California Leafy Greens Research Program

Final Report

April 1, 2018 to March 31, 2019

Project Title: IMPROVING THE POST-HARVEST QUALITY AND SHELF LIFE OF LETTUCE

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ABSTRACT

Poor shelf of whole baby leaf lettuce results in significant post-harvest losses, which impact the economic value and sustainability of production – up to 45% of fruit and vegetable production may be lost in this way, but at the same time, post-harvest quality and shelf life is not a priority for big seed houses and breeding programs. Thus, bespoke research is required from academia and commodities. Alongside this, consumers are becoming increasingly aware of the importance of good nutrition in promoting health. In this project, we aim to increase the post-harvest quality of baby leaf lettuce through genetic improvement. By characterizing the variation in post-harvest quality traits, including shelf life and nutritional quality, we use tools such as quantitative trait loci (QTL) mapping and genome wide association studies (GWAS) to link phenotype to genotype, to elucidate the genetic architecture of these traits which will inform marker-assisted breeding programs. During the first year of this new project, we have undertaken two field trials to assess the post-harvest quality of two populations of lettuce; a wild x cultivated RIL population developed by the Michelmore group and a population of diverse cultivars and breeding lines from the USDA. We have identified robust QTL for shelf life which have mapped alongside those obtained in previous mapping studies, providing strong targets for breeding in different environments and provide candidate genes for further exploration. Genome-edited lines, in which candidate genes for shelf life have been knocked-out, were assessed for performance, indicating an increase in shelf life of more than 3 days. Having developed a successful research pipeline, we will continue to utilize genome-editing to functionally investigate candidate genes for post-harvest quality.

OBJECTIVES

1. To deliver a refined set of candidate genes that underpin QTL for shelf life and nutritional quality, following mining of the lettuce genome and analysis of our extensive data-sets.
2. To fine map these candidate genes - QTL associations using an association genetics approach already underway in the Michelmore Laboratory with a wide population and undertaking GBS (genotyping by sequencing) and association analysis to confirm trait-gene associations.

3. To use all biotechnological tools available but likely to be CRISPR-Cas, to provide proof of concept for the functionality of the selected target genes, including gene stacking.
4. To develop robust markers, made available to screen large pools of germplasm for future molecular breeding.

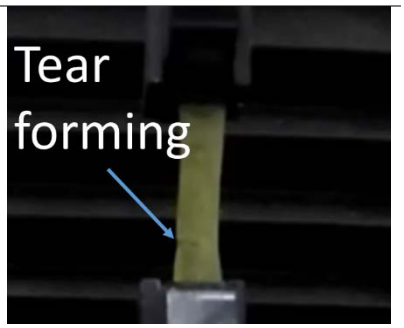
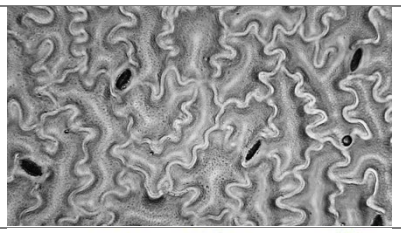
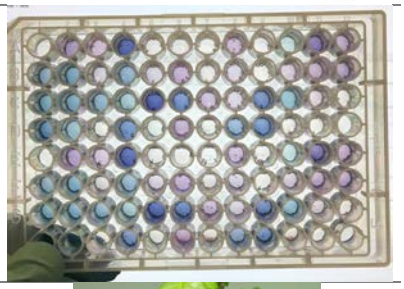
PROCEDURES

Objective 1

Previous research in the laboratory of Gail Taylor has utilized the core lettuce mapping population and resources (Truco *et al.*, 2013) developed by the Michelmore laboratory to identify Quantitative trait loci (QTL); regions of the genome associated with the phenotype of interest. This is a population of recombinant inbred lines (RILs) developed from a cross between the Salinas cultivar and the expected wild progenitor of lettuce, *Lactuca serriola*. Using our extensive phenotype data on this population (see Table 1) collected from field trials on commercial farms in the United Kingdom and Portugal, as well as greenhouse trials, we conducted analyses to identify candidate genes for shelf life and nutritional quality traits. QTL from each of the trials were mapped together to identify genomic regions of interest. Positions where QTL for shelf life measured in different environments and estimated to control a high percentage of the phenotypic variation were selected for analyses (Figure 1A). Other positions of interest included those in which QTL for shelf life mapped to the same position in the genome as QTL for cell wall traits, such as cell wall strength, elasticity and plasticity. Molecular markers boarding these QTL regions were located in the lettuce genome and all predicted genes were retrieved using the lettuce genome resource (LGR: http://lgr.genomecenter.ucdavis.edu/, Figure 1B). Genes were annotated to determine predicted gene function by using the Basic Local Alignment search Tool (BLAST) against the NCBI database. Genes involved in cell wall biosynthesis, carbohydrate metabolism and transportation, cellular senescence, abiotic or biotic stress response or hormone response were selected as candidates for shelf life. Genes involved in phytonutrient biosynthetic pathways were selected as candidates for nutritional quality.

Additional field trials were also conducted in Davis, California from June-July to collect phenotype data for QTL mapping, using the core mapping population, consisting of 307 F_8 RILs and the parent lines. Lettuce was planted in a four block, fully randomized design in 5 ft. by 120 ft beds (Figure 2A). Four replicates of RIL were planted in each block in a random order, with a row of romaine border plants (cultivar Parris Island) surrounding each block and 12 replicates of each parent (Figure 2B), totaling 5,008 experimental plants. Seeds were planted into 200 cell flats in PRO-MIX HP high porosity soil (Premier Tech Horticulture, USA), irrigated twice daily using a mist spray nozzle, under 16 hour day length (supplemental artificial light given when necessary). Seedlings were transplanted at two weeks into beds treated with BALAN* DF preemergence herbicide and a granular Ammonium Sulphate 21:0:0 fertilizer. Plants were irrigated three times daily (approx. 30 minute duration) using microsprinklers for the first two weeks after transplanting, after which watering was reduced to twice daily. Plant biomass was harvested after 4 weeks field growth and traits were assessed, as described in Table 1.

Table 1 Summary of traits measured and a brief description of experimental methods.

Trait	Analyses	
Shelf life	Evaluation of the number of days after harvest until the first signs of visible damage following mock-processing (washing and spin drying) and storage at 10°C. Damage found included yellowing, bruising, necrosis and pinking/browning	
Cell wall strength, elasticity and plasticity	Tensiometer analysis. The tensiometer pulls a leaf section three times which informs: 1) elasticity and plasticity 2) elasticity and 3) leaf strength where elasticity is the reversible stretching, plasticity is the initial irreversible stretching and the leaf strength is an indication of the cell wall strength and subsequent toughness of the leaf	
Leaf physiology	Imprints of the cell patterning on the leaf surface revealing the size and ratio of epidermal cells to stomata. Some of the leaves had fewer big cells and some of the leaves had more smaller cells, both with a range of stomata numbers	
Leaf morphology	Leaf area images for differences in leaf sizes, partly determined by leaf shape	
Nutritional quality	FRAP- Total antioxidant capacity analysis using a color change assay	
Pigment analysis	In the 2018 trial a CCM200 (Opti-Sciences) was used which measures the chlorophyll content of a leaf. In the 2019 trial a Polypen (Photo Systems Instruments) was used which measures additional pigment data such as greenness, and anthocyanin content	

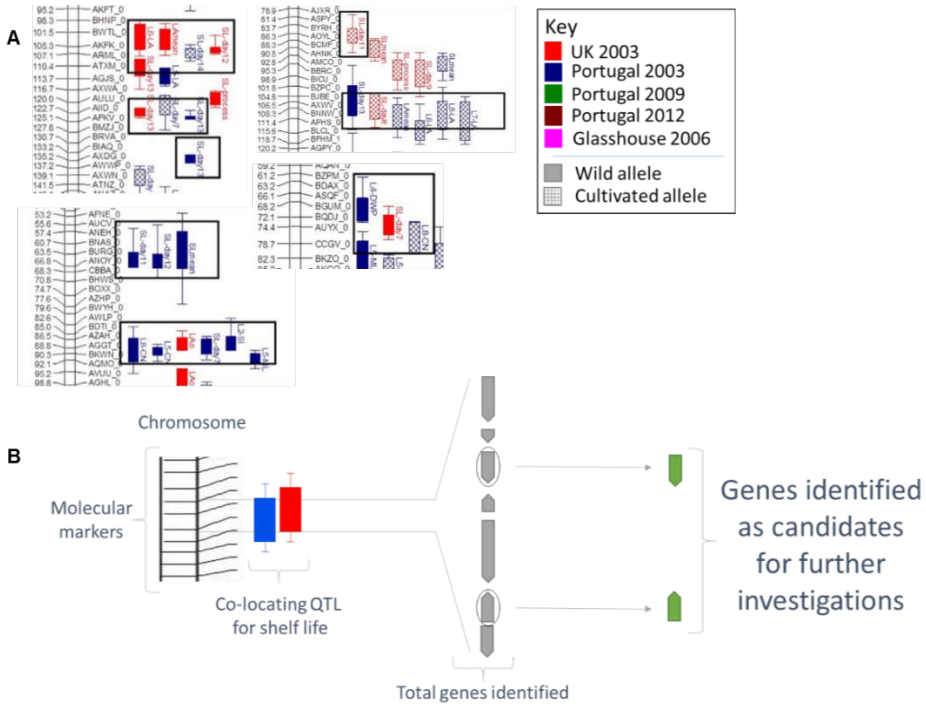


Figure 1 Candidate genomic regions in which QTL measured in different trials for shelf life and related traits mapped to the same chromosome position were identified (A) and candidate genes were selected (B).

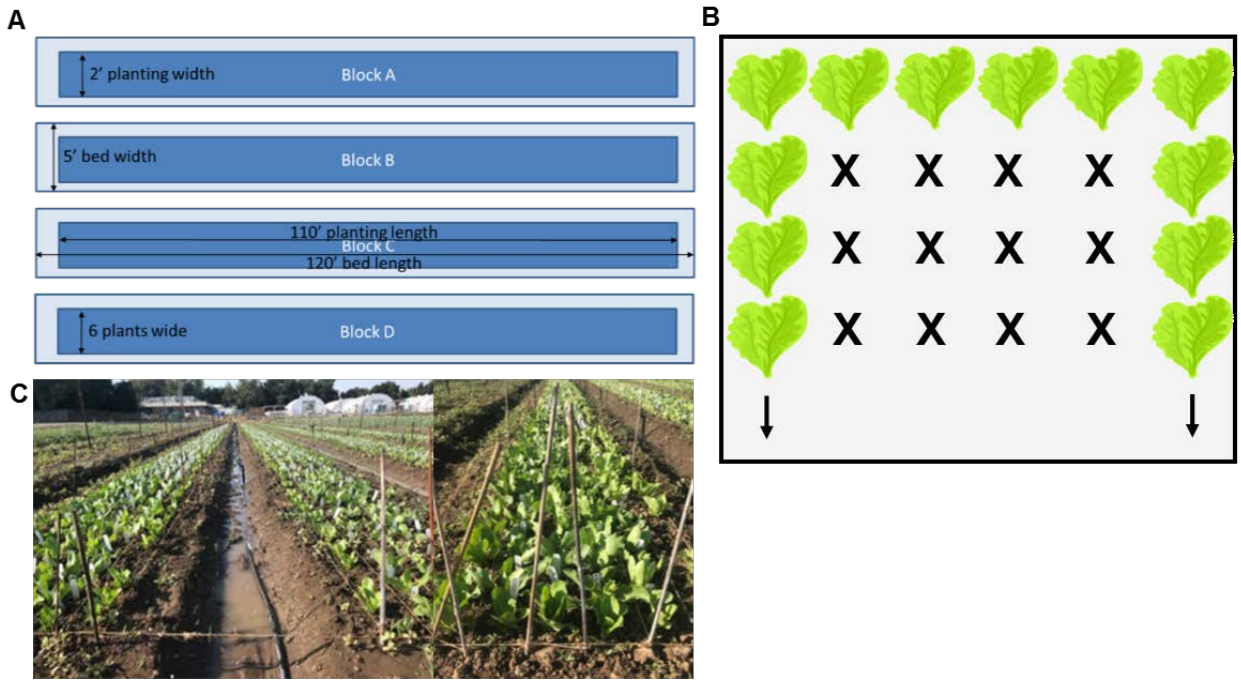


Figure 2 Planting plan for the 2018 field trial in Davis, California. Four block (1 bed per block) randomized design utilized (A), with a 10cm spacing and border row of plants surrounding each bed (B). Microsprinkler irrigation with one pipe in the furrows between beds A-B, B-C and C-D (C).

Objective 2

In order to conduct genome wide association studies, a population of diverse germplasm is required. ***In collaboration with Ivan Simko***, we have gained access to a wide population of 493 baby leaf accessions including breed lines and cultivars for association genetics studies. This population has been planted on farm in Davis, California in April-May 2019, in a three block, fully randomized design in 5 ft. by 166 ft beds. Each block consisted of 493 lines, planted in a square of four replicates, which were randomized through the bed, with 24 additional replicates (six squares of four plants) of *L. sativa* and *L. serriola* (the parents of the core RIL mapping population, for reference), totaling ***6036 experimental plants***. As described previously, each block was surrounded by a row of romaine border plants (cultivar Parris Island) and seeds were sown and irrigated in 200 cell flats before transplanting to the field at 2 weeks. Plants were irrigated three times daily (approx. 30 minute duration) using sprinklers for the first 24 hours, after which irrigation was changed to four times daily (approx. 20 minute duration) using microsprinklers until harvest. Plant biomass was harvested after 3 weeks field growth. Traits measured were as described in table 1 with minor modifications. Trait assessment modifications included the removal of tensiometer and leaf physiology traits and shelf life assessment was also modified to include an "end of shelf life" measure where bags were assessed for days until severe damage (i.e. approx. 50% leaf damage).

We are also working to ***procure a diverse germplasm collection from the Centre for Genetic resources, the Netherlands (CGN) comprising of 449 accessions largely of wild origin***. Species include: *Lactuca sativa* (128), *L. serriola* (198), *L. saligna* (47), *L. virosa* (49) and *L. georgica* (12). This population will be grown in Davis and Salinas during 2019 and trait data will be collected for association genetics studies.

Objective 3

CRISPR/Cas9 genome editing was previously utilized in collaboration with the Michelmore laboratory to develop lettuce lines with gene knockout mutations in six shelf life candidate genes. These genes encode xyloglucan endotransglucosylase/hydrolase (XTH) enzymes, which function in modifying, through extending and shortening, xyloglucan tethers between cellulose microfibrils in the cell wall (Figure 3A). This extension of these xyloglucan chains leads to cell expansion, modifying cell wall strength and plastic and elastic properties. The six XTH genes targeted were found within QTL for shelf life on chromosomes 4 and 8 in previous candidate gene mining studies, as described in Objective 1. Guide RNAs (gRNAs), 20bp in length, guided the Cas9 double-stranded DNA endonuclease to regions within the first exon of each gene (Figure 3B). Each gRNA was cloned into a custom construct (modified from Fauser et al., 2014, Plant Journal, by the Brady laboratory, UC Davis), containing genes encoding *Streptococcus pyogenes* Cas9 driven by the constitutive Ubiquitin 4-2 promoter and terminated by *Arabidopsis* Heat Shock Protein 18, and a kanamycin resistance gene as a selectable marker, controlled by the nopaline synthase promoter and terminator. Three gRNAs were targeted to each gene, in an effort to maximize the efficiency of gene knockout. Constructs were transformed into *Agrobacterium tumefaciens* and then into lettuce cultivar Salinas by the UC Davis Plant Transformation Facility (https://ptf.ucdavis.edu/). Mutant lines were identified by amplicon sequencing of the primary transformants.

Efforts are now focused on trialing mutant lines and measuring phenotypic traits (as described in Table 1), to determine the effect of gene mutation on shelf life. Ten replicates of each of the six mutant lines (denoted A-F), one negative control line (G) which had been transformed with *Agrobacterium* but had no mutation in the XTH genes targeted and the wild type Salinas were grown in the greenhouse in 1 gallon pots filled with PRO-MIX HP, under 16 hour day length (supplemental artificial light given when necessary) watered by drip irrigation three times daily (approximate flow rate of 33 mL each watering). Trait data were collected following 6 weeks growth.

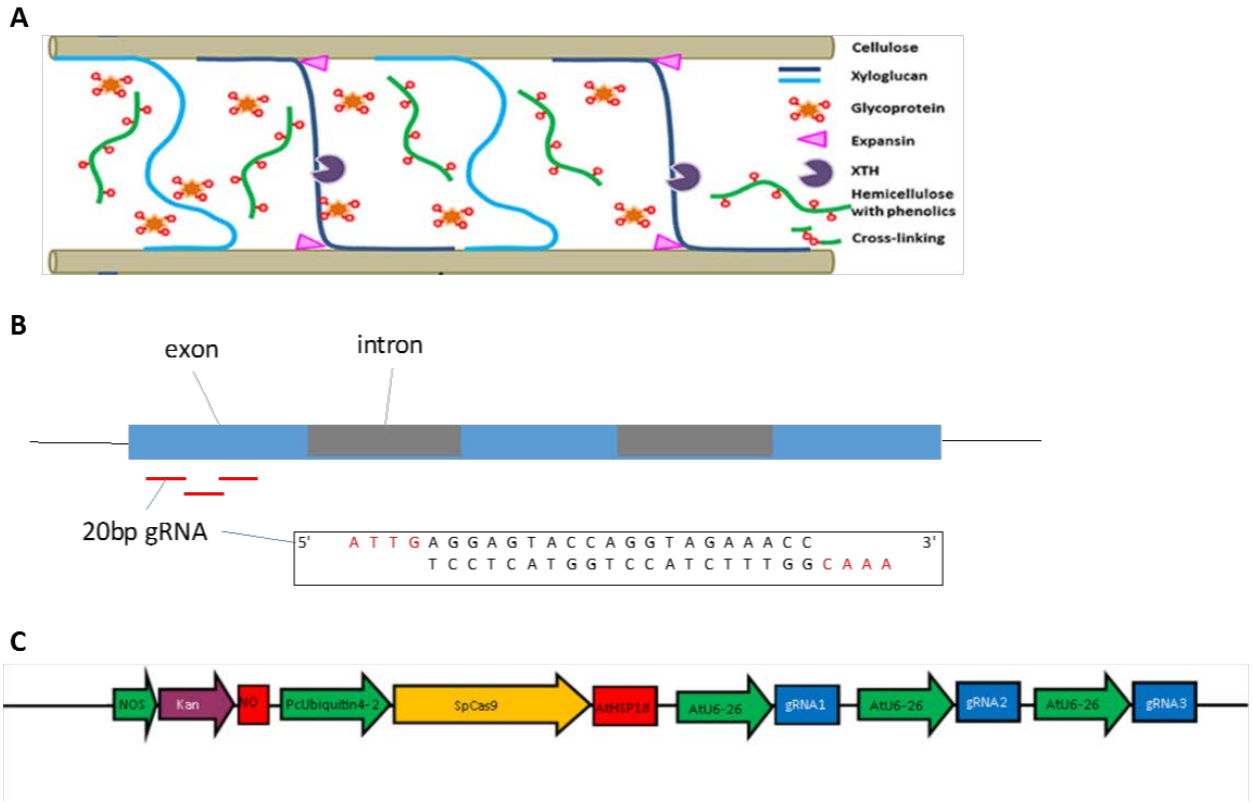


Figure 3 Cartoon demonstrating the role of XTH enzymes in cell wall modification (A). Schematic overview of the 20bp gRNA target (B) and custom constructs (C) used to generate gene knockout mutants.

Objective 4

We have developed high resolution melt (HRM) analysis as a rapid and inexpensive single nucleotide polymorphism (SNP) genotyping tool following marker-assisted breeding in lettuce. This highly sensitive PCR-based approach detects subtle changes in the melting temperature of small double-stranded DNA fragments resulting from polymorphism in the sequence. Firstly, primers are designed to amplify approximately 200 bp of DNA at the site of the molecular marker, using a PCR reagent containing an intercalating double-stranded DNA binding dye. The PCR amplicon is then subjected to increases in temperature from 60-95°C in 0.3°C increments. Dissociation of the double-stranded dye as a result of DNA strand separation causes a reduction

in fluorescence, measured by a quantitative PCR machine. This generates a melt curve, analysed to identify the melting temperature of the fragments, which is altered by the DNA sequence. We previously generated a collection of backcross lines in which a RIL from the Salinas x *L. serriola* mapping population demonstrating high antioxidant content was backcrossed with a Lollo Rosso cultivar, Rampage, in an attempt to introgress a large-effect QTL for antioxidant status for which wild alleles contributed to the trait (Damerum *et al.*, 2015). HRM was utilized to assess the genotypes of these crosses.

RESULTS AND DISCUSSION

Objective 1

In total, 228 genes were identified as potential gene candidates (Figure 4). These included cell wall modifying proteins and enzymes (expansins, xyloglucan endotransglucosylase-hydrolases), enzymes involved in plant cell wall carbohydrate biosynthesis (cellulose, glucan and xylose synthases), auxin and ethylene response transcription factors and phenolic acid biosynthesis (flavonoid-3-hydroxlase, ferulate-5-hydroxylase). Genes identified will be further explored in functional investigations, as described in Objective 3.

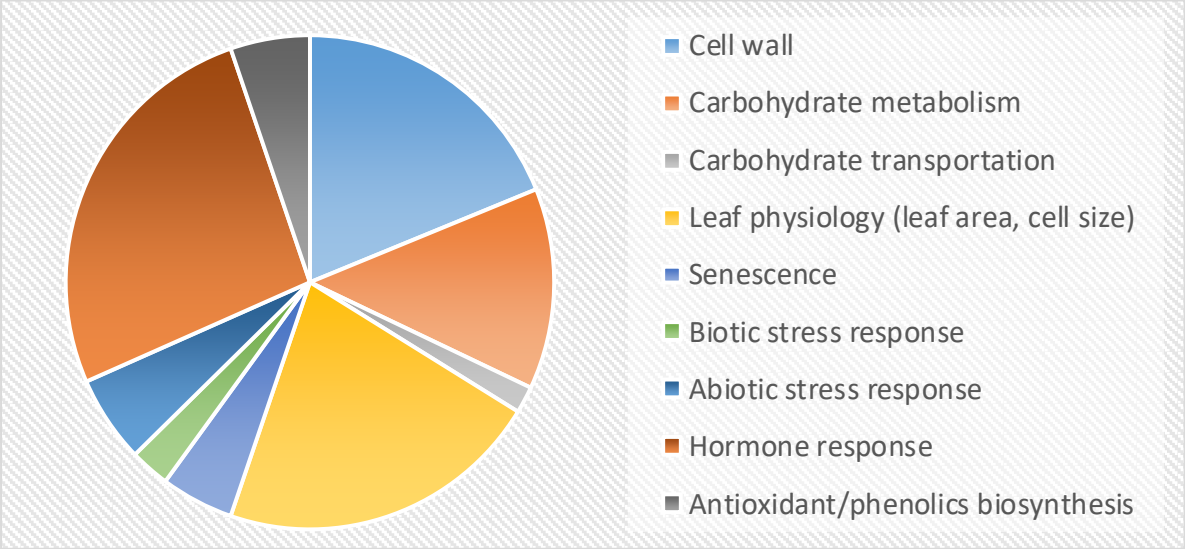
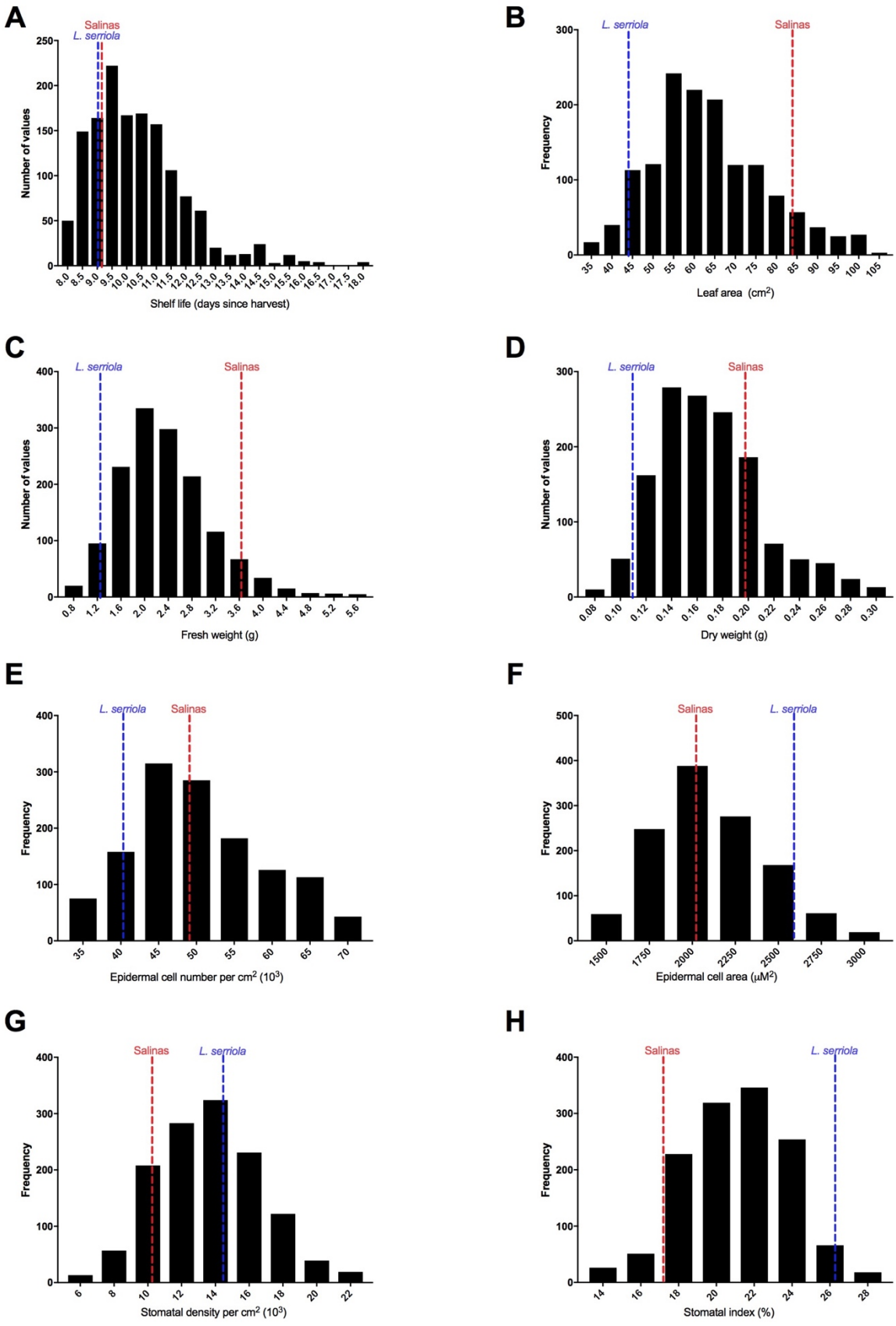


Figure 4 Functional groups of genes identified in candidate gene mining.

The June-July 2018 RIL trial was successful and trait data were collected for QTL analyses. The RILs varied in shelf life, ***measured as the first signs of deterioration (such as bruising, browning, pinking, yellowing)***, from ***5-15 days post harvest*** and positive transgressive segregants (RILs with longer shelf life than either parent) were observed (Figure 5A) for this trait and all others under investigation (Figure 5B-L). Shelf life (SL) was found to be positively correlated cell wall strength (maximum load, ML) and negatively correlated with leaf area (LA) and cell wall elasticity (E; Figure 6). This indicates that leaves with a smaller cell area and

stronger, less elastic cell walls (also referred to as reversible extensibility) have the longest shelf life. ***QTL for SL mapped to chromosome 6, co-locating to the same position as a QTL for SL detected in a UK trial (Figure 7, indicated in red)***. Ongoing efforts are working to re-plot all existing QTL data collected from the core mapping population using the same consensus genetic map.


A
Salinas
L. serriola
Number of values
Shelf life (days since harvest)
B
L. serriola
Salinas
Frequency
Leaf area (cm2)
C
L. serriola
Salinas
Number of values
Fresh weight (g)
D
L. serriola
Salinas
Number of values
Dry weight (g)
E
L. serriola
Salinas
Frequency
Epidermal cell number per cm2 (103)
F
Salinas
L. serriola
Frequency
Epidermal cell area (μM2)
G
Salinas
L. serriola
Frequency
Stomatal density per cm2 (103)
H
Salinas
L. serriola
Frequency
Stomatal index (%)

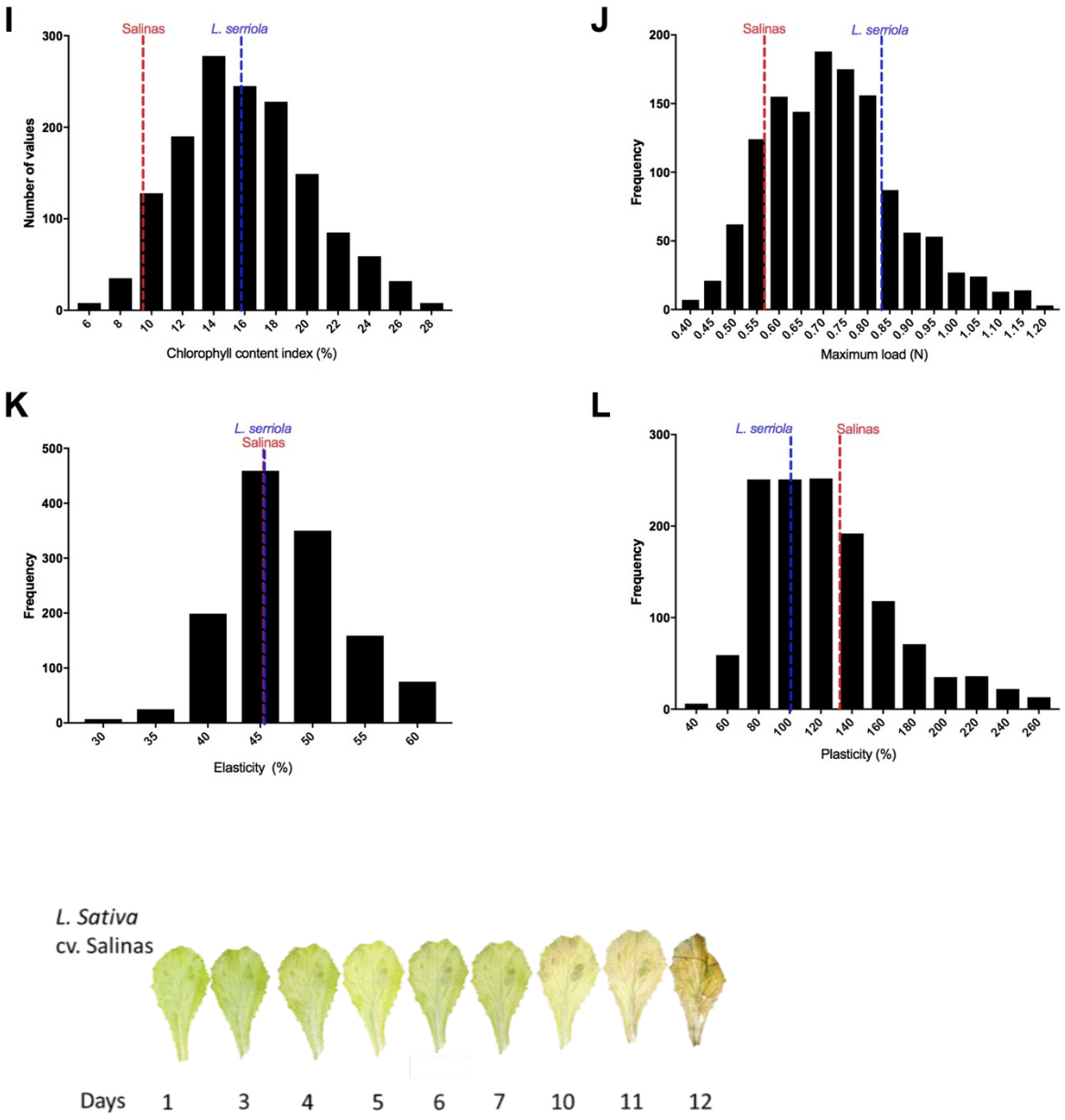


Figure 5 Variation of traits in the RIL population (A-L) with an example of the deterioration observed after mock-processing of the cultivated parent (in days post-processing, 72 hours after harvest).

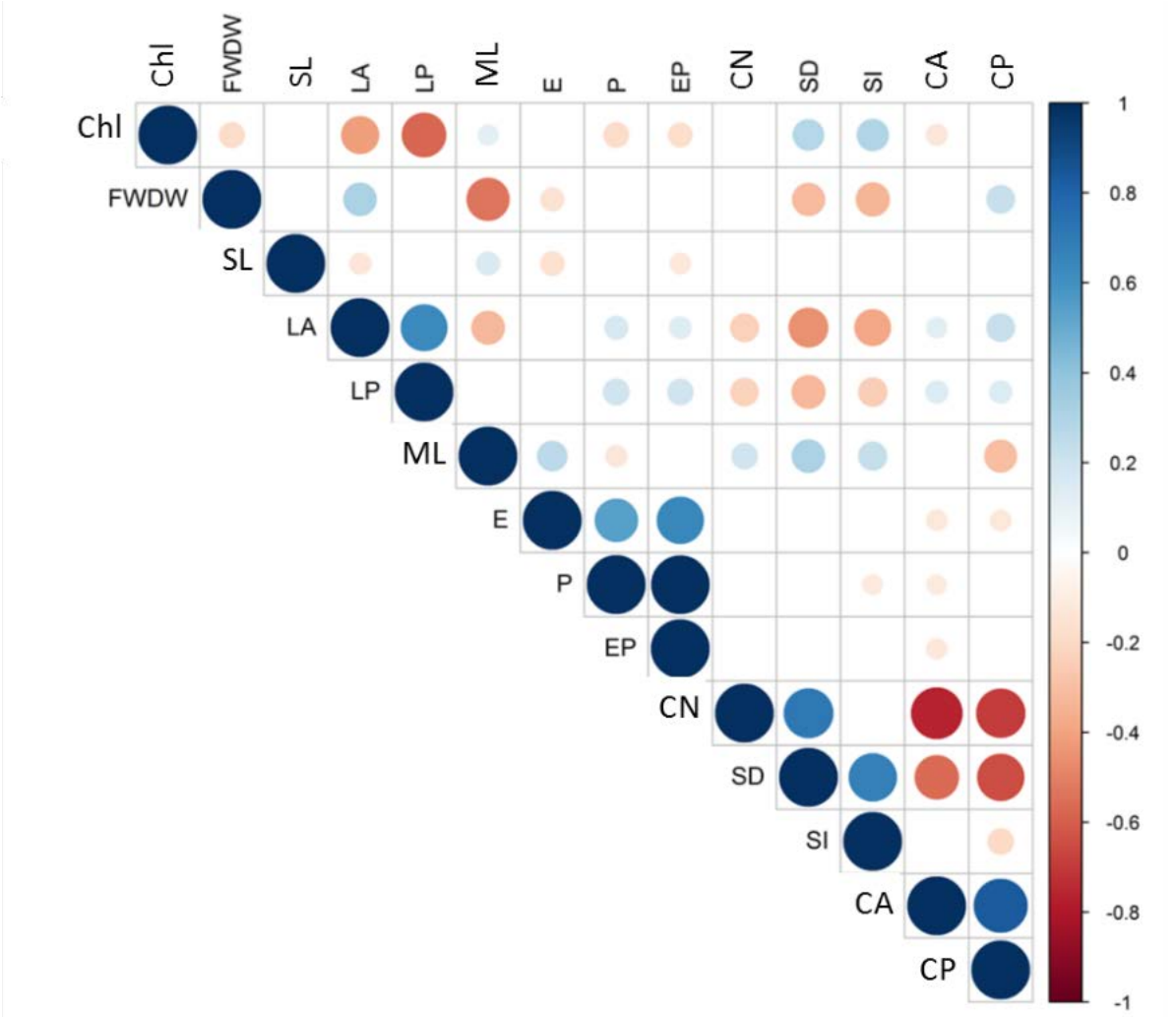
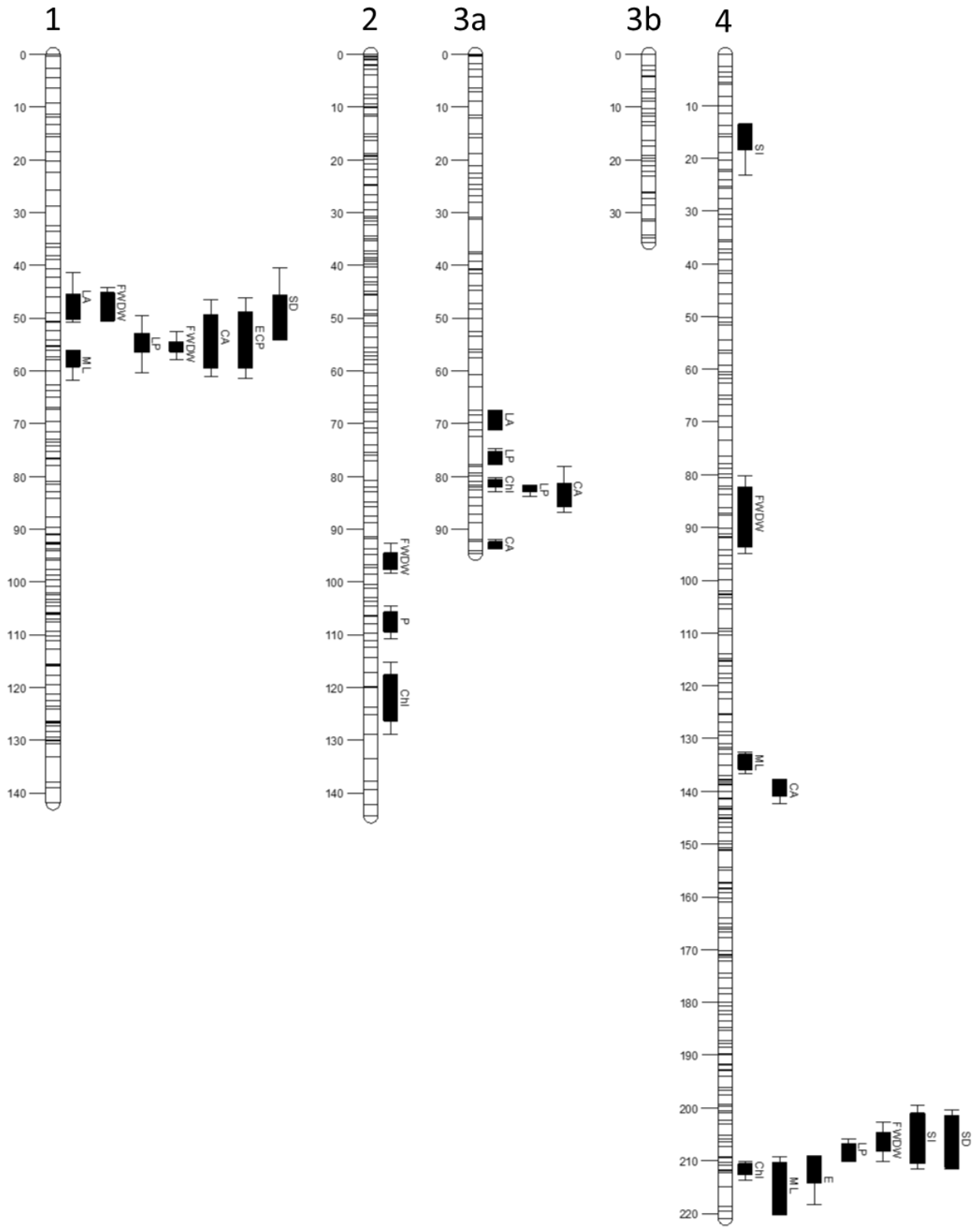


Figure 6 Spearman's rank correlation. Circles indicate significant (P<0.05) correlation between traits, with color scale demonstrating positive (blue) or negative (red) correlation for traits: Chl=chlorophyll content, FWDW=ratio of fresh to dry weight, SL=shelf life (days since harvest), LA=leaf area, LP=leaf perimeter distance, ML=cell wall strength (maximum load), E=elasticity, P=plasticity, EP=ratio of elasticity to plasticity, CN= cell number, SD= stomatal density, SI=stomatal index, CA=cell area, CP=cell perimeter distance.


1
2
3a
3b
4
LA
FWDW
ML
LP
FWDW
CA
ECP
SD
FWDW
P
Chl
LA
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LP
FWDW
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SD

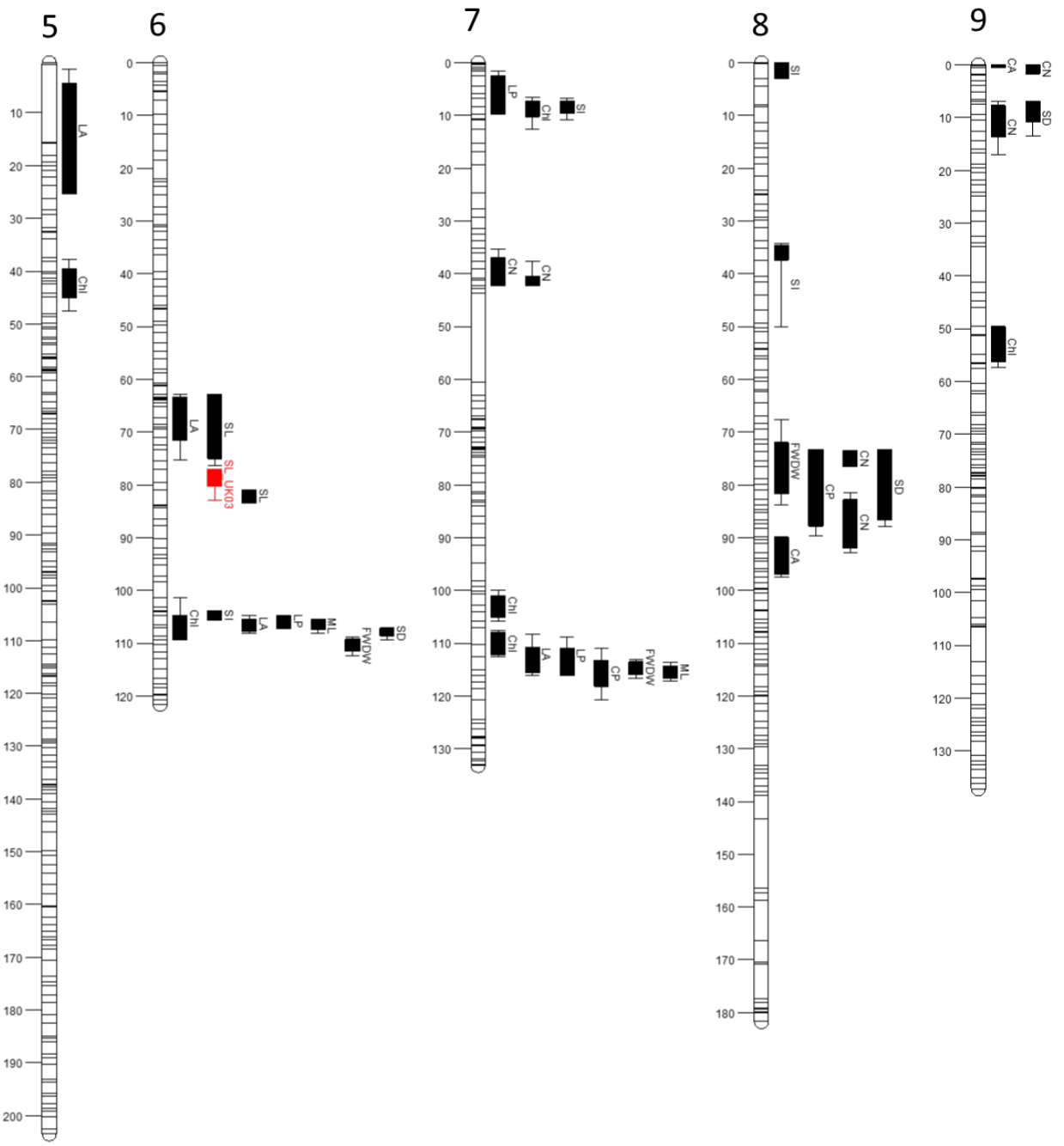

Figure 7 QTL identified in the June-July 2018 trial using the core mapping population. Bars indicate linkage groups, with scale to the left of the bar given in cM and linkage group number above each bar. QTL are shown to the right of each bar, with the box and error bars demonstrating the 1-LOD and 2-LOD intervals, respectively. Red QTL indicates those identified in previous trials conducted in the UK. See Figure 6 legend for abbreviations.

Objective 2

USDA Wide Population for GWAS Our first trial ***in collaboration with Ivan Simko*** at from USDA Salinas was planted on the Davis campus in March 2019 and completed in May 2019. In this trial, we have measured the following traits, many of which have not been measured previously: shelf life, yield, cell wall strength, elasticity and plasticity and nutritional quality, including antioxidant content and various spectra, such as NDVI, chlorophyll, carotenoid and anthocyanin content. Analyses of these data is on-going and will be completed by September 2019.

Objective 3

Phenotypic analyses have now been undertaken in three generations and indicated that mutant lines, in which XTH gene activity has been reduced, ***have an increase in shelf life from 0.5-3 days when compared to the wild type Salinas*** (Figure 8A). This was achieved with no significant differences in leaf area (Figure 8B) or chlorophyll content, excluding line E, which had a significantly higher chlorophyll content compared to the wild type Salinas (Figure 8C). Line G, the negative control line, demonstrated reduced shelf life and chlorophyll content, which may indicate mutations incurred during whole plant regeneration from tissue culture, or off-target mutations by Cas9. On-going investigations involve more extensive phenotyping of these lines, including measurement of XTH activity and cell wall strength and selection of lines in which Cas9 has segregated, as these lines are not deemed "transgenic" and can be used in future breeding programs.

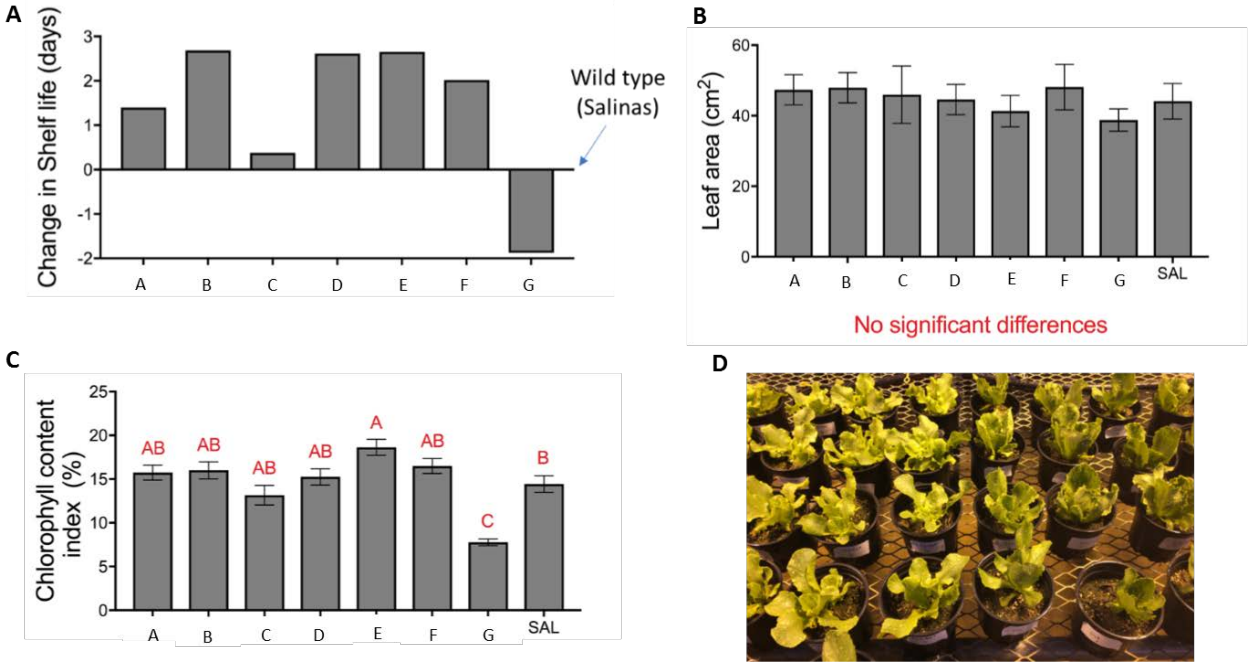

Figure 8 Phenotyping XTH gene knockout mutants in preliminary trials identified shelf life increase compared to wild type (A), with no significant differences in leaf area (B) and minimal differences in chlorophyll content (C). Lines in greenhouse on day of harvest (D).

Objective 4

In a proof of concept study, we were able to generate a novel backcross line, carrying a heterozygous allele for markers within a large-effect QTL for antioxidants, for which wild lettuce alleles increased trait value. HRM was used to genotype the line after 4 generations of backcrossing of a RIL from the Salinas x *L. serriola* population to the cultivar Rampage. Figure 9B demonstrates representative HRM curves of the Donor parent (RIL), recurrent parent (Rampage) and the BC_4 line, carrying a heterozygous allele for a molecular marker at the QTL site. The BC_4 line demonstrated significantly higher antioxidant content than either parent (Figure 9C).

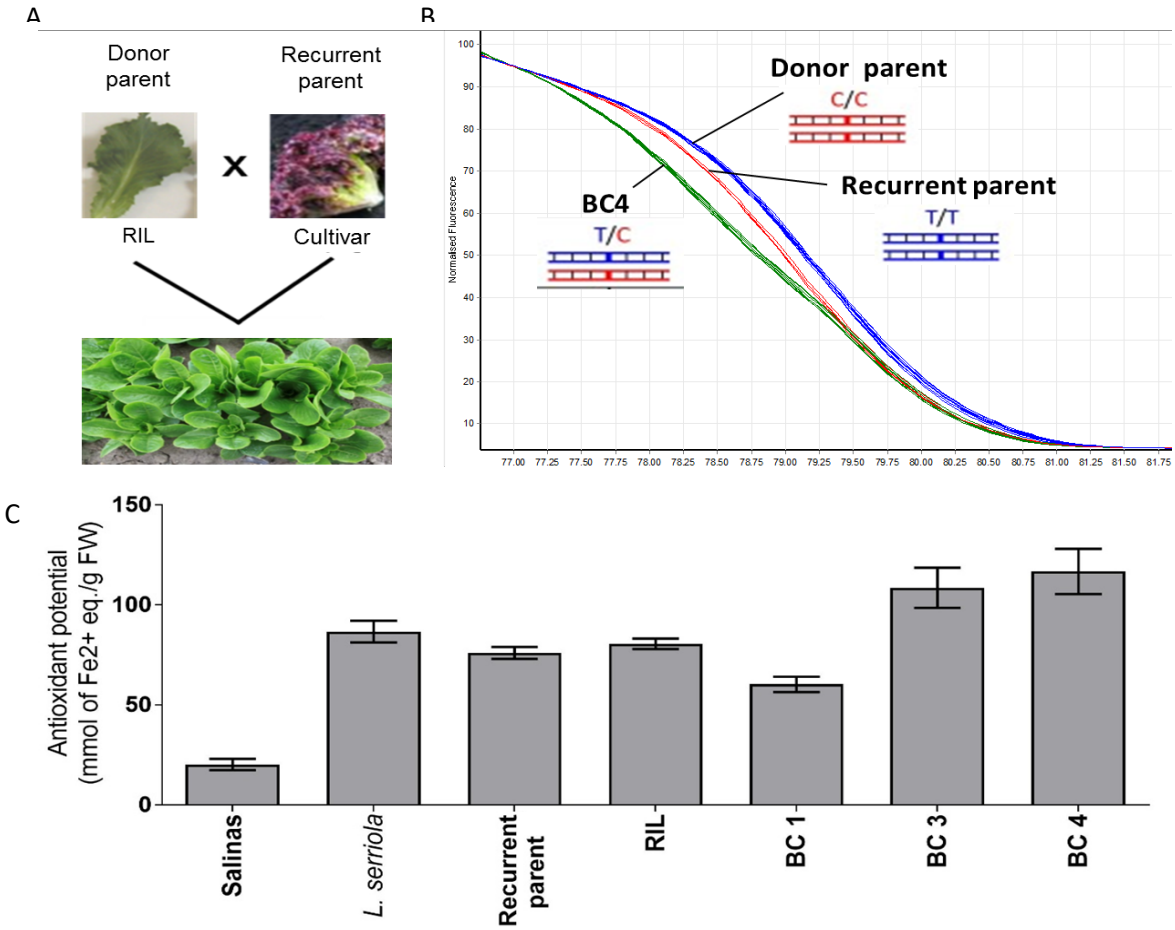

Figure 9 Marker-assisted breeding approach. A high antioxidant RIL was backcrossed with cultivar Rampage to develop novel breeding lines (A). HRM revealed a heterozygous genotype at the large-effect antioxidant QTL in the BC_4 generation (B) which demonstrated significantly higher antioxidant content (C).

Conclusions

In this first year of California Leafy Greens funding, we have made significant progress towards our short and long-range objectives, conducting multiple field trials in Davis, California, and planning Salinas trials, collecting extensive phenotype data on two different populations, developing methods for HRM genotyping and gene knockout by CRISPR/Cas genome editing and identifying candidate genes for the improvement of shelf life. In the upcoming year, we will continue to characterize baby leaf lettuce material for post-harvest traits and work to employ developed methods for gene editing and marker-assisted breeding programs.

Our overall aim to develop significant pre-breeding resources on traits not routinely analysis by large seed houses remains intact.

Literature

Damerum A, Selmes SL, Biggi GF, Clarkson GJ, Rothwell SD, Truco MJ, Michelmore RW, Hancock RD, Shellcock C, Chapman M a, *et al.* 2015. Elucidating the genetic basis of antioxidant status in lettuce (Lactuca sativa). *Horticulture Research* **2**: 1–13.

Truco MJ, Ashrafi H, Kozik A, van Leeuwen H, Bowers J, Reyes Chin Wo S, Stoffel K, Xu H, Hill T, Van Deynze A, *et al.* 2013. An Ultra High-Density, Transcript-Based, Genetic Map of Lettuce. *G3: Genes | Genomes | Genetics* **3**: 617–631.