Novel temporal, fine-scale and growth variation phenotypes in roots of adult-stage maize (*Zea mays* L.) in response to low nitrogen stress

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ABSTRACT

There is interest in discovering root traits associated with acclimation to nutrient stress. Large root systems, such as in adult maize, have proven difficult to be phenotyped comprehensively and over time, causing target traits to be missed. These challenges were overcome here using aeroponics, a system where roots grow in the air misted with a nutrient solution. Applying an agriculturally relevant degree of low nitrogen (LN) stress, 30-day-old plants responded by increasing lengths of individual crown roots (CRs) by 63%, compensated by a 40% decline in CR number. LN increased the CR elongation rate rather than lengthening the duration of CR growth. Only younger CR were significantly responsive to LN stress, a novel finding. LN shifted the root system architectural balance, increasing the lateral root (LR)-to-CR ratio, adding ~70 m to LR length. LN caused a dramatic increase in second-order LR density, not previously reported in adult maize. Despite the near-uniform aeroponics environment, LN induced increased variation in the relative lengths of opposing LR pairs. Large-scale analysis of root hairs (RHs) showed that LN decreased RH length and density. Time-course experiments suggested the RH responses may be indirect consequences of decreased biomass/demand under LN. These results identify novel root traits for genetic dissection.

Key-words: aeroponics; architecture; corn; crown root; lateral root; root hair; second-order lateral root; stochastic variation.

INTRODUCTION

Nitrogen is one of the most limiting nutrients in global production of maize (*Zea mays* L.) (Ladha *et al.* 2005) prompting interest in optimizing maize root architecture for efficient nutrient uptake under low nitrogen (LN) stress conditions (Fitter 1991; Dorlodot *et al.* 2007; Lynch 2007; Garnett *et al.* 2009). Such targeted improvement can be facilitated by understanding how maize roots respond to

LN both spatially and temporally, particularly at adult stages of development (Liu *et al.* 2009).

The root system of adult-stage maize plants can consist of >3 km of roots (Mengel & Barber 1974) with a diversity of organs that change dynamically during development. At the seedling stage, the embryonic maize root system consists of a single primary root and a variable number of branched seminal roots (Fig. 1a). Later in development, below-ground shoot-borne crown roots (CRs) form the majority of the root system required for nutrient acquisition (Hochholdinger *et al.* 2004) (Fig. 1b). Additional above-ground shoot-borne brace roots insure anchorage of the stem (Fig. 1c). Lateral roots (LRs), which initiate from the numerous CRs, form an extensive underground branch network, including secondary, tertiary and higher orders of branching that increase the surface area of the root system (Forde & Lorenzo 2001; Malamy 2005). At the finest scale, crown and LRs initiate root hairs (RHs), which uptake water and nutrients.

Various studies have shown that maize alters its root architecture in response to LN stress. Using hydroponics and semi-hydroponic growth systems, LN stress was shown to increase the root to shoot ratio, total root length (TRL) and lengths of the CR and LR systems, while reducing CR number (Feil *et al.* 1990; Eghball & Maranville 1993; Chun *et al.* 2005; Wang *et al.* 2005; Liu *et al.* 2008). Similar root responses to LN were found in field studies (Mackay & Barber 1985; Pan, Jackson & Moll 1985; Durieux *et al.* 1994; Liu *et al.* 2009).

Despite the above-mentioned painstaking efforts, gaps remain in our understanding of the response by maize roots to LN stress. Many of the published studies were limited to the seedling stage, often included length but not architectural details, and/or lacked measurements of the root types that contribute most to root function, namely, higher orders of LRs and RHs. For example, only first-order LRs have been studied in adult maize, or measurements of all LRs have been averaged, even though the finer roots might have unique responses to nutrient stress. Many field studies have required broken roots to be collected from the soil and root system architecture to be reconstructed virtually (Maizlish, Fritton & Kendall 1980; Mackay & Barber 1986; Eghball & Maranville 1993; Durieux *et al.* 1994). As most previous studies involved destructive phenotyping, also missing have
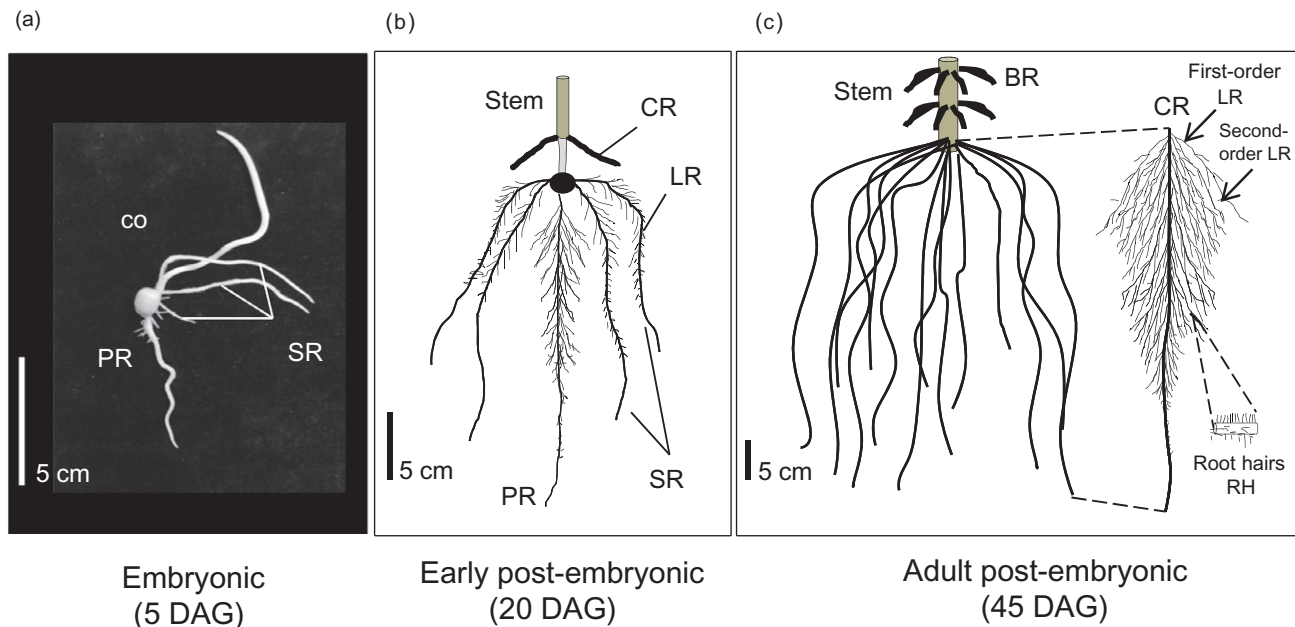
Figure 1. Schematic representation of (a) maize embryonic and (b, c) post-embryonic root system development at progressive days after germination (DAG). (a) The primary root (PR) and seminal roots (SR) initiate from the embryo. (b) Lateral roots (LR) initiate from PR and SR, while crown roots (CRs) initiate from the stem. (c) The root system in adult maize consists of multiple crown roots and their LR that undergo multiple orders of branching. The various root organs terminate in root hairs (RHs). Structural brace roots (BR) that initiate above-ground are also shown. co, coleoptile.

been time-course descriptions of maize root responses to nutrient stress.

Gaps in maize root phenotyping have been caused by diverse technical challenges. In semi-hydroponics, containers restrained root growth, while in hydroponics, fine root branching and RH growth were impaired (Tuberosa *et al.* 2002). Field studies have suffered from heterogeneity in the N stress itself, incomplete tracking of whole root systems, and root breakage during excavation, particularly of fine root tissues.

In this study, we undertook to provide a complete description of the effects of LN stress on the maize root system, of adult-stage plants, at all scales of development: macro (centimetre to metre, CRs), meso (millimetre to centimetre, LRs) and micro (micrometre to millimetre, higher order LRs and RHs). We also undertook to provide time-course measurements to add a temporal understanding of important root responses. To overcome the challenges experienced in earlier studies, an aeroponics system for maize was engineered and optimized in combination with assays that permitted non-destructive root phenotyping. Aeroponics is a growth system in which plants are grown by spraying roots suspended in the air with a nutrient solution (Waisel 1996) (Fig. 2). Aeroponics permits clean, fine-scale phenotyping of roots and creates a nearly uniform rhizosphere environment to separate intrinsic responses from environmental variation. Although aeroponics is not a new technique, its application for the study of maize root architecture has not previously been reported. Rather than studying responses to nitrogen starvation, following dose-response experiments,

we imposed a real-world level of nitrogen stress (8 mM total N) associated with deficiencies that maize would likely encounter in an agricultural field (Wolt 1994). Using these experimental parameters, here we report novel temporal, fine-scale and growth variation responses to LN by root systems of adult-stage maize plants.

MATERIALS AND METHODS

Aeroponics growth system

Multiple parameters were optimized to create an aeroponics system for maize (see Supporting Information). A complete sketch of the final aeroponics system is shown (Fig. 2a). Independent systems were constructed in parallel for independent replicates (Fig. 2b). Each system consisted of six 133 L aeroponic barrels (90 cm deep) and each barrel was used to grow two plants. Within each barrel, the nutrient solution was misted from the lid onto the root system at regular 1 min intervals (10 s of spray every 50 s). The nutrient solution was pumped from a 100 L tank by a Geyser ® submersible utility pump (4770 L h^{-1}, Simer, Delavan, WI, USA) regulated by a time delay relay (CB-1004B-70, Tyco Electronics, Fuquay-Varina, NC, USA) (Fig. 2a). The barrels were placed onto an artificial sloping floor to allow the nutrient solution to drain back by gravity flow to a feedback pipe system, where it was first collected in a 5 L black container and then pressurized back to the 100 L nutrient solution tank using a smaller submersible pump (PE-A series, 303 L h^{-1}, Giant, Toledo, OH, USA). One microjet sprinkler
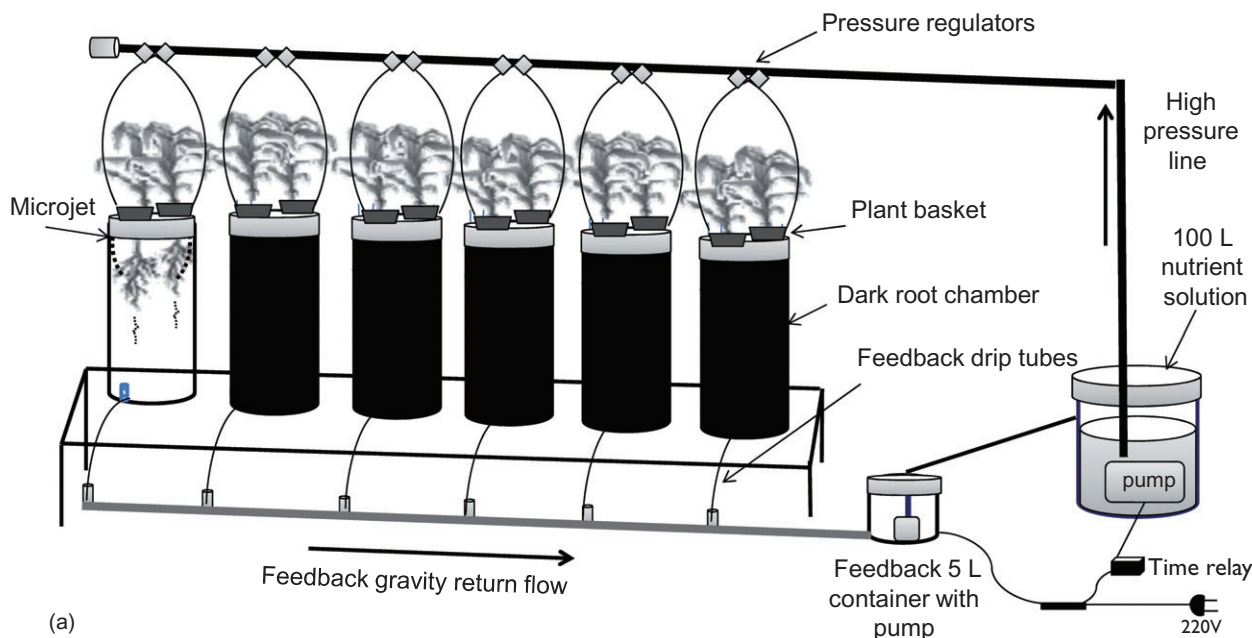
Figure 2. Aeroponics set-up for maize. (a) Schematic of the customized closed-loop aeroponics system. Microjets sprayed the root system 10 s every 50 s in a 180° range. (b) Identical but completely independent systems were constructed in parallel, each with their own 100 L nutrient solution tank, to permit multiple nitrogen treatments or replicates. Aeroponics permitted post-embryonic maize root and reproductive development. Shown are (c) the extensive brace root system (d) the large, long whole root system at 75 d after transplanting (DAT), and (e, f) ears and tassel obtained from aeroponically grown maize at 75 and 90 DAT, respectively.

was allocated per plant to provide uniform misting between root systems. The pressurized solution was pumped to two microjets/barrel (Kalyx, Camden, NY, USA) each capable of spraying 98 L h⁻¹ with a 180° range, to produce an atomized nutrient solution spray.

A pre-germinated seedling was suspended in a basket, and two baskets were fitted onto openings cut into the lid of each barrel. Each basket consisted of a 13-cm-diameter pot with a black nylon net replacing the bottom. The primary root was placed through a hole in the net and the seed was covered with bleach-treated white aquarium stones allowing the primary and subsequent roots to be misted without light penetrating into the root chamber.

Nutrient solutions and nitrogen treatments

To determine the appropriate high nitrogen (HN) and LN concentrations, an initial nitrogen dose-response experiment was conducted in aeroponics using total N concentrations ranging from 4 to 20 mM (Supporting Information Table S1). This concentration range was chosen based on dry matter accumulation and root:shoot biomass ratio results using semi-hydroponics (McCullough *et al*. 1994; Echarte, Rothstein & Tollenaar 2008). Our initial experiment showed that in aeroponics, 8 mM was not significantly different from 4 mM total N, and both caused a ~25% reduction in shoot biomass and significantly increased

the root:shoot biomass ratio (Supporting Information Table S1). Based on this experiment, 8 and 20 mM total N were selected as the LN and HN concentrations, respectively. The final LN and HN nutrient solutions both contained: 0.1 mM K_2SO_4, 1 mM KCl, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 0.04 mM H_3BO_3, 0.02 mM $MnSO_4$, 0.7 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.5 μM $NH_4Mo_7O_{24}$, 1 mM Fe-DTPA. Seven days after planting (three-leaf tip stage), 3 g of ethylenediaminetetraacetic acid (EDTA)-chelated micronutrient mix (Plant-Prod #7906B7B, Plant Products Co, Canada) were added per 100 L of the above solution to achieve a final concentration of 2.1 ppm Fe (5% EDTA chelated and 2% DTPA chelated), 0.6 ppm Mn, 0.12 ppm Zn, 0.03 ppm Cu, 0.39 ppm B and 0.018 ppm of Mo. The HN treatment consisted of 6 mM $Ca(NO_3)_2$ and 4 mM NH_4NO_3 (20 mM total N), while the LN treatment had 2 mM $Ca(NO_3)_2$ and 2 mM NH_4NO_3 (8 mM total N). $CaCl_2$ was used to balance calcium ions between HN and LN. The pH of the solutions was maintained on a daily basis in the pH 5.7–6.3 range.

Experimental design for macro-, meso-, microscale aeroponics studies

Maize inbred line W22 seeds were surface sterilized using 6% bleach (NaCl0) for 5 min, washed for 20 min with sterile water and then germinated in the dark with dH_2O-moistened filter paper and 1 mL of concentrated fungicide (Maxim XL™, Syngenta, Waterloo, NE, USA). Uniformly germinated seeds were transferred to the aeroponics growth system in a glass greenhouse under a mixture of high pressure sodium and metal halide lamps (800 μmol m^{-2} s^{-1}, at pot level), 16 h photoperiod, and 28 °C day/20 °C night regime, during March–June in the Crop Science Building Greenhouse Facility, University of Guelph.

Four identical, but independent aeroponics systems were constructed in parallel. For each system (replicate), a 100 L nutrient solution fed 12 plants distributed among six barrels. Plants were grown under high N (20 mM total nitrogen) or low N (8 mM total nitrogen) for 30 d (12 leaf tips on average) with two replicates arranged in a randomized block design. The experiment was repeated two times ($n = 48$ per nitrogen treatment).

Experimental design for Turface® versus aeroponics comparison

Inbred line SG200 and Hybrid SG150 (Syngenta) were germinated as described earlier. Two identical aeroponics systems, one for HN and one for LN, were constructed in a glass greenhouse and placed side-by-side with 18 cm pots containing Turface® (same growth conditions as above). In aeroponics, each system consisted of two sets of three barrels, each containing two plants, in which six hybrid and six inbred seedlings were distributed randomly. Each set of three aeroponics barrels was considered as a replicate (three plants per genotype × nitrogen treatment), and there were two replicates ($n = 6$). For Turface®, each replicate consisted

of three pots per genotype x nitrogen treatment, and there were 2 replicates, arranged in a randomized plot design. The same nutrient solution (described earlier) was used in both growth systems and replenished frequently (see Supporting Information). For Turface®, the application rate was 1.5 L per day compared with ~400 mL min^{-1} for the whole aeroponics system. Plants grown in aeroponics and Turface were germinated, harvested and analysed simultaneously.

CR measurements

Each root system was harvested, weighed and flat-stored in trays containing 50% ethanol at –20 °C. It was then defrosted, floated in water in 30×42 cm transparent plastic trays, and scanned using a Large Area scanner (LA2400, Hewlett Packard, Palo Alto, CA, USA). Root traits were measured using WinRhizo software root diameter analysis (Version PRO 2007, Regent Instruments Inc., Ontario, Canada). Images were analysed for TRL per plant. Image analyser was set up to analyse root traits per diameter class allowing analysis of LRs (LR < 0.2 mm) and CR (> 0.5 mm) separately. When destructive measurements were performed, embryonic and post-embryonic axial (adult, crown) roots along with their respective laterals were separated for accuracy and to prevent overlapping during scanning. Brace roots were excluded. Shoot and root tissues were then dried and weighed.

Measurements of CR elongation rate

Maize inbred line W22 seeds were germinated and transplanted into the aeroponic system as described above. Twelve plants were grown under high N (20 mM total nitrogen) or low N (8 mM total nitrogen) for 25 d. CRs were labelled daily from the HN treatment as they initiated and compared with CR initiated on the same day from the LN treatment. Each day, three to five pairs of CR were labelled, randomly taken from the plant populations. At 24 h before harvest, each labelled CR was dyed with 1 mM neutral red solution (pH 7). The elongation rate was calculated by measuring new growth (non-stained length) over a 24 h period. At harvest, the length of each individual CR was measured from the HN treatment and compared with the CRs from the LN treatment that initiated at the same time. We previously tested various concentrations to ensure that staining had no effect on root growth (data not shown).

LR measurements

At 15 d after transplanting (DAT), one newly initiated CR on each of 48 HN- and 48 LN-treated plants was labelled by tying a thread around it. At 30 DAT, these labelled roots were harvested, flat stored in 50% ethanol at –20 °C in a transparent plastic bag (Ziploc®, Johnson & Son, Inc., Racine, WI, USA), and later scanned. Because the first- and second-order LRs were not differentiated accurately by the WinRhizo software, we highlighted each LR order in different colors using Paintshop Pro 9 and then analysed the image

again in WinRhizo using color class analysis. As CRs varied in length, LRs were sampled on each CR as follows: each CR was divided into 3 segments above the elongation zone (basal, middle, apical), and then each segment was further divided into 3 subsegments; the middle subsegment, representing one-ninth of each CR, was directly measured, and then multiplied by three to give the score for that segment.

Analysis of growth variation in LR pairs

Maize inbred line W22 seeds were germinated and transplanted into the aeroponic system under similar conditions as described earlier. Plants were grown under high N (20 mM total nitrogen) or low N (8 mM total nitrogen). Synchronously initiating CRs were labelled 5 DAT (one per plant, $n = 12$ per treatment). Fifteen days later, all labelled CRs were harvested. The CR axis was divided into three segments as described in the mesoscale measurement section. First-order LRs were measured in the basal segment. The lengths of pairs of adjacent 180°-opposing LRs were recorded and the ratios of the root pairs (longer/ shorter) are presented. This method approximately normalizes for different LR ages within the segment. It was difficult to use this methodology to measure variation in second-order LR growth as second-order LRs rarely initiated opposite to one another.

Three-dimensional (3D) root modelling

3D modelling of the aeroponic data (Fig. 3d,e) was performed using 3D studio Max software (V9, Autodesk, San Rafael, CA, USA). The model was constructed using the root system quantitative data collected under high and LN treatments at 30 DAT. CR average length, number and first-order LR branching data are represented. For ease of viewing, the root systems shown were scaled down 3.5-fold and hence only represent ~30% of each root system.

RH measurements

The first sampling examined the effect of HN and LN at 30 DAT (Fig. 7). At 15 DAT, one newly initiated CR on each treated plants was marked as above. At 30 DAT, for each plant, a 5-cm-long CR segment beginning 15 cm away from the elongation zone was harvested and stored in deionised water at 4 °C until analysis. From each CR segment, RH measurements were taken from four first-order LRs.

In the second sampling, the effect of plant age and hence size on RH traits was quantified (Fig. 8). RHs were measured from newly initiated first-order LRs at different time points during the development of an individual CR under HN. A total of 24 plants (1 CR/plant) were sampled at 7, 14, 21, 28 and 35 DAT, four first-order LRs were removed in a region beginning 5 cm away from the CR elongation zone and stored in deionised water at 4 °C.

For both experiments, LRs were stained using 0.1% Trypan blue solution for 2 min and then washed in dH₂O for 1 min. In the first experiment (Fig. 7), RH density (RHD)

was measured by counting RHs on a full semi-circular RH plane under a light microscope (Zeiss, 100X) on a 2 mm LR segment. Each measurement was then multiplied by two for an estimate of the total RH number per cylindrical LR segment. These measurements were used to extrapolate the total RH length per root system.

In the second experiment (Fig. 8), for RH density and length measurements five images per LR were captured using Northern Eclipse software (v5.0, Empix Imaging Inc., Ontario, Canada). Pictures were exported to *ImageJ* software (V1.40g, NIH, Bethesda, MD,USA); the scale in the Analyse function was set to 100 μm based on a micrometre. Average RH number, length per 100 μm of LR (ARHL) and total RH length per 100 μm of LR (TRHL), were measured by digitally tracing every RH in *ImageJ*. Only protruding RHs in side-profile (Fig. 7a,b) were traced, not those overlapping each LR and hence the measurements are underestimates. Average RH radius (RHr) was measured by drawing a perpendicular line across the middle of the RH and dividing it by two. RH surface area (RHSA) and volume (RHV) were calculated as follows:

$$\text{RHSA } (\mu\text{m}^2/\mu\text{m of LR}): (\pi \times \text{RHr}^2) + (2 \times \pi \times \text{RHr} \times \text{ARHL}) \times \text{RHD}$$

$$\text{RHV } (\mu\text{m}^3/\mu\text{m of LR}): (\pi \times \text{RHr}^2 \times \text{ARHL}) \times \text{RHD}$$

where RHSA, RH surface area; RHr, RH radius; ARHL, average RH length; RHD, RH density; and RHV, RH volume.

Statistical analyses

Statistical analyses were performed using the MIXED procedure of the SAS statistical software package (Version 9.1, Statistical Analysis System, SAS Institute, Cary, NC, USA). Residuals were screened for normality using the Shapiro Wilk normality test. Homogeneity among treatments, experimental units and outliers were identified and removed from the analysis using Lund's test. Unbalanced two-way analysis of variance and partition were calculated to determine the effect of nitrogen on each root trait using the *F*-test with a Type I error rate set at 0.05.

RESULTS

Aeroponics permits root phenotyping to adult stages

The home-made aeroponics system (Fig. 2a, b) permitted phenotyping of a complete set of maize root traits at adult stages, including brace roots (Fig. 2c), CRs (Fig. 2d), first and second-order LRs and RHs. Root systems were very highly branched, voluminous and achieved considerable depths: 30 d after transfer (DAT) plants could develop up to 80 CRs, and an average of 400 m of LRs; these plants developed vigorously up to the 12–14 leaf tip stage. Plants could be further grown to reproductive stages at ~45 DAT
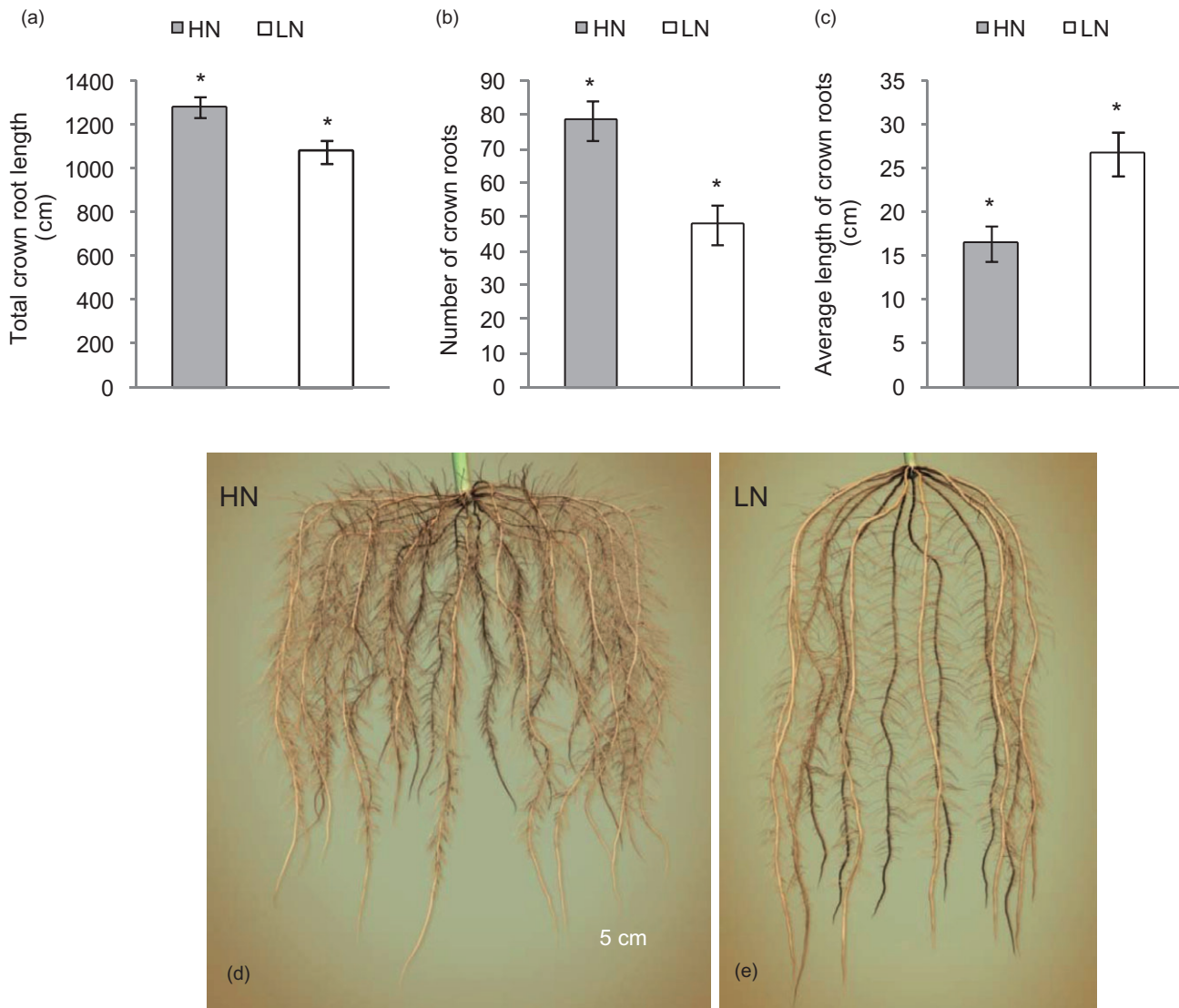
Figure 3. Low nitrogen stress increases average crown root length with a compensatory decrease in crown root number in adult maize. Shown are macro-scale root measurements at 30 d after transplanting (DAT) in maize inbred line W22. (a) Total length of crown roots; (b) number of crown roots; (c) average length of each crown root; (d,e) three dimensional modelling of macro-scale root architecture under (d) high nitrogen and (e) low nitrogen. Shown is a 28% scaled down version representation using 3D studio Max software. HN, high nitrogen; LN, low nitrogen. (*) indicates statistical significance between HN and LN at $P = 0.05$.

(Fig. 2e) achieving physiological maturity at 90 DAT (Fig. 2f).

An agriculturally relevant level of nitrogen stress alters biomass allocation and root system architecture in adult-stage plants

The effect of a mild but agriculturally relevant level of LN stress was first evaluated. Plants were grown under constant HN (20 mM) or LN (8 mM), with weekly replenishment of the nutrient solutions (see Supporting Information). LN-treated shoots showed classic symptoms of nitrogen stress including premature leaf senescence, and yellowing leaves with increased anthocyanin (Supporting Information

Fig. S1). Plants were phenotyped at 30 DAT (corresponding to 12 leaf tips). LN-treated plants showed a significant reduction in the shoot dry weight by 25% while the root dry weight increased by 12% compared with the HN control (Table 1). Taking into account all root types, the TRL increased from 485 m under HN to 553 m under LN (Table 1). The investment in biomass per unit root length [specific root length (SRL)] significantly decreased under LN (Table 1). As described in the following sections, significant growth effects were observed on CRs, LRs and RHs (Figs 3–7). These results demonstrate that an agriculturally relevant level of LN stress alters biomass allocation and root system architecture in adult-stage maize plants grown in aeroponics.

Table 1. Change in dry biomass allocation and total root length under high and low nitrogen treatments in maize inbred line W22 growing in aeroponics

	Shoot dry weight (g/plant)	Root dry weight (g/plant)	Root/shoot ratio	Total root length (m)	Specific root length (cm of root/g)
High nitrogen (20 mM)	45.40 ± 4.24[a]	10.20 ± 0.66[a]	0.22 ± 0.06[a]	485	4542.50 ± 58.90[a]
Low nitrogen (8 mM)	33.97 ± 4.23[b]	11.50 ± 0.51[b]	0.34 ± 0.05[b]	553	4816.03 ± 59.97[b]

Values are least square means from analysis of variance ± standard errors ($n = 24$). Means within a column followed by the same letter are not significantly different at $P = 0.05$.

LN causes CRs to be longer but fewer in number

CR are responsible for anchorage and long-distance nutrient exploration. In contrast to TRL, total CR length significantly decreased, by 16%, under LN compared with HN (Fig. 3a). The number of CR decreased by 40% from 78 in HN to 47 in LN (Fig. 3b); however the average length of each CR increased by 63% from 16.4 cm under HN to 26.7 cm under LN (Fig. 3c). The macro-scale impact of LN was modelled in a 3D image (Fig. 3d,e).

## LN stress causes younger CRs to elongate at a higher rate

CRs initiated asynchronously and throughout development. Given that CR were longer under LN than HN, either LN increased the CR elongation rate or caused CR to elongate for a greater duration, because of earlier initiation or delayed senescence. Aeroponics permitted time-course measurements to distinguish between these hypotheses. To eliminate the impact of LN causing earlier CR initiation, only pairs of CR that synchronously initiated between the HN and LN treatments were studied. Each day for 20 d following the onset of the differential nitrogen treatments, synchronously initiating CR were labelled in both nitrogen treatments, allowed to grow and then all CR were simultaneously measured at harvest (Fig. 4a). There were 3–10 pairs of CR sampled each day per treatment. This methodology mimicked a CR time course but prevented having to re-measure the same individual CR daily, which was previously observed to be damaging (data not shown). Comparing CR initiated on the same day, the LN-treated CRs were significantly longer than their HN-treated siblings beginning 9 d after they initiated (Fig. 4b).

CR elongation rates were then measured. All CR in the age series were simultaneously stained with Neutral Red dye 24 h before harvest; measurements of unstained tissue at the CR tip revealed the elongation rate for CR of different ages, again mimicking a time course (Fig. 4c). Comparing CR of the same age, LN-treated roots had a significantly higher elongation rate than their HN-treated siblings (Fig. 4d). There was no evidence that LN-treated CR elongated for a greater duration than their HN-treated siblings; rather, significantly higher CR elongation rates in the LN-treatment only persisted until an individual CR reached 18 d of age (Fig. 4d). The lack of growth stimulation in older LN-treated CR did not appear to be caused by premature senescence, as older roots continued to grow >1 cm d⁻¹. We conclude that LN-treated plants have longer CR than HN-treated plants because of a higher elongation rate when the CR are young. The distribution of CR lengths shows the architectural impact of this response within the study group: LN stress eliminated short CR in favour of a new class of long CR (Fig. 4e,f).

CRs in adult maize display low intrinsic variation in growth

An adult maize plant has >80 CR initiating at different times during development. We wondered whether different CRs within a genotype and nutrient treatment have an intrinsic growth rate regardless of their timing of initiation or even position on different plants. Aeroponics created an opportunity to test this hypothesis in one maize genotype (inbred W22) given that our aeroponics system created a near uniform rhizosphere environment with ideal nutrition and gas exchange conditions and without any physical barriers. CR growing in HN elongated at an average rate of 0.98 cm day⁻¹ (range, 0.66 –1.2 cm day⁻¹) (Fig. 4d). CR that

Figure 4. Time-course measurements demonstrate that crown roots have an intrinsic growth rate, but that low nitrogen stress causes younger crown roots to elongate at a higher rate. (a) Time-course methodology used to measure root growth without having to re-measure the same individual crown root daily. Each day, synchronously initiating LN/HN pairs of crown roots were labelled, allowed to grow and then all crown roots were simultaneously measured at harvest. (b) Lengths of crown roots as a function of age. The solid black vertical line represents the time when the difference between nitrogen treatments was significant. Within a nitrogen treatment, the two vertical dotted lines indicate the time-period of significantly increasing growth, whereas before and after showed a statistical growth plateau. (c) Picture of the dye staining method used to quantify the elongation rates of crown roots of different ages. (d) Measurements of the mean crown root elongation rate. In the time interval after the solid vertical line, the means between the two nitrogen treatments were not significantly different. (e,f) Resulting effect on the length distribution of crown roots included in the time-course study for plants treated with (e) high nitrogen and (f) low nitrogen. The coefficient of variation (CV) of crown root lengths based on length class is shown as a percentage above each category. HN, high nitrogen; LN, low nitrogen.
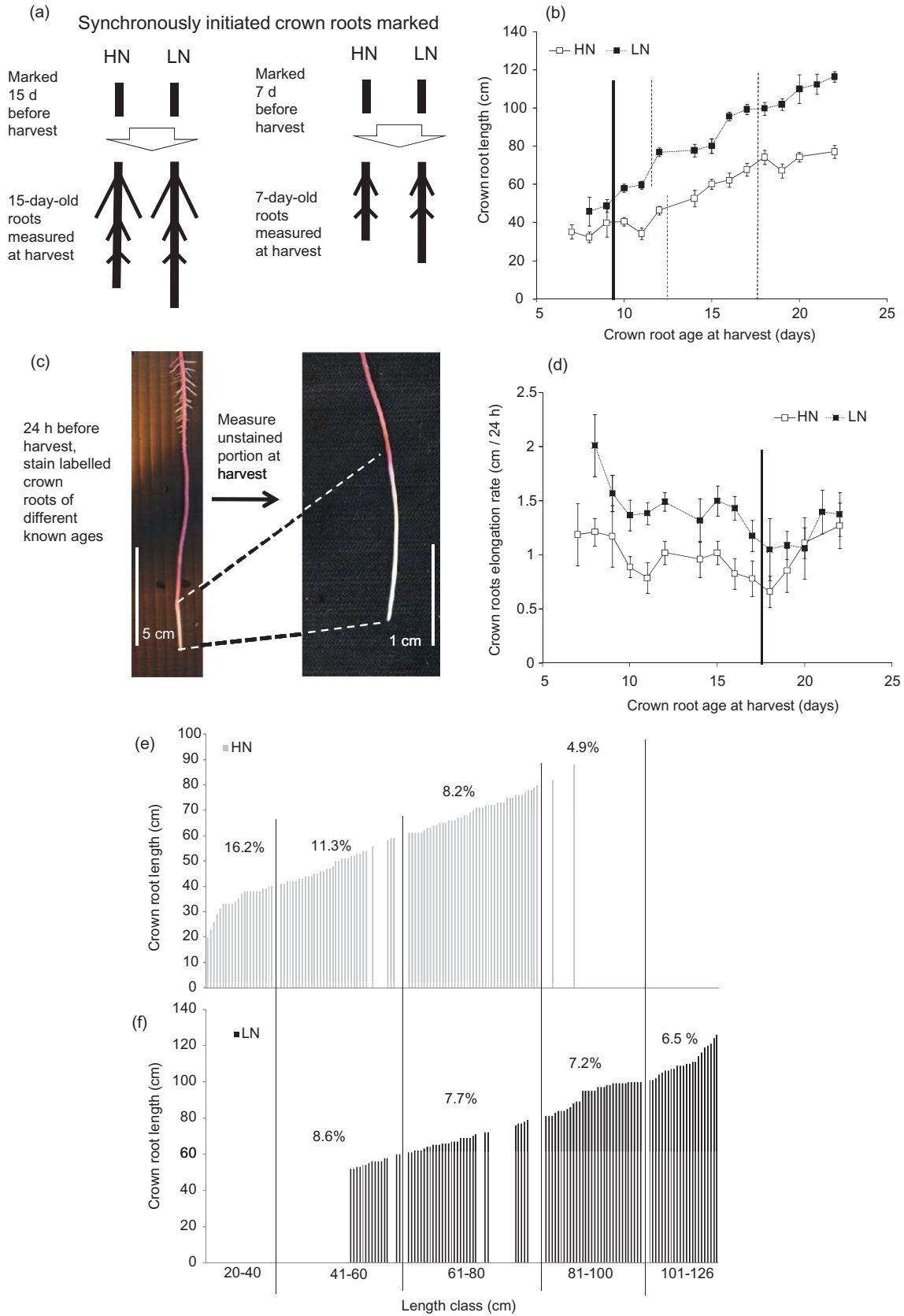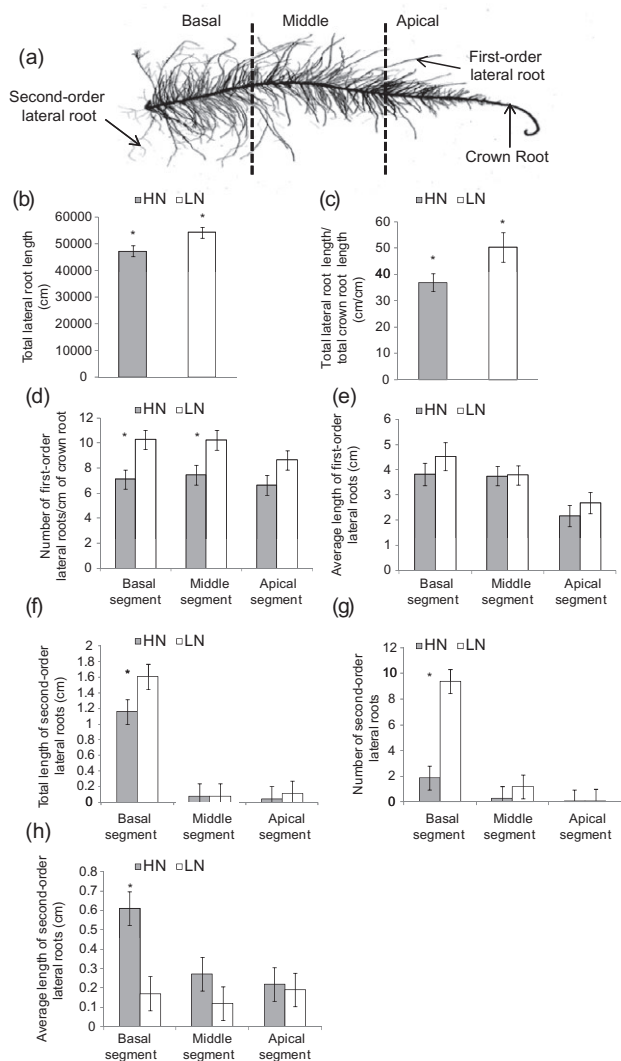
Figure 5. Low nitrogen stress increases the density of first- and second-order lateral roots (LR) in adult maize (30 DAT, inbred W22). (a) Synchronously initiated HN/LN crown root pairs were divided into three segments as indicated, and first- and second-order LR were measured within each segment. Shown are measurements for: (b) total LR length; (c) total LR length per unit of crown root length; (d) density of first-order LR per unit of crown root length; (e) calculated average length of first-order LR; (f) total second-order LR length per unit of crown root length; (g) density of second-order LR per unit of crown root length; (h) calculated average length of second-order LR. HN, high nitrogen; LN, low nitrogen. (*) indicates statistical significance between HN and LN at $P = 0.05$.

initiated on the same day in the same environment showed surprisingly low variation in their lengths, particularly when older CR were compared, in spite of being sampled on different phytomers from different plants growing in different barrels [coefficient of variation (CV) range = 2.6–5.7% for HN) (Supporting Information Fig. S2). Though the average CR growth rate was higher in LN-treated plants [1.37 cm day^{-1} (ranging, 1.1–2.0 cm day^{-1})], nutrient stress did not cause CR lengths to become more variable

(CV = 1.8–3.6% for LN) (Supporting Information Fig. S2) even when grouped by length class rather than by age (%CV, Fig. 4e,f).

## Higher orders of LR branching are part of the root response to LN

Next we tested the effects of LN stress on LR. LN triggered a 13% increase in absolute total LR length but a 36% increase in total LR length per unit CR length and thus shifted the architectural balance of the root system (Fig. 5a–c). We asked whether this increase was caused by an increase in first-order LR number or size and whether higher orders of LR responded similarly, as responses of the latter root types have not been separately reported in previous maize nitrogen studies. Fine root length and branching were quantified by hand-tracing roots from scanned images (Fig. 5a). For comparison purposes, each CR maturation zone was divided into three equal segments above the elongation zone (basal, middle, apical) as these initiate LR progressively later (Fig. 5a). LN caused a modest but significant increase in the density of first-order LR in the basal and middle segments (Fig. 5d) but no change in the calculated average length of first-order LR (Fig. 5e). With respect to second-order LR, their total length increased by 38% in the basal segment in response to LN (Fig. 5f), mainly caused by a dramatic 4.9-fold increase in their density in that segment (Fig. 5g). In contrast, the calculated average length of each second-order LR decreased under LN (Fig. 5h). We conclude that LN stress increases the density of first- and second-order LR, with the latter increase being particularly large.

Opposing first-order LRs differ in length

Similar to the analysis of CR growth, the uniformity and ideal rhizosphere environment provided by aeroponics allowed us to examine whether first-order LR in adult maize plants initiate and grow at a stable, intrinsic rate. To compare the LR emerging from pericycle tissue of the same approximate age, the lengths of the closest 180°-opposing LR were measured and their ratios were compared (Fig. 6a). Random LR pairs were selected from the oldest (basal) CR segment from different CRs on different plants. Under ideal nutrient conditions, defined here as HN, the majority of LR pairs deviated from a ratio of one, with 31% being two- to fivefold different in length from one another (Fig. 6b, right-side). This result shows that opposing LR in adult maize initiate and/or grow asynchronously even within a near uniform rhizosphere environment.

LN induces more variation in the relative lengths of opposing LRs

In parallel to the above experiment, the ratios of opposing LRs under LN were also measured to determine if nutrient
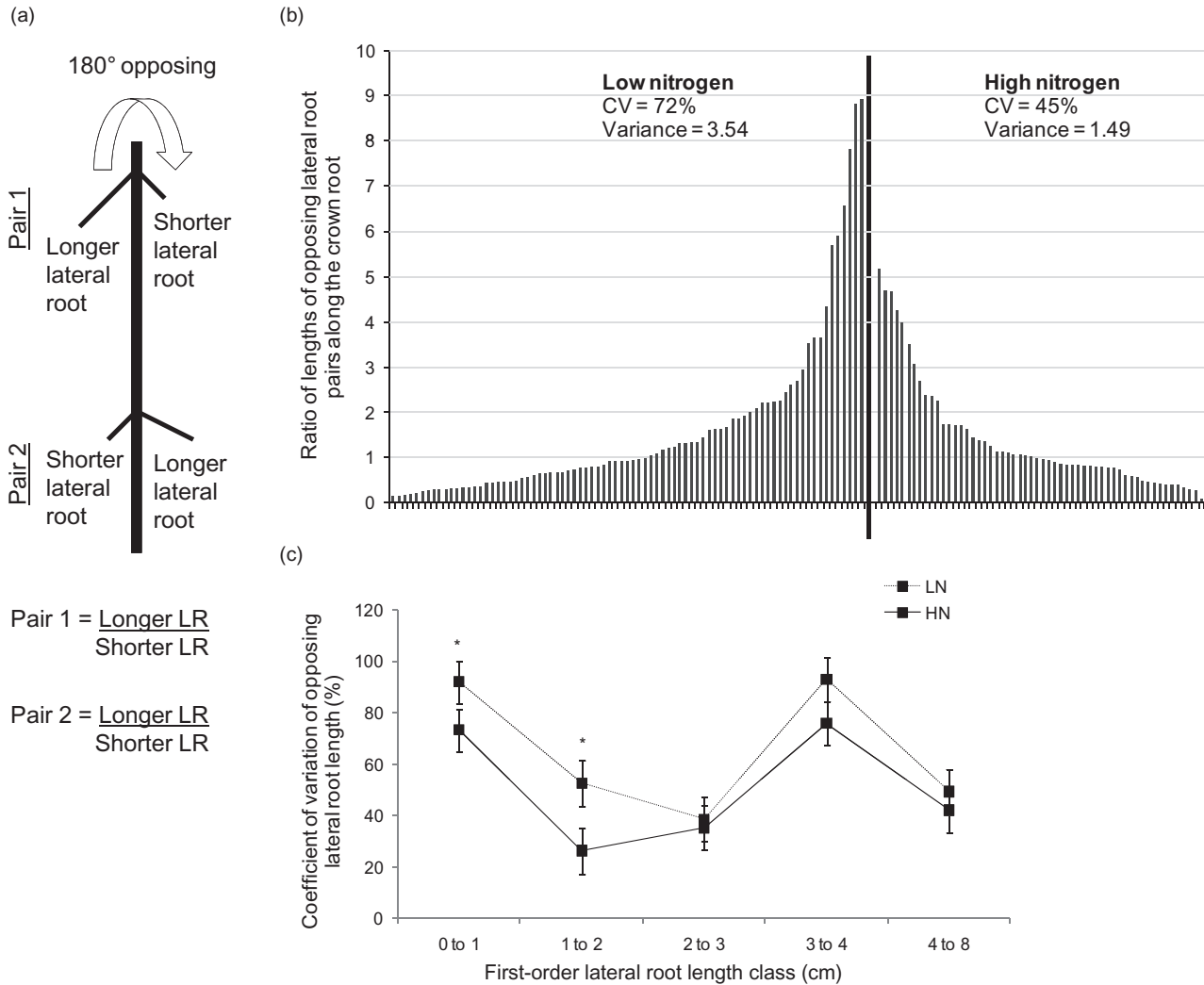
Figure 6. Low nitrogen causes increased variation in the relative lengths of opposing lateral roots in adult maize (30 DAT, inbred W22). (a) Schematic to show the methodology used. (b) Measurements of the ratios of 180°-opposing lateral root pairs along the crown root basal segment. (c) Coefficient of variation (CV) of the ratio of lengths of opposing first-order lateral roots organized by length class of the longer lateral root of each pair. C.V = coefficient of variation. HN = high nitrogen; LN = low nitrogen; (*) indicates statistical significant differences between HN and LN at $P = 0.05$.

stress alters the stability of LR initiation and/or growth. Only LR pairs of LN-treated CRs that initiated on the same day as HN-treated CRs were compared, again limited to the CR basal segment. LR differed more in length relative to their closest 180°-opposing sibling under LN than HN (variance increased ~2.4-fold under LN) (Fig. 6b) even when the same size classes of LR were compared (Fig. 6c). We conclude that LN stress causes greater variability in LR initiation and/or growth.
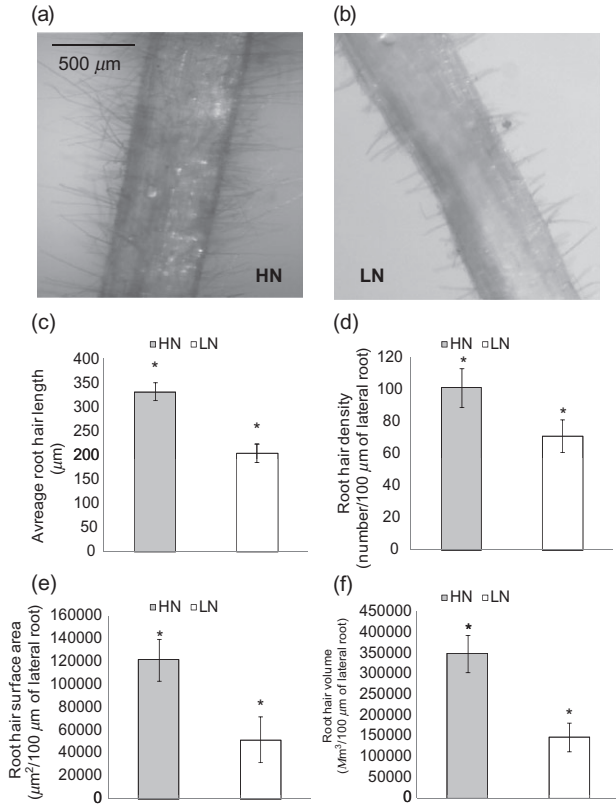
LN reduces average RH length and density

The effect of LN stress on RH has not been systematically reported in maize. Aeroponics made large-scale RH observations possible (Fig. 7a,b). Thirty DAT, measurements of 96 000 individual RH were taken from microscope images.

To estimate RH density, the number of RH on the full half semi-spherical plane of each first-order lateral RH zone was scored and then extrapolated. LN stress was found to significantly reduce RH elongation (Fig. 7c), density (Fig. 7d), surface area (Fig. 7e) and volume (Fig. 7f). The average length of each RH decreased by 38% from 332 μm under HN to 204 μm under LN (Fig. 7c). Pictures of the RH nitrogen response clearly illustrate these results (Fig. 7a,b). We conclude that LN-stressed adult maize plants have fewer and shorter RHs.
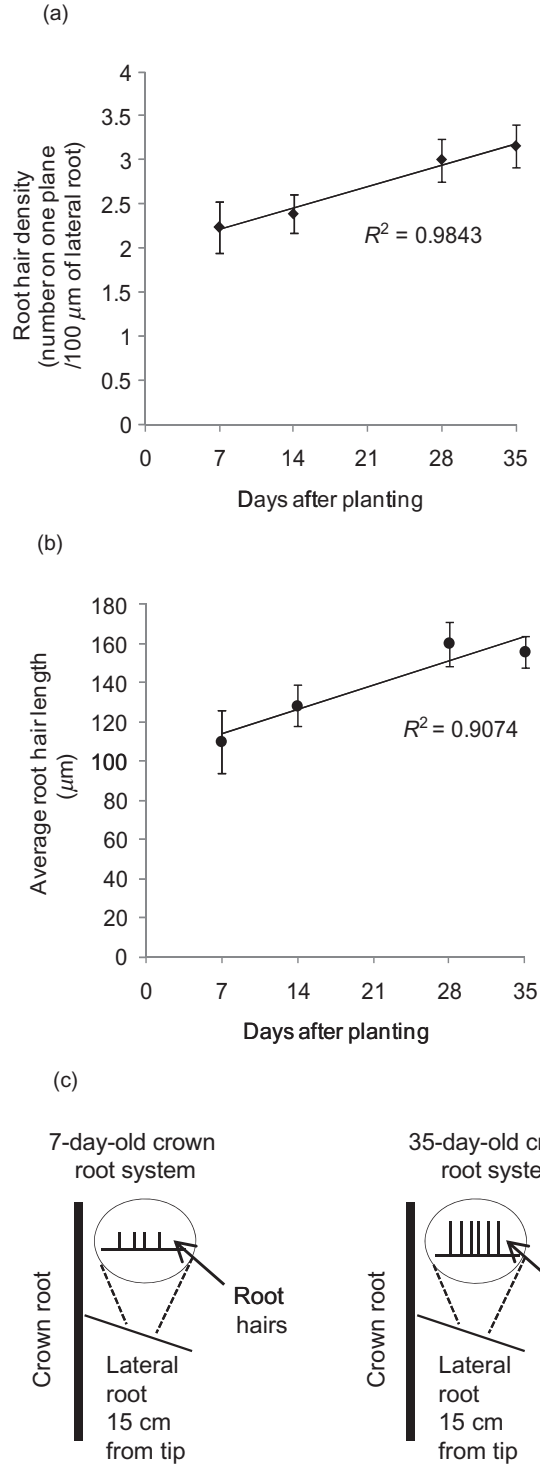
RHs initiated on younger, smaller plants are smaller and less dense than on older plants

We hypothesized that diminished RH phenotypes observed under LN were indirect responses to decreased

(a)

(b)

(c)

□HN □LN

(d)

□HN □LN

(e)

□HN □LN

(f)

□HN □LN

Figure 7. Low nitrogen reduces average root hair (RH) length and density in adult maize (30 DAT, inbred W22). (a,b) Pictures showing RH lengths and density on a first-order lateral root under (a) high nitrogen and (b) low nitrogen. (c) Average RH length (d) RH density (e) total RH surface area, and (f) total RH volume of all root hairs along a 100-μm long segment of first-order lateral roots. Each value from (c-f) is the average RH measurement from 960 digital scans from 48 plants per nitrogen treatment. The standard error is shown. HN, high nitrogen; LN = low nitrogen; LR = lateral root; (*) indicates statistical significance between HN and LN at $P = 0.05$.

(a)

(b)

(c)

7-day-old crown root system

35-day-old crown root system

Root hairs

Root hairs

Crown root

Crown root

Lateral root 15 cm from tip

Lateral root 15 cm from tip

Figure 8. In a high nitrogen treatment, root hairs (RH) on younger (lower biomass) plants are shorter and fewer than those on older plants (maize inbred W22), mimicking the low nitrogen treatment. As an individual crown root aged, new RH from first-order lateral roots were measured at a constant distance (15 cm) from the crown root tip. Shown are the (a) RH density, and (b) average RH length, at different days after transplanting; (c) schematic summary of changes in RH development along a single crown root: RH density and length increased linearly with age ($P = 0.032$ and $P = 0.045$ respectively).

plant demand for nutrients as LN-treated plants have reduced biomass (Table 1). To test this hypothesis, under optimal nitrogen (HN), RH from plants were measured as they aged and hence as the plants grew in size. Specifically, RH from first-order LRs were sampled at a consistent distance (15 cm) from tips of individual CRs as they developed. RH from a total of 1920 digital images were traced and analysed. Compared with RH that initiated on young plants (7 DAT), RH that initiated on older CRs (from 35 DAT), and hence on larger plants, were ~40% more dense (Fig. 8a) and longer (Fig. 8b). This data is summarized schematically (Fig. 8c). These results demonstrate that RH traits are dynamic and change during maize development. Furthermore, diminished RH phenotypes observed under LN correlate with reduced plant age and hence biomass.

The LN root response in aeroponics is consistent with substrate-grown maize at the macro and mesoscales

A potential criticism of this study is that the results obtained are irrelevant to the real world since roots grew in the air without a substrate. In a parallel study, we compared root responses from aeroponics with those from roots grown in Turface©, an inert clay-based substrate (Supporting Information Fig. S3 and Table S2). Turface has been used extensively to evaluate maize physiology and yield potential (Echarte *et al*. 2008). Turface permitted the same nutrient solution to be used as in aeroponics to allow comparisons to be made, though only a subset of macro- and meso-scale responses were possible to measure in Turface. An inbred and hybrid maize genotype were grown in aeroponics and Turface side by side in the same greenhouse under LN and HN (Supporting Information Fig. S3a, b). The root:shoot mass ratio and number of CRs were not significantly different between Turface and aeroponics, though the lengths of all roots (crown and laterals) were 3 to 5-fold larger in aeroponics than in Turface (Supporting Information Table S2). Because the roots supported a greater shoot mass in aeroponics, it was thus important to normalize root traits to plant biomass. Using this normalization, maize grown in both aeroponics and Turface showed a similar investment in CR length or number (Supporting Information Fig. S3c). Aeroponics emphasized the decreased investment in CRs under LN, whereas this decrease was not significant in Turface (Supporting Information Fig. S3c). Similarly, aeroponics amplified the increase in LR length under LN compared with Turface (Supporting Information Fig. S3d, e and Table S2). Though some responses by maize to LN were exaggerated in aeroponics, the data suggest that the macro and mesoscale root responses observed in aeroponics are consistent with those in substrate-grown maize.

DISCUSSION

For the first time, the comprehensive response to LN stress by an intact root system of an adult maize plant has been described at all scales of development, macro, meso and micro. This was possible as aeroponics allowed growth of plants to adult stages and permitted clean phenotyping of near-intact large root systems (Fig. 2). Since the adult maize plants had higher orders of root branching and extensive RHs, novel fine-scale root responses could be uncovered (Figs 5f-h, 6 and 7). Without the need for excavation, time-course responses by maize roots and RHs could also be analysed (Figs 4 and 8; Supporting Information Fig. S2). Finally, as aeroponics created a uniform rhizosphere without gas exchange constraints or physical barriers, the uniformity of intrinsic growth rates of different maize root organs could be measured (Figs 4d-f and 6; Supporting Information Fig. S2). These results are summarized (Fig. 9).

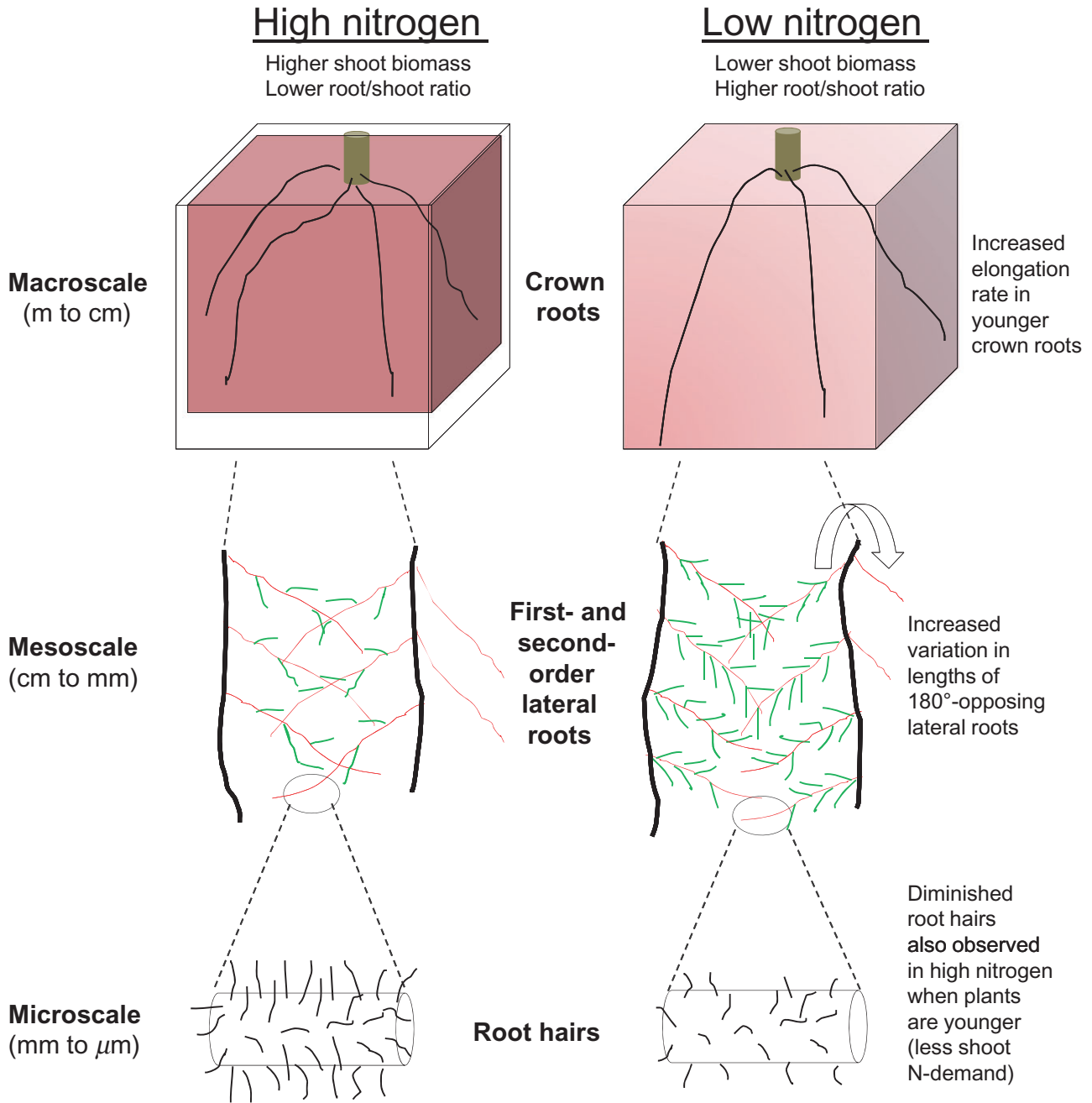## CRs in maize have a stable, intrinsic growth rate in a uniform environment

CRs provide the backbone of the root system in adult maize. Nitrogen has been shown to alter the growth and architecture of maize at the macroscale, including CR (Maizlish *et al*. 1980; Feil *et al*. 1990; Schortemeyer, Feil & Stamp 1993; Vamerali *et al*. 2003; Chun *et al*. 2005; Wang *et al*. 2005; Liu *et al*. 2008, 2009; Liu *et al*. 2009). Previous studies conducted with soil-grown maize showed that CR lengths and elongation rates within plants of the same age varied widely (Pellerin & Pages 1994; Pellerin & Tabourel 1995). However, in these studies, variation was also observed in shoot growth and in the physio-chemical properties of the soil surrounding the root tip. Using the near uniform rhizosphere environment created by aeroponics, the current study has now shown that CR initiating at different times during development and on different plants have a surprisingly uniform growth rate of ~1 cm day^{-1} under non-limiting nitrogen conditions (Fig. 4d). CR initiated on the same day from sibling plants varied by only <20% in length after 22 days of growth (Supporting Information Fig. S2). It thus appears that a maize CR has an intrinsic daily growth rate, though one that can be modulated by the environment (Fig. 4d; Supporting Information Fig. S2). In future studies, it will be interesting to compare the intrinsic growth rates of CR of different maize genotypes. For example, it has been shown that nitrogen use efficient maize genotypes have a larger and deeper root system under field conditions (Liu *et al*. 2009), suggestive of enhanced CR growth.

LN increases the CR elongation rate but only in younger roots

Consistent with results obtained from maize grown in Turface (Supporting Information Fig. S3 and Table S2), in hydroponics (Wang *et al*. 2005) and in the field (Liu *et al*. 2009), LN caused individual CRs to elongate (Figs 3c-e and 4), with a simultaneous dramatic decrease in CR number (Fig. 3b,d-e). As CR are responsible for long-distance foraging, the former response may be an adaptive trait to explore a wider or deeper soil volume for nitrogen, while the latter may be to balance the resulting energy demand. In the current study, time-course experiments combined with a dynamic dye-staining method (Fig. 4c), permitted a deeper understanding of this response. Specifically, the results showed that constant exposure to LN stress causes the absolute growth rate of CR to increase, but only when CR are young (Fig. 4d), a novel finding. Consistent with this observation, in maize seedlings grown in hydroponics, high nitrate was previously shown to inhibit CR growth by reducing cell elongation in the root elongation zone (Tian *et al*. 2008).

Higher orders of LRs are part of the LN stress response in maize

LRs in maize have typically been quantified as a single entity. However, whereas LN caused only a modest increase

High nitrogen
Higher shoot biomass
Lower root/shoot ratio

Low nitrogen
Lower shoot biomass
Higher root/shoot ratio

Macroscale
(m to cm)

Crown roots

Increased elongation rate in younger crown roots

Mesoscale
(cm to mm)

First- and second-order lateral roots

Increased variation in lengths of 180°-opposing lateral roots

Microscale
(mm to μm)

Root hairs

Diminished root hairs **also observed** in high nitrogen when plants are younger (less shoot N-demand)

Figure 9. Summary of the effects of nitrogen on macro, meso and micro-scale root traits in adult-stage maize plants. First-order lateral roots are shown in red and second-order lateral roots in green. HN, high nitrogen; LN = low nitrogen.

in the density of first-order LR (Fig. 5d), it caused a 6-fold increase in the density of second-order LR (Fig. 5g) resulting in a 1.7-fold increase in total second-order LR along this region (Fig. 5f). LN thus appears to cause a fundamental shift in the fine-scale architecture of an adult maize root system. Stimulation of second-order LR for local nitrogen acquisition was recently suggested in 7-day-old maize seedlings growing in hydroponics (Liu *et al*. 2010). Similarly, in rice seedlings grown in substrate, an increase in the ratio of LR that further branch compared with LRs that do not branch was observed in response to LN (Tanaka, Yamauchi & Kono 1995). Though very limited data are available on finely branched cereal roots, together, these results suggest that fine roots may play a more significant role in resource acquisition in cereals than previously appreciated and should not be omitted from nutrient studies.

Opposing LRs vary in length, and LN induces more variation

In contrast to older CRs that varied by only <20% in length even though they initiated on different plants (though on the same day) (Supporting Information Fig. S2), LRs that initiated on the same plant, 180° opposite to one another, showed up to fivefold variation in length under optimal nitrogen (HN) (Fig. 6b). This result shows that adjacent LR in adult maize initiate and/or grow asynchronously even when the rhizosphere environment is highly uniform and when there are no physical barriers. Interestingly, LN stress further increased the relative length differences between 180°-opposing LR to up to ninefold (Fig. 6b, c), suggesting that nutrient stress disrupts LR initiation and/or elongation. In a previous study of field-grown maize, variation was observed in the density of LR emerging from the same region along an axile root near the branching zone (Pagès & Pellerin 1994). However, as this root variation varied with soil depth, there was concern that variation in soil physical properties might have been responsible, not intrinsic variation in growth. Variation in LR traits has also been reported among genetically identical Arabidopsis plants (Forde 2009). Among possible explanations, it has been speculated that variation in LR growth, termed stochastic variation, may be adaptive, for example to assist in the foraging of nutrient patches in the soil (Forde 2009).

LN stress reduces RH length and density, perhaps as an indirect result of decreased total plant nitrogen demand

In this study, we undertook a large-scale analysis of the response of RH to LN stress as this had not been systematically studied in adult maize. The RH number in adult maize plants decreased by 2.2-fold under LN and the total RH length decreased by 3.6-fold compared with HN (Fig. 7). These results were counter-intuitive and contrasted responses by maize to low phosphate in which RH density and length have been observed to increase (Zhu, Zhang & Lynch 2010). As a possible explanation, we undertook a time-course experiment, and the results were consistent with the diminished RH traits in LN being indirect consequences of decreased shoot biomass and hence total plant nitrogen demand (Table 1, Fig. 8). Additional experiments will be needed to clarify this issue.

Prospects of aeroponics for future root studies

Aeroponics has been used to grow maize seedlings for physiological studies on nitrification (Padgett & Leonard 1993), to examine the root elongation zone (Pellerin & Tabourel 1995; Freundl, Steudle & Hartung 2000) and for genotype screening for disease resistance (du Toit *et al.* 1997), but prior to this study it has not been applied to studies of cereal root system architecture. When we compared roots grown in aeroponics with those grown in pots with clay substrate, the root responses were found to be similar (Supporting Information Fig. S3 and Table S2), suggesting that aeroponics is a relevant growth system for the study of root architecture. Aeroponics, however, is an artificial growth system and caution should be used in extrapolating results to the field. Furthermore, our aeroponics system did not allow some important root traits to be measured, such as CR angle.

Nevertheless, it is of interest to note that the aeroponically grown roots displayed more root plasticity in response to nitrogen than those grown on substrate (Supporting Information Fig. S3 and Table S2). The underlying reasons may have been the improved gas exchange in aeroponics, the lack of physical impedance in aeroponics compared with plants grown in substrate, and the smaller pots used in the substrate experiment (Supporting Information Fig. S3a), which may have constrained growth responses. As a result of the exaggerated phenotypes, the aeroponically grown maize displayed a greater genotype (inbred versus hybrid) by environment (LN versus HN) interaction (G × E) for all root traits compared with Turface (Supporting Information Table S2). The general lesson here for future studies of large root systems is that improving the conditions for root growth is essential to reveal the effect of genotype in response to stress regardless of the growth system used.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Exposure to 8 mM total nitrogen causes classic symptoms of low nitrogen stress to shoots of maize inbred W22 growing in aeroponics under the conditions noted. Shown are representative (a) shoots and (b) leaves of plants exposed to 20 mM total N (HN, left) and 8 mM total N (LN, right) at 20 DAT.

Figure S2. Variation in crown root growth is unaffected by low nitrogen stress. Synchronously initiating LN/HN crown root pairs from different positions on different plants were

measured as described in Fig. 4a. The dataset shown corresponds to Fig. 4b,e,f. Shown are the lengths of individual crown roots on sibling plants grouped by crown root age at harvest. Above each age cluster are the coefficients of variation of crown root lengths. Triangles indicate the mean length for that age class (HN means, LN means). HN, high nitrogen; LN, low nitrogen.

Figure S3. The low nitrogen root response in aeroponics is consistent with substrate-grown maize at the macro and mesoscales. Roots of two maize genotypes (hybrid SG150 and its inbred parent SG200) grown in aeroponics were compared withthose growing in an inert clay substrate (Turface), side by side in the same greenhouse. (a, b) Pictures of (a) a maize shoot and (b) maize roots grown in Turface. Measurements of (c) crown root length and (d) total lateral root length, both normalized to biomass; (e) comparison of the ratio of lateral root length normalized to crown root length. HN, high nitrogen; LN, low nitrogen; (*) indicates statistical significance between HN and LN at $P = 0.05$. Also refer to Supporting Information Table S2 for detailed measurements.

Table S1. Dose-response curve of total N on dry biomass accumulation and root:shoot ratio in maize using aeroponics.

Table S2. Comparison of the effects of growing maize plants in aeroponics versus solid substrate (Turface-containing pots) when treated with high nitrogen (HN) or low nitrogen (LN).

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