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Association analysis of genes involved in maize (Zea mays L.) root development with seedling and agronomic traits under contrasting nitrogen levels

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Association analysis of genes involved in maize (*Zea mays* L.) root development with seedling and agronomic traits under contrasting nitrogen levels

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Abstract

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Abstract

Genotypes with large and well distributed root system might have the potential to adapt to soils with limited nutrient availability. For this purpose, an association study (AS) panel consisting of 74 diverse set of inbred maize lines were screened for seedling root traits and adult plant root traits under two contrasting nitrogen (N) levels (low and high N). Allele re-sequencing of *RTCL*, *RTH3*, *RUM1*, and *RUL1* genes related to root development was carried out for AS panel lines. Association analysis was carried out between individual polymorphisms, and both seedling and adult plant traits, while controlling for spurious associations due to population structure and kinship relations. Based on the SNPs identified in *RTCL*, *RTH3*, *RUM1*, and *RUL1*, lines within the AS panel were grouped into 16, 9, 22, and 7 haplotypes, respectively. Association analysis revealed several polymorphisms within root genes putatively associated with the variability in seedling root and adult plant traits development under contrasting N levels. The highest number of significantly associated SNPs with seedling root traits were found in *RTCL* (19 SNPs) followed by *RUM1* (4 SNPs) and in case of *RTH3* and *RUL1*, two and three SNPs, respectively, were significantly associated with root traits. *RTCL* and *RTH3* were also found to be associated with grain yield. Thus considerable allelic diversity is present within the candidate genes studied and can be utilized to develop functional markers that allow identification of maize lines with improved root architecture and yield under N stress conditions.

Keywords: Nitrogen, Maize, Root traits, Grain yield, Resequencing, Gene based association mapping

Introduction

Nitrogen (N) is a macronutrient crucial for maximizing grain yield. Globally, N fertilizer demand in 2010 was 103.9 million tons and is expected to increase to 111.0 million tons in 2014 at an annual growth rate of 1.8 percent (FAO 2010). Among all crops, cereals utilize the majority (about 65%) of fertilizer N (FAO 2006). However, the N fertilizer used by cereals is only about 33% (Raun and Johnson 1999; Tilman et al. 2002). The remainder is lost in a combination of leaching, surface runoff, gaseous release from soil, and de-nitrification processes. This leads to environmental pollution and increased input costs to farmers (Tilman et al. 2002; Arregui and Quemada 2008). A societal challenge is to balance crop productivity for feeding a growing world population along with reducing negative effects of N pollution due to agriculture activity. This can be achieved through improving nitrogen use efficiency (NUE) of agricultural crops and/or by adopting precision farming.

There are several definitions for NUE in plants (Moll et al. 1982; Ortiz-Monasterio et al. 1997; Muurinen et al. 2007). One of the most commonly used NUE calculation is the grain yield per unit of available N. This NUE definition is also

referred to as Agronomic efficiency (AE) or yield efficiency. NUE in maize involves complex interactions between various physiological processes such as N uptake by roots, N assimilation by shoots and roots, N utilization by ear shoot and seeds, and N remobilization from source tissues. Substantial genetic variation for NUE in maize was found in exotic, U.S., and European germplasm (Pollmer et al. 1979, Laffitte and Edmeades 1994; Agrama et al. 1999; Presterl et al. 2003; Worku et al. 2007). Selection for NUE based on seedling root traits was seldom considered as a selection criterion for improving NUE in maize (Tuberosa and Salvi 2007). Our focus in this study was on genes involved in the root development for three reasons: (1) the root system has been shown to be important in relation to NUE (Hirel et al. 2007), (2) simple assays at the seedling stage allow description of effects on the root directly caused by the gene, increasing the chance of finding meaningful associations with root development, and (3) alterations in the root system have been shown to affect drought tolerance in addition to NUE (Ribaut et al. 2007). Thus, beneficial alleles in terms of NUE for genes affecting root development might act pleiotropic on drought tolerance and potentially uptake of other nutrients.

The root system in maize is determined by embryonic and postembryonic roots formed during different stages of the development (Abbe and Stein 1954; Hochholdinger 2009). Embryonic roots include primary root and seminal roots. The postembryonic root system includes crown roots formed at consecutive underground nodes and lateral roots which emerge from major root types (Hochholdinger 2009). The number and length of seminal, crown, and lateral roots are genotype dependent (Feldman 1994). The root volume, vertical distribution, and rooting depth of a plant are important key parameters determining its potential for water and nutrient acquisition (e.g. Lynch and Ho 2005; Hund et al. 2009). Root size is an important factor for the uptake of essential nutrients such as phosphorous (P), calcium (K) and N (Barber and Mackay 1986; Marschner 1998). Therefore, genotypic variation in root traits provides an opportunity to address economic and pollution consequences of N fertilizer application through selection and breeding for N efficient genotypes.

Root morphology is influenced by the amount of N fertilizer applied (Eghball and Maranville 1993). Several studies have reported adaptation strategies for N deficiency (Narayanan and Reddy 1982; Anuradha and Narayanan 1991; Bailigar et al. 2001; Monaco et al. 2003; Bonifas et al. 2005; Li et al. 2009). Root morphology is relevant for efficient acquisition of N by plants. An increase in soil N is generally associated with a reduced root to shoot ratio (Maizlisch et al. 1980, Narayanan and Reddy 1982; Anuradha and Narayanan 1991). In contrast, Barber and Mackay (1986) showed that N fertilization can enhance root growth and root dry weight. In another study, high nitrate concentrations (2 and 4 mM) increased total length of lateral roots (Wang et al. 2004), but decreased total

primary root length. The increase of the root system characteristics such as root length, number of primary roots, and the elongation rate of first order laterals (Maizlisch et al.1980) in response to low N availability suggests that roots are sinks of photo-assimilates when nutrients are limiting factors to plant growth, especially N and P. This might be an adaptation strategy to increase absorption efficiency when these nutrients are limited (Horst et al. 2001).

Several maize mutants affected in root development have been identified, and respective genes have been cloned (Hochholdinger 2009). These include *RTCS* (rootless concerning crown and seminal roots), *RTH1* (root hairless 1), *RTH3* (root hairless 3), and *RUM1* (rootless with undetectable meristems 1). *RTCL* (*RTCS* like gene) is regarded as a paralogue of *RTCS* and *RUL1* (*RUM1* like gene-1) as a paralogue of *RUM1* (Wen et al. 2005; Woll et al. 2005; Taramino et al. 2007; von Behrens et al. 2011). The maize *rtcs* mutant affects crown roots, seminal and shoots borne roots initiations. The *rth1* mutant fails to elongate root hairs, and the *rum1* mutant is deficient in the initiation of seminal roots and lateral roots at the primary roots. *RTCS* encodes a 244 amino acids (aa) long Lateral Organ Boundaries (LOB) domain protein located on chromosome 1S. *RTCS* is orthologous to the rice genes *CRL1* and *ARL1* on chromosome 3 with a 259 aa protein LOB domain (Inukai et al. 2005; Liu et al. 2005). During evolution, *RTCS* is duplicated as *RTCL* gene which maps on chromosome 9. The paralogous *RTCL* gene displays 72% sequence similarity with *RTCS* at protein level. Both these genes promoters share auxin responsive elements and they are preferentially expressed in roots (Taramino et al. 2007). *RTH1* encodes a SEC3 homologue (Wen et al. 2005). In yeast (*Saccharomyces cerevisiae*) and mammals, SEC3 is a part of the exocyst complex, which ropes together exocytotic vesicles prior to their fusion.*RTH3* belongs to the COBRA-like gene family (Hochholdinger et al. 2008). Members of this plant-specific glycosylphosphatidylinositol anchored protein coding gene family are involved in cell expansion and cell wall biosynthesis (Brady et al. 2007). Most recently, von Behrens et al. (2011) isolated and characterized *RUM1* gene which controls initiation of seminal and lateral roots. *RUM1* located on chromosome 3 encodes a polypeptide of 269 aa, which is a monocot specific AUX/IAA protein (von Behrens et al. 2011). *RUL1* is a closely related Aux/IAA protein coding gene and is localized on chromosome 8. *RUL1* encodes a polypeptide of 273 aa that displays 92% identity with *RUM1* on the amino acid level.

Two most commonly used methods to dissect complex traits in plants are linkage analysis and association mapping. Linkage analysis uses a well characterized pedigree to identify the non-random association between the genotype and phenotype, whereas association mapping utilizes ancestral recombinations in unrelated individuals and linkage disequilibrium to identify the associations between genotype and phenotype (Zhu et al. 2008; Ersoz et al. 2009).

Major limitations of linkage mapping compared to association analyses are poor resolution in detecting QTL in the order of 10-30 centimorgans (cM) due to fewer recombinations within pedigrees (Alpert and Tanksley 1996; Stuber et al. 1999), longer time needed to produce a mapping population, and only two alleles per locus to be studied (one from each parent in a bi-parental mapping population) (Jannink et al. 2001). Association analyses exploit historical recombinations and natural variation among unrelated individuals to carry out high resolution mapping. The first candidate gene-based association mapping study in plants associating individual *dwarf8* polymorphisms with flowering time of maize was carried out by Thornsberry et al. 2001, and has been followed by numerous association mapping studies in maize (Breseghello et al. 2006; Andersen et al. 2008; Zhu et al. 2008; Krill et al. 2010; Zhang et al. 2010; Chen et al. 2010; Brenner et al. 2010) and in other crop plants (Thornsberry et al. 2001; Gupta et al. 2005; Kim et al. 2005; Garcés-Claver et al. 2007). Gene-based association studies ultimately lead to the identification of quantitative trait polymorphisms (QTPs) with causal genetic effect on agronomic traits, which can be converted into functional markers (Andersen and Lübberstedt 2003). Linkage mapping has been extensively used to detect QTL controlling root development in maize under different abiotic stress conditions (Tuberosa et al. 2003; Zhu et al. 2005a and b; Liu et al. 2008; Ruta et al. 2010). However, there are no reports on using association analysis to dissect the genes controlling root development in maize. In this study, our objectives were i) to examine nucleotide diversity for candidate genes related to root development in a diverse set of maize inbred lines, ii) to study the extent of LD for these genes, and iii) to test for associations between individual polymorphisms and seedling root, and adult plant traits in maize grown under contrasting N conditions.

Materials and Methods

Plant materials

Allele re-sequencing of candidate root genes *RTCL, RTH3, RUM1,* and *RUL1* was carried out in a diverse set of maize inbred lines. Inbred lines used in this study composed of 104 expired plant variety protection (PVP) lines and public inbred lines such as all Nested Association Mapping (NAM) founder lines, 2009 released Germplasm Enhancement of Maize (GEM) lines and some lines used in a maize diversity study. Due to the wide range in the maturity in this mapping population, we selected 74 lines from this population that had suitable maturity to grow well in Iowa. We refer to these lines as association study (AS) panel. The rationale for using expired PVP lines in this study is to capture a substantial fraction of genetic variation present in current elite germplasm. The public lines were chosen to enable detection of SNP and INDEL polymorphisms.Seed of AS lines were obtained from the North

Central Regional Plant Introduction Station, Ames, IA. All 74 AS lines were selfed at the Agronomy Farm, Iowa State University in summer 2009 to produce seed of equal quality for all genotypes for this study.

Cigar roll culture and assessment of root parameters

Seedling root characteristics in AS lines were studied using paper roll tests described by Woll et al. (2005). Seeds were first surface sterilized with Clorox® solution (6% sodium hypochlorite) for 15 minutes. After surface sterilization, seeds were washed three times with sterile water. Thereafter, seeds were placed on brown germination paper (Anchor Paper, St. Paul, MN, USA) pre-moisturized with fungicide solution Captan® (2.5g/l), and afterwards rolled up vertically. Rolled germination papers were kept in 2 l glass beakers containing autoclaved Hoagland nutrient solution (Hershey 1994) with contrasting levels of Nitrate (NO_3^-). High N (HN) Hoagland solution contained 15 mM of $NO_{3-,}$ whereas Low N (LN) Hoagland solution contained 1.5 mM NO_3^-. Other macro- and micro-nutrients remained the same in both N treatments (Supp. Table 1). Experiments were carried out in a growth chamber at a photoperiod of 16/8 h at 25/22 $^{\circ}$C (light/darkness) with photosynthetically active radiation of 200 µmol photons m^{-2} s^{-1}. The relative humidity in the growth chamber was maintained at 65%. The experimental design was a Randomized Complete Block Design (RCBD) with split-plot arrangement of treatments. The experiment involved 74 AS lines and two N levels (high N: HN, low N: LN). Nitrogen level was the main plot and line was the sub-plot factor. The experiment was repeated twice and each experiment had two replicates. Each replicate was represented by three healthy seedlings. After 14 days, growth of the seedlings was stopped by preserving seedlings in 30% ethanol. Primary root length (PRL), total length of seminal roots (SRL) and total length of crown roots (CRL) was measured manually using a ruler. Total length of lateral roots (LRL) was measured using image analysis software (WinRhizo Pro 2009, Regent Instruments, Quebec, Canada). Dry weight measurements including shoot (SDW) and root dry weight (RDW) was measured after drying roots and shoots at 80°C for at least 48 hrs. Total length of roots (TRL) was estimated by summing PRL, SRL, CRL, and LRL. Total seedling biomass (TSB) was estimated by summing RDW and SDW.

Field study

In summer 2010, field trials were conducted in two locations: Agronomy research station, Iowa State University, Ames, IA (Ames) and at DuPont Pioneer research station, Marion, IA (Marion) in a RCBD design in two row plots. Due to wide range in the flowering time, all 74 lines in the AS panel were divided into seven maturity groups and were planted in the order of their flowering to reduce shading effects. Lines within each maturity group were

randomized before planting in the field. In the low N (LN) field, no N was applied at Ames and 56 kg N ha^{-1} was applied at Marion. In the high N (HN) field, 250 kg N ha^{-1} was applied at Ames and 269 kg N ha^{-1} at Marion. At both locations, planting density was 69,187 plants ha^{-1} and each inbred line was planted in two-row plots, which were 5.64 and 5.31 m long, and spaced 0.76 m apart at Ames and Marion, respectively. Adult plants traits such as anthesis to silking interval (ASI), leaf chlorophyll content (CHLMET), and plant height (PHT) were measured at both locations and treatments. ASI was measured by calculating the difference in growing degree units (GDU's) between anthesis and silking time (McMaster and Wilhelm 1997). Days to anthesis (DA) and days to silking (DS) were recorded as the number of days from sowing to the day when 50% of anthers extruded outside the glumes and when silk became visible, respectively. Chlorophyll content was measured from the flag leaf 15 days after silking using a chlorophyll meter SPAD-502 (Minolta Camera Co., Osaka, Japan). PHT was estimated as the distance between the ground surface and the tip of the central tassel spike. Grain yield was recorded on a plot basis using hand and machine harvest at Ames and Marion, respectively.

Phenotypic data analysis

Phenotypic data were recorded on a plot basis (seedling traits: means of three seedlings per inbred line; adult plant traits: two-row plots) for all studied traits. Best linear unbiased prediction estimates (BLUP) (Piepho et al. 2008) for the seedling and adult plant traits were calculated and used in the association study. Agronomic efficiency (AE) for TRL, RDW, TSB, and grain yield was calculated as follows: (performance in HN – performance in LN)/ (difference in N applied). Path coefficient analysis was performed by examining PRL, CRL, SRL, and LRL as independent variables for major contributors to RDW and SDW, and RDW and SDW as independent variables for TSB trait. Path coefficient analysis was performed using the statistical program Amos (Arbuckle 2006). Through path analysis, it is possible to determine the direct influence of one variable over another, and at the same time correlation coefficients can be separated into components of direct and indirect effects. The advantage of partitioning correlation coefficients into direct and indirect effects is that it provides actual information on contribution of each trait (independent variable) on a dependent variable, and thus provides information to make selection for improvement of traits of interest (Khan and Dar et al. 2009). Complete phenotypic analysis of seedling and adult plant traits is presented elsewhere (Abdel-Ghani et al. 2013).

DNA extraction, PCR amplification, and allele-resequencing

DNA was extracted from fresh leaf tissues of three week old AS lines using the Maxi CTAB method (Saghai-Maroof et al. 1984). For allele re-sequencing of candidate root genes: *RTCL*, *RTH3*, *RUM1*, and *RUL1*, forward and reverse primers were designed using the respective gene sequences from the B73 maize genome (http://www.ncbi.nlm.nih.gov/). The Primer3 program (http://frodo.wi.mit.edu/primer3/) was used to design the primer pairs for resequencing of candidate genes. Forward and reverse primer pairs used to amplify the genes with their annealing temperature, and GenBank accession numbers of templates used to design primers are listed in Table 1. PCR reactions were carried out in 50 μl volumes under the following conditions: 50 ng template DNA, 250 nM of each primer, 250 nM dNTPs, 2 U Taq polymerase and 250 μM $MgCl_2$. Reactions were performed for each primer pair using the following PCR program in a thermocycler (MJ research, California, USA): an initial 94 °C denaturation step for 2 min followed by 35 cycles of 94 °C for 30 sec (denaturation step), 57.5 °C for 30 sec (annealing step), and 72 °C for 90 sec (elongation step). The final extension step was followed by 72 °C incubation for 10 min. Amplified DNA fragments were resolved by gel electrophoresis (Biorad) using 1% agarose gels in Tris-EDTA (TE) buffer. Gels were stained with 0.5 µg of ethidium bromide per ml. The running time was 90 min at 120 mV voltage. Finally, gels were visualized and photographed by UV illuminator system (Alphainnotech, California, USA). For each gel, the first lane was specified for a 100 bp DNA ladder (Promega, USA), the second lane and the third lane were specified for positive and negative controls. The entire genome sequence of *RTCL* (828 bp) containing two exons with 420 and 279 bp, respectively, spaced by one intron with 129 bp was amplified (Supp. Fig. 1). In case of *RTH3*, *RUM1*, and *RUL1* genes, only partial amplification of genes succeeded. Due to their suspected role in agronomic traits, we anticipated increased levels of linkage disequilibrium for these genes due to selection, and assumed that partial sequences would still be valuable for this association analysis. The total length of the *RTH3* gene is 3145 bp, consisting of 2001bp open reading frame (ORF) (Supp. Fig. 2). Out of 2001 bp of the ORF, 714 bp were successfully amplified for our association study. *RUM1* gene is composed of 5 exons spaced by 4 introns with 2696 bp in total (Supp. Fig. 3). Intron 4 and exon 5 were partially amplified in this study: 225 out of 461 bp in intron 4 and 207 out of 315 bp in exon 5. *RUL1* gene is composed of 6 exons and 5 introns with the total size of 2952 bp (Supp. Fig. 4). In this gene, intron 5 and exon 6 were partially amplified (411 bp in total): 84 bp out of 507 and 327 out of 391bp were amplified in intron 5 and exon 6, respectively.

Amplified fragments of *RTH3*, *RUM1*, and *RUL1* genes were obtained for all 74 inbred lines in the AS panel, whereas in *RTCL* gene, amplicons were obtained from 69 lines in the AS panel. For sequencing, 10 µl of the

amplified fragments were first cleaned using 2 units of shrimp alkaline phosphatase and 2 units of exonuclease I at 37 °C for 1 h followed by 72 °C for 15 min to deactivate the enzymes. Amplified gene products were then labeled for sequencing using the ABI Prism® BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Labeling reactions were performed in 10 µl reaction volume containing 1 µl of PCR product, 1 µl of BigDye Terminators, 0.256 µl of 50 mM original PCR primers (Forward or Reverse), 1.75 µl of 5× sequencing buffer and 6µl deionized distilled water. The thermocycler cycle sequencing reaction was performed using the following cycling parameters: 96°C 2 min, 25 cycles of 96°C for 30 sec, 50°C for 1 min and 60°C for 4 min, followed by 4°C for storage. Sequencing reactions were precipitated using ethanol and dried thoroughly before re-suspending in ABI Hi-Dye formamide, and run on Applied Biosystems 3730 DNA Analyzer with a 96-capillary array. Sequencing was performed for each amplified fragment using forward and reverse primers separately. Sequences were trimmed and assembled using Sequencher version program 4.10 default parameters with the exception of a minimum match percentage of 100% (Gene Codes Corporation, Ann Arbor, MI, USA). In order to maximize read lengths and to obtain sequences of quality > 98%, two replicates of forward and reverse reads for each amplified fragment were aligned to get consensus sequences of gene fragments from individual lines in the AS panel.

Haplotype, population structure, and association analysis

DnaSp Version 4 (Rozas et al. 2003) was used to analyze the haplotype diversity among candidate genes based on the SNPs in the amplified fragment sequences of the AS panel. For all studied traits, maximum, minimum, and range of phenotypic values of lines representing individual haplotypes were calculated. To identify the population structure within the mapping population, lines in the AS panel were genotyped with 101 SNP markers distributed evenly across the maize genomeusing the Sequenom MassARRAY® System at the Genomic Technologies Facility (http://www.plantgenomics.iastate.edu/) at ISU. Population structure (Q) was estimated from SNP data using *Structure 2.0* software program (Pritchard et al. 2000). The Q matrix estimating membership coefficients for each individual in each subpopulation was produced with burn-in length of 50.000 followed by 50.000 iterations for each of clusters (K) varying from 1 to 20; each K was run 20 times. The admixture model was applied with independent allele frequencies. To identify the most probable value of K, an ad hoc (ΔK) statistic (Evano et al. 2005) based on the second order rate change of P (X|K) was used, the posterior probability of the data with respect to a given K.

TASSEL 2.10 software (Bradbury et al. 2007) was used to asses LD at the candidate gene loci and to evaluate associations between individual polymorphisms and mean phenotypic values using a Mixed Linear Model (MLM) (Yu 2006). The MLM accounts for overall population structure (Q), but also for finer scale relative kinship (K). Loiselle kinship coefficients (Loiselleet al. 1995) between lines (a K matrix) were estimated using the same 101 SNP markers in the *Tassel 2.10* program. Population structure (Q) and kinship coefficients (K) were included as covariates in the MLM analysis. SNPs with a frequency of less than 5% were excluded from analyses. To control the multiple testing of the SNP markers, false discovery rate was set at 0.05 (Storey et al. 2004). Motifs in the *RTCL*, *RTH3*, *RUM1*, and *RUL1* genes were searched using a PLACE (Plant cis-acting regulatory DNA elements) database (Higo et al. 1999) to identify which of the significantly associated SNPs might alter motif sequences in the candidate genes.

Results

Phenotypic data

In this study, 74 maize lines were evaluated for seedling and adult plant traits under contrasting N levels. Analysis of variance (ANOVA), broad sense heritability estimates, phenotypic correlation among various pairs of seedling and adult plant traits measured under contrasting N levels have been reported elsewhere (Abdel-Ghani et al. 2013). ANOVA results revealed significance of lines and N levels on seedling and adult plant traits. On average, lines under low N exhibited enhanced root growth compared to shoot growth and *vice versa*. SDW was significantly higher under HN (mean = 110.02 mg seedling^{-1}) compared to LN (mean = 86.71 mg seedling^{-1}) conditions. RDW and TRL were significantly higher under LN as compared to the HN treatment. Under LN treatment, average RDW and TRL were 51.74 mg and 193.3 cm seedling^{-1}, respectively, whereas under HN treatment, average RDW and TRL were 47.4mg and 174.6cm seedling^{-1}, respectively. Adult plant traits measured in the field were significantly (*P=0.01*) affected by lines and N levels, and their interaction effect. Inbred lines responded to N stress by reduced chlorophyll content and plant height, and increased ASI. Average grain yield was reduced under LN 3.2 and 2.2 times compared to HN at Ames and Marion, respectively.

Path coefficient analysis

Path coefficients were partitioned into direct and indirect effects (Table 2), with RDW and SDW as dependent variables for TRL components (PRL, CRL, SRL, and LRL), and TSB as dependent variable for RDW and SDW seedling traits. Path coefficient analyses revealed that all TRL related components have a positive direct effect on

root and shoot DW under low and high N. SRL was the major contributor to RDW under HN and LN (contributions= 36.3 and 47.7%, respectively). The remaining direct contributions from PRL, CRL, and LRL on RDW ranged from 14.8 to 23.4% and 11.7 to 27.3% under HN and LN, respectively. Under HN, all TRL components indirectly and positively contributed to TSB with percentages ranging from 19.5% for PRL to 31.0% for SRL, while under LN the major indirect contribution to TSB came from SRL (44.8%) and LRL (33.3%), followed by CRL (12.2%) and PRL (9.9%). The direct contribution of RDW to TSB was higher under LN (38.9%), than under HN treatment, while SDW contributed less to the TSB under LN (61.1%) than under HN (69.2%).

Population structure, linkage disequilibrium, and haplotype diversity

The ad hoc statistic (ΔK) calculated for each K was highest at K=2, with a sharp decrease between K=2 to 3, and much lower between K=3 to 20. Based on the ad hoc statistic values, lines in the AS panel were grouped into two sub-populations (K=2), which agrees with the pedigree and breeding history of the lines used in this study. Out of 74 AS lines, the majority of them (57 lines) belonged to group 1 (non Stiff Stalk Synthetic, OH07-Midland, and Iodent lines).12 lines belonged to group 2 (Stiff Stalk Synthetic lines) (Fig. 1). The other five lines were found mixed with lines from two groups, as they had membership values <60% to any of the above two groups.

LD was estimated between all pairs of polymorphic sites in the sequenced region of the four candidate genes. LD levels decayed very rapidly within the sequenced regions of the *RTCL, RUM1,* and *RUL1* amplified gene products (Fig. 2).The extent of LD across all these three candidate genes was 191, 140, and 194 bp for *RTCL*, *RUM1*, and *RUL1* genes using $r^2 = 0.2$ as a critical threshold estimated from a logarithmic equation. In case of *RTH3*, LD persisted ($r^2 > 0.25$) over the length of the sequenced region considering all 14 polymorphisms within the amplified fragment.

Analysis of haplotype diversity for the 74 maize inbred lines revealed 16, 9, 22, and 7 haplotypes for the *RTCL*, *RTH3*, *RUM1,* and *RUL1* genes, respectively (Table 3). Maize lines containing these haplotypes showed wide range of phenotypic values for TRL, RDW, TSB and grain yield measured under HN and LN condition. The range of haplotype means for all of these traits was larger for RUM1 gene compared to other three genes (Table 3).

Association of root and agronomic traits with *RTCL*

Resequencing of 828 bp of the *RTCL* gene from 69 AS lines resulted in the identification of 45 SNPs. 19 out of 45 polymorphisms in the *RTCL* gene were significantly associated (P=0.05) with at least one of the seedling root traits under HN and LN treatment by MLM analysis. 13 SNPs were significantly associated (P=0.05) with at least one of

the adult plant traits under HN and LN treatment. Those SNPs were distributed as follows: thirteen in exon1, five in the intron and thirteen in exon 2 (Table 4). Ten SNPs in exon 1 and seven SNPs in exon 2 caused non-synonymous changes in the protein sequence, while the remaining SNPs in the two exons caused synonymous changes. 17 non-synonymous SNPs in exon 1 and 2 associated with seedling and adult plant traits lead to amino acid changes (Table 4). Overall, *RTCL* is significantly associated with seedling traits such as RDW, TRL, and TSB under HN and LN. Under field conditions, *RTCL* was found to be associated with grain yield under HN (Marion location) and LN (Ames location). SNPs at the sites 290, 296, 298, 373 were found to be significantly associated with seedling root traits RDW, TRL, and TSB under HN. SNPs at the sites 413, 473, 531 and 547 were significantly associated with TRL and RDW measured under HN condition. Moreover, SNP at 531 site was also associated with RDW and TRL under LN condition. Under LN, a SNP at the site 736 was associated with all three seedling root traits. SNPs at the sites 317, 320, and 324 were associated with RDW under both HN and LN. SNP at the site 554 was associated with yield at Marion under HN. In case of LN, 11 SNPs in *RTCL* were associated with yield measured in Ames. None of these SNPs were associated with seedling root traits. Using B73 as reference sequence, nine SNP's (204, 290, 317, 320, 332, 597, 708, 711, and 759) were significantly associated either with seedling or adult plant traits affected motifs in the *RTCL* gene. These motifs represent the binding factors of several regulating elements (Supp. Table 3). Moreover, non-synonymous SNPs at 290, 317, and 320 also affected the LOB domain amino acids in the *RTCL* gene (data not shown).

Association of root and agronomic traits with *RTH3*

In the *RTH3* gene, 15 SNPs were detected. By MLM analysis, 2 out of 15 polymorphisms in the *RTH3* gene were significantly associated (P=0.05) with at least one of the seedling and 6 polymorphisms with adult plant traits. All SNPs that showed significant associations were in the exon region of the gene, and they caused synonymous changes in the protein sequence (Table 5). Overall, *RTH3* was significantly associated with the grain yield under HN (Marion location). In case of seedling traits, *RTH3* was found to be associated with RDW under HN condition and TSB under both HN and LN. SNPs at sites 180, 234, 465, 492, 519, and 600 were significantly associated with grain yield measured under HN (Marion). A SNP at site 621 was significantly associated with TSB both under HN and LN. SNPs at 600 and 621 affected the motifs representing regulating factors in the *RTH3* (Supp. Table 3). Since these SNPs were synonymous in nature, they did not affect the COBRA domain in the *RTH3* gene.

Association of root and agronomic traits with *RUM1*

Resequencing of 432 bp of *RUM1* from 74 AS lines resulted in the identification of 12 SNPs. Four out of 12 polymorphisms in *RUM1* were significantly associated (P=0.05) with seedling root traits RDW and TSB (Table 6). A non-synonymous polymorphism at site 63 (Val→Ala) in exon 5 was significantly associated with RDW and TSB under LN. A polymorphism in intron 4 (site 251) was associated with RDW both under HN and LN.

Association of root and agronomic traits with *RUL1*

Resequencing of 411bp of *RUL1* from 74 AS lines resulted in the identification of 6 SNPs. Three out of six SNPs in *RUL1* were associated with TRL under HN (Table 7). Out of these three SNPs, SNP at site 311 was a synonymous mutation, and the other two SNPs (sites 336 and 389) were non-synonymous mutations (Thr→Ile and Ser→Gly, respectively).

Discussion

The historic 2012 drought in the US (http://droughtmonitor.unl.edu/) has re-enforced the importance of water and nutrient management in agriculture. With respect to plant nutrients, N is the major input cost to farmers. A better management of fertilizer application through split application of fertilizers (application during planting and flowering stage), and planting hybrids that are nitrogen use efficient might be helpful in reducing input costs. Genetic selection of nitrogen use efficient maize is facilitated by a better understanding of molecular and physiological processes controlling maize productivity under LN. In this study, we studied maize seedlings and adult plants under contrasting N levels to explore the genetic differences among inbred lines, and tested for associations of polymorphisms in genes affecting root development with both root and yield traits.

Decay of linkage disequilibrium within genes affecting root development

The extent of LD in maize lines across three candidate genes (*RTCL*, *RUM1*, and *RUL1*) was less than 200 bp using $r^2 = 0.20$ as a critical threshold estimated from separate analyses of sequences ranging in length between 441 and 831 bp. In case of *RTH3*, LD persisted along the partially sequenced part of the gene (714 bp). This rapid decay of LD in *RTCL*, *RUM1*, and *RUL1* is in agreement with the low LD commonly seen in cross-pollinated plants such as maize since it has a higher effective recombination rate because of its out crossing mating system which leads to a rapid decay of LD. Extended LD at the gene loci might arise due to the several processes of population genetics, including selection, population size and population bottlenecks (Flint-Garcia et al. 2003). Extended LD might also indicate relatedness between individuals maize lines, but in our study maize lines originated from different genetic

background. Also, LD can be a signature of a selection sweep, i.e. a local reduction of nucleotide variation, caused by the rapid fixation of beneficial mutation (Kim and Nielsen 2004).*RTH3* might be under strong indirect selection, as direct selection to increase yield and plant biomass may cause indirect selection pressure on root hairs, which are critical for water and nutrient uptake. In replicated yield trials, it was found that the *rth3* mutation causes a 35% reduction in grain yield (Hochholdinger et al. 2008). Similar effects of indirect selection have been reported in other studies of maize (Bänziger and Lafitte 1997; Bolaños and Edmeades 1996).Given the large length of sequence amplified in *RTH3* gene, nucleotide diversity was low compared to other three genes studied in this work (Kumar et al. 2013, – in preparation). Thus, the distinct LD pattern at the *RTH3* loci reflects genetic drift or selection sweeps within the AS panel lines. Moreover, LD decay can also vary substantially from gene to gene within the same population as reported by many authors (Remington et al. 2001; Palaisa et al. 2003 and 2004), which was also observed in this study.

Path coefficient analysis

Path coefficient analysis showed positive influences of different root traits on RDW and SDW under both N levels. Under HN, CRL, SRL, and LRL together had a direct effect of 85.1% on RDW (Table 2). The strong effect of these traits is due to the total length of these roots compared to PRL. Under LN, the direct effect of SRL and LRL increased, while that of CRL decreased due to its reduced length (Table 2). This suggests that for increasing RDW under HN, selection needs to be based on CRL, SRL, and LRL values, whereas under LN, selection for SRL and LRL might be sufficient. Similar observations were made in case of TSB, where the indirect effect of SRL and LRL on TSB increased from HN to LN, but decreased in case of CRL. In our study, both RDW and TSB had positive effects on TSB (Table 2). The increase in the direct effect of RDW on TSB from HN to LN was due to the increase in root development compared to shoot development from HN to LN. Under N starvation, an increased R:S ratio might be due to enhanced assimilate translocation from shoot to root (Maizlisch et al. 1980; Chun et al. 2005; Tian et al. 2005; Wang et al. 2003). In these studies, N starvation increased root surface area, consumption of assimilates, and reduced the amount of N transported to shoots. In our study under HN, a significant increase in shoot weight might be due to reduced assimilate availability for roots, since plants do not need to waste energy to expand their root system for N acquisition, which leads to a reduced R:S ratio. Thus, an increased R: S ratio seems to be a general response of maize seedlings to reduced N fertilizer levels.

Evaluation of root genes

Various QTL mapping studies have been conducted for above ground traits in maize (Neuffer et al. 1997). The challenge involved in studying below ground traits such as roots is that they are very variable depending on soil conditions, and the difficulty involved in their extraction from soil without damages. Thus, most of the genetic mapping studies used seedling assays in paper roll to map the root traits in maize (e.g., Wen and Schnable 1994; Hertz et al. 1996; Hochholdinger and Feix 1998; Hochholdinger et al. 2001; Woll et al. 2005). Through map-based cloning, *RTCL* was the first gene to be isolated in maize involved in initiation and maintenance of seminal and crown roots (Taramino et al. 2007). In our gene based association study, *RTCL* was found to be associated with maize root development (Table 4). Apart from seedling traits (TRL, RDW, TSB), *RTCL* was associated with grain yield both under HN and LN, thus it re-confirms the previous results that *RTCL* affects root development in maize. The *RTH3* gene was found to be associated with root hair elongation (Hochholdinger et al. 2008). Moreover, in replicated field trials the *rth3* mutant showed a significant yield decrease. In our association study, more polymorphisms in *RTH3* were significantly associated with grain yield than seedling traits under HN. The previous study in which the effect of the *rth3* mutant on grain yield was detected, was also conducted under HN field conditions (Hochholdinger et al. 2008).

Map-based cloning of *RUM1* revealed that the gene is associated with seminal and lateral root formation (von Behrens et al. 2011). Through our study, *RUM1* was found to be associated with RDW and TSB, both under HN and LN conditions, thus confirming the role of this gene in root development of maize seedlings. Another gene *RUL1* – a paralogue of *RUM1* has been sequenced but its function is still unknown (von Behrens et al. 2011). Through our gene based association study, it was found that *RUL1* affects TRL in maize under HN. However, it was not found to be significantly associated with TRL under LN conditions, possibly due to genotype by environment interactions. Several SNPs from the candidate genes were commonly associated with both RDW and TSB traits. RDW is a component of TSB, and this explains the frequent common QTPs affecting both RDW and TSB traits.

Molecular physiological basis of SNP– trait associations

In the current study, SNPs from the candidate root genes in maize were surveyed for their association with seedling root traits and adult plant traits under contrasting N levels. Of the four candidate genes surveyed in this study, *RTCL* showed the highest number of significant associations with seedling and adult plant traits measured under different N levels. A similar high number of associations were found for *RTCL* and seedling root traits measured in 6-, 10-

and 14-day-old seedlings (Kumar et al. 2013, in preparation). Map-based cloning revealed that *RTCS* encodes an auxin-inducible LOB domain transcription factor that is involved in the early events of root development that leads to the initiation and maintenance of seminal and shoot-borne root primordia (Taramino et al. 2007). Maize mutants with impaired LOB domain genes have reduced seminal and crown roots (Iwakawa et al. 2002; Liu et al. 2005; Inukai et al. 2005). *RTCS* gene is orthologous to rice *CRL1* and *ARL1* involved in root development (Inukai et al. 2005; Liu et al. 2005). *RTCS* and its paralogue *RTCL* share auxin responsive promoter elements, and they are preferentially expressed in roots (Taramino et al. 2007). These characteristics make *RTCL* a potential candidate gene associated with seedling and adult plant traits in maize. Another reason for high number of significant associations is that in our study whole *RTCL* gene was sequenced, which helped to completely evaluate the role of polymorphisms in the gene on seedling and adult plant traits. *RTH3* gene belongs to COBRA – like gene family (Hochholdinger et al. 2008) specifically involved in cell expansion and cell wall biosynthesis (Brady et al. 2007). In our study, even though we did not evaluate the association of *RTH3* gene with root hair formation, we found that this gene affects total seedling biomass and grain yield. This significant association with plant biomass and grain yield might be due to the role of root hairs in water and nutrient uptake (Hochholdinger et al. 2008). *RUM1*is Aux/IAA inducible and encodes a protein that contains four conserved domains, and a bipartite nuclear localization sequence (von Behrens et al. 2011). A deletion of six nucleotides in the first exon and insertion of a truncated Mu element in the second exon led to the suppression of embryonic seminal roots, and post-embryonic lateral roots at the primary root. *RUM1* is auxin-inducible, and encodes a protein that localizes to the nucleus. Phylogenetic analyses revealed that *RUL1* represents a paralogue of *RUM1*; based on colinearity of the genomic regions surrounding these two genes and the observation that the corresponding chromosomal regions resulted from an ancient duplication event (von Behrens et al. 2011).This might be the reason for significant association of polymorphisms of *RUM1* with RDW and TSB in our study. *RUL1* is a paralogue of *RUM1* sharing 92% identify at the amino acid level, and also codes an Aux/IAA protein that is auxin inducible. The functional role of *RUL1* gene is still unknown. Our study suggests that*RUL1*is associated with TRL under HN conditions. Thus, both *RUM1* and *RUL1* are involved in root development at the seedling stage in maize. In our study, we did not find significant associations of *RUM1*and *RUL1*with adult plant traits such as grain yield. This might be due to the partial amplification of both*RUM1* and *RUL1* gene, and thus presence of respective associated polymorphisms in other parts of the gene was not evaluated. Another reason could be that seminal root formation by *RUM*1 becomes obsolete in adult plants. So, its role in affecting grain yield might

be minimal (Hochholdinger 2009). In the present study, QTPs associated with grain yield in *RTCL* and *RTH3* were different from those associated with seedling root traits TRL, RDW, and TSB. Moreover, QTPs associated with grain yield in *RTCL* and *RTH3* were also different from those associated with TRL and RDW measured in 6-, 10- and 14-day-old seedlings (Kumar et al. 2013, in preparation). However, we did identify QTPs that were associated with both seedling root traits (TRL, RDW and TSB) measured under contrasting N levels, and in 6-, 10- and 14-day-old seedlings (TRL and RDW). Absence of QTPs associated with both grain yield and seedling root traits might be due to the complex nature of the quantitative trait grain yield, which is influenced by multiple factors in the field. Also, we didn't notice any tendency of beneficial alleles always coming from either expired plant variety protection lines or public inbred lines, but that both the sub-sets of lines contributed those beneficial alleles.

Targets for Functional marker development

Functional markers are DNA markers derived from polymorphic sites within genes, causally involved in phenotypic trait variation (Anderson and Lübberstedt 2003). One of our future objectives of this study is to develop functional markers for seedling root traits and adult plant traits (grain yield) under contrasting N levels. Developing such markers requires functional characterization of allelic variants for different traits of interest. In our study, polymorphisms in genes encoding proteins affecting root development at seedling stage in maize were used for our association study. Out of the four candidate genes studied, polymorphisms in *RTCL* gene showed a significant association with RDW, TRL, TSB, and grain yield. Within *RTCL*, SNPs at sites 317 and 320 are promising candidates to derive functional markers for RDW selection based on large effect on the trait (Supp. Table 2). These SNPs not only create non-synonymous changes in protein sequence, but alter motif sequences and LOB domain in *RTCL* gene. For TSB and TRL selection, SNPs at sites 317 and 531 are candidates to derive functional markers, respectively. In case of *RTH3*, a synonymous mutation at position621 is a candidate for TSB. This SNP also affects the motif representing regulating elements of *RTH3* gene. For grain yield, a synonymous SNP at site 600 is a candidate, as it also affects the motif sequence in the *RTH3*gene. Apart from these SNPs, selection of beneficial haplotypes associated with higher seedling traits TRL, RDW, TSB, and grain yield would lead to development of maize lines with better root architecture and grain yield under limited N condition.

Conclusions

By MLM analysis, 19, 2, 4, and 3 SNPs from *RTCL*, *RTH3*, *RUM1*, and *RUL1*respectively,were associated with seedling roots traits under HN and LN conditions. Moreover, polymorphisms in *RTCL* and *RTH3*were also

associated with grain yield under contrasting N levels. These polymorphisms could be useful in the development of functional markers for improvement of root characteristics and yield in maize under limited N condition.

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Table
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Table 1 Name of the amplified genes, chromosome localization, primer sequence and expected size of amplified products

Candidate gene	Chromosome	GenBank accession no.	Primer Sequence (5' - 3')	Ta (°C)	Expected Products (bp)	Gene size (bp)
RTCL (*RTCS-like* gene)	9	EF051733.1	F: GCAAGAACACGAAGTGATCG R: TTCCGGCACTACGAGTAAGT	57	975	828
RTH3 (*roothairless 3*)	1	AY265855.1	F: GTACCCGCATACAGACCTCCT R: ACAGCTTAGTCCGGTTCAGGT	57	722	3145
RUM1 (rootless with undetectable meristems 1)	3	EU968452.1	F: ACGGATCACGTCACAGACATAC R: GGCTGGTAGCTGAGCATAAACT	54.3	443	2696
RUL1 (RUM1-like 1)	8	BT036405.1	F: TCTTGACATCAGGAACCATCAG R: AAGACCCACAGACTATCGCATT	54.3	451	2952

Table 2 The direct, indirect, and % contribution of various traits (between brackets) to root dry weight, shoot dry weight and total seedling biomass (TSB) using path coefficient analysis

		High N			Low N				High N	Low N
		RDW	SDW	TSB	RDW	SDW	TSB		TSB	TSB
Primary root length	Direct effect	0.175 (14.8%)	0.221 (21.8%)	-	0.136 (11.7%)	0.072 (8.5%)	-	Indirect effect	0.232 (19.5%)	0.114 (9.9%)
Crown root length		0.300 (25.4%)	0.228 (22.6%)	-	0.155 (13.3%)	0.096 (11.3%)	-		0.280 (23.5%)	0.140 (12.2%)
Seminal root length		0.428 (36.3%)	0.286 (28.3%)	-	0.556 (47.7%)	0.359 (42.2%)	-		0.370 (31.0%)	0.514 (44.8%)
Lateral root length		0.276 (23.4%)	0.276 (27.3%)	-	0.319 (27.3%)	0.324 (38.0%)	-		0.310 (26.0%)	0.380 (33.1%)
Root dry weight (RDW)		-	-	0.345 (30.8%)	-	-	0.459 (38.9%)		-	-
Shoot dry weight (SDW)		-	-	0.776 (69.2%)	-	-	0.721 (61.1%)		-	-

TSB = Total seedling biomass

Table 3 Number of haplotypes based on single nucleotide polymorphisms (SNPs) in the *RTCL*, *RTH3*, *RUM1* and *RUL1* genes in maize, and minimum, maximum and ranges of lines for haplotypes representing each gene.

			RTCL (16 haplotypes)			*RTH3* (9 haplotypes)			*RUM1* (22 haplotypes)			*RUL1* (7 haplotypes)		
			HN	LN	AE	HN	LN	AE	HN	LN	AE	HN	LN	AE
Total root length		Min	199.8	216.9	2.63	182.0	211.9	2.27	198.1	220.8	4.16	179.7	208.6	3.45
		Max	135.0	156.1	-0.62	165.0	183.3	0.69	111.6	144.4	-0.91	135.0	156.3	-0.21
		Range	64.8	60.8	3.25	17.0	28.6	1.59	86.5	76.4	5.07	44.7	52.3	3.66
Root dry weight		Min	59.88	65.72	0.87	50.43	56.63	0.46	61.8	71.45	1.28	50.57	58.52	0.81
		Max	28.54	28.24	-0.39	41.91	46.5	-0.09	28.13	28.73	-0.38	28.13	35.35	-0.35
		Range	31.34	37.48	1.25	8.52	10.13	0.54	33.68	42.72	1.65	22.44	23.17	1.16
Total seedling biomass		Min	207.01	173.88	2.88	164.73	149.73	2.88	198.98	180.36	3.3	168.43	162.03	3.0
		Max	107.43	80.90	0.3	141.32	124.25	0.45	105.32	70.54	-1.46	110.67	109.96	0.05
		Range	99.57	92.98	2.59	23.41	25.48	2.43	93.66	109.82	4.76	57.75	52.07	2.94
Grain Yield	Ames	Min	3.035	1.067	9.1	2.653	1.039	7.94	3.386	1.184	10.36	2.451	1.184	6.31
	2010	Max	1.494	0.173	4.4	2.021	0.522	5.38	0.966	0.172	2.61	1.578	0.367	4.41
		Range	1.538	0.893	4.7	0.632	0.518	2.56	2.42	1.012	7.74	0.872	0.818	1.9
Grain Yield	Marion	Min	2.773	1.261	8.14	2.628	1.136	7.0	2.67	1.371	8.63	2.773	1.105	7.83
	2010	Max	1.452	0.743	2.93	1.714	0.498	4.23	0.704	0.502	0.54	1.398	0.722	1.54
		Range	1.321	0.518	5.21	0.913	0.638	2.77	1.966	0.868	8.09	1.375	0.382	6.29

Table 4 Polymorphic sites of *RTCL* associated with key related traits (root dry weight, root to shoot ratio and total root length) identified by MLM under high nitrogen level (HN) and low nitrogen level (LN).

Site	SNP	Amino acid change	E/I	High N	Low N
204	A→C	Syn.	E1	-	YIELD-A
232	A→C	Met-Leu	E1	-	YIELD-A
290	T→A	Leu-His	E1	RDW;TRL;TSB	-
296	A→G	Asp-Gly	E1	RDW;TRL;TSB	-
298	A→T	Ser-Cys	E1	RDW;TRL;TSB	-
307	T→A	Ser-Thr	E1	-	YIELD-A
317	C→T	Pro-Leu	E1	RDW;TSB	RDW;TSB
320	T→C	Val-Ala	E1	RDW	RDW
324	G/C/T	Syn	E1	RDW	RDW
332	A→C	Asp-Ala	E1	TRL	-
357	G/A/T	Syn	E1	RDW	-
373	G→A	Asp-Asn	E1	RDW;TRL;TSB	-
413	C→A	Thr-Lys	E1	RDW;TRL	-
473	A→G	-	I1	RDW;TRL	-
481	A→G	-	I1	-	YIELD-A
531	G→C	-	I1	RDW;TRL	RDW;TRL
543	G→A	-	I1	RDW;TSB	-
547	T→G	-	I1	RDW;TRL	-
554	C→T	Ala-Val	E2	YIELD-M	-
576	C→T	Syn.	E2	-	YIELD-A
597	G→T	Syn	E2	RDW;TSB	-
632	A→G	Glu-Gly	E2	RDW;TSB	-
648	G/A/T	Syn	E2	-	YIELD-A
694	G→A	Gly-Arg	E2	-	YIELD-A
703	C→G	Arg-Gly	E2	RDW;TSB	-
704	G→A	Arg-Gln	E2	-	YIELD-A
708	A→T	Syn	E2	-	YIELD-A
711	A→T	Syn	E2	-	YIELD-A
736	C→A	His-Asn	E2	RDW	RDW;TRL;TSB
759	C→G	Syn	E2	-	YIELD-A
799	T→G	Trp-Gly	E2	RDW	RDW;TSB

E = Exon, I = Intron, RDW = Root Dry Weight, TRL = Total Root Length, TSB= Total Seedling Biomass, YIELD-M = Grain Yield measured at Marion-IA, YIELD-A = Grain Yield measured at Ames-IA

Table 5 Polymorphic sites of *RTH3* associated with key related traits (root dry weight, root to shoot ratio and total root length) identified by MLM under high nitrogen level (HN) and low nitrogen level (LN).

Site	SNP	Amino acid change	E/I	High N	Low N
180	G→T	Syn	E	YIELD-M	-
234	C→T	Syn	E	YIELD-M	-
417	G→C	Syn	E	TSB	-
465	C→A	Syn	E	YIELD-M	-
492	G→T	Syn	E	YIELD-M	-
519	G→A	Syn	E	YIELD-M	-
600	T→C	Syn	E	YIELD-M	-
621	G→A	Syn	E	RDW;TSB	TSB

E = Exon, RDW = Root Dry Weight, TSB= Total Seedling Biomass, YIELD-M = Grain Yield measured at Marion-IA

Table 6 Polymorphic sites of *RUM1* associated with key related traits (root dry weight, root to shoot ratio and total root length) identified by MLM under high nitrogen level (HN) and low nitrogen level (LN).

Site	SNP	Amino acid change	E/I	High N	Low N
63	C→T	Val-Ala	E5	-	RDW;TSB
251	T→A	-	I4	RDW;TSB	RDW
302	T/G/A	-	I4	-	TSB
381	T→C	-	I4	RDW	

E = Exon, I = Intron, RDW = Root Dry Weight, TSB= Total Seedling Biomass

Table 7 Polymorphic sites of *RUL1* associated with key related traits (root dry weight, root to shoot ratio and total root length) identified by MLM under high nitrogen level (HN) and low nitrogen level (LN).

Site	SNP	Amino acid change	E/I	High N	Low N
311	G→A	Syn	E6	TRL	-
336	C→T	Thr-Ile	E6	TRL	-
389	A→G	Ser-Gly	E6	TRL	-

E = Exon, TRL = Total Root Length

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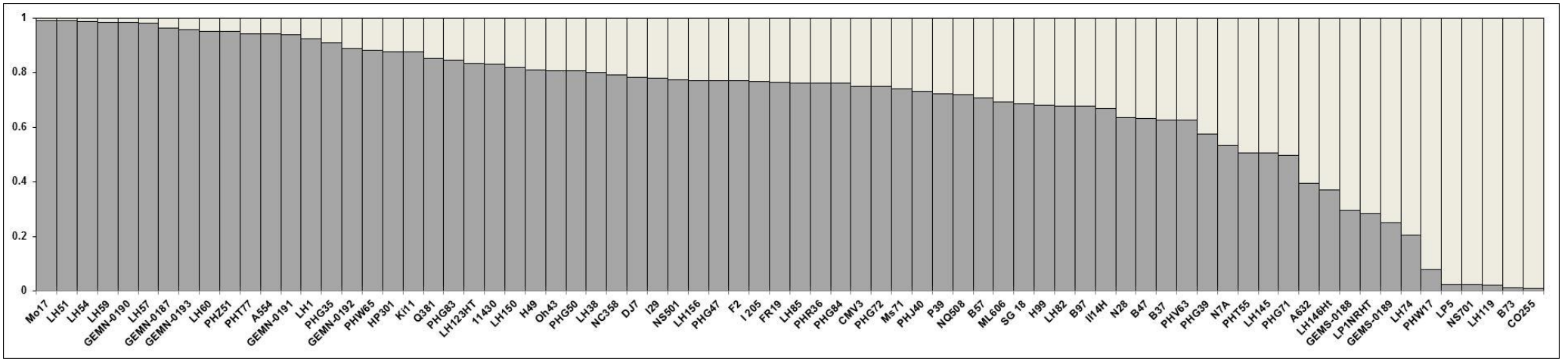

Fig. 1 Population structure estimates based on 101 SNPs evenly distributed across the maize genome. *Vertical bars* represent individual maize lines. The area of 2 different colors (grey and white) illustrates the proportion of either each subpopulation based on these SNPs markers

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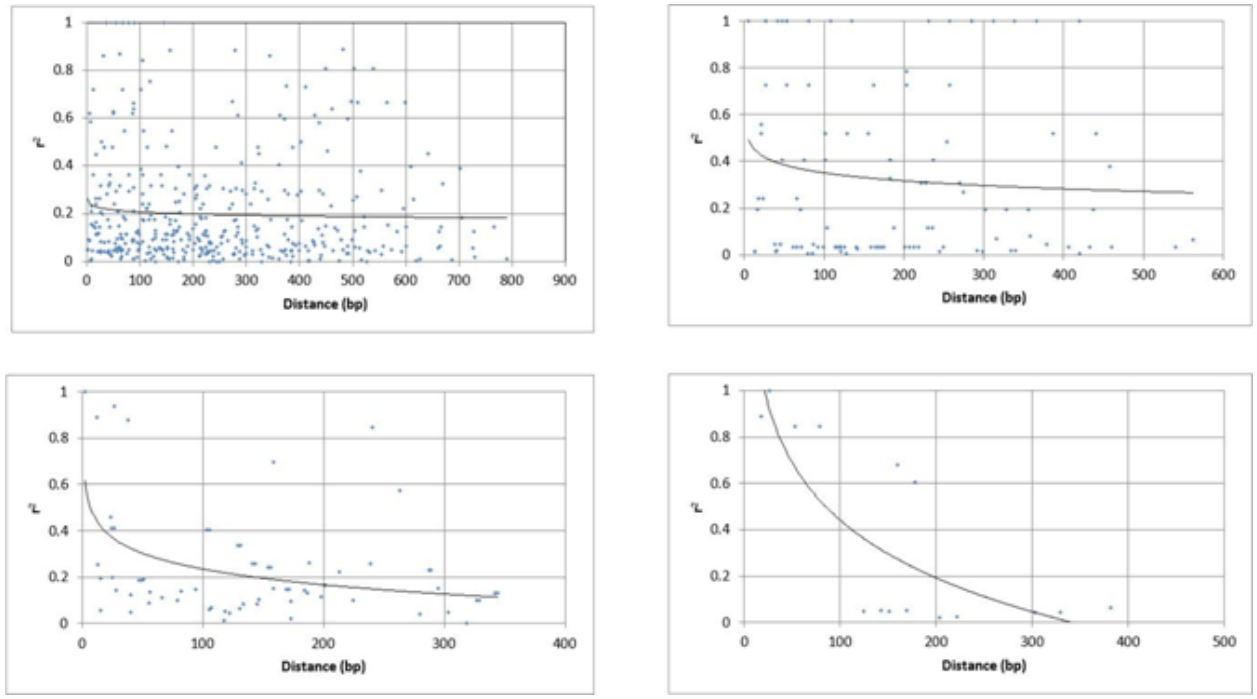

Fig. 2 Plots of squared correlations of allele frequencies (r^2) against weighted distance between polymorphic sites in four root development genes: *RTCL*(top left), (*b*) *RTH3* (top right), (*c*) *RUM1* (bottom right),and (*e*) *RUL1*(bottom right).

Supplementary material

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