Project Title: Increasing nitrogen use efficiency in lettuce by screening root growth and nitrogen uptake during early growth.

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Abstract

Lettuce requires relatively high amounts of nitrogen (N) fertilizers to ensure adequate growth and quality in the harvested crop. Providing excess N can lead to leaching of nitrates into groundwater. Further, N fertilizers release nitrous oxides (NO_2) into the atmosphere that contribute significantly to greenhouse gases that are driving climate change. Improving nitrogen use efficiency (NUE) in lettuce is necessary to reduce the environmental impacts of lettuce production and ensure the economic sustainability of the lettuce industry in California. Our long-term goal is to develop breeding lines with improved NUE. Identifying the genetic and physiological basis of NUE is the first step to developing an efficient breeding program because it allows breeders to directly select for factors which have the greatest impact on NUE. Nitrogen use efficiency is a complex trait controlled by efficiencies in N uptake and assimilation with substantial genotype x environmental variation. To simultaneously gain insight into its physiological basis and identify loci associated with NUE, a recombinant inbred line (RIL) of lettuce was screened using a gel-based assay that allowed measurement of N uptake and assimilation, root growth rates and length under a uniform environment. Seeds from the F_{10} Diplomat x Margarita RIL population were germinated and transferred to gel plates containing complete Murashige and Skoog (MS) basal salts with nitrogen supplied as NH_4NO_3 and KNO_3^- . Seedlings were grown under high or low N, defined as 100% N and 50% N, respectively. Root length and leaf nitrogen content were affected by N level supplied. Using averages for the DxM RIL, root growth rates and root length were lower under high N than low N. Leaf N concentration (dwt basis) was lower in the low N treatment than the high N treatment. N metabolism, measured by $\delta^{15}N$ isotope discrimination indicated distinct differences between high and low N. Genotypes that accumulated high leaf N levels when grown under high N failed to do so when grown under low N. Quantitative trait loci were identified for five nitrogen metabolism traits, three under high N and two under low N. The results suggest that gel assays are an efficient method to screen for N uptake and assimilation and selections for improved NUE should be made under low N. The differences in N metabolism among the genotypes at high versus low N may be attributed to efficiencies in gene expression and/or protein turnover. If so, this would allow direct selection for alleles which contribute to the efficiencies.

Objectives

The objectives of this project were to: 1) determine growth rates of roots of the DxM RIL grown under non-limiting and limiting N conditions; 2) determine NUE in the DxM RIL population grown under reduced nitrogen conditions using nitrogen isotope discrimination.

Procedures

Objective 1: Determine growth rates of roots of the Diplomat x Margarita (DxM) RIL grown under non-limiting and limiting N conditions.

Seeds of each genotype (~152 families plus two parental lines) of the DxM RIL were germinated on 23 cm x 23 cm agarose plates. Each plate contained two families each with 10 seeds. The experiment was independently replicated three times and the parental lines replicated as experimental controls to measure environmental variations due to light and temperature differences among shelves which held the plates. Plates were positioned vertically and grown under continuous light (140 µmol m^{-2} s^{-1}) in a controlled environment room held at 22 °C. The agarose medium consisted of full strength Murashige and Skoog basal salts (Phytotechnology Laboratories, Shawnee Mission, KS) with three different nitrogen formulations, as follows. The non-limiting N treatment consisted of MS salts with N supplied as NH_4NO_3 and KNO_3, designated as 1x N treatment. The N-limiting treatment, designated as 0.5 x N, consisted of MS salts with N supplied as both NH_4NO_3 and KNO_3 at half-concentration relative to the 1X N treatment. The third formulation contained full strength MS basal salts with 1x KNO_3 without N_4NO_3 and is designated as the no N_4NO_3 treatment. The third treatment was designed to provide information on the contribution of ammonium to N accumulation in leaf tissues and determine if genetic variation exists. Root growth was assessed every 48 h for 10 to 14 days, depending on N treatment, by photographing each plate with a digital camera. Root growth rates and overall length for each seedling were determined using Root Detection software (version 0.2.1). The images of each plate were visually inspected to ensure start points for root measurements at day zero were accurately identified by the software. Likewise, the measurements for subsequent measurements were manually inspected to ensure accuracy. For each RIL family, the overall root length and relative growth rate of ten seedlings were averaged for each of the three experiments. Genotypic differences in root growth rates and overall length were assessed using analysis of variance (PROC GLM, SAS ver. 9.4). Average root length and growth rates were mapped as quantitative trait loci (QTL). For each experiment the average values of these variables were used to identify QTL using JMP Genomics 7.0 with analysis parameters set as composite interval mapping, Haley-Knott regression algorithm, 2 cM test steps, 1000 permutations and a LOD threshold level of 2.5.

Objective 2: Determine NUE in the Diplomat x Margarita recombinant inbred line (DxM RIL) population grown under reduced nitrogen conditions using nitrogen isotope discrimination.

Plants grown in Objective 1 were harvested at the on day 9 of the experiment, the agarose rinsed from the roots, and biomass determined. For each treatment, leaves were removed, oven dried, crushed in a ball mill and prepared for isotope analyses to determine carbon and nitrogen discrimination ($\delta^{13}C$ and $\delta^{15}N$, respectively). The carbon and nitrogen discrimination data are used as proxies for water and nitrogen use efficiency, respectively. Since plants were not grown under water deficit, water use efficiency ($\delta^{13}C$) is not limited by stomata closure, but instead is likely to be a measure of the genotype's ability to regenerate a rate limiting enzyme of

photosynthesis, RUBISCO. The complete experiment containing all genotypes and three nitrogen treatments was repeated three separate times. For each experiment, nitrogen uptake and assimilation of each RIL family is represented by evaluations of ten seedlings. The data for each RIL family and N treatment includes leaf dry weight, amount of leaf carbon and nitrogen, and isotope discrimination of carbon and nitrogen. For the isotope discrimination samples, the ten seedlings of each RIL family in each experiment were blended together and used as a single sample to reduce experimental costs. Treatment differences in the concentration of carbon and nitrogen and isotope discrimination were assessed using analysis of variance (PROC GLM, SAS ver. 9.4). For each experiment the average values of these variables were used to identify QTL using JMP Genomics 7.0 with analyses parameters including composite interval mapping implementing the Haley-Knott regression algorithm, 2 cM test steps, 1000 permutations and a LOD threshold level of 2.5.

Results and Discussion.

Root Growth

To assess the effect of nitrogen concentration seedlings were grown in gel plates containing full nitrogen (1 x N) or half-strength nitrogen (0.5 x N), photographed every 48 h and root length analyzed using Root Detection software (Figure 1). For each treatment, after obtaining the images and computing root lengths the bottom 5% of the population was filtered from the analyses to remove low vigor seedlings. Root length was significantly affected by nitrogen treatment (Prob > F, <0.0001). The root length of RIL families grown under low nitrogen (0.5 x nitrogen) were significantly longer than those grown in high nitrogen (Prob > F, <0.0001). The DxM RIL population was grown in media containing only KNO3 (No NH4NO3 treatment) to assess the contributions of NH_4 on root length. Differences were observed in root length between the high N treatment (1 x N) and the "No NH4NO3" treatment (Prob > F, < 0.0001), whereas no differences were detected between the low N (0.5 x N) and the "No NH4NO3" treatment (Prob > F = 0.1069). These results indicate that when averaged across the entire DxM RIL population, root growth is sensitive to nitrate (NO_3) concentration but not ammonium (NH_4). However, significant genetic variation exists which suggests a possible role in contributing to root length in some genetic lines, which will be discussed below.

In addition to treatment differences, genotypic differences in root length were significantly different within each of the three nitrogen treatments (Prob > F, <0.0001). Using the dataset with the lowest 5% of the population filtered from each treatment, the RIL population grown under the 'no NH_4NO_3' treatment had the greatest range in root length (range = 4.8 cm, 3.5-fold difference). The 0.5 x N treatment had the lowest variation (range = 3.4 cm; 2.8-fold difference), while the 1 x N treatment was intermediate between the two other treatments (range = 3.9 cm, 3.2-fold difference). The maximum root lengths were much greater in the 'no NH_4NO_3' treatment than either of the two N treatments containing NH_4NO_3. However, the variation among the genotypes was higher, which explains why no statistical differences were detected in root length between the low N and the 'no NH_4NO_3' treatments. Since there are

different uptake mechanisms for NH_4 and NO_3 and different genes involved in assimilation, this observation suggests that these differences in uptake and assimilation can be taken advantage of to increase NUE. Our Leafy Green project next year specifically examines this possibility by targeting specific genes that are associated with NH_4 and NO_3 uptake and assimilation.

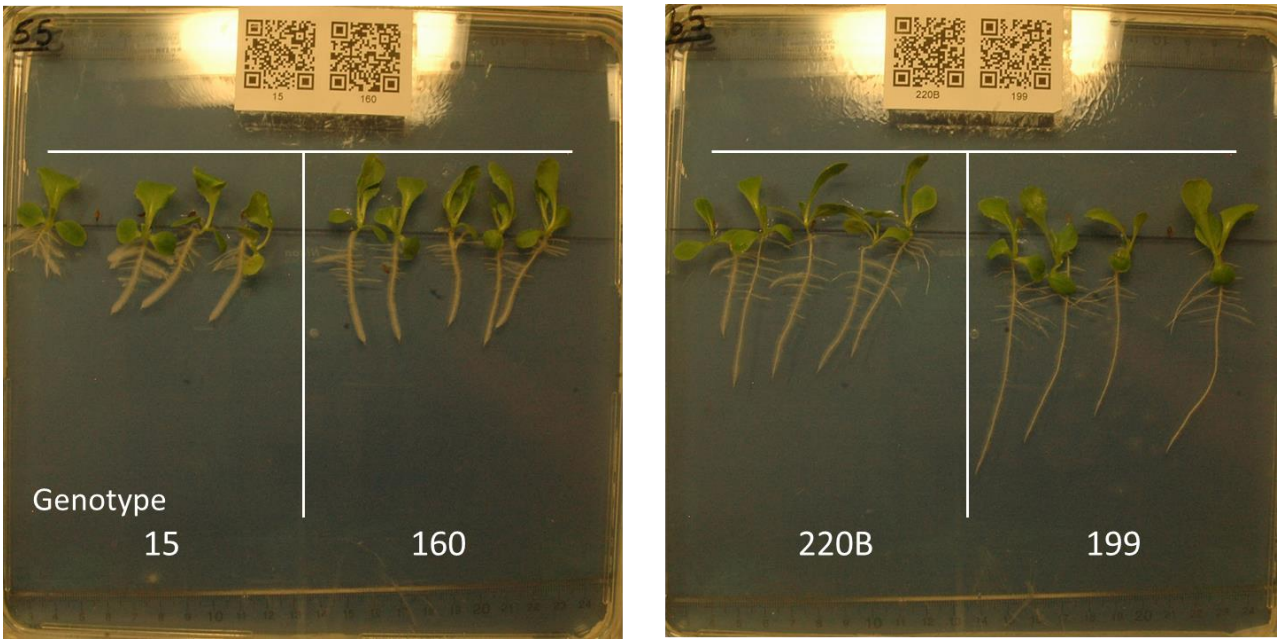

Figure 1. The Diplomat x Margarita F_{10} RIL population was screened for root growth, nitrogen uptake and assimilation was accomplished using gel plate assays. This assay virtually eliminates environmental variation and is suitable to assess genetic potential of germplasm. The image above shows differences in overall root length and architecture (secondary roots) in four RIL families grown under the high nitrogen (1 x N) treatment. Digital images were taken of seedlings every 48 hours and overall length and growth rates determined. In each experiment, n = 1480 for each treatment. Experiments were replicated three times.

Nitrogen Uptake and Metabolism

Plant size is controlled by nitrogen uptake and assimilation of nitrogen from inorganic to organic forms. Our objective was to measure the amount of leaf nitrogen in each RIL family and by doing so have quantitative measure of the genetic potential for uptake and assimilation. Previously, we have measured considerable variation in soil nitrogen in lettuce fields and a wide range of nitrogen metabolism values and leaf nitrogen concentration was observed in cultivars and genotypes replicated as checks. For example, variation in nitrogen metabolism ($\delta^{15}N$) values in the parental line 'Diplomat' planted in the field in Yuma were 6.5 times greater than Diplomat

evaluated with gel assays (n=22 observations for Diplomat in the field studies and n = 12 for gel assays.)

The $\delta^{15}N$ values are a measure of the overall nitrogen metabolism occurring in the lettuce plant. In this report the $\delta^{15}N$ values is used as a proxy for nitrogen metabolism since it represents an integration of nitrogen metabolism over the life of the plant and similarly is an integration of the biochemical reactions associated with nitrogen metabolism. Nitrogen metabolism (via isotope discrimination) of the DxM RIL population was evaluated under 1.0 x N, 0.5 x N and without NH_4NO_3. The population average for nitrogen metabolism among the treatments was significantly different (Prob > F < 0.0001), with $\delta^{15}N$ values of 1.8 and 7.6 for the 1.0 x N and 0.5 x N treatments, respectively. Thus, by this measure, it is readily apparent that nitrogen metabolism is quite different in lettuce population grown at high versus low nitrogen. The ranked order of $\delta^{15}N$ values of the RIL population was not conserved between high and low N, indicating the complexity by which lettuce utilizes N. Importantly, leaf growth is dependent on an adequate supply of nitrogen, and the population average for the 0.5xN treatment was 66.8 ug / mg dwt, which was 89% of that measured in the 1.0 x N population. The genetic variation in N metabolism and N concentration is considerable. N metabolism ranged from 0.92 to 4.09 in the 1.0 x N treatment, and from 5.9 to 9.3 in the 0.5xN treatment. Likewise, N concentration ranged from 51 to 90 µg / mg dry weight for the 1.0 x N treatment and from 46 to 81 µg / mg dry weight in the 0.5x N treatment. Thus, despite having different metabolism, many genotypes had the potential to assimilate N and accumulate N in the leaves. However, again, the ranked order of the RIL population was not conserved between the high and low N treatments. These data strongly suggest that selections for improved nitrogen uptake and assimilation should be made under a low N treatment. However, sampling from growers fields in Yuma and Salinas, we have not found any indication of lettuce crops being grown anywhere near the low N conditions used in this study.

The DxM RIL population grown in gel assays without NH_4NO_3 had a N metabolism population average of 1.96, which was statistically different from that measured under the 1.0 x N treatment (i.e., $\delta^{15}N$ values of 1.96). Nitrogen metabolism ranged from 0.9 to 5.5, a greater range than that observed in the 1.0 x N treatment. Nitrogen concentration in the no NH_4NO_3 treatment were only 65% of that measured for the population average of those grown at 1.0 x nitrogen, despite having similar N metabolism values and range. Nitrogen is supplied as KNO_3 in the 'no NH_4NO_3' at the same concentration as the 1.0 x N treatment. Thus, two possibilities exist. Either NH_4 – specific reactions are not being reflected in the nitrogen metabolism values, or N metabolism is shifted to compensate for the lack of NH_4. Our next set of studies will reveal which of the two is correct. Since wide genetic variation exists in this trait, this too may be a strategy to exploit. It is interesting to note that the effect of NH_4 is manifested through leaf N concentration and through root growth. The 'no NH_4NO_3 treatment had root lengths that were similar to the 0.5 x N, which were both greater than that measured for the 1.0 x N treatment.

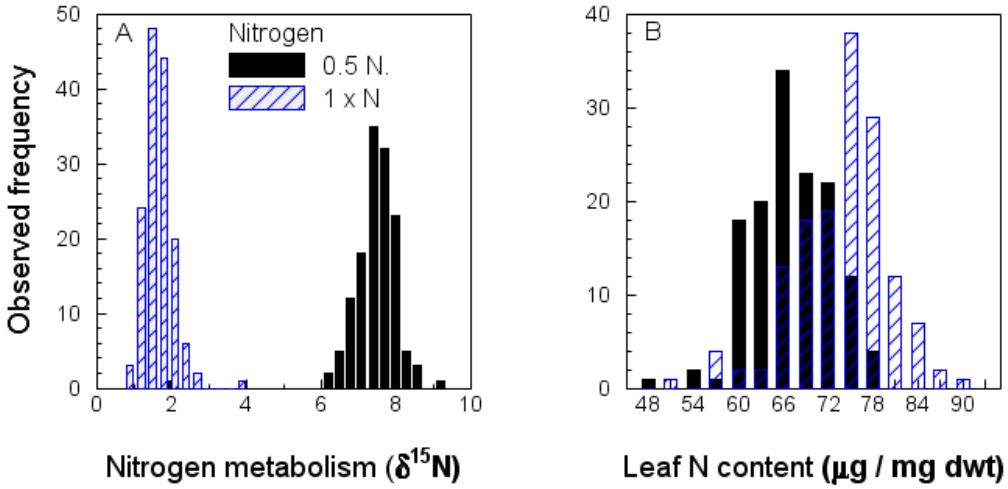

Figure 2. The frequency distribution of nitrogen assimilation and leaf nitrogen content of the DxM RIL displays distinctly different N metabolism at high versus low nitrogen (A, 1.0 x N, 0.5 x N, respectively). Although the population means are significantly different, the frequency distribution of leaf N in the DxM RIL shows considerable overlap with between the high and low N treatments (B). These results indicate lettuce metabolism in lettuce differs between high and low N but also indicate a few genetic lines perform well under low N. However, the top performing genotypes under high N are not the top performing genotypes under low N. Together, these results suggest that to adapt cultivars to low N field conditions, selections must be made under low N. Details of the plate assays are provided in the procedures section.

QTL Mapping

Quantitative trail loci were identified for five traits associated with nitrogen and carbon metabolism (Table 1). These include water use efficiency, carbon concentration, root length, nitrogen metabolism and nitrogen concentration. No two traits mapped under both N conditions, offering additional support that the underlying physiology and metabolism in lettuce is different under high N than under low N. At 1 x N concentration, WUE, carbon concentration and root length mapped, while at 0.5 x N concentration, nitrogen metabolism and nitrogen concentration mapped. Although this project is centered on improving N uptake and assimilation, biomass accumulation is directly related to carbon metabolism through nitrogen's dominant role in photosynthesis. Thus, improvements in NUE must also assess the effects of the efficiency by which biomass is added by assessing carbon and water use efficiency.

Table 1. Quantitative trait loci associated with nitrogen metabolism and leaf N accumulation in the Diplomat x Margarita recombinant inbred line. Seedlings of the DxM RIL population were assessed using gel plate assays to identify genetic potential under high N (1 x N) or low N (0.5 x N) without confounding environmental effects that occur under field conditions. Root length, leaf nitrogen content, leaf N and C content and isotope discrimination were obtained for each genotype and the experiment was replicated three times. Note that the same traits did not map at both high and low N. Each locus mapped to a unique location. Detailed experimental information is provided within the Procedures section.

Nitrogen	Trait	Linkage Group	LOD	PVE[z]
1.0 x	WUE(δ^{13}C)	2	3.2	12.1
	C concentration	6	3.6	13.3
	Root length	4	3.6	12.4
0.5 x	N metabolism (δ^{15}N)	4	3.3	12.7
	N concentration	7	3.8	14.3

[z]PVE – percent variation explained by the QTL.