Nitrogen depletion enhances endodermal suberization without restricting transporter-mediated root NO$_3^-$ influx

V.J. Melino$^{a,b,1}$, D.C. Plett$^{a,c,1}$, P. Bendre$^a$, H.C. Thomsen$^{c,d}$, V.V. Zeisler-Diehl$^e$, L. Schreiber$^e$, H.J. Kronzucker$^{a,f}$

$^a$ School of Agriculture and Food, The University of Melbourne, Melbourne, VIC, 3010, Australia
$^b$ Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology (KAUST), Thuwal, 23955-6900, Saudi Arabia
$^c$ School of Agriculture, Food and Wine, The University of Adelaide, Urrbrae, SA, 5064, Australia
$^d$ Carlsberg Research Laboratory, J.C. Jacobsens Gade 4, 1799, Copenhagen V, Denmark
$^e$ Department of Ecophysiology, Institute of Cellular and Molecular Botany, University of Bonn, 53115, Bonn, Germany
$^f$ Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada

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ABSTRACT

Roots vary their permeability to aid radial transport of solutes towards xylem vessels in response to nutritional cues. Nitrogen (N) depletion was previously shown to induce early suberization of endodermal cell walls and reduce hydraulic conductivity of barley roots suggesting reduced apoplastic transport of ions (Armand et al., 2019). Suberization may also limit transcellular ion movement by blocking access to transporters (Barberon et al., 2016). The aim of this study was to confirm that N depletion induced suberization in the roots of barley and demonstrate that this was a specific effect in response to NO$_3^-$ depletion. Furthermore, in roots with early and enhanced suberization, we assessed their ability for transporter-mediated NO$_3^-$ influx. N depletion induced lateral root elongation and early and enhanced endodermal suberization of the seminal root of each genotype. Both root to shoot NO$_3^-$ translocation and net N uptake was half that of plants supplied with steady-state NO$_3^-$ Genes with predicted functions in suberin synthesis (HvHORST) and NO$_3^-$ transport (HvNRT2.2) were induced under N-deplete conditions. N-deplete roots had a higher capacity for high-affinity NO$_3^-$ influx in early suberized roots than under optimal N$_3$. In conclusion, NO$_3^-$ depletion induced early and enhanced suberization in the roots of barley, however, suberization did not restrict transcellular NO$_3^-$ transport.

1. Introduction

Radial movement of water and nutrients from the soil through roots involves passing across concentric layers of epidermis, endodermis and cortical cells before reaching the vasculature system for long-distance translocation to the shoot (Sattelmacher, 2001). Nutrient ions and water can move across plasma membranes, via membrane-bound transporters and channels (transcellular route), or plasmodesmata, from one protoplast to another (symplastic route). Alternatively, they can move within the extracellular space outside the plasma membrane (apoplastic route). The composite transport model explains that the pathways are arranged in parallel allowing nutrient ions to switch between them depending on the resistance for solute flow (Steudle and Frensch, 1996; Steudle and Peterson, 1998). This enables a form of regulation to meet shoot nutrient demand (Plett et al., 2020).

The presence of endodermal barriers is dependent on the stage of development. Early in the development of fully elongated endodermal cells, aligned patches of lignin are seen that later fuse to form Casparian strips. In mature cells, Casparian strips terminate the apoplastic pathway, forcing water and nutrients to move via the symplastic/transcellular route. In later stages of root development, suberin, a bio-polyester, deposits in the inner surface of primary endodermal cell wall forming a hydrophobic, waxy layer and deposition increases with root age (Meyer and Peterson, 2013). Suberin and casparian bands are both

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$^1$ These authors should be considered joint first author
root barriers but whilst the latter only restricts apoplastic passage of nutrient ions, suberin, based on its deposition between the plasma membrane and the plasmastema, may also restrict access of nutrient ions to their transporters (the transcellular route) but leaving the symplastic route open to the passage of ions (Barberon et al., 2016; Geldner, 2013).

Suberization appears to be an adaptive response to nutrient stress. This concept was supported by the ionomic studies of Barberon et al. (2016) using transgenic line over-expressing the Cuticle Destructing Factor 1 (AtCDEF1). The ELTP::CDEF1 and CASP1::CDEF1 lines have a functional Casparian band but no detectable suberin (Naseer et al., 2012), and a lower potassium (K) concentration in the shoot. Additionally, deficiencies in the macro-nutrients sulphur (S), K, and phosphorous (P) resulted in enhanced suberization of wild type Arabidopsis plants (Barberon et al., 2016; Li et al., 2020). Early development of suberin lamellae was also observed in the K-deficient akor mutant and the S-deficient sultr1;1 sultr1;2 mutant (Barberon et al., 2016).

In contrast, deficiencies in the micro-nutrients zinc, iron (Fe) and Mn resulted in delayed deposition of suberin lamellae (Barberon et al., 2016; Chen et al., 2019; Sijmons et al., 1985). Discontinuous (patchy) suberization was observed in the Fe deficient mutant, irr1, and in the Fe and Mn deficient, rampl mutant (Barberon et al., 2016). These results may indicate that radial transport of micronutrients predominantly occurs across the endodermis via the apoplastic route, although the cation exchange capacity of the apoplast may result in root accumulation of these aforementioned nutrient ions. Barberon (2017) also suggests that enhanced endodermal suberization prevents leakage of some macro-nutrients (i.e. S, K, and P) from the stele to the apoplast of cortical cells.

Under conditions of prolonged drought or deficiencies of certain nutrients, root cortical senescence results in increased deposition of aliphatic suberin (Schneider et al., 2017), and, the endodermis may in some conditions become the outermost protective layer of the root (Meyer and Peterson, 2013). Aliphatic suberin, which is composed of primary alcohols, fatty acids, α-ω dicarboxylic acids (diacids), and ω-hydroxy acids (ω-HA acids), is thought to prevent movement of water and gases due to its high hydrophobicity (Schreiber et al., 1999) whilst aromatic suberin, whose most abundant constituents are ferulic and coumaric acids, has a greater protective role against pathogens as well as preventing solute movement (Kreszies et al., 2018).

The extracellular space around the cell wall is water-filled, enabling ions such as nitrate (NO$_3^-$) to move along with water apoplastically, through cell-wall interstitial spaces and characterized by very rapid turn-over kinetics for ions such as NO$_3^-$ (Kronzucker et al., 1995a, 1995b; Siddiqi et al., 1991). However, NO$_3^-$ is also able to cross the plasma membrane via both low-affinity nitrate transporter systems (LATS) and high-affinity nitrate transporter systems (HATS). LATS function significantly only when external NO$_3^-$ concentrations are high (> 1 mM) and often display linear transport kinetics (Kronzucker et al., 1995a, 1995b; Siddiqi et al., 1990). Nitrate transporters of the NPF/NRT1, the nitrate transporter 1/peptide transporter family (Leran et al., 2014), function as LATS but the dual-affinity transporter AtNPF6.3/NRT1.1 can switch its mode of action dependent on its phosphorylation state which varies as a function of external NO$_3^-$ (Liu and Tsay, 2003). The high-affinity nitrate pathway is considered to dominate over the low-affinity pathway in oilseed rape (Malagoli et al., 2004), dwarf maize (Garnett et al., 2013) and wheat (Melino et al., 2015) at lower levels of external N supply. HATS function is confined to lower external NO$_3^-$ concentrations (0.01–1 mM) and displays saturable transport activity and both constitutive and inducible components (Siddiqi et al., 1990).

Armand et al. (2019) recently demonstrated that low N caused enhanced and developmentally early deposition of suberin lamellae in barley roots with a concomitant decrease in root hydraulic conductivity when compared to the control supplied with both NH$_4^+$ and NO$_3^-$ Suberization is considered to create a barrier to reduce back-flow of water and thereby NO$_3^-$ losses from the stele to the apoplast of the cortex (Kreszies et al., 2018). On the other hand, in roots of rice, low ammonium (NH$_4^+$) supply, when ammonium was the sole N source, resulted in reduced suberin and lignin content as compared to the control, whereas high NH$_4^+$ supply enhanced suberin and lignin content (Ranathunge et al., 2016).

Broadly, we aim here to understand the effect of N nutrition on transport pathways of NO$_3^-$ from the root to the shoot. Specifically, the aim of this project was to assess whether N-depletion induced suberin composition changes in early suberized barley roots using NO$_3^-$ supply to the control as the sole source of N to avoid complications with differences in the effect of NH$_4^+$ doses seen by Ranathunge et al. (2016). Furthermore, in roots with early and enhanced suberization, we assessed whether suberin deposition could reduce transporter mediated NO$_3^-$ influx (transcellular route).

Three malt barley cultivars that are currently grown and used as parents in breeding programs were selected to assess whether the root architecture and biochemical responses to N-depletion were consistent or whether genetic variation for this trait exists. One of these cultivars, Scarlett, was previously used in a study demonstrating enhanced cell wall suberization under osmotic stress (Kreszies et al., 2019). The results of this present study showed that reduced N translocation to the shoot under N-deplete conditions was correlated with early and enhanced cell wall suberization and induction of the high-affinity NO$_3^-$ transport system. The evidence suggests that early and enhanced suberization under N-deplete conditions does not affect transcellular routes for NO$_3^-$ transport in barley.

2. Materials and methods

2.1. Plant material and growth conditions

Three barley (Hordeum vulgare L. spp. vulgare) cultivars were selected for this experiment, Australian cultivars Bass and La Trobe and the German cultivar Scarlett. Bass and La Trobe are Australian malt varieties that attract a higher premium than other varieties on the export market (Robertson, D. Senior barley Trader). Cultivar La Trobe has a higher predicted yield than cv. Bass across multiple environments and years in Australia (app.nvtonline.com.au, 2019). Scarlett is a German variety with good malt quality (Friedt et al., 2010). Plants were grown in a growth chamber (Conviron) at day/night conditions of 20 °C/15 °C, 16 h/8 h with a constant RH of 60 % and light intensity of 200 μmol m$^{-2}$ s$^{-1}$ at plant height. Four seeds per collar were surface sterilized in 1% (v/v) sodium hypochlorite for 15 min and germinated in acid-washed sand for three days before transfer to 14 L hydroponic systems with modified Johnson’s nutrient solution. Solutions contained 0.5 mM K$_2$SO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM MgSO$_4$, 0.2 mM CaSO$_4$, 25 μM H$_3$BO$_3$, 20 μM FeEDTA, 10 μM CaCl$_2$, 0.5 μM ZnSO$_4$, 0.5 μM MnSO$_4$, 0.125 μM CuSO$_4$, and 0.125 μM Na$_2$MoO$_4$ (pH adjusted to 6.3–6.5, using 1 M NaOH). Nitrogen (N) in the form of 0.75 mM Ca(NO$_3$)$_2$ (1.5 mM N final) was supplied to both the treatment and control for 14 d before transfer of the treatment group to N deplete conditions (Switch, 0 mM N final) for seven days whilst the control group remained at steady-state NO$_3^-$ (1.5 mM N final). Nitrate losses in the nutrient solution were measured via the rapid colorimetric method of Cataldo and Schrader (1975) by nitration of salicylic acid. Nitrate concentration was maintained at < 15 % loss by changing the nutrient solutions on days 3, 7, 9, 11, and daily from 12 to 21 days after sowing (DAS). Plants were thinned to two plants per collar at 9 DAS and hydro clay pebbles were used to cover the roots. Plants were imaged, and chlorophyll measurements (SPAD-502Plus) were recorded at 20 DAS. All plants were harvested at 21 DAS for fresh and dry weights.
2.2. Root nitrate influx

This protocol can be found online at https://dx.doi.org/10.17504/protocols.io.biibkcan. Plants were grown as described above with some modifications; plants were thinned to one plant per collar at 9 DAS and at 18 DAS, plants were removed from their collars and two plants were bundled together at the stem (Fig. S1). Plant roots were identified and separated according to types as defined by Watt et al. (2008). Three seminal roots originating from the embryo (embryonic roots) were bundled together (Fig. S1). These bundled embryonic roots (ER) would later be used specifically for \(^{15}N\) labelling whilst the \textquoteleft{}remaining roots\textquoteright{} (RR, scutella node axile roots, coleoptile node axile root and newly formed adventitious roots) would not be labelled but would otherwise be similarly treated. A split-root design was used so that all pre-label, label and wash steps were performed in 50 mL falcon tubes with aeration (Fig. S1). The plants were returned to their original growth conditions (steady-state \(\text{NO}\textsubscript{3}^-\) at 1.5 mM N or Switch at 0 mM N). At 21 DAS, these plants were moved to a freshly prepared pre-wash solution (unlabeled matching solution) for 5 min before transfer of the ER to a matching solution labelled with 0.1 mM N in the form of K\(^{15}\text{NO}_3\) (10 atom %) for 10 min. The K\(^{15}\text{NO}_3\) was balanced across all solutions at 1 mM final using K\(_2\text{SO}_4\). The ER and the RR were then transferred to their own matching unlabeled post-wash solution for 2 min in separate 50 mL falcon tubes to avoid cross-contamination of the label. Finally, the ER and the RR were moved for a final 5-s dip in 0.01 M Ca\(_2\text{SO}_4\). Plants were then removed and divided into shoots, ER and RR for drying at 70 °C for 72 h prior to nitrogen analysis. This experiment was initiated three hours after the start of the light period with randomization of cultivars and replicates, and all samples were completed in 30 min. Two independent experiments were undertaken, each with four replicates per genotype by treatment group.

2.3. Nitrogen isotope analysis

Elemental analysis of the total nitrogen content of dried and homogenized root and shoot samples was performed on a Thermo Flash 2000 H T (elemental analyser) paired to a Thermo Delta V Advantage (mass spectrometer). Nitrogen analysis was performed in the TrACEES Facility (University of Melbourne) on an elemental analyzer (ANCA GSL) paired to an isotope mass spectrometer (Secron Hydra 20–20). Data was calibrated against acetanilide (ThermoFischer) and acquired using Callisto v6.27.

2.4. Root architecture

Roots were cut from seeds and scanned on a flat-bed scanner (Epson Expression 10000 XL) using the parameters described by Melino et al. (2015). Image processing was first performed in FIJI Image J (http://imagej.net/Fiji) before root architecture was analysed via RootGraph (Cai et al., 2015). RootGraph enabled seminal roots to be separated from lateral roots for analysis of root volume, root length, root surface area, and root tip number.

2.5. Root segmentation

For RNA isolation, histochemical analysis and chemical analysis of suberin, the longest seminal root originating from the embryo was identified from apical to basal ends as zones A, B, C and D (Fig. 1). Section D was not used for analysis as described below. The root tip was cut from the top 0.5 cm away from the tip and discarded.

2.6. Histochemical detection of Casparian bands and suberin lamellae

The longest seminal root from each of two plants was pooled and placed into fixative solution for segmentation. A 0.5-cm length from the top, middle, and base of each of the zones was then used for histochemical analysis. Only zones A, B and C were used for histochemical analysis, as almost all endodermal cells were fully suberized in both the treatment and control groups in zone C and, therefore, this was assumed to be similar for zone D. Root segments were embedded in 5% (w/v) agarose, and 200 μM sections were cut using a vibratome (Leica VT1000 S). Sections were stained with either 0.01 % (w/v) Fluorol Yellow 088 (Sigma) or 0.1 % (w/v) berberine hemi-sulphate for 1 h in the dark at 80 °C. Sections were counter-stained in 0.5 % (w/v) Aniline Blue for 1 h in the dark at RT (Brundrett et al., 1988, 1991; Lux et al., 2005). Cross-sections were visualized by confocal microscopy (Nikon C2+) and images acquired and processed using NIS-Elements (Nikon). Histochemical results were confirmed by analysis of roots harvested from two independent experiments.

2.7. Chemical analysis of root suberin

For each replicate, four plants were pooled and dissected into three root zones (A, B, and C) prior to chemical extraction of lipids. All steps were performed at RT. Root segments were vacuum infiltrated with 10 mL of an enzyme solution containing 10 mM citric acid, 1 mL cellulase solution (Deltagen, Australia), 1 mL pectinase solution (Deltagen, Australia), and 100 mM sodium azide. Samples were enzymatically digested and treated as described previously (Kreszies et al., 2019). The
roots were washed with chloroform:methanol (1:1) for soluble extraction of lipids and continuously shaken for two weeks prior to air-drying overnight. Dried samples were used for trans-esterification, and gas chromatographic analysis and mass spectrometric identification were performed as described previously (Kreszies et al., 2019). The data was normalized to endodermal surface area (cm²) (Kreszies et al., 2019).

2.8. RNA isolation and cDNA preparation

Two hours after the start of the light period, roots were sectioned into zones A, B, C, and D and immediately snap-frozen in liquid nitrogen. Root zones A, B, and C were ground into a homogeneous powder, and 100 mg was used for RNA isolation. RNA was isolated using Ribozol (Ambion) according to the manufacturer’s instructions, and quality was confirmed by spectrophotometry (NanoDrop One, Thermo Fischer) and gel electrophoresis. 6 μg of RNA was DNase-treated using the Ambion DNA-freeTM kit; 1 μg of DNase-treated RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad).

2.9. Gene targets and expression

The barley suberin biosynthetic genes (HvHORST and HvRALPH) were identified based on orthology to Arabidopsis and rice orthologs. Maize NRT2 genes, previously shown to be responsive to N supply (Garnett et al., 2013), were selected and used in the phylogenetic analysis (Fig. S5), alongside other NRT2 orthologs as previously identified by Plett et al. (2010). qPCR primers were designed and gene stability determined according to recommendations by Vandesompele et al. (2002). Each qPCR reaction contained 20 ng of cDNA, 1 x SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.25 μM each of forward and reverse primer and nuclease-free water, to a final volume of 10 μL. qPCR was performed using a thermocycler (Bio-Rad, CFX96 Touch), and the data was analysed using CFX-Maestro 1.1 (Bio-Rad, 2017). Single amplicons were verified by gel electrophoresis, melt curve analysis, and sequencing prior to qPCR. Three stably expressed genes were used as reference genes; HvCyclophilin and HvGapDH (Burton et al., 2004) and HvActin (Sui et al., 2020) were used for the calculation of a normalization factor as described by Vandesompele et al. (2002). Normalized expression (ΔΔCq) was calculated according to Pfaffl (2001) and the average of four biological replicates calculated. All gene targets, primers, and annealing temperatures are presented in Table S1.

2.10. Statistical analysis

Statistical tests were performed using GENSTAT (V15.0, VSN International Ltd, Hemel Hempstead, UK). All data were assessed for homoscedasticity, non-correlations of means and variances, and normality of distribution. Outliers were identified on a residual plot (>2.5 standard deviations from the mean). Significant differences between groups were made using one- or two-way ANOVA. Null hypotheses were tested using Tukey’s post-analysis test.

3. Results

3.1. Plant growth responses to N depletion

Barley plants were grown with a steady-state supply of nitrate (NO3), provided at 1.5 mM for 14 d before a treatment group was moved to N starvation for seven days (0 mM, Switch), while the control group was maintained at a steady state of 1.5 mM NO3. Plants grown under N-deplete condition had a reduced number of tillers, above-ground biomass (Table 1), and chlorophyll content (Fig. 2). Additionally, root dry weight, but not fresh weight, was increased (Table 1) as reflected in the root architecture data. Plants of cv. Bass grown under N-deplete conditions had a higher root biomass (fresh and dry weight) than cv. La Trobe (Table 1) suggesting early root vigor in this growth system. Plants grown under N-deplete conditions (0 mM N, Switch) had a longer seminal root than plants grown under steady-state conditions (1.5 mM N) (Figs. 1 and 3A). When considering the sum of the length of all axile roots, cultivars La Trobe and Scarlett had twice the total axial root length (Fig. 3B) as compared to plants grown at steady-state NO3, contributing to a greater total root surface area, RSA (Fig. 3C). In N-deplete conditions, cv. La Trobe and cv. Scarlett, but not cv. Bass, increased their density of lateral roots (Fig. 3D) but not their length or number (Fig. S2). This enhanced lateral root density also contributed to an increased RSA (Fig. 3C).

3.2. Histochemical analysis of endodermal root barriers

Suberized root barriers are known to develop as the root ages and the endodermis differentiates (Barberon, 2017); however, the effect of NO3 nutrition specifically on this developmental process is unknown. Roots were segmented into quarters and 1/3 of the total root length (zones A, B, and C) was segmented further for sectioning and histochemical staining (Fig. 1). From the root apex, Zone A was 0–25 % of the root length, zone B was 25–50 % of the root length, and zone C was 50–75 % of the root length (Fig. 3A). Suberin lamellae deposition in the endodermis was visualized by histochemical means and, as expected from previous studies, was observed in the outer edges of the endodermal cell wall (Kreszies et al., 2019). The brightest fluorescence was in a U-shape, with more on the cortex-facing side of the endodermal cell wall than the stele-facing side, which is in-line with the tertiary stage of development (Schreiber et al., 1999), (Fig. 3B). In roots exposed to steady-state NO3 (1.5 mM N), suberin lamellae were either not detected (cv. Scarlett) or very few cells were suberized (cv. La Trobe and cv. Bass) in zone A, patchy suberization was evident in zone B, and complete suberization (cv. La Trobe and cv. Bass) or > 90 % suberization (cv. Scarlett) in zone C (Fig. 3B and C).

In contrast, under N-deplete conditions, roots of cultivars Scarlett and Bass showed early induction of suberin lamellae in zone A relative to

<table>
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<th>Table 1</th>
<th>Summary of the growth responses of barley plants 21 DAS to the nitrogen (N) treatments. Plants were grown for 14 d with steady-state NO3 (1.5 mM N), a treatment group was then switched to N starvation for 7 d (Switch, 0 mM N). Data is the mean ± SEM of four independent experiments, n = 16-18. Different letters in a single column indicate statistical significance at p &lt; 0.05. The probability value (p-values) for main effects and interactions are reported.</th>
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<td>Genotype</td>
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<td>Bass</td>
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roots grown under steady-state NO\textsubscript{3} conditions (Fig. 4B). In the zone closest to the root apex, there was a 55% increase in suberized endodermal cells of cv. Bass and a 97% increase in cv. Scarlett when plants were grown under N-deplete conditions as compared to steady-state NO\textsubscript{3} (Fig. 4B and C). Likewise, early suberization was seen in root zone B of cv. Scarlett and La Trobe but not Bass when plants under N-deplete conditions, accounting for a >17% increase in suberized endodermal cells when compared to plants grown with steady-state NO\textsubscript{3} (Fig. 4B and C). There was no significant difference between the number of suberized endodermal cells in zone C between the N-deplete and steady-state conditions, which is not unexpected given that endodermal cells in this zone were 90–100% suberized (Fig. 4C). There was also no difference in suberization of lateral roots in zone C (Fig. S2).

In a preliminary experiment, we tested whether suberization of the endodermis in zone B of cv. Scarlett also occurred at a low steady-state supply of N (0.5 mM N final) as compared to steady-state NO\textsubscript{3} control (1.5 mM N final). There was no difference in endodermal suberization at these two treatment levels (Fig. S3). The presence of Casparian strips was determined histochemically on the same root sections as for suberin detection. Casparian bands appear as flecks in root zone A of all cultivars but were brighter and longer in root zones B and C of all cultivars (Fig. S3). From these images, there was no evidence that N starvation altered Casparian strip formation in comparison to steady-state NO\textsubscript{3} (Fig. S3).

3.3. N-depletion induces aliphatic and aromatic suberin

Suberized cells contain polyaliphatic and polyaromatic domains
covalently linked together (Kreszies et al., 2018). Chemical isolation of the cell walls from each of the root zones are presented as either aromatic or aliphatic suberin. The main aliphatic suberin monomers released from rice and corn are primary alcohols, fatty acids, α-ω diacids and ω-OH acids (Schreiber et al., 2005). The predominant aromatic suberin monomers are ferulic and coumaric acid (Kreszies et al., 2018). Here these data (Fig. 5) are presented as the total amount of aliphatic suberin or aromatic suberin per endodermal surface area of each zone (Kreszies et al., 2019). The aliphatic suberin content increased in root zone C in all genotypes grown under N-deplete conditions (0 mM N, Switch) as compared to steady-state conditions (1.5 mM N), ($p < 0.05$, Fig. 5). Scarlett was the only cultivar, when grown under N-deplete conditions, where an increased aliphatic suberin content was observed in both root zones A and B as compared to steady-state conditions ($p < 0.05$, Fig. 5). Cultivars La Trobe and Scarlett grown in N-deplete conditions had a greater aromatic suberin content in root zone C than under steady-state conditions ($p < 0.05$, Fig. 5). Scarlett grown under N-deplete conditions was the only cultivar to show induction of aromatic suberin content in root zone B ($p < 0.05$, Fig. 5).

Fig. 4. Induction of suberin deposition in the endodermal cells of roots from N starved plants of the cultivars Bass, La Trobe and Scarlett. B. Three barley genotypes were grown for 14 d with steady-state external NO$_3^-$ (1.5 mM N). At 14 DAS, half of the replicates were switched to N starvation (Switch, 0 mM N) whilst the other half remained at stead-state N. All roots were harvested at 21 DAS, stained with fluorol-yellow 088 (yellow) and counter-stained with aniline blue (blue) and fluorescence was detected with a confocal microscope, a composite image is shown. A, diagram of the seminal root that was taken for sectioning. The root was divided into quarters with zones A (apex, 0-25 %), zone B (25-50 %), zone C (50-75 %) and zone D (base, 75-100 %, which was not used for analysis). B, Microscopy images from three genotypes at zones C, B and A (100 μm scale-bars are shown). C, Graphs showing % of endodermal cells which are suberized in zone C (top-panel), B (middle-panel) and A (bottom-panel). For this purpose, four biological replicates were assessed with 8-10 sections photographed per sample. Data shown is the mean ± standard error of the mean. Different letters above means indicate statistically significant differences between means (cultivars and treatments) at $p \leq 0.05$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 5. The amount of aliphatic (top-panel) and aromatic (bottom-panel) suberin chemically isolated from the cell wall of roots of the 21-day old barley plants of the cultivars Bass, La Trobe and Scarlett. Three barley genotypes were grown for 14 days with steady-state external NO$_3^-$ (1.5 mM N). At 14 DAS, half of the replicates were switched to N starvation (Switch, 0 mM N) whilst the other half remained at steady-state NO$_3^-$. The root was divided into quarters with zones A (apex, 0-25 %), zone B (25-50 %) and zone C (50-75 %) used for chemical isolation of suberin from the endodermal cell wall. Suberin content is expressed as amount per unit of endodermal surface area along the length of the root zone. Four biological replicates were used to calculate the mean. Data shown is the mean ± standard deviation. Different letters above means indicate significant difference between means within a single zone at $p < 0.05$ according to a T-test analysis.
3.4. N-depletion induces expression of suberin synthesis genes

Suberin biosynthesis in Arabidopsis has been well characterized and involves a number of enzymes. Two genes coding for proteins in the cytochrome P450 monoxygenase family are involved specifically in synthesis of the aliphatic fatty acid ω-hydroxy acid monomers. RALPH belongs in the cytochrome P450 monoxygenase family (CYP86B1) (Compagnon et al., 2009), as does HORST (CYP86A1) (Höfer et al., 2008). Here, we assessed whether differences in transcriptional response of putative orthologous genes relate to the zones where early suberization occurred. Similar to the histochemical and chemical results, the longest seminal root originating from the embryo was segmented into quarters (Fig. 1A) and each segment used separately for RNA isolation. There was a 1.6 to 2-fold increase in HvRAPLH transcripts in zone C of cultivars Scarlett and La Trobe when exposed to N-deplete conditions relative to steady-state NO3− (Fig. 6 B and Fig. S4). By contrast, a 20 % decrease in HvRAPLH transcripts observed in root zone C of the cultivar Bass grown under N-deplete conditions relative to steady-state NO3− (Fig. 6B and Fig. S4). There were no significant changes in HvRAPLH expression in response to N depletion in zones A and B. By contrast, HvHORST expression was significantly increased in zones A, B, and C in all three cultivars. The greatest induction of HvHORST was seen in zone A of N-deplete roots, where there was a 34-fold induction in Scarlett, a 32-fold induction in La Trobe, and a 13-fold induction in Bass (Fig. 6B and Fig. S4).

3.5. Identification of high-affinity nitrate transporters in barley

Barley orthologs of NRT2 genes were previously identified without any extensive phylogenetic comparison to grasses and model plant species (Han et al., 2016; Trueman et al., 1996; Vidmar et al., 2000a, 2000b). We identified the putative barley orthologues of the Arabidopsis NRT2 genes and compared them to the grass genomes of rice, sorghum, Brachypodium, and maize (Fig. S5). Similar to the findings of Plett et al. (2010), which did, however, not include barley, it was not possible to separate NRT2 genes simply based on sequence homology to Arabidopsis genes. The barley NRT2 encoding genes NRT2.1, NRT2.2, NRT2.3, and NRT2.4 clustered together and were more similar to the Brachypodium distachyon NRT2 genes of the same name (Fig. S5). HvNRT2.5 clustered more closely with the NRT2.5 genes of the other grasses examined (Fig. S5).

3.6. Nitrate transporters differentially respond in suberized zones

The presence of suberized root barriers enables a plant to regulate movement of ions via the symplastic pathway. Active uptake of NO3− at low external concentrations occurs via high-affinity nitrate transport systems in the NRT2 family. Gene expression analysis was performed on RNA isolated from zones A, B, and C of the longest seminal root originating from the embryo (Fig. 1). Transcriptional responses are shown as log2-transformed fold change as a response to the N-depletion (Switch, 0 mM N) relative to that of the steady-state NO3− control (1.5 mM N), (Fig. 6A), and as absolute normalized relative quantities (Fig. S6). Transcription abundance of HvNRT2.1 was increased in cv. Scarlett (zone B), and cv. Bass (zone A) in response to N-depletion. This was in contrast, to a ≥ 30-fold decrease in transcript abundance of HvNRT2.1 in all root zones of cv. La Trobe (Fig. 6A). Transcript abundance of HvNRT2.2 was strongly induced in response to N-deplete conditions in all root zones of cultivars Scarlett and Bass but only in root zone C of La Trobe (Fig. 6A). Transcript abundance of HvNRT2.3 was reduced in response to N-depletion in all root zones of Scarlett, in root zones A and B of La Trobe but unchanged in all root zones of cv. Bass (Fig. 6A). Transcription of HvNRT2.4 was reduced in all root zones of cv. La Trobe and in zone C of cv. Bass but not in cv. Scarlett under N-deplete conditions relative to steady-state NO3− (Fig. 6A). Abundance of HvNRT2.3 transcripts were significantly higher in cv. Scarlett than in cultivars La Trobe and Bass, irrespective of the NO3− treatment (Fig. S6).

3.7. Nitrate influx is induced under N-starvation

The effect of early suberization in the seminal roots of cultivars Scarlett (zone A and B), Bass (zone A), and La Trobe (zone B) on the rate of movement of N from the external medium and into roots was unknown. In order to explore this, seminal roots originating from the embryo of 21-d old barley plants were exposed to 0.1 mM N in the form of K15NO3, a concentration which is known to stimulate the high-affinity

![Fig. 6. Transcriptional response of selected suberin biosynthetic and nitrate transport genes in 21-day old barley roots in response to a switch from steady-state NO3− (1.5 mM N) to N starvation (0 mM N, Switch) for 7 days. A. a diagram of the root zones of which Zone C (basal, 50-75 %), Zone B (middle, 25-50 %) and Zone A (apex, 0-25 %) are relevant to this analysis. B. Log2 transformed fold change in response to the treatment was calculated using the Normalized Relative Quantity (NRQ) and the following equation: log2(NRQ of Switch/NRQ of 1.5 mM N). HvRAPLH encodes a putative cytochrome P450 monoxygenase (CYP86B1), HvHORST encodes a putative cytochrome P450 monoxygenase (CYP86A1), HvNRT2.1, HvNRT2.2, HvNRT2.3 and HvNRT2.4 encode members of the high affinity nitrate transport (NRT2) family. One-way ANOVA was performed to compare the treatment response within a zone for a single target gene and this significance is denoted in black font. Shading intensity (see legend) is not representative of statistical significance but demonstrates induction (red) or reduction (blue) of transcript abundance of a gene target in response to the Switch treatment (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).]
nitrate transport system (Kronzucker et al., 1995a, 1995b; Siddiqi et al., 1990). N-depletion stimulated NO$_3^-$ influx by 3-fold in cv. Bass, but by 6-fold in cv. La Trobe and cv. Scarlett, although there was no statistically significant difference between genotypes at steady-state NO$_3^-$ so this difference in stimulation could not be confirmed (Fig. 7A). Over the 10 min labelling period, 7–20% of $^{15}$N was translocated from the root to the shoot (Fig. 7B). There was a significant decrease in translocation of $^{15}$N from the roots to the shoots in the Switch treatment (main effect when genotypes were averaged together, $p = 0.0254$) when compared to the controls but there was no significant treatment effect within a single genotype nor between genotypes (Fig. 7B). There was also no significant difference in nitrate influx measured between genotypes grown in either the Switch or steady-state NO$_3^-$ growth conditions (Fig. 7A). After 21 d of growth, the N content in plants grown under N-deplete conditions (Switch, 0 mM N) was half that of plants supplied with steady-state NO$_3^-$ (1.5 mM N), and there were no genotypic differences (Fig. 7C).

4. Discussion

Plants must continuously acclimate to keep pace with spatiotemporal fluctuations in N availability. Conditions of water deficit, causing reduced transpirational water fluxes, can impair the acquisition of nitrate and ammonium (Plett et al., 2020). Plants use a myriad of strategies to overcome localised N deficiencies which, aside from adjustments in the expression of genes encoding N transporters, their abundance and regulation, includes changes in root system architecture, the organization of root anatomy, and the chemical composition of roots (Plett et al., 2020).

In this study, barley plants were either exposed to steady-state NO$_3^-$ (1.5 mM N) or seven-days of N starvation (0 mM N, Switch). Plants grown for 7 days without N were moderately deficient in that growth was limited when compared to the control plants (Table 1). In our present study, the N content of N-deplete plants was half that of plants grown at steady-state NO$_3^-$ (1.5 mM N). N-deplete plants had approximately 75 mg of N per g dry weight or 7.5% N (Fig. 7C) which is growth limiting (see Table 1) and slightly higher than the N level (6.1%) considered critical for maximum shoot and grain yield in barley (Reuter and Robinson, 1997). Deficiency symptoms such as yellowing or senescence of the oldest leaf were not visible by eye but a slight and significantly reduced chlorophyll content was measured (Fig. 2) suggesting that photosynthesis was reduced. Similar to the results by Armand et al. (2019), there was a reallocation of resources in the barley plants with a reduced shoot mass and increased root mass (Table 1).

Increased dry mass of N-deplete roots was accounted for by longer growing at 1 mM NO$_3^-$ supplied to barley roots caused enhanced and early endodermal suberization and lignification as compared to the control (Armand et al., 2019). In a preliminary experiment, we did not find any significant difference between suberization of the endodermis of cv. Scarlett at a concentration of 0.2 mM N as reflected by the nitrate depletion rates with that of Armand et al. (2019) suggests that endodermal suberization depends on the severity of the N deficiency and that lower than 0.5 mM N is required to induce this deficiency. It was not technically feasible in this experimental design to maintain a steady-state NO$_3^-$ concentration of 0.2 mM N as reflected by the nitrate depletion rates shown in Fig S3 B.

![Figure 7](image-url)
Instead, we tested a period of short-term (7 days) N starvation, which induced suberization of endodermal cell walls in barley roots as compared to a control supplied with steady-state NO₃⁻ (Fig. 4) and we provide quantitative evidence of this (Fig. 5). Aliphatic and aromatic suberin monomers from roots were collected, chemically degraded, and analysed quantitatively by GC–MS. The histochemical analysis showed that a greater number of suberized endodermal cells were present in root zone A of cv. Scarlett under N-deplete conditions than under control conditions (Fig. 4), which was supported by increased aliphatic and aromatic suberin (Fig. 5). However, the chemical data shows increased suberin content in the distal root zone C for cultivars La Trobe and Bass whereas this was visualized in basal root zone A histochemically. This difference may be due to the fact that the histochemical data is reliant on representative 0.5 cm segments of each root zone to be sectioned whilst the chemical data used the entire length of the root zone including the lateral roots for chemical extraction. The effect of the treatment on lateral root suberization is inconclusive in root zone B due to technical issues with sectioning sufficient replicates. However, the thicker lateral roots sectioned from root zone C of cv. Scarlett showed near complete or complete suberization patterns in both treatment groups (Fig. S2) suggesting that the increased aliphatic and aromatic content measured in zone C of N-deplete cv. Scarlett roots (Fig. 5) was the result of enhanced suberization of the seminal root alone.

Considering that the seminal root of plants grown under N-deplete conditions are longer than those grown under steady state NO₃⁻ (Fig. 3A), we must consider that developmental differences exist between the root zones as also visualized by lateral root differences in cv. Scarlett zone B (Fig. 1). Considering that suberization occurs with developmental aging, we would therefore expect to see more suberization in the endodermis of control roots rather than those rapidly elongating under the N-deplete conditions. However, the results of this study show that the N-deplete zone B roots of cv. Scarlett were in fact more suberized (Fig. 5) providing conclusive evidence that enhanced suberization is a specific effect of N-depletion (Fig. 5). It is also clear from both the histochemical and chemical analyses that cv. Scarlett had the most pronounced and earliest induction of endodermal suberin lamellae of all the genotypes in response to N starvation (Figs. 4 and 5).

Aliphatic suberin is mainly composed of ω-hydroxy acids and α,ω-diacids of carbon chain-length C₆–C₄₂ in Arabidopsis and up to C₅₂ in barley and rice (Kreszies et al., 2018). The oxygenation of fatty acids is performed by NADPH-dependent cytochrome P450 monooxygenases in barley and rice (Kreszies et al., 2018). The oxygenation of fatty acids results in maize (Zimmermann et al., 2000), however a negative correlation between suberin amount and solute permeability is not consistently found (Ranathunge and Schreiber, 2011).

In this study, the relationship between early induction of root barriers and nitrate transport was assessed. In Arabidopsis, root NO₃⁻ uptake at low external NO₃⁻ concentrations relies on five members of the nitrate transporter 2 (NRT2) family; NRT2.1 and NAR2.1 (NRT3.1) (Okamoto et al., 2006), NRT2.2 and NRT2.4 (Kiba et al., 2012), and NRT2.5 (Lezhneva et al., 2014). The significant separation of the phylogenetic tree of NRT2s in monocots and dicots makes direct comparison of gene functions difficult (Plett et al., 2010). In maize, ZmNRT2.1, ZmNRT2.2 and ZmNRT3.1 showed the largest response to low nitrate provision (Garnett et al., 2015). Despite the importance of understanding how NO₃⁻ moves via the symplastic route in response to external NO₃⁻ provisions, this information in grasses is limited. Phylogenetic analysis shows that the putative barley NRT2 encoding genes HvNRT2.1, HvNRT2.2, HvNRT2.3, and HvNRT2.4 cluster together but are well separated from Arabidopsis and other dicots, making it difficult to directly assign functions to the barley genes based on phylogeny alone, without further functional characterisation. HvNRT2.5 clustered with that of the other grasses and of the Arabidopsis NRTs, and shared the greatest orthology with AtNRT2.5, suggesting a similar function. AtNRT2.5 has a role in adult plants, where it can transport NO₃⁻ across the epidermis and cortex of roots (Lezhneva et al., 2014).

Both Arabidopsis NRT2.1 and NRT2.2 were shown to be strongly inducible at the transcriptional level by NO₃⁻ provision to NO₃⁻-deplete plants, whilst AtNRT2.4 was only modestly induced and AtNRT2.5 repressed (Okamoto et al., 2003). In the present study, we assessed the response of HvNRT genes involved in root NO₃⁻ uptake in young plants focusing on those that were NO₃⁻-responsive. HvNRT2.1 and HvNRT2.2 were both transcriptionally induced by NO₃⁻ starvation in zone B of cv. Scarlett (Fig. 6). There were, however, considerable variation in the responses of HvNRT2.1 and HvNRT2.2, which were dependent on genotype and root zone. HvNRT2.3 and HvNRT2.4 were either repressed or unchanged in response to N starvation (Fig. 6). Amino acids such as glutamine are known to down-regulate HvNRT2 expression (Vidmar et al., 2000a, 2000b) and down-regulate NO₃⁻ transport physiologically, in addition to NO₃⁻ itself, via negative feedback mechanisms (Kronzucker et al., 1995a, 1995b). Also, it is important to consider post-translational regulation of NRT2s at the protein level, which in the case of Arabidopsis NRT2.1, requires the NAR2.1 protein to be expressed and present at the plasma membrane (Wirth et al., 2007). Considering that two of the NRTs and HvHORST were transcriptionally induced in the same root zone where the aliphatic and aromatic suberin accumulated, it is interesting to speculate on a coordinated signal from ABA. ABA has been shown to regulate NRT2/NAR genes under limited NO₃⁻ supply in wheat roots (Wang et al., 2020) whilst Barberon et al. (2016) observed that ABA supply to Arabidopsis roots induced an early and continuous expression of a suberin biosynthetic gene and an increase in root suberin content.

The activity of the high-affinity NO₃⁻ transport (HAT) system at 21 DAS in the seminal roots was measured using a split-root design which enabled us to focus on the roots where early and enhanced suberization under N-deplete conditions was identified. The NO₃⁻ HAT activity is the sum of the activities of all root NO₃⁻ transporters that operate at low external NO₃⁻ concentrations and should reflect the maximum HAT capacity. High-affinity NO₃⁻ influx was induced in all genotypes by greater than 3-fold in response to N-depletion (Fig. 7 A), which is similar to results in maize (Garnett et al., 2015). NO₃⁻ -responsive transcriptional networks, including those involved in nitrate transport, is highly variable across the life cycle of maize (Plett et al., 2016), and presumably in barley as well. To obtain a better idea of the sum of the activities of all NO₃⁻ transporters, translocation of ¹⁵Ν-labelled NO₃⁻ from the root to the shoot (Fig. 7 B) and net nitrogen uptake (N content, Fig. 7 C) was assessed. The root to shoot translocation of NO₃⁻ was reduced in N-deplete plants and this was correlated with early and enhanced endodermal suberization. Armand et al. (2019) also identified reduced transpiration rates of barely grown at low-N conditions, which would
similarly result in reduced root to shoot translocation of NO$_3$.

There is substantial evidence to show that roots have enhanced or delayed suberization of the endodermis in response to nutritional cues. However, the movement of nutrients such as S and K radially via the symplastic route is likely to dominate over the apoplastic route. This was demonstrated using the enhanced suberin 1 (esb1) mutant, which, despite a reduced transpiration rate and higher root suberin, had enhanced shoot S and K content (Baxter et al., 2009). The role of early and enhanced suberin in plants deficient in macronutrients may, instead, prevent the leakage of ions from the stele, which could otherwise readily occur under conditions of reduced transpiration, such as during the night cycle, low nutrient or drought conditions when the chemical water potential gradient is directed outwards towards the soil. The role of suberin in preventing NO$_3$ back-flow from the stele to cortical apoplast was however not the focus of this present study.

Here we support and build upon the findings of Armand et al. (2019) that nitrogen deficiency induces early and pronounced development of suberin lamellae in the endodermal cells of barley roots by using both qualitative and quantitative measurements of suberin monomers. Unlike Armand et al. (2019), we did not find an increase in the formation of Casparian bands under N deficiency (Fig. S3). Further studies using barley mutants defective in suberization are required to understand the complexity of the composite model of root radial transport of nutrients. We suggest that induction of the high-affinity nitrate transport system in the same root and zones where early endodermal suberization occurred is part of an adaptive strategy by plants to maintain NO$_3$ influx via the transcellular route but limit stelar NO$_3$ efflux; the latter was not studied here.

5. Conclusion

Reduced root to shoot translocation of NO$_3$ under N-deplete conditions occurs despite induction of the high-affinity NO$_3$ transport system. We speculate that this is due to blocking of the apoplastic transport route, however, whether this was due to reduced transpiration or enhanced endodermal suberization, or a combination of both is inconclusive. Maintenance of shoot N in plants grown under optimal N nutrition must therefore rely on both the apoplastic and symplastic/transcellular transport routes. It was suggested that suberization also limits transcellular ion movement by blocking access to transporters (Barberon et al., 2016). However, induction of HAT NO$_3$ influx under N-deplete conditions suggests that suberization does not limit transcellular routes for NO$_3$ transport operating at low N levels in barley.

CRediT authorship contribution statement

V.J. Melino: Conceptualization, Methodology, Software, Validation, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing.
D.C. Plett: Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.
P. Bendre: Investigation, Validation, Formal analysis.
H.C. Thomsen: Validation, Formal analysis, Investigation.
V.V. Zeisler-Diehl: Validation, Formal analysis, Investigation.
L. Schreiber: Conceptualization, Methodology, Formal analysis, Data curation, Writing - review & editing, Supervision.
H.J. Kronzucker: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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