Project Title:

Breeding for increased water and nitrogen use efficiency.

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Abstract:

Nitrogen and water use efficiency are complex traits affected by nitrogen (N) uptake, remobilization and assimilation. If genetic variation exists in these traits, it can be exploited to develop improved germplasm for use in breeding programs. To provide evidence for a genetic basis for nitrogen use efficiency (NUE), a diverse collection of lettuce germplasm was screened under both non-limiting and limiting N to identify genotypes that accumulated high or low leaf N. To test the hypothesis that genotypes with high leaf N concentrations would have gene expression signatures distinct from those with low leaf N, the expression of genes involved in N metabolism, mobilization and assimilation was assessed. The contrasting genotypes were grown under limiting or non-limiting N conditions and 48 candidate genes were assayed in leaf and root tissues. Under non-limiting N, minor differences in gene expression were observed between genotypes that accumulated high leaf N versus those that had low leaf-N concentrations. Conversely, large differences in gene expression were observed between genotypes that accumulated high-N versus those that were low-N accumulators. Four genes appeared to be particularly influential and should be further considered for their potential to improve NUE in lettuce.

Objectives:

The objective of this study was to determine if key genes in the N metabolic pathway are correlated to N uptake, assimilation and leaf N content.

Procedures:

To identify genes that control nitrogen metabolism, uptake, remobilization and assimilation in lettuce, a diverse collection of germplasm including commercial cultivars, inbred lines, and closely related *Lactuca* species were grown in agarose-based gel medium with nitrogen at two concentrations, designated as limiting-N or non-limiting N. Seeds of each genotype (132 genotypes) were germinated on 23 cm x 23 cm agarose plates. Each plate contained two genotypes each with five seeds. Plates were positioned vertically on shelves and grown under continuous light (140 µmol m^{-2} s^{-1}) in a controlled environment room held at 22 °C. Parental lines from a recombinant inbred line (Diplomat x Margarita cross) mapping population were replicated as experimental controls to estimate light and temperature variation among the shelves. The agarose medium consisted of full strength Murashige and Skoog (MS salts) basal salts (Phytotechnology Laboratories, Shawnee Mission, KS) with two different nitrogen levels, The non-limiting N treatment consisted of full-strength MS salts with N supplied as NH_4NO_3 and KNO_3. The N-limiting treatment consisted of MS salts with N supplied as both NH_4NO_3 and KNO_3 at one-half the concentration of the non-limiting N treatment. The non-limiting N treatment supplied N at levels where growth or N metabolism would not be limited whereas the

limiting N treatment was designed to alter N metabolism. N isotope discrimination verified that N metabolism differed significantly between the two N treatments. We hypothesized that gene expression would provide an insight into the biological basis for the different N metabolism and if the causative genes could be identified, they would serve as the basis by which to select for improved NUE.

Since N uptake is influenced by root morphology, root growth was assessed by imaging roots every other day for 14 days by photographing each plate with a digital camera. Root Detection software (version 0.2.1) was used to trace roots from which root growth rates, secondary branching and overall length for each seedling were derived. Images of each plate were visually inspected to ensure accuracy of the software-traced root images. In cases where the traces were not accurately depicted by the software the root traces were corrected by hand. For each genotype, the overall root length and relative growth rate of the five seedlings were averaged for each of the experimental treatments. The entire experiment was replicated three times.

Experimental and genotypic differences in root growth rates and overall length were assessed using analysis of variance (PROC GLM, SAS ver. 9.4). In previous projects supported by the CLGRB, average root length and growth rates were mapped as quantitative trait loci (QTL) indicating a genetic basis for root growth rates and length. The experimental results described herein were designed to explore and identify genetic diversity of root growth characteristics.

The data collected on each genotype and N treatment included leaf dry weight, root length, and secondary branching, At the end of the 14 day growth period, seedlings were harvested, the agarose rinsed from the roots, and biomass determined. From this screening, the top 5% and bottom 5% genotypes of the population in terms of leaf N accumulation were identified. For each treatment and genotype, seedlings were harvested and the leaves separated from roots to allow gene expression to be performed on each tissue separately. The tissues were dipped in liquid nitrogen and held in -80° C freezers until processed for RNA extraction using Sigma Spectrum Plant Total RNA kits (Sigma-Aldrich, St. Louis, MO). For each N accumulation phenotype (high leaf N or low leaf N) the RNA of six genotypes (each consisting of five seedlings) grown under limiting N or non-limiting N was pooled.

Gene expression of 48 genes associated with N uptake, remobilization and assimilation was assessed under limiting-N and non-limiting N. In addition to the N-metabolism genes, an additional four genes whose expression was not affected by the experimental N treatments were used as reference (normalization) genes. The candidate genes associated with N metabolism, assimilation, uptake and remobilization were identified from the literature, primarily from experimental evidence using Arabidopsis, maize and rice. The lettuce homolog was retrieved from the lettuce genome resource, Lettuce GBrowse version 3.2 (https://lgr.genomecenter.ucdavis.edu/). Since most of the candidate genes were members of gene families, primers were designed to bind to regions of the target genes which would differentiate the intended target gene from those within the gene family. To quantify transcript abundance of the candidate genes, a multiplexed quantitative PCR platform was employed (GeXP system, Beckman-Coulter, La Brea, CA). The 48 N-associated genes were assessed

using two different gene expression panels, with each panel comprised of 24 genes of interest plus the four normalization genes.

Differences in gene expression were assessed using analysis of variance (PROC GLM, SAS ver. 9.4). To get a global view of how gene expression was affected by genotype and N treatment, the expression data were analyzed using using principal component analysis (PCA) using JMP Genomics, version 8 software.

Results and Discussion:

The leaf N concentration of the lettuce population grown under limiting N was ranked and the genotypes comprising the top 5% and bottom 5% were identified. Under non-limiting N, the top 5% genotypes (n=6) had 136% of the leaf N compared to the bottom 5%, (data not presented). Of the six genotypes comprising the top 5%, three were commercial cultivars, two were *L. serriola* accessions and one was a *L. saligna* accession. When the population was grown under limiting N, the genotypes comprising the top 5% had leaf N concentrations 177% of that of the bottom 5% genotypes (Table 1). Under limiting N, none of the top genotypes were commercial cultivars; five of the six were *L. serriola* accessions and one was a *L. saligna* accession. Further, no commercial cultivars were in the top 10% of the screened population. Since all selections are made under commercial growing conditions, as might be expected, commercial cultivars are efficient at accumulating leaf N when N is abundant.

Table 1. Leaf nitrogen (N) content of the top 5% and bottom 5% of a diverse lettuce population screened for nitrogen uptake and assimilation. The population consisted of 34, 65, 21 and 12 genotypes and accessions of *Lactuca saligna*, *L. sativa*, *L. serriola* and *L. virosa*, respectively. Of the 65 *L. sativa* genotypes, 57 were commercial cultivars. The population was ranked by leaf N content under limiting N and mean values and standard deviations (in parenthesis) for the genotypes comprising the top and bottom 5% were calculated. The plants were grown under controlled conditions and evaluated for leaf N content after 14 days of growth under non-limited or limited N concentrations.

	Leaf Nitrogen (µg/mg dwt)	
Distribution	Non-limiting	Limiting
Top 5%	76.0 (5.4)	75.5 (1.2)
Bottom 5%	55.7 (10.1)	42.7 (3.2)

Significantly, none of the 57 commercial cultivars evaluated were as efficient as the unimproved accessions when N was in limited supply. The genotypes comprising the best 5% under limiting N, had 93% of the leaf N concentration of the genotypes comprising the best 5% grown under non-limiting N (76 µg/mg dwt vs 81.5 µg/mg dwt, respectively). These data support the possibility that alleles from the closely related and sexually compatible *Lactuca* species could be identified and introgressed to develop commercial cultivars with improved N use efficiency.

The large difference in leaf N concentration between the top and bottom 5% suggests that N metabolism differs between the two groups. To test that hypothesis, the expression of genes associated with N uptake and assimilation between the two phenotypic groups was quantified. To improve the probability of producing unambiguous results, RNA used to quantify gene expression of the genotypes comprising the top 5% was pooled, and likewise, RNA was pooled from the genotypes from the bottom 5% of the population; a process termed bulked segregant expression analysis. Thus the pooled RNA represents the average expression of the genotypes comprising each bulked pool. After normalization against reference genes, the expression data were analyzed by PCA. Briefly, PCA is a process by which the complexity of a data set is reduced into a small number of unrelated, non-redundant variables that largely still represents the original data set. The data are arranged in multiple dimensions (x, y, z, etc.) such that the first principal component includes the standardized original variables that together comprise the greatest possible variance. The principal component data are graphically represented in multiple dimensions, ordered by the amount of variance captured in that dimension. By definition, the first component represents the largest amount of variation and is therefore of greatest interest. Variation diminishes in successive components and usually only the first two or three components are meaningful and graphically presented. Thus, a complex data set can be reduced to summarize the features of the data without sacrificing meaningful variation, and further, it can be used to identify the most influential observations. In our case, this means PCA can be used to quickly determine if the gene expression was affected by N treatments and if so, identify the genes most affected by N treatment.

The results from the PCA analysis indicated that there was little difference in gene expression between the top and bottom 5% of the population when N was not limiting (Figure 1). The locations in PC space of the top and bottom 5% are almost identical in location along the x-axis (i.e., first dimension), and slightly separated along the y-axis (i.e., second dimension); both groups are in the same quadrant. In contrast, PCA indicates a clear separation of gene expression among the top and bottom leaf N accumulating groups when the plants are grown under limiting N (Figure 1). The groups are separated both in the x- and y-axes and are in different quadrants. This is a clear indication that the overall N metabolism between the two groups differs and is driven by differences in gene expression of key N genes.

The data indicated minor differences in gene expression between the high N accumulating and low N accumulating groups but very large differences in expression between the groups when grown under limiting N. The next step was to analyze the data to identify genes that could influence this observation. If differentially expressed genes exist, it would strongly suggest sequence difference in a gene that is present in one group but absent in another and is probable that this would affect N accumulation and assimilation. A sequence difference, possibly a single nucleotide polymorphism, would provide an efficient DNA-based selection method to improve NUE in commercial lettuce. To explore this possibility and identify influential genes, the data were further analyzed using PCA analysis and the variation in gene expression plotted. Genes that are clustered together have similar expression and can generally be assumed as not causative to N accumulation. Those that are separated from the others are ones that are of interest and should be further explored.

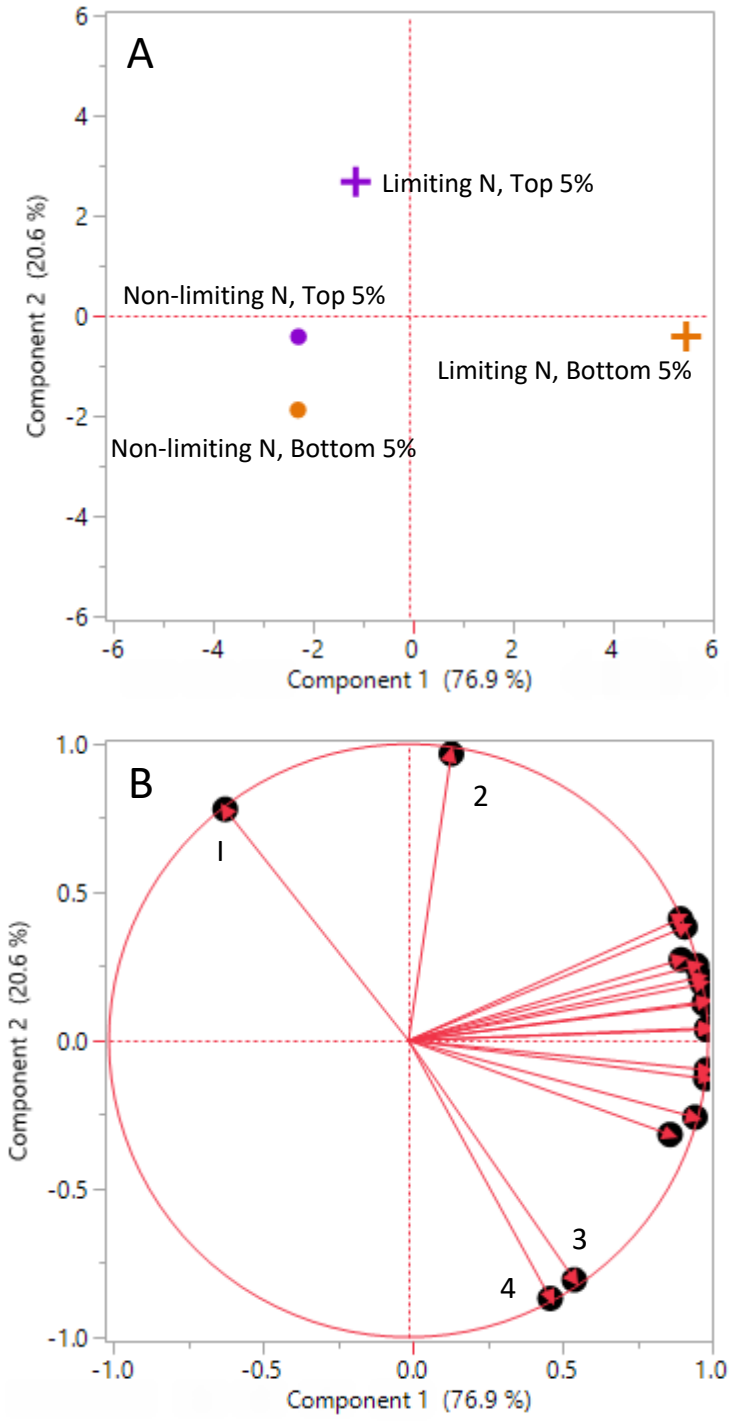

Figure 1. Principal component analysis (PCA) visually displays similarities and differences in gene expression in response to treatments (A) and identifies genes whose expression have greater influence (B) on leaf N concentration.

(A) Gene expression clustered by N treatments indicated similar expression between the top and bottom 5% under non-limiting N, but different expression when grown under limiting N. The gene expression of plants grown under limiting N treatments is indicated by "+" symbols, while plants grown under non-limiting N treatments are represented by "●" symbols. Genotypes representing the highest leaf N concentration (top 5% of the population) are represented by blue-colored symbols while the genotypes comprising the bottom 5% of leaf N concentration are colored orange.

(B) PCA clustered by genes indicated four genes, labeled 1-4, are influential in N uptake and assimilation and are differentially expressed under limiting or non-limiting N treatments.

Water and nitrogen are primary inputs that affect size and yield in lettuce and the application of both can be controlled by the grower. Our data presented herein and elsewhere, indicate that some commercial cultivars are amongst the best genotypes in accumulating leaf N when N is provided at amounts that do not limit growth such as under commercial growth conditions. Unimproved germplasm with the capacity to accumulate and concentrate high levels of leaf N were also identified. In this screening, four commercial cultivars were ranked in the top 10% in leaf N concentration. (Note that 10% of the screened population is equivalent to 13 genotypes.) Since all breeding programs have made selections under non-limiting N conditions, this is not a surprising finding. However, when N is limited, under both controlled and field conditions, none of the 57 commercial cultivars tested ranked in the top 10%. Of the 13 genotypes comprising the top 10% when grown under limiting N, two were *L. saligna*, one was *L. virosa* and the remaining ten were *L. serriola* accessions.

Adapting lettuce cultivars to use less water and nitrogen will help mitigate the negative effects of a warming environment and lessen the environmental impact of producing the crop. Nitrogen and water use efficiency in lettuce are complex traits affected by physiological traits that include nitrogen uptake, remobilization and assimilation but also are influenced by root morphology and growth. Genetic variation of these traits can be exploited and the genetic underpinning identified and used to develop improved germplasm for use in breeding programs.

With previous support from the CLGRB a recombinant inbred line (RIL) developed in our lab was evaluated in the field and under controlled conditions and quantitative trail loci (QTL) associated with whole-plant water use efficiency, nitrogen metabolism, nitrogen use efficiency and biomass accumulation were identified. The experimental results reported herein identified genotypes that accumulate and assimilate N to high leaf N concentrations when grown under limiting N, significantly above those of commercial cultivars. These species are sexually compatible with commercial lettuce and can be used to donate useful alleles to improve NUE.

Expression of genes associated with N uptake and assimilation indicated differences in gene expression between genotypes which accumulated high leaf N and those that did not have high leaf N concentrations. Four genes were identified as candidates for further investigation since they appear to influence leaf N concentration. We hypothesize that sequence variation (i.e., mutations) affect the ability to uptake and assimilate N under low N, and if this is true, an efficient and exact screening and selection tool can be developed to rapidly introgress and develop cultivars with improved NUE.