


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

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Funding information

Australian Research Council, Grant/Award Number: Grant Number: FT170100195; Deutsche Forschungsgemeinschaft, Grant/Award Number: GRK 2064

Abstract

Wild barley, *Hordeum vulgare* spp. *spontaneum*, has a wider genetic diversity than its cultivated progeny, *Hordeum vulgare* spp. *vulgare*. Osmotic stress leads to a series of different responses in wild barley seminal roots, ranging from no changes in suberization to enhanced endodermal suberization of certain zones and the formation of a suberized exodermis, which was not observed in the modern cultivars studied so far. Further, as a response to osmotic stress, the hydraulic conductivity of roots was not affected in wild barley, but it was 2.5-fold reduced in cultivated barley. In both subspecies, osmotic adjustment by increasing proline concentration and decreasing osmotic potential in roots was observed. RNA-sequencing indicated that the regulation of suberin biosynthesis and water transport via aquaporins were different between wild and cultivated barley. These results indicate that wild barley uses different strategies to cope with osmotic stress compared with cultivated barley. Thus, it seems that wild barley is better adapted to cope with osmotic stress by maintaining a significantly higher hydraulic conductivity of roots during water deficit.

KEYWORDS

apoplast, barley, osmotic stress, root, suberin, transcriptomics, water deficit, water transport, wild barley

1 | INTRODUCTION

Climate change will lead to longer and more frequent drought periods as well as more extreme weather conditions in the future. This will lead to significant yield losses of crops (Challinor et al., 2014; Kang, Khan, & Ma, 2009). *Hordeum vulgare* L. is known to be one of the most tolerant crop species towards abiotic stresses such as drought and salinity (Colmer, Flowers, & Munns, 2006; Kosová, Vítámvás, & Prášil, 2014). As a crop plant, barley is almost

as important as wheat, maize, and rice (Mascher et al., 2016; Mayer et al., 2012). However, because of its early domestication around 10,000 years ago and modern breeding strategies to achieve higher yields, much of its allelic variation has been lost. Reduced genetic diversity is often linked to a higher susceptibility towards various environmental stresses (Nevo & Chen, 2010; Tanksley & McCouch, 1997). Modern cultivated barley, *H. vulgare* ssp. *vulgare*, is derived from its wild progenitor *H. vulgare* ssp. *spontaneum*, which originates from the fertile crescent (Badr et al., 2000; Harlan & Zohary, 1966).

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Therefore, wild barley is adapted to a range of arid to semiarid habitats and exhibits a wider diversity than cultivated barley. However, still today, wild and cultivated barley can be crossed, and progenies are fully fertile. Thus, beneficial traits of wild barley can be reintroduced to cultivated barley (Ellis et al., 2000; Gunasekera, Santakumari, Glinka, & Berkowitz, 1994).

One trait that could be crucial for plant survival during periods of water deficit is root suberization. Plant roots are the organs that take up water from the surrounding soil, they sense soil water status, they transduce signals during water deficit, and they play a central role in adjusting the plant's capacity to take up water during water deficit (Zingaretti, Inácio, de Matos Pereira, Antunes Paz, & de Castro França, 2013). According to the composite transport model, water transport in plant roots can occur through the apoplastic, symplastic, and transcellular pathways (Steudle, 2000a; Steudle & Peterson, 1998). Symplastic and transcellular pathways are often referred together as the cell-to-cell pathway. Resistances of these pathways can be regulated and modified by suberin deposition and plasma membrane-bound aquaporins (Kim et al., 2018; Kreszies, Schreiber, & Ranathunge, 2018; Steudle, 2000a, 2000b; Steudle & Peterson, 1998). The hydrophobic biopolymer suberin is the main component of apoplastic barriers of roots, and it plays a significant role in water and nutrient transport (Franke & Schreiber, 2007; Ranathunge, Schreiber, & Franke, 2011). Suberin can be found in distinct cell layers such as the endodermis and exodermis of primary roots. The increase of suberization of roots in response to abiotic stresses such as water deficit, salinity, or hypoxia has been shown in the past (Barberon et al., 2016; Enstone, Peterson, & Ma, 2002; Hose, Clarkson, Steudle, Schreiber, & Hartung, 2001; Kotula, Schreiber, Colmer, & Nakazono, 2017; Kreszies et al., 2019; Krishnamurthy et al., 2009; Ranathunge, Lin, Steudle, & Schreiber, 2011). The biopolymer suberin contains an aliphatic and an aromatic domain, which are cross-linked via ester bounds. The hydrophobic aliphatic domain establishes the barrier properties for water transport, whereas it is suggested that the aromatic domain connects the polyester to the primary cell wall (Graça, 2015; Kolattukudy, Kronman, & Poulouse, 1975; Zimmermann, Hartmann, Schreiber, & Steudle, 2000). The aliphatic domain contains mainly long chain fatty acid derivatives with ω -hydroxy acids and α,ω -dicarboxylic acids, primary fatty acids, and alcohols as predominant substance classes. The aromatic components are mostly coumaric and ferulic acids (Bernards, 2002; Ranathunge, Schreiber, & Franke, 2011).

In a recent study, we reported the effect of osmotic stress on the development of root suberization in seminal roots of the barley cultivar Scarlett (Kreszies et al., 2019). In the study presented here, we investigated three additional modern barley cultivars (Golden Promise, Morex, and Barke) and three selected wild barley accessions (ICB181160 [Iran], ICB181243 [Pakistan], and ICB181466 [Jordan]). It was the aim to compare the response of modern cultivars to osmotic stress with the response of the wild barley, which we hypothesized to be more stress-tolerant. In fact, our findings indicate that the wild barley accessions use different strategies to cope with osmotic stress compared with the modern cultivated barley plants. All cultivated barley varieties showed a similar reaction towards osmotic stress, increasing the amount of suberin

in roots as an adaptation to prevent uncontrolled passive water loss from the root to the surrounding environment. In contrast, wild barley accessions had a series of different strategies to deal with osmotic stress, ranging from suberization of specific root tissues or only specific root zones, or no changes in suberization at all. Whereas in cultivated barley, water transport was significantly reduced in response to osmotic stress and suberization enhanced, in roots of wild barley, water uptake rates did not change at all and remained constant.

2 | MATERIAL AND METHODS

2.1 | Plant material and growth conditions

Seeds of cultivated barley (*H. vulgare* spp. *vulgare*) varieties Barke, Morex, and Golden Promise and of wild barley accessions (*H. vulgare* spp. *sponataneum*) ICB181160 (Iran), ICB181243 (Pakistan), and ICB181466 (Jordan) were stratified for 1 week at 4°C. For simplicity, wild barley accessions are referred to by the name of their country of origin. Seeds were germinated in the dark at 25°C and covered with wet filter papers. Three days later, seedlings were transferred to an aerated hydroponic system containing half-strength Hoagland solution (Hoagland & Arnon, 1950) in a climatic chamber under long day conditions (16/8 hr, light/dark), an air temperature of 23/20°C (day/night), and a relative humidity of 50–65%. When plants were 6 days old (3 days of germination and 3 days of growth), they were transferred to osmotic stress or control solution for further 6 days until they were 12 days old. At this stage, they had two leaves and five to six seminal roots.

2.2 | Water deficit application induced by osmotic stress through PEG8000

Osmotic stress was applied when the plants were 6 days old. Plants were moved from half-strength Hoagland solution (20 mOsmol·kg⁻¹ or -0.05 MPa of osmotic pressure) to half-strength Hoagland solution adjusted to a defined water potential of -0.8 MPa by adding 25.4% (w/w) PEG8000 (Roth, Karlsruhe, Germany; Michel, 1983). This water potential of the medium of -0.8 MPa mimicked water deficit or physiological drought of plants. The water potentials of the nutrient solution as well as the nutrient solution containing PEG8000 were verified using both an osmometer (Gonotec Osmomat 030; Gonotec GmbH, Berlin, Germany) and a WP4C Water Potential Meter (METER Group, USA).

2.3 | Histochemical detection of Casparian bands and suberin lamellae in roots

Cross-sections were made over the whole length of seminal roots using a cryostat microtome (Microm HM 500M, Microm International, Walldorf, Germany). Formation and development of Casparian bands over the root length was studied by staining cross-sections with 0.1% (w/v) berberine hemisulfate for 1 hr and with 0.5% (w/v) aniline blue for 30 min (Brundrett, Enstone, & Peterson, 1988). Suberin lamellae were stained with 0.01% (w/v) lipophilic fluoral yellow 088 for 1 hr (Brundrett, Kendrick, & Peterson, 1991). Cross-sections were

observed under an epifluorescence microscope using an ultraviolet filter set (excitation filter BP 365, dichroic mirror FT 395, barrier filter LP 397; Zeiss, Oberkochen, Germany). Pictures were taken with a Canon EOS 600D camera at ISO 200 or 400 for 1- to 2-s exposure time.

2.4 | Chemical analysis of barley root suberin

Seminal roots were divided into three zones: the root tip zone A (0–25% of total root length), the transition zone B (25–50% of total root length), and the basal zone C (50–100% of total root length) as described earlier (Kreszies et al., 2019). Zone A was the youngest part of the root including the root apex. Here, only Casparian bands were present but no suberin lamellae deposited in the endodermis. In zone B, all endodermal cells had Casparian bands, but only a limited number of cells had suberin lamellae depositions. In zone C, which was the mature part of the root close to the root base, all endodermal cells were characterized by the presence of Casparian bands and suberin lamellae. For each replicate, 10 segments of each zone were pooled together. Lateral roots, which started to develop in the zone C, were removed with a razor blade. For gas chromatography, root segments were enzymatically digested, soluble lipids extracted and the remaining samples were transesterified using BF_3 -methanol as described earlier (Kolattukudy & Agrawal, 1974; Kreszies et al., 2019; Zeier & Schreiber, 1997, 1998). Three independent biological replicates were used for each experiment.

2.5 | Root pressure probe experiments

Root pressure probe experiments were conducted with the end segments/apical part of the seminal roots lacking lateral roots (zones A and B together) as described earlier (Kreszies et al., 2019; Ranathunge et al., 2017; Steudle, Oren, & Schulze, 1987). Plants grown in -0.8 MPa PEG8000 solution were transferred back to half-strength Hoagland nutrient solution at least 1 hr before the measurements. Hydrostatic experiments were carried out by moving the metal rod forward and backward to induce radial water flow out or into the root. The subsequent pressure changes were used to calculate the hydrostatic hydraulic conductivity (L_p , (Hy)). Osmotic experiments were induced by exchanging the nutrient solution with nutrient solution containing 30-mM NaCl (60 mOsmol $\cdot\text{kg}^{-1}$). This resulted in a biphasic change in pressure, where the rapid water phase was used to calculate the osmotic hydraulic conductivity (L_p , (Os)), and the slower solute phase was used to calculate the solute permeability (P_{sr}) and the reflection coefficient (σ_{sr}) for NaCl. Five independent biological replicates were used for each experiment.

2.6 | Determination of osmotic potential and proline in barley roots

For the measurement of the osmotic potential, five seminal roots were ground in a mixer mill (Retsch MM400; Retsch GmbH, Haan, Germany) at a frequency of 30 rounds s^{-1} for 1 min. Subsequently, samples were

centrifuged at 10,000 rpm for 2 min, and the concentration of the supernatant was measured with a freezing point osmometer (Gonotec Osmomat 030; Gonotec GmbH, Berlin, Germany). The resulting concentration in mOsmol $\cdot\text{kg}^{-1}$ was converted to osmotic potential using the van't Hoff equation: $\Psi = MiRT$ with M = concentration in molarity, i = van't Hoff factor, R = ideal gas constant, T = absolute temperature (K). Three biological replicates were used for each experiment. Proline measurements were performed photometrically as previously described by Bates, Waldren, and Teare (1973), where seven biological replicates were used for each subspecies.

2.7 | RNA isolation and cDNA library construction

RNA was isolated from the three root zones of five 12-day-old seminal roots of wild barley Pakistan (ICB181243) grown under stress or control conditions. RNA was isolated from root samples frozen in liquid nitrogen with the RNeasyPlus Universal Mini Kit (Qiagen, Venlo, Netherlands). Each zone by treatment combination was harvested in four biological replicates. RNA integrity was determined with the Agilent RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA integrity number ≥ 8.0 were used for subsequent experiments. cDNA libraries for RNAseq were constructed with the TruSeq RNA sample preparation kit as described by the manufacturer (Illumina, San Diego CA, USA). Each library was indexed with an Illumina TruSeq Adapter. Cluster preparation and paired-end sequencing were performed according to the manufacturer's protocol (HiSeq 4000, Illumina).

2.8 | Processing of raw reads and analysis of differentially expressed genes (RNA-sequencing)

Raw sequencing 100 bp paired-end reads were quality trimmed by removing low-quality reads and adapter sequences by CLC genomics Workbench Version 12.0 (<https://www.qiagenbioinformatics.com/>) as previously described (Kreszies et al., 2019). Reads with a minimum length of 40 bp that mapped uniquely and exceeded a threshold of similarity $\geq 90\%$ and length fraction $\geq 80\%$ were retained for mapping to the high-confidence annotation of the genome sequence (Mascher et al., 2017; ftp://ftp.ensemblgenomes.org/pub/plants/release-42/gff3/hordeum_vulgare/; v2.42). Read counts were normalized by sequencing depth and \log_2 -transformed to meet the assumptions of a linear model and adjusted for heteroscedasticity (Law, Chen, Shi, & Smyth, 2014). The Bioconductor package limma (Smyth, 2005) in R (R Version 3.4.0, limma_3.32.2) was used to test the data quality via a multidimensional scaling plot. To assess differences in gene expression between osmotic stress treatment and control in each root tissue of the Pakistan wild barley and for the comparison between the Pakistan wild barley and the cultivar Scarlett (Raw Data from Kreszies et al., 2019, SRA accession: SRP136092), linear models were fitted, including a fixed effect for tissue and treatment or tissue and genotype and an interaction effect for both terms. After shrinking the variances over all genes in the fitted model towards a common value by

using an empirical Bayes approach (Smyth, 2004), pair-wise comparisons between stress treatment and control treatment for each tissue and between the different genotypes were computed. According to Benjamini and Hochberg (1995), p values were adjusted for multiplicity with a false discovery rate (FDR) of $\leq 5\%$. The raw sequencing data have been deposited at the NCBI sequence read archive (Scarlett SRA accession: SRP136092; ICB181243 [Pakistan] SRA accession: PRJNA543388).

2.9 | Functional annotation and GO analysis

For Gene Ontology (GO) analysis of differentially expressed genes, the AgriGOv2.0 toolkit (Tian et al., 2017) was used for Singular Enrichment Analysis (SEA) by comparing the lists of differentially expressed genes. The cross comparison of SEA (SEACOMPARE) tool was used to combine the SEA results. The gene list from Kreszies et al. (2019) and EnsemblPlants (Kersey et al., 2018) was used for identification of putative barley orthologous.

2.10 | Statistical analysis of chemical and physiological data

Data analysis and statistical tests were performed with Origin Pro 9. A normal distribution of the data was tested with the Shapiro–Wilk test. Significant differences between means were tested with two-sample t test, one-way analysis of variance (ANOVA; Fisher LSD) or two-way ANOVA (growth conditions vs. genotype; Fisher LSD). All tests were performed with a significance level of 0.05.

3 | RESULTS

3.1 | Root and shoot length, biomass, root morphology, and anatomy

Under control conditions, seminal roots of wild barley accessions were significantly longer in most cases compared with roots of cultivated barley. Roots exposed to osmotic stress were always significantly shorter than roots from control conditions (Figure 1a). Similarly, the shoots of wild barley accessions were significantly longer than the shoots of cultivated barley. In response to osmotic stress, the shoots of all barley species were significantly shorter compared with control conditions (Figure 1b). Because the reduction of shoot lengths in response to osmotic stress was more pronounced than reduction of the root lengths, root/shoot ratios significantly increased for all investigated barley lines, with the exception of Pakistan (Figure 2, Table S1).

Seminal roots of all investigated barley cultivars and wild types showed one large central late metaxylem vessel together with eight to nine early metaxylem vessels (Figure 3, Figure S1). On average, the central cortex had five cell layers. Endodermal Casparian bands were visible in all barley seminal roots at about 5–10% distance from the root tip. These bands were continuous in the radial endodermal cell wall over the length of the root. When exposed to osmotic stress,

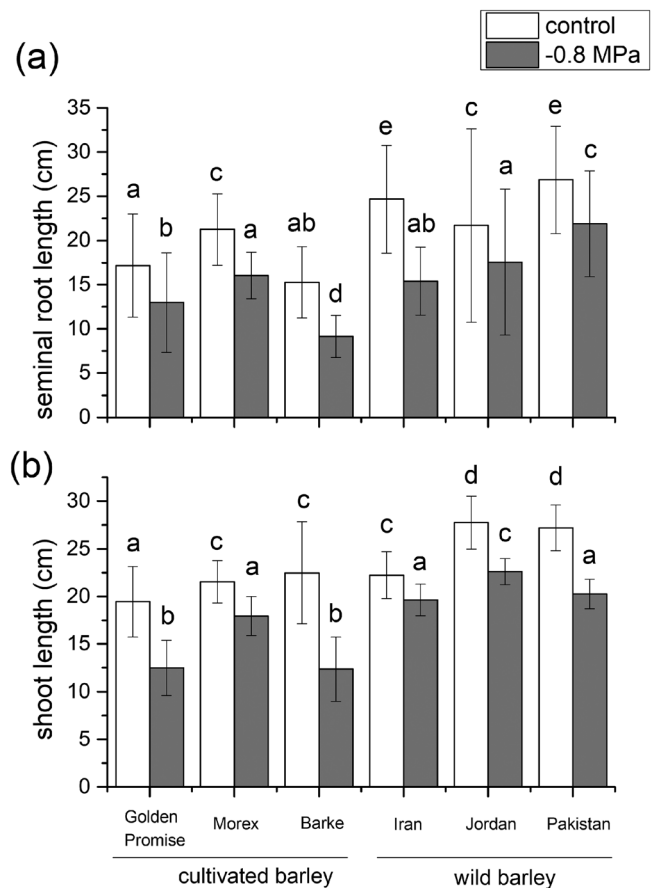


FIGURE 1 (a) Seminal root lengths and (b) shoot lengths of 12-day-old cultivated and wild barley plants, either grown under control conditions or osmotic stress at a water potential of -0.8 MPa. All roots from osmotic stress were significantly shorter than control roots, and all wild barley accession roots were significantly longer than cultivated barley, except for Jordan (ICB181466). The bars represent the mean values with standard deviation of (a) more than 150 individual seminal roots ($n > 150$) or (b) 20 individual plants ($n = 20$). Different letters indicate significant differences between means at a significance level of 0.05 by one-way analysis of variance (Fishers LSD test)

approximately 20% of seminal roots of Jordan plants induced an exodermis with Casparian bands (Figure 3b,c) and suberin lamellae (Figure 3e,f) near the root base. All other wild and cultivated barley accessions did not develop an exodermis neither under control nor osmotic stress conditions (Figure 3a, d, Figure S1).

3.2 | Chemical analysis of suberin of barley seminal roots in response to osmotic stress

For chemical analysis of suberin, barley seminal roots were divided into three root zones based on the degree of endodermal suberization detectable in fluorescence microscopy (zone A: no suberization visible; zone B: patchy suberization visible; zone C: full suberization). Single monomer classes of the aliphatic suberin fraction were alcohols (alc), fatty acids (fa), α,ω -dicarboxylic acids (diacids), and ω -hydroxy acids (ω -OH acids). The $C_{18:1}$ diacid and ω -OH acids

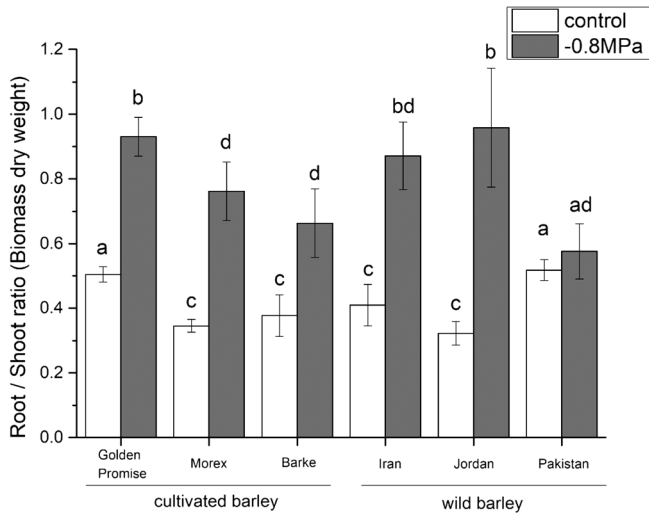


FIGURE 2 Root/Shoot ratio from dry weight biomass of 12-day-old cultivated and wild barley plants, either grown under control or osmotic stress at a water potential of -0.8 MPa. The bars represent the mean values with standard deviation of five independent biological replicates ($n = 5$). Different letters indicate significant differences between means at a significance level of 0.05 by one-way analysis of variance (Fishers LSD test)

($C_{18:1}$ and C_{24} ω -OH acids) were the most abundant aliphatic suberin constituents in barley seminal roots. The chain lengths varied from C_{16} to C_{26} . Aromatic suberin components were essentially composed of coumaric and ferulic acids. There were no major detectable differences in substance classes or single monomer composition between control and osmotic stress, or between wild and cultivated barley plants (Figures S2 and S3).

Barley seminal roots showed a significant increase in total aliphatic (Figure 4) and aromatic suberin amounts (Figure S3) during maturation from zone A to C. Comparing the effect of osmotic stress on the degree of aliphatic suberization between the cultivars and wild barley accessions, there were no significant differences observed in zone A. In zone B, osmotic stress increased aliphatic suberization by twofold only in the modern cultivars compared with their controls, whereas there was no change in the wild barley subspecies. In zone C, all cultivated and wild barley plants, with the exception of Jordan, showed a significant increase in aliphatic suberization in response to osmotic stress (Figure 4).

3.3 | Hydraulic conductivity, solute permeability, and reflection coefficient of barley seminal roots in response to osmotic stress

Hydraulic conductivities (L_{p_r}), solute permeabilities (P_{sr}), and reflection coefficients (σ_{sr}) for NaCl were measured in Morex (cultivated barley) and Pakistan (wild barley) comparing plants exposed to osmotic stress (-0.8 MPa) to control conditions. The hydrostatic L_{p_r} , which gives the overall root water uptake, was significantly lower in the wild accession Pakistan compared with the modern cultivar Morex under control conditions. Enhanced root suberization under osmotic stress significantly decreased the hydrostatic L_{p_r} of Morex. In Pakistan, where aliphatic suberin was not induced in the zones of A and B in response to osmotic stress, the hydrostatic L_{p_r} was not decreased (Table 1 and Figure 4). The osmotic L_{p_r} , which gives the water uptake via the cell-to-cell path, was the same in roots of Morex and Pakistan under control conditions. With

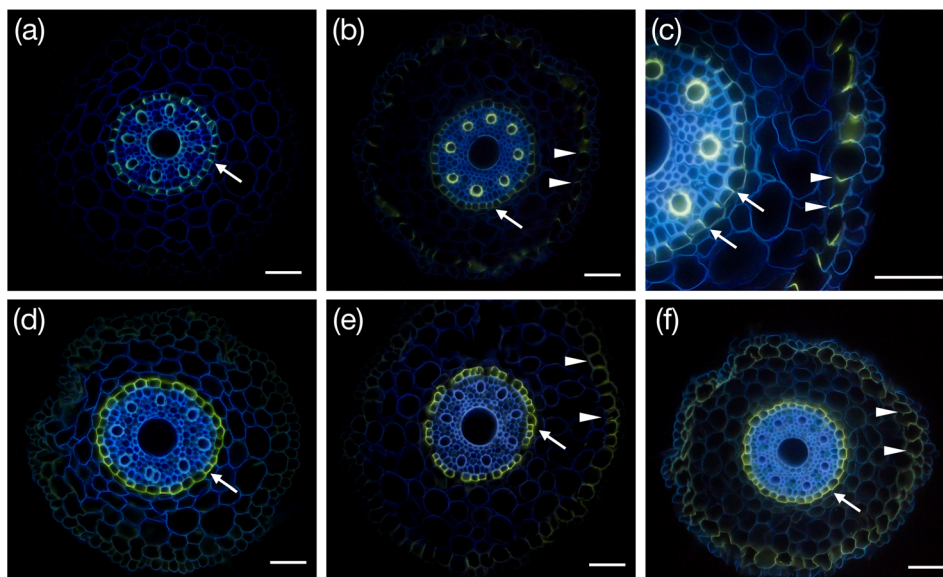


FIGURE 3 Cross-sections of the basal part (zone C) of Jordan (ICB181466) seminal roots. All cross-sections were stained either with berberine aniline blue (a–c) or fluoro yellow 088 (d–f). An inducible exodermis in Jordan occurs in 20% of the seminal roots in response to osmotic stress (b, c, e, and f). The presence of Casparian bands is indicated by a yellowish fluorescence (a, b, and c; arrows: endodermis, arrowheads: exodermis). The presence of a suberin lamellae is indicated by a bright yellowish fluorescence (d, e, and f; arrows: endodermis, arrowheads: exodermis). Bars = 50 μ m

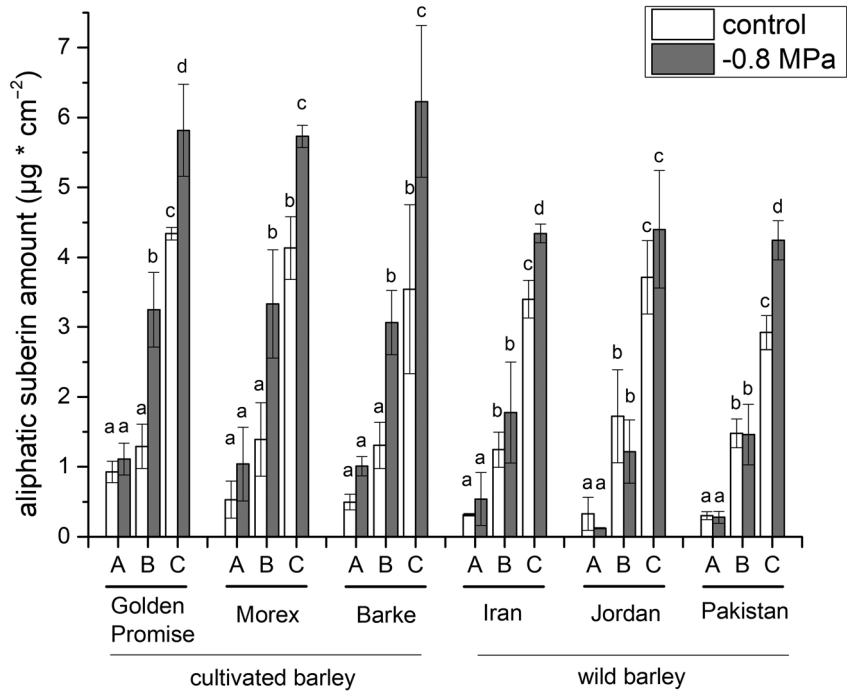


FIGURE 4 Total amounts of aliphatic suberin in barley seminal roots grown either under control conditions or at a water potential of -0.8 MPa. The roots were divided into three root zones: the root tip zone A (0–25% of the root length), the transition zone B (25–50% of the root length), and the basal zone C (50–100% of the root length). The bars represent the mean values with standard deviation of three independent biological replicates ($n = 3$). Different letters indicate significant differences between means at a significance level of 0.05 by one-way analysis of variance (Fishers LSD test)

TABLE 1 Hydrostatic and osmotic hydraulic conductivity (L_{pr}), solute permeability (P_{sr}), and reflection coefficient (σ_{sr}) for NaCl of individual barley seminal roots grown under control or osmotic stress (water potential of -0.8 MPa)

Parameters	Morex		Pakistan	
	Control	Osmotic stress (-0.8 MPa)	Control	Osmotic stress (-0.8 MPa)
Hydrostatic L_{pr} ($10^{-8} \text{ m}\cdot\text{s}^{-1}\cdot\text{MPa}^{-1}$)	9.75 ± 3.36 a	3.39 ± 1.95 b	6.29 ± 1.8 c	6.49 ± 2.5 c
Osmotic L_{pr} ($10^{-8} \text{ m}\cdot\text{s}^{-1}\cdot\text{MPa}^{-1}$)	2.45 ± 1.77 a	3.05 ± 1.91 a	2.87 ± 0.96 a	4.60 ± 2.26 a
Hydrostatic/Osmotic	4.68 ± 2.39 a	1.11 ± 0.36 b	2.14 ± 0.87 ab	1.42 ± 0.54 ab
Solute permeability P_{sr} (10^{-9} m s^{-1})	0.57 ± 0.81 a	0.41 ± 0.51 a	0.94 ± 0.81 a	2.25 ± 1.70 a
Reflection coefficient (σ_{sr})	0.57 ± 0.08 a	0.61 ± 0.17 a	0.29 ± 0.14 b	0.31 ± 0.10 b

Note. Values are given as means with standard deviation of five independent replicates ($n = 5$). Different letters indicate significant differences at 0.05 level in one-way analysis of variance (Fishers LSD test).

enhanced root suberization in response to osmotic stress, the osmotic L_{pr} was not significantly decreased in both Morex and Pakistan. Yet, there was a slight trend that osmotic stress slightly increased the osmotic L_{pr} of Pakistan. Consequently, the ratio of hydrostatic to osmotic L_{pr} was significantly lower in the presence of osmotic stress in Morex, which was due to the decreased hydrostatic L_{pr} (Table 1).

The solute permeability P_{sr} of control roots, measured by treating roots with $60 \text{ mOsmol}\cdot\text{kg}^{-1}$ NaCl was similar for both Morex and Pakistan (Table 1). In Morex, the osmotic stress did not change the P_{sr} for NaCl. In Pakistan, there was a trend that the P_{sr} for NaCl increased in response to osmotic stress; however, these changes were not statistically significant. The reflection coefficient (σ_{sr}) for the passive selectivity of roots for NaCl was twofold higher in Morex

compared with Pakistan, but there were no changes in the σ_{sr} in response to osmotic stress (Table 1).

3.4 | Osmotic potential and proline concentration in barley roots

We further tested whether barley seminal roots undergo osmotic adjustment in response to osmotic stress. In parallel, concentration of proline, which is known to act as a major compatible solute for osmotic adjustment in plants, was measured for the two modern cultivars, Scarlett and Morex, and the wild barley Pakistan. In response to osmotic stress, the osmotic potentials in roots decreased about two-fold from -0.6 to -1.2 MPa (Figure 5a). Proline concentrations

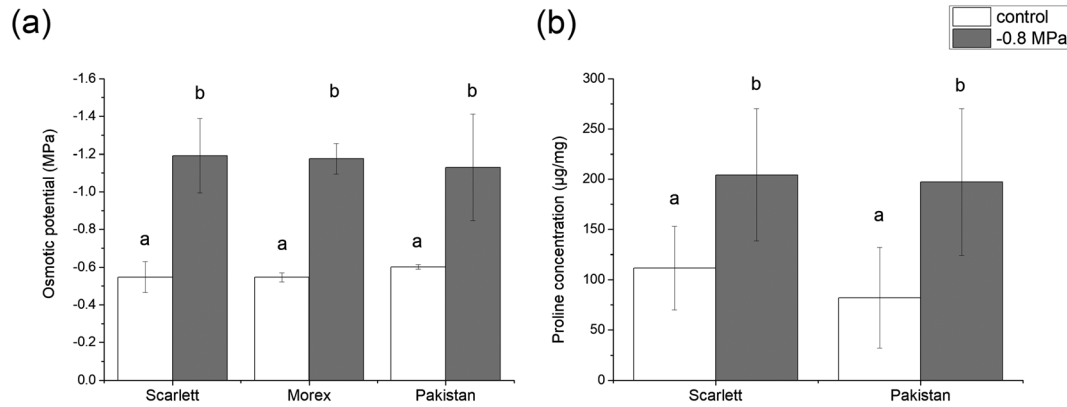


FIGURE 5 (a) Osmotic potential and (b) proline concentration of barley seminal roots under control and osmotic stress with a water potential of -0.8 MPa. The bars represent mean values with standard deviation ($n = 3$ for osmotic potential and $n = 7$ for proline concentration). Different letters indicate significant differences between means at a significance level of 0.05 by one-way analysis of variance (Fishers LSD test)

increased about twofold (Figure 5b) in both modern cultivars and the wild barley.

3.5 | Transcriptome analysis of barley roots using RNA-sequencing

The wild barley Pakistan was selected to identify global gene expression because it showed no pronounced response to osmotic stress, like enhanced suberization and decreased hydrostatic Lp, as all investigated modern cultivars did. For RNA-Seq analysis, total RNA was extracted from the three root zones (A, B, and C) from control and osmotically stressed plants. Differentially expressed genes with $FDR \leq 5\%$ resulted in an upregulation of total 3,504 unique genes and downregulation of 4,570 unique genes in wild barley Pakistan. Differential gene expression was root zone specific with 488, 536, and 829 unique upregulated genes and 666, 1,588, and 503 unique downregulated genes in the zones of A, B, and C, respectively (Figure 6a, Table S2).

Further, a functional categorization and singular enrichment analysis of GO terms was performed with AgriGO v.2 (Tian et al., 2017). GO

terms were assigned to functionally categorize the differently expressed genes according to their biological processes, cellular component, and molecular function. The results showed 550 enriched GO terms comparing the differently expressed genes in the three root zones of wild barley Pakistan under control and osmotic stress. From this, 109 enriched GO terms were derived from the upregulated differently expressed genes and 261 enriched GO terms from the downregulated differently expressed genes. These included 25 GO terms connected with the term "regulation" such as "regulation of cellular process," "regulation of biological process," and "biological regulation" as most enriched ones. These include 48 GO terms connected with the term "transport" such as "protein transport," "metal ion transport," or "ion transport" and so forth (Table S3). Transcripts of suberin biosynthesis genes and aquaporin genes were found in all three root zones of wild barley Pakistan, but they were not differentially expressed in response to osmotic stress (Table S4).

Comparing the RNA-Seq data of the wild barley Pakistan with published RNA-Seq data from the modern cultivar Scarlett (Kreszies et al., 2019) grown under exactly the same experimental conditions, at $FDR \leq 5\%$ and $|\text{Log}_2\text{FC}| \geq 1$, resulted in 5,749 and 5,730 unique

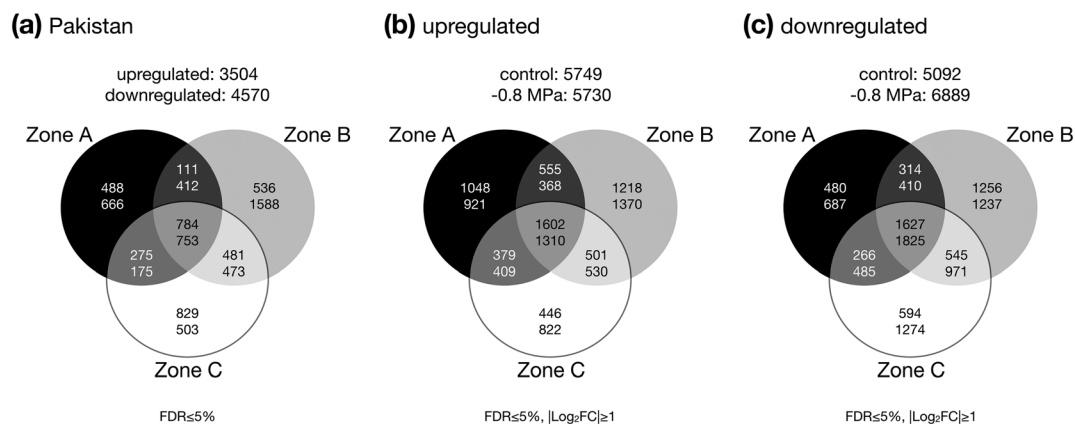


FIGURE 6 Numbers of differentially expressed genes in barley seminal root zones. (a) Overlap of upregulated genes (upper numbers) and downregulated genes (lower numbers) in Pakistan (ICB181243) root zones of A, B, and C in response to osmotic stress. (b) Overlap of upregulated genes under control (upper numbers) or at water potential of -0.8 MPa (lower numbers) between Pakistan and Scarlett. (c) Overlap of downregulated genes under control (upper numbers) or at the water potential of -0.8 MPa (lower numbers) between Pakistan and Scarlett

upregulated genes and 5,092 and 6,889 unique downregulated genes in control and osmotic stress, respectively (Figure 6b,c, Table S4). The functional categorization and singular enrichment analysis of GO terms of the comparison between wild barley Pakistan and modern cultivar Scarlett showed 717 enriched GO terms. From this, 157 enriched GO terms were derived from the upregulated differentially expressed genes and 316 enriched GO terms from the downregulated differentially expressed genes. Here, 39 GO terms connected with the term “regulation” such as “regulation of primary metabolic process,”

“regulation of cellular metabolic process,” “regulation of gene expression,” and so forth. Further, 57 GO terms were connected with the term “transport” such as “transmembrane transport” or “ion transport” (Table S3). Furthermore, aquaporin genes were significantly upregulated in wild barley Pakistan compared with the modern cultivar Scarlett (Figure 7). Important aquaporin genes such as HORVU3Hr1G085020 (putative CYP86A1 homologue) and HORVU1Hr1G042810 (putative CYP86B1 homologue) were downregulated in Pakistan compared with Scarlett. Some of the other

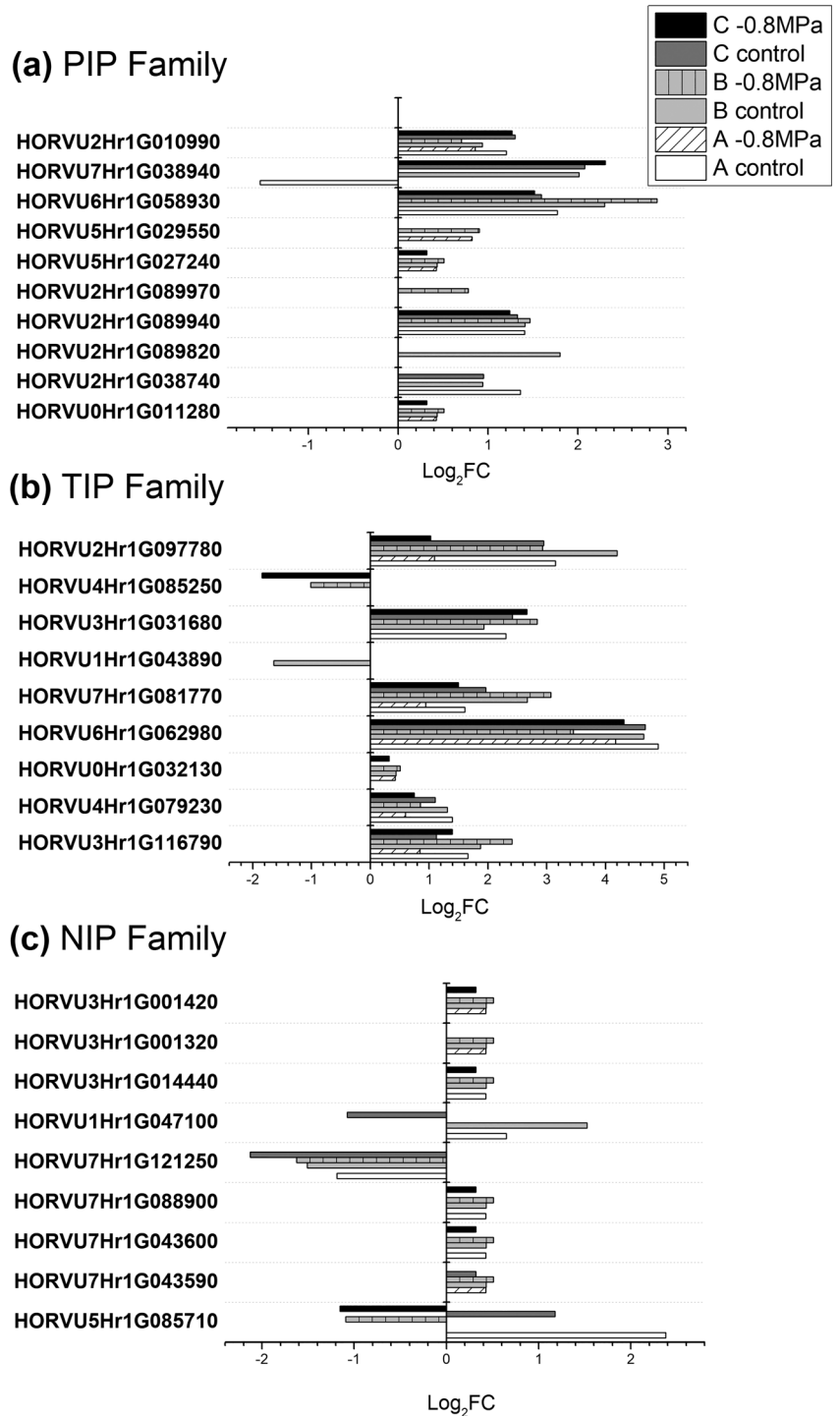


FIGURE 7 Differentially expressed aquaporin genes in the root zones of A, B, and C between Pakistan (ICB181243) in comparison to Scarlett grown either under control conditions or osmotic stress with a water potential of -0.8 MPa. (a) PIP family, (b) TIP family, and (c) NIP family. Missing bars indicate that there are no significant differentially expressed genes at false discovery rate $\leq 5\%$

suberin biosynthesis genes, for example, members of the Ketoacyl-CoA Synthase family were upregulated and/or downregulated (Table S4).

The key gene of proline biosynthesis (Pyrroline-5-carboxylate synthase 1; HORVU1Hr1G072780) was significantly upregulated in all three root zones in response to osmotic stress both in wild barley Pakistan and modern cultivar Scarlett (Table S1; Table S4).

4 | DISCUSSION

Plant roots are the first organs sensing declining soil water potential. In a recent study (Kreszies et al., 2019), we found that roots of the modern barley cultivar Scarlett, exposed to osmotic stress (-0.8 MPa), specifically responded with an enhanced endodermal suberization and reduced hydraulic conductivity of the roots. Parallel transcriptome studies indicated that genes involved in suberin biosynthesis were specifically upregulated in response to osmotic stress. Results indicated that in response to water deficit, the root apoplast was sealed with suberin and water uptake was exclusively possible via the cell-to-cell pathway, which ensures further water uptake into the plant and avoids water loss to the dry soil environment under drought conditions. Here, we extended our investigations including three additional modern barley cultivars (Golden Promise, Morex, and Barke) and compared them with three wild barley accessions (Iran, Jordan, and Pakistan), which are the ancestors of the modern cultivars.

In barley seedlings, seminal roots, but not adventitious roots, predominantly contribute to the overall root water uptake (Knipfer & Fricke, 2011); thus, their development and root length play an important role in response to osmotic stress. Hence, it is remarkable that seminal roots of wild barley accessions were on average always longer than those of the modern barley cultivars, in both control and stress conditions (Figure 1). Consequently, root/shoot ratios were always higher (Figure 2), which is a common drought-induced plant response in grasses (Zhou et al., 2018). Obviously, this developmental trend forming longer roots is genetically fixed in wild barley because in the fairly artificial hydroponic growth system used here, formation of longer roots should not represent an advantage, neither under control nor under stress conditions. Only when grown in soil longer roots should represent an advantage under drought conditions (Ahmed, Passioura, & Carminati, 2018; Naz, Ehl, Pillen, & Léon, 2012). Longer roots might get access to deeper soil layers, where more water should still be available compared with upper soil layers (Lynch & Wojciechowski, 2015). But it must be kept in mind that agricultural land and soil can be very heterogeneous, and long roots would not give an advantage when the soil is shallow (Ahmed et al., 2018).

A more vigorous root system, including longer roots together with a high variation of root traits, was in fact reported for several other wild barley accessions from the Middle East (Arifuzzaman et al., 2014; 2016; Naz et al., 2012; Naz, Arifuzzaman, Muzammil, Pillen, & Léon, 2014). During breeding programs in the last decades, the focus was largely on increasing aboveground traits, such as yield and grain

filling, rather than their root growth (Koevoets, Venema, Elzenga, & Testerink, 2016). This could explain why roots tend to be shorter and less vigorous in most modern cultivars because more carbon needs to be allocated to the aboveground biomass. As long as environmental conditions (soil moisture and nutrient levels) are ideal, limited root development is irrelevant; under stress, however, this could become highly relevant for survival.

So far it has been described that very different from other crops, for example, wheat, maize, and rice (Ranathunge, Lin, et al., 2011; Ranathunge, Schreiber, Bi, & Rothstein, 2016; Schreiber, Franke, Hartmann, Ranathunge, & Steudle, 2005), barley does not form an exodermis characterized by Casparian bands and suberin lamellae (Coffey et al., 2018; Gitto & Fricke, 2018; Jackson, 1922; Knipfer & Fricke, 2011; Kreszies et al., 2019; Ranathunge et al., 2017). Thus, it was an exciting observation that in response to osmotic stress, the wild barley accession Jordan formed an exodermis in the basal parts of about 20% of the investigated seminal roots (Figure 3). This suggests again that during breeding of modern cultivars, concentrating on the aboveground part of the plants and yield, important root traits contributing to stress tolerance potentially got lost in modern barley cultivars.

The induction or strengthening of an exodermis was for example very pronounced when rice and cotton plants were exposed to oxygen deficiency (Ranathunge, Schreiber, & Franke, 2011) and salinity (Krishnamurthy et al., 2009). Currently, the only barley species that has been reported to form an exodermis is *Hordeum marinum*. This is a wetland plant, and similar like rice, it generally forms an exodermis and reinforces it in response to oxygen deficiency to prevent radial oxygen loss when grown in stagnant or waterlogged conditions (Kotula et al., 2017). The formation of an exodermis in the wild barley accession Jordan is currently under further detailed investigation. It is hypothesized that wild barley, which is inherently more drought tolerant than modern cultivars, needs much stronger stress signals than -0.8 MPa water deficiency for the formation of an exodermis in all roots and not only in about 20% of the roots.

The qualitative suberin composition in terms of substance classes and single detected suberin monomers was identical between cultivated and wild barley, and it also fits to published data of other barley cultivars such as Golf (Ranathunge et al., 2017) and Scarlett (Kreszies et al., 2019). This suggests that the root suberin monomer composition is genetically well conserved in barley even under stress conditions. As it was also described for other crop species (e.g., maize and rice), suberization in both cultivated and wild barley increased over the root length and correlated with root maturity (Kotula, Ranathunge, Schreiber, & Steudle, 2009; Ranathunge, Schreiber, & Franke, 2011; Schreiber et al., 2005).

However, there were remarkable differences in root suberin amounts between modern cultivars and wild barley accessions. On average, all modern barley cultivars had higher suberin amounts compared with wild accessions in all three root zones (Figure 4). In response to osmotic stress, the modern cultivars showed a significantly increased suberization in root zones B and C (Figure 4). Most remarkably, wild barley accessions showed a significantly delayed root

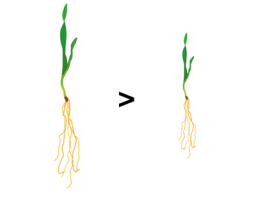
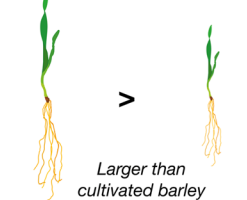
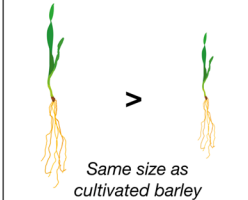
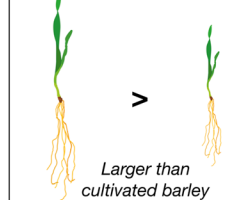
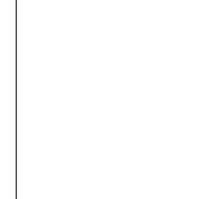
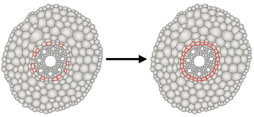
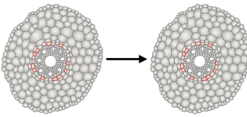
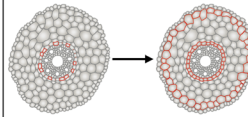
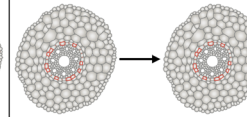

Accessions Traits	Cultivated barley		Wild barley					
	Golden Promise, Morex, Barke, Scarlett		Pakistan (ICB 181243)		Jordan (ICB 181466)		Iran (ICB 181160)	
Root and Shoot Length	<i>Control</i>	<i>-0.8 MPa</i>	<i>Control</i>	<i>-0.8 MPa</i>	<i>Control</i>	<i>-0.8 MPa</i>	<i>Control</i>	<i>-0.8 MPa</i>
								
Biomass Ratio (DW)	R/S < R/S		R/S = R/S		R/S < R/S		R/S < R/S	
Suberization Pattern								
Osmotic Adjustment	Proline < Proline		Proline < Proline		Proline < Proline		Proline < Proline	
Gene Expression	Proline biosynthesis genes (Scarlett ↑) = Proline biosynthesis genes (Pakistan ↑)				Suberin biosynthesis genes (Scarlett ↑) > Suberin biosynthesis genes (Pakistan →)			
	Aquaporin genes (Scarlett →) < Aquaporin genes (Pakistan →)							
Hydraulic Conductivity	L _p (Hy) > L _p (Hy) L _p (Os) = L _p (Os)		L _p (Hy) = L _p (Hy) L _p (Os) < L _p (Os)					

FIGURE 8 Summary scheme comparing cultivated and wild barley in response to -0.8 MPa osmotic stress. In all accessions, root and shoot lengths were reduced under osmotic stress. Root/shoot biomass ratio increased in response to osmotic stress. Cultivated barley showed an enhanced amount of suberin in the endodermis, where passage cell number declined due to suberization. In the wild barley from Pakistan and Iran, this effect was not very pronounced and there was no increase in aliphatic suberin. In Jordan, in response to osmotic stress, approximately 20% of the seminal roots developed a suberized exodermis, which was missing in all other accessions. All lines showed osmotic adjustment in their seminal roots, including an increase in proline levels. Similarly, RNA-Seq experiments revealed that proline biosynthesis genes were significantly upregulated in both Scarlett and Pakistan (upward arrows). In the modern cultivar Scarlett, the suberin biosynthesis genes were upregulated (upward arrow), but not in the wild barley from Pakistan (sideward arrow). The aquaporin genes were not upregulated in both Scarlett and Pakistan in response to osmotic stress (sideward arrows), but when directly comparing the gene expression between them, it was significantly higher in Pakistan roots compared with Scarlett roots in control as well as in osmotic stress. Osmotic stress significantly decreased the hydrostatic hydraulic conductivity (L_p , (Hy)) of roots in cultivated barley, whereas osmotic hydraulic conductivity (L_p , (Os)) was not changed. In the wild barley from Pakistan, osmotic stress did not change the L_p , (Hy), whereas it slightly increased the L_p , (Os) of seminal roots [Colour figure can be viewed at wileyonlinelibrary.com]

suberization when exposed to osmotic stress compared with cultivated barley. Very different from modern cultivars, none of the wild barley accessions showed a significant increase in root suberization in zone B in response to osmotic stress (Figure 4). This missing increase in root suberization strongly corresponds to the transcriptome studies, which showed that key genes of the suberin biosynthesis pathway in the wild barley from Pakistan were not differentially expressed between control and osmotic stress (Table S2). The opposite was in fact described for the modern barley cultivar Scarlett, where enhanced root suberization occurred in parallel to the pronounced induction of the suberin biosynthesis pathway (Kreszies et al., 2019). This indicates again that different from modern barley cultivars, wild barley obviously follows other more diverse strategies (longer roots, induction of an exodermis, delayed suberization) to cope with water deficit created by the osmotic stress.

As a further strategy dealing with osmotic or drought stress, plants can adjust their internal water potential in response to changes of the external soil/medium water potential by accumulating compatible solutes (osmotic adjustment; Turner, 2018). In barley, osmotic adjustment is achieved by accumulating proline (Muzammil et al., 2018). Genes involved in proline biosynthesis were in fact upregulated in response to osmotic stress in both the wild barley Pakistan and the modern cultivar Scarlett (Table S2), and proline concentrations were increased in stressed plants (Figure 5). As a consequence, osmotic potentials in roots were decreased (more negative) in stressed roots in both wild and cultivated barley (Figure 5). Thus, in terms of osmotic adjustment in response to stress, both modern barley as well as wild barley are equally efficient.

In response to osmotic stress, overall root hydraulic conductivity (hydrostatic L_p), consisting of the cell-to-cell path and the apoplastic

path of water transport, significantly decreased in the modern cultivar Morex (Table 1). A similar behaviour was described for the modern cultivar Scarlett (Kreszies et al., 2019). This reduced hydrostatic L_p , correlated with a significantly enhanced suberization of seminal roots starting in root zone B (Figure 4). This can be interpreted that upon enhanced suberization, the apoplastic water uptake is to a larger extent blocked, and the cell-to-cell path essentially remains as major transport route for root water uptake. This fits to the observations with Pakistan, where the hydrostatic L_p was not different between control and osmotic stress and where root suberization was not significantly enhanced in response to osmotic stress (Figure 4). Thus, Pakistan having significantly longer roots compared with Morex (Figure 1) and lacking a pronounced suberization in zone B (Figure 4) can keep its overall radial hydraulic conductivity in the presence of osmotic stress. Radial water uptake in barley roots mostly occurs through the weakly suberized younger root zone including the root tip, whereas water uptake is significantly decreased in the strongly suberized basal part of the root (Ranathunge et al., 2017; Sanderson, 1983). Thus, in Morex with an enhanced suberization, a decreased hydrostatic L_p can be expected.

In both the wild and the cultivated barley, the osmotic L_p (cell-to-cell path of water transport) did not change under osmotic stress, and a constant water flow through the cell-to-cell path was kept constant (Table 1). There was even a slight tendency, especially in Pakistan, that osmotic L_p increased in response to osmotic stress (Table 1), and this might represent even an adaptation of root hydraulic conductivity to osmotic stress ensuring sufficient water uptake. It was in fact found, in previous studies with *Sorghum bicolor* and tomato studying the effect of silicon on plant stress tolerance, that overall water permeability of roots increased after adding either PEG6000 or NaCl to the hydroponic solution. This leads to an overall better plant performance (Liu et al., 2014, 2015; Shi et al., 2016), and it was suggested to be due to enhancement of aquaporin activity, which regulates the osmotic L_p via water transport through the cell-to-cell path.

The cell-to-cell water flow is based on the permeability of the cell membranes, in which water is mainly crossing through the plasma membrane aquaporins. The contribution of this path to the water flow can reversibly be regulated by modulating the activity of aquaporins within short time periods in barley roots (Kaneko et al., 2015). Although our RNA-Seq data did not indicate changes of aquaporin gene expression in Pakistan during 6 days of adaptation to osmotic stress (Table S2), the overall expression of aquaporin genes in the wild accession Pakistan was significantly higher compared with the modern cultivar Scarlett (Figure 7, Table S4). This indicates that the wild accession Pakistan could be better adapted dealing with osmotic stress on the symplastic level, whereas the modern cultivar Scarlett shows more pronounced stronger responses to osmotic stress on the apoplastic level (enhanced suberization). Furthermore, the GO term "transport", for example "ion transport," was also enriched in Pakistan in comparison with Scarlett, indicating again that water and solute transport are somewhat differently regulated between wild and cultivated barley in response to osmotic stress. This is confirmed by the observation that the GO term "regulation" was highly, but very differently, enriched in

response to osmotic stress in wild and cultivated barley, respectively (Table S3).

Not only water permeability, but also solute permeability (P_{sr}) can be reduced by enhanced root suberization (Steudle, 2000a, 2000b; Steudle & Peterson, 1998). In the modern cultivar Morex, the P_{sr} for NaCl was slightly reduced in the roots of osmotically stressed plants. Most interestingly, different from Morex, the wild accession Pakistan again showed a different response because the P_{sr} of roots for NaCl was slightly increased under osmotic stress. This could be due to the fact that sodium ions might also move into the root slipping through the ion channels up to a certain extent or potentially through nonselective aquaporins (Byrt et al., 2017; Kourghi et al., 2017). Finally, it is known that the P_{sr} is inversely correlated with the passive selectivity of the root for solutes given by the reflection coefficient (σ_{sr}) (Steudle, 2000a). This is also found here because Pakistan has a lower σ_{sr} and a higher P_{sr} , whereas in Morex, it is exactly the opposite (Table 1).

In conclusion, this study shows that cultivated and wild barley roots show different chemical and morphological responses to osmotic stress (Figure 8). In modern barley cultivars, osmotic stress leads to significantly enhanced root suberization, whereas this response was much weaker in the wild accessions. However, in the wild accession Jordan, the induction of an exodermis could be observed in response to osmotic stress. Enhanced suberization of the endodermis significantly reduced the radial water transport in modern cultivars, whereas wild accessions with very weak suberization kept their water transport constant under osmotic stress. Different from modern cultivars, wild types were characterized by a generally higher expression of aquaporin genes. Thus, wild barley is better adapted to osmotic stress by maintaining constant water uptake rates even under osmotic stress than that of cultivated barley. These various beneficial traits of wild barley accessions could be selected for future breeding programs developing more drought stress tolerant crops.

ACKNOWLEDGEMENTS

The authors gratefully thank Dr Ali Naz, Professor Jens Leon, and Karola Müller (Department of Plant Breeding, University of Bonn) for providing barley seeds and the help with propagation of wild barley accessions. We thank Jonas Wallraff for technical assistance. Financial support by the Deutsche Forschungsgemeinschaft (GRK2064) to TK, NS, AO, JAB, FH, LS and the Future Fellowship from the Australian Research Council (ARC Grant Number: FT170100195) to KR is highly appreciated.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Suberin lamellae of barley cultivar Morex roots grown under (a) control or (b) osmotic stress at water potential of -0.8 MPa, stained with fluoral yellow 088.

Figure S2. Total aromatic suberin amount in 12-days-old barley seminal roots grown either under control conditions or osmotic stress at a water potential of -0.8 MPa.

Figure S3. Amounts of monomers of aliphatic suberin in Zone B (25–50% root length) of the modern cultivar Morex and the wild barley Pakistan grown either under control conditions or osmotic stress at a water potential of -0.8 MPa.

Table S1. Shoot and root dry weight of 12-days-old cultivated and wild barley plants, either grown under control conditions or osmotic stress at a water potential of -0.8 MPa.

Table S2: Complete list of differentially expressed genes for Pakistan

Table S3: Cross comparison of enriched GO terms among differentially expressed genes in the barley seminal root zones A, B and C in response to osmotic stress.

Table S4: Complete list of differentially expressed for Pakistan vs Scarlett

How to cite this article: Kreszies T, Eggels S, Kreszies V, et al. Seminal roots of wild and cultivated barley differentially respond to osmotic stress in gene expression, suberization, and hydraulic conductivity. *Plant Cell Environ.* 2020;43:344–357. <https://doi.org/10.1111/pce.13675>