

# The Nitrogen Adaptation Strategy of the Wild Teosinte Ancestor of Modern Maize, *Zea mays* subsp. *parviglumis*

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## ABSTRACT

Examination of wild ancestors can identify which traits have been altered by selection as possible targets for genetic improvement. We investigated the whole plant response to low nitrogen (LN), especially below ground, by the wild ancestor of modern maize (*Zea mays* L.), Balsas teosinte (*Zea mays* subsp. *parviglumis* H. H. Iltis & Doebley). Teosinte responded to LN by reducing the shoot N concentration and increasing the root:shoot biomass ratio. The lengths of individual crown roots and the total lateral root length increased, compensated by reduced crown root number. Low N caused a decrease in total root hair (RH) length and increased expression of high affinity nitrate transporters. To facilitate future mapping studies, these results were compared to a modern inbred ("W22") used as the parent in a modern maize × teosinte population and extensively employed in maize domestication studies. The adaptations to LN in teosinte and W22 were surprisingly conserved, but the strategies employed were often different. To reduce total RH length, teosinte reduced RH density whereas W22 reduced average RH length. To achieve reduced shoot biomass in response to LN, teosinte reduced tiller number and hence leaf number whereas W22 reduced average leaf size. Since tiller crown roots initiate from stem tissue, teosinte used tiller plasticity to reduce crown root number whereas modern maize reduced crown root number independently of tillering. We discuss the implications of these results for maize domestication.

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**Abbreviations:** CR, crown root; DAT, days after transplanting; EDTA, ethylenediaminetetraacetic acid; HN, high nitrogen; LN, low nitrogen; LR, lateral root; mRNA, messenger RNA; NUE, nitrogen use efficiency; NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilization efficiency; PCR, polymerase chain reaction; RH, root hair.

**A**RCHAEOLOGICAL, botanical, and molecular genetic evidence show that early agriculturalists in the Balsas River Valley in southwest Mexico began the process of maize (*Zea mays* L.) domestication from teosinte ~9000 yr ago through selection and crop improvement (Hastorf, 2009; Piperno et al., 2009; Ranere et al., 2009; Sluyter and Dominguez, 2006). The closest living wild relative of modern maize, indigenous to Mexico and Central America, is known as Balsas teosinte (*Zea mays* subsp. *parviglumis* H. H. Iltis & Doebley) (Doebley, 1990, 2004; Fukunaga et al., 2005; Matsuoka et al., 2002). Balsas teosinte plants differ dramatically from modern maize by having a larger shoot with multiple branches (tillers) each tipped with an inflorescence producing few seeds rather than a single stalk with one to two large seed-bearing cobs (Doebley, 2004; Doebley et al., 1997).

Although domestication has caused dramatic changes in the shoot size and architecture of maize, its responses to the environment have also been altered. Above ground, shading by leaves of adjacent teosinte plants was shown to decrease tillering in Balsas teosinte whereas modern maize has largely lost this architectural strategy as it typically has few or no tillers (Doebley et al., 1995; Lukens and Doebley, 1999). Similarly, in the Balsas River Valley,

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Wilkes (1977) has observed that tillering in teosinte is reduced in apparent spots of low soil fertility, allowing teosinte to decrease its shoot nutrient requirements while most modern maize genotypes do not have this option. Thus, an indirect consequence of domestication has been loss in the ability of modern maize to respond to competition and low soil fertility using shoot tiller plasticity.

The native habitat of Balsas teosinte, the Balsas River Valley, is mountainous, has well-drained soils, and >80% of the rain falls intensively between June and October. As a result, annual flushing of mobile soil nutrients, especially N, may occur combined with seasonal fertilization from runoff and organic matter decomposition (Hastorf, 2009) resulting in a nutrient-variable environment. In addition, in the Balsas River Valley, teosinte competes with tropical deciduous trees, other grasses, and annual dicots [e.g., *Bidens* spp., *Coreopsis* spp., and *Tithonia diversifolia* (Hemsl.) A. Gray] for soil nutrients (Fukunaga et al., 2005; Iltis et al., 1979; Piperno et al., 2007; Ruiz Corral et al., 2008; Wilkes, 1977). In contrast, modern maize is cultivated with low interspecies competition and has been grown and selected with added fertilizers applied at regular intervals.

Given the changes associated with maize domestication, including changes in competition, habitat, and cultivation practices, one unexplored hypothesis is that domestication may have not only altered maize shoot architecture but may have also changed the morphology and physiology of the root system. The architecture of the teosinte root system has not been reported, but the root system of modern maize is large, complex, and plastic. At the seedling stage, the embryonic root system of modern maize consists of a single primary root and a variable number of branched seminal roots (Fig. 1A). Subsequently in development, thick crown roots (CRs) initiate from the shoot below ground and form the backbone of the adult root system (Hochholdinger et al., 2004) (Fig. 1B). Additional brace roots also initiate from the shoot but above ground to ensure anchorage of the stem (Fig. 1C). Lateral roots, which initiate from the CRs, form an expansive underground branch network including secondary, tertiary, and higher orders of branching (Fig. 1C). Finally, the crown and lateral roots (LRs) initiate root hairs (RHs) that interact with soil to take up water and nutrients (Fig. 1C).

The root system of modern maize visibly responds to nutrient stress—of particular interest is N—which limits maize yields worldwide (Sinclair, 1998). Low N (LN) has been reported to alter modern maize root growth and architecture (Chun et al., 2005; Feil et al., 1990; Liu et al., 2008, 2009; Maizlish et al., 1980; Schortemeyer et al., 1993; Vamerali et al., 2003; Wang et al., 2005). In general, these experiments show that LN treatment increases the total length of LRs while limiting the numbers of seminal and CRs (axial roots). Morphologically, modern maize also responds to LN by decreasing shoot growth relative to

root growth, to reduce nutrient demand while maintaining nutrient uptake; this results in an increased root:shoot biomass ratio (Ding et al., 2005; Echarte et al., 2008; McCullough et al., 1994; Rajcan and Tollenaar, 1999).

Physiologically, the roots of modern maize acclimate to changes in external N by altering the expression of N uptake transporters (Quaggiotti et al., 2003; Trevisan et al., 2008). The most important N source for maize is nitrate, but nitrate concentrations in soils can vary spatially and temporally from a few hundred micromoles to 20 mmol (Dechorgnat et al., 2011). Plants have evolved different transporter systems to cope with this wide ecosystem variation (Crawford and Glass, 1998). At high external nitrate, there is a low-affinity transport system that is thought to be constitutively expressed. At low external nitrate, there is a high-affinity transport system, members of which can be constitutively expressed or inducible by LN (Crawford and Glass, 1998). Within each gene family, a plant may have multiple paralogs that perform similar functions but are expressed in different tissues, enabling N uptake from the soil, xylem loading from the root, and transport to shoot organs including unloading in leaves (Dechorgnat et al., 2011). The genome of modern maize (inbred B73) appears to encode two low affinity nitrate transporters (*ZmNrt1.1* and *ZmNrt1.2*) and three high affinity nitrate transporters (*ZmNrt2.1*, *ZmNrt2.2*, and *ZmNrt2.3*) (Quaggiotti et al., 2003, 2004; Trevisan et al., 2008) (Maize Genome Project, 2010). Some NRT2 transporters need to interact with a second protein encoded by the *Nar2/Nrt3* gene family to be functionally active (Okamoto et al., 2006; Orsel et al., 2006). Modern maize (inbred B73) appears to encode two NAR2 proteins (*ZmNar2.1* and *ZmNar2.2*) (Maize Genome Project, 2010).

Although several N responses in modern maize have been well characterized, the responses by Balsas teosinte have not been systematically characterized above ground and no phenotyping has been undertaken below ground. Furthermore, the N transporter genes of teosinte have not been reported or characterized. Strategies that maintain fitness in Balsas teosinte under N stress may provide novel traits and genetic targets to improve N acquisition in modern maize. For example, although some modern maize genotypes can form aerenchyma to facilitate oxygen transport to roots after the plants are flooded, the teosinte genotypes *Zea nicaraguensis* (H.H. Iltis & B.F. Benz) and *Z. luxurians* (Durieu & Asch., R.M. Bird) form aerenchyma even at the seedling stage under nonflooded conditions, identifying a hypermorphic trait for possible introgression into modern maize (Mano et al., 2007). Examination of N stress responses in Balsas teosinte may also provide insights into how plant architecture and physiology coevolved during domestication (Hancock, 2005; Ross-Ibarra et al., 2007).

Here we investigated both the shoot and root morphological and physiological responses of Balsas teosinte to LN and high nitrogen (HN), with the major source of N being in

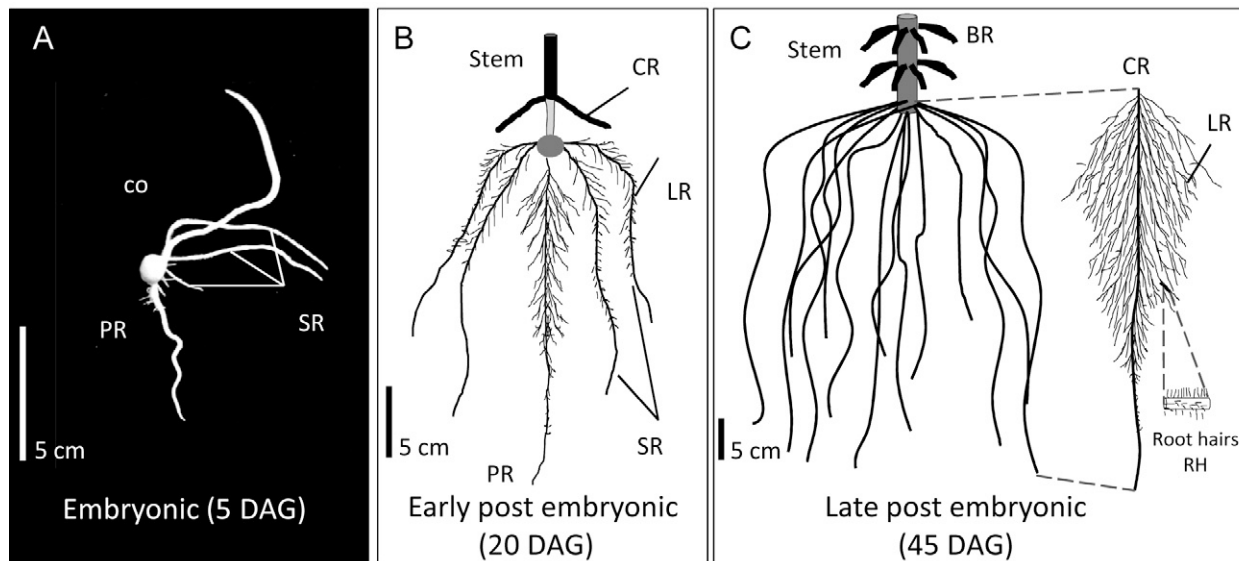


Figure 1. Schematic diagrams of root system development in modern maize at the (A) embryonic stage and (B and C) postembryonic stages at successive days after germination (DAG). (A) The primary root (PR) and seminal roots (SR) initiate from the embryo. (B) Lateral roots (LRs) initiate from SR and PR while crown roots (CRs) initiate from the stem. (C) The mature root system comprises multiple CRs and their LR, which undergo branching. Structural brace roots (BRs) and root hairs (RHs) are also shown. CO, coleoptile.

the form of nitrate. Morphologically we focused on dynamic changes in root architecture in response to LN. Physiologically, as Balsas teosinte was found to have high nitrogen uptake per unit root length under LN, we attempted to amplify and monitor the expression of genes encoding all three nitrate transporter families (*NRT1*, *NRT2*, and *NAR2*) in teosinte to elucidate if domestication affected plant responses to LN by altering the regulation of these transporters. These responses were compared to modern maize inbred ‘W22’. This specific inbred was chosen to facilitate future genetic studies as a W22 × Balsas teosinte mapping population was previously generated by Doebley and colleagues (Briggs et al., 2007; Doebley et al., 1995; Lukens and Doebley, 1999). Furthermore, W22 shoots have already been extensively characterized in maize domestication studies (Briggs et al., 2007; Doebley et al., 1995; Lukens and Doebley, 1999). For root morphology studies, we employed aeroponics, a growth system in which roots are suspended in the air and misted with a nutrient solution. In maize, aeroponics has been used for physiological studies on nitrification (Padgett and Leonard, 1993), to examine the root elongation zone (Freundl et al., 2000; Pellerin and Tabourel, 1995), and for genotype screening for disease resistance (du Toit et al., 1997). Aeroponics allows maize plants to be grown to maturity and hence permits examinations of connections between tillering and root system architecture, root systems at late growth stages, and measurements of fine roots and RHs in a uniform rhizosphere environment with minimal experimental noise (Gaudin et al., 2011). Further, responses by maize roots to nutrient stress in aeroponics have been shown to be similar to substrate-grown maize (Gaudin et al., 2011). Using aeroponics, we show that the responses to LN by Balsas teosinte are surprisingly similar to W22 but the physiological and

morphological mechanisms used to achieve these responses are often different.

## MATERIALS AND METHODS

### Plant Materials

*Zea mays* subsp. *parviglumis* Balsas (Balsas teosinte) seeds (ID 9477) (Doebley, 1990; Matsuoka et al., 2002) were obtained from CIMMYT from an open pollinated population. Maize inbred line W22 (Briggs et al., 2007; Doebley et al., 1995; Lukens and Doebley, 1999) was obtained from the Maize Genetic Stock Center (accession NSL 30053, lot 04ncai02; USDA, North Central Station, Ames, IA).

### Plant Growth System

Maize plants were grown in a custom-made aeroponics growth system where plant roots were suspended and misted in the air with a nutrient solution in a closed loop. Using a nylon net (0.6 by 0.3 cm, Plant Products, Brampton, ON, Canada) to suspend seeds, pairs of plants were suspended onto 133-L black barrels containing internal microjets that were connected to a nutrient solution tank; the solution was replaced weekly. Four independent but identical aeroponics systems were constructed side by side. For each system, a 100-L nutrient solution fed 12 plants distributed among six barrels. Nutrient delivery was optimized for each genotype according to the root size and in some cases developmental stage. Uniform misting among barrels was achieved by matching the number of barrels with the pressure of the submersible pump (e.g., 6 barrels were chosen instead of more). Two microjets, one flanking each of the two root systems, were used in each barrel allowing uniform misting of the complete root system and percolation on the full root length, even the most inner roots. Spray uniformity was maximized by placing the microjets at the height equivalent to the bottom of

the seed net, resulting in spraying roots from above. Sprinklers with a 5 to 20  $\mu\text{m}$  droplet size and 180 degree spraying pattern also allowed uniformity. The flow rate at each microjet was measured to be  $16.5 \pm 0.8 \text{ mL s}^{-1}$ . To ensure that each root system was constantly moistened and to meet the plant transpiration demand at 30 d after planting, the frequency of misting was optimized to 10 s of misting per min. Finally, the pH and temperature of the nutrient solution were checked daily and the solutions were kept at  $\pm 2^\circ\text{C}$  from room temperature. To avoid temperature rise, the solution tank was covered with a white plastic garbage bag during the summer. Using these optimized conditions, plants exhibited no signs of water stress or accumulation of salts on the root surface and had low plant-to-plant variability. In addition, aeroponics permitted nondestructive sampling of the large postembryonic root system of maize.

## Growth Conditions

Seeds were surface sterilized using 20% bleach with 0.05% Tween 20 for 5 min and washed twice for 10 min each with water. Teosinte fruit cases were cut closest to the radicle with a nail clipper to improve the homogeneity of germination. Seedlings were germinated in the dark with distilled  $\text{H}_2\text{O}$ -soaked filter paper with 1 mL of Maxim XL fungicide (Syngenta Crop Protection, Greensboro, NC). Uniformly germinated seedlings were transferred to the aeroponics growth system in a glass greenhouse under a mixture of high pressure sodium and metal halide lamps ( $800 \mu\text{mol m}^{-2} \text{ s}^{-1}$  at pot level), 16-h photoperiod, and 28/20°C day/night regime during January through March 2009. Six plants per genotype were grown under either HN (20 mmol) or LN (8 mmol, see below) for 35 d (12 leaf tips on average for W22) with two replicates arranged in a randomized block design. The experiment was repeated two times ( $n = 24$ ).

The HN and LN nutrient solutions both contained 1 mmol  $\text{MgSO}_4$ , 0.1 mmol  $\text{K}_2\text{SO}_4$ , 1 mmol  $\text{KCl}$ , 2 mmol  $\text{KH}_2\text{PO}_4$ , 0.04 mmol  $\text{H}_3\text{BO}_3$ , 0.02 mmol  $\text{MnSO}_4$ , 0.7  $\mu\text{mol}$   $\text{ZnSO}_4$ , 0.3  $\mu\text{mol}$   $\text{CuSO}_4$ , 0.5  $\mu\text{mol}$   $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24}$ , and 1 mmol ferric diethylenetriaminepentaacetic acid (Fe-DTPA). Seven days after planting (3-leaf-tip stage for W22), 3 g ethylenediaminetetraacetic acid (EDTA)-chelated micronutrient mix (Plant-Prod #7906B7B, Plant Products, Brampton, ON, Canada) was added per 100 L of the above solution for a final concentration of 2.1  $\text{mg L}^{-1}$  Fe (5% EDTA chelated and 2% diethylenetriaminepentaacetic acid [DTPA] chelated), 0.6  $\text{mg L}^{-1}$  Mn, 0.12  $\text{mg L}^{-1}$  Zn, 0.03  $\text{mg L}^{-1}$  Cu, 0.39  $\text{mg L}^{-1}$  B, and 0.018  $\text{mg L}^{-1}$  Mo. The HN treatment contained 6 mmol  $\text{Ca}(\text{NO}_3)_2$  and 4 mmol  $\text{NH}_4\text{NO}_3$  while the LN treatment had 2 mmol  $\text{Ca}(\text{NO}_3)_2$  and 2 mmol  $(\text{NH}_4)_2\text{NO}_3$ . Calcium ions were balanced using 5.5 mmol  $\text{CaCl}_2$ . The LN treatment was chosen according to previous experiments that showed a 30% biomass reduction compared to HN in other maize genotypes in our aeroponics system (data not shown). The pH of the solutions was maintained on a daily basis in the 5.7 to 6.3 range.

## Plant Measurements

Given the dramatic differences in leaf number between Balsas teosinte and W22, all comparisons were normalized by age rather than phenological stage. At 35 d after transplanting (DAT), shoots were analyzed for biomass partitioning between stems and leaves after 48 h of drying at 82°C. The number of emerged leaf tips and tillers were counted. All leaf blades were

harvested and the total green leaf area was measured with a LI-3000 leaf area meter (LI-COR, Lincoln, NE).

Root systems were harvested at 35 DAT, weighed, and flat-stored in trays containing 50% ethanol at  $-20^\circ\text{C}$ . Twelve hours before root scanning, roots were thawed and floated in water in 30 by 42 cm transparent plastic trays, and then they scanned using a Large Area scanner (LA2400, Hewlett-Packard, Palo Alto, CA). Root traits were quantified using WinRhizo software root diameter analysis (Version PRO2009; Arsenault et al., 1995). Scans were analyzed for total root length per plant and the image analyzer was set up to measure length per diameter class allowing analysis of LRs (LR < 0.2 mm) and CRs (CR > 0.5 mm) separately. Brace roots were excluded. The CR number was scored by counting their initiation in the crown region. The root mass was measured following drying (82°C for 48 h).

At 15 DAT, one newly initiated CR from each plant was labeled for RH measurements. At 35 DAT, a 5-cm long CR segment beginning 15 cm distal to the elongation zone was removed and stored in deionized water at 4°C until processing. For each CR segment, RH were measured from four first order LRs. Trypan blue was used to stain LRs by adding a 0.1% Trypan blue solution to roots for 2 min followed by washing with distilled  $\text{H}_2\text{O}$  for 1 min. Root hair density was measured by counting RH on the full semicircular plane of a 2-mm LR segment under a light microscope (100x, Zeiss, Heidelberg, Germany). This measurement was then multiplied by two for an estimate of the total RH number per LR segment and further extrapolated to estimate the total RH length per root system. Root hair lengths were measured using a light microscope (MZ8, Leica, Wetzlar, Germany) with a 1 mm stage micrometer with 100 divisions (0.1  $\mu\text{m}$  per division); four images per LR were taken using Northern Eclipse software (v5.0; Empix Imaging, 2004). Images were exported to ImageJ software (V1.40 g; Abramoff et al., 2004). The scale in the Analyze function was set to 37 pixels per 100  $\mu\text{m}$  based on the micrometer. Total RH length per 100  $\mu\text{m}$  of LR was quantified by digitally tracing individual RH in ImageJ; only protruding RH in side profile were traced. The RH measurements are robust as RH were traced from a total of 960 digital images per N treatment per genotype. Digital tracing of ~30 RH per image was used to quantify average lengths and thus a total of ~60,000 RH were quantified.

Total leaf N content was measured using the Dumas combustion method (Dumas, 1831). The ammonium and nitrate inorganic fractions were measured using the standard spectrophotometric methods (650–660 nm) from the USEPA (1983, 1993). All three measurements were performed on the apical half of the last fully expanded leaf on the main stem. Eight pools of three plants each were quantified. Nitrogen use efficiencies (NUEs) in W22 and Balsas teosinte were estimated as follows: shoot N utilization efficiency (NUtE) = shoot dry weight/shoot total N content (where shoot total N content was estimated as N content per gram of leaf dry weight  $\times$  total shoot dry biomass), shoot N uptake efficiency (NUpE) = shoot total N content/N supply (20 mmol or 8 mmol of total N), and NUE = NUtE  $\times$  NUpE.

## Nitrogen Transporter Expression

The expression of major nitrate transporter genes was examined by first isolating RNA from LRs and associated RHs in a zone 15 to 20 cm away from the CR tip at 35 DAT. Polymerase chain reaction

(PCR) efficiencies were determined by a series of 10-fold complementary DNA (cDNA) dilutions. Polymerase chain reaction primers corresponding to all seven nitrate transporter genes in modern maize (*ZmNrt1.1*, *ZmNrt1.2*, *ZmNrt2.1*, *ZmNrt2.2*, *ZmNrt2.3*, *ZmNar2.1*, and *ZmNar2.2*) were designed using primers from the literature (Quaggiotti et al., 2003, 2004; Trevisan et al., 2008) and from the maize genome database (Maize Genome Project, 2010). Initial attempts were made to amplify transporter orthologs from Balsas teosinte root messenger RNA (mRNA) in the absence of relevant teosinte DNA sequence. Sequencing of amplicons showed that only *Nrt1.1*, *Nrt1.2*, *Nrt1.3*, and *Nar2.1* orthologs were successfully amplified from teosinte roots. Subsequent root expression analysis was limited to these four genes using the following highly purified salt-free primers: *Nrt1.1* (gi|37778585): forward 5'-CTGTCTGGCACCGTGATTGT-3', reverse 5'-CGTAGCTGACTGCCACCTAA-3'; *Nrt1.2* (gi|63397127): forward 5'-TGTTCTCGGCGTGGTGAA-3', reverse 5'-CCTCTGTACCTGACGGAGCAA-3'; *Nrt2.3* (gi|63397156): forward 5'-CTTCTTACCACGTCAGCTACT-3', reverse 5'-GCCATGATGCCATGTTCTC-3'; *Nar2.1* (gi|63397072): forward 5'-GCGGGTGGCGCAAGT-3', reverse 5'-TTGAACTGGCAGCCTTGT-3'; and *Tubulin* (gi|195610153): forward 5'-GAGTGCATTTTCGATCCACATCG-3', reverse 5'-GTTGTTGGCTGCATCCTCCTTC-3'. Amplification conditions were as follows: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing (53°C for *Nar2.1* and 60°C for *Nrt2.3*, *Nrt1.1*, *Nrt1.2*, and *Tubulin*) for 30 sec, and extension at 72°C for 1 min. As we were concerned about possible DNA target polymorphisms in teosinte versus W22 creating artifacts, StepOne software (v2.2.2; Applied Biosystems, 2010) was used to measure the efficiency of primer annealing and amplification for each primer set in both genotypes using the  $\Delta\Delta C_t$  method (Pfaffl et al., 2002) and these efficiencies were taken into account in all data shown. The relative expression ratio of the target genes was calculated based on real-time PCR efficiency and transporter expression was normalized to *Zea mays*  $\alpha$ -tubulin-3 (Genbank-EU954789.1) as previously described (Liu et al., 2009). These results were verified independently using REST (relative expression software tool) (Pfaffl et al., 2002).

## Statistical Analysis

Statistical analyses were performed using the MIXED procedure of the SAS statistical software package (Version 9.1; SAS Institute; 2010) with replications and repetitions as random effects and N treatment and genotypes as fixed effects. Residuals were tested for normality using the Shapiro Wilk normality test; Lund's test was used to identify and remove outliers. Unbalanced two-way ANOVA and partition were calculated using *F*-test, and Tukey's test was used for multiple pairwise comparisons with a type I error of 0.05.

## RESULTS

### Root:Shoot Biomass Ratio

Under nonlimiting HN, the root:shoot biomass ratio was not significantly different between teosinte and W22 (Table 1) while under LN the root:shoot ratio increased to a similar extent in Balsas teosinte and W22 resulting from

decreased shoot biomass but increased root biomass (Table 1). In response to LN, the shoot biomass of Balsas teosinte decreased by 43%, which was similar to the 49% decline in W22. However, the basis of this decline was different: in Balsas teosinte, the tiller number and leaf number declined dramatically (−65% and −40% respectively) whereas the average leaf dry weight remained unchanged. In contrast, in W22, the tiller number was always zero and the leaf number remained unchanged but the average leaf dry weight decreased (−40%). Similarly, LN caused both Balsas teosinte and W22 stems to have decreased biomass (39 and 46%, respectively), but in W22 this was not due to reduced tiller stem number (Table 1).

### Nitrogen Responses

Balsas teosinte and W22 showed a similar decrease in leaf total N concentration (65 and 70%, respectively) in response to LN (Fig. 2A). In terms of the inorganic N storage pool, total leaf nitrate and ammonium similarly declined in both teosinte and the modern inbred (83 and 78%, respectively) (Fig. 2B).

Despite no difference in leaf N concentration between the two genotypes (Fig. 2A), the estimated total shoot N content was approximately twofold higher in Balsas teosinte compared to W22 due to the greater shoot biomass of teosinte (Fig. 2C and D). Given this difference in N demand, these genotypes were investigated for possible differences in N uptake or utilization, the two components of NUE. As shoot NUE is defined as shoot biomass per unit of N supplied (Hirel et al., 2007; Moll et al., 1982; Raun and Johnson, 1999), it was not surprising that teosinte had a higher shoot NUE than the smaller W22 inbred; more importantly, both genotypes showed similar increases in NUE in response to LN (+56 fold for teosinte and +50 fold for W22) (Fig. 2E). The shoot NUtE increased in both teosinte and W22 in response to LN, with the increase being greater in the modern inbred (+233% for W22 and +182% for teosinte) (Fig. 2F). Similarly, in response to LN, both genotypes showed increases in shoot NUpE (+144% for W22 and +193% for teosinte; Fig. 2G).

### Root System Architecture and Nutrient Dynamics

In response to LN, the developmental mechanisms responsible for the decrease in shoot biomass were different between Balsas teosinte and W22. Since the architecture of a root system is critical for efficient nutrient uptake (Fitter, 1991; Lynch, 1995, 2007; Moll et al., 1982), we asked whether the increase in root biomass also resulted from different architectural adaptations. Under HN, similar to the above ground high-tillering phenotype (Fig. 3A and 3B), the Balsas teosinte root system was bushier than the modern inbred (Fig. 3A and 3C). Teosinte had ~ 50% more CRs than W22 (91 vs. 61, respectively) (Fig. 4A),

**Table 1. Dry biomass allocation and shoot traits under high and low N treatments in a modern maize inbred line and its wild teosinte ancestor. Values are least square means from ANOVA ± SE (n = 24) 35 d after transplanting.**

|  | Dry weights (g per plant) |             |             |             |             |                     | Shoot traits     |                 |                                    |                |                      |                      |
|--|---------------------------|-------------|-------------|-------------|-------------|---------------------|------------------|-----------------|------------------------------------|----------------|----------------------|----------------------|
|  | Total                     | Shoot       | Stem        | Leaves      | Root        | Root to shoot ratio | No. of leaf tips | No. of tillers  | Total leaf area (cm <sup>2</sup> ) | Avg. leaf area | Avg. leaf dry weight | Avg. stem dry weight |
| <i>Balsas teosinte (Zea mays subsp. parviglumis)</i> |                           |             |             |             |             |                     |                  |                 |                                    |                |                      |                      |
| High N (20mmol)                                      | 71.6 ± 3.8a <sup>†</sup>  | 57.8 ± 5.2a | 31.8 ± 2.4a | 25.6 ± 1.8a | 13.8 ± 0.6a | 0.28 ± 0.05a        | 67.9 ± 3.2a      | 16.7 ± 1.2a     | 1048.6 ± 97.4a                     | 15.2 ± 1.1a    | 0.31 ± 0.1a          | 1.9 ± 0.08a          |
| Low N (8 mmol)                                       | 50.3 ± 3.8b               | 33.1 ± 5.2b | 19.1 ± 2.4b | 12.8 ± 1.8b | 17.3 ± 0.6b | 0.52 ± 0.05b        | 38.0 ± 3.0b      | 5.7 ± 1.1b      | 504.0 ± 92.7b                      | 12.1 ± 1.1b    | 0.29 ± 0.1a          | 3.1 ± 0.08b          |
| <i>Modern inbred line (Zea mays subsp. mays)</i>     |                           |             |             |             |             |                     |                  |                 |                                    |                |                      |                      |
| High N (20mmol)                                      | 45.8 ± 2.8b               | 35.4 ± 2.2b | 20.4 ± 1.8b | 14.3 ± 0.9b | 10.4 ± 0.6c | 0.31 ± 0.06a        | 12.9 ± 3.5c      | 0               | 1154.5 ± 100.7a                    | 94.8 ± 7.2b    | 1.1 ± 0.1b           | 20.4 ± 1.2c          |
| Low N (8 mmol)                                       | 32.7 ± 2.8c               | 18.2 ± 2.2c | 11.2 ± 1.8c | 8.1 ± 0.9c  | 14.6 ± 0.6d | 0.61 ± 0.05b        | 11.6 ± 3.2c      | 0               | 691.5 ± 95.9b                      | 58.2 ± 7.2c    | 0.6 ± 0.1c           | 11.2 ± 1.2d          |
| G <sup>‡</sup>                                       | **                        | **          | *           | *           | *           | NS <sup>§</sup>     | *                | NA <sup>¶</sup> | NS                                 | **             | **                   | **                   |
| N <sup>#</sup>                                       | **                        | *           | *           | *           | *           | *                   | *                | NA              | *                                  | *              | *                    | *                    |
| G × N <sup>††</sup>                                  | **                        | *           | NS          | *           | NS          | NS                  | **               | NA              | NS                                 | *              | *                    | *                    |

\*Significant at p = 0.05.

\*\*Significant at p = 0.01.

<sup>†</sup>Means within a column followed by the same letter are not significantly different under high and low N at p = 0.05.

<sup>‡</sup>G, genotype.

<sup>§</sup>NS, not significant.

<sup>¶</sup>NA, not applicable.

<sup>#</sup>N, nitrogen treatment.

<sup>††</sup>Interaction between genotypes and N treatment.

which were 32% shorter in teosinte (Fig. 4B). Under LN, both teosinte and W22 decreased CR number (−65 and −42%, respectively) (Fig. 4A and 4D through 4G). Both genotypes also increased the average length of individual CRs, but this increase was only 33% in W22 compared to 285% in teosinte (Fig. 4B). Taken together, the total CR length increased by 33% in teosinte in response to LN but decreased by 20% in modern maize (Fig. 4C).

In teosinte, the CRs originate from the base of individual tillers (Fig. 5A). As noted above, there was a large decline in CR number in teosinte under LN. In response to LN, a similar reduction in the number of shoot tillers was observed in teosinte (Table 1; Fig. 5B and 5C). There was a strong positive correlation between declines in the number of tillers per plant in teosinte and the number of corresponding CR (Fig. 5D). We conclude that the decline in CR number in Balsas teosinte in response to nutrient stress is related to a decline in shoot branching. However, W22 also responded to LN with a reduction in CR number similar to teosinte (Fig. 4A) despite having a single stem with no tillers even under HN (Fig. 2A and 2C; Table 1).

The different orders of LR represent 95% of the total root length excluding RHs (Fig. 6A and 6B). In response to LN, we observed similar increases in total root length in Balsas teosinte and W22 (11 and 15%, respectively) (Fig. 6A) resulting from proportionally similar increases in LR length (Fig. 6B). We asked whether W22 and teosinte compensated for this increased metabolic demand. In response to LN, Balsas teosinte and W22 both produced more length of roots per unit biomass (specific root length) (Fig. 6C) demonstrating that both genotypes compensate for increasing root scavenging perhaps by decreasing the overall root system thickness. However, the modern inbred compensated to a greater extent than Balsas teosinte (+58 and +24%, respectively) (Fig. 6C).

We also calculated the N uptake per unit root length as the ratio of N taken up divided by the total LR length. In both Balsas teosinte and W22, the N uptake per unit LR length declined six- to eightfold in response to LN (Fig. 6D), possibly reflecting the conserved increased cost of having to scavenge more soil due to declining external N. Interestingly but opposite to prediction, under LN, N uptake per unit LR length was actually 60% higher in teosinte than the modern inbred (Fig. 6D).

Root hairs contribute negligibly to the root:shoot biomass ratio but may be major sites of nutrient uptake. Representative images of the responses of Balsas teosinte and modern maize to N stress are shown (Fig. 7A through 7D) along with quantitative measurements (Fig. 7E through 7G). In response to LN, the total RH length decreased in both teosinte and W22 (−53 and −52%, respectively) (Fig. 7E), but the underlying mechanisms were different: in teosinte it was due to decreased average root length (Fig. 7F) whereas in W22 the RH density declined (Fig. 7G).

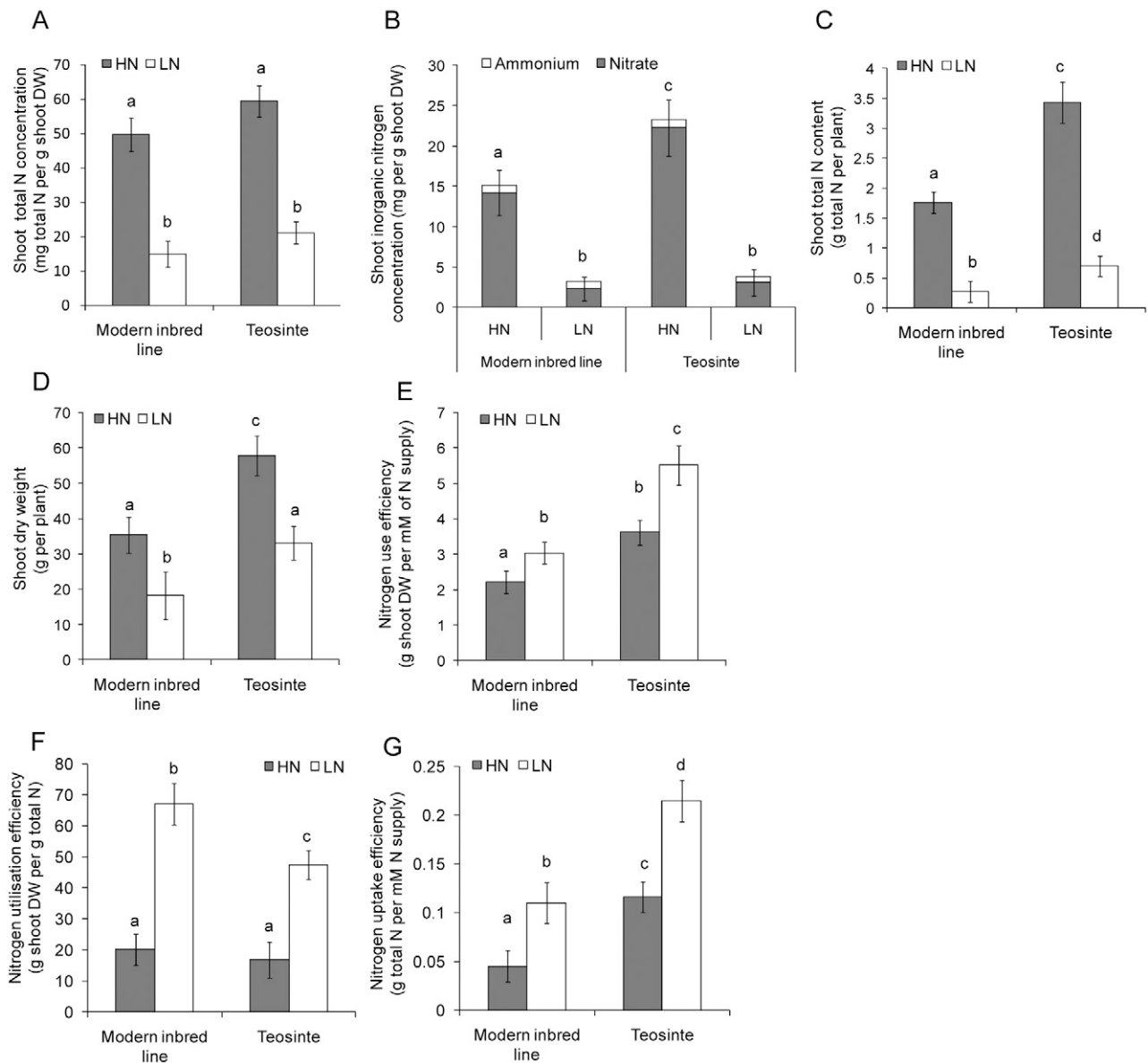


Figure 2. Effect of low N (LN) treatment on total N concentration, N content, N uptake, and N use efficiencies in W22 and Balsas teosinte at 35 d after transplanting. (A) Leaf total N concentration per gram of dry leaf tissues. (B) Leaf inorganic N (nitrate and ammonium) concentration per gram of dry leaf tissues. (C) Shoot total N content. (D) Shoot dry weight. (E) Nitrogen use efficiency (N uptake efficiency  $\times$  N utilization efficiency). (F) Shoot N utilization efficiency (shoot dry weight/shoot total N content). (G) Shoot N uptake efficiency (shoot total N content/N supply). The standard error is shown. Similar letters indicate that results are not significantly different at  $p = 0.05$ . DW, dry weight; HN, high nitrogen.

### Nitrate Uptake Transporters

Absolute transcript levels (Supplemental Fig. S1) were used to calculate whether the plasticity of transporter gene expression was conserved between teosinte and W22 in response to changing N (Fig. 8). Orthologs of four of the seven known nitrate transporter genes in modern maize (B73) could be successfully PCR amplified from teosinte root mRNA (*Nrt1.1*, *Nrt1.2*, *Nrt2.3*, and *Nar2.1*). In terms of the low affinity transporters, *ZmNrt1.2* showed no significant change in both genotypes in response to LN while *ZmNrt1.1* was upregulated in W22 but downregulated in teosinte (Fig. 8). In terms of the high affinity transporters, involved in adapting to LN, they were similarly

upregulated in response to LN in both teosinte and W22 (Fig. 8). However, *ZmNrt2.3* was upregulated twofold more in teosinte than in the modern maize inbred in response to LN. *ZmNar2.1* showed a proportional increase under LN in both genotypes (Fig. 8) but the absolute transcript levels were approximately eightfold higher in teosinte than W22 under both HN and LN (Supplemental Fig. S1).

### DISCUSSION

Maize is well known to require high amounts of N for optimal yield. Here we examined how the closest living wild ancestor of modern maize, Balsas teosinte, responded to LN stress to identify traits that may have been altered during

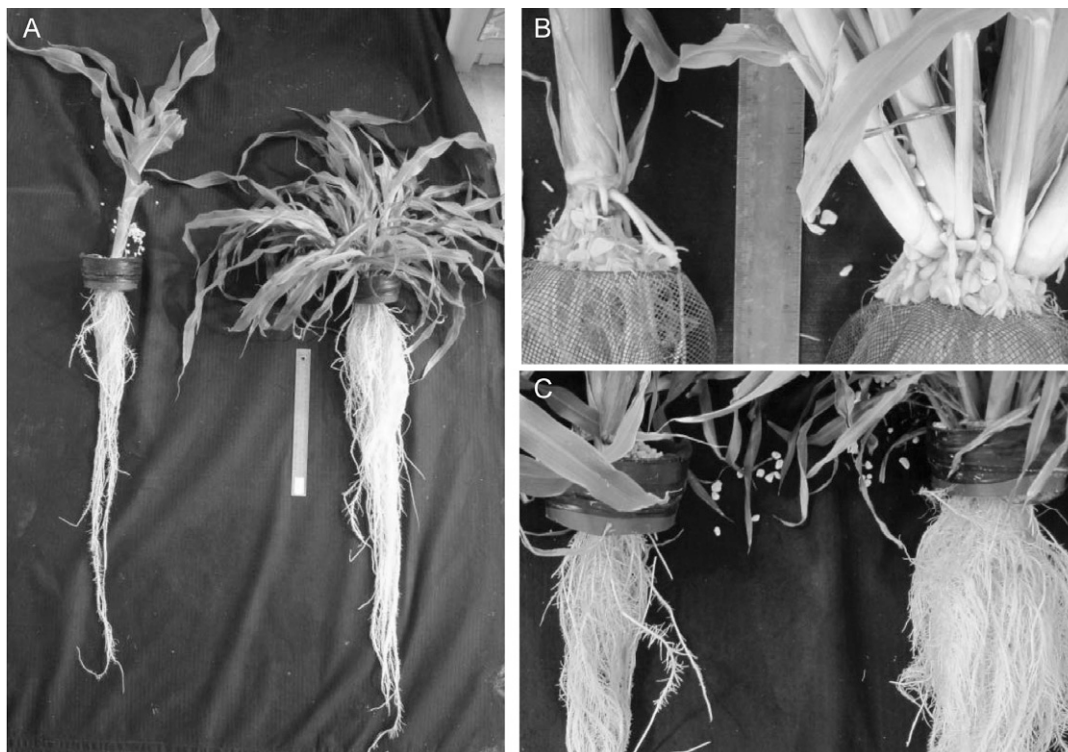


Figure 3. Comparison of the root system of W22 and Balsas teosinte. (A) W22 (left) and Balsas teosinte (right) at 35 d after transplanting (DAT) into an aeroponics growth system. (B) Crown root region from W22 (left) and Balsas teosinte (right). (C) Basal part of the root system in W22 (left) and Balsas teosinte (right) at 35 DAT.

domestication and breeding and to facilitate future studies using a teosinte  $\times$  W22 mapping population. Above ground, Balsas teosinte harbors numerous tillers that bear many leaves, resulting in a large, bushy plant. In contrast to teosinte, the shoot of the modern maize genotype used in this study, W22, has less biomass and a single stem with fewer leaves. Balsas teosinte grows in the wild in a variable and challenging mountainous environment in subtropical southwestern Mexico. This environment is subject to seasonal fertilization from recessional flooding and mineralization, extended dry seasons, and interspecies competition for nutrients (Fukunaga et al., 2005; Hastorf, 2009; Iltis et al., 1979; Piperno et al., 2007; Ruiz Corral et al., 2008; Wilkes, 1977; Zhu et al., 2010). Compared to teosinte, W22 was bred in the temperate, fertile, highly productive plains of the northern United States (Wisconsin), cultivated as a monoculture. W22 is separated from Balsas teosinte by 9000 yr of domestication and artificial selection (Hastorf, 2009; Piperno et al., 2009; Ranere et al., 2009; Sluyter and Dominguez, 2006).

Given their extreme differences in plant habitat, size, and morphology, we hypothesized that Balsas teosinte and W22 would differ in their adaptation strategies to changing N, especially underground. Surprisingly, we found considerable conservation in the fundamental responses by these genotypes to LN stress in terms of the change in biomass allocation (Table 1), shoot morphology (Table 1), root architecture (Fig. 4A, 6A, and 6B), RH length (Fig. 7E), and regulation of N transporters (Fig. 8). The

decline in CR number in response to LN in both Balsas teosinte and W22 was consistent with responses by a diversity of modern maize genotypes (Chun et al., 2005; Liu et al., 2008, 2009; Wang et al., 2005). The increase in LR length in Balsas teosinte in response to LN was not only proportionally similar in W22 but is consistent with reported LR responses across a diversity of modern maize inbreds and hybrids (Chun et al., 2005; Liu et al., 2009; Wang et al., 2005). The decline in the shoot N concentration in response to LN was also conserved in Balsas teosinte in comparison to W22 (Fig. 2A through 2C). Furthermore, the absolute leaf N concentrations under LN and HN were also surprisingly conserved (Fig. 2A and 2B). The decline in leaf N concentration in response to LN has previously been shown in several different maize landraces and inbreds (Lafitte et al., 1997; Liu et al., 2009; Niu et al., 2007; Pérez Leroux and Long, 1994; Vos et al., 2005). Similar results have been reported in pre- and post-domesticated wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Wacker et al., 2002). Increased NUtE and NUtP in response to LN similarly appear to have been conserved across maize domestication given results from W22 (Fig. 2E through 2G) and other studies using modern maize (Liu et al., 2009; Martins et al., 2008; Prestler et al., 2002; Worku et al., 2007). We conclude that 9000 years of selective breeding do not appear to have altered many fundamental developmental and physiological responses to N stress in maize.



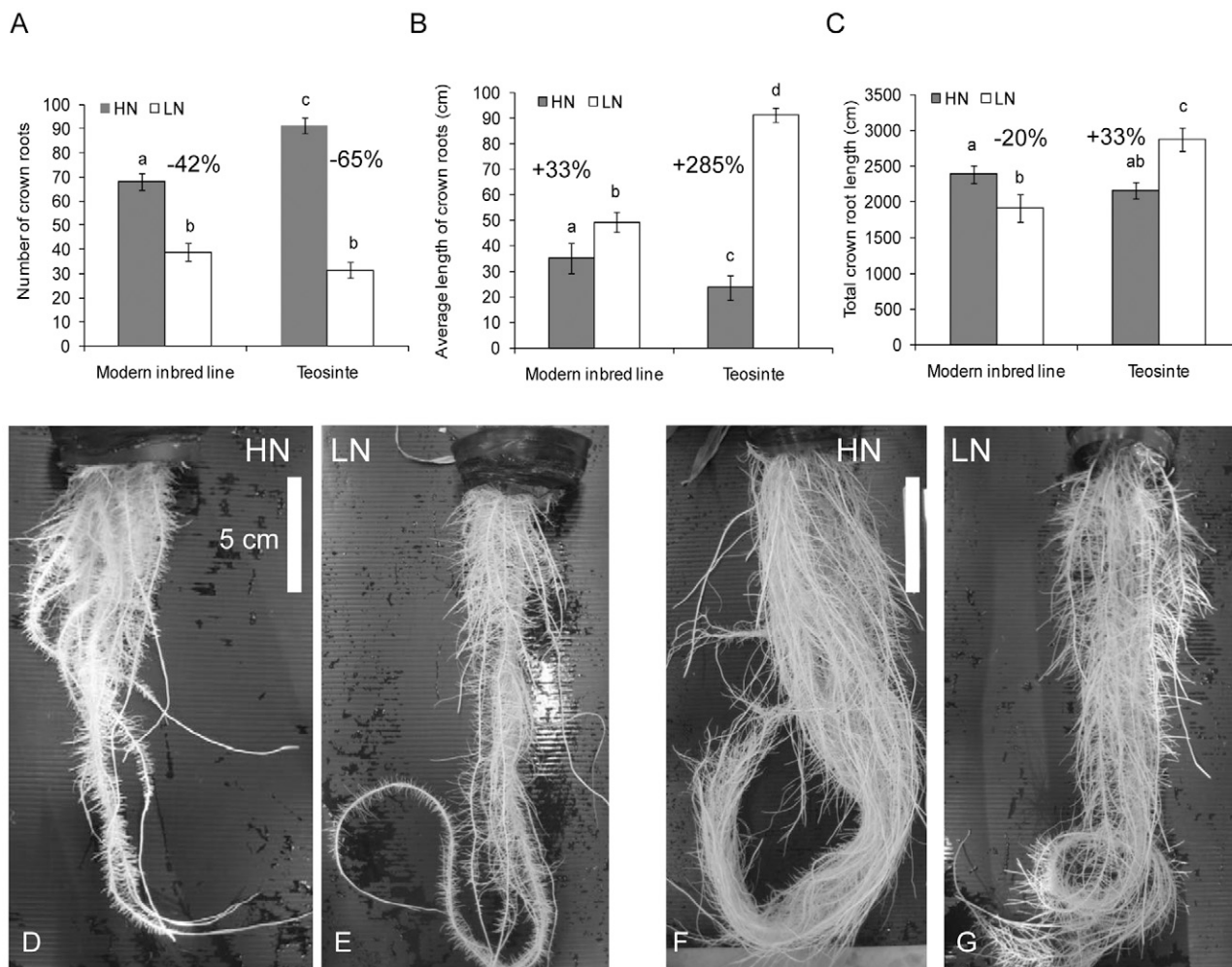


Figure 4. Effect of low N (LN) on root traits at 35 d after transplanting in W22 and Balsas teosinte. Shown are measurements for (A) number of crown roots (CRs), (B) average length of each CR, (C) total CR length. (D through G): Representative pictures of (D and E) W22 root system under (D) high N (HN) and (E) LN and (F and G) Balsas teosinte under (F) HN and (G) LN at 35 DAT. Percentages indicate the effect of LN treatment as a percentage of the HN control. Identical letters indicate that results are not significantly different at  $p = 0.05$  ( $n = 24$ ).

### Divergence of Underlying Developmental Mechanisms

Despite apparent conservation by Balsas teosinte and W22 in many responses to LN, we found that the underlying developmental strategies to achieve these responses were often different (summarized in Fig. 9). Balsas teosinte reduced tiller number and leaf number to achieve decreased shoot biomass whereas W22 decreased leaf size and stem weight (Table 1). Second, since CRs originate from the base of tillers, teosinte reduced its CR number by decreasing tiller number whereas the modern inbred decreased CR number without tiller plasticity (Fig. 4 and 5). One possibility is that Balsas teosinte reduces the number of CR under LN to counterbalance the metabolic cost of their elongation, using tiller plasticity as the mechanism. If so, these shifts were likely unavoidable consequences of artificial selection by ancient agriculturists in Mexico for increased apical dominance.

With respect to leaf area, once maize lost the capacity to dramatically reduce leaf number by reducing tiller number,

the plant had to develop an alternative strategy to reduce shoot mass in response to nutrient stress. Domestication may have resulted in a shift in the meristem that primarily perceives N stress, from the shoot axillary branch (tiller) meristem located in the leaf axil (teosinte) to the leaf growth meristem located at the base of each leaf. This would result in a smaller leaf size (Tardieu et al., 2000) as was observed in W22 under LN. Consistent with these results, in diverse modern inbreds and hybrids, LN was shown to cause decreased leaf area without decreasing leaf number (D'Andrea et al., 2006, 2009) by affecting the leaf elongation zone (Tóth et al., 2002; Vos et al., 2005). Vos (2005) has noted that in plants without tiller plasticity (maize, Brussels sprouts [*Brassica oleracea* L.], and sunflower [*Helianthus annuus* L.]), N limitation causes a reduction in leaf size while in plants that possess tiller plasticity (potato [*Solanum tuberosum* L.] and pearl millet [*Pennisetum glaucum* (L.) R. Br.]), leaf number changes rather than leaf size. It is important to note that, in modern maize, tiller meristems do exist but are in a state of permanent repression

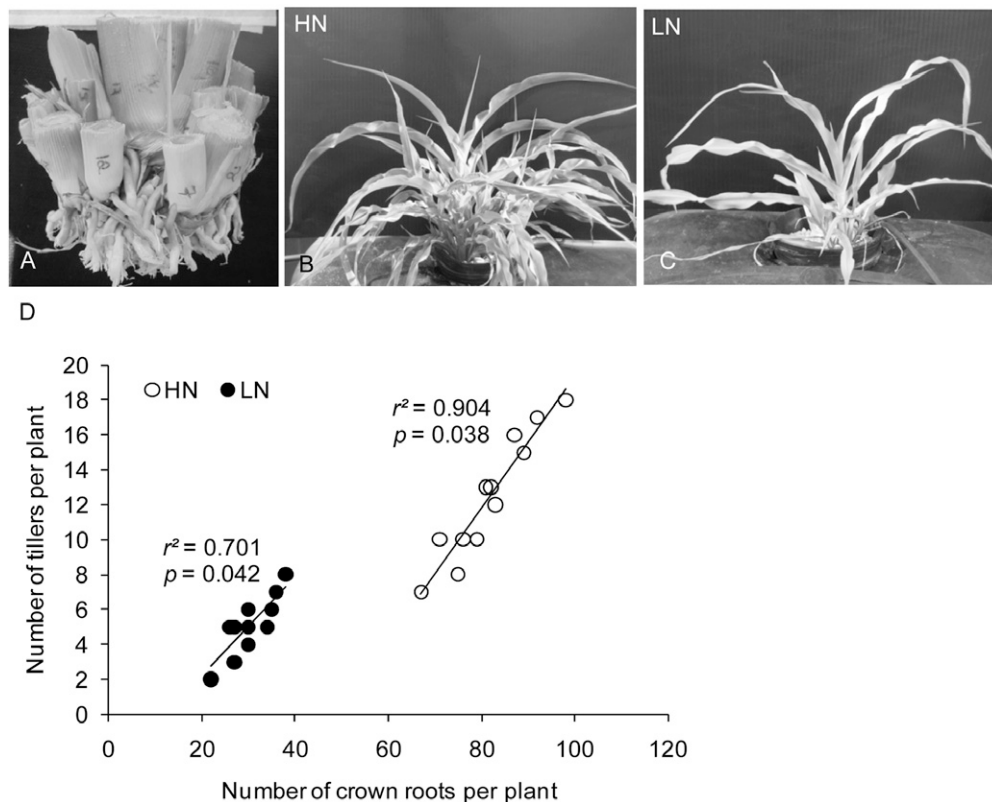


Figure 5. Effects of N concentration on shoot branching (tillering) relative to the number of crown roots (CRs) in Balsas teosinte. (A) The CR region in teosinte showing the base of several tillers and root initiation from each tiller. (B and C) Above-ground teosinte shoot branching under (B) high N (HN) and (C) low N (LN) at 25 d after transplanting (DAT). (D) The correlation between number of tillers per plant and number of CRs per plant at 35 DAT.

and are no longer responsive to the environment (Doebley et al., 1997; Doust, 2007).

Modern maize apparently has the ability to reduce CR number in response to N stress despite losing tillering plasticity. We observed that W22 reduced CR number under LN (Fig. 4A) despite having a single stem regardless of the N concentration (Fig. 3A). During maize evolution, a shift in signaling had to occur from repressing axillary tiller bud outgrowth under LN (teosinte) to directly repressing adventitious root meristems (modern maize). In wheat, barley, and rice (*Oryza sativa* L.), domestication and genetic improvement also altered tiller number with corresponding alterations in CR number, but the result was opposite to maize: breeding of dwarf and semidwarf varieties in these other crops increased both tiller and CR number (Hockett, 1986; Lo et al., 2008; MacKey, 1979) leading to a more extensive, shallow root system (Chloupek et al., 2006; Evans, 1993; Waines and Ehdaie, 2007; Yoshida et al., 1982).

### Ecological Significance

Similar to previously studied modern maize genotypes (Ding et al., 2005; Echarte et al., 2008; McCullough et al., 1994; Rajcan and Tollenaar, 1999), both teosinte and W22 responded to LN by decreasing shoot biomass while maintaining root biomass, presumably to decrease overall nutrient requirements but preserve resource allocation to the

root system. What appears to be a universal LN response in the genus *Zea* is not however a universal plant response: in a survey of ~130 species, no consistent pattern was found in the root:shoot plasticity in response to N (Reynolds and D'Antonio, 1996) nor among species adapted to different soil fertilities (Campbell et al., 1991; Grime et al., 1991).

Crown roots are responsible for long distance searching from the stem by positioning LR and RH in nutrient-rich patches. Under HN conditions, W22 had fewer CRs but they were significantly longer than Balsas teosinte, resulting in a higher proportion of roots being deeper (Fig. 4 and 9). It has been suggested that increased yields are associated with the abundance of finer roots in deeper horizons than at the surface soil layer (King et al., 2003). However, despite the higher grain yield of W22 compared to Balsas teosinte, CRs elongated only 30% in the modern inbred in response to LN compared to 285% in teosinte (Fig. 4B). Since CRs grow both vertically and horizontally (Fig. 9), this difference may reflect an ecological shift from interspecies competition for soil nutrients in the wild to intraspecies competition under cultivation in modern maize. This is because a weak correlation exists between increased root length density and nitrate uptake in maize monocultures while more roots give a competitive advantage when interspecies competition occurs (Robinson, 2001; Robinson and Fitter, 1999).

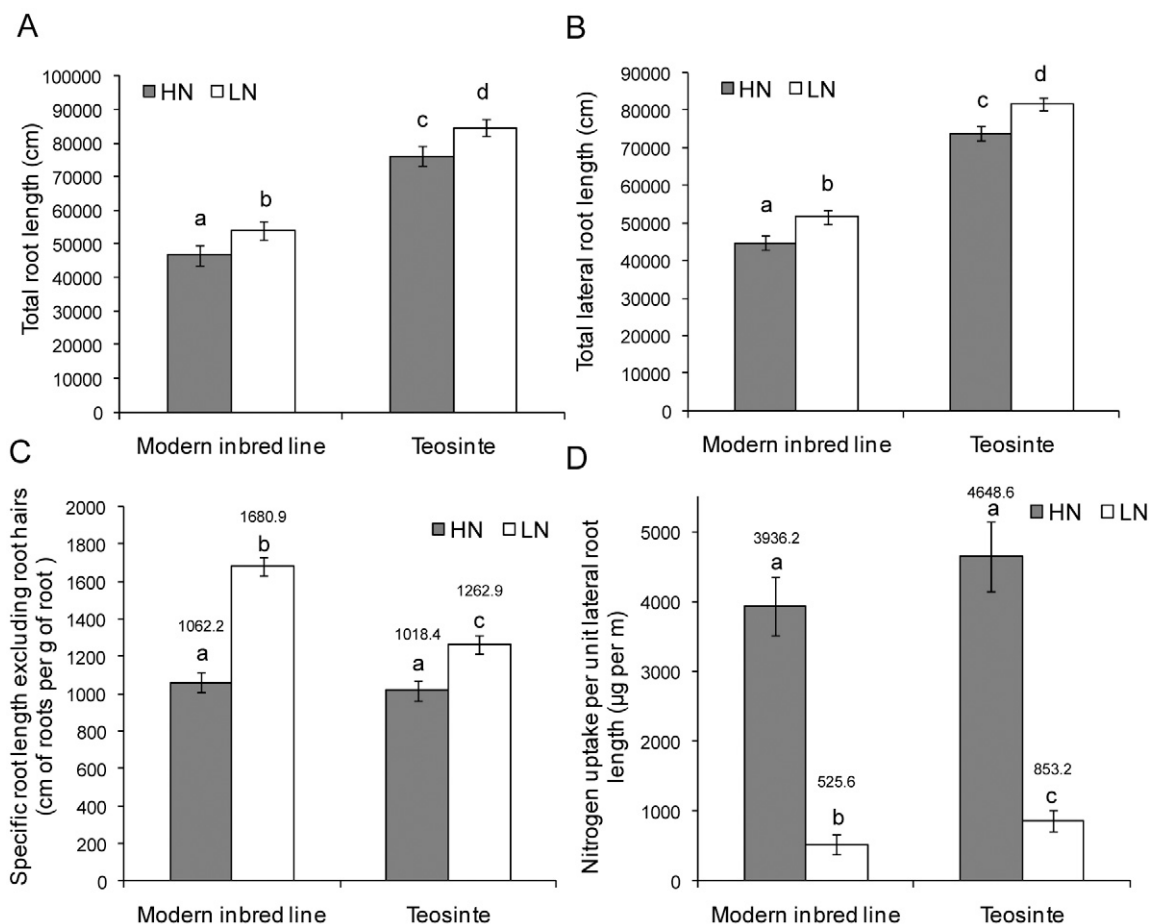


Figure 6. Effect of low N (LN) on root length at 35 d after transplanting in W22 and Balsas teosinte. Shown are measurements made with the WinRHIZO software (Arsenault et al., 1995) for (A) total root system length, (B) total lateral root length, (C) specific root length, and (D) N uptake per unit lateral root length. Identical letters indicate that results are not significantly different at  $p = 0.05$  ( $n = 24$ ). HN, high nitrogen.

Elongated CRs in teosinte may therefore confer higher fitness in the wild.

Additionally, our data infer that in teosinte, above-ground competition may create a coordinated response below ground and that N and light signaling pathways must interact to regulate the response. As noted earlier, in teosinte, a higher plant density or low soil fertility have been observed to reduce shoot tillering (Doebley et al., 1995). However, prolific tillering in teosinte may increase its competitiveness during the vegetative phase under high fertility conditions. In higher plants in general, high soil fertility is associated with high vegetative growth and hence increased competition for sunlight whereas low fertility results in increased competition primarily below ground (Newman, 1973). Since tiller and CR number are correlated in teosinte (Fig. 5D), the above- and below-ground responses to competition may be ecologically connected.

We also observed differences in RH plasticity. In response to LN, teosinte decreased the average RH length whereas W22 reduced its RH density (Fig. 7). Ecologically, decreased average RH length decreases the depletion zone perimeter around LRs (Bhat and Nye, 1973; Lewis and Quirk, 1967). It will be valuable to understand

the costs versus benefits of altering RH length versus density, although other factors affecting the root depletion zone such as soil physiochemical processes, symbiotic activity, and the higher CR system density of teosinte (Fig. 4) may be part of the explanation (Barber, 1984). It is also important to remember that RH help regulate not only N uptake but also water and other nutrients including immobile ions such as phosphate, which must be balanced by RH breaks acting as potential pathogen entry points to epidermal cells (Genre et al., 2009).

Finally, at the molecular level, in response to LN, we observed differences in the expression of a subset of nitrate transporters in Balsas teosinte and W22. In particular, expression of the low affinity transporter gene *Nrt1.1* decreased threefold in teosinte but increased twofold in W22 under LN (Fig. 8; Supplemental Fig. S1). The low affinity transporters are generally not thought to be transcriptionally altered by exposure to LN (Glass et al., 2002). In *Arabidopsis thaliana* (L.) Heynh., however, *Nrt1.1* has been shown to switch from functioning as a low affinity nitrate transporter to a high affinity nitrate transporter under LN conditions, caused by a posttranscriptional modification (Ho et al., 2009; Liu et al., 1999). It may be that different *Zea mays* genotypes

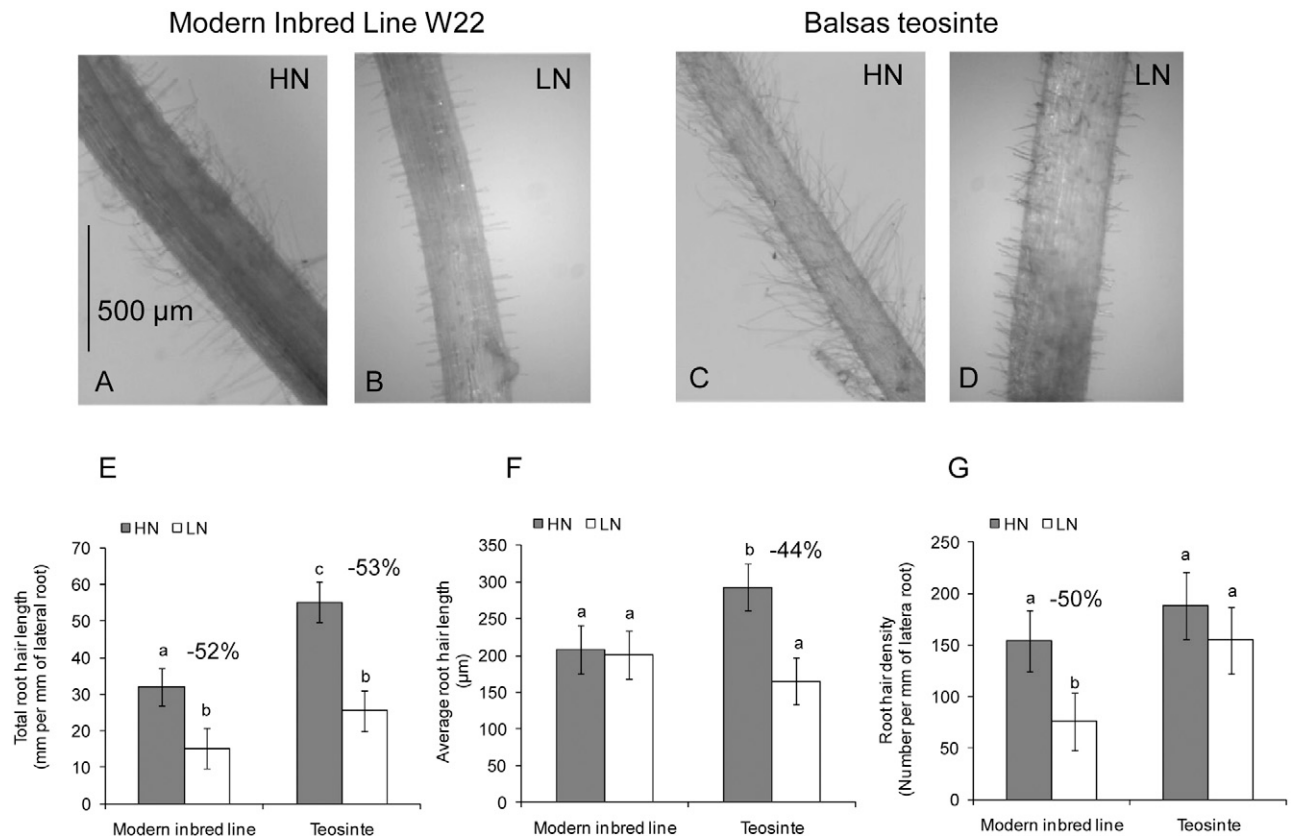


Figure 7. Effect of low N (LN) on root hair (RH) traits at 35 d after transplanting (DAT). (A through D) Representative pictures showing RH lengths and density on a first order lateral root of (A and B) W22 under (A) high N (HN) and (B) LN and (C and D) Balsas teosinte under (C) HN and (D) LN. Measurements for (E) total RH length per millimeter of first order lateral roots, (F) average RH length, and (G) RH initiation density. The standard error is shown. Percentages indicate the effect of LN treatment as a percentage of the HN control. Identical letters indicate that results are not significantly different at  $p = 0.05$ .

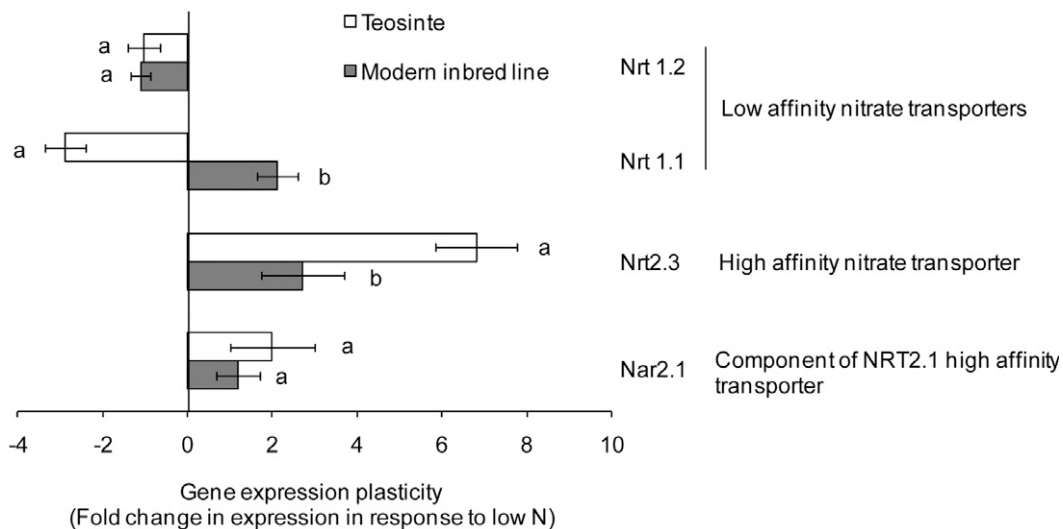


Figure 8. Comparative RNA expression of nitrate transporters on lateral roots samples and their associated root hairs at 35 d after transplanting based on real-time quantitative polymerase chain reaction (PCR). Shown is the change in nitrate transporter gene expression under low N compared to the high N treatment. Real-time quantitative PCR results were normalized using the maize  $\alpha$ -tubulin-3 gene (Genbank EU954789.1). Absolute levels of gene expression are shown in Supplemental Fig. S1.

use plasticity of *Nrt1* expression to comodulate this switch mechanism to adapt to changing N conditions. In contrast to *Nrt1*, at least some high affinity transporter genes (*Nrt2*) have previously been shown to be transcriptionally activated

by a shift to LN in maize and other species (Trevisan et al., 2008), consistent with their ecological function to scavenge N when scarce (Glass et al., 2002). We similarly found that expression of *Nrt2.3* increased several fold in both teosinte

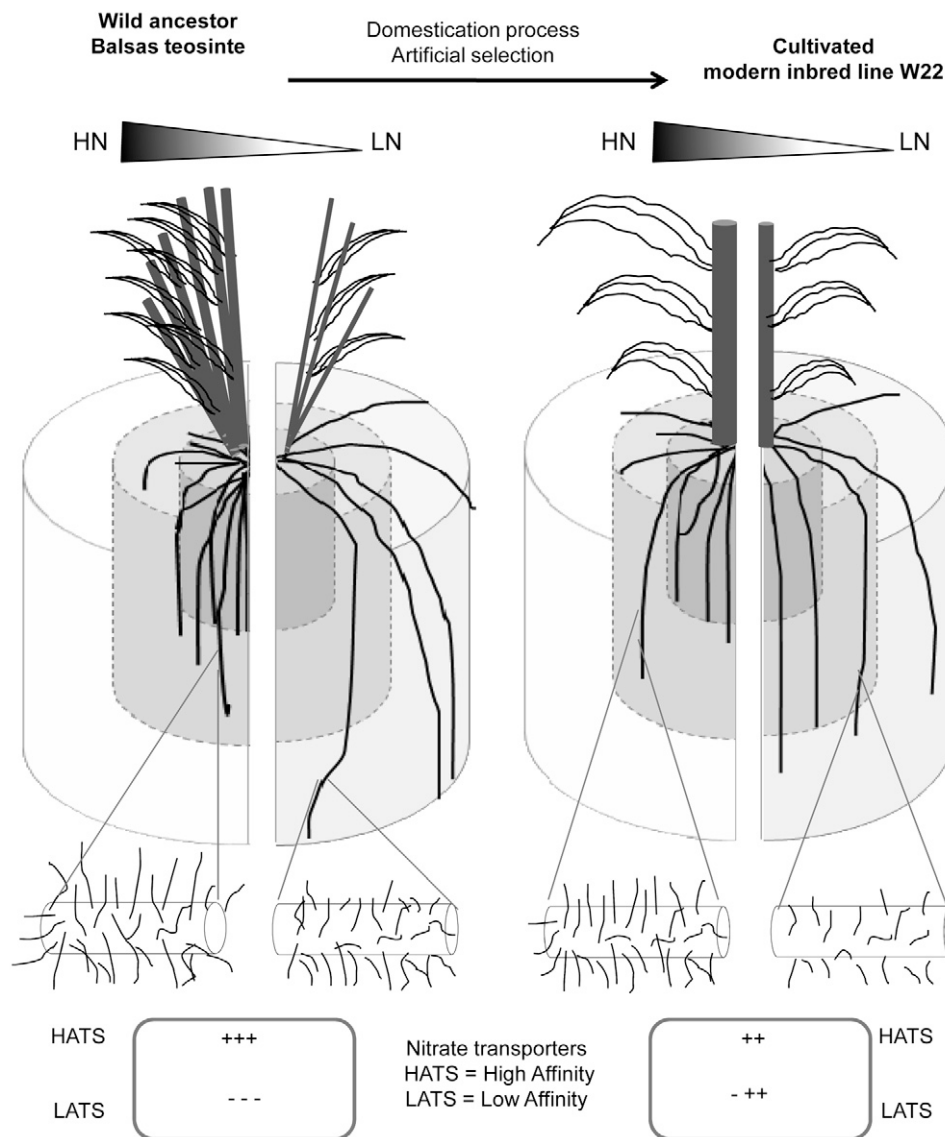


Figure 9. Summary model of the low N (LN) adaptation strategy of Balsas teosinte compared to W22. Shown are the shoot, crown roots (CRs), and root hairs (RHs) as well as the regulation of nitrate transporter genes. HATS, high affinity transport system; HN, high nitrogen; LATS, low affinity transport system.

and W22 in response to LN although the level of inducibility was greater in teosinte (Fig. 8; Supplemental Fig. S1). Although previous studies implicated *NRT2.1* and *NRT2.2* in nitrate uptake and xylem loading in maize roots (Trevisan et al., 2008), the expression or function of *NRT2.3* has not previously been reported in maize. Our results show that *Nrt2.3* is highly expressed in *Zea mays* roots (Supplemental Fig. S1) and that it is highly inducible by nitrate, thus making it a member of the inducible high affinity transport system (Glass et al., 2002). Based on results primarily from *Arabidopsis thaliana*, high affinity transport systems are generally thought to function when nitrate levels are extremely low (<1 mmol) (Glass et al., 2002). In our study, however, the LN treatment consisted of 6 mmol nitrate (8 mmol total N), suggesting that *Nrt2* genes in different species may be inducible at different low-threshold concentrations of nitrate (Hormoz, 2000). In particular, our *Nrt2* expression data suggest that

Balsas teosinte may be more adapted to a lower or more variable soil N ecosystem than W22 (Fukunaga et al., 2005; Hasstorf, 2009; Iltis et al., 1979; Piperno et al., 2007; Ruiz Corral et al., 2008; Wilkes, 1977). *NRT2* was recently shown to physically interact with cotransporter *NAR2* at the plasma membrane of *Arabidopsis thaliana* plants, forming a tetramer consisting of two subunits each of *NRT2* and *NAR2* (Yong et al., 2010). Not surprisingly, then, we found that expression of *Nar2.1* appeared to be coregulated with *Nrt2.3* (Fig. 8; Supplemental Fig. S1), which is consistent with results from *Arabidopsis* (Okamoto et al., 2006; Orsel et al., 2006). As to why only four of the seven known maize nitrate transporters were detected in teosinte roots, it may be that some of these transporters are not expressed in teosinte roots or that teosinte has DNA sequence polymorphisms overlapping the maize-derived PCR primers used.

## CONCLUSIONS

This study attempted to identify N stress traits in maize, which may have been altered during 9000 yr of human selection as possible targets for future genetic improvement. The adaptations to LN stress in Balsas teosinte and the W22 were surprisingly conserved, but the strategies employed were often different perhaps reflecting the unique ecology and shoot architecture of these genotypes. It may now be possible to map these polymorphic traits using existing Balsas teosinte × W22 mapping populations. With respect to general statements about the implications of these results for maize domestication, a wider study involving more modern and ancestral genotypes is needed to confirm the generality of the responses observed in this study.

## Acknowledgments

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