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Early transcriptomic plasticity in barley (*Hordeum vulgare* L.) seminal roots in response to abiotic stress

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Content

Abbreviations

ABA	Abscisic acid
ABC	ATP-binding cassette
ABRE	ABA responsive element
ANOVA	Analysis of variance
AP2/ERF	APETALA 2/ethylene responsive factor
AREB/ABF	ABA-RESPONSIVE ELEMENT BINDING FACTORS
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
bZIP	Basic-domain leucine zipper
Ca^{2+}	Calcium ion
cDNA	Complementary DNA
CDPK	Calcium-dependent protein kinases
CesA	Cellulose synthase
Cl^-	Chloride ion
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
DREB/CBF	Dehydration-responsive element binding
ERF	Ethylene responsive factor
FAO	Food and agriculture organization
FC	Fold-change
FDR	False discovery rate
GO	Gene ontology

GPI	Glucose-6-phosphate isomerase
H^+	Hydrogen ion
HD-ZIP	Homeodomain leucine-zipper
HKT	High-affinity K^+ transporter
HSF	Heat shock factor
K^+	Potassium ion
LBD	Lateral organs boundaries domain
MAPK	Mitogen-activated protein kinase pathway
MDS plot	Multi-dimensional scaling plot
MPa	Mega pascal
MPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
MYB	MYB (myeloblastosis)-domain
Na^+	Sodium ion
NAC	NO APICAL MERISTEM, Arabidopsis thaliana ACTIVATING FACTOR 1/2, CUP-SHAPED COTYLEDON 2
NRT1/PTR	Low-affinity nitrate transporter /peptide transporter
OSCA1	Reduced-hyperosmolality-induced-$[Ca^{2+}]_i$-increase 1
PEG	Polyethylene glycol
PFP	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase
PP2C	Protein phosphatase 2C
RLK	Receptor-like kinase
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species

SLAC1	Slow anion channel-associated 1
SnRK2	Sucrose non-fermenting 1-related protein kinase 2
SOS	Salt overly sensitive
TALE	Three-amino-acid-loop-extension
TF	Transcription factor
WGCNA	Weighted gene co-expression network analysis
WRKY	WRKY DNA-binding domain
XTH	Xyloglucan endotransglycosylase/hydrolase

1 Zusammenfassung / Summary

1.1 Zusammenfassung

Wasserdefizit und Bodenversalzung wirken sich negativ auf das Pflanzenwachstum aus und stellen somit eine große Bedrohung für die landwirtschaftliche Produktion und die Ernährungssicherheit dar. Bereits einzelnes Auftreten führt zu enormen wirtschaftlichen Schäden, die Kombination beider Stressoren kann sogar zu einem völligen Ernteverlust führen. Aufgrund der globalen Erwärmung ist zu erwarten, dass Extremwetterereignisse, wie z. B. Dürren in Zukunft häufiger auftreten. Dies hat zur Folge, dass Flächen, auf denen vorher Regenfeldbau betrieben wurde, von künstlicher Bewässerung abhängig werden. Dies verstärkt eine Versalzung des Bodens und begünstigt somit das Auftreten von Stresskombinationen. Daher ist es unerlässlich, die zugrundeliegenden Prozesse der pflanzlichen Stressantwort zu untersuchen, wobei nicht nur Reaktionen der gesamten Wurzeln, sondern auch die der spezifischen Wurzelzonen berücksichtigt werden sollten, um ein ganzheitliches Bild zu erhalten.

In der vorliegenden Studie untersuchten wir zuerst die Auswirkungen von Wasserdefizit (- 0,8 MPa), erhöhtem Salzgehalt (150 mM NaCl) und deren Kombination auf das Transkriptom ganzer Seminalwurzeln von Gerstenkeimlingen nach 6 h und 24 h Stress-Exposition. Anschließend untersuchten wir die Wirkung desselben Wasserdefizits auf das Transkriptom der einzelnen Wurzelzonen: Wurzelhaube und Meristem, Streckungszone und Wurzelhaarzone nach 6 h, 24 h und 48 h Behandlung.

Mittels RNA-Sequenzierung identifizierten wir hunderte Gene, die als Reaktion auf die Kombination verschiedener Stressszenarien, Zeitpunkte oder Wurzelzonen differenzielle Expression zeigten. In der Gesamtwurzel konnten wir einen Zusammenhang zwischen der Dauer der Stressbehandlung und der Anzahl an differenziell exprimierten Gene feststellen. Im Vergleich zur Stressdauer von 6 h, wurden Hunderte von zusätzlichen Genen erst zum späteren Zeitpunkt, d. h. 24 h nach Exposition, aktiviert. Die Anzahl an differentiell exprimierten Genen, die wir in der Stresskombination identifizierten, überstieg deutlich die Summe der Gene aus den individuellen Stressszenarien, was für eine nicht-additive Aktivierung zusätzlicher Gene spricht. Im Gegensatz dazu stellten wir in der Wurzelzonen-spezifischen Transkriptomanalyse verschiedene Phasen mit jeweils unterschiedlichen Ausmaßen der Reaktion fest. Solch eine spezifische Aktivierung wurde von uns auch in den einzelnen Wurzelzonen beobachtet. Hier war die Streckungszone am stärksten von der Stressexposition betroffen, was die Aktivierung vieler Gene bedingte, die in anderen Zonen keine Stressreaktion zeigten.

Eine Zuordnung der identifizierten Gene in ihren biologischen Kontext zeigte, dass obwohl viele der identifizierten Prozesse und Stoffwechselwege zwischen den Behandlungen

übereinstimmen, die Richtung der Modulation und die daran beteiligten Gene für das jeweilige Szenario spezifisch waren.

Zusammenfassend waren es Veränderungen im Redoxsystem, die Regulierung von Transportern, posttranslationale Modifikationen durch Proteinkinasen, Anpassungen im Energiestoffwechsel, Umstrukturierung der Zellwände und Modulation von Transkriptionsfaktoren, die die Veränderung in Reaktion auf abiotischen Stress prägten. Viele dieser beobachteten Anpassung waren spezifisch im Hinblick auf das Stressszenario, die Dauer der Exposition und die betrachtete Wurzelzone.

Die hier dargestellten Ergebnisse liefern auf Transkriptomebene neue Einblicke in die komplexen und dynamischen Reaktionen von Gerstenwurzeln auf abiotischen Stress und heben die Bedeutung von kombinatorischen Expositionsszenarien sowie die Berücksichtigung einer räumlich und zeitlichen differenzierten Auswertung hervor.

1.2 Summary

Water deficit and soil salinity negatively affect plant growth and thus pose major threats to agricultural production and food security. Already one of these factors results in economic damage, while a combination of both stressors often has detrimental effects leading to total yield loss. Due to global warming, extreme weather events like droughts are expected to increase in the future. Formerly rain-fed land will become dependent on irrigation, further enhancing soil salinization, leading to constant abiotic stress combinations. Thus, it is imperative to examine and understand the processes and mechanisms that shape whole-root and root zone-specific responses to these abiotic constraints to secure food productivity under unfavorable environmental conditions. In this study, we first investigated the effects of water deficit (- 0.8 MPa), salinity (150 mM NaCl) and their combination on the transcriptome of whole seminal roots of barley seedlings after 6 h and 24 h of stress exposure. Then, we examined the effect of the same water deficit on the transcriptome of the individual root zones: root cap and meristem, elongation zone and differentiation zone after 6 h, 24 h and 48 h of treatment.

RNA-sequencing revealed hundreds of genes that are differentially regulated in response to either treatment-by-time or root zone combination. We observed a treatment-wise temporal conservation of responsive genes in the whole-root study, with hundreds of additional genes activated at the later time point. This was not confirmed in the root zone-wise approach. Instead, we identified different phases of modulation with varying extents. The combination of stresses affected a unique set of differentially expressed genes, that were not differentially expressed in either individual stress scenario and whose extend exceeded the sum of the individual applications. Root zone-wise, we also detected several unique sets of differentially expressed genes according to their time-by-zone combination. Overall, the elongation zone was the most affected root zone.

Grouping the identified differentially expressed genes into a biological context showed that processes and pathways were shared between different treatments and root zones, yet their modulation and the involved genes remained specific. In general, alterations to the redox system, regulation of transporters, post-translational modification by protein kinases, adaptations in the energy metabolism, cell wall reorganization and modulation of transcription factor activity shaped the plant responses to abiotic stresses in a treatment, time and root zone-specific manner.

The findings presented here provide new insights into complex, dynamic transcriptomic responses, emphasizing the importance of combinatorial stress research and considering spatial and temporal resolution.

2 Introduction

2.1 Significance of barley in agriculture and scientific research

Barley (*Hordeum vulgare* L.) is an economically important crop with versatile uses. Scientifically it belongs to the monocotyledonous family of *Poaceae*, more commonly known as grasses. Since barley was one of the first cereals to be domesticated, it has played a crucial role in human civilization for thousands of years. Barley originated approximately 10,000 years ago from an area called the Fertile Crescent, a region comprising parts of what is nowadays Iran, Iraq, Israel, Lebanon, Jordan, Syria and Turkey (Kilian *et al.*, 2010, **Figure 1**).

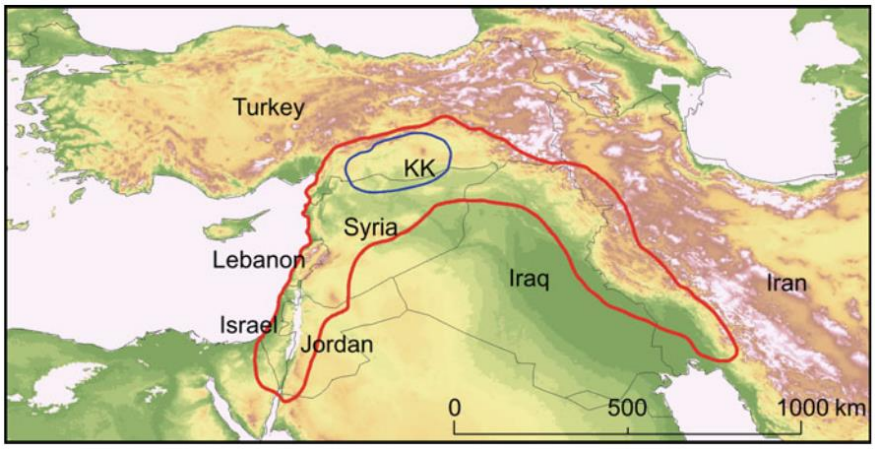

Figure 1: Map of the Fertile Crescent (red line) and the 'core area' (blue line) of domestication (Kilian *et al.*, 2010).

Since then, barley has been distributed around the globe and is now cultivated in most areas of the world, including highly productive and subsistence agricultural systems across diverse environments (**Figure 2A**). Today, barley ranks fourth in a comparison of global cereal production with over 150 million tons produced in 2022/23 (Statista, 2023).

During domestication, three different types of barley were generated that show differences in their responses to photoperiod and their need for vernalization. Winter varieties are sown in the fall, and require vernalization and long day lengths to initiate flowering (Kling *et al.*, 2004). In contrast, spring varieties are sown in spring and require shorter day lengths to initiate flowering, but no vernalization (Miralles *et al.*, 2021). So-called facultative varieties can be sown both in spring and fall as they can adapt their growth and flowering time to varying day lengths, offering higher flexibility (Zitzewitz *et al.*, 2005). While spring barley varieties are often used as malting barley for beverage production, winter barley is commonly used as a fodder crop (Diepenbrock *et al.*, 2009).

The barley genome is diploid (2n) and consists of seven chromosome pairs with a size of around 5.1 gigabases. A breakthrough in barley research was achieved in 2012 with the release of the first draft of the barley genome sequence with over 26,000 annotated high-confidence gene models (The International Barley Genome Sequencing Consortium, 2012). This first draft of the spring variety Morex was improved twice, leading to the publication of the most recent version MorexV3, containing over 35,000 high-confidence gene models (Mascher *et al.*, 2021).

Barley is self-pollinating and possesses naturally high adaptability to diverse environmental conditions (Newton *et al.*, 2011), which made it an excellent candidate for experimental research even before the genome sequence was drafted. In combination with the availability of natural and induced mutants, wild accessions and landraces, barley is widely used as a model plant in various fields, including physiology, genetics, biochemistry, biotechnology and phytopathology (Harwood, 2019).

2.2 Abiotic stresses and their impact on agriculture

Environmental constraints, or abiotic stressors like extreme temperature, flooding, soil salinity, or drought pose major threats to agricultural production. Exposure of crops to stress affects a multitude of processes which in turn impact plant development, growth, yield and nutritional quality (Wang and Frei, 2011).

According to the Food and Agriculture Organization (FAO), drought poses the most dominant threat to agricultural production (FAO, 2021a). Meteorologically, drought is defined as a condition with below-normal precipitation compared to the average condition in a region. In agriculture, drought occurs when such a precipitation deficit limits water availability for crops and natural vegetation through soil moisture deficits (Wilhite and Glantz, 1985). A collaboration between various organizations and experts founded the Global Drought Monitor Portal to meet the need for coordinated assessment and monitoring of drought conditions worldwide (Heim and Brewer, 2012). They provide the Global Drought Monitor, a tool that offers near real-time information at a high-resolution spatial scale to not only asses present conditions but also help in predicting future drought events. As of June 2023, large areas, spanning all continents are facing drier than normal conditions (**Figure 2B**), this includes many agriculturally used regions, such as Europe and North America.

Another important factor that limits agricultural productivity is high soil salinity. Soil salinity is a naturally occurring phenomenon in many arid and semi-arid regions due to high water evaporation rates that lead to salt accumulation in the upper soil layers. Irrigation-based agriculture in these regions is a principal contributor to food safety. However, the combination of several factors, including the use of low-quality irrigation water, poor drainage systems and a prevalence of improper land further drives human-induced secondary salinization, leading to land degradation or even desertification (Devkota *et al.*, 2022). Based on data submitted to the FAO that covers 73% of the global land area, 4.4% of topsoils (0 – 30 cm) and 8.7% of subsoils (30 – 100 cm) are already affected by soil salinity (**Figure** 2**C**). Estimates project an additional land loss of around 1.5 million hectares of farmland annually due to soil salinization (FAO, 2021b).

A comparison of drought-affected areas and regions of high soil salinity shows a co-occurrence of both stresses, including major barley production sites (**Figure 2**). Plants subjected to a combination of multiple abiotic stresses show enhanced developmental constraints and oftentimes such combinations prove to be more destructive to agricultural production than single-stress exposure (Mittler, 2006).

In the context of global warming, abiotic stresses, in particular droughts are expected to increase (United Nations Office for Disaster Risk Reduction, 2021). This will result in more frequent drought periods with higher intensity, even in areas not yet affected by such limitations (Dai, 2012; Naumann *et al.*, 2021). In turn, around 35% of rain-fed land is expected to be dependent on irrigation in the future (Rosa *et al.*, 2020). While adopting irrigation-based farming is necessary to meet the demand for global food supply, it also aggravates the potential of soil salinization (Singh, 2015).

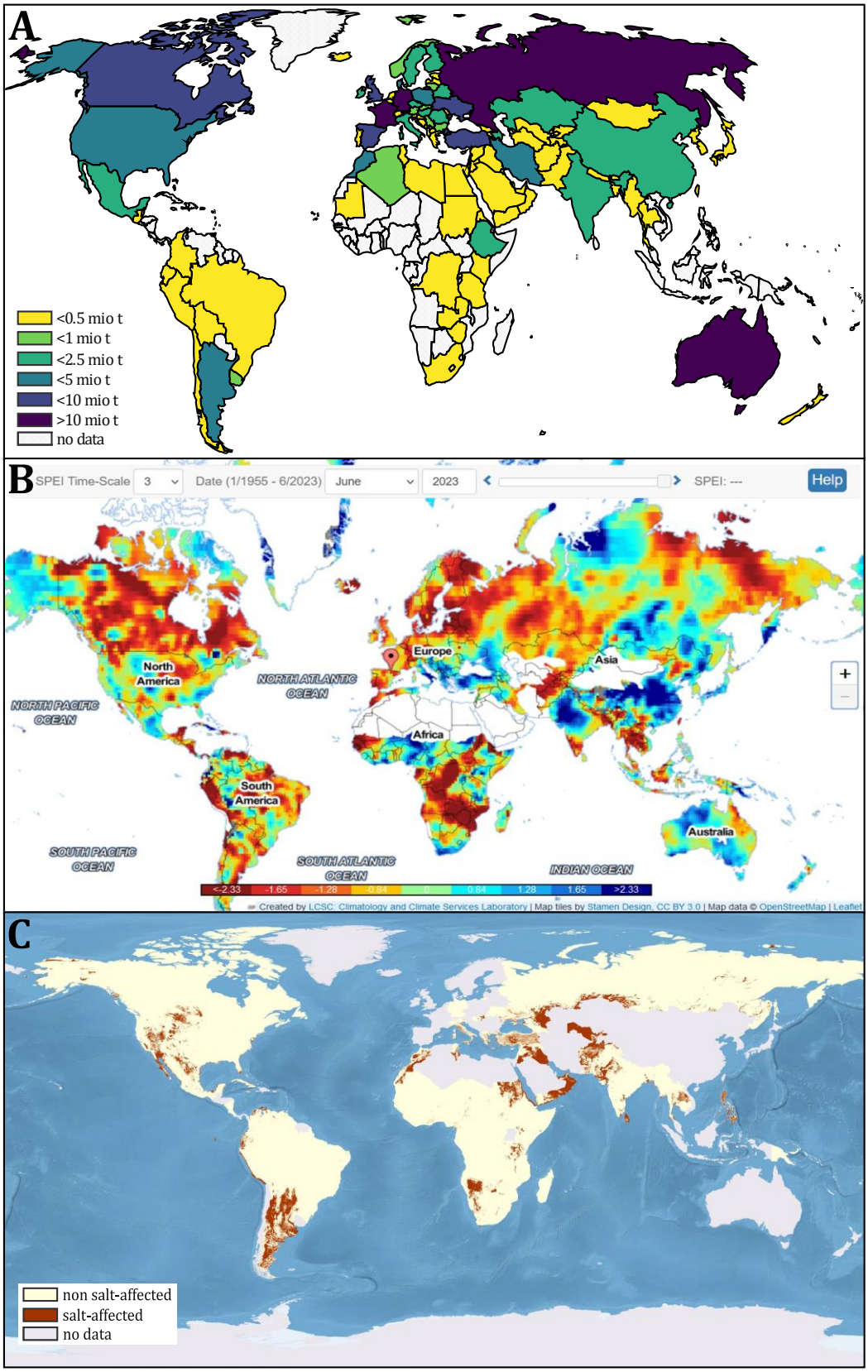

Figure 2: Geographical distribution of barley production, drought areas and soil salinity. A) World barley production in 2021 in million tons per year. Adapted from Food and Agriculture Organization of the United Nation; OurWorldInData.org/agricultural-production (Ritchie et al., 2023). **B)** Global drought monitor for June 2023 based on the Standardized Precipitation Evapotranspiration Index indicating areas with drought (red colors) and wet (blue color) conditions (LCSC: climatology and climate services laboratory, 2023). **C)** Salt-affected soil at 30-100 cm depth (FAO, 2021b).

2.3 Molecular responses of plants to abiotic stress

2.3.1 Water deficit

Plants take up water via their roots, from where it is transported through a xylem network to shoots and leaves. This axial transport is driven by root pressure, capillary action and transpiration along a gradient of water potentials. The difference between water potentials creates the flow of water from higher to lower potentials (Kim *et al.*, 2014). Based on this hydraulic principle, the lower water potential in root cells leads to the uptake of water from the surrounding soil. At field capacity, which refers to the maximum amount of water retained in the soil against gravity, the water potential in the soil is - 0.033 MPa. Further drying of the soil decreases the water potential up to the permanent wilting point at - 1.5 MPa, where plants are no longer able to extract water from the soil (O'Green, 2013). At this stage, the uptake of water from the soil is inadequate to meet the demands due to evapotranspiration in leaves, resulting in water deficit conditions.
Water deficit has adverse effects on plants, reducing plant growth, photosynthetic rate and ultimately crop yield (Shinozaki, 2003; Kapoor *et al.*, 2020).

Plants perceive water deficit as a decrease in osmotic potential which triggers a complex network of stress-responsive alterations affecting physiology, metabolism, biochemical processes, gene expression and ultimately morphology. While the identification of an actual osmosensor is still pending, a variety of sensing mechanisms have been proposed. One potential osmosensor, OSCA1 (reduced-hyperosmolality-induced-$[Ca^{2+}]_i$-increase 1), located in the meristem of roots and encoding a hyperosmolality-gated transmembrane calcium channel, was found to affect the influx of Ca^{2+} in response to changes in osmotic potentials (Yuan *et al.*, 2014). *OSCA1* responds to plasma membrane tension or high extracellular osmotic potential, facilitating Ca^{2+} influx, which then acts in downstream signaling (Liu *et al.*, 2018). Additionally, prolonged water deficit can lead to plasmolysis, the detachment of plasma membranes from cell walls, which activates receptor-like kinases within the plasma membrane and affects downstream cell wall-related genes (Lindner *et al.*, 2012). However, this serves as a secondary sensor system and is not directly involved in the initial water deficit perception.

Subsequent signals are transduced through the involvement of Ca^{2+}, reactive oxygen species (ROS), mitogen-activated protein kinases (MAPKs) and phytohormones that interplay in a highly coordinated and complex signaling network (Lamers *et al.*, 2020).

The phytohormone abscisic acid (ABA) plays a central role, orchestrating various response pathways that are crucial for survival. The accumulation of ABA under water deficit conditions triggers an increase in ROS production, which, in turn, enhances ABA biosynthesis (Li *et al.*, 2022). ROS are highly reactive oxygen-containing molecules generated as by-products of photosynthesis. While these oxidizing agents are considered harmful to plants, they also serve as important signaling molecules in growth, development and environmental responses. At cellular

homeostasis, ROS scavenging antioxidants like superoxide dismutase, peroxidase or glutathione balance the level of ROS to protect cells from oxidative damage. However, this equilibrium is disturbed under water deficit conditions, leading to an increase in ROS production, which results in unwanted oxidizing reactions in proteins, DNA, or membranes (Miller *et al.*, 2010; Das and Roychoudhury, 2014). While water deficit initially leads to osmotic stress, the increased production of ROS adds oxidative stress as a secondary constraint (Tripathy and Oelmüller, 2012). Plants employ various measures to counteract the overproduction of ROS. Rising levels of ROS are accompanied by an increased synthesis of antioxidants that perform enzymatic and non-enzymatic ROS scavenging to restore redox homeostasis (Nadarajah, 2020). Moreover, ABA and ROS are involved in regulating the production of compatible solutes, such as proline, sugars, or glycine betaine. These small organic molecules accumulate in the cytosol, changing the osmotic potential of cells and maintaining turgor pressure while preventing water loss. Due to their antioxidant activity, compatible solutes also participate in ROS scavenging and the prevention of oxidative cell damage (Cuin and Shabala, 2007).

Additionally, both ROS and ABA activate the MAPK pathway, a cascade of protein kinases that phosphorylate downstream targets, such as enzymes, signaling proteins and transcription factors, thereby altering the expression of stress-responsive genes. The cross-talk between ABA, ROS and the MAPK pathway is complex and involves regulation by positive and negative feedback in response to stress (Danquah *et al.*, 2014; Jalmi and Sinha, 2015; Li *et al.*, 2022).

Moreover, ABA increases cytosolic Ca^{2+} levels which activates calcium-dependent protein kinases (CDPKs). These kinases possess a calcium-sensing domain and a kinase effector domain that allows them to sense the changes in the cytosolic Ca^{2+} concentration and convert this information into physiological responses. Their involvement in all types of abiotic stress reactions has been researched in various species, including the model plant Arabidopsis, but also in major crops, such as rice, maize, wheat, or barley (Li *et al.*, 2008; Y Yang *et al.*, 2017; Mittal *et al.*, 2017). CDPKs interact with their target proteins via phosphorylation. One of these interaction partners, the guard cell-specific SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1), controls stomatal aperture and is targeted by CDPKs upon drought stress which in turn leads to stomatal closure, mediated by ABA. This process also involves the aforementioned MAPK pathway and ABA-activated Sucrose Non Fermenting 1-related protein kinase 2 (SnRK2), a kinase that regulates ion channel activity (Geiger *et al.*, 2010; Zhang *et al.*, 2021).

This ABA-mediated closure of stomata is one of the first responses to water deficit conditions and is triggered directly by soil dehydration, independent of leaf turgor pressure (Hoshika *et al.*, 2013). By closing their stomata, plants conserve water that would be lost through evapotranspiration. However, this leads to a decrease in the photosynthetic rate while increasing the photorespiration rate, leading to an overproduction of ROS (Pirasteh-Anosheh *et al.*, 2016).

ABA also plays an important role in growth regulation by interacting antagonistically with the phytohormone ethylene (Müller, 2021). Under normal conditions, ethylene inhibits root growth by stimulating auxin biosynthesis, which limits cell elongation (Růzicka *et al.*, 2007). The accumulation of ABA in roots under water deficit blocks ethylene biosynthesis, permitting continued root growth, albeit at a slower rate and enabling the plant to reach deeper soil layers, accessing new water resources (Sharp and LeNoble, 2002).

All these processes involve the alteration of gene expression, which is often mediated by transcription factors. Transcription factors are regulatory proteins, controlling DNA binding in gene promoters and thus modulating the transcription of target genes. They offer a dynamic switch between increasing or decreasing expression levels that allow for fast responses to environmental cues.

ABA-RESPONSIVE ELEMENT BINDING FACTORS (AREBs/ABFs), belonging to the basic-domain leucine zipper (bZIP) transcription factor family, play a crucial role in plant responses to water deficit (Fujita *et al.*, 2011). After activation of AREB/ABF transcription factors via phosphorylation by SnRK2, they bind to the ABA-responsive element (ABRE) within the promoter region and activate target genes depending directly on the ABA level (Hrmova and Hussain, 2021). Other transcription factors that are involved in ABA-mediated regulation, but lack the ABRE, include members of the NAC (NO APICAL MERISTEM, Arabidopsis thaliana ACTIVATING FACTOR 1/2, CUP-SHAPED COTYLEDON 2), homeodomain leucine-zipper (HD-Zip), WRKY, or MYB (myeloblastosis) families. Though gene activity of these families can be induced by ABA, they additionally show direct responsiveness to abiotic stressors (Rushton *et al.*, 2012; Nuruzzaman *et al.*, 2013; Valdés, 2014; Yao *et al.*, 2020).

Heat shock factors (HSFs), activated in response to various abiotic and biotic stresses, regulate the production of heat shock proteins that function as chaperones, stabilizing proteins to ensure correct folding or refolding of damaged structures. Though HSFs were conventionally perceived as ABA-independent, emerging research suggests additional activation in an ABA-mediated manner (Huang *et al.*, 2016; Andrási *et al.*, 2021).

Dehydration-responsive element binding (DREB) transcription factors, belonging to the class of AP2/ERF (APETALA2/Ethylene Responsive element binding Factor) proteins, are activated directly by abiotic stresses without the direct involvement of ABA, though exogenous application of ABA also induces their activity under experimental conditions. They bind to the dehydration-responsive element within the promoter region and thus regulate the expression of water deficit-responsive genes (Lata and Prasad, 2011).

In summary, water deficit leads to changes in the osmotic potential within plant cells which is perceived by osmosensors, activating a network of stress-responsive mechanisms. The phytohormone ABA is the key regulator, orchestrating these responses. A complex cross-talk

between ABA, other phytohormones, ROS, CDPKs and MAPKs mediates growth alterations, stomatal closure and gene expression changes via transcription factors to adapt and survive under environmental constraints.

2.3.2 High salinity

Under saline conditions, higher concentrations of Na^+ and Cl^- ions are dissolved in soil water and passively taken up by the inflow of water into roots. This excessive intake of Na^+ and Cl^- ions changes the osmotic potential in cells and leads to osmotic stress and secondary oxidative stress, similar to the stress experienced under water deficit conditions. However, it also induces an ionic stress component due to the resulting Na^+ accumulation (Ma *et al.*, 2020).

These cations can bind to negatively charged glucuronic acids within glycosyl inositol phosphoryl ceramides, a major component of the plasma membrane, or to rhamnogalacturonan-II, a cell wall component, leading to changes in tension, both activating calcium influx channels (Lamers *et al.*, 2020). While osmotic and ionic stress both activate Ca^{2+} channels, the pattern and location of Ca^{2+} influxes are stress-type specific (Whalley and Knight, 2013; Choi *et al.*, 2014). The increased level of intracellular Ca^{2+} then activates the Salt Overly Sensitive (SOS) pathway, aiming at the exclusion of excess Na^+ from the cell (Lamers *et al.*, 2020; Wang *et al.*, 2022). In the first step, a calcium-binding protein, SOS3, activates the protein kinase SOS2 and forms a complex that then phosphorylates SOS1, a Na^+/H^+ antiporter in the plasma membrane. SOS1 actively exports Na^+ ions out of the cell and helps to regain and maintain ion homeostasis (Quintero *et al.*, 2002). The activity of SOS pathway genes is regulated by several transcription factors, including bZip, MYB and WRKY transcription factors but also by MPK3 and MPK6, both members of the MAPK pathway, indicating an essential role of MAPKs in mediating water deficit and salt stress responses (Rolly *et al.*, 2020; Ma *et al.*, 2022; Ali *et al.*, 2023).

Moreover, plants can sequester Na^+ into vacuoles via a vacuolar Na^+/H^+ antiporter, that shows increased activity at higher Na^+ levels. Studies in salt-tolerant and salt-sensitive species showed, that the activity of this antiporter was correlated with better salinity tolerance. Sequestration of Na^+ from the cytoplasm into vacuoles alters the osmotic potentials. To maintain osmotic balance, plants accumulate osmoprotectants in their cytoplasm, similar to those accumulated under water deficit conditions (Tester and Davenport, 2003; Keisham *et al.*, 2018).

2.3.3 Combined abiotic stress exposure

Under field conditions, plants often experience combinations of abiotic stresses. Seasons without sufficient precipitation are often accompanied by high temperatures or soil salinity due to poor irrigation water usage, resulting in detrimental effects on plant growth, development and

ultimately crop yield (Mittler, 2006). Various studies suggest that the response of plants to a combination of stresses is more complex and unique, as they exceed the sum of individual stress responses, underscoring the importance of research in this area to enhance crop productivity (Mittler, 2006; Ahmed *et al.*, 2013; Pandey *et al.*, 2015; Zandalinas *et al.*, 2021; Mohammadi Alagoz *et al.*, 2023; Da Ros *et al.*, 2023).

Studies in wheat showed, that individual exposure to drought and heat negatively affected yield traits, such as grain number and spike fertility, defined as the reproductive capability of a cluster of flowers arranged on a stem. Their combination resulted in even greater yield losses than their additive effects alone, indicating a co-occurrence of different growth-limiting mechanisms (Prasad *et al.*, 2011; Suzuki *et al.*, 2014; Mahrookashani *et al.*, 2017). Comparable effects have been demonstrated on plant growth and photosynthetic rates in barley, though the impact of drought on photosynthetic activity was found to be less significant compared to that of heat or the combined stress (Rollins *et al.*, 2013).

Moreover, a stress combination can result in conflicting responses. Plants counteract heat stress by increasing stomatal conductance for more efficient cooling. Under water deficit conditions, stomata are closed to save water and thus, cooling via increased transpiration is not possible, leading to increased leaf temperature. Though proline is commonly accumulated in response to water deficit, it is replaced by sucrose in a combination of water deficit and heat stress, to prevent proline toxicity caused by elevated temperatures in Arabidopsis (Rizhsky *et al.*, 2004). These antagonistic effects also occur in other multiple stress combinations: increased transpiration under heat stress could result in enhanced salt uptake if combined with salinity. A combination with nutrient deficiency could weaken the ROS scavenging system since essential resources and energy are limited (Mittler and Blumwald, 2010).

While this phenomenon has been studied for many combinations, studies examining the effects of combined salt and water deficit stress in crops remain scarce (Suzuki *et al.*, 2014). Exposure of cultivated and wild barley to a combination of salt and drought stress revealed a higher tolerance in wild barley, though both genotypes showed a reduction in growth and photosynthetic rates. Higher tolerance was achieved by higher levels of osmoprotectants and ROS scavenging enzymes, better water-use efficiency and improved uptake of K^+ over Na^+ (Ahmed *et al.*, 2013). Experiments in potatoes and maize showed that a combination of drought and salinity reduced chlorophyll and leaf water content more than either individual stress and that the severity of the effects was strongly dependent on the growth stage at stress exposure (Wen *et al.*, 2022).

2.4 The barley root system

The root system anchors the plant to the ground and enables the acquisition of water and nutrients from the surrounding soil (Jackson, 2005). Barley, as a monocotyledonous plant, has a fibrous root system that forms embryonic seminal roots from the embryo radicle during germination, post-embryonic lateral roots that emerge from the seminal roots and nodal roots from the lower tillers (Liu *et al.*, 2020).

Along their longitudinal axis, roots can be separated into several distinct zones (**Figure 3**) and each of these root zones has its own functions (Ishikawa and Evans, 1995). The root cap encloses the tip of the root and serves as a protective layer. Additionally, so-called statoliths, which are starch-filled organelles within columella cells play a role in gravity-sensing and thus root growth orientation (Leitz *et al.*, 2009). The quiescent center is located at the most distal part of the root meristem and consists of undifferentiated, non-dividing cells, that are crucial for the maintenance of the meristem (Kirschner *et al.*, 2017). The meristematic zone is characterized by cell division with smaller, rapidly dividing cells as the provider for root growth, while in the elongation zone, root cells rapidly increase in length but remain undifferentiated (Hayashi *et al.*, 2013). The differentiation into more specialized cells takes place in the differentiation zone, the most basal part of the root. Post-embryonic lateral roots emerge from phloem-pole cells within the pericycle of the differentiation zone, increasing the area of the root system. Some epidermal cells in the differentiation form root hairs which are tubular extensions, further expanding the root surface area to enhance water und nutrient uptake (Pierre-Jerome *et al.*, 2018).

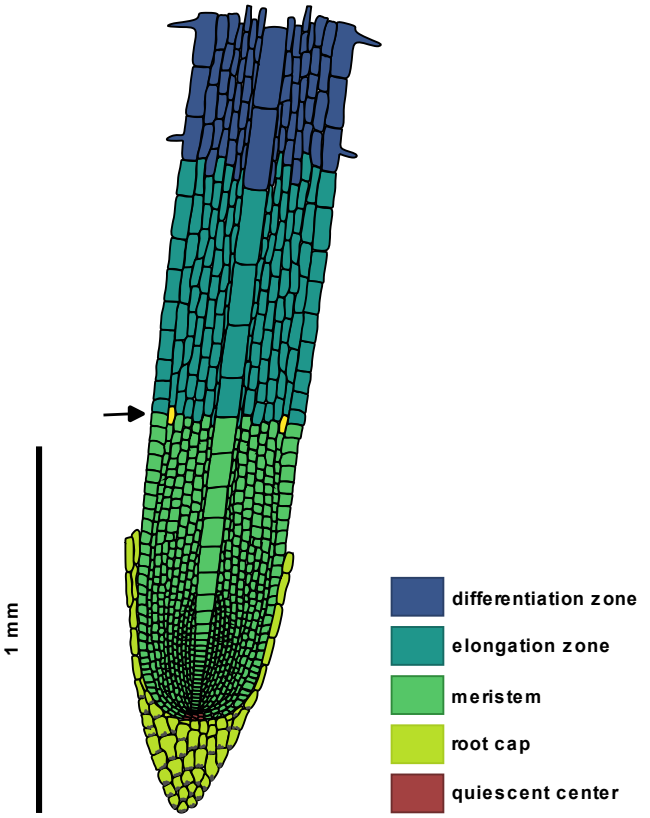

Figure 3: Schematic of a barley root with longitudinal root zones. Each zone is depicted in a different color. Transition between the meristem and the elongation zone is highlighted with an arrow and only the last not elongated cells in the outer cortex layer are colored yellow.

2.5 RNA-seq to study abiotic stress transcriptomics in barley

While research conducted in Arabidopsis has been instrumental in the identification of many molecular pathways associated with abiotic stress responses, major crops, including barley, wheat, maize and rice belong to the monocot family, making them genetically different from the dicotyledon Arabidopsis plants. Consequently, their stress response mechanisms may diverge from those identified in Arabidopsis and involve different genes.

With the emergence of RNA-sequencing (RNA-seq) in 2008, researchers gained a powerful tool for direct transcript-level quantification, enabling a more precise examination of gene expression changes compared to microarray approaches (Nagalakshmi *et al.*, 2008). Total RNA from any tissue or cell type is isolated and transcribed to complementary DNA (cDNA) using reverse transcriptase and random primers. Sequencing libraries are constructed by ligation of adapters to the ends of each cDNA fragment and subsequent amplification of these fragments via polymerase chain reaction. The obtained libraries are then sequenced on high-throughput platforms, resulting in millions of short reads. These reads can either be used for *de novo* genome assembly or, if a reference genome is available, directly aligned to it. Based on this alignment, transcript levels and differential gene expressions can be computed (Kukurba and Montgomery, 2015). Furthermore, unsupervised clustering methods, like weighted gene co-expression network analysis (WGCNA) construct networks of correlated genes based on their expression profiles, facilitating the identification of key genes and regulators in response cascades (Langfelder and Horvath, 2008).

Many studies utilized RNA-seq to examine transcriptional changes in barley in response to water deficit or salt stress. While some studies focused on seedling roots, even distinguishing between root zones, the majority either assessed only a single time point or investigated the transcriptomic response to individual stresses (Zeng *et al.*, 2016; Hill *et al.*, 2016; Janiak *et al.*, 2017; Janiak *et al.*, 2019; Kreszies *et al.*, 2019; Nefissi Ouertani *et al.*, 2021; Mahalingam *et al.*, 2022).

Despite the undertaken efforts to understand the underlying mechanisms of barley stress responses and the significant progress that has been made, there remain many unknowns, which are probably also due to the high complexity of abiotic stress responses. To bridge this knowledge gap and enable future food security, additional research is required. This also includes approaches covering multiple time points, stress combinations and zone-specific responses at the transcriptome level.

2.6 Aims

The overall objective of this doctoral thesis was to investigate the effects of water deficit and salinity on the transcriptome of young barley seedlings at the whole-root and root zone levels.

In detail the following hypotheses were tested:

1) Transcriptomic changes upon abiotic stress are treatment and time-dependent.

2) The extent of transcriptomic adjustments in response to combined stresses cannot be predicted based on single-stress exposure.

3) Water deficit and /or high salinity affect different metabolic pathways.

4) Water deficit responses show spatiotemporal plasticity at the root zone level.

3 Results

3.1 Transcriptomic analysis of barley seminal roots subjected to a combination of water deficit and salt stress

To identify stress-responsive genes in barley roots that shape the underlying response mechanisms to water deficit, salinity and a combination of both, we grew pre-germinated seedlings of the barley variety Scarlett for two days in paper rolls and then subjected them to moderate water deficit (PEG8000: -0.8 MPa), salt stress (NaCl: 150 mM) and a combination of both. Seedlings used as a control group were grown in half-strength Hoagland solution throughout the experiment. Seedlings used for phenotyping were transferred to custom-build germination pouches that allow image acquisition without disturbing the roots. We collected samples for RNA-seq after 6 h and 24 h of treatment, before morphological changes became apparent, to capture genetic adaptations that shaped the observed phenotypic alteration.

3.1.1 Phenotypic response to abiotic stress treatments

Total root length was measured based on images before and after treatment for seven consecutive days and differences in length between treated and control plants were assessed for each time point separately by ANOVA. After four days of treatment, combined stress significantly reduced root length, while water deficit exhibited a significant root length reduction on day five. In contrast, salinity did not induce a significant effect.

3.1.2 Differential gene regulation in response to abiotic stress treatments

We extracted total RNA from barley roots at 6 h and 24 h of treatment in four biological replicates each and subjected them to RNA-seq. We quality-trimmed the obtained raw reads, mapped them to the previous version of the barley reference genome (IBSC v2.0) and quantified the expression of gene models.

The transcriptomic kinship relation between samples, visualized in a multidimensional scaling plot showed, that libraries derived from the same treatment-by-time point combination clustered closely together, suggesting that the transcriptomic divergence is mainly driven by stress type, followed by duration. We next identified genes that show expression changes in response to stress treatment by calculating pair-wise contrasts of treatment groups against the control groups for each time point by treatment combination (water deficit 6 h, salt 6 h and combined 6 h against control 6 h; water deficit 24 h, salt 24 h and combined 24 h against control 24 h). All genes that exceeded a $|\log_2 FC| \geq 1$ and FDR <5% were considered as differentially expressed genes (DEGs). In general, the combined treatment regulated a higher number of genes (4845 DEGs at 6 h and 8105 DEGs at 24 h) compared to the single treatments, independent of stress duration. In the

water deficit treatment, 1560 DEGs at 6 h and 7094 DEGs at 24 h were identified, respectively. The lowest number of DEGs (953 DEGs at 6 h and 1802 DEGs at 24 h) was identified in the salt treatment. The number of DEGs increased with prolonged stress-exposure in all treatments. Though the direction of regulation varied within stress type and time point, 60-80% of the DEGs showed consistent expression changes over time. Comparison of DEG sets from all treatments at 6 h and 24 h showed that only 12-15% were conserved between treatments. Remarkably, the number of uniquely regulated genes was highest in the combined treatment at both time points, indicating that exposure to multiple stresses triggers genes that are not responsive if only individual stresses are applied.

3.1.3 Assessment of stress-responsive metabolic pathways

We assigned Gene Ontology (GO) terms (Ashburner *et al.*, 2000; Aleksander *et al.*, 2023) to DEGs to characterize their functions and gain insights into processes regulated by water deficit, high salinity and a combination of both. Analysis of GO term enrichment revealed 63 GO terms enriched after 6 h of treatment, from which half the terms were specific to one treatment while the other half was shared between at least two treatments.

The combinatorial treatment showed the highest number of uniquely enriched GO terms and shared a substantial number of GO terms that were also enriched in either one of the single treatments in response to short-term stress exposure. Enriched GO terms, shared between combined and salt stress treatment included several catalytic activities and metabolic processes. Terms shared between combined and water deficit treatment were mainly involved in oxidative stress response. GO terms that were enriched independently of stress-type were related to transcriptional regulation, such as ‘regulation of gene expression’ and ‘transcription factor activity’.

The majority of enriched GO terms identified after 24 h were shared between water deficit and combined treatments or between all treatments. Interestingly, no terms were shared between high salinity and the combined treatment exclusively. Commonly enriched terms were mostly related to oxidative stress responses and regulation of transcription. Since regulation of gene expression via transcription factor activity was highly enriched at both time points, we examined the distribution of transcription factors within our dataset more closely.

3.1.4 Prevalence of transcription factor families

We employed the Plant Transcription Factor Database (Jin *et al.*, 2017) to identify all transcription factors within the RNA-seq dataset and counted them family-wise. Next, we compared the prevalence of transcription factor families within the DEG sets against the distribution in all active genes and calculated overrepresentation scores. In total, we identified 12 families enriched after

6 h and 18 families enriched after 24 h. Among these enriched families, bHLH, ERF and HSF transcription factors were overrepresented at both time points in response to all treatments. Interestingly, while some families were specifically overrepresented in response to salt treatment and combined treatment at 24 h, this was not the case for water deficit.

These results are described in detail in the following publication:

"Transcriptomic reprogramming of barley seminal roots by combined water deficit and salt stress"

Alina Osthoff, Petra Donnà Dalle Rose, Jutta Baldauf, Hans-Peter Piepho and Frank Hochholdinger
BMC Genomics 2019, **20**: 325
DOI: https://doi.org/10.1186/s12864-019-5634-0

3.2 Analysis of transcriptomic plasticity of barley seedlings subjected to water deficit at the root zone level

The plant root is the first organ to perceive water deficit and initiates a multitude of response mechanisms to ensure survival. While whole-root approaches already examined many of these mechanisms, the individual responses in each one of the longitudinal root zones remain largely unknown. To gain a comprehensive overview of the spatial dynamics shaping water deficit responses, we examined the transcriptome of young barley seedling roots subjected to moderate water deficit (PEG8000, -0.8 MPa) at the level of longitudinal root zones

3.2.1 Phenotypic assessment of water deficit

We grew barley seedlings in custom-built phenotyping boxes that facilitated measurement without disturbing the roots and measured the length of the three longest seminal roots per plant for seven days and calculated the average root length for each individual. Plants were either watered with a nutrient solution (control group) or with a nutrient solution containing PEG8000 (-0.8 MPA, water deficit). Pairwise comparisons revealed that the root growth rate was negatively affected by water deficit conditions after two days, resulting in a length reduction of 15 to 20% between days three and seven compared to the control group.

3.2.2 Spatiotemporal plasticity of the seminal root transcriptome upon water deficit

We subjected barley seedlings to water deficit and sampled seminal roots of the treatment and the control group after 6 h, 24 h and 48 h, to examine transcriptional changes, before phenotypical alterations became apparent. The roots were divided into their three longitudinal root zones: root cap and meristem, elongation zone and differentiation zone and subsequently sequenced. Then, we quality-trimmed the obtained raw reads and used a pseudo alignment approach to quantify gene model abundance. A spatial arrangement of samples showed, that the factor root zone explained 58% of the overall variance.

We calculated differential expression changes between control and water deficit samples for each time point by root zone combination individually, to focus on the treatment effect rather than the effect introduced by differences in root zones. Unexpectedly, the number of DEGs did not increase with prolonged exposure to water deficit. Instead, we found the highest number of DEGs at 6 h and the lowest at 24 h of treatment. A comparison of DEG sets reveals that a large proportion of them were unique to their treatment-by-time point by root zone combination, though we observed an overlap of DEGs between 6 h and 48 h of treatment across root zones and some conserved DEGs within each zone over different time points.

3.2.3 Enrichment analysis of metabolic pathways among differentially expressed genes

We utilized the Gene Ontology Knowledgebase (Ashburner *et al.*, 2000; Aleksander *et al.*, 2023) to assign molecular functions and biological pathways to identified DEGs and performed enrichment analysis to detect modulated water deficit responses.

Analysis of GO terms enrichment after 6 h of water deficit revealed a high number of enriched terms that were shared between at least two root zones or even commonly conserved across all zones. Many commonly enriched terms were associated with 'transmembrane transport' and stress response, such as 'oxidoreductase activity', 'heme binding', 'response to oxidative stress', or 'cellular oxidant detoxification'. Moreover, we identified several cell wall-related GO terms enriched in at least two root zones with contrasting directions of regulation, suggesting zone-specific roles within the water deficit response. After 24 h, the number of enriched GO terms was comparably lower, with many terms shared between the elongation and the differentiation zone. Those also encompassed the before mentioned stress-responsive terms. We identified 'carbohydrate metabolic process' and 'heme binding' as the only commonly enriched terms. Interestingly, these terms were also the only common terms identified after 48 h of treatment, though the total number of enriched terms was higher compared to 24 h. Among the terms shared between two root zones, we also found the stress-responsive terms and 'transmembrane transport'.

Based on these observations we suggest a root zone and time-dependent response in barley roots under water deficit. The number of GO terms, that were enriched in response to the different treatment-by-time combinations, further highlighted the complexity of the response mechanisms.

3.2.4 Identification of weighted gene co-expression network analysis modules correlated with water deficit

We utilized a weighted gene co-expression network analysis (WGCNA) approach to gain a systems-level understanding of gene expression patterns in response to water deficit. Since we were mainly interested in the treatment effect, we constructed each network separately for all three root zones.

We identified 21 co-expression modules in the data obtained from the root cap and meristem and correlated them to the water deficit traits. Next, we selected the modules with the highest positive correlation coefficient for each time point and analyzed those more in detail. A comparison of their module eigengene expressions between control and water deficit samples suggested a triggered response to the water deficit treatment. We observed a strong correlation ($r > 0.5$) of module membership against gene significance in those selected modules which further indicates that genes within the modules are likely associated with water deficit treatment. In the

elongation zone data, we found 23 co-expression modules in total. The three selected modules displayed the same eigengene expression patterns that we already observed in the root cap and meristem, while the correlation between module membership and gene significance was even higher (r> 0.6). Co-expression analysis in the differentiation zone revealed also 23 modules with the previously observed triggered water deficit response in the selected modules based on their treatment correlation coefficients. Notably, the observed correlations between module membership and gene significance varied strongly between these modules and ranged from r = 0.31 to r = 0.8.

3.2.5 Differential hub gene analysis highlights the modulation of the energy metabolism

We identified genes with a module membership and gene significance value >0.8 in each of the selected modules. Since these genes are highly connected within their respective module and strongly associated with the water deficit trait, we used them as hub genes for further analyses.

We compared the list of hub genes with a list of differentially expressed genes from the previous analysis to identify differential hub genes that were consistently associated with water deficit and characterized them according to GO categories. Interestingly, the number of such differential hub genes was considerably lower in all differentiation zone modules.

Enriched GO terms in differential hub genes identified at 6 h in the root cap and meristem and the elongation zone were associated with a variety of different processes and functions. These included cell wall, stress response and transport-related terms, mainly in the root cap and meristem and various metabolic and regulatory processes in the elongation zone. Later time points showed a predominant involvement of energy metabolism-related terms in all three root zones, suggesting a modulation of carbohydrate and sugar metabolic processes to meet the energy demand under prolonged water deficit exposure.

These results are described in detail in the following publication:

"Spatiotemporal transcriptomic plasticity in barley roots: Unravelling water deficit responses in distinct root zones"

Alina Klaus, Caroline Marcon and Frank Hochholdinger

BMC Genomics, 2024, **25**:79

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4 Discussion

Agricultural production faces a significant challenge as the occurrence and severity of abiotic stresses intensify due to global warming (Naumann *et al.*, 2021). Particularly water deficit and high salinity are major threats affecting barley growth and yield (FAO, 2021b, 2021a). Even individual occurrences have detrimental effects on plants, leading to reduced growth, reduced photosynthetic activity and ultimately yield loss. However, when plants are exposed to both stresses simultaneously, these effects are amplified, resulting in growth arrest and possible death (Mittler, 2006).

The root is the first organ that encounters water deficit and soil salinity, by perceiving changes in ion concentration and water potential, making the root the ideal organ to study early stress responses. Roots can be separated along their longitudinal axis into three developmental zones with undifferentiated, dividing cells in the root meristem at the tip, elongating cells in the middle and mature cells at the basal part (Ishikawa and Evans, 1995). Though the sensors for water deficit and salinity are presumably only localized in the meristematic zone, the whole root is involved in the signaling cascade (Takahashi and Shinozaki, 2019; Hartmann *et al.*, 2021).

To ensure food safety, it is crucial to better understand the underlying mechanisms that shape the responses of plants to abiotic stresses with a special focus on combinatorial stress and root zone-specific responses. To this end, we firstly subjected barley seedlings to moderate water deficit (- 0.8 MPa), high salinity (150 mM NaCl) and a combination of both and examined whole-root responses and transcriptomic changes in the cultivar Scarlett and secondly performed a root zone-wise examination of transcriptomic reprogramming under moderate water deficit (- 0.8 MPa) in the reference cultivar Morex. Based on our phenotypic findings, we chose to examine changes in the transcriptomic landscape in barley roots before visible physiological effects were detectable. Thus, we aimed to capture initial stress responses that shape plant responses to water deficit and salinity already after 6 h, 24 h and 48 h of exposure.

4.1 Individual and combined water deficit and salinity negatively affect root growth

Under water deficit conditions and high salinity, plants conserve carbohydrates by decreasing the growth rates of above and below-ground organs. During the early phase, shoot growth is strongly decreased while roots continue to elongate albeit at slower rates, to access deeper water resources (Bartels and Sunkar, 2005; Bogeat-Triboulot *et al.*, 2007).

In our study, exposure of barley seedlings to moderate water deficit caused a significant reduction in root length after three days of treatment in Morex and after five days in Scarlett. This

visible reduction was preceded by a decrease in the average root growth rate from 3.8 cm*day^{-1} to 3.3 cm*day^{-1} on the second and 2.3 cm*day^{-1} on the third day of treatment in Morex. However, growth rates became more similar between control and water deficit conditions after five days of treatment, comparable to observations of Boudiar *et al.* (2020), indicating a temporal dynamic response of root growth rate regulation, depending on stress duration.

Treatment with high salinity did not significantly decrease root length, though a negative effect was visible, indicating a higher salt tolerance in the cultivar Scarlett. While a reduced root elongation in Arabidopsis and a salt-susceptible barley landrace under salt stress was caused by inhibition of cell production and shorter meristems, a salt-tolerant cultivar did not show decreased meristem length, but a reduction in the cell elongation rate (Shelden *et al.*, 2016). This comparatively higher salinity tolerance could be attributed to a more efficient Na^+ exclusion mechanism, higher levels of osmoprotectants and selective uptake of K^+ over Na^+ from the soil (Munns, 2002; Shelden and Roessner, 2013; Yousefirad *et al.*, 2020; Shelden *et al.*, 2020).

The combination of water deficit and salinity significantly decreased the root length already after four days compared to control plants and salt-stressed plants, indicating that the severity of the stress combination is mainly caused by water-deficit effects, that are enhanced by co-occurrence of high salinity. Similar results were obtained in a large-scale study on maize and potatoes, where a decrease in leaf area index, chlorophyll content and photosynthetically absorbed radiation was comparable between a drought and a drought-salt-combination treatment, but was less severe in salt-stressed plants (Wen *et al.*, 2022).

4.2 Modulation of differential gene expression under individual and combined water deficit and salinity

4.2.1 Transcriptomic divergence is driven by root zone, stress duration and stress type

We performed RNA-seq to characterize responses to different abiotic stresses and durations in a whole-root and a root zone-specific approach. Examination of sample relationships in an MDS plot revealed, that mainly stress duration, followed by stress type explained gene expression variations in the whole-root approach as we observed a clear distinction between all libraries from 6 h and 24 h treatments, while separation based on stress type was not as clear. Other studies also highlighted the dominant effect of treatment duration over other factors, explaining up to 40% of the observed variance when exploring differences in gene expression (Opitz *et al.*, 2014; Liang *et al.*, 2021). Interestingly, libraries from 24 h water deficit and combined stress clustered closely together with a clear distinction from salt-stressed samples, suggesting that water deficit mechanisms also shape the responses to combined stress exposure, as already

indicated in the observed root growth responses. This spatial arrangement was also observed by Mahalingam *et al.* (2022) in a multifactorial stress approach with drought and heat stress in barley, where they not only showed a clear separation between samples drawn from 1 day and 5 days of treatment but also found that combined stress and drought stress samples formed a cluster, distinct from single heat stress samples.

However, the effect of time on the observed transcriptomic divergence was almost undetectable in our second approach. Instead, 58% of the variance was explained by the factor root zone. All samples showed a clear separation, mirroring the distribution of the root zones along the root axis. This was in line with findings in transcriptomic studies conducted in barley and papaya roots and leaves and maize primary roots (Opitz *et al.*, 2016; Hill *et al.*, 2016; Gamboa-Tuz *et al.*, 2018; Kreszies *et al.*, 2019; Nefissi Ouertani *et al.*, 2021; Wei *et al.*, 2022). This predominant effect of root zone or tissue over all other examined factors, like duration and treatment, emphasizes the importance of zone-specific approaches to unmask distinct mechanisms and responses that would be overlooked in whole-root approaches.

4.2.2 The extent of transcriptomic adjustments is controlled by multiple factors

Identification of differentially expressed genes (DEGs) is a powerful tool to examine transcriptomic adjustments in response to external stimuli. To this end, we computed pairwise comparisons between control and treated samples in two separate approaches. First, we explored the effect of water deficit and salinity on the whole-root transcriptome after 6 h and 24 h of treatment separately. Second, we investigated the effect of water deficit on the root zone-specific transcriptomes at 6 h, 24 h and 48 h of treatment.

Various studies in maize, pearl millet and barley under water deficit showed, that prolonged exposure increased the number of DEGs (Opitz *et al.*, 2014; Ji *et al.*, 2021; Mahalingam *et al.*, 2022). This is in line with our observations at the whole-root transcriptome level, where 24 h of treatment drastically increased the number of DEGs independent of stress type in comparison to 6 h. Moreover, combined stress resulted in the highest number of stress-responsive genes, suggesting the activation of additional mechanisms to counteract negative effects. This possible link between stress complexity and increased gene responsiveness was also detected in grapevine under drought and heat stress and in Arabidopsis under salinity, drought and heat (Shaar-Moshe *et al.*, 2017; Tan *et al.*, 2023).

In contrast, prolonged stress exposure did not increase the number of DEGs identified at the root zone level. Instead, we observed drastic changes in gene expression after 6 h of treatment, compared to a relatively stable phase at 24 h, followed by another more dynamic phase after 48 h of water deficit. This inconsistent response was also detected in a heat stress experiment in pearl

millet and in drought-stressed cotton, where initial responses were accompanied by a higher number of DEGs compared to later time points (Huang *et al.*, 2021; L Chen *et al.*, 2022). Moreover, Liang *et al.* (2021) showed that the timing and extent of gene modulation could be linked to drought tolerance in rice. They observed a stronger response towards drought exposure at an earlier time point compared to a later time point in a tolerant genotype, while the susceptible genotype exhibited a later and more extensive overreactive response, causing an additional disturbance in plant homeostasis. Considering the different genotypes that we used in our study, the detected variations in water deficit responses may be caused by this genetic variation. Based on previous studies, Morex, the cultivar used in the zone-specific study, is considered moderately drought-tolerant compared to the drought-susceptible cultivar Scarlett, which we used in the whole-root approach (Khalili *et al.*, 2016; Gol *et al.*, 2021).

The extent of transcriptomic adjustments was not evenly distributed among the different root zones. While the number of DEGs was considerably lower in the root cap and meristem, we identified substantial numbers in the differentiation zone and the highest numbers in the elongation zone, independent of time point, indicating a strong spatial response pattern. These spatial differences were also identified in wheat and maize roots under water deficit, with more stress-responsive genes located in the elongation zone (Opitz *et al.*, 2016; Wang *et al.*, 2021). Thus, the elongation zone appears to be of particular importance for mediating water deficit responses (Sharp *et al.*, 2004).

4.2.3 Modulation of distinct gene sets upon abiotic stress is spatiotemporal and treatment-specific

We compared sets of DEGs identified in both approaches to find commonly and uniquely regulated genes and observed an overlap of 60% (salt stress) to 80% (combined stress) of DEGs between 6 h and 24 h of treatment in the whole-root approach. This high level of conservation over time was also detected in tomato roots under hypoxia and maize roots under water deficit treatment (Opitz *et al.*, 2014; Safavi-Rizi *et al.*, 2020), suggesting that initial responses, triggered already after short-term treatment, are still affecting response mechanisms after longer exposure. However, root zone-wise comparisons across time points did not mirror this level of conservation, though sets from the more dynamic phases at 6 h and 48 h shared a substantial number of DEGs in the elongation and differentiation zone. This time point specificity was in line with findings from Liu *et al.* (2021) in maize and P Chen *et al.* (2022) in cotton, indicating distinct phases of water deficit responses, involving unique sets of DEGs for each phase.

While the majority of DEGs were unique to their root zone-by-time point combination, we still found some shared genes across different root zones, mainly between elongation and

differentiation zones after 6 h of treatment. In contrast, Kreszies *et al.* (2019) found a larger overlap between drought-responsive DEGs of the meristem and differentiation zone in barley under water deficit. However, while the experimental setup was comparable to the one applied in our study, their differentiation between the root zones was based on proportions of whole-root length, omitting transition zones. Since we separated zones based on measurements conducted by Kirschner *et al.* (2017) and included also transition zones, differences in zone-specificity may be explained by the experimental approaches. The small overlap with DEGs identified after 24 h of treatment, might be a result of the overall low number of stress-responsive genes.

When examining the degree of conservation between different stress types, we found little overlap between water deficit, salinity and their combination at 6 h. Instead, exposure to combined stress uniquely regulated a set of over 3,000 DEGs, making up 65% of all genes identified as differentially expressed after 6 h. This amount represents more than the number of DEGs from both individual stresses combined, implying a strong non-additive effect of stress combination. Our observation aligns with studies in melon, Arabidopsis and tobacco, subjected to various combinations of biotic and abiotic stresses, that also showed unique and non-additive responses towards multifactored stress scenarios (Rizhsky *et al.*, 2002; Villalba-Bermell *et al.*, 2021; Zandalinas *et al.*, 2021). After 24 h, we still observed a large proportion of genes unique to the combination treatment, though we also found a considerable overlap between water deficit and the stress combination. This might be due to the predominant effect of water deficit over salinity in the acclimation of barley to co-occurring stresses. Similar findings were reported in Arabidopsis subjected to a combination of salinity and heat and tomato exposed to drought and heat stress. In both studies, the response to one factor dominated the response to the stress combination (Suzuki *et al.*, 2016; Zhou *et al.*, 2017).

Taken together, our results indicate that water deficit, salinity, or a combination trigger dynamic, specific and complex spatiotemporal response mechanisms, suggesting a high plasticity of the barley root transcriptome towards stress.

4.3 Abiotic stress-induced alterations of ABA-dependent and independent pathways and processes

4.3.1 Peroxidases are universal targets for modulation under abiotic stress

To provide a more comprehensive understanding of the alterations caused by abiotic stress treatments and to add a more sophisticated biological context to identified DEGs, we performed Gene Ontology (GO) enrichment. We identified various significantly enriched biological processes and molecular functions that are shared among DEGs.

Oxidative stress is a common response to all kinds of abiotic and biotic stresses, caused by a disequilibrium between ROS production and ROS scavenging (Huang *et al.*, 2019). To restore homeostasis and counter negative effects, plants employ antioxidants, like oxidoreductases, peroxidases, glutathione S-transferases and reductases, or catalases (Singhal *et al.*, 2015; He *et al.*, 2017). Their universal importance is reflected by the enrichment of GO terms covering 'oxidoreductase activity', 'peroxidase activity', 'response to oxidative stress' and 'antioxidant activity', observed in response to almost all treatment-by-time combinations in our whole-root study. These redox-related GO terms were also enriched in all root zones after 6 h and in the elongation and differentiation zone at later time points. Moreover, differential hub genes, identified by integrating DEG analysis and WGCNA also revealed these stress-responsive terms as significantly enriched, with peroxidases as the most prominent differentially expressed hub genes.

Peroxidases play a crucial role in the detoxification of ROS, like hydrogen peroxides, or singlet oxygen (Caverzan *et al.*, 2012). At lower concentrations, ROS may function as signaling molecules in stress acclimation, while higher concentrations can lead to cell death, underlining the complexity of ROS responses under abiotic stress (Tamás *et al.*, 2010). In our study, we observed a non-uniform regulation of differentially expressed peroxidases. While the majority was up-regulated in the meristem and down-regulated in the differentiation zone, we found that both up and down-regulation were observed in the elongation zone. This inconsistent directional regulation is in line with findings in wheat exposed to osmotic stress, though here only a few selected peroxidases were examined with regard to genotypes differing in their drought tolerance (Csiszár *et al.*, 2012). Studies in Arabidopsis showed that the accumulation of singlet oxygen in the root meristem and hydrogen peroxide in the elongation zone is crucial for cell division and elongation, respectively (Tsukagoshi *et al.*, 2010). Imbalance between these ROS due to abiotic stress, can disrupt the transition between these two processes and impair root growth (Huang *et al.*, 2019). Considering this dynamic balance, the varying regulation of peroxidases, observed in our study, may reflect this mechanism to restore ROS balance.

4.3.2 Alteration of transport-related genes is treatment-specific

Plants maintain cellular homeostasis by active and passive transport of ions and molecules across the cell membrane. These channels, carriers and transporters are common targets of modulation upon exposure to abiotic stress (Osakabe *et al.*, 2014; Jarzyniak and Jasiński, 2014).

At the root zone level, we found several significantly enriched GO terms related to the transport system across all root zones and time points in response to water deficit. These included 'transmembrane transport', 'metal ion transport', 'channel activity' and 'transmembrane

transporter activity'. Transport-associated GO terms were also identified among differential hub genes in the root cap and meristem at 6 h, highlighting the importance of transport system modulation in the early phases of water deficit responses to relocate substrates and transmit signals (Mohammadi *et al.*, 2008; Osakabe *et al.*, 2014). While we also identified 'transporter activity' and 'transport' as enriched GO terms in our whole-root approach in response to short and long-term water deficit and long-term combination treatment, salinity did not show this response.

During salinity, plants activate the Salt Overly Sensitive pathway to exclude excess Na^+ from cells via a Na^+/H^+ antiporter, which showed no salt-specific regulation (Lamers *et al.*, 2020; Wang *et al.*, 2022). However, we observed the up-regulation of a high-affinity K^+ transporter, facilitating the translocation of Na^+ into leaves (Mian *et al.*, 2011). This efficient compartmentalization was identified as a key mechanism in salt tolerant lines and may explain the marginal changes of salinity on root growth in our study (Han *et al.*, 2018; Gharaghanipor *et al.*, 2022).

Water deficit decreases the availability of water from the soil, which in turn affects the expression and activity of aquaporins. Aquaporins are a large group of major intrinsic proteins, that transport water, metal ions and gases across membranes, allowing roots a rapid alteration of water permeability in response to environmental stimuli (Javot and Maurel, 2002). We found aquaporins mainly down-regulated in the elongation and differentiation zone at all time points and up-regulated in the meristem after 6 h of treatment, among them several differential hub genes. This bi-directional regulation was in line with other studies in barley and Arabidopsis under water deficit (Alexandersson *et al.*, 2005; Kurowska *et al.*, 2019). While a down-regulation of aquaporins could be beneficial to prevent water loss, up-regulation in dividing tissues, like the meristem, could be important to maintain cell turgor and stability (Knipfer *et al.*, 2011). Furthermore, many aquaporins were differentially expressed in the water deficit and the combined treatment, but not in the salinity treatment after 24 h of water deficit treatment at the whole-root level. This insensitivity to modulation by salt stress compared to water deficit was also observed by Jang *et al.* (2004) in Arabidopsis under abiotic stresses. Though many studies examined the effect of abiotic stress on the expression of aquaporins, their regulation remains quite inconclusive and strongly depended on stress type, severity, duration and species (Molina *et al.*, 2008; Yepes-Molina *et al.*, 2020).

The transport of ABA, a phytohormone well known to play a crucial role in mediating and coordinating plant stress responses involves various transporters (Tuteja, 2007; G Chen *et al.*, 2022; Muhammad Aslam *et al.*, 2022). Of these, ATP-binding cassette (ABC) transporters and low-affinity nitrate transporter /peptide transporters (NRT1/PTR) were differentially regulated mainly under water deficit and combined stress treatment. While ABA signaling is also involved in salt stress responses by modulating Ca^{2+} to induce the SOS pathway or regulating the

metabolism of antioxidants, we could only identify a few ABA transporter genes among salt stress DEGs (Gong *et al.*, 2004). This is in contrast to findings by Nefissi Ouertani *et al.* (2021) who identified various differentially expressed ABA transporters in salt-stressed barley roots after short (2 h) and long-term (24 h) treatment.

4.3.3 Protein kinases commonly regulate stress responses via ABA

Reversible protein phosphorylation is a key mechanism in regulating plant responses to stress. This posttranslational modification targets already present proteins and thus offers rapid reactions to all kinds of stimuli. Protein kinases catalyze the transfers of a phosphoryl group from ATP to target proteins by forming an ester bond with serine, threonine, or tyrosine residues. The counter-reaction is catalyzed by phosphatases (Damaris and Yang, 2021).

'Protein phosphorylation' was one of the largest enriched GO terms in the elongation and differentiation zone upon water deficit and was also significantly enriched in all treatments in the whole-root approach. Additionally, related terms covering phosphorylation and dephosphorylation activities were also identified in differential hub genes. Genes associated with these terms were unspecific protein kinases or family proteins, but also serine/threonine protein kinases, mitogen-activated protein kinases (MAPK), calcium-dependent protein kinases (CDPK) and receptor-like kinases (RLK). Most of the differentially expressed protein kinases were down-regulated in both approaches, which was consistent with findings in jute plants under PEG treatment and rice under drought, suggesting that many protein kinases act as negative regulators within the water deficit response complex (Wu *et al.*, 2015; Z Yang *et al.*, 2017). In contrast, the up-regulation of protein kinases particularly in the elongation zone and in the whole-root after long-term salt treatment might be due to the involvement of specific RLKs in cell elongation, which could be linked to the superior root growth of salt-stressed plants compared to the other treatments (Zhu *et al.*, 2023).

Furthermore, the ABA pathway is strongly regulated by protein kinases and phosphatases. Depending on their subclass, protein phosphatase 2C family proteins (PP2C) are known to regulate ABA signaling via SnRK2, act as negative regulators in the MAPK pathway, or promote stem-cell identity in meristems (Hirayama and Umezawa, 2010; Fuchs *et al.*, 2013). Under stress conditions, increased ABA levels lead to the inactivation of PP2Cs (Park *et al.*, 2009). In total, we identified over 40 different stress-responsive PP2Cs in our whole root approach and 18 PP2Cs in the root zone-specific approach with two of them also found in the hub genes of the elongation zone. This was in line with findings from other stress-related studies and further highlights the key role of ABA signaling and its feedback mechanisms in plant stress responses (Ahmed *et al.*, 2013; Opitz *et al.*, 2014; Ji *et al.*, 2021; Nefissi Ouertani *et al.*, 2021; Wu *et al.*, 2023).

4.3.4 Water deficit alters the energy metabolism in a time-dependent manner

Protein modifications, like most other biological processes, are energy-consuming and require ATP, the main energy source in cells. ATP is produced via photophosphorylation in chloroplasts and in the absence of light through the breakdown of carbohydrates in the respiratory metabolism (Allen, 2002; Fernie *et al.*, 2004). The activity of involved enzymes shows a high variability between tissues, and developmental stages but also in response to abiotic stress (Millar *et al.*, 2011; Araújo *et al.*, 2012).

Accordingly, we identified many genes with relation to energy metabolism as differentially regulated in both transcriptomic approaches, indicating that adjustments in the energy metabolism are important targets for stress acclimation. GO term analysis in the whole-root approach revealed more general terms like 'carbohydrate metabolic process', or 'ATP binding' which involved mainly protein kinases of various kinds. In contrast, differential hub genes showed a strong prevalence for more specific energy metabolism-related processes.

We identified proteolysis as a key term in the meristem after 6 h of water deficit. Proteolysis, the degradation of proteins into peptides or amino acids by proteases, plays an important role in controlling many cellular processes and functions. Under water deficit, increased ROS levels may damage proteins and lipids, which are in turn degraded by proteases to remobilize amino acids as a source of nitrogen (D'Ippólito *et al.*, 2021). Studies in rice suggested that up-regulation of proteases in roots leads to an increase in nutrient mobilization and thus enhanced drought tolerance, whereby an increase in wheat leaves was considered as a marker for susceptibility (Kohli *et al.*, 2012). Moreover, protease-driven oxidation of amino acids may also contribute significantly to the energy status of plants under stress conditions (Hildebrandt *et al.*, 2015). While the involvement of proteases in plant stress response is well established, the underlying mechanisms and functions, especially in roots remain a subject of research. The observed unique regulation suggests that up-regulation of proteases provides either a rapid energy or nitrogen resource during the initial phase of water deficit to maintain meristematic activity until alternative resource-providing mechanisms are mobilized.

These alternative mechanisms encompass carbohydrate and sugar catabolic and metabolic processes, that provide energy and carbon during stress conditions. Glycolysis generates ATP by oxidizing glucose in a series of enzymatic reactions (Plaxton, 1996). The first enzyme, pyrophosphate-fructose 6-phosphate 1-phosphotransferase (PFP), was a central differential hub gene in the elongation and differentiation zone, while the second enzyme glucose-6-phosphate isomerase (GPI), was identified in differential hub genes in the meristem and elongation (Chen *et al.*, 2020). The involvement of both enzymes in the modulation of carbohydrate metabolic

processes during water deficit adaptation was also observed in drought-tolerant ryegrass and transgenic Arabidopsis(Lim *et al.*, 2014; Pan *et al.*, 2016).

4.3.5 Cell wall reorganization is a key process in abiotic stress response

The plant cell wall is a complex structure composed of cellulose, hemicellulose and pectin, though the composition depends on cell type (Alberts *et al.*, 2002). It provides structural integrity, acts as a barrier against external stresses and enables cell-to-cell-transport and communication via receptors, pores and channels (Brett and Waldron, 1990; Zambryski, 2004; Hamann, 2012). Upon exposure to environmental stress, the cell wall undergoes extensive remodeling to enable differential growth responses, like maintenance of root growth and reduced shoot growth (Tenhaken, 2014).

The importance of cell wall-related processes was evident in both transcriptomic approaches, as seen in the GO enrichment and supported by a large network of differential hub genes in the meristem after 6 h of water deficit.

Xyloglucan endotransglucosylase/hydrolases (XTHs), cell wall modifying enzymes, were the most abundant. They can change the extensibility of cell walls by hydrolyzing or transferring xyloglucans and show high responsiveness to various abiotic stimuli (Xu *et al.*, 1995; Xu *et al.*, 1996; Cho *et al.*, 2006; Cheng *et al.*, 2021). The expression of XTHs in transgenic Arabidopsis conferred enhanced drought and salinity tolerance by altering cell growth and root architecture, supporting their role in abiotic stress response acclimation (Cho *et al.*, 2006; Han *et al.*, 2023). In our study, the direction of XTH regulation was time, treatment and root zone-dependent and thus strongly varied between different combinations. This undirected, time and tissue-specific regulation was also observed in wheat roots and shoots (Han *et al.*, 2023). In soybean seedlings, XTHs activity in the shoot elongation zone was decreased, resulting in growth inhibition due to increased inflexibility of cell walls (Wu *et al.*, 2005). Taken together, this suggests specific modulation targets for different XTHs within the cell wall, depending on stress type, tissue and duration.

Another important cell wall-related enzyme is cellulose synthase (CesA). It produces cellulose, the most frequent macromolecule in the cell wall, which is the main load-bearing component, providing structural support and is considered a major carbon sink (Richmond, 2000; Delmer and Haigler, 2002). We observed contrasting responses, with increased CesA expression after 6 h and 48 h in the meristem and elongation zone, but decreased activity in the differentiation zone and in response to all treatments in the whole-root approach. Spatial differences along the root axis were also reported by Kozlova *et al.* (2020), though gene read counts and protein abundance also varied between individual genes. Several studies suggest the regulation of CesA activity by abiotic

factors to dynamically balance cell expansion and resource usage at lower photosynthetic efficiency, explaining the diverging modulations we observed in our studies (Wang *et al.*, 2016).

Expansins, likewise involved in cell wall loosening and extension, were also identified as DEGs in all root zones and treatments in both experiments. Their expression was increased in response to salt treatment and in the meristem of water deficit roots but mainly decreased in all other treatments or root zones. The superior performance of salt-stressed plants and the observed up-regulation of expansins are in line with findings in transgenic rice, where overexpression of Expansin7 resulted in enhanced tolerance against salinity by specifically coordinating accumulation of K^+ over Na^+, increased antioxidant activity and enhanced elongation of metaxylem root cells (Jadamba *et al.*, 2020). Samalova *et al.* (2023) showed that in Arabidopsis roots the expansin *EXPA1* was primarily expressed in the root cap and the meristem, while *EXPA10* showed stronger expression in the transition and elongation zone, indicating that the expression of different expansins followed a spatial distribution, providing a context for the observed differences between root zones in our study.

4.3.6 Transcription factor activity shows universal and treatment-specific modulation

Transcription factors are master regulators of gene expression, closely interacting with plant hormones to orchestrate plant development and responses to different stimuli by binding to specific DNA sites (Strader *et al.*, 2022).

The conserved up-regulation of GO terms related to transcription factors in our whole root approach, independent of stress type and duration, further reflects this universal involvement of transcription factors in plant stress responses. Although we identified over 300 regulated transcription factors at the root zone level, analysis of GO terms only showed the enrichment of ‘sequence-specific DNA binding’ in the meristem after 6 h of water deficit, but no enrichment in other root zone-by-time point combinations. This is in contrast to findings by Opitz *et al.* (2016), where ‘transcription factor activity’ was significantly overrepresented in all root zones in maize seedlings under water deficit. A closer examination of all differentially expressed transcription factors revealed, that the prevalence of each family was not consistently universal, but also dependent on treatment, root zone and time point.

The plant hormone ABA and its associated transcription factors (AREB/ABF) play a crucial role in mediating gene expression changes to ensure plant survival under stress. AREB /ABFs belong to the family of bZIP transcription factors, which are known for their involvement in various developmental processes and stress responses (Gai *et al.*, 2020). Additionally, MYB, WRKY, HD-Zip, ERF and HSF transcription factors are also known to be at least partially regulated in an ABA-dependent manner (Hrmova and Hussain, 2021). In our whole-root approach, we found bZIP

transcription factors to be the most prevalent family, enriched in response to almost all treatment-by-time combinations. Notably, the number of differentially expressed bZIP transcription factors in response to water deficit was highest after 6 h, which was in line with our findings from the root zone-wise approach. There, bZIP transcription factors prevailed in the elongation zone, after 6 h of treatment, while almost none were identified at later time points or at all in the meristem. This temporal plasticity might be attributed to their involvement during the early phases of stress response, as they are crucial for initiating ABA-dependent mechanisms (Gai *et al.*, 2020). The high diversity within this family might also explain the observed variations in the expression of bZIP transcription factors across our treatments and root zones. Studies in Arabidopsis revealed, that regulation and effect under abiotic stress were not consistent and depended on the individual gene, reflecting the complexity of bZIP transcription factor regulation in response to abiotic stresses (Liu *et al.*, 2008; Yang *et al.*, 2009; Rolly *et al.*, 2020).

Under salt stress conditions, ethylene response factors (ERFs) emerged as the predominant transcription factor family. Additionally, ERFs were also enriched across all other treatment-by-time combinations and after 6 h and 48 h of water deficit in all root zones, suggesting an important role in mediating plant responses to water deficit and particularly salt stress. ERFs can be activated in an ABA or ethylene-dependent manner. As with bZIPs, the direction of modulation of ERFs depends on stress type and tissue (Abiri *et al.*, 2017). Transgenic rice, overexpressing *OsERF106MZ* showed strong growth inhibition in shoots with increased ROS levels and simultaneously promoted root growth when exposed to high salinity (Chen *et al.*, 2021; Chen *et al.*, 2023). This suggests, that the up-regulation of ERF transcription factors in our 6 h salt treatment might be contributing to the observed maintenance of root growth. ERFs are also known to be involved in water deficit responses.

Though HD-Zip transcription factors were overrepresented in the water deficit treatment after 6 h and in the salt treatment after 24 h, we detected only a few differentially HD-Zip transcription factors among our root zone-level DEGs, mainly in the elongation zone. As they are involved in limiting cell growth under stress conditions, their specific regulation in the elongation zone could be associated with the observed retardation of growth (Harris *et al.*, 2011). Consequently, salt stress affected root growth at later time points, supporting the regulation of these transcription factors after 24 h but not after 6 h of treatment.

The highest number of uniquely overrepresented families, including LBD, MYB, NAC and TALE, was found after 24 h of combined salt and water deficit treatment. This activation of additional transcription factor families further highlights the intense constraints that plants suffer under such combinatorial exposure.

While we identified many transcription factors among the differentially expressed genes and the hub genes in the root zone-level approach, we only found a few of them in the differential hub

gene analysis. This was mainly due to low module membership, which would infer a low correlation of gene expression profiles with module Eigengene (Langfelder and Horvath, 2008). As discussed, the expression of transcription factors was not modulated at one specific time point but rather during the whole response. Since we divided the traits by treatment duration, this could lead to a lower contribution to the module-trait relationships at single time points and thus resulted in the exclusion of many of them in the differential hub gene analysis.

4.4 Concluding remarks

This doctoral thesis aimed to assess the transcriptomic plasticity of young barley roots under abiotic stresses. We investigated the effect of water deficit, salinity and their combination on whole seminal roots, and studied the effect of water deficit in distinct root zones.

The observed phenotypical differences between the examined cultivars Scarlett and Morex in regards to root growth under water deficit conditions underline the impact of the respective genetic background. To gain a more sophisticated understanding of water stress responses, testing various cultivars with different tolerances should be considered. This would also enable researchers to find key genes or gene groups that confer enhanced tolerances.

In the future, when crops face increasing environmental constraints with various combinations, single-stress treatments are not sufficient to test for desirable breeding traits. As seen in our whole root approach, transcriptomic adjustments to such combinations are larger and unique and thus deserve more awareness in future crop breeding programs.

Likewise, the spatial resolution approach revealed diverse mechanisms in differing extents activated in response to water deficit across distinct root zones, that were not detected in the whole-root approach. To date, studies examining the effect of abiotic stresses at the root zone level are scarce and often limited to the model plant Arabidopsis. To expand our knowledge, researchers would benefit from such studies in other crops, with higher agricultural impact.

While the transcriptome analyses provided many interesting genes for future research, the actual cause-effect relationships between these genes and abiotic stress remain hypothetical and require further experimental validation. Moreover, alterations in protein activity extend beyond these detected transcriptional changes, since post-transcriptional modifications also significantly contribute to the dynamic modulation of processes.

In the face of global warming and the need to ensure food security, the breeding of resistant crops is indispensable. The data presented in this study can be accessed by the research community and it serves as an initial point for future research and breeding initiatives. This study is the first comprehensive analysis considering the combination of water deficit and salinity, offering detailed transcriptional quantification on a root zone-specific scale in young barley roots.

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6 Publications and presentations

6.1 Publication related to this thesis

6.1.1 Transcriptomic reprogramming of barley seminal roots by combined water deficit and salt stress

Alina Osthoff, Petra Donnà Dalle Rose, Jutta Baldauf, Hans-Peter Piepho and Frank Hochholdinger

BMC Genomics 2019, **20**: 325
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Own contribution: I performed and analyzed the RNA-seq experiment together with PDR and JB. I performed the phenotyping experiment, conducted all downstream analyses, interpreted the data, designed all figures and drafted the manuscript. HPP contributed to the sequencing design and supported the statistical analyses. Coordination of the study and revision of the manuscript was done by FH.

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BMC Genomics

RESEARCH ARTICLE Open Access

Transcriptomic reprogramming of barley seminal roots by combined water deficit and salt stress

Alina Osthoff[1], Petra Donà dalle Rose[1], Jutta A. Baldauf[1], Hans-Peter Piepho[2] and Frank Hochholdinger[1*]

Abstract

Background: Water deficit and soil salinity substantially influence plant growth and productivity. When occurring individually, plants often exhibit reduced growth resulting in yield losses. The simultaneous occurrence of these stresses enhances their negative effects. Unraveling the molecular mechanisms of combined abiotic stress responses is essential to secure crop productivity under unfavorable environmental conditions.

Results: This study examines the effects of water deficit, salinity and a combination of both on growth and transcriptome plasticity of barley seminal roots by RNA-Seq. Exposure to water deficit and combined stress for more than 4 days significantly reduced total seminal root length. Transcriptome sequencing demonstrated that 60 to 80% of stress type-specific gene expression responses observed 6 h after treatment were also present after 24 h of stress application. However, after 24 h of stress application, hundreds of additional genes were stress-regulated compared to the short 6 h treatment. Combined salt and water deficit stress application results in a unique transcriptomic response that cannot be predicted from individual stress responses. Enrichment analyses of gene ontology terms revealed stress type-specific adjustments of gene expression. Further, global reprogramming mediated by transcription factors and consistent over-representation of basic helix-loop-helix (bHLH) transcription factors, heat shock factors (HSF) and ethylene response factors (ERF) was observed.

Conclusion: This study reveals the complex transcriptomic responses regulating the perception and signaling of multiple abiotic stresses in barley.

Keywords: Barley, Combined stress, RNA-Seq, Salt stress, Seminal roots, Transcriptome, Water deficit

Background

Natural abiotic stresses such as water deficit and high soil salinity are major factors threatening global crop production [1, 2]. Exposure of plants to these osmotic stresses results in loss of turgor and as a consequence potential disruption of membranes and proteins accompanied by rising levels of reactive oxygen species (ROS) [3]. This, in turn, leads to growth inhibition and loss of yield [4]. While shoot growth is reduced under these stress conditions, roots continue to elongate at a slower rate to ensure survival by extracting water and nutrients from deeper soil layers [5]. Perpetuated root growth is mainly regulated by abscisic acid (ABA), which interacts with auxin, cytokinin, and ethylene in a hormonal network [6]. In addition to physiological alterations, the effects of either water deficit or salinity on gene expression patterns in roots have been studied. For instance, mature chickpea roots displayed several sets of differentially expressed genes in response to either water deficit or salinity at different developmental stages [7]. Microarray experiments in roots and leaves of three-week-old barley plants subjected to both stress conditions individually demonstrated, that the number and function of differentially expressed genes strongly depend on stress type and duration [8]. A study comparing gene expression levels in salt and osmotic-stressed barley leaves and roots came to the same conclusion [9]. While these studies surveyed the transcriptomic response to individual stress types, the simultaneous occurrence of several stress types under field conditions can lead to more severe responses [10]. Combinatorial abiotic stress application

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typically results in negative and in a few instances in positive physiological interactions between stress types [11]. For instance, a combination of salt and heat stress in Arabidopsis led to a negative effect by significantly reducing biomass and rosette diameter and lower survival rate that exceeded the decreases under single stress conditions [12]. Similarly, tobacco showed reduced respiration under water deficit, while heat shock and combined stress treatments enhanced this response [13]. In barley, plant growth and chlorophyll content reflecting the photosynthetic rate, water, and osmotic potential were reduced when subjected to either water stress, salinity or a combination of both. Yet, plants were more vulnerable to the combinatorial treatment of these stress factors [14]. In contrast, exposing tomato plants to combined heat and salinity had a positive effect leading to a significantly increased protection from the harmful effects of the individual application of salinity by accumulating trehalose and glycine betaine [15].

On the molecular level, it was demonstrated in early cDNA microarray studies in tobacco that the effects of combined water deficit and heat shock cannot be deduced by characterizing responses to single stress treatments [13]. Similarly, in Arabidopsis, the comparison of differentially regulated genes revealed a large overlap between heat and combined stress treatments but also a substantial treatment specificity [12]. In line with observations on the gene expression level, metabolic profiling of maize shoots and leaves treated by a combination of water deficit and salinity stress demonstrated that metabolic adjustments to combined stress were not additive when compared to single stress factors [16].

Barley is better adapted to abiotic stresses than other cereal species such as wheat or maize and can thus be grown in harsher environments [17]. This makes barley an ideal model plant to study abiotic stress adaptions. The usage of high molecular weight organic osmotica such as mannitol or PEG8000 (polyethylene glycol) to establish defined water potentials allows studying plant responses under controlled conditions. It was previously demonstrated that PEG8000 solution can be utilized to mimic water deficit [18–21]. Water deficit treatment of -0.8 MPa is in the mid-range of naturally occurring, plant-usable soil water potentials [22]. Similarly, NaCl concentrations of 150 mM in soil water is considered as moderate salinity and observed in many agricultural regions of the globe [23]. In the present study, we subjected 3-day-old barley seedlings to either PEG8000 solution with a water potential of -0.8 MPa to mimic water deficit, 150 mM NaCl to simulate salt stress or a combination of both. In these seedlings, we monitored root growth for eight consecutive days. Based on the results of these phenotyping experiments, we analyzed samples of seminal roots 6 h and 24 h after stress induction by RNA-Sequencing (RNA-Seq). The aim of this study was to explore the early transcriptomic reprogramming of barley seminal roots exposed to individual and combinatorial stresses at two time points. This study will provide candidate genes for further genetic analyses that might be helpful for marker-assisted barley breeding programs.

Results

Phenotypic response to abiotic stress treatments

Seedlings of the barley spring cultivar Scarlett germinated for 2 days under control conditions were subjected to water deficit (PEG8000: -0.8 MPa), salt stress (NaCl: 150 mM) or a combination of both at T_0 for 7 days (Fig. 1). To investigate the effect of the abiotic stress factors on seminal root development, total root length per treatment was determined relative to roots grown under control conditions (Fig. 1). By day four (T_4), total seminal root length of seedlings subjected to combined stress treatment was significantly shorter than that of control plants (Fig. 1). By day five (T_5), plants subjected to water deficit displayed also significantly reduced total root length relative to control plants. In contrast, although a substantial decrease in total root length was monitored in salt-stressed plants these differences were not statistically significant compared to control plants within 7 days of treatment.

Mapping of RNA-sequencing reads to the barley reference genome

We monitored global changes in the seminal root transcriptomes of young barley seedlings subjected to water deficit, high salinity and a combination of both for 6 h and 24 h. These treatments correspond to time points $T_{0.25}$ and T_1 in Fig. 1. Hence, at both time points, no morphological differences between control plants and plants subjected to the three types of stress were detectable. Total RNA from four biological replicates per treatment-by-time point combination was extracted from seminal roots, converted into cDNA and subjected to RNA-Seq. The workflow of the RNA-Seq experiment and downstream analyses are summarized in Additional file 1: Figure S1. After quality trimming, between 67 and 76% of the obtained sequences per library mapped to the barley reference genome (Additional file 2: Table S1). After removal of stacked reads, i.e. reads that share identical 5′ coordinates, orientation and length, on average 60% of the remaining reads mapped successfully in pairs to the set of 39,734 high confidence gene models of barley version IBSC v2.0 [63].

Transcriptomic relationships of RNA-Seq samples

Transcriptomic relationships between the type and duration of stress treatment were determined in a multidimensional scaling (MDS) plot (Fig. 2). Replicated samples of treatment-by-duration combinations clustered

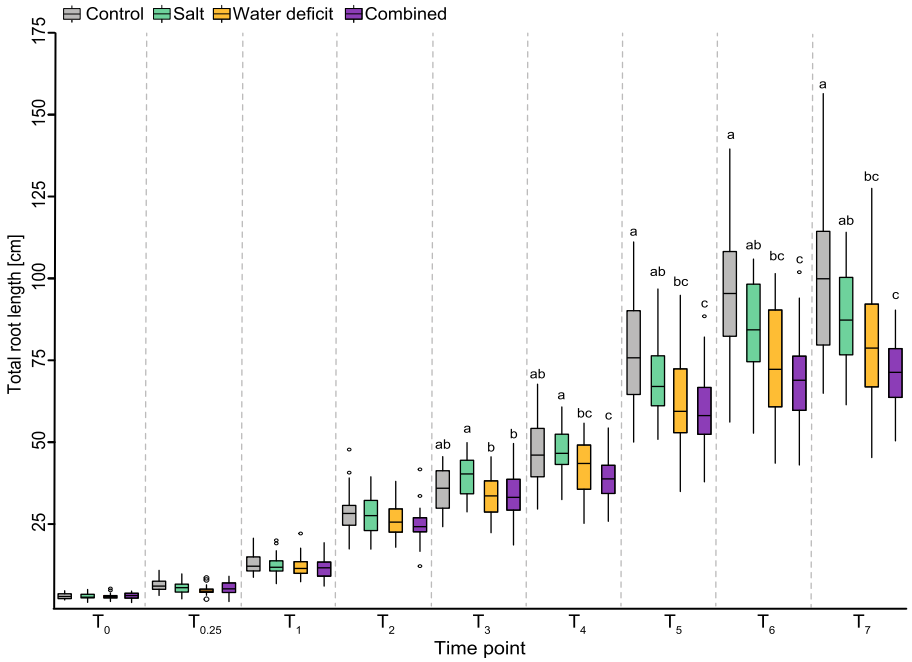

Fig. 1 Comparison of total root length between control, salt, water deficit and combined stress from stress induction (T_0) until 7 days of treatment (T_7). Significant differences ($\alpha = 0.05$) of means at each time point were calculated by ANOVA and indicated with small letters. Means not sharing any letter are significantly different

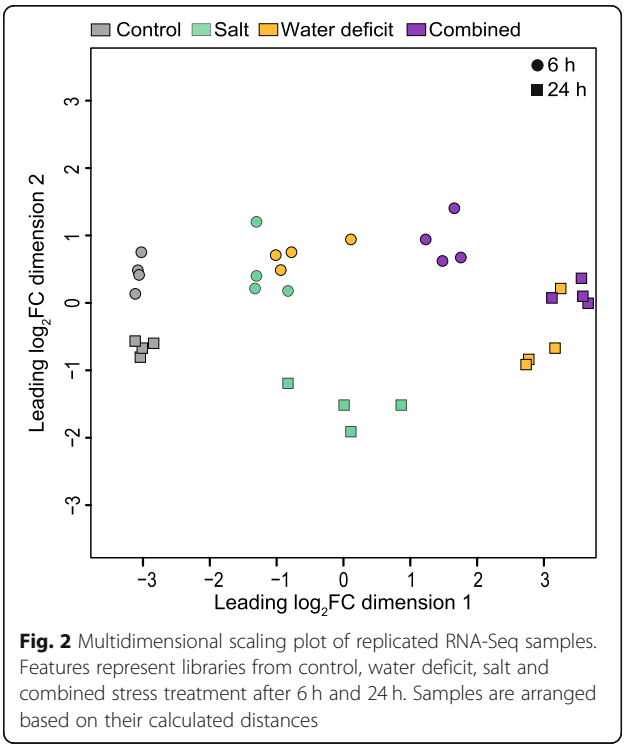

Fig. 2 Multidimensional scaling plot of replicated RNA-Seq samples. Features represent libraries from control, water deficit, salt and combined stress treatment after 6 h and 24 h. Samples are arranged based on their calculated distances

closely together. Moreover, samples subjected to short and long-term stress as well as distinct stress treatments are separable in the MDS plot, demonstrating that the observed transcriptomic divergence is driven by stress type and duration. For both stress durations, control and combined stress samples are positioned most distantly apart, while individually salt stressed and water stressed samples cluster between the control and combined treatment.

Identification of differentially expressed genes

Differentially expressed genes (DEGs) were computed in three pairwise contrasts between control and stress treatment samples for short- and long-term response. The number of DEGs (FDR ≤5% and $|\log_2 FC| > 1$) between control and stress treatment for the three treatment by two time point combinations are depicted as volcano plots (Fig. 3a). A comprehensive list of these DEGs is provided in Additional file 3: Table S2. The number of DEGs varied between treatment-by-time combinations. Under both, short and long-term stress exposure, the salt stress treatment resulted in the smallest number of DEGs (953 at 6 h and 1802 at 24 h). The most severe impact on gene expression was observed in the combined water deficit and salt treatment with 4845 DEGs at 6 h and 8105 DEGs after 24 h. After short-term treatment, the total number of genes differentially regulated in the combined treatment was substantially higher

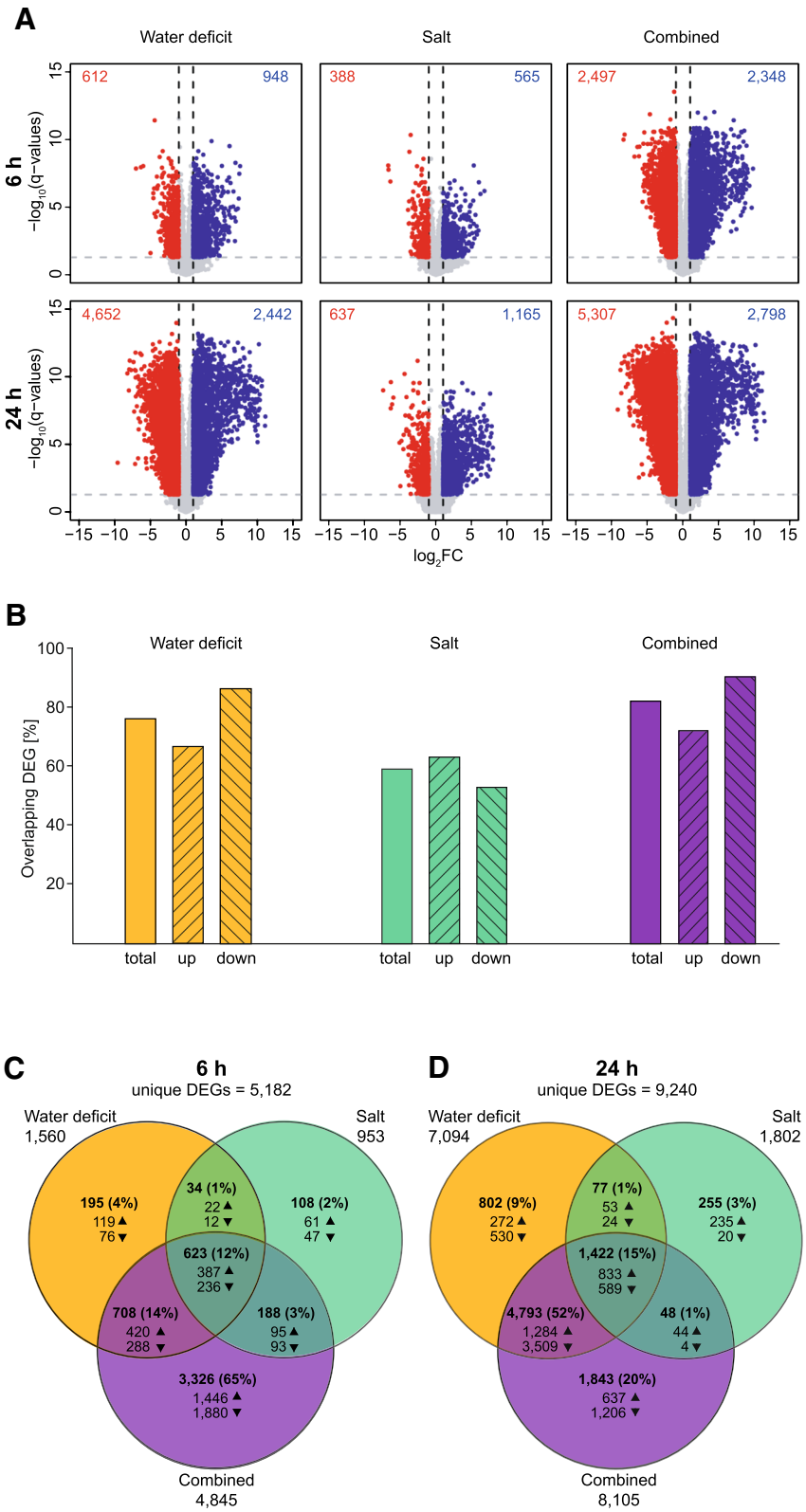

Fig. 3 (See legend on next page.)

(See figure on previous page.)
Fig. 3 Overview of differentially expressed genes (DEGs) between control and stress-treated samples. **a** Volcano plots depict DEGs for each treatment-by-time combination. Up-regulated DEGs are indicated by blue dots, down-regulated DEGs are indicated by red dots. Total number of DEGs are shown in the upper left and right corner for significant up- and down-regulated DEGs. DEGs that do not exceed the threshold of $|\log_2 FC| > 1$ and FDR ≤1% are depicted in grey. **b** Overlap of DEGs at 6 h that are also differentially expressed at 24 h for each treatment in percent. Bars show overlaps of all DEGs, up-regulated and down-regulated DEGs separately. **c** Venn diagram showing the overlap between DEGs responsive to water deficit, salt and combined stress after 6 h of treatment. Arrows indicate number and direction of DEGs. **d** Venn diagram showing the overlap between DEGs after 24 h of treatment. Arrows indicate number of up- and down-regulated DEGs

than the sum of genes differentially regulated by water deficit or salt stress alone. Furthermore, the direction of regulation depended on stress type and duration. Between 60% (salt treatment) and 80% (combined treatment) of genes that were differentially expressed after 6 h, were also responsive after 24 h of treatment (Fig. 3b). Among these, 70 to 75% of up-regulated DEGs and 55 to 95% of the down-regulated DEGs were conserved over time. Cross-comparison between the different gene sets after 6 h showed that the highest proportion of genes (65%) was unique to the combination treatment, while water deficit (4%) and salt (2%) treatment resulted in less uniquely expressed genes (Fig. 3c). This indicates that the combined treatment does not only result in the additive regulation of genes differentially expressed in the two single stress treatments. Instead, a substantial number of genes was only regulated by the combined stress but not by the individual stress factors. A set of 623 DEGs (12%) was responsive to all three treatments, pointing towards regulatory changes that were unaffected by stress type. Long-term stress response showed a strong overlap (52%) of genes responsive to water deficit and combined stress treatments, which is in line with the distribution of samples in the MDS plot in Fig. 2. Nevertheless, 20% of DEGs were specific for combined stress (Fig. 3d), while only 9 and 3% of DEGs were unique to water deficit and salt treatment, respectively.

Assessment of stress-responsive pathways

Gene Ontology (GO) terms were assigned to DEGs to functionally characterize stress-responsive processes and functions. GO terms were analyzed for singular enrichment and obtained results were cross-compared with the SEACOMPARE tool. A full list of enriched GO terms in all treatment-by-time combinations is provided in Additional file 4: Table S3. In total, 63 GO terms responsive to short-term stress remained after filtering with REVIGO. Half of these terms were treatment-specific, while the other half was shared between two or more treatments (Table 1). The highest number of unique treatment-specific GO terms was observed for the combinatorial treatment. A substantial number of GO terms was commonly enriched between combined stress and each of the single stresses but not between water deficit and salinity. Shared terms of biological processes and molecular functions that were responsive to salt and combined treatment were commonly down-regulated. This included several catalytic activities such as 'transferase activity' (GO:0016740) but also metabolic processes like 'phosphorus metabolic process' (GO:0006793). Mutual DEGs between water deficit and combined stress were identified to be involved in oxidative and general stimulus responses. While 'oxidoreductase activity' (GO:0016491) and 'oxidation-reduction process' (GO:0055114) were down-regulated, responses to stimuli were up-regulated. The last set of treatment-independent terms showed up-regulation in all biological processes mainly involved in 'transcription' (GO:0006351), 'regulation of gene expression' (GO:0010468) and the corresponding functional term 'transcription factor activity' (GO:0003700). Genes involved in binding and hydrolase activity were down-regulated.

GO terms assigned to DEGs after long-term exposure were mostly shared between two or more treatments (Table 2). A considerable number of commonly enriched terms was identified between water deficit and combined treatment, which is in support with the MDS plot (Fig. 2) and in line with the overlap of differentially expressed genes in Fig. 3d. Among functional terms, several transferase activities and metabolic processes were down-regulated, while 'regulation of gene expression' (GO:0010468) and 'ion binding' (GO:0043167) were up-regulated. Up-regulation of 'developmental process' (GO:0048856) and its child term was shared between water deficit and salt treatment, in contrast to salt and combined treatment, which showed no overlapping terms. Terms covered by 'catalytic activity' (GO:0003824) such as oxidoreductases, that were previously only enriched in short-term combined stress were enriched independent of stress type after long-term exposure. Furthermore, if terms were shared between two or more stress types, the direction of regulation was largely preserved. Up-regulation of the term 'transcription factor activity' (GO:0003700) was even conserved in all treatment-by-time combinations.

Distribution of transcription factors in differentially expressed gene sets

Transcription factors (TFs) within the RNA-Seq dataset were identified via the Plant Transcription Factor Database. In total, 924 of 2620 known barley TFs were

Table 1 Enriched functional GO terms among DEGs responding to 6 h short-term treatment

GO term[a]	Description[b]	Treatment[c]		
		D	S	SD
GO:0005215	**transporter activity (2)**			
GO:0036094	**small molecule binding (4)**			
GO:0005524	ATP binding (9)			
GO:0032502	**developmental process (2)**			
GO:0048856	anatomical structure development (3)			
GO:0005975	**carbohydrate metabolic process (4)**			
GO:0003824	**catalytic activity (2)**			
GO:0008146	sulfotransferase activity (5)			
GO:0016758	transferase activity, transferring hexosyl groups (5)			
GO:0016705	oxidoreductase activity, acting on paired donors, incorporation/ reduction of molecular O_2 (4)			
GO:0043167	**ion binding (3)**			
GO:0043169	cation binding (4)			
GO:0097159	**organic cyclic compound binding (3)**			
GO:1901363	**heterocyclic compound binding (3)**			
GO:0046983	**protein dimerization activity (4)**			
GO:0005200	**structural constituent of cytoskeleton (3)**			
GO:0050896	**response to stimulus (2)**			
GO:0006950	response to stress (3)			
GO:0051704	**multi-organism process (2)**			
GO:0065007	**biological regulation (2)**			
GO:0000003	**reproduction (2)**			
GO:0007017	**microtubule-based process (3)**			
GO:0018130	**heterocycle biosynthetic process (5)**			
GO:0019438	**aromatic compound biosynthetic process (5)**			
GO:1901362	**organic cyclic compound biosynthetic process (5)**			
GO:0034654	nucleobase-containing compound biosynthetic process (6)			
GO:0071705	**nitrogen compound transport (5)**			
GO:0009664	**plant-type cell wall organization (5)**			
GO:0016740	**transferase activity (3)**			
GO:0016773	phosphotransferase activity, alcohol group as acceptor (5)			
GO:0097367	**carbohydrate derivative binding (3)**			
GO:1901265	**nucleoside phosphate binding (4)**			
GO:0000166	nucleotide binding (5)			
GO:0043531	*ADP binding (9)*			
GO:0022414	**reproductive process (2)**			
GO:0044706	**multi-multicellular organism process (3)**			
GO:0008037	**cell recognition (3)**			
GO:0006793	**phosphorus metabolic process (4)**			
GO:0043412	**macromolecule modification (5)**			
GO:0006468	protein phosphorylation (8)			
GO:0020037	**heme binding (5)**			
GO:0043565	**sequence-specific DNA binding (6)**			

[a]Only non-redundant terms (similarity ≤0.5) with FDR ≤5% are shown for identified molecular processes (white background) and molecular functions (grey background)
[b]Indented terms belong to the same cluster as the above listed higher-ranking term. Numbers in parentheses indicate the level of the GO term
[c]Treatments are water deficit (D), salt (S) and combined (SD). The direction of regulation is represented by blue (down-regulation), red (up-regulation), and grey (not significantly regulated)

Table 2 Enriched functional GO terms among DEGs responding to 24 h long-term treatment

GO term[a]	Description[b]	Treatment[c]		
		D	S	SD
GO:0003885	**D-arabinono-1,4-lactone oxidase activity (6)**			
GO:0005507	**copper ion binding (7)**			
GO:0044262	**cellular carbohydrate metabolic process (4)**			
GO:0050896	**response to stimulus (2)**			
GO:0006950	response to stress (3)			
GO:0071705	**nitrogen compound transport (5)**			
GO:0000041	**transition metal ion transport (8)**			
GO:0030410	**nicotianamine synthase activity (5)**			
GO:0004866	**endopeptidase inhibitor activity (6)**			
GO:0072350	**tricarboxylic acid metabolic process (7)**			
GO:0072351	tricarboxylic acid biosynthetic process (8)			
GO:0051179	**localization (2)**			
GO:0019222	**regulation of metabolic process (4)**			
GO:0006351	**transcription, DNA-templated (6)**			
GO:0008152	**metabolic process (2)**			
GO:0032502	**developmental process (2)**			
GO:0048856	anatomical structure development (3)			
GO:0016740	**transferase activity (3)**			
GO:0016757	transferase activity, transferring glycosyl groups (4)			
GO:0008146	sulfotransferase activity (5)			
GO:0016773	phosphotransferase activity, alcohol group as acceptor (5)			
GO:0005200	**structural constituent of cytoskeleton (3)**			
GO:0043167	**ion binding (3)**			
GO:0043169	cation binding (4)			
GO:0043531	ADP binding (5)			
GO:0046983	**protein dimerization activity (4)**			
GO:0005975	**carbohydrate metabolic process (4)**			
GO:0010468	**regulation of gene expression (6)**			
GO:0006468	**protein phosphorylation (8)**			
GO:0008037	**cell recognition (3)**			
GO:0071554	**cell wall organization or biogenesis (3)**			
GO:0045229	**external encapsulating structure organization (4)**			
GO:0000003	**reproduction (2)**			
GO:0022414	reproductive process (3)			
GO:0051704	**multi-organism process (2)**			
GO:0044706	multi-multicellular organism process (3)			
GO:0006810	**transport (4)**			
GO:0003824	**catalytic activity (2)**			
GO:0016491	oxidoreductase activity (3)			
GO:0016705	*oxidoreductase activity, acting on paired donors, incorporation/ reduction of molecular O_2 (4)*			
GO:0016684	*oxidoreductase activity, acting on peroxide as acceptor (4)*			
GO:0004601	*peroxidase activity (5)*			

[a]Only non-redundant terms (similarity ≤0.5) with FDR ≤5% are shown for identified molecular processes (white background) and molecular functions (grey background)
[b]Indented terms belong to the same cluster as the above listed higher-ranking term. Numbers in parentheses indicate the level of the GO term
[c]Treatments are water deficit (D), salt (S) and combined (SD). The direction of regulation is represented by blue (down-regulation), red (up-regulation), purple (up and down-regulation), and grey (not significantly regulated)

expressed in the present dataset. The prevalence of these TFs in the 56 TF families was used as a reference distribution to identify deviations in the family distributions of the DEG datasets for each treatment-by-time combination (Fig. 4). Short-term 6 h stress treatment resulted in the enrichment of 12 treatment-by-TF family combinations (Fig. 4a) while 24 h stress led to the enrichment of 18 such combinations (Fig. 4b). At both stress treatment durations, 6 h and 24 h, bHLH, ERF, and HSF TF families were enriched at all three stress treatment combinations (Fig. 4a and b). In addition, the bZIP TF family was over-represented after water deficit and combined treatment at both time points (Fig. 4a and b). At 6 h, only the HD-ZiP TF family was enriched specifically upon water deficit treatment. At 24 h the bZIP, G2-like, HD-ZIP TF families were specifically enriched upon salt stress, while the LBD, MYB, NAC and TALE TF families were enriched upon combined salt and water deficit treatment. Remarkably, while several TF families were particularly enriched after 24 h after salt treatment, no water deficit-specific enrichment of TF families was observed after 24 h.

Discussion

Under field conditions, crops are often subjected to simultaneous abiotic stresses such as water deficit, heat or high salinity [10, 11]. Understanding the molecular responses to these combined stresses that have detrimental effects on crop productivity is necessary for sustainable agriculture under changing global climatic conditions [11]. In the present study, we surveyed the individual and simultaneous effects of water deficit (PEG8000, -0.8 MPa) and high salinity (150 mM NaCl), on root development and the global transcriptome profiles of barley seminal roots.

Growth arrest as a response to salinity or water deficit in aboveground parts of plants is a common mechanism

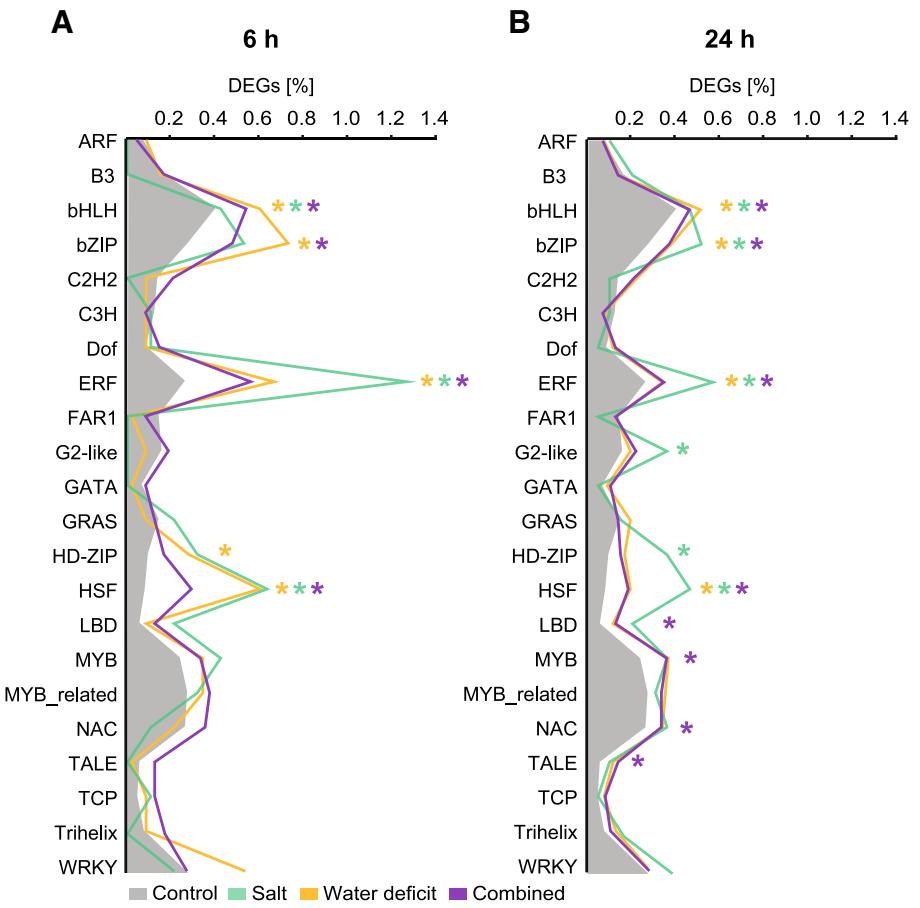

Fig. 4 Prevalence of transcription factor (TF) families after salt, water deficit and combined stress for 6 h (**a**) and 24 h (**b**). Only families with ≥15 expressed members are shown. Grey background represents the family distribution among all expressed genes. Colored lines represent treatment-specific distributions of differentially expressed TFs as a percentage of all differentially expressed genes in the treatment. Significant deviations from the background distribution were calculated by Fisher's exact tests (α ≤0.05) and are indicated by asterisks

to conserve carbohydrates and thus maintain the energy supply. However, roots continue to elongate albeit at a lower rate to access water stored in deeper soil layers [24]. In the present study, we also observed a reduction in root growth rate. Roots of plants grown under combined stress and water deficit conditions continued to elongate but were significantly shorter in comparison to the roots of control plants after four or more days of treatment (Fig. 1). These phenotypic adjustments were also monitored in 12-day-old barley seminal roots, which were significantly shorter upon water deficit conditions of -0.8 MPa compared to control conditions [18]. In maize seedlings, phenotypic plasticity of primary roots in response to water deficit was even faster than in barley as demonstrated by a 30% reduced elongation within 24 h under the same -0.8 MPa water deficit regime as applied in the present study [20]. In contrast to water deficit, exposure to 150 mM NaCl solution did not affect root elongation in barley seedlings in the present study (Fig. 1). This supports the notion that salt tolerance of barley is linked to better root growth rates to provide an additional surface for sequestration of toxic ions that accumulate due to raising Na^+ level within the plant [25].

To characterize the transcriptomic landscape of barley seminal roots and its adaptions in response to different abiotic stresses, seminal roots exposed to stress conditions for 6 h and 24 h were analyzed by RNA-Seq. Although significant developmental differences became only visible after 4 days in barley, previous studies showed, that transcriptomic adaptions are detectable already after a few hours of treatment and preceded the later observed phenotypic effects [8, 26, 27]. The number of identified DEGs, based on pairwise comparisons between control and stress samples, varied substantially between the duration of treatment with 5182 DEGs after 6 h (Fig. 2c) and 9240 DEGs after 24 h (Fig. 2d). This increase over time was for instance also reported in maize [20] and Arabidopsis [28]. Differences in the number of differentially regulated genes were also observed between different stress types. In line with their moderate impact on root elongation, salt treatment resulted in a considerably lower number of DEGs than water deficit at both time points (953 vs 1560 at 6 h and 1802 vs 7094 at 24 h; Fig. 3a). This remarkably low quantity of salt-responsive genes is in line with the previously demonstrated salt tolerance of barley [8]. Previous research suggested a possible link between the number of responsive genes and their association with the complexity and intensity of the imposed stress treatment. For instance, experiments in soybean exposed to different levels of water deficit showed that more severe stress treatment leads to an increased number of DEGs [29]. Furthermore, changes in stress complexity by applying multiple biotic and abiotic stresses to Arabidopsis were also positively correlated with the number of responsive genes [30]. In contrast to this, exposure of *Brachypodium distachyon* to triple stress (heat, water deficit, and salinity) did not increase the number of DEGs compared to double stress combinations [31]. The results obtained in this study support the notion that the duration of individual or combined stresses increases the number of differentially expressed genes (Fig. 3a). At the same time, genes regulated by these stresses at different time points displayed a substantial degree of conservation of 60 to 80% (Fig. 3b). Similar proportions of conservation of stress-responsive genes were also discovered in maize roots subjected to 6 h and 24 h of water deficit [20]. This finding supports the notion that certain molecular stress responses, which are already established after short-term exposure to stress, are still important after long-term exposure. Comparison of the DEG sets for each time point across treatments revealed that 65% of all short-term stress-responsive genes were unique to the combined stress treatment and as such not predictable by single-stress responses. This is consistent with findings in Arabidopsis ecotypes that also exhibited non-additive effects for plants subjected to combinatorial stress for 61% of the identified DEGs [32]. A similar pattern was observed in *Dianthus spiculifolius* subjected to cold and water stress, in which approximately half of the stress-responsive genes were unique to the combinatorial treatment after 24 h [33]. In the present study, only 20% of differentially expressed genes were unique to the combinatorial stress after 24 h, while a substantial overlap of regulated genes with water deficit regulated genes was observed. This notion is supported by recent studies in Arabidopsis suggesting that the response to one stress dominates the acclimation responses to a combination of stresses due to an extensive overlap between DEGs [12, 34].

Plant stress responses and adaptive processes to single and multiple stresses are orchestrated by a complex network of cross-talk between signaling pathways and sensors [10]. To gain further insight into biological processes and molecular functions that showed a stress-response, GO terms were assigned to the DEGs and analyzed for enrichment. Enriched GO terms observed in comparable studies in 12-day-old barley roots and young maize roots exposed to the same water deficit treatment are in accordance with the results of the present study. For instance, GO terms related to stress or stimulus responses and oxidoreductase activity were also highly over-represented in both experiments [18, 20, 26]. Moreover, exposure of Arabidopsis and chickpea plants to drought treatment resulted in an identical enrichment [7, 28]. Thus, indicating similar global patterns of stress response across species.

The abundance of enriched GO terms for both time points (Tables 1 and 2) further demonstrates the

complexity of stress responses with the involvement of many different pathways. Water deficit and high salinity share a common osmotic component caused by a lowered water potential in the root vicinity [35] leading to identical responses and mutually enriched GO terms. The direction of regulation is highly conserved among these commonly enriched terms except for term GO:0009665 'plant-type cell wall organization' which shows different directions between short and long-term stress and also between salt and other stress types. Within this GO term, expansins are highly represented. Continuous growth and development are based on constant loosening and remodeling of the cell wall that enables expansion [36]. Expansins play an important role in the regulation of these perpetual plasticity changes within cell walls [37]. Extensive studies in maize roots subjected to low water potentials linked enhanced gene expression of several expansins in the root growth zone to the maintenance of root growth under stress. Thus, alterations of root growth triggered by adjustment to water deficit are most likely due to the gene-specific regulation of expansin levels [38]. The regulation of expansin expression is in line with the previously described root growth adaptions. Under salt stress, expansins are up-regulated and thus, roots continue to elongate. This, in turn, can be linked to a better adaptation of barley to salt exposure [25]. In contrast, prolonged exposure to water deficit conditions and combined stress leads to down-regulation of expanins and to decreased root elongation as observed in later time points.

Another group of over-represented GO terms corresponded to antioxidant (GO:0016209) and oxidoreductase activity (GO:0016491) and their respective child terms. Genes associated with these functions are mainly involved in the scavenging of reactive oxygen species (ROS), known to harm plant cells subjected to different stresses [39]. Exposure to salt and water deficit resulted in a significant down-regulation of genes encoding oxidoreductases, glutathione reductases, and peroxidases. Down-regulation of involved enzymes was also observed in abiotically stressed Arabidopsis plants in which the direction of regulation strongly depended on the stress type underscoring the complexity of the ROS-induced network [40]. Several studies have examined expressional changes of genes involved in these processes when subjected to different stresses. In contrast to our study, 6 days of water deficit conditions in young barley roots resulted in the up-regulation of genes involved in oxidoreductase activities [18]. These findings suggest that genes involved in the ROS network are under developmental regulation and thus show different regulation over time.

Transcription factors (TFs) control the activity of downstream target genes. The GO term 'transcription factor activity' showed up-regulation in all investigated stress types and time points. In barley, 2620 TFs are classified in 56 families. Among those 924 TFs were active in seminal roots surveyed in the present study. A major proportion of these TFs are located within the bHLH, MYB-related and bZIP families [41].

Heat shock factors (HSFs) were significantly over-represented in all treatment-by-time combinations. HSFs control the expression of Heat-Shock-Proteins (HSPs) [42] that function as chaperones to protect proteins under heat stress [24, 43]. Nevertheless, it has been demonstrated that HSFs also play a role in general stress responses such as water deficit and combinations of non-thermal stresses [42, 44–46]. Experiments in barley showed that exposure to multiple abiotic stresses including water deficit and salinity resulted in the up-regulation of multiple HSFs. Of those, two candidates were subjected to qRT-PCR to validate the expressional changes [47]. We checked for these candidate genes in the present RNA-Seq dataset and found both genes HORVU4Hr1G090090 and HORVU4Hr1G090850 significantly up-regulated in all treatment-by-time combinations and with the highest fold change in the combined treatments. This alteration in modulation severity was also observed in experiments in Arabidopsis, that showed strong induction of HSFA7B by a combination of salt, osmotic and heat stress, while it was induced less severely under single heat stress [34]. The overexpression of *At*HSP17.6A in transgenic Arabidopsis plants led to enhanced osmotic stress tolerance [48]. Consequently, the expression of HSFs correlates with osmotic stress tolerance. Therefore, modulating the expression of HSF and HSP encoding genes might be an effective strategy for breeding plants with enhanced tolerance to abiotic stress.

Another over-represented family at all treatment-by-time combinations were ERF TFs. ERFs are involved in many developmental and physiological processes [49] but also act in response to wounding [50] and in abiotic stress response. Like HSF they have the potential to improve crop tolerance to abiotic stresses as demonstrated by transgenic plants overexpressing certain ERFs that are more resistant to salinity, cold and water stress [51, 52]. In the present study, genes identified as ERF were both up and down-regulated. While a swift induction of stress accelerates ethylene production, a moderate change results in inhibition of ethylene biosynthesis which in turn leads to different regulatory directions in gene expression [29]. Exposure of rice to high salinity and water deficit leads to the induction of two ERFs known as DREB1A and DREB2A [53]. In the present study, the barley homolog to DREB2A HORVU1Hr1G060490 was only slightly induced in long-term water deficit and combined stress. The closest barley

homolog for DREB1A HORVU5Hr1G080450 was significantly down-regulated in response to both water deficit treatments but not by any other treatment-by-time combination.

The phytohormone ABA mediates gene expression by induction of ABA-dependent TFs from the bZIP and bHLH families. Both have regulatory functions in numerous developmental and physiological processes, including stress response [54, 55]. Enrichment of bHLH among differentially expressed genes was observed in all treatments and durations, while the bZIP family was enriched in all conditions except the salt treatment at 6 h. It was previously demonstrated in Arabidopsis that the large family of bHLH TFs contains members that regulate cell elongation and thus have a direct effect on the root development [56]. The function of these bHLH genes in barley needs to be elucidated in future genetic analyses.

Conclusions

This data provides a starting point to understand the complex molecular mechanisms involved in the perception and signaling of multiple abiotic stresses in barley. Moreover, candidate genes identified here are a resource for further detailed genetic studies. Understanding the molecular networks underlying the signaling of combinatorial stresses will also be helpful for the identification of possible breeding targets for improved barley stress tolerance.

Material and methods

Plant material, growth conditions, and treatment

For phenotyping and transcriptome experiments, seeds of the German spring barley cultivar Scarlett were stratified in Petri dishes on soaked filter paper at 4 °C for 3 days to synchronize germination. Subsequently, seeds were transferred to germination paper (Anchor Paper Co, Saint Paul, USA) and grown in half-strength Hoagland solution [57] in growth cabinets (Conviron, Winnipeg, Manitoba, Canada) at 16 °C at night (8 h) and 20 °C at day (16 h). After 2 days of growth under control conditions, Hoagland solution was replaced and complemented with either PEG8000 solution (-0.8 MPa) to simulate water deficit, NaCl solution (150 mM) to induce salt stress or a combination of both.

Phenotypic evaluation of seedlings

To facilitate image-based phenotyping, stratified seedlings were grown in germination pouches in custom-built boxes that were manufactured in-house for this purpose. Each box consists of 25 slots to fit growth pouches at an angle of 15° to ensure root growth along the germination paper and not the pouch foil. In each pouch, one seedling was grown. This experimental system enables easy handling without disturbing the roots when documenting them. The replicates for each treatment (control, water deficit, high salinity and combined) were allocated to the boxes in a randomized block design. In total, 25 replicates per treatment were measured in four boxes (blocks). To avoid exposure of the roots to light, the boxes were closed with lids until the initiation of shoot growth. Then, the top of the boxes was covered with aluminum foil sparing the emerging shoots. Seedlings were imaged prior to stress induction and after stress induction every 24 h for eight consecutive days. Total root length was measured with WinRHIZO Pro (Version 2009b, Regent Instruments, Canada) based on pixel color classifications. Obtained values were log_2-transformed to meet the assumptions for an ANOVA. The block model included boxes as blocks (B) and pouches as plots (P) (B/P = B + B*P) with B*P as the residual error term. The following treatment model was applied: DxS = D + S + D*S with D = water deficit (control vs treated) and S = salt (control vs treated). The R package *ggpubr* (R Version 3.4.0, [58], ggpubr_0.1.6, [59]) was used for visual representation of the data. Statistical evaluation was performed with the packages *car* (car_2.1–6, [60]) for ANOVA and *agricolae* (agricolae_1.2–8, [61]) for Tukey's tests.

RNA isolation, cDNA library construction and RNA-Sequencing

For RNA extraction from seminal roots, ten seeds were grown in paper rolls as previously described [62]. Samples were harvested after 6 h and 24 h of treatment, immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. For each replicate, ten roots were pooled. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. RNA quality was checked with a Bioanalyzer (Agilent RNA 6000 Nano Chip, Agilent Technologies, Santa Clara, CA, USA). RIN (RNA integrity number) values ≥7.5 were obtained for all collected samples indicating their high quality and integrity. cDNA libraries for transcriptome sequencing were constructed according to the TruSeq RNA Sample Preparation protocol (Illumina, San Diego CA, USA). Library indexing, cluster preparation, and paired-end sequencing were performed according to the manufacturer's instructions (Illumina). In total, 32 libraries with four biological replicates per treatment were sequenced on an Illumina HiSeq 4000 sequencer resulting in 100 bp paired-end reads.

Processing of raw sequencing data

RNA-Seq reads were processed with CLC Genomics Workbench (Version 10.0.1; https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/). The raw sequencing data were deposited in NCBI's sequencing read archive under accession number SRP133479 (https://www.ncbi.nlm.nih.gov/sra/SRP133479). Low-quality reads

and adapter sequences were removed from the dataset by trimming. Only reads with a length of ≥40 bp were retained for further analyses. The remaining reads were mapped to the barley reference genome of the genotype morex [63], ftp://ftp.ensemblgenomes.org/pub/plants/release-36/fasta/hordeum_vulgare/dna/; Hv_IBSC_PGSB_v2) allowing large gaps of up to 50 kb to span introns. Reads were mapped successfully when they matched uniquely with ≥80% of their length and ≥ 90% identity to the reference sequences. By mapping RNA-seq reads of the barley genotype Scarlett to the reference genotype Morex we introduce a mapping bias which is reflected by the average mapping rates of 72% in Additional file 3: Table S2. By this approach, we toss out many true positives (Additional file 2: Table S1). However, without a Scarlett reference genome at hand, we cannot decide which of these unmapped reads would map to unique and which would map to multiple positions in the Scarlett genome. Therefore, de novo assembly of unmapped reads could introduce substantial false positive rates that might affect gene expression patterns. We, therefore, decided to exclude reads that do not map to the Morex reference genome from further analyses. Stacked reads, i.e. read pairs that have identical 5′ coordinates, orientation and length, were removed from the dataset. Subsequently, the remaining reads were mapped to the set of high-confidence gene models [63], ftp://ftp.ensemblgenomes.org/pub/plants/release-36/gff3/hordeum_vulgare/; Hv_IBSC_PGS_v2.36). Only reads that matched with ≥90% of their sequence length and ≥ 90% identity to the longest transcripts of the high confidence gene models were considered as mapped. Multi-mapping reads that mapped to more than one position were excluded from subsequent analyses.

Multidimensional scaling analysis

To assess the quality of the data, samples were clustered in a multidimensional scaling plot (MDS plot) using the plotMDS function implemented in the Bioconductor package *limma* in R (R Version 3.4.0, limma_3.32.2, [64]). Resulting distances between paired samples were displayed as the leading $\log_2$-fold change, which is defined as the estimated root-mean-square deviation for the top 500 genes with the largest standard deviation among all samples. This analysis provided a visual representation of sample relationships by spatial arrangement.

Statistical assessment of differential gene expression

To meet the assumptions of a linear model, the obtained read counts were normalized by sequencing depth and $\log_2$-transformed. The mean-variance relationship was estimated and used to assign precision weights to each observation to adjust for heteroscedasticity [65]. A linear model was fitted to assess differences in gene expression between control and stress treatments at 6 h and 24 h. The model included a fixed effect for treatment and time and the interaction of both terms. To estimate the variability over all genes and to shrink the variances towards a common value, an empirical Bayes approach was applied [66]. The contrasts.fit function of the R package *limma* was used to compute pairwise comparisons between each stress and control treatment at 6 h and 24 h and between short and long-term stress induction for each treatment. To correct the calculated *p*-values of the performed pairwise *t*-tests for multiplicity, the false discovery rate (FDR) was adjusted to ≤5% [67].

Gene ontology (GO) and transcription factor analyses

To gain better insight into stress-responsive pathways, GO categories were assigned to differentially expressed genes with the web-based agriGO v2.0 software [68]. Singular enrichment analysis identified over-represented categories by comparing GO terms of up and down-regulated differentially expressed genes separately to the set of all expressed genes based on Fisher's exact test. To correct for multiple testing, the resulting *p*-values were adjusted by controlling the FDR ≤5% [67]. The obtained results were combined and cross-compared with the SEACOMPARE tool implemented in the agriGO v2.0 software [68]. REVIGO [69] was used to filter redundant GO terms based on their similarity. Only terms with a similarity of ≤0.5 were kept.

Transcription factors were identified by sequence similarity searches of proteins from IBSC (The International Barley Sequencing Consortium) v1.0 annotation [70] deposited in the Plant Transcription Factor Database v4.0 [41] versus the barley gene annotation IBSC v2.36 [63] via blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). All expressed transcription factors within the RNA-Seq dataset were separated for short and long-term responsive genes and assigned to 56 families. The same classification was performed for transcription factors identified as differentially expressed in each treatment-by-time combination. Significant shifts between the expected background distribution of all expressed transcription factors and the observed distribution of differentially expressed transcription factors were determined by Fisher's exact test (α ≤0.05) for 6 h and 24 h separately.

Additional files

Additional file 1: Overview of the experimental workflow of the RNA-Seq experiment. (PDF 130 kb)

Additional file 2: Overview of RNA-Seq output and mapping results. (XLSX 16 kb)

Additional file 3: Comprehensive list of the differential expression analysis. (XLSX 3317 kb)

Additional file 4: Overview of all identified over-represented Gene Ontology (GO) terms. (XLSX 28 kb)

Abbreviations
DEGs: Differentially expressed genes; GO: Gene ontology; TFs: Transcription factors

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Availability of data and materials
The raw sequencing data is deposited in NCBI's sequencing read archive under accession number SRP133479 (https://www.ncbi.nlm.nih.gov/sra/SRP133479). The datasets supporting the conclusions of this article are included within the article and its Additional files.

Authors' contributions
AO performed the phenotyping experiment and wrote the manuscript. AO, PDR, and JAB performed and analyzed the RNA-Seq experiment. HPP supported the statistical analyses. FH conceived and coordinated the study and helped to draft the manuscript. All authors have read and approved the manuscript for publication.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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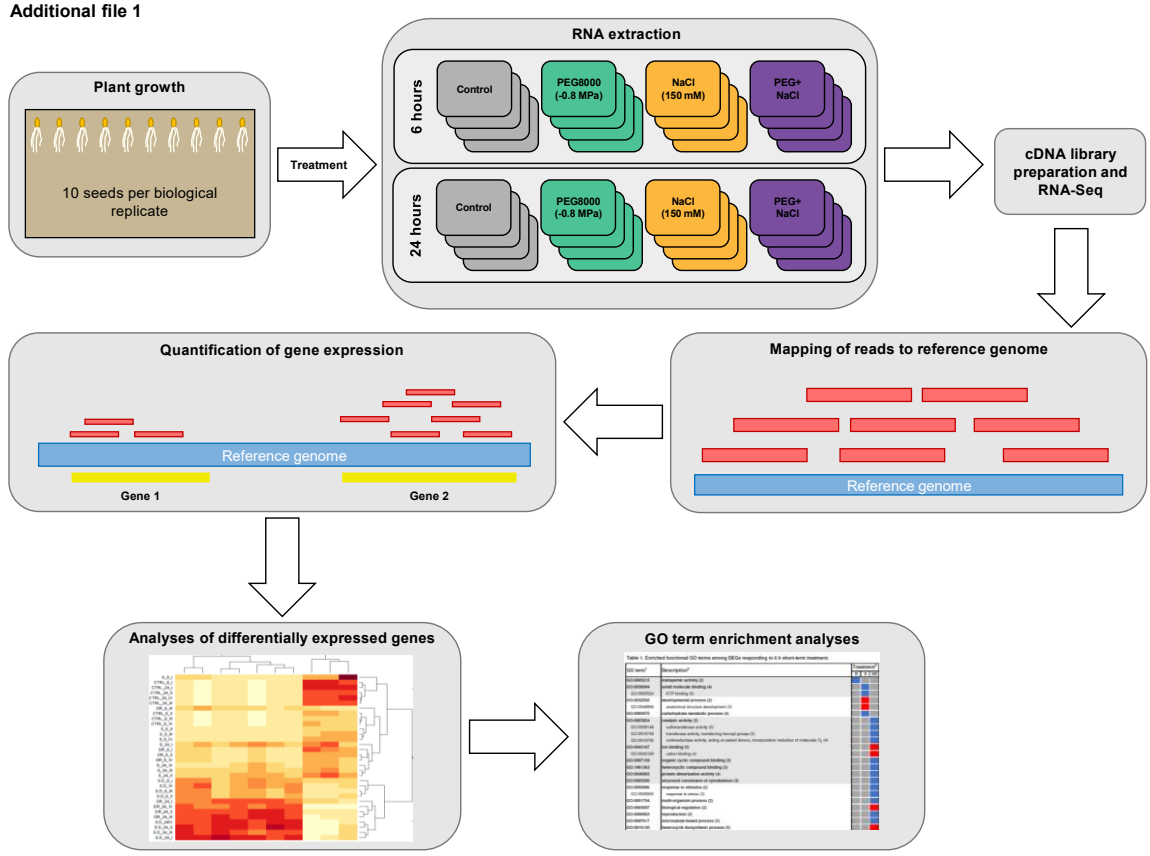
Supplementary data

Additional file 1



Additional file 1. Overview of the experimental workflow of the RNA-seq experiment.

Additional file 2. Overview of RNA-seq data output and mapping results.

control	Time point	Replicate	Number of reads							
			Total - raw	Total - after quality trimming [1]	Uniquely mapped on genome [2]	Mapping rate on genome [%]	Uniquely mapped on genome without stacks [3]	Mapping rate on genome without stacks [%]	Uniquely mapped in pairs without stacks on gene set [4]	Mapping rate pairs without stacks [%]
control	6 h	1	48,124,602	48,119,962	35,128,867	73.0	23,440,514	66.73	21,067,902	89.88
		2	45,505,026	45,504,410	33,463,757	73.5	22,294,720	66.62	19,754,434	88.61
		3	59,136,840	59,136,100	43,984,539	74.4	28,438,768	64.66	25,229,178	88.71
		4	41,329,194	41,325,048	30,379,545	73.5	21,908,722	72.12	19,824,876	90.49
control	24h	1	46,981,080	46,976,424	34,313,841	73.0	22,782,882	66.40	20,389,028	89.49
		2	47,042,300	47,041,746	34,388,603	73.1	22,649,180	65.86	20,349,688	89.85
		3	40,932,052	40,931,506	29,846,740	72.9	20,214,278	67.73	18,114,150	89.61
		4	44,473,236	44,468,750	32,742,492	73.6	22,852,592	69.79	20,121,086	88.05
PEG8000 (D)	6 h	1	46,681,242	46,676,630	33,901,366	72.6	22,990,718	67.82	20,373,794	88.62
		2	42,486,916	42,486,244	28,317,307	66.7	19,916,030	70.33	17,702,950	88.89
		3	50,821,478	50,820,836	37,495,289	73.8	24,997,016	66.67	22,439,862	89.77
		4	46,852,998	46,848,472	34,667,337	74.0	23,818,990	68.71	20,938,154	87.91
PEG8000 (D)	24h	1	44,885,784	44,881,306	30,946,953	69.0	21,029,342	67.95	18,586,452	88.38
		2	42,283,708	42,283,190	30,576,696	72.3	20,933,028	68.46	18,614,724	88.93
		3	48,494,384	48,493,718	33,925,369	70.0	21,629,536	63.76	19,168,180	88.62
		4	41,918,812	41,914,778	28,532,624	68.1	19,053,120	66.78	17,048,638	89.48
NaCl (S)	6 h	1	43,141,314	43,137,074	32,855,537	76.2	22,342,478	68.00	19,595,708	87.71
		2	45,816,240	45,815,578	34,381,309	75.0	23,224,924	67.55	20,839,668	89.73
		3	39,285,764	39,285,074	29,114,797	74.1	20,558,008	70.61	18,368,246	89.35
		4	42,868,144	42,863,938	31,892,010	74.4	22,518,634	70.61	19,757,886	87.74
NaCl (S)	24 h	1	43,920,524	43,916,316	32,091,883	73.1	22,183,612	69.13	19,882,916	89.63
		2	42,347,408	42,346,844	30,898,120	73.0	21,228,600	68.71	19,010,070	89.55
		3	46,319,840	46,319,218	34,099,663	73.6	22,377,996	65.63	19,712,436	88.09
		4	43,105,462	43,101,048	31,793,393	73.8	22,319,930	70.20	19,894,626	89.13
PEG + NaCl (SD)	6 h	1	39,272,568	39,268,712	28,550,253	72.7	19,823,468	69.43	17,738,150	89.48
		2	47,901,474	47,900,744	35,539,667	74.2	24,447,538	68.79	21,679,898	88.68
		3	44,855,256	44,854,702	32,973,974	73.5	22,427,662	68.02	19,951,988	88.96
		4	44,870,188	44,896,295	33,288,473	74.1	23,460,650	70.48	20,478,536	87.29
	24 h	1	43,963,142	43,958,896	31,035,174	70.6	21,265,064	68.52	18,720,524	88.03

PEG + NaCl (SD)	2	45,962,914	45,962,308	32,027,840	69.7	21,004,992	65.58	18,783,742	89.43
	3	40,543,214	40,542,700	28,451,662	70.2	18,875,082	66.34	16,533,632	87.60
	4	47,196,362	47,191,870	32,107,243	68.0	20,503,730	63.86	18,137,566	88.46
Average		44,978,733	44,977,201	32,616,010	72.5	22,109,744	67.9	19,650,272	88.88
Minimum		39,272,568	39,268,712	28,317,307	66.7	18,875,082	63.8	16,533,632	87.60
Maximum		59,136,840	59,136,100	43,984,539	76.2	28,438,768	72.1	25,229,178	90.5

[1] Trimming by quality scores and ambiguous nucleotides of sequence ends

[2] Mapped to the IBSC v2 reference genome (Mascher et al., 2017)

[3] Removal of stacked reads i.e. redundant reads sharing same start and end coordinates, direction and sequence

[4] Mapped to the annotated gene model v2.36

Online supplementary data

Additional file 3. Comprehensive list of the differential expression analysis. For differentially expressed (DE) genes the log2FC and the q-value (adjusted p-value) are noted. Functional annotation is based on the annotation provided by Mascher et al., 2017.

Additional file 4. Overview of all identified over-represented Gene Ontology (GO) terms. Terms are separated into three ontologies: biological process (P), molecular function (M) and cellular component (C). P-values are shown for up and down-regulated DEGs separately.

6.1.2 Spatiotemporal transcriptomic plasticity in barley roots: Unravelling water deficit responses in distinct root zones

Alina Klaus, Caroline Marcon and Frank Hochholdinger

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Own contribution: I carried out all experiments and isolated the RNA. I conducted downstream and statistical analyses, interpreted the results and designed the figures. I wrote the manuscript with revisions from FH and CM.

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RESEARCH Open Access

Spatiotemporal transcriptomic plasticity in barley roots: unravelling water deficit responses in distinct root zones

Alina Klaus[1], Caroline Marcon[1] and Frank Hochholdinger[1*]

Abstract

Background Drought poses a major threat to agricultural production and thus food security. Understanding the processes shaping plant responses to water deficit is essential for global food safety. Though many studies examined the effect of water deficit on the whole-root level, the distinct functions of each root zone and their specific stress responses remain masked by this approach.

Results In this study, we investigated the effect of water deficit on root development of the spring barley (*Hordeum vulgare* L.) cultivar Morex and examined transcriptomic responses at the level of longitudinal root zones. Water deficit significantly reduced root growth rates after two days of treatment. RNA-sequencing revealed root zone and temporal gene expression changes depending on the duration of water deficit treatment. The majority of water deficit-regulated genes were unique for their respective root zone-by-treatment combination, though they were associated with commonly enriched gene ontology terms. Among these, we found terms associated with transport, detoxification, or cell wall formation affected by water deficit. Integration of weighted gene co-expression analyses identified differential hub genes, that highlighted the importance of modulating energy and protein metabolism and stress response.

Conclusion Our findings provide new insights into the highly dynamic and spatiotemporal response cascade triggered by water deficit and the underlying genetic regulations on the level of root zones in the barley cultivar Morex, providing potential targets to enhance plant resilience against environmental constraints. This study further emphasizes the importance of considering spatial and temporal resolution when examining stress responses.

Keywords Barley, Differential hub genes, Gene expression, RNA-seq, Root zones, Water deficit, WGCNA

Background

Barley (*Hordeum vulgare* L.) ranks fourth in global cereal production with 145.9 mio tons per year in 2021/22 [1]. It is used in various food products and beverages but mainly serves as fodder for livestock [2]. Barley is considered resilient against environmental constraints like salinity [3], or water deficit [4].

Nevertheless, global warming and thus prolonged drought periods [5] pose a major threat to global barley production [6]. Understanding the mechanisms underlying drought stress response and tolerance is essential to cope with these negative effects and will help to improve food security. Extensive research has been carried out to unravel the mechanisms of drought responses in plants and thus to increase crop tolerance [7–9]. Upon water deficit, plants initiate a multitude of molecular and physiological responses that aim to prevent detrimental

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effects caused by water loss. The phytohormone abscisic acid (ABA) was identified as a key player, orchestrating many regulatory processes, like stomatal aperture [10], including the regulation of gene expression via ABA-responsive element binding factors [11]. In contrast, dehydration-responsive element binding (DREB) proteins are part of the ABA-independent response complex but also act in gene regulatory processes [12]. The plasticity of gene expression enables the precise modulation of processes and pathways involved in stress response to water deficit and is the focus of many studies including crop species like maize [13], wheat [14], rice [15] and barley [16]. The study of transcriptomics facilitated by sequencing technologies, like RNA-sequencing [17], allows for studying drought responsiveness of all active genes of a tissue or organ. Moreover, advanced analytical tools, such as weighted gene co-expression network analysis (WGCNA, [18]), enabled the identification of stress-responsive gene groups by examining the expression patterns of genes. WGCNA is a systems biology method for describing correlations among large and quantitative data sets, such as RNA-seq. It can be used as an unsupervised analysis method to find modules of genes highly correlated in their expression pattern, which then can be associated with specific conditions or traits [18]. This correlation facilitates the network-based identification of candidate genes related to specific root zones and drought treatments. The genes, which are among the most highly connected ones within a module detected by WGCNA, are referred to as hub genes [18]. By integrating differential gene expression analysis and WGCNA, a comprehensive understanding of the complex molecular interactions underlying water deficit responses can be gained. Roots are the first plant organ to encounter water deficit. Therefore, they offer an ideal model to study early transcriptomic adaptations [19]. Roots can be separated into more specialized longitudinal root zones with distinct functions. While the root cap protects the root tip, the meristem harbors stem cells and thus provides new cells for growth. In the elongation zone, cells elongate, while in the differentiation zone, the most basal part of the root, cell differentiation takes place [20]. Though, it is well-established that each root zone exhibits distinct functions, many studies examine the effect of water deficit responses on the whole root level and thus, zone-specific mechanisms may be entirely masked [21].

In the present study, we focused on the effect of water deficit, simulated by polyethylene glycol (PEG8000), on root morphology and the root transcriptome of barley seedlings. High molecular weight organic osmotica such as PEG8000 (polyethylene glycol 8000), which cannot enter plant cells, can be utilized to mimic water deficit [22]. This allows generating defined water potentials to study plant responses under controlled water deficit conditions. Water deficit treatment of -0.8 MPa is in the mid-range of naturally occurring, plant-usable soil water potentials thus representing moderate drought stress [23]. We divided the root into three distinct longitudinal developmental zones: root cap and meristem, elongation zone and differentiation zone and performed RNA-sequencing after 6 h, 24 h and 48 h of water deficit. By this approach, we aim to provide a comprehensive overview of the spatiotemporal dynamics of gene expression patterns and fill the knowledge gap regarding zone-specific responses.

Methods

Plant material, growth conditions, and treatment

We pre-germinated seeds of the spring barley variety Morex for two days at 4 °C and then either transferred them to germination paper rolls [24] for RNA-sequencing or to germination paper-covered panels fitting into custom-built boxes [25] for phenotyping. We grew the plants in a climate chamber (Conviron, Winnipeg, Canada) at 20 °C at night (8 h) and 22 °C (16 h) at day and watered them with half-strength Hoagland solution [26]. After two days, we renewed the nutrient solution for control plants or exchanged it for a polyethylene glycol (PEG8000, Roth, Karlsruhe, Germany) solution with a water potential of -0.8 MPa to simulate moderate water deficit for stressed plants [27].

Assessment of root growth under water deficit conditions

To ensure comparability, we selected only the three longest seminal roots per plant and measured the root length for seven consecutive days. We then calculated the average root length and growth rate of each plant at each time point and determined differences between control and water deficit plants by ANOVA in RStudio [28]. A mixed-effects model was used to analyze the data: lme(trait ~ treatment * time, random = ~ 1 | id) with average root length or average root growth as trait responding to the interaction of the fixed-effect terms time (day 0 to day 7) and treatment (control and water deficit), correcting for a random-effect term id, that was used as an identifier for each plant. We performed *post-hoc* analyses with the emmeans package [29], which calculates the estimated marginal means (EMM) of the fitted model with treatment set as the specs argument and separated by time. Pairwise comparisons between EMMs of each treatment group were calculated and adjusted for multiplicity with the adjust = "bonferroni" option. This way we handled every time point separately without neglecting the longitudinal character of the data. We used the package ggpubr [30] to visualize the data.

RNA isolation and RNA-sequencing

We harvested root samples of seedlings grown in paper rolls for 6 h, 24 h and 48 h after water deficit stress induction. We then separated the roots into three distinct root zones: root cap and meristem, elongation zone and differentiation zone and immediately froze them in liquid nitrogen. The boundaries between the meristematic zone and the elongation zone were estimated based on previously analyzed longitudinal sections by Kirschner et al. [31], where the transition started around 1 mm from the root tip, while the boundaries towards the differentiation zone were marked by the first appearance of root hairs. In total, we obtained 54 samples with three biological replicates for all treatment-by-root zone-by -time point combinations with a pool of 30 roots for each biological replicate. We extracted total RNA with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and assessed RNA quality and integrity with a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and a BioAnalyzer (Agilent RNA 6000 Nano Chip, Agilent Technologies, Santa Clara, CA, USA). All samples exceeded a RNA integrity number value of 8.1. The RNA samples were sequenced on a NovaSeq 6000 sequencing platform (Novogene, Cambridge, UK) using a paired-end 150 bp strategy.

Processing of raw sequencing data

We performed quality trimming of raw reads obtained from Novogene with trimmomatic v0.39 [32]. Trimmomatic was run in paired-end mode with the following options: ILLUMINACLIP:adapter.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:60. Reads > 60 bp were retained for subsequent processing. We then quantified transcript abundances with the pseudo alignment tool kallisto v0.46.0 [33] using the kallisto quant command with default options. The index was built from the transcriptome of Morex v3 (Hv_Morex.pgsb.Jul2020.HC.cds.fa; https://doi.ipk-gatersleben.de/DOI/b2f47dfb-47ff-4114-89ae-bad8dcc515a1/21172880-2956-4cbb-ab2c-5c00bceb08a2/0). We used the tximport package [34] to import transcript abundance quantification files from kallisto to R Studio including the option countsFromAbundance = "lengthScaledTPM" to account for gene length and sequencing depth biases between samples. The resulting counts were then used for subsequent analyses. Kallisto uses expectation maximization instead of aligning reads to the reference, thus, reads that would be omitted due to multi-mapping in other approaches are now equally distributed between compatible transcripts. Hence, we filtered out gene models showing this equal distribution of counts across samples in R studio [28] before downstream analyses. The raw sequencing data were deposited in the NCBI SRA under BioProject accession number PRJNA988922.

Analysis of differentially expressed genes

We analyzed the obtained read counts as previously described in Osthoff et al. [25]. In brief, we filtered read counts to only include active genes, i.e. genes with more than 0.4 counts per million reads in at least 3 samples. Then, we defined a linear model including a fixed effect for the combined factor treatment, time and root zone and transformed it with voom [35]. For visual representation of the sample relationships by spatial arrangement, we used a multi-dimensional scaling plot. We employed the R package limma [36] to fit the linear model and shrink the standard errors towards a common value with an empirical Bayes approach [37]. Contrasts between control and water deficit samples were always drawn from the same time point and root zone to mainly focus on the treatment effect. We adjusted the false discovery rate (FDR) to < 5%. Only gene models that displayed a $|\log_2 FC| \geq 1$ and FDR < 5%, were considered significantly differentially expressed.

Weighted gene co-expression network analysis

We used a weighted gene co-expression network analysis [18] to find clusters of highly connected genes within the dataset derived from RNA sequencing of the different root zones. To set the focus on the treatment response, we carried out the co-expression analysis separately for each root zone. We filtered each count matrix for active genes by a cut-off ≥ 50 reads per gene model and used the function pickSoftThreshold with networkType = "unsigned" to pick the best-fitting power value for calculating the adjacency matrix. The selected powers were eight for the meristematic, seven for the elongation and 12 for the differentiation zone matrix. We manually constructed the networks by first calculating an adjacency matrix. Then, we calculated the topological overlap matrix using the TOMsimilarity command on the adjacency matrix. We subtracted the values of the topological overlap matrix from one to calculate the dissimilarity matrix. To generate a clustered gene tree based on the dissimilarity matrix we used the command flashClust. We set the minClusterSize to 30 to avoid small clusters and used a dynamic approach to form clusters of branches that are highly similar with cutreeDynamic and the deepSplit = 2 option. We converted cluster allocations to a color scale and used this color scale to calculate the module Eigengenes. Module Eigengenes represent the overall expression patterns of genes within their modules. We calculated the dissimilarity of these eigengenes and

clustered them with flashClust. Then, we merged close modules with cutHeight set to 0.3 for all root zones. For visualization of the obtained hierarchical clustering, we employed the plotDendroAndColors function. To identify modules that are correlated to the water deficit treatment, we computed the Pearson correlation coefficient between module eigengenes and traits (treatment-root zone-combinations). Since the examined trait data was qualitative and not quantitative, we used a presence-absence matrix in the correlation analyses.

We used the module membership or intramodular connectivity, which is the correlation between an individual gene and the respective module eigengene and the gene significance, which is the correlation between the expression of an individual gene and the trait, to identify hub genes within modules that showed a significant correlation with water deficit treatments. High gene significances indicate a higher biological relevance of the gene regarding the trait of interest, while high module membership indicates that a gene is highly connected to other genes within a selected module [18]. All genes that showed a module membership and a gene significance > 0.8 were considered hub genes. We then compared these hub genes to the sets of differentially expressed genes to identify differential hub genes that are consistently associated with water deficit across different analysis methods. To determine whether the observed overlap deviates from the expected overlap, we used either Fisher's exact test ($n < 5$) or a chi-square test ($n \geq 5$).

Gene Ontology (GO) enrichment analysis

To further decipher the function of identified differentially expressed genes and differential hub genes, we used the Gene Ontology knowledgebase [38, 39]. We assigned ontology terms to the differentially expressed genes and carried out an enrichment analysis using the topGO package [40] in R studio. The degree of enrichment was determined with Fisher's exact test. We used the algorithm = "weight01" option to reduce the number of false positives without missing too many true positives. Then, we filtered the obtained lists of enriched terms with REVIGO [41] to remove redundant terms with default settings. For visualization of significantly enriched gene ontology terms identified in the differential expression analyses, we used ggplot2 [42]. For visualization of enriched ontology terms identified in the co-expression analyses, we used Cytoscape [43]. To ensure accessibility, colors were chosen according to the viridis scale [44].

Results

Water deficit leads to decreased growth rates resulting in root length reduction

To study the response of seminal root growth to water deficit, we monitored the average root length and growth rate of barley seedlings under control and moderate water deficit conditions (PEG8000, -0.8 MPa) for seven days and calculated significant pairwise contrasts between treatment groups based on their estimated marginal means (EMMs; Fig. 1). Between three and seven days of treatment, the average root length of water-deficit

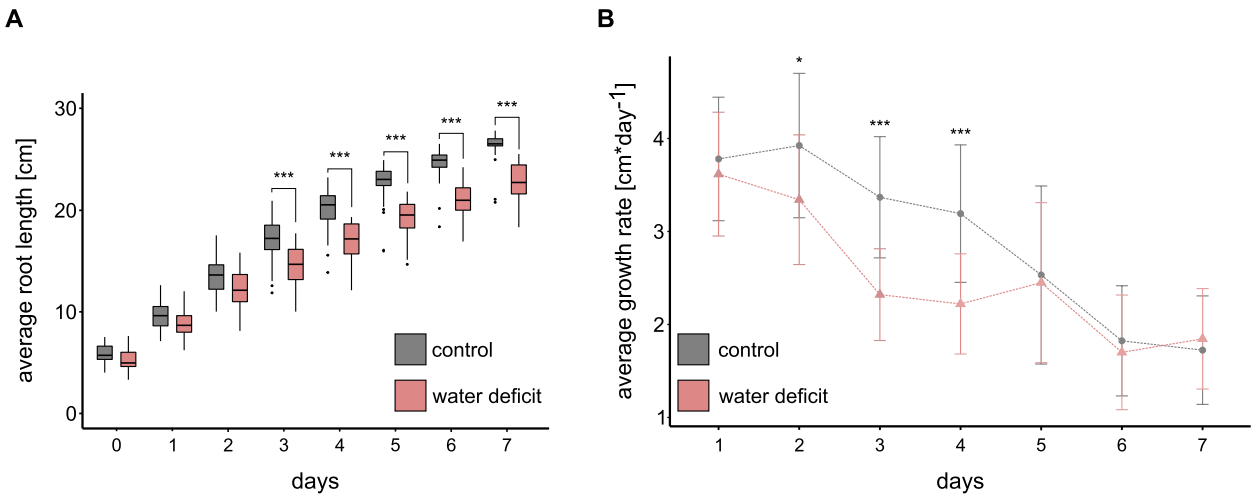

Fig. 1 Effect of water deficit on barley seedling root traits. Values are averaged over the three longest roots for each sample. Grey color represents control, red color water deficit samples with $n = 25$ plants each. Differences between control and water deficit samples were determined by ANOVA using a mixed linear model that included the random effect term id used as a plant identifier (lme(trait ~ treatment*time, random = ~ 1|id). Post hoc analyses were carried out based on estimated marginal means for each time point separately in pairwise comparisons between control and water deficit samples. **A** Comparison of root length. **B** Comparison of root growth rates. Bars represent the standard deviation of the means. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$

plants was between 15 to 20% shorter compared to the control group (Fig. 1A). For average root growth, we observed significant differences already after two days of treatment. From day five until the end of the experiment on day seven, both groups exhibited similar growth rates (Fig. 1B).

Developmental and zone-dependent plasticity of the seminal root transcriptome upon water deficit

We surveyed the transcriptomic dynamics of barley seminal root zones subjected to water deficit (-0.8 MPa) and to control conditions for 6 h, 24 h and 48 h (see methods), hence before phenotypic changes in root length between the treatments were manifested (compare with Fig. 1A, 0 – 2 days). Changes in root growth rates were already visible after 48 h of treatment (Fig. 1B, day 2) and adaptations that underlie these changes in growth rate are thus included in the transcriptomic analysis of time point 48 h. Subsequently, we sampled three different longitudinal zones of seminal roots: the root cap and meristem, the elongation zone and the differentiation zone for all treatment-by-time point combinations in three biological replicates. For RNA-sequencing, we isolated RNA from these samples and converted them into cDNA libraries for sequencing (see methods). We then pseudo-aligned the obtained reads to the reference genome annotation of the barley cultivar Morex (v3) with an average overall rate of 87%. Details regarding individual pseudo-alignment rates and quality-based removals are summarized in Table S1. After removing duplicated, lowly expressed and inactive gene models (see methods), each library retained > 20 million reads for further analyses (Figure S1). We explored the transcriptomic kinship relation between samples in a multidimensional scaling (MDS) plot (Fig. 2A). The spatial arrangement of samples on the x-axis mirrored the distribution of root zones along the root axis from the root tip to the differentiation zone and explained 58% of the overall variance. Replicated samples from each root zone clustered together under control and water deficit conditions. Hence, differences between seminal root zones were more distinct than those between treatments. To identify genes differentially regulated in response to water deficit treatment, we computed pairwise contrasts between treated and control samples for each root zone-by-time point combination. The total number of differentially expressed genes ($|\log_2 FC| > 1$ and FDR < 5%) varied widely between root zones and time points (Fig. 2B, Figure S2). The highest number of water deficit-responsive genes (6580) was identified after 6 h of treatment. After 24 h of treatment, the number of water-deficit-responsive genes declined to 983 and increased again to 4091 differentially expressed genes after 48 h. When comparing sets of genes differentially expressed between control and water deficit conditions, large proportions of these genes were unique for their respective zone-by-treatment duration combination (Fig. 2C, highlighted in black). We observed a substantial overlap of genes differentially expressed between control and water deficit conditions at the same time point in different root zones (Fig. 2C, highlighted in green). Similarly, we identified genes, that were differentially expressed between control and water deficit conditions after different treatment durations in only one root zone (Fig. 2C, highlighted in blue).

GO enrichment analysis highlights the complex and dynamic nature of water deficit responses

We performed Gene Ontology (GO) enrichment analyses to identify significantly enriched biological processes (Fig. 3) and molecular functions (Figure S3) among the differentially expressed genes in the three analyzed root zones in the time course of water deficit treatment.

After 6 h, all enriched terms related to biological processes were up-regulated in the root cap and meristem, while in the differentiation zone 14 of 17 (82%) of enriched terms were down-regulated. In the elongation zone, 79% (15/19) enriched GO terms were up-regulated, but the direction of regulation within each term varied as seen by the lower average $\log_2$ fold changes between 0 and 1 Fig. 3). Some stress-responsive GO terms were enriched in all three zones (GO:0006979; GO:0042744; GO:0098869). Another commonly affected term was 'transmembrane transport' (GO:0055085), which mainly included aquaporins, ABC transporters, NRT1/PTR family proteins and WAT1-related proteins. Several terms related to cell wall formation and maintenance (GO:0042546; GO:0071555; GO:0009664) were enriched in one or more root zones. These terms mainly encompassed cellulose synthases, expansins and xyloglucan endotransglucosylases. While those genes were strongly up-regulated in the root cap and meristem, they showed strong down-regulation in the differentiation zone, suggesting the maintenance of continued growth through cell wall remodeling and induction of cell division in the meristem.

At 24 h, fewer differentially expressed genes were observed resulting in a lower number of enriched GO terms. The general stress response terms (GO:0006979; GO:0042744; GO:0098869) and the term 'transmembrane transport' (GO:0055085) were also enriched but only in the elongation zone and the differentiation zone. The only commonly enriched term in all root zones after 24 h was 'carbohydrate metabolic process' (GO:0005975).

After 48 h, 'carbohydrate metabolic process' (GO:0005975) was again the only term shared by all root zones, while 80% (8/10) of enriched GO terms of

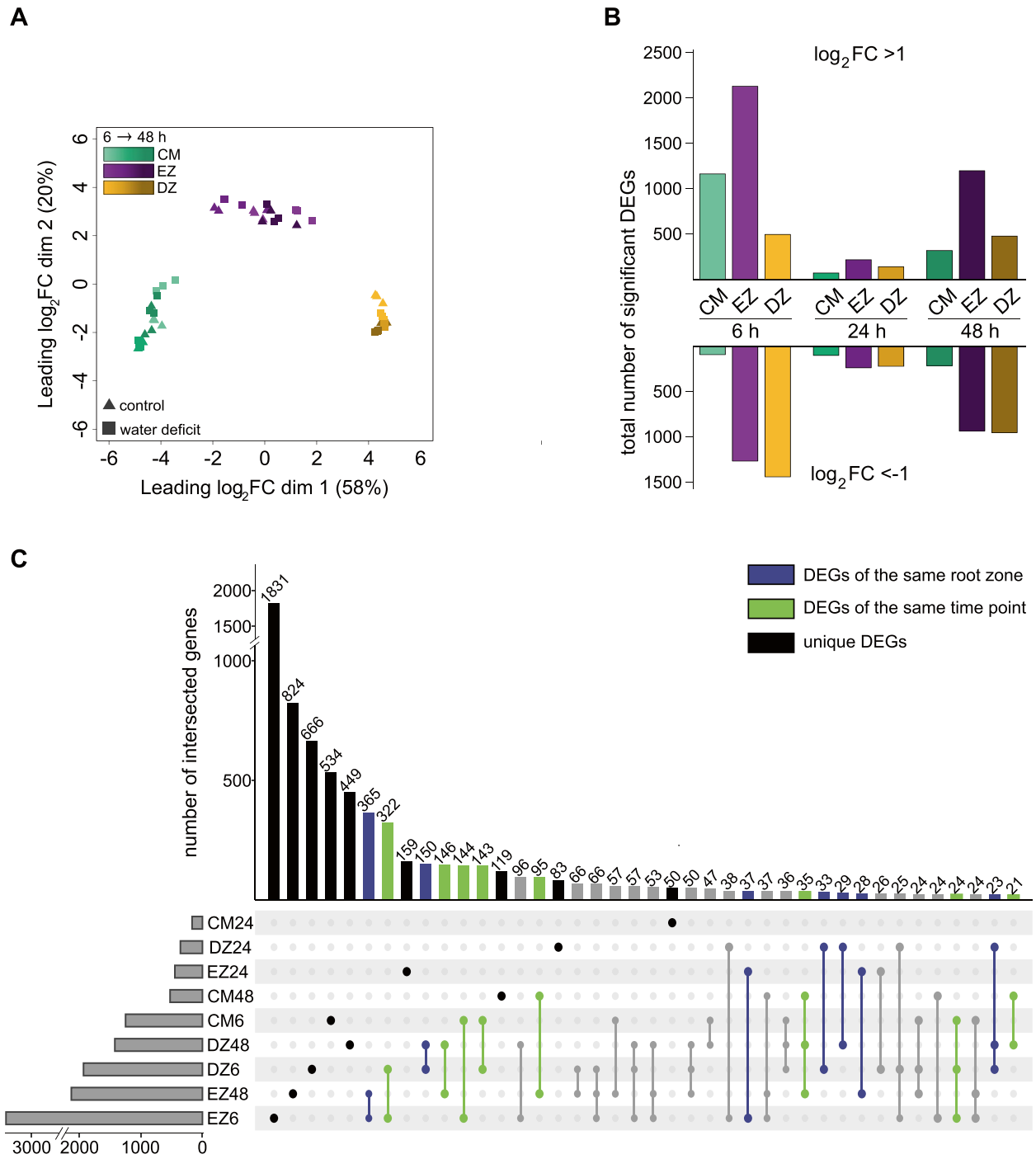

Fig. 2 Sample relationship and differential gene expression. **A** Multidimensional scaling plot of seminal root tissue transcriptomes. **B** Number of differentially expressed genes (DEGs) identified in each root zone-by-time point combination. Bars represent up-regulated ($log_2FC > 1$) and down-regulated ($log_2FC < -1$) DEGs with an FDR < 5%. **C** Comparison of DEGs across root zones and time points. Intersections are marked by connecting lines between samples. The total number of intersected genes is indicated above each bar. DEGs that are unique to their root zone-by-time combination are marked in black. Intersections of DEGs from the same root zone across different time points are marked in blue, intersections of DEGs from the same time point across different root zones are marked in green, all other intersections are marked in grey. CM: root cap and meristem; EZ: elongation zone; DZ: differentiation zone

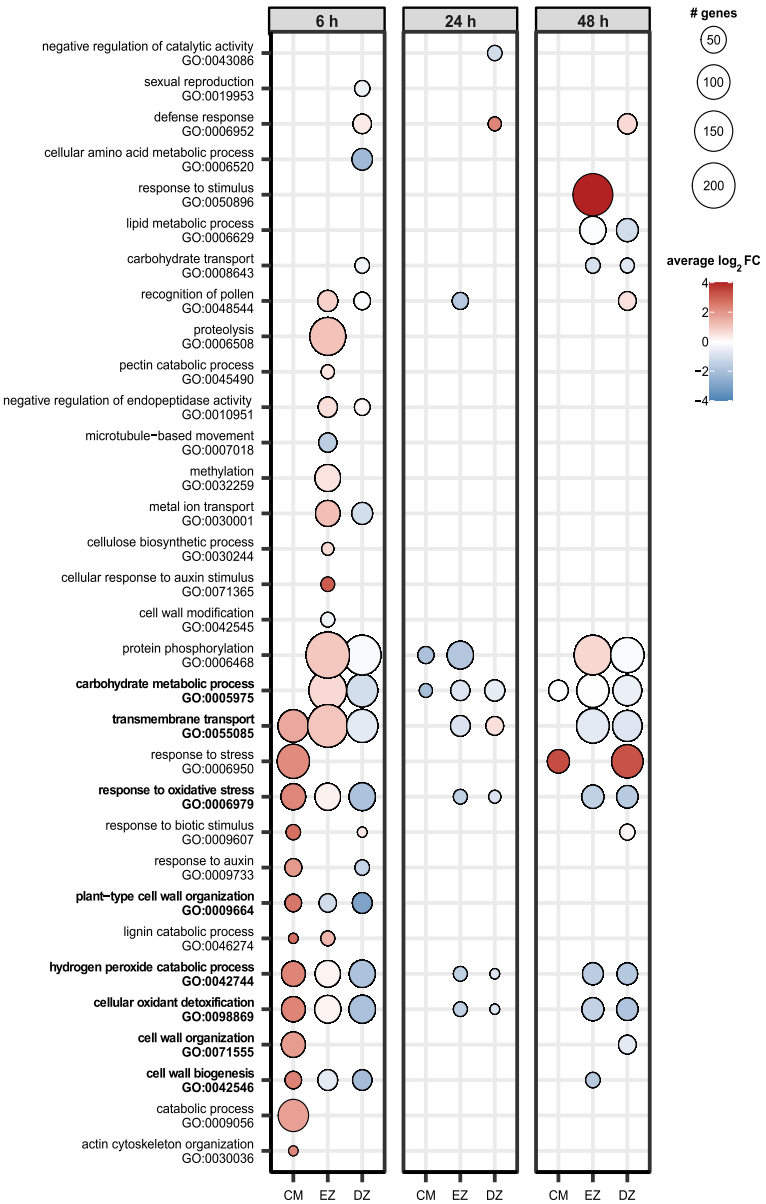

Fig. 3 Enriched biological processes based on gene ontology (GO) of differentially expressed genes. Only GO terms with ≥ 10 associated genes are shown. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ). The circle size reflects the number of DEGs associated with the respective term and the color indicates the average $\log_2$FC of these DEGs. Only significantly enriched terms with $p < 0.05$ based on Fisher's exact test are shown. Bold terms are referred to in the accompanying text

the elongation zone were shared with the differentiation zone. This also encompassed the previously found general stress-related biological processes (GO:0006979; GO:0042744; GO:0098869) and transmembrane transport (GO:0055085).

All enriched molecular functions identified after 6 h of water deficit were up-regulated in the root cap and meristem while 94% (15/16) were down-regulated in the differentiation zone. In the elongation zone 77% (20/26) of enriched molecular functions at that time point were upregulated (Figure S3). Many of the terms were enriched in at least two root zones after 6 h of water deficit. This included some stress-related terms (GO:0004601, GO:0016491, GO:0020037) cell wall-related terms (GO:0016762, GO:0016758) and 'transmembrane transporter activity' (GO:0022857). However, a vast number of terms were specific to the elongation zone. Many of these terms were highly specific child

terms (GO:0016702, GO:0016614), that derived from the more commonly enriched term 'oxidoreductase activity' (GO:0016491). After 24 h, 'heme binding' (GO:0020037) a stress-related term was the only one enriched molecular function in all root zones. The few other enriched terms were more root zone-specific. This zone-specificity was also observed in enriched molecular functions after 48 h of water deficit. Still, some stress-related terms (GO:0004601, GO:0016491, GO:0020037) and 'transmembrane transporter activity' (GO:0022857) were shared between at least two zones. In summary, these results support the notion, that the response of barley roots to water deficit is root zone and time-dependent, as many different biological processes and molecular functions were enriched across root zones and time points, highlighting the complex and dynamic nature of these responses.

Weighted gene co-expression analysis identifies modules highly correlated with water deficit

For each of the three root zones, we conducted a weighted gene co-expression network analysis (WGCNA) to identify clusters of highly connected genes (i.e. co-expressed gene modules), associated with water deficit treatment of 6 h, 24 h and 48 h to focus on the treatment effect. To each module, we assigned a specific color name to distinguish between different modules. This provides a complementary approach to the differential expression analysis, by gaining a systems-level understanding of expression patterns.

Setting the minimum module size to 30 genes per module, we identified 21, 23 and 23 distinct co-expression modules in the root cap and meristem, the elongation zone and the differentiation zone, respectively (Fig. 4A, D, G). For each module, we calculated its correlation coefficient with the duration of water deficit (Fig. 4A, D, G: color of the matrix cells) and highlighted significant modules (Fig. 4A, D, G: number of p-values in the cells). For downstream analyses, we selected the positively correlated module with the highest significant correlation coefficient for each tissue-by-treatment duration combination (Fig. 4A, D, G: modules highlighted in bold). A comprehensive list of all active genes and their respective module affiliation is provided in Table S3.

Further examination of the module eigengene expression within these selected modules revealed, that in most instances the module eigengene expression was higher in water deficit samples than in control samples at the same time point (Fig. 4B, E, H), indicating that these modules may exhibit a triggered response to water deficit treatment. To understand the relationship between gene expression and the trait water deficit, we assessed the relationship between module membership and gene significance, by calculating the correlation between these two measures for each selected module (see methods). The results showed a strong correlation ($r > 0.5$) for 8 of 9 modules (Fig. 4C, F, I. These findings suggest that genes with the highest module membership and gene significance in these selected modules are likely associated with water deficit treatment at the respective time points.

Differential hub gene analysis highlights the root zone specificity of processes and functions under water deficit

To further investigate the underlying mechanisms that shape water deficit responses, we identified hub genes in each of the modules selected in Fig. 4B, E, H. Hub genes are genes that are highly connected within their co-expression network and are strongly associated with the correlated trait. The identification of hub genes is a key step for reducing the complexity of the analysis and prioritizing the most significant genes. Thus, we set a threshold of ≥ 0.8 for module membership and gene significance, to find all hub genes within the selected modules that showed a high correlation with water deficit treatments. A comprehensive overview of all identified hub genes is listed in Table S4. The number of hub genes varied from very low numbers in all differentiation zone modules (2–28 genes) to up to 418 hub genes in one of the modules of the root cap and meristem. In general, the number of hub genes was lower in 24 h modules than in the 6 h and 48 h modules. We compared the hub genes with the list of previously identified differentially expressed genes. This allowed us to find differentially expressed hub genes that are highly connected, biologically important and consistently associated with water deficit under the same condition. Then we calculated if the observed overlap differed from the expected overlap with Fisher's exact test or Pearson's chi-square tests and found a significant overrepresentation of differentially expressed hub genes in six of the nine modules (Figure S4). To identify enriched biological processes and molecular functions covered by the significantly overrepresented differentially expressed hub genes (Figure S4), we performed a functional enrichment analysis of gene ontology (GO) terms for these six comparisons (Fig. 5). In the root cap and meristem modules subjected to 6 h of water deficit (CM6) or 48 h of water deficit (CM48) gene ontology terms mainly fitted into networks associated with energy metabolism, or stress response. While differential hub genes from CM6 also corresponded to cell wall and transport, hub genes from CM48 were additionally associated with stress responses and protein regulation (Fig. 5A, B). In the elongation zone modules subjected to 6 h (EZ6), 24 h (EZ24), or 48 h (EZ48) of water deficit, hub genes were consistently associated with protein metabolism. In EZ6 we additionally identified GO terms

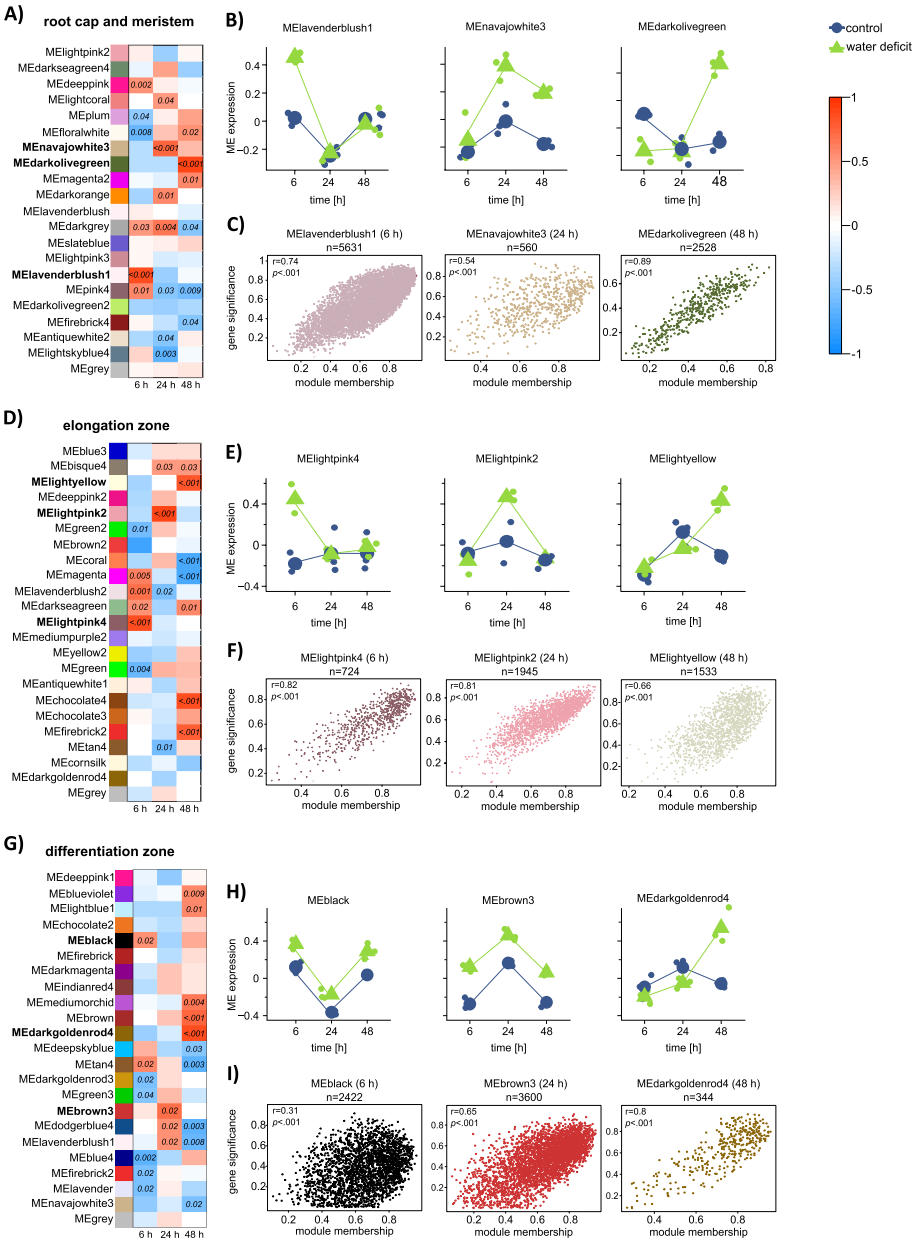

Fig. 4 Module-treatment-correlation analysis results. **A**, **D**, **G** Module trait-correlation matrix for co-expression networks derived from the root cap and meristem (**A**), the elongation zone (**D**) and the differentiation zone (**G**). Each column represents a different treatment duration (6 h, 24 h and 48 h of water deficit) and each row represents one co-expression module identified by distinct color names. The color within the matrix cells shows the correlation coefficient between -1 (blue) and 1 (red). Only significant p-values are reported in the cells. One module with the highest significant trait correlation for each time point was chosen for further analyses and marked with bold labels. **B**, **E**, **H** Module eigengene expression pattern over treatment duration in the three selected modules for the root cap and meristem (**B**), the elongation zone (**E**) and the differentiation zone (**H**). Color indicates control (blue) and water deficit samples (green). **C**, **F**, **I** Gene significance versus module membership scatter plots for selected WGCNA modules from root cap and meristem (**C**), elongation zone (**F**) and differentiation zone (**I**). The Pearson correlation coefficient (r) is calculated and reported with the respective p-value

related to various metabolic responses. In contrast, EZ24 and EZ48 hub genes were enriched for energy metabolism and stress-related terms (Fig. 5 C-E). Finally, the differential hub genes from the differentiation zone after 48 h of water deficit, were associated with either energy metabolism or protein regulation (Fig. 5F). Overall, the examined responses reflect only a small portion of adaptations occurring during water deficit treatment, since we

Fig. 5 Gene ontology (GO) enrichment analyses of differential hub genes. **A** Root cap and meristem (CM) after 6 h (CM6/MElavenderblush1). **B** CM after 48 h (CM48/MEdarkolivegreen). **C** Elongation zone (EZ) after 6 h (EZ6/MElightpink4). **D** EZ after 24 h (EZ24/MElightpink2). **E** EZ after 48 h (EZ48/MElightyellow). **F** Differentiation zone (DZ) after 48 h (DZ48/darkgoldenrod4). Differential hub genes are represented by grey dots and connected to enriched biological processes (dark grey diamond), and /or enriched molecular functions (light grey hexagon). GO terms associated with similar functions are circled and labeled accordingly

compared only genes from those modules with the highest Pearson correlation coefficients towards the treatment with the identified differentially expressed genes. Nevertheless, we consider the identified differential hub genes highly relevant, as they showed a strong association with water deficit response based on two different approaches. In summary, these results suggest that although a variety of differential hub genes are involved in water deficit responses, most fit into one of five main categories, with energy metabolism being of particular importance at later time points.

Discussion

Drought is a significant challenge to agricultural production, which is expected to intensify in occurrence and severity in the foreseeable future due to global warming [45]. Thus, understanding the mechanisms underlying plant responses to water deficit and developing tolerant varieties will improve crop productivity and ensure food safety. Extensive studies on the effects of water deficit on whole roots or root systems have been conducted in major cereals such as rice [46], wheat [47], and barley [48]. However, they do not provide insights into root zone-specific transcriptomic responses to water deficit. To understand the molecular responses to drought stress along the root from the division of root cells via their elongation up to their differentiation is important because they are the underlying causes of whole root system adaptation. In this study, we contribute to closing this knowledge gap by examining the impact of water deficit on barley seminal root development and the transcriptomic response of different seminal root zones of barley.

We observed that root length significantly decreased in barley seedlings after three days of water deficit (Fig. 1A). The detrimental effect of water scarcity on root elongation is consistent with the results of studies in wheat and maize where seminal root lengths were significantly reduced upon water deficit [49, 50]. Moreover, our results showed that root growth rates were already significantly affected on the second day of treatment (Fig. 1B), reflecting an immediate response to changes in water availability, preceding the later observed reduction in root length. Notably, both control and drought-treated plants exhibited similar growth rates after five days. This trend was also observed in barley plants under drought treatment, which showed an equal growth rate at later time points which was comparable to the growth rate of control plants [51]. These findings indicate that the plants adapt dynamically to drought stress over time, which may be influenced by complex feedback signals, triggering the different response phases.

We performed RNA-sequencing of three different root zones of seminal roots after 6 h, 24 h and 48 h of water deficit treatment to further understand the genetic regulation underlying the observed phenotypic changes. Our results showed that the root zone was the main driver for the observed transcriptomic divergence (Fig. 2A). These findings are in accordance with previous studies that have documented that comparable tissue types were the major drivers shaping the transcriptomic landscape in maize primary roots [52] and barley seminal roots [53, 54]. The unique transcriptomic landscape of the different root zones further underlines the relevance of spatial resolution to identify the distinct molecular processes that shape water deficit responses in roots.

We computed pairwise contrast between control and water deficit samples for each time point-by-root zone combination individually to identify genes differentially expressed upon water deficit. We found that the number of differentially expressed genes varied between the three time points (Fig. 2B), indicating a temporal response of barley seedlings to water deficit. In contrast to other studies, the number of responsive genes did not increase over time, which was previously observed after 6 h compared to 24 h in whole roots of barley [25] and maize [50] or after 24 h, 48 h, 96 h and 144 h in pearl millet [55]. Instead, we observed highly dynamic responses after 6 h and 48 h with a higher number of responsive genes compared to a stagnant phase at 24 h, where the number of differentially expressed genes was comparably low in the surveyed root zones. This suggests that roots may adapt to water deficit conditions depending on the duration of exposure. Such a change in gene activity over time was also observed in cotton seedlings subjected to PEG treatment, where the largest number of responsive genes was identified only 3 h after stress induction and the lowest number of responsive genes was detected after 24 h [56].

When comparing the differentially expressed genes in the three root zones across the duration of drought stress, we showed that the majority of genes were unique to the respective root zone-by-stress duration combination but also observed overlapping differentially expressed genes at different treatment durations (Fig. 2C). This is in line with results observed in maize under drought stress, where a large number of differentially expressed genes showed a high time point specificity but also some overlap of drought-responsive genes at more time points [57]. Apart from this temporal response, we also found a spatial response, in which we observed differences in differentially expressed gene numbers between the three root zones (Fig. 2C). Our analysis showed, that the number of responsive genes was always lowest in the root cap and meristem and highest in the elongation zone. This is in line with results from maize seedlings subjected to water

stress treatment, where the elongation zone and the cortex were most strongly affected by water deficit [52]. These significant gene expression changes in response to water deficit suggest, that the elongation zone is particularly important for water deficit responses as it might be involved in mediating and maintaining root growth [58] or in adjusting the root structure to cope with water deficit. As observed for the temporal response, some differential genes were shared across root zones, though these were only a minority in comparison to the total number of differentially expressed genes identified in the distinct zones. Such a spatial response was also reported in a comparable experimental setup where the transcriptomes of seminal root zones of barley were subjected to long-term drought stress of 12 days [54]. Regarding the direction of regulation, regulatory directions change between different time points and root zones. This directional change over time was also observed in pearl millet roots under drought stress [55], where it was hypothesized that the regulation of biosynthetic genes especially at later time points may contribute to energy conservation mechanisms since photosynthetic rates under stress are impaired and plants have to prioritize protection over growth to ensure survival [59]. Taken together, these results indicate that water deficit triggers a dynamic and complex spatiotemporal response cascade.

Translocation of various molecules across membranes is crucial for osmotic adjustment under low water conditions [60]. We demonstrated that the gene ontology (GO) terms 'transmembrane transport' and 'transmembrane transporter activity' were enriched in all root zones at different time points (Fig. 3, Figure S3). Gene models associated with these terms involved aquaporins, ABC transporters and NRT1/PTR family proteins (Table S2).

Aquaporins are major intrinsic proteins that enable the transport of water, gases, metal ions and small neutral solutes across membranes. Alteration of transcript and protein abundance leads to changes in transporter activity, which can have versatile effects under water deficit depending on the aquaporin gene [61]. We found most aquaporins to be up-regulated in the meristematic and elongation zone and a few were down-regulated in the differentiation zone. This aligns with the vital role of aquaporins in root growth, supported by the observation that aquaporins are up-regulated in meristems and growing root tissues in barley [62] and broad beans [63]. Moreover, we observed that aquaporins were upregulated at 6 h and 24 h of stress and only a few were down-regulated after 48 h. This is in line with findings in drought-stressed rice and chickpeas, where aquaporins showed complex regulation under water deficit [64, 65].

Several studies showed that ABC transporters [9, 66] and NRT1/PTR family proteins [67] transport the phytohormone ABA, which in turn regulates aquaporin abundance and activity [68, 69]. In our study, we observed differential expression of these two transporter types, which supports the well-established role of an ABA-dependent signaling pathway during water deficit response [70–72]. High ABA levels induce the transcription of enzymes which are important for maintaining the cell redox homeostasis by scavenging reactive oxygen species (ROS) such as oxidases, reductases and peroxidases [73, 74]. We found some stress-responsive GO terms, entailing various of these enzymes (Fig. 3, Figure S3). Among them, peroxidases were the most prominent differentially-regulated genes. The non-uniform differential regulation of genes involved in the redox system was also detected in drought-stressed maize [57] and suggests a complex mechanism for regulating cell redox homeostasis, cell wall integrity and cell growth under water deficit conditions. In summary, our analysis of differential gene expression proposes that water deficit triggers a dynamic and sophisticated spatiotemporal response system involving the alteration of various functions and pathways.

To complement our differential expression analysis, we employed a weighted gene co-expression analysis to identify co-expressed gene modules associated with water deficit treatment. We conducted the analyses separately for each root zone, to focus on the treatment effect. For each root zone-by-duration of drought stress combination, we selected the module with the strongest positive correlation toward treatment for further downstream analysis. Comparisons of eigengene expression in the selected modules revealed a treatment-specific pattern. We then identified hub genes in each selected module, which are the regulatory key genes [18] and identified a significant overrepresentation of differentially expressed hub genes in six of the nine modules (Figure S4). GO enrichment analysis of differential hub genes in these six modules revealed unique and conserved GO term categories in the modules identified in different root zone-by-drought treatment combinations. Most GO term categories were present in most root zone-by-treatment combinations. For instance, the categories "energy metabolism" modulating carbohydrate and sugar metabolic processes (Fig. 5A, C-F) or "protein metabolism" (Fig. 5B-F) were present in five of six combinations and "stress response" in four of six combinations (Fig. 5A, B, D, E) highlighting the importance of GO terms within these categories in drought stress response. Importantly, the individual GO terms and subsequently the hub genes in these conserved categories were different, suggesting a wide range of functional adjustments triggered by water deficit. In contrast to these conserved categories "cell wall-related" functions and "transport" were uniquely

observed in the root cap and the meristem at 6 h (Fig. 5A). Cell walls are dynamic interfaces with the environment and undergo remarkable adaptive alterations in their composition and structure under stress [75]. Up-regulation of cell wall-related genes was also observed in maize [52] and wheat [47] roots under drought stress. The balance between ROS and peroxidases plays a crucial role in cell wall loosening and growth maintenance. The prominence of peroxidases as a group of differentially expressed hub genes exclusively in the root cap and meristem at 6 h (Fig. 5A) might highlight their pivotal role in the initial response to water deficit in the early stages [47, 57]. The enrichment of GO terms associated with transport solely among differential hub genes in the root cap and meristem at 6 h suggests that up-regulation of transporters might be associated with the relocation of essential substrates to maintain cellular homeostasis [47] and enable the transmission of signal molecules necessary to trigger the initial water stress-responsive pathways [76]. Taken together, the integration of differential expression and co-expression network analysis provides a comprehensive overview of the processes and functions underlying plant responses to water deficit stress.

Conclusion

Our research highlights the spatiotemporal response cascades in barley seedling root zones triggered by water deficit for two days. The observed root zone and time-specific mechanisms further underline the importance of investigating stress response mechanisms in a zone-specific manner instead of whole root systems, while considering the temporal dynamics. Our findings contribute to a better understanding of distinct and dynamical changes that shape the plant responses to water deficit and might provide additional targets to enhance plant resilience and reduce the negative impacts of water deficit in agriculture.

Abbreviations

ABA	Abscisic acid
CM	Root cap and meristem
DREB	Dehydration-responsive element binding
DZ	Differentiation zone
EMM	Estimated marginal means
EZ	Elongation zone
GO	Gene ontology
MDS plot	Multidimensional scaling plot
PEG8000	Polyethylene glycol
PFP	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase
ROS	Reactive oxygen species
WGCNA	Weighted gene co-expression network analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10002-0.

Additional file 1: Figure S1. Library sizes of RNA-sequencing samples derived from three different root zones and time points. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ). Blue bars represent control sample libraries, red bars represent water deficit sample libraries. The shade reflects the time point (6 h, 24 h or 48 h) with darker shades for later time points.

Additional file 2: Figure S2. Volcano plots of differentially expressed genes. Significantly up-regulated (FDR < 5%, $\log_2$FC > 1) differentially expressed genes (DEGs) are shown in yellow, down-regulated (FDR < 5%, $\log_2$FC < -1) DEGs are shown in purple. The total number of DEGs are shown in the upper left and right corners of each panel. DEGs that do not exceed the significance threshold are depicted in grey. DEGs were calculated between control and water deficit samples for each root zone and time point (6 h, 24 h and 48 h) separately. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ).

Additional file 3: Figure S3. Enriched molecular functions based on gene ontology (GO) of differentially expressed genes. Only GO terms with ≥ 10 associated genes are shown. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ). The circle size reflects the number of DEGs associated with the respective term and color indicates the average $\log_2$FC of these DEGs. Only significantly enriched terms with $p < 0.05$ based on a Fisher's exact test are shown.

Additional file 4: Figure S4. Venn diagrams with a significant overrepresentation of differential hub genes. Comparison of hub genes (yellow circle) and differentially expressed genes (blue circle) from the corresponding root zone-time point combinations. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ). DEGs were identified after 6 h, 24 h and 48 h. Deviations between expected and observed overlap were calculated based on either Fisher's exact test ($n < 5$) or Pearson's chi-square test ($n \geq 5$) with $p < 0.05$.

Additional file 5: Table S1. Overview of RNA-sequencing raw read output and consecutive pseudo alignment results.

Additional file 6: Table S2. List of significantly differentially expressed genes between control and water deficit samples after 6 h, 24 h and 48 h of treatment in three distinct root zones.

Additional file 7: Table S3. List of all active genes and their assigned modules including gene significance (GS) for the water deficit treatments at 6 h, 24 h and 48 h (D6, D24 and D48) and their functional description.

Additional file 8: Table S4. List of all identified hub genes within selected modules that show a high correlation with water deficit at one respective time point including gene descriptions.

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Authors' contributions

A.K. performed the experiments, analyzed the data and drafted the article. C.M. edited the manuscript. F.H. conceived and coordinated the study and edited the manuscript. All authors approved the final draft.

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Availability of data and materials

The dataset generated and analyzed during the current study is available in the NCBI SRA repository under BioProject accession number PRJNA988922 (https://www.ncbi.nlm.nih.gov/sra/PRJNA988922). All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
Barley seeds were obtained from Dr. Li Guo (University of Bonn). The cultivar 'Morex' can be used for non-commercial purposes without permission, according to current guidelines and legislation of the EU. All local, national or international guidelines and legislation were adhered to in the production of this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Publisher's Note

Supplementary data

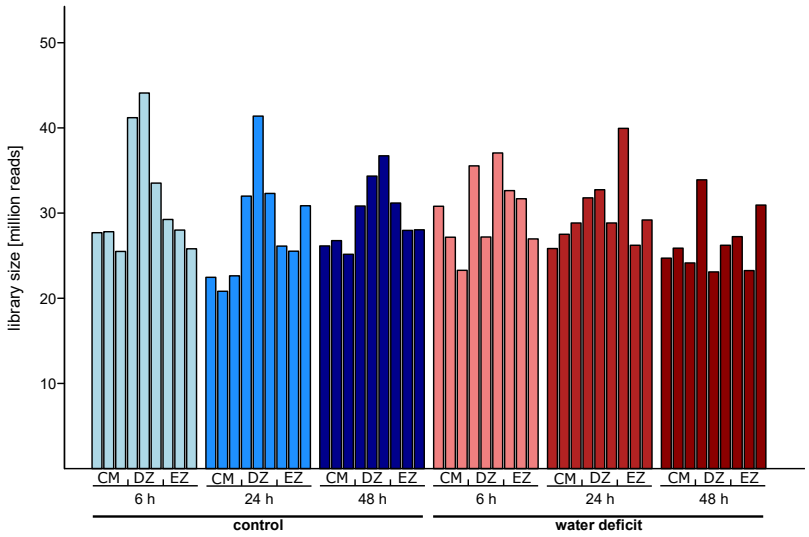

Fig S1. Library sizes of RNA-sequencing samples derived from three different root zones and time points. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ). Blue bars represent control sample libraries, red bars represent water deficit sample libraries. The shade reflects the time point (6 h, 24 h or 48 h) with darker shades for later time points.

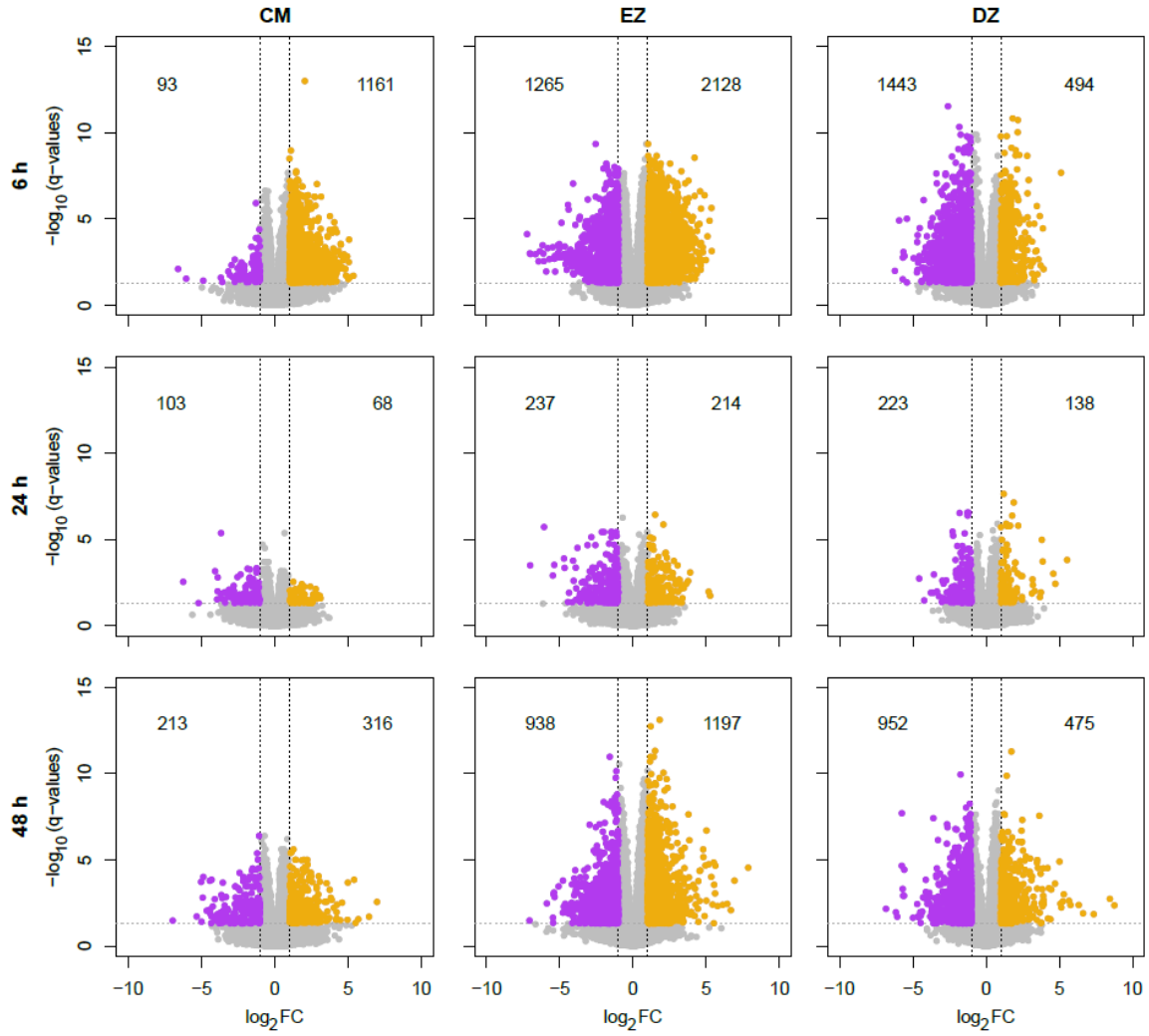

Fig S2. Volcano plots of differentially expressed genes. Significantly up-regulated (FDR <5%, log_2FC >1) differentially expressed genes (DEGs) are shown in yellow, down-regulated (FDR <5%, log_2FC <-1) DEGs are shown in purple. The total number of DEGs are shown in the upper left and right corners of each panel. DEGs that do not exceed the significance threshold are depicted in grey. DEGs were calculated between control and water deficit samples for each root zone and time point (6 h, 24 h and 48 h) separately. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ).

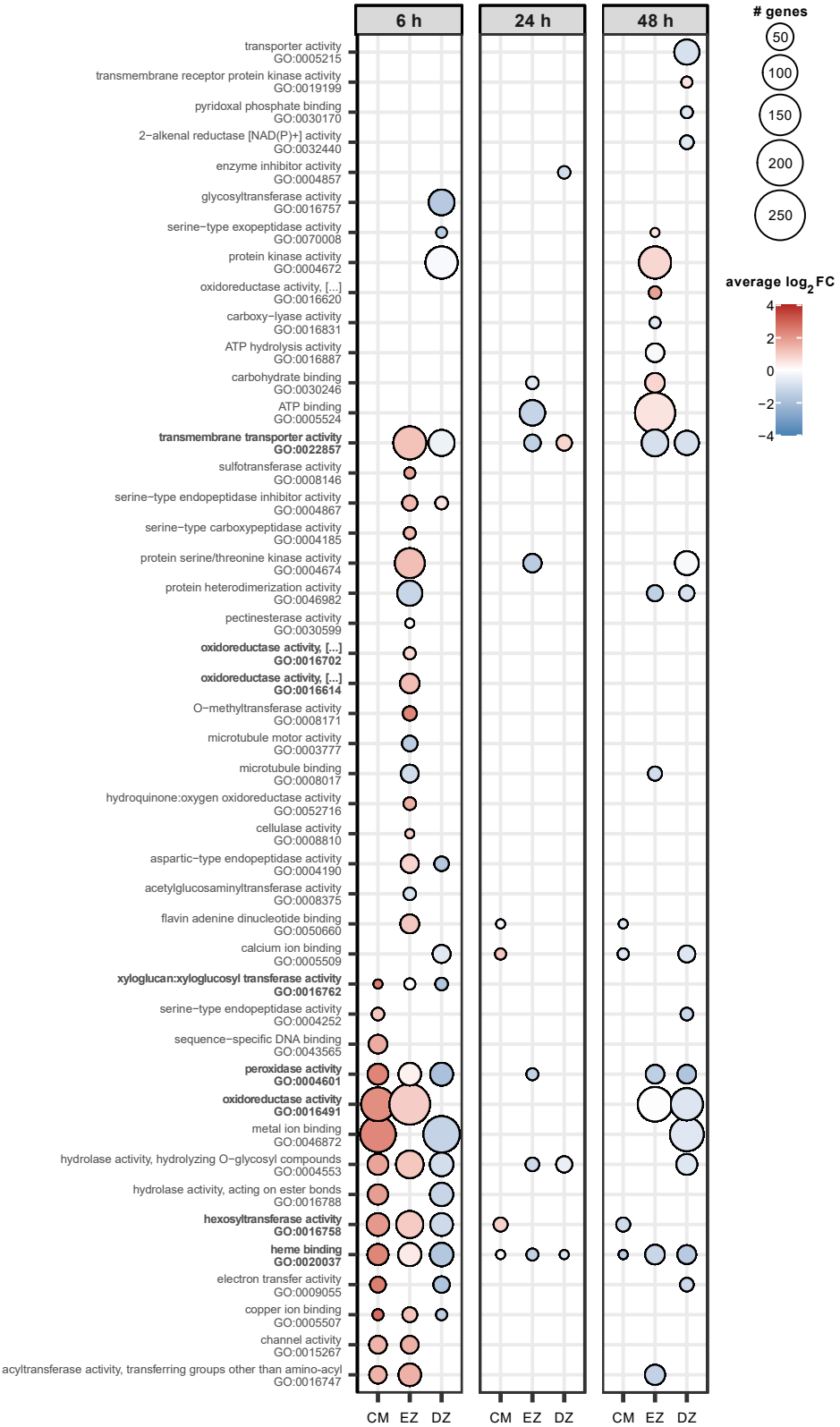

Fig. S3. Enriched molecular functions based on gene ontology (GO) of differentially expressed genes. Only GO terms with ≥10 associated genes are shown. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ). The circle size reflects the number of DEGs associated with the respective term and color indicates the average log_2FC of these DEGs. Only significantly enriched terms with $p < 0.05$ based on a Fisher's exact test are shown.

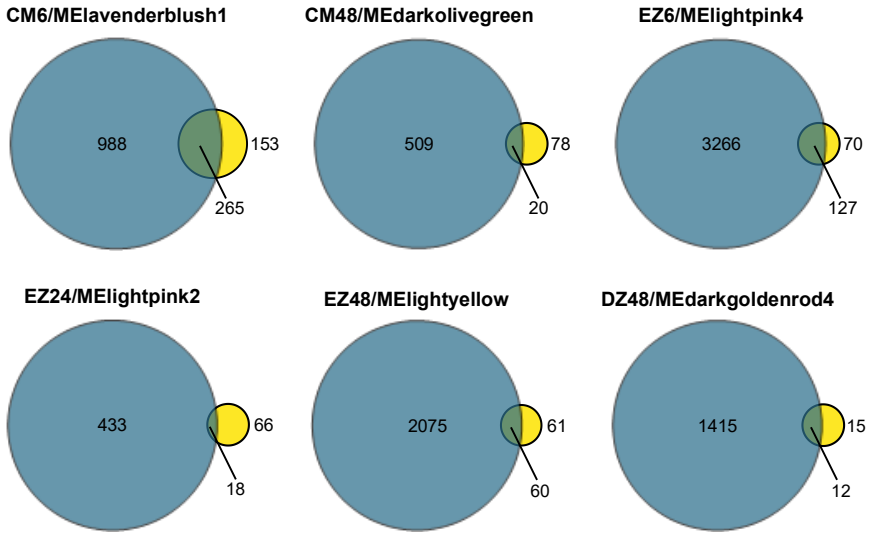

Fig. S4. Venn diagrams with a significant overrepresentation of differential hub genes. Comparison of hub genes (yellow circle) and differentially expressed genes (blue circle) from the corresponding root zone-time point combinations. The root zones are root cap and meristem (CM), elongation zone (EZ) and differentiation zone (DZ). DEGs were identified after 6 h, 24 h and 48 h. Deviations between expected and observed overlap were calculated based on either Fisher's exact test ($n < 5$) or Pearson's chi-square test ($n \geq 5$) with $p < 0.05$.

Table S1. Overview of RNA-sequencing raw read output and consecutive pseudo alignment results.

sample	time point	root zone[1]	replicate	total - raw	total - after quality trimming	total - pseudo aligned	pseudo alignment rate on transcriptome [%]
control	6 h	CM	1	38,271,433	37,163,856	32,841,636	88.4
		CM	2	37,898,712	36,728,501	32,465,389	88.4
		CM	3	34,907,334	33,985,123	30,077,560	88.5
control	24 h	CM	1	45,845,418	44,373,838	38,761,395	87.4
		CM	2	41,267,338	40,119,651	35,083,869	87.4
		CM	3	49,371,893	47,878,113	41,672,825	87.0
control	48 h	CM	1	37,072,122	35,964,237	31,513,125	87.6
		CM	2	38,270,661	36,880,113	32,543,651	88.2
		CM	3	35,220,661	34,174,479	30,257,729	88.5
water deficit	6 h	CM	1	42,932,448	41,487,410	36,446,539	87.8
		CM	2	37,874,233	36,547,198	32,079,574	87.8
		CM	3	32,596,295	31,524,285	27,503,289	87.2
water deficit	24 h	CM	1	30,689,031	29,806,782	26,151,913	87.7
		CM	2	33,672,426	32,737,765	28,842,195	88.1
		CM	3	32,090,683	31,074,814	27,171,978	87.4
water deficit	48 h	CM	1	33,838,342	32,882,276	29,000,108	88.2
		CM	2	35,189,529	34,255,444	30,340,358	88.6
		CM	3	33,275,519	32,323,703	28,336,664	87.7
control	6 h	EZ	1	33,275,877	31,984,443	28,894,358	90.3
		EZ	2	38,068,089	36,720,916	32,286,535	87.9
		EZ	3	36,501,929	35,457,759	31,025,494	87.5

control	24 h	EZ	1	48,450,221	46,738,095	41,181,859	88.1
		EZ	2	42,746,342	41,408,019	36,008,218	87.0
		EZ	3	39,836,075	38,508,770	34,055,354	88.4
control	48 h	EZ	1	40,726,853	39,364,195	34,125,205	86.7
		EZ	2	37,212,735	35,950,622	30,946,295	86.1
		EZ	3	36,293,885	35,110,348	30,647,077	87.3
water deficit	6 h	EZ	1	41,278,973	40,042,854	35,469,171	88.6
		EZ	2	40,192,129	39,139,500	34,455,089	88.0
		EZ	3	34,763,731	33,708,607	29,282,607	86.9
water deficit	24 h	EZ	1	29,294,253	28,414,685	24,355,731	85.7
		EZ	2	37,418,864	36,446,301	31,665,116	86.9
		EZ	3	32,048,785	31,156,152	26,665,332	85.6
water deficit	48 h	EZ	1	34,298,728	33,304,255	29,506,710	88.6
		EZ	2	29,414,056	28,462,759	25,143,561	88.3
		EZ	3	38,914,805	37,841,922	33,565,400	88.7
control	6 h	DZ	1	52,335,006	50,856,898	44,631,985	87.8
		DZ	2	56,297,907	54,503,122	47,602,516	87.3
		DZ	3	42,736,536	41,391,519	36,135,719	87.3
control	24 h	DZ	1	44,996,093	43,651,274	38,700,707	88.7
		DZ	2	39,792,327	38,757,327	34,126,031	88.1
		DZ	3	38,351,949	37,121,165	32,418,175	87.3
control	48 h	DZ	1	40,341,175	39,119,596	33,444,779	85.5
		DZ	2	44,144,996	42,763,789	37,302,682	87.2
		DZ	3	46,695,821	45,294,801	39,630,812	87.5
	6 h	DZ	1	45,654,602	44,261,690	38,265,693	86.5

water deficit		DZ	2	35,027,790	33,987,585	29,250,908	86.1
		DZ	3	47,278,180	45,710,822	39,816,696	87.1
water deficit	24 h	DZ	1	42,770,651	41,526,560	34,734,978	83.6
		DZ	2	39,336,347	38,232,417	32,390,090	84.7
		DZ	3	29,343,570	28,540,654	24,249,198	85.0
water deficit	48 h	DZ	1	42,851,421	41,674,128	36,559,801	87.7
		DZ	2	30,380,301	29,498,209	25,107,025	85.1
		DZ	3	33,937,559	32,862,075	28,286,058	86.1
	Average			38,764,679	37,581,878	32,833,755	87.4
	Minimum			29,294,253	28,414,685	24,249,198	83.6
	Maximum			56,297,907	54,503,122	47,602,516	90.3

[1] CM= root cap and meristem, EZ= elongation zone, DZ= differentiation zone

Online supplementary data

Table S2. List of significantly differentially expressed genes between control and water deficit samples after 6 h, 24 h and 48 h of treatment in three distinct root zones.

Table S3. List of all active genes and their assigned modules including gene significance (GS) for the water deficit treatments at 6 h, 24 h and 48 h (D6, D24 and D48) and their functional description.

Table S4. List of all identified hub genes within selected modules that show a high correlation with water deficit at one respective time point including gene descriptions.

6.2 Publications unrelated to this thesis

- **Population genetics revealed a new locus that underwent positive selection in barley**

  Stephan Reinert, **Alina Osthoff**, Jens Léon and Ali Ahmad Naz

  International Journal of Molecular Science 2019, **20**: 202. doi: 10.3390/ijms20010202

- **Osmotic stress enhances suberization of apoplastic barriers in barley seminal roots: analysis of chemical, transcriptomic and physiological responses**

  Tino Kreszies, Nandhini Shellakkutti, **Alina Osthoff**, Peng Yu, Jutta Baldauf, Viktoria Zeisler-Diehl, Kosala Ranathunge, Frank Hochholdinger and Lukas Schreiber

  New Phytologist 2019, **221**(1): 180-194. doi: 10.1111/nph.15351

- **Seminal roots of wild and cultivated barley differentially respond to osmotic stress in gene expression, suberization and hydraulic conductivity**

  Tino Kreszies, Stella Eggels, Viktoria Kreszies, **Alina Osthoff**, Nandhini Shellakkutti, Jutta Baldauf, Viktoria Zeisler-Diehl, Frank Hochholdinger, Kosala Ranathunge and Lukas Schreiber

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- **Cuticular transpiration is not affected by enhanced wax and cutin amounts in response to osmotic stress in barley**

  Nandhini Shellakkutti, Priya Dharshini Thangamani, Kiran Suresh, Johanna Baales, Viktoria Zeisler-Diehl, **Alina Klaus**, Frank Hochholdinger, Lukas Schreiber and Tino Kreszies

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- **High robustness of cytosolic glutathione redox potential under combined salt and osmotic stress in barley as revealed by the biosensor Grx1-roGFP2**

  Finja Bohle, **Alina Klaus**, Hendrik Tegethof, Markus Schwarzländer, Frank Hochholdinger, Andreas J. Meyer, Ivan F. Acosta and Stefanie J. Müller-Schüssele

  bioRxiv 2022. doi: 10.1101/2022.12.22.521445v1

- **ENHANCED GRAVITROPISM 2 coordinates molecular adaptations to gravistimulation in the elongation zone of barley roots**

  Li Guo, **Alina Klaus**, Marcel Baer, Gwendolyn Kirschner, Silvio Salvi and Frank Hochholdinger

  New Phytologist 2023, **237**(6)*: 180-194. doi: 10.1111/nph.18717*

- **Bacterium-enabled transient gene activation by artificial transcription factors for resolving gene regulation in maize**

  Mingxia Zhao, Zhao Peng, Yang Qin, Tej Man Tamang, Ling Zhang, Bin Tian, Yueying Chen, Yan Liu, Junli Zhang, Guifang Lin, Huakun Zheng, Cheng He, Kaiwen L, **Alina Klaus**, Caroline Marcon, Frank Hochholdinger, Harold Trick, Yunjun Liu, Myeong-Je Cho, Sunghun Park, Hairong Wei, Jun Zheng, Frank White and Sanzhen Liu

  The Plant Cell 2023, **35**(8)*: 2736-2749. doi: 10.1093/plcell/koad155*

6.3 Presentations at conferences

6.3.1 Oral presentations

- „Root zone-specific transcriptomic reprogramming of barley roots in response to water deficit"
  ISRR11/ ROOTING21
  Riga, Latvia (3rd – 7th July 2023)

6.3.2 Poster presentations

- "Root zone-specific transcriptomic reprogramming of barley roots in response to water deficit"
  ISRR11/ ROOTING21
  Riga, Latvia (3rd – 7th July 2023)

- "Tissue-specific transcriptomic reprogramming of Barley roots subjected to drought"
  IBGS 13
  Virtual (8th – 12th March, 2021)

- "Transcriptomic adaptions in young barley roots exposed to a combination of salt and drought stress"
  ISRR10
  Jerusalem, Israel (8th – 12th July, 2018)

- "Root transcriptomic responses to water deficit and salt stress during early barley development"
  German Plant Breeding Conference
  Wernigerode, Germany (28th February – 3rd March, 2018)

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