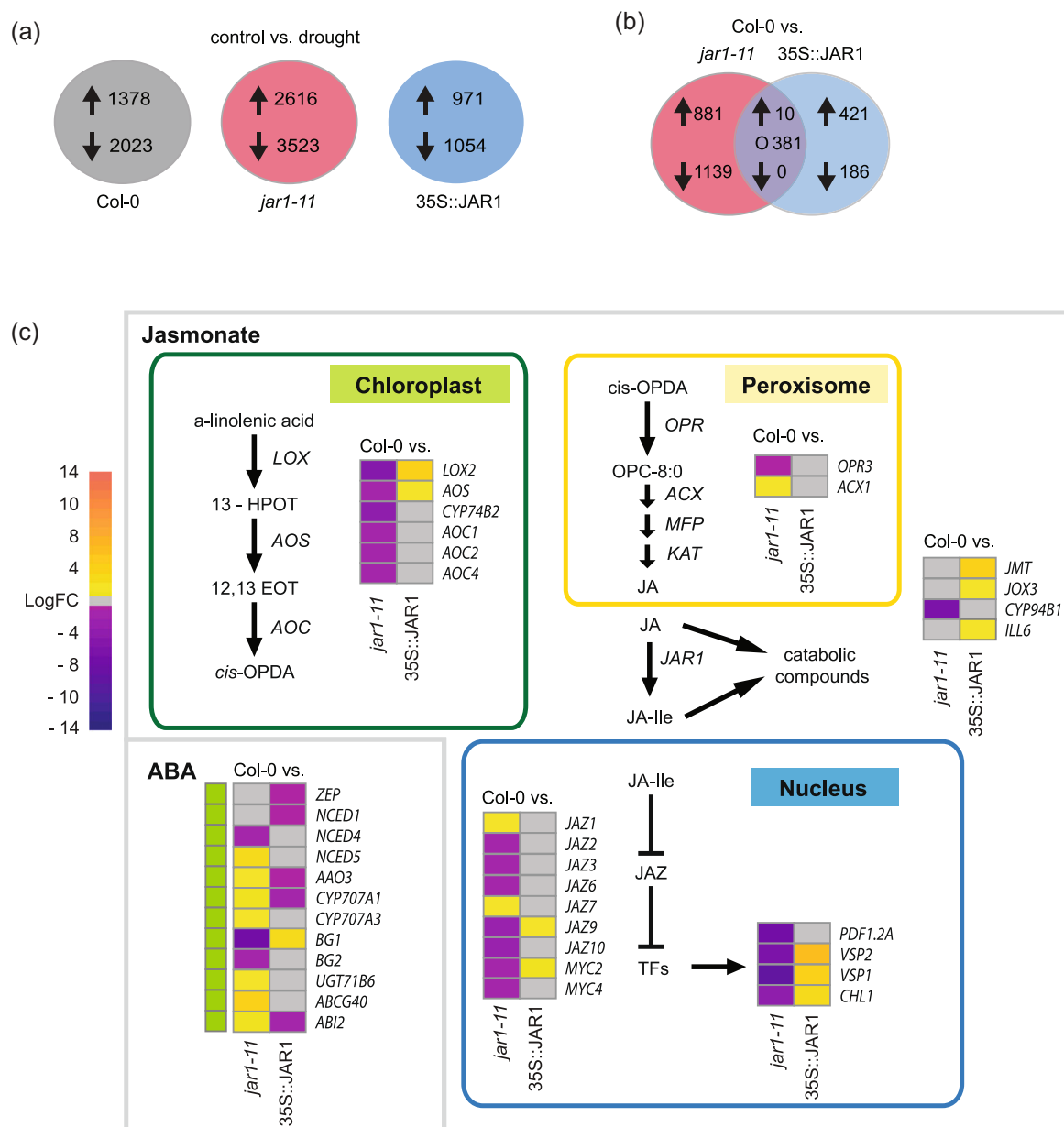


FIGURE 5 JASMONATE RESISTANT 1 (JAR1)-dependent changes in gene expression in rosette leaves under progressive drought. (a) Number of differentially expressed genes (DEGs; DESeq, adjusted $p < 0.01$ and $|\text{LogFC}| \geq 1$) between control and drought conditions in Col-0, *jar1-11* and 35S::JAR1. Arrows indicate up- and downregulation. (b) Venn diagram of DEGs (DESeq, adjusted $p < 0.01$ and $|\text{LogFC}| \geq 1$) in *jar1-11* and 35S::JAR1 compared with Col-0 under drought conditions. Arrows indicate up- and downregulation. 'O' indicates counter-regulated genes. (d) Heat maps of genes involved in jasmonate biosynthesis, catabolism and signalling response depicted by cell compartments, as well as abscisic acid (ABA) biosynthesis, catabolism and signalling response compared between Col-0 and either *jar1-11* or 35S::JAR1, all under drought conditions. Data were analysed using a cut-off of false discovery rate (FDR) < 0.05 and $|\text{LogFC}| \geq 0.5$.

Cluster 1 relate to water transport, whereas Clusters 2 and 4 clearly represent the detrimental effect of drought on the photosynthetic machinery. Genes related to growth regulation were affected on several levels from general regulation of growth (Cluster 1) to cell wall biosynthesis and remodelling (Cluster 3).

Cytokinin response was also negatively affected by drought, especially in *jar1-11*. By contrast, Cluster 5 comprises genes upregulated in all three lines with the highest upregulation in *jar1-11*. Many of these genes represent drought stress responses such as ABA-dependent and independent genes related to water deprivation.

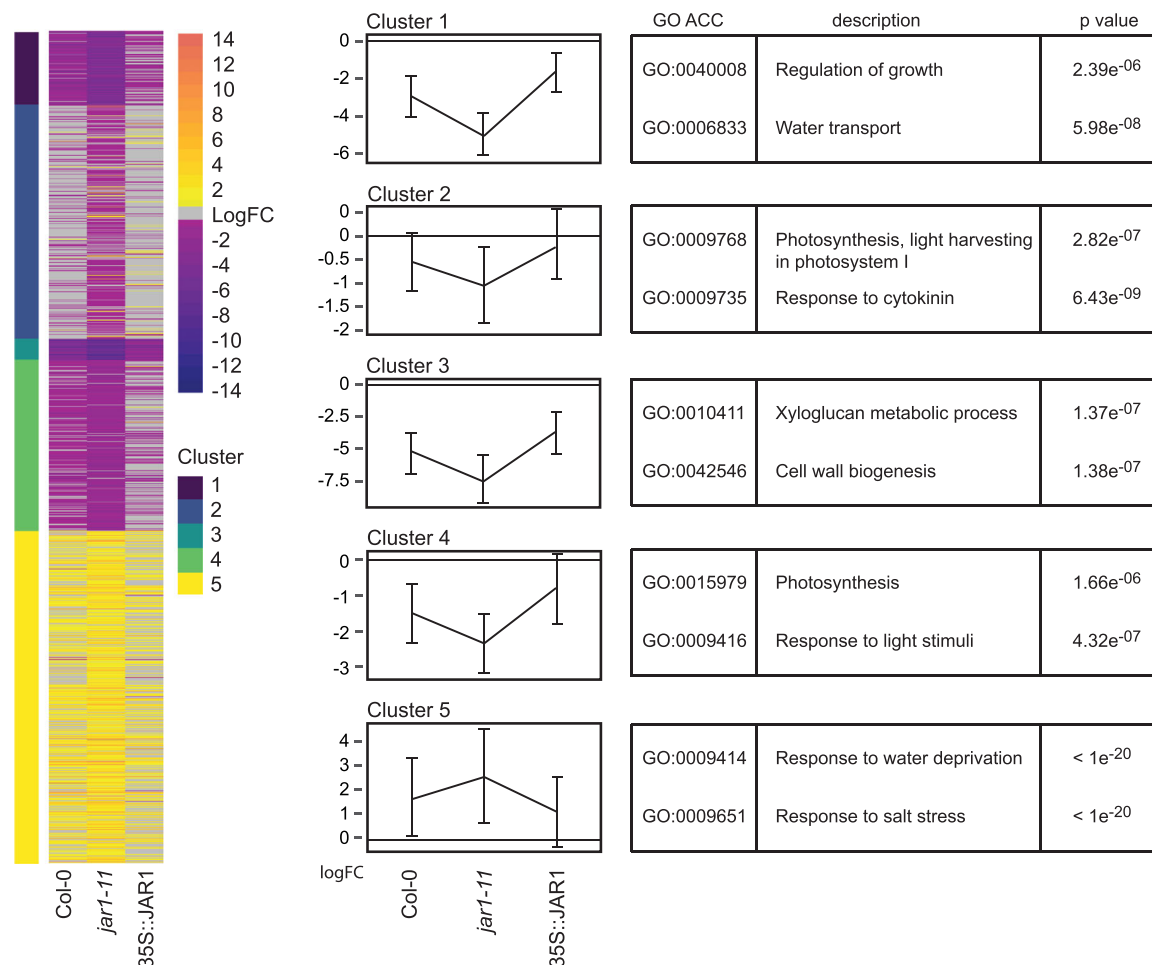


FIGURE 6 JASMONATE RESISTANT 1 (JAR1)-dependent transcriptomic variations between drought stress and control conditions. Heat map (left) and K-means clustering (middle) of genes up- or down-regulated under drought stress compared with control conditions in the different plant genotypes. K-means clustering analysis was performed to produce the clusters (DESeq, adjusted false discovery rate (FDR) < 0.01 and LogFC ≥ 1) and the thin lines represent the mean expression profiles for each cluster (middle). Only genes that are differentially expressed in at least one of the comparisons were used for the cluster analysis. The top two Gene Ontology (GO) terms for each cluster with p are listed (right).

3.5 | JAR1-dependent modulation of drought related features and processes

To better explain the different performance of the *jar1-11* and 35S::JAR1 plants under drought, we looked for specific features that would affect water use efficiency. Our RNA-seq analysis had revealed that expression of the two myrosinases (β -thioglucoside glucosylhydrolases) TGG1 and TGG2 was highly elevated in the 35S::JAR1 line under normal growth conditions (Figure 3b and Supporting Information: Data Set S4). These myrosinases were shown to be involved in ABA- and MeJA-induced stomatal closure downstream of ROS production (Islam et al., 2009; Rhaman et al., 2020). In line with this, leaves from 35S::JAR1 plants grown under control conditions displayed a lower stomatal aperture diameter when challenged (Figure 7a). The analysis also revealed a higher stomatal density in *jar1-11* compared with Col-0 and 35S::JAR1 (Figure 7b and Supporting Information: Figure S6). Thus, JAR1-

mediated JA-Ile formation affects both the aperture and density of stomata, which together can affect the transpirational water loss.

Flavonoids, such as anthocyanins, have been suggested to scavenge ROS and anthocyanin biosynthesis was shown to be induced by MeJA application (Shan et al., 2009). Accordingly, 35S::JAR1 plants showed higher anthocyanin levels under control conditions compared with Col-0 and *jar1-11* (Figure 7c). In addition, although anthocyanin levels increased significantly in all three plant lines upon drought, the highest increase was observed in 35S::JAR1 plants. Moreover, some genes coding for enzymes involved in GSH synthesis or the ascorbate-GSH cycle were shown to be induced by MeJA application (Sasaki-Sekimoto et al., 2006; Xiang & Oliver, 1998). In our RNA-seq data, very little difference in expression could be observed between Col-0, *jar1-11* and 35S::JAR1 under non-stress conditions (Supporting Information: Data Set S4). However, under drought conditions, differential expression of several genes involved in this process could be observed. Most prominently, *jar1-11* showed

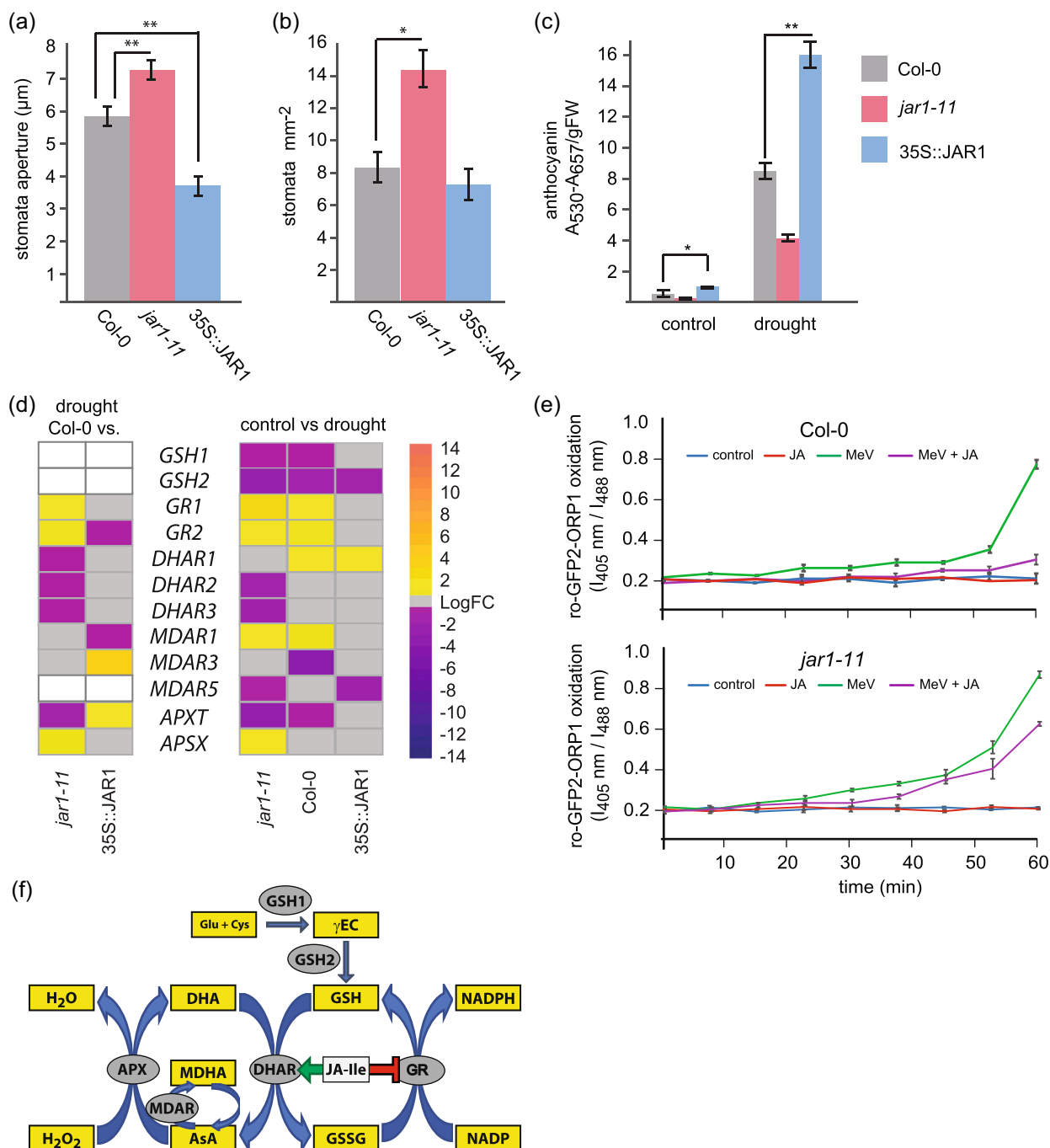


FIGURE 7 Effect of jasmonoyl-L-isoleucine (JA-Ile) on stomatal regulation, anthocyanin content and MV-induced changes in redox status. Number of stomata (a) and stomatal aperture (b) measured on leaf No. 6 of plants grown under control conditions at Day 21. Data represent means $\pm$ SE from three biological replicates ($n = 3$). For stomatal numbers, each replicate quantified leaves from 5 to 6 individual plants. For stomatal aperture, each replicate quantified 90 to 100 stomata in leaves from 6 to 10 individual plants. Data were analysed by one-way analysis of variance (ANOVA) ($*p < 0.05$, $**p < 0.01$) followed by multiple comparisons (Tukey's honest significant difference [HSD] test). (c) Anthocyanin content of different plant genotypes determined in rosette leaves of 32-day-old plants grown under control and drought stress conditions. Data represent means $\pm$ SE from three replicates ($n = 3$), each containing three pooled individual plants. Data were analysed by one-way ANOVA ($*p < 0.05$, $**p < 0.01$) followed by multiple comparisons (Tukey's HSD test). (d) Heat maps of differentially expressed genes (DEGs) involved in the ascorbate-glutathione cycle in *jar1-11* and 35S::JAR1 compared with Col-0 under drought conditions (left) or between control and drought conditions in Col-0, *jar1-11* and 35S::JAR1 (right). Data were analysed using a cut-off of false discovery rate (FDR) < 0.05 and $\text{LogFC} \geq 0.5$. White boxes indicate genes whose changes did not meet the cut-off criteria. (e) Real-time monitoring of redox status using cytosolic roGFP2-Orp1 redox sensors in Col-0 and *jar1-11* leaf cells upon treatment with 10 mM methyl viologen (MeV) and/or 100 μM JA. roGFP2 was excited at wavelengths 405 and 488 nm, and the emission was detected from 505 to 530 nm. Ratios were calculated as the ratiometric image of 405/488 nm. After each run, representative samples were calibrated with 10 mM dithiothreitol (DTT) (ratio = 0.18) and 10 mM H_2O_2 (ratio = 1.20). Mean ratios $\pm$ SE of different time points represent data from three replicates, each including three individual seedlings.

lower expression of all DHARs, the dehydroascorbate reductases that converts GSH to GSSG, and higher expression of GR1 and GR2, GSH reductases that convert GSSG back to GSH (Figure 7d, left panel). Expression of these genes is not much altered in 35S::JAR1 compared with Col-0; however, under drought conditions, the expression of DHAR1, the most-highly expressed DHAR isoform, was increased in Col-0 and 35S::JAR1 (Figure 7d, right panel).

To elucidate possible JAR1-mediated effects on ROS scavenging in vivo, we used plants carrying the genetically encoded H₂O₂ sensor roGFP-Orp1 (Nietzel et al., 2019). Treatment of leaf tissue from Col-0 plants with 10 mM methyl viologen (MeV), which was shown to lead to oxidative stress and the generation of ROS (Schwarzländer et al., 2009), resulted in a strong oxidative shift of the sensor in both Col-0 and *jar1-11* (Figure 7e, green lines). Application of 100 µM JA, given together with MeV, reduced the MeV-induced increase in H₂O₂ levels nearly back to control levels in Col-0 but resulted only in a minor decrease of sensor oxidation in *jar1-11* (Figure 7e, magenta lines). This indicates that JAR1-mediated transformation of JA to JA-Ile is required to reduce MeV-induced ROS and a similar effect would be expected during stress-induced ROS production.

4 | DISCUSSION

Plants are constantly exposed to various biotic and abiotic stresses and to combat their detrimental effect, a balance between optimum fitness and resistance mechanism is mandatory. Jasmonate signalling is known to play a role in many developmental and stress-related processes, and in the current work, we used a TDNA insertion mutant in the *JAR1* locus (*jar1-11*) and a novel transgenic line expressing JAR1.1-YFP under the 35 S promoter (35S::JAR1) to alter the endogenous JA-Ile content of *Arabidopsis*. The *jar1-11* mutant showed a strong reduction in *JAR1* transcripts compared with Col-0 (Figure 1a), but a basal level of full-length transcripts is retained despite the disruption of the *JAR1* locus within an exon after about 1/3 of the coding region. It was also shown recently, that a protein encoded by the GH3.10 locus can convert JA to JA-Ile (Delfin et al., 2022). Thus, *jar1-11* is not a null allele, nevertheless, *jar1-11* plants showed a clear reduction in JA-Ile content and nearly null expression of the jasmonate-dependent defence marker *VSP1*, supporting that JAR1 is the major enzyme involved in JA-Ile formation. Moreover, in the 35S::JAR1 line, strongly increased *JAR1* transcript levels result in an about 10-fold increase in JA-Ile content, together with upregulation of *VSP1* and *VSP2*. Thus, these lines are a great resource to study the effects of varying JA-Ile levels on plant growth and stress responses.

4.1 | JAR1 overexpression distorts jasmonate homeostasis

In the *jar1-11* and 35S::JAR1 lines used in this study *JAR1* expression is altered constitutively. Therefore, the effects of altered JAR1

content are already observed under normal growth conditions. Increased content in JA-Ile and its derivatives under these conditions in the 35S::JAR1 lines indicates that JAR1 is not only a key enzyme in jasmonate biosynthesis, but also seems to represent a rate-limiting step of JA-Ile formation (Figure 2). In the wild type, the early onset of drought stress increases *JAR1* expression and thus JA-Ile levels, however, they still remain below that of 35S::JAR1. In *jar1-11*, drought leads to an increase in JA levels, showing that jasmonate synthesis is induced but JA-Ile cannot be produced. However, it is likely that factors other than just the amount of JAR1 protein control JA-Ile levels, especially under drought conditions. As shown here and described before the basal level of the precursor *cis*-OPDA is almost 200 times higher compared with JA-Ile (de Ollas et al., 2015a). Under drought conditions, the level of *cis*-OPDA decreased but still remained much higher than the increased content of JA-Ile. This indicates that JA formation from *cis*-OPDA is not the limiting factor for JA-Ile synthesis. However, the decrease in *cis*-OPDA is at a similar magnitude as the combined increase in JA, JA-Ile and their derivatives, such as 12-OH-JA, 12-OH-JA-Ile and 12-COOH-JA-Ile, all of which accumulate to a greater extent than JA-Ile itself. 12-OH-JA and 12-OH-JA-Ile were both found to modulate JA-Ile-mediated gene expression, including genes involved in jasmonate biosynthesis (Jimenez-Aleman et al., 2019; Poudel et al., 2019). They could thus play a role in balancing JA-Ile homeostasis as well as responses induced by JA-Ile signalling. This fit well with recent findings from Marquis et al. (2022) on the *jao2* mutant, in which changes in JA catabolism affect JA-Ile formation and signalling. Especially intriguing is the general high amount of the JA-derivative 12-O-JA-Glc and its further JAR1-dependent increase under drought. 12-O-JA-Glc has been shown to accumulate 24 h after wounding of tomato leaves and it was suggested that it also is part of the pathway to remove accumulated JA and JA-Ile under stress (Miersch et al., 2008). Although our study only shows the content of jasmonates at a single (and early) time point during the progressive drought stress, the data strongly support the notion of a continuous flow of JA-Ile synthesis and removal that is enhanced under stress conditions. Constitutive expression of JAR1 distorts this balance, resulting in higher JA-Ile levels.

4.2 | Effects of constitutive elevation of JA-Ile on drought resistance and priming

Our study shows that the *jar1-11* mutant (and also *jar1-12*) is more susceptible to progressive drought stress (Figure 4B), whereas 35S::JAR1 plants display only a mild drought stress phenotype. The higher tolerance of 35S::JAR1 is likely based on changes induced by the elevated JA-Ile content. However, JA-Ile content in the 35S::JAR1 line is increased constitutively and not only in response to drought stress. Thus, this resistance could be based on JA-Ile induced changes that happen long before the onset of the drought stress. On the other hand, different JA-Ile levels observed under drought stress could also alter the plant's short term response in a

favourable manner. Indeed, our results indicate that both factors play a role in the better drought resistance of the 35S::JAR1 plants (Supporting Information: Figure S7).

4.2.1 | JAR1-related alterations in plant growth and development

The differences in *JAR1* transcripts and JA-Ile levels in the transgenic lines manifested themselves in opposite phenotypic alterations compared with Col-0 already under non-stress conditions (Figure 1). Overexpression of *JAR1* resulted in shorter and wider leaves, a similar phenotype achieved by treating Col-0 plants with exogenous MeJA application (Figure S5D). This is in agreement with previous findings that MeJA application on *Arabidopsis* seedlings leads to cell cycle arrest, which resulted in reduced leaf growth (Noir et al., 2013; Zhang & Turner, 2008). However, the initial stunted growth observed in 35S::JAR1 seems to be superseded at a later stage by increased radial growth of older leaves. Accordingly, expression of the cell cycle controlling gene *CYC1.2*, which was found to be down-regulated after exogenous MeJA application in young seedlings (Zhang & Turner, 2008), was upregulated in the older leaves of the 35S::JAR1 plants used for RNA-seq analysis in our experiments (Supporting Information: Data Set S3). 35S::JAR1 plants also seem to have higher expression levels of the transcriptional co-activator genes *GIF1* and *GRF5* (Supporting Information: Data Set S3), which regulate the development of leaf size and shape (Kim & Kende, 2004; Lee et al., 2009). Mutants in the *GIF1* locus have narrower leaf blades compared with Col-0 indicating that *GIF1* regulates lateral leaf expansion. Increased expression of *GIF1* and *GRF5* in 35S::JAR1 could be due to the decreased expression of the *MYC4* TF, which was shown to bind the promoter of *GIF1* and down-regulate its activity (Liu et al., 2020). Reduced leaf growth will reduce the water requirement of the plant and thus can give the 35S::JAR1 plants an advantage under drought conditions.

On the other hand, the *jar1-11* plants show early flowering similar to mutants of the *AOS* and *OPR3* loci that are affected in jasmonate synthesis upstream of *JAR1* (Supporting Information: Figure S3C). By contrast, 35S::JAR1 plants flower several days later than *jar1-11* and Col-0 plants. Although there is no difference between the lines with regard to other parameters related to reproductive success (Table S2), a shorter reproductive cycle will likely be of advantage under favourable growth conditions.

4.2.2 | Cross-talk between jasmonate and ABA

Even though *MYC2* is considered a master regulator of jasmonate signalling (Dombrecht et al., 2007), it was shown previously that not only JA-Ile but also ABA could induce the expression of *MYC2*. Moreover, the effect of both hormones applied together was much stronger (Lorenzo et al., 2004). This would explain the only slight increase of *MYC2* levels in 35S::JAR1 under control conditions

(Supporting Information: Data Set 3) despite the high level of JA-Ile, because ABA levels are not elevated. Under drought conditions, when ABA levels are high, expression of *MYC2* increases in 35S::JAR1 together with genes involved in JA synthesis. This supports a model proposed by Liu et al. (2016), in which exposure to drought activates transcription of *MYC2* via both ABA and jasmonate, which in the form of a positive feedback loop leads to further activation of JA synthesis and subsequently further elevated expression of jasmonate-dependent genes.

Although drought-induced ABA accumulation was evident in all three lines, it was significantly enhanced in *jar1-11* compared with 35S::JAR1 (Figure 2h). Differences in ABA level corresponded to opposite alterations in the expression of genes related to ABA biosynthesis. However, increase in expression of genes related to ABA biosynthesis in *jar1-11* was accompanied by upregulation of genes involved in ABA degradation. In addition, *ABI2*, a negative regulator of ABA signalling (Merlot et al., 2001), showed reduced expression in *jar1-11*. A likely explanation is that the *jar1-11* plants evoke mechanisms to attenuate the effects of a surplus in ABA that accumulates in the absence of JA-Ile. This could be one way in which jasmonate signalling helps to keep the balance between drought protection and growth.

4.2.3 | Jasmonate signalling regulates physiological systems involved in drought adaptation and stress response

Better drought resistance of 35S::JAR1 plants likely stems from the relatively high RWC that they retained compared with Col-0, while the loss of RWC was highest in *jar1-11* (Figure 4c). This in turn is a consequence of the variance in stomatal density and stimuli induced stomata closing observed between the plant lines already under non-stress conditions (Figure 7a and Supporting Information: Figure S6). This difference is also in accordance with previous studies showing that exogenously applied MeJA negatively regulates stomatal development and positively regulates stomatal aperture (Han et al., 2018; Hossain et al., 2011). The regulation of stomatal aperture, however, is a very complex process. The higher expression of *TGG1* and *TGG2* in 35S::JAR1 might play a role, since these myrosinases were shown to be involved in ABA- and MeJA-induced stomatal closure downstream of ROS production (Islam et al., 2009). Although plants cannot simply adjust stomata number under drought in fully developed leaves, lesser stomatal aperture of the 35S::JAR1 plants will attenuate water loss (Supporting Information: Figure S7).

Additionally, 35S::JAR1 plants might cope better with drought stress induced accumulation of H_2O_2 and other ROS (Noctor et al., 2014). Controlled redox regulation is important to remove cytotoxic ROS levels, while sustaining ROS-dependent regulatory circuits. We could show that external addition of JA alleviates MeV-induced H_2O_2 production in Col-0 but not in the *jar1-11* mutant (Figure 7d), where JA cannot be converted into JA-Ile. Previously, external MeJA application was reported to induce some genes involved in the

ascorbate-GSH cycle, one of the major mechanisms to adjust cytosolic H₂O₂ levels (Sasaki-Sekimoto et al., 2006; Xiang & Oliver, 1998). In our study, we observed upregulation of both *DHAR1* and *GR1* under drought in Col-0. DHAR and GR are responsible for the conversion of GSH to GSSG and back, respectively, a central reaction of the ascorbate-GSH cycle (Figure 7e). We did not see any difference in the expression of ascorbate-GSH cycle genes under non-stress conditions in 35S::JAR1, despite the increase in JA-Ile levels. However, *DHAR1* and *GR1/2* expression was differentially regulated in *jar1-11* and 35S::JAR1 under drought. Together, our data suggest that rather than generally inducing its activity, JA-Ile might adjust the flow through the ascorbate-GSH cycle under drought conditions.

4.3 | JA-Ile-mediated global transcriptome changes

Cluster analysis of the RNA-seq data identified hubs of altered gene expression between *jar1-11*, Col-0 and 35S::JAR1 under drought conditions. Many of these fall into categories that can be easily related to drought responses, such as photosynthesis and water transport, or they represent known genes related to drought or general stress. For each of these individual genes and clusters subsequent studies will have to show whether their expression is directly altered by JA-Ile and they are thus involved in jasmonate-related drought susceptibility and tolerance. Changes in their expression could also be a manifestation of the different drought phenotypes and thus an indirect effect. In this context, it should be noted that even under control conditions, 35S::JAR1 plants showed downregulation of certain drought (*RD29A*, *ERD7*, *LEA14* and *GCR2*) and cold-responsive (*COR15B*) genes; however, further studies have to show whether this has an effect of the observed drought resistance of these plants.

Overall, our data show that constitutive deregulation of jasmonate homeostasis provides *Arabidopsis* with better drought resistance. They provide insight into the effects that changes in JA-Ile content have on various morphological and physiological traits that can be related to drought. The results further indicate that priming, that is, changes happening long before the onset of the drought stress, as well as direct stress responses both shape the drought resistance of 35S::JAR1 (Supporting Information: Figure S7). These findings are in line with results from Marquis and coworkers (Marquis et al., 2022) showing that modulating JA turnover improved the resistance of *Arabidopsis* to drought and that the drought tolerance of the *jao2* mutant requires JA-Ile formation by JAR1. Thus, constitutively altering jasmonate homeostasis can be a way to adapt plants to better withstand drought but possible detrimental variations in growth and life-cycle length under more favourable conditions have to be considered.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

- Barrs, H.D. & Weatherley, P.E. (1962) A re-examination of relative turgidity technique for estimating water deficits in leaves. *Australian Journal of Biological Sciences*, 15(3), 413–428. <https://doi.org/10.1071/Bi9620413>
- Bell, E., Creelman, R.A. & Mullet, J.E. (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 92(19), 8675–8679. <https://doi.org/10.1073/pnas.92.19.8675>
- Bhosale, R., Jewell, J.B., Hollunder, J., Koo, A.J., Vuylsteke, M. & Michael, T. et al. (2013) Predicting gene function from uncontrolled expression variation among individual wild-type *Arabidopsis* plants. *The Plant Cell*, 25(8), 2865–2877. <https://doi.org/10.1105/tpc.113.112268>
- Capella, A.N., Menossi, M., Arruda, P. & Benedetti, C.E. (2001) COI1 affects myrosinase activity and controls the expression of two flower-specific myrosinase-binding protein homologues in *Arabidopsis*. *Planta*, 213(5), 691–699. <https://doi.org/10.1007/s004250100548>
- Cheng, C.Y., Krishnakumar, V., Chan, A.P., Thibaud-Nissen, F., Schobel, S. & Town, C.D. (2017) Araport11: a complete reannotation of the *Arabidopsis thaliana* reference genome. *Plant Journal*, 89(4), 789–804. <https://doi.org/10.1111/tpj.13415>
- Core Team, R. (2020) *R: a language and environment for statistical computing*. R Foundation for Statistical Computing. www.R-project.org
- Creelman, R.A. & Mullet, J.E. (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences of the United States of America*, 92(10), 4114–4119. <https://doi.org/10.1073/pnas.92.10.4114>
- Datla, R.S., Hammerlindl, J.K., Panchuk, B., Pelcher, L.E. & Keller, W. (1992) Modified binary plant transformation vectors with the wild-type gene encoding NPTII. *Gene*, 122(2), 383–384. [https://doi.org/10.1016/0378-1119\(92\)90232-e](https://doi.org/10.1016/0378-1119(92)90232-e)
- Delfin, J.C., Kanno, Y., Seo, M., Kitaoka, N., Matsuura, H., Tohge, T. et al. (2022) AtGH3.10 is another jasmonic acid-amido synthetase in *Arabidopsis thaliana*. *Plant Journal*, 110(4), 1082–1096. <https://doi.org/10.1111/tpj.15724>
- Devoto, A., Ellis, C., Magusin, A., Chang, H.S., Chilcott, C., Zhu, T. et al. (2005) Expression profiling reveals COI1 to be a key regulator of

- genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. *Plant Molecular Biology*, 58(4), 497–513. <https://doi.org/10.1007/s11103-005-7306-5>
- Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B. et al. (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *The Plant Cell*, 19(7), 2225–2245. <https://doi.org/10.1105/tpc.106.048017>
- Du, H., Liu, H. & Xiong, L. (2013) Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. *Frontiers in Plant Science*, 4, 397. <https://doi.org/10.3389/fpls.2013.00397>
- Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J.M., Gimenez-Ibanez, S., Geerinck, J. et al. (2011) The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of Jasmonate responses. *The Plant Cell*, 23(2), 701–715. <https://doi.org/10.1105/tpc.110.080788>
- Gao, X.P., Wang, X.F., Lu, Y.F., Zhang, L.Y., Shen, Y.Y., Liang, Z. et al. (2004) Jasmonic acid is involved in the water-stress-induced betaine accumulation in pear leaves. *Plant, Cell and Environment*, 27(4), 497–507. <https://doi.org/10.1111/j.1365-3040.2004.01167.x>
- Han, X., Hu, Y.R., Zhang, G.S., Jiang, Y.J., Chen, X.L. & Yu, D.Q. (2018) Jasmonate negatively regulates stomatal development in *Arabidopsis* cotyledons. *Plant Physiology*, 176(4), 2871–2885. <https://doi.org/10.1104/pp.17.00444>
- Harb, A., Krishnan, A., Ambavaram, M.M. & Pereira, A. (2010) Molecular and physiological analysis of drought stress in *Arabidopsis* reveals early responses leading to acclimation in plant growth. *Plant Physiology*, 154(3), 1254–1271. <https://doi.org/10.1104/pp.110.161752>
- Hickman, R., Van Verk, M.C., Van Dijken, A., Mendes, M.P., Vroegop-Vos, I.A., Caarls, L. et al. (2017) Architecture and dynamics of the jasmonic acid gene regulatory network. *The Plant Cell*, 29(9), 2086–2105. <https://doi.org/10.1105/tpc.16.00958>
- Hossain, M.A., Munemasa, S., Uraji, M., Nakamura, Y., Mori, I.C. & Murata, Y. (2011) Involvement of endogenous abscisic acid in methyl Jasmonate-induced stomatal closure in *Arabidopsis*. *Plant Physiology*, 156(1), 430–438. <https://doi.org/10.1104/pp.111.172254>
- Islam, M.M., Tani, C., Watanabe-Sugimoto, M., Uraji, M., Jahan, M.S., Masuda, C. et al. (2009) Myrosinases, TGG1 and TGG2, redundantly function in ABA and MeJA signaling in *Arabidopsis* guard cells. *Plant and Cell Physiology*, 50(6), 1171–1175. <https://doi.org/10.1093/pcp/pcp066>
- Jimenez-Aleman, G.H., Almeida-Trapp, M., Fernández-Barbero, G., Gimenez-Ibanez, S., Reichelt, M., Vadassery, J. et al. (2019) Omega hydroxylated JA-Ile is an endogenous bioactive jasmonate that signals through the canonical jasmonate signaling pathway. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, 1864(12), 158520. <https://doi.org/10.1016/j.bbalip.2019.158520>
- Kim, J.H. & Kende, H. (2004) A transcriptional coactivator, AtGIF1, is involved in regulating leaf growth and morphology in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 101(36), 13374–13379. <https://doi.org/10.1073/pnas.0405450101>
- Kinoshita, A. & Richter, R. (2020) Genetic and molecular basis of floral induction in *Arabidopsis thaliana*. *Journal of Experimental Botany*, 71(9), 2490–2504. <https://doi.org/10.1093/jxb/eraa057>
- Koo, A.J. (2018) Metabolism of the plant hormone jasmonate: a sentinel for tissue damage and master regulator of stress response. *Phytochemistry Reviews*, 17(1), 51–80. <https://doi.org/10.1007/s11101-017-9510-8>
- Lee, B.H., Ko, J.H., Lee, S., Lee, Y., Pak, J.H. & Kim, J.H. (2009) The *Arabidopsis* GRF-INTERACTING FACTOR gene family performs an overlapping function in determining organ size as well as multiple developmental properties. *Plant Physiology*, 151(2), 655–668. <https://doi.org/10.1104/pp.109.141838>
- Liu, N., Staswick, P.E. & Avramova, Z. (2016) Memory responses of jasmonic acid-associated *Arabidopsis* genes to a repeated dehydration stress. *Plant, Cell and Environment*, 39(11), 2515–2529. <https://doi.org/10.1111/pce.12806>
- Liu, Y., Du, M., Deng, L., Shen, J., Fang, M. & Chen, Q. et al. (2019) MYC2 regulates the termination of jasmonate signaling via an autoregulatory negative feedback loop. *The Plant Cell*, 31(1), 106–127. <https://doi.org/10.1105/tpc.18.00405>
- Liu, Z.P., Li, N., Zhang, Y.Y. & Li, Y.H. (2020) Transcriptional repression of GIF1 by the KIX-PPD-MYC repressor complex controls seed size in *Arabidopsis*. *Nature Communications*, 11(1), 1846. <https://doi.org/10.1038/s41467-020-15603-3>
- Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J. & Solano, R. (2004) Jasmonate-insensitive1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *The Plant Cell*, 16(7), 1938–1950. <https://doi.org/10.1105/tpc.022319>
- Marquis, V., Smirnova, E., Graindorge, S., Delcros, P., Villette, C., Zumsteg, J. et al. (2022) Broad-spectrum stress tolerance conferred by suppressing jasmonate signaling attenuation in *Arabidopsis* JASMONIC ACID OXIDASE mutants. *The Plant Journal*, 109(4), 856–872. <https://doi.org/10.1111/tpj.15598>
- Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A. & Giraudat, J. (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant Journal*, 25(3), 295–303. <https://doi.org/10.1046/j.1365-313x.2001.00965.x>
- Meyer, A. J., Brach, T., Marty, L., Kreye, S., Rouhier, N., Jacquot, J. P., & Hell, R. (2007). Redox-sensitive GFP in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer. *Plant Journal*, 52(5), 973–986. <https://doi.org/10.1111/j.1365-313X.2007.03280.x>
- Michaels, S.D. & Amasino, R.M. (2001) Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. *The Plant Cell*, 13(4), 935–941. <https://doi.org/10.1105/tpc.13.4.935>
- Miersch, O., Neumerkel, J., Dippe, M., Stenzel, I. & Wasternack, C. (2008) Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. *New Phytologist*, 177(1), 114–127. <https://doi.org/10.1111/j.1469-8137.2007.02252.x>
- Nietzel, T., Elsässer, M., Ruberti, C., Steinbeck, J., Ugalde, J.M., Fuchs, P. et al. (2019) The fluorescent protein sensor roGFP2-Orp1 monitors in vivo H₂O₂ and thiol redox integration and elucidates intracellular H₂O₂ dynamics during elicitor-induced oxidative burst in *Arabidopsis*. *New Phytologist*, 221(3), 1649–1664. <https://doi.org/10.1111/nph.15550>
- Noctor, G., Mhamdi, A. & Foyer, C.H. (2014) The roles of reactive oxygen metabolism in drought: not so cut and dried. *Plant Physiology*, 164(4), 1636–1648. <https://doi.org/10.1104/pp.113.233478>
- Noir, S., Bömer, M., Takahashi, N., Ishida, T., Tsui, T.L., Balbi, V. et al. (2013) Jasmonate controls leaf growth by repressing cell proliferation and the onset of endoreduplication while maintaining a potential stand-by mode. *Plant Physiology*, 161(4), 1930–1951. <https://doi.org/10.1104/pp.113.214908>
- de Ollas, C., Arbona, V. & Gomez-Cadenas, A. (2015a) Jasmonic acid interacts with abscisic acid to regulate plant responses to water stress conditions. *Plant Signaling & Behavior*, 10(12), e1078953. <https://doi.org/10.1080/15592324.2015.1078953>
- de Ollas, C., Arbona, V. & Gomez-Cadenas, A. (2015b) Jasmonoyl isoleucine accumulation is needed for abscisic acid build-up in roots of *Arabidopsis* under water stress conditions. *Plant, Cell and Environment*, 38(10), 2157–2170. <https://doi.org/10.1111/pce.12536>

- Poudel, A.N., Holtsclaw, R.E., Kimberlin, A., Sen, S., Zeng, S., Joshi, T. et al. (2019) 12-Hydroxy-Jasmonoyl-L-Isoleucine is an active jasmonate that signals through CORONATINE INSENSITIVE 1 and contributes to the wound response in *Arabidopsis*. *Plant and Cell Physiology*, 60(10), 2152–2166. <https://doi.org/10.1093/pcp/pcz109>
- Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L. & Riechmann, J.L. (2001) Regulation of flowering in *Arabidopsis* by an FLC homologue. *Plant Physiology*, 126(1), 122–132. <https://doi.org/10.1104/pp.126.1.122>
- Rhaman, M.S., Nakamura, T., Nakamura, Y., Munemasa, S. & Murata, Y. (2020) The myrosinases TGG1 and TGG2 function redundantly in reactive carbonyl species signaling in *Arabidopsis* guard cells. *Plant and Cell Physiology*, 61(5), 967–977. <https://doi.org/10.1093/pcp/pcaa024>
- Sasaki-Sekimoto, Y., Taki, N., Obayashi, T., Aono, M., Matsumoto, F. & Sakurai, N. et al. (2006) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in *Arabidopsis*. *Plant and Cell Physiology*, 47, S233.
- Savchenko, T.V., Rolletschek, H. & Dehesh, K. (2019) Jasmonates-mediated rewiring of central metabolism regulates adaptive responses. *Plant and Cell Physiology*, 60(12), 2613–2620. <https://doi.org/10.1093/pcp/pcz181>
- Schwarzländer, M., Fricker, M.D. & Sweetlove, L.J. (2009) Monitoring the in vivo redox state of plant mitochondria: effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge. *Biochimica Et Biophysica Acta-Bioenergetics*, 1787(5), 468–475. <https://doi.org/10.1016/j.bbabi.2009.01.020>
- Shan, X.Y., Zhang, Y.S., Peng, W., Wang, Z.L. & Xie, D.X. (2009) Molecular mechanism for jasmonate-induction of anthocyanin accumulation in *Arabidopsis*. *Journal of Experimental Botany*, 60(13), 3849–3860. <https://doi.org/10.1093/jxb/erp223>
- Smirnova, E., Marquis, V., Poirier, L., Aubert, Y., Zumsteg, J., Ménard, R. et al. (2017) Jasmonic acid oxidase 2 hydroxylates jasmonic acid and represses basal defense and resistance responses against *Botrytis cinerea* infection. *Molecular Plant*, 10(9), 1159–1173. <https://doi.org/10.1016/j.molp.2017.07.010>
- Staswick, P.E. & Tiryaki, I. (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. *The Plant Cell*, 16(8), 2117–2127. <https://doi.org/10.1105/tpc.104.023549>
- Suza, W.P. & Staswick, P.E. (2008) The role of JAR1 in Jasmonoyl-L-isoleucine production during *Arabidopsis* wound response. *Planta*, 227(6), 1221–1232. <https://doi.org/10.1007/s00425-008-0694-4>
- Tayyab, N., Naz, R., Yasmin, H., Nosheen, A., Keyani, R., Sajjad, M. et al. (2020) Combined seed and foliar pre-treatments with exogenous methyl jasmonate and salicylic acid mitigate drought-induced stress in maize. *PLoS One*, 15(5), e0232269. <https://doi.org/10.1371/journal.pone.0232269>
- Tukey, H.B. & Hamner, C.L. (1949) Form and composition of cherry fruits (*Prunus-Avium* and *P-Cerasus*) following fall applications of 2,4-Dichlorophenoxyacetic acid and naphthalene acetic acid. *Proceedings of the American Society for Horticultural Science*, 54(Dec), 95–101.
- Ullah, C., Schmidt, A., Reichelt, M., Tsai, C.-J. & Gershenzon, J. (2022) Lack of antagonism between salicylic acid and jasmonate signalling pathways in poplar. *New Phytologist*, 235(2), 701–717.
- Ullah, C., Tsai, C.J., Unsicker, S.B., Xue, L., Reichelt, M., Gershenzon, J. et al. (2019) Salicylic acid activates poplar defense against the biotrophic rust fungus *Melampsora larici-populina* via increased biosynthesis of catechin and proanthocyanidins. *New Phytologist*, 221(2), 960–975.
- Vercruysse, J., Baekelandt, A., Gonzalez, N. & Inzé, D. (2020) Molecular networks regulating cell division during *Arabidopsis* leaf growth. *Journal of Experimental Botany*, 71(8), 2365–2378. <https://doi.org/10.1093/jxb/erz522>
- Wang, X., Li, Q., Xie, J., Huang, M., Cai, J. & Zhou, Q. et al. (2021) Absciscic acid and jasmonic acid are involved in drought priming-induced tolerance to drought in wheat. *Crop Journal*, 9(1), 120–132. <https://doi.org/10.1016/j.cj.2020.06.002>
- Wasternack, C. & Song, S.S. (2017) Jasmonates: biosynthesis, metabolism, and signaling by proteins activating and repressing transcription. *Journal of Experimental Botany*, 68(6), 1303–1321. <https://doi.org/10.1093/jxb/erw443>
- Widemann, E., Miesch, L., Lugan, R., Holder, E., Heinrich, C., Aubert, Y. et al. (2013) The amidohydrolases IAR3 and ILL6 contribute to Jasmonoyl-Isoleucine hormone turnover and generate 12-Hydroxyjasmonic acid upon wounding in *Arabidopsis* leaves. *Journal of Biological Chemistry*, 288(44), 31701–31714. <https://doi.org/10.1074/jbc.M113.499228>
- Wu, G., & Poethig, R.S. (2006). Temporal regulation of shoot development in *Arabidopsis* thalianaby miR156 and its target SPL3. *Development*, 133(18), 3539–3547. <https://doi.org/10.1242/dev.02521>
- Wu, Z., Fang, X., Zhu, D. & Dean, C. (2020) Autonomous pathway: FLOWERING LOCUS C repression through an antisense-mediated chromatin-silencing mechanism1 [CC-BY]. *Plant Physiology*, 182(1), 27–37. <https://doi.org/10.1104/pp.19.01009>
- Xiang, C.B. & Oliver, D.J. (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *The Plant Cell*, 10(9), 1539–1550. <https://doi.org/10.1105/tpc.10.9.1539>
- Yang, S., Vanderbeld, B., Wan, J. & Huang, Y. (2010) Narrowing down the targets: towards successful genetic engineering of drought-tolerant crops. *Molecular Plant*, 3(3), 469–490. <https://doi.org/10.1093/mp/ssq016>
- Zander, M., Lewsey, M.G., Clark, N.M., Yin, L., Bartlett, A., Saldierna Guzmán, J.P. et al. (2020) Integrated multi-omics framework of the plant response to jasmonic acid. *Nature Plants*, 6(3), 290–302. <https://doi.org/10.1038/s41477-020-0605-7>
- Zhang, Y. & Turner, J.G. (2008) Wound-induced endogenous jasmonates stunt plant growth by inhibiting mitosis. *PLoS One*, 3(11), e3699. <https://doi.org/10.1371/journal.pone.0003699>
- Züst, T. & Agrawal, A.A. (2017) Trade-offs between plant growth and defense against insect herbivory: an emerging mechanistic synthesis. *Annual Review of Plant Biology*, 68, 513–534. <https://doi.org/10.1146/annurev-arplant-042916-040856>

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Appendix 4

Loss-of-function of the drought-induced genes *GASA3* and *AFP1* confers enhanced drought tolerance in *Arabidopsis thaliana*

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Abstract

Prolonged drought is a major challenge in plant growth, severely affecting development and yield. Enhancing drought tolerance is thus a highly desired goal for agriculture. Here, we report that the loss-of-function of two drought-induced genes, *GASA3* and *AFPI*, significantly enhances drought tolerance in *Arabidopsis thaliana*. While constitutive expression of *GASA3* and *AFPI* increased drought sensitivity compared to wild type (WT) plants, a *gasa3afp1* double mutant exhibited superior drought tolerance compared to the single mutants. Enhanced drought tolerance of *gasa3*, *afp1* and *gasa3afp1* is likely due to reduced water loss caused by smaller stomatal apertures and thus lower transpiration rates. Moreover, *gasa3* and *afp1* mutants accumulated higher levels of abscisic acid (ABA) under drought conditions than WT plants, concomitant with a stronger up-regulation of ABA-responsive genes such as *RD29A/B*, *ABF2/3*, and *ABI5*. The stronger ABA increase in the mutants seems to result from hydrolysis of abscisic acid-glucosyl ester (ABA-GE) from vacuolar stores via the β -glucosidase *BG2* rather than by *de-novo* biosynthesis. Promoter analysis revealed the presence of ABA-responsive and drought stress-related cis-acting elements within the *GASA3* and *AFPI* promoter regions. RT-qPCR confirmed that the expression of both genes increased under drought. However, *GASA3* induction was significantly reduced in the absence of *AFPI*, suggesting that *AFPI* is involved in the modulation of *GASA3* expression. Our findings identify a novel *AFPI/GASA3*-driven control circuit that negatively regulates drought tolerance by suppressing stomatal closure and attenuating ABA signalling.

1. Introduction

Plants as sessile organisms are exposed to constantly changing environmental conditions. Successful plant development and adaptation is thus determined by long-time genetically inherited programs that are fine-tuned by short-term responses to abiotic and biotic stresses. For that purpose, plants contain a number of intercommunicating signalling networks to coordinate their responses to various external and internal stimuli (Signorelli, 2022). These signalling pathways balance the often-contrary needs of growth, development and stress protection and thus modify the stress response accordingly (Claeys & Inzé, 2013; Verma *et al.*, 2016). However, stress response often comes at the cost of reduced yield (Zhu, 2016). To select for traits that provide yield stability under environmental challenges, it is crucial to gain an in-depth understand of the mechanisms of stress response.

Drought stress caused by limited water availability is considered as one of the major abiotic stresses that negatively affects plant growth, development and reproductivity (Claeys & Inzé,

2013; Tenorio Berrío *et al.*, 2022). Accordingly, plants have developed various physiological and morphological adaptations to reduce water loss and optimize water use efficiency. Resistance mechanisms to drought comprise a wide range of cellular processes including global reprogramming of transcription, post-transcriptional modification of RNA and post-translational modification of proteins, ultimately leading to adaptive alteration of metabolism and plant development (Yang *et al.*, 2010).

ABA is an essential phytohormone that regulates plant adaptation to drought (Muhammad Aslam *et al.*, 2022). Plant exposure to drought stress induces the elevation of the ABA content, leading to stomatal closure and activation of drought related genes (Nakashima *et al.*, 2014). The biosynthesis of ABA starts in the plastids, from β -carotene, leading to xanthoxin, which is transported to the cytosol. Xanthoxin is then converted to ABA-aldehyde, which is subsequently oxidized to ABA (Wu *et al.*, 2023). The core ABA-signaling network consist of three major components: the ABA receptors PYRABACTIN RESISTANT/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR), negative regulators in form of protein phosphatases 2C (PP2Cs), positive regulators such as sucrose non-fermenting 1-related protein kinases (SnRKs) and basic leucine zipper (bZIP) transcriptional activators such as ABA-INSENSITIVE 5 (ABI5) and ABI5 homologous ABA-RESPONSIVE ELEMENT BINDING FACTORS (ABFs), also known as AREBs for ABA-RESPONSIVE ELEMENT BINDING PROTEINS (Ali *et al.*, 2020). In the absence of ABA, high PP2C activity results in deactivation of SNRK2 by dephosphorylation (Hirayama & Umezawa, 2010). In the presence of ABA, a complex between PYR/PYL/RCAR and PP2CA is formed, and SNRK2 is activated by phosphorylation. Phosphorylated SNRK2 subsequently phosphorylates ABI5/ABFs which then bind to *cis*-elements in target gene promoters known as ABREs (ABA-responsive elements) to activate gene expression (Choi *et al.*, 2000; Ali *et al.*, 2020).

ABI5 BINDING PROTEINS (ABPs) interact with AREBs such as ABI5 and promote their degradation by E3 ubiquitin ligase, hence negatively regulating ABA signalling (Lopez-Molina *et al.*, 2003; Wei *et al.*, 2022; Vittozzi *et al.*, 2024). In Arabidopsis, four ABPs have been identified and ABP1 and its close homolog ABP2 have been shown to repress bZIP activation of certain ABRE-regulated genes (Lynch *et al.*, 2022). Moreover, the *MEDIATOR-OF-OsbZIP46-DEGRADATION-AND-DEACTIVATION* (MODD), an ortholog of ABP3 in rice, was shown to be involved in negative regulation of drought tolerance (Tang *et al.*, 2016).

The *GIBERELLIC ACID STIMULATED ARABIDOPSIS* (*GASA*) family in Arabidopsis comprises genes with homology to *GA-STIMULATED TRANSCRIPT 1* (*GAST1*) from tomato

(*Solanum lycopersicum*). *GASA* genes encode low-molecular-weight peptides, also called SNAKINs, that have been shown to play various roles in plant development as well as plant stress regulation (Bouteraa *et al.*, 2023). Of the 14 *GASA* family members in Arabidopsis, *AtGASA4* has been shown to positively regulate heat stress tolerance (Ko *et al.*, 2007), whereas *AtGASA5* has been demonstrated to negatively regulate thermotolerance (Zhang & Wang, 2011). Furthermore, *AtGASA14* has been shown to positively regulate salt stress tolerance by reducing ROS accumulation (Sun *et al.*, 2013). *AtGASA3* so far has only been proposed to have increased transcript levels in seeds during desiccation (Aubert *et al.*, 1998), without further characterization of any role during abiotic stress, particularly drought.

In this study, we showed that the loss-of-function of two drought-induced genes, *GASA3* and *AFPI*, led to a strong increase in drought tolerance of *Arabidopsis thaliana*. Phenotypic analyses under drought conditions showed that while single as well as *gasa3afp1* double mutants have enhanced drought tolerance, the constitutive overexpression lines have a reduced tolerance compared to WT plants. Expression of *GASA3* and *AFPI* is induced by drought and ABA according to RT-qPCR, however, induction of *GASA3* remains rather low in the absence of *AFPI*. Furthermore, we detected a reduced water loss most likely caused by smaller stomatal apertures and thus transpiration rates in *gasa3* and *afp1* single mutants as well as in *gasa3afp1* double mutants, suggesting an involvement of these two genes in suppressing stomatal closure. Additionally, we show that *gasa3* and *afp1* plants accumulate higher levels of ABA under drought conditions than WT, concomitant with a further increase in the expression of ABA-responsive genes. Increased expression of the vacuolar β -glucosidase *BG2* and repression of genes involved in ABA synthesis suggest that the elevated ABA levels are caused by hydrolysis of abscisic acid-glucosyl ester (ABA-GE). Overall, our results indicated that *GASA3* and *AFPI* are negative regulators of drought stress tolerance in Arabidopsis via the ABA-signalling pathway, with *AFPI* involved in the modulation of *GASA3* expression.

2. Materials and Methods

2.1. Plant material and growth conditions

This study was performed using the Columbia ecotype of *Arabidopsis thaliana* (Col-0) and all transgenic lines were generated in this background. *gasa3* (SAIL_198_A11) and *afp1* (SAIL_13_C02) T-DNA insertion mutants were obtained from Nottingham Arabidopsis Stock Center (NASC, UK). The *aba2-1* mutant (Cheng *et al.*, 2002; Lin *et al.*, 2007) was a kind gift from Prof. Wan-Hsing Cheng, University of Taiwan. For most experiments, plants were directly

placed into standard plant potting soil pre-treated with Confidor WG 70 (Bayer Agrar, Germany). For some experiments, sterilized seeds were sown on ½ MS (Murashige and Skoog medium, Duchefa Biochemie, The Netherlands) plates with 1% (w/v) sucrose and 0.6% (w/v) phytigel. Plants were grown in climate-controlled rooms under long day conditions (LD; 16h light / 8 h dark) with a light intensity of 100 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Philips TLD 18W lamps of alternating 830/840 light temperature).

2.2. Generation of transgenic lines

For the generation of 35S::*GASA3-YFP* or 35S::*AFPI-YFP* lines, the coding sequences of *GASA3* and *AFPI* without the stop codon were cloned into the pBIN19 vector (Datia *et al.*, 1992) in frame with the coding region of *YFP*. The resulting expression cassettes were stably inserted into the genome of Col-0 plants using *Agrobacterium tumefaciens* and floral dipping (Zhang *et al.*, 2006). Two independent T3 lines were selected by BASTA resistance, and constitutive expression was confirmed by RT-qPCR. Two *gasa3afpl* double mutant lines originating from independent crosses were generated by crossing homozygous single mutant lines. All primers used for cloning and screening are listed in Supplementary Table S1.

2.3. Plant phenotyping

For phenotyping of the different plant lines under drought stress, seeds were first germinated in batches on soil for a week. Subsequently, the seedlings were transferred into single pots filled with 100 grams of soil and kept well-watered until day 18. Before discontinuation of watering, individual pot weights were measured and all other pots were set to the pot with the highest weight using tap water. Plants were exposed to progressive drought or well-watered control conditions (50 ml of tap water each other day) for up to 12 days. The position of the plant pots was randomized throughout the experiments to avoid positional effects on the plant growth.

Two crucial parameters were closely monitored: Soil Water Content (SWC) and Real Leaf Water Content (RWC) of the rosette leaves. For measuring the soil water content as a percentage, the following formula was used: $\{(\text{pot weight during measurement}) - (\text{empty pot weight})\} / \{(\text{initial pot weight}) - (\text{empty pot weight})\} \times 100$. RWC was determined using previously published protocols (Barrs & Weatherley, 1962; Bouchabke *et al.*, 2008). by measuring three different weights from whole rosettes (without reproductive tissue): the fresh weight (FW), the turgid weight (TW, after submerging the rosette in water overnight), and the dry weight (DW; measured after drying the rosettes at 72°C for 3 days). The formula applied for RWC expressed as percentage was: $(\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$.

2.4. Gene induction analysis

The expression of *GASA3* and *AFPI* was investigated after treatment with different compounds. For that purpose, 8 ml of either 100 μ M ABA (Sigma-Aldrich, USA), 100 μ M methyl jasmonate (MeJA, SERVA, Germany), 100 μ M gibberellic acid (GA₃, Duchefa Biochemie, Netherlands) or 20 % (w/v) polyethylene glycol (PEG)-6000 (Carl Roth GmbH, Germany) were applied directly onto ½ MS plates with 21-day old plants and incubated for 0, 1, 3, 6, 9 or 24 hours under LD conditions. Whole seedlings were frozen using liquid nitrogen, ground into a fine powder and used for total RNA extractions as describe below in 2.7.

2.5. Stomatal measurements and estimation of transpiration rate

Stomatal aperture was quantified following a modified version of a previously established protocol (Eisele *et al.*, 2016). Briefly, 7th or 8th leaves of 32-day old plants grow on soil were incubated for 2 hours with imaging buffer (10 mM MES, pH 6.15, 5 mM KCl, 50 μ M CaCl₂). Epidermal peels were carefully separated from the mesophyll and fixed to a glass slide using medical adhesive tape. Images were taken under Bright Field settings using a Leica SP8 Lightning using the integral LAS X software and were further processed using the Fiji/ImageJ software (Schindelin *et al.*, 2012). For estimation of stomatal density, the diameter of the field of view (FV) was calculated (πr^2) and used to normalize the count. Transpiration rate ($\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) was quantified using a LiCOR LI 6000 porometer/fluorometer (LI-COR Environmental GmbH, Germany).

2.6. Quantification of ABA

For ABA measurements, whole plant rosettes were frozen in liquid nitrogen, and ground into a fine powder. The extraction and quantification followed a previously established protocol (Pan *et al.*, 2010). Briefly, 50 mg of rosette tissue were harvested and immediately frozen in liquid nitrogen and homogenized with a pre-cooled mortar and pestle. Samples were handled using only liquid nitrogen throughout harvesting ensuring minimal damage due to repetitive freeze-thawing. This was followed by the addition of 500 μ l extraction solvent (2-propanol/water/conc. HCl in a ratio 2:1:0.002, v/v/v) and 25 ng D₆-ABA, addition of 1 ml dichloromethane and phase separation, removal of the lower phase, and nitrogen assisted drying of the upper phase. The dried matter was resuspended in 0.1 ml of methanol:0.1% formic acid in water (1:1, v/v). Phytohormones were separated on a reverse phase C18 Gemini HPLC column and analysed using a QTRAP 6500+ LC-MS/MS system (Sciex, Germany). Data evaluation was carried out using the MultiQuantTM 3.0.2 software (Sciex, Germany). The

concentrations of ABA were determined relative to the internal standards, and expressed as ng/g F.W. All used solvents were of HPLC grade or LC-MS grade.

2.7. Estimation of total anthocyanin content

Anthocyanins were quantified according to a previous protocol (Nakata & Ohme-Takagi, 2014). The rosettes of 32-day old plants (control and drought) were flash frozen using liquid nitrogen and pulverized into a fine powder. Based on the fresh weight of the samples, approximately 5 volumes of extraction buffer (45 % methanol, 5 % acetic acid) were added and vortexed thoroughly. The mixture was centrifuged two times at 12000g for 5 min and absorbances of the supernatants were recorded at 530 and 637 nm. The amount of anthocyanin per gram fresh weight ($\text{g}^{-1} \cdot \text{F.W.}^{-1}$) was calculated by the formula: $(\text{Abs}_{530}/\text{g F.W.}) = [\text{Abs}_{530} - (0.25 \times \text{Abs}_{637})] \times 5$.

2.8. RNA-extraction, cDNA synthesis and RT-qPCR

For RNA extraction of soil-grown plants, whole rosettes were harvested and ground into a fine powder using liquid nitrogen. RNA was extracted from 100 mg of this powder using the Roboklon Plant RNA Kit (Roboklon GmbH, Berlin, Germany). The quality of the RNA was assessed using a Nanodrop Spectrophotometer or by separation on a 1% agarose gel. cDNA synthesis was carried out from at least 500 ng RNA using the Revert Aid First strand cDNA kit (ThermoFisher Scientific, USA) and oligodT₁₈ primers. The reaction was carried out for 1 hour at 42 °C, followed by termination by heating at 72 °C for 10 min.

RT-qPCR was carried out on a Bio-Rad CFX96 touch system (Bio-Rad Laboratories, Germany). Gene expression data were analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) and normalized to the geometric means of two reference genes: *AtACT2* and *AtTUB2* (Vandesompele *et al.*, 2002). All primers used are listed in Supplementary Table S1. Unless otherwise mentioned in the figure legends, for RT-qPCR analyses, 32-day-old plants either under control or drought conditions were used.

2.9. Statistical analyses

Statistical analyses were conducted using R version 4.3.2 (R Core Team, 2023; <https://www.r-project.org/>). A two-tailed Student's *t*-test ($P < 0.05$) was used to compare drought and control samples, employing the base *t.test()* function. For datasets involving multiple groups or treatments, one-way or two-way ANOVA was performed, followed by Tukey's HSD test ($P < 0.05$), using the R packages *agricolae*, *tidyverse*, and *ggplot2*. For two-way ANOVA both capital

and small letters were used, where the capital letters signified variance due to treatment (ABA or drought) and the small letters depicted variance due to genotypic differences.

3. Results

3.1. *GASA3* and *AFPI* expression is strongly induced by progressive drought

While screening for drought-responsive genes using the RNA-seq dataset of a recent study in *Arabidopsis thaliana* (Mahmud *et al.*, 2022), we identified two highly drought induced genes: *GASA3* (AT4G09600) and *AFPI* (AT1G69260). *GASA3* belongs to the gibberellic acid-stimulated (GAS) *Arabidopsis* family implicated in a wide range of functions like plant growth, development and fruit ripening (Vittozzi *et al.*, 2024; Bouteraa *et al.*, 2023). *AFPI*, on the other hand, is best studied during germination and has been shown to promote the degradation of the bZIP transcription factor ABI5, which is known to promote the expression of ABA-responsive genes (Lopez-Molina *et al.*, 2003; Wei *et al.*, 2022).

We thus analysed the expression of *GASA3* and *AFPI* during a time-course of 14 days of progressive drought compared to well-watered plants. As before (Mahmud *et al.*, 2022), water withholding was started when soil-grown plants were 18 days old. RT-qPCR showed that under well-watered conditions the expression of *GASA3* and *AFPI* was very low and showed not significantly changes during the course of the experiment (Figure 1a). By contrast, expression of *GASA3* and *AFPI* increased with a fold change (FC) of about 10 after 6 and 7 days of water withholding, respectively. Expression of *AFPI* further increased gradually to a FC>60, while *GASA3* expression showed an exponential increase to an FC>1000 on day 12 and >2000 on day 14 (Figure 1a). These results confirm the RNA-Seq data from the previous study (Mahmud *et al.*, 2022) but also show that onset of gene induction occurs early on after water withholding.

3.2. *GASA3* and *AFPI* negatively regulate drought tolerance in *Arabidopsis*

To evaluate the effect of *GASA3* and *AFPI* on drought tolerance, we analysed the growth phenotype of homozygous T-DNA insertion lines for *gasa3* and *afp1*, a *gasa3afp1* double mutant and lines expressing YFP-tagged *AFPI* and *GASA3* under control of the 35S promoter in the WT background, which we refer to as 35S::*GASA3* and 35S::*AFPI* (Supplementary Figure 1). RT-qPCR analyses confirmed complete lack of expression of the respective gene in *gasa3* and *afp1* (Supplementary Figure 1b) and constitutively elevated expression in 35S::*GASA3* and 35S::*AFPI* (Supplementary Figure 1d). No difference in growth was observed compared to WT up to 14 days of water withholding (Figure 1b). However, clear differences

could be observed upon longer drought periods. While the WT showed signs of wilting on day 18 and was nearly completely wilted on day 20, *afp1* plants only showed strong wilting on day 21 and *gasa3* plants on day 24. Plants from the 35S::*GASA3* and 35S::*AFPI* lines wilted a bit earlier than the WT plants, while the double mutant lasted even longer than the single mutants. All in all, these results show that despite being induced under drought, both *GASA3* and *AFPI* have a negative impact on drought tolerance, which to a certain degree is additive.

3.3. *GASA3* and *AFPI* affect water loss through modulation of the stomatal aperture

Water loss through transpiration is an important factor related to drought tolerance. To, determine the rate of water loss, the RWC of the rosette leaves was measured in the different lines on day 14 of drought, when all plants still looked similarly healthy, and on day 18, when differences in the drought tolerance was clearly visible (Figure 1b). Already on day 14 the lines showed a difference in RWC, with the single and double mutants still retaining more than 80 % RWC, while RWC in the 35S::*GASA3* and 35S::*AFPI* lines dropped to around 60% (Figure 2a). On day 18, single and double mutants still retained RWCs of over 80 %, while RWC dropped below 60% in the wild type and below 30% in the 35S::*GASA3* and 35S::*AFPI* lines (Figure 2a).

The number of stomata per leaf area as well as their apertures determines the transpiration rate. While the stomata number is a fixed trait determined during development, stomata aperture is regulated dynamically in response to various parameters and the process encompasses multiple signaling pathways (Araújo *et al.*, 2011). With regards to stomata density, a discernible difference was only observed in the double mutant plants, which possess significant fewer stomata per mm (Figure 2b). By contrast, stomatal aperture in the different plants was very much in line with the observed phenotype, with the smallest aperture observed in the single and double mutant and the largest in the 35S::*GASA3* and 35S::*AFPI* lines (Figure 2c). This is also in line with the observation that loss of *GASA3* or *AFPI* increases the transcript levels of two genes associated with stomatal closure, the beta-thioglucoside glucohydrolase *TGG1* (Islam *et al.*, 2009) and the slow (S)-type anion channel *SLAC1* (Deng *et al.*, 2021), even under control conditions (Supplementary Figure S2a). Furthermore, in the leaves of *gasa3*, *afp1* and *gasa3afp1* the transpiration rates were lower than those in the WT and the 35S::*GASA3* and 35S::*AFPI* lines, under drought as well as under control growth conditions (Figure 2d). Together, these results suggest that regulation of stomata aperture is the cause behind the alteration in transpiration and thus the better drought tolerance of the *gasa3* and *afp1* single and *gasa3afp1* double mutant plants.

3.4. Expression of *GASA3* and *AFPI* depends on ABA

In silico analysis of the *GASA3* and *AFPI* promoter regions (-1kb) shows the presence of various cis-elements known to confer response to plant hormones and abiotic stresses (Supplementary Figure S3). These include the Abscissic Acid Responsive Element (ABRE), of which a single one was detected in the *GASA3* promoter region and four in the *AFPI* promoter region.

In order to examine whether ABA regulates the expression of *GASA3* and *AFPI*, we analysed WT seedlings grown for 21 days on ½ MS phytagel plates that were treated with exogenous ABA. We also included MeJA and GA₃ since cross-talk between ABA and jasmonate has been described in drought response (de Ollas & Dodd, 2016; Mahmud et al. 2022) and GASA proteins were originally identified in relation to GA signaling (Shi *et al.*, 1992). We first treated the seedlings with 20% PEG to confirm that both genes are also upregulated under these growth conditions when drought is mimicked (Figure 3a and b). Moreover, addition of 100 µM ABA increased the expression of *GASA3* and *AFPI*, while neither MeJA nor GA₃ had any inducing effect. A similar picture emerged when gene expression was analysed separately in roots and shoots indicating that drought and ABA induction of *GASA3* and *AFPI* is not specific for photosynthetic tissues (Supplementary Figure S4).

We furthermore analysed the transcript levels of *GASA3* and *AFPI* under progressive drought in the *aba2-1* mutant, which is impaired in ABA biosynthesis (Cheng *et al.*, 2002). Compared to WT, no induction of either *GASA3* and *AFPI* could be observed in *aba2* (Figure 4a and b) but induction was restored by addition of external ABA (Figure 4c and d). These results support a direct role of ABA in the drought-induction of *GASA3* and *AFPI*.

3.5. *GASA3* and *AFPI* affect ABA signaling via release of ABA-GE

As described (Mahmud *et al.*, 2022), we found an elevated ABA content in the WT plants after 14 days of drought (Figure 5a). However, the increase in ABA content was stronger in the *gasa3* and *afp1* mutant lines. Analysis of the expression of several genes related to ABA in *gasa3*, *afp1* and the WT (Figure 5b-d) showed that the expression of ABA-responsive genes, such as *ABF2*, *ABF3*, *RD29A*, and *RD29B*, was much stronger induced in *gasa3* and *afp1* compared to WT (Figure 5d). By contrast, the drought induction of *PP2CA*, which forms an important negative feed-back loop of ABA response, is suppressed in the mutants (Figure 5d). Similarly, the expression of *ZEP/ABA1* and *ABA2*, whose gene products catalyse key steps in ABA biosynthesis (Chen *et al.*, 2020), was induced under drought in the WT but suppressed in *gasa3* and *afp1* (Figure 5b), suggesting that the mutants do not produce the surplus ABA by de-

novo biosynthesis from β -carotene under drought. ABA can also be generated by activation of ABA-GE stored in the endoplasmic reticulum and vacuole via the β -glucosidases BG1 and BG2 respectively (Xu *et al.*, 2012; Han *et al.*, 2020). We observed an up-regulation of BG2 but not BG1 in both mutants under drought, a response that is absent in WT plants (Figure 5c). These findings suggest that the increased ABA levels in *gasa3* and *afp1* derive from conjugated ABA-GE stored in the vacuole.

3.6. *AFP1* acts as upstream regulator of *GASA3*

Our data so far raise the question, whether *GASA3* and *AFP1* function in the same drought response pathway. To address this question, we investigated the expression of *AFP1* in the *gasa3* mutant and *vice versa* (Figure 6a and b). Upon progressive drought, *AFP1* was induced in the *gasa3* mutant line to a level even a bit higher than in WT (Figure 6a) but *GASA3* induction was strongly reduced in the *afp1* mutant (Figure 6b). To confirm that the reduce GASA expression is indeed caused by a lack of *AFP1*, we introduced the 35S::*AFP1* construct into the *afp1* mutant background. This resulted in a low constitutive expression of *AFP1* under control conditions and a similar drought sensibility as the WT (Figure 6c). At the same time, strong induction of *GASA3* under drought was restored (Figure 6d). These data suggested that *AFP1* positively modulates *GASA3* expression under drought stress and that *GASA3* might be the key effector that drives drought susceptibility.

4. Discussion

Plants have evolved various cellular and molecular mechanisms that enhance their acclimation to drought stress. In this study, we investigated the roles of *GASA3* and *AFP1* in the drought stress response, revealing a partial interdependent relationship between these two genes, in which *GASA3* is a downstream component of *AFP1* mediated signalling.

GASA3 and *AFP1* exhibit a drought-dependent increase in transcript levels (Figure 1a) and loss-of-function mutants showed that *GASA3* and *AFP1* are negative regulators of drought tolerance in Arabidopsis (Figure 1b). Moreover, the *gasa3afp1* double mutants displayed a further enhanced drought tolerance, suggesting an at least partial additive function of *GASA3* and *AFP1*. Our data further suggests that *GASA3* and *AFP1* negatively regulate drought tolerance through a mechanism that is primarily driven by stomatal movement rather than differences in stomata development. However, differences in other drought related traits not analysed in this study might affect the drought phenotype of the different lines. These could include traits

pertaining to leaves as well as roots, since *AFPI* and *GASA3* are induced by drought in both tissues.

While *GASA3* and *AFPI* expression was induced by ABA (Figure 3), the ABA content was significantly increased under drought stress in *gasa3* and *afp1* plants compared to WT. This suggests that *GASA3* and *AFPI* might be part of a negative feedback loop, regulating ABA biosynthesis in Arabidopsis (Figure 7). However, *de-novo* biosynthesis of ABA seems to be rather suppressed in the absence of *GASA3* and *AFPI*. Instead, increased expression of *BG2* suggests that the mutants generate ABA from conjugated ABA-GE stored in the vacuole. Consistent with the higher accumulation of ABA, *gasa3* and *afp1* plants showed an up-regulation of core ABA-responsive genes such as the ABREs *ABF2*, *ABF3*, and *ABI5*, which are crucial regulators of the ABA-induced transcriptional network (Choi *et al.*, 2000; Vitozzi *et al.*, 2024), or *RD29A*, a key component of ABA mediated drought response (Msanne *et al.*, 2011; Jia *et al.*, 2012). *gasa3* and *afp1* plants also show an increase in anthocyanin production during drought that may contribute to their enhanced drought tolerance (Supplementary Figure 3c) since anthocyanins function as ROS-scavenging antioxidant and several studies have revealed a positive link between anthocyanin levels and drought tolerance in Arabidopsis (Nakabayashi *et al.*, 2014). Overall, our finding fit well into current models on the role of ABA in drought response (Figure 7). The higher ABA level observed in the *gasa3* and *afp1* mutants would result in an increased phosphorylation of SnRK2, which then phosphorylates ABREs, resulting in increased expression of ABA-responsive genes. These include the S-type anion channel *SLAC1* that contributes to stomata closure, while expression of its counterplayer *KAT-1* is repressed (Takahashi *et al.*, 2017). SnRK2 also phosphorylates both *SLAC1* and *KAT-1*, leading to an activation of the former and inhibition of the latter, which ultimately results in stomata closure.

Loss of *afp1* and *gasa3* moreover reduces the ABA-dependent induction of *PP2CA*, thereby preventing the negative feedback on SnRK2 (and thus *SLAC1* and *KAT-1*) phosphorylation, further enhancing the effect of the increased ABA content (Figure 7).

In our study, we observed little difference in drought tolerance and related traits (RWC, stomata aperture etc.) between the *gasa3* and *afp1* mutants. The obvious reason is the lack of strong *GASA3* induction in the *afp1* mutant (Figure 6). While both genes can be induced by ABA, the expression of *GASA3* remains very low in the absence of *AFPI*. AFPs have been shown to bind to bZIP type transcription factors, thereby targeting them for proteasomal degradation (Lopez-Molina *et al.*, 2003). *AFPI*-dependent increase in expression of *GASA3* could thus involve degradation of a *GASA3* repressor. Independent of the exact nature of this regulation, our data

indicate that *GASA3* is the key effector and *AFP1* the regulator of the *AFP1/GASA3* dependent modulation of drought susceptibility (Figure 7). Further studies are needed to elucidate the exact mechanisms behind *GASA3* and *AFP1* dependent regulation in drought stress responses. This should include the expression of *GASA3* in the *afp1* mutant background driven by a drought induced promoter that is not regulated by *AFP1*. Since we could not observe a growth phenotype of the *afp1* and *gasa3* mutant under stress-free growth conditions and their expression under drought results in a reduced tolerance, their drought induction remains enigmatic. More studies are required that more closely resemble natural conditions including repeating cycles of mild drought and watering.

Authors contribution statement

SB contributed to conceptualization, investigation (responsible for most experimental work), formal analysis (responsible for statistical analysis), validation, visualization, and writing - original draft as well as review & editing. BT, SL, DCR, and YS contributed to investigation (gene expression, phenotyping, promoter analysis). KG and PD contributed to investigation (hormone measurements) and writing - review and editing. FC contributed to conceptualization, formal analysis, validation, visualization, supervision, and writing - original draft as well as review & editing. UCV contributed to conceptualization, validation, visualization, funding acquisition, project administration, supervision, and writing - review & editing. All authors contributed to the article and approved the submitted version.

Conflict of Interest

The authors have no conflicts to declare.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

- Ali A, Pardo JM, Yun D-J. 2020.** Desensitization of ABA-Signaling: The Swing From Activation to Degradation. *Frontiers in Plant Science* **11**.
- Araújo WL, Fernie ,Alisdair R., and Nunes-Nesi A. 2011.** Control of stomatal aperture. *Plant Signaling & Behavior* **6**: 1305–1311.
- Aubert D, Chevillard M, Dorne A-M, Arlaud G, Herzog M. 1998.** Expression patterns of GASA genes in Arabidopsis thaliana: the GASA4 gene is up-regulated by gibberellins in meristematic regions. *Plant Molecular Biology* **36**: 871–883.
- Barrs H, Weatherley P. 1962.** A Re-Examination of the Relative Turgidity Technique for Estimating Water Deficits in Leaves. *Australian Journal of Biological Sciences* **15**: 413–428.
- Bouchabke O, Chang F, Simon M, Voisin R, Pelletier G, Durand-Tardif M. 2008.** Natural Variation in Arabidopsis thaliana as a Tool for Highlighting Differential Drought Responses. *PLOS ONE* **3**: e1705.
- Bouteraa MT, Ben Romdhane W, Baazaoui N, Alfaifi MY, Chouaibi Y, Ben Akacha B, Ben Hsouna A, Kačániová M, Čavar Zeljković S, Garzoli S, et al. 2023.** GASA Proteins: Review of Their Functions in Plant Environmental Stress Tolerance. *Plants* **12**.
- Chen K, Li G-J, Bressan RA, Song C-P, Zhu J-K, Zhao Y. 2020.** Absciscic acid dynamics, signaling, and functions in plants. *Journal of Integrative Plant Biology* **62**: 25–54.
- Cheng W-H, Endo A, Zhou L, Penney J, Chen H-C, Arroyo A, Leon P, Nambara E, Asami T, Seo M, et al. 2002.** A Unique Short-Chain Dehydrogenase/Reductase in Arabidopsis Glucose Signaling and Absciscic Acid Biosynthesis and Functions. *The Plant Cell* **14**: 2723–2743.
- Choi H, Hong J, Ha J, Kang J, Kim SY. 2000.** ABFs, a Family of ABA-responsive Element Binding Factors. *Journal of Biological Chemistry* **275**: 1723–1730.
- Claeys H, Inzé D. 2013.** The Agony of Choice: How Plants Balance Growth and Survival under Water-Limiting Conditions. *Plant Physiology* **162**: 1768–1779.
- Datia RSS, Hammerlindl JK, Panchuk B, Pelcher LE, Keller W. 1992.** Modified binary plant transformation vectors with the wild-type gene encoding NPTII. *Gene* **122**: 383–384.
- Deng Y, Kashtoh H, Wang Q, Zhen G, Li Q, Tang L, Gao H, Zhang C, Qin L, Su M, et al. 2021.** Structure and activity of SLAC1 channels for stomatal signaling in leaves. *Proceedings of the National Academy of Sciences* **118**: e2015151118.
- Eisele JF, Fäßler F, Bürgel PF, Chaban C. 2016.** A Rapid and Simple Method for Microscopy-Based Stomata Analyses. *PLOS ONE* **11**: e0164576.
- Han Y, Watanabe S, Shimada H, Sakamoto A. 2020.** Dynamics of the leaf endoplasmic reticulum modulate β -glucosidase-mediated stress-activated ABA production from its glucosyl ester. *Journal of Experimental Botany* **71**: 2058–2071.
- Hirayama T, and Umezawa T. 2010.** The PP2C–SnRK2 complex. *Plant Signaling & Behavior* **5**: 160–163.

Islam MM, Tani C, Watanabe-Sugimoto M, Uraji M, Jahan MdS, Masuda C, Nakamura Y, Mori IC, Murata Y. 2009. Myrosinases, TGG1 and TGG2, Redundantly Function in ABA and MeJA Signaling in Arabidopsis Guard Cells. *Plant and Cell Physiology* **50**: 1171–1175.

Jia H, Zhang S, Ruan M, Wang Y, Wang C. 2012. Analysis and application of RD29 genes in abiotic stress response. *Acta Physiologiae Plantarum* **34**: 1239–1250.

Ko C-B, Woo Y-M, Lee DJ, Lee M-C, Kim CS. 2007. Enhanced tolerance to heat stress in transgenic plants expressing the GASA4 gene. *Plant Physiology and Biochemistry* **45**: 722–728.

Kumar A, Singh A, Kumar P, Sarkar AK. 2019. Gibberellic Acid-Stimulated Transcript Proteins Evolved through Successive Conjugation of Novel Motifs and Their Subfunctionalization. *Plant Physiology* **180**: 998–1012.

Lin P-C, Hwang S-G, Endo A, Okamoto M, Koshiba T, Cheng W-H. 2007. Ectopic Expression of ABSCISIC ACID 2/GLUCOSE INSENSITIVE 1 in Arabidopsis Promotes Seed Dormancy and Stress Tolerance. *Plant Physiology* **143**: 745–758.

Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* **25**: 402–408.

Lopez-Molina L, Mongrand S, Kinoshita N, Chua N-H. 2003. AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. *Genes & Development* **17**: 410–418.

Lynch T, Née G, Chu A, Krüger T, Finkemeier I, Finkelstein RR. 2022. ABI5 binding protein2 inhibits ABA responses during germination without ABA-INSENSITIVE5 degradation. *Plant Physiology* **189**: 666–678.

Mahmud S, Ullah C, Kortz A, Bhattacharyya S, Yu P, Gershenzon J, Vothknecht UC. 2022. Constitutive expression of JASMONATE RESISTANT 1 induces molecular changes that prime the plants to better withstand drought. *Plant, Cell & Environment* **45**: 2906–2922.

Msanne J, Lin J, Stone JM, Awada T. 2011. Characterization of abiotic stress-responsive Arabidopsis thaliana RD29A and RD29B genes and evaluation of transgenes. *Planta* **234**: 97–107.

Muhammad Aslam M, Waseem M, Jakada BH, Okal EJ, Lei Z, Saqib HS, Yuan W, Xu W, Zhang Q. 2022. Mechanisms of Absciscic Acid-Mediated Drought Stress Responses in Plants. *International Journal of Molecular Sciences* **23**.

Nakabayashi R, Yonekura-Sakakibara K, Urano K, Suzuki M, Yamada Y, Nishizawa T, Matsuda F, Kojima M, Sakakibara H, Shinozaki K, *et al.* 2014. Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *The Plant Journal* **77**: 367–379.

Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K. 2014. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Frontiers in Plant Science* **5**.

Nakata M, Ohme-Takagi M. 2014. Quantification of Anthocyanin Content. *Bio-protocol* **4**: e1098.

- de Ollas C, Dodd IC. 2016.** Physiological impacts of ABA–JA interactions under water-limitation. *Plant Molecular Biology* **91**: 641–650.
- Pan X, Welti R, Wang X. 2010.** Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography–mass spectrometry. *Nature Protocols* **5**: 986–992.
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. 2012.** Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**: 676–682.
- Shi L, Gast RT, Gopalraj M, Olszewski NE. 1992.** Characterization of a shoot-specific, GA3- and ABA-Regulated gene from tomato. *The Plant Journal* **2**: 153–159.
- Signorelli S. 2022.** Plant Responses to Stress and Environmental Stimulus. *Agronomy* **12**.
- Sun S, Wang H, Yu H, Zhong C, Zhang X, Peng J, Wang X. 2013.** GASA14 regulates leaf expansion and abiotic stress resistance by modulating reactive oxygen species accumulation. *Journal of Experimental Botany* **64**: 1637–1647.
- Tang N, Ma S, Zong W, Yang N, Lv Y, Yan C, Guo Z, Li J, Li X, Xiang Y, et al. 2016.** MODD Mediates Deactivation and Degradation of OsbZIP46 to Negatively Regulate ABA Signaling and Drought Resistance in Rice. *The Plant Cell* **28**: 2161–2177.
- Tenorio Berrío R, Nelissen H, Inzé D, Dubois M. 2022.** Increasing yield on dry fields: molecular pathways with growing potential. *The Plant Journal* **109**: 323–341.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002.** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**: research0034.1.
- Verma V, Ravindran P, Kumar PP. 2016.** Plant hormone-mediated regulation of stress responses. *BMC Plant Biology* **16**: 86.
- Vittozzi Y, Krüger T, Majee A, Née G, Wenkel S. 2024.** ABI5 binding proteins: key players in coordinating plant growth and development. *Trends in Plant Science* **29**: 1006–1017.
- Wei J, Li X, Song P, Wang Y, Ma J. 2022.** Studies on the interactions of AFPs and bZIP transcription factor ABI5. *Biochemical and Biophysical Research Communications* **590**: 75–81.
- Wu W, Cao S, Shi L, Chen W, Yin X, Yang Z. 2023.** Absciscic acid biosynthesis, metabolism and signaling in ripening fruit. *Frontiers in Plant Science* **14**.
- Xu Z-Y, Lee KH, Dong T, Jeong JC, Jin JB, Kanno Y, Kim DH, Kim SY, Seo M, Bressan RA, et al. 2012.** A Vacuolar β -Glucosidase Homolog That Possesses Glucose-Conjugated Absciscic Acid Hydrolyzing Activity Plays an Important Role in Osmotic Stress Responses in Arabidopsis. *The Plant Cell* **24**: 2184–2199.
- Yang S, Vanderbeld B, Wan J, Huang Y. 2010.** Narrowing Down the Targets: Towards Successful Genetic Engineering of Drought-Tolerant Crops. *Molecular Plant* **3**: 469–490.

Zhang X, Henriques R, Lin S-S, Niu Q-W, Chua N-H. 2006. Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols* **1**: 641–646.

Zhang S, Wang X. 2011. Overexpression of *GASA5* increases the sensitivity of *Arabidopsis* to heat stress. *Journal of Plant Physiology* **168**: 2093–2101.

Zhu J-K. 2016. Abiotic Stress Signaling and Responses in Plants. *Cell* **167**: 313–324.

Figure legends:

Figure 1: Effect of *GASA3* and *AFPI* on drought tolerance in *Arabidopsis thaliana*.

(a) Relative expression of *GASA3* and *AFPI* in WT plants at various days of progressive drought stress (DS) on soil. Water withholding was started when the plants reached an age of 18 days. Data represent means $\pm$ SE of three independent biological repeats (n=3). Statistical analyses were carried out using two-tailed student T-test between drought and control (*P<0.05, **P<0.01, ***P<0.001). (b) Drought stress phenotype of WT, *gasa3* and *afp1* single mutants, a *gasa3afp1* double mutant, and lines expressing 35S::*GASA3* and 35S::*AFPI* in WT background at different days of progressive drought stress (DS). The images are representative of several individual experiments

Figure 2: Effect of *GASA3* and *AFPI* on stomata regulation and leaf relative water content.

(a) Leaf relative water content (% RWC) of plants at days 14 and 18 of progressive drought stress (DS). Data represents means $\pm$ SE of three independent replicates (n=3). Assessment of (b) stomatal density and (c) stomatal aperture measured on leaves No. 7 and 8 of plants grown under control conditions for 32 days. For stomatal density, each replicate quantified leaves from two individual plants. For stomatal aperture, each replicate quantified 20 stomata in leaves from two individual plants. (d) Transpiration rates in leaves of 32 day-old plants grown under control and drought conditions. Data represent means $\pm$ SE from three biological replicates (n = 3). For all measurements the statistics were carried out using ANOVA and Tukey's Post-Hoc HSD tests (P<0.05).

Figure 3: Induction of *GASA3* and *AFPI* expression by PEG and various hormones.

Relative expression of (a) *GASA3* and (b) *AFPI* in 21-day old WT seedlings grown on ½ MS plates treated with either ddH₂O, 100 μ M ABA, 100 μ M MeJA, 100 μ M GA₃ or 20% PEG-6000. Data represent means $\pm$ SE of three independent biological replicates (n=3). Statistical analyses were performed with one-way ANOVA and Tukey's Post-Hoc HSD tests (P<0.05).

Figure 4: ABA-dependency of *GASA3* and *AFPI* expression.

(a) Relative expression of *GASA3* and *AFPI* in WT and *aba2* mutant plants grown on soil under control and progressive drought conditions. (b) Relative expression of *GASA3* and *AFPI* in 21-day old WT and *aba2* seedlings grown on ½ MS plates treated with either ddH₂O or 100 µM ABA for 24 hours. Data represent means ± SE of three biological replicates and statistical analyses were carried out with two-way ANOVA along with Tukey's Post-Hoc HSD tests (P<0.05).

Figure 5: Effect of *GASA3* and *AFPI* on ABA biosynthesis and the ABA-mediated drought response.

WT, *gasa3* and *afp1* mutant plants grown under control and progressive drought conditions were investigated for (a) Endogenous ABA content of rosette tissue as well as relative expression of genes involved in (b) ABA biosynthesis, (c) ABA-GE activation, and (d) ABA-responses. Data represent means ± SE of three independent replicates (n=3). Statistical significance was estimated with two-way ANOVA and Tukey's HSD analyses (P<0.05).

Figure 6: Role of *AFPI* in the expression of *GASA3*.

Relative expression of (a) *GASA3* and (b) *AFPI* in WT, *gasa3* and *afp1* mutant plants grown under control and progressive drought conditions. (c) Relative expression of *AFPI* and drought phenotype of WT, *afp1* and two lines expressing 35S::*AFPI*-YFP in the *afp1* mutant background (C_*afp1*). DS: drought stress. The images are representative for several individual experiments. (d) Relative expression of *GASA3* in WT, *afp1* and C_*afp1* lines grown under control and progressive drought conditions. RT-qPCR data represent means ± SE of three biological replicates (n=3), where statistical analyses were carried out using two-way ANOVA (time period of drought and genotype) and Tukey's Post-Hoc HSD tests (P<0.05).

Figure 7: Model of ABA regulation of drought tolerance in WT compared to *gasa3* and *afp1* mutants.

ABA-dependent protein phosphorylation and transcriptional regulation of ABA-responsive genes are at the core of ABA-dependent drought response. Increase in ABA synthesis and inhibition of the PP2CA negative feedback loop in the absence of *gasa3* and *afp1* ultimately result in increased expression of certain ABA-responsive genes as well as stomata closure via SLAC1 and KAT1 phosphorylation. Red arrows indicate changes in content of ABA or transcripts upon drought. Created in <https://BioRender.com>

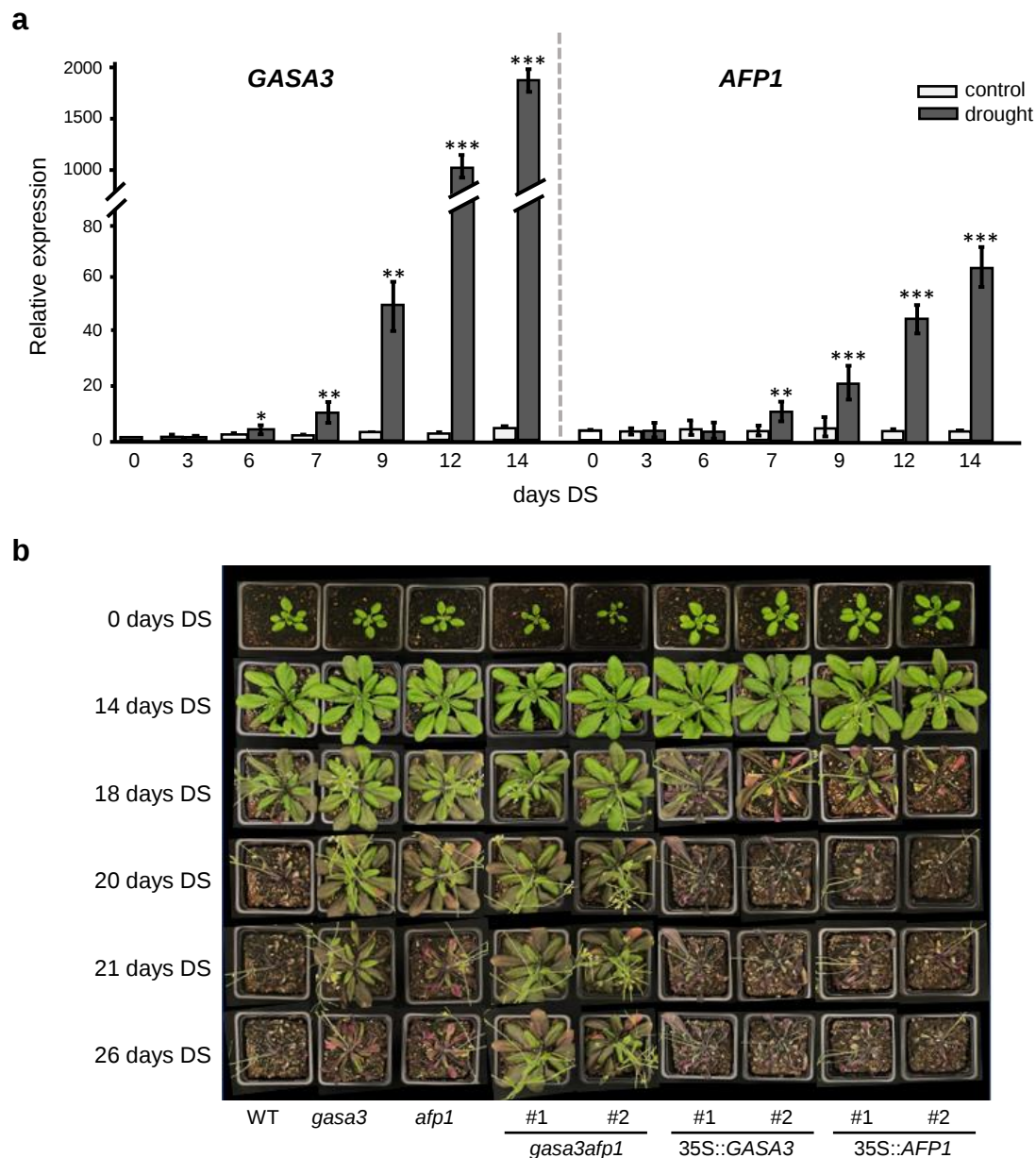


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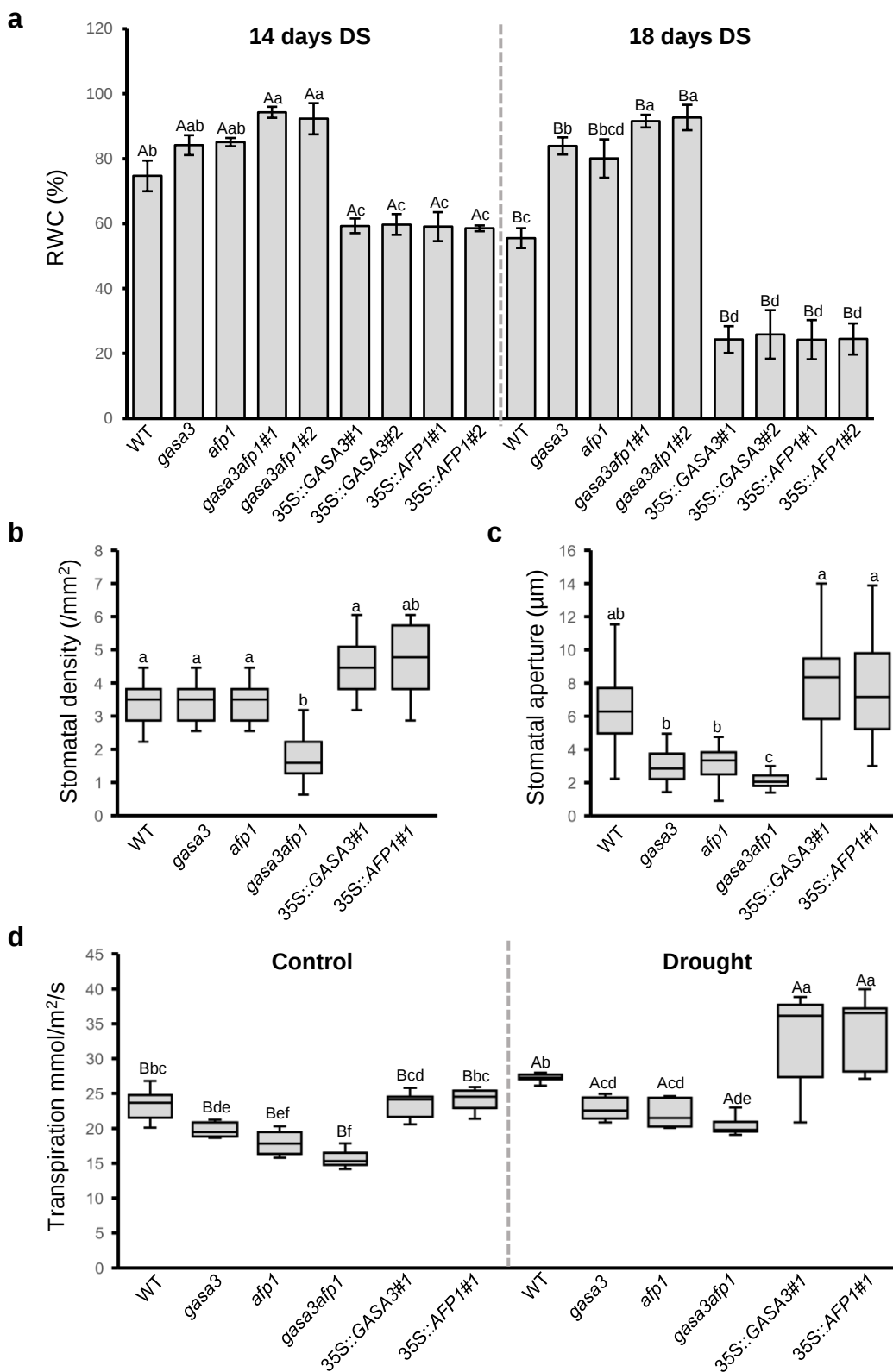


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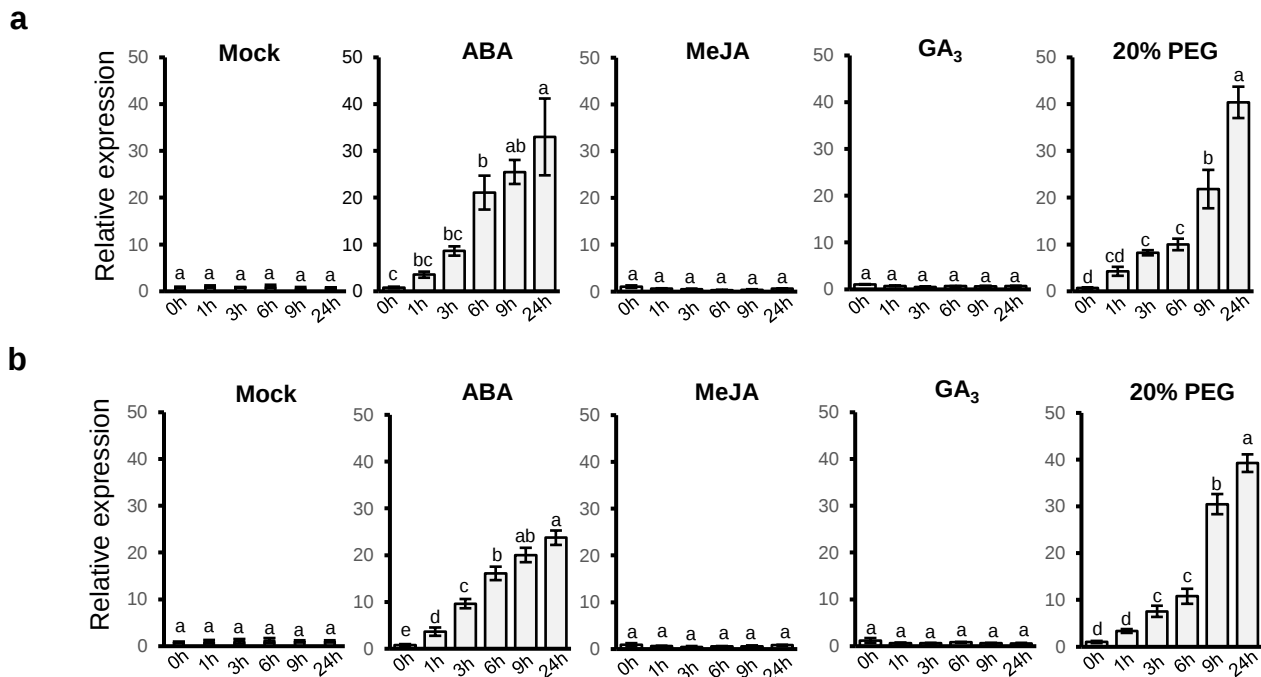


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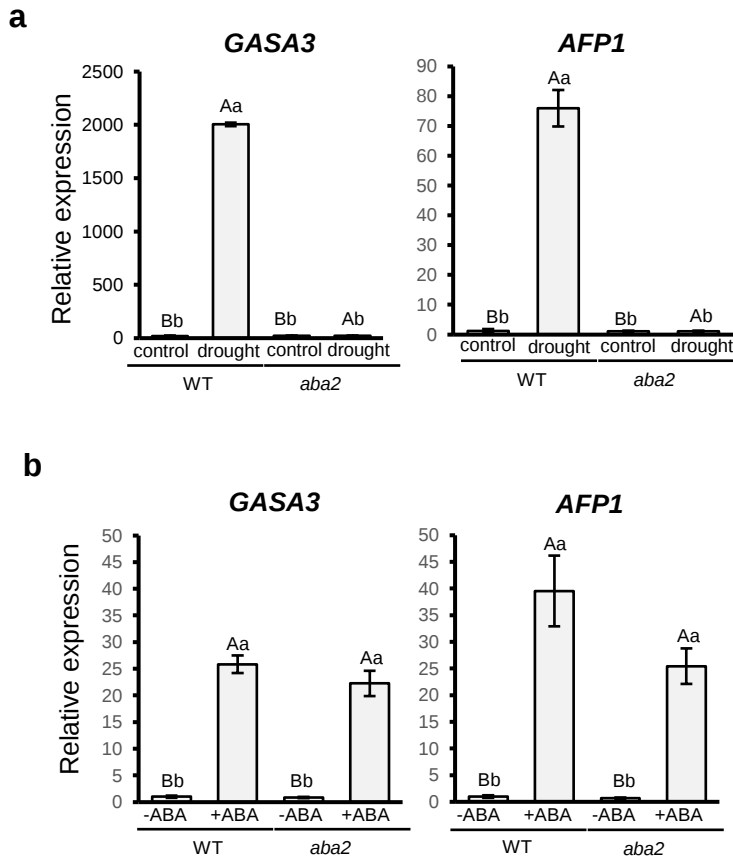


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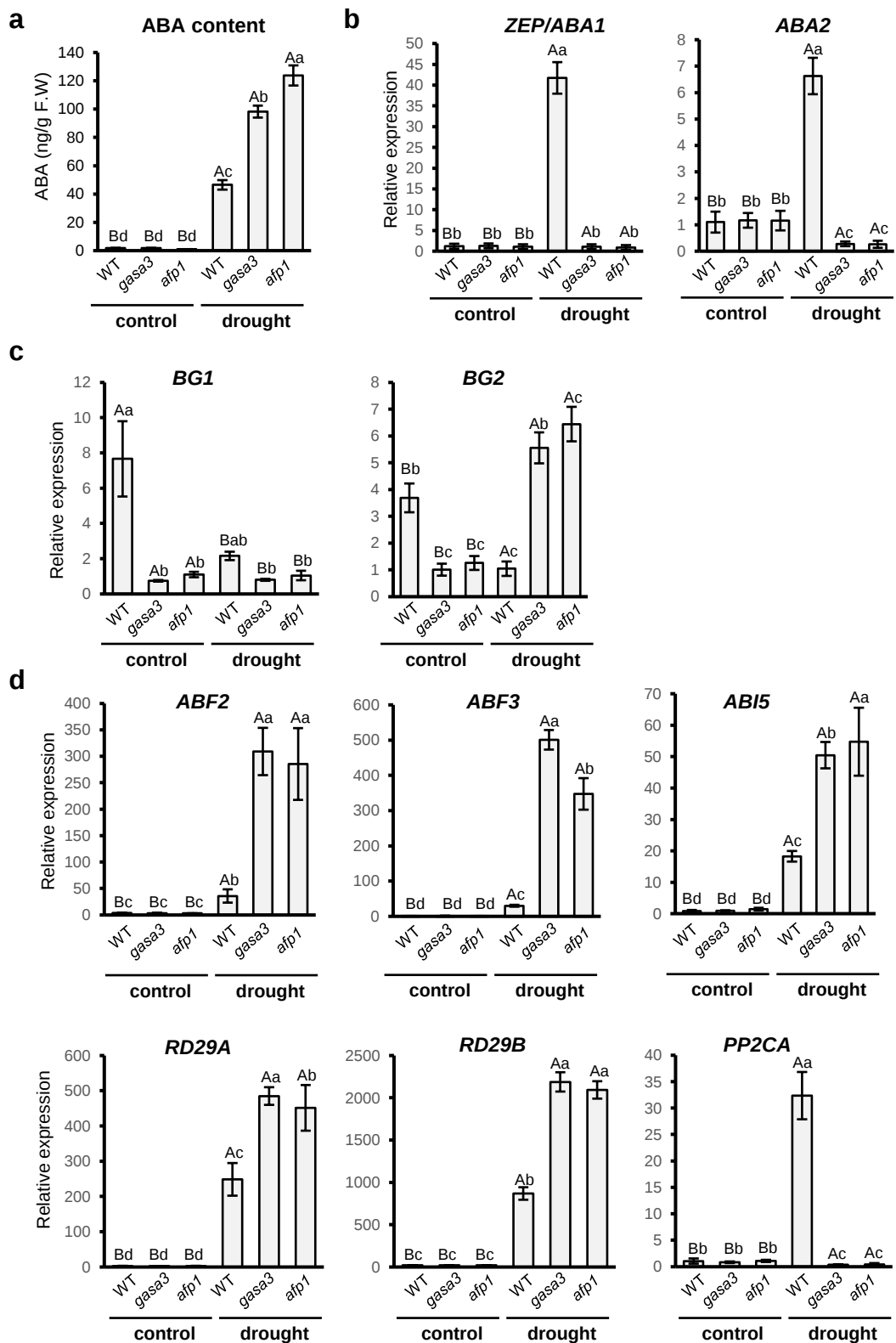


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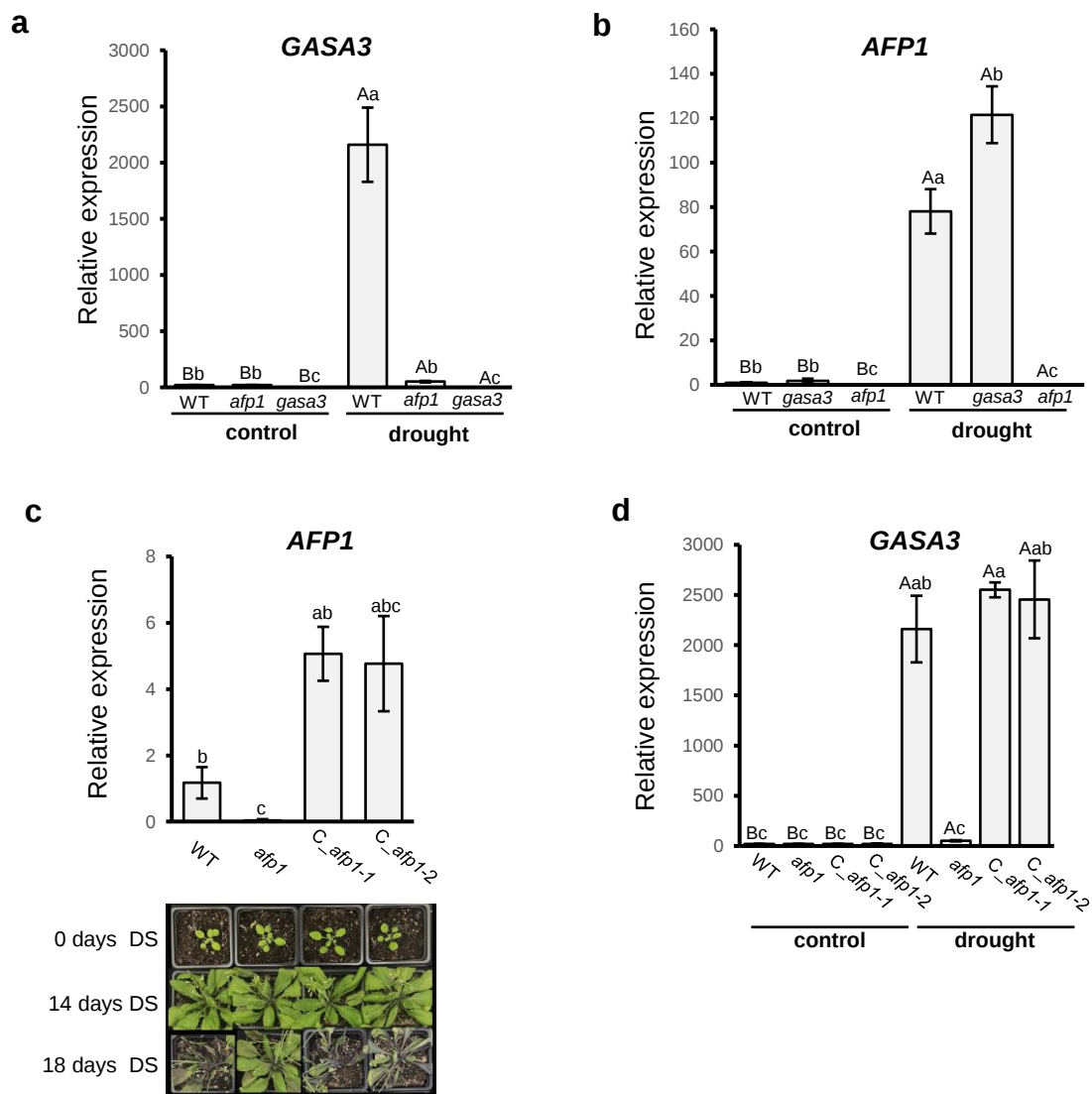


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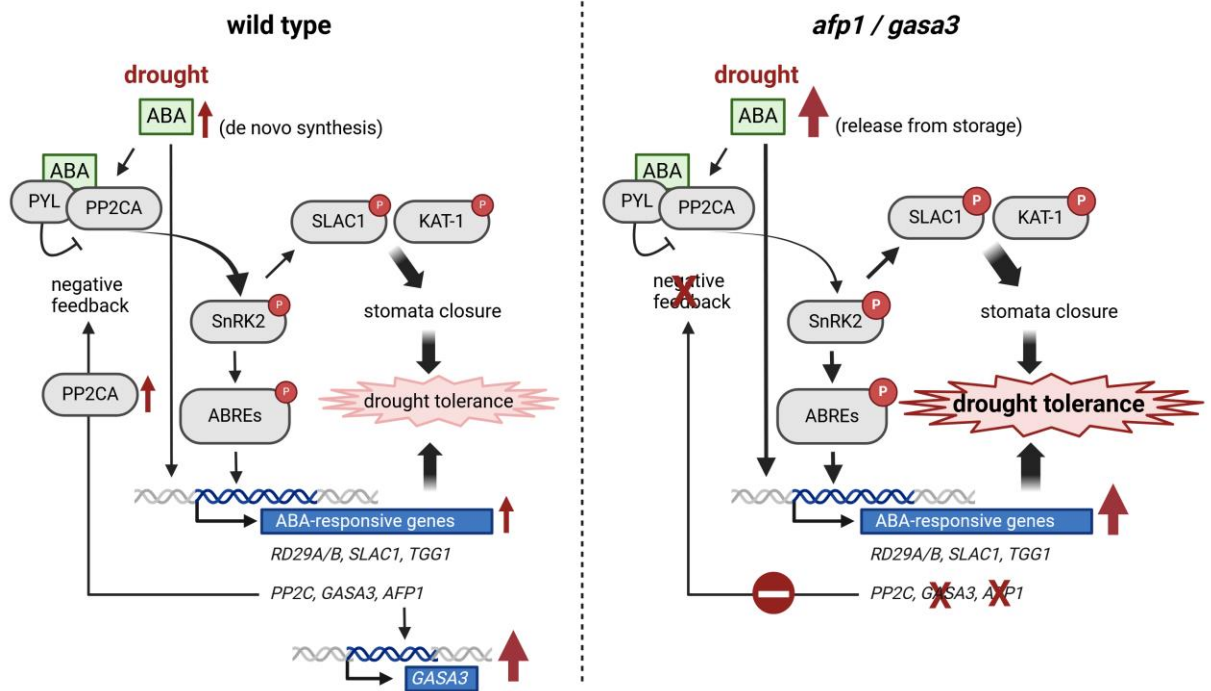


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