

Sustainable use of water in baby leaf cropping systems

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Summary

Water resources are crucial for crop production but are becoming more unpredictable due to climate change and human population growth. It is therefore vital that the water requirements of this sector are reduced. Consequently, we are applying a combined agronomic and genetic approach focused on a mapping population of 214 recombinant inbred lines. This population has been produced using the cultivated lettuce (*Lactuca sativa* cv. Salinas) and the wild subspecies (*L. serriola* US96US23) as parents. *L. sativa* is thought to have originated from *L. serriola*, yet the two show clear morphological differences, due to the domestication of the former. Alongside thermography, a range of plant-based measurements have been applied to determine plant water use under drought in a glasshouse experiment in order to elucidate the genetic basis of water use efficiency. We have identified phenotypic variation in the mapping population that will enable us to map quantitative trait loci (QTL), which will form the basis for improving commercial crops using marker assisted selection.

Key words: Water-use, lettuce, sustainable, irrigation, QTL

Introduction

Cultivated lettuce is eaten widely throughout the world and is of significant economic value with the UK and USA's gross production of lettuce and chicory reaching 117,000 MT and 4,360,400 MT respectively (FAO, 2007). However, water resources are beginning to limit this production due to competition from other sectors, mainly driven by a growing human population, alongside the unpredictable effects of climate change (IPCC, 2007). Ensuring efficient irrigation, both in terms of scheduling and application of water, during the cultivation of these drought sensitive salad crops is therefore crucial, as is the efficiency with which this water is utilised by the crop. The latter, genetic, approach is the target of this paper. Although the genetic control of water use traits has been focussed on previously in a variety of crops and progress has been made (Hong *et al.*, 2000; Rebetzke *et al.*, 2002; Juenger *et al.*, 2005; Monneveux *et al.*, 2006; Richards, 2006; Tuberosa & Salvi, 2006; Chaves *et al.*, 2007; Flexas *et al.*, 2010), lettuce has not yet been targeted for improvement of these traits.

The lettuce genome is not yet fully sequenced, however an Affymetrix high density GeneChip® microarray has been designed to detect single feature polymorphisms (SFPs) in over 35,000 lettuce genes. The tiling array comprises 6,553,600 cells, each of which contains a 25 bp DNA probe, which

has been designed using lettuce expressed sequence tags (ESTs). For each of the 35,000 genes, there is a large number of probes (up to 2,000) allowing the identification of DNA polymorphisms (Gupta *et al.*, 2008). The identification of SFPs occurs through labelling of DNA, which is then hybridised to the tiling array, and therefore the probes within it (Gupta *et al.*, 2008). This has led to the development of a high density molecular map specific to the recombinant inbred line (RIL) population, which contains over 10,000 Single Feature Polymorphisms (SFPs) throughout the nine major linkage groups, which span 1,342 cM (Kozik *et al.*, 2009). Once genetic diversity has been measured in the RIL mapping population it will be possible to find links between DNA markers and important agronomic traits. Further to this, candidate genes which underlie quantitative traits such as water use efficiency can be identified for future marker assisted selection in breeding programmes (Truco *et al.*, 2007). This approach has been previously used in lettuce to identify genes regulating root architecture (Johnson *et al.*, 2000), seed and seedling traits (Argyris *et al.*, 2005; Argyris *et al.*, 2008; Schwember & Bradford, 2010), shelf-life and processability (Zhang *et al.*, 2007) and disease resistance (McHale *et al.*, 2009). This analysis has thus far not been applied to water use traits in lettuce, apart from those associated with root architecture, since previous work has focused on economic traits rather than those which would benefit the environmental impact of crop production. It is only now, with shifts in water availability and crop requirements that water, as a resource, is taking on significant economic value. Moreover, owing to the limited nature of this resource and given that it is key in the provision of many ecosystem services, it is one that should be preserved for current and future generations (UNMillenniumProject, 2005).

The focus of this study is to identify variation across a mapping population for water use efficiency (WUE) in lettuce, from which quantitative trait loci can be identified and candidate genes elucidated. These genes have the potential to be used in future breeding programs where a crop that produces 'more leaf per litre' will be developed.

Materials and Methods

Experimental design

The mapping population of 214 RILs is derived from the parents *L. serriola* (US96US23), which is a wild lettuce, and *L. sativa* cv. Salinas, a cultivated lettuce. The seed material used for both the parents and the RILs was supplied by R W Michelmore (University of California, Davis). Five seeds of each RIL were planted for germination in a 7.5 cm × 7.5 cm pot in a 1:1 mixture of vermiculite (Sinclair) and Humax nursery stock compost (Professional range). Five pots of each of 209 RILs and 10 of each parent were planted. Once germinated the plants, which had at least three replicates, were thinned to one plant per pot and arranged in a random design with three replicates of each RIL and six of each parent in trays where they were watered from the base for the duration of the experiment. The randomised design was constructed using the statistical software package Minitab 15.0 for Windows (Minitab Inc., Philadelphia), with two rows of guard plants (green romaine cv. Lexington) surrounding.

Diurnal measurements of the parents

Diurnal stomatal conductance and transpiration were measured on the parents of the RILs at 26 days of growth under well-watered conditions. Six plants of each parent were used for the experiment where measurements of transpiration, stomatal conductance, leaf temperature, quantum flux and relative humidity were taken using a portable steady state porometer LI-1600 (LI-COR Biosciences, Germany) with data being handled manually. After each leaf had been measured with the porometer it was placed on a scaled background for infra-red imaging of the fifth leaf using a TH9100WR (NEC, Metrum). These measurements were taken at ambient temperature every two hours between 5 am and 11 pm and were conducted on the abaxial side of the third leaf. Leaf number was based on the order of leaf development, with leaf one being the oldest and leaf six

the youngest. Sampling consisted of alternating between replicate parents to minimize the effect of taking measurements at different times.

Drought trial of full recombinant inbred line population

At 27 days of growth, water was withheld for 4 days prior to the start of measurements, where the plants were measured between 9 am and 3 pm using a stratified method where a replicate of each line was measured before the next replicate of the same line was sampled and so on. The aim of this was to reduce the effect of the plants maturing over the course of the 5 day experiment. A Delta-T ML2× ThetaProbe connected to an HH2 moisture meter (Delta-T Devices, Cambridge, UK) was employed to ensure the plants were maintained between 10% and 20% moisture (percent volume) during the experiment so that measurements could be taken with the LI-1600 and TH9100WR as previously explained (data not shown). Plants were watered sparingly from above every two days to ensure that they did not over-dry as this may have caused irreparable damage to the plants.

Between the 37th and 40th days of growth all plants were harvested for biomass data. The plants were cut at the stem base and weighed for fresh weight using a top loading Sartorius Analytic balance (AC120S, Sartorius, USA). These samples were initially oven dried at 80°C for 30 h before air drying for one month prior to a further 48 h of oven drying at 80°C to remove residual moisture so that the plants could then be weighed for dry weight. Dry plant material was stored in paper bags within airtight plastic boxes which also contained small bags of silica.

Results

Diurnal patterning of the parent lines

The parents of the recombinant inbred lines (*L. sativa* and *L. serriola*) showed significant variation in their diurnal pattern of stomatal response both in terms of transpiration (Fig. 1) and stomatal conductance (data not shown).

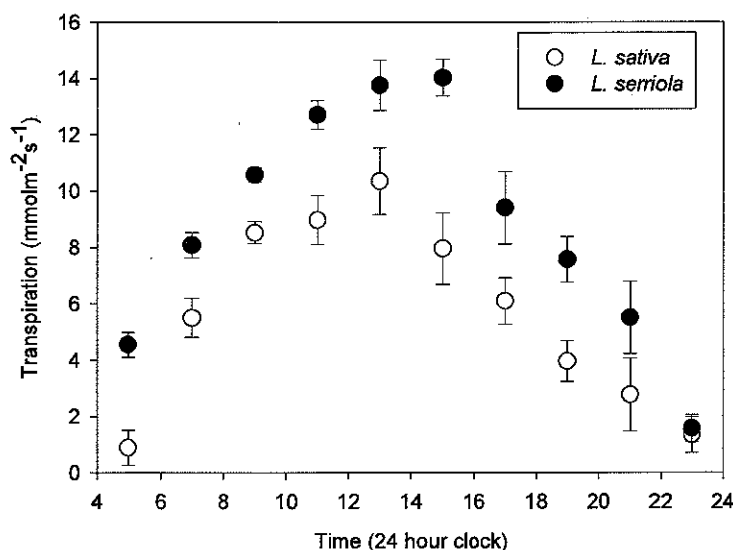


Fig. 1. Diurnal transpiration patterns of the parents *Lactuca sativa* and *L. serriola* measured over an 18 h day with standard error bars.

The diurnal pattern of transpiration and stomatal conductance rose from 5 am until between 1 pm and 3 pm when it declined until measurements ceased at 11 pm. The differences observed for these two responses were each tested using a two way analysis of variance. Significant differences were found between time points for each parent with respect to transpiration ($F_{9,99} = 47.58$,

$P < 0.0001$) while the parents also differed significantly from one another in terms of this trait ($F_{11,99} = 11.14$, $P < 0.0001$). This was also true of the parents' diurnal stomatal conductance with variation between time points ($F_{9,99} = 27.59$, $P < 0.0001$) and between the parents ($F_{11,99} = 12.58$, $P < 0.0001$) (data not shown).

Trait correlations under mild drought conditions

Trait correlations were conducted upon the means for each genotype in order to eliminate variability introduced by sampling throughout, and over, days. Normality was confirmed with the Shapiro-Wilk test for all data sets and when data was not normally distributed it was transformed using a \log_{10} transformation.

Trait frequency distributions under mild drought conditions

The means of the data from the glasshouse drought experiment were taken forward for trait frequency analysis and normality was again confirmed using Shapiro-Wilk tests.

The distribution of traits in the mapping population all followed a normal bell-shaped distribution (Fig. 2) and are thus indicative of quantitative traits, which can be analysed using QTL analysis. The plots also show substantial variation with relation to stomatal conductance, indirect leaf temperature measurement and freshweight, with genotypes varying from 50–450 $\text{mmol m}^{-2} \text{s}^{-1}$, 21–27°C and 1.0–4.5 g respectively.

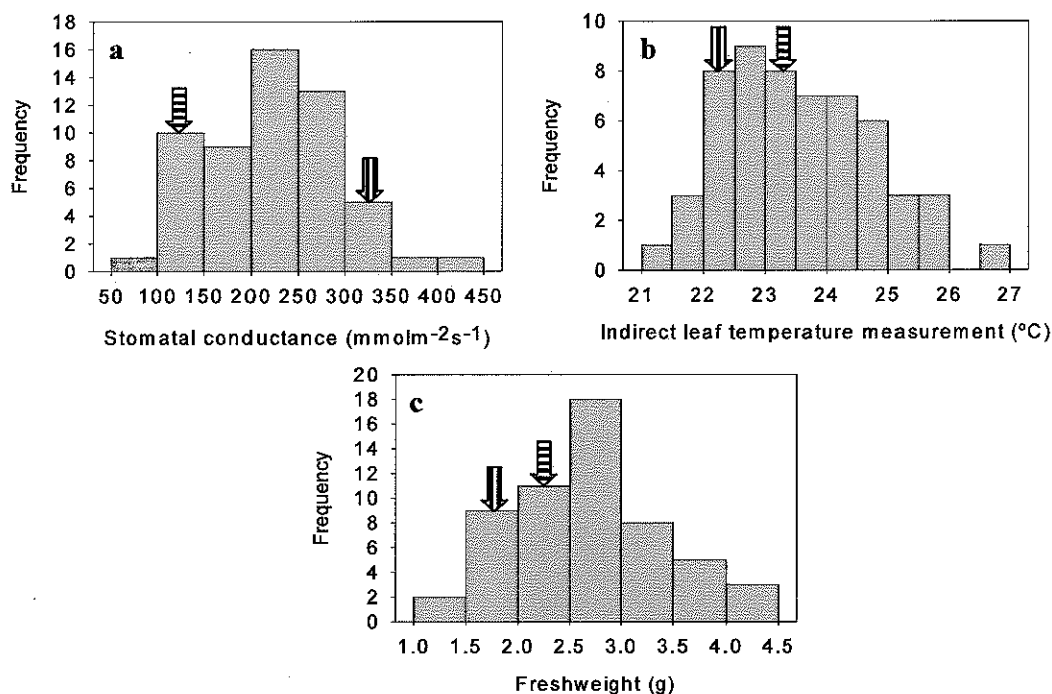


Fig. 2. Frequency plots of the distribution of traits in the recombinant inbred line mapping population under glasshouse mild drought conditions. Stomatal conductance (a), indirectly measured (infra-red imaging) leaf temperature (b) and fresh weight (c). Horizontally striped arrows indicate *L. sativa* and vertically striped arrows refer to *L. serriola*.

The large variation in leaf temperature can be most clearly seen when contrasting thermal images are viewed alongside one another (Fig. 3). The genotypes shown in Fig. 3 exhibited one of the lowest and one of the highest leaf temperatures of the recombinant inbred lines mapping population and were imaged on a single day thus reducing the likelihood of environmental interference. The mean temperature of these plants varied by 5.4°C, with average temperatures of 20.6°C and 26.0°C respectively. The trial also showed other lines varying by 11.7°C with some extremes ranging from 18.9°C to 30.6°C.

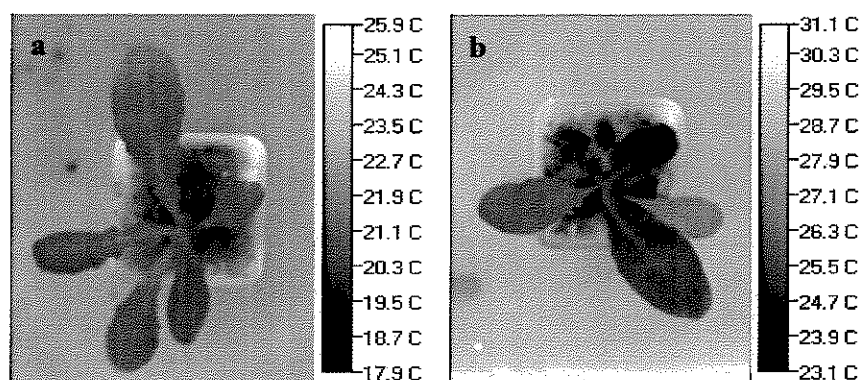


Fig. 3. Thermal images of recombinant inbred lines showing contrasting leaf temperatures when imaged on a single day. The wild parent *L. serriola* demonstrated one of the lowest leaf temperatures during the drought trial (a) and one of the recombinant inbred lines showed the highest leaf temperatures (b). The grey-scale palettes indicate the temperature variation of each corresponding image.

Table 1. Correlation matrix for all traits when the recombinant inbred lines were subjected to drought conditions in the glasshouse. All trait correlations are parametric Pearson's regressions upon the means. $P < 0.05$ *; $P < 0.005$ **; $P < 0.0005$ ***

Traits	Transpiration	Stomatal conductance	Direct leaf temperature (Porometry)	Indirect leaf temperature (Infra-red imaging)	Fresh weight	Dry weight	Fresh: Dry weight
Transpiration	-	55.9% ***	10.3% ***	2.0% *	5.2% **	0% ns	10.6% ***
Stomatal conductance	55.9% ***	-	3.5% *	6.7% ***	4.8% ***	0.2% ns	13.9% ***
Direct leaf temperature (Porometry)	10.3% ***	3.5% *	-	69.5% ***	0% ns	0% ns	0.1% ns
Indirect leaf temperature (Infra-red imaging)	2.0% *	6.7% ***	69.5% ***	-	0.3% ns	0.1% ns	2.9% *
Fresh weight	5.2% **	4.8% ***	0% ns	0.3% ns	-	1.1% ns	0% ns
Dry weight	0% ns	0.2% ns	0% ns	0.1% ns	1.1% ns	-	3.6% *
Fresh: Dry weight	10.6% ***	13.9% ***	0.1% ns	2.9% *	0% ns	3.6% *	-

Discussion

Precipitation patterns are expected to shift in coming centuries with an increasing frequency of drought periods, some of which will become more serious in terms of intensity (IPCC, 2007). To adapt to this changing environment it is necessary for plant breeders to better understand the mechanisms underlying crop responses to drought. Drought has long been a major abiotic stress limiting crop production worldwide and has thus been the focus of many breeding efforts in the past. However, these efforts have been impeded due to the complexity of the physiological basis of water use, complicated by its intricate links with yield, especially under water limited conditions (Passioura, 2002; Blum, 2005). Stomatal closure, while acting to decrease transpiration so as to

maintain homeostatic water potential and avoid severe dehydration (Tardieu, 2005), also limits biomass accumulation through decreasing CO₂ uptake. When less carbon is able to enter the plant, photosynthetic capacity will be intrinsically limited and this causes a conundrum in terms of creating high biomass, drought resistant plants (Blum, 1998). If plants are modified, either through breeding, evolution or engineering, to have decreased stomatal conductance then heat stress cannot be buffered through increased transpiration and its associated cooling effects (Jones *et al.*, 2002), leading to problems with plant growth and development. If these contradictions are serious then it may be that drought resistance is not a trait that can be achieved through the manipulation of a classical resistance process regulated by a series of genes but rather a trade-off limited by other constraints. Despite the difficulty in disentangling this relationship between plant water use and yield, there has recently been progress in breeding wheat in Australia for a future with a drier climate (Richards, 2006; Hochman *et al.*, 2009).

The diurnal pattern of transpiration for both parents, showed a classic response over the course of an of an 18 h day, with increased opening of the stomata from 5 am until 1 pm in line with the rising levels of sunlight experienced. Transpiration then decreased steadily as the stomata began to close until measurements ceased at 11 pm. However, the two parental lines differed significantly from each other throughout this time course. The wild, *L. serriola*, transpired at a higher rate, with increased stomatal conductance, when compared to the cultivated *L. sativa* (Figs 1 and 2a) and this is likely to be due to commercial breeding in *L. sativa*. As a cultivated crop *L. sativa* has been selected to develop as a succulent leafed plant which will accumulate high biomass as this is where its economic value lies. To produce such a crop, selection for plants which preserve more of their water content to contribute to fresh weight has been common and often this has indirectly selected for plants with low transpiration rates. Furthermore modern cultivars are generally selected for high input systems, where water is not a limiting factor while wild relatives have often adapted to survive under drought conditions (Tuberosa & Salvi, 2006). Leaf temperature was lower in *L. serriola* when compared to that of *L. sativa* (Figs 2b and 3) also indicating the higher transpiration of the former, which allows the wild parent to cool more rapidly. Wild progenitors of cultivated crop species are often able to survive in more stressful and unpredictable soil environments, with respect to water, when compared to that of their cultivated relatives (Chapin *et al.*, 1989) and often possess resistance genes which could usefully be introgressed into cultivated lines (Tanksley & Nelson, 1996).

The mapping population demonstrated a significant positive correlation between both measures of leaf temperature with transpiration and stomatal conductance and this follows the physiology of the plant with respect to water relations (Table 1). In response to leaf temperature rising, transpiration is increased in order to dissipate excess heat. However, although the plant can afford to do this while water is not limited (Campalans *et al.*, 1999; Chaves *et al.*, 2003), it would be expected that under drought the plant would not be able to respond in this way. When this is the case, heat stress will occur alongside the drought stress and this is often fatal to the plant (Chaves *et al.*, 2003). With increased stomatal conductance (and transpiration) there is a slight decrease in fresh weight under drought conditions, while there is no effect on dry weight and a positive correlation with the fresh to dry weight ratio (Table 1). Clearly as plants transpire more they lose water which would otherwise have contributed to their fresh weight meaning that plants with a higher transpiration attain lower fresh weights but with unaffected dry weights. It is clear that in a commercial cropping situation lower transpiration rates would be preferable so that more biomass could be yielded of a higher quality. This quality would be in terms of processability since a higher water content in the leaf means that the cells are more turgid and also in terms of texture since the leaves would be thicker and firmer (Clarkson *et al.*, 2003). The relationships between water use traits and biomass accumulation are of critical importance for breeding purposes when applied to crops since yield must not be sacrificed in order to attain a drought resistant crop and it is a question of maintaining yield under stress conditions that is important (Tardieu, 2005).

Traditionally breeding programmes have selected for phenotypes of interest, which can be altered by environmental effects and thus may not always reflect the genetics of the plant. A move towards selection of specific molecular markers allows the evaluation of genetic and environmental effects and interactions making it possible to uncover markers with limited environmental regulation. Furthermore this approach also means that the trait can be selected for at any stage of development and so the process of breeding may be accelerated (Francia *et al.*, 2005) thus creating a high throughput system of breeding. The identification of variation within the mapping population with respect to these water use traits (Figs 2a, b and c) allows for quantitative trait loci analysis to be undertaken, which will identify areas of the genome using marker information which control water use in lettuce. Furthermore marker assisted selection (MAS) can be applied to these QTL, with optimal success when a genetic map which has markers tightly linked to the QTL of interest is used and when there are plentiful recombinations between these markers and the remaining genome. This approach also has the ability to screen a large number of individuals time and cost effectively which is also of crucial in modern breeding programs (Francia *et al.*, 2005).

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