

## **SCREENING POTENTIAL LETTUCE BREEDING LINES FOR IMPROVED NUTRIENT USE EFFICIENCY**

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### **Abstract**

We have unraveled a physiological mechanism by which plants utilize a proton pyrophosphatase (H<sup>+</sup>-PPase) transporter to alter root activity and nutrient and water uptake. We have shown enhanced nutrient use efficiency under greenhouse and field conditions for plants genetically modified using molecular techniques, including romaine lettuce. However, consumers remain disinclined to accept food crops modified using molecular approaches. More recently, in preliminary screening we have found variation in transporter expression among existing lettuce lines, suggesting a prospect for achieving enhanced nutrient efficiency using traditional breeding techniques. Despite its potential as a groundbreaking advance in nutrient and water management, no efforts have been made to explore natural variation in H<sup>+</sup>-PPase expression in any crop species. The objective of this research is to perform preliminary screening of lettuce lines and evaluate growth on a recombinant inbred line to sub-optimal nutrient levels. The long term objective of this research is to identify breeding lines that can be used to develop more nutrient efficient lettuce types by traditional breeding techniques. Overall, the results from this preliminary analysis indicate a possible breeding path to improved nutrient use efficiency. Field studies are planned this coming season with funding from another source.

### **Introduction**

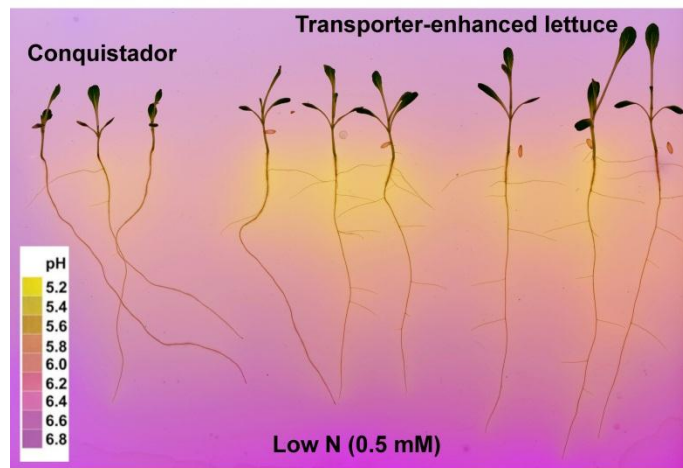
Growers in the desert of the southwestern United States use a range of cultivars depending on planting date (September through January) and risk of disease incidence. Thus, most of the yield improvements in lettuce over the past two decades have been due to breeding efforts for climatic adaptation (heat and cold stress, light adaptation) and disease resistance. In contrast, the prospect of improving nutrient and water use efficiencies of lettuce through breeding has received little attention. Previous research focused on yield has shown that range of lettuce genotypes and phenotypes respond similarly to high nutrient applications (Nagata et al. 1992; Sanchez and El Hout, 1995). However, little is known about the abilities of different lettuce cultivars to produce under water and nutrient resource limitations.

Lettuce produced in the desert receives large annual applications of nitrogen (N) and phosphorus (P) fertilizers. Amounts of N applied range from 200 to 400 kg/ha and crop recoveries are generally less than 50%. There are numerous possible fates of fertilizer applied N in addition to the desired outcome of crop uptake (Sanchez and Dorege, 1996;

Havlin et al., 2005). The urea and ammonium components of the N fertilizer might be lost through ammonia volatilization. The nitrate-N might be lost to leaching with irrigation water below the crop root zone possibly impairing surface and ground water. Nitrate might also be lost as N<sub>2</sub> and N<sub>2</sub>O gasses via denitrification processes affecting air quality and climate. Furthermore, all forms of N might be immobilized into the organic soil fraction by the soil microbial population where availability to the crop is delayed. Nitrogen fertilizer production depends on natural gas availability and prices.

Amounts of P applied to crop production systems often approach and exceed 200 kg P/ha and crop recoveries of P fertilizers are generally less than 20%. While much of the added P is converted to insoluble forms in the calcareous soils of the region (Sanchez, 2007), some of it is potentially carried off in runoff and drainage water into receiving surface waters having adverse ecological effects (Izuno et al., 1991). Further, erratic fertilizer pricing over the past several years has created incentives for improved efficiency. Approximately three years ago, the costs of mono-ammonium phosphate (MAP), a formulation widely used for desert vegetable production, exceeded \$1,200.0 per ton. Although costs have since declined, rapid increases are anticipated as the world economy recovers and resource demand in the developing world regains momentum. In addition, world P reserves are rapidly declining and there is concern that a shortage of P fertilizers will ultimately compromise world food production (Vaccari, 2009).

Work within our research group has shown that the mechanism for increased root and shoot growth in plants with enhanced transporter expression under drought or nutrient-poor conditions is related to auxin and sucrose transport to root systems and pathways of nutrient uptake within root cells (Li et al. 2005; Paez-Valencia et al. 2011; Zhang et al. 2011). Specifically, the H<sup>+</sup>-PPase protein complex regulates plasma membrane H<sup>+</sup>-ATPase in a manner that correlates with apoplastic pH alterations and rhizosphere acidification (Li et al. 2005; Paez-Valencia (in prep); Yang et al. 2007). Rhizosphere acidification is a central mechanism for plant mineral nutrition since it contributes to nutrient solubility and the plasma membrane proton motive force that allows transport of nutrient ions across root tissues. For example, in experimental laboratory trials, transporter-enhanced lettuce seedlings outgrow controls and show augmented rhizosphere acidification capacity when germinated in low nitrate or phosphate media (Figures 1). Evidence for augmented nutrient uptake is also found in transporter-enhanced *Arabidopsis*, rice and tomato plants (Gaxiola et al. 2011;



**Figure 1.** Root acidification capacity is augmented in transporter-enhanced lettuce. Lettuce seedlings that over-express H<sup>+</sup>-PPase outperform controls when grown in low NO<sub>3</sub><sup>-</sup> media.

Paez-Valencia (in prep); Yang et al. 2007). Interestingly, preliminary data on phloem sucrose exudation rates in transporter-enhanced *Arabidopsis* plants showed a two-fold increase when compared to controls (Gaxiola, in prep). We postulate that the enhanced capacity for nutrient uptake is energized by the greater availability of sugars brought about by augmented phloem transport. The commercial feasibility of using lettuce genetically modified by molecular techniques is currently being investigated by our group.

While we possess the technology to transfer the H<sup>+</sup>-PPase trait to plants using molecular techniques, society remains disinclined to consume genetically modified food. The enhancement of this H<sup>+</sup>-PPase expression trait through traditional breeding techniques would be more readily accepted by the public. More recently, we have obtained evidence that this trait may exist in existing lettuce lines based on variation in rhizosphere acidification capacity (Figure 2).

Seeds of both transporter-enhanced and 20 conventional cultivars were grown in nitrate limiting media in the presence of a pH indicator (Figure 2). These preliminary findings suggest that conventional lettuce cultivars will display phenotypes similar to our well-characterized transporter-enhanced lines, including improved performance under drought and low-nutrient conditions. The objective of this research is to perform some preliminary in-vitro screening of inbred lettuce lines for H<sup>+</sup>-PPase expression.

## **Materials and Methods**

Seventy-four inbred lettuce (*Lactuca sativa*) lines derived from the cross between the parental lines *Parade vs. Pavane* were used in these experiments. These breeding lines were grown in nutrient agar media containing sub-optimal levels nitrogen (0.5mM nitrate-N). In experiment one, the lettuce were grown under short days, consisting of 12 hours light at 400 mΣ and 12 hours dark, and under long days consisting of 16 hours light at 1,400 mΣ and 8 hours dark.

Lettuce seeds were grown in 10 x 10 inch media plates containing 0.5 strength Hoagland's nutrient solution modified to provide sub-optimal levels of N. Bromocresol purple dye was utilized as a pH indicator to monitor acidification associated with the root rhizosphere. The nutrient solution was adjusted to a pH of 6.8 and 2-N-morpholino ethanesulfonic (MES) was used as a buffer at a concentration of 1 mM. The media plates contained five lettuce seeds from each of nine inbred lines.

There were eight separate media plates for each experiment, and once the seeds from each parental and inbred line were plated as described above, the plates were incubated in a growth chamber. The growth of each plant, its leaves color, and root acidification were monitored on a regular basis through constant observation and photographic records. At the end of each experiment, fresh weights from shoots and roots were evaluated.

QTL analyses were conducted with the data using multiple intervals mapping in the program QGene 4.3 (<http://coding.plantpath.ksu.edu/qgene/>). Permutation was used to determine LOD significance levels.

## Results and Discussion

The results from experiment 1 (short days) and experiment 2 (long days) were similar. Only the short day data are shown in Figures

The parental breeding line *Pavane* had enhanced root acidification and shoot weight compared to *Parade*, when grown under N limiting conditions (Figure 2 through 6). The average shoot weights were 0.068 and 0.051 g for *Pavane* and *Parade*, respectively. The total biomass of shoots ranged from 0.03g to 0.09g among inbred lines. Interestingly, the shoot weights for some of the stronger inbred lines exceeded that of *Pavane*. For example, lines B9, B17, C1, and C21 produced shoot weights of 0.074, 0.073, 0.084, and 0.074 g, respectively. The shoot and root weight data were generally correlated (data not shown) but there were exceptions, indicating root to shoot ratio may be important as well.

The results from QTL mapping are shown in Tables x, x, and x and Figure x). There are many different methods for QTL mapping. In this case we used three methods, single marker regression (the simplest), composite interval mapping (the most commonly used), and multiple interval mapping (the most complicated). The results were not the same for each method, and I'm still trying to figure out why. Each method does not typically yield identical results, but they should be approximately the same. I have a different software package that may help resolve this, but I will need to reformat the three data files to do this. I'll email you when I get it done.

Composite interval mapping detected a significant QTL for shoot weight using the mean weight from the two experiments. Higher shoot weight was inherited from *Parade*. These were the only QTL for shoot weight that we found, and composite interval mapping found no QTL for root weight.

Single marker regression and multiple interval mapping detected significant QTL for root weight in experiments 1 and 2, and using the mean of these experiments... No QTL for shoot weight were found using single marker regression or multiple interval mapping. In all cases the QTL for root weight were on linkage group 2 and higher root weight was inherited from *Parade*. An advantage of multiple interval mapping is that it provides more precise estimates of the QTL location on the linkage group. With the root weight data, the positions of the QTL in experiments 1 and 2 are both on linkage group 2, but not the same spot on linkage group 2 (Figure 6) In this graph, the x-axis shows the order of the molecular markers on linkage group 2. The y-axis is the proportion of the phenotypic variation explained ( $R^2$ ) by each position on the linkage group. Higher the peaks on the graph equates to a stronger correlations between the molecular marker and higher root weight. The peaks indicate the approximate location of the QTL. For experiment 2, the root weight QTL location is between markers ANEX and BHOD. For experiment 1, the root weight QTL location is between markers BHUT and ~APOY. As it stands right now, this indicates that we may have 2 different QTL for the same trait. These results

can be influenced by a few aberrant root weight values due to environmental effects or miss-scoring the trait. More data is needed to clarify these effects.

Overall, the results from this preliminary analysis indicate a possible breeding path to improved nutrient use efficiency. Field studies are planned this coming season with funding from another source.

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Table 1. Results for single marker regression for shoot and root weight in incubation experiment.

Trait	QTL Mapping Method	Position Linkage Group	2 LOD interval cM	Additive Effect	R2	Allele increasing weights
Root weight (Expt. 1)	SMR	2a	12.7-36.8	-0.001	0.40	Parade
Shoot weight (Expt. 1)	SMR	None found				
Root weight (Expt. 2)	SMR	2a	12.7-33.2	-0.002	0.40	Parade
Shoot weight (Expt. 2)	SMR	None found				
Root weight (combined)	SMR	2a	12.7-36.8	-0.002	0.18	Parade
Shoot weight (combined)	SMR	None found				

Table 2. Results for composite marker regression for shoot and root weight in incubation experiment.

Trait	QTL Mapping Method	Position Linkage Group	cM	Additive Effect	R2	Allele increasing weights
Root weight (Expt. 1)	CIM	None found				
Shoot weight (Expt. 1)	CIM	None found				
Root weight (Expt. 2)	CIM	None found				
Shoot weight (Expt. 2)	CIM	None found				
Root weight (combined)	CIM	None found				
Shoot weight (combined)	CIM	7a 8	34-36 56-60	0.004 0.006	0.27 0.22	Parade Parade



**Table 3.** Results for multiple interval marker regression for shoot and root weight in incubation experiment.

Trait	QTL Mapping Method	Position Linkage Group	2 LOD interval cM	Additive Effect	R2	Allele increasing weights
Root weight (Expt. 1)	MIM	2a	34-36	-0.001	0.40	Parade
Shoot weight (Expt. 1)	MIM	None found				
Root weight (Expt. 2)	MIM	2a	16-18	-0.002	0.19	Parade
Shoot weight (Expt. 2)	MIM	None found				
Root weight (combined)	MIM	2a	16-18	-0.002	0.26	Parade
Shoot weight (combined)	MIM	None found				



Figure 2. Reference pH colors used in rhizosphere acidification screening.

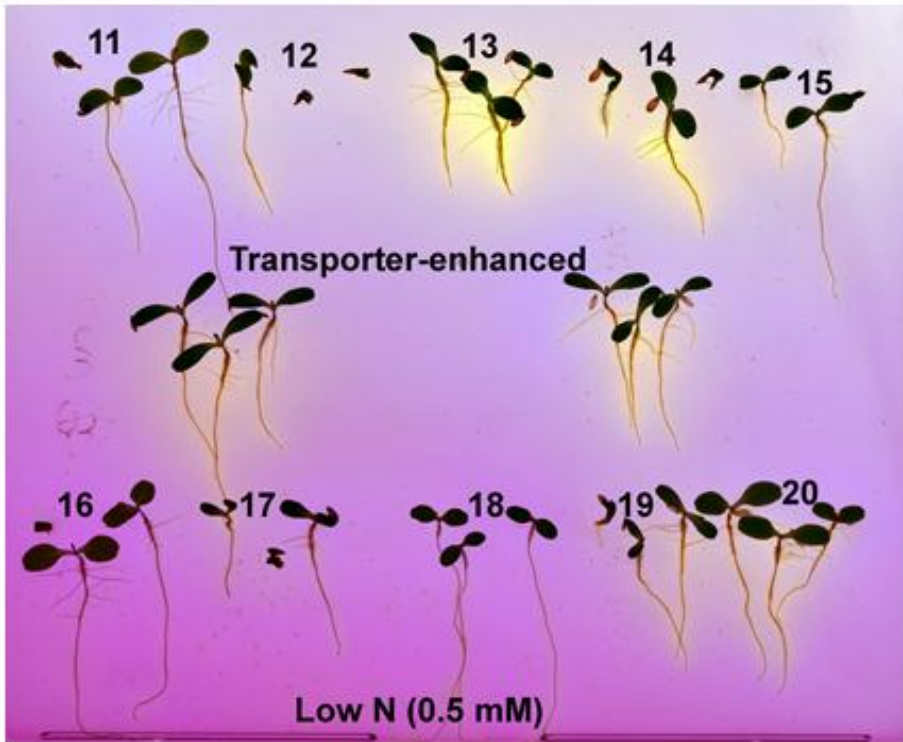


Figure 2. In vitro evaluation of selected lettuce lines from a USDA-ARS collection. Note the lower vigor and less rhizosphere acidification of Parade (#12) compared to Pavane (#13). It was recombinant inbreds from this cross used in these studies.

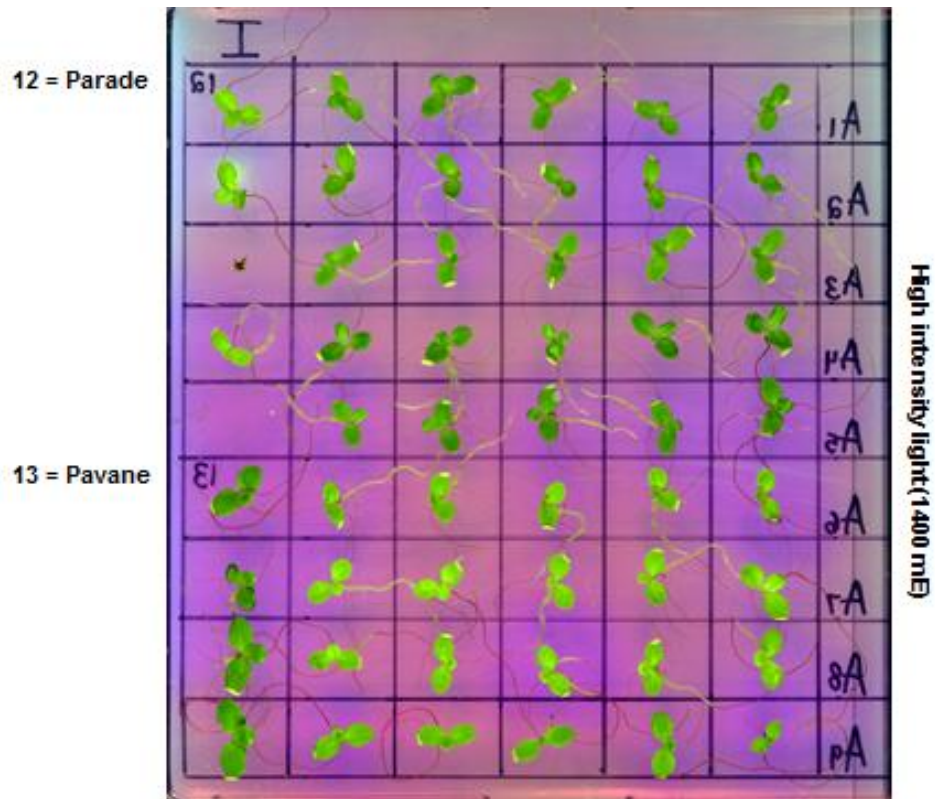


Figure 3. Photograph showing early growth of a subset of the RILs. The first column is the parents. Left of this column are nine of the inbreds resulting from the parental cross.

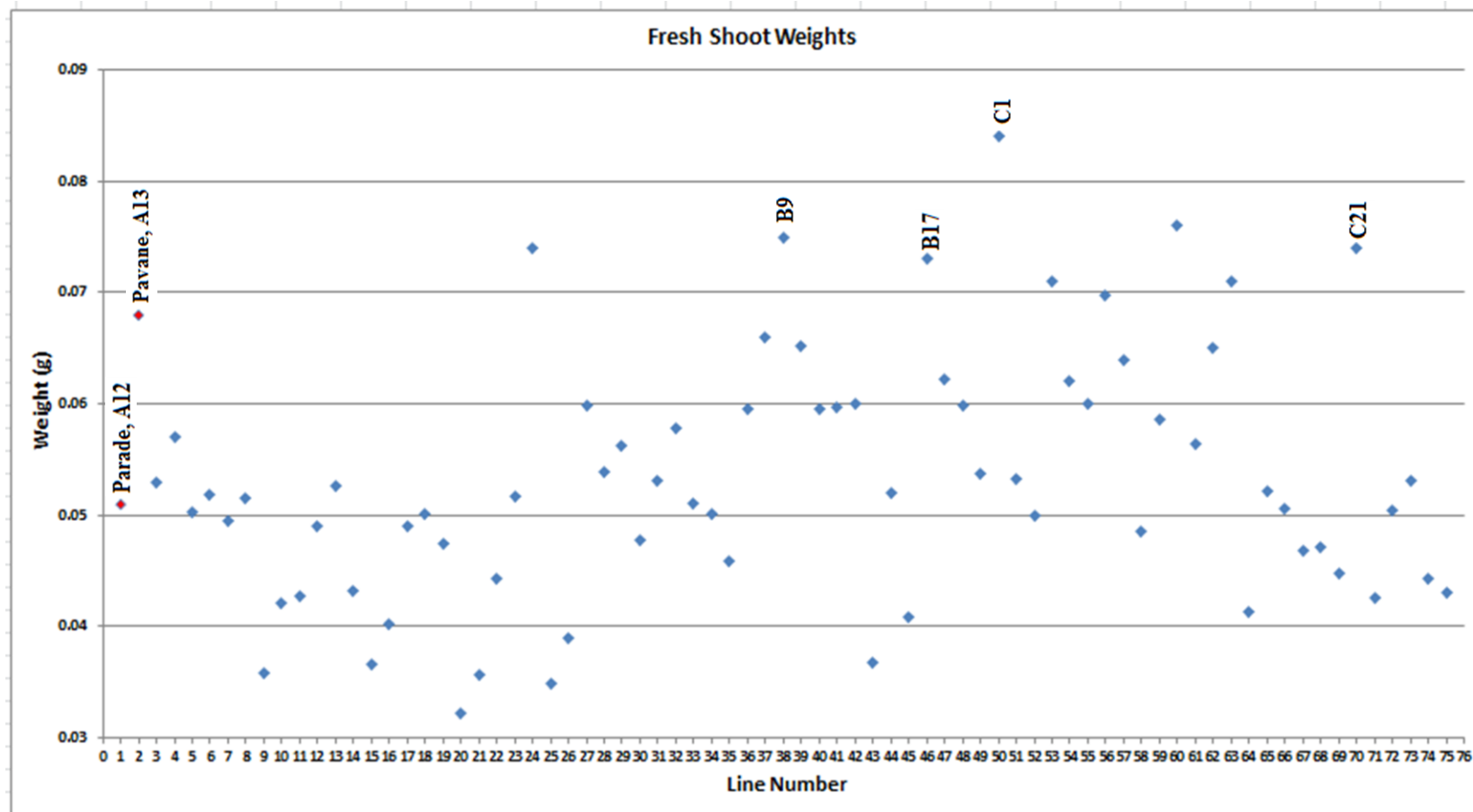


Figure 4. Mean fresh shoot weights of inbred lines in short day experiment.

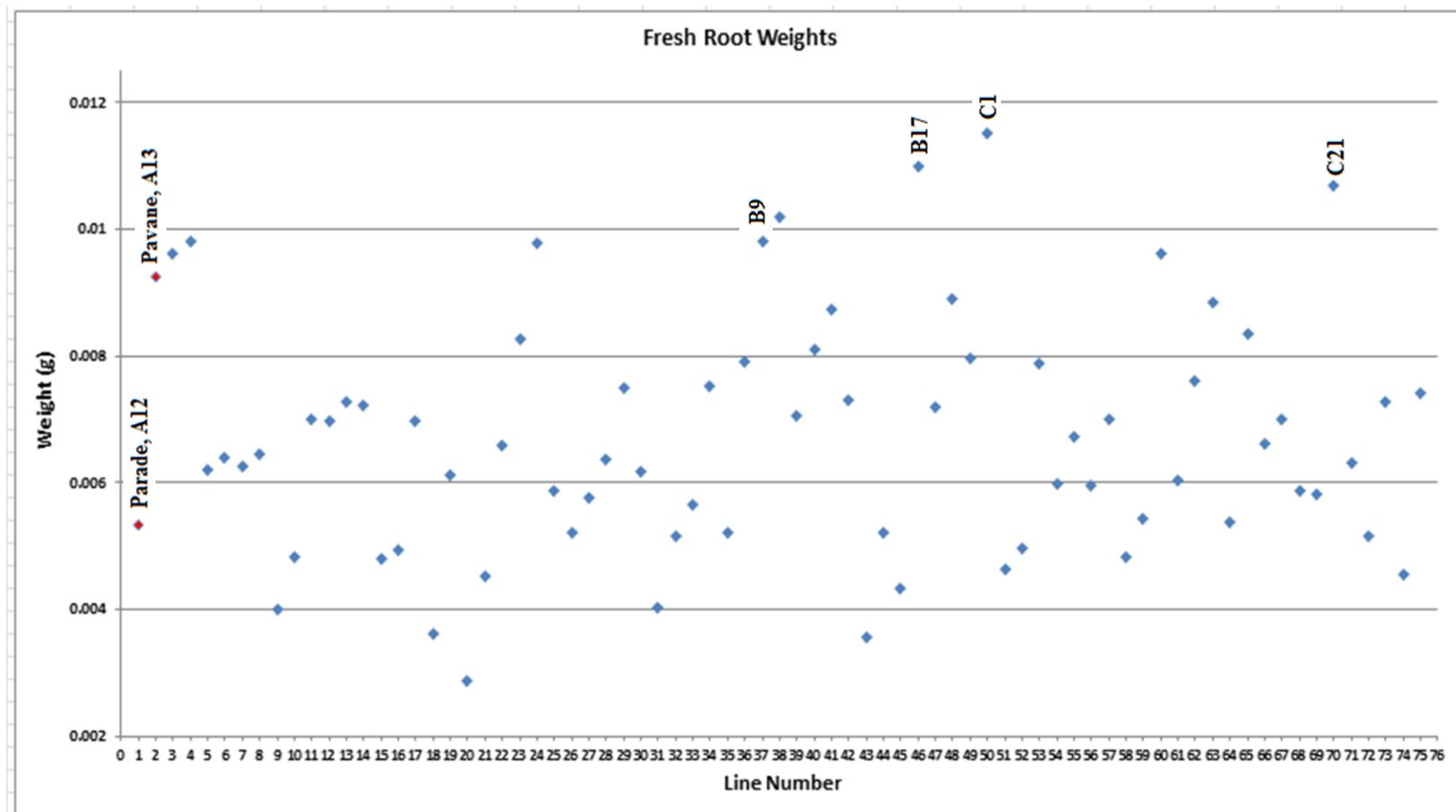


Figure 5. Mean fresh root weights of inbred line in short day experiment.

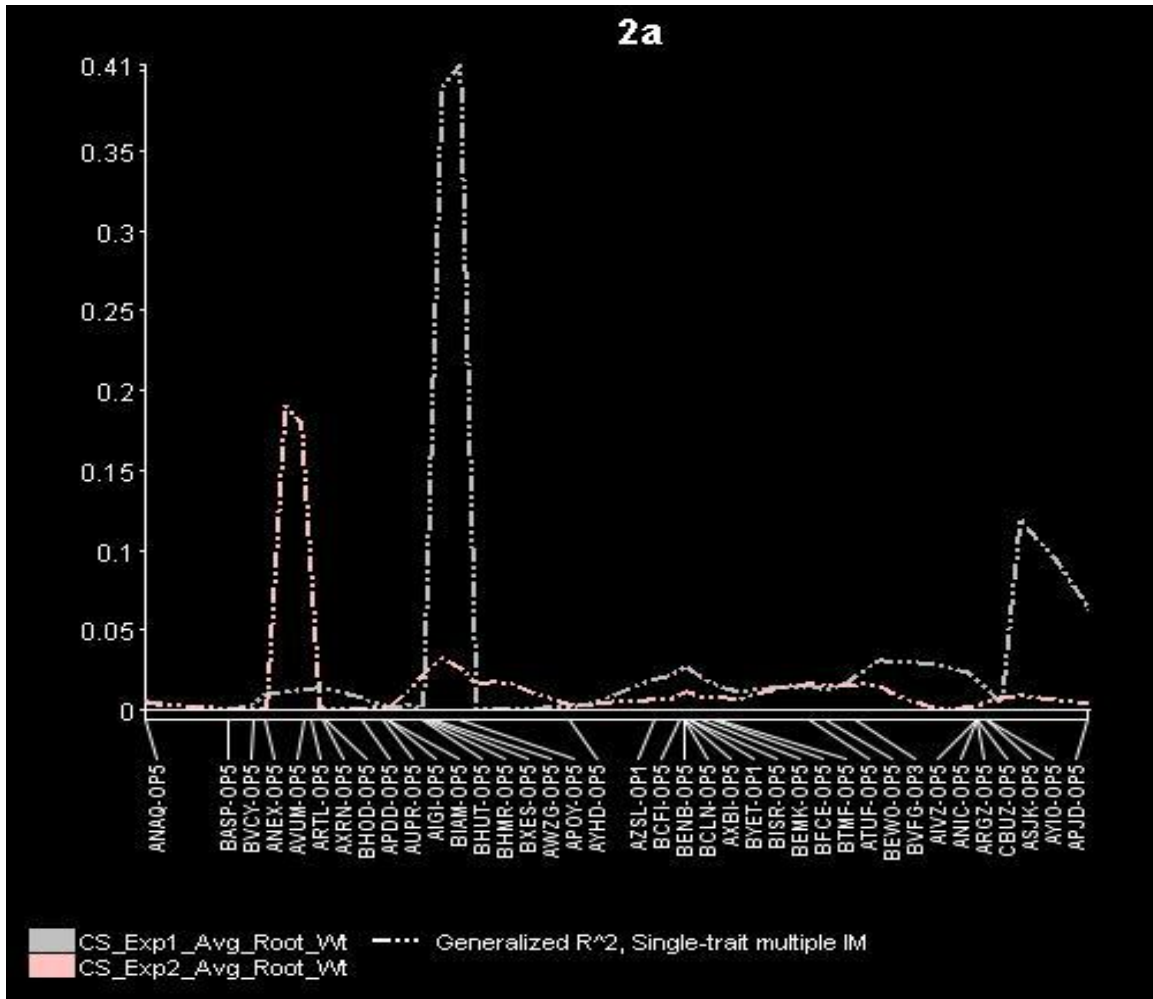


Figure 6. Relationship between order of molecular marker on linkage group 2 (x axis) versus the phenotypic variation by each position on the linkage group.