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

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Summary

- Barley (Hordeum vulgare) is more drought tolerant than other cereals, thus making it an excellent model for the study of the chemical, transcriptomic and physiological effects of water deficit. Roots are the first organ to sense soil water deficit. Therefore, we studied the response of barley seminal roots to different water potentials induced by polyethylene glycol (PEG) 8000.
- We investigated changes in anatomical parameters by histochemistry and microscopy, quantitative and qualitative changes in suberin composition by analytical chemistry, transcript changes by RNA-sequencing (RNA-Seq), and the radial water and solute movement of roots using a root pressure probe.
- In response to osmotic stress, genes in the suberin biosynthesis pathway were upregulated that correlated with increased suberin amounts in the endodermis and an overall reduction in hydraulic conductivity (Lp). In parallel, transcriptomic data indicated no or only weak effects of osmotic stress on aquaporin expression.
- These results indicate that osmotic stress enhances cell wall suberization and markedly reduces Lp of the apoplastic pathway, whereas Lp of the cell-to-cell pathway is not altered. Thus, the sealed apoplast markedly reduces the uncontrolled backflow of water from the root to the medium, whilst keeping constant water flow through the highly regulated cell-to-cell path.

Introduction

Climate changes and extreme weather conditions, such as drought, will become more intensive in the future (Melillo et al., 2014). This will have a major impact on agricultural productivity. Compared with other abiotic stresses, drought accounts for the highest crop losses (Boyer, 1982). Barley (Hordeum vulgare) is more drought tolerant than other crop plants, and represents the fourth most abundant cereal after wheat, maize and rice (http://faostat.fao.org). Other than drought, barley is also fairly resistant to other abiotic stresses, such as salinity, alkalinity and cold, and can survive better under nonoptimal environmental conditions (Colmer et al., 2006; Kosová et al., 2014). These unique properties make barley a model crop for the study of the effect of abiotic stresses in general. Drought starts with a decrease in the soil water potential. Consequently, plant roots are the first organs which sense drought and have to cope with water deficiency (Zingaretti et al., 2013).

The main function of roots is water and nutrient uptake, which is highly dependent on anatomical structures, growth conditions and plant age. Water and solute uptake of plant roots is best described by the composite transport model. According to the model, there are three major pathways for water and solute transport in roots: (1) the apoplastic (cell walls), (2) the symplastic and (3) the transcellular pathway. The last two are also referred to as the cell-to-cell pathway. The apoplastic pathway can be blocked by Casparian bands and suberin lamellae in endodermal and exodermal cell walls. The cell-to-cell pathway can additionally be regulated by aquaporins (Peterson & Cholewa, 1998; Steudle & Peterson, 1998; Steudle, 2000a,b).

The formation of the biopolyester suberin has been shown to be enhanced by abiotic (Hose et al., 2001; Enstone et al., 2002; Krishnamurthy et al., 2009; Ranathunge et al., 2011a; Barberon et al., 2016; Kotula et al., 2017) and biotic (Lulai et al., 1998; Thomas et al., 2007; Ranathunge et al., 2008; Lanoue et al., 2010) stresses. The suberin lamellae contain polyaliphatic and polyaromatic domains, which are polylimerized (Kolattukudy et al., 1975; Bernards, 2002). The aliphatic monomers are primary alcohols, fatty acids, α–ω dicarboxylic acids (diacids) and ω-hydroxy acids (ω-OH acids), whereas the aromatic components are ferulic and

Key words: apoplast, barley, osmotic stress, root, suberin, transcriptomics, water deficit, water transport.
coumaric acids (Schreiber et al., 1999; Graça, 2015). Caspian bands are mainly composed of lignin and partly of suberin (Schreiber, 1996; Zeier & Schreiber, 1998; Schreiber et al., 1999; Naseer et al., 2012). Lignin consists of syringyl, guaiacyl and p-hydroxyphenol monomers which form a complex aromatic biopolymer (Fraser & Chapple, 2011; Lupoi et al., 2015).

Here, the effect of water deficit induced by osmotic stress through polyethylene glycol (PEG) 8000 on suberized barrier development in barley roots, and its physiological effects, are reported. Apoplastic barrier development along the root using microscopy and histochemical studies of barley roots grown under different low water potentials were investigated. Subsequently, changes in root suberization and global gene expression patterns during the different root developmental stages in response to osmotic stress were quantified. Finally, the effect of osmotic stress on water and solute transport in roots using a root pressure probe was studied. These findings indicate that an increased amount of suberin could be an effective adaptation to water deficit as a result of scaling of roots and prevention of uncontrolled passive water loss from the root to the dry soil by backflow via the nonselective apoplastic pathway. At the same time, roots maintain the uptake of water through the cell-to-cell pathway.

Materials and Methods

Plant material and growth conditions

Seeds of barley (Hordeum vulgare L. spp. vulgare cv Scarlett) were stratified for 1 wk at 4°C. They were then germinated in the dark at 25°C covered with wet filter paper. After 3 d, seedlings were transferred into an aerated hydroponic system containing half-strength Hoagland solution in a climatic chamber under long-day conditions (16 h : 8 h, light : dark), an air temperature of 23°C : 20°C (day : night) and a relative humidity of 50–65%. When the plants were 6-d-old, stress treatment was applied for another 6 d in all experiments described; thus plants were grown for 12 d (Fig. 1a) and, at this stage, they had two leaves and five to six seminal roots.

Water deficit application induced by osmotic stress through PEG 8000

Low water potentials were applied when the plants were 6-d-old (Fig. 1a). Plants were moved from half-strength Hoagland solution (20 mOsmol kg⁻¹ or −0.04 MPa of osmotic pressure) to half-strength Hoagland solution adjusted to a defined water potential with PEG 8000 (Roth, Karlsruhe, Germany) simulating water deficit induced by osmotic stress. The water potential of the medium was reduced to −0.4, −0.8 and −1.2 MPa by adding 17.5%, 25.4% and 31.6% (w/w) PEG 8000 (Michel, 1983). The water potentials of the nutrient solutions with different levels of PEG 8000 were measured using a WP4C Water Potential Meter (Meter Group Inc., Pullman, WA, USA).

The simulation of water deficit by PEG 8000 treatment represents a widely accepted experimental approach offering various important advantages. An exactly defined and homogeneous osmotic potential acting on the roots can be adjusted. As, in nature, water stress during drought mostly occurs in a combination with heat and high light, PEG treatment allows water deficit to be examined separately (Kramer and Boyer, 1995; Verslues et al., 2006; Frolov et al., 2017). In addition, for our experiments, hydroponic culture was essential because only with this approach could root transport properties be measured using the pressure probe technique.

Histochemical detection of Caspian bands and suberin lamellae in roots

Cross-sections were made at 1-cm increments along the whole seminal root using a cryostat microtome (Microm HM 500M,
Microm International, Walldorff, Germany). To detect the development of Casparian bands over the root length, cross-sections were stained with 0.1% (w/v) berberine hemisulfate for 1 h and with 0.5% (w/v) aniline blue for 30 min (Brundrett et al., 1988). Suberin lamellae were stained with 0.01% (w/v) lipophilic fluorol yellow 088 for 1 h (Brundrett et al., 1991). Cross-sections were analyzed by epifluorescence microscopy using an ultraviolet (UV) filter set (excitation filter BP 365, dichroic mirror FT 395, barrier filter LP 397; Zeiss, Oberkochen, Germany). Photographs were taken with a Canon EOS 600D camera (Canon Inc., Tokyo, Japan) at ISO 200 or 400 for 1–2 s.

Chemical analysis of barley root suberin

The seminal roots were divided into three zones – A, B and C – based on the previous microscopic investigations (Fig. 1b). Zone A (0–25% of total root length) was the youngest part of the root, which included the root apex. In this zone, only Casparian bands were present in the endodermis, but no suberin lamellae were deposited. Zone B (25–50%) was the transition zone, in which all endodermal cells had Casparian bands, but only a limited number of cells had suberin lamellae depostitions. Zone C (50–100%) was the mature part of the root close to the root base, in which all endodermal cells were characterized by the presence of Casparian bands and suberin lamellae (Fig. 1b).

For each replicate, 10 segments of seminal roots from each of the three zones were pooled together. The root segments were enzymatically digested for 3 wk with 0.5% (w/v) cellulase and 0.5–% (w/v) pectinase at room temperature under continuous shaking (Zeier & Schreiber, 1997). The enzyme solution was replaced four times within the 3 wk and roots were vacuum infiltrated with the solution. Subsequently, isolated cell walls were washed in borate buffer and then transferred to 1 : 1 (v/v) chloroform : methanol for soluble lipid extraction at room temperature under continuous shaking for 2 wk. The chloroform : methanol solution was replaced four times. Finally, samples were dried on polytetrafluoroethylene (PTFE) in a desicator containing activated silica gel. The dried samples were subjected to transesterification with BF$_3$-methanol to release suberin monomers (Kolattukudy & Agrawal, 1974). Gas chromatographic analysis and mass spectrometric identification were performed as described earlier (Zeier & Schreiber, 1997, 1998). Suberin amounts were referred to the endodermal surface area. The endodermal area was calculated for each root zone: $A = 2\pi \cdot r \cdot L$ (r, endodermis radius; L, length of the individual root zone). Three biological replicates were used for each experiment.

RNA isolation

For RNA isolation, five seminal roots from five 12-d-old barley plants grown under control or −0.8 MPa osmotic stress conditions were pooled. Samples of each of the three root zones were taken for specific transcriptome analysis. In contrast with samples taken for chemical analysis, only half of each zone was collected (Fig. 1b). The samples were collected in 2-ml reaction tubes with sterile steel beads inside. The samples were frozen in liquid nitrogen and ground with a mixer mill (Retsch MM400; Retsch GmbH, Haan, Germany) at a frequency of 30 rounds s$^{-1}$ for 1 min. RNA was isolated with the RNeasyPlus Universal Mini Kit (Qiagen, Venlo, the Netherlands). RNA quality was analyzed via a NanoDrop (Thermo Fischer Scientific, Wilmington, Delaware, USA) and Agilent RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA, USA) Bioanalyzer. For all samples, a RNA integrity number $\geq$ 9.1 was detected. Four biological replicates were used for each experiment.

Processing of raw reads and analysis of differentially expressed genes

Raw sequencing data of 100-bp paired-end reads, obtained with an IlluminaHiSeq 4000 sequencer (BGI Tech Solutions, Hong Kong, China), were processed with CLC Genomics WORKBENCH v.10.0.1 (https://www.qiagenbioinformatics.com/) for further analyses. After quality trimming for low-quality scores and ambiguous nucleotides, only reads with a length of $> 40$ bp were retained for mapping. These reads were mapped to the barley reference genome, ENSEMBLPLANTS: Hv_IBSC_PGSB_v2, v.2.36 (Mascher et al., 2017, ftp://ftpensemblgenomes.org/pub/plants/release-36/fasta/hordeum_vulgare/dna/), allowing large gaps of up to 50 kb to span introns. Only reads that matched uniquely with $\geq 80\%$ of their length and an identity of $\geq 90\%$ to the reference genome were considered as mapped. Stacked reads, that is, read pairs that have identical start and end coordinates and orientation, were merged into one. Subsequently, the remaining reads were mapped to the high-confidence annotation of the genome sequence (Mascher et al., 2017, ftp://ftp.ensemblgenomes.org/pub/plants/release-36/gff3/hordeum_vulgare/; v2.36). Sequences had to match with $\geq 90\%$ of their length and $\geq 90\%$ similarity to the set of high confidence gene models. Reads with more than one hit were excluded from subsequent read counting. Before differential expression analysis, read counts were normalized by sequencing depth and log$_2$-transformed to meet the assumptions of a linear model. Furthermore, the mean–variance relationships were estimated and used to assign precision weights to each observation to adjust for heteroscedasticity (Law et al., 2014). To test 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 (Smyth, 2005) in R (R v.3.4.0, limma_3.32.2). Distances between sample pairs were displayed as the leading log fold changes (log$_2$FC), which are defined as the estimated root-mean-square deviation for the top 500 genes with the largest SD among all samples. This analysis provided a visual representation of sample relationships by spatial arrangement. To assess differences in gene expression between osmotic stress treatment and control in each root tissue, a linear model including a fixed effect for treatment and tissue and an interaction effect was applied. An empirical Bayes approach was used to estimate the variability over all genes in the fitted model and to shrink the variances towards a common value (Smyth, 2004). The contrast.fit function of the R package limma was used to compute pairwise comparisons between osmotic stress treatment and control for each tissue. To correct calculated $P$ values of the performed pairwise $t$-tests for multiplicity, the
Functional annotation and gene ontology (GO) analysis

Annotations were retrieved from ENSEMBLPLANTS (Kersey et al., 2016; http://plants.ensembl.org/index.html) and the IPK Barley BLAST server (Deng et al., 2007; http://webblast.ipk-gatersleben.de/barley_ibsc/downloads/). AGRIGO v.2.0 (Tian et al., 2017) was used for singular enrichment analysis (SEA) by comparing the list of differentially expressed genes with the customized annotated reference from the IPK Barley BLAST server. The cross-comparison of the SEA (SEACOMPARE) tool was used to combine the SEA results.

Putative barley orthologs of suberin, lignin, fatty acid elongation and aquaporin genes are based on known mutants described in Arabidopsis and rice (Fraser & Chapple, 2011; Ranathunge et al., 2011; Li-Beisson et al., 2013; Vishwanath et al., 2015; Kreszies et al., 2018). The barley genes were retrieved via the IPK Barley BLAST server (Deng et al., 2007) and the orthologous search from ENSEMBLPLANTS (Kersey et al., 2016).

Root pressure probe experiments

Root pressure probe experiments were conducted with the end segments/apical part of the seminal roots lacking lateral roots (zone A and zone B together) as described earlier (Steudle et al., 1987; Ranathunge et al., 2017). The measurements were only performed for plants grown in control and −0.8 MPa treatment conditions. Plants grown in −0.8 MPa PEG 8000 solution were transferred back to half-strength Hoagland nutrient solution at least 1 h before root pressure probe measurements. Between 2 and 4 h after fixing to the pressure probe, roots reached the steady-state root pressure. In the hydrostatic experiments, water flow was induced by moving the micrometer screw forward and backward, and thus inducing radial water flow out of or into the root. The subsequent pressure changes were used to calculate the hydraulic conductivity (Lp) of the roots from the half-times of water exchange (t½):

\[
L_p = \frac{\log_2(t)}{t_{\text{½}}} = L_p \times \frac{A_r \times P_{\text{sw}}}{V_x}
\]

kₚ is the rate constant of permeation of solutes. Here t½ is the half-time of solute exchange and Vx is the volume of functional xylem in the root. It was 1.5% measured in the cross-sections of seminal roots. The total root volume was calculated with the conductive root length and the root diameter. Reflection coefficients (σₑ) of NaCl were calculated with:

\[
\sigma_{\text{te}} = \frac{\Delta P}{\Delta \pi_{\text{sw}}} \exp(k_{\text{sw}} \times t_{\text{min}})
\]

ΔPₑ is the maximum change in root pressure and tₑ is the time which is required to reach the minimum root pressure. Δπₑ is the change in the osmotic pressure of the medium, which is calculated as Δπₑ = R·T·Cₑ, with R universal gas constant, T absolute temperature and Cₑ osmolarity of the solute (60 mOsmol kg⁻¹).

At the end of each measurement, roots were cut close to the seal to check the proper fixation of the root: if the root pressure did not drop rapidly down to zero and if there was no drastic decrease in tₑ to approximately one order of magnitude faster than during hydrostatic pressure relaxations, the roots were discarded. This usually happens as a result of overtightening of the roots at the fixing point of the pressure probe that blocks the xylem vessels.

Statistical analysis of chemical and physiological data

Data analysis and statistical tests were performed with ORIGIN Pro 9. Normal distribution of the data was tested with the Shapiro–Wilk test. As all data were normally distributed, we tested for statistical significance of differences between means of plants grown under different water potentials at a significance level of 0.05: two-sample t-test for root pressure probe experiments and analysis of variance (Fisher’s least significant difference, LSD) for chemical analyses.

Results

Root morphology and anatomy

The average length of 12-d-old barley seminal roots decreased with increasing osmotic stress treatments (−0.4, −0.8 and −1.2 MPa) (Fig. 2). The reduction in root length at −0.4 MPa (21.5 ± 4.0 cm) was not statistically significantly different from control conditions (22.9 ± 5.5 cm), whereas the root length was...
significantly reduced at −0.8 MPa (19.2 ± 6.9 cm) and −1.2 MPa (19.3 ± 3.6 cm). The seminal root length was not significantly different for the two lowest water potential treatments of −0.8 and −1.2 MPa (Fig. 2).

Endodermal Casparian bands were visible even near the root apex as small dot-like structures (Fig. 3a,e). Starting at 12.5% of the root length, they developed to continuous bands in the radial cell wall (Fig. 3). There were no obvious differences between the control (Fig. 3a–d) and water-stressed plants (−0.8 MPa) in the development of Casparian bands (Fig. 3e–h). Casparian bands were not detected in the rhizodermis of control and water-deficit plants, even in the older root zones, where Casparian bands were completely developed in the endodermis. Thus, barley seminal roots fail to develop an exodermis, even under osmotic stress conditions.

The suberin lamellae in the endodermis started to deposit further back from the root tip than the Casparian bands and were not detectable at 12.5% of the total root length (Fig. 4a,e,i,m). In control and all osmotic stress treatments, the first appearance of single suberized cells was observed at 25% of the root length (Fig. 4b,f,j,n). At 37.5% of the total root length, there was patchy development of suberization detected in the endodermis of both control and osmotic stress treatments (Fig. 4c,g,k,o). At higher osmotic stress levels of −0.8 and −1.2 MPa, the number of suberized cells in the endodermis was higher than in the control (Fig. 4k,o). At 50% of the root length, the endodermal cells were fully suberized (complete ring of suberized cells) in control and all osmotic stress treatments (Fig. 4d,h,l,p).

Chemical analysis of suberin of barley seminal roots in response to different osmotic stress levels

For chemical suberin analysis, barley seminal roots were divided into the three zones A, B and C (Fig. 1b) based on endodermal suberization (Fig. 4). Aliphatic suberin in barley seminal roots was composed of the four monomer classes: alcohols (alc), fatty acids (fa), α–ω-dicarboxylic acids (diacids) and ω-hydroxy acids.
(ω-OH acids) (Fig. 5). The most abundant aliphatic suberin monomers were the C_{18:1} diacid and ω-OH acids (C_{18:1} and C_{24} ω-OH acids) (Figs 5, 6). The chain length of the different suberin monomers varied from C_{16} to C_{26} (Fig. 6). Aromatic suberin components were composed of coumaric and ferulic acids (Fig. S1). There were no significant differences in substance classes (Fig. 5) or single monomer composition (Fig. 6) between control and osmotic stress conditions.

However, the absolute (Figs 5, 6) and relative (Fig. S2) amounts of substance classes changed over the length of the root from zone A to zone C in all treatments (control and osmotic stress conditions). This change was pronounced, in particular, for the total amounts of aliphatic (Fig. 7a) and aromatic (Fig. 7b) suberin. Barley seminal roots showed a significant increase in total aliphatic and aromatic suberin (Fig. 7a,b) from zones A to C (Fig. 5), which correlated well with the suberin histochemical observations (Fig. 3). Comparing the severity of osmotic stress treatments with the degree of aliphatic suberization, there was no significant difference between treatments in zone A (Fig. 7a). In zone B, mild osmotic stress (−0.4 MPa) did not significantly enhance suberization in comparison with the control. However, stronger osmotic stress treatments of −0.8 MPa and −1.2 MPa increased the aliphatic suberin amounts by two-fold compared with the control and −0.4 MPa (Fig. 7a). In zone C, all water stress treatments significantly increased the aliphatic suberin amounts compared with the control, but there was no significant change in aromatic suberin amounts.

Fig. 4 Development of suberin lamellae in the endodermis of barley seminal roots. Suberin lamellae in different zones (Fig. 1b) of roots grown under different water potentials were stained with fluorol yellow 088. The presence of suberin lamellae is indicated by a bright yellow fluorescence. At a distance of 12.5%, no suberin lamellae are visible (a, e, i, m). At 25% of relative root length, the first single, only partially suberized, cells (arrows) are visible (b, f, j, n). At 37.5% of relative root length, a patchy suberization is visible, which is stronger in roots grown in the presence of (k, o) −0.8 MPa and −1.2 MPa compared with (c) control and (g) −0.4 MPa. At a distance of 50%, the endodermis is complete suberized in all growth conditions (d, h, l, p). Bars, 50 μm.
To identify global gene expression changes in barley seminal roots with respect to suberin development, total RNA was extracted from the three root zones (A, B and C) from control and −0.8 MPa conditions (Fig. 1b) and subjected to RNA-Seq.

We chose a water potential of −0.8 MPa for the stress treatment, because the responses of roots for growth and suberization were more pronounced compared with −0.4 MPa, but not different from the treatment with −1.2 MPa (Figs 2, 4, 7).

RNA-Seq yielded, on average, 35 million reads for each of the four biological replicates per zone by treatment combination. In an MDS plot, the replicate samples of the three root zones and the control vs stress conditions clustered separately, and were thus clearly distinguishable (Fig. 8a). The analysis of differentially expressed genes with FDR ≤ 5% showed that, in total, 5531 unique genes were upregulated and 5146 unique genes were downregulated. However, the response to osmotic stress was also root zone specific with 1101, 1139 and 1204 unique upregulated genes and 750, 2980 and 227 unique downregulated genes in zones A, B and C, respectively (Fig. 8b; Table S2). Functional categorization was performed using preliminary annotated barley GO terms from the IPK Barley BLAST server (Deng et al., 2007), and the identification of significantly enriched GO terms by singular enrichment analysis with AGRIGO v.2 (Tian et al., 2017). The analysis showed 95 unique enriched GO terms when comparing the differentially expressed genes between the three root zones under control and stress conditions (Table S3). Significantly enriched biological processes in response to osmotic stress shared by the three root zones were (1) organic acid metabolic process, (2) carboxylic acid metabolic process and (3) oxoacid metabolic process (Table S3).

A significant upregulation of barley suberin genes in control as well as in −0.8 MPa treatments was detected in all three root zones (Fig. 9). In most cases, the highest expression was in zone B (Fig. 9). In total, more suberin genes were upregulated in zones B and C with higher log2FC values compared with zone A (Fig. 9). On average, the expression of aquaporin genes was 50 times higher than barley suberin-associated genes in barley roots. In addition, in contrast with suberin genes, the expression of the majority of barley aquaporin genes was not significantly different in response to osmotic stress, in which few genes were up- or downregulated. Only HORVU1Hr1G047100, a putative NIP5;1 ortholog (portable aquaporin for boric acid and water), was highly upregulated in all three root zones (Table S4). Genes from the phenylpropanoid pathway, which are involved in the biosynthesis of lignin, which is part of the composition of Casparian bands and is heavily deposited in the central cylinder of roots, were also found to be upregulated (Table S4).

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

Similar to the RNA-Seq analysis, we chose a water potential of −0.8 MPa to compare the hydraulic conductivity (Lp) and solute permeability of barley seminal roots between control and osmotic stress conditions (Table 1). The hydrostatic Lp was significantly reduced by 2.5-fold (from $8.11 \times 10^{-8}$ to $3.19 \times 10^{-8}$ m s$^{-1}$ MPa$^{-1}$) in response to osmotic stress. By contrast, the osmotic Lp, did not change in response to osmotic stress (Table 1). Thus, the ratios of hydrostatic:osmotic Lp,
declined in the osmotic stress treatment and showed that there is a shift of water flow from the apoplastic pathway to the cell-to-cell pathway during osmotic stress treatment (−0.8 MPa).

The solute permeability ($P_{sr}$) of roots for NaCl was also reduced by the osmotic stress treatment compared with the control, but was not statistically significant because of the high variability among the water-stressed roots (Table 1). There was no change in the reflection coefficient ($r_{sr}$) for NaCl in response to osmotic stress treatment compared with the control (Table 1).

**Discussion**

Plant roots are the first organs to sense water deficit in dehydrating soil and thus play a crucial role in plant drought responses. In this approach, multifaceted techniques were used to test the hypothesis that an increased suberization of barley roots could represent an efficient response to water deficit by limiting uncontrollable, passive water loss from roots to the dry soil. By adding different concentrations of PEG 8000 to the nutrient solutions of hydroponically growing barley plants, specific water potentials from mild (−0.4 MPa) to more severe (−0.8 and −1.2 MPa) water deficit were adjusted.

One of the most important parameters in seedling root system architecture in response to osmotic stress is the seminal root length, because barley seminal roots contribute to overall root water uptake during early development (Knipfer & Fricke, 2010). At more negative water potentials of −0.8 and −1.2 MPa, barley roots developed 10% significantly shorter seminal roots compared with control and mild osmotic stress treatment (−0.4 MPa) (Fig. 2). This phenotypic alteration in seminal roots is probably a result of osmotically driven reduced cell elongation and organ development in declining water potentials (Yamaguchi & Sharp, 2010), resulting in reduced root length.

Detailed knowledge of the anatomy of the developmental stages along the root was important for our further analyses, including chemical, transcriptomic and water transport measurements and their interpretations (Steudle & Peterson, 1998; Steudle, 2000b; Kreszies et al., 2018). The suberin lamellae were only visible in the endodermis and we detected no exodermis, not even under the most severe osmotic stress conditions.

**Fig. 6** Amounts of monomers of aliphatic suberin detected in barley seminal roots grown under control conditions or at a water potential of −0.4, −0.8 or −1.2 MPa. The roots were divided into three root zones from the apical root tip (a) zone A over (b) zone B to the basal part (c) zone C. The bars represent mean values with ± SD of three biological replicates. Different letters indicate significant differences between means at a significance level of 0.05 in one-way ANOVA (Fisher’s least significant difference, LSD). In (a) zone A, no significant difference were detected. alc, primary alcohols; α-OH, α-hydroxy acids.
values with \( /C6 /C0 \) or ANOVA (Fisher's least significant difference, LSD).

abiotic stresses (Hose et al. observed previously in plant roots as a general response towards suberization in response to osmotic stress (Fig. 4), which was tochemical observations show that barley roots undergo strong % of the endodermal cells were suberized (Fig. 4d,h,l,p). The his-

Fig. 7

The bars represent mean SD of three biological replicates. Different letters indicate significant differences between means at a significance level of 0.05 in one-way ANOVA (Fisher’s least significant difference, LSD).

Nevertheless, this observation is of major interest as there is an ongoing debate as to whether the chemical composition of Casparian bands is exclusively pure lignin or a mixture of lignin as the major component and suberin occurring in minor amounts. In isolated Casparian bands of Clivia miniata, Monstera deliciosa, soybean, pea and maize, mainly lignin, but also suberin, was detected by GC-MS analyses (Karahara & Shibaoka, 1992; Schreiber, 1996; Zeier & Schreiber, 1997, 1998; Zeier et al., 1999; Thomas et al., 2007). Indeed, just recently, direct Raman scattering microscopic investigations of Casparian bands in maize roots reported that they are composed of both polymers, lignin and suberin (Man et al., 2018). However, it was concluded from promoter \( /C211 \)-glucuronidase (GUS) assays of suberin genes with specific endodermal expression in
Arabidopsis roots that Casparian bands are exclusively composed of lignin, but not suberin (Naseer et al., 2012).

A final conclusion regarding the presence or absence of suberin as an additional polymer in Casparian bands cannot be drawn at the moment for barley roots, as different results have been obtained from different species and different experimental approaches. Caution should be exercised when transferring results obtained from Arabidopsis to other plant species, including crop plants. Such simple and direct one-to-one correlations may not always be valid (Kreszies et al., 2018). However, future experimental approaches with higher resolution, allowing, for example, the direct analysis of the chemical composition of Casparian bands of Arabidopsis roots, might help to answer this question. Alternatively, the best option would be an endodermis-specific transcriptomic analysis by RNA-Seq, in combination with chemical analyses of isolated and purified endodermal cell walls, which would provide a higher sensitivity and accuracy than qualitative histochemical staining techniques.

The results of our RNA-Seq analysis in barley roots displayed root zone-specific differential gene expression in response to osmotic stress. This is in agreement with the recently published data for maize and rice roots (Shiono et al., 2014b; Opitz et al., 2016). It was obvious that transition zone B (25–37.5%) showed the highest expression of suberin biosynthesis genes in barley roots for both control and osmotic stress conditions (Fig. 9). This confirmed the microscopic observations (Fig. 4) and chemical analyses (Fig. 7), indicating that, in zone B, there was a rapid and pronounced increase in endodermal suberization. In response to the adaptation to water stress (~0.8 MPa), suberin genes were often significantly up-regulated in zone B compared with the control (Fig. 9), leading to faster and greater root suberization. This can be interpreted as a strategy of the root to efficiently block the apoplastic pathway, preventing uncontrolled water losses from the root to the surrounding medium/soil.

During the developmental transition of the root from zone A to B, there was a pronounced shift in suberin monomer composition from monofunctional fatty acids to ω-OH and diacids (Fig. S2). This can also be explained by the higher expression of suberin biosynthesis genes, such as HORVU3Hr1G085020 and HORVU1Hr1G042910, which are directly located after fatty acid synthesis in the suberin biosynthesis pathway (Figs 9, S3). In zone C, in which the highest amount of suberin (Fig. 7) and a completely suberized endodermis were detected (Fig. 4), the expression of suberin biosynthesis genes was lower than in zone B, but not completely turned off (Fig. 9). Our data show that there is a maximum amount of about 7 μg cm⁻² of aliphatic suberin in barley seminal roots in response to osmotic stress (Fig. 7). As roots failed to develop an induced exodermis in barley under osmotic stress...
Fig. 9 Expression patterns of most highly upregulated suberin biosynthesis genes in barley roots obtained by RNA-sequencing (RNA-Seq). The roots were divided into three root zones from the apical root tip zone A over zone B to the basal part zone C. Transcripts per million (TPM) for the root zones A, B and C of selected genes and their log2FC in response to osmotic stress are given. The log2FC values are given when control and PEG 8000-treated roots display significantly different expression levels at a significance level of 0.05 in pairwise t-tests. ns, not significant. (a, b) Cytochromes P450 converting fatty acids into ω-hydroxy acids and ω-ω dicarboxylic acids. (c, d) Long-chain acyl-CoA synthetases (LACS). (e–g) Alcohol-forming fatty acyl-CoA reductase (AlcFAR). (h–l) Ketoacyl-CoA synthase (KCS) from the fatty acid elongation complex. (m, n) Cytochromes P450 synthesizing coumaric and ferulic acids. (o, p) Aliphatic suberin feruloyl transferase linking aliphatic and aromatic suberin monomers to suberin building units (ASFT/BAHD).
Osmotic Lpr (10⁻⁸ m s⁻¹ MPa⁻¹) and reflection coefficient (σr) for NaCl of individual barley seminal roots grown under control or osmotic stress conditions (water potential of −0.8 MPa)

| Parameter                  | Control               | (osmotic stress)     |
|----------------------------|-----------------------|----------------------|
| Hydrostatic Lp (10⁻⁸ m s⁻¹ MPa⁻¹) | 8.11 ± 2.37a          | 3.19 ± 1.45b         |
| Osmotic Lp (10⁻⁸ m s⁻¹ MPa⁻¹)       | 3.15 ± 3.0a           | 3.59 ± 1.91a         |
| Hydrostatic/osmotic         | 4.27 ± 2.58a          | 1.11 ± 0.36b         |
| Solute permeability Pp (10⁻⁹ m s⁻¹) | 2.24 ± 1.54a          | 0.61 ± 0.61a         |
| Reflection coefficient (σr) | 0.38 ± 0.06a          | 0.38 ± 0.17a         |

Values are given as means ± SD of eight independent replicates (n = 8). Different letters indicate significant differences at a significance level of 0.05, analyzed using a two-sample t-test.

Table 1

In conclusion, this multifaceted study showed that water deficit, mimicked by different osmotic potentials through PEG 8000 treatment, markedly upregulated the suberin biosynthesis genes in barley seminal roots. By contrast, there was no or minimal effect on the expression of aquaporin genes, which are the regulatory components of water transport through the plasma membrane. The upregulation of suberin biosynthesis genes resulted in an increased endodermal suberization, thus reducing water movements through the apoplastic cell walls to prevent uncontrolled water losses from the root to the dry soil/medium. By contrast, water transport through the cell-to-cell path remained unaffected, and thus maintained further efficient water uptake from the soil into the central cylinder of the root. In the future, barley mutants might help to identify further suberin genes and to verify their functions. This could help us to better understand how altered suberin compositions and amounts in roots affect/regulate water and solute transport, and will aid in the improvement of future breeding programs to develop drought-tolerant barley cultivars.

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Author contributions

T.K. and N.S. performed microscopy. T.K., N.S. and V.V.Z-D. performed and analyzed the gas chromatography experiments. T.K., A.O., P.Y. and J.A.B. performed and analyzed the RNA-Seq experiments. T.K. and K.R. performed root pressure probe experiments. L.S., K.R. and F.H. designed and supervised the experiments. T.K., K.R. and L.S. wrote the manuscript. All authors read and approved the final manuscript.

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### Supporting Information

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

**Fig. S1** Amounts of aromatic monomers in barley seminal roots grown under control conditions or at a water potential of −0.4, −0.8 or −1.2 MPa.

**Fig. S2** Relative amounts of aliphatic suberin monomers in barley seminal roots grown under control conditions or at a water potential of −0.4, −0.8 or −1.2 MPa.
**Fig. S3** Hypothetical pathway for suberin biosynthesis in barley roots in response to osmotic stress.

**Table S1** Complete list of transcript per million (TPM) values

**Table S2** Complete list of differentially expressed genes

**Table S3** Cross-comparison of enriched gene ontology (GO) terms amongst differentially expressed genes in the barley seminal root zones A, B and C in response to osmotic stress

**Table S4** Differentially expressed genes (DEGs) and transcript per million (TPM) values of barley suberin, aquaporin, lignin and fatty acid elongation genes

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