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RESEARCH PAPER

QTLs for shelf life in lettuce co-locate with those for leaf biophysical properties but not with those for leaf developmental traits

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Abstract

Developmental and biophysical leaf characteristics that influence post-harvest shelf life in lettuce, an important leafy crop, have been examined. The traits were studied using 60 informative F_9 recombinant inbed lines (RILs) derived from a cross between cultivated lettuce (*Lactuca sativa* cv. Salinas) and wild lettuce (*L. serriola* acc. UC96US23). Quantitative trait loci (QTLs) for shelf life co-located most closely with those for leaf biophysical properties such as plasticity, elasticity, and breakstrength, suggesting that these are appropriate targets for molecular breeding for improved shelf life. Significant correlations were found between shelf life and leaf size, leaf weight, leaf chlorophyll content, leaf stomatal index, and epidermal cell number per leaf, indicating that these pre-harvest leaf development traits confer post-harvest properties. By studying the population in two contrasting environments in northern and southern Europe, the genotype by environment interaction effects of the QTLs relevant to leaf development and shelf life were assessed. In total, 107 QTLs, distributed on all nine linkage groups, were detected from the 29 traits. Only five QTLs were common in both environments. Several areas where many QTLs co-located (hotspots) on the genome were identified, with relatively little overlap between developmental hotspots and those relating to shelf life. However, QTLs for leaf biophysical properties (breakstrength, plasticity, and elasticity) and cell area correlated well with shelf life, confirming that the ideal ideotype lettuce should have small cells with strong cell walls. The identification of QTLs for leaf development, strength, and longevity will lead to a better understanding of processability at a genetic and cellular level, and allow the improvement of salad leaf quality through marker-assisted breeding.

Key words: Biophysical, biomechanical properties, leaf development, lettuce, microbiology, post-harvest, QTLs, shelf life.

Introduction

Pre-packed baby salads, consisting of lettuce, beets, herbs, and spinach, have become a popular and profitable product due to public demand for healthy and convenient food, with a US annual market value in excess of US$2 billion, but the commercial value of these crops is affected

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Abbreviations: AGR, absolute growth rate; CHL, total chlorophyll; DW, dry weight; DWP, percentage dry weight; E, elasticity; ECA, epidermal cell area; ECN, epidermal cell number; EST, expressed sequence tag; FW, fresh weight; LA, leaf area; LG, linkage group (chromosome); LOD, logarithm of odds; ML, maximum load; OSM, osmolality; P, plasticity; P+E, total deformation potential; QTL, quantitative trait locus; RIL, recombinant inbred line; RGR, relative growth rate; SLA, specific leaf area; SD, stomatal density; SI, stomatal index; SL_H, shelf life from day of harvest; XTH, xyloglucan endotransglycosylase/hydrolase.

by a relatively short shelf life. There is a need to extend shelf life, but understanding the genetic basis of post-harvest shelf life in leafy crops is limited, although likely to be linked to pre-harvest leaf traits. Recent research has suggested that the 'ideal' leaf for processability and shelf life is likely to be characterized by small cells, with favourable water relations (high solute potential) and limited cell wall extensibility and loosening. Other favourable traits include increased leaf thickness and a waxy cuticle (Clarkson *et al*., 2003). Understanding of the genetic determinants of such traits in a leafy crop such as lettuce is extremely limited, despite the economic value of this and other leafy salads. However, many of these characteristics are tractable in model plants such as *Arabidopsis* (Kessler and Sinha, 2004) and it is now possible to utilize information from model systems linked to emerging genomic resources in crops to develop informed molecular plant improvement programmes (Zamir, 2001).

Quantitative trait locus (QTL) analysis is a useful tool in such an approach, not only providing DNA markers linked to agronomic traits, but also for elucidating fundamental mechanisms of genetic control of leaf growth (Asins, 2002; El-Lithy *et al*., 2004). Clear progress has been achieved using QTL mapping to improve several crop agronomic traits, such as rice yield (Xing *et al*., 2002), tomato size and quality (Fridman *et al*., 2002; Frary *et al*., 2000), and bean disease resistance (Kelly *et al*., 2003). There have been only three QTL studies in lettuce, reporting the improvement of root water use efficiency (Johnson *et al*, 2000), seed traits (Argyris *et al*., 2005), and disease resistance (Jeuken and Lindhout, 2002) but none has considered leaf development and post-harvest traits. Recently, lettuce backcross inbred lines have been developed for exploration of the *Lactuca saligna* (wild lettuce) germplasm (Jeuken and Lindhout, 2004).

Cell division and expansion both contribute to final leaf size and shape (Wang *et al*., 2000; Dengler and Kang, 2001; Wyrzykowska *et al*., 2002; Taylor *et al*., 2003; Tsukaya, 2003), providing targets for future manipulation and breeding. Leaf cell expansion is determined at the primary cell wall by loosening and reassembly (Cosgrove *et al*., 2002), and this is driven primarily by internal osmotic pressure generated by water uptake. Expansion also depends on cell wall composition and the degree of association between its different components. The plant cell wall is an important structure, providing essential mechanical strength and rigidity, and protecting against pathogens and dehydration (Cosgrove, 2001). The cell wall has two conflicting characteristics: tensile strength and stability versus structural plasticity. A number of important agronomic properties of plants are influenced by the cell walls, for example nutrient absorption and insect resistance. Hazen *et al*. (2003) reported QTLs for sugar composition of the cell walls in maize. They identified a few candidate genes involved in the essential process of cell wall biosynthesis. It was suggested that xyloglucan endotransgluscosylases/hydrolases (XTHs) serve important roles in both assembly and loosening of the cell wall, together enabling long-term plant cell expansion with minimal loss of wall strength (Thompson and Fry, 2001). Investigating the role of the cell wall is likely to lead to identification of the candidate genes involved in the leaf processability. Similarly, the plant cell cycle may also provide further candidate genes. To date, the coordination of cell division and cell expansion during the growth process is still unclear, but several control points in the plant cell cycle have been shown to have an effect on leaf size (Wang *et al*., 2000).

Substantial genomic resources are now available for lettuce, including >68 197 expressed sequence tags (ESTs), providing a unigene set of 22 185 (http://cgpdb.ucdavis.edu/), but these have yet to be employed to improve our understanding of leaf growth, development, and crop improvement. Recently, a new genetic linkage map has been developed based on the mapping population used in this study. The full mapping population includes 130 F_9 recombinant inbred lines (RILs), derived from a cross between cultivated lettuce (*L. sativa* cv. Salinas) and wild lettuce (*L. serriola* acc. UC96US23). The 60 most informative lines (containing the most recombination events) were used in this study. One advantage of such a genetic resource is the opportunity it provides to examine genotype by environment interactions (G×E)—crucial for any programme of plant improvement, where robust QTLs, irrespective of environment, provide a starting point for candidate gene discovery and testing (Maloof, 2003).

The aim of this study is to develop a new 'ideal ideotype' lettuce with extended shelf life to meet the growing market niche. This study is the first approach to identify QTLs for leaf shelf life (processability) and discuss the possible candidate genes involved in processability, and thus provides the first scientific basis on which future crop improvement strategies may be developed. Here (i) evidence is presented of QTLs for pre-harvest leaf development traits and their relationship to post-harvest shelf life; and (ii) the G×E is assessed by comparing QTL discovery at two contrasting field sites, one in northern, temperate, Europe and the other in southern, Mediterranean, Europe.

Materials and methods

Plant material and field sites

The mapping population was derived from a cross between cultivated lettuce (*L. sativa* cv. Salinas) and wild lettuce (*L. serriola* acc. UC96US23). From 113 F_9 RILs, 60 highly informative recombinant lines were selected from the genetic map development to be used in this study using MapPop (Vision *et al*. 2000) and GenoPlayer (http://compgenomics.ucdavis.edu/genoplayer/). This provided a population that had nearly as many recombination breakpoints and was therefore as informative as a population of ~90 RILs. Two contrasting field sites were used in this study. The

Portugal site was at Boavista farm, near Odemira (latitude 37°36′N, longitude −8°38′N), while the UK site was at Pinglestone Farm, near Winchester (latitude 51°6′N, longitude 1°10′N). The climate during the growing season of each crop was similar in Portugal (average minimum–maximum temperature during the growing season: 6–23 °C, average precipitation: 36–53 mm) to that in the UK (temperature 7–22 °C, precipitation: 41–56 mm) (http://weather.co.uk/). The field trials were designed with three blocks. In each block, three replicates of 62 lines (60 F_9 RILs and two parents) were randomized using the statistical software package Minitab 13.0 for Windows (Minitab Inc., Philadelphia, PA, USA). There were four plants of the same line in each replicate plot, with 10 cm between each plant. Two rows of Cos lettuce were planted around each block to avoid 'edge effects'. In each field trial, an average of six replicates was available following germination and establishment for each of the traits measured. The field trials were in commercial farms with standard industry maintenance.

Leaf traits data recording

The plants in two sites were harvested at similar maturity, 7 weeks after planting. In the Portugal field trial, eight leaves, starting from the youngest leaf which was big enough to take two discs of 10 mm diameter, were sampled and labelled in accordance with the leaf development age from 1 (youngest) to 8 (oldest) in series. In the UK field trial, only leaves 3, 6, 7, and 8 were sampled for further measurements.

Leaf area and growth rate

Images of labelled leaves 1–8 in the Portugal trial were taken using a digital camera (Nikon Coolpix 5000), against a white background and linear scale bar. In the UK trial, images of leaves 3, 6, 7, and 8 were obtained using the same procedure. During the growing period in the UK field trial, at ~5 weeks after planting, an additional set of digital images from replicate plants was taken on labelled leaves twice, with a 4 d gap. Pairs of leaf images were used to determine absolute growth rates (AGRs) ($mm^2\ h^{-1}$) and relative growth rates (RGRs) ($mm^2\ mm^{-2}\ h^{-1}$), as described by Taylor *et al*. (2003). Leaf images were used for leaf area measurement, using 'Metamorph' (Metamorph version 5, Universal Image Corporation, Philadelphia, PA, USA). The AGR of leaves was calculated using the equation $AGR=(LA_f–LA_0)/h$, where LA_f and LA_0 were the second and the first areas (mm^2) measured, and h the hours between two measurements. The RGR was calculated as $RGR=(LA_f–LA_0)/LA_0/h$.

Chlorophyll content

A leaf disc (10 mm diameter) was taken from leaves 1, 4, and 7 of each plant from the Portugal trial, to the left of the mid-rib vein, at ~10 mm from the leaf tip, avoiding major veins. Leaf samples were stored in separate microfuge tubes with 0.5 ml of dimethylformamide (DMF) and kept in the dark at 4 °C for >48 h. Leaf 7 was taken for this measurement in the UK trial. Chlorophyll pigment quantity was measured by absorption at 647 nm and 664 nm in a spectrophotometer (U-2001, Hitachi, Wokingham, UK). The chlorophyll concentration in DMF was calculated according to the following formula (Moran, 1982): $C_i=7.04\ A_{664}+20.27\ A_{647}$, where C_i is the chlorophyll concentration in the DMF solution in $\mu g\ ml^{-1}$. From the sample disc area and the measured chlorophyll concentration, total chlorophyll content was calculated using the following formula: $C_t=C_i\times0.5$/disc area, where C_t is total chlorophyll content in $\mu g\ mm^{-2}$.

Leaf fresh weight, dry weight, and specific leaf area

Leaf thickness measurements, inferred from specific leaf area (SLA), were made on complementary leaf discs from the same leaves as chlorophyll content; however, this time to the right of the mid-rib vein (data not shown). After two disc samples for chlorophyll content and transverse section measurement were taken, the leaf fresh weight (FW) was measured on a portable balance (AM/ACB150, Camlab Ltd, Cambridge, UK), the leaves were stored in a paper bag (30 cm×25 cm), sent back to the UK, and dried in a oven at 85 °C for dry weight (DW). Only leaf 7 was taken for this measurement in the UK trial. The percentage dry weight (DWP) was calculated and indicates the percentage water content. The DW measurements were also used to calculate the SLA, according to the following formula: SLA=(leaf area−2×disc area)/leaf DW.

Epidermal cell area

At harvest, a leaf disc of 10 mm diameter was taken from leaves 2, 5, and 8 in the Portugal trial, parallel to the mid-rib vein, at ~10 mm from the leaf tip. Leaf 8 was collected for this measurement in the UK trial. Epidermal cell imprints were made by painting the adaxial surface of the leaf disc with clear nail varnish (No. 17, Boots, Nottingham, UK) which was left to dry for ~15 min. Once the leaf disc was dry, clear sticky tape was pressed firmly onto the dried varnish to obtain an imprint. The imprint was peeled from the disc and transferred to a glass microscope slide (Ferris *et al*., 2001). Images were captured using a light digital microscope (Zeiss axiophot 2) at ×200 magnification. The images were imported into the 'Metamorph' program for analysis. Ten cells per image were chosen at random, with the exception of those bordering a stomata complex, which were ignored; from each, the mean epidermal cell area (ECA) for each sample leaf was calculated. An estimation of epidermal cell number (ECN) per leaf was calculated from leaf area/mean epidermal cell area. The number of whole stomata per field of view was counted, from which stomatal density (SD) was calculated as the number of stomata per mm^2. The stomatal index (SI) was calculated using the following formula: SI (%)=[stomata]/[total cells+stomata]×100.

Cell sap osmolality

Leaf 6 of each plant was wrapped in aluminium foil, immediately frozen, and sent back to the UK in dry ice before storing at −80 °C for sap analysis. The sap was collected from the stored leaf 6 by placing it in a 0.5 ml centrifuge tube with a needle hole in the bottom and centrifuging for 15 s at 16 000 *g*. The sap was tested using the Wescor 5100C vapour pressure osmometer (Wescor Inc., Logan, UT, USA).

Shelf life

The leaves of each line were harvested after the above experiment samples were collected and sent back to the laboratory. The leaf samples collected from the Portugal site were transported back to the UK in a commercial refrigerated lorry (Vitacress Salads Ltd), which took 36 h. For the UK trial, the leaves were washed and packaged the next day after harvesting. Leaves were washed in 20.0 l of distilled water in a Hotpoint Supermatic Plus 9404 twin tub washing machine (General Domestic Appliances Ltd, Peterborough, UK) for 1 min on the lowest setting and dried for 20 s in the spin compartment, simulating the washing processing in the factory and that used previously (Clarkson *et al*., 2005). The leaves were randomly selected and packed in 5 g aliquots in a zip-sealed polythene bag. Where sufficient leaf material was available, five replicate bags for each line were produced. All the bags were stored in the dark at 7 °C. Shelf life was determined through a visual assessment of the bags every day. When breakdown, bruising, or damage was seen in the pack, this bag was rejected. To compare two trials consistently, the shelf life day value was counted from the day the material was processed to the day when the material bag was rejected, noted as SL_P. The whole period from harvest was

truncated individually every day to assess each line condition. The data on each RIL condition were treated as binary in nature and were recorded as good condition (score 1) or bad (being rejected) (score 0). The data were collected separately each day and used as discrete/binary data for analysis.

Cell wall biophysical properties

The oldest leaf from each line was stored in methanol immediately after harvest and transported to the laboratory. Before use, samples were fully rehydrated by two 15 min washes in dH_2O prior to cutting leaf strips (7 mm wide) with razor blades parallel and adjacent to the mid-vein of each leaf. The leaf strip was mounted on Instron grips (High Wycombe, Bucks, UK) using folded sticky labels at each end of the strip with a 10 mm working distance and then subjected to two sequential loads of 400 mN before being pulled to breakpoint. Measurements of plasticity, elasticity, and breakstrength were calculated using Bluehill software (Instron), the former by taking a chord modulus from 300 mN to 330 mN on each successive tissue loading event.

QTL analysis

A dense molecular marker linkage map developed for the RIL population was used to select a set of framework markers for these QTL studies (http://cgpdb.ucdavis.edu/database/genome_viewer/viewer/MAP2_JMR3). A set of 1334 markers was used as a framework map for QTL analysis (http://cgpdb.ucdavis.edu/database/supplemental_data/). Framework markers were selected to maximize genome coverage and marker information content. Forward stepwise regression and backward elimination regression methods were used to identify significant markers for each trait. A composite interval mapping method was employed to increase resolution and reduce background marker effect (Zeng, 1994). QTLs were detected using the computer program QTL Cartographer version 2.5 (Basten *et al*., 1994, 2002); all of the significant markers were selected to control the genetic background, and walk speed was set at 2 cM for all traits. The threshold for the logarithm of odds (LOD) score for declaring QTL significance at $P \leqslant 0.05$ was estimated by permutation analysis for each trait using 1000 iterations (Churchill and Doerge, 1994) and was found to be 3.7. A single LOD drop off was used to calculate QTL confidence intervals. The graphical representation of the linkage maps and QTLs was prepared using MapChart (Voorrips, 2002).

All the traits measured and calculated values were analysed using mean values for each RIL. The normal distribution was tested using the Anderson–Darling normality test of the software Minitab 13.0 for Windows. The correlation among all the leaf development traits and leaf processability was analysed using the above software. All the trait values were assessed using one-way analysis of variance (ANOVA) to test the significant difference between two parents *L. serriola* (US96US23) and *L. sativa* cv. Salinas. The significant effects are shown as $*P < 0.05$ and $**P < 0.01$.

Physiological assessment of good and poor lines

Three lines that were found to perform well and three that performed badly in the Portugal and UK trials for shelf life, plus the two parental lines *L. sativa* and *L. serriola*, were subsequently sown in Aguilas, Spain (latitude 37°24′N, longitude −1°35′N; average minimum–maximum temperature during growing season, 7–23 °C; average precipitation, 1–21 mm) and harvested when 6 weeks old. Lines were planted in randomized triplicate blocks and edged with Cos lettuce to remove any edge effects. They were shipped back to the UK and processed in a commercial wash line at Vitacress Salads Ltd prior to packaging in 50 g bags. In order to provide quantitative data on crop quality to correlate with visual symptoms of leaf deterioration, the lines were assessed by three different methods by staff in independent laboratories as follows. (i) Visual assessment was performed at Vitacress Salads Ltd on a daily basis on a 10-point scale, with overall judgement being an average of qualities such as leaf blackening, bruising, waterlogging, and tearing. (ii) Membrane leakage was measured to give quantitative analysis of cell integrity and the change in membrane permeability throughout shelf life. Leakage may be due to tearing of the leaf, tissue collapse, or by the action of bacteria. Membrane leakage was quantified by means of conductivity measurements on replicate 20 g samples of leaves from each line in 200 ml of deionized water before and after a 3 h incubation. The material was subsequently dried down, and DW was recorded so that conductivity was expressed in µS per gDW. (iii) Bacterial load was hypothesized to contribute to loss of product quality as the blackening seen in some leaves is reminiscent of infection. Bacterial contamination was assessed by processing 25 g of leaves in a pulsifier (Pul100E, Microgen Bioproducts Ltd, Camberley, Surrey, UK) with 225 ml of phosphate-buffered saline (PBS) solution for 2 min. Either 50, 100, or 1000 µl were plated, and coliform units were counted the following day.

Results

Trait performance

The mapping population was characterized at two separate field sites, in the UK and Portugal, which had similar temperature ranges and water availability, but were in North and South Europe, respectively, leading to different light intensity and humidity at each site. The wild lettuce *L. serriola* has longer, narrower, more hairy leaves than the cultivated lettuce *L. sativa*. The segregation of these traits among the 60 RILs gives rise to a wide range of phenotypes. Some leaves were smooth and round in shape, while others were hairy or prickly, with curly edges. In the Portugal field trial, each trait was measured at different leaf development stages, except cell sap osmolality (OSM) and SL_H, while only one measurement was taken for each trait in the UK trial. Additionally, AGR and RGR traits were only assessed in the UK trial.

To compare the Portugal and UK field trials, only the leaf development stage trait measurements were summarized (Table 1). Generally, the performance of the traits measured between two parents in the Portugal field trial showed the same trends in the UK field trial. *Lactuca serriola* had smaller values than *L. sativa* for leaf area (LA), FW, DW, SLA, ECN, SL_H, elasticity (E), and plasticity (P), but larger values for DWP, ECA, SD, SI, and maximum load (ML). The differences between the parents were significant at $P \leqslant 0.05$, except for the traits of chlorophyll content (CHL), OSM, P, and E in the UK trial, and P and E in the Portugal trial. The means of the RILs for the traits were approximately equal to the mean of the two parental lines in most traits such as DW, DWP, and SLA. The 18 traits expressed transgressive segregation in both parental directions in the field trials. The means of RILs in the Portugal trial had much higher values for CHL, ECN, SD, and the biophysical traits than those of the UK field trial, but were lower for SLA and

Table 1. *Mean and range values for measured traits of the mapping population and parents*

Traits	Field site	Parents			RILs		
		L. serriola	*L. sativa*	Mean	Minimum	Maximum	Mean
Leaf area (LA) (mm^2) (mean of leaves 3, 6, 7, 8)	Portugal	3677	5878	4778	2001	5956	4108
	UK	3016	4954	3986	2317	5910	4283
Leaf fresh weight (FW) (mg)	Portugal	1.52	3.97	2.75	0.99	3.55	2.19
	UK	1.07	2.64	1.86	1.16	3.45	2.08
Leaf dry weight (DW) (mg)	Portugal	0.23	0.36	0.29	0.12	0.41	0.26
	UK	0.11	0.19	0.15	0.10	0.26	0.17
Leaf dry weight as a percentage of fresh weight (DWP) (%)	Portugal	15.25	9.32	12.28	9.34	15.05	12.11
	UK	10.47	7.04	8.75	7.26	11.46	8.40
Specific leaf area (SLA) ($mm^2\ mg^{-1}$)	Portugal	18.64	21.72	20.18	16.94	23.21	19.76
	UK	29.76	32.16	30.96	23.04	37.94	30.66
Chlorophyll content (CHL) ($\mu g\ mm^{-2}$)	Portugal	0.47	0.36	0.42	0.33	0.65	0.46
	UK	0.20	0.22	0.21	0.15	0.26	0.20
Epidermal cell area (ECA) (μm^2)	Portugal	1294	970	1132	602	1626	1082
	UK	4034	2988	3511	1772	4177	2939
Epidermal cell number per leaf (ECN) ($\times 10^6$ cells per leaf)	Portugal	3.87	9.32	6.60	1.77	10.04	5.75
	UK	0.75	2.03	1.39	0.88	3.95	2.00
Stomata density (SD) (no. of stomata mm^{-2})	Portugal	195.23	160.72	177.98	87.50	315.31	191.32
	UK	84.53	59.52	72.02	53.57	134.52	84.45
Stomata index (SI) (%)	Portugal	19.32	12.84	16.08	10.84	21.11	16.16
	UK	25.16	15.08	20.12	15.17	23.70	18.92
Cell sap osmolality (OSM) ($mmol\ kg^{-1}$)	Portugal	492	467	480	389	625	464
	UK	423	425	424	367	469	412
Shelf life (SL) (d)	Portugal	9.33	12.60	10.97	6.00	14.40	9.73
	UK	7.20	9.20	8.20	5.00	13.40	8.38
Absolute growth rate (AGR) ($mm^2\ h^{-1}$)	Portugal	–	–	–	–	–	–
	UK	5.79	11.36	8.58	3.61	17.79	9.43
Relative growth rate (RGR) ($\times 10^{-3}\ mm^2\ mm^{-2}\ h^{-1}$)	Portugal	–	–	–	–	–	–
	UK	8.55	16.02	12.29	4.84	14.09	9.12
Maximum load (ML) (N)	Portugal	1.44	0.80	1.24	0.97	0.39	1.36
	UK	1.03	0.57	0.8	0.82	0.55	1.40
Plasticity+elasticity (P+E) (% extension)	Portugal	7.15	8.11	7.63	7.93	2.44	14.06
	UK	4.14	3.53	3.84	4.91	2.54	10.15
Plasticity (P) (% extension)	Portugal	4.47	4.81	4.64	3.11	1.38	6.08
	UK	2.39	1.80	2.10	2.10	1.15	3.88
Elasticity (E) (% extension)	Portugal	2.67	3.30	2.99	4.82	0.87	9.20
	UK	1.75	1.72	1.74	2.81	1.21	6.56

ECA (by approximately one-half and one-third of the value, respectively) than those of the UK trial. The mean values for LA, FW, SI, and OSM were similar in the two field trials. ARG and RGR were only assessed in the UK trial. *Lactuca sativa* had about twice the value for AGR and RGR than *L. serriola*. The mean of the RILs for AGR was close to the mean of the two parents, but the RGR of *L. sativa* was higher than the maximum RIL value.

Normal distributions were observed for most of the traits (Fig. 1). ANOVAs showed that there was significant genetic variability for all the above traits in this population (data not shown, $P < 0.05$), thus permitting further QTL analysis.

Shelf life

On average, the shelf life of the population in the Portugal trial was higher than that of the UK trial (9.73 d versus 8.38 d). The shelf life of the RILs was variable, ranging from 6 d to 14.40 d in the Portugal field trial and from 5 d to 13.40 d in the UK trial (Fig. 2, Table 1). There was a normal distribution among the population for shelf life (Fig. 1j). RIL 1 had the longest shelf life of 14.4 d in Portugal, while this was 12.8 d in the UK and was the second best performing line in this trial. RIL 112 only lasted 10 d in Portugal, but it was the best line in the UK trial, lasting 13.40 d. RIL 121 was the worst line in terms of the shelf life in the Portugal trial, only lasting 6 d, and RIL 89 was the worst line in the UK trial, lasting 5 d. The shelf life of two parents was also investigated; while the shelf lives of *L. serriola* and *L. sativa* in Portugal were 9.33 d and 12.60 d, respectively, they were much shorter at 7.20 d and 9.20 d in the UK. The total number of lines in good condition each day is shown in Fig. 3. Due to lack of sufficient leaf samples for some of the lines, only 56 lines were assessed for the Portugal trial and 60 lines for the UK. These binary data sets were used for further QTL analysis, revealing an additional seven shelf life QTLs (Table 3).

A subset of the best (RILs 5, 15, and 74) and worst (RILs 19, 32, and 89) lines were grown in Aguilas, Spain and harvested after 6 week's growth at an equivalent stage to commercially grown lettuce. Qualitative assessment of shelf life indicated that the lines selected as good performers did indeed look better than those assessed as

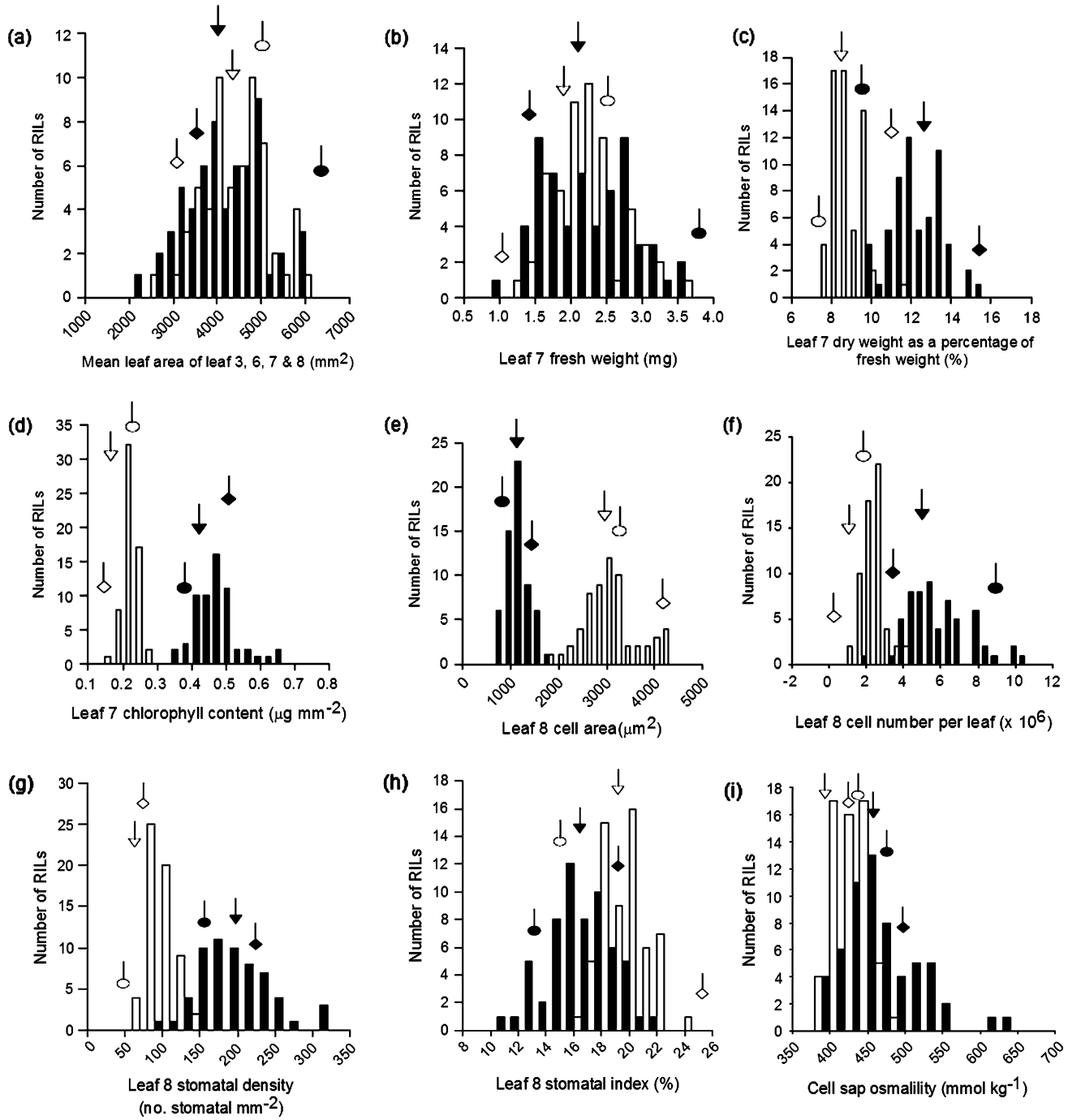

Fig. 1. Distributions of selected traits in the RIL mapping population: (a) Mean leaf area of leaves 3, 6, 7, and 8, (b) Leaf 7 fresh weight, (c) leaf 7 dry weight as a percentage of fresh weight, (d) leaf 7 chlorophyll content, (e) leaf 8 epidermal cell area, (f) leaf 8 cell number per leaf, (g) leaf 8 stomatal density, (h) leaf 8 stomatal index, (i) cell sap osmolality, (j) shelf life, (k) maximum load, (l) plasticity+elasticity, (m) elasticity, and (n) plasticity. The field sites are indicated as either Portugal (filled bar) or the UK (open bar). The mean values of the parents *L. serriola* and *L. sativa*, and of the RILs are indicated by arrows.

poor in previous trials based on visual characteristics such as bruising and waterlogging of the leaf tissue (Fig. 4a). Both parental lines scored well and appeared to last as well as the three good lines. However, membrane leakage assessed by conductivity measurements indicated that the wild parent *L. serriola* had less permeable membranes than *L. sativa* at day 10. This may be due to the growth rate of *L. serriola* being slower than that of *L. sativa*, leading to smaller, more robust leaves at the time of harvest, and therefore a reduced tendency towards membrane leakage. Of the RILs classified as poor, two (19 and 89) had higher conductivity than all the good

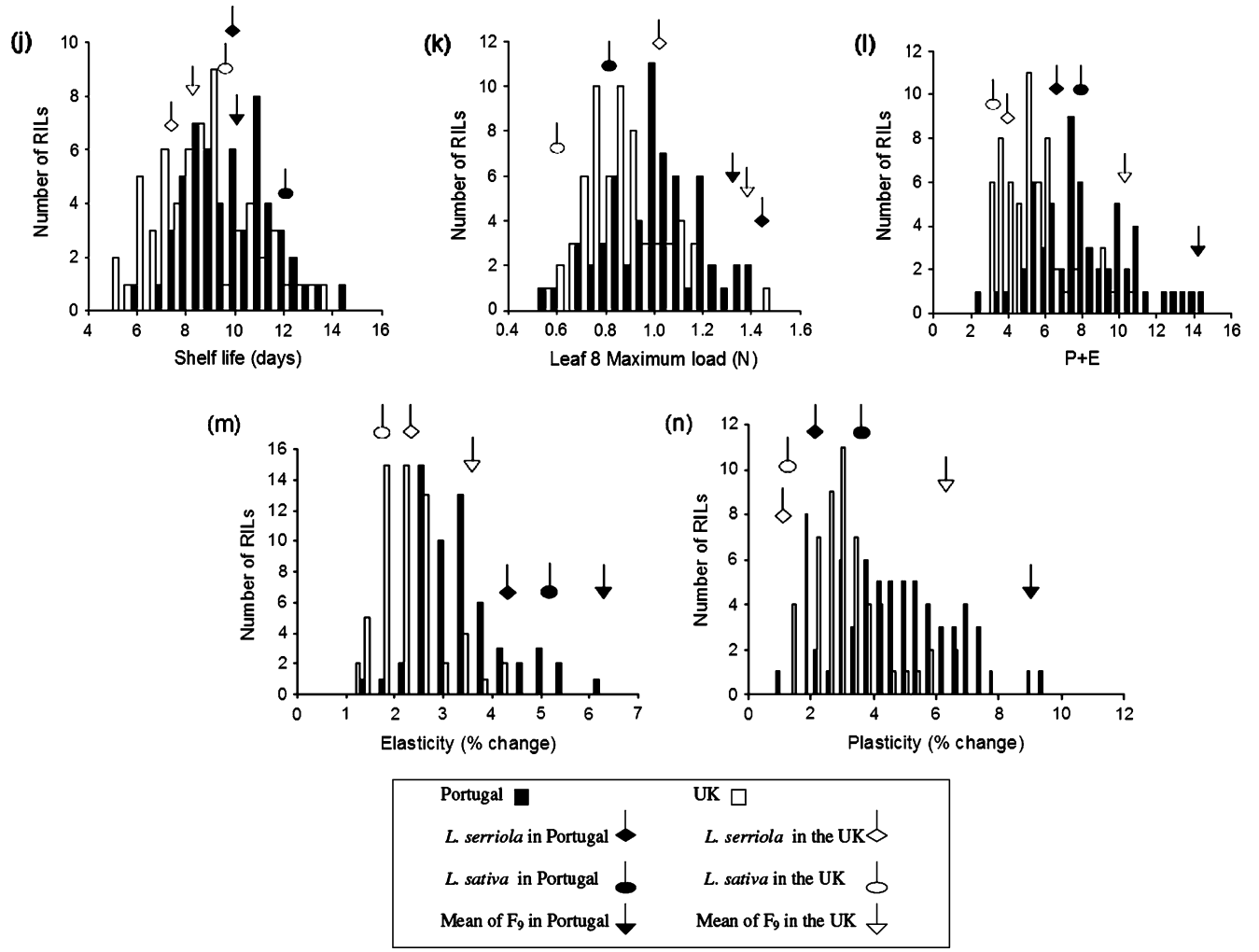

Fig. 1. (*Continued*).

lines, but RIL 32 had very low leakage, despite its poor appearance (Fig. 4b). Examination of coliform units on each RIL showed good correlation with qualitative measurements of appearance, and indicates that bacterial colonization may be a significant contributor to poor shelf life. All of the poor lines showed approximately double the amount of coliform units at day 10 compared with the good lines (Fig. 4c). Prior measurements indicated that coliform units on a leaf line did not change significantly during storage at 4 °C (data not shown); thus, the counts indicated the amount of bacterial load that persisted after processing. Differences between lines may be due either to different levels of colonization in the field, perhaps variable depending on the secondary products produced by each leaf, or to the leaf morphology (e.g. convolutions of the epidermal surface), making the washing process more or less efficient at removing bacteria, or to an interaction of the two factors.

Correlation among the traits

Correlations between the traits in both field trials were calculated using Pearson's correlation coefficient analysis (Table 2). There is a consistent correlation between most pairs of traits in the two field trials. For example, LA had a highly significant positive phenotypic correlation with FW (0.87; 0.85, Portugal and the UK respectively), DW (0.91; 0.86), and ECN (0.61; 0.69), while LA had a negative phenotypic correlation with SI (−0.27; −0.29). FW had the highest positive correlation with DW at both trials (0.90; 0.93), and ECA had the highest negative correlation with ECN (−0.69; −0.70) and SD (−0.65, −0.79) in both trials. Surprisingly, in the UK field trial, SL showed a highly significant positive correlation with LA (0.41), FW (0.36), DW (0.36) (P <0.01), ECN (0.28), and ML (0.30) (P <0.05), and a significant negative correlation with E (−0.38) (P <0.01), CHL (−0.29), SI (−0.27), P (−0.29), and P+E (−0.34) (P <0.05). The only

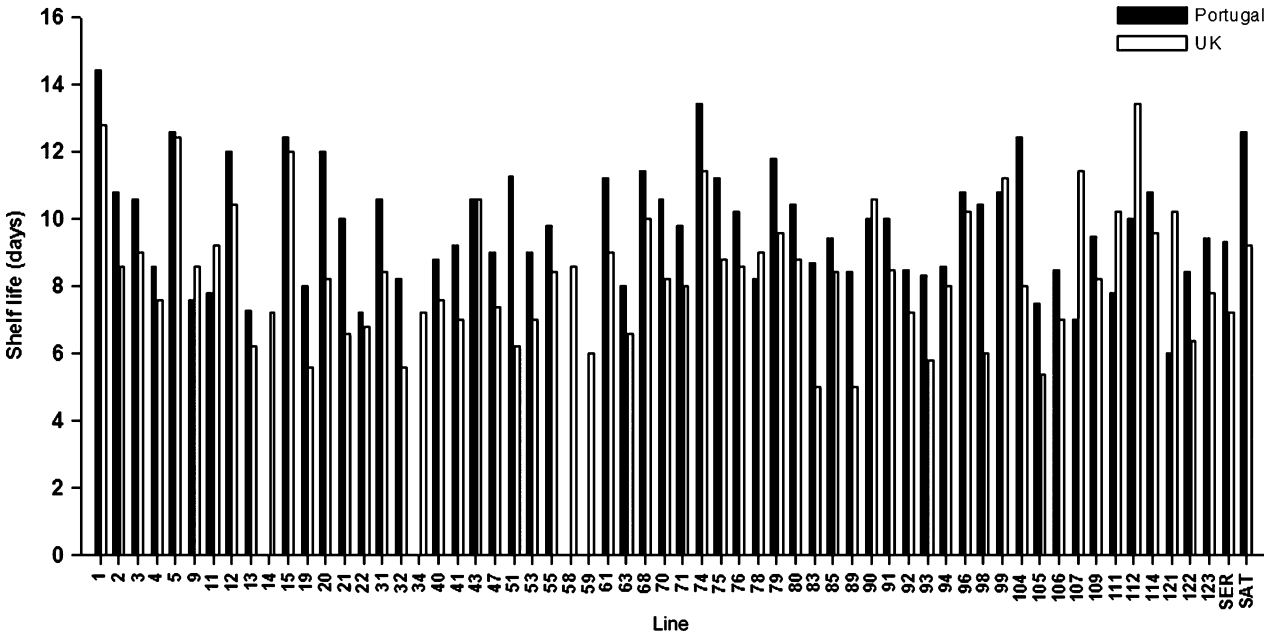

Fig. 2. Shelf life of the RIL mapping population in two field trials: Portugal (filled bar) and the UK (open bar). Five replicates of most lines were kept at 7 °C in a fridge, and shelf life was determined through a visual assessment. When breakdown, bruising, or damage was seen in the pack, the bag was rejected. SER, *L. Serriola*; SAT, *L. Satira*.

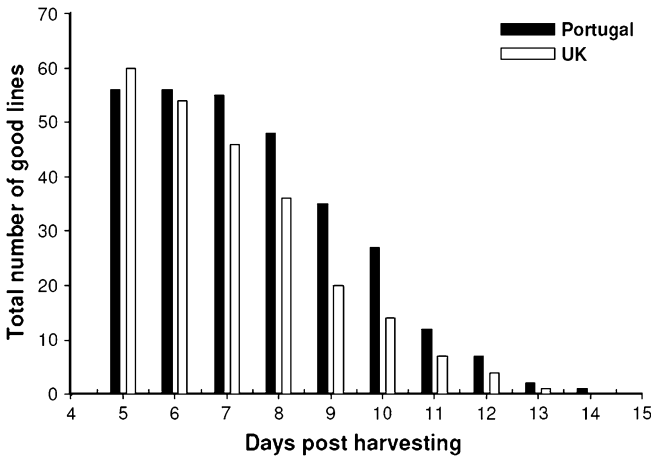

Fig. 3. Post-harvest rejection of the RIL mapping population in two field trials: Portugal (filled bar) and the UK (open bar). The total number of lines in good condition was assessed each day during the shelf life period (n=5).

significant correlation between SL and developmental traits detected in the Portugal trial was for OSM (0.27), but this was not detected in the UK. However, SL also showed a significant negative correlation with elasticity (E; −0.26) in the Portugal trial, correlating with the relationship found in the UK between these two traits.

QTL analysis

A genetic map containing 1334 AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat) markers was used for composite interval mapping analysis [http://cgpdb.ucdavis.edu/database/supplemental_data (MAP2_JMR3)]. All the traits, including the different leaf development stage measurements, were analysed. A total of 107 QTLs with significant effects were detected for the 29 traits, distributed on all nine of the linkage groups (Table 3, Fig. 5, Supplementary Fig. S5 available at *JXB* online). Individual QTLs accounted for 10.83–66.34% of the phenotypic variation in this population. Among these detected QTLs, 61 significant QTLs were identified for the leaf traits measured in the Portugal trial (filled bars in Fig. 5 and Supplementary Fig. S5, available at *JXB* online) and 45 for the UK trial (open bars in Fig. 5 and Supplementary Fig. S5, available at *JXB* online). Five QTLs located to overlapping regions of the same linkage groups in both field trials. These common QTLs were for SD on LG1, DWP on LG4 and LG8, shelf life at day 8 (SL_d8) on LG8, and ML on LG4. The total number of QTLs identified in the UK trial was less than in the Portugal trial, perhaps due to fewer leaf developmental stage samples being tested in the UK. Three different developmental stage samples were assessed for most of the traits in Portugal, while only one leaf sample was assessed in the UK. The maximum number of QTLs per trait for each leaf age was four.

Leaf growth and development (Table 3)

QTLs for leaf area from the Portugal trial were identified on seven chromosomes, but strong overlapping QTLs for leaves 2, 3, and 4 were found on LG3 (30.0–34.8 cM,

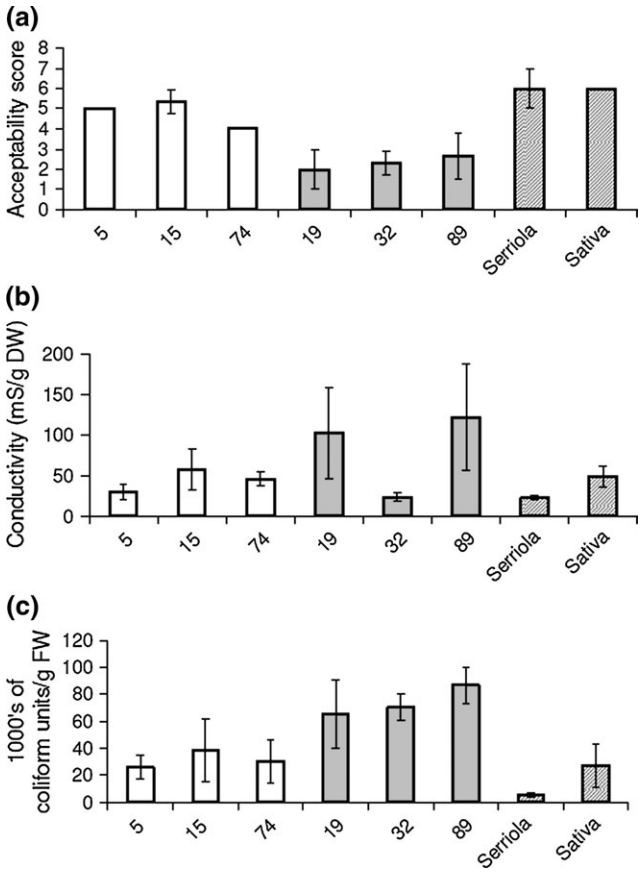

Fig. 4. Quantitative measurements of shelf life from selected RILs 10 d after harvest (7 d after processing). RILs selected as performing well in both previous trials in Portugal and the UK (5, 15, and 74) are indicated by white bars, RILs selected as poor performers in the same trials (19, 32, and 89) as grey bars, and the bars relating to the parental lines are hatched. (a) Acceptability score (out of 10) based on visual assessment (n=3). (b) Conductivity measurements (an an indicator of membrane leakage) (n=6). (c) Bacterial counts (coliform units per g FW) (n=4). Error bars are the SEM.

1A02-270–LK1225) and for leaves 4–8 also on LG3 (82.0–91.8 cM, E45/M49-F-275–LE1011). The increasing allele for leaves 4 and 8 was from *L. serriola*, but from *L. sativa* for leaves 5, 6, and 7. The total phenotypic variance explained by these identified QTLs was between 14% and 21% in young and semi-mature leaves and 29% in mature leaves in the Portugal trial. Only two QTLs were identified for leaf area in the UK trial, one on LG4 (127–138 cM, LE4022) from the *L. serriola* allele for leaves 7 and 8, and another for leaf 8 on LG9 (47.0–48.7 cM, E35/M60-F-157) from the *L. sativa* allele, accounting for 20–25% of the phenotypic variability between them. No QTLs were found for LA from young or semi-mature leaves in the UK trial. AGR and RGR were only measured in the UK trial. A QTL for AGR and one for RGR were both found on LG2 for leaves 3–6 (around markers LE0371 and LE3023, respectively). The QTL for AGR co-located with a QTL for SI, and the QTL for RGR with one for OSM. The QTL for AGR on LG7 also co-located with the first LA measurement taken during development (LAf_UK), ECA in the Portugal trial, and CHL from the UK, spanning a region from 99.1 cM to 111.4 cM (markers LK1513–M6982). The latter two traits were derived from *L. serriola*, the former from *L. sativa*, and accounted for 13–37% of the phenotypic variation.

For FW and DW there were a number of overlapping QTLs. The two traits co-located on LG2 with one for LA (132.4–134.8 cM, E33/M59-F-121–E33/M59-F-226). Again the three traits (FW, DW, and LA) co-located to a QTL on LG3 (82.0–91.8 cM, E45/M49-F-275–LE1011). A QTL for DWP co-located with those for DWP in the Portugal trials on LG4, and also with one for SI (81.8–88.5 cM, E35/M49-F-296–1A04-137), with the increasing alleles coming from *L. serriola* in all cases. The same allele accounted for QTLs for DWP, ECA, and SI on LG6 (123.6–131.1 cM, L2219–LE3085), and a strong overlapping QTL hotspot was identified on LG8 that encompassed the above three traits, plus SLA, ECN, DW, and FW (56.1–70.6 cM, LE1345–1A09-212). With the exception of ECN, DW, and FW, the allele came from *L. serriola* in the remainder, correlating with the allelic origin of the same traits on LG4 and LG6 and accounting for up to 20% of the phenotypic variation.

Leaf biophysical properties (Table 3)

Instron analysis of P, E, and ML (breakstrength) of the leaves revealed several QTLs. One QTL for ML co-located on LG4 in both the Portugal and UK trials (22.2–28.9 cM, LE4021–E33/M59-f-364) and accounted for up to 28% of the phenotypic variation, derived from the *L. sativa* allele in both cases. Another QTL for maximum load in the UK trial co-located with a strong shelf life QTL from the same experiment on LG6. As predicted, QTLs for P+E, representing the total deformation potential of the tissue, co-located with those for either P or E calculated independently, thereby showing which type of stretch the deformation was largely due to. E in both trials co-located with other developmental traits in hotspots on LG7 and LG8, in both cases derived from the *L. sativa* allele. However, QTLs for increased P were attributed to *L. serriola* alleles in the UK trial, but to *L. sativa* in the Portugal trial, and in both cases were clearly associated with hotspots concerned with shelf life on LG5 (21.2–61.0 cM, 1A09-212–LE9015) and LG8 (112.7–124.1 cM, L0248–E35/M49-F-267<N>).

Shelf life (Table 3)

Shelf life QTLs for the Portugal trial were found on LG5 (47.6–61.0 cM, E44/M48-F-085–LE9015) and LG8 (94.4–96.0 cM–E35/M59-F-530). These coincided with QTLs derived from the binary data on days 10 and 12 of shelf life. Additional QTLs were identified for day 9 (LG6) and day 10 (LG2). Chromosome 6 proved to be a hotspot for shelf life data derived from the UK trial,

Table 2. *Pearson's correlation coefficient of trait means of the RIL mapping population in two field trials*

*Correlation is significant at the 0.05 level (two-tailed); **correlation is significant at the 0.01 level; and ***correlation is significant at the 0.001 level.

Field site	Traits[a]	LA	FW	DW	DWP	SLA	CHL	ECA	ECN	SD	SI	OSM	SL	ML	P+E	P	E
Portugal	LA	1															
UK		1															
Portugal	FW	0.87***	1														
UK		0.85***	1														
Portugal	DW	0.91***	0.90***	1													
UK		0.86***	0.93***	1													
Portugal	DWP	−0.16	−0.46***	−0.06	1												
UK		−0.16	−0.41**	−0.07	1												
Portugal	SLA	−0.06	−0.27*	−0.41**	−0.22	1											
UK		0.07	−0.26*	−0.40**	−0.29*	1											
Portugal	CHL	−0.18	−0.14	−0.02	0.26*	−0.35**	1										
UK		−0.36*	−0.02	−0.08	−0.15	−0.41**	1										
Portugal	ECA	0.02	−0.12	0.01	0.32	−0.04	−0.09	1									
UK		−0.07	−0.16	−0.18	0.07	0.21	−0.28*	1									
Portugal	ECN	0.61***	0.62***	0.51***	−0.42**	0.09	−0.20	−0.69***	1								
UK		0.69***	0.67***	0.69***	−0.15	−0.12	−0.09	−0.70***	1								
Portugal	SD	−0.27*	−0.17	−0.28*	−0.18	0.06	0.14	−0.65***	0.32*	1							
UK		−0.16	−0.03	−0.01	0.04	−0.20	0.24	−0.79***	0.48***	1							
Portugal	SI	−0.27*	−0.32*	−0.30*	0.17	−0.02	0.12	0.31*	−0.42**	0.48***	1						
UK		−0.29*	−0.29*	−0.22	0.28*	−0.09	−0.02	0.19	−0.30*	0.41**	1						
Portugal	OSM	−0.18	−0.29*	−0.08	0.58**	−0.27*	0.13	0.43**	−0.38**	−0.23	0.20	1					
UK		−0.22	−0.25*	−0.12*	0.40**	−0.29	0.17	0.17	−0.35**	−0.08	0.23	1					
Portugal	SL	0.14	0.07	0.07	−0.01	0.158	−0.19	0.14	0.11	−0.06	0.03	0.27*	1				
UK		0.41**	0.36**	0.36**	−0.10	0.07	−0.29*	0.04	0.28*	−0.20	−0.27*	−0.09	1				
Portugal	ML	0.02	−0.08	0.09	0.44	−0.25	0.19	0.36	−0.35	−0.40	−0.05	0.11	−0.04	1			
UK		0.19	−0.26*	−0.24	−0.14	0.28*	−0.19	0.30*	0.06	−0.28*	−0.14	−0.09	0.30*	1			
Portugal	P+E	0.18	0.32*	0.14	−0.50***	0.17	−0.21	−0.49***	0.55***	0.27*	−0.33*	−0.47***	−0.23	−0.47***	1		
UK		0.02	0.16	0.18	0.07	−0.27*	0.14	−0.09	−0.24	0.26*	0.33*	0.11	−0.34*	0.10	1		
Portugal	P	0.21	0.34*	0.19	−0.45***	0.11	−0.19	−0.41**	0.50***	0.23	−0.30*	−0.42***	−0.20	−0.48***	0.98***	1	
UK		0.04	0.17	0.19	0.10	−0.22	0.11	−0.07	−0.22	0.25*	0.30*	0.08	−0.29*	0.10	−0.31*	1	
Portugal	E	0.09	0.23	0.03	−0.52***	0.26*	−0.22	−0.56***	0.55***	0.33*	−0.34*	−0.49***	−0.26*	−0.44***	0.91***	0.79***	1
UK		−0.07	0.10	0.14	0.01	−0.33	0.18	−0.11	−0.25*	0.26*	0.34*	0.14	−0.38**	0.08	−0.39**	0.98***	1

[a] Trait abbreviation: LA, mean leaf area of leaves 3, 6, 7, and 8 (mm^2); FW, leaf 7 fresh weight (mg); DW, leaf 7 dry weight (mg); DWP, leaf 7 dry weight as a percentage of fresh weight (%); CHL, leaf 7 chlorophyll content (μg mm^{-2}); ECA, leaf 8 epidermal cell area (μm^2); ECN, leaf 8 epidermal cell number per leaf ($\times 10^6$); SD, leaf 8 stomata density (no. of stomata mm^{-2}); SI, leaf 8 stomata index (%); OSM, leaf 6 cell sap osmolality (mmol kg^{-1}); SL, shelf life period, counted from harvesting to the day before being rejected (d); ML, maximum load of leaf strip before breakage (N); P+E, plasticity+elasticity of leaf strip (% extension); P, plasticity (% extension); E, elasticity (% extension).

Table 3. *QTLs detected by composite interval mapping for all leaf traits assessed in the RIL mapping population in two field trials*

Trait[a]	Position[b] (cM)	Marker[c]	Portugal field trial				UK field trial			
			Leaf no.[d]	LOD[e]	Additive[f]	Variance[g] (%)	Leaf no.	LOD	Additive	Variance (%)
LA	LG1: 98.6–102.3	LK1072	3	5.65	130.03	21.14				
	LG2: 132.9–134.4	E33/M59-F-226	7	4.68	469.04	15.54				
	LG3: 3.7–4.4	LM0075	4	3.74	−171.73	10.83				
	LG3: 30.0–31.3	1A02-270	2,3,4	4.19	119.36	14.62				
	LG3: 34.5–34.8	LK1225	4	3.77	−224.66	21.95				
	LG3: 82.0–84.0	E45/M49-F-275	4,8	5.21	−247.68	21.31				
	LG3: 83.3–91.8	LE1011	5,6,7	7.41	362.33	29.73				
	LG4: 127–138	LE4022					7,8	5.49	−630.39	25.53
	LG7: 78.3–84.1	E44/M49-F-328	1	4.05	33.39	20.77				
	LG8: 73.7–74.6	LE0460	8	4.02	451.26	13.01				
	LG9: 47.0–48.7	E35/M60-F-157					8	5.89	677.96	20.69
	LG9: 75.5–76.4	LE3171	7	5.55	471.59	16.26				
LAf_UK	LG4: 90.6–91.3	E35/M60-F-286						4.46	−210.58	16.48
	LG7: 99.2–102.4	LE0463						8.16	307.24	36.95
LAo_UK	LG7: 93.5–96.8	LK1513						6.31	138.08	29.06
AGR	LG2: 64.5–67.7	LE0371					3–6	3.88	1.10	13.34
	LG7: 99.1–104.0	LK1513						5.97	1.46	22.81
RGR	LG2: 96.5–96.9	LE3023					3–6	3.22	−1.08	21.80
FW	LG2: 132.4–132.8	E33/M59-F-121	4	4.66	−0.1	16.54				
	LG3: 82.9–90.3	LE1011	4,7	6.75	−0.14	24.99				
	LG4: 131.2–141.4	1A09-349<N>					7	4.10	−0.21	16.57
	LG5: 131.2–133.6	1A01-203					7	5.06	0.24	21.45
	LG7: 68.4–70.9	E35/M49-F-590	1	5.33	0.01	24.56				
	LG8: 68.0–69.2	1A20-148	4,7	7.06	0.12	24.49				
DW	LG2: 132.6–134.8	LK0017	7	4.50	−0.03	17.02				
	LG3: 82.8–96.0	LE1011	4,7	8.10	−0.04	31.25				
	LG4: 82.8–96.0	1A15-333	4	5.04	−0.69	21.26				
	LG5: 131.6–138.6	LE0369					7	4.48	0.02	18.50
	LG7: 78.0–84.0	E44/M48-F-328	1	4.33	0.00	19.75				
	LG8: 68.0–68.9	1A20-148					7	4.71	0.02	16.94
DWP	LG1: 92.0–95.1	LE0488	7	3.99	0.46	11.04				
	LG3: 32.6–33.1	1A20-141					7	5.46	−0.33	16.70
	LG4: 100.5–103.0	1A15-333	4	5.04	−0.68	21.26				
	LG4: 81.8–82.8	E35/M49-F-296					7	4.61	−0.30	12.89
	LG4: 85.0–86.8	1A04-137	7	6.48	−0.66	19.99				
	LG6: 123.6–128.6	L2219	7	3.82	−0.44	10.89				
	LG8: 56.1–58.0	LE1345	1	4.06	−0.91	16.06				
	LG8: 69.6–70.2	LE0460	7	5.98	−0.54	19.27				
	LG9: 39.4–40.0	E54/M48-F-307					7	4.11	0.29	12.29
SLA	LG1: 52.2–55.9	1A01-121	4	4.59	−1.17	21.08				
	LG2: 8.1–18.2	M1282	1	4.30	−1.46	17.79				
	LG8: 58.4–59.6	1A02-132<N>					7	5.16	−1.44	19.31
	LG8: 86.2–87.7	E33/M59-F-216	1	4.40	1.81	19.12				
CHL	LG3: 34.5–34.8	LK1225					7	4.79	0.01	20.32
	LG4: 57.4–58.2	Sf2979					7	4.64	0.01	12.56
	LG7: 108.6–111.4	M6982					7	4.85	−0.01	14.86
	LG9: 9.9–12.6	LK1355	1	3.83	0.021	19.02				
ECA	LG1: 84.3–85.5	E33/M59-F-391					8	5.65	248.96	18.14
	LG3: 34.7–34.8	E33/M59-F-208	2	4.30	−60.25	17.63				
	LG4: 105.9–106.7	L1371	5	4.93	−74.20	15.76				
	LG5: 21.2–26.4	1A09-212	8	4.69	−94.45	16.66				
	LG5: 44.5–47.4	LK1046					8	4.28	−203.66	12.37
	LG6: 125.7–131.9	1A09-410<N>	2	4.66	−61.71	15.63				
	LG7: 10.6–20.6	E38/M54-F-304	5	3.96	60.00	10.63				
	LG7: 100.9–110	E35/M60-F-114	2	3.87	−51.55	12.56				
	LG8: 22.6–23.7	LE0040B					8	5.35	−255.93	15.74
	LG8: 66.2–69.3	E33/M59-F-342	5	4.08	−72.72	14.89				
ECN	LG1: 20.4–23.4	LE7032					8	3.77	0.23	11.89
	LG1: 88.5–90.0	1A17-253<N>	2	6.42	−0.49	21.68				
	LG4: 5.3–10.5	1A08-254					8	5.86	−0.30	20.90
	LG7: 87.8–92.0	M2395					8	6.23	0.33	24.71
	LG8: 66.7–69.4	1A20-148	2,8	9.01	0.71	40.66				
	LG8: 70–70.6	1A09-212	5	5.80	0.54	21.45				
SD	LG1: 82.7–85.2	1A17-253< N >					8	7.63	−9.47	25.09
	LG1: 89.6–94.4	E33/M59-F-391	8	6.46	−22.16	21.22				

Table 3. (*Continued*)

Trait[a]	Position[b] (cM)	Marker[c]	Portugal field trial				UK field trial			
			Leaf no.[d]	LOD[e]	Additive[f]	Variance[g] (%)	Leaf no.	LOD	Additive	Variance (%)
	LG4: 0.0–2.5	1A15-253<N>	5	4.83	−23.13	13.91				
	LG7:0.0–1.1	E44/M49-F-248	5	5.68	−25.37	16.71				
	LG8: 9.6–14.8	LE9214	5,8	6.37	20.91	19.33	8	5.52	7.69	17.40
SI	LG1: 68.1–70.3	1A20-191					8	5.82	−0.74	18.52
	LG2: 71.3–74.1	M4833					8	3.94	0.62	14.39
	LG4: 85.2–88.5	1A04-137	2	6.18	−1.88	23.10				
	LG6: 117.6–119.9	E44/M49-F-081	8	4.21	−0.96	17.03				
	LG6: 128.1–131.1	LE3085	2	4.33	−1.44	14.85				
	LG7: 33.9–39.6	E44/M48-F-134	5	4.60	−1.21	17.19				
	LG8: 66.1–66.5	E45/M48-F-490	2	5.29	−1.68	20.08				
OSM	LG2: 97.3–97.8	E35/M59-F-200						7.62	15.52	37.85
SL_P	LG5: 47.6–50.3	E44/M48-F-085		5.49	−0.84	17.93				
	LG6: 84.6–86.5	E44/M49-F-246						6.34	0.98	22.36
	LG8: 94.1–96.0	E35/M59-F-530		4.92	0.78	19.52				
SL_d8	LG5: 34.4–39.3	E44/M48-F-292		5.43	−0.20	22.91				
	LG6:103.8–107.8	LE3092						7.25	0.28	30.19
	LG8: 104.3–106.1	LK1388						4.62	0.25	20.63
	LG8: 121.6–124.1	E35/M49-F-267<N>		4.39	−0.17	19.05				
SL_d9	LG6: 40.0–42.4	1A12-515<N>		4.86	0.21	17.00				
	LG6: 84.8–86.6	E44/M49-F-246						7.97	0.26	29.84
	LG8: 112.7–115.1	LE0248						5.90	0.25	21.91
SL_d10	LG2: 9.6–16.6	M1282		4.94	0.22	16.10				
	LG6:84.8–86.6	E44/M49-F-246						4.51	0.18	17.53
	LG8: 94.1–96.0	E35/M59-F-530		4.66	0.24	16.00				
SL_d11	LG1: 51.8–54.0	LE0243						7.16	0.17	25.62
	LG6: 6.9–10.4	E51/M49-F-206						6.53	0.15	21.25
	LG6: 85.8–87.8	E44/M49-F-246						4.22	0.1251	66.34
SL_d12	LG5: 52.5–61.0	LE9015		6.23	−0.20	28.40				
	LG5: 93.6–95.6	1A18-239<N>						6.26	−0.13	20.15
ML	LG4: 22.2–22.9	LE4021						4.02	0.07	17.46
	LG4: 26.1–28.9	E33/M59-F-364		6.21	0.10	27.58				
	LG5: 30.8–32.1	E35/M59-F-210						3.80	−0.06	14.03
	LG6: 92.1–94.6	LE3108						3.71	−0.06	12.93
	LG8: 0.6–3.5	1A15-569<N>		5.68	−0.09	18.51				
P+E	LG8: 66.7–68.7	E33/M59-F-342		4.12	1.08	16.69				
	LG8: 116.6–119.7	LK1471						4.46	−0.80	19.26
P	LG5: 41.2–42.2	LE1076		4.32	0.77	18.57				
	LG8: 116.6–118.7	LK1471						4.29	−0.52	17.79
E	LG2: 84.2–89.9	E35/M60-F-354		3.87	−0.38	15.32				
	LG7: 97.8–100.1	LE1120						5.34	0.34	25.61
	LG8: 66.7–69.5	E33/M59-F-342		3.99	0.40	17.61				

[a] Trait abbreviation: LA, leaf area at maturity (mm^2); LAf_UK, first leaf area measurement during development in the UK trial (mm^2); LAo_UK, second leaf area measurement during development in the UK trial (mm^2); AGR, absolute growth rate ($mm^2\ h^{-1}$); RGR, relative growth rate ($\times 10^{-3}$ $mm^2\ mm^{-2}\ h^{-1}$); FW, leaf fresh weight (mg); DW, leaf dry weight (mg); DWP, leaf dry weight as a percentage of fresh weight (%); CHL, chlorophyll content ($\mu g\ mm^{-2}$); ECA, epidermal cell area (μm^2); ECN, epidermal cell number per leaf ($\times 10^6$); SLA, specific leaf area ($\mu g/mm^2$); SD, stomatal density (no. of stomata mm^{-2}); SI, stomatal index (%); OSM, cell sap osmolality (mmol kg^{-1}); SL-P, shelf life period, counted from processing to the day before being rejected (days). The day number after trait SL indicated the days after harvesting where the QTL for shelf life was detected; ML, maximum load of leaf strip before breakage (N); P+E, plasticity+elasticity of leaf strip (% extension); P, plasticity (% extension); E, elasticity (% extension).

[b] Position indicated by the linkage group number, and the significant QTL interval over the threshold estimated by permutation analysis of each trait using 1000 iterations.

[c] Markers that are the nearest marker to the QTL.

[d] Leaf no. indicated at which leaf development stage the QTL was detected. NA, not assessed in the field trial for the individual trait.

[e] LOD: log of the odds score. To convert LR to LOD values, LOD=0.217 LR.

[f] Additive effect indicates which parental allele causes an increase in the trait value. Positive values indicate that the cultivated (*L. sativa*) allele increases trait values, and negative values indicate that the wild-type (*L. serriola*) allele increases trait values.

[g] Variance indicated the percentage of phenotypic variance in the mapping population explained by the detected QTL.

with overall shelf life plus binary data for days 9–11 accounted for by a region between 84.6 cM and 87.8 cM (E44/M49-F-246 the closest marker in all cases) derived from the *L. sativa* allele. This hotspot was also associated with ML of the tissue in the same trial. QTLs for SL_d8 were found on LG8 in both the Portugal and UK trials, although there was a gap of ~15 cM between the two QTLs. These QTLs were located slightly below the hotspot for developmental traits discussed above, but co-located with one associated with leaf P. QTLs for SL_d12

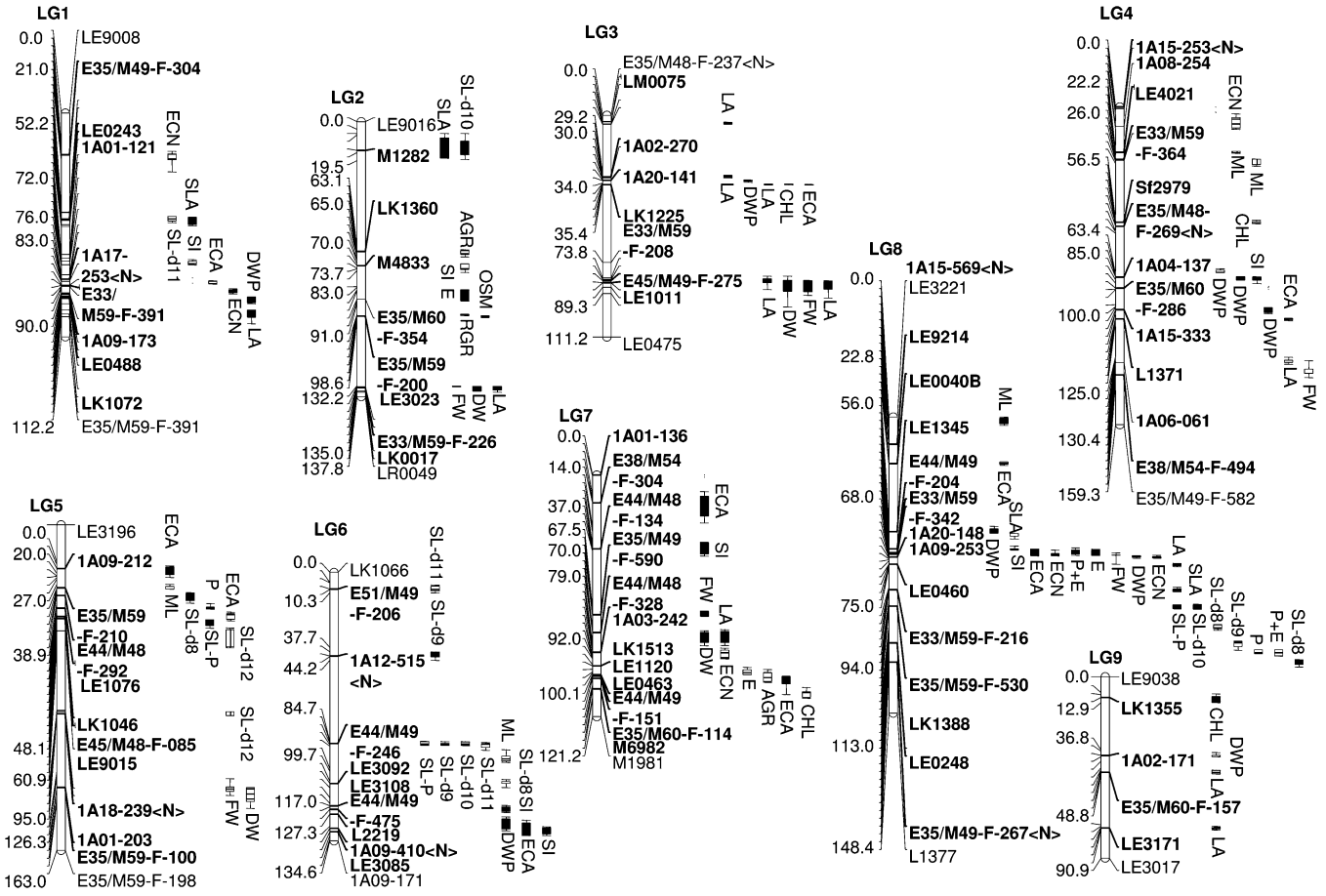

Fig. 5. QTL distributions on the molecular linkage map of the RIL mapping population based on composite interval mapping. Map positions are given in cM, listed on the right of each linkage group. A marker in bold indicates the nearest marker to the QTL for the trait of interest. For trait abbreviations, see Table 3. The leaf number is given after each trait where trait is dependent on development stage. The day number given after the trait SL indicates the days after harvesting where the QTL for shelf life was detected. The length of the bars indicates the LOD interval over the significant threshold for each QTL, and the line extensions of each bar indicate a one LOD lower than significant LOD support confidence interval for each QTL threshold. Filled bars represent the QTLs detected in the Portugal trial, open bars represents the QTLs detected in the UK trial, and patterned bars represent QTLs detected in both trials. The full data set is shown in Supplementary Fig. S5 available at *JXB* online.

also located to the same chromosome (LG5) in both trials, with a 32 cM interval between them, but both were derived from the *L. serriola* allele and accounted for 20–28% of the phenotypic variation. Hotspots for shelf life thus show correlation with biophysical traits, but they locate to different regions of the genome due to the effect of the climate in which they are grown.

QTL hotspots

From the above analysis of multiple phenotypic traits associated with leaf development and post-harvest shelf life performance, several regions have been identified where there are overlapping QTLs, known as hotspots (Fig. 5). There is one such region on LG1 accounting for developmental traits spanning 19 cM and another 4 cM QTL for shelf life located 16 cM away. LG2 has several small QTLs accounting for developmental traits. There are two major hotspots for developmental traits on LG3 that are both highly discrete, spanning 5 cM and 10 cM, the first one accounting for traits from the Portugal and UK trials. LG8 is another major hotspot for developmental traits from both countries, with several QTLs for shelf life located on the same LG at 15–53 cM distance from the developmental one. QTLs for developmental traits rarely co-located with those for shelf life in large hotspots, although there were small regions on LG1, 2, and 5 with overlapping QTLs. A QTL hotspot for shelf life traits was identified on LG5 that co-located with biophysical properties of plasticity and breakstrength. Leaf biophysical properties and shelf life again co-located in hotspots on LG6 and LG8, in all cases co-locating with QTLs for ECA, indicating that small cell size is a major contributing factor to increased leaf strength.

Discussion

Shelf life and leaf development characteristics

This study is the first to report QTLs for leaf shelf life in lettuce and to link this post-harvest trait to pre-harvest leaf

developmental and biophysical characteristics. Additionally, distinct clusters (hotspots) have been identified on the lettuce genome that relate exclusively to developmental characteristics, and others that are specific to post-harvest performance. This may be due to fortuitous trait linkage, or the segregation of a unique QTL controlling multiple traits because of related metabolic regulation. In some cases, these hotspots are almost adjacent to each other on the genome, and a larger sample size may provide more accurate relative locations. Correlation coefficients between the trait data associated with each RIL indicate that there appears to be an association of longer shelf life with larger leaves, lower chlorophyll content, higher cell number per leaf, and lower stomatal index in the Portugal field trial (Table 2). The maximum number of QTLs associated with each trait for each leaf age is four, corresponding to the trends observed by Kearsey and Farquar (1998).

Shelf life in the Portugal trial was mostly accounted for by QTLs on LG5 and LG8, whereas in the UK trial LG6 represented up to 66% of the phenotypic variation of this trait (Table 3). In most cases, the *L. sativa* allele was responsible for increased shelf life. The differences may be due to the 3 d delay to processing from the Portugal trial while the material was transported back to the UK, whereas the UK trial was processed within 24 h of harvest. These findings are novel since most previous reports on plant shelf life are related to fruit spoiling and softness, notably in tomato (Causse *et al*., 2002; Fridman *et al*., 2002; Rodriguez *et al*., 2005). However, studies of tomato fruit shelf life have shown that QTLs for post-harvest performance can be distinct from any other fruit quality trait (Rodriquez *et al*., 2005), and co-locations of QTL for texture attributes and organoleptic traits of flavour and taste were infrequent (Causse *et al*., 2002). Thus it may be necessary to look further at pre-harvest conditions to understand post-harvest performance and genetic regulation. One possible area of investigation that has received little attention in relation to post-harvest performance is that of bacterial colonization. Bacteria are known to continue to metabolize and produce exo enzymes such as proteases, cellulases, and hydrolases even at refrigerated temperatures when replication has ceased. These enzymes actively degrade plant tissue such as the cell wall, and the effect is increased when temperature allows replication. Bacteria are able to enter the plant leaf through damaged regions, such as those caused by washing, and through stomatal pores (CW Keevil, A Sihota, and J Warner, University of Southampton, UK, unpublished data). The data indicate a strong correlation between bacterial load on the leaves and shelf life, but it is not known at this stage if the pre-harvest bacterial load varies, potentially because different RILs have variable antibacterial qualities, or if variability in leaf surface morphology affects the efficiency with which bacteria are removed from the leaf surface during post-harvest processing. Unpublished evidence (CW Keevil, A Sihota, and J Warner, University of Southampton, UK) has shown that bacteria are highly efficient at colonizing furrows between epidermal cells in lettuce leaves and thereby resist dislocation from the leaf surface during the washing process.

The leaf shelf life is also associated with cell wall biochemistry, as well as rates of cell expansion and production. This was clearly reflected in the association of shelf life QTLs with leaf biophysical properties in the present study. Increased leaf cell expansion relates to the stimulation in cell wall loosening (Cosgrove, 2001). Brummell *et al.* (1999) reported that suppression of α-expansin expression in ripening tomato fruit resulted in firmer fruit with fewer breakdowns of some wall components. It also improved shelf life, but had no effects on fruit size (Brummell *et al*., 2002), indicating that the properties of the fruit wall were affected more than fruit expansion. Kaku *et al.* (2002) showed that XET enzyme expression increased the cell wall extensibility of epidermal tissues by hydrolysing xyloglucans within the native cell wall architecture; XET activity has been found in many plant tissues and correlates closely with growth rates in many cell types (Thompson and Fry, 2001), and would therefore be expected to influence post-harvest performance if these structures are detached from the parent plant. Unlike developmental QTLs in ripening tomato fruit, there is a strong association between QTLs for wall strength and traits associated with good organoleptic and shelf life performance in apple species. Apples with high compression and wedge fracture test results were also crisp and juicy (King *et al*., 2001), and QTLs for the elastic properties of tomato flesh co-located with those for organoleptic properties such as firmness, sweetness, and meltiness (Causse *et al*., 2002).

Stomatal density or stomatal index correlates with epidermal cell area, cell number, and dry and fresh weights in hotspots on LG1, LG4, LG6, and LG8, and is usually linked to *L. serriola* alleles. The lower percentage of dry weight compared with fresh weight in *L. sativa* leaves indicates that there was a higher water content than in *L. serriola* leaves. The small cells of *L. sativa* with a higher percentage of water had higher turgidity, although *L. sativa* had higher osmolality than *L. serriola* in the Portugal trial, possibly due to osmolality being derived from a mixture of cell types. QTLs for shelf life are found on the same chromosome as those for epidermal cell area (LG5 and LG6) and stomatal index (LG6). The stomatal index shows the configuration of a leaf to adjust stomatal opening and closure with water availability. The traits OSM, DWP, and SI relate to cell water, thus indicating that leaf processability involves traits related to leaf cell water relations (Clarkson *et al*., 2003).

The QTLs for chlorophyll content also correlate with developmental traits. Lower chlorophyll concentration

was measured in the oldest leaves rather than the newly emerged leaves in this lettuce population as it was harvested before the rosette could form a 'head' typical of an iceberg-type lettuce in which light is excluded from the youngest leaves. Measurements suggested that the older leaves were in the process of senescence. Several genetic mutants and potential regulator components have been identified for leaf senescence in *Arabidopsis* (Lim *et al*., 2003), which will assist the future beneficial analysis of the senescence rate and its genetic basis in lettuce.

Clusters of QTLs

The relatively small population size necessitated by the operational constraints of the experiment and the consequent significance level of this study only allow the detection of QTLs of large effect (Kearsey and Farquhar, 1998). Only 60 RILs of the most informative lines were analysed in this study, although there are 130 RILs in the whole population. The fact that much of the variation was left unexplained in this study suggested that there are probably more QTLs with smaller effects that could not be detected. If QTL peaks are situated close to each other and the LOD support intervals overlap, multiple QTLs can be regarded potentially as a single QTL with pleiotropic effects. However, in most studies, each QTL has been counted independently as this does not imply functional or genetic associations between genes on a similar chromosome location (Xu *et al*., 2004). Several clusters of QTLs were detected in this study (Fig. 5), on linkage groups 1, 3, 5, 6, 7, and 8, and each QTL has been counted separately. For example, QTLs for traits of LA, DWP, SLA, ECA, ECN, and SD mapped to a region on LG1 (83–102 cM), while QTLs for traits of LA, DW, CHL, ECA, ECN, and AGR all map to a similar position on LG7 (78–111 cM). Similarly clustered QTLs have been reported in other studies for leaf traits (Rae *et al*., 2004), rice seed traits (Xu *et al*., 2004), and fruit quality traits (Causse *et al*., 2002). The result showing the clustering of QTLs is consistent with the high correlation coefficients among the traits (Table 2). For example, LA, AGR, SLA, FW, and DW are groupings of physiologically associated traits. CHL is presented as total chlorophyll content per mm^2. The larger (LA or SLA) leaves, with greater thickness, usually had higher chlorophyll content. Therefore, it is not surprising that QTLs for CHL were clustered with QTLs for LA, SLA, and DW leaf traits. It is consistent with the report that growth traits (plant area, dry weight, and relative growth rate) co-located at five genomic regions in an *Arabidopsis* RIL population (El-Lithy *et al*., 2004) in addition to the finding of co-location of QTLs for leaf area, specific leaf area, and chlorophyll fluorescence.

This clustering of QTLs has important implications for plant breeding programmes. For example, clustered QTLs on LG1 showed pleiotropic effects on LG1 for leaf area, dry weight (as a percentage of fresh weight), cell area, and cell number. In *L. sativa*, all of the QTLs showed increased values for desirable traits. For this kind of QTL cluster, the selection of the ideal genotype of one QTL region could simultaneously improve several other traits positively. For other QTL clusters, where both desirable and undesirable traits map together, fine mapping and analysis of near-isogenic substitution lines is necessary to determine whether there are multiple QTLs or a single QTL with pleiotropic effects. If the latter is the case, it would be difficult to select for an improved genotype.

Leaf development-specific QTLs

In the Portugal field trial, QTL effects for 10 leaf traits (LA, FW, DW, DWP, SLA, CHL, ECA, ECN, SD, and SI) were analysed in three different developmental stages (young, semi-mature, and mature). None of the 75 QTLs was identified in all three different developmental stage leaves, although young and semi-mature leaves did map QTLs for LA on LG3. QTLs are therefore dependent on leaf development stage, indicating that some loci may have an overall effect on plant growth, while others only specifically regulate certain processes during a specific phase of growth. Similarly, QTLs for growth-related traits at the top of chromosome 3 of *Arabidopsis* were found mainly for the earlier phase (El-Lithy *et al*., 2004). In this study, QTLs for ECN and/or ECA co-located with QTLs for LA in several places on the genome, but rarely for the same leaf developmental stages; however, the co-location of QTL for LA and FW/DW was commonly for the same leaf developmental stage. This suggests that the leaf size at the stage measured was largely changing as a consequence of cell expansion through water uptake. Research on *Arabidopsis* mutants has also confirmed the importance of both cell production and cell expansion in leaf growth (Kim *et al*., 2002), and Donnelly *et al*. (1999) showed that cell division occurs only after cells have reached a certain size, supporting the view from this study that the leaves are growing by means of cell expansion rather than division. The relative importance of cell division and cell expansion varies among plant species and is under genetic control (Taylor *et al*., 2003). The present results show that cell expansion is highly responsive to environmental conditions, as the epidermal cell area was significantly different between two trials (Table 1). Other studies have reported that leaf cell expansion appeared extremely sensitive to environmental conditions, and QTLs for different environments were identified (Ferris *et al*., 2001, 2002).

QTL×environment interaction

QTL effects may be environmentally sensitive, and this sensitivity results in phenotypic plasticity (Gurganus *et al*., 1999). In this study, QTLs for leaf development, biophysical properties, and shelf life have been studied in two different environments. In the Portugal trial, 61 QTLs

were detected, and in the UK trial 45 were found. Of these, five QTLs were common to both trials and were assumed to be independent of the environment (Table 3, Fig. 5). The number and contribution of each QTL that has significantly different effects across the environments would be associated with substantial G×E interaction effects. Further analyses would reveal whether a QTL detected in one environment but not in the other indicates a real QTL×E interaction.

In summary, this study demonstrated substantial progress in using QTL mapping to understand the genetic basis of variation in plant growth and morphology. The ideal ideotype lettuce for improved shelf life is one with strong cell walls, and low levels of leaf plasticity, traits that are linked to, or possibly even brought about by, small cell area. These physiological traits were associated with the best performing lines with regard to shelf life in the Spanish trial. The QTL mapping provides opportunities for future functional research of not only specific loci but also their interactions with other loci (epistasis) and the environment (G×E interactions). The most important finding in this study was the detection of QTLs for shelf life and the association with leaf biophysical traits. Although the minor QTLs were subject to variable genomic locations in different climactic growing conditions, the trait associated with shelf life, leaf strength, and cell area was consistent in each environment. Mapping of candidate genes, such as members of the XTH gene family that are linked to cell wall biosynthesis, is currently in progress in order to identify the genetic basis of processability. The QTL information offers new targets for investigating the molecular regulation of shelf life, and the marker density of the lettuce map allows transference of significant QTL regions to other mapping and breeding populations, if the selected markers are polymorphic, after QTL stability is confirmed. Further fine mapping will enable a greater precision of QTL location and, as more ESTs are positioned on the map, the underlying genetics of shelf life control will be elucidated.

Supplementary data

The supplementary data mentioned herein are available at *JXB* online.

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