Identification of nitrogen-dependent QTL and underlying genes for root system architecture in hexaploid wheat

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Running title – Nitrogen-dependent wheat root QTLs and underlying genes.

Highlight

Using a hydroponic pouch phenotyping system, nitrogen-dependent root QTLs were identified in wheat. For a candidate N-dependent root angle QTL an upregulated NPF family gene was identified likely transporting nitrate or ABA as part of a N-dependent response affecting root angle.

Abstract

The root system architecture (RSA) of a crop has a profound effect on the uptake of nutrients and consequently the potential yield. However, little is known about the genetic basis of RSA and resource dependent response in wheat (*Triticum aestivum* L.). Here, a high-throughput hydroponic root phenotyping system was used to identify N-dependent root traits in a wheat mapping population. Using quantitative trait locus (QTL) analysis, a total of 55 QTLs were discovered for seedling root traits across two N treatments, 25 of which were N-dependent. Transcriptomic analyses were used on a N-dependent root angle QTL on chromosome 2D and 17 candidate genes were identified. Of these N-dependent genes a nitrate transporter 1/peptide transporter (NPF) family gene was upregulated making it an interesting candidate for N signalling and response processes for root angle change. The RNA-seq results provide valuable genetic insight for root angle control, N-dependent responses and candidate genes for improvement of N capture in wheat.

Key Words

RNA-seq, Root angle, Root system architecture, Savannah Rialto doubled-haploid population, QTL, Nitrate, Nitrogen, NPF, wheat.

Abbreviations

ABA, Abscisic acid; BLUEs, best linear unbiased estimates; BLUPS, best linear unbiased predictions; DAG, days after germination; DH, doubled haploid; LOD, logarithm of odds; nabim, National Association of British & Irish Millers; NPF, peptide transporter family; NRT, nitrate transporter; NUE; nitrogen use efficiency; PTR, proton-dependent oligopeptide transporter; QTL, quantitative trait locus; RNA-seq, RNA sequencing technology; RSA, root system architecture; RSML, Root System Markup Language.

Introduction

Nitrogen (N) is an essential macronutrient for plant growth and development with agriculture greatly dependent on synthetic N fertilisers for enhancing productivity. Global demand for fertilisers is projected to rise by 1.5% each year reaching 201.7 million tonnes in 2020, over half of which is for nitrate fertilizers (118.8 million tonnes) (FAO, 2017). However, there are compelling economic and environmental reasons to reduce N fertiliser use in agriculture, particularly as the N fixing process is reliant on unsustainable fossil fuels (Dawson *et al.*, 2008).

The availability of nutrients is spatially and temporally heterogeneous in the soil. Roots therefore need to forage for such resources. The spatial arrangement of the root system, called the root system architecture (RSA) (Hodge *et al.*, 2009), has a profound effect on the uptake of nutrients and consequently the potential yield. Optimisation of the RSA could significantly improve the efficiency of resource acquisition and in turn increase the yield potential of the crop. An improvement in N use efficiency (NUE) by just 1% could reduce fertiliser losses and save ~$1.1 billion annually (Delogu *et al.*, 1998; Kant *et al.*, 2010).

Understanding the contribution of root traits to root system architecture and function is of importance for crop improvement. Artificial growth systems are widely used in plant phenotyping as they are generally high throughput, allow precise control of environmental parameters and are easy to replicate. Using these systems quantitative trait loci (QTL) have been identified in major crops for root system architectural traits (Ren *et al.*, 2012; Clark *et al.*, 2013; Atkinson *et al.*, 2015; Zurek *et al.*, 2015). Understanding the genetic basis of RSA in cereals is very complex and therefore identifying QTL is useful for precisely linking phenotypes to regions of a chromosome. With the development of high-throughput RNA sequencing technology (RNA-seq), identified QTL can now be further dissected to the gene level. Using RNA-seq, a substantial number of genes and novel transcripts have been identified in cereal crops including rice, sorghum, maize and wheat (Oono *et al.*, 2013; Gelli *et al.*, 2014; Akpinar *et al.*, 2015; Yu *et al.*, 2015). To our knowledge, there are no other studies that have identified genes related to nitrate response or root angle change in wheat.

The aim of this study was to identify root traits and genes that relate to N uptake and plasticity. To achieve this a germination paper-based system was used to phenotype a wheat

doubled haploid (DH) mapping population under two N regimes. Here were present genomic regions and underlying genes that we propose may control a N-dependent root angle response in wheat.

Materials and methods

Plant materials

A winter wheat doubled haploid mapping population comprised of 94 lines was used for root phenotyping. The population was derived from a cross between cultivars Savannah and Rialto F1 plants (Limagrain UK Ltd, Rothwell, UK). Both parents are UK winter wheat cultivars that were on the AHDB recommended list. Savannah is a National Association of British & Irish Millers (nabim) Group 4 feed cultivar first released in 1998. Rialto is nabim Group 2 bread-making cultivar first released in 1995.

Seedling phenotyping

Wheat seedlings were grown hydroponically using the system described in Atkinson *et al.* (2015) (Fig. S1). Seeds from the Savannah × Rialto doubled haploid (S×R DH) mapping population were sieved to a seed size range of 2.8–3.35 mm based on mean parental seed size. Seeds were surface sterilised in 5% (v/v) sodium hypochlorite for 12 minutes before three washes in dH2O. Sterilised seeds were laid on wet germination paper (Anchor Paper Company, St Paul, MN, USA) and stratified at 4°C in a dark controlled environment room for 5 days. After stratification seeds were transferred to a controlled environment room at 20/15°C, 12 hour photoperiod, 400 μmol m^{-2} s^{-1} PAR and kept in a light-tight container. After 48 hours uniformly germinated seedlings with ~5 mm radicle length were transferred to vertically orientated seedling pouches.

Seeds for 94 lines from the S×R DH mapping population were grown hydroponically either in high N (3.13 mM NO_3^-) or low N (0.23 mM NO_3^-) modified Hoagland's solution (Table S1). The experimental design was a randomised block comprised of 94 genotypes split over 11 experimental runs with a target of 20 replications per genotype (n = 8 - 36). Roots and shoots of each seedling were individually imaged 10 days after germination (DAG) resulting in 6924 images. The root system architecture of each seedling was extracted from the images and stored in Root System Markup Language (RSML, Lobet *et al.*, 2015) using the root tracing software RootNav (Pound *et al.*, 2013). Root traits were quantified using RootNav standard functions and additional measurements as described in Atkinson *et al.* (2015). The shoot length and area were extracted from the shoot images using the colour threshold tool in FIJI software package (Schindelin *et al.*, 2012).

Quantitative trait locus mapping

Detection of QTL was conducted using the R Statistics package "R/qtl" (Broman *et al.*, 2003). The map used was a high-density Savannah × Rialto iSelect map obtained from Wang *et al*. (2013) with redundant and closer than 0.5 cM markers stripped out. Before data processing, the best linear unbiased estimates (BLUEs) or best linear unbiased predictions (BLUPs) were calculated for the traits if necessary. QTL were identified based on the extended Haley-Knott algorithm (Haley & Knott, 1992). The threshold logarithm of the odds (LOD) scores were calculated by 1000 × permutation test at $p < 0.05$ level (Churchill & Doerge, 1994). The threshold for declaring presence of a QTL was a LOD score of 2.0. The annotated linkage map was generated using R Statistics package "LinkageMapView" (Ouellette *et al.*, 2018).

RNA-sequencing of candidate QTL

RNA-seq was used to identify underlying genes for a candidate QTL with expression levels changed by N treatment. For the candidate QTL two pooled root samples were immediately frozen after collection using liquid nitrogen and stored at -80°C. Each pool was made up from four individual lines (3 plants per line), one pool was comprised of lines that had the candidate QTL (Group A) and the second pool did not have the QTL (Group B). The lines were selected based on largest phenotypic differences for trait associated with candidate QTL. 500–1000 mg of frozen root tissue was homogenised using a pestle and mortar with liquid nitrogen. The homogenised tissue powder was then transferred to a 2 mL microcentrifuge tube and 1 mL of TRIzol© reagent added. The sample was homogenised and incubated at room temperature for 5 minutes to permit complete dissociation of the nucleoprotein complex. 200 μL of chloroform was added and the tube shaken vigorously for 15 seconds for phase separation. The tube was then incubated for 2–3 minutes at room temperature before centrifuged at 13000 × g at 4°C for 5 minutes. After centrifugation, 500 μL of the aqueous phase was transferred to an RNAse-free tube with 250 μL 70% ethanol and vortexed. Once settled the pellet was removed and resuspended in 1000 μL of RNAse-free water. To analyse RNA quality and purity 1 μL of nucleic acid sample was quantified using a NanoDrop™ 2000c with values above 500 ng μL^{-1} or higher accepted. The samples were then stored at -80°C for RNA-seq. Illumina Paired-End Multiplexed RNA sequencing was performed by Source Bioscience (Nottingham, UK).

Differential gene expression analysis was conducted using the IWGSC RefSeq v1.1 assembly (International Wheat Genome Sequencing Consortium, 2018) (http://plants.ensembl.org/Triticum_aestivum/) and the TGAC v1 Chinese Spring reference sequence (Clavijo *et al.*, 2017). Raw sequencing reads were trimmed for adapter sequence and for regions where the average quality per base dropped below 15 (Trimmomatic version 0.32) (Bolger *et al.*, 2014). After trimming, reads below 40 bp were eliminated from the dataset. Trimmed reads were aligned to the reference sequences assembly using splice-aware aligner HISAT2 (Pertea *et al.*, 2016). Uniquely mapped reads were selected, and duplicate reads filtered out. Unmapped reads across all samples were assembled into transcripts using MaSuRCA software and sequences 250 bp or larger taken forward (Zimin *et al.*, 2013). Unmapped reads were re-aligned to these assembled transcripts individually and added to their sample specific reads while the assembled transcripts were combined with the reference sequence and GTF annotation for downstream investigations. StringTie software was used to calculate gene and transcript abundances for each sample across the analysis specific annotated genes (Pertea *et al.*, 2016). Finally, DEseq was used to visualise results and identify differential expression between samples (Anders & Huber, 2010). Differentially expressed genes were compared between the IWGSC RefSeq v1.1 and TGAC v1 reference assemblies to identify overlap using BLAST (BLASTN, e-value 1e-05, identity 95%, minimum length 40bp) (Altschul *et al*., 1990). The top matches for each gene between the reference sequences were used to allow an integrative and comprehensive annotation of genes.

Phylogenetic analysis

A phylogenetic analysis of protein families was conducted to compare the protein sequences of *A. thaliana*, *O. sativa L.* and *T. aestivum L.* proton-dependent oligopeptide transporter (NPF) families (also known as the NRT1/PTR family). *A. thaliana* sequences were obtained from (Léran *et al.*, 2014). Using the latest genome for *T. aestivum L*. (IWGSC RefSeq v1.1 assembly) and *O. sativa L.* (MSU Release 7.0, Kawahara *et al.*, 2013, https://phytozome.jgi.doe.gov/) a HMM profile search was conducted (Krogh *et al.*, 2001). The resulting list of proteins were scanned using Pfam (El-Gebali *et al.*, 2019). Only single gene models of candidate genes with PTR2 domains were retained. The protein sequences were used to generate a maximum-likelihood tree using the software RAxML (Stamatakis, 2014). The exported tree file (.NWK) was then visualised using the R package “ggtree” (Yu

et al., 2017) and used for phylogenetic tree construction. The exported tree file (.NWK) was visualised using the R package “ggtree” (Yu *et al.*, 2017).

Results

Root phenotypic variation in a wheat double haploid population

The phenotypic trait values for the parental lines, Savannah and Rialto, under two N regimes are summarised in Table 2. Significant differences between the parents were observed in root traits only with no significant shoot length or shoot area differences (p = ns). For the root traits measured, differential responses to N treatment were also observed (Fig. 1 a-c). For all root length and size traits (except for lateral root length under low N) Savannah was significantly larger ($p < 0.05$) than Rialto under both high and low N treatments. There was no significant effect of N supply on root traits in Rialto except for a reduction in seminal root count. Savannah, however, showed significant reduction in lateral root length, convex hull area and maximum root depth under low N. There were no significant root angle differences between the parents or N treatments. Lines within the Savannah × Rialto (S×R) DH population showed transgressive segregation with trait values more extreme than the parents (Fig. 1 a-c).

Root QTL detection in the S×R DH population

A total of 55 QTLs were discovered for seedling root traits across both N treatments (Fig. 2, Table 3), of which 36 came from Savannah, and 19 from Rialto. QTLs were found on chromosomes 1A, 1B, 2D, 3B, 4D, 6D, 7A and 7D, with 23 QTLs located on 6D. Twenty-three QTLs were identified under the low N treatment and 32 for the high N treatment. Nine QTLs were found to be only present in the low N treatment, 18 QTLs were found only in the high N treatment and 14 QTLs (28 total) were present in both N treatments. Phenotypic variation explained by QTLs varied from 3.8 to 82.9%. Of the QTLs found, N treatment dependent root angle QTLs (RAE1001/751, LOD 3.0/2.6 respectively) were identified on chromosomes 2D, 3B and 4D. Nitrogen-dependent root size QTLs were found on chromosomes 1A, 6D and 7D. For chromosomes 6D and 7D, N treatment independent QTLs were found for root size and vigour. N-dependent QTLs were also found on chromosomes 6D and 7D that co-localised with other N independent root size QTLs.

RNA-seq analysis

A seminal root QTL (RAE1001) was selected for RNA-sequencing analysis as it had the smallest peak confidence region (25 cM) for an N-dependent QTL (Table 4). As there was no

single clear enriched region from the QTL analyses for the trait, the whole chromosome was considered for analysis. A total of 3299 differentially expressed genes were identified in the analysed groups. 1857 differentially expressed genes showed significant ($p < 0.05$) up-regulation in Group A (with the QTL) compared to Group B (without QTL). Of these, 88 gene candidates resided on chromosome 2D. Additionally, MaSuRcA transcript assemblies were considered that were identified as significantly ($p < 0.05$) up-regulated in Group A compared Group B on chromosome 2D bringing the total to 93 (88 plus five) differentially expressed candidate sequences (Table S2). The inclusion of these *de novo* assembled transcript sequences in the analysis factors for varietal specific genes responsible for this phenotype that are not present in the Chinese Spring based reference sequences. Of the 93 differentially expressed candidate sequences, 17 candidate genes were consistently expressed across the Group A replicates verses zero reads mapping in one or more Group B replicates and were therefore considered as our primary candidates (Table 4). There were also 1442 differentially expressed genes that showed significant ($p < 0.05$) down-regulation in Group A (with the QTL) compared to Group B (without QTL). Of these, 65 were annotated as residing on chromosome 2D (Table S2).

Phylogenetic analysis

For the candidate N-dependent root angle QTL (RAE1001) an upregulated NPF family gene, TraesCS2D02G348400, was identified. A phylogenetic analysis of protein families was conducted comparing NPF family protein sequences of *A. thaliana*, *O. sativa L.* and *T. aestivum L* (Fig. S2). A total of 53 *A. thaliana* proteins, 130 *O. sativa L.* proteins and 391 *T. aestivum L.* proteins were aligned using MUSCLE with 1000 bootstrap interactions and 20 maximum likelihood searches (Edgar, 2004). The candidate *T. aestivum L.* protein TraesCS2D02G348400 is situated in a monocot specific sub-clade within the NPF4 clade (Fig. 3). This clade includes *A. thaliana* NPF members AtNPF4.1, AtNPF4.2, AtNPF4.3, AtNPF4.4, AtNPF4.5, AtNPF4.6 and AtNPF4.7. In addition, the candidate protein is closely related to a rice nitrate(chlorate)/proton symporter protein LOC_Os04g41410.

Discussion

N-treatment dependent root QTLs

In this study, a total of 55 QTLs were discovered for seedling root traits across both N treatments (LOD > 2.0) (Fig. 2, Table 3). Of these loci, nine root QTLs were only detected in the low N treatment, 18 QTLs were found only in the high N treatment and 14 QTLs (28 total) were present in both N treatments.

In the literature, there are previously described QTL regions associated with architectural root traits. On chromosome 1A QTL were found in this study for lateral root traits under low N conditions. Interestingly chromosome 1A has been previously associated with lateral root length in wheat and rice (Ren *et al.*, 2012; Beyer *et al.*, 2018). It appears that there are underlying genes on chromosome 1A that could be related to plasticity, tolerance and/or lateral root development (An *et al.*, 2006; Landjeva *et al.*, 2008; Ren *et al.*, 2012; Guo *et al.*, 2012; Zhang *et al.*, 2013; Liu *et al.*, 2013; Sun *et al.*, 2013). This region has also been correlated to NUp in S×R field trials (Atkinson *et al.*, 2015) which would make the chromosome region an important candidate for further study. On chromosomes 2D, 3B and 4D, N-dependent root angle QTLs were identified in wheat grown in hydroponics. QTLs on these chromosomes have been described in other studies but very few of these have measured root angle or distribution traits. From comparison with other studies that found root QTLs on chromosome 2D it appears there is an underlying gene for seminal root development and/or plasticity (An *et al.*, 2006; Zhang *et al.*, 2013; Liu *et al.*, 2013). For chromosome 3B, other studies have found QTLs affecting root size and stress related traits or genes relating to N plasticity, uptake or mobilisation (An *et al.*, 2006; Habash *et al.*, 2007; Guo *et al.*, 2012; Zhang *et al.*, 2013; Bai *et al.*, 2013). For chromosome 4D, comparing with other studies that found QTLs on this chromosome there appears to be an underlying root development and/or root plasticity gene (Zhang *et al.*, 2013; Bai *et al.*, 2013).

RNA-sequencing of candidate root QTL

A low N-treatment dependent seminal root angle QTL (LOD 3.0) on chromosome 2D was targeted for transcriptomic analysis. A total of 17 candidate upregulated genes were identified that were upregulated in this region (Table 4). A more detailed list of the genes identified are

given in Fig. S2. Two of the three genes with highest log changes plus four others have unknown function. Point mutation detection and mutant generation with TILLING or RNAi represent the next step to functionally characterise these genes.

One promising candidate from root transcriptomic analyses was a nitrate transporter 1/peptide transporter (NPF) family gene, NPF4 (TraesCS2D02G348400). This gene was upregulated in a N-dependent manner related to a root angle QTL. In *A. thaliana* and *O. sativa L.*, NPF family genes have important roles in lateral root initiation, branching and response to nitrate (Remans *et al.*, 2006; Krouk *et al.*, 2010; Fang *et al.*, 2013). However, no studies have reported genes controlling root angle change in wheat, to date. A phylogenetic analysis of protein families was conducted comparing the protein sequences of *A. thaliana*, *O. sativa L.* and *T. aestivum L.* to the candidate protein. The candidate *T. aestivum L.* protein is situated in a monocot specific sub-clade within the NPF4 clade and is closely related to a rice nitrate(chlorate)/proton symporter protein (LOC_Os04g41410) (Fig. 3). Members of this clade are known for transporting the plant hormone abscisic acid (ABA) (AtNPF4.1 and AtNPF4.6) and have been demonstrated to have low affinity nitrate transport activity (AtNPF4.6) (Huang *et al.*, 1999; Kanno *et al.*, 2012). ABA is known to be a key regulator in root hydrotropism, a process that senses and drives differential growth towards preferential water potential gradients (Antoni *et al.*, 2016; Takahashi *et al.*, 2002). Hydrotropism has been demonstrated to be independent of the auxin induced gravitropism pathway and can compete in root angle changes against gravity (Dietrich *et al.*, 2018). Based on the experiments presented here, we propose that the enhanced ABA flux via the upregulated NPF4 gene could be driving a low N-dependent shallow root angle change while competing with the gravitropism pathway. As root angle is a determinant of root depth, pursuing this gene function is important agronomically for improving foraging capacity and uptake of nitrate in deep soil layers.

In summary, we found 55 root QTLs using a wheat seedling hydroponic system, 25 of which were N-treatment dependent. Using transcriptome analyses we found an upregulated NPF family gene likely transporting nitrate or ABA as part of a N-dependent response affecting root angle. These findings provide a valuable genetic insight for root angle control, N-dependent responses and candidate genes for improvement of N capture in wheat.

Supplementary data

Table S1. Composition of ¼ Hoagland's nutrient solution.

Table S2. Full list of up- and downregulated genes ($p < 0.05$) for a seminal root angle QTL located on chromosome 2D.

Fig. S1. High-throughput hydroponic phenotyping system for seedling root & shoot traits. (A) Growth assembly. (B) Image acquisition. (C) Example image of a wheat root grown on germination paper 10 DAG. (D) Root system extraction to RSML database using RootNav software. (E) Measurement of root traits from RSML database. (F) Example image of a wheat shoot 10 DAG. (G) Shoot image colour thresholding & shoot measurement using Fiji. (H) Example of QTL peak extracted from phenotyping data & mapping data with rQTL.

Fig. S2. Phylogenetic tree of protein families comparing the NPF family protein sequences of *A. thaliana*, *O. sativa L.* and *T. aestivum L*.

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Table 1. Definition of root traits measured using RootNav.

Acronym	Definition	Software	Units
RTLA	Total length of all roots	RootNav	mm
RTLS	Total length of seminal roots	RootNav	mm
RTLL	Total length of lateral roots	RootNav	mm
RSC	Number of seminal roots	RootNav	Dimensionless (Count)
RLC	Number of lateral roots	RootNav	Dimensionless (Count)
RMW	Maximum width of the root system	RootNav	mm
RMD	Maximum depth of the root system	RootNav	mm
RWDR	Width-depth ratio (MW/MD)	RootNav	Dimensionless (Ratio)
RCMX	Root centre of mass- horizontal co-ordinate	RootNav	mm
RCMY	Root centre of mass - vertical co-ordinate	RootNav	mm
RCH	Convex hull - area of the smallest convex polygon to enclose the root system	RootNav	mm^2
RCHCX	Convex hull centroid - horizontal co-ordinate	RootNav	mm
RCHCY	Convex hull centroid - vertical co-ordinate	RootNav	mm
RAE1	Angle of emergence between the outermost seminal roots measured at 30 px	RootNav	Degrees (°)
RAE2	Angle of emergence between innermost pair of seminal roots measured at 30 px	RootNav	Degrees (°)
RAE951	Angle of emergence between outermost pair of seminal roots measured at 95 px	RootNav	Degrees (°)
RAE952	Angle of emergence between innermost pair of seminal roots measured at 95 px	RootNav	Degrees (°)
RAE251	Angle of emergence between outermost pair of seminal roots measured at first quartile of total length	RootNav	Degrees (°)
RAE252	Angle of emergence between innermost pair of seminal roots measured at first quartile of total length	RootNav	Degrees (°)
RAE501	Angle of emergence between outermost pair of seminal roots measured at second quartile of total length	RootNav	Degrees (°)
RAE502	Angle of emergence between innermost pair of seminal roots measured at first quartile of total length	RootNav	Degrees (°)
RAE751	Angle of emergence between outermost pair of seminal roots measured at third quartile of total length	RootNav	Degrees (°)
RAE752	Angle of emergence between innermost pair of seminal roots measured at third quartile of total length	RootNav	Degrees (°)
RAE1001	Angle of emergence between outermost pair of seminal roots measured at root tip	RootNav	Degrees (°)
RAE1002	Angle of emergence between innermost pair of seminal roots measured at root tip	RootNav	Degrees (°)
SH	Shoot height	FIJI	mm
SA	Shoot area	FIJI	mm^2

Table 2. Seedling phenotypic values for the S×R doubled haploid population and parents under two N regimes (*n* = 18, range = 8 to 36). Trait units as Table 1. Note: shoot data available for low N treatment only.

Trait	Treat	Savannah	Rialto	DH population			
		Mean ± SE	Mean ± SE	Mean ± SE	Range	Kurt	Skew
TLA	LN	536 ± 49	360.4 ± 24	485.7 ± 28	286.1 − 891	-0.5	0.5
	HN	668 ± 48	360 ± 28	479 ± 27	244 − 811	-0.5	0.3
TLS	LN	503 ± 39	353 ± 24	461 ± 24	280 − 791	-0.8	0.4
	HN	574 ± 32	345 ± 25	448 ± 23	240 − 651	-1	0
TLL	LN	33 ± 14	7.5 ± 2	25 ± 5	2.6 − 99	1.6	1.5
	HN	94.6 ± 23	15.4 ± 5	31.4 ± 6	1.5 − 176	7.1	2.3
RAE1	LN	85.7 ± 9	93.3 ± 6	92.4 ± 2	70 − 121	0.3	0.5
	HN	101 ± 10	103 ± 7	93.1 ± 4	56.7 − 140	0.3	0.4
RAE2	LN	49.1 ± 12	55.4 ± 6	60.5 ± 2	38.9 − 85	1.2	0.3
	HN	73.5 ± 6	62.8 ± 8	62.9 ± 2	39.7 − 95	0.3	0.5
LRC	LN	11.6 ± 7	4 ± 2	9.7 ± 1	1.7 − 28	0.6	1.2
	HN	20.9 ± 4	4.9 ± 1	9.1 ± 1	0.5 − 39	3.7	1.7
SRC	LN	4.7 ± 0.3	4.8 ± 0.1	4.6 ± 0.1	3.8 − 5	-0.1	-0.4
	HN	4.7 ± 0.2	5.2 ± 0.1	4.7 ± 0.1	3.8 − 5	0	-0.6
RCH	LN	11216 ± 2759	4540 ± 700	8893 ± 1015	2824 − 21774	-0.4	0.7
	HN	17832 ± 2498	4193 ± 792	9026 ± 950	2530 − 22836	-0.1	0.7
RMW	LN	108 ± 21	68.9 ± 7	94.3 ± 6	52.4 − 161	-0.7	0.5
	HN	138 ± 15	71.8 ± 7	89 ± 5	55.1 − 158	-0.2	0.7
RMD	LN	177 ± 15	113 ± 8	154 ± 8	94.7 − 240	-1.3	0.3
	HN	232 ± 15	118 ± 9	165 ± 8	89.5 − 246	-0.8	0
RWD	LN	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0	0.4 − 1	0.1	-0.2
	HN	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0	0.3 − 1	0.6	0.2
RCMX	LN	-0.9 ± 3	-3.4 ± 2	-2.7 ± 1	-14.2 − 2	3.3	-1.1
	HN	-2.3 ± 2	-0.5 ± 2	-1.9 ± 0	-9.6 − 1	0.9	-0.9
RCMY	LN	55.1 ± 4	36 ± 3	49.6 ± 3	29.2 − 73	-1.4	0.1
	HN	61.2 ± 3	30.6 ± 3	50.3 ± 3	23.5 − 73	-1	-0.2
RCHCX	LN	1.4 ± 5	-4.4 ± 2	-4.4 ± 1	-19.2 − 3	1.9	-0.9
	HN	-4.2 ± 5	-0.5 ± 2	-4.1 ± 1	-13.5 − 2	-0.6	-0.4
RCHCY	LN	85.3 ± 9	52.1 ± 4	74.2 ± 5	41.9 − 119	-1.3	0.3
	HN	103 ± 6	46.9 ± 4	76.2 ± 4	34.8 − 121	-0.9	0
RAE951	LN	81.4 ± 11	83.8 ± 6	82.2 ± 2	63.4 − 104	-0.1	0.1
	HN	95.9 ± 8	93.9 ± 6	87.9 ± 2	59.6 − 112	0	-0.3
RAE952	LN	46.7 ± 12	48.4 ± 5	51.9 ± 2	34.2 − 72	0	0
	HN	68.5 ± 9	54.4 ± 7	58.9 ± 2	35 − 88	0.4	0.2
RAE251	LN	76.8 ± 12	85.4 ± 6	78.5 ± 2	59.7 − 94	-0.5	-0.2
	HN	89.4 ± 7	92.2 ± 6	84 ± 2	60.2 − 104	-0.1	-0.2
RAE252	LN	50.4 ± 12	48.6 ± 5	49.1 ± 1	29.5 − 63	-0.4	-0.2
	HN	63.1 ± 8	55.9 ± 6	56.1 ± 2	36.8 − 83	0.1	0.3

RAE501	LN	71.8 ± 13	78.9 ± 6	73.2 ± 2	53.8 − 90	-0.2	0
	HN	87.9 ± 7	85.4 ± 7	79.4 ± 2	59.3 − 99	-0.1	-0.2
RAE502	LN	49.1 ± 10	47.5 ± 4	46.2 ± 1	28.7 − 61	-0.2	0
	HN	56.4 ± 9	54.8 ± 5	50.4 ± 2	28.2 − 70	-0.1	0
RAE751	LN	69.1 ± 12	74.7 ± 6	71.8 ± 2	56.6 − 91	0.2	0.3
	HN	87.2 ± 8	81.6 ± 7	76.7 ± 2	55.6 − 96	0	-0.1
RAE752	LN	50 ± 9	46 ± 4	46 ± 1	30.4 − 59	-0.2	0
	HN	53.7 ± 9	49.4 ± 6	47 ± 2	26.6 − 63	-0.3	-0.2
RAE1001	LN	68.6 ± 11	71.6 ± 6	71.3 ± 2	55.7 − 92	0.5	0.5
	HN	87.2 ± 8	76.5 ± 7	74.4 ± 2	53.2 − 97	0.4	0
RAE1002	LN	49.3 ± 8	43.8 ± 4	45.4 ± 1	32 − 59	-0.2	-0.1
	HN	51.8 ± 8	42.5 ± 6	43.5 ± 2	21.4 − 59	0.2	-0.4
SH	LN	72.4 ± 7	69.1 ± 3	75.8 ± 0	51 − 90	0.2	-0.4
SA	LN	137.9 ± 8	153.7 ± 5	165.8 ± 1	85.4 − 271	0.2	-0.4

Table 3. QTLs for wheat seedling traits detected in the S×R DH population grown in hydroponics. Trait units as Table 1. Note: shoot data available for low N treatment only.

Trait	Treat	QTL	Interval[a]	Site[b] (cM)	LOD[c]	Additive[d]	H2[e] (%)
RTLA	LN	6D	BobWhite_c7090_522–BS00023964	5.0	27.4	-229	65.0
		7D	wsnp_Ku_c416_869895–BS00028760_51	26.0	8.4	-107	11.3
	HN	6D	BobWhite_c7090_522–BS00023964	4.4	23.0	-1275	57.4
		7D	wsnp_Ku_c416_869895–BS00028760_51	27.0	8.7	-703	14.3
RTLS	LN	6D	BobWhite_c7090_522–BS00023964	5.0	33.5	-198	70.5
		7D	wsnp_Ku_c416_869895–BS00028760_51	26.0	11.3	-86	12.1
	HN	6D	BobWhite_c7090_522–BS00023964	4.4	24.8	-1068	59.9
		7D	wsnp_Ku_c416_869895–BS00028760_51	27.0	9.4	-580	14.4
RTLL	LN	1A	BS00004043–BS00000226	215.0	2.3	-9.0	6.2
		6D	BobWhite_c7090_522–BS00023964	8.0	13.4	-31.2	48.0
	HN	6D	BS00009514–BS00023964	4.4	6.3	-208	28.0
RAE1	HN	3B	BobWhite_c22370_352–wsnp_RFL_Contig3336_3426054	178.8	2.2	-11.0	10.8
RAE2	HN	3B	GENE-1154_396–wsnp_RFL_Contig3336_3426054	178.8	2.8	-8.2	13.3
RLC	LN	1A	BS00004043–BS00000226	216.0	4.9	-2.4	8.6
		6D	BobWhite_c7090_522–BS00023964	5.0	19.6	-9.4	52.8
		7D	wsnp_Ku_c416_869895–BS00028760_51	22.0	6.0	-4.4	10.9
	HN	6D	BS00009514–BS00023964	4.4	8.8	-8.5	36.5
RSC	LN	6D	BS00009514–BS00022787	4.4	3.2	0.2	13.1
		7D	wsnp_Ku_c416_869895–IAAV4624	23.0	3.8	-0.2	15.8
	HN	7A	Excalibur_c48636_283–wsnp_RFL_Contig2864_2688208	12.0	2.9	0.2	13.7
RCH	LN	6D	BobWhite_c7090_522–BS00023964	4.4	31.1	-8464	80.0
	HN	6D	BobWhite_c7090_522–BS00023964	4.4	18.4	-287837	53.5
		7D	wsnp_Ku_c416_869895–Kukri_c46303_512	34.0	4.2	-133799	8.3
RMW	LN	1B	IAAV3905–wsnp_RFL_Contig3951_4390396	12.5	3.6	-8.9	5.0
		6D	BobWhite_c7090_522–BS00023964	4.4	26.7	-48.5	72.8
	HN	4D	wsnp_Ex_c9440_15657149–wsnp_Ku_c16354_25219645	23.9	3.1	68.8	7.1
		6D	BS00009514–BS00023964	4.4	16.4	-230	54.6
RMD	LN	6D	BobWhite_c7090_522–BS00023964	4.4	31.5	-71.4	75.1
		7D	wsnp_Ku_c416_869895–BS00021859	27.0	3.7	-20.6	3.8
	HN	6D	BobWhite_c7090_522–BS00023964	4.4	21.8	-384	58.8
		7D	wsnp_Ku_c416_869895–BS00028760_51	30.0	5.2	-169	8.6
RMWD	HN	4D	wsnp_Ex_c9440_15657149–BS00065168	4.8	3.1	0.1	14.6
RCMX	LN	6D	BS00009514–BS00023964	6.0	2.3	1.6	11.2

	HN	1A	GENE-0249_122–BS00075532_51	145.0	4.1	-11.1	16.6
		6D	BS00009514–BS00023964	22.0	4.0	9.8	16.3
RCMY	LN	6D	BobWhite_c7090_522–BS00023964	4.4	31.9	-23.1	80.8
	HN	6D	BobWhite_c7090_522–BS00023964	4.4	19.5	-123	63.5
RCHCX	LN	6D	BS00009514–BS00023964	4.4	2.3	2.5	11.2
	HN	6D	BS00009514–BS00023964	18.0	3.2	13.6	12.9
		7D	wsnp_Ku_c416_869895–IAAV4624	21.0	3.2	16.5	13.1
RCHCY	LN	6D	BobWhite_c7090_522–BS00023964	4.4	34.1	-40.0	82.9
	HN	3B	BS00064778–BS00075879	216.2	4.9	45.2	6.8
		6D	BobWhite_c7090_522–BS00023964	4.4	25.0	-215.8	62.1
		7D	wsnp_Ku_c416_869895–Kukri_c46303_512	32.0	5.8	-91.8	8.2
RAE951	HN	3B	RAC875_c5799_224–wsnp_Ra_c7158_12394405	178.8	2.8	-7.7	13.3
RAE251	LN	2D	BS00049876_51–BS00066132_51	117.0	1.4	3.8	7.1
	HN	3B	BobWhite_c22370_352–wsnp_CAP11_c323_263800	178.8	3.6	-7.7	17.0
RAE252	HN	4D	wsnp_Ex_c9440_15657149–BS00065168	0.8	2.8	6.5	13.4
RAE501	HN	4D	wsnp_Ex_c9440_15657149–BS00065168	0.8	2.9	6.3	14.0
RAE502	HN	4D	wsnp_Ex_c9440_15657149–BS00065168	0.8	3.0	6.4	14.2
RAE751	LN	2D	BS00010393–BS00066132_51	160.0	2.6	5.1	12.5
	HN	4D	wsnp_Ex_c9440_15657149–BS00024014	0.8	2.9	6.3	13.8
RAE752	HN	4D	wsnp_Ex_c9440_15657149–BS00065168	0.8	2.1	5.3	10.4
RAE1001	LN	2D	BS00010393–BS00066132_51	160.0	3.0	5.5	14.3
	HN	4D	wsnp_Ex_c9440_15657149–BS00024014	0.8	2.4	6.0	11.9

[a] Chromosome region of the QTL defined by two flanking markers

[b] Genetic position of the QTL peak value

[c] Logarithm of the odds value

[d] Additive effects of putative QTL; a positive value indicates that positive alleles are from Savannah; negative values indicate positive alleles are from Rialto

[e] Trait heritability

Table 4. Candidate genes for seminal root angle QTL located on chromosome 2D that were consistently expressed across the Group A replicates verses zero reads mapping in one or more Group B replicates. Gene naming convention according to IWGSC RefSeq v1.1.

Gene	Log_2 fold change	p value	Functional annotation
TraesCS2D02G509700	1.73	0.002	Peroxidase
TraesCS2D02G344400	1.45	0.013	Unknown
MSTRG.42598 (TGACv1)	1.31	0.041	Unknown
TraesCS2D02G441300	1.29	0.037	AAA domain UvrD/REP helicase N-terminal domain
TraesCS2A02G111200	2.12	2.5E-05	Kelch motif
TraesCS2B02G126600	2.21	9.5E-06	Unknown
TraesCS2D02G487000	1.53	0.008	DUF wound-responsive family protein
TraesCS2D02G088100	1.29	0.036	C2H2-type zinc finger
TraesCS2D02G129100	1.36	0.036	Legume lectin domain
TraesCS2D02G330200	1.44	0.013	Unknown
MSTRG.40366 (TGACv1)	2.02	8.9E-05	Unknown
TraesCS2D02G108500	1.38	0.026	Peroxidase
TraesCS6A02G175000	1.66	0.002	Nuclear pore complex scaffold, nucleoporin
TraesCS2D02G270000	1.66	0.002	Helix-loop-helix DNA-binding domain
TraesCS2D02G511200	1.41	0.025	Peroxidase
TraesCS4B02G057100	1.48	0.013	Unknown
TraesCS2D02G348400	1.88	3.6E-04	NPF4

Figure legends

Fig. 1. Phenotypic variation in seedling traits for S×R doubled haploid under low high N treatments. Organised smallest (left) to largest (right) for low N treatment. (A) Total length of all roots (RTLA). (B) Total length of lateral roots (RTLL). (C) Angle of emergence between outermost pair of seminal roots measured at root tip (RAE1001). (n = 18, range = 8 to 36).

Fig. 2. Molecular linkage map showing position of QTLs detected in the S×R DH population grown in hydroponics. QTLs and confidence regions for all root traits are colour labelled for low N-dependent (blue), high N-dependent (red) and N treatment independent (green) (LOD > 2.0).

Fig. 3. Phylogenetic tree of protein families comparing the protein sequences of *A. thaliana*, *O. sativa L.* and *T. aestivum L.* NPF family proteins to an identified candidate *T. aestivum L.* protein. The candidate *T. aestivum L.* protein is situated in a monocot specific outgroup within a NPF4 protein clade (highlighted in red). Branch lengths are proportional to substitution rate.

Fig. 1.

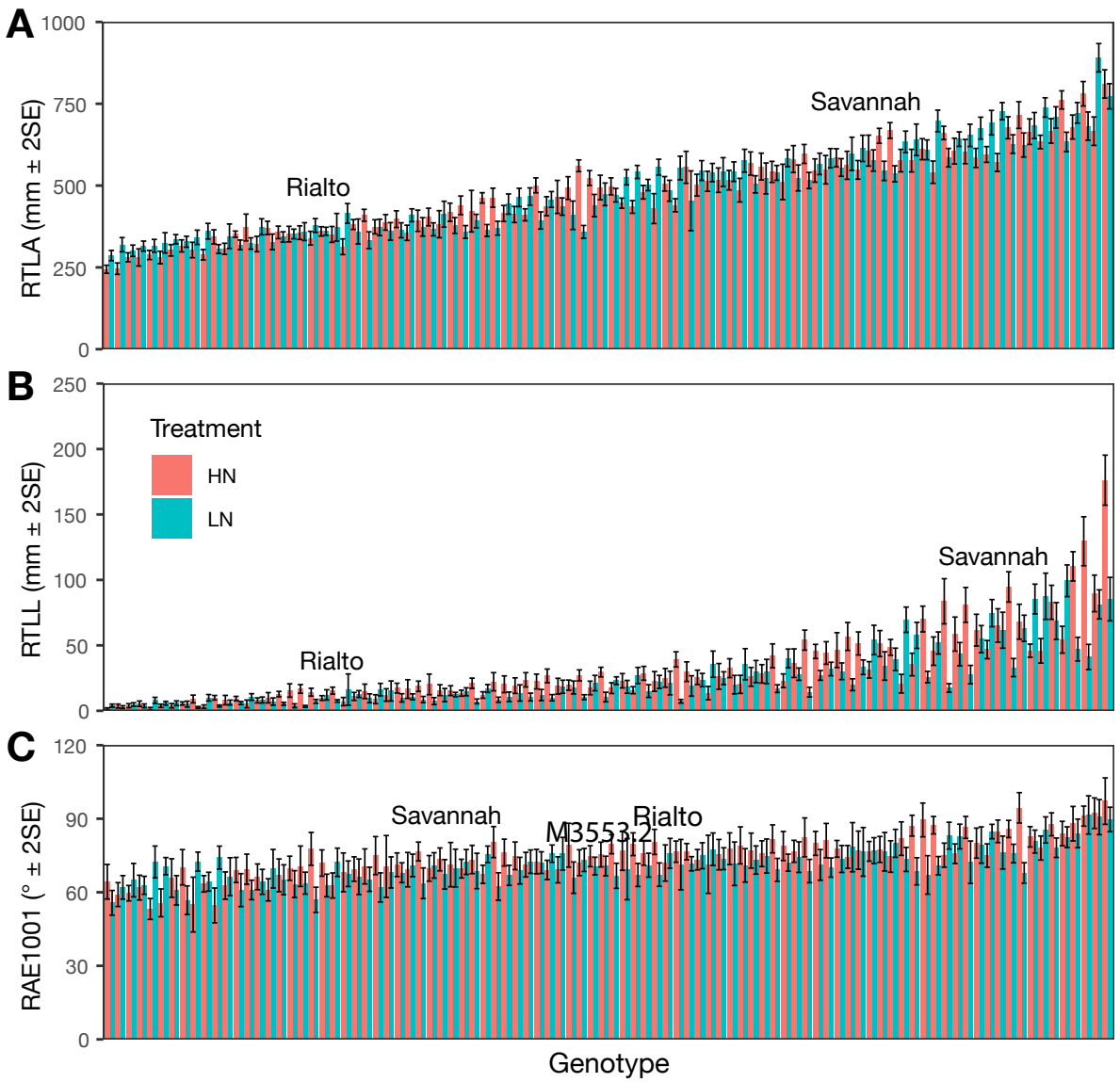
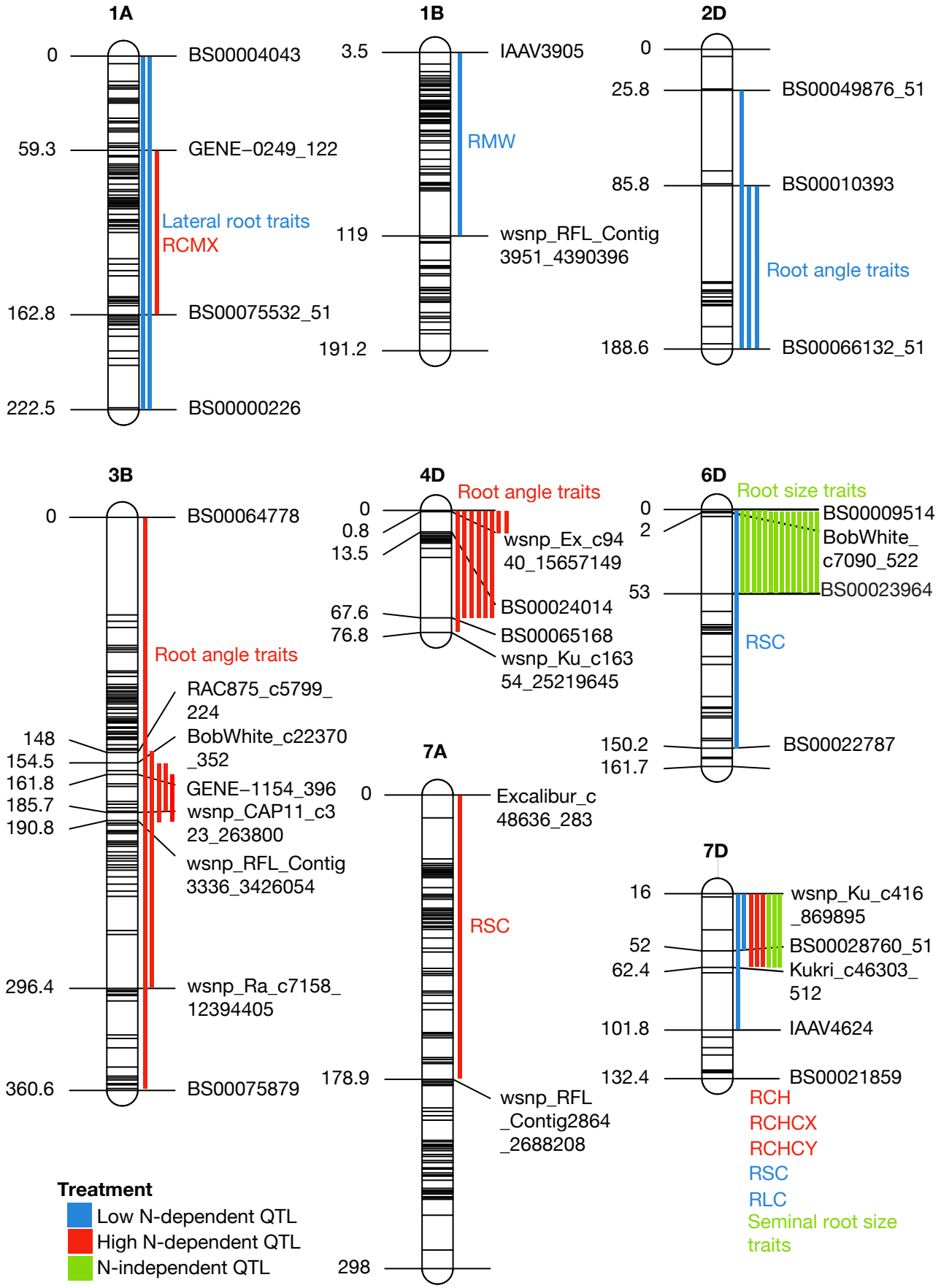

A
RTLA (mm ± 2SE)
1000
750
500
250
0
Rialto
Savannah
B
RTLL (mm ± 2SE)
250
200
150
100
50
0
Treatment
HN
LN
Rialto
Savannah
C
RAE1001 (° ± 2SE)
120
90
60
30
0
Savannah
M3553.2
Rialto
Genotype

Fig. 2.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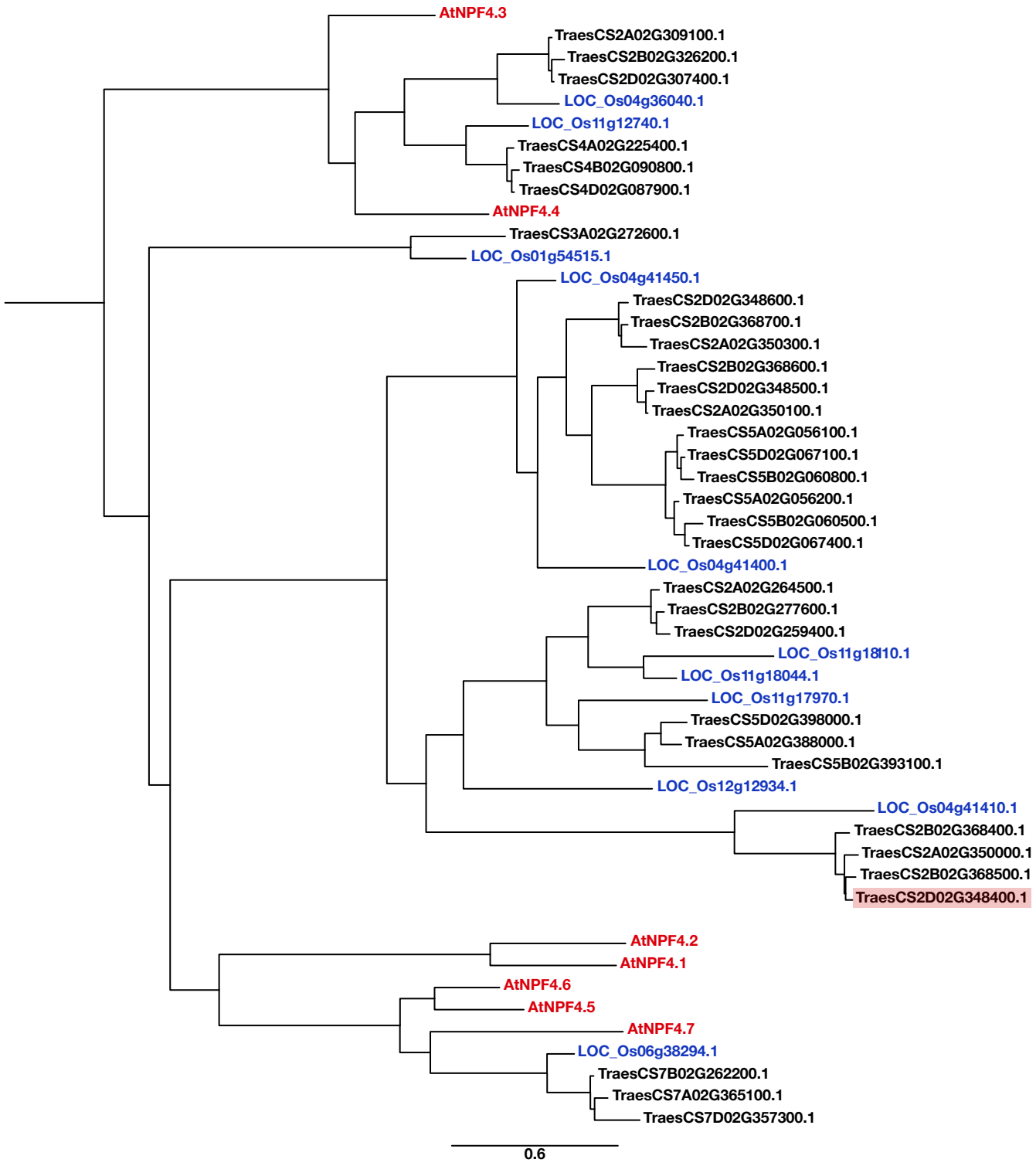
0
BS00004043
59.3
GENE−0249_122
Lateral root traits
RCMX
162.8
BS00075532_51
222.5
BS00000226
1B
3.5
IAAV3905
RMW
119
wsnp_RFL_Contig
3951_4390396
191.2
2D
0
25.8
BS00049876_51
85.8
BS00010393
Root angle traits
188.6
BS00066132_51
3B
0
BS00064778
Root angle traits
RAC875_c5799_
224
148
154.5
161.8
185.7
190.8
BobWhite_c22370
_352
GENE−1154_396
wsnp_CAP11_c3
23_263800
wsnp_RFL_Contig
3336_3426054
296.4
wsnp_Ra_c7158_
12394405
360.6
BS00075879
4D
Root angle traits
0
0.8
13.5
wsnp_Ex_c94
40_15657149
67.6
76.8
BS00024014
BS00065168
wsnp_Ku_c163
54_25219645
6D
Root size traits
0
2
BS00009514
BobWhite_
c7090_522
53
BS00023964
RSC
150.2
161.7
BS00022787
7A
0
Excalibur_c
48636_283
RSC
178.9
wsnp_RFL
_Contig2864
_2688208
298
7D
16
wsnp_Ku_c416
_869895
52
BS00028760_51
62.4
Kukri_c46303_
512
101.8
IAAV4624
132.4
BS00021859
RCH
RCHCX
RCHCY
RSC
RLC
Seminal root size
traits
Treatment
Low N-dependent QTL
High N-dependent QTL
N-independent QTL

Fig. 3.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TraesCS2B02G368600.1
TraesCS2D02G348500.1
TraesCS2A02G350100.1
TraesCS5A02G056100.1
TraesCS5D02G067100.1
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AtNPF4.1
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